Published in final edited form as:

Gut. 2015 December ; 64(12): 1913–1920. doi:10.1136/gutjnl-2014-307748.

Epigenetic upregulation of metabotropic glutamate receptor 2 in the spinal cord attenuates estrogen-induced visceral hypersensitivity

Dong-Yuan Cao, Guang Bai, Yaping Ji, and Richard J. Traub

Department of Neural and Pain Sciences, University of Maryland School of Dentistry, UMB
Center to Advance Chronic Pain Research, Baltimore, MD 21201

Abstract

Objective—Epigenetic mechanisms are potential targets to relieve somatic pain. However, little is known whether epigenetic regulation interferes with visceral pain. Previous studies show that estrogen facilitates visceral pain. This study aimed to determine if histone hyperacetylation in the spinal cord could attenuate estrogen facilitated visceral pain.

Design—The effect of the histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA) on the magnitude of the visceromotor response (VMR) to colorectal distention was examined in ovariectomized rats with/without estrogen replacement. An additional interaction with the metabotropic glutamate receptor 2/3 (mGluR2/3) antagonist LY341495 was tested. The levels of acetylated histone and mGluR2 mRNA and protein were analyzed. The binding of acetylated H3 and estrogen receptor alpha (ER α) to the *GRM2* promoter were measured by chromatin immunoprecipitation (ChIP) coupled with qPCR.

Results—In ovariectomized rats 17 β -estradiol (E2), but not safflower oil, increased the magnitude of the VMR to colorectal distention. SAHA attenuated the E2-facilitated VMR, but had no effect in safflower oil-treated rats. Subsequent spinal administration of LY341495 reversed the antinociceptive effect of SAHA in E2 rats. In addition, SAHA increased mGluR2 mRNA and protein in the spinal dorsal horn following E2, but not vehicle, treatment. In contrast, neither E2 nor SAHA alone altered mGluR2 mRNA. SAHA increased binding of H3K9ac and ER α to the same regions of the *GRM2* promoter in E2-SAHA treated animals.

Conclusion—Histone hyperacetylation in the spinal cord attenuates the pro-nociceptive effects of estrogen on visceral sensitivity, suggesting that epigenetic regulation may be a potential approach to relieve visceral pain.

Address correspondence to: Richard J. Traub, Ph.D., Department of Neural and Pain Sciences, University of Maryland School of Dentistry, 650 West Baltimore Street, Baltimore, MD 21201, Phone: 410-706-5117, Fax: 410-706-0865, rtraub@umaryland.edu.

Contributors DYC: data acquisition, data analysis and drafting of manuscript. GB: data acquisition, data analysis, study design, critical reading of manuscript. YJ: data acquisition, data analysis and critical reading of manuscript. RJT: study concept and design, obtained funding, study supervision and critical reading of manuscript. All authors read and agreed on the final manuscript.

Disclosures: The authors report no conflicts of interest.

Keywords

visceral pain; estrogen receptor; epigenetics; histone deacetylase inhibitor; chromatin immunoprecipitation

Introduction

Visceral pain is one of the most common reasons for clinical visits and it impacts the quality of life of millions of people. Currently, visceral pain is poorly managed because the underlying mechanisms remain largely unclear. In general, women exhibit a higher morbidity of functional pain syndromes such as irritable bowel syndrome (IBS) than men and estrogen is thought a critical player in IBS severity^{1,2}. Our previous studies in animal models have demonstrated that estrogen facilitates colorectal distention-evoked visceral pain through activation of estrogen receptor alpha (ER α) in the spinal cord^{3,4}. ER α is a nuclear receptor that primarily functions as a transcription factor to regulate promoter activity⁵. Therefore, regulation of gene expression is critical to mediate estrogen's effects on visceral sensitivity, although little is known of the underlying mechanism.

Recently, it has been reported that gene transcription or expression is regulated not only by genetic but also by epigenetic mechanisms which are independent of genomic DNA sequences and impacted largely by environmental changes^{6,7}. Three molecular mechanisms, i.e., DNA methylation, chromatin remodeling and non-coding RNAs are involved in epigenetic regulation⁶⁻⁸. Histones are basic structures of chromatin and post-translational modification of histones remodels chromatin structure regulating transcriptional activity⁷. Acetylation of lysine residues in the N-terminal of histones is a well-studied histone modification. Increase in histone acetylation following pharmacological inhibition of histone deacetylase (HDAC) has been found to attenuate inflammatory and neuropathic pain in animal studies⁹⁻¹³. However, it remains largely unknown whether histone acetylation is critical to modulate visceral sensitivity.

In the present study we tested our working hypothesis that epigenetic mechanisms are critical to modulate visceral pain at the spinal level. We applied HDAC inhibitors to the spinal cord in ovariectomized rats receiving 17 β -estradiol (E2) and found that HDAC inhibition increased histone acetylation in the spinal dorsal horn and attenuated E2-induced visceral hypersensitivity. We also observed that HDAC inhibition significantly increased binding of acetylated histone 3 and ER α to selective regions of the promoter of the metabotropic glutamate receptor 2 (mGluR2) gene (*GRM2*) and upregulated mGluR2 mRNA and protein. Blocking mGluR2 activity disrupted the anti-nociceptive effect of HDAC inhibition. These data suggest that HDAC inhibition interacts with E2-activated ERs to modulate visceral sensitivity.

Materials and Methods

Animals

Experimental protocols were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee and adhered to guidelines for experimental

pain in animals published by the International Association for the Study of Pain. Female Sprague-Dawley rats weighing 225–250 g were obtained from Harlan. Rats were housed in pairs with free access to food and water at 25°C with 12 h/12 h alternating light/dark cycle.

Surgery

Rats were anesthetized with 55 mg/kg ketamine, 5.5 mg/kg xylazine, and 1.1 mg/kg acepromazine. Ovariectomies (OVx) were performed by a dorsolateral approach. In order to direct drugs to the spinal cord, the atlanto-occipital membrane was slit and a polyethylene catheter (32 g, ReCathCo, Allison Park, PA, USA) was inserted in the subdural space to the level of the lumbosacral spinal cord (L6-S2). After catheter placement, electromyogram (EMG) electrodes made from Teflon-coated 32 gauge stainless steel wire (Cooner Wire Company, Chatsworth, CA, USA) were stitched into the ventrolateral abdominal wall. The electrode leads were tunneled subcutaneously and exteriorized at the back of the neck with the catheter. Rats were individually housed and allowed 10–14 days to recover from surgery.

Visceromotor response (VMR)

Rats were fasted for 18–24 hours prior to recording the VMR. Water was available *ad libitum*. On the day of testing, rats were briefly sedated with isoflurane and a 5–6 cm balloon attached to Tygon tubing was inserted into descending colon and rectum through the anus. The secured end of the balloon was at least 1 cm proximal to the external anal sphincter and the tubing was taped to the tail. Rats were loosely restrained in acrylic tubes and given 30 min to recover from sedation. The EMG signals were recorded with a CED 1401 and analyzed using Spike 2 for windows software (Cambridge Electronic Design, Cambridge, UK). Colorectal distention trials (CRD; each trial was 5 distentions to 60 mmHg CRD, 20 sec duration, 3 min interstimulus interval) was produced by inflating the distention balloon with air under computer control. The recorded EMG signal was rectified in Spike 2 and the area under the curve (AUC) calculated. The response for each distention stimulus was the AUC of the EMG for the 20 s before distention (background) subtracted from the AUC during the 20 s distention. The responses of the 5 distentions during a trial were averaged for the response for that trial.

Tissue collection

Rats were euthanized with CO₂ and decapitated at selected time points after treatment. The spinal cord was removed by pressure ejection with ice cold saline as described previously¹⁴. Using the lumbar enlargement as a landmark, the L6 to S2 section of the spinal cord was isolated. The dorsal part of isolated spinal cord was dissected, frozen on dry-ice and saved at –80°C until use.

Western blots analysis

Tissues were homogenized in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS) supplemented with 1 mM Na₃VO₄ and protease inhibitor cocktail (Roche, Branford, CT, USA). The homogenates were centrifuged at 14,000×g for 10 min at 4°C and the supernatant was collected. Protein contents in supernatants were measured using the Bradford method. After denaturing protein

samples were fractionated 25 µg per lane on 4–12% SDS-NuPAGE gel and blotted to nitrocellulose membrane. The membranes were incubated with primary antibody (1:1,000) directed against acetylated lysine 9 on histone 3 (H3K9ac, Cell Signaling Technology, Danvers, MA, USA) or mGluR2 (Abcam Inc, Cambridge, MA, USA) at 4°C overnight. The membranes were further incubated for 1 hour in blocking buffer with IRDye 800CW goat anti-rabbit secondary antibody (Li-Cor Biosciences, Lincoln, NE, USA). Washed membranes were scanned and analyzed with the Li-Cor Odyssey System (Li-Cor Biosciences, Lincoln, NE, USA). Blots were then stripped for 30 min and reprobed with antibodies for pan-histone 3 (1:1000, Cell Signaling Technology) or β-actin primary antibody (1:5,000, Sigma, St. Louis, MO, USA) and IRDye 800CW goat anti-rabbit secondary antibody or IRDye 680LT goat anti-mouse secondary antibody (1:20,000, Li-Cor Biosciences) with same processes as above.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the dissected L6–S2 dorsal spinal cord using absolutely RNA miniprep kit (Stratgene, La Jolla, CA, USA) and reverse transcribed into cDNAs using SuperScript II Reverse Transcriptase kit with random primers (Invitrogen, Grand Island, NY, USA). Quantification of rat mGluR2 and mGluR3 mRNAs were completed by SYBR green-based real-time PCR (qPCR). PCR primers were designed based on mRNA sequences to cross at least one Exon/Exon junction (Genbank accession number of NM_001105711 for mGluR2 and NM_001105712 for mGlu3) using Primer3 software. Three pairs of primers per mRNA were evaluated and one each (mGluR2, forward, 5'-CGTGAGTTCTGGGAGGAGAG, reverse, 5'-GCGGACCTCATCGTCAGTAT; mGluR3, forward, 5'-GTGGTCTTGGGCTGTTTGT, reverse, 5'-GCAGCATGTGAGCACTTTGT) with comparable PCR efficiency to that of the GAPDH mRNA (forward, 5'-TCACCACCATGGAGAAGGC, reverse, 5'-GCTAAGCAGTTGGTGGTGCA) was chosen. qPCR was completed in Maxima SYBR Green/Rox qPCR Master Mix (Thermo Scientific, Waltham, MA, USA) on the Eppendorf Mastercycler Real-plex system (Eppendorf, Hauppauge, NY, USA) and C_T values were obtained from the system. The efficiency of PCR was calculated from the slope of the standard curve of serially diluted cDNA and was found within the range of 90–110%. Relative level of mRNA was calculated using the C_T method after normalization to GAPDH mRNA as described previously ¹⁵.

Chromatin immunoprecipitation (ChIP)

ChIP was performed using a previously described method with the following modifications ¹⁶. Half of the dissected spinal dorsal horn was minced in 1xPBS (pH7.4) supplemented with protease inhibitors and subjected to DNA-protein cross-link by incubation with 1.5% paraformaldehyde for 20 min at room temperature. After homogenization on ice, the homogenate was centrifuged and the resulting pellet incubated in swelling buffer (25 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5% NP-40) on ice for 15 min and subjected to a second homogenization. Nuclei were isolated by centrifugation, broken in lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, protease inhibitor cocktail, 100 µg/ml valproic acid) and sonicated in the Covaris M2 system to yield DNA fragments around 500 bp. For immunoprecipitation, 3 µg of polyclonal antibodies specific to H3K9ac or H3K18ac (Cell Signaling Technology, Danvers, MA,

USA) or to ER α (Millipore, Temecula, CA, USA) were incubated overnight with 200 μ g protein at 4°C. The antibody-antigen complexes were pulled down by protein A/G-agarose followed by successive washes once each with low salt buffer (20 mM Tris-HCl, pH 8.0, 150 mM KCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100), high salt buffer (the same as low salt buffer except 500 mM KCl), LiCl buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 1 mM EDTA, 1% NP-40, 1% sodium dodecyl sulfate) and twice in TE buffer (pH 8.0). The complex was eluted into solution of 100 mM NaHCO₃-1% SDS. After reverse cross-link in 250 mM NaCl at 65°C for 6 ~12 hours, DNA was purified by a Qiaquick column (Qiagen, Frederick, MD, USA). Lysate equal to 5% of the immunoprecipitate was directly subjected to reverse cross-link and Qiaquick column purification without antibody immunoprecipitation and served as input. Two percent of eluted DNA was subjected to qPCR with SYBR green (Thermo Scientific, Waltham, MA, USA) in duplicate using primers covering five different regions in the *GRM2* promoter (figure 4A). qPCR was completed on the Eppendorf RealPlex2 system with a program of initial denaturing at 95°C for 4 min followed by cycling at 95°C for 15 sec and 57~64°C for 30 sec.

Two negative controls were performed. First, in a mock ChIP, IgG purified from non-immunized rabbit serum (VWR, Philadelphia, PA, USA) was added to replace antibody during immunoprecipitation for initial studies of ChIP specificity. Second, a 187-bp intergenic locus in rat chromosome 4 was examined by qPCR from ChIP elutes and inputs of every sample, which was then used for calculation. Primers used for this negative locus are forward, 5'-TGCAAACCTTCACACGTCCTC, and reverse, 5'-GGGGTGGAAATTTCTGGTTT. Antibody-enriched experimental DNA over the negative control was calculated using the Ct method with a normalization of immunoprecipitation data to relevant input of each sample for the promoter regions and the intergenic locus¹⁷ (supplemental figure 2). Six rats per group were tested for each pair of primers in ChIP.

Hormones and drugs

E2 was dissolved in safflower oil to a concentration of 50 μ g/100 μ l. E2 (100 μ l) or safflower oil was injected subcutaneously. SAHA (Cayman Chemical, Ann Arbor, MI, USA), made fresh, was dissolved in 20% dimethyl sulfoxide (DMSO) to a final concentration of 5–80 μ g/20 μ l. The HDAC inhibitor trichostatin A (TSA) and mGluR2/3 antagonist LY341495 (Tocris Bioscience, Bristol, UK) were also dissolved in 20% DMSO. For intrathecal injection (i.t.), 20 μ l volume of drug was injected followed by a 10 μ l saline flush.

Data analysis

All data are expressed as mean $\pm$ SEM. Data were analyzed by one or two way ANOVA as appropriate. Significant ANOVA results were further analyzed using Bonferroni's multiple comparison test and reported in the figures. *t*-test was used for two group comparison. *p* < 0.05 was considered significant.

Results

HDAC inhibitors attenuate the E2-facilitated VMR and increase histone acetylation

Ten to fourteen days following ovariectomy the baseline VMR was recorded. Rats were subsequently injected with E2 or safflower oil and the VMR recorded again 4 hours later. Rats were then injected with SAHA or vehicle and the VMR recorded over the next 2 hours. There was no difference in the baseline response (labeled OVx in figure 1A, one way ANOVA, $p > 0.05$) between treatment groups. Following E2, but not oil injection, there was a significant increase in the magnitude of the VMR as previously reported (t -test, $p < 0.05$; figure 1C)^{3,4}.

The HDAC inhibitor SAHA or its vehicle DMSO was then intrathecally injected. The VMR trial was recorded starting 30, 60, 90, and 120 min after injection of SAHA/vehicle. SAHA at doses of 20 and 80 μ g reversed the E2-facilitated VMR for at least 2 hours (one way RM ANOVA for each treatment group, $p < 0.0001$ and 0.01 , respectively; figure 1A). A lower dose of SAHA (5 μ g) or vehicle (20% DMSO) had no effect on the E2-facilitated VMR (one way RM ANOVA, $p > 0.05$ for both). In the absence of E2 (safflower oil treated ovariectomized rats), SAHA showed no effect on the magnitude of the VMR compared to the OVx or post oil injected state (one way RM ANOVA, $p > 0.05$).

Within each treatment group the response post SAHA remained stable over the 2 hour recording period so the response was averaged and normalized to the E2 or oil response prior to SAHA/DMSO injection. Comparison between treatment groups show that 20 and 80 μ g SAHA significantly attenuated the magnitude of the VMR compared to 5 μ g SAHA or DMSO in E2-treated rats or 80 μ g SAHA in oil-treated rats (one way ANOVA, $p < 0.005$, figure 1D).

The reversal of the E2-facilitated VMR by HDAC inhibition was confirmed by i.t. administration of another HDAC inhibitor, TSA (20 μ g, one way RM ANOVA, $p < 0.005$, supplemental figure 1). A lower dose of TSA (5 μ g) had no effect (one way RM ANOVA, $p > 0.05$). Similar to SAHA, TSA had no effect on the VMR in the absence of E2 (one way RM ANOVA, $p > 0.05$).

Western blots were used to confirm that SAHA inhibited HDAC and increased global histone acetylation in the spinal cord. Rats were treated with E2 or safflower oil for 4 hours followed by SAHA (80 μ g) or DMSO treatment for 30 min or 2 hours before tissues were collected for Western blot analysis. SAHA significantly increased H3K9ac in E2-treated animals compared to the E2 + DMSO (two way ANOVA, $p < 0.001$ at 30 min post SAHA; $p < 0.01$ at 2 hours post SAHA, figure 2). SAHA had no effect on histone acetylation in safflower oil treated animals compared to the oil + DMSO ($p > 0.05$ at both 30 min and 2 hours post SAHA). Interestingly, E2 alone did not alter the level of histone acetylation (E2 + DMSO group vs oil + DMSO group, $p > 0.05$ at both 30 min and 2 hours post DMSO).

These data show that E2-induced visceral hypersensitivity is attenuated by spinal administration of HDAC inhibitors. In addition, the HDAC inhibitor increased global histone acetylation in the presence of E2.

HDAC inhibitor attenuation of the E2-facilitated VMR is mGluR2 mediated

The simplest explanation for the inhibitory effect of SAHA on visceral sensitivity is an increase in spinal inhibitory processing. Considering that HDAC inhibitors attenuated nociceptive responses in the formalin test through upregulation of mGluR2¹¹, we tested the hypothesis that SAHA upregulates mGluR2 to strengthen inhibitory processing and in turn attenuate visceral hypersensitivity. Results of qRT-PCR revealed that the mGluR2 mRNA level significantly increased 2 hours after SAHA (80 µg) injection in E2-treated rats (two way ANOVA, $p < 0.05$; figure 3A). In comparison, injection of SAHA following oil treatment at that time point had no effect on mGluR2 mRNA. There were no significant differences in mGluR2 mRNA among groups at 30 min and 1 hour after SAHA injection (two way ANOVA, $p > 0.05$). Consistent with the mRNA change, mGluR2 protein expression also significantly increased 2 hours after SAHA injection in E2-treated rats in comparison to DMSO treated rats (two way ANOVA, $p < 0.05$, figure 3B). However, no difference in the expression level of mGluR3 mRNA at any time point (figure 3C) or protein at 2 hours (not shown) was revealed after SAHA administration (two way ANOVA, $p > 0.05$).

Since the transcription rate is sensitive to histone acetylation and directly impacts mRNA levels, we applied ChIP to examine the interaction between acetylated histones to the *GRM2* promoter following E2 + SAHA treatment. The rat *GRM2* promoter was defined for the region upstream of and surrounding the transcriptional start site (TSS) or the first base of exon 1 which has 397 bp sequences as provided by the University of California Santa Cruz genome database (genome.ucsc.edu). We searched putative *cis*-elements within ± 2 Kb of the TSS using TRANSFAC software (BIOBASE Biological Database, Qiagen, Frederick, MD, USA) and designed primers to cover five regions containing motifs for potential positive transcription factors (figure 4A). After optimizing the PCR program, we were able to obtain a single band of the expected size from each pair of primers from gDNA precipitated with an antibody against H3K9ac in ChIP of naïve animals (figure 4C). The specificity of these primers was also confirmed by the single peak in melting curves of SYBR green qPCR (supplemental figure 3). In comparison, these primers were unable to generate signal or band from negative control of IgG mock ChIP elute (figure 4C). We also examined an intergenic locus as negative control for calculation of ChIP results. This locus does not contain binding sites for estrogen receptors, and exhibited a low binding to acetylated histone 3 (Supplemental figure 2). Our results demonstrate that E2 + 80 µg SAHA treatment significantly increased binding of H3K9ac to three regions (A1, 4, 5) of the *GRM2* promoter (*t*-test, $p < 0.05$) while binding of H3K9ac to the other two regions and binding of H3K18ac to all five regions revealed no significant change compared to E2 + DMSO (figure 4D).

Interestingly, atypical or half estrogen response elements (aERE) were found in the A2 (TGACC, -1106/-1102), A4 (GTGAC, -503/-499) and A5 (GGGTCA, 1133/1138) regions of the *GRM2* promoter. It has been reported that more than 50% of estrogen receptor-bound sequences are atypical sites¹⁸. To examine whether these aEREs are functional, we performed ChIP with a polyclonal antibody against ER α and found that regions A2, A4 and A5 were precipitated by this antibody (figure 4E). After normalization to the input, A5 had the highest binding capacity. Furthermore, similar to the increase in H3K9ac binding, SAHA

significantly increased ER α binding to A4 and A5 (t -test, $p < 0.05$). In comparison, the low binding level of ER α to A2 was not altered by SAHA, consistent with no change in H3K9ac binding to this region. These data indicate that hyperacetylation of K9 in histone 3 is associated with increased binding of ligand-bound ER α to the *GRM2* promoter and with upregulation of *GRM2* transcription.

Due to the observed increase in mGluR2 expression and the binding of H3K9ac and ER α to the *GRM2* promoter in E2-SAHA treated animals, we next determined whether there was any functional significance of these changes. Thirty minutes following SAHA (80 μ g) injection and establishing attenuation of the E2-facilitated VMR, the mGluR2/3 antagonist LY341495 (20 nmol, i.t.) was injected (figure 5). Starting 5 minutes later the VMR was recorded over the next hour. LY341495 gradually reversed the attenuation of the VMR evoked by SAHA, and the magnitude of the VMR was significantly increased at 35 and 50 min after LY341495 application (i.e., 105 and 120 min after SAHA application) compared with the VMR at same time point in E2 plus SAHA treated animals receiving DMSO (vehicle for LY341495)(two way RM ANOVA, $p < 0.01$ for treatment; figure 5).

Discussion

In the present study, we show that increasing histone acetylation by spinal administration of HDAC inhibitors attenuated E2-induced visceral hypersensitivity. We further demonstrated SAHA reversed this E2-induced hypersensitivity by increasing mGluR2 expression in the spinal cord and that SAHA treatment significantly increased binding of H3K9ac to three of five tested regions in the *GRM2* promoter. More importantly, SAHA also significantly increased binding of E2-activated ER α specifically to the *GRM2* promoter regions which contain aERE and exhibited increased binding to H3K9ac. Spinal administration of the mGluR2/3 antagonist LY341495 reversed the effects of SAHA, confirming that epigenetic regulation of mGluR2 by HDAC inhibitors may contribute to attenuation of visceral pain. Previous reports have shown that the inhibitory effect of HDAC inhibitors on somatic pain may be due to epigenetic regulation of mGluR2 in the spinal cord^{11,19} and activation of estrogen receptors triggers mGluR2/3 signaling to decrease cAMP response element-binding protein (CREB)^{20,21}. Our data indicate that estrogen receptors interact with mGluR2 in the spinal cord to regulate visceral pain through epigenetic mechanisms. To the best of our knowledge, this is the first report to show that an epigenetic mechanism modifies visceral pain at the spinal cord level and that the access of ligand-bound ER α to the promoter of an antinociceptive gene is enhanced by hyperacetylated histone interacting with the same promoter regions. Our findings provide a new approach to relieve existing visceral pain involving estrogen function.

Our previous studies found that estrogen increases sensitivity of the VMR to colorectal distention through activation of spinal ER α ^{3,4}. Furthermore, E2 increased expression of the N-methyl-D-aspartate (NMDA) receptor subunit GluN1 and phosphorylation of GluN1 in the spinal cord, suggesting genomic and nongenomic actions of ER in facilitating visceral pain¹⁴. In the nucleus ER α bound and activated by estrogen is generally considered a regulator (activator or repressor) of transcription or promoter activity via two different mechanisms⁵. First, activated ER α directly binds an ERE on a promoter²² and regulates

transcription. Second, ER α binds to a half ERE, termed atypical or nonclassical ERE (aERE), and further interacts with nearby DNA bound-transcription factors including members of the NF κ B, Sp, SRF and Ap families^{5,18,23}. Consistent with this concept, the GluN1 gene *Grin1* promoter contains an aERE and the elements for Sp and Ap factors²⁴.

In the present study, we identified several aERE elements from the promoter or regulatory region of the *GRM2* gene. We found that ligand-bound ER α could not alter *GRM2* transcription until histone 3 became hyperacetylated by SAHA and then binding of activated ER α to this promoter increased (figure 4E). As indicated by our ChIP data, ER α binds only to these aERE elements that are located in the region(s) whose binding to acetylated H3 is enhanced by E2 + SAHA (A4, A5 in figures 4D, E). An interaction between ER α and hyperacetylated histones is further supported by the observation that histone hyperacetylation by SAHA did not alter the nociceptive response in control animals in the absence of E2 (figure 1). It has been observed previously that binding of transcription factors such as NF κ B to the promoter may trigger histone acetylation surrounding ERE sites resulting in recruitment of ER^{23,25}. On the other hand, in most cases, binding of ER to the promoter recruits factors with histone acetylase activity increasing histone acetylation on the promoter and recruiting other transcription factors for transcription regulation^{25,26}. Here we provide a novel mechanism in which access of ligand-bound or activated ER α to aERE on the promoter depends first on acetylation of K9 on histone 3 binding to sequences encompassing these elements and this mechanism is important for expression of the anti-nociceptive gene, *GRM2*. This novel mechanism of ER α action supports the notion that histone acetylation allows transcription factors to access promoters and regulate transcription⁶⁻⁷. In addition, this regulation may explain why E2 is antinociceptive under some conditions (e.g., histone hyperacetylation increasing mGluR2) while it is pronociceptive under other conditions (e.g., increasing NMDA receptor function).

It has been reported that acetylated histones bind transcribed genes in a bell-shape centering on the TSS²⁷. Our ChIP data showed acetylated histone 3 binding starting approximately 1kb upstream of the *GRM2* TSS without a robust increase closer to the *GRM2* TSS. There are three possible reasons to account for this different pattern. First, relatively large amplicons were examined in our qPCR, which differs from small tiled amplicons used for mapping acetylated histone binding to the genome. Thus, the large amplicon may mask any sudden change in acetylated histone binding. Second, this acetylated histone 3 binding distal to the *GRM2* TSS might suggest multiple TSS which may be either an alternative TSS of the *GRM2* gene or a TSS for an unknown gene²⁸. Lastly, the spinal cord is composed of a heterogeneous population of cells. It is known that *GRM2* is expressed only in a limited portion of dorsal horn neurons (Allen Institute.org) while our samples of spinal cord consist of a large number of multiple types of cells including neurons and glia. This may mask the pattern of acetylated histone binding reported in homologous populations of cultured cells²⁷. Nevertheless, our ChIP data combined with the pharmacological results support the notion that SAHA plus E2 treatment altered binding of acetylated histone to the *GRM2* promoter and thus regulated mGluR2 expression and impacted E2-related visceral hypersensitivity.

Further, our finding that spinal administration of HDAC inhibitors attenuated estrogen-induced facilitation of visceral pain is consistent with recent reports that increasing spinal histone acetylation attenuates hyperalgesia in neuropathic and inflammatory pain models ^{10,12}. Intrathecal application of HDAC inhibitors does not increase histone acetylation in dorsal root ganglion ¹², suggesting that the spinal cord instead of the dorsal root ganglion may be the main epigenetic regulation region in the present study.

In this study we showed that spinal mGluR2 is the downstream target of the inhibition of HDAC for attenuation of visceral pain. We also recognize possible roles of other genes as targets mediating the effects of histone hyperacetylation on the pain circuitry through epigenetic modulation. For example, HDAC inhibition in the nucleus raphe magnus enhanced GABA activity and thus relieved persistent pain ⁹. While the studies mentioned above focused on somatic pain, one recent study showed the contribution of epigenetic regulation to visceral pain. Tran et al. reported that application of the HDAC inhibitor TSA into the cerebral ventricles attenuated chronic psychological stress-induced visceral hypersensitivity ²⁹. In addition, this study focused on a noxious distention pressure as determined in normal rats. Future studies should also evaluate the effect of HDAC inhibitors on less intense distention pressures experienced during normal digestive functioning.

HDAC inhibitors have shown potential therapeutic efficacy in many rodent models of neurodegenerative diseases for neuroprotective function, preventing or delaying neuronal dysfunction. In addition, HDAC inhibitors including SAHA have been approved or are under evaluation in clinical trials for several neurological disorders and cancer ^{30–32}. The present study together with other studies ^{9–12} provides evidence that HDAC inhibitors may be used for analgesia on both somatic and visceral pain. Spinal administration of HDAC inhibitors did not affect visceral pain in normal rats, suggesting that increasing histone acetylation may have specific function on pathophysiological condition and HDAC inhibitors may have few side effects when they are used for treatment ¹⁰.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to thank Ms. Jane Karpowicz and Sangeeta Pandya for technical assistance.

This work was supported by National Institutes of Health (NIH) grant R01 NS 37424 to RJT.

Abbreviations

AUC	area under the curve
aERE	atypical estrogen response elements
ChIP	chromatin immunoprecipitation
CRD	colorectal distention

DMSO	dimethyl sulfoxide
E2	17 β -estradiol
EMG	electromyogram
ERα	estrogen receptor alpha
H3K9ac	acetylated lysine 9 on histone 3
HDAC	histone deacetylase
IBS	irritable bowel syndrome
LY341495	(2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid
mGluR2/3	metabotropic glutamate receptor 2/3
OVx	ovariectomy
SAHA	suberoylanilide hydroxamic acid
TSA	trichostatin A
TSS	transcriptional start site
VMR	visceromotor response

Reference List

1. Mulak A, Tache Y, Larauche M. Sex hormones in the modulation of irritable bowel syndrome. *World J Gastroenterol.* 2014; 20(10):2433–2448. [PubMed: 24627581]
2. Traub RJ, Ji Y. Sex differences and hormonal modulation of deep tissue pain. *Front Neuroendocrinol.* 2013; 34:350–366. [PubMed: 23872333]
3. Ji Y, Tang B, Traub RJ. Spinal estrogen receptor alpha mediates estradiol-induced pronociception in a visceral pain model in the rat. *Pain.* 2011; 152:1182–1191. [PubMed: 21392887]
4. Ji Y, Murphy AZ, Traub RJ. Estrogen modulates the visceromotor reflex and responses of spinal dorsal horn neurons to colorectal stimulation in the rat. *J Neurosci.* 2003; 23(9):3908–3915. [PubMed: 12736360]
5. Safe S, Kim K. Non-classical genomic estrogen receptor (ER)/specificity protein and ER/activating protein-1 signaling pathways. *J Mol Endocrinol.* 2008; 41(5):263–275. [PubMed: 18772268]
6. Graff J, Kim D, Dobbin MM, et al. Epigenetic regulation of gene expression in physiological and pathological brain processes. *Physiol Rev.* 2011; 91(2):603–649. [PubMed: 21527733]
7. Portela A, Esteller M. Epigenetic modifications and human disease. *Nat Biotechnol.* 2010; 28(10):1057–1068. [PubMed: 20944598]
8. Varela MA, Roberts TC, Wood MJ. Epigenetics and ncRNAs in brain function and disease: mechanisms and prospects for therapy. *Neurotherapeutics.* 2013; 10(4):621–631. [PubMed: 24068583]
9. Zhang Z, Cai YQ, Zou F, et al. Epigenetic suppression of GAD65 expression mediates persistent pain. *Nat Med.* 2011; 17(11):1448–1455. [PubMed: 21983856]
10. Bai G, Wei D, Zou S, et al. Inhibition of class II histone deacetylases in the spinal cord attenuates inflammatory hyperalgesia. *Mol Pain.* 2010; 6:51. [PubMed: 20822541]
11. Chiechio S, Zammataro M, Morales ME, et al. Epigenetic modulation of mGlu2 receptors by histone deacetylase inhibitors in the treatment of inflammatory pain. *Mol Pharmacol.* 2009; 75(5):1014–1020. [PubMed: 19255242]

12. Denk F, Huang W, Sidders B, et al. HDAC inhibitors attenuate the development of hypersensitivity in models of neuropathic pain. *Pain*. 2013; 154(9):1668–1679. [PubMed: 23693161]
13. Denk F, McMahon S. Chronic Pain: Emerging Evidence for the Involvement of Epigenetics. *Neuron*. 2012; 73(3):435–444. [PubMed: 22325197]
14. Tang B, Ji Y, Traub RJ. Estrogen alters spinal NMDA receptor activity via a PKA signaling pathway in a visceral pain model in the rat. *Pain*. 2008; 137:540–549. [PubMed: 18068901]
15. Bai G, Ambalavanar R, Wei D, et al. Downregulation of selective microRNAs in trigeminal ganglion neurons following inflammatory muscle pain. *Mol Pain*. 2007; 3:15. [PubMed: 17559665]
16. Liu A, Hoffman PW, Lu W, et al. NF-kappaB site interacts with Sp factors and up-regulates the NR1 promoter during neuronal differentiation. *J Biol Chem*. 2004; 279(17):17449–17458. [PubMed: 14970236]
17. Mukhopadhyay A, Deplancke B, Walhout AJ, et al. Chromatin immunoprecipitation (ChIP) coupled to detection by quantitative real-time PCR to study transcription factor binding to DNA in *Caenorhabditis elegans*. *Nat Protoc*. 2008; 3(4):698–709. [PubMed: 18388953]
18. Mason CE, Shu FJ, Wang C, et al. Location analysis for the estrogen receptor-alpha reveals binding to diverse ERE sequences and widespread binding within repetitive DNA elements. *Nucleic Acids Res*. 2010; 38(7):2355–2368. [PubMed: 20047966]
19. Chiechio S, Copani A, De PL, et al. Transcriptional regulation of metabotropic glutamate receptor 2/3 expression by the NF-kappaB pathway in primary dorsal root ganglia neurons: a possible mechanism for the analgesic effect of L-acetylcarnitine. *Mol Pain*. 2006; 2:20. [PubMed: 16764720]
20. Boulware MI, Weick JP, Becklund BR, et al. Estradiol Activates Group I and II Metabotropic Glutamate Receptor Signaling, Leading to Opposing Influences on cAMP Response Element-Binding Protein. *J Neurosci*. 2005; 25(20):5066–5078. [PubMed: 15901789]
21. Boulware MI, Mermelstein PG. Membrane estrogen receptors activate metabotropic glutamate receptors to influence nervous system physiology. *Steroids*. 2009; 74(7):608–613. [PubMed: 19070630]
22. Jin VX, Sun H, Pohar TT, et al. ERTargetDB: an integral information resource of transcription regulation of estrogen receptor target genes. *J Mol Endocrinol*. 2005; 35(2):225–230. [PubMed: 16216904]
23. Pradhan M, Baumgarten SC, Bembinster LA, et al. CBP mediates NF-kappaB-dependent histone acetylation and estrogen receptor recruitment to an estrogen response element in the BIRC3 promoter. *Mol Cell Biol*. 2012; 32(2):569–575. [PubMed: 22083956]
24. Bai, G.; Hoffman, PW. Transcriptional Regulation of NMDA Receptor Expression. In: Van Dongen, AM., editor. *Biology of the NMDA Receptor*. Boca Raton, FL: CRC press; 2009.
25. DiRenzo J, Shang Y, Phelan M, et al. BRG-1 is recruited to estrogen-responsive promoters and cooperates with factors involved in histone acetylation. *Mol Cell Biol*. 2000; 20(20):7541–7549. [PubMed: 11003650]
26. Chen H, Lin RJ, Xie W, et al. Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase. *Cell*. 1999; 98(5):675–686. [PubMed: 10490106]
27. Wang Z, Zang C, Rosenfeld JA, et al. Combinatorial patterns of histone acetylations and methylations in the human genome. *Nat Genet*. 2008; 40(7):897–903. [PubMed: 18552846]
28. Kapranov P, Willingham AT, Gingeras TR. Genome-wide transcription and the implications for genomic organization. *Nat Rev Genet*. 2007; 8(6):413–423. [PubMed: 17486121]
29. Tran L, Chaloner A, Sawalha AH, et al. Importance of epigenetic mechanisms in visceral pain induced by chronic water avoidance stress. *Psychoneuroendocrinology*. 2013; 38(6):898–906. [PubMed: 23084728]
30. Jiang Y, Langley B, Lubin FD, et al. Epigenetics in the nervous system. *J Neurosci*. 2008; 28(46):11753–11759. [PubMed: 19005036]
31. West AC, Johnstone RW. New and emerging HDAC inhibitors for cancer treatment. *J Clin Invest*. 2014; 124(1):30–39. [PubMed: 24382387]
32. Slingerland M, Guchelaar HJ, Gelderblom H. Histone deacetylase inhibitors: an overview of the clinical studies in solid tumors. *Anticancer Drugs*. 2014; 25(2):140–149. [PubMed: 24185382]

Significance of this study

What is already known on this subject?

- Women are more susceptible to functional pain syndromes including irritable bowel syndrome.
- Estrogen facilitates visceral pain.
- Epigenetic mechanisms are potential targets to relieve somatic pain.

What are the new findings?

- HDAC inhibitors attenuate estrogen-induced visceral hypersensitivity in the rat.
- HDAC inhibitors upregulate mGluR2 mRNA and protein in the spinal cord.
- HDAC inhibitors increase binding of histone 3 hyperacetylated at lysine 9 and ligand-bound estrogen receptor alpha (ER α) to the same regions of the *GRM2* promoter.
- An mGlu2/3 receptor antagonist reversed the inhibitory effect of HDAC inhibitors.

How might it impact on clinical practice in the foreseeable future?

- Inhibition of HDAC may be a potential approach to relieve visceral pain, a defining characteristic of irritable bowel syndrome.

Figure 1.

The magnitude of the VMR following intrathecal injection of the HDAC inhibitor SAHA or vehicle in E2- or oil-treated rats. (A) SAHA (20 and 80 μg) attenuated the E2-facilitated VMR. *, **, *** $p < 0.05, 0.01, 0.001$ vs E2 response in each treatment group. $n = 5-10$ in each treatment group. (B) Example EMG recordings from a rat in the 20 μg SAHA group showing the baseline response (top; OVx), 4 hours post E2 (middle; E2) and 30 min post SAHA injection (bottom; SAHA). Two distentions are indicated by the 20 sec horizontal lines under the bottom trace. (C) Average magnitude of the VMR in Oil or E2-treated rats normalized to their OVx (baseline) response. Data were derived from the OVx and E2/oil points in panel A, the E2 data were pooled from all E2-treated groups. E2, but not oil,

increased the magnitude of the VMR. * $p < 0.05$ vs oil. (D) Average magnitude of the VMR from 30 min to 2 hours post SAHA or DMSO (vehicle) injection normalized to E2/oil response. ** $p < 0.01$ vs E2 + 20 μg SAHA; #,## $p < 0.05, 0.01$ vs E2 + 80 μg SAHA.

Figure 2.

Intrathecal injection of SAHA, but not its vehicle DMSO, increased the acetylation level of H3K9ac in E2 treated rats at 30 min (A) and 2 hours (B) post SAHA injection. **, *** $p < 0.01, 0.001$ vs E2 + DMSO. #, ## $p < 0.05, 0.01$ vs oil + SAHA. $n = 2$ for oil + DMSO group and $n = 4$ for other groups at 30 min post SAHA injection. $n = 4$ for oil + DMSO group and $n = 3$ for other groups at 2 hours post SAHA injection.

Figure 3.

Intrathecal injection of SAHA increased mGluR2 mRNA (A) and mGluR2 protein expression (B), but not mGluR3 mRNA (C), in E2 treated rats 2 hours post SAHA. * $p < 0.05$ ($n = 3-5/\text{group}$).

Figure 4.

HDAC inhibition on binding of acetylated histone 3 or ER α to the *GRM2* promoter. (A) PCR primers designed to cover the proximal transcriptional regulatory region are shown schematically. (B) Primer sequences, relation to TSS, amplicon size and relevant *cis*-elements. (C) Sample of PCR amplicons fractionated on agarose gel. (D) Following IP with an antibody to H3K9ac, binding to five regions of the *GRM2* promoter was quantified. SAHA increased H3K9ac enriched amplicons 1, 4 and 5 compared to DMSO in E2-treated rats. (E) Following IP with an antibody to ER α , binding to three regions of the *GRM2*

promoter was quantified. SAHA increased ER α binding to amplicons 4 and 5 compared to DMSO in E2-treated rats. *, **, *** $p < 0.05, 0.01, 0.001$ for E2 + SAHA compared to E2 + DMSO, n = 6 for each group.

Figure 5.

The magnitude of VMR following i.t. injection of the mGlu2/3 receptor antagonist LY341495 (n = 9) or vehicle (n = 8) in E2+SAHA treated rats. *,*** $p < 0.05$, 0.001 vs E2+SAHA+DMSO.