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Epigenetic Regulation of Genes That Modulate Chronic Stress-Induced Visceral Pain in the Peripheral Nervous System

Shuangsong Hong,* Gen Zheng,* and John W. Wiley

Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan

BACKGROUND & AIMS: Chronic stress alters the hypothalamic–pituitary–adrenal axis, increases gut motility, and increases the 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 hour of 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 also was associated with up-regulation in DNMT1-associated methylation of the *Cnr1* promoter and down-regulation 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. **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.

In human and animal models, chronic stress is associated commonly with enhanced abdominal pain (visceral hyperalgesia) and altered bowel function.[1,2] Despite considerable clinical and preclinical research, the pathophysiology of chronic stress-induced visceral hyperalgesia remains poorly understood. Alterations in corticotropin releasing factor 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 showed that chronic stress induces reciprocal changes in the antinociceptive endocannabinoid receptor 1 (CNR1) (down-regulation) and pronociceptive transient receptor potential vanilloid type 1 (TRPV1) (up-regulation) pain pathways in nociceptive L6–S2 dorsal root ganglion (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] and activation of CNR1 inhibits TRPV1 function.[7] How chronic stress modulates these pathways is unknown.

Epigenetics refers to a variety of processes that 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 focused primarily on the central nervous system. For example, chronic stress alters the level of circulating glucocorticoids and DNA methylation at CpG islands on the

*Authors share co-first authorship.

Abbreviations used in this paper: ChIP, chromatin immunoprecipitation; CNR1, cannabinoid receptor 1; CNS, central nervous system; CORT, corticosterone; CRD, colorectal distension; DNMT, DNA (cytosine-5-)-methyltransferase; DRG, dorsal root ganglion; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRE, glucocorticoid response element; HPA, hypothalamic-pituitary-adrenal; MeDIP, methylated DNA immunoprecipitation; NR3C1, glucocorticoid receptor; qPCR, quantitative polymerase chain reaction; siRNA, small interfering RNA; TRPV1, transient receptor potential vanilloid type 1; VMR, visceromotor response; WA, water avoidance.

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glucocorticoid receptor (NR3C1) gene promoter in specific regions of the brain, resulting in altered NR3C1 expression and function in the hypothalamic–pituitary–adrenal (HPA) axis.[13,14] Little is known presently about the role of epigenetic mechanisms in modulating peripheral sensory nerve function. A subset of patients with irritable bowel syndrome show altered expression of specific noncoding microRNAs in intestinal mucosa that inhibits 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 (weight, 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-hour 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 tank filled with water (25°C) to 1 cm below the height of the platform. The animals were maintained in 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

For knockdown of the targeted genes in rat DRG neurons in situ, the predesigned small interfering RNA (siRNA) or the nontargeting 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 nmol/L/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 (Austin, TX) and used for gene knockdown: *Nr3c1* (#s127819), *Cnr1* (#s129266), *Dnmt1* (#s136451), *Trpv1* (#s136297), and *Ep300* (#s220365). Verification of region-specific gene knockdown at the spinal DRG levels is shown in the Supplementary Figures using *Trpv1* siRNA.

Visceromotor Response to Colorectal Distension

Measurement of the visceromotor response (VMR) to colorectal distension (CRD) was conducted in separate groups of rats on day 11 after 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 awake animals. CRD was conducted at 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, 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 Assay

The methylated DNA immunoprecipitation (MeDIP) assay was performed using the EpiQuik Tissue MeDIP Kit (Epigentek, Farmingdale, NY), according to the manufacturer's instructions. Briefly, the genomic DNA was sheared to random fragments between 200 and 1000 bp. Immunoprecipitation was performed using a monoclonal antibody against 5-methylcytosine (Epigentek) for the sample or normal mouse IgG as the negative control. The immunoprecipitated samples were treated with proteinase K for 1 hour at 65°C and the methylated DNA was recovered by phenol-chloroform extraction, followed by ethanol precipitation. Quantitative polymerase chain reaction (qPCR) amplification was performed. The relative changes in the methylation levels were normalized to the input DNA.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) 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 mol/L glycine for 5 minutes. The cell lysate was incubated for 10 minutes at 4°C and the crude nuclear extract was collected by centrifugation at 600 × g for 5 minutes at 4°C. The DNA was sonicated to random fragments between 200 and 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 finally was 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 area under the curve, were normalized as the 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 2-way analysis of variance followed by Bonferroni post-test comparisons. The 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 ± SEM. A P value less than .05 was considered statistically significant.