Title: Selective boosting of transcriptional and behavioral responses to drugs of abuse by histone deacetylase inhibition

Abbreviated title: HDACi and drugs of addiction interaction

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Abstract

Histone acetylation and other modifications of the chromatin are important regulators of gene expression and, consequently, may contribute to drug-induced behaviors and neuroplasticity. Previous studies have shown that a reduction on histone deacetylase (HDAC) activity results on the enhancement of some psychostimulant-induced behaviors. In the present study, we extend those seminal findings by showing that the administration of the HDAC inhibitor sodium butyrate enhances morphine-induced locomotor sensitization and conditioned place preference. In contrast, this compound has no effects on the development of morphine tolerance and dependence. Similar effects were observed for cocaine and ethanol-induced behaviors. These behavioral changes were accompanied by a selective boosting of a component of the transcriptional program activated by chronic morphine administration that included circadian clock genes and other genes relevant in addictive behavior. Our results support an specific role for histone acetylation and the epigenetic modulation of transcription at a reduced number of biologically relevant *loci* on non-homeostatic, long lasting, drug-induced behavioral plasticity.

Introduction

Behavioral changes observed after chronic exposure to drugs of abuse, such as tolerance, dependence and addictive behavior, appear or are maintained long after the drugs have been cleared from the organism and cannot be accounted by acute effects of the interaction of drugs with their primary molecular targets. Although these clinically relevant phenomena notably differ in their temporal persistence, all of them arise from the ability of drugs to promote persistent structural and functional changes in the central nervous system (Chao and Nestler, 2004). These phenomena are usually referred as "drug-induced neuroplasticity" and depend on changes in gene expression (McClung and Nestler, 2008).

There is a growing interest in the possible functional consequences of covalent modifications of the chromatin in the appearance and maintenance of behavioral changes (Sweatt, 2009), including the development and manifestation of addictive behavior (Renthal and Nestler, 2008b; Tsankova *et al*, 2007). Changes in the structure of the chromatin could underlay long-lasting changes on neuronal gene expression and ultimately contribute to explain the persistence of addictive behavior. Recent studies have provided initial support to this hypothesis. For example, the administration of psychostimulants, such as cocaine, at dosages that promote conditioned place preference (CPP) or locomotor sensitization result in histone hyperacetylation at specific *loci* relevant in the development of addictive behavior (Kumar *et al*, 2005; Levine *et al*, 2005; Renthal *et al*, 2008a; Renthal *et al*, 2007) and perhaps also at the bulk chromatin level (Kalda *et al*, 2007). Furthermore, genetic studies have established a functional role for histone acetyltransferase (HAT) (Levine *et al*, 2005) and histone deacetylase (HDAC) (Renthal *et al*, 2007) activities in the mechanisms of action of psychostimulants, a view also supported by pharmacological experiments with HDAC

inhibitors (HDACi) (Kalda *et al*, 2007; Kumar *et al*, 2005; Renthal *et al*, 2007).

Because most of the research conducted in this area has focused on psychostimulants such as cocaine or amphetamine, here we explore the effect of HDAC inhibition on drugs of abuse belonging to three different pharmacological families: cocaine, ethanol and morphine. We found that the HDACi sodium butyrate selectively enhanced some, but not all, behavioral responses to chronic administration of these drugs. We also extended previous studies by performing a detailed biochemical and gene profiling analysis of striatal tissue, which showed that morphine, as cocaine, induces striatal histone H3 phosphorylation, and indicated that the effects on behavior are not associated to global changes in gene expression or chromatin acetylation, but to the specific modulation of relevant *loci* and genetic programs.

Methods and Materials

Subjects

Male Swiss-Albino mice, 6-8 weeks old, were purchased from Janvier España, S.A. (Madrid, Spain). Subjects housing, care and experimental manipulation followed the national guidelines and approved by the Institutional Animal Care and Use Committees.

Behavioral procedures

Sensitization. Locomotor sensitization induced by ethanol, cocaine and morphine was evaluated using a protocol divided into two phases: Induction and challenge. The induction phase involved six trials on alternate days, one trial per day. On each one of these trials mice received an injection of saline or sodium butyrate (100, 150 or 300 mg/kg) followed by a second injection of ethanol (2.5 g/kg), morphine (20 mg/kg) or cocaine (10 mg/kg). Based on the results of previous studies (Kumar et al., 2005) both treatments were separated by a 20 minutes delay in the case of ethanol or cocaine

experiments. However, attending to the need of using a longer period in morphine related experiments (see below), sodium butyrate was simultaneously administered with morphine. Immediately after this second injection mice were placed in open-field chambers, consisting of glass cylinders of 25 cm in diameter, and locomotion was registered by a computerized video-tracking system (SMART; Panlab SL; Spain). The duration of these testing sessions was restricted to 20 minutes in the case of ethanol and cocaine, whereas it was prolonged to 60 minutes in the case of morphine. On the other hand, the challenge phase consisted of a single trial conducted 7 days after the last test of the induction phase. In this case, all animals received a single injection of ethanol (2.5 g/kg), cocaine (10 mg/ kg) or morphine (20 mg/kg) and locomotion was assessed as in the treatment phase. *Conditioned place preference (CPP).* Morphine-induced CPP was assessed using four black acrylic chambers (30 x 15 x 20 cm). Tactile cues (interchangeable grid and hole floors) were used as conditioned stimuli. The behavioral procedure was divided into three consecutive phases. First, initial preference was assessed in three successive daily tests by placing each animal in the CPP chambers (floor divided with half grid, half holes) for 15 min. The individual scores of the third test were used to match two groups that did not differ according to their initial preference for any of both floors. The second phase of this procedure consisted of six trials (20 min duration; one trial per day), corresponding to three morphine/CS+ pairings and three saline/CS- pairings. In each one of the 6 conditioning sessions, mice received an injection of saline or sodium butyrate (150 mg/kg) twenty minutes before of receiving the corresponding injection of morphine (20 mg/kg) or saline. Immediately after this second injection mice were confined into the conditioning chamber prepared with the corresponding CS+ or CS- floor for 20 minutes. The third phase consisted of a single test (duration: 15 min) conducted the day after the last conditioning session. In

this case the floor of the conditioning chambers was divided (half grid, half holes) and the time spent in each floor was assessed by a video-tracking system (SMART, Panlab SL, Spain). *Tolerance.* The development of tolerance to the analgesic effects of morphine was assessed using the tail-flick procedure using an automated analgesiometer (Ugo Basile, Italy). The procedure had three main phases: First, drug-naïve mice were first tested to establish their individual pain thresholds in a single assay (infrared intensity: 50). Accordingly to these initial values mice were matched in two pre-treatment (saline vs. sodium butyrate) groups. In the second phase, that started twenty-four hours later, mice received an acute saline or sodium butyrate (300 mg/ kg; i.p.) injection and, 20 minutes later, all mice received a saline injection. The latency to withdraw the tail from the heat focus was evaluated in a single assay performed 10 minutes after the saline injection. Finally, the third phase of this experiment addressed the analgesic effects of morphine and the development of tolerance to this effect. This phase consisted of 6 trials. (one trial per day, on consecutive days). Each trial was identical to that described for phase 2, but mice were treated with morphine (5 mg/kg; i.p.) instead of saline 10 min before of assessing the latency to flick the tail. The development of tolerance to the motor impairing effects of ethanol (2.5 g/kg) was assessed using a commercial rota-rod for mice (rota-rod 9756;Ugo Basile, Italy). First, ethanol-naïve mice were trained in the rota-rod under a constant acceleration schedule (3.75-37.5 rpm over 5 min) till they were able to stay in the rod for 120s for two of three consecutive trials. All mice learned this ability after two training sessions (5 trials per session) conducted in consecutive days. Twenty-four hours after this training phase, mice were randomly assigned to a pre-treatment group and received an acute saline or sodium butyrate (300 mg/ kg) injection. Twenty minutes after this treatment, mice received a saline injection and 10 min later were tested once in the rota-rod under the

same schedule used in the training phase. This test was conducted to assess a possible effect of sodium butyrate in motor coordination *per se*. The third phase of this experiment addressed the motor impairing effects of ethanol and the development of tolerance to this effect and consisted of 6 trials (one trial per day, on consecutive days). These trials were identical to that previously described but ethanol (2.5 g/kg) instead of saline was administered to saline or sodium butyrate pretreated mice. *Dependence/withdrawal.* Opioid dependence was induced by repeated injections of morphine based on the procedure described by (Maldonado *et al*, 1997). Mice received morphine injections twice a day (9 AM and 9PM) with progressively increasing doses (20, 40, 60, 80 and 100 mg/kg, i.p.) in their home cages for 5 consecutive days. Saline or sodium butyrate (150 mg/kg) was intraperiotneally injected immediately before each morphine injection. On the 6^{th} day, mice were pre-treated with saline or sodium butyrate followed by a last morphine (100 mg/kg, i.p.; 9 AM) injection. Ninety minutes later mice were individually placed in cubic metacrylate boxes (40 x 40 x 40 cm). Fifteen minutes later, morphine withdrawal was precipitated by a subcutaneous naloxone injection (1mg/kg) and mice behavior was videotaped for 15 additional mice. Several signs of the morphine withdrawal syndrome were evaluated by an observer blind to treatment conditions. Ethanol dependence was induced by repeated injection of ethanol accordingly to a self-developed experimental protocol adapted from (Gililland and Finn, 2007) using the reduction of locomotion as an index of ethanol withdrawal intensity (Kliethermes, 2005). Our experimental procedure had two main phases, namely, dependence induction and withdrawal assessment. In the first one, four separate groups of mice received in their home cages an injection of saline or sodium butyrate (150 mg/ kg; IP) immediately followed by an ethanol (4 g/kg; IP) or saline challenge. This treatment was repeated twice a day (9 AM and 9PM) for 5 consecutive days. The

second phase consisted in a single test session conducted 15 hours after the last saline/ ethanol injection. In this test, mice locomotion was assessed during 20 in an open field and following the same experimental conditions described above.

Western blotting and immunohistochemistry

Mice striatal tissue was rapidly dissected from anterior 6mm coronal sections using a chilled acrylic mouse brain slicer matrix (Zivic Instruments) with 2 mm coronal section slice intervals, frozen in dry ice and stored at -70ºC until further protein extraction. Western blot and immunohistochemistry analyses were performed as previously described (Lopez de Armentia *et al*, 2007). See supplementary methods for additional details.

Microarray and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses

For microarray analysis, striatal tissue was dissected as described above and placed on RNAlater solution (Qiagen, Venlo, The Netherlands). Equal amounts of total RNA from four animals were pooled, processed and hybridized to Mouse Gene 1.0 ST genechips (Affymetrix, Santa Clara, CA). Three to six biological replicates were prepared for each experimental condition (saline-saline, N=6; saline-morphine, N=3; butyrate-morphine, N=3). Microarray data were processed, normalized and statistically analyzed using GeneSpring GX. This dataset will be accessible at the GEO database upon manuscript acceptance. For qRT-PCR, cDNA was prepared from eight independent mice per group. Real-time quantitative PCR was performed using ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) and amplified using SYBR GreenER qPCR reagent mix (Invitrogen, Carlsbad, CA). Each sample was assayed in duplicate and normalized using GAPDH levels. Primer sequences for qRT-PCR amplification were

designed using Primer Express® Software v2.0 (Applied Biosystems) and are available on request.

Statistics

All data are depicted as mean ± SEM. Most data were analyzed using ANOVAs, followed by Tuckey HSD posthoc tests because it does not requires a significant interaction between factors and it is highly conservative against type I error (Wilcox et al., 1980). Experimental designs involving the comparison of multiple dependent variables were analyzed using between-groups MANOVA. Comparisons involving only two means were compared by means of Student's t test for independent samples. For clarity, details of statistical comparisons that did not yield significant ($p<0.05$) differences are not fully described in the results section.

Results

Sodium butyrate administration enhances cocaine-induced locomotor sensitization.

To confirm and extend previous findings on the ability of HDACi to enhance psychostimulants-induced behaviors, we assessed the effects of sodium butyrate on cocaine-induced locomotor sensitization. Our experimental protocol was divided in two phases: induction and challenge (see figure 1A for details). Mice were divided into eight groups and injected either with saline or cocaine (10 mg/kg, ip) in the presence or absence of sodium butyrate pretreatment (100, 150 or 300 mg/kg, ip). The administration of sodium butyrate enhanced the development of cocaine-induced locomotor sensitization without affecting locomotion in saline treated mice (figure 1B). A three-way ANOVA (pretreatment x treatment x days) revealed that the (cocaine) treatment as well as the days factors reached statistical significance ($F_{1,85}$= 144.08, $p<0.001$; $F_{5,425}$ = 9.16, $p<0.001$) whereas the pretreatment (sodium butyrate) factor did

not. Further, all bifactorial ($F_{3,85}$= 3.37, $p<0.05$; $F_{5,425}$= 10.73, $P<0.001$; $F_{15,425}$=2.56, $p<0.001$) as well as the three way interaction ($F_{15,425}$=1.99, $p<0.05$) yielded a significant effect. Tuckey HSD posthoc comparisons revealed that repeated cocaine injections in saline pretreated mice lead to a progressive enhancement of locomotion, confirming the development of locomotor sensitization ($p<0.05$). This behavior was boosted in mice pre-treated with sodium butyrate. Notably, the effect of sodium butyrate was very persistent. A challenge test conducted 7 days later, in which the same eight groups of mice received cocaine (10 mg/kg, ip), but not sodium butyrate, revealed that the mice that had received moderate (150 mg/kg) or high (300 mg/kg) doses of sodium butyrate concurrently with cocaine during the treatment phase exhibited higher locomotion in the challenge test than those that were pretreated with saline (figure 1C). Two-way ANOVA (pretreatment x treatment) comparing the locomotor scores in this test revealed a significant effect for both factors ($F_{3,85}$= 3.57, $p<0.05$; and $F_{1,85}$= 56.84, $p<0.001$, respectively) as well as for their interaction ($F_{3,85}$= 3.63, $p<0.05$), whereas posthoc comparisons showed significant increases for both sodium butyrate concentrations ($p<0.01$). As expected, regardless of their pre-treatment conditions, all the mice that did not receive cocaine during the sensitization induction phase showed in the challenge phase enhanced locomotion in response to acute cocaine administration (figure 1C, compare white bars to dashed line).

Sodium butyrate administration enhances ethanol-induced locomotor sensitization but not ethanol tolerance or withdrawal

We next investigated the effects of sodium butyrate in ethanol-induced behaviors. First, we evaluated the effects of this HDACi on ethanol (2.5 g/ kg, ip)-induced sensitization using the same experimental scheme previously described for cocaine (figure 2A). As

depicted in figure 2B, we observed that sodium butyrate enhanced ethanol-induced locomotor sensitization. A three way ANOVA (pretreatment x treatment x days) revealed a significant effect of the three main factors ($F_{3,106}$= 4.39, $p<0.001$; $F_{1,106}$= 162.98, $p<0.001$; $F_{5,503}$= 19.16, $p<0.001$, respectively). Two bi-factorial interactions reached statistical significance (pretreatment x treatment: $F_{3,106}$= 6.21, $p<0.001$; treatment x days: $F_{5,530}$= 29.43, $p<0.001$). Posthoc comparisons revealed that all doses of sodium butyrate significantly enhanced ethanol-induced locomotion as compared to the saline pretreated group (100 mg/ kg: $p<0.05$, 150 mg/ kg: $p<0.001$; 300 mg/ kg: $p<0.001$, respectively). The same sodium butyrate doses did not affect the daily scores of saline treated mice (figure 2C). Again, these differences between groups were persistent and were expressed in a challenge test conducted 7 days later at which all mice received a single ethanol injection (figure 2C). A two way ANOVA (pretreatment x treatment) comparing the locomotor scores on this challenge test revealed that both main factors as well as their interaction reached statistical significance ($F_{3, 92}$= 3.51, $p<0.05$; $F_{1, 92}$= 86.17, $p<0.001$; $F_{3, 92}$= 5.60, $p<0.01$; respectively). Thus, mice that had received sodium butyrate and ethanol during the sensitization induction phase exhibited higher locomotion than the group that received saline as a co-adjuvant treatment of ethanol ($p<0.01$ in all cases). All mice that had been treated with saline during the induction phase equally reacted to an acute ethanol administration.

In addition to locomotor sensitization, the repeated administration of ethanol can cause other relevant behavioral adaptations, such as tolerance and withdrawal symptoms. We extended our study to these other behavioral effects of ethanol and found that sodium butyrate co-administration did not affect the development of tolerance to the motor incoordinating effects of ethanol (figure 2D). Similarly, we also observed that the co-administration of this iHDAC during the induction of alcohol

dependence did not affect the hypolocomotion associated to ethanol withdrawal (figure 2E). These effects were not caused by changes in ethanol pharmacokinetics (supplementary table 1).

Sodium butyrate administration enhances morphine-induced sensitization and CPP, but not tolerance or withdrawal.

To extend and confirm this intriguing dissociation on the effects of HDACis on different drug-induced behavioral phenomena, we assessed the effect of sodium butyrate on several behavioral effects of chronic morphine administration. First, we evaluated the effects of this HDACi on morphine-induced sensitization using the same protocol described for cocaine and ethanol (figure 3A). A three-way ANOVA (pretreatment x treatment x days) revealed a significant effect of the three main factors ($F_{3,87}$= 12.12, p<0.001; $F_{1,87}$= 36.66, p<0.001 and $F_{5, 435}$= 41.01, p<0.001, respectively). The dyadic interactions treatment x days ($F_{5,435}$= 47.22, p<0.001) and pre-treatment x treatment ($F_{3,87}$= 10.99, p<0.001) reached statistical significance. Tuckey HSD posthoc comparisons revealed that all mice treated with morphine displayed a significant increase in locomotion across days, and this effect was significantly boosted in mice pre-treated with moderate (150 mg/kg, p<0.05) or high doses of sodium butyrate (300 mg/kg, p<0.01). The same doses of sodium butyrate did not affect locomotion of saline-treated mice. As observed for cocaine- and ethanol- treated mice, the co-administration of sodium butyrate (150 or 300 mg/kg) selectively enhanced the development of morphine-induced locomotor sensitization (figure 3B). Notably, as observed for cocaine- and ethanol- treated mice, the effects of sodium butyrate persisted and were still apparent in a challenge test conducted 7 days after the last sodium butyrate injection. As in previous experiments, in the challenge phase all mice received

morphine, but not sodium butyrate. We found that those mice that had concurrently received intermediate or high doses of sodium butyrate and morphine during the induction phase exhibited higher locomotion than the group that had received saline as the co-adjuvant of morphine (figure 3C; $p<0.01$).

We also compared the interaction between a sodium butyrate dose (300 mg/kg) and two doses of morphine (10 and 20 mg/kg) that differed in their ability to promote locomotor sensitization under the treatment conditions described above (figure 3D). A three way ANOVA (pre-treatment x morphine dose x days) revealed a significant effect of all three main factors ($F_{1,20}=6.05$, $p<0.05$; $F_{1,20}=56.62$, $p<0.001$; $F_{5,100}=11.01$, $p<0.001$; respectively) as well as the bifactorial interactions pretreatment x morphine dose ($F_{1,20}=6.20$, $p<0.05$) and pretreatment x days ($F_{5,100}=3.72$; $p<0.001$). Follow up Tuckey HSD comparisons revealed that the administration of sodium butyrate did not affect the locomotor scores observed after repeated injections of a low dose of morphine (10 mg/ kg) but it significantly boosted the acute and chronic effects of a higher dose (20 mg/kg, $p<0.01$). We further analyzed these effects of sodium butyrate by calculating the linear regression equations describing the dynamic changes in morphine-induced locomotion across the different days of the induction phase. The equations corresponding to the regression lines describing the changes of morphine-induced locomotion over time in each group were: SM10= 9283.4 + 774.69x, $r^2=0.953$; BM10= 9092.0 + 825.47x, $r^2=0.683$); SM20= 20101.4 + 2284.9x, $r^2=0.941$; BM20= 32289.4 +3280.3x, $r^2=0.857$). These results indicated that the intercept and the slope values of the regression line of the two groups treated with a low dose of morphine unable to trigger locomotor sensitization were almost identical and independent of the pretreatment conditions. Conversely, in mice receiving a higher morphine dose, both the intercept and the slope values of the regression line corresponding to the group of mice

co-treated with sodium butyrate were larger than in the group co-treated with saline. These results indicate that sodium butyrate did not only enhanced morphine locomotion but also exerted a positive modulation of the mechanisms leading to the sensitization of this response with repeated morphine administration. Taken together these data support an interactive effect rather than a merely additive effect of sodium butyrate and morphine in locomotor sensitization.

We also examined other addiction-related behaviors and found that sodium butyrate enhanced morphine-induced CPP (figure 3E, $t_{23}= 2.30$, $p< 0.05$). In contrast, but in close parallelism to the results obtained in experiments involving repeated ethanol administration, the same doses of this HDACi did not affect the development of tolerance to the analgesic effects of morphine (figure 3F) nor the intensity of morphine withdrawal (figure 3G).

Acute administration of drugs of abuse does not modify bulk chromatin acetylation in the striatum, but triggers histone H3 phosphorylation

We assessed the ability of the drugs morphine, ethanol and cocaine, at the concentrations used in our behavioral experiments (respectively, 20 mg/kg, 2.5 g/kg and 10 mg/kg), to induce changes in the acetylation state of bulk chromatin in striatal neurons. To this end, we assessed acetylation of the four nucleosome histones, H2A, H2B, H3 and H4, as well as phosphorylation of histone H3. Although the administration of sodium butyrate increased bulk histone aceylation in a dose dependent manner (figure S1), we found that neither one of the three drugs had a significant effect on bulk histone acetylation levels. We, however, confirmed that cocaine administration increased phosphorylation of histone H3 at Ser10 ($p<0.05$, figure 4A) (Brami-Cherrier *et al*, 2005; Kumar *et al*, 2005; Stipanovich *et al*, 2008). This increase in H3

phosphorylation was probably also responsible of the increase in H3 phosphoacetylation observed after cocaine administration (Brami-Cherrier *et al*, 2005; Kumar *et al*, 2005; Stipanovich *et al*, 2008). Similar results (figure 4B) were obtained in response to higher doses of morphine (60 mg/kg), ethanol (6 g/kg i.p.) and cocaine (40 mg/kg i.p.). To confirm these results and, at the same time, gain spatial resolution and sensitivity in our assays, we performed immunohistochemistry experiments in brain sections of animals treated with high doses of morphine, cocaine and ethanol using antibodies against the same histone modifications. As observed in the western blot analyses of striatal extracts, we could not detect significant changes in histone acetylation for neither one of the four nucleosome histones (figure S2), but we found striking differences in the abitity of drugs to enhance histone H3 phosphorylation (figure 4C and figure S3). Thus, whereas phospho-H3 antibody in brain sections of control mice only labeled proliferating cells in the subventricular zone (figure S3), the injection of cocaine caused, as previously reported (Brami-Cherrier *et al*, 2005), a robust increase in histone phosphorylation in broad regions of the dorsal striatum, nucleus accumbens and olfactory tubercle (supplementary table 2). Interestingly, we found that morphine increased histone H3 phosphorylation in the same brain regions than cocaine, although the magnitude and number of cells affected was lower, which may explain the absence of significant changes in response to morphine in our western blot analysis. Ethanol injection did not cause any obvious change in striatal histone H3 phosphorylation.

We also explored the interaction at the level of histone acetylation between sodium butyrate and acute administration of cocaine (10mg/kg), ethanol (2.5 g/kg) or morphine (20 mg/kg). Sodium butyrate at the dose used in our behavioral analyses did not cause a significant increase in histone acetylation, neither alone nor in combination with either one of these drugs (figure S4A). In the case of morphine, we also examined

whether changes in bulk chromatin acetylation became evident after chronic drug administration. For this purpose, mice received the same dose and treatment previously described for behavioral sensitization and the tissue was collected 48h after the last morphine injection. Repeated morphine injection did not cause changes in the bulk level of acetylation of neither one of the four nucleosome histones or in the phosphorylation of histone H3 as examined by western blot analysis (figure S4B). The lack of significant changes in bulk chromatin acetylation after drug administration does not discard that more subtle changes could take place at specific genomic *loci* or restricted neuronal populations.

Previous co-administration of sodium butyrate alters the transcriptional response to morphine re-exposure

To further investigate the molecular bases of sodium butyrate action on long-lasting behavioral responses to morphine, we screened for potential substrates of their interaction by performing a genome-wide comparison of the striatal transcriptome after chronic administration of morphine in the absence or presence of sodium butyrate. To this end, additional groups of mice underwent the same protocol described in the sensitization experiments (figure 5A). Striatal RNA was extracted 1 h after the morphine challenge in groups of four animals that received either saline (N=6), or morphine (N=3), or the sodium butyrate-morphine co-treatment (N=3) during the sensitization induction phase. One-way ANOVA of microarray data identified 240 differentially expressed probe sets, corresponding to 197 identified genes (figure 5B and supplementary table 3). Most of these genes (80%) were upregulated in response to the morphine challenge including both genes previously identified as important in neuroplasticity, such as *Arc* and *Nfkbia*, and novel genes that may be highly relevant in

additive behavior, such as *Ttr* and *Kcnj13*, which showed the largest response to chronic morphine administration in our study (figure S5). The list of genes altered in the mice that received the morphine-sodium butyrate co-treatment (BM group) was very similar to that obtained for the mice that only received morphine (SM group). Interestingly, in 105 genes (53% of those identified in the ANOVA), the sodium butyrate pretreatment led to an increase in expression when compared to animals treated with morphine alone. This upregulation was significant for 26 genes (13%), which represent interesting candidates to mediate HDACi effects, only 14 genes (7%) showed the opposite behavior. Of note is the presence, among the genes with an increased transcriptional response to morphine, of several circadian clock genes, namely *Per1*, *Rev-erbα* and *Cry1*, whose expression was consistently and significantly affected by the HDACi pretreatment, indicating that the previously described transcriptional feedback loop involving these genes was enhanced (figure 5C). Other genes highly relevant in the context of drug addiction, such as *c-fos, nr4a1, Zbtb16* and *fosB*, also showed the same trend toward enhanced upregulation by co-administration of sodium butyrate and morphine. We validated relevant changes by qRT-PCR (figures 5D-E), confirming the boosting effect of sodium butyrate on a component of the transcriptional response to morphine.

Discussion

Four important conclusions can be drawn from this study. First, the HDAC inhibitor sodium butyrate enhances some behavioral responses to drugs of abuse, such as locomotor sensitization or conditioned place preference. This interaction was observed for drugs of abuse of different pharmacological families, including psychostimulants, opioids and ethanol. Second, the modulatory effects of histone acetylation cannot be

generalized to all the behavioral effects derived from chronic exposure to these drugs of abuse (e.g. tolerance and dependence). Third, we show that morphine, as previously demonstrated for cocaine, induces the phosphorylation of histone H3 in a restricted population of striatal neurons. In contrast, we could not detect significant changes in the acetylation state of bulk chromatin in striatal neurons triggered by either drug. Fourth, gene profiling analysis indicates that the interaction between morphine and HDACi first revealed at the behavioral level had a clear transcriptional correlate on specific *loci* highly relevant in neuroplasticity and addiction.

More specifically, our data suggest that HAT/HDAC activities have a prominent and selective role on the development of long-lasting behavioral effects, such as CPP and sensitization. These effects arise from non-homeostatic neuroplastic responses to drugs of abuse and are considered highly relevant in the development and maintenance of addictive behavior. Conversely, acetylation-related processes seem less relevant in the development of drug tolerance and dependence (as measured by the intensity of drug withdrawal), which result from transient homeostatic adaptations occurring within the cells and circuits directly stimulated by each drug and are not considered core symptoms of addiction (Hyman *et al*, 2006). This view is in agreement with previous pharmacological and genetics studies on psychostimulants-induced sensitization and CPP (Bilbao *et al*, 2008; Kalda *et al*, 2007; Kumar *et al*, 2005; Levine *et al*, 2005; Renthal *et al*, 2007), as well as with the few preceding studies indicating that HDACis do not facilitate the development of tolerance (Wang *et al*, 2007) and that histone acetylation might be more involved in the expression than in the induction of drug dependence (Pandey *et al*, 2008). Future studies should further explore this intriguing dissociation.

This study also provides the most comprehensive analysis to date of posttranslational modifications of histones in the chromatin of striatal neurons in response to drugs of abuse. Previous analyses on histone acetylation in response to cocaine (Brami-Cherrier *et al*, 2007; Brami-Cherrier *et al*, 2005; Cassel *et al*, 2006) or ethanol (Kim and Shukla, 2006; Pandey *et al*, 2008) have produced somewhat conflicting results. Despite the dose-dependent hyperacetylation observed in response to sodium butyrate, our analysis failed to reveal significant changes in bulk chromatin acetylation after acute administration of cocaine, ethanol or morphine to saline or sodium butyrate pretreated mice. These negative findings might result of the insufficient sensitivity of the techniques used here (i.e. western blot and immunohistochemistry) to detect small increases over already high basal levels of histone acetylation, or more likely, of the unsuitability of these techniques to reveal subtle modifications of the chromatin that are presumably restricted to the promoters of specific genes in the nuclei of particular neuronal ensembles that are part of a large and cellularly-heterogeneous brain structure like the striatum. On the other hand, in agreement with previous studies (Brami-Cherrier *et al*, 2005; Kumar *et al*, 2005; Stipanovich *et al*, 2008), we found that cocaine administration caused the phosphorilation and/or phosphoacetylation of histone H3. The identification of these changes using western blot and immunohistochemical procedures was probably favored by the very low basal level of H3 phosphorylation and/or phosphoacetylation in saline-treated mice. Further, our results also present the first evidence indicating that the activation of this signaling pathway is not exclusive of cocaine, since morphine administration also increased H3 phosphorylation in several brain areas although to a lower extent than cocaine. In contrast, ethanol administration did not result in any change on bulk histone acetylation/phosphorylation identifiable by western blot or immunistochemical procedures. Ethanol could still promote changes in

histone H3 phosphorylation that are below the sensitivity of our assays. Alternatively, it is also possible that ethanol does not activate the signaling pathway that leads to H3 phosphorylation, but can still interact with sodium butyrate.

We also observed that HDACi and morphine interact on the regulation on the transcription of several genes. Thus, our microarray analysis, in addition to confirm a number of transcriptional targets of morphine (Korostynski *et al*, 2007; McClung *et al*, 2005) and reveal novel ones, provided a short list of candidate genes to play a role in HDACi-mediated enhancement of non homeostatic behavioral responses to morphine. Particularly remarkable is the case of circadian clock genes. In this regard, we observed an enhanced upregulation of *Per1* in mice that received the sodium butyrate as coadjuvant treatment of repeated morphine injections. Although we did not assess chromatin acetylation at this specific locus, it has been shown that both HDACi (Naruse *et al*, 2004) and cocaine (Renthal et al., 2009) can induce histone acetylation at the *Per1* promoter and increase its transcription. Interestingly, the enhanced expression of *Per1* in mice co-treated with sodium butyrate and morphine was associated to increased downregulation of *Cry1* and upregulation of *Rev-erbα* (figure 5C). These changes are in good agreement with current models indicating that *Rev-erbα* acts as a potent repressor of *Cry1* expression and that *Cry1* works as a negative regulator of *Per1* expression (Etchegaray *et al*, 2003). The potential behavioral relevance of these changes in *Per1* expression is highlighted by studies showing that both mice and *Drosophila* mutants, lacking respectively *Per1* or *Per*, failed to show sensitization to cocaine (Abarca *et al*, 2002; Andretic *et al*, 1999), and the reduction of Per1 activity in mice by DNAzyme led to a reduction in CPP for morphine (Li *et al*, 2008). The mechanisms by which circadian clock genes regulate addictive behavior remain however elusive have not been fully elucidated (Perreau-Lenz *et al*, 2007), but probably involve their regulatory role on

dopamine receptor responsiveness (Andretic and Hirsh, 2000).

Although circadian clock genes are appealing candidates for mediating the interaction between sodium butyrate and morphine, the transcriptional program activated by chronic morphine is broad (Korostynski *et al*, 2007; McClung *et al*, 2005), and its interaction with sodium butyrate complex (figure 5 and figure S5). Beside circadian genes, our results show that morphine and sodium butyrate interact on the regulation of the expression of several other transcription factors that may lead to further transcriptional changes, such as fosB, the activity-regulated transcription factors Npas4 and Nr4al and the transcriptional repressor Zbtb16 that positively regulates the ERK pathway and can potentially enhance drug effects. Genes encoding proteins involved in neurite outgrowth and structural changes (*Mpp7, Btg2, Cdc42ep2, Rem2, Cdh9*) or that may contribute to the enhanced behavioral response observed during sensitization, such as the translation enhancer Rbm3, were also differentially regulated. All these genes represent interesting candidates for further analysis of epigenetic regulation of addiction-related behaviors.

Taken together, our behavioral, histone posttranslational modification and gene profiling studies support a scenario in which drug-induced changes in the chromatin would be restricted to specific genomic *loci* relevant in neuronal plasticity, rather than global, genome-wide, changes in chromatin acetylation and gene expression. These *loci* are the most likely sites where HDACi and drugs of abuse interact to promote non-homeostatic neuroadaptations that underlie behavioral phenomena relevant in the context of addictive behavior, such as CPP and sensitization. This view is supported by a number of recent studies exploring changes in histone phosphorylation and acetylation at the promoters of *Per1, c-fos, fosB, bdnf* and *NK1R*, among other genes, in response to the acute administration of HDACi or drugs of abuse (Kumar *et al*, 2005; Levine *et al*,

2005; Renthal *et al*, 2008a; Renthal *et al*, 2009; Renthal *et al*, 2007; Russo *et al*, 2009; Schroeder *et al*, 2008). However, it should be also noted that HDACs do not only acetylate nucleosome histones, but a much wider range of cellular proteins and can therefore influence cell physiology and animal behavior also through non-genomic mechanisms (Glozak *et al*, 2005; Spange *et al*, 2009). Indeed, some behavioral consequences observed after the administration of HDACi might be difficult to reconcile with the temporal requirements of the genomic actions of these compounds, which imply not only gene transcription but also the synthesis of proteins *de novo* and their transport to cellular structures/organelles. In agreement with this view, others and we (Kumar et al., 2005) have observed a rapid enhancement of cocaine-, ethanol- or morphine-induced locomotion when these drugs were injected simultaneously or shortly after (i.e. 20 min) HDACi administration. Therefore, it is possible that these compounds influence drug-induced behaviors by epigenetic and non-epigenetic mechanisms. Initial support for this dual action of HDACi was obtained in our detailed analysis on the effects of sodium butyrate on morphine-induced locomotor sensitization which revealed that this compound affected both the intercept and the slope of the regression line describing the progressive enhancement of locomotion observed after repeated administration of this drug. Future research should further explore the relative contribution of the different molecular changes triggered by sodium butyrate on the behavioral effects of its co-administration with drugs of abuse.

In summary, our results confirm and extend previous reports on the ability of HDACi to modify the behavioral effects of drugs of abuse belonging to different pharmacological families. This modulatory activity is complex and might imply different mechanisms when considering acute *vs.* chronic drug administration. HDAC inhibition also seems to differentially affect addiction-related behaviors associated to

non homeostatic neuroplasticity and those resulting from homeostatic adaptations to the drug. Future studies should explore further how drugs of abuse can trigger changes in histone acetylation and phosphorylation at specific genomic *loci* and determine whether these transient chromatin modifications can lead to more stable and specific molecular marks, such as histone or DNA methylation (Borrelli *et al*, 2008) that could better account for the persistence of addiction features revealed in our locomotor sensitization studies, in which the boosting effect of HDAC inhibition was maintained even one week after the last sodium butyrate injection. Our microarray screen has revealed a number of interesting candidates for such in depth epigenetic analysis.

Disclosure/ Conflicts of interest

The authors declare that they do not have any conflicts of interest.

Acknowledgments

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Figure legends

Figure 1. Effects of sodium butyrate administration on cocaine-induced locomotor sensitization. A. Scheme of the behavioral training and treatment groups used for the sensitization experiment. The development and expression of locomotor sensitization was evaluated using a protocol divided into two phases: induction and challenge. **B.** Sodium butyrate enhanced the ability of repeated cocaine (10 mg/kg) injections to promote locomotor sensitization, but it did not alter locomotion in saline treated mice. **C**. The effects of sodium butyrate were persistent and those mice receiving this HDACi as co-adjuvant during the induction phase exhibited higher locomotion when re-exposed to cocaine in the challenge phase 7 days later. B100, B150 and B300 denotes pretreatment with 100, 150 or 300 mg/kg of sodium butyrate, respectively. The horizontal line of panel 1C depicts average locomotion of saline treated groups at the induction phase. In all panels +: $p<0.05$, * $p< 0.01$ as compared to SC group.

Figure 2. Effects of sodium butyrate administration on ethanol-induced behaviors. A. Scheme of the behavioral training and treatment groups used for the sensitization experiment. The development and expression of locomotor sensitization was evaluated using a protocol divided into two phases: induction and challenge. **B.** Co-administration of sodium butyrate and ethanol (2.5 g/kg) increased the development of locomotor sensitization (in all panels of this figure :+: $p<0.05$, * $p< 0.01$ as compared to SE group). **C.** Mice that had received sodium butyrate and ethanol during the induction phase exhibited higher locomotion when re-challenged 7 days later with ethanol (2.5 g/kg) than those that had been only exposed to ethanol. B100, B150 and B300 denotes pretreatment with 100, 150 or 300 mg/kg of sodium butyrate, respectively. The horizontal line depicts average locomotion of saline treated groups at the induction phase. **D.** The administration of sodium butyrate (300 mg/kg) did not modify the motor-

incoordinating effects of ethanol (2.5 g/kg) or the development of tolerance as this treatment was repeated. **E.** Fifteen hours after the termination of an intense ethanol administration regimen (4 g/kg, twice daily; 5 days) a significant reduction on spontaneous locomotion was observed ($p<0.05$). The magnitude of this ethanol withdrawal sign was not modified in mice that had received sodium butyrate (150 mg/kg) with each ethanol injection.

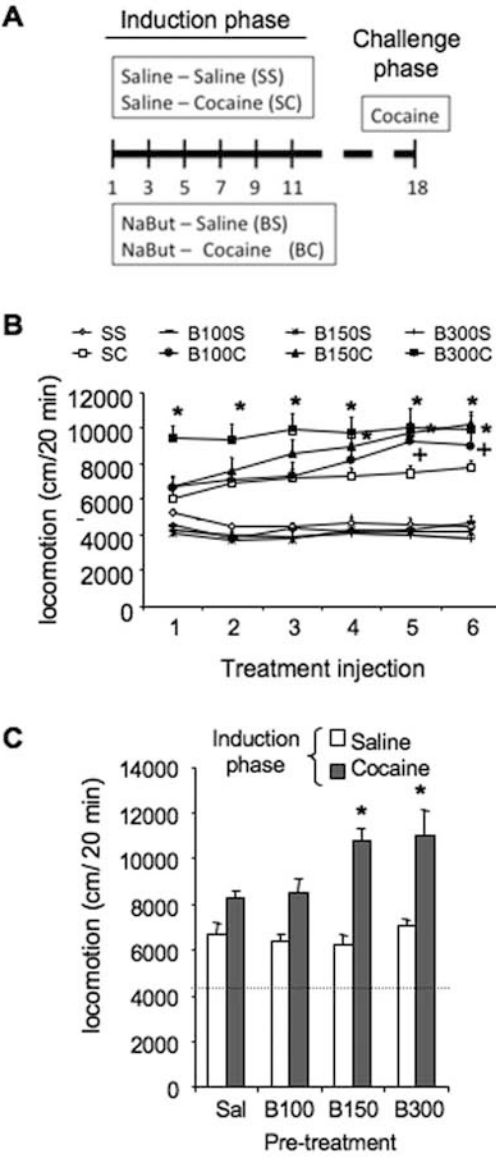
Figure 3. Effects of sodium butyrate administration on morphine-induced behaviors. A. Scheme of the behavioral training and treatment groups used for the sensitization experiment. The development and expression of locomotor sensitization was evaluated using a protocol divided into two phases: induction and challenge. **B.** The development of morphine-induced locomotor sensitization was enhanced in mice treated with intermediate (150 mg/kg) or high (300 mg/kg) doses of sodium butyrate. **C.** Expression of locomotor sensitization in the same 8 groups of mice seven days after the termination of the induction phase. All mice were challenged with a single morphine (20 mg/kg) injection. The horizontal line depicts the averaged locomotion score of all groups receiving saline injections during the last test of the induction phase. The boosting effect of sodium butyrate still persisted when the mice were re-exposed to morphine 7 days after the last injection. B100, B150 and B300 denotes pretreatment with 100, 150 or 300 mg/kg of sodium butyrate, respectively. **D.** The analysis of locomotor sensitization in four additional groups of mice receiving sodium butyrate (0 or 300 mg/kg) and morphine (10 or 20 mg/kg) revealed that repeated administration of the lower dose of morphine did not result in locomotor sensitization regardless of the co-adjuvant (saline or sodium butyrate) treatment. In contrast, repeated administration of a higher dose of morphine resulted on the development of locomotor sensitization, an effect that was boosted by the administration of sodium butyrate. These data were

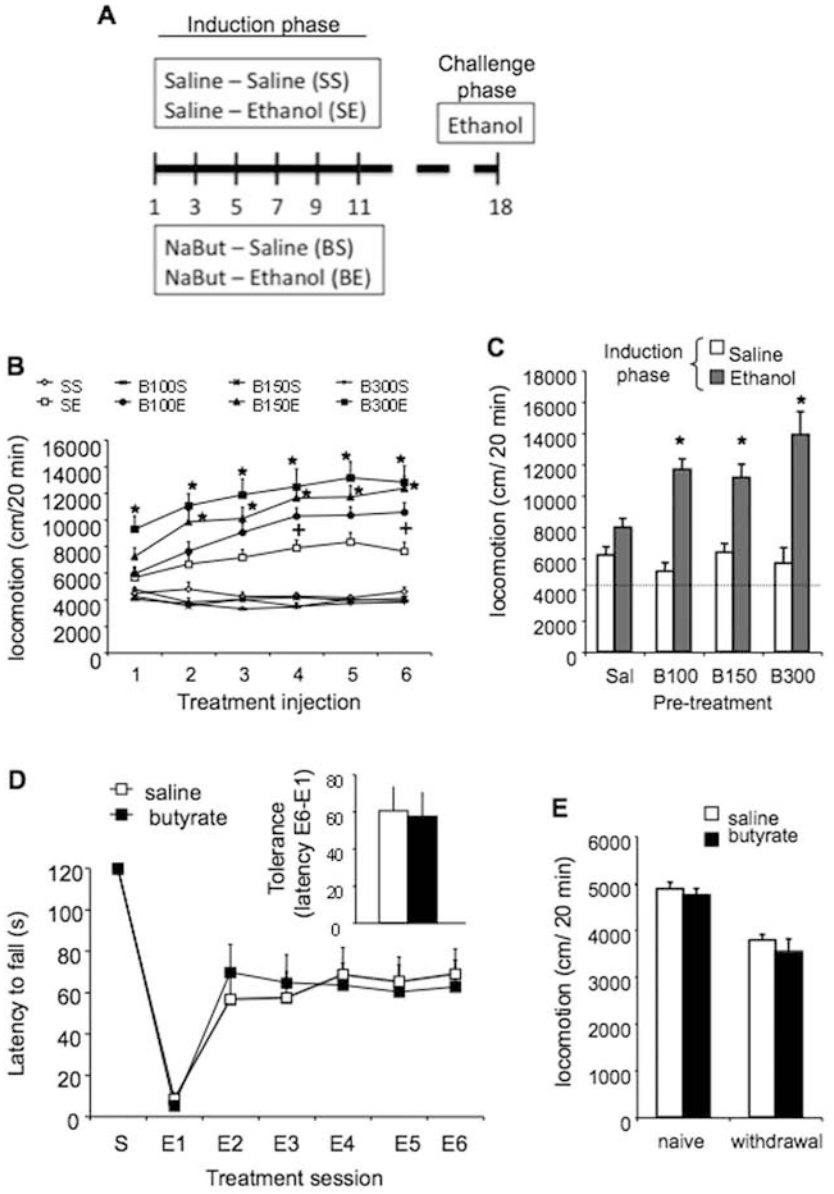
further analyzed by a linear-regression-based procedure (straight lines on the figure; see results section for further details). **E.** Sodium butyrate (150 mg/kg) increases morphine-induced CPP. **F.** Sodium butyrate (300 mg/kg) does not affect the development of tolerance to the analgesic effects of morphine (5 mg/kg; tail-flick test). **G.** Effects of sodium butyrate (150 mg/kg) on naloxone-precipitated morphine withdrawal. For all panels of this figure: + $p<0.05$ and * $p<0.01$ as compared to SM group.

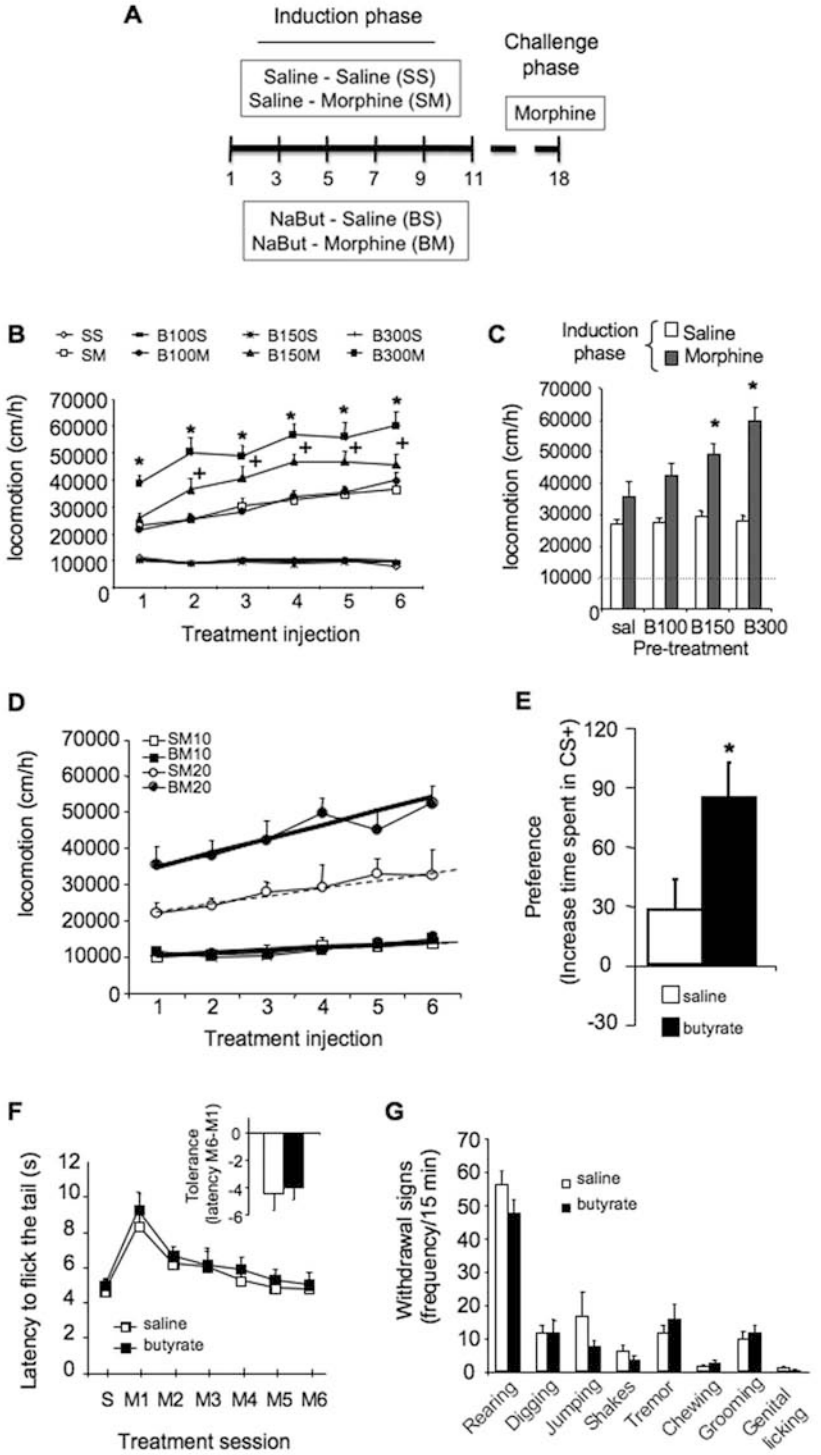
Figure 4. Modification of striatal bulk chromatin by drugs of abuse. A-B. Upper schemes: Drug administration dose (**A**: low, **B**: high) and protocol. Left: Bar graph summarizing data from immunoblots analysis. Data (mean±SEM; 4 mice per group) are expressed relative to saline-treated control subjects, after normalization to β-actin. * $p<0.05$. Right: Representative immunoblots. **C.** Representative immunostaining of coronal sections showing phospho-H3 reactivity at the medial portion of the dorsal striatum. Histone modification was analyzed 30 min after injection of saline (0.9% NaCl), morphine (60 mg/kg), ethanol (6 g/kg) and cocaine (40 mg/kg) intraperitoneal administration (3 mice per experimental condition were analyzed and produced similar results). Scale bar: 100 μm. The diagram at the left indicates the striatal area showed in the pictures.

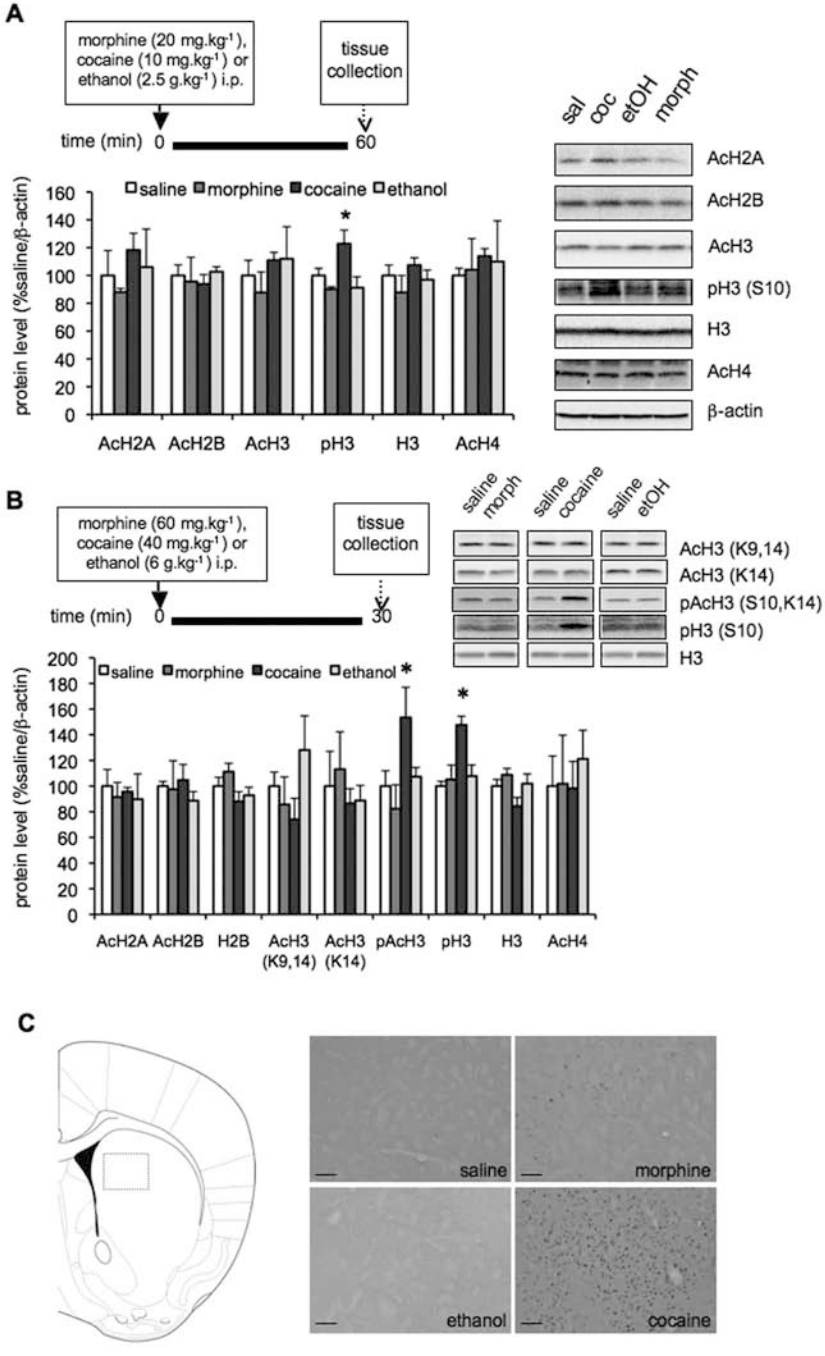
Figure 5. Chronic inhibition of HDAC alters the genetic program activated by morphine administration. A. Scheme of the regime injections and treatment groups used in the microarray experiment. **B.** Hierarchical clustering of the 197 genes that were significantly affected by treatment in one-way ANOVA of microarray data and showed a fold change larger than 1.3 in at least one comparison between treatments. Candidate gene names for further validation are shown. **C.** Several circadian rhythms genes showed a differential regulation in response to morphine (20 mg/kg) as result of their differential treatment during the sensitization induction phase. The upper inset shows

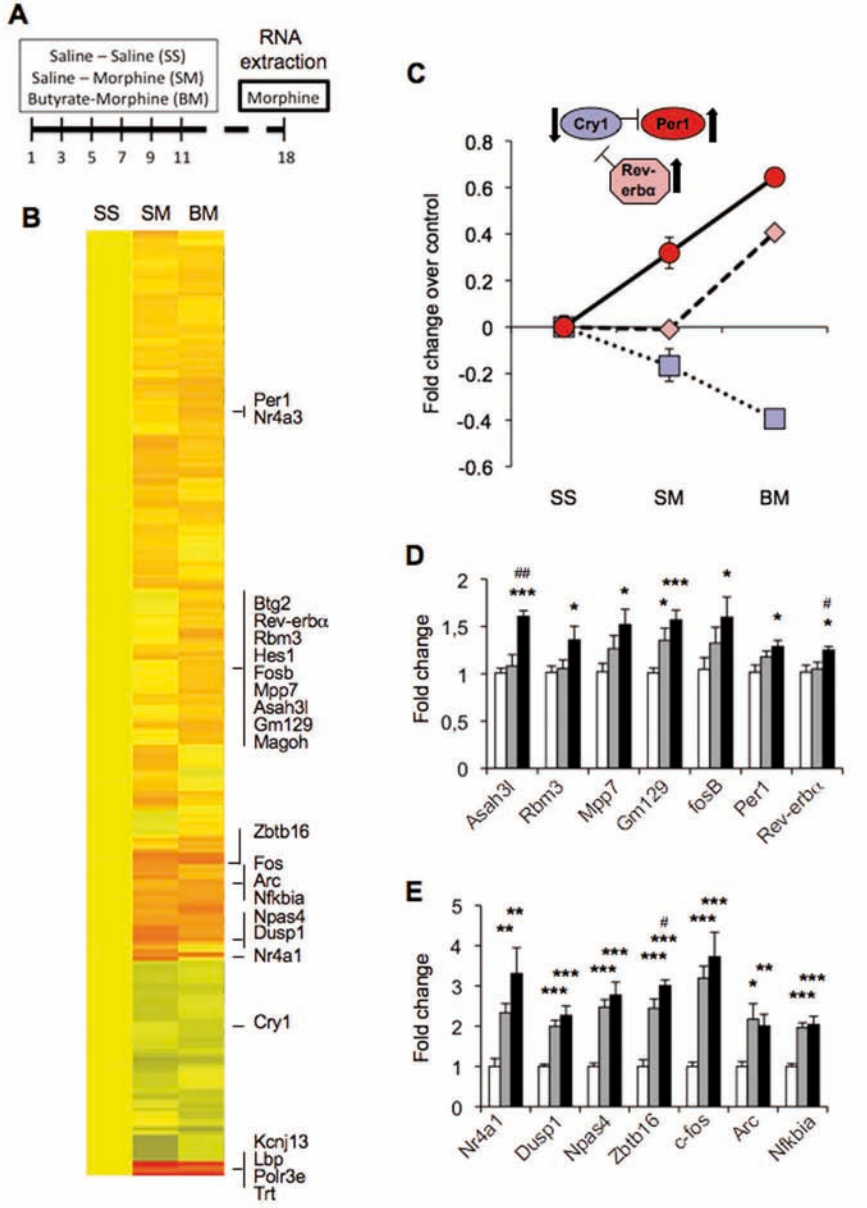
that the changes in expression found in our analysis are in agreement with current models of their regulatory interactions (Etchegaray *et al*, 2003). **D-E** Quantitative real-time RT-PCR of selected transcripts identified in our screening as selectively upregulated by the co-treatment (**D**) or equally induced by morphine or morphine plus sodium butyrate (**E**). (*, **, ***: $p<0.05$, $p< 0.01$ or $p<0.001$ as compared to SS group; (#, ##: $p<0.05$ or $p< 0.01$ as compared to SM group). Data are presented as fold change (mean ± SEM; 8 mice per group).


A
Induction phase
Challenge phase
Saline – Saline (SS)
Saline – Cocaine (SC)
Cocaine
1 3 5 7 9 11
18
NaBut – Saline (BS)
NaBut – Cocaine (BC)
B
SS
SC
B100S
B100C
B150S
B150C
B300S
B300C
locomotion (cm/20 min)
12000
10000
8000
6000
4000
2000
0
1 2 3 4 5 6
Treatment injection
C
Induction phase
Saline
Cocaine
locomotion (cm/ 20 min)
14000
12000
10000
8000
6000
4000
2000
0
Sal B100 B150 B300
Pre-treatment


A
Induction phase
Saline – Saline (SS)
Saline – Ethanol (SE)
Challenge phase
Ethanol
1 3 5 7 9 11
18
NaBut – Saline (BS)
NaBut – Ethanol (BE)
B
SS
SE
B100S
B100E
B150S
B150E
B300S
B300E
locomotion (cm/20 min)
Treatment injection
C
Induction phase
Saline
Ethanol
locomotion (cm/ 20 min)
Sal
B100
B150
B300
Pre-treatment
D
saline
butyrate
Tolerance (latency E6-E1)
Latency to fall (s)
S
E1
E2
E3
E4
E5
E6
Treatment session
E
saline
butyrate
locomotion (cm/ 20 min)
naive
withdrawal


A
Induction phase
Challenge phase
Saline - Saline (SS)
Saline - Morphine (SM)
Morphine
1 3 5 7 9 11 18
NaBut - Saline (BS)
NaBut - Morphine (BM)
B
SS
SM
B100S
B100M
B150S
B150M
B300S
B300M
locomotion (cm/h)
Treatment injection
C
Induction phase
Saline
Morphine
locomotion (cm/h)
sal B100 B150 B300
Pre-treatment
D
SM10
BM10
SM20
BM20
locomotion (cm/h)
Treatment injection
E
Preference
(Increase time spent in CS+)
saline
butyrate
F
Latency to flick the tail (s)
Tolerance
(latency M6-M1)
saline
butyrate
S M1 M2 M3 M4 M5 M6
Treatment session
G
Withdrawal signs
(frequency/15 min)
saline
butyrate
Rearing
Digging
Jumping
Shakes
Tremor
Chewing
Grooming
Genital licking


A
morphine (20 mg.kg-1), cocaine (10 mg.kg-1) or ethanol (2.5 g.kg-1) i.p.
tissue collection
time (min) 0 60
saline morphine cocaine ethanol
protein level (%saline/β-actin)
AcH2A AcH2B AcH3 pH3 H3 AcH4
sal coc etOH morph
AcH2A
AcH2B
AcH3
pH3 (S10)
H3
AcH4
β-actin
B
morphine (60 mg.kg-1), cocaine (40 mg.kg-1) or ethanol (6 g.kg-1) i.p.
tissue collection
time (min) 0 30
saline morphine cocaine ethanol
protein level (%saline/β-actin)
AcH2A AcH2B H2B AcH3 (K9,14) AcH3 (K14) pAcH3 pH3 H3 AcH4
saline morph
saline cocaine
saline etOH
AcH3 (K9,14)
AcH3 (K14)
pAcH3 (S10,K14)
pH3 (S10)
H3
C
saline
morphine
ethanol
cocaine


A
Saline – Saline (SS)
Saline – Morphine (SM)
Butyrate-Morphine (BM)
RNA extraction
Morphine
1 3 5 7 9 11 18
B
SS SM BM
Per1
Nr4a3
Btg2
Rev-erbα
Rbm3
Hes1
Fosb
Mpp7
Asah3l
Gm129
Magoh
Zbtb16
Fos
Arc
Nfkbia
Npas4
Dusp1
Nr4a1
Cry1
Kcnj13
Lbp
Polr3e
Trt
C
Cry1
Per1
Rev-erbα
Fold change over control
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
SS SM BM
D
Fold change
2
1,5
1
0,5
0
Asah3l Rbm3 Mpp7 Gm129 fosB Per1 Rev-erbα
E
Fold change
5
4
3
2
1
0
Nr4a1 Dusp1 Npas4 Zbtb16 c-fos Arc Nfkbia

SUPPLEMENTARY DATA

Supplementary Methods

Measure of blood alcohol levels

Mice were sacrificed by decapitation and 1 ml of trunk blood was collected in heparinized microcentrifuge tubes and immediately placed in a microcentrifuge where the samples were spun down for 5 min at 5000 rpm. 160 µl of supernatant were mixed with 1.44 ml of TCA (20%) and the mixture was spun down again to obtain a clear, protein-free supernatant. This protein-free serum samples were stored at −80°C until chromatographic analysis using a CE Instruments GC 8000 gas chromatograph (Polyethylene glycol column: 122–7032 DB-WAX, 30 m × 0.25 mm in J&W Scientific) with an HS-850 headspace analyzer. Nitrogen was used as a carrier gas (flow rate 84 ml/min). The injector temperature was set to 90°C and the oven temperature was 60°C. The retention time for ethanol was 3.9 min and the detection limit 0.5 µg/ml.

Antibodies

We used the following antibodies: α-H2B, α-H3 and α-AcH3 (Lys14) (Abcam, MA, USA); α-AcH2A (Lys 5,9,13,15), α-AcH2B (Lys 5,12,15,20), α-AcH3 (lys 9,14), α-pAcH3 (Ser10, Lys14), α-AcH3 (Lys14), pH3 (Ser 10) and α-AcH4 (Lys 5,8,12,16) (Millipore, Billerica, MA, USA); α-AcH2A (lys 5) (Cell Signaling Technology, Beverly, MA, USA); and α-β-actin (Sigma-Aldrich, Barcelona, Spain). In addition, we also raised and used rabbits antisera obtained against acetylated peptides corresponding to the N-terminal tail of each one of the four nucleosome histones. The specificity of the different polyclonal antisera was compared to that of commercial antibodies and evaluated by competition assays in the presence of increasing amounts of acetylated or unacetylated peptides (data not shown).

Supplementary Figure legends

Supplementary Figure S1. Striatal histone acetylation by the HDAC inhibitor sodium butyrate. Bar graph showing striatal histone acetylation is dose-dependent increased 30 min after inhibiting HDAC activity by intraperitoneal injection of NaBt (600 mg/kg or 1.2 g/kg). Data (mean±SEM) are expressed relative to saline-treated control subjects, after normalization to β-actin (n=3 per group). *=$p < 0.05$. Panels at the right show representative immunoblots demonstrating dose-dependent increase of histone acetylation in the striatum by sodium butyrate.

Supplementary Figure S2. Absence of drug-mediated modifications at bulk histone acetylation levels. Coronal sections covering the rostral and medial portion of the dorsal striatum, of mice subjected to acute morphine (60 mg/kg), ethanol (6 g/kg), cocaine (40 mg/kg) or saline intraperitoneal administration were stained using anti-Acetyl histone H2A, H2B, H3 and H4. Scale bar: 100 μm

Supplementary Figure S3. Morphine and cocaine-mediated phosphorylation of histone H3. Representative coronal sections showing immunoperoxidase labeling for phospho-H3 at dorsal striatum (caudate putamen) (A, C and E) or ventral striatum (nucleus accumbens and olfactory tubercle) (B, D and F) of mice subjected to saline (A, B), morphine (60 mg/kg) (C, D), or cocaine (40 mg/kg) (E, F). There is a substantial labeling in dorsal and ventral striatum in cocaine-treated mice (E, F). The immunolabeling is also pronounced, both in dorsal and ventral striatum, upon morphine treatment (C, D). Note the labeling of proliferating/mitotic cells in the subventricular zone of both saline and drug treated animals (A, C, E). Scale bar: 200 μm

Supplementary Figure S4. Bulk histone acetylation by drug administration in the presence of sodium butyrate. A. Intraperitoneal NaBt (300 mg/kg) was given 15 min before an acute administration of morphine (20 mg/kg); cocaine (10 mg/kg); or ethanol

(2.5 g/kg). Mice were sacrificed and brain removed for micro-dissection of the striatum 15 min after drug administration (upper scheme). Left: Quantification of acetylated histones levels after sodium butyrate treatment either alone or in combination with the different drugs. Data (mean ± SEM) are expressed relative to saline-treated control subjects after normalization to β-actin (*n*=3-4 per group). Right: Representative immunoblots of modified histones extracted from striatum of mice subjected to the indicated treatment and dose. **B.** Morphine (20mg/kg) was administered on alternate days by intraperitoneal injection and the development of locomotor sensitization was monitorized. Mice were sacrificed and the striatum dissected 48 h after the fifth injection (upper scheme). Left: Bar graph summarizing data from immunoblots analysis. Data (mean ± SEM) are expressed relative to saline-treated control subjects, after normalization to β-actin (*n* = 4 per group). Right: Representative immunoblots showing absence of change in striatal histone acetylation levels in mice subjected to chronic administration of morphine.

Supplementary Figure S5. A. Forty-five genes showing the strongest transcriptional response to the morphine challenge after chronic morphine administration. The response of these genes to morphine challenge in the group of chronic morphine administration in presence of sodium butyrate is also shown. **B-C.** Graphs showing normalized signal intensity (log scale) for *Trt* (B), *Kcnj13* (C) and *Lbp* (D) across the different microarrays used in this study (SS, saline-saline; SM, saline-morphine; BM, butyrate-morphine). **E.** Pahtway created with the 197 genes significantly altered by treatment upon ANOVA analysis using Pathways Studio 5.0 software; only direct interactions were considered and unlinked entities were excluded. Note the high degree of causative relations and interaction among the differentially expressed genes upon drug treatment (51 genes out of 176 entities are directly interconnected).

Supplementary Table 1

	Saline	Sodium butyrate	P value
Induction phase	200.47 ± 3.81	207.59 ± 9.12	0.59
Challenge phase	233.058 + 10.54	243. + 3.54	0.38

The administration of sodium butyrate does not affect blood alcohol levels (mg/ Dl). Locomotor sensitization induced by ethanol was evaluated using a protocol divided into two phases: Induction and challenge. The induction phase involved six trials on alternate days, one trial per day. On each one of these trials mice were pre-treated with saline or sodium butyrate (300 mg/kg) and, 20 min later, injected with ethanol and immediately placed in an open field for 20 min. The challenge phase consisted of a single trial conducted 7 days after the last test of the induction phase. In this case, all animals received a single injection of ethanol (2.5 g/kg) and locomotion was assessed as in the preceding phase. Blood samples were collected from different groups (n=4-6, per group) of lightly anesthetized mice immediately after the end of the 6^{th} trial or immediately after the end of the challenge test. Blood alcohol levels (mg/ Dl) were evaluated by gas chromatography (see Supplementary Methods for details). Data are presented as the mean ± SEM and were compared by means of two separate Student's t test for independent samples.

Supplementary Table 2

	saline	morphine	ethanol	cocaine
motor cortex	-	-	-	-
somatosensory cortex	-	-	-	-
insular cortex	-	-	-	-
piriform cortex	-	-	-	-
lateral septum	-	-	-	+
lateral ventricle (SVZ)	+	+	+	+
striatum (caudate putamen)	-	++	-	+++
nucleus acumbens core	-	+	-	++
nucleus acumbens shell	-	++	-	+++
olfactory tubercle	-	+	-	++

Relative effects of saline, morphine (40 mg/kg), ethanol (4 g/kg) and cocaine (20 mg/kg) acute administration on H3 phosphorylation levels. Three mice per treatment were used in this analysis.

Supplementary Table 3. Genes significantly altered by treatment (One-way ANOVA, FC>1.3, p<0.05)

FC [SM] vs [SS]	*p*-value	FC [BM] vs [SS]	*p*-value	[BM] vs [SM] % change	*p*-value	gene symbol
1.86	0.001	2.41	0.001	29.85	NS	***Nr4a1***
1.69	<0.001	2.17	<0.001	28.47	0.043	***Dusp1***
1.58	0.001	1.97	<0.001	24.89	0.047	***Npas4***
1.90	<0.001	2.26	<0.001	18.85	NS	***Zbtb16***
1.49	<0.001	1.75	0.001	18.09	NS	***Fos***
1.32	<0.001	1.51	<0.001	14.12	NS	*2010002N04Rik*
1.39	<0.001	1.58	<0.001	13.81	0.015	*Trp53inp1*
1.36	0.004	1.52	<0.001	11.62	NS	*Arl4d*
1.33	0.049	1.47	0.029	10.78	NS	*ND6*
1.40	0.028	1.54	0.004	10.16	NS	*Cdh19*
1.52	0.004	1.66	0.001	9.03	NS	***Arc***
1.62	<0.001	1.76	<0.001	8.81	NS	*Adipor2*
1.34	<0.001	1.45	<0.001	8.52	NS	*Sult1a1*
1.38	<0.001	1.50	<0.001	8.40	NS	*Slc2a1*
1.95	<0.001	2.11	<0.001	7.79	NS	*Cdkn1a*
1.74	<0.001	1.87	<0.001	7.59	NS	*Pdk4*
1.39	<0.001	1.50	<0.001	7.51	NS	*Tsc22d3*
1.30	0.048	1.39	0.002	7.36	NS	*Fut11*
1.32	<0.001	1.41	<0.001	6.98	NS	*Rhou*
1.43	<0.001	1.53	<0.001	6.89	NS	*Fkbp5*
1.56	<0.001	1.67	<0.001	6.82	NS	***Nfkbia***
1.30	0.002	1.39	<0.001	6.29	NS	*Dclre1b*
1.31	<0.001	1.39	<0.001	6.25	NS	*Fzd4*
2.46	<0.001	2.61	<0.001	6.13	NS	*Polr3e*
1.31	0.001	1.39	<0.001	5.91	NS	*Mfsd2*
1.39	<0.001	1.48	<0.001	5.84	NS	*Errfi1*
1.31	<0.001	1.36	<0.001	3.41	NS	*Snf1lk*
1.30	0.005	1.34	0.003	3.31	NS	*Mertk*
1.32	<0.001	1.36	0.001	2.92	NS	*Sdpr*
1.62	<0.001	1.66	<0.001	2.82	NS	*Tiparp*
1.69	0.002	1.73	<0.001	2.75	NS	*Hspa8*
1.68	0.003	1.70	<0.001	1.08	NS	
1.47	<0.001	1.51	<0.001	2.62	NS	*Cldn1*
1.49	<0.001	1.51	<0.001	1.90	NS	*Zfp189*
1.47	<0.001	1.48	0.001	0.86	NS	*Pkp2*
1.31	0.007	1.31	NS	0.25	NS	*Ptpn3*
1.33	0.001	1.31	0.017	-1.48	NS	
1.49	0.002	1.48	0.001	-0.46	NS	*Plekhf1*
1.32	<0.001	1.31	<0.001	-0.78	NS	*Rhpn2*
2.09	<0.001	2.07	<0.001	-1.01	NS	*Sgk1*
1.33	0.001	1.31	0.001	-1.28	NS	*Adamts1*
1.43	<0.001	1.41	<0.001	-1.75	NS	*Klf15*
1.30	<0.001	1.28	<0.000	-2.10	NS	*Wdr52*
1.43	<0.001	1.39	<0.001	-2.66	NS	*Ucp2*
1.48	0.001	1.43	0.001	-3.81	NS	*Txnip*
1.45	<0.001	1.40	<0.001	-3.97	NS	*Cab39l*
1.42	0.001	1.36	0.002	-4.45	NS	*S3-12*
1.33	0.001	1.26	<0.001	-5.30	NS	*Slc4a2*
1.31	<0.001	1.24	0.004	-5.32	NS	*Bbox1*
1.78	<0.001	1.68	<0.001	-5.52	NS	*Clic6*
1.50	0.008	1.41	0.009	-5.85	NS	*Zic2*
1.47	<0.001	1.38	0.001	-6.42	NS	*2310016C16Rik*
1.39	0.001	1.30	0.008	-6.70	NS	*Ak7*
1.33	0.002	1.24	0.006	-6.84	NS	*Gjb6*
1.33	<0.001	1.24	0.002	-7.01	NS	*AB041803*
1.54	<0.001	1.42	<0.001	-7.75	0.024	*F5*
1.38	<0.001	1.26	<0.001	-8.44	NS	*Acss3*
1.34	<0.001	1.23	0.001	-8.48	NS	*F3*
1.34	0.001	1.21	0.003	-9.41	NS	*Rnf152*

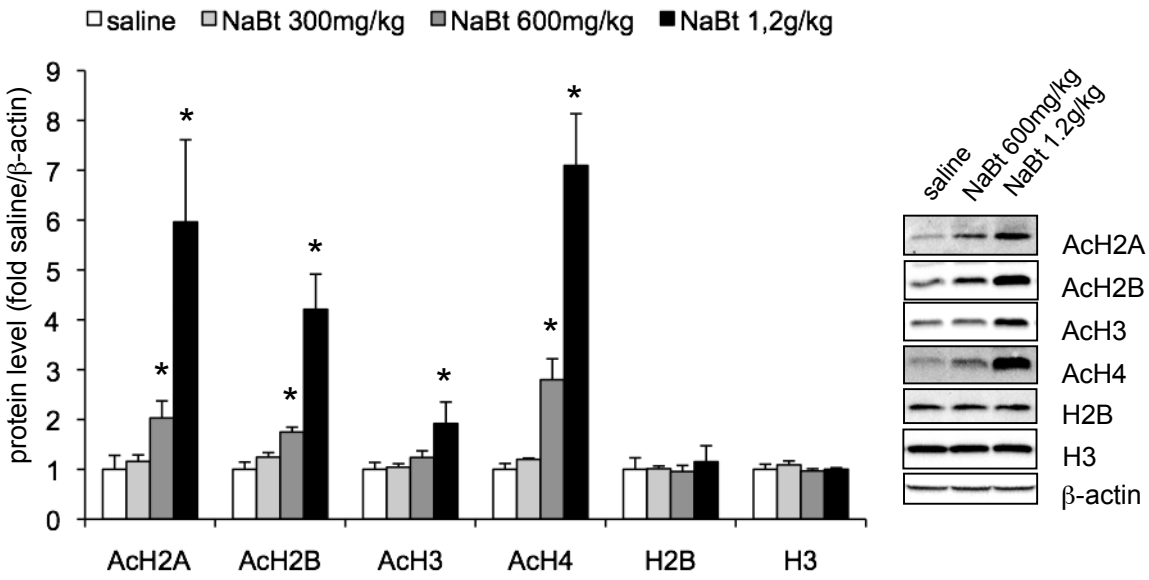
1.46	<0.001	1.32	<0.001	-9.42	NS	*Ddit4*
1.34	<0.001	1.21	<0.001	-9.79	0.040	*Cldn2*
1.34	<0.001	1.21	0.001	-10.03	0.023	*Pcolce2*
1.44	<0.001	1.29	<0.001	-10.12	NS	*Spint2*
1.37	0.001	1.23	0.018	-10.64	NS	*Zic5*
1.34	<0.001	1.19	0.003	-10.75	NS	*D19Ertd652e*
1.57	<0.001	1.39	<0.001	-11.34	NS	*C230095G01Rik*
1.37	< 0,001	1.21	0.005	-11.73	NS	
1.41	0.004	1.32	0.003	-6.31	NS	*A330023F24Rik|C03*
1.31	< 0,001	1.32	0.001	1.25	NS	
1.31	0.006	1.16	0.022	-11.93	NS	*Tmem22*
1.33	0.002	1.16	0.013	-12.30	NS	*Morn2*
1.53	<0.001	1.34	<0.001	-12.38	NS	*Trpm3*
1.40	0.001	1.23	0.014	-12.40	NS	*Ccdc135*
2.20	<0.001	1.92	<0.001	-12.80	NS	*Kl*
1.45	0.002	1.26	0.024	-12.97	0.049	*Slc13a4*
1.31	<0.001	1.14	NS	-13.18	NS	*Col8a1*
1.41	<0.001	1.22	0.005	-13.38	NS	*Kcne2*
1.66	0.003	1.43	0.029	-13.61	NS	*AI506816*
1.40	0.003	1.21	0.029	-13.86	NS	*Trpc4*
1.56	0.033	1.34	NS	-13.90	NS	*Agxt2l1*
1.38	<0.001	1.19	0.003	-13.94	NS	*Slc4a5*
1.56	<0.001	1.34	0.003	-14.01	NS	*Rasd1*
1.34	0.017	1.14	NS	-14.39	NS	*Ptgds*
1.80	<0.001	1.54	<0.001	-14.58	NS	*Igf2*
1.32	0.013	1.12	0.046	-15.24	NS	*Hap1*
1.39	0.007	1.17	NS	-15.29	NS	*Tbrg3*
1.31	0.002	1.09	NS	-16.46	NS	*Nnat*
1.72	<0.001	1.44	0.002	-16.71	NS	*Otx2*
1.32	0.013	1.10	NS	-16.72	NS	*Dgkk*
1.50	<0.001	1.24	0.003	-17.05	NS	*Calml4*
1.43	0.003	1.18	NS	-17.06	NS	*Ngb*
1.61	0.014	1.30	NS	-19.35	NS	*Zic1*
1.34	0.035	1.08	NS	-19.51	NS	*Itih3*
1.55	<0.001	1.24	0.002	-19.62	NS	*Igfbp2*
2.07	<0.001	1.66	<0.001	-19.85	NS	*Enpp2*
1.37	0.009	1.09	NS	-20.15	NS	*Cbln1*
13.41	<0.001	10.68	<0.001	-20.33	NS	*Ttr*
1.51	0.009	1.19	0.026	-21.04	NS	*6430550H21Rik*
1.83	<0.001	1.44	<0.001	-21.11	0.005	*Slc2a12*
1.32	0.004	1.04	NS	-21.19	NS	*LOC675636*
2.23	<0.001	1.73	<0.001	-22.47	NS	*Sostdc1*
1.36	<0.001	1.06	NS	-22.50	0.001	*LOC244958*
1.41	0.022	1.09	NS	-22.82	NS	*Agt*
1.58	0.002	1.20	NS	-23.62	NS	*1500015O10Rik*
2.27	<0.001	1.73	<0.001	-23.79	NS	*Prlr*
1.65	<0.001	1.34	<0.001	-18.88	<0.001	
1.45	0.039	1.10	0.009	-23.87	NS	*Nts*
1.43	0.038	1.08	NS	-24.11	NS	*1100001E04Rik*
1.50	0.008	1.14	0.034	-24.21	NS	*Gpr165*
1.95	<0.001	1.47	<0.001	-24.31	NS	*Folr1*
1.31	0.021	-1.02	NS	-24.81	NS	*Irs4*
1.92	0.003	1.44	0.005	-25.26	NS	*Calb2*
1.52	0.007	1.12	NS	-26.12	NS	*Baiap3*
1.75	0.004	1.29	NS	-26.54	NS	*Slc17a6*
3.19	0.000	2.33	<0.001	-27.00	0.013	*Lbp*
1.54	0.005	1.11	NS	-28.31	NS	*AW551984*
5.96	<0.001	4.20	<0.001	-29.62	NS	*Kcnj13*
1.46	0.023	-1.11	0.101	-36.31	0.059	*Oxt*
1.15	0.030	1.65	<0.001	43.68	0.005	***Asah3l***
1.10	NS	1.57	0.001	42.36	0.018	***Mpp7***
1.15	0.040	1.58	<0.001	37.50	0.023	
1.06	NS	1.41	<0.001	33.08	0.006	

1.08	NS	1.42	<0.001	31.71	0.032	***Rbm3***
1.09	NS	1.33	0.001	22.16	0.008	
1.01	NS	1.33	0.001	32.00	0.006	***Rev-erbα***
1.21	NS	1.60	0.026	31.73	0.008	***Gm129***
1.15	NS	1.49	0.008	29.96	0.001	
1.00	NS	1.31	0.012	31.33	0.049	*Btg2*
1.12	NS	1.43	0.005	28.32	NS	*Magoh*
1.25	0.004	1.56	<0.001	25.27	0.012	***Per1***
1.20	NS	1.49	0.008	24.37	NS	*Ssh2*
1.09	0.018	1.33	<0.001	22.60	0.017	*Hes1*
1.14	0.013	1.39	<0.001	21.93	0.034	*Bmp2*
1.16	NS	1.41	0.025	21.71	NS	*LOC280487*
1.16	NS	1.41	0.025	21.31	NS	
1.16	NS	1.40	0.025	21.26	NS	
1.16	NS	1.40	0.026	21.18	NS	*LOC100043775|LOC*
1.16	NS	1.40	0.025	21.02	NS	
1.16	NS	1.41	0.025	20.98	NS	
1.16	NS	1.40	0.025	20.97	NS	
1.12	NS	1.36	0.026	21.08	0.049	*Tmem16b*
1.18	NS	1.43	0.004	21.06	NS	*Acp1|LOC631286*
1.16	NS	1.40	0.025	20.89	NS	*LOC280487*
1.09	NS	1.30	0.005	19.66	NS	*Hdx*
1.17	NS	1.37	0.003	16.97	0.040	*Gpr3*
1.23	0.009	1.44	<0.001	16.89	NS	*Kcna5*
1.16	NS	1.34	0.003	16.05	NS	*5730403M16Rik*
1.27	0.001	1.47	0.001	15.45	NS	*Nr4a3*
1.14	0.002	1.31	0.001	15.16	NS	***Fosb***
1.27	0.002	1.45	<0.001	14.58	NS	*Stard13*
1.20	<0.001	1.37	<0.001	13.80	NS	*Gadd45g*
1.15	0.022	1.31	<0.001	13.63	NS	*Mt2*
1.24	0.005	1.41	0.002	13.52	NS	*Slc25a13*
1.28	<0.001	1.45	<0.001	13.12	NS	*Nt5e*
1.15	0.002	1.30	<0.001	13.07	0.011	*Snx24*
1.25	0.002	1.40	0.001	12.10	NS	*Ppp1r3g*
1.24	0.009	1.38	<0.001	11.48	NS	*Bcl6*
1.22	0.003	1.34	0.001	9.82	NS	*Phactr4*
1.22	0.031	1.34	0.001	9.55	NS	*Net1*
1.26	0.002	1.38	<0.001	9.51	NS	*Pxdn*
1.20	0.002	1.31	0.001	9.25	NS	*Usp54*
1.28	0.003	1.39	0.001	8.41	NS	*Camk1g*
1.22	<0.001	1.32	<0.001	8.25	NS	*Nostrin*
1.23	0.003	1.34	0.001	8.23	NS	*Sap30*
1.24	<0.001	1.32	<0.001	6.11	NS	*Lifr*
1.24	<0.001	1.31	<0.001	6.04	0.005	*2310007D09Rik*
1.24	0.002	1.30	0.001	4.84	0.006	*Dyrk3*
1.27	0.001	1.32	0.001	4.12	0.018	*2610301F02Rik*
-1.48	0.002	-1.67	<0.001	-12.61	NS	*Cldn5*
-1.34	0.013	-1.37	0.007	-1.97	NS	*EG329126*
-1.32	0.030	-1.34	0.030	-1.29	NS	*2610044O15Rik*
-1.32	<0.001	-1.30	<0.001	1.58	NS	*Cxcl12*
-1.33	0.004	-1.30	<0.001	2.07	NS	*Cckbr*
-1.30	0.001	-1.27	0.001	2.58	NS	*Rgs20*
-1.55	<0.001	-1.51	<0.001	3.05	NS	*Kdr*
-1.35	<0.001	-1.31	<0.001	3.48	NS	*Gbp4*
-1.43	<0.001	-1.36	<0.001	4.95	NS	*P2ry13*
-1.50	<0.001	-1.42	<0.001	5.18	NS	*Rasgef1b*
-1.40	<0.001	-1.32	<0.001	6.28	NS	*Slc40a1*
-1.32	<0.001	-1.23	0.001	6.89	NS	*Sh3rf2*
-1.30	<0.001	-1.21	<0.001	6.89	NS	*Jag1*
-1.36	<0.001	-1.26	0.002	7.39	NS	*Hmgb1*
-1.30	<0.001	-1.18	0.006	9.44	NS	*9430020K01Rik*
-1.33	0.003	-1.19	0.010	10.76	NS	*Gbp3*
-1.35	0.001	-1.19	0.008	12.11	NS	*Tgfa*

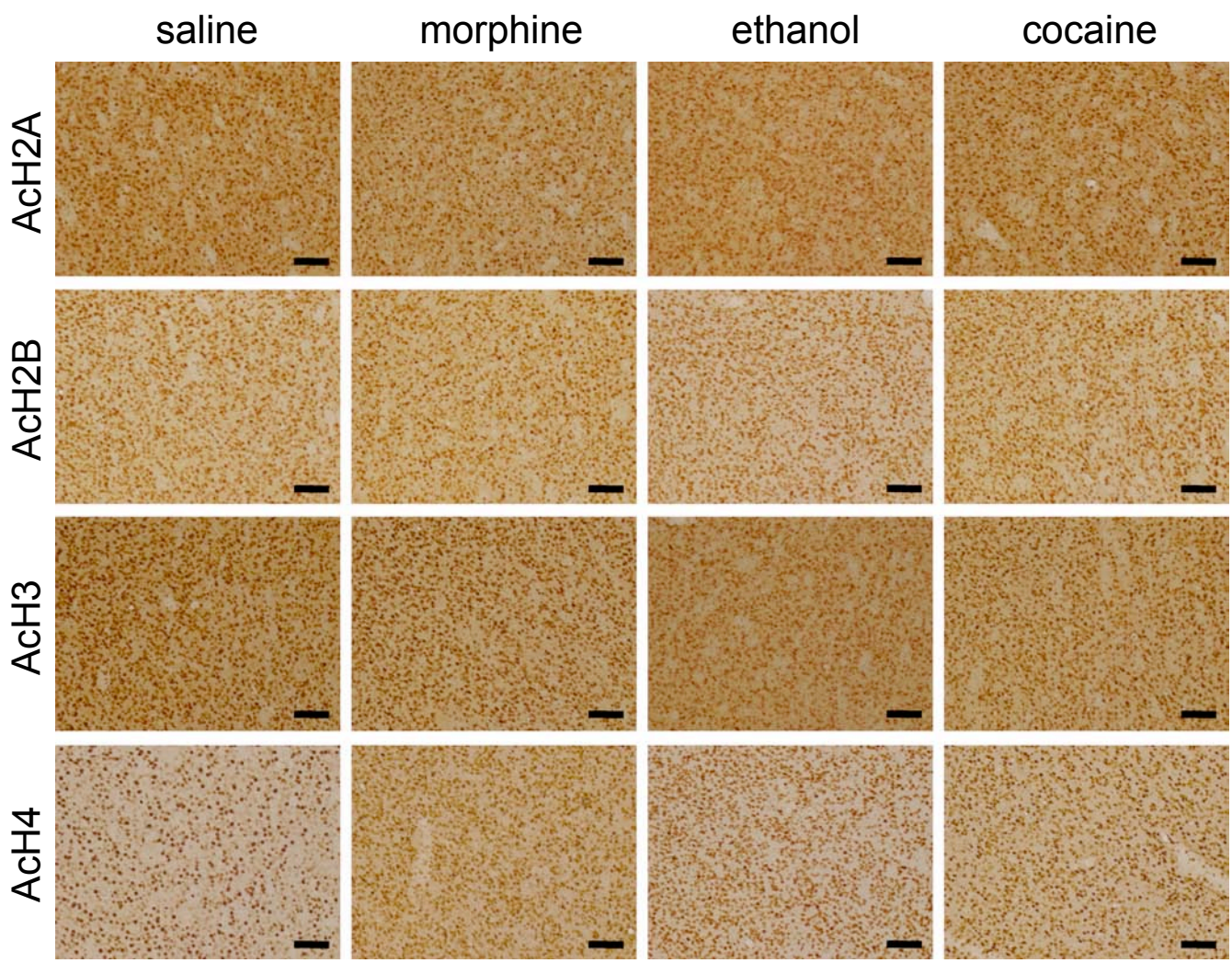
-1.37	<0.001	-1.20	0.010	12.46	NS	*Cdc42ep1*
-1.35	<0.001	-1.18	0.001	12.82	0.047	*Cdh9*
-1.38	0.003	-1.20	0.022	13.14	NS	*Rspo2*
-1.36	0.001	-1.17	0.010	13.57	NS	*Kcnv1*
-1.37	<0.001	-1.16	0.006	15.30	0.045	*1110032E23Rik*
-1.36	<0.001	-1.15	0.024	15.60	NS	*Myo3b*
-1.46	<0.001	-1.18	0.029	19.16	NS	*Trim59*
-1.32	0.001	-1.05	NS	20.66	0.016	*Rasl11b*
-1.31	0.006	-1.03	NS	21.49	0.045	*Cntnap3*
-1.31	0.007	-1.03	NS	21.62	NS	*Ephb6|Mon2*
-1.68	0.008	-1.24	NS	26.18	NS	
-1.68	0.008	-1.24	NS	26.20	NS	
-1.68	0.008	-1.24	NS	26.21	NS	
-1.68	0.008	-1.24	NS	26.24	NS	*BC003993*
-1.68	0.008	-1.24	NS	26.24	NS	
-1.68	0.008	-1.24	NS	26.24	NS	
-1.68	0.008	-1.24	NS	26.25	NS	
-1.01	NS	-1.37	0.003	-35.21	NS	*LOC676959*
-1.10	NS	-1.42	<0.001	-29.99	NS	*Sdf2l1*
-1.11	NS	-1.32	<0.001	-19.57	NS	*N6amt2*
-1.12	0.015	-1.31	<0.001	-17.10	0.001	***Cry1***
-1.12	NS	-1.30	0.001	-16.23	0.008	*Chchd2*
-1.15	NS	-1.33	<0.001	-15.24	0.023	
-1.22	0.005	-1.38	<0.001	-13.48	0.008	*Eif2c4*
-1.21	0.001	-1.30	<0.001	-7.99	0.041	*Cdc42ep2*
-1.23	0.037	-1.32	0.001	-7.71	NS	*B930094E09Rik*
-1.23	0.004	-1.32	<0.001	-7.19	NS	*Magef1*
-1.24	NS	-1.31	0.036	-5.58	NS	*LOC632394*
1.07	NS	1.10	0.002	3.26	0.025	*Klhl1*
-1.07	NS	1.09	0.042	13.79	0.019	*Slc39a1*
-1.14	0.048	1.16	0.037	23.08	0.005	*Rem2*
-1.15	0.049	1.19	NS	25.37	0.042	*Thbs4*
1.26	0.022	-1.10	NS	-26.47	0.028	*Gpr101*

Table showing genes significanly affected by morpine challenge after sodium butyrate or saline pretreatment before morphine administration during the induction phase (one-way ANOVA, FC>1.3, p<0.05) sorted by percentage of change between BM and SM conditions. Selected genes for further validation by quantitative real-time RT-PCR are shown in bold.

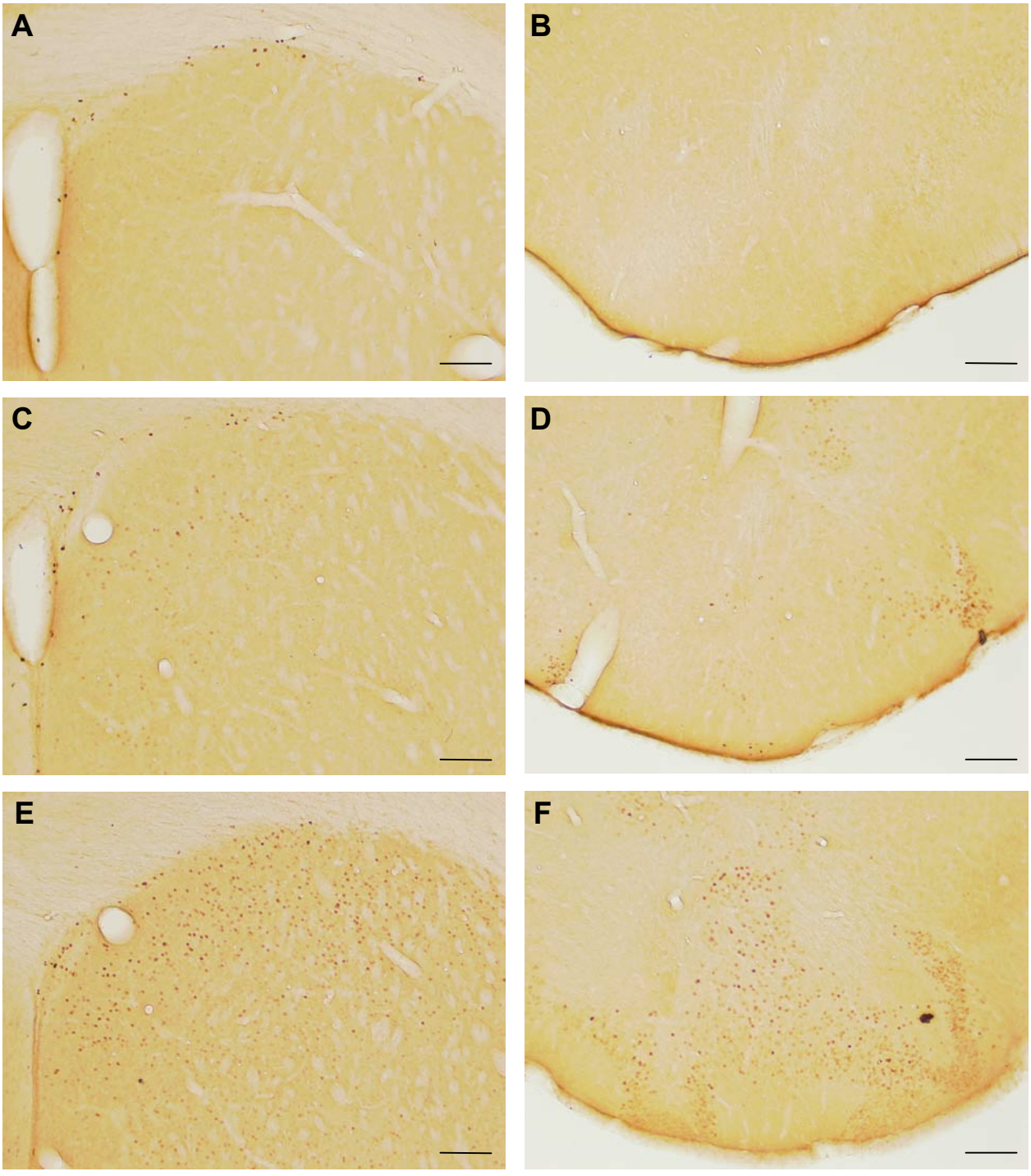
Supplementary Figure S1



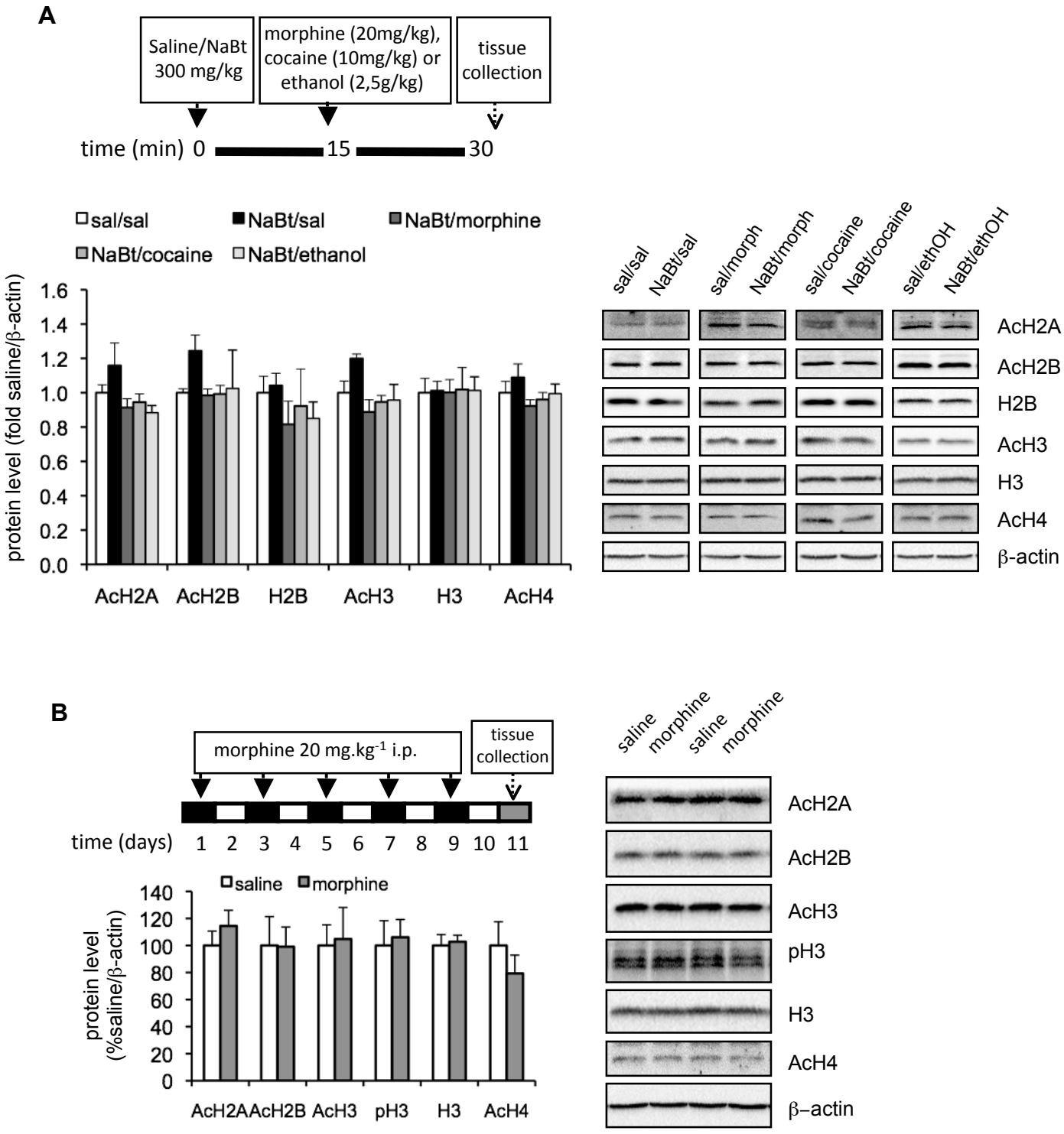
Supplementary Figure S2



Supplementary Figure S3



Supplementary Figure S4

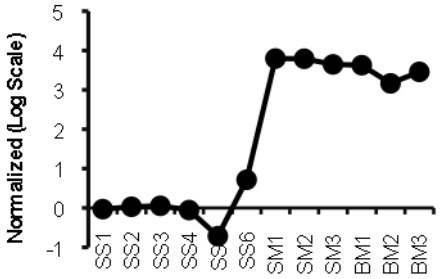


Supplementary Figure S5

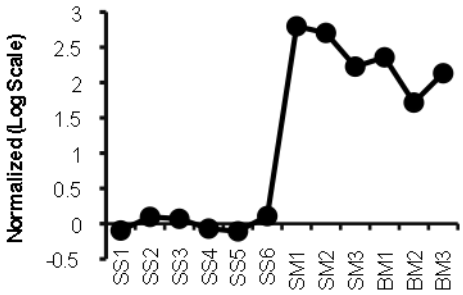
A

FC [SM] vs [SS]	*p*-value	FC [BM] vs [SS]	*p*-value	gene symbol
13,41	< 0,001	10,68	< 0,001	*Ttr*
5,96	< 0,001	4,20	< 0,001	*Kcnj13|LOC100045137*
3,19	< 0,001	2,33	< 0,001	*Lbp*
2,46	< 0,001	2,61	< 0,001	*Polr3e*
2,27	< 0,001	1,73	< 0,001	*Prlr*
2,23	< 0,001	1,73	< 0,001	*Sostdc1*
2,20	< 0,001	1,92	< 0,001	*Kl*
2,09	< 0,001	2,07	< 0,001	*Sgk1*
2,07	< 0,001	1,66	< 0,001	*Enpp2*
1,95	< 0,001	2,11	< 0,001	*Cdkn1a*
1,95	< 0,001	1,47	< 0,001	*Folr1*
1,92	0,003	1,44	0,005	*Calb2*
1,90	< 0,001	2,26	< 0,001	*Zbtb16*
1,86	0,001	2,41	0,001	*Nr4a1*
1,83	< 0,001	1,44	< 0,001	*Slc2a12*
1,80	< 0,001	1,54	< 0,001	*Igf2*
1,78	< 0,001	1,68	< 0,001	*Clic6*
1,75	0,004	1,29	NS	*Slc17a6*
1,74	< 0,001	1,87	< 0,001	*Pdk4*
1,72	< 0,001	1,44	0,002	*Otx2*
1,69	< 0,001	2,17	< 0,001	*Dusp1*
1,69	0,002	1,73	< 0,001	*Hspa8*
1,68	0,003	1,70	< 0,001	*Hspa8*
1,66	0,003	1,43	0,029	*AI506816*
1,65	< 0,001	1,34	< 0,001	*Prlr*
1,62	< 0,001	1,76	< 0,001	*Adipor2*
1,62	< 0,001	1,66	< 0,001	*Tiparp*
1,61	0,014	1,30	NS	*Zic1|LOC100044533*
1,58	< 0,001	1,97	< 0,001	*Npas4*
1,58	0,002	1,20	NS	*1500015O10Rik*
1,57	< 0,001	1,39	< 0,001	*C230095G01Rik*
1,56	< 0,001	1,67	< 0,001	*Nfkbia*
1,56	< 0,001	1,34	0,003	*Rasd1*
1,56	0,033	1,34	NS	*Agxt2l1*
1,55	< 0,001	1,24	0,002	*Igfbp2*
1,54	0,005	1,11	NS	*AW551984*
1,54	< 0,001	1,42	< 0,001	*F5*
1,53	< 0,001	1,34	< 0,001	*Trpm3*
1,52	0,004	1,66	0,001	*Arc*
1,52	0,007	1,12	NS	*Baiap3*
1,51	0,009	1,19	0,026	*6430550H21Rik*
1,50	0,008	1,41	0,009	*Zic2*
1,49	< 0,001	1,51	< 0,001	*Zfp189*
1,49	< 0,001	1,75	0,001	*Fos*
-1,55	< 0,001	-1,51	< 0,001	*Kdr*
-1,68	0,008	-1,24	NS	*BC003993*
-1,68	0,008	-1,24	NS	*BC003993*
-1,68	0,008	-1,24	NS	*BC003993*
-1,68	0,008	-1,24	NS	*BC003993*
-1,68	0,008	-1,24	NS	*BC003993*
-1,68	0,008	-1,24	NS	*BC003993*
-1,68	0,008	-1,24	NS	*BC003993*

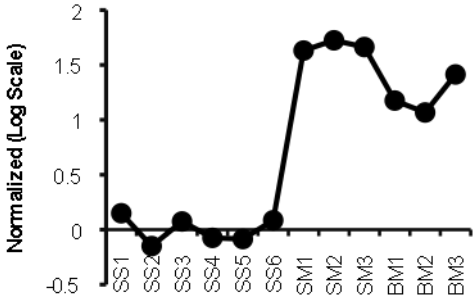
B



C



D



E