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Histone hyperacetylation modulates spinal type II metabotropic glutamate receptor alleviating stress-induced visceral hypersensitivity in female rats

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Abstract

Stress is often a trigger to exacerbate chronic pain including visceral hypersensitivity associated with irritable bowel syndrome, a female predominant functional bowel disorder. Epigenetic mechanisms that mediate stress responses are a potential target to interfere with visceral pain. The purpose of this study was to examine the effect of a histone deacetylase inhibitor, suberoylanilide hydroxamic acid, on visceral hypersensitivity induced by a subchronic stressor in female rats and to investigate the involvement of spinal glutamate receptors. Three daily sessions of forced swim induced visceral hypersensitivity. Intrathecal suberoylanilide hydroxamic acid prevented or reversed the stress-induced visceral hypersensitivity, increased spinal histone 3 acetylation and increased mGluR2 and mGluR3 expression. Chromatin immunoprecipitation (ChIP) analysis revealed enrichment of H3K9Ac and H3K18Ac at several promoter *Grm2* and *Grm3* regions. The mGluR2/3 antagonist LY341495 reversed the inhibitory effect of suberoylanilide hydroxamic acid on the stress-induced visceral hypersensitivity. In surprising contrast, stress and/or suberoylanilide hydroxamic acid had no effect on spinal NMDA receptor expression or function. These data reveal histone modification modulates mGluR2/3 expression in the spinal cord to attenuate stress-induced visceral hypersensitivity. HDAC inhibitors may provide a potential approach to relieve visceral hypersensitivity associated with irritable bowel syndrome.

Keywords

Visceral pain, histone deacetylase inhibitor, colorectal distention, spinal cord, visceromotor response, stress, hyperalgesia, epigenetics

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Introduction

Visceral hyperalgesia is a main symptom of irritable bowel syndrome (IBS).[1] Accumulating clinical and experimental evidence indicates that stress is a trigger and exacerbating factor for IBS and induces visceral hypersensitivity in animal models.[1–4] Furthermore, the effect of experimental stress on pain is more robust in females and estrogen dependent.[4–10] A three-day forced swim (FS) paradigm is a subchronic stressor that induces somatic nociceptive hypersensitivity in male rats and increases medullary dorsal horn neuron processing of noxious temporomandibular joint stimuli in females.[11–14] However, its effect on visceral hypersensitivity has not been tested.

Epigenetic mechanisms modulate behavior by regulating genomic activity without altering the DNA sequence. Chromatin remodeling regulates gene

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expression by increasing or decreasing histone acetylation via histone acetyltransferase and histone deacetylase (HDAC) activity, respectively. Inhibition of HDACs prevents deacetylation indirectly enhancing histone acetylation, and is therapeutically useful for the treatment of cancer and neurological disease.[15–17] Preclinical evidence has shown that infusion of HDAC inhibitors into the nucleus accumbens or hippocampus increases histone acetylation and exerts robust antidepressant-like effects.[18,19] The mode of anti-depressant activity exhibited by HDAC inhibitors has been shown to be through regulation of cAMP response element binding protein (CREB), brain-derived neurotrophic factor, and serotonin 2 A receptor in the hippocampus, amygdala, nucleus accumbens, frontal cortex, or ventrolateral orbital cortex.[20,21]

Recently, epigenetic mechanisms were identified in pain processing. In several somatic pain models, HDAC inhibitors attenuated hyperalgesia, in some cases by increasing inhibitory mechanisms.[22–25] Systemic or local administration of HDAC inhibitors into the cerebral ventricles or central amygdala significantly attenuated early-life stress, chronic psychological stress or corticosteroid-induced visceral hypersensitivity.[26–28] Increased expression of the transient receptor potential vanilloid type 1 is accompanied by histone hyperacetylation of its promoter in L6–S2 dorsal root ganglion neurons of chronically stressed male rats.[29] However, it is well known that stress affects visceral sensitivity (i.e. IBS) in women to a greater extent than men, yet the effects of epigenetic modulation of stress-induced visceral hypersensitivity in females remain unknown.

In the present study, we documented that three days of FS evoked visceral hypersensitivity in intact female rats and determined the contribution of epigenetic regulation of type II metabotropic glutamate receptors at the level of the spinal cord.

Materials and methods

Animals

Experimental protocols were approved by the University of Maryland 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 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. In order to direct drugs to the spinal cord, the atlantoccipital membrane was slit and a polyethylene catheter (32 g, ReCathCo, Allison Park, PA) 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) 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.

Forced swim

Subchronic stress in intact female rats was induced by a FS paradigm.[8,11–13,30,31] Rats were placed in a cylindrical container (30 cm diameter) filled to 20 cm with 25°C–26°C water. Rats swam for 10 min on the first day and 20 min on the next two days. The immobility time of rats was recorded during the first 5 min of each swim session. Immobility was defined as the rat moving gently; not struggling to remain floating, but maintaining its head above water.[32] For sham FS, rats remained in their home cages. Pretreated rats were injected with drug/vehicle 30 min prior to each FS. Posttreated rats were injected with vehicle/drug starting one day after the last FS.

Visceromotor response

Rats were fasted for 18–24 h prior to recording the visceromotor response (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 (each trial was five distentions to 60 mmHg, 20 s duration, 3 min interstimulus interval) was produced by inflating the distention balloon with air under computer control (B482CM-1 Valve controller, University of Iowa, Iowa City, IA). The EMG was analyzed by rectifying the signal, subtracting the background activity of the same duration before each distention stimulus, and averaging the response from the five distentions for each trial. The baseline VMR was recorded two or three days before the first day of FS. The response to FS was recorded one day after the last FS.

Tissue collection

One day post FS, rats were euthanized with CO_2 and decapitated. The spinal cord was removed by pressure ejection with ice cold saline as described previously.[33] The L6 to S2 dorsal segment of the spinal cord was isolated, half of which was used for protein extraction and half for RNA extraction. Colorectal distention was not used in the naïve and FS cohorts so as not to have confounding effects of distention. However, animals were given colorectal distention (60 mmHg, 20 s duration, 3 min interstimulus interval for 1 h) in suberoylanilide hydroxamic acid (SAHA) or DMSO pretreated groups to match VMR recording protocols.

Western blots analysis

Tissues were homogenized in RIPA buffer (50 mMTris-HCl, pH 8.0, 150 mMNaCl, 1 mM EDTA, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS) supplemented with 1 mM Na_3VO_4 and protease inhibitor cocktail (Roche, Branford, CT). The homogenates were centrifuged at 14,000 × g for 10 min at 4°C, and the supernatant was collected. Protein concentration in supernatants was 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 (1:1000) directed against acetylated lysine 9 on histone 3 (H3K9Ac, Cell Signaling Technology, Danvers, MA), mGluR2 (abcam, Cambridge, UK), mGluR3 (Novus Biologicals, Littleton, CO), or GluN1 (Santa Cruz Biotechnology, Dallas, TX) at 4°C overnight. The membranes were further incubated for 1 h in blocking buffer with IRDye 800CW goat anti-rabbit secondary antibody (Li-Cor Biosciences, Lincoln, NE). Washed membranes were scanned and analyzed with the Li-Cor Odyssey System (Li-Cor Biosciences, Lincoln, NE). Blots were then stripped for 30 min and reprobed with antibodies against pan-histone (1:1000, Cell Signaling Technology, Danvers, MA) or β-actin (1:5000, Sigma, St. Louis, MO) for normalization.

Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from the L6–S2 dorsal spinal cord using absolutely RNA miniprep kit (Stratgene, La Jolla, CA) and reverse transcribed into cDNAs using SuperScript II Reverse Transcriptase kit with random primers (Invitrogen, Grand Island, NY). Quantification of rat mGluR2 and mGluR3 mRNAs were completed by SYBR green-based real-time polymerase chain reaction (PCR). 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 XM_008762682 for mGluR3) using Primer3 software. Three pairs of primers per mRNA were evaluated and one each (mGluR2, forward, 5′-CG TGAGTTCTGGGAGGAGAG, reverse, 5′-GCGGAC CTCATCGTCAGTAT; mGluR3, forward, 5′-GTGGT CTTGGGCTGTTTGTT, reverse, 5′-GCAGCATGTG AGCACTTTGT; GluN1, forward, 5′-GCAGGTGGA GTTGAGTACCATGTAC, reverse, 5′-CAGCTTGT TGTCCCGCACA) with comparable PCR efficiency to that of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA (forward, 5′-TCACCACCATGGA GAAGGC, reverse, 5′-GCTAAGCAGTTGGTGGTG CA) was chosen. Real-time PCR was completed in Maxima SYBR Green/Rox qPCR Master Mix (Thermo Scientific, Waltham, MA) on the Eppendorf Mastercycler Real-plex system (Eppendorf, Hauppauge, NY), 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 $\Delta\Delta C_T$ method after normalization to GAPDH mRNA as described previously.[34]

Chromatin immunoprecipitation

ChIP was performed using a previously described method.[35,36] Briefly, dissected L6–S2 spinal dorsal horn was minced in 1 × PBS (pH 7.4) supplemented with protease inhibitors and subjected to DNA-protein crosslink by incubation with 1.5% paraformaldehyde for 20 min at room temperature. Tissues underwent homogenization and isolation of nuclei. Isolated nuclei were sonicated to yield DNA fragments around 500 bp. For immunoprecipitation, 3 μg of polyclonal antibodies specific to H3K9Ac or H3K18Ac (Cell Signaling Technology, Danvers, MA) were mixed with 200 μg proteins of the lysates for overnight incubation at 4°C. In negative controls, IgG purified from non-immunized rabbit serum was added to replace antibody. The antibody–antigen complexes were pulled down by protein A/G-agarose followed by successive washes and final elution into solution of 100 mM $NaHCO_3$-1% SDS. After reverse crosslink, DNA was purified using a Qiaquick column. Lysate equal to one quarter of the immunoprecipitate was directly subjected to reverse crosslink and served as input. Eluted DNA was subjected to real-time PCR with SYBR green (Thermo Scientific, Waltham, MA) in duplicates for the *Grm2* (4), *Grm3* (4), and *Grin1* (1) promoters (number of primer pairs in parentheses; sequences shown in Table 1). PCR 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 s and 57°C–64°C for 30 s. Relative levels of bound DNA were analyzed using the $\Delta\Delta C_T$ method as described previously with a normalization of immunoprecipitation data to

Table 1. Primer sequences and location of amplicons on the promoters of genes examined in ChIP assay.

Gene	Amplicon	bp	Sequence	Direction	To TSS
GRM2[a]	A1	338	GTATGGCTGGGCTCTCTCTG	Forward	−1509/−1490
			AGGCCTTTTCTCTGGACACA	Reverse	−1191/−1172
	A2	245	CCATTAGCAGTTGGGCTTGT	Forward	−1282/−1263
			GGGCTCATCATCCCTACTCA	Reverse	−1054/−1038
	A3	164	AGCTCTGAGGCCAAATCTCA	Forward	−986/−967
			TGAGCAGAGGGAGGACAGTT	Reverse	−842/−823
	A4	208	TGACCTCCCCCTCCTAATCT	Forward	−533/−514
			TGGACTCGGGAAGAGAGCTA	Reverse	−345/−326
GRM3[b]	A1	230	TCTCCTCTAGCCCTCCCTTT	Forward	245/264
			GGTTGGTCCTCTTCATCCGA	Reverse	455/474
	A2	221	AGGATGTCAGAAGGTCCGTG	Forward	43/62
			AAGGGAGGGCTAGAGGAGAA	Reverse	244/263
	A3	205	GTGTCGGATGAAGAGGACCA	Forward	452/471
			TCAACTTTCCTCGCTTTGCC	Reverse	636/655
	A4	238	CCACTGCTCCGAAATTCACC	Forward	−176/−156
			CACGGACCTTCTGACATCCT	Reverse	43/62

[a]The transcription start site or the 5′ end of exon 1 of the rat *GRM2* gene is obtained from UCSC Genome Browser.
[b]Exon 1 of *GRM3* contains 559 bpgenomic sequences (Genebank accession number of XM_008762682 and the rat genome annotation release 105). Intronic sequences are counted following these of exon 1.

relevant input of each sample and a desert region.[34,36] Six rats per group were tested for each pair of primers in ChIP.

Hormones and drugs

SAHA (Cayman Chemical, Ann Arbor, MI), made fresh, was dissolved in 10% dimethyl sulfoxide (DMSO) to a final concentration of 40 µg/10 µl. SAHA (40 µg) or 10% DMSO (vehicle) was injected intrathecally (i.t.) 30 min before each FS for three days. The glutamate mGluR2/3 antagonist (alphaS)-alpha-amino-alpha-[(1 S,2 S)-2-carboxycyclopropyl]-9H-xantine-9-propanoic acid (LY341495; 20 nmol, Tocris Bioscience, Bristol, UK) was also dissolved in 10% DMSO. The NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (APV, 30 nmol, Sigma, St. Louis, MO) was dissolved in saline. For i.t. injection, 10 µl volume was injected followed by a 10 µl saline flush. Drug doses were taken from previous studies in our lab.[36,37]

Data analysis

Data were analyzed using Prism 6. All data are expressed as mean ± SEM. In all experiments, the baseline data refers to the VMR recorded prior to any manipulation (drug injection, stress). For the VMR data raw area under the curve, data (magnitude of VMR) are presented if statistical comparison were within group (one-way ANOVA). If different experimental groups were compared, data were normalized to equalize the baseline and show the magnitude of change by treatment. The mean of the raw baseline values was calculated, and individual baseline values were normalized to this mean value. All other data for each rat were normalized to the baseline response from that rat. Data were analyzed by one-way or two-way ANOVA as appropriate. *t* test was used for two group comparison. $p < 0.05$ was considered significant.

Results

Subchronic stress-induced visceral hypersensitivity in intact female rats

Three days of FS significantly increased the magnitude of the VMR one day after the last FS compared with baseline, a state of stress-induced visceral hypersensitivity (paired *t* test, $t_{23} = 3.199$, $p = 0.0040$, Figure 1(a)). Consistent with FS as a stressor, immobility time, measured during the first 5 min of each FS, increased on the second and third day compared with the first day (one-way RM ANOVA, $F_{2,62} = 24.48$, $p < 0.0001$; Figure 1(b)).

Stress-induced visceral hypersensitivity is blocked/reversed by an HDAC inhibitor at the level of the spinal cord

Stress was reported to affect DNA methylation and chromatin remodeling to alter behavior.[38,39] Here, we

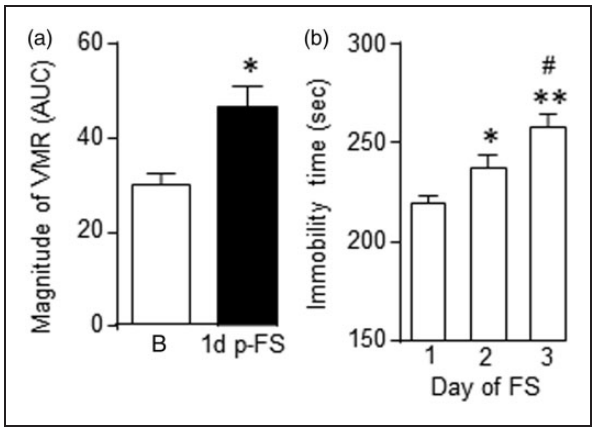

Figure 1. Stress induces visceral hypersensitivity. (a) The magnitude of the VMR increased one day following the last FS (1 d p-FS) compared to baseline (B). *$p < 0.005$ vs. baseline ($n = 24$). Data were pooled from all animals tested before and after FS without drug treatment. (b) Immobility time during the first 5 min of each FS increased daily. *$p < 0.001$, **$p < 0.0001$ vs. day 1; #$p < 0.0001$ vs. day 2 ($n = 32$). Immobility time observations included eight rats whose tissues were collected for Western blot, but the VMR was not recorded.

investigated whether spinal application of the HDAC inhibitor SAHA could prevent or reverse visceral hypersensitivity evoked by subchronic stress. In pretreated rats, there was a significant effect of SAHA vs. DMSO (two-way RM ANOVA, treatment, $F_{1,33} = 6.887$, $p = 0.0131$; Figure 2(a)). FS increased the magnitude of the VMR in rats pretreated with 10% DMSO. In contrast, there was no increase in visceral sensitivity in rats pretreated with SAHA. These results indicate that inhibition of spinal HDACs prevents the development of visceral hypersensitivity evoked by FS stress. In control rats, three daily i.t. injections of SAHA in the absence of stress did not affect the magnitude of the VMR measured one day after the last injection compared with baseline (paired t test, $t_{17} = 1.267$, $p = 0.2224$; Figure 2(b)), indicating that the inhibitory effects of SAHA on visceral sensitivity are specific to the pathologic condition.

We also determined whether SAHA or vehicle reversed the stress-induced visceral hypersensitivity after it developed. As expected, FS increased visceral sensitivity measured one day after the last FS (FS: $p < 0.05$ compared with baseline for both groups, Figure 2(c) and (d)). Visceral hypersensitivity was not affected 30 min following a single injection of SAHA (day 1, Figure 2(c)). However, the three daily injections significantly reversed the visceral hypersensitivity produced by FS (one-way RM ANOVA, $F_{3,36} = 4.344$, $p = 0.0103$; Figure 2(c)). Multiple injections of vehicle (10% DMSO) had no effect on visceral hypersensitivity induced by FS as visceral sensitivity remained elevated compared to baseline (one-way RM ANOVA, $F_{3,21} = 3.789$, $p = 0.0257$, Figure 2(d)).

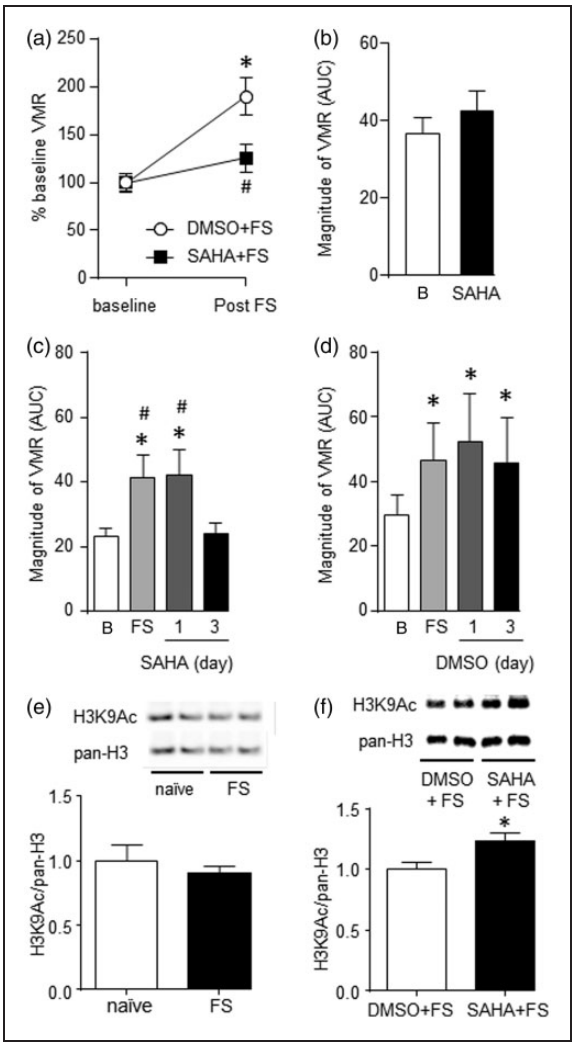

Figure 2. SAHA prevents/attenuates stress-induced visceral hypersensitivity and increases histone acetylation in the LS spinal cord. (a) The magnitude of VMR one-day post FS following SAHA or DMSO pretreatment. Data were normalized to each rat's baseline response. #$p < 0.01$ vs. DMSO + FS group; *$p < 0.001$ vs. baseline; $n = 16$ for SAHA + FS group and $n = 19$ for DMSO + FS group. (b) The magnitude of the VMR one day following three daily injections of SAHA in the absence of FS ($n = 18$). Data were pooled from all animals administered SAHA in the absence of stress. (c, d) The magnitude of the VMR following one or three daily injections of SAHA or DMSO starting one day after the last FS. *$p < 0.05$ vs. baseline (B), #$p < 0.05$ vs. three-day post FS. $n = 13$ for FS + SAHA group, $n = 8$ for FS + DMSO group. (e) FS had no effect on histone acetylation. (f) SAHA prior to FS

To confirm that SAHA-induced chromatin remodeling contributed to the attenuation of the stress-induced visceral hypersensitivity, the effect of SAHA on histone acetylation was determined. As a proxy for HDAC inhibition at multiple lysine sites on nuclear histones, the relative level of H3K9Ac was determined. Compared to naïve rats, FS did not alter the global

level of H3K9Ac in the dorsal spinal cord (t test, $t_8 = 0.6701$, $p = 0.5217$; Figure 2(e)). However, consistent with its action as an HDAC inhibitor, SAHA prior to each FS increased H3K9Ac compared to DMSO pretreatment (t test, $t_{11} = 2.542$, $p = 0.0274$; Figure 2(f)).

SAHA increases inhibitory processing attenuating stress-induced visceral hypersensitivity

The simplest explanation for the antinociceptive effect of SAHA at the level of the spinal cord is an increase in inhibitory processing. We previously reported that SAHA reversed estradiol-dependent facilitation of the VMR in ovariectomized rats by increasing mGluR2 in the spinal cord.[36] In the present study, we determined if this similarly occurs in intact females in response to stress. Rats were pretreated with SAHA or DMSO prior to FS. Following measurement of the post-stress VMR (FS in Figure 3(a)), the mGluR2/3 antagonist LY341495 (20 nmol) was administered i.t. and the VMR measured at 30, 60, and 90 min. There was a significant effect of the mGluR2/3 antagonist on the VMR comparing rats pretreated with SAHA or DMSO (two-way RM ANOVA: treatment × time, $F_{4,48} = 3.322$, $p = 0.0176$; Figure 3(a)). SAHA pretreatment prior to each FS prevented the development of visceral hypersensitivity. i.t. injection of LY341495 reversed the inhibitory effect of SAHA inducing visceral hypersensitivity (Figure 3(a)). In contrast, in rats pretreated with DMSO, there was a FS-induced increase in visceral sensitivity that remained elevated but did not further increase following LY341495 (Figure 3(a)). Furthermore, the effect of LY341495 was significantly greater in the SAHA pretreated rats compared to the DMSO pretreated rats. In naïve females (no DMSO/SAHA, no FS), LY341495 had no effect on visceral sensitivity (one-way RM ANOVA, $F_{3,15} = 0.6644$, $p = 0.5867$; Figure 3(b)), nor in females that were treated with SAHA but were not stressed (one-way RM ANOVA, $F_{4,24} = 2.014$, $p = 0.1245$; Figure 3(c)). These data suggest that i.t. SAHA increases expression of mGluR2/3 in the spinal cord contributing to the attenuation of stress-induced visceral hypersensitivity. This was confirmed by Western blot and Quantitative reverse transcription polymerase chain reaction data. SAHA pretreatment increased mGluR2 mRNA ($t_{15} = 3.87$, $p = 0.0015$; Figure 4(a)) and mGluR2 protein expression ($t_{11} = 6.462$, $p < 0.0001$; Figure 4(b)), and mGluR3 mRNA ($t_{12} = 3.334$, $p = 0.006$; Figure 4(c)) and mGluR3 protein expression ($t_{11} = 5.92$, $p < 0.0001$; Figure 4(d)).

Since the increase in H3K9Ac in the spinal cord by SAHA (Figure 2(f)) was nonspecific, but SAHA increased mGluR2/3 expression, we determined if SAHA led to an enrichment of acetylated histones at promoters of *Grm2* or *Grm3*. A ChIP assay revealed pretreatment with SAHA enriched H3K9Ac and H3K18Ac at three of the four sites tested on the *Grm2* promoter compared to DMSO (Figure 5). The *Grm3* promoter showed enrichment at one of the four sites to H3K9Ac and three of the four sites to H3K18Ac (Figure 6).

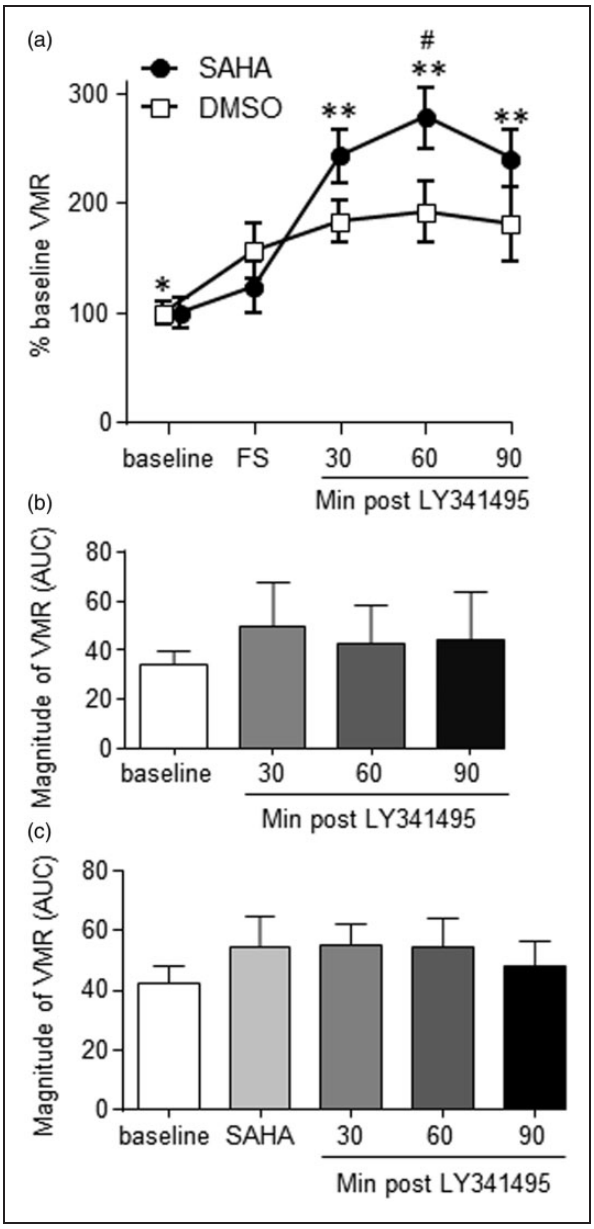

Figure 3. The mGluR2/3 antagonist LY341495 reversed the effect of SAHA on visceral sensitivity. (a) Baseline was recorded prior to start of the stress paradigm (offset for clarity). SAHA or DMSO was injected i.t. 30 min prior to each FS. One day after the last FS, the VMR was recorded (FS). LY341495 was injected i.t. and the VMR recorded 30, 60, 90 min later. LY341495 reversed the inhibitory effect of SAHA but had no effect following DMSO + FS. $^{*}p < 0.05$, $^{**}p < 0.0001$ vs. post FS; $^{\#}p < 0.05$ vs. DMSO. (b) LY341495 did not affect visceral sensitivity in naïve female rats without SAHA or stress, nor (c) in SAHA treated females without stress. $n = 6$–7 per group.

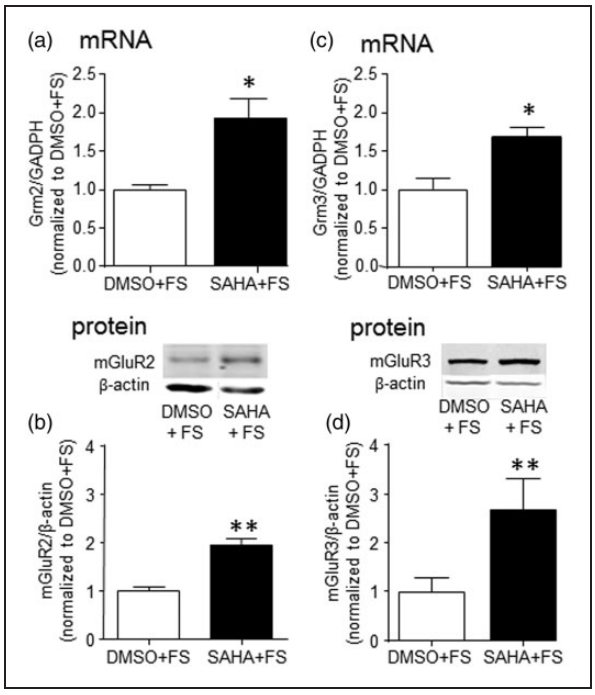

Figure 4. SAHA increased mGluR2 and mGluR3 mRNA (a, c) and protein (b, d) expression in stressed rats. $^{*}p < 0.01$, $^{**}p < 0.0001$ vs. DMSO + FS group. $n = 6$–9 per group.

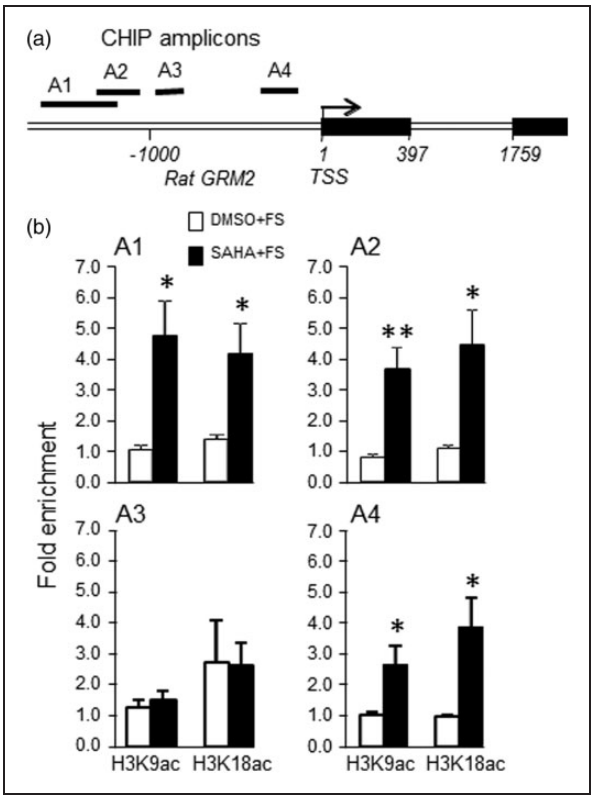

Figure 5. ChIP assay for *Grm2* and acetylated histone 3. (a) PCR primers designed to cover the proximal transcriptional regulatory region shown schematically (sequences shown in Table 1). (b) PCR amplicons of four regions of the *Grm2* promoter were quantified and normalized to relevant input and a desert region. $n = 6$ for each group. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with DMSO pretreatment group for amplicons 1–4 for H3K9Ac or H3K18Ac. TSS: transcription start site.

These data confirm that in addition to any nonspecific effects, SAHA acted specifically to increase histone acetylation at the mGluR2/3 promoters increasing receptor expression.

Stress and SAHA have no effect on spinal NMDA receptor activity

As an alternative to increasing inhibitory processing, a decrease in excitatory processing could contribute to the SAHA evoked decrease in stress-induced hypersensitivity. In female rats, visceral nociception and colonic inflammation-induced hypersensitivity are modulated by estradiol and are NMDA receptor dependent. However, the role of spinal NMDA receptors in stress-induced visceral hypersensitivity and modulation by HDAC inhibitors is unknown. Following FS stress, there was a significant increase in *Grin1* mRNA in the spinal cord ($t_8 = 3.130$, $p = 0.0140$; Figure 7(a); $n = 5$/group), but there was not an increase in GluN1 protein expression ($t_8 = 1.930$, $p = 0.0897$; Figure 7(b); $n = 5$/group). SAHA pretreatment decreased *Grin1* in the spinal cord following stress ($t_{15} = 2.372$, $p = 0.0315$; Figure 7(c); $n = 8$–9/group), but there was no change in GluN1 protein ($t_{12} = 1.179$, $p = 0.2636$ Figure 7(d); $n = 6$–8/group).

As a functional measure, 30 nmol APV was injected i.t., and the effects on the VMR were determined (Figure 7(e)). APV attenuated or strongly trended toward significant attenuation of the VMR compared to baseline and/or post-FS in experimental groups sham FS (RM ANOVA, $F_{2,26} = 3.948$, $p = 0.0318$, $n = 14$), FS ($F_{2,20} = 7.953$, $p = 0.0029$, $n = 11$), SAHA + sham FS ($F_{2,22} = 7.574$, $p = 0.0031$, $n = 12$) and SAHA + FS ($F_{2,16} = 3.440$, $p = 0.0572$, $n = 9$). However, consistent with the lack of change in protein expression, APV produced equivalent attenuation of the VMR when compared to the post-stress response (one-way ANOVA, $F_{3,32} = 0.3585$, $p = 0.7833$: Figure 7(f)). These data suggest that spinal NMDA receptors may not contribute to stress-induced hypersensitivity nor is there modulation of NMDA receptors by SAHA.

Discussion

In the present study, we demonstrated that increasing histone acetylation by spinal administration of SAHA, an HDAC inhibitor, intervened in the development of visceral hypersensitivity evoked by subchronic FS stress. Previous reports have shown that analgesic effects of HDAC inhibitors may due to epigenetic regulation of

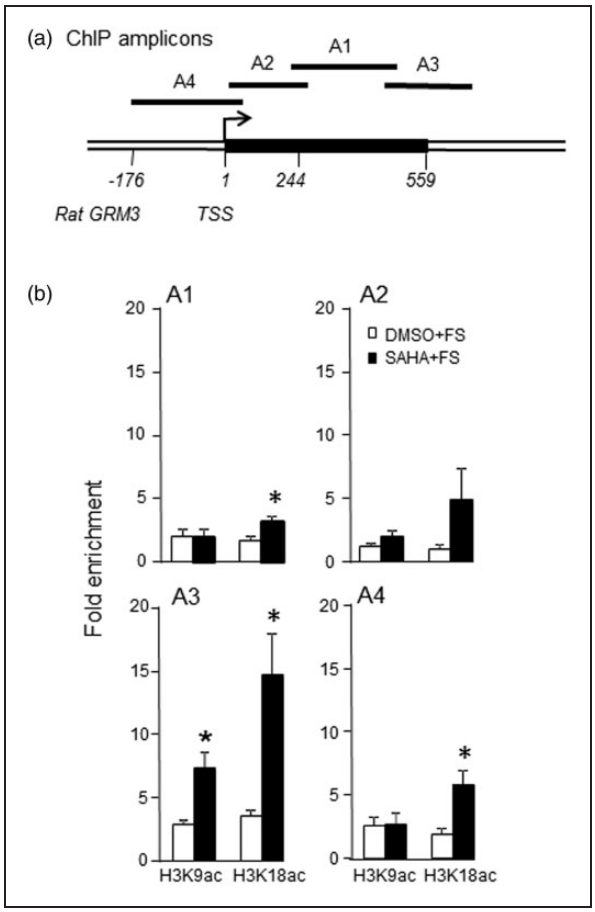

Figure 6. ChIP assay for *Grm3* and acetylated histone 3. (a) PCR primers designed to cover the proximal transcriptional regulatory region shown schematically (sequences shown in Table 1). (b) PCR amplicons of four regions of the *Grm3* promoter were quantified as Figure 5. $n = 6$ for each group. $^{*}p < 0.05$ compared with DMSO pretreatment group for amplicons 1–4 for H3K9Ac or H3K18Ac. TSS: transcription start site.

mGluR2 in the spinal cord.[23,36] Consistent with this, our results show that: (1) spinally administered SAHA blocked stress-induced visceral hypersensitivity from developing, which was reversed by an mGluR2/3 antagonist; (2) SAHA increased acetylation of histone 3 and enhanced enrichment of acetylated histone 3 to the *Grm2 and Grm3* promoters in the spinal cord of stressed rats; and (3) SAHA upregulated mGluR2 and mGluR3 expression, suggesting that HDAC inhibitors could be used to modulate spinal mGluR2 and mGluR3 expression and relieve stress-induced visceral hypersensitivity. Surprisingly, however, spinal NMDA receptors did not contribute to stress-induced visceral hypersensitivity or SAHA-evoked antihypersensitivity in this animal stress model.

Visceral hypersensitivity is one of the chief pathophysiological symptoms of IBS. These patients report greater pain, lower thresholds, and expanded areas of referred pain compared to healthy volunteers.[40–43] Furthermore, female patients report greater pain and discomfort compared to male patients.[44,45] Similarly, in rodents, the magnitude of the VMR is greater in females compared to males across several, but not all, species and strains.[37,46–48] In addition, female rats have more robust responses to colonic inflammation compared to males.[37] For this reason, we focused on females in this study.

Stress and visceral hypersensitivity

Stress is a major trigger to develop and exacerbate visceral pain.[49] It has been well established that chronic stress such as repeated water avoidance stress, heterotypic intermittent stress, or neonatal maternal separation produce visceral hypersensitivity.[4,10,50–53] Here, we demonstrate that a much shorter FS stress (3 days vs. 10 days or longer) induces visceral hypersensitivity in intact female rats. Previous studies showed that subchronic FS stress-induced somatic hyperalgesia that persists for one to two weeks.[11,54,55] FS stress increased phospho-CREB and c-Fos in the anterior insula cortex of male rats and increased the functional magnetic resonance imaging signal in the insula of female rats to colorectal distention that is estrogen dependent.[31,56] In addition, we have used the same stress paradigm in a temporomandibular disorder-IBS comorbid pain model to induce E2-dependent visceral hypersensitivity that persists for months.[8] At the cellular level, repeated FS enhanced both the background firing rate and stimulation-activated response magnitude in spinomedullary dorsal horn neurons, indicating that spinal neurons may contribute to the development of hyperalgesia following repeated psychophysical stress.[14,57] These data support that multiday FS is a valid stress-inducing paradigm in addition to an assay for despair.[32]

Epigenetics, stress, and pain

Posttranslational acetylation of histones and other transcription factors contributes to chromatin remodeling and transcriptional regulation in response to external stimuli including stress and pain. For example, in the prefrontal cortex and hippocampus, the acetylating agent L-acetylcarnitine (LAC) increased levels of acetylated H3K27 bound to the *Grm2* promotor. This upregulated mGluR2 expression and reduced stress behaviors.[58] In the amygdala corticosteroid injection models stress-induced hypersensitivity which is attenuated by HDAC inhibitors.[27] Neuropathic and inflammatory pain decreases GABAergic signaling in the brainstem contributing to hyperalgesia. HDAC inhibitors recover GABAergic function reversing the increased pain.[22]

At the level of the spinal cord, upregulating spinal inhibition by increasing mGluR2/3 expression has been

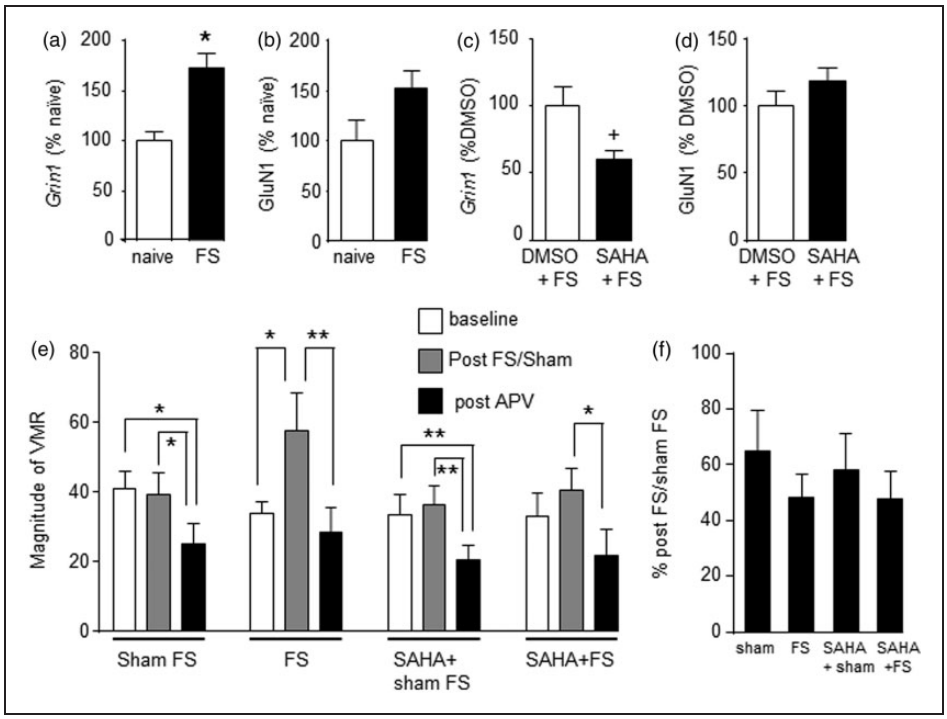

Figure 7. Spinal NMDA receptors do not contribute to the effects of stress and/or SAHA on visceral hypersensitivity. mRNA (a, c) and protein (b, d) expression in the LS spinal cord. Data were normalized to naïve or DMSO + FS as appropriate. * $p < 0.05$ vs. naïve, + $p < 0.05$ vs. DMSO + FS. (e) The effect of i.t. APV on the VMR. *$p < 0.05$, **$p < 0.01$. (f) The effect of i.t. APV normalized to the post FS/sham FS for each experimental group shown in Panel e. There was no difference between groups.

used to attenuate inflammatory and neuropathic pain.[59,60] LAC or HDAC inhibitors increased NFκB family transcriptional activity upregulating mGluR2 evoked attenuation of inflammatory pain in mice.[23,61] HDAC inhibitors also attenuated visceral sensitivity following estradiol replacement in ovariectomized rats by increasing mGluR2 expression.[36]

In the present study, stress had no effect on global acetylation of histone 3 in the spinal cord, yet there was a significant increase in visceral sensitivity. Furthermore, i.t. SAHA had no effect on the VMR to acute colorectal distention. However, in stressed rats, i.t. SAHA increased histone acetylation, upregulated mGluR2 and mGluR3 expression and attenuated the stress-induced visceral hypersensitivity. Furthermore, we confirmed a spinal site of action by demonstrating that SAHA increased histone acetylation and enriched hyperacetylated histone 3 binding to several sites on the mGluR2 and mGluR3 promoters followed by upregulated mRNAs and proteins, although possible regulation of transcriptional activity in DRG cells cannot be ruled out.[62] These data suggest that histone hyperacetylation in the dorsal horn is not necessary for stress to induce visceral hypersensitivity, at least in the short time points, but altering the balance of histone acetylation/deacetylation by HDAC inhibitors resolves stress-induced hypersensitivity.

In addition to examining epigenetic modulation of inhibitory mechanisms, the effects of stress and SAHA on spinal NMDA receptors were examined. Since NMDA receptors modulate visceral sensitivity,[37,63] spinal NMDA receptor function is modulated by estrogen[33,64] and stress/depression are modulated by NMDA receptors,[65,66] we examined epigenetic regulation of spinal NMDA receptors by stress in female rats. Stress increased GluN1 message in the spinal cord, but there was no significant increase in GluN1 protein. Further, there was no change in the inhibitory effect of an NMDA receptor antagonist. These data suggest that stress-induced visceral hypersensitivity is not dependent on an increase in spinal NMDA receptor activity in this model. In addition, while SAHA increased mGluR2/3 activity, there was no effect on NMDA receptor activity.

Therapeutic potential for HDAC inhibitors in stress and chronic pain

HDAC inhibitors have shown potential therapeutic efficacy in many rodent models of neurodegenerative diseases for neuroprotective function, preventing or delaying neuronal dysfunction, and are being tested for several neurological disorders in preclinical and clinical studies.[15,67,68] The finding that spinal administration of

HDAC inhibitors blocked the development of visceral hypersensitivity after repeated FS stress is consistent with recent reports that increasing spinal histone acetylation attenuates hyperalgesia development in neuropathic and inflammatory pain models.[23–25] The present study together with other studies[22–25,27] 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 sensitivity 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.[25]

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Author contributions

DYC, GB, YJ and JK conducted experiments and collected the data; DYC, GB, YJ, and RJT analyzed the data; DYC and RJT wrote the manuscript.

Declaration of Conflicting Interests

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