NEUROSCIENCE

Prior alcohol use enhances vulnerability to compulsive cocaine self-administration by promoting degradation of HDAC4 and HDAC5

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Addiction to cocaine is commonly preceded by experiences with legal or decriminalized drugs, such as alcohol, nicotine, and marijuana. The biological mechanisms by which these gateway drugs contribute to cocaine addiction are only beginning to be understood. We report that in the rat, prior alcohol consumption results in enhanced addiction-like behavior to cocaine, including continued cocaine use despite aversive consequences. Conversely, prior cocaine use has no effect on alcohol preference. Long-term, but not short-term, alcohol consumption promotes proteasome-mediated degradation of the nuclear histone deacetylases HDAC4 and HDAC5 in the nucleus accumbens, a brain region critical for reward-based memory. Decreased nuclear HDAC activity results in global H3 acetylation, creating a permissive environment for cocaine-induced gene expression. We also find that selective degradation of HDAC4 and HDAC5, facilitated by the class II–specific HDAC inhibitor MC1568, enhances compulsive cocaine self-administration. These results parallel our previously reported findings that the gateway drug nicotine enhances the behavioral effects of cocaine via HDAC inhibition. Together, our findings suggest a shared mechanism of action for the gateway drugs alcohol and nicotine, and reveal a novel mechanism by which environmental factors may alter the epigenetic landscape of the reward system to increase vulnerability to cocaine addiction.

INTRODUCTION

Loss of control over drug use, typified by continued use despite aversive consequences, is a hallmark characteristic of cocaine addiction (1). The finding that only a small proportion (~21%) of cocaine users progress to compulsive use (2) highlights the importance of both genetic and environmental factors in conferring vulnerability to cocaine addiction. Prior use of either alcohol or nicotine ranks high among the environmental factors known to influence subsequent use of cocaine. Thus, in 2015, 91% of cocaine users, 18 to 49 years old, had first used alcohol before using cocaine and 5.1% had started both drugs at the same age; 85% had first used nicotine, and 5.8% had started both nicotine and cocaine at the same age (3). These behavioral regularities in drug involvement have given rise to the gateway hypothesis, which proposes that certain drugs, such as alcohol, nicotine, and marijuana, increase the risk of subsequently using other drugs, such as cocaine (4). However, the mechanisms by which these initial drug experiences increase vulnerability to cocaine use have only recently begun to be understood (5, 6).

Using a sequential drug administration paradigm that models the stages of drug abuse seen in human populations, we previously found that mice preexposed to nicotine show an enhanced response to the rewarding properties of cocaine (5). Nicotine primes the brain to cocaine-induced changes by modifying chromatin structure and enhancing cocaine-induced gene expression and long-term synaptic depression in the striatum. The reverse is not observed: Cocaine has no effect on nicotine-induced behavior or gene expression, supporting the hypothesis of a unidirectional progression of drug use (5). If the different gateway drugs operate through similar biological mechanisms in a rodent model, one would predict that alcohol, a gateway drug, would also potentiate the effects of cocaine. We therefore asked: Does prior alcohol use enhance the behavioral effects of cocaine in a rodent model of addiction and, if so, is the priming process of alcohol mediated by cellular and molecular mechanisms that are similar to those for nicotine? Moreover, is the effect of alcohol on cocaine consumption also unidirectional, as it is with nicotine?

To study the effects of alcohol on cocaine-related behaviors, we again used a sequential drug administration paradigm, with voluntary access to both alcohol and cocaine (Fig. 1A). In this paradigm, daily alcohol use (10% ethanol, 2 hours per day; see Fig. 1A and table S1) precedes the start of daily cocaine self-administration by 10 days. Because most drug users progress to illicit drug use by adding the illicit drug to their preexisting drug regimen (as opposed to switching) (4), daily alcohol use is continued throughout the duration of the cocaine self-administration paradigm. To avoid acute behavioral, metabolic, or pharmacokinetic interaction between alcohol and cocaine (7–11), we restricted access to alcohol and cocaine to different time periods during the dark (wake) cycle (Fig. 1A). We assessed the effect of prior alcohol exposure on three key indices of cocaine addiction observed in humans: (i) persistence of drug seeking in the absence of reward, (ii) motivation for drug use, and (iii) compulsivity. Conversely, we explored the directionality of drug action by reversing the sequence of drug exposure and testing the effect of prior cocaine self-administration on subsequent alcohol preference. Furthermore, to determine whether alcohol creates a permissive epigenetic environment for cocaine-induced gene expression, we used the same alcohol exposure paradigm (10% alcohol, 2 hours per day)

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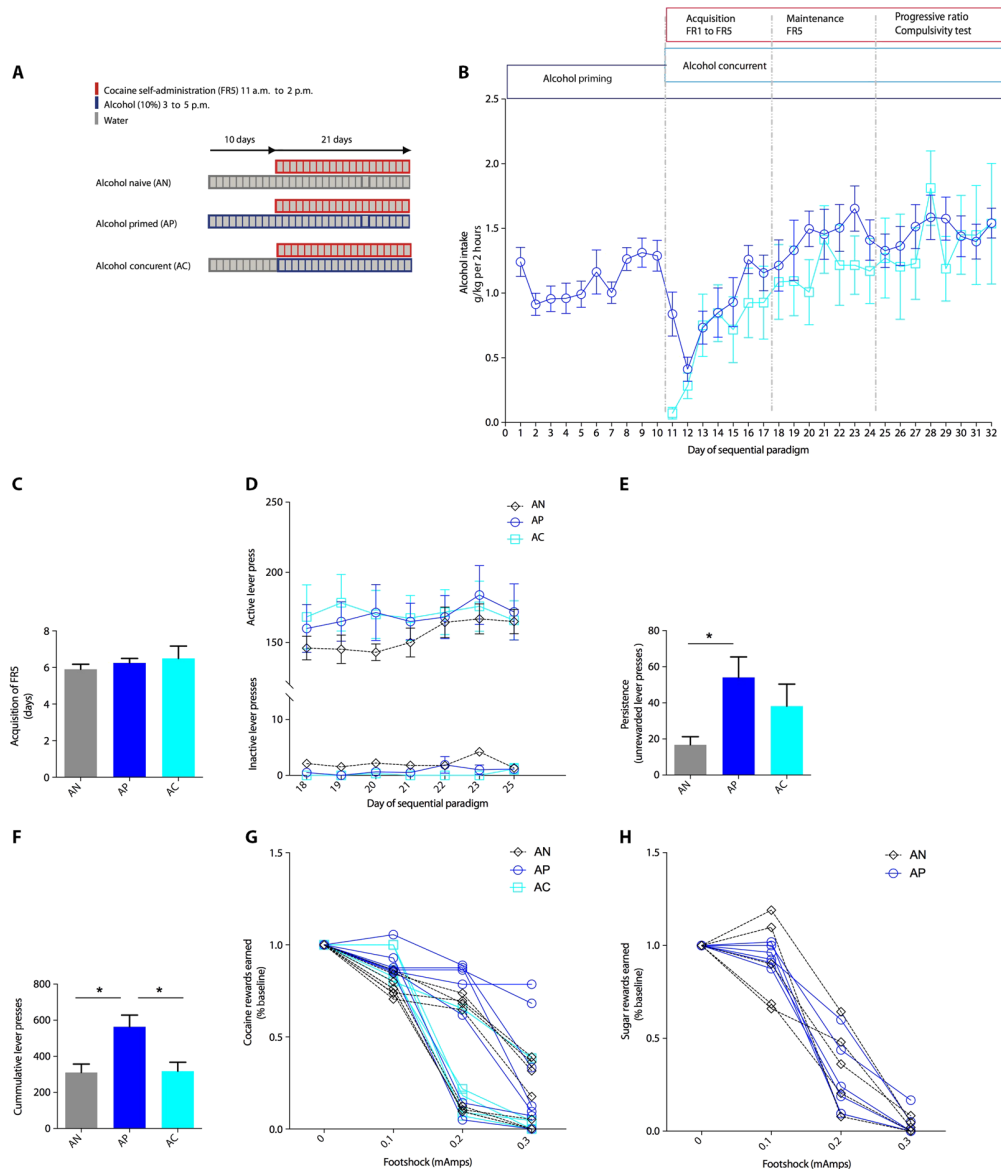
Fig. 1. Prior alcohol use enhances persistence, motivation, and compulsivity for cocaine self-administration. (**A**) Drug treatment paradigm to study alcohol and cocaine coadministration. Access to voluntary alcohol (10% alcohol, 2 hours per day) and voluntary cocaine (self-administration, 0.8 mg/kg per injection) was restricted to 3 p.m. to 5 p.m. and 11 a.m. to 2 p.m., respectively, to avoid metabolic interaction between the two drugs. (**B**) Alcohol self-administration. Animals in the alcohol-primed group began drinking alcohol on day 1 of the paradigm; animals in the alcohol-concurrent group began drinking on day 11. All groups start cocaine self-administration (0.8 mg/kg per infusion) on day 11. The average alcohol intake during the alcohol-priming period was 1 g/kg during the first 5 days, increasing to 1.2 g/kg during the second 5 days. Alcohol intake was similar in the alcohol-primed versus alcohol-concurrent groups from days 11 to 32 [two-way repeated-measures (RM) analysis of variance (ANOVA): Treatment group: $F_{1,12} = 1.398$, $P = 0.26$; Treatment day: $F_{21,252} = 10.71$, $P < 0.0001$; Interaction: $F_{21,252} = 1.14$, $P = 0.303$; $n = 6$ to 8 per group]. (**C**) Prior exposure to alcohol does not affect the acquisition of lever pressing on an FR5 schedule of reinforcement. Animals were started on FR1 and gradually increased to FR3 (1 to 2 days) and then to FR5. Animals reached FR5 after 5.9, 6.2, and 6.6 days for water control, alcohol-primed, and alcohol-concurrent groups, respectively (one-way ANOVA: $F_{2,21} = 0.63$, $P = 0.5425$, not significant; $n = 6$ to 8 per group). (**D**) Prior alcohol exposure does not enhance lever pressing for cocaine reward (0.8 mg/kg per injection) during the maintenance phase of cocaine self-administration. The analysis showed no main effect for treatment group, treatment day, or an interaction between the two factors (two-way ANOVA: Group: $F_{2,123} = 1.119$, $P = 0.3298$, not significant; Treatment day: $F_{6,123} = 1.44$, $P = 0.20$, not significant; Interaction: $F_{12,123} = 0.16$, $P = 0.9995$, not significant). (**E**) Alcohol preexposure enhances persistence of cocaine seeking during unrewarded time-out sessions, averaged over the last 3 days of the maintenance phase (B) (one-way ANOVA: $F_{2,19} = 3.66$, $P = 0.045$; Tukey post hoc: $P = 0.047$, alcohol primed versus alcohol-naïve; $n = 6$ to 8 per group). (**F**) Alcohol preexposure enhances motivation for cocaine self-administration in a progressive ratio schedule of reinforcement (one-way ANOVA: $F_{2,20} = 6.45$, $P = 0.007$; Tukey post hoc analysis: $P < 0.01$, control versus alcohol-primed; $P < 0.01$, concurrent versus alcohol-primed; $n = 6$ to 8 per group). (**G**) Alcohol preexposure enhances compulsive cocaine self-administration. Successive increases of the footshock intensity resulted in a decrease in lever pressing for all groups. Alcohol pretreated animals have significant resistance to footshock (two-way RM ANOVA: Group: $F_{2,19} = 4.76$, $P = 0.02$; Footshock: $F_{3,57} = 67.94$, $P < 0.0001$; Footshock × Group interaction: $F_{6,57} = 1.829$, $P = 0.1095$; $n = 6$ to 8 per group). The baseline number of cocaine infusions (at 0.0 mA) did not differ between groups (AN, 19.67 ± 2.10; AP, 19.7 ± 2.32; AC, 20.00 ± 2.46). (**H**) Alcohol preexposure does not alter shock-resistant lever pressing for sugar pellets in food-restricted animals (two-way RM ANOVA: Group: $F_{1,9} = 0.003$, $P = 0.96$, not significant; Footshock: $F_{3,27} = 131.3$, $P < 0.0001$; Footshock × Group interaction: $F_{3,27} = 0.64$, $P = 0.59$, not significant; $n = 5$ to 6 per group). Baseline amount of sugar pellet reward (at 0.0 mA) did not differ between groups (AN, 46.60 ± 3.3; AP, 49.33 ± 2.15). *$P < 0.05$. Data are means ± SEM.

to examine the epigenetic effects of daily alcohol use in the nucleus accumbens, a brain region critical for mediating cocaine addiction–like behaviors.

RESULTS
Prior alcohol use does not enhance acquisition or amount of cocaine self-administration
To distinguish the effects of alcohol preexposure from alcohol co-exposure, we compared the outcomes of cocaine self-administration in the alcohol-primed group ("AP"; Fig. 1A) with two control groups: (i) an alcohol-naïve (AN) group that drank water instead of alcohol and (ii) an alcohol-concurrent (AC) group that started using alcohol and cocaine on the same day. Animals in the AP and AC groups did not differ in their daily alcohol intake patterns (Fig. 1B). Prior exposure to alcohol did not increase the daily intake of cocaine during the acquisition or maintenance phase of the sequential paradigm (fig. S1). Prior exposure to alcohol did not affect the acquisition rate [duration of time required to learn to lever press for cocaine injection on a fixed ratio of 5 (FR5)]. We found that the animals learned to lever press on an FR5 schedule after 5.9, 6.2, and 6.6 days in the alcohol-naïve, alcohol-primed, and alcohol-concurrent groups, respectively (Fig. 1C). Similarly, the groups did not differ in the average number of lever presses for cocaine during a 7-day maintenance phase (Fig. 1D). Overall, our behavioral findings on the effect of prior alcohol use on the early stages of cocaine self-administration (days 11 to 21; Fig. 1A) are consistent with the observations of Fredriksson et al. (12). Using a 12-day cocaine self-administration protocol, they reported no change in acquisition of cocaine self-administration in animals that had an extensive 7-week history of prior alcohol use.

Prior alcohol use enhances cocaine addiction–like behaviors
Cocaine addiction is characterized not only by the choice to self-administer cocaine but also by the complex behaviors that define addiction, such as increased motivation for the drug, persistence of drug seeking in the absence of reward, and continued drug use despite negative consequences. To determine whether animals with a history of prior alcohol use have enhanced cocaine addiction–like behaviors, we assayed these key behavioral indices using an approach modeled after Deroche-Gamonet et al. (13) and Belin et al. (14). To examine the persistence of cocaine seeking in the absence of reward, we measured the number of lever presses during "no drug periods" of daily cocaine self-administration (two 15-min intervals during which the levers are available but are not rewarded). Animals in the alcohol-naïve group averaged 18 lever presses during the time-out periods. Animals in the alcohol-primed group had significantly enhanced persistence in comparison to alcohol-naïve animals, averaging 58 lever presses. Animals in alcohol-cocaine concurrent control averaged 38 lever presses during the time-out period but were not significantly different from the alcohol-naïve group (Fig. 1E).

We next measured motivation for cocaine self-administration on a progressive ratio schedule of reinforcement, where the animal was required to make increased number of lever presses to earn each subsequent intravenous cocaine reward (0.8 mg/kg per injection), until it reached the breakpoint where it ceased lever pressing (15). We found that alcohol-naïve animals worked for cocaine until an average breakpoint of 310 lever presses, whereas alcohol-primed animals worked for cocaine until they reached an average breakpoint of 563 lever presses. Animals in the alcohol-concurrent group had an average

breakpoint of 317 lever presses, similar to the alcohol-naïve group (Fig. 1F).

One of the core characteristics of drug addiction in humans is compulsivity, as reflected in continued drug use despite negative consequences (13, 16). Prior studies have found that most rats stop self-administering cocaine when challenged with an aversive footshock 20 days after initiating cocaine self-administration (16). But when rats have had long-term exposure to cocaine (~60 days of daily cocaine self-administration), a subset of animals continue to seek and take cocaine despite an aversive footshock (13, 17). We reasoned that exposure to alcohol before cocaine would reveal a compulsive phenotype that would not otherwise occur in the absence of priming by alcohol.

To test for compulsive drug use, we introduced an aversive 2-s foot shock (0.1 to 0.3 mA) to the cocaine self-administration session, with an increase in shock intensity occurring every 40 min. We found that animals in the alcohol-naïve and alcohol-concurrent groups declined similarly, earning 73 and 76% of baseline reward at 0.1 mA, 39 and 31% of baseline reward at 0.2 mA, and 16 and 14% of baseline reward at 0.3 mA. By contrast, animals preexposed to alcohol were significantly more resistant to punishment, earning 90, 68, and 29% of baseline reward at 0.1, 0.2, and 0.3 mA of footshock, respectively (Fig. 1G).

Alcohol is a nonspecific and potent neurotoxin, and could decrease sensitivity to footshock by decreasing peripheral pain sensitivity or fear memory. We found, however, that alcohol use did not enhance compulsive lever pressing for sugar pellets in food-restricted animals (Fig. 1H). Thus, the priming effects of alcohol on cocaine compulsivity appear to be mediated by processes that do not generalize to natural rewards. Together, our results indicate that voluntary alcohol use increases vulnerability to cocaine by enhancing persistence of drug seeking, motivation, and continued use despite negative consequences.

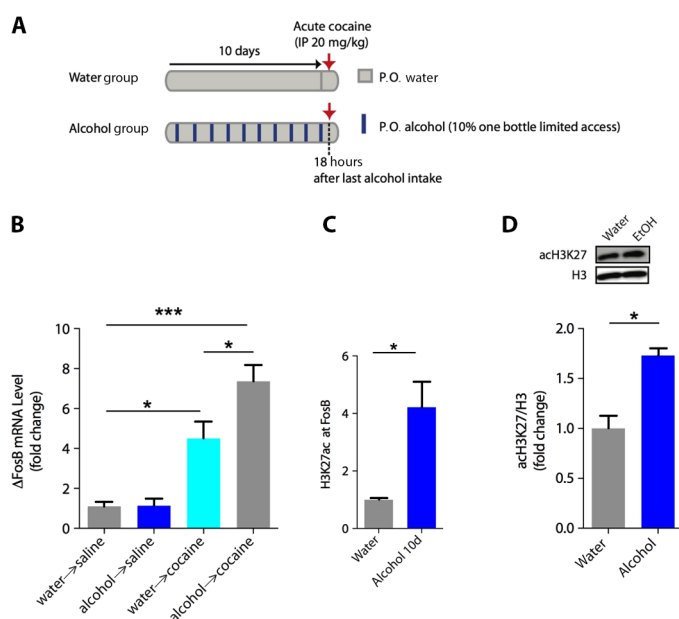### Prior cocaine use decreases preference for alcohol
To determine whether prior exposure to cocaine enhances alcohol preference, we reversed the order of drug exposure so that cocaine self-administration preceded the start of voluntary alcohol use by 10 days (fig. S2A). Animals in the control group with no prior history of cocaine showed increasing preference for alcohol in a two-bottle free choice paradigm, whereas animals with a prior history of cocaine use actually had decreased alcohol intake and decreased preference for alcohol (fig. S2, B and C).

When we analyzed the daily alcohol intake of animals in our sequential alcohol-to-cocaine paradigm (Fig. 1B), we found that animals in the alcohol-primed group had a transient but significant decline in daily alcohol intake during the first 2 days of cocaine self-administration, and after reaching a nadir of alcohol intake, the animals required 4 to 5 days before reaching the prior baseline of daily alcohol intake of 1 to 1.2 g/kg per day (fig. S3). Animals that started alcohol self-administration concurrently with cocaine use also required 4 to 5 days before reaching a daily alcohol intake of 1 to 1.2 g/kg. This is much slower than cocaine-naïve animals that began drinking 1 g/kg by the second day of alcohol use (alcohol-primed group, days 1 to 5). These results suggest that cocaine use may decrease the preference for alcohol even without a priming period.

Our findings are consistent with previous reports that prior cocaine use does not enhance alcohol use in rats (18) and that, in mice, prior cocaine use results in decreased alcohol preference (19). Together, our findings are consistent with a unidirectional cross-sensitization as predicted by the gateway hypothesis: from alcohol to cocaine, but not from cocaine to alcohol.

Alcohol use creates a permissive epigenetic environment for cocaine-induced gene expression

To gain insight into the molecular mechanisms by which prior alcohol use potentiates the behavioral effects of cocaine self-administration, we examined the epigenetic effects of alcohol use in the nucleus accumbens, a brain region critical for addiction-related learning and memory. In our previous studies with nicotine, we found that nicotine inhibits histone deacetylase (HDAC) activity in the striatum. Inhibition of HDAC activity resulted in global histone acetylation and a permissive epigenetic environment for cocaine-induced gene expression (4). To determine whether alcohol exposure similarly primes the transcriptional response to cocaine, we allowed animals to drink alcohol for 10 daily 2-hour sessions and followed this with a single injection of cocaine (20 mg/kg intraperitoneally) 18 hours after the last alcohol ingestion (Fig. 2A). Accumulation of the ΔFOSB protein in the nucleus accumbens has been found to have a key role in enhancing cocaine self-administration (20), and is induced by cocaine under voluntary (self-administered) conditions as well as experimenter-administered conditions (21). We

now asked: Does prior alcohol use enhance the cocaine-induced transcription of this key marker? We carried out quantitative polymerase chain reaction (PCR) using primers specific to the ΔFosB splice variant and found that alcohol preexposure significantly enhanced cocaine-induced expression of ΔFosB in the nucleus accumbens compared to alcohol-naïve animals that also received an injection of cocaine (Fig. 2B).

To determine whether alcohol facilitates acetylation locally at the FosB gene locus, we next performed chromatin immunoprecipitation (ChIP) in lysates of the nucleus accumbens of alcohol-treated animals. A search of the encyclopedia of DNA elements (ENCODE) revealed that acetylation of H3 lysine 27 (H3K27) in the first intronic region of FosB (the gene giving rise to the splice variant ΔFosB) may be a key epigenetic regulatory mark for this gene in several tissues, including brain (fig. S4) (22). We found that alcohol facilitated robust acetylation of H3K27 residues locally at the FosB gene—fourfold above the (water) control group (Fig. 2C). Because the level of global H3 acetylation in the nucleus accumbens has been found to be positively correlated with motivation for cocaine self-administration in rats [although no such effect has been observed for H4 acetylation (23)], we next immuno-blotted total histone lysates and found that alcohol also increased global acetylation of H3K27 in the nucleus accumbens (Fig. 2D).
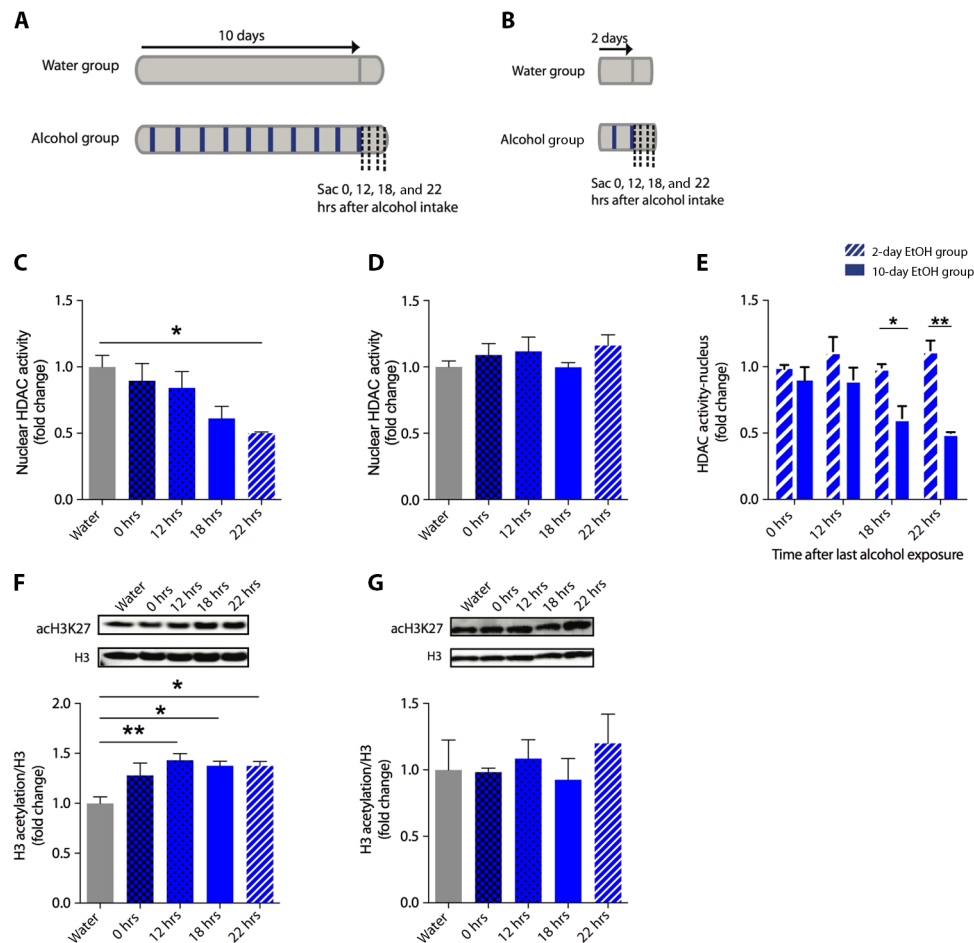
Alcohol use promotes decreased nuclear HDAC activity in the nucleus accumbens

To determine whether the local acetylation at the FosB locus and the global acetylation in the nucleus accumbens are due to changes in HDAC activity, we next tested the enzymatic HDAC activity in nuclear lysates of the nucleus accumbens of animals exposed to 10 days of daily alcohol use. Prior studies have found that the effects of alcohol on HDAC activity are dynamic and highly sensitive to both the acute exposure and abstinence stages of alcohol use (24). Therefore, we tested HDAC activity in nucleus accumbens lysates of animals sacrificed at sequential time points after the 10th daily 2-hour alcohol session (on day 10) (Fig. 3A). To determine whether the effects are specific to long-term alcohol exposure, we performed a similar analysis of nuclear HDAC activity in the nucleus accumbens of animals after only 2 days of alcohol exposure (Fig. 3B).

Ten days, but not 2 days, of alcohol use resulted in a significant decrease in HDAC activity in comparison to water control (Fig. 3, C and D). Ten days of alcohol use caused a progressive decrease in HDAC activity after the end of the alcohol drinking session (Fig. 3E). We observed no significant change in HDAC activity at the time of acute alcohol exposure ("time 0," during which blood alcohol levels were 193 ± 43 μg/ml; see table S1) and a significant decrease in HDAC activity at time points during which no serum alcohol was detectable (18 and 22 hours; Fig. 3E and table S1).

In line with this decrease in HDAC activity, we also observed an increase in global H3K27 acetylation in the nucleus accumbens, starting at 12 hours after the last alcohol intake (Fig. 3F). Two days of alcohol use did not have a significant effect on H3K27 acetylation in the nucleus accumbens (Fig. 3G). The finding that short-term alcohol use does not affect HDAC activity or histone acetylation suggests that HDACs behave quite differently under short-term versus long-term exposure conditions, fostering a permissive epigenetic environment only after long-term exposure. Together with the findings observed in our earlier studies with nicotine, the present findings suggest that HDAC inhibition, global acetylation in the nucleus accumbens, and specific hyperacetylation of key genes in the nucleus accumbens may be general mechanisms of action for gateway drugs (5).

Fig. 2. Alcohol use creates a permissive epigenetic environment for cocaine-induced gene expression. (**A**) Diagram of sequential drug administration paradigm. Animals drank alcohol in a limited access paradigm [alcohol 10% (v/v); 2 hours per day; average intake, 1.11 ± 0.1 g/kg per day; n = 14] for 10 days. An acute cocaine injection (20 mg/kg intraperitoneally) was given 18 hours after the last alcohol ingestion to avoid pharmacokinetic interaction between alcohol and cocaine. (**B**) A single cocaine injection causes increased expression of the ΔFosB transcript in alcohol-naïve animals (one-way ANOVA: $F_{3,20}$ = 20.15, $P < 0.0001$; Sidak post hoc: water/saline-injected versus water/cocaine-injected, $P < 0.05$), whereas alcohol pretreated animals have significantly enhanced cocaine-induced ΔFosB induction ($P < 0.05$, alcohol/cocaine-injected versus water/cocaine-injected; $P < 0.0001$, alcohol/cocaine-injected versus water/saline-injected, alcohol/saline-injected; n = 6 to 7 per group). (**C**) ChIP experiment for acetylated histone H3K27 at the FosB region of interest shows increased acetylation in animals treated with 10% alcohol, 2 hours per day for 10 days (average intake, 1.1 ± 0.1 g/kg per day, n = 5, $P < 0.01$ versus water control, n = 3 to 5 per group). (**D**) Immunoblotting experiment for H3K27 acetylation in animals that drank for 10 consecutive days [10% (v/v) alcohol, 2 hours per day; average intake, 0.98 ± 0.12 g/kg per day; n = 5] and euthanized 18 hours after the last alcohol ingestion shows global increase in H3K27 acetylation in the nucleus accumbens ($P < 0.05$, 0 hours versus water control; n = 4 to 5 per group). P.O., per oral. $*P < 0.05$, $**P < 0.01$, $***P < 0.0001$. Data are means ± SEM.

Fig. 3. Long-term, but not short-term, alcohol use promotes progressive decrease of nuclear HDAC activity following alcohol cessation. (**A**) Diagram of 10-day voluntary drinking protocol. Alcohol-treated animals were sacrificed at sequential time points after the last alcohol ingestion on day 10: 0 (end of alcohol drinking session), 12, 18, and 22 hours after the last alcohol ingestion (average intake, 0.95 ± 0.05 g/kg per day; $n = 20$). (**B**) Short-term (2-day) alcohol exposure paradigm (average alcohol intake, 1.07 ± 0.16 g/kg per day; $n = 20$). (**C**) Ten-day exposure to alcohol causes a decrease in nuclear HDAC activity in the nucleus accumbens (one-way ANOVA: $F_{4,19} = 3.669$, $P = 0.0224$; Sidak post hoc: $P = 0.026$, water versus alcohol 22 hours after treatment). (**D**) Two-day exposure to alcohol does not result in decreased nuclear HDAC activity (one-way ANOVA: $F_{4,18} = 0.794$, $P = 0.5442$). (**E**) HDAC activity decreases progressively following cessation of alcohol treatment in animals treated with alcohol for 10 days (two-way ANOVA: Time after treatment cessation: $F_{3,32} = 3.165$, $P = 0.038$; Treatment group: $F_{1,32} = 17.18$, $P = 0.0002$; Interaction: $F_{3,32} = 4.715$, $P = 0.0078$; Sidak post hoc: no significant change at 0 and 12 hours, $P = 0.035$ at 18 hours, and $P = 0.0003$ at 22 hours; $n = 4$ to 5 per group). (**F**) Immunoblotting experiment for H3K27 acetylation shows enhancement of global H3K27 acetylation at 12, 18, and 22 hours after the last alcohol ingestion (one-way ANOVA: $F_{4,16} = 5.45$, $P = 0.0058$; Sidak post hoc: $P = 0.0033$, $P = 0.006$, $P = 0.010$ at 12, 18, and 22 hours, respectively, versus water control group; $n = 4$ to 5 per group). (**G**) Two-day exposure to alcohol does not result in enhanced H3K27 acetylation in the nucleus accumbens (one-way ANOVA: $F_{4,14} = 0.3981$, $P = 0.8067$; $n = 4$ to 5 per group). $*P < 0.05$, $**P < 0.01$. Data are means $\pm$ SEM.

Alcohol use decreases nuclear HDAC4 and HDAC5 levels in the nucleus accumbens

We next asked: Which specific HDACs are involved in alcohol-induced acetylation, and how are these inhibited by alcohol? Eleven HDACs have been identified in mammals. An extensive body of literature has demonstrated key roles of these enzymes in integrating a diverse array of molecular responses to stimulants such as cocaine (*25*, *26*). We focused on the class IIa HDACs HDAC4 and HDAC5 because they have been previously implicated in regulating the behavioral effects of cocaine, including cocaine self-administration (*23*, *27*, *28*). The class II HDACs shuttle between the cytoplasm and nucleus in an activity-dependent manner (*29*, *30*), and are heavily regulated by post-translational modifications such as phosphorylation, carbonylation,

SUMOylation, and ubiquitination (*31*). Although the class II HDACs carry a mutation in their catalytic domains that render them inactive as deacetylases (*32*, *33*), genetic and pharmacological knockdown of class II HDACs causes decreased HDAC activity (*34*) and global histone hyperacetylation and enhanced neuronal plasticity (*35*–*37*). Class II HDACs mediate histone acetylation by forming a multiprotein co-repressor complex that includes the class I HDAC, HDAC3 (*38*). HDAC3 is a potent deacetylase. Focal deletion of HDAC3 has been found to enhance long-term memory (*39*) and to facilitate conditioned place preference to cocaine (*40*). The catalytically inactive C terminus of HDAC4 is crucial for the recruitment and deacetylase activity of the multiprotein complex, which forms only in the nucleus of the cell. Thus, class II HDACs may function as activity-dependent scaffolds that target

the co-repressor complex to specific promoters (38). HDAC4 has been found to be critical for activity-dependent regulation of H3K27 deacetylation in specific gene promoter regions such as BDNF (brain-derived neurotrophic factor) (41).

We first asked: Does either HDAC4 or HDAC5 interact with the FosB gene locus? To address this question, we performed ChIP experiments in the rat striatum with antibodies against HDAC4 and HDAC5 and found that HDAC4, but not HDAC5, interacted with the FosB locus (fig. S5A). This finding is consistent with that of Wang et al. (23), who reported that overexpression of HDAC4 in the nucleus accumbens resulted in a decreased expression of FosB. Having found that HDAC4 is present at the FosB gene locus, we next examined the specific regulation of HDAC4 by asking whether alcohol regulates acetylation at the FosB promoter by altering the subcellular localization of HDAC4 in the nucleus accumbens. Another class IIa HDAC, HDAC5, limits cocaine reward by shuttling from the cytoplasm to the nucleus following acute exposure to cocaine (28). Although purified class II HDACs have weak deacetylase activity (32), Fischle et al. (38) found that, in vivo, nuclear HDAC4 (but not cytoplasmic HDAC4) exists in an enzymatically active multiprotein complex. To explore the effects of alcohol use on subcellular localization of HDAC4, we performed immunohistochemistry on striatal slices of animals exposed to alcohol for 10 days (fig. S5, B and C). We found that an acute exposure to alcohol (time 0) did not result in a significant change in HDAC4 subcellular localization compared to control animals. The HDAC4 stain completely and precisely overlaid the nuclear stain in 81% of nucleus accumbens cells in both the water control group and the acutely exposed alcohol group (time 0). By contrast, 18 hours after the last alcohol exposure on day 10 (when blood alcohol was not detectable; table S1), a significantly lower number of cells had HDAC4-positive nuclei, suggesting that HDAC4 accumulated less in the nucleus during the alcohol abstinence interval (fig. S5, B and C).

We next immunoblotted for HDAC4 in nuclear and cytoplasmic fractions of nucleus accumbens cells from animals sacrificed at four successive time points after the last alcohol ingestion (0, 12, 18, and 22 hours). Similar to immunohistochemical stains, we found that acute alcohol exposure (time 0) did not lead to a change in the nuclear accumulation of HDAC4, whereas the lack of alcohol resulted in a significant decrease in the nuclear accumulation of HDAC4 at 18 and 22 hours after the last alcohol ingestion (Fig. 4A). In parallel, we also performed immunoblotting studies of HDAC5 following a 10-day alcohol exposure, which revealed a similar decrease in nuclear accumulation 18 hours after the last alcohol ingestion (fig. S6B). Although HDAC4 may be a specific regulator of FosB, it is possible that other HDACs may be involved and may account for the changes that we observed in nuclear HDAC activity and global histone acetylation.

### Decrease of nuclear HDAC4 is facilitated by proteasome-mediated degradation
Surprisingly, we found that alcohol-induced decrease of nuclear HDAC4 accumulation was not paralleled by a comparable increased accumulation in the cytoplasmic compartment, as might be expected for an enzyme that shuttles between the nucleus and the cytoplasm (Fig. 4, A and B). In addition, quantitative analysis of HDAC4 mRNA revealed no change at the level of transcription in response to alcohol exposure (Fig. 4C). These findings suggested that the observed decrease in nuclear HDAC accumulation might be due to the degradation in the nucleus, not to shuttling to the cytoplasm. Studies by Potthoff et al. (42) have demonstrated that the activity-dependent differentiation of

mature skeletal muscle is mediated by selective proteasomal degradation of class IIa HDACs in the nucleus of muscle fibers. To test for this possibility, we placed in-dwelling cannulae into the nucleus accumbens of rats bilaterally and allowed the animals to drink either alcohol or water for 10 days. Twelve hours after the last exposure to alcohol, we injected a covalent inhibitor of the proteasome in one hemisphere [one-time injection of 200 μM lactacystin, 0.4 μl into the nucleus accumbens, as described by Massaly et al. (43)] and injected dimethyl sulfoxide (DMSO) vehicle in the contralateral, control hemisphere. Lactacystin rescued the decrease in nuclear HDAC activity (Fig. 4D) and kept nuclear HDAC4 and HDAC5 protein levels at control levels, with no change in the vehicle-treated contralateral hemisphere (Fig. 4E and fig. S6B). Lactacystin infusion did not affect HDAC4 or HDAC5 accumulation in the cytoplasm, suggesting that the degradation of these HDACs is occurring in the nuclear compartment (Fig. 4F and fig. S6C). Proteasome-mediated degradation of HDAC4 in the nuclear compartment has been found to mediate growth factor–induced cell motility (44) and activity-dependent striatal muscle differentiation (42). Our findings therefore suggest that, in addition to the well-described phosphorylation-dependent shuttling of the class II HDAC (28), other posttranslational modifications, such as SUMOylation (45) and ubiquitinization (44), may have key mechanistic roles in the way in which class II HDACs regulate vulnerability to drug addiction. Moreover, these data suggest that the class II HDACs, HDAC4 and HDAC5 may act as nodal regulators, integrating environmental stimuli (alcohol use) with behavioral response (potentiated reward-based learning, resulting in addiction-like behavior).

MC1568 promotes selective degradation of class II HDAC in the nucleus accumbens and enhances compulsivity for cocaine self-administration
To determine directly whether nuclear degradation of HDAC4 and HDAC5 causes an increase in addiction-like behavior, similar to the behaviors induced by alcohol (Fig. 1), we treated animals to the class II–specific HDAC inhibitor MC1568 (46, 47). MC1568 selectively inhibits class II HDAC activity by promoting nuclear import and proteasome-mediated degradation of HDAC4 and HDAC5 in the nucleus of the cell (48). Several other HDAC inhibitors, including suberoylanilide hydroxamic acid, valproic acid, and quinidine, have been found to inhibit HDAC activity by promoting proteasome-mediated degradation of HDAC4, HDAC2, and HDAC1, respectively (48–50). Systemic administration of MC1568 is well tolerated by mice, and crosses the blood-brain barrier, at doses of up to 6.5 mg/kg (51). In addition, MC1568 promotes global H3 hyperacetylation and enhances neurite outgrowth in dopaminergic and sympathetic neurons in vitro (37).

Although the effects of MC1568 on class II HDACs have been well characterized in cancerous and noncancerous tissue (34, 46, 48), the effects of this drug on HDAC4 and HDAC5 have not been described in the nucleus accumbens in vivo. Thus, we first characterized the effects of MC1568 in the nucleus accumbens. We treated animals to 10 daily injections of MC1568 (0.5 mg/kg) and tested for nuclear HDAC activity in the nucleus accumbens 18 hours after the last treatment. We found that MC1568 causes a 35% decrease in nuclear HDAC activity (Fig. 5B). To determine whether MC1568 selectively decreases class II HDACs, we immunoblotted nuclear and cytoplasmic fractions of the nucleus accumbens cells for class II HDACs, HDAC4 and HDAC5, and for class I HDACs, HDAC1 and HDAC2. We found that 10 days of daily exposure to MC1568 caused a significant decrease in nuclear HDAC4 and HDAC5 in the nucleus accumbens, with no significant change in
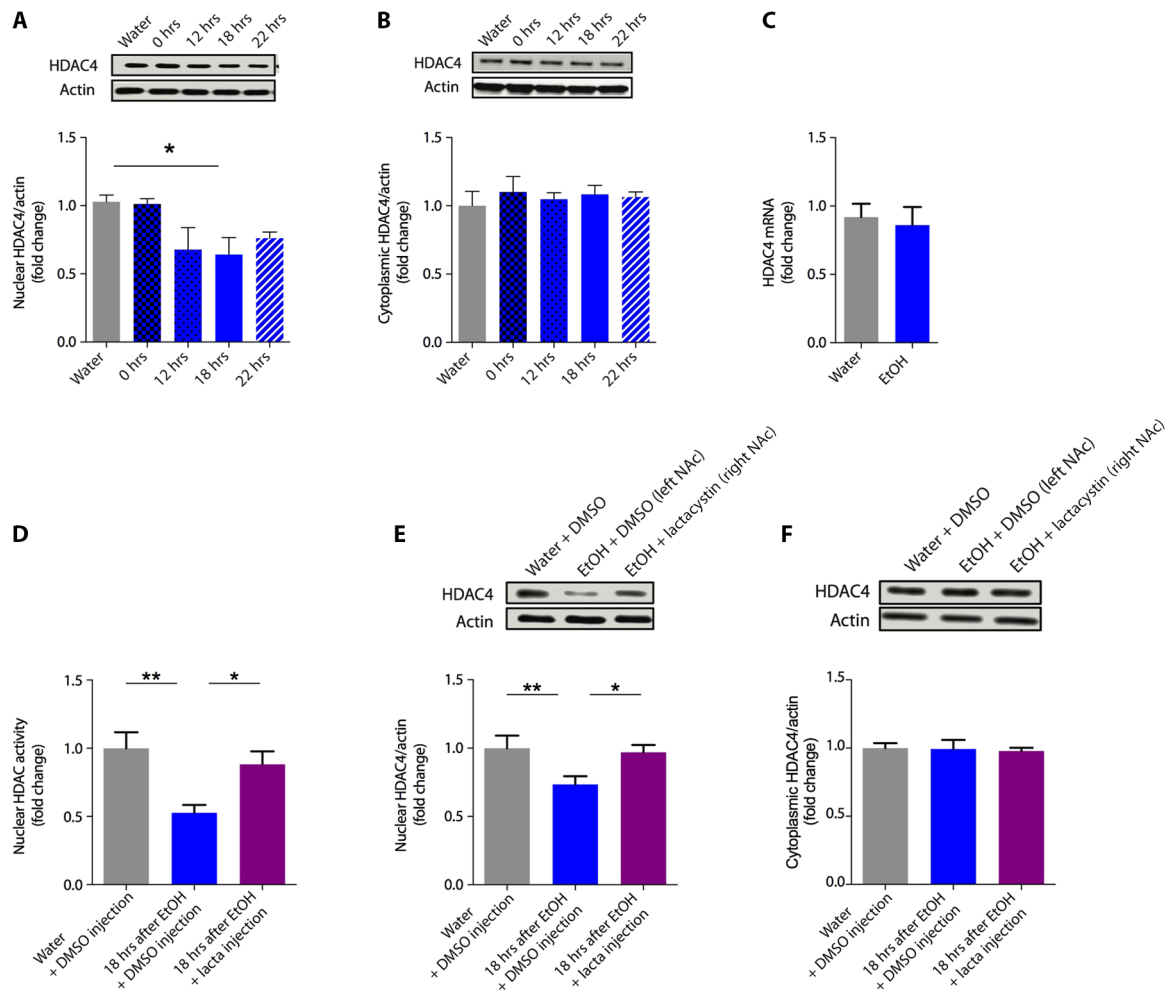
Fig. 4. Alcohol use promotes degradation of nuclear HDAC4 and HDAC5 in the nucleus accumbens. (**A**) Immunoblotting experiment for HDAC4 in the nuclear lysates of nucleus accumbens cells shows that alcohol cessation following 10 days of alcohol use (Fig. 3A) is associated with significantly decreased levels of HDAC4 in the nucleus accumbens 18 hours after the last alcohol ingestion (one-way ANOVA: $F_{4,17} = 4.37$, $P = 0.0130$; Sidak post hoc: $P < 0.05$, alcohol/18-hour cessation versus water control; $n = 4$ to 5 per group). (**B**) Alcohol use is not associated with changes in HDAC4 levels in the cytoplasmic fractions of nucleus accumbens cells (one-way ANOVA: $F_{4,20} = 0.5071$, $P = 0.7310$; $n = 4$ to 5 per group). (**C**) Quantitative real-time PCR (qRT-PCR) analysis for HDAC4 mRNA in nucleus accumbens lysates after 10 days of alcohol use shows no change in HDAC4 mRNA 18 hours after the last alcohol ingestion ($P > 0.05$, alcohol pretreated versus water control; $n = 4$ to 5 per group). (**D**) Intra-nucleus accumbens delivery of the proteasomal inhibitor lactacystin rescues the decrease in HDAC activity observed following alcohol cessation (one-way ANOVA: $F_{2,13} = 7.97$, $P = 0.0055$; Sidak post hoc: $P < 0.01$, water versus alcohol; $P < 0.05$, alcohol versus alcohol + lactacystin; $n = 6$ to 8 per group). (**E**) Intra-nucleus accumbens delivery of the proteasomal inhibitor lactacystin rescues the decrease in nuclear HDAC4 observed following alcohol cessation (one-way ANOVA: $F_{2,13} = 7.97$, $P = 0.0055$; Sidak post hoc: $P < 0.01$, water versus alcohol; $P < 0.05$, alcohol versus alcohol + lactacystin; $n = 6$ to 8 per group). (**F**) Alcohol treatment and lactacystin infusion had no significant effects on HDAC4 levels in the cytoplasm (one-way ANOVA: $F_{4,20} = 0.507$, $P = 0.7310$; $n = 6$ to 8 per group). *$P < 0.05$, **$P < 0.01$. Data are means ± SEM.

HDAC1 and HDAC2 (Fig. 5C). We also observed no changes in the cytoplasmic accumulation of HDAC4 and HDAC5 (Fig. 5D). These findings are consistent with previous studies showing that MC1568-mediated inhibition of class II HDACs is facilitated by the degradation of HDAC4 and HDAC5 in the nucleus (48, 52).

To explore the effect of selective degradation of HDAC4 and HDAC5 on cocaine addiction–like behaviors, we administered MC1568 (0.5 mg/kg), or vehicle, daily for 10 consecutive days before cocaine self-administration (Fig. 5A). Drug treatments were continued concurrently with cocaine self-administration but administered at a different time during the dark cycle (Fig. 5A). Treatment with MC1568 did not increase the daily intake of cocaine during the acquisition or maintenance

phase of the sequential paradigm (fig. S7). We tested motivation for cocaine on a progressive ratio schedule of reinforcement, at three cocaine doses (0.8, 0.4, and 0.2 mg/kg), on days 15, 17, and 19 of cocaine self-administration, respectively. As expected, animals had fewer cumulative lever presses for cocaine with each successive decrease in cocaine concentration (23). Animals treated with the selective class II HDAC inhibitor, however, had significantly higher motivation for cocaine at a unit dose of 0.4 mg/kg and no significant difference in motivation at the lowest unit dose of 0.2 mg/kg (Fig. 5E). We measured persistence of cocaine seeking in the absence of the drug and found that animals exposed to MC1568 had significantly higher persistence than the control group (Fig. 5F).
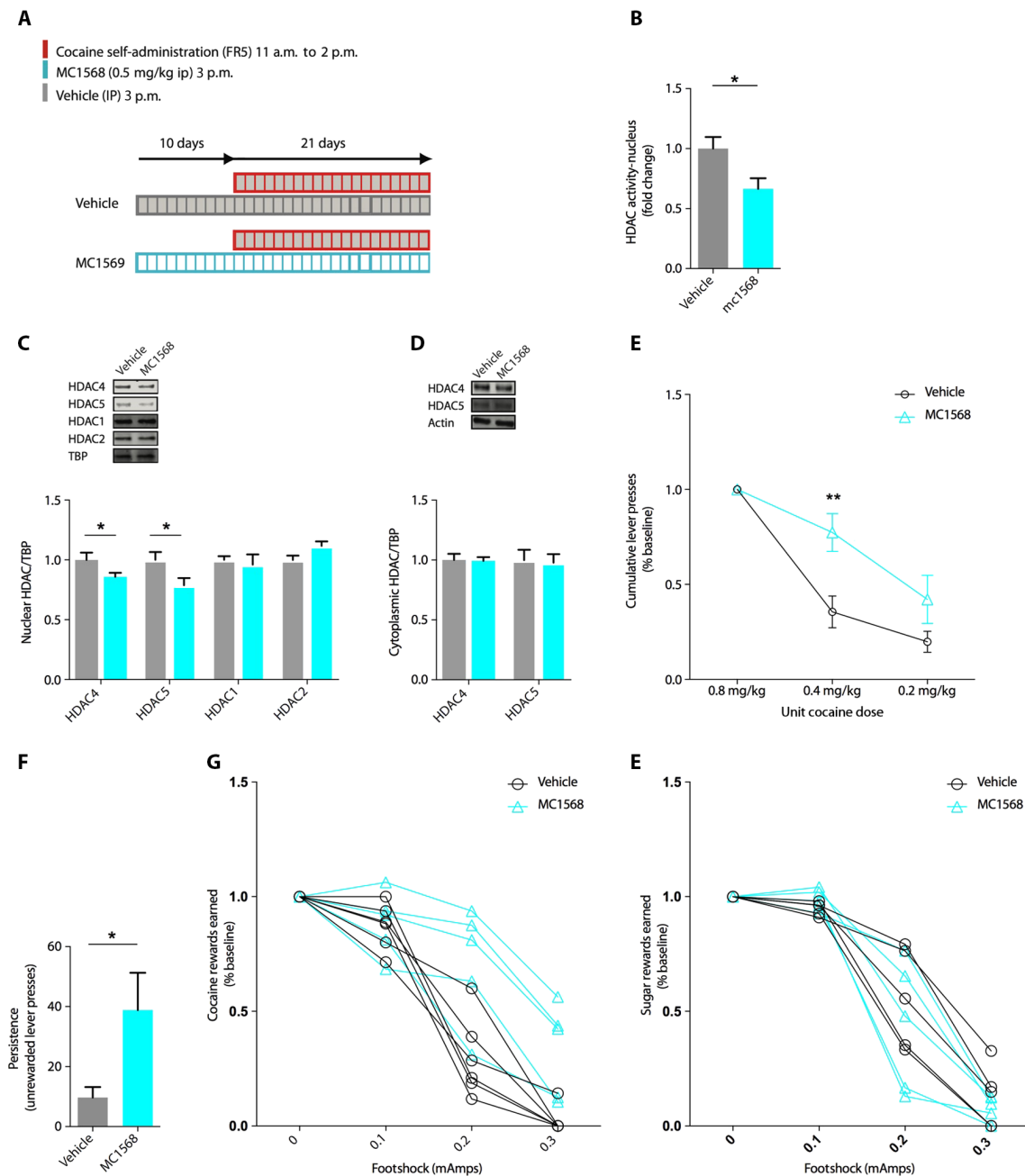
Fig. 5. Selective degradation of HDAC4 and HDAC5 by the class II–specific HDAC inhibitor MC1568 enhances compulsive cocaine self-administration.
(**A**) Treatment paradigm to test molecular and behavioral effects of the class IIa selective HDAC inhibitor MC1568. (**B**) HDAC activity assay of nuclear lysates of nucleus accumbens cells isolated from animals following 10 daily (0.5 mg/kg intraperitoneally) treatments shows 35% decrease in HDAC activity in comparison to control (vehicle-treated) animals ($P < 0.01$ versus vehicle control; $n = 5$ per group). (**C**) Immunoblot for HDAC1, HDAC2, HDAC4, and HDAC5 of nucleus accumbens lysates after 10-day treatment with MC1568 (0.5 mg/kg) shows selective decreases of nuclear accumulation of HDAC4 and HDAC5 ($P < 0.05$), and no significant change in HDAC1 or HDAC2 ($P > 0.5$ versus vehicle control; $n = 7$ to 8 per group). (**D**) MC1568 did not change cytoplasmic accumulation of HDAC4 or HDAC5. (**E**) Animals in the MC1568 treatment group had significantly higher motivation in a progressive ratio schedule of reinforcement at a unit cocaine dose of 0.4 mg/kg (two-way RM ANOVA: Treatment group: $F_{1,10} = 7.33$, $P = 0.022$; Unit cocaine dose: $F_{2,20} = 56.51$, $P < 0.0001$; Interaction: $F_{2,20} = 5.08$, $P = 0.0164$; Tukey post hoc: $P < 0.01$ at 0.4 mg/kg; $n = 5$ to 6 per group), with no significant difference at a lower unit dose of 0.2 mg/kg. (**F**) MC1568 enhances persistence of cocaine seeking during unrewarded time-out sessions ($P < 0.05$, $n = 6$ per group), averaged across 3 days before progressive ratio testing. (**G**) MC1568-treated animals are more compulsive, with significantly higher percentage of rewards earned during 0.2 and 0.3 mA of punishment (two-way RM ANOVA: Group: $F_{1,9} = 7.396$, $P = 0.023$; Footshock: $F_{3,27} = 96.94$, $P < 0.0001$; Footshock × Group interaction: $F_{3,27} = 3.98$, $P = 0.019$; $P < 0.05$ at 0.2 and 0.3 mA; $n = 5$ to 6 per group). The average cocaine infusions at baseline (0.0 mA) did not differ between groups: vehicle, 19.17 ± 1.77; MC1568, 18.80 ± 2.35. (**H**) MC1568 does not alter shock-resistant lever pressing for sugar pellets in food-restricted animals (two-way RM ANOVA: Group $F_{1,10} = 0.2159$, $P = 0.65$, not significant; Footshock $F_{3,30} = 137.6$, $P < 0.0001$; Footshock × Group interaction: $F_{3,30} = 0.3396$, $P = 0.7968$; $n = 6$ per group). Baseline amount of sugar pellet reward (at 0.0 mA) did not differ between groups (AN, 53.40 ± 0.67; AP, 53.20 ± 0.73). TBP, TATA-binding protein; IP, intraperitoneal. *$P < 0.05$, **$P < 0.01$. Data are means ± SEM.

To determine whether selective degradation of class II HDACs enhances compulsivity of cocaine use, we used the variable shock paradigm described in Fig. 1, where we introduced an aversive 2-s footshock to the standard self-administration session, with an increase in shock intensity occurring every 40 min. Under mild aversive condition (0.1 mA of footshock), control and MC1568-treated animals did not differ, earning 83% of baseline reward. However, at 0.2 mA of punishment, the MC1568 group earned almost twice as much reward (65% versus 37% of baseline for drug-treated and vehicle, respectively); at 0.3 mA of footshock, the control animals earned no drug reward, whereas the MC1568 group earned 35% of baseline reward (Fig. 5G). We found that MC1568 did not enhance resistance to punishment in food-restricted animals pressing for sugar pellets (Fig. 5H). These data indicate that MC1568 does not disrupt fear circuits involved in decision-making, and suggest that, like alcohol, the priming effect induced by the selective HDAC inhibitor does not generalize to natural rewards. Together, these findings further substantiate our observation that the degradation of HDAC4 and HDAC5 increases vulnerability to cocaine addiction–like behaviors.

DISCUSSION

Several risk factors for cocaine addiction have been identified. These include endogenous (presumably genetic) factors (such as trait impulsivity and novelty seeking), exogenous (environmental) factors (such as prior use and dependence on nicotine, alcohol, or marijuana), and psychiatric disorders (such as lifetime personality disorder or attention deficit hyperactive disorder). Controlling for multiple predictors, alcohol dependence, together with mood disorder, is second in importance—after cannabis dependence—as predictors of cocaine dependence (2). Although many rodent studies have tested cross-sensitization between different drug classes, the significance of these studies to drug addiction vulnerability is often confounded by behavioral models that focus on the early stages of drug experimentation rather than on addiction. Here, we have used a rodent model of compulsive cocaine use to test, on both a behavioral and molecular level, the epidemiological hypothesis that alcohol acts as a gateway drug to enhance cocaine addiction, and whether the priming effect is unidirectional or bidirectional.

Our data indicate that animals with a history of alcohol use have increased vulnerability to developing key indices of cocaine addiction found in humans: motivation for cocaine and continued cocaine use despite aversive consequence. The reverse is not true: Cocaine use does not enhance preference for alcohol. Our results are not completely in accord with epidemiological evidence that, in humans, prior use of alcohol is a significant risk factor for subsequent use of cocaine, and not only the addictive phenotypes (4). However they are consistent with epidemiological evidence that alcohol use is a significant risk factor for the transition from cocaine use to cocaine dependence (2).

Although the synergistic effects of simultaneous alcohol and cocaine use have been well documented in human (10) and rodent studies (8), our results indicate that alcohol use can enhance the addictive properties of cocaine independently of metabolic, pharmacokinetic, or acute behavioral interaction of the two drugs. Our findings indicate that a prior history of alcohol use is required for the enhancement of cocaine addiction–like behavior, and that priming by alcohol is a metaplastic effect, whereby exposure to this gateway drug initiates intracellular events that alter the epigenome, creating a permissive environment for cocaine-induced learning and memory, thereby enhancing the addictive potential of cocaine. Cocaine can still be addictive without

prior alcohol exposure, although in human populations, as noted in the introduction, very few individuals initiate cocaine use without prior use of alcohol or tobacco.

Moreover, our findings suggest that the two gateway drugs, alcohol and nicotine, act through similar molecular mechanisms to increase vulnerability to cocaine. Both drugs inhibit nuclear HDAC activity, resulting in increased histone acetylation in the nucleus accumbens and the creation of a permissive environment for cocaine-induced ΔFosB expression, and likely other genes. We have now also obtained the first insights into the mechanism of this inhibition: HDAC inhibition and the subsequent histone acetylation are promoted by proteasome-mediated degradation of HDAC4 and HDAC5. The finding that HDAC inhibition does not occur after short-term exposure to alcohol may explain why individuals who drink more rarely and then use cocaine do not go on to meet the criteria for cocaine dependence.

The repressive properties of class II HDACs on drug-related plasticity and behavior have been well described: HDAC5 overexpression in the nucleus accumbens has been found to inhibit cocaine reward in a conditioned place preference paradigm (53), whereas overexpression of HDAC4 in the nucleus accumbens inhibits conditioned place preference and motivation for cocaine self-administration (23, 27). It has been suggested that processes that alter these "brakes" on cocaine-induced gene regulation may tip the balance from recreational to addictive drug use (28). Upon entering the nucleus, HDAC4 forms a multiprotein complex with SMART/n-COR and HDAC3. This complex has been proposed to form a "molecular break pad" on molecular events conferring learning; therefore, removal of essential components of the complex represents a critical step to the formation of persistent memories (54). Here, we present evidence that a history of alcohol use—an environmental risk factor strongly associated with subsequent illicit drug use in the population—increases vulnerability to cocaine addiction–like behavior in rodents by promoting proteasome-mediated degradation of nuclear HDAC4 and HDAC5. It will therefore be of interest to determine whether other drugs, such as marijuana, also act via nuclear HDAC regulation.

HDAC4 acts as a fulcrum between opposing processes in many tissues: differentiation versus apoptosis, and cell growth and motility versus cell cycle arrest (31). Not surprisingly, HDAC4 is heavily regulated by posttranslational modifications. Our finding of markedly different nuclear accumulation profiles of HDAC4 following short-term versus long-term alcohol use is consistent with the central role of HDAC4 as a biological switch for vastly different neuronal outcomes. It will be important to determine which posttranslational modifications and upstream modifying agents (that is, E3 ligases) mediate these changes in the nucleus accumbens.

Our results highlight a key role for HDAC4 and HDAC5 in increasing vulnerability to cocaine addiction. However, because we tested the link between HDAC4 and cocaine compulsivity using systemic administration of the selective class II HDAC inhibitor, we cannot rule out the possibility that brain regions other than the nucleus accumbens may also be affected by MC1568, acting to facilitate the addictive phenotype. Cocaine-induced changes in the dorsal striatum and the prefrontal cortex have both been found to play a key role in the transition to addiction-like behavior (55).

Together, our findings indicate that alcohol consumption increases vulnerability to compulsive cocaine use by promoting proteasome-mediated degradation of HDAC4 and HDAC5 in the nucleus accumbens. This degradation results in increased histone acetylation and creates a permissive epigenetic environment for cocaine-induced gene expression.

MATERIALS AND METHODS
Animals
Sprague-Dawley rats (8 to 12 weeks, weighing 280 to 300 g at the beginning of experiments) (Taconic Laboratories) were used for all studies. Rats were single-housed on a reverse light/dark schedule (10 a.m. lights off, 10 p.m. lights on). All experiments were conducted during the dark cycle and in accordance with institutional guidelines.

Drugs
Cocaine HCl (provided by the National Institute on Drug Abuse) was dissolved in 0.9% NaCl to a concentration of 5 mg/ml and filtered through 0.2-μm filters. Lactacystin (Sigma) was dissolved in 2% DMSO to a final concentration of 200 μM, as described by Massaly *et al.* (*43*). MC1568 (ApexBio) was dissolved in sterile saline/0.3% Tween/5% DMSO.

Alcohol
Animals were acclimated to ad libitum water access in 100-ml graduated feeding tubes (Dyets) for 4 days before initiation of alcohol treatment protocol. Alcohol treatments occurred each day, 3 p.m. to 5 p.m. (approximately 5 hours after the start of the dark cycle). We used a one-bottle, limited alcohol access paradigm, to obtain stable alcohol intake between animals, with fast acquisition of alcohol drinking. In the one-bottle paradigm, animals voluntarily drink from one bottle of 10% alcohol in their home cages, approximately 5 hours into the dark cycle. Animals were water-restricted for 18 to 24 hours before the first day of alcohol exposure to stimulate drinking. In all experiments, an overnight water bottle was left with the animal after the 2-hour alcohol treatment, to be removed the next morning at approximately 10:30 a.m. No liquids of any kind were given to animals between the hours of 10:30 a.m. and 3 p.m. to control for the animals that undergo behavioral studies and do not drink during this time period due to cocaine self-administration (11 a.m. to 2 p.m.).

Sequential paradigm
Serum alcohol, cocaine, and cocaethylene levels in the sequential drug administration paradigm: When taken simultaneously, alcohol and cocaine can have synergistic effects: (i) Alcohol ameliorates the anxiogenic effects of cocaine, resulting in higher quantities of cocaine use during coadministration (*7*, *8*); (ii) alcohol and cocaine form a metabolic by-product in the liver, cocaethylene, which by itself is euphorigenic (*9*, *10*); and (iii) alcohol can inhibit cocaine metabolism, resulting in higher serum cocaine concentrations (*9*, *11*). Access to the two drugs was therefore restricted to different times during the dark cycle, thereby limiting behavioral, metabolic, and pharmacokinetic interaction.

General behavioral methods
Catheter surgery
Under ketamine (75 to 95 mg/kg intraperitoneally) and xylazine (5 mg/kg intraperitoneally) anesthesia, the rat was prepared for surgery by shaving the hair at the surgery site and prepping the skin with a three times alternating scrub of dilute betadine and alcohol. A 2.5-cm incision was made through the skin on the dorsal surface, 0.5 cm posterior to the midscapular level and perpendicular to the rostral-caudal axis of the rat. Another 2.5-cm incision was made ventrally on the area of the neck overlying the right jugular vein parallel to the rostral-caudal axis. The distal end of the catheter (Camcaths) was threaded subcutaneously from the dorsal incision to the ventral incision, and the tip of the catheter was inserted into the right jugular vein and tied with suture. Animals received

carprofen (5 mg/kg) subcutaneously for analgesia at the end of surgery and for 7 days after surgery. Animals received gentamicin (5 mg/kg) subcutaneously for 7 days after surgery. All animals were allowed to recover 5 to 7 days before initiation of cocaine self-administration. The catheter was flushed daily with 0.1 ml of heparinized saline immediately before and after each self-administration session.

Self-administration apparatus
Cocaine self-administration studies were performed in Med Associates Operant Chambers (St. Albans, VT), equipped with two retractable levers (active and inactive) located 8 cm above the floor. A cue light was placed above each lever, and a house light was placed on the back wall. Chambers were also equipped with a metal floor attached to a shock generator. All test chambers were housed in sound-attenuating chambers. The operant chambers were controlled using MED-PC software.

Acquisition of lever pressing
Food restriction. Rats were placed on a food-restricted diet immediately before (1 day) and during lever press training. Rats were fed a restricted amount of food (20 g of chow per day) each day of lever press training. Rats were weighed each day, and their weights were recorded to ensure that no rat drops below 85% of their ad libitum feeding weight. Animals are returned to ad libitum diet after lever press training and before alcohol or cocaine self-administration studies.

Lever press training. Lever pressing was shaped in daily 1-hour sessions (11 a.m. to 12 p.m.), under an FR1, which resulted in the delivery of a sucrose pellet (Dyets) and illumination of a cue light directly over the lever. Sugar pellet delivery resulted in retraction of active and inactive lever, a 20-s time-out period. Animals were trained on the FR1 schedule until they earned more than 50 sugar pellets during the 1-hour session (1 to 3 days).

Acquisition and maintenance of cocaine self-administration
The self-administration session (2.5 hours) was composed of three drug components (40 min each) punctuated by two 15-min time-out (no drug) periods. Drug sessions were signaled by illumination of the house light and insertion of two levers, active and inactive. "No drug" periods were signaled by house light turning off. During the no drug periods, levers remained extended, but lever presses had no consequence. During the drug period, one lever press turned on the cue light located above it and then, 1 s later, switches on the infusion pump. The cue light remains on for a total of 4 s. The infusion volume was 40 μl (2-s infusion) and contained cocaine (0.8 mg/kg). Each infusion was followed by a 20 s time-out period. Training began with FR1 (one lever press results in an infusion of cocaine). Animals remained on FR1 for 2 to 4 days, followed by FR3 (1 to 3 days), and then FR5. Animals were graduated from one fixed ratio to the next if they earned more than 30 infusions per day. Animals were maintained on an FR5 schedule for the duration of the experiment, 7 days per week, and interrupted only for behavioral testing (progressive ratio and compulsivity; Fig. 1A). All animals were given one to two sessions to re-baseline after a testing procedure before proceeding to another test.

Progressive ratio schedule
After 7 days of stable maintenance cocaine self-administration, animals were tested on a progressive ratio schedule of reinforcement. The number of lever presses required for a cocaine infusion was increased after each infusion according to the following progression: 10, 20, 30, 45, 65, 85, 115, 145, 185, 225, 275, 325, 385, 445, 515, 585, 665, 745, 835, 925, 1025, 1125, 1235, 1345, 1465, and 1585. The cumulative number of lever presses that the rat performs before it ceases lever pressing is referred to as the breaking point. The session was ended after 3 hours or when a period of 30 min elapsed after the previously earned infusion.

Persistence of lever pressing in the absence of reward

Persistence of lever pressing during the two 15-min time-out sessions was averaged over the last 3 days of the maintenance period (days 22, 23, and 24 of sequential paradigm).

Resistance to punishment: Cocaine

Resistance to punishment was tested after 20 days of stable cocaine self-administration. This test of compulsivity consisted of four 40-min drug sessions, each separated by a 15-min time-out period. The first 40 min session proceeded as a maintenance FR5 session. During the second 40-min drug session, the first lever press of the FR5 sequence activated a blue warning light that remained on for 60 s. The fourth lever press in the FR5 sequence resulted in a footshock of 0.1 mA for 2 s, whereas the fifth lever press resulted in the delivery of a drug injection (0.4 mg/kg), followed by retraction of levers. After the first lever press, an animal had 60 s to finish the FR5 sequence; failure to do so caused the blue warning light to turn off, and the lever count to reset back to 0. The third and fourth 40-min sessions proceeded as the second, with the intensity of the footshock increasing to 0.2 and 0.3 mA, respectively.

Resistance to punishment: Sucrose pellets

Animals were acclimated to a restricted food schedule (free access to food from 1 p.m. to 3 p.m.) for 3 days before initiation of lever pressing for natural reward. Lever pressing was shaped on an FR1 as described above. Following acquisition of lever pressing, animals underwent maintenance lever pressing for sucrose pellets on an FR1 schedule of reinforcement using a 75-min paradigm composed of three sucrose access intervals, 20 min each, separated by two 7.5-min "time-out" periods. The schedule of reinforcement was increased over 7 days to FR5. Testing for compulsivity occurred similar to cocaine compulsivity tests: The first 20-min session was unpunished, during the second 20-min session, the first lever press of the FR5 sequence activated a blue warning light that remained on for 60 s. The fourth lever press in the FR5 sequence resulted in a footshock of 0.1 mA for 2 s. The third and fourth sucrose access session proceeded as the second, with the intensity of the footshock increasing to 0.2 and 0.3 mA, respectively.

mRNA expression by qRT-PCR
RNA preparation and complementary DNA synthesis

Following water, alcohol, and/or cocaine treatment, animals were sacrificed and brains were flash-frozen in isopentane. Nucleus accumbens punches (1 mm diameter) were obtained, and RNA was extracted using TRIzol reagent (Life Technologies) with the Direct-zol RNA Miniprep, as instructed by the manufacturer (Zymo Research). RNA was treated with deoxyribonuclease I (Invitrogen) before complementary DNA synthesis using the SuperScript III First-Strand Synthesis kit, as instructed (Invitrogen).

Primers

Rat PCR primers were ordered from Sigma. The following primers were used: HDAC5, 5′-TTCTTCAACTCCGTAGCC-3′ (forward) and 5′-TCCCATTGTCGTAGCG-3′ (reverse); HDAC4, 5′-TGAGAGAC-GGAGCAGCCCCC-3′ (forward) and 5′-GGCGCTGCTACATGCG-GAGT-3′ (reverse) (the annealing temperature used was 60°C); and ΔFosB, 5′-AGGCAGAGCTGGAGTCGGAGAT-3′ (forward) and 5′-GCCGAGGACTTGAACTTCACTCG-3′ (reverse). The housekeeping gene used was rat *GAPDH* (forward, 5′-AGGTCGGTGTGAACG-GATTTG-3′; reverse, 5′-TGTAGACCATGTAGTTGAGGTCA-3′). Samples were run in triplicates using SYBR Green Real-Time PCR Master Mix (Bio-Rad) on the Chromo4 Real-Time PCR Detection System (Bio-Rad).

Chromatin immunoprecipitation

ChIP experiments were performed to study histone modifications (HM ChIP) and DNA interactions with HDAC ChIP at the FosB gene using a ChIP assay kit (EMD Millipore). Briefly, for HM ChIP, nucleus accumbens punches were homogenized and cross-linked for 10 min at room temperature in the presence of 1% formaldehyde. The cross-linking reaction was quenched with an excess presence of glycine. Following washes, the cell pellet was resuspended in SDS lysis buffer and sonicated to shear the DNA to approximately 1000 base pairs (bp). The sonicated cell supernatant was diluted, precleared with protein A agarose/salmon sperm DNA beads, and then incubated overnight at 4°C in the presence of 5 μg of an anti-H3K27 acetylation antibody (Active Motif, #39133). The antibody/histone complex was collected with protein A agarose/salmon sperm DNA beads and washed five times according to the assay's protocol. The histone complex was then eluted from the antibody, histone-DNA cross-links were reversed overnight, and the eluate was treated with proteinase K. DNA was recovered using the Chromatin IP DNA Purification Kit (Active Motif), and the samples were analyzed in triplicates using qRT-PCR as previously described. Data were calculated as % input [$100*2^{\wedge}(\text{Adjusted input} - C_t (\text{IP})$] and were then transformed to fold change differences relative to the water group.

The primer pair used was targeting the first intron of the rat FosB gene to capture the region showing elevated H3K27 acetylation levels based on ENCODE's mouse data (forward, 5′-ACGACGACCTT-CAATTCCCC-3′; reverse, 5′-ATTCTTGTCAGGATCCGGCG-3′) (see fig. S4). For the HDAC ChIP analyses, the same procedure was used as before with the following exceptions: Dorsal striatal punches ($n = 8$) were homogenized and dual cross-linked with 5 mM disuccinimidyl glutarate for 1 hour, followed by 1% formaldehyde for 10 min at room temperature. Samples were sonicated to approximately 1000 bp, pooled, and divided equally into four samples that were incubated with 5 μg of either one of the following antibodies: HDAC4 (Santa Cruz Biotechnology, sc-11418 X), HDAC5 (Santa Cruz Biotechnology, sc-133106), rabbit immunoglobulin G (IgG) (Cell Signaling Technology, 27295), and mouse IgG (Santa Cruz Biotechnology, sc-2025). Both HDAC antibodies were first confirmed to work in IP experiments to not cross-react with each other. The eluted DNA was analyzed using qRT-PCR using the same primers that capture the first intron of FosB, which is the region showing elevated H3K27 acetylation levels. Because only the HDAC4 and input samples gave a detectable signal (C_t values for HDAC5 and both IgGs were undetectable), the data were visualized by running the samples on a 1% agarose gel.

Subcellular fractionation and immunoblotting

Nucleus accumbens punches were processed using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific) to extract a cytoplasmic fraction and a nuclear fraction. The protocol was modified to also obtain a separate histone fraction: Following extraction of the cytoplasmic and nuclear fractions, the pellet was resuspended in nuclear extraction reagent and incubated with 300 U of micrococcal nuclease, in the presence of 5 mM $CaCl_2$, for 15 min at 37°C. To confirm efficient subcellular fractionation, Western/immunoblotting experiment was performed for proteins known to be present in all fractions (HDAC4; Santa Cruz Biotechnology, sc-11418), present in the cytoplasm (mitochondrial HSP60; Abcam, ab45134), present in the nucleus and cytoplasm (β-actin; Abcam, ab6276), and present in the histone fraction (histone H3; Abcam, ab10799). The results of the subcellular fractionation experiment are presented in fig. S8. For all Western blotting experiments, equal amounts of nuclear, cytoplasmic, or histone

extracts were run on 4 to 20% gradient TGX precast gels (Bio-Rad), transferred to polyvinylidene difluoride membranes, and immunoblotted with the antibodies of interest: acetyl-H3K27 (Active Motif, #39133), HDAC1 (Abcam, ab7028), HDAC2 (Abcam, ab12169), HDAC4 (Santa Cruz Biotechnology, sc-11418), and HDAC5 (Santa Cruz Biotechnology, sc-133106). Total H3 (Abcam, ab10799) was used as loading control for the histone fractions, and β-actin (Abcam, ab6276) was used as loading control for the nuclear and cytoplasmic fractions.

HDAC activity assays
HDAC activity was measured using the Epigenase HDAC Activity Assay, according to the manufacturer's protocol (EpiGentek).

Immunohistochemistry
Perfusion and brain slicing
Animals were perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS) after being anesthetized with ketamine. Anterior to posterior coronal slices at 40 μm were obtained approximately between bregma 2.7 mm and 1.7 mm in frontal regions of the brain that are within the span of the nucleus accumbens. Brain slices are then suspended in a mixture of 30% glycerol and 30% ethylene glycol in 0.1 M tris buffer at pH 7.4 and stored at −20°C.

Immunohistochemistry
Brain slices were permeated with 1% Tween in PBS and blocked with 0.5% Tween and 10% fetal bovine serum in PBS. Slices were incubated with polyclonal HDAC4 primary antibody (Abcam, ab1437), anti-rabbit Alexa Fluor 647 secondary antibody (Invitrogen), and a nuclear stain Hoechst 33342 (Thermo Fisher Scientific). Slices were mounted on Superfrost Plus Micro Slides (VWR) with Vectashield HardSet mounting medium (Vector Laboratories). The nucleus accumbens regions of these slices were visualized by confocal microscopy. The localization of HDAC4 was categorized as predominantly nuclear or both nuclear and cytoplasmic for each visible in situ neuron under experimenter-blind conditions.

Intracranial surgery and microinfusion
Stereotactic surgery
Animals were anesthetized with a ketamine/xylazine cocktail and head-fixed in a stereotactic device. The incision site was first cleaned with betadine scrub, and ophthalmic ointment was placed on the eyes. An incision was made along the midline to expose the skull. Bilateral holes were drilled above the nucleus accumbens to allow insertion of 26-gauge cannulae 1 mm dorsal to the nucleus accumbens (anterior-posterior, +1.6; medial-lateral, ± 1.2; dorsal-ventral, −6.5). Surgical screws were implanted in the skull to aid the dental cement in securing both cannulae in place. Animals were given carprofen (5 mg/kg) postoperatively and allowed to recover for 3 to 5 days.

Microinfusion
Following the 10-day alcohol regimen, animals received infusions of the proteasome inhibitor lactacystin. Twelve to 15 hours after the last exposure to alcohol, 33-gauge microinjectors were inserted into the guide cannulae with 1 mm projection to deliver either lactacystin (200 μM) or vehicle (2% DMSO in artificial cerebrospinal fluid) to each side of the nucleus accumbens. Pharmacological agents (0.4 μl per hemisphere) were infused at a rate of 0.4 μl/min. Microinjectors were kept in place to allow diffusion of the drug for 10 min before retracting the microinjectors. Six hours later, animals were euthanized and brains were prepared for tissue extraction.

Statistical analyses
For behavioral analyses with more than one time point, group comparisons were performed using RM ANOVA, followed by Tukey or Sidak post hoc tests. For all remaining comparisons, Student's t test was used to compare alcohol-treated groups to the untreated (water) control group. Student's t test was also used to compare the alcohol + DMSO group with the water and the alcohol + lactacystin groups, respectively. Potential outliers were excluded from the statistical analyses. All statistical analyses were performed using GraphPad Prism, and a P value of less than 0.05 was considered significant.

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Science Advances

Prior alcohol use enhances vulnerability to compulsive cocaine self-administration by promoting degradation of HDAC4 and HDAC5

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