Neonatal maternal deprivation sensitizes voltage-gated sodium channel currents in colon-specific dorsal root ganglion neurons in rats

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1Institute of Neuroscience, Laboratory for Translational Pain Medicine, Department of Neurobiology, Soochow University, Suzhou, People’s Republic of China; and 2Division of Gastroenterology, the Second Affiliated Hospital, Soochow University, Suzhou, People’s Republic of China

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Hy, Xiao, Zhu, Li, Hu, Jiang, and Xu. Neonatal maternal deprivation sensitizes voltage-gated sodium channel currents in colon-specific dorsal root ganglion neurons in rats. Am J Physiol Gastrointest Liver Physiol 304: G311–G321, 2013. First published November 8, 2012; doi:10.1152/ajpgi.00338.2012.—Irritable bowel syndrome (IBS) is a common gastrointestinal disorder characterized by abdominal pain in association with altered bowel movements. The underlying mechanisms of visceral hypersensitivity remain elusive. This study was designed to examine the role for sodium channels in a rat model of chronic visceral hyperalgesia induced by neonatal maternal deprivation (NMD). Abdominal withdrawal reflex (AWR) scores were performed on adult male rats. Colon-specific dorsal root ganglion (DRG) neurons were labeled with DiI and acutely dissociated for measuring excitability and sodium channel current under whole-cell patch-clamp configurations. The expression of NaV1.8 was analyzed by Western blot and quantitative real-time PCR. NMD significantly increased AWR scores, which lasted for ~6 wk in an association with hyperexcitability of colon DRG neurons. TTX-resistant but not TTX-sensitive sodium current density was greatly enhanced in colon DRG neurons in NMD rats. Compared with controls, activation curves showed a leftward shift in NMD rats whereas inactivation curves did not differ significantly. NMD markedly accelerated the activation time of peak current amplitude without any changes in inactivation time. Furthermore, NMD remarkably enhanced expression of NaV1.8 at protein levels but not at mRNA levels in colon-related DRGs. The expression of Nav1.9 was not altered after NMD. These data suggest that NMD enhances TTX-resistant sodium activity of colon DRG neurons, which is most likely mediated by a leftward shift of activation curve and by enhanced expression of Nav1.8 at protein levels, thus identifying a specific molecular mechanism underlying chronic visceral pain and sensitization in patients with IBS.

dorsal root ganglion; neonatal maternal deprivation; irritable bowel syndrome; visceral pain; voltage-gated sodium channel
malities of neuronal excitability (10, 38) and abnormal expression and function of VGSCs (13, 16, 19). Human and rodent studies have identified several channels as pivotal for enhanced neuronal excitability in peripheral sensory neurons (15, 17), including VGSCs NaV1.7, NaV1.8, and NaV1.9, with the latter two resistant to TTX (6, 16).

As part of an ongoing investigation, we focused on changes in the membrane properties and excitability of colon-specific DRG neurons associated with time course of NMD-induced visceral hypersensitivity in response to CRD. Furthermore, we specifically examined changes in Na\(^+\) channel conductance and the iron properties in these neurons and expression of NaV1.8 in colon-related DRGs. Our findings indicated that NMD treatment resulted in enhanced neuronal excitability, which was associated with a potentiation of TTX-resistant (TTX-R) sodium channel currents and upregulation of NaV1.8 expression, therefore providing the mechanistic insight into chronic visceral pain from a neurobiological perspective and a potential molecular target for therapy.

Parts of this work have been published previously in an abstract form (29).

Materials and Methods

Induction of CVH. 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 Soochow University and were in accordance with the guidelines of the International Association for the Study of Pain. Chronic visceral hyperalgesia (CVH) was induced by a NMD rat model, as described previously (3, 11). In brief, pups for the NMD group were separated from the maternity cages and placed in isolated cages with an electric blanket to keep them warm (32°C) for 180 min daily from postnatal days (PND) 2 to 15. After the separation period, pups were returned to their maternity cages. Pups for the control (Con) group were not exposed to handling and were maintained in their maternity cages with the dam. On approximately PND 21–22, the sex of pups from both groups was determined. Female pups were culled, and male pups were weaned and housed in individual cages in the same way. Experiments were conducted at 7–15 wk of age. For the above two groups, a total of 48 rats (n = 22 for behavioral studies, n = 10 for excitability recordings, n = 4 for Na\(^+\) current recordings, n = 7 for Western blot analysis, and n = 5 for PCR assays) in the Con group and a total of 45 rats (n = 22 for behavioral studies, n = 9 for excitability recordings, n = 3 for Na\(^+\) current recordings, n = 7 for Western blot analysis, and n = 4 for PCR assays) in the NMD group were used.

Behavioral testing for nociceptive responses. CVH was measured at the age of 7 wk by grading the behavioral response of rats to CRD as described previously (1, 40, 44). Briefly, under mild sedation (1% Brevital, 25 mg/kg, intraperitoneally), 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 with a phagmynometer. The balloon was inflated to 20, 40, 60, and 80 mmHg for 20 s followed by 2 min of 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).

Cell labeling. Colon-specific DRG neurons were labeled by injection of 1,1-dioleoyl-3,3,3', 3-tetramethylinocarbocyanine methane-sulfonate (DiI, Invitrogen) into the colon wall (44). In brief, when the rats were 6 wk old, animals were anesthetized with ketamine (80 mg/kg ip) plus xylazine (≈5–10 mg/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 ~1 μl volume at 10 sites on the exposed colon extending from the level of the bladder to ~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 normal saline following each injection. The colon was gently swabbed prior to 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, NMD (7 wk or 15 wk) or age-matched control rats were killed by cervical dislocation, followed by decapitation (43). DRGs (T\(_1\)–L\(_2\) or L\(_4\)–L\(_5\)) were bilaterally dissected out and transferred to an ice-cold, oxygenated fresh dissecting solution, containing (in mM) 130 NaCl, 5 KCl, 2 KH\(_2\)PO\(_4\), 1.5 CaCl\(_2\), 6 MgSO\(_4\), 10 glucose, and 10 HEPES, pH 7.2 (osmolarity 305 mosM). After removal of the connective tissue, the ganglia were transferred to a 5 ml dissecing solution containing collagenase D (1.8–2.0 mg/ml; Cell Groove, Becton Dickinson) and pronase E (0.04 mg/ml; Boehringer-Mannheim, Indianapolis, IN). Ten minutes later, ganglia were transferred to a 4 ml cold incubation solution (13 mg/ml), and incubated for an additional 10 min. After thorough washing, DRGs were transferred to a 15 ml incubation solution (5 mg/ml) and incubated for 10 additional min. The ganglia were then triturated using a firepolished Pasteur pipette and allowed to adapt for 30 min. The triturated cell suspension was filtered through a 70-μm cell strainer, and 100–400 μl was transferred to the recording chamber for patch-clamp recordings.

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).
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 dissecting 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 acid-cleaned glass coverslips.

Coverslips 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 an external solution containing (in mM) 130 NaCl, 5 KCl, 2 KH2PO4, 2.5 CaCl2, 1 MgCl2, 10 HEPES, 10 glucose, with pH adjusted to 7.2 with NaOH, osmolarity 295–300 mosM. Recording pipettes were pulled from borosilicate glass tubing by 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, 1 Na-GTP, 10 glucose, pH 7.2, adjusted with CsOH, osmolarity 292 mosM. Resting potential (RP) and APs were recorded. The voltage was clamped at −60 mV by a HEKA EPC10 patch-clamp amplifier (HEKA; Lambrecht/Pfalz, Germany).

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Capacitive transients were corrected by using capacitive cancellation 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 with 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 glucose, 5 EGTA, and 1 MgCl2, pH 7.4, adjusted with tetraethylammonium-OH; osmolarity 300 mosM. The patch electrode had a resistance of 3–5 MΩ at room temperature with an external solution containing (in mM) 60 NaCl, 80 choline chloride, 0.1 CaCl2, 10 Hepes, 10 glucose, 5 EGTA, and 1 MgCl2, pH 7.4, adjusted with tetraethylammonium-OH; osmolarity 300 mosM. The peak TTX-R was measured as the peak of the transient component of the subtracted current at every given voltage.

Western blotting. DRGs (T13–L2 or L4–L5) from NMD-treated rats (7 or 15 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% Na deoxycholate, 0.1% SDS, PMSF (10 μM/l), and aprotinin (30 μl/ml; Sigma). The cell lysates were then microcentrifuged at 15,000 rpm for 30 min at 4°C. The concentration of protein in homogenate was determined by using a BCA reagent (Beyotime). Twenty micrograms of proteins for NaV1.8 or NaV1.9 studies were loaded onto a 10% Tris-HCl SDS-PAGE gel (Bio-Rad, Hercules, CA). After electrophoresis, the proteins were electrotransferred onto polyvinylidifluoride membranes (Millipore) at 200 mA for 2 h at 4°C. The membranes were incubated in 25 ml of blocking buffer (1×TBS and 5% wt/vol fat-free dry milk) for 2 h at room temperature. The membranes were then incubated with the primary antibodies for 2 h at room temperature. Primary antibodies used were rabbit anti-NaV1.8 or NaV1.9 (1:200; Alomone Labs, Jerusalem, Israel) and mouse anti-actin (1:1,000; Chemicon, Temecula, CA). After incubation, the membranes were washed with TBST (1×TBS and 5% Tween 20) three times for 15 min each and incubated with anti-rabbit peroxidase-conjugated secondary antibody (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-mouse horseradish peroxidase-conjugated secondary antibody (1:4,000; Chemicon) for 2 h at room temperature.

Fig. 2. Changes in membrane properties of colon-specific dorsal root ganglion (DRG) neurons from rats 7 wk after NMD. A: representative DRG neuron retrogradely labeled by 1, 1'-dioleyl-3,3',3'-tetramethylindocarbocyanine methanesulfonate (DiI) viewed under fluorescence microscope and bright field. Scale bar: 50 μm. B: NaV significantly depolarized the resting membrane potential (RP) in DiI-labeled DRG neurons (**P < 0.001, Mann-Whitney test). C: NMD resulted in a marked reduction of the rheobase in DRG neurons (***P < 0.001, 2-sample t-test).
The membrane was then washed with TBST three times for 15 min each. The immunoreactive proteins were detected by enhanced chemiluminescence (ECL kit; Amersham Biosciences, Arlington Heights, IL). The bands recognized by primary antibody were visualized by exposure of the membrane onto an X-ray film. All samples were normalized to β-actin as control. For quantification of Nav1.8 or Nav1.9 protein levels, the photographs were digitalized and analyzed with a scanner (Bio-Rad imaging system Bio-Rad GelDoc XRS+).

### Table 1. Membrane characteristics of colon-specific DRG neurons in control and NMD-induced chronic visceral hypersensitivity rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NMD</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size, μm</td>
<td>27.8 ± 0.5 (n = 28)</td>
<td>26.6 ± 0.3 (n = 41)</td>
<td>NS</td>
</tr>
<tr>
<td>RP, mV</td>
<td>−49.7 ± 0.8 (n = 28)</td>
<td>−45.2 ± 0.5 (n = 41)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Rheobase, nA</td>
<td>0.24 ± 0.02 (n = 28)</td>
<td>0.17 ± 0.01 (n = 41)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AP, 2×rheobase</td>
<td>1.0 ± 0.3 (n = 25)</td>
<td>2.6 ± 0.5 (n = 40)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>AP, 3×rheobase</td>
<td>2.0 ± 0.1 (n = 21)</td>
<td>3.5 ± 0.5 (n = 36)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Rin, MΩ</td>
<td>674.0 ± 47.5 (n = 28)</td>
<td>703.5 ± 31.1 (n = 41)</td>
<td>NS</td>
</tr>
<tr>
<td>AP threshold, mV</td>
<td>−16.8 ± 2.2 (n = 28)</td>
<td>−18.4 ± 1.1 (n = 41)</td>
<td>NS</td>
</tr>
<tr>
<td>AP amplitude, mV</td>
<td>75.7 ± 2.8 (n = 28)</td>
<td>71.0 ± 2.1 (n = 41)</td>
<td>NS</td>
</tr>
<tr>
<td>AP overshoot, mV</td>
<td>26.0 ± 2.5 (n = 28)</td>
<td>25.8 ± 1.9 (n = 41)</td>
<td>NS</td>
</tr>
<tr>
<td>AP duration, ms</td>
<td>3.3 ± 0.2 (n = 28)</td>
<td>3.3 ± 0.2 (n = 41)</td>
<td>NS</td>
</tr>
<tr>
<td>Latency, ms</td>
<td>72.8 ± 19.2 (n = 28)</td>
<td>55.1 ± 14.1 (n = 41)</td>
<td>NS</td>
</tr>
<tr>
<td>AP, ramp 300 pA</td>
<td>7.7 ± 1.5 (n = 15)</td>
<td>16.2 ± 1.8 (n = 17)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AP, ramp 500 pA</td>
<td>14.9 ± 1.5 (n = 21)</td>
<td>23.7 ± 2.3 (n = 23)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE, with sample size in parentheses. DRG, dorsal root ganglion; NMD, neonatal maternal deprivation; NS, no significance; RP, resting membrane potential; AP, action potential; Rin, input resistance. P values were determined by 2-sample t-test or Mann-Whitney test where appropriate.
nia (CA) according to the manufacturer’s instructions. RNA was then immediately isolated, precipitated, washed, and dissolved in RNase-free water at −80°C. RNA purity and concentration were determined spectrophotometrically. RNA was only used if the ratio between spectrophotometer readings (260 nm:280 nm) was between 1.8 and 2.0. A reverse transcription and first-strand cDNA synthesis was performed with MMLV-RT reverse transcriptase (Invitrogen). The first-strand cDNA was checked by using the housekeeping gene to assess the quality of the reverse transcription. Real-time PCR (ABI Prism 7500) was performed in a 96-well plate with 12.5 ml 2×SYBR Green master mix (Takara, Otsu, Japan). Amplification conditions involved a preincubation at 95°C for 1 min followed by amplification of the target DNA for 40 cycles (95°C for 10 s and 60°C for 20 s) and fluorescence collection at 60°C. Melting curve analysis was performed at a linear temperature transition rate of 0.5°C/s from 60 to 95°C with continuous fluorescence acquisition. All experiments were repeated three times for reproducibility. The relative gene expression (RGE) was normalized to GAPDH and calculated as follows: RGE = 2^(-ΔΔCt), where t represents the level of experimental groups, N represents the control group whose expression level was calculated, and ΔΔCt is the difference of threshold cycle (Ct) between the gene of interest and GAPDH.

Data analysis. Rise times (Ta) of TTX-R currents were obtained by measuring the activation time between 10 and 90% of the peak value. The half-decay times of current inactivation (Tin) were obtained by measuring the time of half-decay phase of the currents. The membrane conductance (G) at each command potential (mV) was determined by dividing the measured membrane current (I) by the driving force as follows: G = I/(Vm − EK), where EK is the equilibrium Na+ potential and was calculated to be +78 mV (external [Na+] = 60 mM and internal [Na+] = 3 mM; where [Na+] is Na+ concentration). Activation data (G-V curve) were fitted by the following modified Boltzmann equation: G/G_{max} = 1/[1 + exp(-(V_{1/2} − V_{m})(k))], where G_{max} is the fitted maximal conductance, V_{1/2} is the membrane potential for half-activation, and k is the slope factor. Steady-state inactivation of currents was fitted with the following negative Boltzmann equation: I/I_{max} = 1/[1 + exp(-(V_{1/2} − V_{m})(k))], where I_{max} is maximal current. Inactivation data were plotted as I/I_{max} vs. the prepulse voltage used to generate the inactivation curves.

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 or Mann-Whitney test or Friedman ANOVA followed by Mann-Whitney test or Dunn’s post hoc test, as appropriate. A P value less than 0.05 was considered statistically significant.

RESULTS

Persistent enhancement of AWR scores in NMD rats. CVH was determined by measuring the AWR scores in response to CRD at 7–15 wk of age. The AWR scores were significantly higher in NMD-treated rats at 20, 40, 60, and 80 mmHg distention pressures than those in age matched control rats (Fig. 1, A–C, *P < 0.05 vs. control for the same pressure, **P < 0.01 vs. Con for the same pressure, Mann-Whitney test following Friedman ANOVA). The enhanced AWR scores lasted for ~6 wk and returned to normal level at 15 wk of age (Fig. 1D), suggesting that NMD significantly increased visceromotor response to CRD in adult rats compared with controls. The AWR scores of control rats didn’t change significantly when repeatedly measured at the same distention pressure at different time points. However, the AWR scores of NMD-treated rats at 80 mmHg distention pressure was significantly higher when tested at 9 wk and compared with those at 15 wk (n = 8 for each group, *P < 0.05, Dunn’s post hoc test following Friedman ANOVA); the AWR scores of NMD-treated rats at 20–60 mmHg distention pressures didn’t change dramatically when tested at different time points.

Enhanced excitability of colon DRG neurons. To determine the effect of NMD on excitability of colon-specific DRG neurons, we measured passive and active membrane properties of Dil-labeled DRG neurons (Fig. 2A) from control and NMD-treated rats. Patch clamp recordings were performed on colon-specific DRG thoracolumbar neurons (TL, including T13, L1, and L2 DRGs). We observed a significant depolarization in resting membrane potential of TL neurons from NMD rats compared with controls (Con: −49.7 ± 0.8 mV, n = 28, NMD: −45.2 ± 0.5 mV, n = 41, Fig. 2B, **P < 0.001, Mann-Whitney test). Rheobase and pattern of firing in response to depolarizing current stimulations were also determined. Rheobase is the minimal injected current that evokes one AP. The respective average rheobase of colon DRG neurons from NMD-treated animals were much lower than those from the corresponding controls (Con: 0.24 ± 0.02 nA, n = 28, NMD: 0.17 ± 0.01 nA, n = 41, Fig. 2C, **P < 0.01, two-sample t-test). The number of APs in response to a two-times rheobase (2X) and three-times rheobase (3X) current stimulation was significantly increased in DRG neurons from NMD-treated group (Fig. 3, A and B). The number of APs (2X) was 1.0 ± 0.3 APs/300 ms in control rats (n = 25) and 2.6 ± 0.5 APs/300 ms in NMD group (n = 40, Fig. 3B, left columns, *P < 0.05, Mann-Whitney test); the number of APs (3X) was 2.0 ± 0.1 APs/300 ms in control rats (n = 21) and 3.5 ± 0.5 APs/300 ms in NMD group (n = 36, Fig. 3B, right columns, *P < 0.05, Mann-Whitney test). In addition, the number of APs in response to a 300- and 500-pA ramp current stimulation was also remarkably increased in NMD-treated group compared with controls (Fig. 3C). The number of repetitive firing in responding to 300-pA ramp stimulation was 7.7 ± 1.5 APs/1,000 ms in control rats (n = 15) and 16.2 ± 1.8 APs/1,000 ms in NMD group (n = 17, Fig. 3D, left columns, two-sample t-test).
**P < 0.01, Mann-Whitney test); the number of repetitive firing in responding to 500-pA stimulation was 14.9 ± 1.5 APs/1,000 ms in control rats (n = 21) and 23.7 ± 2.3 APs/1,000 ms in NMD group (n = 23, Fig. 3D, right columns, **P < 0.01, two-sample t-test).

Several additional membrane properties were also examined. AP duration (at 0 mV), AP threshold, AP overshoot, and membrane input resistance were not significantly altered in colon-specific TL DRG neurons from rats after NMD treatment (Table 1). Furthermore, NMD treatment did not alter the cell size of colon-specific DRG neurons (Table 1). In both control and NMD-treated groups, the colon-specific DRG neurons were mainly middle-sized neurons (>20 and <35 μm). These data strongly indicated that NMD treatment produced distinct changes in passive and active membrane properties of colon-specific TL DRG neurons.

To determine the effect of NMD on excitability of colon-specific DRG neurons from rats at age of 15 wk, we measured resting membrane potentials and number of APs of DiI-labeled DRG neurons (Con: n = 9; NMD: n = 10) from control and NMD-treated rats. Neither resting membrane potentials (Fig. 4A) nor numbers of APs (Fig. 4B) were significantly different between NMD rats at 15 wk and age-matched control rats. Similarly, we did not observe any significant change in resting membrane potential (Fig. 4C) and number of APs (Fig. 4D) of lumbar DRG neurons (Con: n = 10; NMD: n = 9) in NMD rats at 7 wk compared with age-matched controls.

**Increase in TTX-R sodium current density.** Because changes in spike frequency and activation thresholds suggest an alteration in NaV channels (24, 25, 27, 36), we next performed patch-clamp recordings to examine these currents under voltage-clamp conditions. K+ in the control external solution was replaced with tetraethylammonium and the Ca2+ concentration was reduced to 0.1 mM. K+ current can be blocked by Cs+ in the internal solution, and the F− can maintain the stability of sodium currents and also can block some Ca2+ currents (14). A depolarization step from −70 mV to +50 mV in 10-mV increments with duration of 80 ms activated all NaV channels (Total; Fig. 5A). The peak current-voltage (I-V) curves are shown in Fig. 5D. NMD did not produce any significant change

![Fig. 5. NMD-induced chronic visceral hyperalgesia (CVH) significantly enhanced TTX-resistant (TTX-R) Na+ current density. Currents were measured at same holding potentials (−60 mV). A: for total voltage-gated Na+ (NaV) current, 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: for sustained TTX-R sodium current, the voltage steps were the same as those for the total voltage-gated Na+ (NaV) current. C: currents generated by the protocol were subtracted to produce TTX-sensitive Na+ currents (TTX-S). D: peak currents (Ipeak) of total current. E: TTX-R. F: TTX-S. Currents vs. voltages (I-V) were plotted from the average representative cell. NaV amplitude was measured as the peak of the current at every voltage step. G–I: bar graphs showing the mean peak current densities of total voltage-gated Na+ (NaV) current (G), TTX-R sodium current (H), and TTX-S sodium current (I) from control and NMD rats. The current density (in pA/pF) was calculated by dividing the current amplitude by cell membrane capacitance. NMD treatment significantly increased density of total voltage-gated Na+ (NaV) current in DiI-labeled neurons compared with the control (G: Con, −114.3 ± 20.6 pA/pF, n = 11; NMD, −204.2 ± 25.1 pA/pF, n = 13, *P < 0.05, Mann-Whitney test). Current subtraction revealed that NMD-induced visceral hypersensitivity did not alter the TTX-S sodium current density compared with controls (I); however, the TTX-R sodium current density was significantly increased from −81.8 ± 17.1 pA/pF (n = 11) in the control group to −147.9 ± 24.3 pA/pF (n = 13) in the NMD group (H; *P < 0.05, Mann-Whitney test).
in the reversal potential, which indicates that the ion selectivity of Na\(^+\) channels is not altered by this treatment. However, NMD treatment greatly enhanced peak current density in DiI-labeled neurons compared with the control (Con: \(-114.3 \pm 20.6\) pA/pF, \(n = 11\); NMD: \(-204.2 \pm 25.1\) pA/pF, \(n = 13\), \(\ast P < 0.05\), Mann-Whitney test; Fig. 5G).

To further determine the involvement of TTX-R and TTX-S in the NMD-induced potentiation of total sodium current densities, TTX (0.5 \(\mu\)M) was used to identify TTX-R and TTX-S sodium current in this experiment. At the same voltage protocol as described above, TTX-R sodium currents were recorded in the presence of 0.5 \(\mu\)M of TTX (Fig. 5B). Subtraction of TTX-R sodium currents from total currents yields TTX-S sodium currents (Fig. 5C). The peak \(I-V\) curves for TTX-R current were shown in Fig. 5E and TTX-S currents in Fig. 5F. NMD did not produce any significant change in the reversal potential of TTX-R and TTX-S currents (Fig. 5, E and F). However, TTX-R sodium current density was remarkably enhanced in NMD animals (Con: \(-81.8 \pm 17.1\) pA/pF, \(n = 11\); NMD: \(-147.9 \pm 24.3\) pA/pF, \(n = 13\), \(\ast P < 0.05\), Mann-Whitney test; Fig. 5H), whereas TTX-S sodium current density was not significantly altered (Con: \(-50.0 \pm 11.8\) pA/pF, \(n = 11\); NMD: \(-72.1 \pm 13.9\) pA/pF, \(n = 13\); Fig. 5I).

### Dynamic modification of TTX-R sodium current activation and inactivation.

Because the enhancement of peak current density of TTX-R sodium current was evident, we next mainly characterized the effect of NMD on voltage dependence and gating properties of TTX-R currents of colon-specific DRG neurons. We first analyzed the voltage dependence of activation and inactivation of the isolated TTX-R channels. Activation of current-voltage relationship was constructed from \(I-V\) curves of neurons from control and NMD rats (Fig. 6A). Currents at various test pulses were divided by the driving force for Na\(^+\) (with +78 mV as the value for \(E_{Na}\)) and the resulting conductance was expressed as a percentage of that achieved at +30 mV for each condition. The G-V relationship curves were fitted with a modified Boltzmann equation to derive values for the half-maximal activation potential (\(V_{1/2}\)) and the slope factor (\(k\)). The G-V curve obtained from the control group had a \(V_{1/2}\) of \(-10.1 \pm 3.0\) mV and \(k\) of 3.3 \(\pm 0.5\) (\(n = 8\); Fig. 6, C and D). The G-V curve obtained from the NMD group had a \(V_{1/2}\) of \(-23.6 \pm 2.0\) mV (\(n = 13\)) and \(k\) of 1.7 \(\pm 0.4\) (\(n = 13\)). NMD treatment led to a \(-14\) mV negative shift in the steady-state activation curve (\(***P < 0.001\), two-sample \(t\)-test) and a significant decrease in the slope factor in NMD group (\(\ast P < 0.05\), two-sample \(t\)-test). The shift of the
activation curve in a hyperpolarizing direction permits the channel to open in response to weaker depolarization.

We next determined the effect of NMD on the voltage dependence of steady-state inactivation of TTX-R sodium channels of colon-specific DRG neurons. The membrane potential was held at −60 mV and voltage steps were from −90 mV to +30 mV with 10-mV increments and 80-ms duration. Inactivation curves were obtained by plotting TTX-R sodium current during the test pulse (+20 mV) against the membrane potentials during the conditioning pulse. TTX-R sodium current was normalized to I_{max} during the test pulse to +20 mV after a conditioning pulse to −90 mV. Data were fitted with the negative Boltzmann function (Fig. 6).

The steady-state inactivation obtained from the control group had a V_{1/2} of −37.5 ± 6.3 mV and k of 14.9 ± 1.9 (n = 8; Fig. 6, A and D). In contrast, the V_{1/2} and k of the I-V curve of inactivation from the NMD group were −40.6 ± 4.4 mV (n = 11; Fig. 6C) and 11.4 ± 1.4 (n = 11; Fig. 6D), respectively. NMD treatment did not significantly change the V_{1/2} or the k of the inactivation curve (Fig. 6, A and D), indicating the same channel availability in the potential domain between −50 mV and −10 mV of colon-specific DRG neurons from these two groups of rats.

We then examined the current kinetics of TTX-R sodium currents. As shown in Fig. 7, the current activation time between 10 and 90% (Ta) and the half-decay inactivation time (Tin) were measured in peak TTX-R sodium currents from both control and NMD rats (Fig. 7A). The current’s activation time was 2.0 ± 0.3 ms (n = 8) in controls and 0.96 ± 0.1 ms (n = 13) in NMD rats, respectively. NMD significantly accelerated the activation time (**P < 0.01, Mann-Whitney test). However, no significant change was found in the Tin between control and NMD rats (1.2 ± 0.2 ms, n = 8 in Con vs. 2.0 ± 0.4 ms, n = 13 in NMD, Fig. 7C).

Enhanced NaV1.8 expression. To determine whether the expression of NaV1.8 indeed increased in DRG after NMD, Western blotting assays were performed on DRGs in control and NMD rats. Proteins were isolated from both sides of T13–L2 DRGs of control (n = 4) and NMD (n = 4) rats (7 wk). After the proteins were separated by electrophoresis under denaturing conditions, they were transferred to polyvinylidene difluoride membranes and probed with anti-NaV1.8. Anti-NaV1.8 antibody labeled a 250-kDa protein (Fig. 8A, **P < 0.05, independent sample test). After NMD treatment, the molecular weight of the proteins did not change. However, the level of expression of NaV1.8 was increased significantly (**P < 0.05, two-sample t-test, Fig. 8A). The expression of NaV1.8 from rats 15 wk after NMD was also determined. As expected, NaV1.8 expression was not significantly altered at the age of 15 wk (Fig. 8B, n = 3 for each group). To investigate whether an enhanced TTX-R current is attributed to an upregulation of NaV1.9 expression, we examined protein level of NaV1.9 from NMD rats at ages of 7 and 15 wk. NMD did not markedly change expression of NaV1.9 at these two time points (Fig. 8, C and D). We next determined expression of NaV1.8 in L4–L5 DRGs. NMD did not remarkably change expression of NaV1.8 of L4–L5 DRGs (Fig. 8E). The expression of NaV1.8 mRNA was not significantly changed 7 wk after NMD compared with age-matched controls (Fig. 8F, Con, n = 5; NMD, n = 4).

DISCUSSION

Early life stress has long been implicated in the etiology of IBS. NMD, one form of early life stress, has been found to trigger visceral hyperalgesia and colonic dysfunction in several rodent models (5, 20, 32). Our study further confirmed that NMD produced visceral nociception in Sprague-Dawley rats that was manifested as an increase in AWR scores in response to CRD in adult rats compared with controls. The increased response lasted for −6 wk within our observation time period (Fig. 1). This result was consistent with and in an extension for previous study that NMD reduced the distention pressure threshold to elicit visceral-motor responses to colorectal distention (11).

Since NMD induced CVH that lasted for a relatively long period of time, we examined the neuronal excitability and ionic mechanisms of colon-specific DRG neurons. We first demonstrated an enhanced neuronal excitability from rats 7 wk after NMD. This conclusion was evidenced by our observation of a significant depolarization in the resting membrane potential (Fig. 2B), a lowered current stimulation thresholds (Fig. 2C), and enhanced firing frequencies in response to a standardized stimulation (Fig. 3, A and B) and a ramp stimulation (Fig. 3, C and D) in NMD rats compared with age-matched controls. These are consistent with previous findings (31). However, we extended our observation to the time point of 15 wk. As expected, neuronal excitability returned to normal levels 15 wk after NMD (Fig. 4, A and B), which is correlated with the reversed visceral hypersensitivity at 15 wk (Fig. 1D). In addition, neuronal excitability of non-colon-related DRGs (i.e., L4–L5 DRGs) was not altered after NMD (Fig. 4, C and D). Collectively, these data suggest that NMD enhanced excitability of colon-specific DRG neurons and that the enhanced...
neuronal excitability may contribute to the visceral hypersensitivity in NMD rats. Together with the previous report (31), NMD-induced visceral hypersensitivity is associated with peripheral sensitization, a phenomenon in which primary sensory neurons respond in an abnormal and exaggerated manner to stimulation.

The effect of processes by which sensitization is maintained 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. A recent study reported that NMD suppressed \(I_K\) and expression of Kv1.2 subtype of colon-specific DRG neurons (31). In the present study, we found that NMD treatment significantly increased \(Na^+\) current density in colonic-specific DRG neurons (Fig. 5A). The increase in sodium current density may well contribute to the enhanced excitability of colon-specific neurons in our model. Molecularly, the subunits of mammalian sodium channels have been categorized into nine different subtypes (NaV1.1–NaV1.9) (9, 23). Pharmacologically, sodium channels have been classified according to their sensitivity to the blocker TTX wherein the currents conducted by NaV1.1–1.4, 1.6, and 1.7 are completely blocked, whereas the currents carried by NaV1.5, NaV1.8, and NaV1.9 are resistant or insensitive to TTX. The TTX-S sodium currents exhibit both rapid activation and inactivation properties, whereas the TTX-R subtypes activate and inactivate more slowly. Since DRG neurons express both TTX-S and TTX-R components, we therefore used the TTX to identify which component is involved in potentiation of total sodium currents after NMD treatment. TTX-R (Fig. 5B) but not TTX-S (Fig. 5C) sodium currents density was greatly enhanced in NMD rats compared with controls. This is in agreement with previous reports that TTX-R sodium current is involved in somatic pain (21) and visceral pain (2, 22). Possible mechanisms for the potentiation of TTX-R sodium currents include an increase in single-channel conductance, channel opening probability, and/or upregulation of NaV1.8 or NaV1.9 expression. Because the cell diameter was not altered after the NMD treatment (Table 1), the increase of TTX-R sodium current density cannot be attributed simply to somal hypertrophy of colon-specific DRG neurons. Although single channel properties of colonic-specific neurons after NMD have yet to be studied, our analysis of kinetic properties of TTX-R sodium currents revealed that voltage dependence of the activation was altered after NMD treatment. The G-V curve for TTX-R sodium channels shifted significantly in a hyperpolarized direction following NMD treatment (Fig. 6A). As such, this would permit the TTX-R sodium channel to open in response to weaker depolarization. According to our results, ~50% of TTX-R sodium channels were activated at ~20 mV in NMD group compared with less than 10% in control group. Moreover, the steady-state activation curve for TTX-R channels in NMD rats was shifted closer to the resting membrane potential so that a decreased electrical threshold would contribute to the increased neuronal excitability. Because there was no significant change of the

Fig. 8. Increase in expression of NaV1.8. A: Western blots for NaV1.8 of ganglia from control (Con) and NMD rats. Actin control for each sample was given. Bar graph showing mean density relative to \(\beta\)-actin for NaV1.8 from control and NMD rats. After NMD treatment, the relative densities of NaV1.8 were increased by 208% (Con, 0.24 ± 0.05; NMD, 0.74 ± 0.15, \(n = 4\) for each group; \(*P < 0.05\); 2-sample \(t\)-test). NMD treatment enhances NaV1.8 expression. B: NaV1.8 expression was not significantly altered at age of 15 wk after NMD. C and D: NMD did not markedly change expression of NaV1.9 at age of 7 (C, \(n = 4\) for each group) and 15 wk (D, \(n = 3\) for each group). E: NMD did not remarkably change expression of NaV1.8 of L4-L5 DRGs (\(n = 4\) for each group). F: NMD treatment did not significantly alter mRNA expression of NaV1.8 in T13-L2 DRGs (Con, \(n = 5\); NMD, \(n = 4\)).
steady-state inactivation curve of TTX-R sodium channels (Fig. 6B), these data strongly suggest that the large shift of the activation toward a negative direction may be a major cause for the increase in TTX-R sodium current density in NMD rats (Fig. 5H) and could thus contribute to the enhanced excitability. Of note is that transactivation of VGSCs is not the only NMD-induced mechanism that is likely to be important in the excitation of sensory neurons. Indeed, inhibition of voltage-dependent K+ conductance and expression has been reported to arise from the action of NMD within DRGs (31). Together with previous report that NMD also suppressed I_k and expression of Kv1.2 subtype of colon-specific DRG neurons (31), the enhanced excitability in NMD rats may be due to potentiation of TTX-R sodium channel currents and suppression of I_K channel as well.

The mechanism underlying this activation curve shift is yet unclear but may result from changes in either the function or expression of Na+ channels. We showed that the rise time of TTX-R current was significantly shortened after NMD treatment (Fig. 7). Such change could be the simple consequence of the ~14-mV hyperpolarization shift in TTX-R sodium currents. However, it is important to note that fast inactivation can mimic shortened activation, i.e., produce shortened current rise times. However, this is not the case in this study because the half-decay times were not significantly altered after NMD treatment (Fig. 7C). To better understand the molecular basis of chronic changes in Na+ currents in stressed states, the expression of Nav1.8 and Nav1.9 channels, which are two dominant TTX-R sodium channel proteins in nociceptors, were further determined. NMD treatment significantly enhanced Nav1.8 expression (Fig. 8A) but did not alter Nav1.9 expression in colon-related DRGs (Fig. 8, C and D). This enhancement of Nav1.8 expression would give rise to the potentiation of TTX-R sodium channel current. Findings that NMD did not produce any change in Nav1.8 expression in noncolon DRGs (Fig. 8E) further confirm the specificity of Nav1.8 expression in colon-related DRGs. Since the mRNA level of Nav1.8 was not altered in this study, the upregulation of Nav1.8 protein was unlikely modified at transcriptional level and was most likely regulated at translational level. To date, several inflammatory mediators, including PGE2, nerve growth factor, and hydrogen sulfide (H2S), have been shown to sensitize Nav1.8 sodium channels and therefore to significantly increase the excitability of the nociceptor. H2S could be a good candidate to regulate the function and expression of Nav1.8 channels because a large body of evidence shows that H2S plays an important role in producing sensitization in somatic pain models (37, 42) and in acute and chronic visceral hyperalgesia (38, 41) by affecting ion channels and membrane properties in afferent sensory neurons (30, 35). Further experiments are warranted to explore the possible mediators underlying the sensitization of nociceptors under stressed conditions.

In conclusion, our data demonstrate that NMD significantly increased the visceral motor response to CRD, which lasted for ~6 wk within our observation time period. This CVH is associated with an increased neuronal excitability and potentiation of TTX-R sodium currents in colon-specific DRG neurons. Since hyperexcitability by VGSCs, especially the Nav1.8 subunits not Nav1.9 subunits in sensory neurons are thought to be the source of some types of chronic pain states, inhibition of VGSCs represents a promising avenue for therapeutic intervention in patients with IBS.

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AUTHOR CONTRIBUTIONS
Y.X. analyzed data, prepared figures and the manuscript; L.Z. performed experiments, analyzed data; L.L. performed experiments; C.-Y.H. designed experiments and prepared the manuscript; X.J. designed experiments and prepared the manuscript; G.-Y.X. designed experiments and edited the manuscript.

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