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Neonatal colonic inflammation sensitizes voltage-gated Na\(^+\) channels via upregulation of cystathionine β-synthetase expression in rat primary sensory neurons

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Qu R, Tao J, Wang Y, Zhou Y, Wu G, Xiao Y, Hu C, Jiang X, Xu G. Neonatal colonic inflammation sensitizes voltage-gated Na\(^+\) channels via upregulation of cystathionine β-synthetase expression in rat primary sensory neurons. Am J Physiol Gastrointest Liver Physiol 304: G763–G772, 2013. First published February 28, 2013; doi:10.1152/ajpgi.00466.2012.—The pathogenesis of pain in irritable bowel syndrome (IBS) is poorly understood, and treatment remains difficult. We have previously reported that colon-specific dorsal root ganglion (DRG) neurons were hyperactive in a rat model of IBS induced by neonatal colonic inflammation (NCI). This study was designed to examine plasticity of voltage-gated Na\(^+\) channel activities and roles for the endogenous hydrogen sulfide-producing enzyme cystathionine β-synthetase (CBS) in chronic visceral hyperalgesia. Abdominal withdrawal reflex (AWR) scores were recorded in response to graded colorectal distention in adult male rats as a measure of visceral hypersensitivity. Colon-specific DRG neurons were labeled with 1,1'-dioleyl-3,3,3',3'-tetramethylindocarbocyanine methanesulfonate and acutely dissociated for measuring Na\(^+\) channel currents. Western blot analysis was employed to detect changes in expressions of voltage-gated Na\(^+\) (Na\(_V\)) channel subtype 1.7, Na\(_{1.8}\), and CBS. NCI significantly increased AWR scores when compared with age-matched controls. NCI also led to an ∼2.5-fold increase in Na\(^+\) current density in colon-specific DRG neurons. Furthermore, NCI dramatically enhanced expression of Na\(_{V,1.7}\), Na\(_{V,1.8}\), and CBS in colon-related DRGs. CBS was colocalized with Na\(_{V,1.7}\) or -1.8 in colon-specific DRG neurons. Administration of O-(carboxymethyl)-hydroxylamine hemihydrochloride (AOAA), an inhibitor for CBS, remarkably suppressed Na\(^+\) current density and reduced expression of Na\(_{V,1.7}\) and Na\(_{V,1.8}\). More importantly, intraperitoneal or intrathecal application of AOAA attenuated AWR scores in NCI rats in a dose-dependent manner. These data suggest that NCI enhances Na\(^+\) channel activity of colon DRG neurons, which is most likely mediated by upregulation of CBS expression, thus identifying a potential target for treatment for chronic visceral pain in patients with IBS.

dorsal root ganglion; irritable bowel syndrome; visceral pain; hydrogen sulfide

IRRITABLE BOWEL SYNDROME (IBS) remains a common and challenging disorder for clinicians. It is defined by recurrent symp-

toms of abdominal pain or discomfort associated with alterations in bowel habits. The pathophysiology of pain in IBS involves psychological disorder, altered intestinal motility, and visceral hypersensitivity (13, 29). However, the exact causes of IBS have not been clearly elucidated, and effective therapeutics for the primary symptoms have been unavailable. Recent studies in rodents found that early life trauma in the form of neonatal colonic inflammation (NCI) led to the development of visceral hypersensitivity in adult rats, mimicking main pathophysiological features of IBS in humans (35, 37, 38). Indeed, early traumatic experiences such as acute bacterial gastroenteritis or childhood abuse situations have been shown to increase the risk of IBS development (6, 18, 32). The NCI-induced visceral hypersensitivity is distinct from those of inflammatory pain and neuropathic pain in that it produces visceral hyperalgesia without involving inflammatory responses in the gut mucosa; the latter is characteristic of IBS. Therefore, NCI in rats have been used as an animal model to study the mechanisms of IBS (35).

Evidence showed that NCI involves an increase in excitability of primary afferent nociceptors, which convey peripheral stimuli into action potentials (APs) that propagate to the central nervous system (5, 38). Sensitization of primary sensory neurons is maintained by a number of ion channels such as transient receptor potential channels (35), P2X3 receptors (36), and voltage-gated Na\(^+\), K\(^+\), and Ca\(^{2+}\) channels (3, 19, 39). Both the suppression of K\(^+\) currents and promotion of Na\(^+\) currents appear to contribute to peripheral sensitization in different pain models (3, 19, 31, 41). However, whether voltage-gated Na\(^+\) channels (VGSCs) play a role in NCI-induced visceral pain remains unknown. VGSCs are integral membrane glycoproteins that are essential for generation and conduction of electrical impulses in excitable cells, thus playing a fundamental role in controlling neuronal excitability. Many of the most common neurological disorders, such as epilepsy, migraine, neurodegenerative diseases, and chronic pain, involve abnormalities of neuronal excitability (8, 33) and abnormal expression and function of VGSCs (9, 11, 15). Human and rodent studies have identified several channels as pivotal for enhanced neuronal excitability in peripheral sensory neurons (10, 12), including VGSCs Na\(_{V,1.7}\), Na\(_{V,1.8}\), and Na\(_{V,1.9}\), with the latter two resistant to tetrodotoxin (TTX) (2, 11). Therefore, in this study, we hypothesize that NCI sensitizes VGSCs...
through activation of the cystathionine Β-synthetase (CBS)-hydrogen sulfide (H2S) signaling pathway, thus contributing to visceral hypersensitivity.

As a part of an ongoing investigation (19, 22, 40), we aimed to determine changes in function and expression of VGSC of colon-specific dorsal root ganglion (DRG) neurons in a rat model of NCI-induced visceral hypersensitivity. In addition, we examined changes in expression of the endogenous H2S-producing enzyme CBS in colon-related DRGs since exogenous luminal H2S plays a pronociceptive role in mouse colon (25), and CBS expression was upregulated in this model (40).

Our findings implicate an important role for CBS-H2S signaling in modulation of Na+ channel activities in a rat model of IBS-like visceral hyperalgesia and identify the CBS as a potential molecular target for the treatment of visceral pain under this condition.

MATERIALS AND METHODS

Induction of chronic visceral hyperalgesia. Experiments were performed on male Sprague-Dawley rats. Care and handling of these animals were approved by the Institutional Animal Care and Use Committee of the Soonchun University and were in accordance with the guidelines of the International Association for the Study of Pain. Chronic visceral hyperalgesia (CVH) was induced by NCI, as described previously (35, 37, 40). In brief, 10-day-old pups received an infusion of 0.2 ml of 0.5% acetic acid solution in saline into the colon 2 cm from the anus. Controls received an equal volume of normal saline (NS). Experiments were performed in these rats between 6 and 12 wk of age. A total of 44 rats in the control (CON) group (n = 16 for behavioral studies, n = 8 for Na+ current recordings, n = 17 for Western blot analysis and 3 for immune-staining) and a total of 83 rats for Western blot analysis) were used in this study.

Behavioral testing for nocifensive responses. CVH was measured at the age of 6 wk by grading the behavioral response of rats to colorectal distention (CRD) as described previously (1, 35, 37). Briefly, under mild sedation (1% Brevital, 25 mg/kg ip), a flexible latex balloon (6 cm) attached to a tygon tubing was inserted 8 cm into the descending colon and rectum via the anus and held in place by tapping the tubing to the tail. Rats were placed in small Lucite cubicles and allowed to adapt for 30 min. CRD was performed by rapidly inflating the balloon to constant pressure using a phymgomonometer. The balloon was inflated to 20, 40, 60, and 80 mmHg for 20 s followed by 2 min rest. Behavioral response to CRD was measured by visual observation of the abdominal withdrawal reflex (AWR) by a blinded observer, and AWR scores were scored either 0 (normal behavior), 1 (slight head movement without abdominal response), 2 (contraction of abdominal muscles), 3 (lifting of abdominal wall), or 4 (body arching and lifting of pelvic structures).

To minimize the possible insult from the repetitive distention stimuli of the colon, distention threshold (DT) was measured, as described previously (22). DT was the minimal distention pressure to evoke abdominal viscromotor response. It was recorded in millimeters mercury by giving a steady increase in distention pressure by a phymgomonometer.

Cell labeling. Colon-specific DRG neurons were labeled by injection of 1.1'-dioleyl-3,3',3'-tetramethylindocarbocyanine methanesulfonate (DiI; Invitrogen) into the colon wall (37, 40). In brief, when the rats were 5 wk old, animals were anesthetized with chloral hydrate (0.36 g/kg ip). The abdomen was opened by midline laparotomy, and the colon was exposed. DiI, 25 mg in 0.5 ml methanol, was injected in an ~1-μl volume at 10 sites on the exposed colon extending from the level of the bladder to about 6 cm in an oral direction. To prevent leakage and possible contamination of adjacent organs with the dye, the needle was left in place for 1 min, and each injection site was washed with NS following each injection. The colon was gently swabbed before closing of the abdomen. Animals were returned to their housing and given free access to drinking water and standard food pellets.

Dissociation of DRG neurons and patch-clamp recording. Ten days after DiI injection, NCI (6 or 10 wk) or age-matched control rats were killed by cervical dislocation, followed by decapitation (36). DRGs (T13–L2 or L4–L5) were bilaterally dissected out and transferred to an ice-cold, oxygenated fresh dissection solution containing (in mM): 130 NaCl, 5 KCl, 2 KH2PO4, 1.5 CaCl2, 6 MgSO4, 10 glucose, and 10 HEPES, pH 7.2 (osmolality: 305 mosmol/kgH2O). After removal of the connective tissue, the ganglia were transferred to a 5-ml dissection solution containing collagenase D (1.8–2.0 mg/ml; Roche, Indianapolis, IN) and trypsin (1.2–1.5 mg/ml; Sigma, St. Louis, MO) and incubated for 1.5 h at 34.5°C. DRGs were taken from the enzyme solution, washed, and transferred to 0.5 ml of the dissection solution containing DNase (0.5 mg/ml; Sigma). A single cell suspension was subsequently obtained by repeated trituration through flame-polished glass pipettes. Cells were plated onto acridine-cleaned glass cover slips.

Cover slips containing adherent DRG cells were put in a small recording chamber (1 ml volume) and attached to the stage of an inverted microscope (Olympus IX71) fitted for both fluorescence and bright-field microscopy. DiI-labeled neurons were identified by their fluorescence under the fluorescent microscope. For the patch-clamp recording experiments, cells were continuously superfused (1.5 ml/min) at room temperature with normal external solution containing (in mM): 130 NaCl, 5 KCl, 2 KH2PO4, 2.5 CaCl2, 1 MgCl2, 10 HEPES, and 10 glucose, with pH adjusted to 7.2 with NaOH; osmolality: 295–300 mosmol/kgH2O. Recording pipettes were pulled from borosilicate glass tubing using a horizontal puller (P-97; Sutter Instruments). Unless indicated, patch-clamp pipettes had a resistance of 4–7 MΩ when filled with the pipette solution containing (in mM): 140 potassium gluconate, 10 NaCl, 10 HEPES, 10 glucose, 5 EGTA, and 1 CaCl2, pH = 7.25 adjusted with KOH; osmolality: 292 mosmol/kgH2O. Resting potential and APs were recorded. The voltage was clamped at −60 mV by a HEKA EPC10 patch-clamp amplifier (HEKA). Capacitive transients were corrected using capacitive cancelation circuitry on the amplifier that yielded the whole cell capacitance and access resistance. Up to 90% of the series resistance was compensated electronically. Considering the peak outward current amplitudes of <10 nA, the estimated voltage errors from the uncompensated series resistance would be <10 mV. The leak currents at −60 mV were always <20 pA and were not corrected. The currents were filtered at 2–5 kHz and sampled at 50 or 100 μS/point. Whole cell current and voltage were recorded with a HEKA EPC10 patch-clamp amplifier; and data were acquired and stored on a computer for later analysis using FitMaster (HEKA). Patch-clamp recordings were performed at room temperature (~22°C).

Isolation of NaV currents. To record NaV currents, cells were superfused (2 ml/min) at room temperature with an external solution containing (in mM): 60 NaCl, 80 choline chloride, 0.1 CaCl2, 10 HEPES, 10 tetraethylammonium chloride, 10 glucose, and 0.1 CaCl2, pH = 7.4, adjusted with tetraethylammonium hydroxide, osmolality: 310 mosmol/kgH2O). The patch electrode had a resistance of 3–5 MΩ when filled with the pipette solution containing (in mM): 140 CsF, 1 MgCl2, 5 EGTA, 3 Na-GTP, 10 glucose, and 10 HEPES, pH = 7.2, adjusted with CsOH; osmolality: 285–295 mosmol/kgH2O. The total NaV currents were recorded in response to depolarization steps to different testing potentials from −70 to +50 mV in 10-mV increments with a duration of 80 ms. The peak Na+ current was measured as the peak of the transient component of the current at every given voltage. To control for changes in cell size, the current density (pA/pF) was measured by dividing the current amplitude by whole cell membrane capacitance, which was obtained by reading the value
for whole cell input capacitance cancellation directly from the patch-clamp amplifier.

**Western blotting.** DRGs (T13–L2) from NCI-treated rats (7 or 10 wk) or age-matched control rats were dissected out and lysed in 100 μl of radioimmunoprecipitation assay buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μl/ml phenylmethylsulfonyl fluoride, and 30 μl/ml aprotinin (Sigma). The cell lysates were then microfuged at 15,000 rpm for 30 min at 4°C. The concentration of protein in homogenate was determined using a Bio-Rad protein assay (Hercules, CA). After electrophoresis, the proteins were electrotransferred onto polyvinylidifluoride membranes (Millipore) at 200 mA for 2 h at 4°C. Membranes were incubated in 25 ml of blocking buffer (1× TBS with 5% wt/vol fat-free dry milk) for 2 h at room temperature and then incubated with the primary antibodies for 2 h at room temperature. Primary antibodies used were rabbit anti-NaV1.7 or anti-NaV1.8 (1:200; Alomone Labs, Jerusalem, Israel), mouse anti-CBS (1:1,000; Abnova, Taiwan, China), and mouse anti-actin (1:1,000; Chemicon, Temecula, CA). After incubation, membranes were washed with 1× TBS and 5% Tween 20 (TBST) three times for 15 min each and incubated with anti-rabbit peroxidase-conjugated secondary antibody (1:2,000; Santa Cruz Biotecnology, Santa Cruz, CA) or anti-mouse horseradish peroxidase-conjugated secondary antibody (1:4,000; Chemicon) for 2 h at room temperature. The membranes were then washed with TBST three times for 15 min each. The immunoreactive proteins were detected by enhanced chemiluminescence (ECL kit; Amershams Biosciences, Arlington Heights, IL). The bands recognized by primary antibody were visualized by exposure of the membrane onto an X-ray film. For quantification of NaV1.7, NaV1.8, or CBS protein levels, photographs were digitalized and analyzed using a scanner (Bio-Rad imaging system Bio-Rad GelDoc XRS+). All samples were normalized to β-actin as a control.

**Immunofluorescence study.** One to two weeks after DiI injection, rats were perfused transcardially with 150 ml PBS followed by 400 ml ice-cold 4% paraformaldehyde in PBS. DRG T13 to L2 were removed and rats were perfused transcardially with 150 ml PBS followed by 400 ml 0.1 M glycine (pH 8.0) and 0.1 M sodium meta-sulfite for 1 h. Tissues were simultaneously incubated with CBS (1:200; Abnova) and NaV1.7 (1:200; Alomone Laboratories) or NaV1.8 (1:200; Alomone Laboratories) antibody and then incubated with Alexa Fluor 355 and Alexa 488. Negative control was performed by omitting the primary antibody. Sections were viewed with filter cubes appropriate for DiI (rhodamine filter), Alexa 488, and Alexa 355. Images were captured and analyzed using Metaview software. To ensure that a neuron was counted only one time, serial sections were placed on consecutive slides with at least 50 μm between sections on the same slide.

**Drug application.** O-(carboxymethyl) hydroxylationine hemihydrochloride (AOAA, an inhibitor of CBS) was purchased from Sigma-Aldrich and freshly prepared in 0.9% NS. At the age of 5 wk, AOAA was administrated intraperitoneally at 10 mg/kg body wt, one time per day for a consecutive 7 days. Tissues from these animals were used for patch-clamp recordings or molecular expression experiments. For behavioral test, a single injection at different doses (5, 10, and 20 mg/kg body wt for ip and 0.1, 1, and 10 μg for it) of AOAA was used in the present studies.

**Data analysis.** No neuron with a resting membrane potential more depolarized than −40 mV was included in the data analysis. All data are expressed as means ± SE. Normality was checked for all data before analysis. Statistical significance was determined by two-sample t-test, Mann-Whitney test, or Tukey post hoc test following Kruskal-Wallis ANOVA or Tukey post hoc test following two-way repeated-measures ANOVA or Mann-Whitney test or Dunn’s post hoc test following Friedman ANOVA, as appropriate. A P value <0.05 was considered statistically significant.

**RESULTS**

**Increase in AWR scores in NCI rats.** CVH was determined by measuring AWR scores in response to CRD at 6–12 wk of age from control (CON, n = 10 for each group) and NCI (n = 10 for each group) rats. At the age of 6 wk, the AWR scores were significantly higher in NCI rats at 20, 40, 60, and 80 mmHg distention pressures than those in age-matched controls (P < 0.05 vs. CON, for the same pressure, Mann-Whitney test following Friedman ANOVA; Fig. 1A). The enhanced AWR scores lasted for ~4 wk (P< 0.05 vs. CON, for the same pressure, Mann-Whitney test following Friedman ANOVA; Fig. 1B) and returned to normal levels at 10 and 12 wk (Fig. 1C and D), suggesting that NCI significantly increased the visceromotor response to CRD in adult rats compared with controls. The AWR scores of control rats did not change significantly when repeatedly measured using the same distention pressure at different time points. However, the AWR scores of NCI-treated rats at 80 mmHg distention pressure were significantly higher when tested at 8 wk and compared with those at 12 wk (n = 10 for each group, P < 0.05, Dunn’s post hoc test following Friedman ANOVA); the AWR scores were significantly higher when tested at 12 wk compared with 8 wk (n = 10 for each group, P < 0.05, Dunn’s post hoc test following Friedman ANOVA) compared with controls. The AWR scores of control rats did not change significantly when repeatedly measured using the same distention pressure at different time points. However, the AWR scores of NCI-treated rats at 80 mmHg distention pressure were significantly higher when tested at 8 wk and compared with those at 12 wk (n = 10 for each group, P < 0.05, Dunn’s post hoc test following Friedman ANOVA); the AWR scores were significantly higher when tested at 12 wk compared with 8 wk (n = 10 for each group, P < 0.05, Dunn’s post hoc test following Friedman ANOVA)
of NCI-treated rats at 20–60 mmHg distention pressures did not change dramatically when tested at different time points.

Potentiation of Na⁺ current densities of colon DRG neurons in NCI rats. The previous data showed that NCI significantly increased excitability of colon-specific T13–L2 DRG neurons (38). We next examined the ionic mechanism underlying the enhanced neuronal excitability in this study. Because VGSCs are responsible for the generation and propagation of APs in the membranes of neurons, we measured current densities of VGSCs in DiI-labeled DRG neurons from control and NCI rats (Fig. 2). Colon-specific DRG neurons were labeled by DiI (Fig. 2, A and B). Under current-clamp conditions, the current (I)-voltage (V) relationship was also examined (Fig. 2, C and D). The average expected reversal potentials were 71.28 ± 2.66 (n = 16 neurons) and 66.51 ± 2.47 (n = 16 neurons) mV for control and NCI rats, respectively. NCI treatment did not significantly alter the reversal potential of Na⁺ currents (P > 0.05), indicating that ion permeability was not changed. However, NCI treatment significantly increased the average of peak amplitude of Na⁺ currents in DiI-labeled neurons compared with the control (CON: −115.12 ± 10.67 pA/pF; NCI: −264.43 ± 26.35 pA/pF; n = 16 neurons for each group, *P < 0.05, two-sample t-Test; Fig. 2E). These results support our previous hypothesis that NCI-induced hyperexcitability is mediated, at least in part, by sensitization of Na⁺ channel currents in colon-specific DRG neurons.

Because the AWR scores returned to the baseline level at the age of 10 wk (Fig. 1C), we then measured current densities of VGSCs of DiI-labeled DRG neurons from NCI-treated rats at the age of 10 wk (Fig. 3A). The I-V curve was determined. The average expected reversal potentials were 55.71 ± 1.17 (n = 9 neurons) and 53.66 ± 0.93 (n = 8 neurons) mV for control and NCI rats, respectively. NCI treatment did not significantly alter the reversal potential of Na⁺ currents (P > 0.05; Fig. 3B). Similarly, NCI treatment did not produce any change in the average of peak amplitude of Na⁺ currents in DiI-labeled neurons compared with the control (CON: −99.29 ± 15.30 pA/pF, n = 9; NCI: −97.27 ± 17.85 pA/pF, n = 8; P > 0.05, two-sample t-Test; Fig. 3C). To determine whether NCI treatment affected the function of L4 and L5 DRG neurons, we also examined Na⁺ current density of L4–5 DRG neurons (Fig. 3D). The I-V curve was also examined (Fig. 3E). The average expected reversal potentials were 60.19 ± 0.66 (n = 10 neurons) and 63.11 ± 2.24 (n = 8 neurons) mV for control and NCI rats, respectively. NCI treatment did not significantly alter the reversal potential of Na⁺ currents (P > 0.05). The peak Na⁺ current density of L4–5 DRG neurons was −89.70 ± 16.53 (n = 10 neurons) and −91.44 ± 14.32 (n = 8 neurons) pA/pF from control and NCI rats, respectively. As expected, NCI did not significantly change Na⁺ current density of L4–5 DRG neurons when compared with age-matched control rats at the age of 6 wk (P > 0.05; Fig. 3F).

Enhanced expression of Nav1.7 and Nav1.8 in NCI rats. To determine whether expression of Nav1.7 and Nav1.8 increased in colon-related DRGs after NCI, Western blotting assays were performed. Proteins isolated from both sides of T13–L2 DRGs of control and NCI rats were probed with anti-NaV1.7 or -Nav1.8 antibodies. Anti-NaV1.7 antibody labeled a 226-kDa molecular mass protein (Fig. 4A). Anti-NaV1.8 antibody labeled a 220-kDa molecular mass protein (Fig. 4B). Six weeks after NCI treatment, the relative densitometry of Nav1.7 was 0.13 ± 0.04 (n = 3) and 0.38 ± 0.05 (n = 3) for control and NCI, respectively. The levels of expression of Nav1.7 were increased significantly (P < 0.05, two-sample t-test; Fig. 4A). The relative densitometry of Nav1.8 was 0.05 ± 0.02 (n = 4) and 0.45 ± 0.06 (n = 4) for control and NCI, respectively. The levels of expression of Nav1.8 were increased dramatically (P < 0.05, two-sample t-test; Fig. 4B). The expression of

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**Fig. 2.** Potentiation of Na⁺ current density of colon-specific dorsal root ganglion (DRG) neurons by NCI: A: an example of a 1,1'-dioleyl-3,3',3'-tetramethylindocarbocyanine methanesulfonate (DiI)-labeled DRG neuron (arrow). Star shows the place where a neuron is not labeled by DiI. B: a phase image of the same DRG neuron labeled by DiI is shown on the right (arrow), and the neuron not labeled by DiI is shown on the left (star). Bar = 50 μm. Patch-clamp recordings were performed on DiI-labeled colon neurons. C: an example of voltage-gated Na⁺ (Nav) channels recorded from T13-L2 DRG neurons from control (left) and NCI (right) rats at the age of 6 wk. The membrane potential was held at −60 mV. Voltage steps were from −70 to +50 mV with 10-mV increments and 80 ms duration. D: current (I)-voltage (V) curves for Na⁺ currents of CON and NCI rats (n = 16 neurons for each group). Each point represents the mean ± SE. E: bar graph showing that NCI treatment significantly increased the peak amplitude of Na⁺ currents compared with age-matched control rats (n = 16 neurons for each group, *P < 0.05).
Nav1.7 and Nav1.8 from rats at the age of 10 wk was also determined. As expected, neither Nav1.7 (n = 4 for each group; Fig. 4C) nor Nav1.8 (n = 3 for each group; Fig. 4D) expression was significantly altered at the age of 10 wk. To determine whether NCI affect expression of these proteins in L₄–L₅ DRGs, proteins from lumbar (L₄–5) DRGs were examined from NCI rats and age-matched controls. Similarly, expression of Nav1.7 (n = 3 for each group; Fig. 4E) and Nav1.8 (n = 3 for each group; Fig. 4F) in L₄–5 DRGs was not markedly altered. Thus, NCI upregulates both Nav1.7 and Nav1.8 expression only in colon-related T₁₃–L₂ DRGs at the age of 6 wk.

Antagonism of AOAA treatment on Nav1.7 and Nav1.8 expression in NCI rats. To determine the possible interaction between CBS and Na⁺ channels, we first examined whether CBS was coexpressed in Nav1.7- or Nav1.8-positive colon-specific DRG neurons. Triple-labeling techniques showed that all colon-specific DRG neurons that were immunoreactive for CBS were coexpressed in Nav1.8-positive colon-specific DRG neurons (Fig. 5A). To determine whether NCI induced upregulation of CBS expression in colon-related DRGs, bilateral thoracolumbar (T₁₃–L₂) DRGs were dissected out. As shown in Fig. 5B, NCI dramatically increased CBS expression in T₁₃–L₂ DRGs at 6 wk (n = 3 rats for each group), which is in line with our previous report (40). To determine the role for CBS in the upregulation of Nav1.7 expression, CBS inhibitor AOAA was used in this study as described above. The relative densitometry of Nav1.8 was 0.83 ± 0.07 (n = 3) and 0.37 ± 0.03 (n = 3) for NS and AOAA, respectively. AOAA treatment significantly reduced expression of Nav1.8 (P < 0.05) and control and NCI, respectively. NCI did not significantly alter expression of CBS proteins in L₄–5 DRGs (Fig. 5C).

We then determined whether CBS was coexpressed in Nav1.8-positive colon-specific DRG neurons. Triple-labeling techniques showed that all colon-specific DRG neurons that were immunoreactive for CBS were also positive for Nav1.8 (Fig. 6A). Similarly, all colon-specific DRG neurons that were immunoreactive for Nav1.8 were also positive for CBS (Fig. 5A). To determine whether NCI affected CBS expression in L₄–5 DRGs, the expression of CBS in L₄–5 DRGs from rats at the age of 6 wk was also examined. The relative densitometry of CBS was 0.21 ± 0.02 (n = 3) and 0.17 ± 0.03 (n = 3) for control and NCI, respectively. NCI did not significantly alter expression of CBS proteins in L₄–5 DRGs (Fig. 5C).

Antagonism of AOAA treatment on Na⁺ current density in NCI rats. Because AOAA reversed the expression of Nav1.7 and Nav1.8 channels in NCI rats, we next investigated whether AOAA treatment suppressed current density of VGSCs in colon-specific DRG neurons (Fig. 7A). Rats were divided into the following two groups: AOAA group treated (AOAA at 10 mg/kg body wt ip, one time/day for a consecutive 7 days) and NS group treated with the same volume of NS (ip). The Na⁺ current density was −288.15 ± 23.04 (n = 10) and −108.41 ± 14.66 (n = 10) pA/pF for NS- and AOAA-treated groups, respectively. AOAA treatment significantly reduced Na⁺ current density compared with that of the NS-treated group (P < 0.05; Mann-Whitney test; Fig. 7B and C). However, AOAA treatment did not significantly alter the reversal potentials. The average reversal potentials were 65.76 ± 2.64 (n = 10 neurons) and 59.99 ± 1.11 (n = 10 neurons) mV for NS and AOAA, respectively (P > 0.05), indicating that ion permeability was not changed after AOAA treatment.
Antagonism of AOAA treatment on AWR scores in NCI rats.

We next examined the effect of AOAA treatment on AWR scores in NCI rats. The CBS inhibitor AOAA was administered intraperitoneally. Injection of AOAA significantly reduced AWR scores in NCI rats in a dose-dependent fashion (n = 6 for each group; *P < 0.05 vs. NS, Tukey post hoc test following Kruskal-Wallis ANOVA; Fig. 8A). The optimized dose for AOAA to produce the maximal effect was 10 mg/kg body wt in this study. We then determined the time course of AOAA effects. The effect of AOAA at doses of 10 and 20 mg/kg body wt lasted for ~60 min (n = 6 for each group; P < 0.05 vs. NS, Tukey post hoc test following two-way repeated-measures ANOVA; Fig. 8B). Maximal inhibition was at 30 min. To exclude nonspecific effect, AOAA at 10 mg/kg was intraperitoneally injected into age-matched healthy control rats. Application of AOAA had no significant effects on AWR scores in healthy control rats (n = 6 for the healthy group; Fig. 8C). To further confirm the AOAA effect on NCI rats, AOAA at 0.1, 1, or 10 µg/kg was intrathecally injected (n = 6 for each group; P < 0.05 vs. NS, Tukey post hoc test following two-way repeated-measures ANOVA; Fig. 8D). Injection of AOAA (it) at both 1 or 10 µg increased DT, and this effect wore off 60 min after injection. These data suggest that AOAA did not act as a nonspecific analgesic and that CBS do not normally participate in the responses to CRD in rats under normal conditions.

DISCUSSION

This study demonstrates that NCI produces a significant increase in VGSC activities that is manifested as an upregulation of Nav1.7 and Nav1.8 expression and potentiation of total Na\(^+\) channel current density in colon-specific DRG neurons in adult rats with visceral hypersensitivity. We further show for the first time that inhibition of the endogenous H\(_2\)S-producing enzyme reverses the Na\(^+\) channel activities and visceral hypersensitivity. These results indicate that the endogenous H\(_2\)S signaling pathway might play an important role in modulating VGSC activity in IBS-like visceral hypersensitivity.

We have previously reported that NCI induced CVH, which was associated with enhanced neuronal excitability (38). Mechanisms maintaining sensitization include signal transduction, generation of APs, and neurotransmitter release. These functions are in turn subserved by several different classes of ion channels such as transient receptor potential and voltage-gated Na\(^+\), K\(^+\), and Ca\(^2+\) channels. In the present study, we found that NCI treatment significantly increased Na\(^+\) current density in colonic-specific DRG neurons. This is in agreement
with previous reports that Na\(^+\) current is involved in somatic pain (16, 26) and visceral pain (7, 17, 27, 42). The increase in Na\(^+\) current density is attributed to more Na\(^+\) influx. The increase in Na\(^+\) influx may lead to a greater depolarization of the cell membrane and more APs for a given stimulus, thus enhancing excitability of colon-specific neurons in our model.

The results of our study are significant because they provide further evidence that VGSCs may play an important role in “functional” visceral pain, that is, pain occurring in the absence of overt structural or inflammatory processes. To prove this, we used a previously validated approach of mild colonic irritation in the neonatal period that does not produce significant inflam-

Fig. 5. Antagonism of cystathionine β-synthetase (CBS) inhibitor on the expression of Nav1.7. A: triple-labeling techniques showed that CBS (blue)- and Nav1.7 (green)-like immunoreactivities were colocalized in colon-specific DRG neurons labeled by Dil (red). B: NCI treatment significantly enhanced expression of the endogenous hydrogen sulfide-producing enzyme CBS in T13-L2 DRGs when compared with CON (n = 3 for each group, *P < 0.05). C: administration of the CBS inhibitor O-(carboxymethyl)hydroxylamine hemihydrochloride (AOAA) significantly reduced the expression of Nav1.7 of T13-L2 DRGs from NCI rats at the age of 6 wk. *P < 0.05, n = 3 for each group.

Fig. 6. Antagonism of CBS inhibitor on the expression of Nav1.8. A: triple-labeling techniques showed that CBS (blue)- and Nav1.8 (green)-like immunoreactivities were colocalized in colon-specific DRG neurons labeled by Dil (red). B: administration of the CBS inhibitor AOAA significantly reduced the expression of Nav1.8 of T13-L2 DRGs from NCI rats at the age of 6 wk. *P < 0.05, n = 3 for each group. C: NCI treatment did not significantly alter CBS expression in L4-L5 DRGs (n = 3 for each group).
Fig. 7. Antagonism of CBS inhibitor on Na⁺ current density. A: an example of NaV currents of T13–L2 DRG neurons from normal saline (NS, left)- and AOAA (right)-treated rats with NCI. The membrane potential was held at −60 mV, and voltage steps were from −70 to +50 mV with 10-mV increments and 80 ms duration. B: I–V curves for Na⁺ currents of NS and AOAA rats (n = 10 neurons for each group). Each point represents the mean ± SE. C: bar graph showing that AOAA treatment significantly reduced the peak amplitude of Na⁺ currents compared with the NS group (n = 10 neurons for each group, *P < 0.05). Possible mechanisms for the potentiation of Na⁺ currents include an increase in channel activities and/or upregulation of channel expression. Although channel properties of colonic-specific neurons after NCI have yet to be investigated, our analysis of I–V curves and channel protein expression suggests that upregulation of NaV₁.7 and NaV₁.8 expression might be a major cause for the large increase in total Na⁺ currents after NCI. In this study, total Na⁺ currents increase about 2.3-fold, but NaV₁.7 protein is up ~3-fold, and NaV₁.8 protein is up ~9-fold by Western blot. Although we do not know whether it is necessary that the amount of increase in current density has to be the same as the amount of upregulation of channel protein expression, it is obvious that NaV₁.8 protein expression was greater than NaV₁.7. The detailed mechanism is unknown. NaV₁.8 is the major subtype to mediate TTX-resistant Na⁺ currents while NaV₁.7 mediates TTX-sensitive Na⁺ currents. It is tempting to hypothesize that NaV₁.8 may play an important role in visceral hypersensitivity in terms of expression. Future experiments are needed to identify the TTX-resistant and TTX-sensitive current components. Findings that NCI did not produce any change in NaV₁.7 and NaV₁.8 expression in L₄–L₅ DRGs further confirm the specificity of NaV₁.7 and NaV₁.8 expression in colon-related DRGs. In addition, the visceral sensitivity returned to the baseline level at 10 wk, whereas the level of NaV₁.7 and NaV₁.8 expression was normalized, indi-
cating a good correlation between Na$^+$ channel activities and visceral sensitivity. Of note is that Na$\text{v}_{1.9}$ might play a certain role in this model. However, the enhanced Na$^+$ currents may not be mainly attributed to Na$\text{v}_{1.9}$ activity because these channels remain largely inactivated at the holding potential of $-70 \text{ mV}$.

A unique feature of our study is the systemic in vivo use of the endogenous H$_2$S-producing enzyme CBS inhibitor AOAA in a model of colonic visceral pain. This has previously been used in vivo in a somatic model of complete Freund adjuvant-induced arthritis in rats (26). The reason why we chose AOAA and not hydroxylamine (HA), another CBS inhibitor, is because HA has been reported to have a cyclooxygenase-1 inhibitory action (20). The dose of AOAA used in this study and not hydroxylamine (HA), another CBS inhibitor, is being in a model of colonic visceral pain. This has previously been used in vivo in a somatic model of complete Freund adjuvant-induced arthritis in rats (26). The reason why we chose AOAA for this study was according to our previous study (26). No significant effect was seen in control rats, suggesting that this was not a non-specific analgesic effect. This also suggests that the role of the CBS-H$_2$S pathway in signaling colonic distension may not be as important in healthy as in the sensitized state. This is in keeping with the results of studies directly measuring nerve responses and others that have shown that there is a relatively minor contribution of CBS-H$_2$S signaling to distension-induced nerve firing but that this is significantly enhanced in the presence of colitis. We do not know the mechanism by which H$_2$S modulates Na$^+$ channel activities. It is possible that there are acute inhibitory effects of AOAA on Na$^+$ channel function and chronic effects of AOAA on expression. This conclusion is supported by our previous report that acute application of NaHS, a donor of H$_2$S, enhanced Na$^+$ channel currents and that chronic administration of AOAA significantly attenuated AWR scores in rats after heterotopically intermittent stress (34).

To date, several inflammatory mediators, including PGE$_2$ (17) and nerve growth factor (21, 28), have been shown to sensitize Na$\text{v}_{1.7}$ and Na$\text{v}_{1.8}$ Na$^+$ channels and therefore to significantly increase excitability of the nociceptors. H$_2$S could be a good candidate to regulate the function and expression of Nav channels because a large body of evidence shows that H$_2$S plays an important role in producing sensitization in somatic pain models (24, 26) and in acute hyperalgesia and CVH (40) by affecting ion channels and membrane properties in afferent sensory neurons. Of note is that CBS-induced H$_2$S not only enhances Na$\text{v}_{1.7}$ and Na$\text{v}_{1.8}$ activity but also regulates other ion channels’ excitabilities in DRG neurons such as transient receptor potential A1 and voltage-gated Ca$^{2+}$ channel subtype 3.2 (24, 30), which may also contribute to visceral nociception. Although the effects for H$_2$S are contradictory (4, 14), we provided evidence to show that CBS is involved in regulation of Na$^+$ channel function and expression, and thus mitigating the visceral hypersensitivity. Further experiments are warranted to explore the possible mediators underlying the sensitization of nociceptors under this condition.

In conclusion, our data show that NCI significantly increased the visceromotor response to CRD. The CVH is associated with an increased expression of Na$\text{v}_{1.7}$ and Na$\text{v}_{1.8}$ and potentiation of total Na$^+$ currents in colon-specific DRG neurons. Because inhibition of the endogenous H$_2$S-producing enzyme CBS normalizes Na$^+$ channel activities in primary sensory neurons under functional colonic visceral pain states, inhibition of the CBS-H$_2$S signaling pathway represents a promising avenue for therapeutic intervention in patients with IBS.

REFERENCES


