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Epigenetic Regulation of Genes that Modulate Chronic Stress-induced Visceral Pain in the Peripheral Nervous System

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Abstract

BACKGROUND & AIMS—Chronic stress alters the hypothalamic–pituitary–adrenal axis, increases gut motility, and increases perception of visceral pain. We investigated whether epigenetic mechanisms regulate chronic stress-induced visceral pain in the peripheral nervous systems of rats.

METHODS—Male rats were subjected to 1 hr water-avoidance stress each day, or given daily subcutaneous injections of corticosterone, for 10 consecutive days. L4–L5 and L6–S2 dorsal root ganglia (DRG) were collected and compared between stressed and control rats (placed for 1 hour each day in a tank without water). Levels of cannabinoid receptor 1 (CNR1), DNA (cytosine-5)-methyltransferase 1 (DNMT1), transient receptor potential vanilloid type 1 (TRPV1), and EP300 were knocked down in DRG neurons in situ with small interfering RNAs. We measured DNA methylation and histone acetylation at genes encoding the glucocorticoid receptor (NR3C1), CNR1, and TRPV1. Visceral pain was measured in response to colorectal distention.

RESULTS—Chronic stress was associated with increased methylation of the *Nr3c1* promoter and reduced expression of this gene in L6–S2, but not L4–L5, DRGs. Stress was also associated with upregulation in DNMT1-associated methylation of the *Cnr1* promoter and downregulation of glucocorticoid receptor-mediated expression of CNR1 in L6–S2, but not L4–L5, DRGs. Concurrently, chronic stress increased expression of the histone acetyltransferase EP300 and increased histone acetylation at the *Trpv1* promoter and expression of the TRPV1 receptor in L6–S2 DRG neurons. Knockdown of DNMT1 and EP300 in L6–S2 DRG neurons of rats reduced DNA methylation and histone acetylation, respectively, and prevented chronic stress-induced increases in visceral pain.

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AUTHOR CONTRIBUTIONS

S.H. and G.Z. contributed equally to this work. S.H. and G.Z. designed, performed and analyzed the experiments. S.H. and J.W.W. conceived the study, supervised the research, and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

CONCLUSIONS—Chronic stress increases DNA methylation and histone acetylation of genes that regulate visceral pain sensation in the peripheral nervous system of rats. Blocking epigenetic regulatory pathways in specific regions of the spinal cord might be developed to treat patients with chronic abdominal pain.

Keywords

Visceral hyperalgesia; HPA; epigenetic modification; pain sensitivity

Introduction

In human and animal models, chronic stress is commonly associated with enhanced abdominal pain (visceral hyperalgesia) and altered bowel function.^{1, 2} Despite considerable clinical and pre-clinical research, the pathophysiology of chronic stress-induced visceral hyperalgesia remains poorly understood. Alterations in CRF signaling, the substance P/ neurokinin-1 receptor system, a disrupted intestinal epithelial barrier function and changes in the microbiota correlate with peripheral nociceptor sensitization.^{2, 3} Our previous studies revealed that chronic stress induces reciprocal changes in the anti-nociceptive endocannabinoid receptor 1 (CNR1; down-regulation) and pro-nociceptive endovanilloid TRPV1 (up-regulation) pain pathways in nociceptive L6-S2 DRG neurons that innervate the pelvic organs including the colon based on retrograde labeling and functional studies.^{4, 5} The CNR1 and TRPV1 pain pathways interact;⁶ activation of CNR1 inhibits TRPV1 function.⁷ How chronic stress modulates these pathways is unknown.

Epigenetics refers to a variety of processes which can have long-term effects on gene expression programs without changes in DNA sequence. Interest in epigenetic regulatory pathways has emerged rapidly recently because of their apparent significance in key physiological processes including cell cycle regulation. Important processes in epigenetic control include DNA methylation, catalyzed by DNA methyltransferases (DNMTs) including DNMT1 which is responsible for the maintenance of methylation patterns and DNMT3a and DNMT3b which are responsible for *de novo* methylation patterns, resulting in gene silencing,^{8, 9} and histone acetylation catalyzed by acetyltransferases that promote gene transcription.¹⁰ DNA methylation and histone acetylation are considered “stable yet reversible” because the relevant enzymes are inducible and may be altered by biochemical and environmental modulation.^{11, 12}

Studies of epigenetic regulation in response to stress have been primarily focused on the central nervous system. For example, chronic stress alters the level of circulating glucocorticoids and DNA methylation at CpG islands on the glucocorticoid receptor (NR3C1) gene promoter in specific regions of the brain, resulting in altered NR3C1 expression and function in the HPA axis.^{13, 14} Little is presently known about the role of epigenetic mechanisms in modulating peripheral sensory nerve function. A subset of patients with irritable bowel syndrome (IBS) demonstrate altered expression of specific noncoding microRNAs in intestinal mucosa that inhibit glutamine synthetase, supporting a potential role for epigenetic pathways in this disorder.¹⁵ In the current study, we

investigated a potential role for epigenetic regulatory pathways in chronic stress-enhanced abdominal pain (visceral hyperalgesia).

Materials and Methods

Animals

Male Sprague–Dawley rats (200–220 g) were obtained from Charles River Laboratories (Wilmington, MA). Animals were housed in an animal facility that was maintained at 22°C with an automatic 12 h light/dark cycle. The animals were given a standard laboratory diet and tap water was available *ad libitum*. All experimental procedures were performed in accordance with US National Institutes of Health guidelines and were approved by the University Committee on Use and Care of Animals at the University of Michigan. The experimenter was blinded to animal treatments during the assessment of the behavioral (pain) response.

Chronic water avoidance stress

Repeated exposure of adult rats to water avoidance (WA) stress was conducted as described previously.^{5, 16} The rats were placed on a glass platform in the middle of a Plexiglas tank filled with water (25°C) to 1 cm below the height of the platform. The animals were maintained on the tank for 1 hour in the morning daily for 10 consecutive days. The control (sham-stress) rats were placed similarly for 1 hour daily for 10 days in a tank without water.

Gene knockdown using small interfering RNA (siRNA)

For knockdown of the targeted genes in rat DRG neurons *in situ*, the pre-designed siRNA or the non-targeting negative control siRNA was dissolved in double-distilled water, diluted with the transfection reagent i-Fect (Neuromics, Edina, MN) to achieve a final concentration of 0.4 nM/10 µL, and injected intrathecally at the L6–S2 or L2–L3 spinal region every other day for a total of 5 injections in control and stressed rats during the 10-day stress phase. The following siRNAs were purchased from Ambion and used for gene knockdown: *Nr3c1* (#s127819), *Cnr1* (#s129266), *Dnmt1* (#s136451), *Trpv1* (#s136297), *Ep300* (#s220365). Verification of region-specific gene knockdown at the spinal DRG levels was shown in the Supplemental Data using *Trpv1* siRNA.

Visceromotor response (VMR) to colorectal distension (CRD)

Measurement of the VMR to CRD was conducted in separate groups of rats on day 11 following the completion of the 10-day WA stress procedure, as described previously.^{4, 5} Briefly, the VMR was quantified by measuring electromyographic activity in the external oblique musculature in the awake animals. CRD was conducted to constant pressures of 10, 20, 40, and 60 mm Hg by a custom-made distension control device. The responses were considered stable if there was less than 20% variability between 2 consecutive trials of CRD at 60 mm Hg. The increase in the area under curve (AUC), which is the sum of all recorded data points multiplied by the sample interval (in seconds) after baseline subtraction, was presented as the overall response during the course of the CRD test.

Methylated DNA immunoprecipitation (MeDIP) assay

MeDIP assay was performed using the EpiQuik™ Tissue MeDIP Kit (Epigentek, Farmingdale, NY), according to the manufacturer's instruction. Briefly, the genomic DNA was sheared to random fragments between 200 bp to 1,000 bp. Immunoprecipitation was performed using monoclonal antibody against 5-methylcytosine (Epigentek) for the sample or normal mouse IgG as the negative control. The beads were treated with proteinase K for 1 h at 65°C and the methylated DNA was recovered by phenol-chloroform extraction followed by ethanol precipitation. Q-PCR amplification was performed. The relative changes in the methylation levels were normalized to the input DNA and *Gapdh*.

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation was performed using a chromatin immunoprecipitation kit (EMD Millipore, Billerica, MA). In brief, DRG samples were cross-linked using 1% formaldehyde and terminated by incubation with 0.125 M glycine for 5 min. The cell lysate was incubated for 10 min at 4°C and the crude nuclear extract was collected by centrifugation at 600×g for 5 min at 4°C. The DNA was sonicated to random fragments between 200 bp to 500 bp. The chromatin was subjected to immunoprecipitation using the following antibodies: NR3C1 (#sc-1004; Santa Cruz Biotechnology, Dallas TX), DNMT1 (#13479; Cayman Chemical, Ann Arbor, MI) and acetyl-histone H3 (#P-2012; Epigentek). Normal mouse or rabbit IgG was used as a control. DNA was finally eluted in elution buffer and used for PCR or real-time PCR amplification using the same primer sets with MeDIP-qPCR.

Statistical analysis

To examine the VMR in response to CRD pressures, the electromyographic amplitudes, represented by calculating the AUC, were normalized as percentage of baseline response for the highest pressure (60 mm Hg) for each rat and then averaged for each group of rats. The effects of stress and/or pharmacologic treatment on the VMR to CRD was analyzed using a repeated-measures two-way ANOVA followed by Bonferroni post-test comparisons. Unpaired Student *t* test was used to examine the data for the protein/RNA expression obtained from Western blot, immunohistochemistry and qPCR. Results were expressed as means ± s.e.m. *P* < 0.05 was considered statistically significant.

Results

Chronic WA stress induced region-specific increase in DNA methylation of *Nr3c1* promoter and decrease in NR3C1 expression in DRG neurons

Chronic WA stress induced a significant increase in CpG site (P1) methylation of the glucocorticoid receptor gene (*Nr3c1*) exon 1₇ promoter region (1738 to 1794) in nociceptive L6-S2 DRG neurons innervating the pelvic viscera (Figure 1A; *P* < 0.05), similar to observations in the brain under stress conditions.¹⁷ No significant changes of CpG methylation at this promoter region was observed in L4–L5 DRG neurons that contribute to somatosensory innervation of the lower extremities (Supplementary Figure 1A). Methylated DNA immunoprecipitation (MeDIP) followed by PCR showed a significant increase in

DNA methylation of the *Nr3c1* promoter region near the transcription start site (TSS) in L6-S2 ($P < 0.01$) but not L4–L5 DRG neurons (Figure 1B and C). Consistently, *Nr3c1* mRNA and protein were decreased in L6-S2 but not L4–L5 DRG neurons of stressed rats (Figure 1D and E), predominantly in DRG neurons with a small soma, which correlate with peripherin-positive C-fibers that transmit pain (nociceptive) signals ($P < 0.05$), but not in DRG neurons with large soma, which correlate with NF-200 positive A-fibers that transmit mechanical sensation (Figure 1F and Supplementary Figure 1B and C). These data indicate that chronic stress induces region-specific DNA methylation of the *Nr3c1* promoter in L6-S2 but not L4–L5 DRGs, which is associated with region- and cell-specific down-regulation of NR3C1 expression in the subpopulation of nociceptive DRG neurons.

NR3C1 regulates *Cnr1* transcription in DRG neuron-derived F11 cells in vitro

Two potential glucocorticoid response elements (GREs) at cannabinoid receptor 1 gene (*Cnr1*) promoter region were identified through sequence blasting. Using the synthesized oligonucleotides identical to *Cnr1* promoter GREs for DNA pull-down assay in F11 cells, we observed that NR3C1 bound to these oligonucleotides and CORT treatment increased the amount of NR3C1 pulled down by these two oligonucleotide sequences as shown in Figure 2A. Luciferase assay showed two *Cnr1* promoter constructs (–376 to +87 and –1022 to +87 from TSS) had higher transcription activities (Figure 2B). NR3C1 knockdown by siRNA down-regulated NR3C1 protein expression and significantly reduced *Cnr1* transcriptions in three of the four *Cnr1* constructs (Figure 2B and C). These results support a role for NR3C1 as a positive transcription factor in modulation of *Cnr1* transcription.

NR3C1 modulates CNR1 expression and function in DRG neurons in situ

Chromatin immunoprecipitation (ChIP) analysis revealed the binding of NR3C1 to *Cnr1* promoter region in rat L6-S2 DRG neurons. The relative amount of NR3C1 binding to *Cnr1* promoter decreased substantially in L6-S2 but not L4–L5 DRGs of stressed rats compared with controls (Figure 3A and Supplementary Figure 2A). Treatment with NR3C1 antagonist RU486 during the stress phase prevented the decrease of NR3C1 binding to the *Cnr1* promoter (Figure 3B and C) and reversed the down-regulation of *Cnr1* mRNA in L6-S2 DRG neurons of stressed rats (Figure 3D). Region-specific delivery of *Nr3c1* siRNA decreased NR3C1 expression and down-regulated CNR1 protein expression in L6-S2 DRGs *in situ* in control rats (Figure 3E). Knockdown of CNR1 by siRNA in L6-S2 DRG neurons of control rats decreased CNR1 expression (Supplementary Figure 2B) and significantly increased the visceral pain response to colorectal distension, similar to the enhanced level observed in stressed rats (Figure 3F).

Chronic WA stress increased DNMT1 expression in L6-S2 DRG neurons

The screening of three DNA methyltransferases revealed a robust increase in DNMT1 in L6-S2 DRG neurons from stressed rats compared with controls; alterations of DNMT3a (decreased) and DNMT3b (increased) were less significant (Figure 4A). Increased DNMT1 was detected in nuclei (Figure 4B) and DNMT1 activity was significantly ($P < 0.05$) increased in L6-S2 DRG neurons in stressed rats (Figure 4C). Immunofluorescence staining demonstrated that the increased level of DNMT1 was evident in small soma L6-S2 DRG

neurons (Supplementary Figure 3A and B). Retrograde labeling using CTB-FITC also showed that DNMT1 was significantly increased in L6-S2 DRG neurons that innervate the colon (Figure 4D and Supplementary Figure 3B). Co-immunoprecipitation experiments revealed that DNMT1 binds to methyl binding protein 2 (MeCP2), and the level of binding increased significantly in L6-S2 DRG neurons from stressed rats (Supplementary Figure 3C), supporting the activation of DNMT1 for CpG methylation under chronic stress conditions. Consistently, CORT treatment in isolated DRGs from healthy control rats increased DNMT1 protein expression in L6-S2 but not L4–L5 DRGs which was prevented by RU486 (Figure 4E).

DNMT1 mediates chronic WA stress-induced *Cnr1* promoter methylation and function

Using MeDIP, we determined that methylation of the *Cnr1* promoter region near the TSS was increased significantly ($P < 0.05$) in L6-S2 DRG neurons from stressed rats compared with controls, and this increase was prevented by RU486 treatment *in situ* (Figure 5A). In contrast, histone H3 acetylation was unchanged in this *Cnr1* promoter region (Supplementary Figure 3D and E). Luciferase assay showed that pre-methylation of the *Cnr1* promoter constructs inhibited *Cnr1* transcription activity in both CORT-treated and untreated transfected cells (Figure 5B). Moreover, gene knockdown of DNMT1 at the L6-S2 level during the chronic stress phase prevented the increase in DNA methylation in the *Cnr1* promoter region (Figure 5C), providing additional evidence that DNMT1 directly mediates methylation of the *Cnr1* promoter in chronic stress. Finally, *Dnmt1* siRNA delivered to L6-S2 level during the stress phase reduced DNMT1 expression, prevented CNR1 down-regulation caused by WA stress (Figure 5D), and significantly decreased ($P < 0.05$) the visceral motor response to colorectal distension at 40 and 60 mm Hg in stressed rats (Figure 5E), supporting an important role for DNMT1 in chronic stress-induced visceral hyperalgesia.

Up-regulation of histone acetyltransferase EP300 enhanced *Trpv1* histone H3 acetylation and visceral pain in Chronic WA stress

The level of acetyl-histone H3 in the *Trpv1* promoter region was significantly ($P < 0.05$) increased in L6-S2 but not L4–L5 DRG neurons of stressed rats, compared to controls, and blocked by RU486 treatment during the stress phase (Figure 6A and B). In contrast, the level of methylation in the same *Trpv1* promoter region in stressed rats was unchanged and knockdown of DNMT1 by siRNA in L6-S2 DRGs did not significantly alter TRPV1 expression (Supplementary Figure 4A and B). Histone acetyltransferase EP300 was significantly increased in L6-S2 DRG neurons of stressed rats compared to controls (Figure 6C). Immunofluorescence staining confirmed this result, and that EP300 and TRPV1 co-localization was significantly ($P < 0.05$) increased in L6-S2 DRG neurons of stressed rats (Supplementary Figure 4C and D). Blocking glucocorticoid receptor with the antagonist RU486 prevented stress-induced up-regulation of EP300 expression in L6-S2 DRG neurons (Supplementary Figure 4E), supporting linkage to stress-axis. ChIP assay showed that EP300 binds directly to *Trpv1* promoter, an action increased by chronic stress (Figure 6D). Targeted delivery of *Ep300* siRNA to L6-S2 DRG levels eliminated EP300 binding to *Trpv1* promoter in stressed rats (Figure 6D), reduced histone H3 acetylation of *Trpv1* promoter (Figure 6E), and prevented the increase of TRPV1 expression caused by chronic WA stress

(Supplementary Figure 4F). Assessment of visceral pain demonstrated that region-specific knockdown of EP300 significantly decreased ($P < 0.05$) visceral motor response to colorectal distension in stressed rats (Figure 6F). Furthermore, knockdown of TRPV1 with siRNA in L6-S2 but not L2–L3 DRG region had a similar preventive effect (Figure 6F and Supplementary Figure 5).

Discussion

In this study, we report several novel findings which support the interpretation that epigenetic regulatory mechanisms play a pivotal role in chronic stress-induced visceral hyperalgesia, affecting nociceptive DRG neurons in a region-specific manner. Specifically, chronic WA stress induced DNA methylation of *Nr3c1* promoter region resulting in down-regulation of its protein expression in a region- and cell (nociceptive)-specific manner in L6-S2 DRG neurons innervating the pelvic organs but not L4–L5 DRG neurons that contribute to the sciatic nerve somatosensory innervation of the lower extremities. NR3C1 acts as a positive transcription factor for the anti-nociceptive endocannabinoid CNR1 receptor in primary sensory DRG neurons. The “maintenance” DNA methyltransferase, DNMT1, is inducible under chronic stress and mediates *de novo* methylation of the *Cnr1* promoter, and down-regulation of CNR1 receptor expression and function. Concurrently, chronic WA stress increased expression of histone acetyltransferase EP300 which enhanced histone H3 acetylation of pro-nociceptive endovanilloid *Trpv1* promoter and receptor expression and function. Moreover, targeted delivery of siRNAs for either DNMT1 or EP300 to the L6-S2 spinal region during chronic stress blocked the changes observed in the specific epigenetic pathways and prevented the enhancement in abdominal pain (visceral hyperalgesia) (summarized in Figure 7).

Glucocorticoid receptor is an important target for epigenetic regulation in the HPA axis. Dysregulation in *Nr3c1* transcription is associated with stress vulnerability. For example, increased DNA methylation in *Nr3c1* promoter(s) is tightly linked to the down-regulation of NR3C1 expression in the brain that influences behavior, mood, learning and memory.^{18, 19} In primary nociceptive DRG neurons innervating the pelvic organs, we observed that chronic stress-induced visceral hyperalgesia is linked to significant increase in DNA methylation of *Nr3c1* promoter and down-regulation of *Nr3c1* transcription and expression in nociceptive DRG neurons. It has been shown that transcriptional control of NR3C1 appears to be tissue specific.²⁰ Consistently, *Nr3c1* promoter methylation increased preferentially in L6-S2 but not L4–L5 DRG neurons in stressed rats, supporting region-specificity of epigenetic regulation. Moreover, NR3C1 bound to GREs in the *Cnr1* promoter and regulated *Cnr1* transcription and expression in DRG neurons, supporting a modulator role of NR3C1 in the peripheral pain pathway. The evidence that chronic stress decreased NR3C1 binding to *Cnr1* promoter in L6-S2 but not L4–L5 DRG neurons, together with the observation that knockdown of CNR1 in L6-S2 DRGs induced visceral hyperalgesia in control rats, further demonstrate region-specific modulation of peripheral pain pathways under conditions of chronic stress. The difference in the *Nr3c1* promoter methylation pattern between L6-S2 and L4–L5 DRGs suggests that different levels of DRGs along the spinal cord may be developmentally “hard-wired”, which may cause the region-specific response under stress conditions. Alternatively, it is possible that the DRGs neurons innervating the

pelvic organs (including the colon) are exposed to a macromolecular milieu that triggers a low-grade inflammatory response and activation of primary afferent pain pathways due to the impaired intestinal and/or bladder barrier function in chronic stress.^{21, 22} Nevertheless, our data support a novel and significant role for epigenetic regulation of peripheral pain pathways in chronic stress. Consistent with this, epigenetic gene regulation in the peripheral sensory system mediates pain perception in mouse models of neuropathic and inflammatory pain.^{23, 24} It should be noted that chronic stress is also associated with changes in receptor expression/function, trophic factors and signal transduction pathways “upstream” to the peripheral nervous system including the spinal cord and CNS. For example, a recent report revealed that DNA methylation of *Nr3c1* and *Crf* genes are differentially altered in the amygdala under conditions of chronic stress that is associated with visceral hyperalgesia.²⁵ This suggests that epigenetic regulatory pathways in the CNS also play a role in modulation of visceral pain. It is unresolved whether the changes observed in the CNS occur independently, or are a response to peripheral input.

Endocannabinoid receptors including CNR1 have been shown to be involved in nociception in models of acute, inflammatory and neuropathic pain.²⁶ A recent report illustrates that the HPA-axis and the endocannabinoid system interact under stressful conditions,²⁷ implicating a role for endocannabinoids in the modulation of visceral pain. Our previous studies demonstrate that chronic WA stress induces corticosterone-mediated enhanced endocannabinoid levels and down-regulation of CNR1 receptor in DRG neurons innervating the colon, and that treatment with a CNR1 (CB1) agonist prevented stress-induced visceral hyperalgesia.^{4, 5} It has been suggested that elevated endocannabinoid levels, possibly induced by enhanced glucocorticoids, reduces the CNR1 expression level.²⁸ Data presented in the current study demonstrate that epigenetic regulation plays an important role in chronic stress-induced down-regulation of CNR1 expression and function in primary nociceptive DRG neurons. First, DNA methylation levels at *Cnr1* promoter region were significantly increased in L6-S2 DRG neurons which were blocked by the corticoid receptor antagonist RU486. Second, *in vitro* methylation of *Cnr1* promoter region significantly decreased *Cnr1* transcription. Third, knockdown DNMT1 decreased *Cnr1* promoter methylation and increased CNR1 receptor expression and function. In addition, NR3C1, as a transcription factor, positively mediated *Cnr1* transcription and expression. *Nr3c1* expression itself was regulated by its promoter DNA methylation status. It should be noted that DNMT1 is generally recognized to maintain methylation patterns.^{9,9} Our observations demonstrate that DNMT1 is inducible under conditions of chronic stress, mediates *de novo* methylation of the *Cnr1* promoter and regulates stress-induced visceral hyperalgesia, indicating that DNMT1 is a potential therapeutic target in treatment of chronic stress-related visceral pain.

It is generally accepted that the endocannabinoid pathway and its receptors can modulate TRP receptor function.^{6, 7} Endocannabinoids, such as anandamide, are elevated in colonic DRGs in WA stress rats,⁴ and can directly activate TRPV1.²⁹ On the other hand, activation of CNR1 activates inhibitory G_{i/o} proteins and leads to inhibition of adenylyl cyclase, reduces production of the second messenger cyclic adenosine monophosphate (cAMP), which subsequently inhibits TRPV1 function through down-regulation of protein kinase-dependent phosphorylation.³⁰ Therefore, the down-regulation of CNR1 receptor expression

observed in DRG neurons inverting the colon in chronic stress rats may reduce the inhibitory effect of CNR1 on TRPV1 function, culminating in enhanced visceral pain sensation.

Several members of the TRP receptor family including TRPV1, TRPV3, TRPV4, and TRPA1 have been implicated in neuropathic and visceral pain.³¹ Our previous study demonstrates that chronic stress increases TRPV1 expression and function in colonic DRG neurons.^{4, 5} Knockdown of TRPV1 in L6-S2 DRG neurons prevented chronic stress-induced visceral hyperalgesia supporting an important role of TRPV1 in chronic stress-induced visceral pain. Consistently, up-regulation of TRPV1 has been reported in a variety of gastrointestinal diseases associated with enhanced visceral pain,³² including the IBS in human patients.³³ It has been suggested that intrinsic modulators can sensitize TRPV1 via phosphorylation and thereby enhance the probability of channel gating by heat or other stimuli.³⁴ However, how the expression level of TRPV1 is up-regulated is largely unknown. We observed that histone acetyltransferase EP300 was significantly increased as well as its binding to *Trpv1* promoter region that correlated to increased expression of TRPV1 observed in L6-S2 DRG neurons in WA stress rats. The increased expression of EP300 might be caused by elevated corticosterone level since glucocorticoids can up-regulate EP300 expression.³⁵ Knockdown EP300 expression not only decreased the amount of EP300 binding to *Trpv1* promoter region but also reduced histone H3 acetylation of the *Trpv1* promoter and prevented visceral hyperalgesia in stress rats. These data indicate a novel role of EP300-mediated histone acetylation that contributes to chronic stress-induced TRPV1 receptor up-regulation and visceral hyperalgesia.

The study of epigenetic regulation of pain pathways underlying chronic stress-induced visceral hyperalgesia was primarily focused on the L6-S2 DRG neurons in this study. Other cell types such as satellite glial cells may also be affected by WA stress.³⁶ L6-S2 DRG neurons innervate the pelvic organs including the colon and bladder. Indeed, it has been reported that chronic stress also alters the function urinary bladder.²¹ The enhanced bowel-bladder interaction under chronic stress conditions may elucidate a potential mechanism underlying stress-associated visceral pain syndrome.³⁷

In summary, we have demonstrated that epigenetic regulatory mechanisms play a potentially pivotal role in chronic stress-induced visceral hyperalgesia, affecting nociceptive DRG neurons in a region- and cell-specific manner. DNMT1-mediated methylation of upstream promoter sites results in down-regulation of anti-nociceptive *Cnr1* expression, which reduces CNR1-mediated inhibition of TRPV1 function; whereas, EP300-mediated histone H3 acetylation results in up-regulation of pro-nociceptive TRPV1 expression and function. These observations support the significance of concurrent epigenetic regulation of the CNR1 and TRPV1 pain pathways in the peripheral nervous system under conditions of chronic stress. These data advocate novel applications of gene silencing to treat chronic abdominal pain using interventions that target peripheral sensory pathways in a region-specific manner, thereby avoiding CNS side-effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations: The abbreviations used in this paper

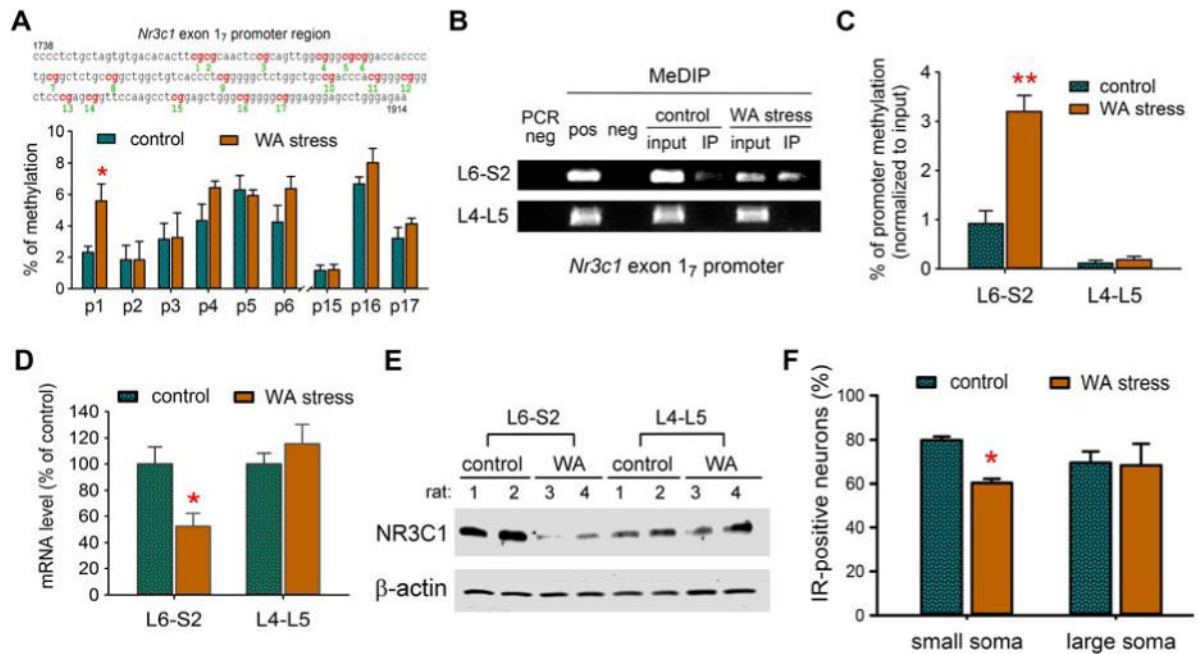
CNR1	cannabinoid receptor 1
ChIP	chromatin immunoprecipitation
CORT	corticosterone
CRD	colorectal distension
CTB	chlorotoxin B
CRF	corticotropin releasing factor
DNMT1	DNA (cytosine-5-)-methyltransferase 1
DRG	dorsal root ganglion
EMG	electromyography
NR3C1	glucocorticoid receptor
GRE	glucocorticoid response element
HPA	hypothalamic-pituitary-adrenal
IBS	Irritable Bowel Syndrome
MeDIP	methylated DNA immunoprecipitation
NR3C2	mineralocorticoid receptor
TRP	transient receptor potential
TRPV1	transient receptor potential vanilloid type 1
VMR	visceromotor response
WA	water avoidance

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**Figure 1.**

Chronic water avoidance (WA) stress induced region-specific DNA methylation at the *Nr3c1* promoter region and down-regulation in NR3C1 expression. (A) Pyrosequencing analysis of DNA methylation in the *Nr3c1* exon 17 promoter region of L6-S2 DRG neurons from stressed and control rats. PCR neg: no input DNA; pos: MeDIP positive control using input DNA prepared from lumbar DRGs of control rats; neg: MeDIP negative control using normal mouse IgG; input: 4% input DNA from relevant DRGs for normalization; IP: MeDIP using anti-mouse 5-methylcytosine antibody. (B) Region-specific changes in DNA methylation of the *Nr3c1* exon 17 promoter region measured by MeDIP and PCR. (C) Percentage change in *Nr3c1* promoter methylation in control and stressed rats. (D) Relative mRNA levels of *Nr3c1* in DRG neurons from control and stressed rats determined by qPCR. (E) Western blots for NR3C1 in different spinal (DRG) regions. (F) Quantification of the percentage of neurons that stained positive for NR3C1 in small vs. large L6-S2 DRG neurons. Data are expressed as mean \pm stand error, n = 6 in each group. * $P < 0.05$, ** $P < 0.01$ between control and WA stress rats.

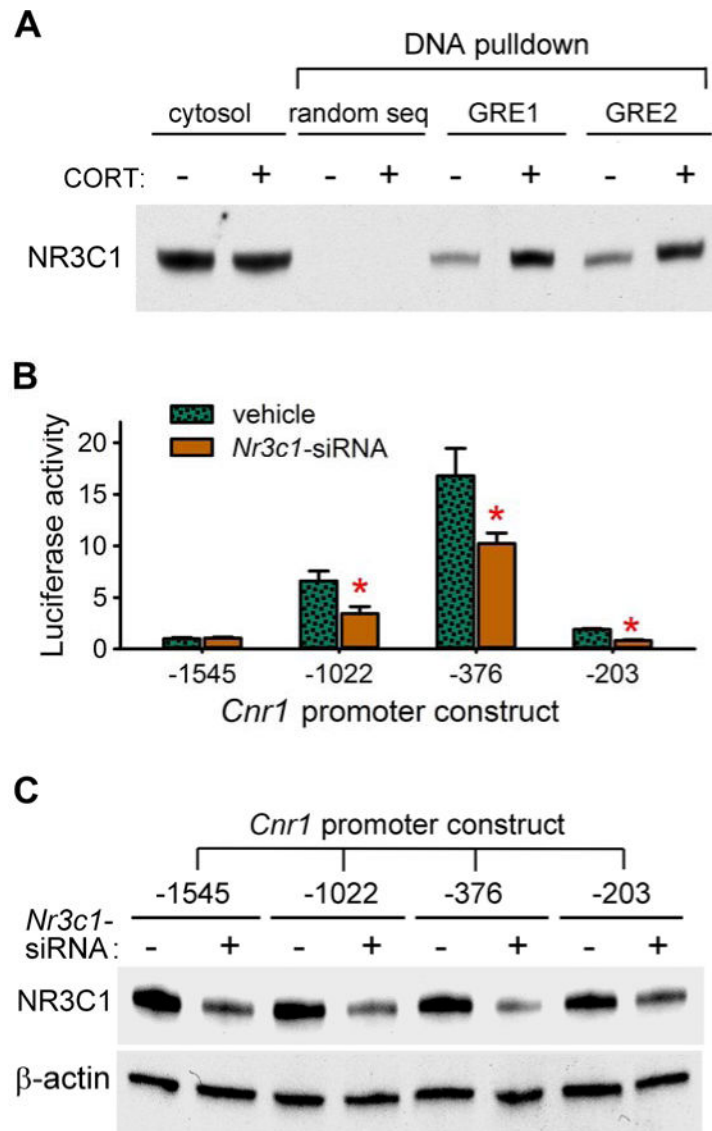
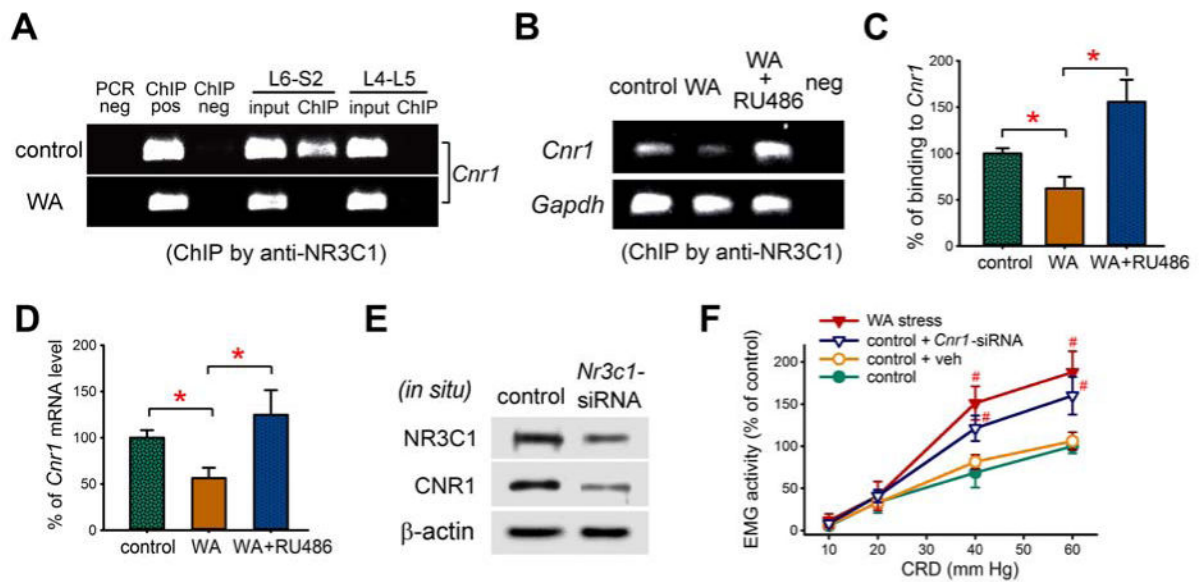


Figure 2.

Cnr1 transcription was regulated by NR3C1 in F11 cells. (A) DNA pull-down assay in F11 cells using synthesized glucocorticoid response elements (GREs). GRE1 (−95 to −124) and GRE2 (−247 to −276) represent two GREs near the *Cnr1* TSS. (B) *Cnr1* promoter transcription activity in F11 cells that were transfected with four different *Cnr1* constructs: −1545 to +87, −1022 to +87, −376 to +87, and −203 to +87 from TSS. Transfected F11 cells were treated with *Nr3c1* siRNA or vehicle. (C) Western immunoblots for NR3C1 in transfected F11 cells treated with/without *Nr3c1*-siRNA. * $P < 0.05$; $n = 4$ for each treatment.

**Figure 3.**

NR3C1 regulated *Cnr1* expression and function in DRG neurons in chronic stress in a region-specific manner. (A) ChIP using anti-NR3C1 antibody followed by PCR for *Cnr1* promoter (–145 to –42) in L6-S2 and L4–L5 DRG neurons from control and stressed rats. PCR neg: no input DNA; ChIP pos: ChIP using input DNA prepared from lumbar DRGs of control rats; ChIP neg: ChIP with normal rabbit IgG. Input: 4% input DNA from relevant DRGs for normalization. (B) ChIP analysis of NR3C1 binding to *Cnr1* promoter in L6-S2 DRG neurons in RU486-treated stressed rats. (C) Percentage of NR3C1 binding to *Cnr1* promoter in L6-S2 DRG neurons. (D) Q-PCR analysis of relative *Cnr1* mRNA levels in L6-S2 DRG neurons. (E) Western blots for NR3C1 and CNR1 in L6-S2 DRG neurons of rats treated with *Nr3c1* siRNA or random sequence siRNA (control) delivered *in situ*. (F) Visceral motor response to colorectal distension in stressed and control rats treated with *Cnr1* siRNA or vehicle (veh) delivered *in situ*. * $P < 0.05$ for stressed rats compared to controls or RU486-treated stressed rats; # $P < 0.05$ for stressed rats or *Cnr1* siRNA-treated controls compared to vehicle-treated controls; two-way ANOVA followed by Bonferroni test; $n = 6–8$ per group.

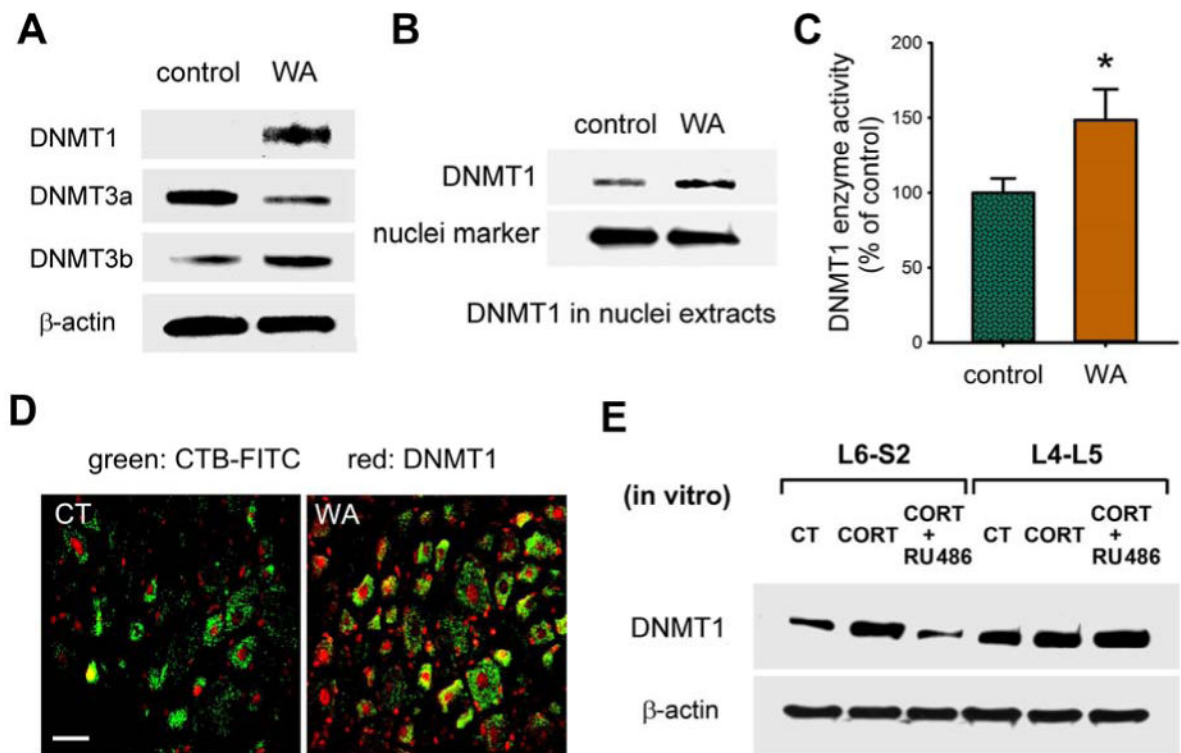


Figure 4. Increased DNMT1 expression and function in L6-S2 DRGs in chronic WA stress. (A) Western blots for DNMTs in L6-S2 DRG neurons from control and stressed rats. (B) Western immunoblots for DNMT1 in isolated nuclei fragmentation from L6-S2 DRGs of the stressed and control rats. (C) DNMT1 activity in nuclei of L6-S2 DRG neurons. (D) Immunofluorescence staining of DNMT1 (shown red) in L6-S2 DRG neurons innervating the colon. CTB-FITC (shown green) was used for retrograde labeling. Scale bar: 40 μ m. (E) CORT treatment of isolated DRGs from control rats with/without RU486. n = 5 in each group.

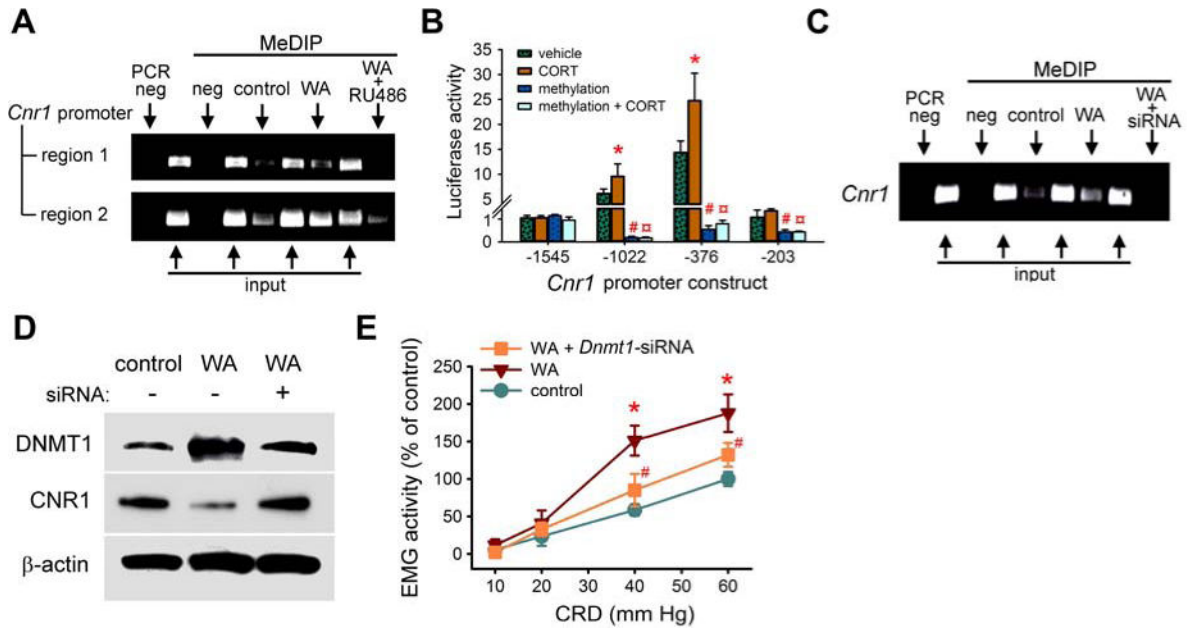


Figure 5. DNMT1 mediates *Cnr1* promoter methylation and visceral pain perception in chronic stress. (A) MeDIP of *Cnr1* promoter region 1 (–376 to –146) and 2 (–145 to –42) in L6-S2 DRG neurons of rats with/without RU486. (B) *Cnr1* promoter transcription activity in constructs treated with CORT or vehicle with/without pre-methylation. * $P < 0.05$ for CORT-treated compared to vehicle; # $P < 0.01$ for pre-methylated compared to control; $\square P < 0.01$ for CORT-treated compared to pre-methylated CORT-treated. (C) MeDIP of *Cnr1* promoter region 2 in L6-S2 DRG neurons from rats treated with/without *Dnmt1* siRNA during WA stress. (D) Western blots for DNMT1 and CNR1 in L6-S2 DRG neurons from rats treated with/without *Dnmt1* siRNA. (E) Visceral pain in stressed rats treated with *Dnmt1* siRNA compared to vehicle. * $P < 0.05$ for stressed rats compared to controls; # $P < 0.05$ for stressed rats compared to *Dnmt1* siRNA–treated stressed rats; two-way ANOVA followed by Bonferroni test. $n = 6–8$ in each group.

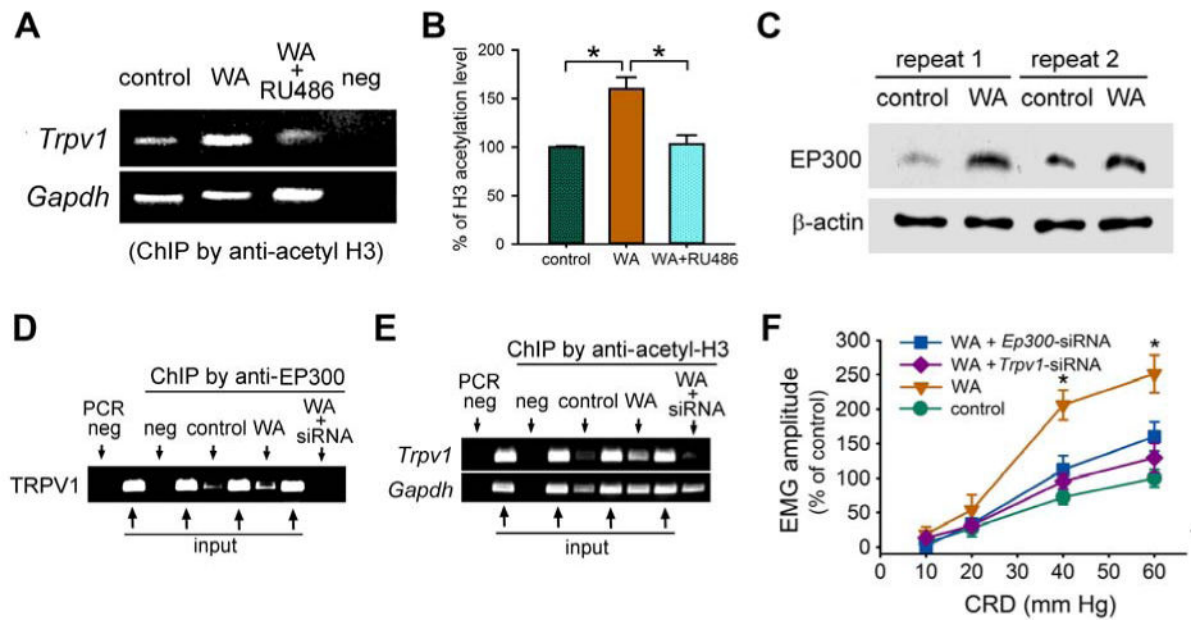


Figure 6.

Histone acetyltransferase EP300 regulates *Trpv1* histone H3 acetylation, protein expression and visceral pain perception in chronic stress. (A) ChIP using anti-acetyl-histone H3 antibody followed by PCR for *Trpv1* promoter in L6-S2 DRG neurons. (B) Percentage of H3 acetylation (normalized to control) at *Trpv1* promoter in groups treated with/without RU486. * $P < 0.05$ for the stressed group compared to the control or RU486-treated group. (C) Western blots for EP300 in L6-S2 DRG neurons from control and stressed rats ($n = 4$ rats per group). (D) ChIP analysis using anti-EP300 antibody followed by PCR for *Trpv1* promoter in L6-S2 DRG neurons from stressed rats treated with *Ep300* siRNA compared to untreated. (E) ChIP analysis using anti-acetyl-histone H3 antibody followed by PCR for *Trpv1* promoter in L6-S2 DRG neurons from stressed rats treated with *Ep300* siRNA compared to untreated rats. (F) Visceral pain assessment in stressed rats treated with *Ep300* siRNA or *Trpv1* siRNA compared to untreated rats. * $P < 0.05$ for stressed rats compared to the control or siRNA-treated group; two-way ANOVA followed by Bonferroni test. $n = 6-8$ in each group.

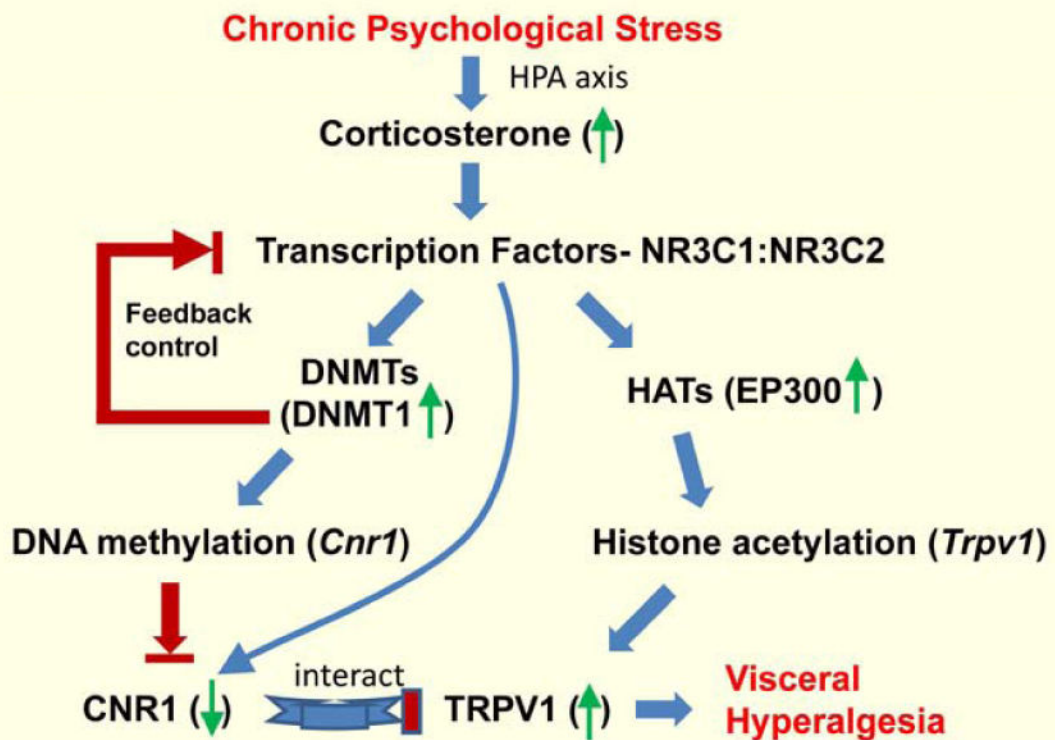


Figure 7.

Proposed epigenetic regulation of CNR1 and TRPV1 pathways underlying chronic stress-induced visceral hyperalgesia in primary nociceptive DRG neurons. Chronic stress increases serum corticosterone, which activates glucocorticoid receptors (NR3C1) and mineralocorticoid receptors (NR3C2). NR3C1 and NR3C2 activation enhances transcription of DNA methyltransferases (DNMTs), i.e. DNMT1, and histone acetyltransferases (HATs), i.e. EP300. This leads to increased methylation of *Cnr1* promoter region, culminating in decreased transcription factor (NR3C1) binding and thus reduced *Cnr1* transcription and expression, and increased histone acetylation of *Trpv1* promoter, resulting in increased *Trpv1* transcription and function. These events occur preferentially in nociceptive DRG neurons innervating the GI tract.