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Epigenetic Regulation of Opioid-Induced Hyperalgesia, Dependence and Tolerance in Mice

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Abstract

Repeated administration of opioids such as morphine induces persistent behavioral changes including opioid-induced hyperalgesia (OIH), tolerance and physical dependence. In the current work we explored how the balance of histone acetyltransferase (HAT) vs. histone deacetylase (HDAC) might regulate these morphine-induced changes. Nociceptive thresholds, analgesia and physical dependence were assessed during and for a period of several weeks after morphine exposure. To probe the roles of histone acetylation, the HAT inhibitor curcumin or a selective HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) was administered daily to groups of animals. Histone acetylation in spinal cord was assessed by Western blotting and immunohistochemistry. Concurrent administration of cucumin with morphine for 4 days significantly reduced development of opioid-induce mechanical allodynia, thermal hyperalgesia, tolerance and physical dependence. Conversely, the HDAC inhibitor SAHA enhanced these responses. Interestingly, SAHA treatment after the termination of opioid administration sustained these behavioral changes for at least 4 weeks. Histone H3 acetylation in the dorsal horn of the spinal cord was increased after chronic morphine treatment, but H4 acetylation was unchanged. Moreover, we observed a decrease in HDAC activity in the spinal cords of morphine treated mice while overall HAT activity was unchanged suggesting a shift towards a state of enhanced histone acetylation.

Keywords

Opioid; tolerance; hyperalgesia; dependence; histone acetylation

Introduction

Opioids currently constitute the cornerstone therapy for moderate to severe pain of many etiologies. One of the more controversial aspects of their use relates to uncertainties about adaptations to chronic use which include concerns over tolerance, opioid-induced hyperalgesia (OIH) and dependence [10, 40]. These adaptive phenomena have been studied for

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decades with notable insights gained from experiments, usually pharmacological, in which the function of a single protein in a known pathway, e.g. receptor, ion channel or kinase, was implicated in one or more of the adaptive processes. Likewise, newer genetic techniques have been used to identify gene candidates contributing to tolerance, OIH and dependence [20, 27, 34]. While very valuable, both approaches fail to identify mechanisms integrating the many adaptive changes which occur after chronic opioid exposure. Epigenetic mechanisms, on the other hand, may be responsible for the integration of these complex cellular changes.

The term "epigenetics" has been conceived of differently by various investigators since the time of introduction of the concept. Recently Bird[8] attempted to provide a contemporary definition updated to take into account more recent aspects of our understanding of these events, "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states." Commonly studied epigenetic processes include the modification of histone proteins by acetylation, phosphorylation and methylation as well as the methylation of DNA, though many other epigenetic mechanisms exist as well. The acetylation of histone at specific lysine residues causes the relaxation of chromatin and, generally, the enhancement of transcription of genes in the relaxed regions [22]. The acetylation process is regulated by the balance of activity of histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes. Epigenetic processes are characterized by the sustained and sometimes heritable changes they can cause. Thus epigenetic processes may be especially well suited to effecting chronic changes in gene expression and function such as are seen after chronic exposure to opioids.

As plausible as epigenetic mechanisms are as controllers of opioid adaptations, relatively little work has been done in this area, and those data are not completely consistent. This is in contrast to the better developed understanding of epigenetics and addiction involving substances like cocaine and alcohol. For example, Matsushita et al. reported that the HAT inhibitor curcumin reduced morphine tolerance [32]. The proposed mechanism was through a reduction in morphine-induced up-regulation of brain derived neurotrophic factor (BDNF). On the other hand, Sanchis-Segura et al. found enhancing effects of the relatively non-specific HDAC inhibitor sodium butyrate on morphine-induced locomotion and conditioned place preference (CPP), but not on tolerance or physical dependence [39]. Finally, Wang et al. using a CPP paradigm different from that of Sanchis-Segura et al. found that sodium butyrate actually inhibited the CPP reinstatement behavior under study [41]. Given the dearth of data on the roles of epigenetic mechanisms in regulating opioid adaptations, we set out to address two questions, 1) Do pharmacological agents regulating histone acetylation alter the behavioral adaptations observed after the chronic administration of morphine?, and 2) Do pharmacological modifiers of epigenetic enzymes alter the state of histone acetylation in the dorsal region of spinal cord tissue, a center for the neuroadaptive effects of opioids?

Materials and Methods

Animal Subjects

All animal experiments were done after approval of protocols by the Veterans Affairs Palo Alto Health Care System Institutional Animal Care and Use Committee (Palo Alto, California, United States) and complied with the Guide for the Care and Use of Laboratory Animals available through the National Academy of Sciences. Male C57BL/6J mice were obtained from Jackson Laboratory (JAX, Bar Harbor, ME) at 7–8 weeks of age. Mice were kept a further 7–10 days from the date of arrival in our animal care facility prior to use to allow for acclimation. Mice were housed 4–6 per cage under pathogen-free conditions with soft bedding and were provided food and water ad libitum with a 12:12 light:dark cycle.

Chronic Morphine Administration

After baseline nociceptive testing, morphine (Sigma Chemical, St. Louis, MO) was subcutaneously administered to mice 10 mg/kg twice per day on day1, 20 mg/kg twice per day on days 2–3 and 40 mg/kg twice per day on day 4 in 50–100 μl volumes of 0.9% NaCl similar to our previous protocols for OIH and tolerance [5, 26–29].

Suberoylanilide Hydroxamic Acid and Curcumin Administration

Suberoylanilide hydroxamic acid (SAHA) was purchased from Cayman Biochemical (Ann Arbor, Michigan), which was dissolved and diluted in a vehicle of DMSO. Animals received SAHA 50 mg/kg or vehicle, daily via i.p. injection. The drug was either given concurrently with morning morphine treatment during the chronic dosing paradigm, or given daily for 6 days after termination of morphine treatment.

Curcumin Administration

Curcumin was purchased from Sigma Chemical (St. Louis, MO), which was dissolved and diluted in a vehicle of DMSO. Animals received curcumin 50 mg/kg or vehicle, daily via i.p. injection. The drug was given concurrently with morning morphine treatment during the chronic dosing paradigm.

Behavioral Measurement

Opioid-induced hyperalgesia: Mechanical allodynia was assessed using nylon von Frey filaments according to the “up-down” algorithm described by Chaplan et al. [11] as previously described [3, 5]. For these measurements, mice were placed on mesh platforms within transparent plastic cylinders. After 15 minutes of acclimation, nylon fibers of sequentially increasing stiffness were applied to the plantar surface of one hind paw, and left in place 5 sec. Withdrawal of the hind paw from the fiber was scored as a response. If no response was observed, the next stiffer fiber was applied to the same paw; if a response was observed a less stiff fiber was applied. Testing continued until 4 fibers had been applied after the first withdrawal response allowing the estimation of the mechanical withdrawal threshold [21]. This data fitting algorithm allowed the use of parametric statistics for analysis.

Heat Hyperalgesia

Paw withdrawal response latencies to noxious heat stimulation were measured using the method of Hargreaves et al[16]. as we have modified for use with mice[25]. In this assay, mice were placed on a temperature-controlled glass platform (29 °C) in a clear plastic enclosure similar to those described above. After 30 min of acclimation, a beam of focused light was directed towards the same area of the hindpaw as described for the von Frey assay. A 20s cutoff was used to prevent tissue damage. In these experiments, the light beam intensity was adjusted to provide an approximate 10s baseline latency in control mice. Three measurements were made per animal per test session separated by at least one minute.

Morphine dose-response

Cumulative morphine dose-response curves were constructed using the tail flick assay and methods similar to those we described previously [5]. For these measurements mice were gently restrained within a cone shaped tube made of cotton toweling. Using a tail-flick analgesic apparatus (Columbus Instruments, Columbus, OH), tail flick latency was measured with 0.1 second precision. A 10 second cutoff time was used to prevent tissue sensitization or damage. Two measurements were made per mouse with the light beam focused on two different points 1cm apart on the tail. The lamp intensity was identical for all animals providing baseline tail flick measurements of 3–4 seconds. For the assessment of tolerance, these dose-response experiments followed 18 hours after the final dose of

morphine given as part of the chronic morphine administration protocol. The cumulative doses of morphine used were 1, 3 and 10 mg/kg. Tail flick latency was determined 25 min after morphine injection as previous experiments established 25min to be the time at which peak morphine effect was achieved. The percent maximal possible effect (%) was determined according to the following formula:

$$\text{The percent maximal possible effect (\%)} = 100^{*}(\text{measured latency} - \text{baseline latency})/(\text{cutoff latency} - \text{baseline latency})$$

Precipitated withdrawal

For physical dependence determinations, mice were challenged a with s.c. injection of naloxone (Sigma Chemical) 10 mg/kg in 100 μl saline as described previously by our laboratory and others [5, 19]. After naloxone administration, mice were placed in transparent plastic cylinders (10 cm in diameter and 40 cm in height, and the number of jumps during the following 15 minutes was counted.

Immunohistochemical Analysis

The methods used for the immunohistochemical localization of aceH3K9 and aceH4K16 were similar to those reported previously [12]. Briefly, mice were sacrificed using carbon dioxide asphyxiation, followed by trans-cardiac perfusion of 20 ml 0.9% NaCl followed by 20 ml of 10% neutrally buffered formalin. The spinal cords were subsequently extruded and fixed in 10% neutrally buffered formalin for 12 h at room temperature followed by overnight incubation in 30% sucrose at 4°C. The lumbar regions of the spinal cords were embedded in OCT medium, and 30 μm sections were made. These were processed using floating slice techniques. Blocking took place overnight at 4 °C with Blocking Buffer including 5% NGS in TBS-T, followed by exposure to the primary antibody, aceH3K9(1:5000, Epigentek, Brooklyn, NY) or aceH4K16 (1:500, Millipore, Temecula, CA). After incubation overnight at 4 °C, slices were rinsed three times in tris–buffered saline (TBS)-Tween-20(TBS-T) and transferred to TBS-T containing FITC-conjugated anti-rabbit or anti-goat second antibody (1:300, Vector Lab Linc & Jackson Immunano Research Lab,) and incubated for 1 h. Sections were then rinsed and mounted on glass slides, followed by the application of coverslips. Confocal laser-scanning microscope was carried out using a Zeiss LSM/510 META microscope (Thornwood, NY). Sections from control and experimental animals were processed in parallel. Control experiments omitting either primary or secondary antibody revealed no significant staining.

Nuclear Protein Extraction

Nuclear protein of lumbar spinal cord tissue was extracted by using cytoplasmic/Nuclear Protein Extraction Kit (BioRad, Hercules, CA, USA). The procedures were performed according to the protocol provided by the manufacturer. Briefly, spinal cord tissue was homogenized in cytoplasmic protein extraction buffer (CPEB) containing protease inhibitors. The homogenate was incubated on ice for 2 minutes to allow any tissue fragments to sediment to the bottom. The supernatant was transferred to a new tube, and centrifuged at 1000× g for 10 min at 4 °C. After washing, the pellet containing nuclei was suspended in 0.5ml of complete protein solubilization buffer (PSB) for Western blot analyses. The concentration of nuclear protein was measured using the DC Protein Assay kit (Bio-Rad, Hercules, CA, USA).

Two-color fluorescent Western Blot Analysis

Western blot analysis was conducted in Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, Nebraska) according to method provided by the manufacture. Equal

amounts of nuclear protein (30 μg) were resolved by Ready 10%Tris-HCl Gel (BioRad, Bio-Rad, Hercules, CA, USA) and transferred onto a Nitrocellulose membrane (Li-membrane. The blot was blocked for 2 hours in Blocking Buffer (LI-COR Biosciences, Lincoln, Nebraska), then incubated with primary antibody (diluted 1:2000) to aceH3K9(Epigentek, Brooklyn, NY) or aceH4K16 (Millipore, Temecula, CA) and goat polyclonal β-actin antibody(Santa Cruz Biotech, Santa Cruz, CA) on a rocking platform at 4 °C for overnight. After washing in TBST, the blots were incubated 1 hour at room temperature in two dye labeled secondary antibodies: goat anti-rabbit IRDye 680 (anti-rabbit (red) against H3K9 or H4K16 primary antibodies) and IRDye 800 (anti-mouse (green), against β-actin. After washing, the membrane was scanned and the fluorescent signal was quantified in the Odyssey system. The data was normalized by β-actin.

HDAC and HAT Activity Assay

Histone deacetylase activity—HDAC activity was measured by the use of a colorimetric assay system (AK-501, Biomol, Exeter, UK) according to the manufacturer's protocol. Briefly, the procedure involved the use of HDAC colorimetric substrate (Color de Lys substrate, 500 μm), possessing an acetylated lysine side chain incubated with a sample containing nuclear extract. Deacetylation due to the sample's HDAC activity produced a chromophore analysed with a colorimetric plate reader at 405 nm. HeLa cell nuclear extract was used as a positive control. A standard curve was prepared, using known amounts of the deacetylated standard (Boc-Lys-pNA) provided. Results are expressed as μm of HDAC per mg of nuclear protein.

Histone acetylase activity—HAT activity was measured by using a colorimetric assay kit manufactured from Epigentek Group (Farmingdale, NY) according to the manufacturer's protocol. Briefly, the procedure was involved in direct measurement of HAT activity by quantifying the amount of acetylated histone substrate produced from a unique histone substrate bound by active HATs in a sample containing nuclear extract. The acetylated histone substrate was recognized with a high affinity anti-acetylated histone antibody. The amount of the acetylated histone was colorimetrically quantitied through an ELISA-like reaction in 96-well microplate. HeLa cell nuclear extract was used as a positive control. A standard curve was prepared, using known amounts of an included HAT standard. Opitcal density was read on a microplate reader at 450nm. Results are expressed as OD value of HAT per mg of nuclear protein.

ChIP Assay

Chromatin was prepared using the Millipore ChIP kit (Waltham, MA). Spinal cord lumbar tissue was dissected and minced on ice, followed by cross-linked using 1% formaldehyde (Sigma, St. Louis, MO) and sonicated for 180s (intervals of 10 s on and 10 s off) on ice using a Branson sonicator with a 2-mm microtip at 40% output control and 90% duty cycle settings. The sonicated chromatin precipitated with specific antibody (5 μg) against aceH3K9 (Millipore)or IgG (Millipore) as negative control. Sonicated chromatin 1% was used for input control. DNA that was released from the bound chromatin using proteinase K, and was purified using the phenol-chloroform method. Target gene promoter enrichment in ChIP samples was measured by quantitative PCR using the ABI prism 7900HT system and SYBR Green. Primer sequences used to amplify the promoter region of BDNF were designed by the Primer 3 program: GCGCGGAATTCTGATTCTGGTAAT (forward), and GAGAGGGCTCCACGCTGCCTTGACG (reverse). Five microliters of input, ChIP, or IgG sample were used in each reaction and were run in duplicate for three biological samples in each condition. Fold enrichment was calculated by using ΔΔCt Method. The data were normalized using input and IgG results.

Quantification of mRNA

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). Complementary DNA was synthesized from total RNA using random hexamer priming and a first strand synthesis system (Invitrogen, Carlsbad, CA). Expression of BDNF gene was determined by quantitative real-time PCR. Target gene primers were: CCATAAGGACGCGGACTTGTAC (forward), and AGACATGTTTGCGGCATCCAGG (reverse). β-actin was used as an internal control. Its primers were AGACATGTTTGCGGCATCCAGG (forward), and CTCTCAGCTGTGGTGGTGAA (Reverse). The expression level of specific genes was analyzed with ΔΔCt Method.

Statistical Analysis

All data are expressed as the means ± standard error of the mean (SEM) unless otherwise noted. The data for mechanical sensitivity, tail flick response, and enzyme activity in spinal cord were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test for multiple comparisons. Simple comparisons of 2 groups involved unpaired t-testing with two-tail p values. $P<0.05$ was considered significant.

Results

The effects of HAT and HDAC inhibitors on OIH

Histone acetylation is reciprically controlled by the HAT and HDAC enzyme systems, and has been observed to control long term adaptations to substances of abuse such as cocaine and alcohol. To determine whether acetylation alters adaptations to opioid administration including the acquisition of OIH, we determined if the HAT inhibitor curcumin or the HDAC inhibitor, SAHA affected OIH induced by chronic morphine treatment. Figure 1A and B present data demonstrating that after 4 days of escalating dose morphine administration mice display thermal hyperalgesia and mechanical allodynia similar to our previous observations [26]. However, if mice received daily curcumin administration along with morphine, OIH was prevented whereas mice receiving morphine and SAHA trended towards enhanced levels of thermal and mechanical sensitization. Neither curcumin nor SAHA altered nociceptive thresholds if administered by themselves.

While SAHA effects on nociception added only slightly to the sensitizing effects of morphine immediately after completion of morphine treatment, we hypothesized that morphine-SAHA co-administration might prolong the duration of the resulting OIH. We therefore treated separate groups of mice with morphine, morphine and SAHA or, SAHA as a control, alone for 4 days. The OIH due to morphine alone was detectable during the first 4 days after morphine administration, and then returned to baseline. However, SAHA if administered with morphine significantly extended the duration of OIH such that the animals had not fully recovered even 28 days after the cessation of morphine administration. SAHA administration alone had no effect in these experiments (Figure 1C).

We next sought to determine if SAHA treatment beginning after establishing OIH would prolong the sensitization seen in mice. Animals first received escalating morphine treatment followed by measurement of mechanical thresholds. The mice then received daily injections of SAHA or vehicle as a control. Mice receiving only vehicle injections after eshablishing OIH quickly recovered, but those receiving the HDAC inhibitor SAHA demonstrated prolonged mechanical sensitization measurable for 28 days (Figure 1D). These data demonstrate that inhibition of HDAC activity with SAHA during or after morphine administration substantially prolongs OIH in mice.

The effects of HAC and HDAC inhibitors on morphine tolerance

Tolerance is a significant maladaptation reducing the clinical effectiveness of opioids. Previous work demonstrates that OIH and tolerance likely share an overlapping set of mechanisms [5]. Figure 2A shows data demonstrating that after 4 days of escalating doses of morphine, mice displayed significant anlagesic tolerance to morphine when compared to vehicle treated animals. However, the co-administration of curcumin with morphine prevented tolerance. In another set of experiments, SAHA co-administration enhanced morphine tolerance as shown in Figure 2B. To determine if SAHA administered after establishing morphine tolerance is effective in maintaining tolerance, SAHA was administered daily to mice after completion of morphine administration. The data in Figure 2C demonstrate that while tolerance has largely resolved 7 days after the completion of morphine administration in control animals, significant tolerance is still evident in the subsequently SAHA treated mice. Importantly, the repeated administration of SAHA or curcumin alone did not alter morphine's analgesic effects.

The effects of HAC and HDAC inhibitors on morphine dependence

Physical dependence occurs predictably after the long term administration of opioids for therapeutic purposes and in opioid addiction. We therefore assessed the role of histone acetylation in determining the extent and duration of physical dependence after 4 days of morphine administration. In our first experiments, animals received morphine plus concurrent treatment with vehicle, curcumin or SAHA for 4days. On day 5 we measured withdrawal responses after naloxone injection. Treatment with curcumin diminished withdrawal responses in comparison with morphine alone. Conversely, SAHA co-administration with morphine enhanced the withdrawal responses as shown in Figure 3A. Neither curcumin nor SAHA administered alone caused the animals to be responsive to naloxone administration.

In light of the prolonged OIH and tolerance observed in morphine-SAHA treated mice, we followed opioid dependence in mice for up to 28 days after completion of morphine or morphine plus SAHA. Figure 3B demonstrates that SAHA extended the withdrawal response for at least 4 weeks after treatment termination, while animals in the control (morphine alone) group displayed withdrawal responses only within the first week after treatment termination. To further refine our understanding of the effects of SAHA on withdrawal responses we first treated animals with morphine according to our regular treatment paradigm, and followed this with daily SAHA treatment as done for animals used in the OIH and tolerance experiments. Figure 3C shows data demonstrating that SAHA treatment after establishing morphine dependence maintains that dependence long-term (up to 28 days).

Alteration of histone acetylation induced by morphine in spinal cord tissue

Since inhibitors of HAT and HDAC significantly affected opioid adaptations, we hypothesized that these behavioral changes may be associated with enhanced histone acetylation in spinal cord tissue. To test this idea we employed immunohistochemistry and western blot approaches to detect acetylated H3K9 and H4K16, which are crucial histone subtypes observed to be hyperacetylated after exposure to other substances of abuse [33]. The time point selected was 18 hours after the final dose of morphine in the chronic dosing paradigm as this was the time point when adaptive changes were maximal. Immunohistochemistry data demonstrated that morphine treatment significantly increased acetylation of H3K9 in the dorsal horn of the spinal cord in comparison with the control group as shown in Figure 4A and B. Western blot data further demonstrated that ace-H3K9 expression was increased in the opioid treated group in comparison with the saline treated control group as shown in Figure 4C and D. However, acetylation of H4K16 was not

significantly regulated after morphine treatment in either immunohistochemical or western blot analyses (Figure 5A–D).

Alteration of HDAC and HAT enzymatic activity in spinal cord tissue

We further addressed the balance of HAT/HDAC enzymatic activity controlling histone acetylation in homogenates of mouse spinal cord tissue. In Figure 6A data are presented showing a moderate and statistically significant reduction in HDAC activity after chronic treatment with morphine. However, no change in HAT activity was detected in this tissue after morphine treatment shown as Figure 6 B. Thus the balance of enzymatic HAT/HDAC activity shifts towards the maintenance of histone acetylation in morphine trated mice.

Epigenetic effects of morphine on BDNF expression

To probe the epigenetic mechanisms supporting OIH, tolerance and physical dependence, we completed studies involving mRNA expression and acetylated H3 ChIP analysis of BDNF in spinal cord tissue. BDNF is a notable biomarker which is well established to be involved in opioid adaptations. Epigenetic regulation of expression via H3 acetylation was demonstrated in other models [42]. Spinal cord tissue was collected from separate groups of mice having undergone escalating morphine injections or saline injections. Tissues were harvested 18 hours after the final dose of morphine, a point at which opioid adaptive effects are maximal.

Figure 7A first shows that BDNF mRNA levels were significantly elevated under our morphine treatment conditions in mouse lumbar spinal cord tissue. Samples processed from separate mice then underwent ChIP analysis. Figure 7B shows that the promoter region of this gene was more strongly associated with acetylated H3 (H3K9) after morphine treatment than under control conditions.

Discussion

Adaptations to chronically administered opioids limit their utility as analgesic agents and contribute to their abuse potential. Understanding the underpinnings of these adaptations may therefore provide insight into methods to prevent or limit the extent of these changes. Epigenetics offers a framework of mechanisms providing an interface between “environmental” conditions (including exposure to drugs) and the genome. These interactions are likely of great relevance to pain and analgesic mechanisms though they are poorly understood at this point [13]. Epigenetic mechanisms do not involve genetic or DNA sequence based differences per se, but share the property of being able to alter behavior and physiology by altering the rate of DNA transcription, or, in the case of micro RNA, translation. In these studies our principal observations were that 1) if given concurretly with morphine, drugs blocking histone acetytransferase (HAT) or histone deacetylase (HDAC) can diminish or enhance OIH, tolerance and physical dependence, 2) inhibitors of HDAC activity if given after opioid adaptations are established can prolonge the adapted state, 3) acetylation of the H3 histone subunit at lysine residue 9 in dorsal horn spinal cord tissue (H3K9) coincides with the behavioral changes, and 4) the overall enzymatic activity of HDAC is reduced while that of HAT is unaltered in spinal cord tisue. Together these observations suggest that epigenetic mechanisms are functionally relevant to the long term adaptations caused by sustained opioid administration.

Though a diverse range of processes can be thought of as “epigenetic,” much of the current interest is in studying the effects of non-sequence modifications of DNA such as methylation and the modification of histones though acetylation, methylation and other processes. In our work we focused on the acetylation of two histone proteins helping to form

the core of DNA-protein nucleosomes as histone acetylation has been shown to modulate the neuroplastic effects of exposure to other drugs of abuse [37]. Both the H3 and H4 histone subunits undergo acetylation at specific lysine residues on the tails of those proteins. Perhaps the best understood effect of such acetylation is the conversion of transcription-restrictive heterochromatin to transcription-permissive euchromatin; histone acetylation often leads to enhanced gene expression [6]. Such modifications are tightly regulated, and are cell type specific [4]. Each of the principal enzyme classes responsible for acetylation, the histone acetyl transferases (HATs) and histone deacetylases (HDACs), have several family members, and the balance of HAT versus HDAC activity governs the overall levels of histone acetylation [7, 35]. It is notable in this regard that we observed a relative decrease in overall HDAC activity after morphine treatment with unchanged overall HAT activity consistent with greater DNA acetylation in response to this drug. We did not explore the duration of altered HDAC enzyme activity, though it is possible that the profound pharmacological inhibition of HDAC activity with SAHA near the time of opioid exposure leads to a relatively persistently changed system.

The field of substance abuse research has increasingly turned to epigenetics to provide mechanisms broadly supporting drug-induced neuroplasticity. For example, it has been observed that the inhibition of histone deacetylation resulting in enhanced acetyl-H3 levels both enhanced conditioned place preference (CPP) for cocaine as well as self-administration [23]. On the other hand, the over-expression of HDAC4 in the nucleus accumbens using a viral vector diminished H3 acetylation and CPP with cocaine [41]. Complementary data were provided in experiments using cAMP-response element binding protein-binding protein (CBP) deficient mice, a strain of mice with reduced HAT activity [24]. These mice displayed less sensitization to chronic cocaine administration than did control mice. Both sets of observations suggest H3 acetylation is critical to the maladaptive changes occurring during chronic cocaine administration. It needs to be mentioned, however, that not all reports directly support this model; the non-selective HDAC inhibitor sodium butyrate was observed to accelerate extinction of cocaine-induced CPP in one set of studies [31].

Additional experiments have demonstrated that an array of genes in the CNS are up-regulated after chronic cocaine administration via an histone acetylation dependent mechanism including the genes for prodynorphin, CamKII, CDK5 and BDNF [9, 23, 41]. We hypothesized that morphine treatment may lead to similar changes. BDNF was of particular interest as this gene is linked to opioid tolerance and dependence[2, 32]. In the current work BDNF was observed to be up-regulated at the mRNA level after morphine treatment, and there was enhanced association with acetylated histone H3 (H3K9). Moreover, the analysis of enhanced histone acetylation near gene promoters after chronic cocaine administration led to the identification of more than 700 genes in nucleus accumbens tissue alone [36]. This suggests that histone acetylation is an epigenetic mechanism capable of broadly coordinating changes in gene expression in the setting of chronic drug exposure. In the future both the identification of epigeneticaly regulated genes and the functional significance of the regulation of specific genes will be important goals.

Epigenetic influences on changes in behavior related to chronic opioid administration have received comparatively little study. Matsushita et al. used curcumin as an inhibitor of CBP's HAT activity during a 6-day chronic morphine administration paradigm and observed a reduction in analgesic tolerance as well as a reduction in BDNF levels in brain tissue [32]. Our data are consistent with this finding in that curcumin inhibited analgesic tolerance in our model when co-administered with morphine while it had no effect on mophine analgesia if given alone. We were also able to extend these observations by showing that curcumin reduced OIH and dependence as well. Unfortunately, curcumin, while a useful probe reagent, is not completely selective for the inhibition of HAT enzymes. This compound also

interacts with HDACs and DNA methyl transferases though perhaps with lower affinity [15, 38].

The non-specificity of curcumin and most other available HAT inhibitors caused us to complement our curcumin studies with additional experiments examining the effects of HDAC inhibition. Our data demonstrate that HDAC inhibition using a selective agent (SAHA) perpetuates rather than speeds resolution of OIH, tolerance and dependence consistent with its function of sustaining histone acetylation. This drug is believed to be a competitive inhibitor of HDACs[14]. In fact, OIH and dependence continued for at least 28 days after administration of HDAC inhibitors, the limit of our observation period. Our data are therefore internally consistent in showing reduced opioid adaptations using HAT inhibition, and enahancement or prologation of adaptations when using HDAC inhibitors. Available reports from the literature are perhaps less consistent in their findings of HDAC inhibition on addiction-related opioid adaptations. One study used the non-selective HDAC inhibitor sodium butyrate administered after completion of morphine conditioning in a CPP paradigm. These investigators reported that sodium butyrate accelerated the extinction of morphine-induced CPP [41]. Jing et al. using a single morphine bolus administration protocol observed that the HDAC inhibitors sodium butyrate and valproic acid slightly reduced the behavioral sensitization to morphine if administered before exposure to this opioid [18]. Somewhat contrary to these observations, another report describes enhanced locomotor sensitization and CPP activity when HDAC inhibitors were co-administered with morphine [39]. Thus not all behavioral consequences of opioid administration are necessarily regulated by epigenetic mechanisms in the same way. Our studies have the strength of looking at several opioid effects in parallel under the same treatment conditions.

Aside from the study of epigenetic mechanisms as regulators of neuroplasticity, epigenetic mechanisms have become therapeutic targets. Curcumin, for example, has been used in a number of clinical trials primarily related to the control of cancer [17, 38]. Used as an oral therapeutic, this naturally occurring compound is well tolerated in doses of up to several grams per day, but suffers from relatively poor oral bioavailability. Limited data suggest that this agent may also be useful as an analgesic independent of its effects in slowing opioid adaptations [1]. A major issue with epigenetic agents is that the number of genes potentially effected by systemic drug administration. There is no reason to think that only genes related to pain and analgesia would be effected, and in fact opposing effects could be caused by a single agent. A major focus in pharmaceutical development related to epigenetic drugs is the targeting of specific isoforms of the disease-relevant enzymes as this may be an avenue towards greater therapeutic efficacy with fewer side effects[30]. In the case of adaptations to the chronic administration of morphine, we do not know at this time which of the many HAT and HDAC family members are involved.

In conclusion our data demonstrate that the regulation of histone acetylation can control the expression of three clinically relevant adaptations to the sustained administration of morphine including tolerance, dependence and OIH. These drug effects may be mediated via control of acetylation of H3K9 in spinal cord tissue. Future work will involve the identification of specific genes, or, more likely, groups of genes subject to this form of epigenetic regulation serving to support opioid-induced neuroplasticity. Blocking histone acetylation during chronic morphine administration may enhance the clinical utility of these drugs.

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Perspective

The current study indicates that epigenetic mechanisms play a crucial role in opioid-induced long-lasting neuroplasticity. These results provide new sight into understanding the mechanisms of opioid-induced neuroplasticity and suggest new strategies to limit opioid abuse potential and increase the value of these drugs as analgesics.

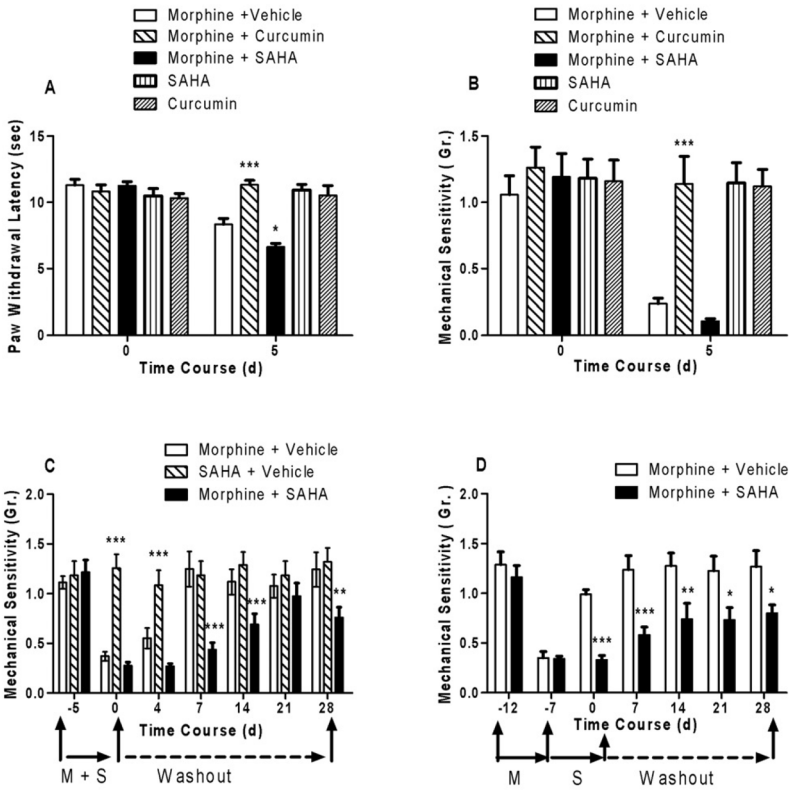

Figure 1.

The pharmacological effects of the histone acetyltransferase (HAT) inhibitor curcumin, and histone deacetylase (HDAC) inhibitor, suberoylanilide hydroxamic acid (SAHA) on heat hyperalgesia (A) and mechanical allodynia (B, C and D) induced by repeated morphine administration. In panel A data are presented representing paw withdrawal latency to heat stimulation after 4 days of morphine administration with concurrent administration of SAHA (50mg/kg, ip, daily), curcumin (50 mg/kg, ip, daily) or vehicle. In panel B data representing measurements of mechanical withdrawal thresholds are displayed from mice that underwent the treatment protocols as described for panel A. In panel C the duration of the effects of SAHA on mechanical allodynia induced by chronic morphine treatment were assessed. Mice received repeated concurrent injection of SAHA with morphine, SAHA with vehicle or vehicle with morphine followed by a 4-week observation period. In panel D animals received daily SAHA or vehicle treatment for 6 days after completion of the standard 4-day-treatment with morphine followed by a 4-week observation period. Data are presented as the means +/− SEM; *P<0.05; **<0.01; ***P<0.001 (comparison with control group). Six mice were used in each group. M: morphine; S: SAHA.

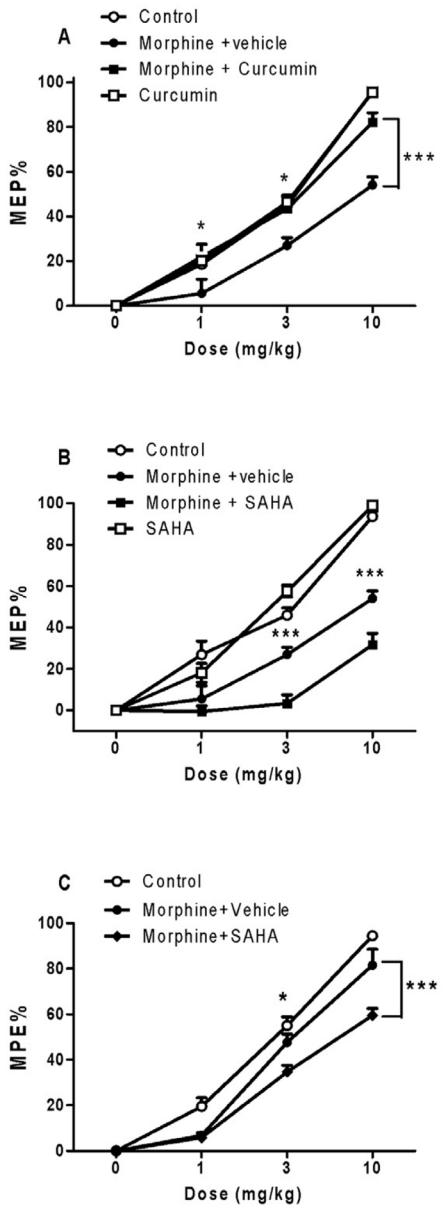

Figure 2.

The effects of the HAT inhibitor curcumin, and HDAC inhibitor SAHA on morphine analgesic tolerance. Morphine dose-response relationships were assessed using the tail flick assay. MPE%: The percent maximal possible effect (%). Panel A presents data for the effect of curcumin on morphine analgesic tolerance relative to morphine treatment with vehicle. Dose-response relationships were measured the day after completion of a 4-day morphine treatment protocol. The effects of curcumin administration alone are also presented, and overlapped with control group*P<0.05; **<0.01; ***P<0.001 (morphine + curcumin vs. morphine alone). Panel B presents the effects of concurrently administered SAHA on morphine analgesia using a protocol similar to that described for panel A. The effects of SAHA alone are also presented. ***P<0.001 (morphine + SAHA vs. morphine alone). Panel C presents the effects of SAHA on morphine analgesic tolerance if administered for 6 days beginning the day after completion of the standard morphine treatment protocol. Dose-response relationships were measured on day #7 after completion of repeated morphine

treatment. *P<0.05; ***P<0.001 (morphine followed by SAHA vs. morphine alone). All data are presented as the means +/− SEM. Six mice were used in each group.

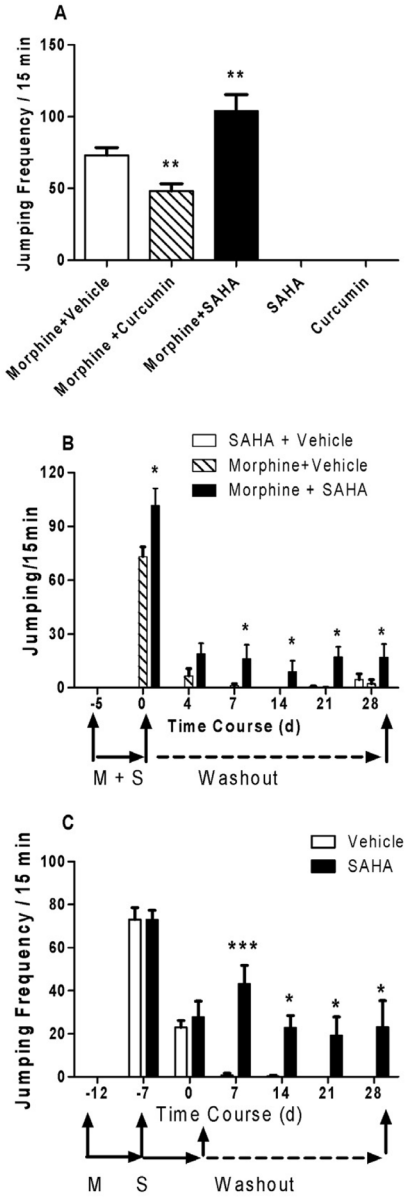

Figure 3.

The effects of the HAT inhibitor curcumin and HDAC inhibitor SAHA on physical dependence induced by morphine treatment. Physical dependence was assessed using the total number of jumps in 15 min. following naloxone injection. Panel A presents the effect of curcumin and SAHA co-administered with morphine on opioid dependence after a 4-day treatment protocol. *P<0.05 (morphine + SAHA vs. morphine alone). Panel B presents the persistent effects of SAHA on opioid dependence during a 4 week observation period after completion of drug administration. *P<0.05 (morphine + SAHA vs. morphine alone). Panel C presents the sustained effects of SAHA on opioid dependence during a 4-week observation period after termination of drug treatments. For these experiments, animals received SAHA treatment for 6 days after completion of the standard 4-day treatment with morphine, followed by a 4-week observation period. *P<0.05; ***P<0.001 (morphine followed by SAHA vs. morphine alone). Data are presented as the means +/− SEM. Six mice were used in each group. M: morphine; S: SAHA.

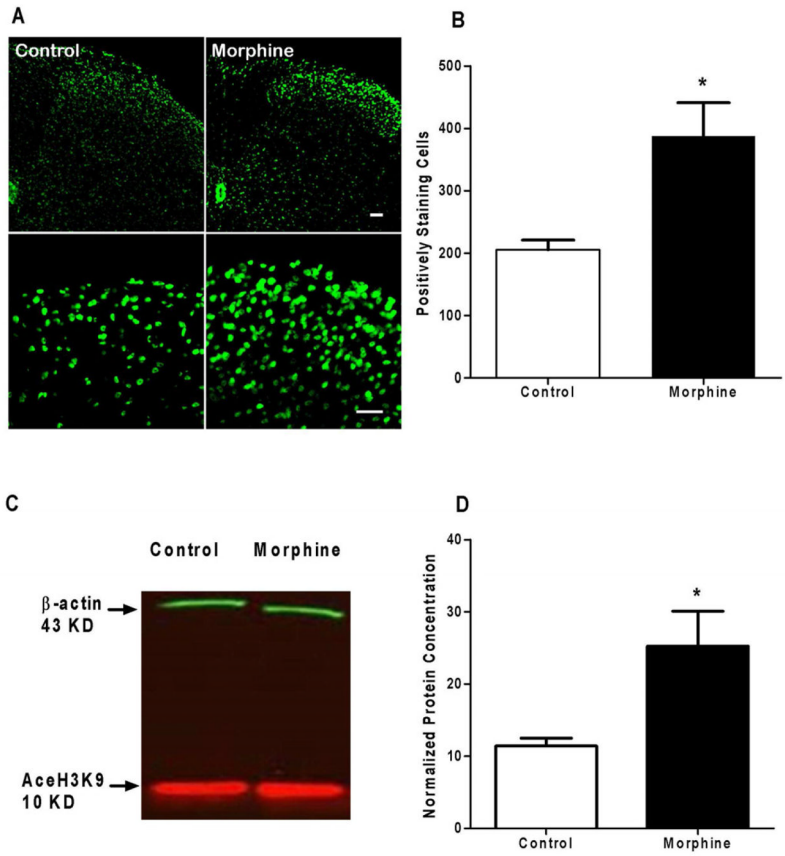

Figure 4.

Expression of aceH3K9 in spinal cord lumbar tissue after 4-days of morphine treatment. Lumbar spinal cord tissue was dissected on the fifth day. Panel A shows expression of aceH3K9 using immunohistochemical analysis. Scale bars are 25 and 50 μm for upper or lower photomicrograph pannels respectively. Panel B shows quantification of positively staining cells in the dorsal horn areas (n=5 animals/group, 3 micrographs/animal). Panel C demonstrates the expression of aceH3K9 using a Western blot assay on lumbar spinal cord tissue, and Panel D shows the quantification of those data presented as the means +/− SEM (n=5 animals/group, each sample run in duplicate). *P<0.05 (comparison with control group).

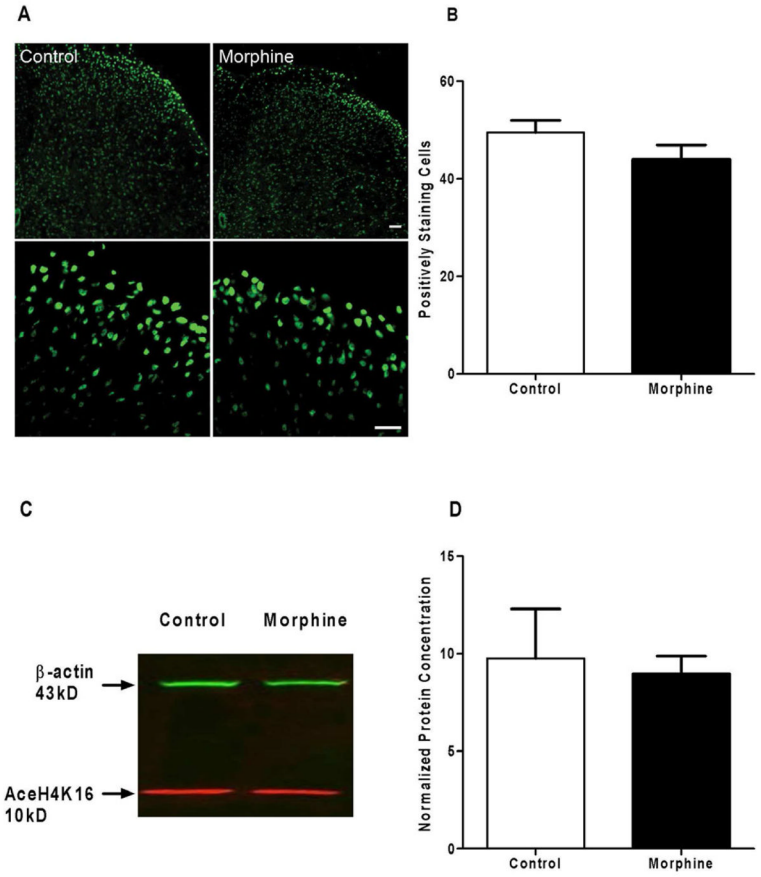

Figure 5.

Expression of aceH4K9 in spinal cord lumbar tissue after 4-days of morphine treatment. Lumbar spinal cord tissue was dissected on the fifth day. Panel A shows expression of aceH4K9 using immunohistochemical analysis. Scale bars are 25 and 50 μm for upper or lower photomicrograph pannels respectively. Panel B shows quantification of positively staining cells in the dorsal horn areas (n=5 animals/group, 3 micrographs/animal). Panel C demonstrates the expression of aceH4K9 using a Western blot assay on lumbar spinal cord tissue, and Panel D shows the quantification of those data presented as the means +/− SEM (n=5 animals/group, each sample run in duplicate).

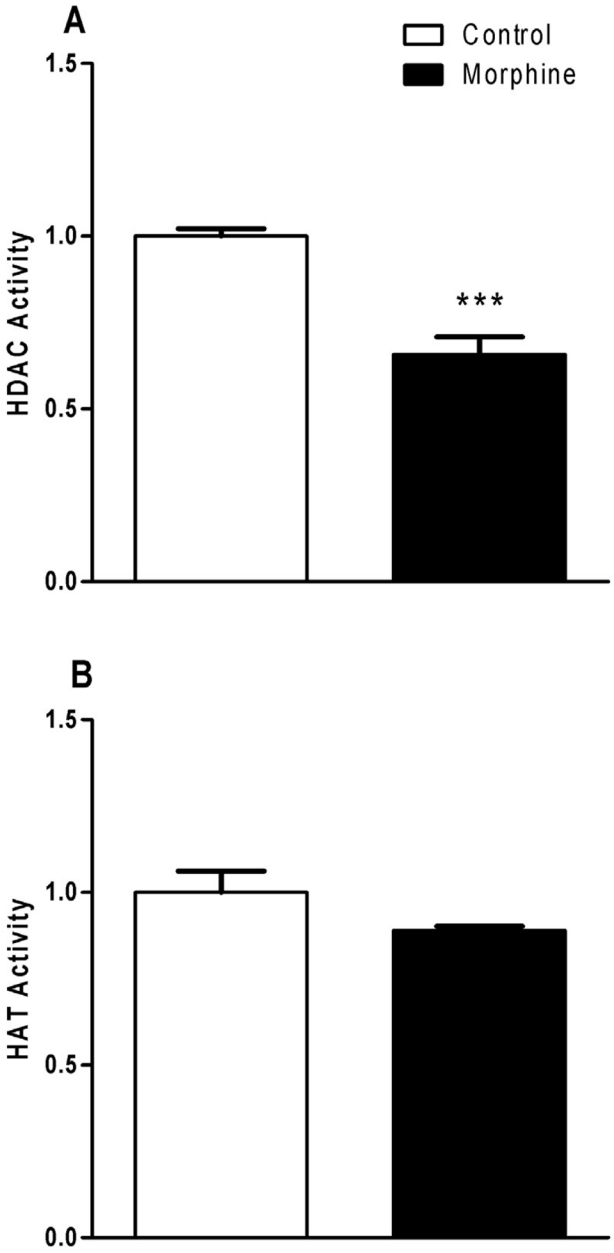

Figure 6.

The spinal cord enzymatic activity of HDAC and HAT after treatment with morphine or saline vehicle. Animal received morphine or vehicle treatment for 4-days. The spinal lumbar tissue was dissected on fifth day. Panel A represents the HDAC activity after treatment while panel B represents the HAT activity in nuclear extracts of spinal cord tissue. Data were normalized to control level of activity. Data are presented as the means +/– SEM (n=5 animals/group). ***P<0.001 (comparison with control group).

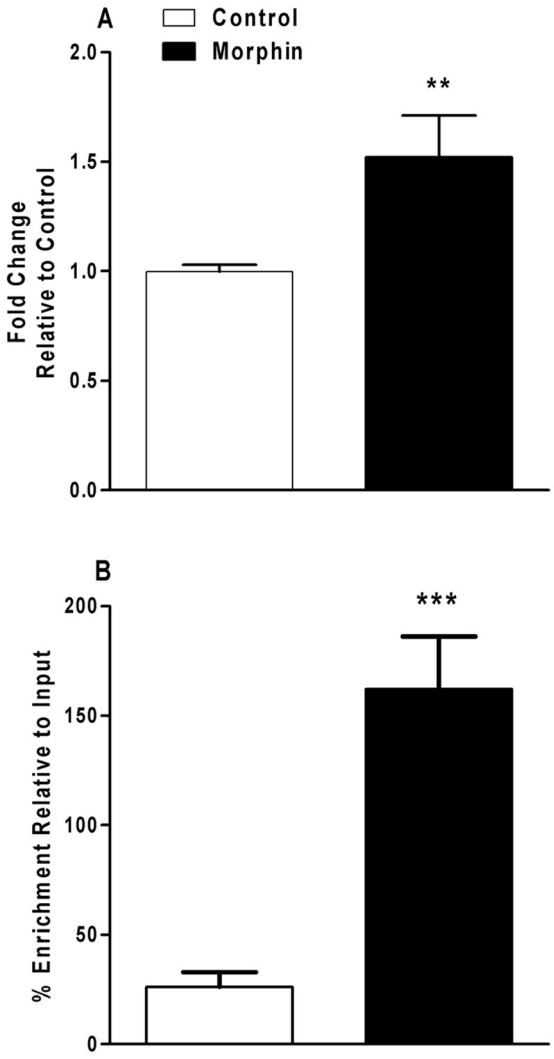

Figure 7.

mRNA and ChIP analysis of BDNF in spinal cord tissue after treatment with morphine or vehicle. BDNF: brain-derived neurotrophic factor. Panel A, mRNA expression of BDNF in lumbar spinal cord tissue induced by 4 days of treatment with morphine or saline. Panel B, changes in BDNF promoter region histone acetylation in spinal cord tissue after chronic morphine treatment detected using ChIP assay and real-time PCR. Data are presented as the means +/− SEM. ** $p<0.01$ and ***<0.001. Five mice were used for mRNA analysis, and three animals for ChIP assay in each group.