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Effects of Trichostatin A on Neuronal mu-Opioid Receptor Gene Expression

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Abstract

In this study, we determined the effects of a histone deacetylase (HDAC) inhibitor, trichostatin A (TSA), on neuronal mu-opioid receptor (MOR) gene expression using human neuronal NMB cells, endogenously expressing MOR. Recruitment of two classes of HDAC, HDAC1 and HDAC2, to MOR promoter region in situ was detected via chromatin immunoprecipitation (ChIP) analysis with NMB cells. Functional analysis using the luciferase reporter gene system showed that TSA induced an approximately 3-fold increase of the promoter activity as compared to the vehicle treated group. Mutation analysis demonstrated that TSA response was mediated by both dsDNA (Sp1/Sp3 binding site) and ssDNA (PolyC binding protein1, PCBP, binding site) elements located in mouse MOR proximal core promoter region, further suggesting the functional importance of this cis-element, which shows high sequence homology between human and mouse MOR genes. ChIP analysis further suggested that TSA enhanced the recruitment of Sp1/Sp3 and PCBP to the promoter region, whereas no significant changes of total proteins were observed in response to TSA using Western blot analysis. Moreover, confocal images showed TSA-induced nuclear hot spots of endogenous PCBP in neuronal cells, whereas no obvious nuclear PCBP hotspot was observed in vehicle treated cells. Taken together, these results suggested that TSA enhanced neuronal MOR gene expression at the transcriptional level. RT-PCR analysis further revealed that TSA also decreased the steady-state level of MOR mRNA in a time-dependent manner by enhancing its instability. Thus, data suggest that TSA, an epigenetic regulator, affects neuronal MOR gene expression at both transcriptional and post-transcriptional levels.

Keywords

neuronal MOR gene expression; proximal promoter; trichostatin A; Sp1/Sp3 and PCBP factors; transcriptional activation; mRNA degradation

1. Introduction

Nuclear DNA is packaged in basic structural units called nucleosomes, which consist of DNA and histones. The resulting chromatin may be in a compact or unfolded form, depending on

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physiological events such as replication, DNA repair or transcription. Two types of enzymes, histone acetyltransferase (HAT) and histone deacetylase (HDAC), can be recruited to the genomic arena and alter the acetylation status of histones. Acetylation and deacetylation of histone lysine residues are thought to fine-tune the DNA accessibility to other molecules (Legube and Trouche, 2003). These enzymes can also physically interact with sequence-specific transcription factors, and modulate local histones at promoter regions of target genes (Saha and Pahan, 2006). Additionally, some of transcription factors, such as TAF1 and CBP, are found to possess HAT or HDAC activity (Ogryzko et al., 1996; Spencer et al., 1997; Hilton et al., 2005).

HDAC inhibitors such as trichostatin A (TSA) have been used as a tool to investigate the dynamic relationship between chromatin structure and gene regulation. In general, HDAC inhibitors augment the acetylation of histones and activate gene transcription (Legube and Trouche, 2003; Sowa et al., 1999; Xiao et al., 1999). HDAC inhibitors also induce the acetylation of non-histone proteins, such as p53 (Gu and Roeder, 1997; Bannister and Miska, 2000).

However, HDAC inhibitors do not result in a generalized transcriptional activation (Gosh et al., 2007; Reid et al., 2005). Various studies (Glaser et al., 2003; Mitsiades et al., 2004; Peart et al., 2005; Van Lint et al., 1996) suggested that HDAC inhibitions can affect up to one fifth of all known genes, with an approximately equal ratio of upregulated versus downregulated genes, suggesting an effect that is somewhat gene-specific. HDAC inhibitors also induce epigenetic modifications, resulting in the alteration of gene expressions, proliferation, differentiation or apoptosis (Legube and Trouche, 2003; Saha and Pahan, 2006). Recently, HDAC inhibitors, such as TSA, have been introduced as a potential cancer treatment and are under phase I and II clinical trials.

Cancer patients suffer from cancer-induced pain, and morphine is clinically used to alleviate severe pain. Morphine-induced analgesia is mediated by mu-opioid receptors (MOR) (Kieffer and Gaveriaux-Ruff, 2002; Kieffer and Evan, 2002), which are mainly found in certain subsets of neurons in the central nervous system (CNS) (Mansour et al., 1995). Thus effects of HDAC inhibitors on the neuronal MOR gene expression would be of not only general interest, but possibly relevant to their clinical usage. MOR-1 gene encodes the most abundant MOR protein in the CNS (Chen et al., 1993; Min et al., 1994; Bare et al., 1994; Koch et al., 1998; Pan et al., 2001). Using deletional and transient transfection assays, three promoters (proximal, distal, and far upstream promoter) of mouse MOR gene were identified (Min et al., 1994; Ko et al., 1997; 2002; Pan et al., 2001). The proximal promoter initiated MOR transcription from four major transcription initiation sites (291 to 268 bp upstream of ATG), which are close to the translation initiation site (ATG) (Min et al., 1994). The distal promoter initiated the transcription from a single transcription initiation site, 794 bp upstream of the translation initiation site (Liang et al., 1995), and the far upstream promoter is located 10Kb upstream of translation initiation site (Pan et al., 2001). The proximal promoter is the main director of MOR transcription in brain (Ko et al., 1997). High sequence homology of the proximal core promoter between human and mouse MOR genes further suggests the functional importance of this region (Ko et al., 2005). In the proximal promoter, several transcription factors, including Sp1 and Sp3 (double-stranded (ds) DNA binding protein) and single-stranded (ss) DNA binding proteins, contribute critically to the neuronal MOR gene expression (Ko et al., 1998; 2001; 2003). Poly C binding protein 1 (PCBP), cloned from a brain cDNA library by yeast one-hybrid screening (Ko and Loh, 2005), is also involved in the MOR gene expression via binding to the ssDNA element of the proximal core promoter (Ko and Loh, 2005; Kim et al., 2005; Malik et al., 2006; Rivera-Gines et al., 2006). In this study, we have investigated the effects of a HDAC inhibitor, TSA, on the neuronal MOR gene expression.

2. Results

Presence of HDAC1/2 in MOR proximal promoter in situ

Recruitment of HDAC1 and HDAC2 to genomic DNA has been discussed (Legube and Trouche, 2003; Saha and Pahan, 2006). We first determined if histone deacetylase (HDAC), HDAC1 or HDAC2, was recruited to the MOR proximal promoter region, the predominant promoter directing MOR transcription in the neuronal system (Ko et al., 1997;2002), using chromatin immunoprecipitation (ChIP) assay with human neuronal NMB cells, endogenously expressing MOR. Chromatin from cells treated with HDAC inhibitor, trichostatin A (TSA), or vehicle (C) was subjected to immunoprecipitation using anti-HDAC1 and -HDAC2 antibodies (Ab), respectively. The immunoprecipitated DNA was then analyzed using PCR with a primer set encompassing the -491bp to -214 bp region of MOR proximal promoter (translation start site ATG designated as +1), including double-stranded (ds) and single-stranded (ss) DNA elements bound by Sp1/Sp3 and PCBP (Fig. 1A).

As shown in Fig. 1B, no precipitated DNA fragment was observed using either no antibody (lanes 1 and 2) or non-specific IgG antibody (lanes 11 and 12). The crude chromatin prior to immunoprecipitation was also examined (indicated as “input” in lanes 8 and 9). Anti-HDAC1 (lanes 4 and 6) and anti-HDAC2 (lanes 5 and 7) antibodies precipitated the 278 bp fragment of the MOR proximal promoter in the presence or absence of TSA. Collectively, ChIP analysis suggested the association of HDAC1 and HDAC2 with the MOR proximal promoter region in situ, indicating that TSA inhibited HDAC1/2.

Influence of TSA on MOR proximal promoter activity

To determine if TSA affected MOR gene transcription, the activity of the proximal core promoter, containing ss and ds binding sites for PCBP and Sp1/3 (Ko et al., 1998;2001;2003; Ko and Loh, 2005; Kim et al., 2005; Malik et al., 2006; Rivera-Gines et al., 2006), was examined. Neuronal NMB cells, transfected with the pL340/300 plasmid (Fig. 2A, left panel) containing a luciferase reporter gene driven by mouse MOR proximal core promoter (a region of high homology between mouse and human genes), were treated with TSA or vehicle (control). Using the luciferase reporter system, function analysis showed that TSA induced an approximately 3-fold increase of activity as compared to control, while no significant increase was observed using the pGL3-basic blank vector. This result suggested that TSA enhanced MOR core promoter activity in neuronal cells.

To test if the ds or ssDNA element mediated the TSA effect, mutant promoters (pLcsp or pLGGT) were used (Fig. 2A, left panel). The pLcsp plasmid, containing a consensus GC box, abolishes the ssDNA structure and the binding of PCBP to its ssDNA element, but retains the Sp1 and Sp3 binding (Ko et al., 2001). Conversely, the pLGGT plasmid, containing a trinucleotide GGT mutation in the ds DNA element, abrogates the binding of both Sp1 and Sp3 transcription factors, but ssDNA structure and the ssDNA binding of PCBP remain intact (Ko et al., 2001). As shown in Fig. 2A, right panel, the promoter activity of wild type construct (pL340/300) was arbitrarily defined as 100 %. Mutation (pLGGT) of both Sp1 and Sp3 binding resulted in an approximately 65±9% reduction in the promoter activity, and the pLcsp mutation resulted in an approximately 34±5% loss of the promoter activity, in response to TSA.

To further confirm that the core proximal promoter is an essential region in response to TSA, plasmids with a longer DNA fragment including the core proximal promoter were tested. The pL450 plasmid containing the -450 to -249 bp region of MOR proximal promoter, and the pL4.7K (-4.7K to -249 bp) plasmid, containing the MOR proximal promoter with a 4.5 kb upstream region were examined. As shown in Fig. 2B, function analysis showed that TSA

treatment resulted in a 3.2-, 3.5-, and 3.0-fold increase in the promoter activity of pL450, pL4.7K and pL340/300, individually, as compared to control groups.

In summary, these data suggested that MOR proximal core promoter, with ds and ssDNA elements, is a key region in response to TSA in neuronal cells.

Effect of TSA on Sp1, Sp3 and PCBP protein levels

To verify that TSA increased the acetylation status in neuronal NMB cells, a positive control using anti-acetyl-lysine antibody (labeled as Acetyl-Lys) was included (Fig. 3, bottom panel). Whole cell lysates (60 µg) from TSA (indicated as TSA) or vehicle treated (indicated as C) cells were first subjected to SDS-PAGE and then Western blot analysis. As shown in Fig. 3, bottom panel, a band with M.W. approximately 53 kDa was observed only in TSA (TSA) but not in vehicle (C) treated samples, suggesting the acetylation of p53 protein upon TSA treatment, in agreement with the previous reports (Gu and Roeder, 1997; Bannister and Miska, 2000).

To determine if an increase of total protein amounts of Sp1, Sp3 or PCBP was associated with the increase of MOR transcription upon TSA treatment, Western blot analysis was performed with anti-Sp1 (indicated as Sp1), Sp3 (indicated as Sp3) or PCBP (indicated as PCBP) antibodies using whole cell lysates. As shown in Fig. 3, no significant differences for all three factors were observed in cells treated with vehicle (C) or TSA (TSA). These results suggested that TSA treatment did not change the total protein amounts of Sp1, Sp3 and PCBP factors in neuronal cells.

Recruitment of Sp1/Sp3 and PCBP to MOR proximal promoter

We next determined if binding of Sp1/Sp3 or PCBP to the MOR proximal promoter region was enhanced by TSA treatment using ChIP assay. Chromatin from NMB cells treated with TSA (indicated as TSA) or vehicle (indicated as C) was subjected to immunoprecipitation using anti-Sp1, Sp3 and PCBP antibody, respectively. The immunoprecipitated DNA was then analyzed using PCR with a primer pair encompassing the proximal promoter region (as shown in Fig. 1A).

As shown in Fig. 4, binding of Sp1, Sp3 (lane 5) and PCBP (lane 8) to the MOR proximal promoter was augmented by TSA as compared to those (lane 2 for Sp1, lane 3 for Sp3 and lane 7 for PCBP) of vehicle (C) treated cells. No significant immunoprecipitated DNA was detected using no antibody (indicated as negative control in lane 9 for C and lane 10 for TSA treated cells) or using non-specific IgG antibody (data not shown). The crude chromatin prior to immunoprecipitation was also examined (indicated as “input” in lanes 11 and 12).

Taken together, these results suggested that TSA promoted the recruitment of Sp1/Sp3 and PCBP factors to the neuronal MOR proximal promoter in situ.

TSA induced nuclear PCBP redistribution

Our previous study showed that PCBP distributes in cytosolic and nuclear regions of neuronal cells, visualized using N2a cells transiently transfected with a plasmid containing GFP-PCBP fusion protein (Berry et al., 2006). We now examined if TSA affected the nuclear distribution of the endogenous PCBP in NMB cells using confocal microscopy with anti-PCBP antibody as the primary antibody and FITC conjugated anti-IgG antibody as the secondary antibody.

As shown in Fig. 5, results showed that TSA triggered PCBP hot spots in the nucleus (panel b, indicated by arrows) as compared to vehicle (C) treated cells with no clear nuclear hot spots (panel a). Nucleus was counterstained by DAPI (panels c and d). Merged images of PCBP and

DAPI staining are shown in panel e for vehicle treated cells and panel f for TSA treated cells. In summary, these results suggested that TSA induced the redistribution of endogenous PCBP in the nucleus of neuronal cells.

The presence of these hotspots could reflect that TSA enhanced the nuclear PCBP binding to chromatin DNA, which might be accompanied by a detectable decrease of nucleoplasmic PCBP protein level. To examine if this decrease occurred, nucleoplasmic extracts from NMB cells treated with TSA or vehicle (C) were subjected to Western blot using anti-PCBP antibody. As shown in Fig. 6, results demonstrated that TSA treatment significantly decreased nucleoplasmic PCBP protein level as compared to the vehicle treated group (C).

Taken together, these results suggested that TSA induced the redistribution (hotspots) of endogenous PCBP to the nucleus of neuronal cells.

TSA affects the steady-state of MOR mRNA

To further understand the effects of HDAC inhibitor on MOR expression, the endogenous level of MOR mRNA was examined. RNA was extracted from cells treated with TSA for various lengths of time, and was then subjected to semiquantitative RT-PCR using MOR specific primers. A β -actin primer pair was used as an internal standard for normalization. The normalized MOR message amount of vehicle treated cells (C) was arbitrarily defined as 100%.

As shown in Fig. 7, a significant decrease of steady state MOR mRNA (indicated as MOR) was observed at 12 hr ($27 \pm 1.4\%$), and it decreased further after 24 hr TSA treatment ($58.4 \pm 3.9\%$), whereas the amounts of endogenous β -actin messages (indicated as β -actin) remained at similar levels. These data thus suggested that TSA treatment resulted in a reduction of the steady-state neuronal MOR messages in a time-dependent manner.

TSA facilitates MOR mRNA degradation

The above data showed that TSA enhanced MOR transcription, but also induced an overall decrease of the steady-state MOR mRNA in neuronal NMB cells. To understand the mechanism underlying this decrease, we tested the effect of TSA on MOR mRNA degradation rate using actinomycin D (Act D), which inhibits mRNA synthesis by blocking RNA polymerase II activity. RNA, extracted from cells treated with Act D for various lengths of time in the presence or absence of TSA, was subjected to semiquantitative RT-PCR using MOR specific primers. A β -actin primer pair was used as an internal standard for normalization purpose. Because Act D treatment reduced mRNA level in addition to TSA treatment, and in order to keep the linear range of the relationship between the PCR cycle number and the amount of amplified fragments generated, two extra PCR amplification cycles (total 32 cycles) were used for probing MOR messages. The linear correlation co-efficiency between amplified MOR message amounts and PCR cycles (30 to 40 cycles) is 0.950 ± 0.027 .

As shown in Fig. 8, TSA facilitated the degradation rate of MOR mRNA in a time-dependent manner (closed squares) as compared to that with no TSA treatment (open circles). These results thus suggested that TSA enhanced the instability of MOR mRNA in neuronal cells.

3. Discussion

This study explored the effects of TSA, a HDAC inhibitor, on neuronal MOR gene expression. HDAC1 and HDAC2 have been well studied and suggested to be involved in the nuclear chromosome remodeling of various types of cells (Saha and Pahan, 2006). Using ChIP analysis (Fig. 1B), we demonstrated that HDAC1/2 were recruited to the region of proximal promoter, the main promoter directing neuronal MOR expression (Ko et al., 1997, 1998, 2002; Choe et al., 1998). Using luciferase reporter system, function analysis (Fig. 2) suggested that TSA

enhanced MOR gene transcription, with ds (Sp1/Sp3) and ssDNA (PCBP) elements as critical TSA responsive sites.

There are at least two possible mechanisms underlying TSA-induced transcriptional activation. First, TSA enhances availability of transcription factors by increasing total amount of proteins. This was not supported by our Western blot analysis. TSA induced no significant differences in total protein levels of Sp1/Sp3 and PCBP (Fig. 3) using whole cell lysates. Studies of the promoters of 5-lipoxygenase and luteinizing hormone receptor genes have also shown no change in Sp1/Sp3 protein levels in response to TSA (Zhang et al., 2006; Schnur et al., 2007).

An alternative possibility is that TSA inhibits HDAC activity, which leads to a disruption of local nucleosomes (de-repression) and facilitating access of transcription factor binding at the MOR promoter. This mechanism was supported by our data. ChIP analysis (Fig. 4) showed that TSA promoted Sp1/Sp3 and PCBP binding to the promoter in situ. In addition, confocal images demonstrated TSA-induced redistribution (hotspots) of endogenous nuclear PCBP (Fig. 5), which resulted in a detectable decrease of nucleoplasmic PCBP protein levels using Western blot analysis (Fig. 6). A punctate nuclear pattern often indicates that the protein is a component of a nuclear complex (Cobb et al., 2000; Riderle et al., 1998; Berube et al., 2000; Leonhardt et al., 2000), which is consistent with the functional roles of PCBP in gene regulation.

It is interesting that confocal images revealed no obvious nuclear hot spots of endogenous PCBP in non-treated NMB cells, but only in TSA treated cells (Fig. 5). In these studies, we used anti-PCBP antibody as the primary antibody and the FITC-conjugated-anti-IgG as the secondary antibody. In our previous studies using the transient transfection with GFP-PCBP fusion protein system, nuclear PCBP hot spots were clearly visualized (Berry et al., 2006) in transfected neuronal cells. We therefore tested transfected cells with TSA using GFP-PCBP fusion protein, and found that TSA treatment resulted in relatively brighter GFP-PCBP hot spots in the nucleus compared to those of untreated cells (data not shown). These results further confirmed that TSA indeed induced the redistribution of nuclear PCBP of neuronal cells, even though some nuclear hot spots were already present in the nucleus of transfected cells without TSA. One possible reason for the absence of endogenous PCBP nuclear hot-spots observed in this study may be the low affinity of anti-PCBP antibody-antigen interaction.

Using RT-PCR, a time-dependent decrease of steady-state MOR messages was also observed in response to TSA (Fig. 7), which could have been due to a decrease of transcription, an increase of mRNA degradation, or both. The first possibility was not supported by experiments showing that TSA enhanced MOR transcription (Fig. 2), especially that regulated by the MOR proximal core promoter, which exhibits high homology between mouse and human. However, it is possible that other inhibitory element(s) located outside the region of the proximal core promoter of human MOR gene function in response to TSA. Such a possibility will be examined in the future. The second possibility was supported by empirical results that TSA facilitated the degradation rate of MOR mRNA (Fig. 8). A similar result was reported for the DNA methyltransferase 1 (DNMT1) gene (Januchowski et al., 2007). The mechanisms underlying this TSA enhancement of neuronal MOR mRNA degradation remain to be elucidated.

TSA facilitated MOR mRNA degradation relatively quickly (Fig. 8), with a 40% decrease at 2 hr and a maximum reduction after 8 hr treatment. In contrast, the overall reduction of MOR mRNAs in response to TSA was much slower (Fig. 7). No significant change was detected at 6 hr and maximum reduction was not observed at 12 hr treatment. This discrepancy can be explained partially by a concomitant TSA-induced increase of neuronal MOR gene transcription. However, the transcriptional upregulation did not completely overcome the downregulation at the post-transcriptional level.

Collectively, these results therefore suggest that TSA affects the MOR gene expression at both transcriptional and post-transcriptional levels in neuronal cells. The opposite effects observed at these two levels may play roles in fine tuning the overall MOR gene expression under a variety of conditions. Further experiments are required to determine if there is any crosstalk between transcriptional and post-transcriptional regulation of the neuronal MOR gene, as well as the relative contribution of regulation at each level in various pathological conditions.

Understanding of the MOR promoter architecture, including chromosome remodeling, and interactions of transcriptional factors and modulators, will provide new insights into mechanisms underlying the MOR gene expression in neuronal cells. It appears that different promoter architectures under different physiological or pathological conditions will be able to establish different levels of gene expression. Therefore, studies of the components involved in MOR gene regulation may lead to identification of potential therapeutic targets for disorders associated with the neuronal MOR gene.

4. Experimental Procedure

Cell culture and whole cell lysate preparation

Human neuroblastoma NMB cells were grown in RPMI medium with 10 % heat-inactivated fetal calf serum in an atmosphere of 10 % CO₂ and 90 % air at 37 °C. Cells were treated with 250 nM trichostatin A (TSA) or vehicle (as the control) for 24 hrs or various time periods as indicated in each figure. To prepare whole cell lysates, cells were harvested and subjected to lysis solution, containing 1% SDS and protease inhibitors, then sonicated.

RNA extraction and RT-PCR

Total RNA of cells was isolated using TriReagent method (Molecular Research Center, Inc., Cincinnati, OH). First strand cDNA was synthesized using total RNA and random hexamers (Promega, Madison, WI) in the presence of MLV reverse transcriptase (Invitrogen, CA). PCR procedure was described previously (Ko and Loh, 2005). To ensure quantitative analysis, the amount of amplified fragments was kept within the linear range of the relationship between the amount of RNA used and the amount of amplified fragments generated. PCR amplification was performed as 1 min at 94 °C, 30 sec at 68 °C, and 30 sec at 72 °C. For detection of the human mu-opioid receptor (MOR) message, PCR amplification was performed using a MOR specific primer pair: upper, 5'-CTG CCC CCA CGA ACG CCA GCA AT -3'; lower: 5'-CTG GAA GGG CAG GGT ACT GGT GG -3'. For detection of human β -actin message, PCR amplification was performed for 17–18 cycles with an upper primer (5'-CCTTCCTGGGCATGGAGTCCTG-3') and a lower primer (5'-GACGCGAGGCCAGGATGGA -3'). PCR products were quantified by Molecular Dynamic Imager System. Paired Student *t*-Test was used for statistical analysis.

Transient transfection and reporter gene activity assay

NMB cells were transfected using the lipofectamine (Invitrogen, CA) method. Briefly, cells at approximately 40% confluency were transfected with individual plasmid. The amount of DNA used was within the linear range of the relationship between the luciferase activity and the amount of DNA. Twenty-four hrs after transfection, cells were treated with TSA or vehicle for 24 hrs. Cells were then washed and lysed with lysis buffer (Promega, Madison, WI). Normalization among different samples is followed the method described by Conn et al. (1996). All transfection experiments were repeated at least three times with similar results. The luciferase activity of each lysate was determined as described by the manufacturers (Promega, WI) using Lumat LB 9507 (Berthold Technologies, Drescher, PA).

Cell fixation and Confocal Microscopy

Cells fixed with 4% paraformaldehyde were perforated using acetone. Cells were incubated with the blocking solution, containing 2% goat serum and 1% BSA in PBS, for 1 hr at R. T. Cells were incubated with anti-PCBP antibody (Santa Cruz Biotechnology, CA) at 4 °C, and then with the FITC-conjugated anti-IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA). A coverslip with cells was placed onto a slide with anti-fade reagent containing DAPI (Vector Laboratory) for the nuclear counterstaining. Images were captured by Fluoview 1000 confocal microscope (Olympus).

Nucleoplasmic extract preparation

Nucleoplasmic extracts were prepared from NMB cells using the method described by Johnson et al. (1995). Briefly, cells were grown to confluence, harvested and washed with phosphate-buffered saline. The following steps were performed at 4 °C. Cells were resuspended in sucrose buffer (0.32 M sucrose, 3 mM CaCl₂, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol (DTT), 0.5 mM PMSF and 0.5% NP-40). The nuclei were then collected by microcentrifugation at 500 × g, and washed with sucrose buffer without NP-40. The nuclei were resuspended in low salt buffer (20 mM Hepes, pH 7.9, 25 % glycerol, 0.02 M KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF), then were lysed by gently mixing with high salt buffer (20 mM Hepes, pH 7.9, 25 % glycerol, 0.8 M KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 1% NP-40 and 0.5 mM PMSF), followed by diluent (2.5 vol. of 25 mM Hepes, pH 7.6, 25 % glycerol, 0.1 mM EDTA, 0.5 mM DTT and 0.5 mM PMSF). The chromatin DNA and its associated proteins were removed by microcentrifugation at 13,690 × g, and the supernatant was then ready for use.

Western blot analysis

Western blot was performed as previously described (Rivera-Gines et al., 2006). Cell lysate (60 µg) mixed with the treatment buffer, containing 62.5 mM Tris-HCl, pH6.8, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol, was boiled for 2 min. The treated extracts were then subjected to SDS-polyacrylamide gel electrophoresis. The gel was electroblotted onto polyvinylidene difluoride membrane (GE healthcare, Piscataway, NJ) in the transfer buffer containing 48 mM Tris-HCl, 39 mM glycine and 20% methanol. The membrane was then incubated with blocking solution, containing 10% dry milk, 0.1% Tween-20 in Tris-buffered saline, overnight at 4 °C, and it was further probed with anti-Sp1, Sp3 or PCBP antibody (Santa Cruz Biotechnology, Inc., CA). Signals were developed following GE Healthcare's instruction (Piscataway, NJ), and quantified by a Molecular Dynamic imager system.

Chromatin immunoprecipitation (ChIP)

This procedure followed Millipore's (CA) instructions (Rivera-Gines et al., 2006). Cells, incubated with 1% formaldehyde at 37 °C for 10 min, were washed twice with ice-cold PBS. Cells were then collected by centrifugation at 4 °C, and resuspended in the cell lysis buffer containing 50 mM Tris-HCl, pH 8, 10 mM EDTA, 1% SDS and protease inhibitors. Cell lysates were sonicated to give a DNA size of approximately 600 bp, and supernatants were diluted with dilution buffer containing 16.7 mM Tris-HCl, pH 8, 1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 0.01% SDS and protease inhibitors. The solutions were precleared with salmon sperm DNA/protein G agarose slurry and then treated with antibody overnight at 4°C. Anti-HDAC1, HDAC2 and anti-goat antibodies were purchased from Santa Cruz Biotech, respectively.

Immune complexes were then collected by adding a salmon sperm DNA/protein G agarose slurry. The beads were washed sequentially in the following buffers: low salt wash buffer (20 mM Tris-HCl, pH 8.1, 150 mM NaCl, 2 mM EDTA, 0.1% SDS and 1% Triton X-100); high

salt wash buffer (20 mM Tris-HCl, pH 8.1, 500 mM NaCl, 2 mM EDTA, 0.1% SDS and 1% Triton X-100); LiCl wash buffer (10 mM Tris-HCl, pH 8.1, 0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, and 1 mM EDTA), and Tris-EDTA buffer. Immuno-complexes were then extracted from the beads using the solution containing 1% SDS and 0.1M NaHCO₃. Cross-linking was reversed by heating the eluates at 65 °C for 4 hrs. The eluates were then digested with proteinase K at 45 °C for 1h, and then further subjected to the phenol/chloroform extraction. The DNA was purified by ethanol precipitation.

The -491 bp to -214 bp region of the human MOR proximal promoter (translation start site designated as +1) was amplified by PCR (1 min at 94 °C, 30 sec at 68 °C, and 30 sec at 72 °C). The pair of primers used in the PCR included the sense primer: 5'-GGCGCTGGAAAATTGAGTGATGTTAGC-3' and the antisense primer: 5'-CCTTAGTAGTTCACAGAGGCTCATC-3'.

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Abbreviations

MOR	mu-opioid receptor
PCBP	poly C binding protein 1
ds	double-stranded
ss	single-stranded
TSA	trichostatin A
HDAC	histone deacetylase
HAT	histone acetyltransferase
CNS	central nervous system
ChIP	chromatin immunoprecipitation
Act D	actinomycin D

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Fig. 1. Association of HDAC1 and HDAC2 in MOR proximal promoter in situ

A, Diagram represents the human MOR proximal promoter region with the ds/ss DNA element labeled by a black box. Locations of a primer pair used in the PCR reaction are indicated by arrows. The expected size of PCR product is 278 bp, which is depicted by a solid line. **B**, ChIP assay was performed using the chromatin prepared from human NMB cells treated with vehicle (indicated as C in lanes 1, 4, 5, 8 and 11) or trichostatin A (indicated as TSA in lanes 2, 6, 7, 9 and 12) for 24 hrs. The soluble chromatin extracts were immunoprecipitated with no antibody (lanes 1 and 2), anti-HDAC1 (lanes 4 and 6), anti-HDAC2 (lanes 5 and 7) or non-specific IgG antibody (lanes 11 and 12), and were then subjected to PCR analysis. “Input” indicates that the crude chromatin extracts prior to immunoprecipitation from control (lane 8) or TSA treated sample (lane 9) were also analyzed. The arrowheads (in lanes 3 and 10) indicate the 200 bp and 300 bp DNA markers, respectively.

Fig. 2. Functional analysis of the wild type or mutant MOR proximal core promoter via the luciferase reporter system

A, NMB cells were transiently transfected with plasmids containing the luciferase reporter gene driven by the wild type (pL340/300) or mutant mouse MOR proximal core promoter, individually. Translation start site is designated as +1. The pLcsp plasmid contains a mutant ss DNA element, and pLGGT plasmid contains the mutated ds DNA element. "X" indicates the mutated ss or ds element (gray box). Twenty-four hrs after transfection, cells were treated with TSA for 24 hrs. Wild type promoter activity was arbitrarily defined as 100%. Data are presented as mean $\pm$ S.E. from at least three preparations (*, $p < 0.01$). **B**, Schematic diagram represents the promoter-luciferase chimeric plasmids, which contain a longer DNA fragment (solid line) located at the 5'-upstream of the core proximal promoter region (–340 to –300 bp). NMB cells were transiently transfected with pL450, pL4.7K and pL340/300 plasmids, individually. Twenty four hrs after transfection, cells were treated with vehicle (control) or trichostatin A for 24 hrs. The promoter activity obtained from control was arbitrarily defined as 100%. Data are presented as mean $\pm$ S.E. from three preparations.

Fig. 3. Western blot analysis of Sp1/Sp3 and PCBP protein levels

NMB cells treated with vehicle (C) or trichostatin A (TSA) for 24 hrs were lysed using 1% SDS with sonication. Equal amounts (60 μ g) of whole cell lysates were subjected to 10% SDS-PAGE (for Sp1, PCBP and Acetyl-Lys) or 9% SDS-PAGE (for Sp3) and sequential to Western blot analysis, probing with anti-Sp1 (indicated as Sp1), Sp3 (indicated as Sp3), PCBP (indicated as PCBP) or anti-acetyl lysine (indicated as Acetyl-Lys) antibodies. Numbered lines indicate the size of protein standards.

Fig. 4. TSA-enhanced recruitment of Sp1/Sp3 and PCCP to MOR proximal promoter in situ
 NMB cells treated with vehicle (C) or trichostatin A (TSA) for 24 hrs were subjected to ChIP analysis. The soluble chromatin extracts were immunoprecipitated with anti-Sp1 (indicated as Sp1), -Sp3 (indicated as Sp3), -PCBP (indicated as PCBP) or with no antibody (indicated as negative control). Immunoprecipitated DNA was analyzed using PCR with a pair of primers shown in Fig. 1A. “Input” indicates that the crude chromatin extracts prior to immunoprecipitation from vehicle treated (lane 11) or TSA treated sample (lane 12) were also analyzed. The arrowheads indicate the 200 bp and 300 bp DNA markers (lanes 1 and 6), respectively.

Fig. 5. TSA-induced redistribution of endogenous PCBP in the nucleus of neuronal cells

Neuronal NMB cells, treated with vehicle (C) or trichostatic acid (TSA) for 24 hrs, were fixed and perforated. Green color signals of endogenous PCBP (indicated as PCBP) in panel a (C) or panel b (TSA) were obtained using anti-PCBP antibody as the primary antibody and FITC conjugated anti-IgG as the secondary antibody. Nuclear PCBP hot spots are indicated by arrows (panel b). Nuclear location was counterstained by DAPI in panels c and d. Merged images (panels e and f) showed the colocalization of PCBP (panels a and b) and nucleus (panels c and d).

Fig. 6. Effect of TSA on protein levels of nucleoplasmic PCBP

Equal amounts (60 μ g) of nucleoplasmic extracts from NMB cells treated with vehicle (C) or trichostatin A (TSA) for 24 hrs were subjected to 10% SDS-PAGE and sequential to Western blot analysis, probed with PCBP (indicated as PCBP) antibodies, respectively. Numbered line indicates the size of protein standard.

Fig. 7. Effect of TSA on the steady state level of MOR mRNA

RNA was prepared from NMB cells treated with TSA for 6, 12 or 24 hrs. RT-PCR was performed using a human MOR-specific primer set with 30 cycle amplification. A pair of human β -actin-specific primers was also included in every PCR reaction as an internal control for normalization purpose. PCR products were resolved using electrophoresis. Sizes of DNA markers are indicated. The normalized MOR message amount of vehicle treated cells (C) at time zero hr was arbitrarily defined as 100%. Quantitative analysis of MOR message levels are presented as mean $\pm$ S.E. from four different preparations. The student paired *t*-test was used. "*" indicated $p < 0.01$.

Fig. 8. TSA facilitated the degradation rate of MOR mRNA

RNAs, from cells treated with actinomycin D (ActD) for 2, 4, 8, 12 or 24 hrs in the absence (open circles) or presence of TSA (closed squares), were subjected to RT-PCR using a human MOR-specific primer set with a 32 cycle PCR amplification. A pair of β -actin-specific primers was included as an internal standard for normalization purpose. Normalized MOR message from vehicle treated cells was arbitrarily defined as 100%. Data are presented as mean $\pm$ S.E. from four preparations (*:p< 0.01).