SUPPLEMENTAL INFORMATION

The Material includes:

A) Supplemental Data

These consist of seven Supplemental Figures S1 to S7 with their legends.

B) Supplemental Methods

C) Supplemental References

A) SUPPLEMENTAL DATA

When we initiated this study our goal was to define the currents involved in the low pH sensitivity of nodose neurons in contrast to the well-studied acid sensitive currents in DRG neurons.
We identified transient and sustained cationic currents during low pH exposure of the nodose neurons which we opted to describe in this Supplement and focus the main manuscript on the novel discovery of the large anionic Cl^- conductance that was induced following brief low pH exposures.

ASIC-like Transient Currents in Nodose Neurons (Figure S1)
As mentioned in the first paragraph of the Results in the main text we identified in nodose neurons transient and sustained cationic currents in response to low pH_o which were similar to those reported on DRG neurons. The transient currents rapidly activated and inactivated despite sustained exposures to acid pH. These were ion channels that most likely belong to the DEG/ENaC family and more specifically to the mammalian subfamily expressed in peripheral nerves in rat and in human brain (1-3), and designated as the Acid-Sensing Ion Channels (ASICs) subfamily (4).
Three ASIC genes and their spliced variants (ASIC1a, -1b, -2a, 2b, and 3) form homo-or hetero – multimeric channels with different pH sensitivities (5, 6). ASIC 1 and 3 homomers are most sensitive to low pH with half maximal activation at ~pH6.5, and are involved in nociception in spinal sensory afferents of DRG neurons and in pH sensitivity of carotid body glomus cells (7). Such currents with high sensitivity to acid pH were rarely seen in nodose neurons (5 of 53 neurons) (Figure S1 A).
ASIC2 homomers, on the other hand, are the least pH sensitive (pH_{50}≈4.5) and participate in cutaneous touch sensitivity (Price et al; 2000) and in mechanosensitivity of aortic baroreceptors of nodose neurons in response to a rise in arterial pressure (8). We have found these relatively acid insensitive ASIC channels expressed in a much larger number of nodose neurons (20/53) (Figure S1B) and more consistently represent the ASIC2 mechanosensitive homomeric channel.

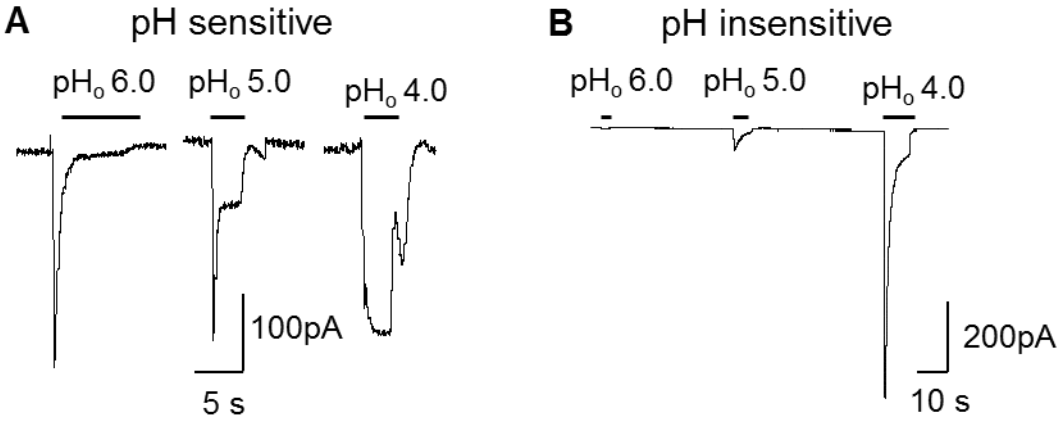


Figure S1. ASIC-like currents with rapidly-activating and desensitizing kinetics.
A. Representative transient acid-evoked currents demonstrating rapid desensitization in response to pH 6.0. The pH 6-evoked current amplitudes were as large as the pH4-evoked amplitudes, suggesting high pH sensitivity. Such currents were seen in only 5/53 nodose neurons and their properties suggest that they are likely ASIC1 and ASIC3 containing channels.
B. A larger number of cells (20/53) displayed transient acid-evoked currents only at lower pH values (5.0 or 4.0) consistent with ASIC2 homomeric channels.

Sustained Inward Currents during Low pH Exposure (Figure S2)

There were also cationic inward currents that were sustained during the period of low pH exposure. Such currents portrayed in Figure S2A in response to pH_o 6.0 were seen in 28 of the 53 cells. Ten of the 20 cells that exhibited the transient ASIC2-like current also expressed this sustained current (Figure S2B). Attempts at assessing the effect of pH_o 6.0 on inward and outward currents examined the contribution of increased inward Ca^{2+} and Na^+ currents and outward Cl^- currents, and decreased outward K^+ currents as reflected in Figure S2C and D, E, and F. Our results suggest that Ca^{2+}, Na^+ and Cl^- currents make a limited contribution to the sustained inward current, but the inhibition of K^+ channels accounts for most of the current. We have reported that inhibition of K^+ currents increases the excitability of nodose neurons (9-11), and abrogates the adaptation of aortic baroreceptor neurons in nodose ganglia (12).

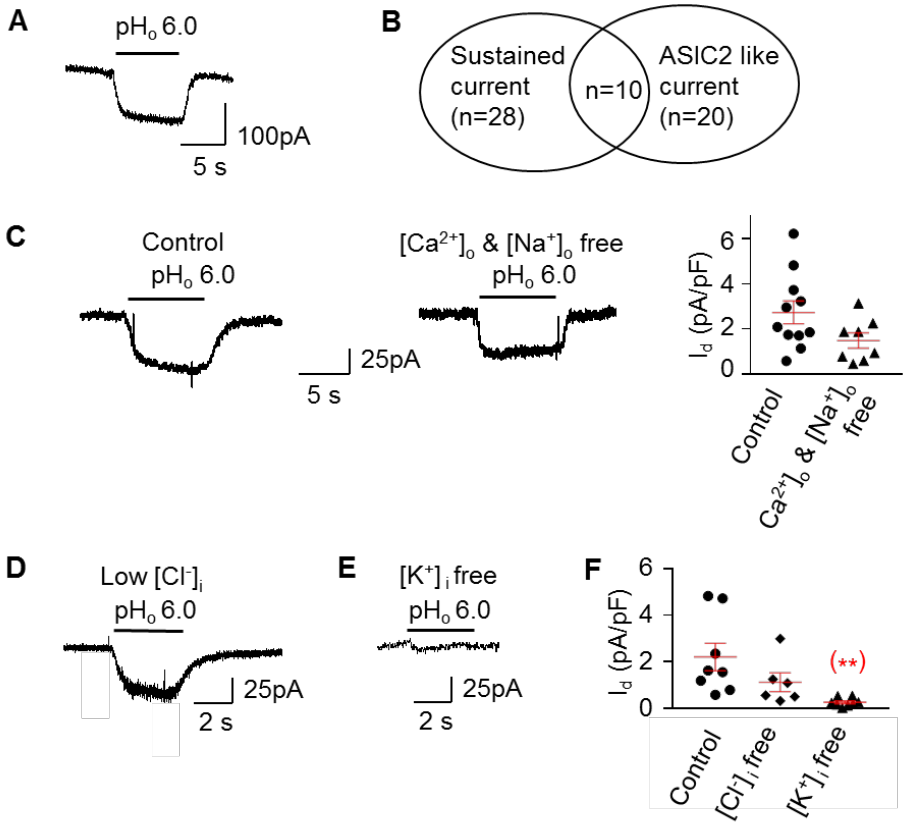


Figure S2. Sustained inward current.
A. Typical sustained acid-evoked current during exposure to pH_o 6.0 seen in 28 of the 53 cells.
B. In ten of these cells, pH 4.0 also activated a transient ASIC2-like current as described above in Suppl. Fig 1B.
C. The tracings and panel show the sustained current in individual neurons partially reduced from 2.7±0.5 (Control, n=3 mice) to 1.5±0.3 pA/pF (n=3 mice) in the absence of extracellular $[Ca^{2+}]_o$ and $[Na^+]_o$ in the solution.
D, E, F. The current is maintained during lowering of intracellular $[Cl^-]_i$ (2.2±0.6 in control, n=3 mice and 1.2±0.4 pA/pF, n=4 mice in the absence of $[Cl^-]_i$) but is essentially eliminated by removing the intracellular $[K^+]_i$ (0.4±0.1 pA/pF, n=4 mice, **p<0.01, unpaired two-tailed student's t test).

Non Additive Effect of the pH_o-Conditioned and Hypoosmolarity Induced Current (Figure S3)

We proposed that pH_o-conditioning may activate the same Cl^- current as hypoosmolarity. We applied pH_o-conditioning together with hypoosmolarity. If pH_o-conditioning and hypoosmolarity activate the same channel, the effect of pHo-conditioning and hypoosmolarity should not be additive. However, if the stimuli activate separate channels, their combined current should be additive. Results below show that the effect of the two stimuli together was not larger than that of single stimuli, implying that pH_o-conditioning and hypoosmolarity are activating the same channel i. e. VRAC (Figure S3A and B). However, the pathway that is used by these two stimuli might be different. The pathway used by pH_o-conditioning is inactivated after one stimulus (Figure S3C), but the hypoosmolarity activation pathway is preserved.

A consideration is whether the pH-conditioned Cl^- current is native of nodose neurons or a product of the cell culture conditions. Two observations should alleviate that concern. One is the fact that the low pH_o-conditioned current is evident in the same neurons under the same culture conditions that show activation of the well-established "native" SWELL1 or VRAC. The second is the fact that the well-established ischemic conditioning known to be induced by VRAC in vivo is reproduced in our cultured neurons by low pH conditioning thus providing strong support for the authenticity of the low pH-induced Cl^- current as native of nodose neurons as is the VRAC.

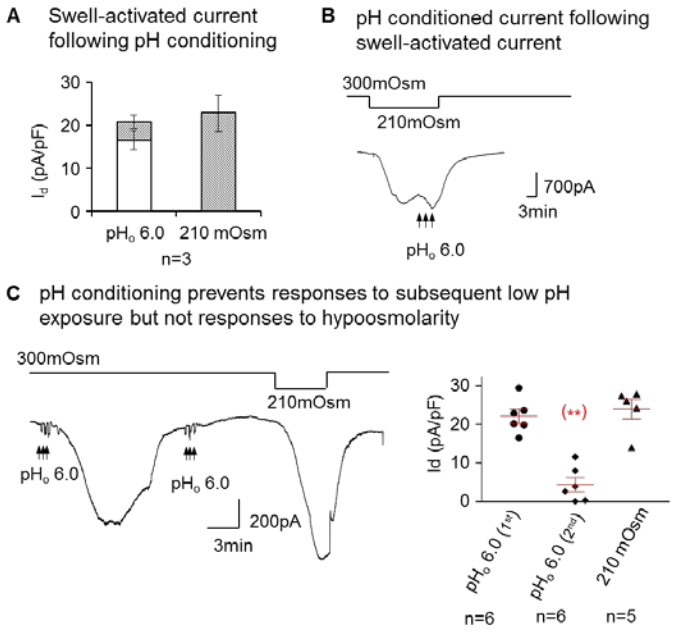


Figure S3. **The Swell-activated and pH-conditioned Cl^- currents are not additive.**
A. The bar graph shows that the activation of the swell current by hypoosmolarity near the peak of the pH_o-conditioned current did not augment the peak current significantly more than the hypoosmolar effect alone (shaded bar).
B. Similarly, activating the pH_o-conditioned current at the peak of the hypoosmolarity-induced current did not significantly increase the peak current.
C. The tracings and panel show that after activation of the current with brief exposures to low pH_o, the neuron is unresponsive to subsequent low pH_o exposures (22.1±1.8 for the first and 4.5±1.8 pA/pF for the second response, n=3 mice, paired two=tailed student's t test, **p<0.001) but remains responsive to hyposmolarity (24.0±2.6 pA/pF, n=3 mice).

Validation of LRRC8A Antibody in HEK293 Cells (Figure S4)

The LRRC8A antibody was obtained in rabbit against peptide QRTKSRIEQGIVDRSE.

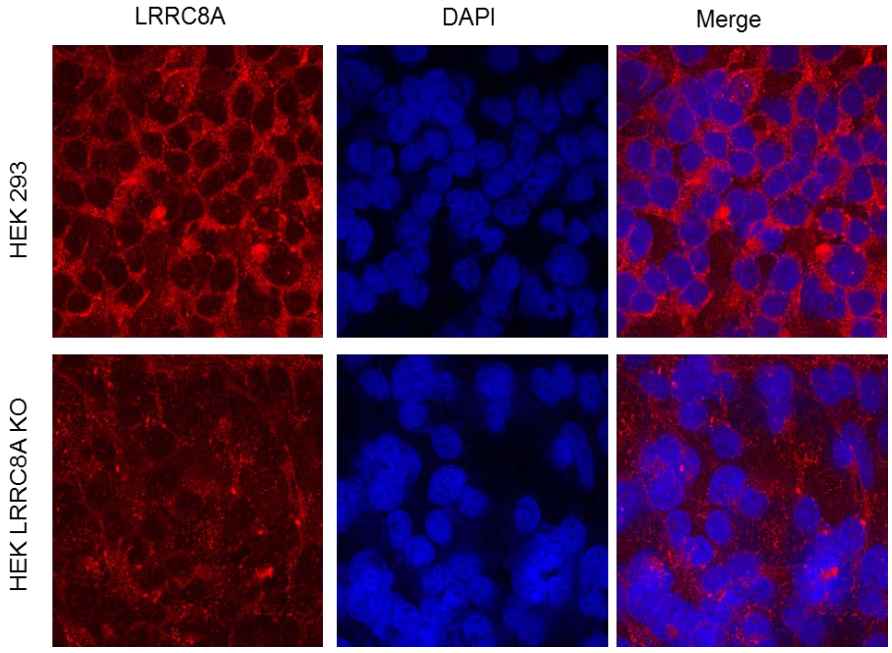


Figure S4. Protein expression of LRRC8A in HEK293 cells

The LRRC8A protein expression was detected by immunostaining. LRRC8A was stained by rabbit anti-LRRC8A (red) and the nucleus was stained by DAPI (blue) (in upper panel). Merger of the images shows the localization of LRRC8A staining primarily on cell membrane. The LRRC8A CRISPR/Cas9 knock out cell line (lower panel) showed only background staining. (Please refer to Figure 3B in the main manuscript for the western blot of LRRC8A protein expression in HEK293 and HEK KO cells)

Activation of VRAC by Hypoosmolarity Does Not Requires Intracellular Alkalinity but Involves AT1 Receptors (Figure S5)

Hypoosmolarity-mediated activation of VRAC has been reported to be dependent on mechanically induced activation of AT1 receptor and the NOX (13). NOX activation requires an intracellular alkaline pH and is inhibited by intracellular acidification (14). To test the dependence of VRAC channel on intracellular pH, we buffered the intracellular pH with 40mM HEPES and kept it at pH_i 6.0. The hypoosmolarity induced VRAC activation which was pronounced at the normal intracellular pH_i of 7.25 was reduced significantly under lower pH_i condition (pH_i 6.0). Inhibition of the hypoosmolarity induced current by Losartan indicated the requirement of AT1 receptor for activation of the VRAC. Although autocrine release of angiotensin II mediate stretch-induced hypertrophy in cardiac myocytes (15), the exact mechanism of hypoosmolarity-induced activation of VRAC in nodose neurons needs further study.

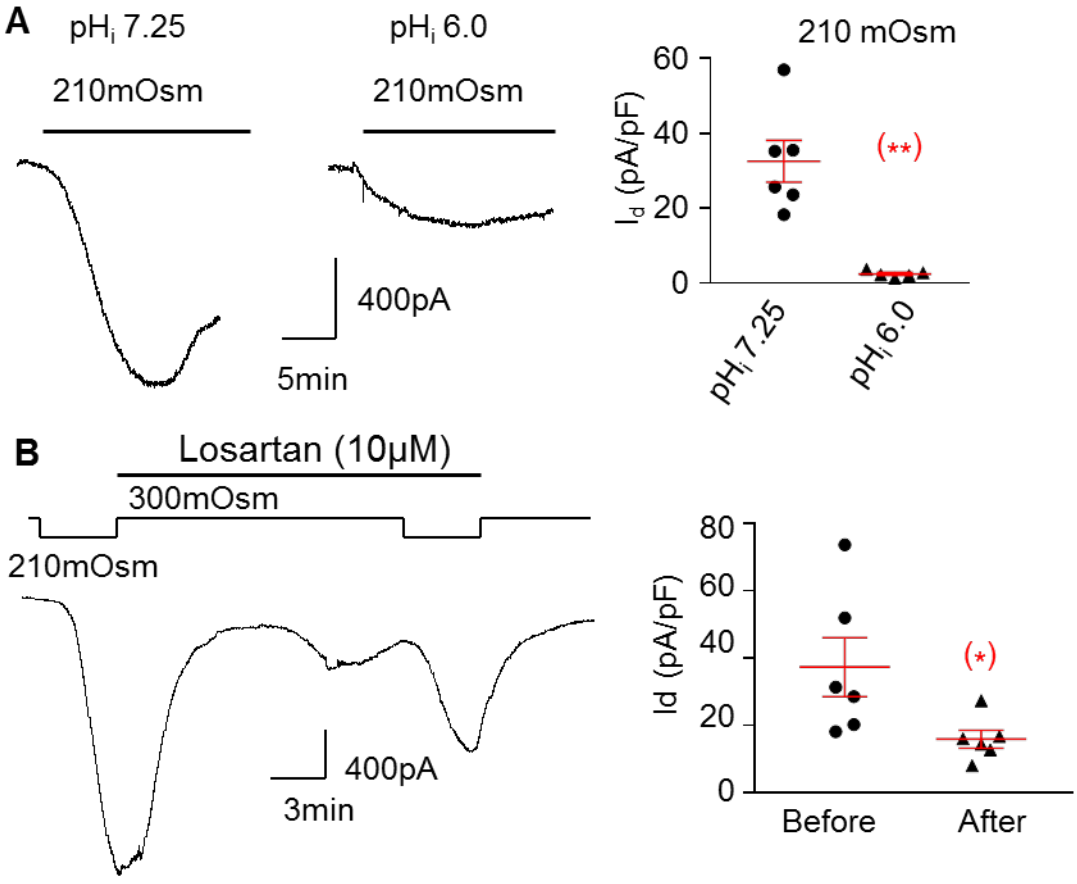


Figure S5. Intracellular acidification and losartan inhibit the hypoosmolarity-induced VRAC current.

A. Tracings and bar graph indicate that lowering intracellular (pipette) pH from 7.25 to 6.0 inhibited the hypoosmolarity-induced current from 32.6±5.6 pA/pF at pH_i 7.25 (n=5) to 2.5±0.4 pA/pF at pH_i 6.0 (n=5), $p<0.01$).

B. The tracings and bar graph represent the responses to 210 mOsm before and after exposure to 10 μM losartan. The decreased response after losartan (37.3±4.6 before and 15.8±2.6 pA/pF after losartan, n=2 mice, *$p<0.05$, paired two-tailed student's t test) suggests that angiotensin II type1 AT_1 receptors contribute to the activation of VRAC during hypoosmolarity.

Intracellular Alkalinity Does Not Activate the VRAC Current in DRG Neurons and HEK Cells (Figure S6)

We observed a hypoosmolarity induced VRAC in the nodose and DRG neurons and HEK293 cells. However low pH_o- conditioned current was only seen in the nodose neurons, but was minimal or absent in the DRG neurons and HEK293 cells (**Figure 3E**). Since the low pH_o-conditioned activation of the VRAC in nodose neurons is mediated by intracellular alkalinity instead of mechanical swelling, we assumed that the HEK cells and DRG neurons might lack the mechanisms for intracellular alkalization, i.e. proton channel and Na^+/H^+ exchanger. We speculated that direct increase in intracellular pH_i might induce the current. We therefore directly increased the intracellular pH by changing the pipette solution pH to 7.6. Results below showed that intracellular alkaline pH did not elicit a current in either HEK cells or DRG neurons, although the hypoosmolarity induced current remained intact in both cell types (Figure S6). Both the alkaline pH_i induced current and the hypoosmolarity induced currents were absent in the HEK LRRC8A KO cells (Figure S6A, C, D). The reason for the absence of low pH_o-conditioned current in the HEK cells and DRG neurons is unclear. Differences in heteromeric subunit combinations of the LRRC8 family members and other components in the vicinity of the channel might contribute to the different responses in different cell types.

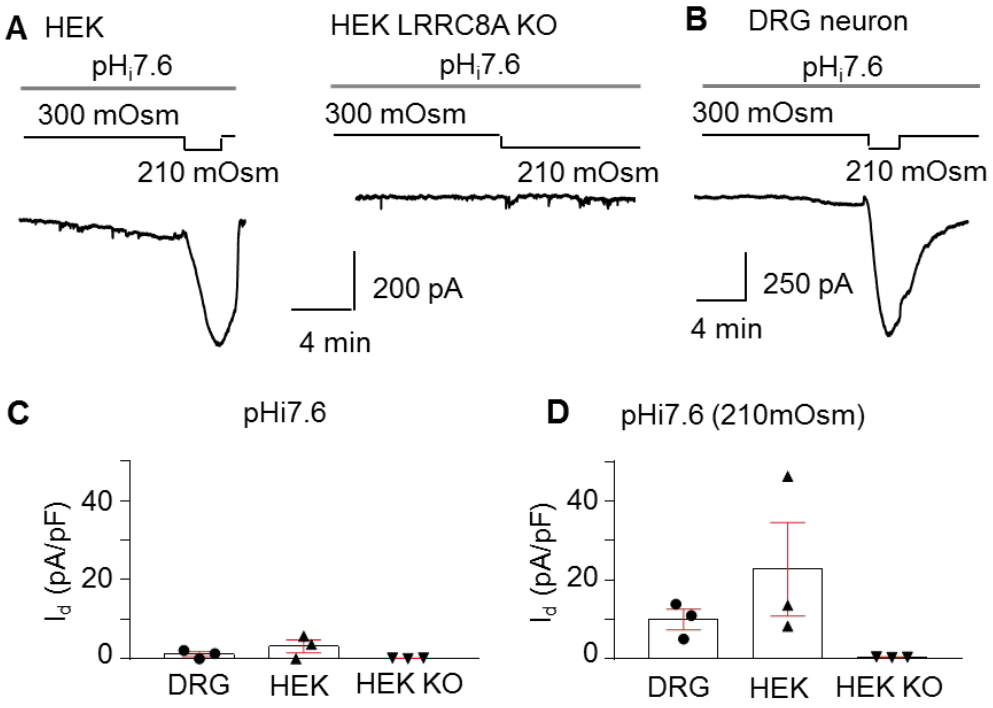


Figure S6. Intracellular alkalinity does not induce VRAC current in HEK293 cells and DRG neurons.
A, B, C. Intracellular alkalinity at pH_i7.6 does not elicit a significant current in wild type HEK cells (3.1±1.7 pA/pF, n=2 batches), in LRRC8A knockout HEK293 cells (0.1±0.1 pA/pF, n=2 cell cultures), nor in the DRG neurons (1.2±0.6 pA/pF, n=2 mice) . **D**. The hypoosmolarity induced current on the other hand, is prominent in HEK cells (22.7±11.9 pA/pF, n= 2 cell cultures) and DRG neurons (10.0±2.6 pA/pF, n=2 mice)**,** but is eliminated by LRRC8A knockout in HEK293 cells (0.4±0.1 pA/pF, n=2 cell cultures) **(A**). Bars include values of individual neurons or cells and their means±SE.

Protective effect of VRAC in simulated ischemia (Figure S7)

The Cl^- channel contributes to the protective effect of ischemic preconditioning in the cardiomyocytes (16). We asked whether the pH_o-conditioned VRAC current plays a similar role in protecting the nodose ganglia neurons from ischemic injury. Ischemia was simulated by oxygen and glucose deprivation plus a low pH_o 5.0. The lactic dehydrogenase (LDH) release was assayed as indicator of cell damage. We found that the LDH release is reduced with pre-activation of the pHo-conditioned current before simulated ischemia, and the protection was eliminated by using VRAC inhibitor tamoxifen (Figure S7A). Figure S7B shows that tamoxifen inhibited the pH_o-conditioned current.

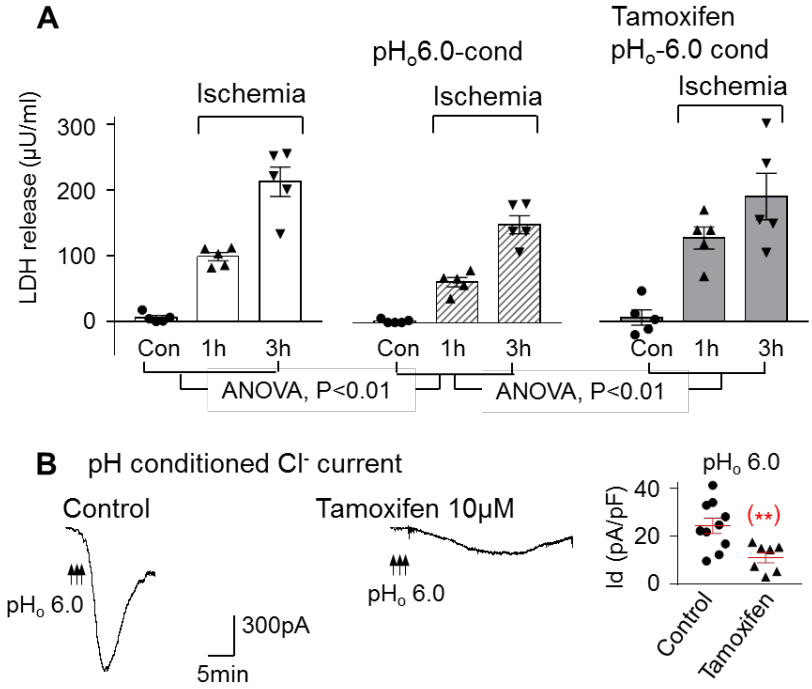


Figure S7. Absolute levels of LDH released during simulated ischemia with both oxygen and glucose deprivation.

A) Nodose ganglia neurons from 5 mice were exposed for 1 to 3 hours to "simulated ischemia" in acidic bathing solutions (pH 5.0) that were oxygen- and glucose-deprived. In the absence of ischemia, the release of LDH from the cultured neurons is negligible over a period of 3 hours in each groups indicated by control values (Con). The release of LDH at 1 and 3 hours of ischemia (104.5±6.6 and 224.8±23.4 µU/ml respectively) shown in the left panel is reduced following activation of the Cl^- current (middle panel) to 65.0±7.5 and 165.4±14.5 µU/ml and restored when tamoxifen was added during the pH_o-conditioning (right panel) to 133.3±17.5 and 199.7±37.3 µU/ml respectively. The values represent mean concentrations ± S.E. in each group (n=5 coverslips) that differenced significantly as indicated (**p<0.01 by ANOVA).

B) The tracings and bar graph show that the pH-conditioned current (24.3±3.1 pA/pF, n=2 mice) is reduced significantly by the blocker of VRAC: tamoxifen (11.1±2.2 pA/pF, n=2 mice, **p<0.01, unpaired two-tail student t test).

B) SUPPLEMENTAL METHODS

Dissociation and Acute Culture of Neurons and HEK293 Cells

Nodose ganglia (NG) and dorsal root ganglia (DRG) were dissociated from 6-8 week-old male C57 BL/6 mice as described previously (17). Mice were anesthetized with 3~5% isoflurane inhalation and their NG and DRG were dissected out through a mid-line neck incision under aseptic condition and placed in ice cold Hibernate A solution (Brainbits, Springfield IL). The ganglia were then transferred to DMEM/F12 media containing 1% penicillin/streptomycin, 10 mM HEPES (Gibco), and supplemented with DNase (0.1 mg/ml), trypsin (1 mg/ml), and collagenase (1 mg/ml) (all from Worthington, Lakewood NJ USA) and incubated at 37° C. The digestion was then stopped after 50-60 min. by adding 6~7 mls of DMEM media containing BSA (1 mg/ml) and the cells were triturated with siliconized pipette until the tissue was well dispersed. The neurons were then centrifuged for 5 minutes at 50-100 G (800 rpm), the supernatant was discarded, and neurons were resuspended in culture medium containing DMEM/F12, 10 mM HEPES, penicillin/streptomycin, and 10% fetal bovine serum (FBS). The culture medium (100 µl) was then transferred to glass coverslips coated with poly-L-lysine (10 µg/ml for 30 min). After incubating overnight, 2 ml of additional culture medium were added to the dishes before the electrophysiologic studies were carried out at room temperature. Euthanasia was performed following guideline of NIH/APS.

Human Embryonic Kidney 293(HEK293) cell line was obtained from American Type Culture Collection (ATCC® CRL-1573™). Cells were maintained between 10% to 90% confluence tissue culture incubator with 37º C and 57% CO_2. The culture medium contains: DMEM, 10% FBS and penicillin/streptomycin. Cells were passed twice a week using 0.25% Trypsin-EDTA.

Electrophysiology

Whole-cell patch clamp technique used for NG and DRG neurons and HEK293 cells was employed as described previously (7, 18) for both current-clamp and voltage-clamp recording using Axopatch 200B amplifier and Pclamp8 software (Axon Instrument, Union City, CA, USA). Patch pipettes with resistance of 3-5MΩ were pulled with micropipette puller (Sutter instruments) and polished (MF830, Narishige, Japan). The pipettes were filled with a solution containing (mM): 121 KCl, 10 NaCl, 2 $MgCl_2$, 10 HEPES, 5 EGTA, 2 K_2-ATP, pH 7.25 adjusted with 1 M tetramethylphenyl-ammonium hydroxide (TMAOH). During recording, coverslips were continuously perfused with bath solution containing (mM): 128 NaCl, 5.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 MES, 5.5 glucose, pH 7.4 and 7.0, 6.5, and 6.0, adjusted with TMAOH. Solutions were delivered with rapid solution exchanger (RSC-200 & EVH-9, Biologic Science Instruments). An equimolar concentration of NMDG-Cl was used to replace NaCl, KCl, or $CaCl_2$, and Cl^- was replaced with aspartate when needed. The permeable cation free solutions contained (mM) 133 NMDG-Cl, 5 Cs-EGTA, 10 HEPES, 2 Na_2-ATP at pH 7.4, 7.25, and 6.0, adjusted with TMAOH. The low Cl^- solution contained (mM) 4 NMDG-Cl and 129 NMDG-Aspartate, 5 Cs-EGTA, 10 HEPES, 2 Na_2-ATP at pH 7.4, 7.25, and 6.0, adjusted with TMAOH. The hypoosmotic solution was made by reducing concentrations of NaCl in the extracellular solution. Mannitol was added to the hypoosmotic solution to make an isotonic control solution. Pipette voltage offset was neutralized prior to the formation of the giga-ohm seal. Membrane resistance and membrane capacitance were determined from current transients elicited by a 5 mV depolarization step from a holding potential of -60 mV using "Membrane Test" mode of Clampex 8.2. The capacitance and over 80% of series resistance was compensated. The voltage-clamp protocols were performed after stabilization of neurons to record the current and a step protocol was applied to obtain the I-V curve. For the step protocol we changed the voltage from -100 mV to 100 mV in 20 mV step-increments. Each voltage step lasted 200 ms with an interval of 1 second. Gap-free current-clamp protocol was used to monitor changes in membrane potential. The recording chamber was grounded through a 3 M KCl Agar Bridge. Junction potential was nulled before the formation of seal. To determine the ion permeability and the reversal potential, the bath solutions were changed after obtaining baseline values with normal solutions. In experiments with "permeable cation free solutions," the pipette was loaded with permeable cation free solution first and the bath solution was switched from regular to permeable cation free after the current size had reached the peak level. The peak level of the current was measured with Clamfit 9.2 (Axon Instrument). The τ of the inactivation of transient currents was fitted with a single exponential equation.

Activation of the Low pH_o-Conditioned Current

Following the early observation that transient exposure to low pHo induced a large sustained current, we planned a specific protocol for all subsequent studies to characterize the current. This consisted of first washing the neurons with solution at pH 7.4, then using a rapid solution exchanger to bathe the cultures with a solution at pH 6.0, for 10 seconds and then switching to pH 7.4 solution for 20 seconds. This exchange to pH 6.0 and then to pH 7.4 was repeated twice before returning to the normal bathing solution at pH7.4 during the recording of the low pH_o-conditioned current which lasted 10 to 15 minutes. This same protocol was applied to studies in which

fluorescent probes were used to detect changes in ROS or intracellular pH during the activation of the current. Control studies were performed using exchange solution at pH 7.4.

Detection of ROS

NG neurons cultured on coverslips were washed using Hank's Balanced Salt Solution (HBSS, from GIBCO Invitrogen) containing (mM): 136.9 NaCl, 5.4 KCl, 4.2 $NaHCO_3$, 1.3 $CaCl_2$, 0.8 $MgSO_4$, 0.4 NaH_2PO_4, 5.6 D-glucose, 10 HEPES, adjusted to pH 7.4 using 1 M NaOH. The coverslip was mounted in a closed perfusion chamber on the inverted fluorescence microscope (Nikon TE2000-U) for fast changes in solution. The neurons were loaded with 10 μM dihydroethidium (DHE) (Invitrogen Co., Molecular Probes) in the bath solution at room temperature. The image was taken with 10X objective lens using computer-controlled 12-bit digital cooled charge-coupled device (CCD) camera (Photometrics Cool SNAP-cf). Data were saved and analyzed using the graphics control software package (MetaMorph 6.3). The fluorescence of DHE was excited at wavelength 535 ± 50 nm and sampled at emission wavelength of 610 ± 75 nm using filter cube (c-FL HYQ TRITC). Images were acquired at a 10 ms exposure time and a 1-5 seconds sampling interval. The average intensity of the background (F_o) was subtracted from the image signals (F) to obtain the relative fluorescence intensity $(F-F_o)/F_o$. Bath solution at pH 6.0 with 10 μM DHE was applied in the same way as for electrophysiological measurements (i.e., three pulses at pH 6.0) after loading DHE for ~15 minutes.

Intracellular pH Measurement

The intracellular pH of NG neurons was monitored with cytoplasmic pH-sensitive fluorochrome BCECF-AM (Molecular Probe by Life Technologies) as reported previously (19). Briefly, neurons cultured on coverslips were loaded with 2 μM BCECF in HBSS for 5 minutes at room temperature and incubated in 37º C for 20 minutes after wash. The coverslip was then mounted in a closed perfusion chamber on an inverted fluorescence microscope (Nikon TE2000-U) (perfusion solution: HBSS with 10 mM HEPES, 10 mM MES and pH adjusted to 7.4 and 6.0 with 1 M NaOH). Fluorescence of BCECF was excited at wavelengths 495 nm and 440 nm and monitored at wavelength 530 nm using lambda DG-4 illumination system (Sutter Instrument). The calibration of intracellular pH was obtained using nigericin (30 μM) and high K^+ buffer (140 mM KCl, 10 mM HEPES and 10 mM MES) adjusted to pH 6.0-9.0 using 1 mM KOH. The ratiometric fluorescence intensity (recorded by NIS-Elements AR 3.10 software, Nikon) was calculated as $F_{ratio} = (F_{495}-F_{0\,495})/(F_{440}-F_{0\,440})$ (F0 refers to fluorescence intensity at baseline) to cancel the effect of bleaching. The relationship of pH value and F_{ratio} was fitted with Hill's equation $F_{ratio}=F_0+a/(1+(PKa/pH_i)^b)$ using Sigma Plot 8.0 software.

The low pH_o-conditioning extracellular solution at pH 6.0 was applied three times, with each time lasting for 10 seconds and with a 20-second interval at pH 7.4 as described above. The intracellular pH was measured at 2-second intervals for a total of ~20 minutes before, during, and after the extracellular exchange in solutions. The pH_i was calculated at baseline, at the peak of the pH drop during the three pulses of extracellular low pH exposure and approximately 10 minutes thereafter when pH_i increased to a plateau level. The $ZnCl_2$ (1 mM) and amiloride (300 μM) solutions adjusted to pH 7.4 were then added 10 minutes after extracellular low pH exposure when pH_i had reached a plateau. The average baseline intracellular pH was obtained by averaging results from 20 NG neurons. The obtained baseline F_{ratio} in all experiments was normalized to the average baseline F_{ratio} from these 20 neurons to calculate the intracellular pH changes.

LDH Release During Simulated Ischemia

Freshly dissociated nodose neurons were cultured for 24 hours in 96-well plates coated with poly-L-lysine with 3-wells in each plate. They were washed with HBSS solution (from Gibco, with Ca^{2+} and Mg^{2+} buffered with 10 mM HEPES and 10 mM MES), adjusted as needed to pH 5.0, 6.0, or 7.4 with HCl and NaOH). The neurons in each plate were randomly divided into three treatment groups exposed to: 1) "simulated ischemia", 2) "simulated ischemia" 10 minutes after activation of the pH_o-conditioned current, and 3) "simulated ischemia" after having added tamoxifen (20 μM) 10 minutes before and 10 minutes after activation of the pH_o-conditioned current.

In the first well, the Cl^- current was not activated and the cell washing protocol for the pH_o-conditioning current was followed except that the 10 sec. exchange solution was 7.4 instead of 6.0. The cells were then incubated in the normal (pH 7.4) bathing solution at 36° C for 10 minutes. Thereafter the normal solution was replaced with 100 μl of acidic solution at pH 5.0 that was oxygen-deprived in an anaerobic chamber filled with 95% N and 5% CO_2 at 36° C to simulate ischemia for 1 to 3 hours. In some experiments, the acidic oxygen-deprived solution was also totally glucose-deprived, while in others we maintained a high glucose content (20 mM) to determine if the additional deprivation of glucose would enhance the neuronal damage from acidosis and oxygen deprivation. The treatment of cells in the second well was the same as the first except for using the pH_o-conditioning protocol to activate the Cl^- channel. Treatment of cells in the third well was the same as the second except for the addition of tamoxifen as mentioned above. The acidic pH 5.0 of HBSS was essentially constant throughout the 3 hours of simulated ischemia, being reduced by only 0.06 pH units.

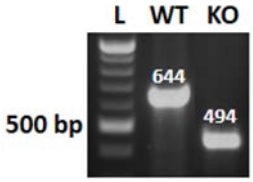
LDH measurements were performed using the Cytotoxicity Detection Kit^{plus} from Roche) on 100 µl supernatant mixed with 100 µl reaction buffer for 25 min. according to the protocol. After the reaction was terminated with 50 µl buffer, the optical density was measured with a microplate reader (Spectra Max Plus, Molecular Device) using 492 nM absorbance. Background absorbance at 620 nM was subtracted.
The total LDH release from lysis of all cells was measured after a lysis buffer was added to a well of control neurons. This allowed us to estimate that exposure to simulated ischemia for 3 hours caused the release of 20 to 30% of the total cellular content of LDH. (Please refer to Suppl. Fig. S7.)

Apoptosis Assay with NMDA
NMDA receptors exist in nodose neurons (Peeters et al 2006), and an exposure of cultured nodose neurons to NMDA induce excitotoxic neuronal damage (Liu et al, 2007). In this experiment, nodose neuronal cultures were treated with 50 µM NMDA for 20, 40, or 60 minutes and examined for neuronal cell apoptosis 20 hours later. NMDA-induced apoptotic injury was determined by counting green fluorescent neurons.
Alexa Fluor 488 annexin V (Molecular Probe), a green fluorescent conjugate (excitation/ emission maximal wavelengths ~495/519 nm) was used as a label of apoptotic neurons. Live cells at a normal physiological state have phosphatidyl serine (PS) located on the cytoplasmic surface of the cell membrane. When apoptosis occurs, the PS is translocated from the inner to the outer surface of the plasma membrane. Annexin V has a high affinity for PS, and is a Ca^{2+}-dependent phospholipid-binding protein (35-36 kDA). When labeled with a fluorophore, it can identify apoptotic cells by binding to PS exposed on the outer surface.
The red fluorescent dye propidium iodide (PI, Molecular Probe) is impermeant to live cells and apoptotic cells, but stains dead cells with red fluorescence, binding tightly to the nucleic acids in the cell. Thus, apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence.
The NG neurons on the coverslips in the 24-well plate were washed with the buffer Hank's Balanced Salt Solution (HBSS from Gibco Invitrogen) containing (mM): 136.9 NaCl, 5.4 KCl, 4.2 $NaHCO_3$, 1.3 $CaCl_2$, 0.8 $MgSO_4$, 0.4 NaH_2PO_4, 5.6 D-glucose, 10 HEPES adjusted to pH 7.4 using 1 M NaOH. Each well was filled to the final volume of 100 µl buffer. The mixture of 5 µl Annexin V and 1 µl PI (100 µg/ml) were added into each well, incubated for 15 min. at room temperature, and finally 400 µl buffer were added into each well according to the protocol provided by the manufacturer.
The neurons in the plate were observed on the inverted fluorescence microscope (Nikon TE2000-U) with 10X objective lens, and the images taken using computer-controlled 12-bit digital cooled charge-coupled device (CCD) camera (Photometrics Cool Snap-cf). The fluorescence of Annexin V was excited at wavelength 480±50 nm and sampled at emission wavelength 535±50 nm using filter cube (c-FL HYZ FITC), and excitation of 535±50 nm and emission of 610 ±50 nm using filter cube c-HYQ TRITC) for PI.
Data are expressed as the percent of green fluorescent cells seen in the cultures after 20 hours without exposure to NMDA (control) and 20 hours after 20, 40, and 60-minute exposures to NMDA. These apoptotic responses were contrasted to those observed in similar cultures after activation of the low pH_o-conditioned current as well as to responses after tamoxifen and the current activation following the same protocol described for the experiments with simulated ischemia. The linearity of the "dose-response" curves to NMDA allowed the statistical analyses of variance between treatments with $p<0.05$ (*) and $p<0.01$ (**) for significant differences.

shRNA Transduction
We tried two different lentiviral shRNAs in this study, shRNA1 and shRNA2, respectively targeting different sequence regions of LRRC8A mRNA (Genbank ReSeq: NM_019594).
Production of lentiviral shRNA1. An LRRC8A shRNA construct was designed to express a hairpin oligonucleotide sequence of CCGGGCACAACATCAAGTTCGACGTCTCGAGACGTCGAACTTGA TGTTGTGCTTTTTG which contains 21 nucleotides, GCACAACATCAAGTTCGACGT, matched to a segment of LRRC8A mRNA (Figure 3C). This was cloned by Dr. Sah's lab into pLKO.1 TRC plasmid cloning vector,(Addgene). We used lentiviruses with empty pLKO.1 TCR cloning vector as controls. The lentiviruses were produced by cotransfection of HEK293T cells with pLKO.1 TRC plasmid carrying LRRC8A shRNA, lentiviral packaging plasmid pCMV-dR8.2 dvpr, and envelope protein plasmid pCMV-VSV-G using FuGENE DNA Transfection Reagent (Promega) . Transfection was allowed for 15 hours followed by a change of culture medium. Two batches of virus particle containing culture supernatants were collected at 24hr intervals. These viral preparations were filtered (0.45µM low protein binding filter) and aliquots stored at -80ºC.
Lentiviral shRNA2. shRNA2 is a pool of 2 different shRNA sequences packaged into lentiviral particles (sc-92566-V) purchased from Santa Cruz Biotechnology Inc., generating the first hairpin sequence(sc-92566A): GATCCCCATCATCAAGGTGATCAATTCAAGAGATTGATCACCTTGATGATGGTTTTT
and the second hairpin sequence(sc-92566-VB): GATCCCCTTCATCCAGATAACTTATTCAAGAGATAA GTTATCTGGATGAAGGTTTTT. Both sequences contain 21 nucleotides targeting at the LRRC8A mRNA

sequences about 2000 nucleotides apart.
Lentiviral CRE and GFP. Lentiviral particles containing CRE or eGFP as control (FIV3.2CMVCre-eGFP VSVG and FIV3.2CMVeGFP VSVG) were obtained from the Viral Vector Core Facility of University of Iowa.
Transduction of Lentivirus to Cultured Neurons. An MOI test was carried out to determine the optimal transduction efficiency for shRNA lentivirus for the cultured mouse nodose neurons. MOI of 200 for shRNA1 or 2 and their control was found to be the best and used in all viral transduction experiments.
For shRNA1 and 2, a time course experiment was carried out to determine the optimum time for harvesting the cultured neurons. We have found that 3 to 5 days of treatment with puromycin (3μg /ml) was effective in killing the non-transduced neurons.
Dissociated nodose ganglia neurons were cultured for 24 hrs, followed by a challenge with lentivirus in serum-free medium for 6 hours. Neurons were then incubated in fresh growth medium at 37°C for 48 hours. Puromycin (3 μg/ml) was then added to eliminate the non-transduced neurons.
LRRC8A knockout in cultured nodose neurons using floxed mice. The LRRC8A floxed mice (unpublished) were obtained from Dr. Rajan Sah's lab. Their nodose neurons were isolated and transduced in culture with lentivirus (as described above) containing a vector expressing CRE recombinase gene or control eGFP (500 MOI for each). GFP was expressed as an indicator of site-specific flox-CRE recombination. Neurons were harvested or recorded 6 days after transduction without treatment of puromycin. The patch-clamp experiments and immunostaining were performed with neurons expressing GFP.

CRISPR/Cas9 Mediated Knockout of LRRC8A in HEK293 Cells:
A double guide knockout approach was used to generate LRRC8A null gene in HEK 293 cells. Guide sequences (OA: CCTGTGCTAACCCCCCTCCTAT and 3A: GATGATTGCCGTCTTCGGGGGG) were designed by a web based tool (http://crispr.mit.edu/) and cloned in a bicistronic vector expressing cas9 (pSpCas9(BB)-2A-Puro) (20, 21). The guides were transfected into HEK293 cells using Lipofectamine 2000 as per manufacturer's instructions. The knockout pool was enriched 48 hours after transfection by addition of puromycin (1 μg/ml) and maintained for 5 days. Individual clones were isolated by serial dilution and confirmed for gene deletion by PCR across the region targeted by the guide pairs. The agarose gel image comparing the gene products obtained by PCR amplification across the target region in non-transfected (wildtype, WT) and double guide RNA transfected (Knockout, KO) HEK 293 cells.



Real-time RT-QPCR of Tissues and Cultured Cells
Total RNA from either HEK293, HEK293 LRRC8A knockout cell line or NG, DRG, aorta, brain stem, heart, small intestine, kidney, skeleton muscle and spleen of 12 week old male mice was extracted using Trizol reagent (Life Technologies). Total RNA of cultured NG neurons was obtained from the culture of 5 mice in each group. RNA samples were treated with DNase (Ambion DNase kit) and further purified using RN-easy Column (Qiagen's). RNA samples were reverse transcribed into cDNA by using AffinityScriptTM QPCR cDNA Synthesis Kit (Life Technologies) according to the manufacturer protocol. QPCR was performed using SYBR-green technology (ABI 7000). The RNA expression was quantified by $\Delta\Delta C_T$ method using the expression of 18S ribosomal as a reference gene. The Forward: 5'-GGC CGT CAC AGC CAA TAG GA-3', and Reverse: 5'-TCG ATC TGC GTC AGG TTG GT-3'(8). Primers were used for LRRC8A gene expression.
The knockdown efficiencies of lentiviral shRNAs or Cre on LRRC8A mRNA and proteins in mouse nodose neurons in culture were tested by QPCR and Western blotting.
Primers used for LRRC8A mRNA in the cultured mouse neurons: Forward: CATGCGGCAGACCATCATCA; Reverse: GTGCAGTCCACGTCGAACTT. This primer pair was designed to check knockdown efficiency, and spans the region of shRNA2 targeting sequence.

Western Blotting of Tissues and Cultured Cells
Tissue samples were collected from the aorta, brain stem, heart, small intestine, kidney, skeleton muscle, spleen or six NG from 3 mice in each group. Cell samples were from cultured neurons from 5 mice in each group, or 1×10^6 cells from cultured HEK293 and HEK293 LRRC8A knockout cell lines. Samples were washed in PBS, and homogenized in lysis buffer (50 mmol/L Tris-HCL, 0.1% sodium dodecyl sulphate (SDS), 100 μg/mL phenyl methyl sulphonyl fluoride, 150 mmol/L NaCl, pH 8.0), 1% (v/v)Triton X-100, protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail set Ⅱ (Calbiochem/EMB Biosciences)). The lysates were sonicated on ice with three bursts of 15 s each (Microson Ultrasonic Cell Disruptor). The protein concentration of the lysate was determined by using a Bradford's Protein Assay (Bio-Rad). Protein (60 μg) was heat- denatured at

95º for 5 minutes after adding an equal volume of SDS sample loading buffer, and run on a 10% polyacrylamide gel (Ready Gel® Tris-HCl gel)(Biorad). The proteins in gel were electrophoretically transferred to a 0.45 μm nitrocellulose membrane (Thermo). Membrane were blocked with 5% (w/v) nonfat milk in TBS-T (0.02 M Tris and 0.15 M NaCl buffer, containing 0.1% Tween 20, pH 7.45) for 1 h at room temperature. The membrane was then incubated with the primary antibody (1:2500 dilution) of the rabbit anti-LRRC8A Polyclonal antibodies against peptide sequence QRTKSRIEQGIVDRSE (from Dr. Sah's lab) in TBS-T, washed twice and incubated with the secondary antibody of (horseradish peroxidase-conjugated goat anti-rabbit IgG 1:5000, Santa Cruz Biotech, Cat# sc-2004). The antibody reactivity was detected by using an enhanced chemiluminescence (ECL) detection system (Pierce Chemical) and exposure to X-ray film (Bioexpress Genemate). After this step, the membrane was stripped with Restore Western Blot Stripping Buffer Sample (Thermo) and reprobed with anti-GAPDH antibody (Cell Signaling, D16H11 XP rabbit mAb) to normalize protein loading by densitometry. Antibody for mouse LRRC8A was obtained from Dr. Sah's lab (University of Iowa) and the method is detailed in the following section.

Immunohistochemistry

Nodose ganglia neurons or HEK293 and HEK293 LRRC8A knockout cell lines cultured on cover slips were washed with PBS twice and then fixed with 100% acetone at -20°C for 15 minutes. The cells were then permeabilized with 0.1% triton X-100 in PBS at room temperature for 15 minutes, change permeabilization medium every 5 minutes. Coverslips were then incubated in blocking buffer with 4% goat serum in PBS and 0.1% triton for 1 hour. The primary LRRC8A and NeuN antibodies in PBS with 4% goat serum and 0.1% triton were then added and cells were incubated in 4°C overnight. The dilution rates were: rabbit antibodies against LRRC8A (from Dr. Sah's lab) 1:2000 for nodose neurons and 1: 500 for HEK293 and HEK293 LRRC8A knockout cell lines, and mouse anti-NeuN antibody from Millipore 1:1000 (MAB377), chicken anti-GFP from Aves (GFP-1020) 1:1000. The coverslips were washed with PBS three times for 10 minutes each time. Coverslips were then incubated in secondary antibody in PBS with 4% goat serum at room temperature for 45 minutes. The dilution rate for secondary antibody is 1:500 for goat anti-rabbited Alexa Fluor® 568 (Life Technologies A21069), 1:500 for goat anti-chicken Alexa Fluor® 488 (Life Technology A11039) and 1:500 for goat anti-mouse Alexa Fluor® 647 (Life Technology A21235). The coverslips were washed with PBS 4 time with 10 minutes each and mounted with mounting medium (Vectashield HardSet antifade mounting medium with DAPI H-1500). Pictures were taken with Zeiss lsm 710 inverted confocal microscopy in the Central Microscopy Facility of the University of Iowa. For pictures for shRNA or flox-CRE knocking down, pictures were taken with the same setting for both shRNA and CRE and control virus transfected neurons. The fluorescence intensity was obtained after subtracting the background; the intensity was then normalized to the average intensity of the control group of the same day.

Chemicals/Solutions

In testing the effects of the various antagonists and antioxidant enzymes on the low pH_o-conditioned current, the experiments were performed in a control group of cells without the antagonists and another group of cells after administration of the antagonists. This was necessary because the response to low pH_o exposures was not reproducible in the same cells; the response to the second exposure to low pH_o tended to be reduced without any intervention. In contrast, the response to hypoosmolarity was very reproducible in the absence of interventions. Therefore, responses before and after the antagonists were tested in the same neuron.

The chemicals used in experiments, including niflumic acid, tamoxifen, apocynin, and amiloride (all from Sigma-Aldrich), were dissolved in DMSO to make a 1000 times stock solution that was diluted in the working solution with 1:1000 ratio before using. The DPI (Sigma-Aldrich) was dissolved in DMSO to make 5 mM stock solution and diluted to 30 μM in bath solution before using. The PEG-catalase and PEG-SOD (all from Sigma-Aldrich) were dissolved in bath solution directly to make a stock solution and stored at 4º C. The $ZnCl^2$ was dissolved in double distilled water to make 1 mM solution and diluted in bath solution as needed. For the electrophysiologic studies, all solutions used for patch-clamp were delivered with a solution changing system (RSC-200 from Science Instrument) connected to an open perfusion chamber with a volume capacity of 1 ml. Each solution was delivered at a constant perfusion rate of 1.5 ml/minute through the perfusion pipette with its tip pointed to the patch-clamped cell. At this continuous perfusion rate, the system allowed a fast exchange from one solution to another around the cell under study without any interruption of flow. Apocynin was administered for 2 hours, and all other chemical treatments were applied for 30 minutes before the interventions. For ROS and intracellular pH imaging, the cells were studied in a closed chamber with a volume capacity of 200 μl and perfused with HBSS solution at the rate of 1.5 ml/minute. This assured a rapid and efficient exchange of solutions in the chamber and around several cells simultaneously. The pH of the solutions was adjusted to the desired level with HCl and NaOH. $ZnCl^2$ and amiloride were added to the perfusion solution before the pH

adjustment.

Statistical Analysis

Data were presented as means ± SE (n = number of mice, coverslips or neuronal cultures). Significance of differences was analyzed by comparison of mean values with student t tests using Excel and Sigma Plot software, or by analysis of variance. Values of $p \leq 0.05$ were considered significant.

In testing the effects of various channel blockers on the responses to low pH, the control responses without the blocker were obtained from cultured cells in one coverslip and the responses with the blocker were from cells cultured in another coverslip but from the same nodose ganglia. The responses could not be tested before and after the blocker in the same cell because they tended to decline with the repeated exposure over time without any blockade. The statistical comparison of the pH responses were carried out using the unpaired t test. In contrast, the responses to hypoosmolarity were very reproducible in the same neuron with repeated exposures; hence the effect of the blocker could be reliably assessed before and after the blocker in the same neuron and appropriately analyzed by a paired t test.

Statistical analyses used in each experiment was indicated in figure legend.

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