Role of the potassium chloride cotransporter isoform 2-mediated spinal chloride homeostasis in a rat model of visceral hypersensitivity

Dong Tang,1 Ai-Hua Qian,1 Dan-Dan Song,1 Qi-Wen Ben,1 Wei-Yan Yao,1 Jing Sun,1 Wei-Guang Li,2 Tian-Le Xu,2 and Yao-Zong Yuan1

1Department of Gastroenterology, Ruijin Hospital, Shanghai, China; and 2Department of Anatomy, Histology and Embryology, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Submitted 20 August 2014; accepted in final form 6 March 2015

IRRITABLE BOWEL SYNDROME (IBS) is a common gastrointestinal disorder that affects up to 30% of the worldwide adult population (20). Visceral hypersensitivity, characterized by a reduced pain threshold and/or exaggerated response to colorectal distension, has been regarded as the most important biological and clinical hallmark of IBS (44). Although mechanisms of visceral hypersensitivity are not fully understood, accumulating evidence indicates that sensitization of visceral sensory transmission, which includes both peripheral and central sensitization, represents a candidate mechanism responsible for visceral hypersensitivity in IBS (24). The important role of peripheral sensitization mediated by colonic dorsal root ganglion (DRG) neurons has been widely explored in many studies (22, 31, 40). However, less is known about central sensitization mechanisms at the spinal level in the pathogenesis of visceral hypersensitivity.

The dorsal horn of the spinal cord is the first central site for gastrointestinal tract nociceptive transmission, acting as a gate control system for noxious processing and visceral pain perception. In the area of the dorsal horn, lamina I is one of the main noxious output pathways from the spinal cord to brain. The visceral nociceptive information in this area is critically modulated by the dynamic balance between inhibition and facilitation (39). Any spinal plastic changes that alter the excitation-inhibition balance may lead to abnormal visceral pain perception. Several lines of evidence have demonstrated that hyperexcitability of spinal lamina I neurons caused by microglia activation (7) neurokinin 1 receptor upregulation (5) or N-methyl-D-aspartate receptor phosphorylation (26) may account for IBS-like visceral hypersensitivity. On the other hand, a deficit in synaptic inhibition, termed “disinhibition,” may also “open the pain gate” of the spinal cord and facilitate nociceptive information transmission to the brain, resulting in pain hypersensitivity (38). One important mechanism underlying disinhibition is the disruption of neuron Cl− homeostasis. The action of inhibitory transmitters, γ-aminobutyric acid (GABA) and glycine, including the strength and the polarity of inhibitory synaptic currents, is crucially dependent on the transmembrane Cl− concentration gradient. This is mainly controlled by two cation-chloride cotransporters (CCCs), the K+−Cl− cotransporter isoform 2 (KCC2) and the Na+−K+−2Cl− transporter isoform 1 (NKCC1) (2). KCC2 is the principal Cl− exchanger in adult neurons whereas NKCC1 normally mediates uptake of Cl−. The net effects of the two transporters are to maintain a low Cl− concentration in neurons. Any factors that result in low KCC2 activity and/or high NKCC1 activity may impair Cl− homeostasis by promoting intracellular Cl− accumulation. The breakdown of the transmembrane Cl− concentration gradient further reduces or even reverses the polarity of GABA/glycine receptor-mediated synaptic currents, thus decreasing the efficacy of inhibition or even switching inhibition to excitation. Cl− homeostasis disruption-mediated disinhibition resulting from derangement in CCC activity and/or expression at the spinal levels has been an important substrate of pain hypersensitivity in several pathological somatic pain states such as neuropathic pain (10) and inflammatory pain (41), making Cl− homeostasis an attractive target for treatment of these disorders. In contrast to somatic pain, little is known about the role of spinal Cl− homeostasis under visceral pain state. By using an inflammatory visceral...
pain model induced by intracolonic instillation of capsaicin in mice, one study shown that painful visceral stimulus rapidly increased spinal NKCC1 activity by phosphorylation and membrane insertion, which might be involved in the generation and maintenance of a hypersensitivity state, respectively (16). Intrathecal injection of the NKCC1 inhibitor bumetanide inhibited capsaicin-induced referred abdominal allodynia (30). Although these data implied a role for spinal NKCC1 in acute inflammatory visceral pain, it is not known whether altered spinal Cl⁻ homeostasis also contributes to pain signaling in common chronic “functional” bowel disorders, such as IBS.

In the present study, we aimed to investigate the role of CCC-mediated spinal Cl⁻ homeostasis in an established chronic water avoidance stress (WAS) model of chronic visceral hypersensitivity.

MATERIALS AND METHODS

Induction of chronic visceral hypersensitivity. All experimental procedures were approved by the Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine and conformed to the guidelines of the International Association for the Study of Pain. All possible efforts were made to minimize the number of animals as well as suffering. Animals were group housed in standard rodent cages at 24°C in a 12-h light-dark room, which was automatically controlled, and had ad libitum access to food and water. Chronic visceral hypersensitivity was induced by the WAS protocol as described before (5–7). Adult male Wistar rats (6–8 wk old) were placed on a platform (8 cm length × 8 cm width × 10 cm height) affixed to the center of a Plexiglas tank (45 cm length × 25 cm width × 35 cm height) filled with water (25°C) to 1 cm below the height of the platform. The animals were maintained on the platform for 1 h daily at 9 to 11 AM in the morning for 10 consecutive days. The sham control rats were subjected to similar procedure in the waterless tank. Experiments were conducted on day 11 of WAS or sham stress procedure.

Western blotting. Western blotting for quantification of protein levels in spinal cord tissue was performed as previously reported (14). The L6–S1 lumbosacral spinal cord and T13–L1 thoracolumbar spinal cord were rapidly dissected, immediately frozen in liquid nitrogen, and stored at −80°C until use. Frozen tissues were homogenized in ice-cold RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% deoxycholic acid, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, and 2 mM EGTA) and supplemented with cocktails of protease and phosphatase inhibitors (at 1:200; Sigma-Aldrich, St. Louis, MO). The lysates were incubated on ice for 30 min and the supernatants were collected following centrifugation at 13,000 g for 20 min at 4°C. Protein concentrations were determined by using a BCA protein assay kit (Bio-Rad Laboratories, Hercules, CA). Gel loading was prepared with 4X sample buffer containing 8% SDS, 8% β-mercaptoethanol, 40% glycerol, and 0.001% bromophenol blue in 0.25 M Tris-HCl, pH 6.8. For KCC2 detection, 0.5% lithium dodecyl sulfate was contained in the sample buffer to solubilize KCC2, and -mercaptoethanol was not included when detecting the oligomeric form of KCC2 (14). The samples were heated at 95°C for 5 min, except for KCC2 detection, because heating may cause transmembrane proteins to form nonspecific higher order complexes (32). Equal amounts of proteins (15 μg) were separated by SDS-PAGE, and transferred to polyvinylidene fluoride membranes. Membranes were blocked in 3% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween 20, pH 7.2–7.4 (TBST) for 1 h at room temperature (RT). The blots were probed overnight at 4°C with the following primary antibodies diluted in 1% BSA/TBST: rabbit-anti-KCC2 (1:1,000, Millipore, Temecula, CA), rabbit-anti-NKCC1 (1:500, Millipore), and mouse-anti-action (1:1,000, Millipore). After washing in TBST, the membranes were then incubated with the secondary anti-bodies, goat anti-rabbit or goat anti-mouse IgG conjugated with horseradish peroxidase (1:1,000, Millipore) for 2 h at RT and rinsed in TBST. Immunoblots were developed with an enhanced chemiluminescence kit (Pierce Biotechnology, Rockford, IL) and an ImageQuant LAS 4,000 Mini system (GE Healthcare Life Sciences, Piscataway, NJ). ImageJ (http://rsb.info.nih.gov/ij) was used to measure signal intensities.

Immunohistochemistry. Immunostaining of the spinal cord tissue was done as previously reported (12). Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) and intracardiac perfusion with saline followed by 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). The spinal cord was removed, postfixed in the same fixative overnight, and cryoprotected in phosphate buffer containing 30% sucrose for at least 3 days at 4°C, and 30-μm transverse sections from L6-S1 level were cut with use of a cryostat (Leica CM1900, Leica, Wetzlar, Germany) at −20°C. For immunofluorescence, sections were blocked in 10% BSA in PBS with 0.3% Triton X-100 for 1 h at RT and then incubated overnight at 4°C with rabbit-anti-KCC2 (1:400, Millipore) dissolved in 5% BSA in PBS with 0.3% Triton X-100. For colabeling studies, rabbit anti-KCC2 (1:400, Millipore) was simultaneously applied with either mouse anti-NeuN (1:200, Millipore), mouse anti-GFAP (1:50, Millipore), guinea pig anti-calciitonin gene-related peptide (CGRP; 1:2,000; a kind gift from Dr. Xingjun Liu, Institute of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences), or mouse anti-OX-42 (1:200; BD Biosciences). After washing in PBS, sections were then incubated with secondary antibodies that included Alexa 568 donkey anti-rabbit IgG (1:200, Invitrogen), Alexa 488 goat anti-mouse IgG (1:200, Invitrogen), Alexa 488 donkey anti-rabbit IgG (1:200, Invitrogen), or Alexa 568 goat anti-guinea pig IgG (1:200, a kind gift from Dr. Xingjun Liu, Institute of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences). Sections from WAS and sham rats were mounted on the same slide and simultaneously immunostained under identical conditions. Images were obtained on a laser scanning confocal microscope (Zeiss LSM510 META, Zeiss, Jena, Germany) with identical parameter settings for the whole series. Lamina boundaries on spinal cord slices were identified as described (25). Analysis of optic density of KCC2 in the superficial spinal dorsal horn was performed with Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD).

Quantitative reverse transcriptase-polymerase chain reaction. Quantitative RT-PCR was performed as previously described (31, 33). Total RNA was extracted from the frozen L6–S1 spinal cord with TRIzol reagent (Invitrogen); 1.5 μg of total RNA from each tested sample was used for cDNA synthesis in a total volume of 20 μl, with oligo(dT) and Superscript II (Life Technologies, Carlsbad, CA) included in the reverse transcription system. Quantitative RT-PCR was performed in 10-μl wells with RT² SYBR Green PCR master mix (Superarray Biosciences, Frederick, MD) on the ViiA 7 Real-Time PCR System (ABI). After incubation at 95°C for 10 min as the initiation of thermal cycling, 40 cycles of PCR were performed, each of which consisted of heating at 95°C for 10 s for melting and 60°C for 1 min for annealing and extension. Each reaction was performed in triplicate. GAPDH was used as a loading control to normalize each sample. PCR primers were as follows: KCC2 forward 5′-GAAGCTTGTAATGCGACATCTG-3′; reverse 5′-CCCTGTGAGGCTCTGGAAGCT-3′; glyceraldehyde-3-phosphate dehydrogenase forward 5′-GGAAACGGTGTCGGCTGTAC-3′; reverse 5′-GAAGTTGGAAGATGGTGAGTGG-3′. Specificity of the PCR products was monitored by agarose gel electrophoresis and melting curve analysis. The relative amount of the KCC2 mRNA transcript was quantified by the 2⁻ΔΔCT method with the 2⁻ΔCT values as the statistical variable for comparison of each group.

Spinal cord slice preparation. The spinal cord slices were prepared as previously described with some modifications (28, 43). Briefly, adult Wistar rats that had either water avoidance or sham stress were anesthetized with pentobarbital sodium (50 mg/kg ip). After brief
transcardiac perfusion, lumbar spinal cord was exposed by a
dorsal laminectomy and placed in ice-cold preoxygenated equimolar
sucrose artificial cerebrospinal fluid (ACSF) with the following com-
position (in mM): 206 sucrose, 3.6 KCl, 0.5 CaCl2, 6 MgCl2, 1.25
Na3HPO4, 25 NaHCO3, 12.5 glucose, 1.3 ascorbate, 2 thiourea, and 3
sodium pyruvate. After the arachnoid and pia mater were removed
under an anatomical microscope, transverse spinal cord slices (400
µm) at L6–S1 level were cut in ice-cold sucrose ACSF by vibratome
/ H9262
under an anatomical microscope, transverse spinal cord slices (400
µm) at L6–S1 level were cut in ice-cold sucrose ACSF by vibratome
/ H9262
and recovered in preoxygenated Krebs solution at 34°C for 40 min, and then at RT for
an additional 1 h before recordings. The Krebs solution contained (in
mM) 117 NaCl, 3.6 KCl, 2.5 CaCl2, 1.25 MgCl2, 1.25 NaHPO4, 25
NaHCO3, and 12.5 glucose (pH 7.4, when bubbled with 95% O2-5%
CO2) and had osmolality of 295–305 mosmol/kg.

Whole-cell patch clamp recordings. The slices were transferred to
the recording chamber and continuously perfused with Krebs solution
at 5.0 ml/min. The procedure for whole-cell voltage-clamp recordings
was performed as previously described with some modifications (14,
42). Neurons located in lamina I of the spinal cord were identified
under an infrared differential interference contrast video microscopy.
All recordings were obtained at RT by using borosilicate glass
microelectrodes that were pulled with a horizontal micropipette puller
(P-97, Sutter Instruments, Novato, CA) and had a resistance of 5–10
MΩ when filled with intracellular solution containing (in mM) 135
potassium gluconate, 5 KCl, 2 MgCl2, 0.5 CaCl2, 5 EGTA, 5 HEPES,
5 ATP-Mg, 0.5 GTP-Na (pH 7.2 adjusted with KOH). Resting
membrane potential (VRest) and cell capacitances were measured after
attaining whole-cell configuration. Focal electrical stimulation
(~50 µm away from the recorded neuron) delivered by concentric
bipolar electrodes (CBARC75, FHC, Brunswick, ME) was used to
evoke inhibitory postsynaptic currents (IPSCs) in the presence of
bath-applied 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione (a specific
AMPA receptor antagonist) and 50 µM D-(-)-2-amino-5-phospho-
oxonic acid (APV, a specific NMDA receptor antagonist). To
evaluate ECl, neurons were voltage clamped at ~60 mV and evoked
inhibitory postsynaptic currents (eIPSCs) were obtained at various
holding potentials from ~93 to ~33 mV in 10-mV steps. By plotting
amplitudes of eIPSCs against holding potentials, we obtained the
current-voltage line, and the ECl was determined as the intercept of the
regression line with the abscissa. The difference between the experimental
ECl measured by whole-cell recordings (10 mM Cl−), and the theoretical
ECl (according to the Hodgkin-Katz-Goldman equation) was considered as an indicator of Cl−
extrusion capacity (9, 14).

Repetitive IPSCs were evoked by trains of stimuli (25 pulses of
200-µs duration each; 50 ms apart) delivered every 20 s at
200-
200-
90 or 0
90 or 0
mV (14). Ten consecutive trains were averaged for subsequent analy-
ysis. For pharmacological inhibition experiments, a potent KCC2
inhibitor [dihydroidenyl]oxy] alkanoic acid (DIOA, 30 µM) was
applied to slices for at least 20 min before data collection. Data were
obtained by using an Axopatch 200B amplifier, filtered at 2 kHz, and
digitized at 5 kHz. The access resistance was monitored during the

\[ \text{Relative protein level} = \frac{\text{IPSC amplitude measured at each } V_h}{\text{IPSC amplitude measured at } V_h = 0} \]

![Figure 1](http://ajpgi.physiology.org/)

**Fig. 1.** K+−Cl− cotransporter isoform 2 (KCC2), and Na+−K+−2Cl− transporter isoform 1 (NKCC1) regulation in the lumbar spinal cord (LS) of water avoidance stress (WAS)-exposed rats. **A:** representative immunoblots (left) for total protein level of KCC2 and NKCC1 in L6–S1 segment of spinal cord from sham and WAS rats. Actin is used as a loading control. **B:** representative immunoblots (left) for total protein level of KCC2 and NKCC1 that is expressed as a ratio over actin in the same sample and normalized to sham rats. **C:** representative immunoblots (left) and quantification data (right) shows quantification of KCC2 oligomer and monomer expressed as a ratio to actin in the same sample and normalized to sham rats. Both oligo-
meric and monomeric forms of KCC2 were significantly reduced in WAS (n = 6) rats compared with sham (n = 6) rats (**P < 0.01).
recording and data were acceptable if access resistance changes were < 20%.

*Noxious CRD.* After sham or WAS, a series of twenty 20-s distensions of 80 mmHg at 2-min intervals between stimulations was performed as described previously (8, 34). Rats were terminally anesthetized with diethyl ether at various time points after the last distension (1, 2, and 3 h). The L6–S1 lumbar sacral spinal cord specimens were collected for Western blotting. Electrophysiological experiments were conducted 2 h after the final distension (see RESULTS for details). The remaining group of sham and WAS rats underwent similar experimental procedures, but without colorectal distention (CRD).

**Surgery.** Intrathecal catheters (PE-10 polyethylene tubing) were implanted as previously described with modification (35). Briefly, following anesthesia with 1% pentobarbital sodium (50 mg/kg ip), the L5 and L6 vertebræ were exposed. A small puncture was made to facilitate catheter insertion into the subarachnoid space. The catheter was advanced in a cranial direction to reach the lumbar segments of spinal cord. The rostral end of the catheter was kept exterior over the forehead and the wound was closed with sutures. After catheter placement, two electrodes made from Teflon-coated, 32-Ga stainless steel wires were implanted in the external oblique muscles and also externalized behind the head (13, 31). Animals were then caged and allowed to recover for at least 5 days before the experiment. Animals that developed signs of motor impairment after catheter placement were excluded from the study. The KCC2 blocker DIOA (30 μg, 0.2% DMSO plus 0.9% NaCl) or vehicle (0.2% DMSO plus 0.9% NaCl) was injected intrathecally in a total volume of 10 μl followed by a 10-μl flush with normal saline.

**Behavioral testing for nocifensive response.** Visceral sensitivity was evaluated by measuring the abdominal withdrawal reflex (AWR) in response to CRD (1). The procedure was performed as previously described (31, 33). Briefly, rats were mildly anesthetized by diethyl ether, and a 5-cm-long flexible latex balloon with the open end secured to an arterial embolectomy catheter (LeMaitre Embolectomy Catheter 6F; LeMaitre Vascular, Burlington, MA) by thread and tape (1 cm wide) was inserted 6 cm into the descending colon and rectum of the fasted sham or WAS rats and held in place by taping the catheter to the tail. The animals were placed in small Lucite cubicles and allowed to adapt for 30 min. Distension was performed by rapidly inflating the balloon to a constant pressure by use of a sphygmomanometer attached to the catheter by a T connector. 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 AWR by two observers blind to the experimental conditions and was performed in triplicate. The AWR was scored as previously described (1). As for pain threshold measurement, distension was applied gradually in 2 mmHg increments, and the threshold was considered as the pressure at which identifiable contraction of the abdominal wall was observable.

For EMG measurements, the electrodes were connected to a BioAmp (AD Instruments, Bella Vista, NSW, Australia), which was connected to a Power Lab (AD Instruments) as an EMG acquisition system (31). Each recording progression consisted of a 20-s predistention baseline activity measurement, a 20-s CRD-evoked response (40 or 60 mmHg), and a 20-s postdistention activity measurement, followed by a 3-min rest between two CRD episodes. The EMG signals were collected and analyzed by Chart 7.5 software (AD Instruments). The response for each CRD stimulus was plotted as the area under the curve (AUC) of the EMG for the 20-s predistention value subtracted from the AUC during the 20-s distention (13).

**Data analysis.** Whole-cell patch data were analyzed by use of Clampfit 10.2 (Molecular Devices, Sunnyvale, CA). All values are given as means ± SE. For the sake of clarity, all quantification histograms for Western blotting, immunofluorescence, and quantitative PCR present values that have been normalized to the sham group (thus the sham group is taken as 1.0 in all the histograms). Student’s t-test was used to compare two groups whereas one-way ANOVA test with a Student-Newman-Keuls post hoc test was used to compare more than two groups. When data did not fit a Gaussian distribution, nonparametric tests were used (i.e., Mann-Whitney test or Kruskal-Wallis test with post hoc Dunn test). Repeated-measures data were analyzed by two-way repeated-measures ANOVA test followed by post hoc Bonferroni comparisons. Statistical analyses were performed with Prism 5.01 software (GraphPad software, San Diego, CA). The level of statistically significant difference was set at P < 0.05, and the P value was adjusted to 0.008 when post hoc Bonferroni was applied, according to the number of times multiple comparisons were made.

**RESULTS**

*Reduced expression of KCC2, but not NKCC1, in lumbar sacral spinal cord of WAS rats.* Given the critical role of CCCs (including KCC2 and NKCC1) for maintaining Cl⁻ homeostasis, we examined the protein levels of KCC2 and NKCC1 in the spinal cord under repeated WAS. After 10 days of WAS, the major Cl⁻ intruder NKCC1 was not significantly affected. By contrast, total KCC2 protein was reduced to ~65% of the control in the L6–S1 spinal cord (sham, n = 6 rats; WAS, n = 7 rats; P = 0.01; Fig. 1A). Because KCC2 oligomerization may be critical for transporter function (3), we further analyzed the KCC2 oligomer and monomer levels in the absence of detergents and found that both oligomeric and monomeric forms of KCC2 were significantly decreased (to ~57% of the sham level for oligomer and ~74% of the sham level for monomer) in the L6–S1 segments of spinal cord from WAS rats (sham, n = 6 rats; WAS, n = 6 rats; both P < 0.01 for oligomer and monomer; Fig. 1B). In contrast, T13–L1 thoracolumbar spinal
cord and other spinal segments that are also involved in visceral nociceptive transmission showed no significant changes of both total and oligomeric forms of KCC2 between the two groups (sham, n = 6 rats; WAS, n = 6 rats; P > 0.05; Fig. 1C). Thus for subsequent experiments we focused on the L6–S1 lumbosacral spinal cord.

In addition, immunohistochemical analysis revealed a significantly decreased intensity of KCC2 labeling in the dorsal horn of L6–S1 lumbosacral spinal cord (sham, n = 5 rats; WAS, n = 5 rats; P < 0.01; Fig. 2, A and B). Furthermore, using a double immunostaining method, we examined the intracellular distribution of KCC2 in the superficial spinal dorsal horn (SDH). The majority of KCC2-positive SDH cells were found to contain NeuN (29) indicating that KCC2 was mainly expressed in neurons (Fig. 3A). By colabeling KCC2 with CGRP, a neuropeptide contained in primary afferent fibers (10), we found that the distribution of KCC2 and CGRP was mostly nonoverlapping (Fig. 3B), which is consistent with a previous report that primary afferent fibers lack expression of KCC2 (10). Moreover, KCC2 did not colocalize with the astrocyte marker GFAP (Fig. 3C) or the microglia marker OX-42 (Fig. 3D). Thus the present results indicated that the decreased expression of KCC2 observed in WAS rats was restricted to SDH neurons rather than the central terminals of DRG neurons or glia cells in SDH.

Further quantitative RT-PCR analysis confirmed the KCC2 downregulation under chronic stress at the transcriptional level (sham, n = 8 rats; WAS, n = 8 rats; P < 0.05; Fig. 2C). Together, these results suggest that chronic WAS may lead to a loss of KCC2 mRNA and protein in the dorsal horn of the L6–S1 lumbosacral spinal cord.

Depolarizing shift of $E_{Cl}$ through KCC2 reduction in lamina I neurons of spinal slices from WAS rats. Using whole cell patch recordings as described before (18), we first examined the effect of DIOA, a KCC2 blocker, on the equilibrium potential of eIPSCs in control slices. After application of DIOA (30 $\mu$M) for 20 min, $E_{Cl}$ was substantially depolarized (sham, $-63.7 \pm 1.1$ mV, n = 10 cells from 5 rats; sham + DIOA, $-51.1 \pm 2.6$ mV, n = 10 cells from 5 rats; P < 0.01; Fig. 4, A and B), indicating that impaired KCC2 activity can disrupt Cl$^{-}$ homeostasis by causing a depolarizing shift of $E_{Cl}$. The DIOA dose (30 $\mu$M) selected here was based on previous in vitro studies (4).

To directly test the changes in Cl$^{-}$ homeostasis following WAS, we measured $E_{Cl}$ in spinal slices obtained from repeatedly stressed animals. In lamina I neurons, we found that WAS induced a significantly depolarizing shift of $E_{Cl}$ (sham, $-62.4 \pm 0.9$ mV, n = 10 cells from 5 rats; WAS, $-51 \pm 1.7$ mV, n = 11 cells from 5 rats; P < 0.01; Fig. 4, A, C, and E). In addition, DIOA (30 $\mu$M) had no significant effect on $E_{Cl}$ of WAS neurons (WAS, $-51 \pm 1.7$ mV, n = 11 neurons from 5 rats; WAS+DIOA, $-49.3 \pm 2.3$ mV, n = 11 neurons from 5 rats; P > 0.05; Fig. 4, A, D, and E), suggesting that the effects of DIOA were inhibited by chronic WA stress. The $V_{R}$ values were not affected by DIOA (30 $\mu$M) both in sham control

Fig. 3. Neuron-specific expression of KCC2 in the dorsal horn of spinal cord. Double immunofluorescence labeling of KCC2 with NeuN (a neuron-specific marker, A, scale bar: 50 $\mu$m), CGRP (a marker of primary afferents, B), GFAP (a marker for astrocytes, C), or OX-42 (a marker of microglia, D). Higher magnification views of the areas marked by the square are shown in far right column. Arrows indicate KCC2-positive cells. Note that KCC2 immunoreactivity was observed in the plasmalemmal region of the cell body. Scale bar: 10 $\mu$m.
Fig. 4. Chronic stress-induced depolarizing shifts of equilibrium potential of evoked inhibitory postsynaptic current (E_{Cl}) in lamina I neurons of L6–S1 lumbar-sacral spinal slices. A: representative examples of whole-cell recording for measuring E_{Cl} in lamina I neurons of L6–S1 spinal slices from sham and WAS rats before and after application of KCC2 inhibitor [(dihydroindanyl)oxy] alkanoic acid (DIOA, 30 μM) for 20 min. Traces of evoked inhibitory postsynaptic currents (eIPSCs) shown are recorded at various holding potentials from −83 mV to −33 mV in the presence of glutamate inhibitors d-(-)-2-amino-5-phosphonovaleric acid (APV; 50 μM) and 6-cyano-7-nitroquinazoline-2,3-dione (CNQX; 10 μM). Note that the gray traces represent GABA response when neurons are clamped at −63 mV. B: effects of DIOA on E_{Cl} in neurons from sham rats. Left, current-voltage relationship (I–V curves) for eIPSCs obtained from spinal slices of sham rats before and after DIOA application. E_{Cl} is determined as intercept of the regression line with the abscissa. Right, summary data for E_{Cl} in neurons shown at left. C–I–V curves for eIPSCs obtained from spinal slices of sham rats and WAS-exposed rats. D: I–V curves for eIPSCs obtained from spinal slices of WAS rats before and after DIOA application. E: summary data for E_{Cl} in neurons shown in C and D. n.s., Not significant. F: summary data for resting membrane potentials (V_{REST}) of recorded neurons shown in B, C, and D (P > 0.05). Data are presented as means ± SE.

(Sham, −57 ± 1.5 mV, n = 10 cells; sham + DIOA, −56.7 ± 1.9 mV, n = 10 cells; P > 0.05; Fig. 4F) and WAS group (WAS, −56.9 ± 1.1 mV, n = 11 cells; WAS + DIOA, −57.2 ± 1.1 mV, n = 11 cells; P > 0.05; Fig. 4F). In addition, V_{REST} was not significantly different between sham and WAS rats (sham, −57.1 ± 1.2 mV, n = 10 cells; WAS, −56.9 ± 1.1 mV, n = 11 cells; P > 0.05; Fig. 4F). Thus E_{Cl} was on average lower (5.3 ± 1.4 mV) and higher than (5.9 ± 2 mV) V_{REST} in sham and WAS rats, respectively. The above results indicated that chronic repeated WAS may induce a depolarizing shift of E_{Cl} in lamina I neurons, which may result from a loss of KCC2 proteins.

Impaired Cl⁻ extrusion capacity of lamina I neurons of spinal slices from WAS rats. To directly estimate KCC2-mediated neuron Cl⁻ extrusion capacity, we used an established method by evaluating collapse of the Cl⁻ gradient during trains of eIPSCs (14, 18). Repeated inhibition of transmission stimulation may lead to progressive amplitude decrease of eIPSCs, known as activity-dependent synaptic depression. Collapse of the Cl⁻ gradient induced by accumulation of intracellular Cl⁻ during repetitive activity may also contribute to decrease of the eIPSC amplitude when the membrane potential is clamped above the Cl⁻ equilibrium potential, such as 0 mV, which favors Cl⁻ influx. Under 0-mV condi-
tions, KCC2-mediated sustained Cl\textsuperscript{−} extrusion is critical to prevent the collapse of the Cl\textsuperscript{−} gradient and thus slow down the depression rate of eIPSC amplitude (Fig. 5A). In contrast, the depression rate of eIPSC amplitude was not affected by the Cl\textsuperscript{−} gradient when neurons were clamped below the Cl\textsuperscript{−} equilibrium potential, such as at −90 mV, which favors Cl\textsuperscript{−} efflux (Fig. 5D).

To isolate the contribution of KCC2-mediated Cl\textsuperscript{−} extrusion during trains of eIPSCs, we conducted experiments while the membrane potential was clamped either at 0 mV or −90 mV. In sham control slices, focal repeated electrical stimulation at 20 Hz induced progressive amplitude decrease of eIPSCs both at 0 mV and −90 mV in lamina I neurons from L6–S1 lumbarosacral spinal cord (Fig. 5, B and E). After application of DIOA (30 μM) to inhibit KCC2 activity for 20 min, the decrease in rate of amplitude of eIPSCs was more significant at 0 mV (sham, n = 11 cells; sham + DIOA, n = 11 cells; F = 34.61, P < 0.0001; Fig. 5C) but was not affected at −90 mV (sham, n = 8 cells; sham + DIOA, n = 8 cells; F = 0.62, P > 0.05; Fig. 5F), indicating that collapse of the Cl\textsuperscript{−} gradient mediated by loss of KCC2 function contributed to activity-dependent depression at 0 mV, but not at −90 mV.

With chronic stress-associated downregulation of KCC2 expression, the depression rate during repetitive synaptic activity at 0 mV might be more pronounced after stress. To test this idea, we performed similar experiments in lumbarosacral spinal slices obtained from the WAS animals. Compared with the sham group, the rate of amplitude decrease of eIPSCs became more rapid following repeated stress at 0 mV (sham, n = 11 cells; WAS, n = 9 cells; F = 17.32, P = 0.0006; Fig. 5C), but not at −90 mV (sham, n = 8 cells; WAS, n = 7 cells; F = 0.09, P > 0.05; Fig. 5F). The difference in depression rates between 0 mV and −90 mV revealed that chronic stress resulted in increased intracellular Cl\textsuperscript{−} accumulation. In addition, bathing with DIOA (30 μM) had little effect on the decay rate of eIPSC amplitude in WAS rats (WAS, n = 9 cells; WAS+DIOA, n = 9 cells; F = 1.44, P > 0.05; Fig. 5C), indicating that the effects of DIOA were inhibited by chronic WA stress. These data suggested that the KCC2-mediated Cl\textsuperscript{−} extrusion capacity was impaired in the lamina I neurons after chronic WAS.

**Disrupted KCC2 expression and function after noxious CRD stimulation in sham but not WAS rats.** To further investigate the role of KCC2 during progression of visceral pain, we performed noxious CRD stimulation on sham and WAS rats. We measured the time-course changes of KCC2 expression after stimulation. A reduction in total KCC2 expression began at 2 h after the final distention in sham rats [sham, n = 8 rats; sham + CRD 1 h, n = 5 rats, P > 0.05 compared with sham group (the same as below); sham + CRD 2 h, n = 6 rats, P < 0.01; sham + CRD 3 h, n = 6 rats, P < 0.01; Fig. 5, A and C], but not in WAS rats (WAS, n = 6 rats; WAS + CRD 1 h, n = 6 rats; WAS + CRD 2 h, n = 6 rats; WAS + CRD 3 h, n = 5 rats, all P > 0.05 compared with WAS group; Fig. 5, A and C). Also, WAS + CRD did not reach statistical significance compared with sham + CRD group at 2 or 3 h after CRD stimulation.

In addition, similar time-course changes were detected in oligomeric (sham; sham + CRD 1 h, P > 0.05; sham + CRD 2 h, P < 0.01; sham + CRD 3 h, P < 0.01; n = 4 rats in each group; Fig. 6, B and D) and monomeric forms (sham; sham + CRD 1 h, P > 0.05; sham + CRD 2 h, P < 0.01; sham + CRD 3 h, P > 0.05; n = 4 rats in each group; Fig. 6, B and E) of KCC2 from sham rats, but not from WAS rats (WAS, WAS + CRD 1 h, WAS + CRD 2 h, and WAS + CRD 3 h, n = 4 rats

![Fig. 5. The Cl\textsuperscript{−} extrusion capacity in lamina I neurons of L6–S1 lumbarosacral spinal slices from WAS-exposed rats. A and D: schematic diagrams show experimental design to isolate the contribution of KCC2-mediated Cl\textsuperscript{−} extrusion to activity-dependent depression of eIPSC amplitude during trains of electric stimulation (25 pulses of 200-μs duration each; 20 Hz) by clamping neurons at 0 mV (A) or −90 mV (D) in the presence of glutamate inhibitors APV (50 μM) and CNQX (10 μM). B and C: representative traces of trains of eIPSCs (average of 10 repetitions) in lamina I neurons of L6–S1 spinal slices from sham and WAS rats with or without DIOA (30 μM) treatment under conditions that neurons are clamped at 0 mV (B). Stimulus artifacts were removed to improve clarity. The depression rate of eIPSC amplitude is plotted in C. Amplitude values of 5th, 10th, 15th, 20th, and 25th eIPSC are normalized to the first eIPSC. **Adjusted P < 0.001, compared with the unstimulated sham group. E and F: representative traces (E) and summary of synaptic depression (F) presented in similar conditions as B, but for neurons clamped at −90 mV. P > 0.05, compared with the unstimulated sham group. Stimulus artifacts were removed to improve clarity in E. V\textsubscript{holding} represents the holding potential at which the neurons are clamped. Data are presented as means ± SE.](http://ajpgi.physiology.org/doi/10.1152/ajpgi.00313.2014)
in each group; all $P > 0.05$ compared with WAS group; Fig. 6, B, D, and E).

Considering that the most striking effects of CRD on KCC2 expression of sham rats were observed at 2 h after stimulation, we chose this time point for subsequent electrophysiological experiments. Consistent with the expression changes of KCC2, $E_{Cl}$ was substantially depolarized (sham, $-64.5 \pm 1.5$ mV, $n = 10$ cells from 5 rats; sham + CRD, $-54.7 \pm 1.9$ mV, $n = 10$ cells from 5 rats; $P < 0.01$; Fig. 6F) at 2 h after stimulation in lamina I neurons from sham rats, but not in neurons from WAS rats (WAS, $-53.1 \pm 1.7$ mV, $n = 10$ cells from 5 rats; WAS + CRD, $-50.9 \pm 1.5$ mV, $n = 10$ cells from 5 rats; $P > 0.05$;
Enhanced visceral nociception in sham rats but not in WAS rats by blockade of KCC2 activity. We conducted behavioral experiments to identify the role of KCC2 downregulation in visceral hypersensitivity in vivo. Visceral sensitivity was measured by using the AWR scores and nociceptive threshold in response to CRD. Compared with sham rats, the WAS rats had significantly higher AWR scores (20 mmHg: sham 0.57 ± 0.09; WAS, 1.08 ± 0.2; P < 0.05; 40 mmHg: sham 1.87 ± 0.15; WAS, 2.46 ± 0.23; P < 0.05; 60 mmHg: sham 2.63 ± 0.1; WAS, 3.13 ± 0.17; P < 0.05; 80 mmHg: sham 3.3 ± 0.13; WAS, 3.7 ± 0.13; P < 0.05; sham n = 10 rats, WAS n = 8 rats; Fig. 7A) and lower nociceptive thresholds (sham, 35.8 ± 1.8 mmHg, n = 10 rats; WAS, 25.8 ± 2.4 mmHg, n = 8 rats; P < 0.01; Fig. 7B), indicating the presence of visceral hyperalgesia and allodynia respectively after WAS.

To investigate the effects of spinal KCC2 downregulation on visceral sensitivity, the KCC2 blocker DIOA (30 μg) was administered directly to the lumbosacral spinal cord in the intact rats via an intrathecal catheter. The DIOA dose (30 μg) was selected according to previous in vivo studies on somatic nociception (10). The results showed that functional blockade of KCC2 caused a rapid and reversible sensitization of visceral nociception, manifested as decreased nociceptive threshold (P < 0.01; Fig. 7C) and enhanced response to CRD at both 40 and 60 mmHg (P < 0.01; Fig. 7, D and E), whereas the vehicle treatments had no effect.

To further examine the possible role of KCC2 in chronic functional visceral hypersensitivity, we performed similar experiments in the WAS rats. The results showed that in the viscerally sensitized rats, DIOA had no significant effect on visceral nociception (Fig. 7, C–E), suggesting that the pronociceptive effects of DIOA were occluded by chronic WAS. The above results indicated that KCC2 downregulation may contribute to the visceral hypersensitivity observed in the repeated WAS rats.

In a series of separate experiments, we recorded EMG signals in response to CRD stimulus as another index for visceral pain sensitivity. Firstly, we confirmed the viscerally sensitized phenotype of WAS rats by showing that the visceromotor response (VMR) amplitude was significantly higher in WAS rats than that in sham rats (20 mmHg: sham 78.1 ± 9.2 μV-s; WAS, 116.4 ± 7.3 μV-s; P < 0.05; 40 mmHg: sham 118.1 ± 9.2 μV-s; WAS, 161.9 ± 9.8 μV-s; P < 0.05; 60 mmHg: sham 165.7 ± 6.9 μV-s; WAS, 214.4 ± 13.7 V-s; P < 0.01).
0.01; 80 mmHg: sham 197.6 ± 10.3 μV·s; WAS, 248.8 ± 13.4 μV·s; P < 0.01; sham n = 6 rats, WAS n = 6 rats; Fig. 8B). Then, after functional blockade of KCC2, we observed a rapid and reversible sensitization of visceral nociception in sham rats, but not in WAS rats, similar to the results of AWR scores. By 20 min after intrathecal injection of DIOA, the VMR to CRD in sham rats were significantly increased by 30 and 36% at 40 and 60 mmHg pressures, respectively (P < 0.05 or P < 0.01 vs. vehicle; Fig. 8A and B). The pronociceptive effects of DIOA gradually diminished, and the VMR returned to the vehicle control level at 2 h after DIOA application (P > 0.05 vs. vehicle; Fig. 8, E and F). DIOA had no effect on VMR to CRD when administered to WAS rats (P > 0.05 vs. vehicle; Fig. 8, E and F).

**DISCUSSION**

Recent evidence indicates that the function and expression of KCC2 can be regulated by acute restraint stress (18), and chronic social defeat stress (27) in specific brain areas such as hypothalamic parvocellular neuroendocrine neurons. However, it is an unanswered question whether stress may modulate Cl⁻ plasticity in other portions of the nervous system. In our visceral hypersensitivity model induced by chronic WAS, we demonstrated that both total and oligomeric KCC2 protein levels were downregulated specifically in the dorsal horn of the L6–S1 lumbosacral spinal cord, but not in the T13–L1 thoracolumbar spinal cord, although both of the segments accept the projection from afferent fibers innervating the colon and rectum (37). The mechanism underlying the region-specific effects of stress remains unknown.

One of the main KCC2 functions in neurons is to modify Cl⁻ homeostasis by maintaining constant intracellular Cl⁻ during channel-mediated ion fluxes, which tend to dispel the transmembrane Cl⁻ concentration gradient (2). In addition, a number of studies have confirmed that the equilibrium potential of the anion-permeable channel-mediated currents (E ço) is a reliable reporter for changes in Cl⁻ homeostasis even in whole-cell conditions (11, 18, 42). Therefore, it is rational to monitor E ço as a “read-out mode” of KCC2 function. The depolarizing shift of E ço observed in chronic stress slices

**Fig. 8.** The visceromotor response (VMR) to CRD stimulus in sham rats and WAS rats after pharmacological blockade of KCC2. A and B: representative (A) and summary data (B) for raw VMR responses to CRD at 20, 40, 60, and 80 mmHg pressures in sham and WAS rats. *P < 0.05 and **P < 0.01, compared with sham group. The time of distention (20 s) is denoted by the horizontal black line below the EMG recordings shown in A. C and D: representative raw VMR responses in sham and WAS rats before and at various time points after DIOA injection at 40 mmHg (C) or 60 mmHg pressure (D). E and F: effects of intrathecal injection of DIOA (30 μg) on VMR in response to CRD at 40 mmHg (E) and 60 mmHg (F) in sham rats (*P < 0.05 and **P < 0.01; sham + vehicle n = 6 vs. sham + DIOA n = 5), and WAS rats (P > 0.05; WAS + vehicle n = 4 vs. WAS + DIOA n = 5). Data are presented as means ± SE.
indicates that neurons are unable to maintain the intracellular Cl⁻ concentration as low as those in sham slices, which are not stressed. This may have resulted from the downregulation of KCC2. The increased intracellular Cl⁻ concentration may have further reduced the GABAergic/glycinergic inhibitory efficacy and enhanced the probability of neurons to be excited. Our results may provide a cellular mechanism for allodynia in which apparent innocuous stimuli are perceived as painful.

Because $E_{Cl^{-}}$ only provides information on the presence of Cl⁻ extrusion (2), we used another index, the depression rate of repetitive eIPSCs, to directly evaluate Cl⁻ extrusion capacity. During repetitive synaptic activity at 0 mV that favors Cl⁻ influx, a Cl⁻ load is imposed on the neuron and sustained KCC2 transport is required to avoid the overload of intracellular Cl⁻. However, the Cl⁻ gradient will collapse when KCC2 transport capacity is saturated during a pulse train, manifesting as a gradual decrease in eIPSC amplitude. The more pronounced decay rate of eIPSC amplitude observed in chronic stress slices indicated that KCC2-mediated Cl⁻ extrusion capacity was impaired. Notably, the length and internal frequencies of stimuli trains we applied (25 pulses of 200 μs each; 50 ms apart) may have, to some extent, mimicked bursts of activity observed in dorsal horn interneurons in vivo (9) including colorectal distention-triggered nociceptive afferent input. Thus the present results may further provide a cellular mechanism for hyperalgesia in which pain perception is exaggerated in response to a noxious stimulus.

By using noxious CRD stimulation as an acute visceral pain model, we found that acute visceral pain caused a time-course disruption of KCC2 expression and function in sham rats. However, we did not observe a significant CRD effect in WAS rats. One possible explanation is that the basal KCC2 expression has been substantially reduced in WAS rats, in which case the expression of KCC2 may have reached a plateau. This may explain why the effects of CRD were not manifested in model rats.

The pronociceptive effect of spinal KCC2 blockade on somatic nociception by DIOA or furosemide has been confirmed in many studies (10, 21, 41). Here, we presented the first evidence that visceral nociceptive transmission is also facilitated after KCC2 activity was inhibited at the spinal level. Although DIOA preferentially inhibits KCC2, it may also antagonize NKCC1 (17). However, evidence has been shown that intrathecal injection of the more selective NKCC1 antagonist bumetamide exerts no influence on the nociceptive transmission in naive rats (30). Thus the sensitization of visceral nociception after DIOA injection can be explained by downregulated KCC2 activity. In addition, the occlusion of pronociceptive effect between DIOA and chronic WAS provides indirect evidence that KCC2 downregulation may be necessary for visceral hyperalgesia and allodynia in WAS rats. However, more direct evidence, such as whether sensitized visceral nociceptive behavior may be relieved after enhancing KCC2 activity in WAS rats, will be needed to further evaluate the contribution of KCC2 to the visceral hyperalgesia. Enhancing KCC2 activity to restore proper synaptic inhibition has emerged as a novel strategy in the conditions involving impaired Cl⁻ transport (23). Recently, a small-molecule compound, CLP257, has been screened as a KCC2-selective analog (15). By rescuing KCC2 plasma membrane expression, the compound was shown to be effective in restoring normal intracellular Cl⁻ concentrations, increasing inhibitory efficacy, and alleviating pain hypersensitivity in a neuropathic pain model. It would be interesting to examine the effects of this compound on visceral pain sensation in our IBS-like chronic stress model.

One limitation of the present study is that cell-type-specific changes were not examined. As a complex neural circuit net, the dorsal horn of spinal cord comprises many different types of neurons, such as projection neurons and interneurons. Although nociceptive projection neurons are concentrated in lamina I, they constitute only ~5% of lamina I neurons, the majority of which are inhibitory or excitatory interneurons (36). The heterogeneity of the various neuronal components indicates that various functional roles may be played by various neurons during nociceptive transmission. Thus it will be necessary to further investigate the Cl⁻ homeostasis changes in different lamina I neurons during chronic stress.

In conclusion, our data demonstrate that WAS-induced chronic functional visceral hypersensitivity is associated with altered Cl⁻ homeostasis and diminished synaptic inhibition in the dorsal horn of spinal cord, which is mediated by KCC2 reduction. These findings identify new mechanisms underlying functional colonic hypersensitivity associated with enhanced stress responsiveness and may pave the way for novel treatment of IBS and related disorders.

ACKNOWLEDGMENTS

We thank Medjaden Bioscience Limited for assisting in the preparation of this manuscript.

GRANTS

The work was supported by the National Nature Science Foundation of China (No. 81170347, 91132303, 81000151).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


