Role of Acid-Sensing Ion Channel 1a in the Secondary Damage of Traumatic Spinal Cord Injury

Rong Hu, MD, PhD,* Bo Duan, PhD,† Dianshi Wang, PhD,‡ Ye Yu, PhD,† Weiguang Li, PhD,‡ Haishui Luo, PhD,* Peigang Lu, MD,* Jiangkai Lin, MD, PhD,* Gang Zhu, MD, PhD,* Qi Wan, MD, PhD,* and Hua Feng, MD, PhD*†

Objective: To determine the cellular and molecular mechanisms by which acid-sensing ion channel 1a (ASIC1a) plays its role in the secondary injury after traumatic spinal cord injury (SCI), and validate the neuroprotective effect of ASIC1a suppression in SCI model in vivo.

Background: Secondary damage after traumatic SCI contributes to the exacerbation of cellular insult and thereby contributes to spinal cord dysfunction. However, the underlying mechanisms remain largely unknown. Acidosis is commonly involved in the secondary injury process after the injury of central nervous system, but whether ASIC1a is involved in secondary injury after SCI is unclear.

Methods: Male Sprague–Dawley rats were subjected to spinal contusion using a weight-drop injury approach. Western blotting and immunofluorescence assays were used to observe the change of ASIC1a expression after SCI. The TUNEL staining in vivo as well as the cell viability and death assays in spinal neuronal culture were employed to assess the role of ASIC1a in the secondary spinal neuronal injury. The electrophysiological recording and Ca2+ imaging were performed to reveal the possible underlying mechanism. The antagonists and antisense oligonucleotide for ASIC1a, lesion volume assessment assay and behavior test were used to estimate the therapeutic effect of ASIC1a on SCI.

Results: We show that ASIC1a expression is markedly increased in the peri-injury zone after traumatic SCI. Consistent with the change of ASIC1a expression in injured spinal neurons, both ASIC1a-mediated whole-cell currents and ASIC1a-mediated Ca2+ entry are significantly enhanced after injury. We also show that increased activity of ASIC1a contributes to SCI-induced neuronal death. Importantly, our results indicate that down-regulation of ASIC1a by antagonists or antisense oligonucleotide reduces tissue damage and promotes the recovery of neurological function after SCI.

Conclusion: This study reveals a cellular and molecular mechanism by which ASIC1a is involved in the secondary damage process after traumatic SCI. Our results suggest that blockade of Ca2+-permeable ASIC1a may be a potential neuroprotection strategy for the treatment of SCI patients.


*Department of Neurosurgery and Key Laboratory of Neurotrauma, Southwest Hospital, Third Military Medical University, Chongqing, China. †Institute of Neuroscience and State Key Laboratory of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China. ‡Departments of Anesthesia and Physiology, University of Toronto, Toronto, Canada and §Department of Physiology and Cell Biology, School of Medicine, University of Nevada, Reno, NV.

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Reprints: Hua Feng, MD, PhD, Department of Neurosurgery and Key Laboratory of Neurotrauma, Southwest Hospital, Third Military Medical University, 30 Gao-tanyan Street, Chongqing 400038, China. E-mail: fenghua8888@yahoo.com.cn.

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Original Article

MATERIALS AND METHODS

Animal Model of SCI

Male Sprague–Dawley rats (~220 grams) were housed on a 12 hour light/dark cycle with standard rat chow and water ad libitum. Each animal was anesthetized with sodium pentobarbital (50 mg/kg i.p.), and a laminectomy was performed at T9 to expose a circle of dura. Spinal contusion injury was produced using the weight-drop injury model.30,31 A 10 grams weight rod with diameter of 2.5 mm was dropped from a height of 10 mm onto the exposed rat spinal
cord. For sham surgery, animals were age-, sex-, and weight-matched undergoing laminectomy without contusion. Body temperatures were maintained at 37°C on a heating pad throughout the procedure. Bladders were manually expressed twice daily until reflex bladder emptying returned. All experiments were carried out in accordance with China Animal Welfare Legislation and were approved by the Institutional Animal Care and Use Committee of Institute of Neuroscience, Shanghai, China.

**Gel Electrophoresis and Immunoblotting**

Spinal cords were quickly removed after animals were anesthetized. Samples of cord (12 mm) were taken from the thoracic level (including the epicenter of injury, 4 mm in length) as well as areas rostral (4 mm) and caudal (4 mm) to the injury site. Immunoblotting procedures were performed as previously described.26 In brief, samples were homogenized and then centrifuged at 13,000 rpm for 15 minutes at 4°C, and the supernatants were boiled for 5 minutes and separated using SDS-PAGE gel containing 7.5% polyacrylamide and transferred to polyvinylidene difluoride (PVDF) membranes (Healthcare, Buckinghamshire, UK). The membranes were blocked with 5% nonfat milk in TBST (20 mM Tris-HCl, pH 7.4, 140 mM NaCl, and 0.1% Tween-20) at room temperature for 1 hour, and then incubated at 4°C overnight with anti-ASIC1a (1:200; Alomone Labs, Jerusalem, Israel), or anti-β-tubulin (1:1000; KangChen, Shanghai, China) antibodies followed by the appropriate secondary antibodies conjugated with hors eradish peroxidase. Washes were performed with TBST. The immunoreactivity was detected using enhanced chemiluminescence (ECL; GE Healthcare). The ECL-exposed films were digitized, and densitometric quantification of immunoreactive bands was performed using ImageJ (Wayne Rasband, National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij).

**Immunohistochemistry**

Experimental rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused with 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). The spinal cord (12 mm, centered at the injury epicenter, including areas rostral and caudal to the epicenter) was removed and cryoprotected overnight in a 30% sucrose solution at 4°C. Longitudinal and transverse frozen sections (30 μm thick) of 12-mm-length spinal cord centered at the lesion epicenter was collected (n = 5 per group). Lesion zone was identified as gray areas of cellular breakdown, debris and tissue fragmentation. Volume quantification was assessed using unbiased stereology and the Stereologer Program (Systems Planning and Analysis, Alexandria, VA). Two different blinded individuals quantitated these lesions, and their scores were averaged.

**Primary Culture of Spinal Cord Neurons**

The cultures were prepared as reported previously with some modification.34–36 In brief, spinal cords of rats were dissociated in Ca2+-free saline with sucrose (20 mM). After removal of the meninges and dorsal root ganglia, spinal cords were minced into 1-mm3 fragments and placed into DMEM containing 0.25% trypsin (Difco, 1:250, Detroit, MI) and 0.01% DNase for 15 minutes at 37°C. After gentle centrifugation (190g, 5 minutes), the supernatant was discarded and the tissue pellet was suspended in serum-containing basal media and triturated through a flame-polished, fine-bore Pasteur pipette. Cells were centrifuged (190g, 3 minutes) and resuspended in fresh growth media and plated (1–2 × 105 cell/mL) on poly-d-lysine (Sigma)-coated cover glasses. The neurons were grown in Dulbecco’s modified Eagle’s medium with L-glutamine plus 10% fetal bovine serum and 10% F-12 nutrient mixture (Invitrogen). After 1 day, Neurobasal medium (1.5 mL, Invitrogen) with 2% B27 (Invitrogen) was replaced every 3 to 4 days. Treatment with 5-fluoro-2-deoxyuridine (20 μg/mL, Sigma) on the 4th day after plating was used to block cell division of nonneuronal cells, which helped to stabilize the cell population. The cultures were maintained at 37°C in a 5% CO2-humidified atmosphere. Neurons were used for patch-clamp recording and Ca2+ imaging 9 to 14 days after plating.

**Electrophysiology**

The electrophysiological recordings were performed in the conventional whole-cell patch-clamp recording configuration under voltage clamp conditions. Patch pipettes were pulled from glass capillaries with an outer diameter of 1.5 mm (Narishige, Tokyo, Japan) on a 2-stage puller (PP-830, Narishige Co. Ltd., Tokyo, Japan). The resistance between the recording electrode filled with pipette solution and the reference electrode was 4 to 6 MΩ. Patch pipettes were filled with the following solution (in mM): 120 KCl, 30 NaCl, 0.5 CaCl2, 1 MgCl2, 5 EGTA, 2 MgATP, and 10 HEPES, pH 7.2. The standard extracellular solution contained (in mM) 150 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, and 10 glucose, buffered to various pH values with 10 mM HEPES, pH 6.0 to 7.4. Membrane currents were measured using a patch clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, CA), filtered at 1 kHz, sampled, and analyzed using a DigiData 1320A interface and a computer with the pCLAMP system (Version 8.0, Axon Instruments, Foster City, CA). The series resistance, estimated from optical cancellation of the capacity transient, was 10 to 30 MΩ, and in most experiments, 70% to 90% series resistance was compensated. The membrane potential was voltage clamped at –60 mV throughout the experiments under voltage clamp conditions. All experiments were carried out at room temperature (22–25°C). All drugs for electrophysiological experiments were purchased from Sigma except for pscalmotoxin 1 (PcTx1 venom), which was obtained as the venom of the South American tarantula Psalmopoeus cambridgei.37

**Ca2+ Imaging**

According to the previously described methods,26,27 intracellular Ca2+ imaging was carried out as follows. Primary cultured spinal neurons grown on 8 × 8 mm glass coverslips were washed 3 times with PBS and then incubated with 1 μM Fura-2-acetoxymethyl ester for 20 minutes at 37°C, they were again washed 3 times and incubated in standard extracellular solution for 30 minutes. Then, coverslips were transferred to a perfusion chamber on an inverted microscope (Nikon TE2000-E, Tokyo, Japan). Experiments were performed by using a 40 × UV fluor immersion objective lens, and images were recorded by a cooled CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan). To block potential Ca2+ entry through voltage-gated Ca2+ channels or glutamate receptors, 10 μM

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nifedipine, 20 μM APV and 10 μM CNQX were added to the bath solution. The fluorescence excitation source was a 75-Watt xenon arc lamp. The fluorescence-ratio images were acquired by using alternating excitation wavelengths (340/380 nm) with a monochromator (Polychrome IV; Till, Munich, Germany). Fura-2 fluorescence was detected at emission wavelength of 510 nm. Digitized images were acquired and analyzed in a personal computer controlled by SimplePCI (Compix, Sewickley, PA). Ratio images (340/380 nm) were analyzed by averaging pixel ratio values in circumscribed regions of cells in the field of view. The data were exported and further analyzed by Origin 7.0.

**TUNEL Staining**

The TUNEL (dT-T-mediated dUTP-X nick end labeling) assay was conducted by using a TUNEL detection kit according to the manufacturer's instructions (In Situ Cell Death Detection Kit, TMR red, Roche, Switzerland).

**Cell Viability and Death Assays with FDA and PI Staining**

The assays were performed according to the methods previously described with some modification. In brief, cultures were rinsed with PBS and incubated with fluorescein diacetate (FDA, 5 μM) and propidium iodide (PI, 2 μM) for 30 minutes, and then rinsed with PBS. Alive (FDA-positive) and dead (PI-positive) cells were viewed and counted on a microscope (Zeiss) equipped with epifluorescence at 580/630 nm excitation/emission for PI and 500/550 nm for FDA. For each slide, 15 random fields were enumerated.

**Intrathecal Administration of Antisense**

For intrathecal delivery of drugs, We performed as previous description. The 18-mer antisense oligonucleotide (AS) was designed in the 5' part of the coding sequence as previously reported (5'-GTG CCC CCT TGG TCA GAG ACA-3'), and the inverted sequence (IS) was used as a control (5'-ACA GAG ACT TCG CCC GTG-3'). In brief, cultures were rinsed with PBS and incubated with fluorescein diacetate (FDA, 5 μM) and propidium iodide (PI, 2 μM) for 30 minutes, and then rinsed with PBS. Alive (FDA-positive) and dead (PI-positive) cells were viewed and counted on a microscope (Zeiss) equipped with epifluorescence at 580/630 nm excitation/emission for PI and 500/550 nm for FDA. For each slide, 15 random fields were enumerated.

**Behavioral Test**

Animals were assigned to treatment groups blindly and functional deficits were measured in a blinded manner. Locomotor function was observed every 3 days after SCI and recorded using the Basso, Beattie, and Bresnahan (BBB) Locomotor Rating Scale.

**Data Analysis**

Results were expressed as mean ± SE. Statistical comparisons were performed using paired or unpaired Student’s t tests or 1-way ANOVA. Statistical significance was placed at P < 0.05.

**RESULTS**

**ASIC1a Expression is Increased in Injured Spinal Cord**

To determine the role of ASIC1a in SCI, we first examined ASIC1a expression in injured cord using the weight-drop SCI model. We performed immunoblotting and immunohistochemical assays to measure the protein expression of ASIC1a in 2 injury regions: injury site (4 mm in length, including injury epicenter) and peri-injury site (4 mm in length, rostral and caudal to injury site, respectively). At peri-injury site (indicated as “P” in Fig. 1A), Western blot analysis showed that ASIC1a expression was markedly increased 3 hours after injury and reached its peak during 12–24 hours, and started to fall 24 hours after injury. ASIC1a expression was recovered to the control level 1 week after injury, and remained for up to 6 weeks (Fig. 1A and B). Unlike the peri-injury site, ASIC1a expression at the injury site (indicated as “I” in Fig. 1A) was significantly decreased at 3 hours after SCI, and the expression level continued to fall without recovery (Fig. 1A and C).

To locate the change of ASIC1a expression after SCI, we performed immunocytochemical labeling of ASIC1a in longitudinal and transverse sections of spinal cords. Our results showed that ASIC1a expression was significantly increased in the peri-injury site after insult (Fig. 2A1 and B1). Statistic analysis showed that ASIC1a expression was increased at 12 hour after SCI (Fig. 2A2 and B2). Although ASIC1a was detectable at the injury site, ASIC1a-positive cells were dramatically decreased due to loss of cells caused by primary injury, which may explain why western blotting data showed decreased ASIC1a level at the injury site after SCI. The specificity of ASIC1a antibody used in this study was tested (see Supplemental Digital Content 1, available at: http://links.lww.com/SLA/A142).
FIGURE 2. Immunocytochemical labeling of ASIC1a expression in injured spinal cord. Immunofluorescent images showing the distribution of ASIC1a (green) in spinal cord. Neurons were double-labeled with neuronal marker NeuN (Red). A, The expression of ASIC1a around the injury site was significantly enhanced after impact. ASIC1a expression was increased at 12 hours, fell back thereafter and recovered to the control level at 7 days after injury. B, Bar graph summarizing the average intensity of ASIC1a expression at different time points. Immunostaining was performed from 9 sections of 3 sham or injured rats per time point, respectively. Data (mean ± SE) were normalized to the level from Sham-operated rats. **P < 0.01, compared with “Sham” (1-way ANOVA). C, Transverse sections showing the distribution of ASIC1a in cord. At 12 hours after SCI, ASIC1a intensity is significantly increased. Scale bars: A, 100 μm; C without Inset panels, 100 μm; C Inset panels, 50 μm.

Taken together, our results indicate an increased expression of ASIC1a at the peri-injury site in the injured spinal cord.

ASIC1a Activity is Enhanced in Spinal Neurons by Hypoxia

To first test whether the increased expression of ASIC1a led to a functional consequence. We recorded ASIC1a-mediated whole-cell currents in cultured spinal neurons at 9 to 14 days in vitro (DIV). At a holding potential of –60 mV, acidic stimulation at pH 6.0 evoked large transient inward currents (I_{6.0}, Fig. 3A1). Consistent with the evidence that ASIC1a is the dominant ASIC distributed in rat spinal dorsal horn,27,28 specific ASIC1a antagonist psalmotoxin 1 (PcTx1 venom, 5 μg/mL) or nonspecific ASIC antagonist amiloride (100 μM) were found to inhibit I_{6.0} in cultured spinal neurons (Fig. 3A1–A3). These data also indicate that the acid-induced currents in cultured spinal neurons are mediated by ASIC1a.

We next examined whether ASIC1a-mediated currents was increased through secondary injury mechanisms. Because ischemia/hypoxia is critically involved in the secondary injury process after SCI,3,10,11 we set up to record ASIC1a-mediated currents in cultured spinal neurons treated with sodium cyanide (NaCN, 10^{-4} M), an agent widely used in the study of hypoxic insult.26,41,42 We showed that the peak amplitude of I_{6.0} was not altered during repeated applications of the same acidic solution. However, after 5 minutes of NaCN treatment, the amplitude of I_{6.0} was markedly increased (Fig. 3B1 and B3). The current amplitude was returned to control level after washout of NaCN (data not shown). We further showed that KN93 (5 μM), an inhibitor of calcium/calmodulin-dependent kinase II (CaMKII), prevented NaCN-induced enhancement of ASIC1a currents, suggesting that regulation of ASIC1a by CaMKII may mediate NaCN-induced enhancement of ASIC1a currents (Fig. 3B2 and B3). This is consistent with previous finding that NaCN enhanced ASIC1a currents through CaMKII phosphorylation of the channels in hippocampal neurons.26

The homomeric ASIC1a channels are permeable to Ca^{2+}.19,20,26 To provide further evidence to support the enhancement of ASIC1a function by NaCN-induced hypoxic insult, we tested whether intracellular Ca^{2+} concentration ([Ca^{2+}]_{i}) is altered in spinal neurons in response to acidic solution (pH 6.0) using Fura-2
fluorescence ratio imaging of Ca$^{2+}$. The voltage-gated Na$^+$ and Ca$^{2+}$ channels, as well as glutamate receptors, were blocked by a cocktail of pharmacological blockers of TTX (1 μM), Nifedipine (10 μM), DL-2-amino-5-phosphovaleric acid (APV, 20 μM) and 6-cyano-7-nitroquinazoline-2,3-dione (CNQX, 10 μM). Elevation of [Ca$^{2+}$]; was detected after acidic stimulation, which was reversibly blocked by ASIC antagonist amiloride (100 μM; Fig. 4A and D). In accordance with the enhancement of ASIC1a-mediated current, treatment of NaCN also markedly enhanced [Ca$^{2+}$]; (Fig. 4B and D), and the effect was abolished by CaMKII antagonist KN93 (5 μM; Fig. 4C and D). Thus, these results suggest that hypoxic insult enhances ASIC1a function and leads to increasing Ca$^{2+}$ influx.

**Increased Activity of ASIC1a Promotes Spinal Cell Death**

To investigate whether ASIC1a is involved in mediating SCI-induced cell death, we performed TUNEL staining to analyze the apoptotic cell death in the injured spinal cord. As shown in Fig. 5A1, in the peri-injury site 39.5% of TUNEL-positive cells are ASIC1a-positive and 48.8% ASIC1a-positive cells are TUNEL-positive. As a control, ASIC2a-labelled cells were not TUNEL-positive (data not shown). Compared with sham control, SCI causes increased apoptotic cell death in the peri-injury site (Fig. 5A2). These results suggest a significant correlation of increased ASIC1a activity with apoptotic cell death in spinal cord injury.

To determine whether increased ASIC1a activity contributed to SCI-induced cell death, we treated the SCI animal with intrathecal injection of ASIC1a antagonists and antisense (AS, targeting to the 5’ part of the coding sequence of ASIC 1a). As shown in Figure 5(B1) and (B2), PcTx1 venom (10 μL, 0.5 mg/ml total protein, ∼25 μg/kg) and amiloride (10 μL, 53.2 μg/mL, ∼0.5 μg/kg) reduced TUNEL-positive cells in the areas close to the injury site, and the downregulation of ASIC1a expression by antisense injection decreased the number of TUNEL-positive cells after injury (Fig. 5B1 and B2).

**FIGURE 4.** ASIC1a-mediated Ca$^{2+}$ influx in spinal neurons. Cultured spinal neurons were bathed in standard extracellular solution containing 1 μM TTX, 10 μM nifedipine, 20 μM APV and 10 μM CNQX. A–C, Representative images (top panel) and fluorescence ratio (340/380, lower panel) showing that: A, ASIC1a antagonist amiloride (100 μM) blocks pH6.0-induced increase of [Ca$^{2+}$];, B, NaCN (10$^{-4}$ M) enhances acid-induced [Ca$^{2+}$]; elevation, and C, KN93 (5 μM), the CaMKII antagonist, blocks NaCN-induced enhancement of ASIC1a-mediated [Ca$^{2+}$]; elevation. D, Summary of results from (A) to (C). Data are mean ± SE (n = 15). **P < 0.01, compared with the ratio induced by pH6.0 before NaCN treatment or in the presence of KN93, respectively; ***P < 0.001, compared with the ratio induced by pH6.0 (1-way ANOVA). SS, standard extracellular solution.
FIGURE 5. Apoptotic cell death in ASIC1a-positive cells after SCI. A1, Samples images showing that TUNEL-positive cells were increased in the region close to the injury site at 24 hours after SCI, and most of the ASIC1a-positive cells were TUNEL-positive (arrows). Inset showing the marked region at high magnification. A2, Summarized data showing that the number of TUNEL-positive cells was increased 24 hours after SCI. Data (mean ± SE) were from 9 sections of 3 rats per group. ***P < 0.001 (Student’s t test), compared with the sham-operated control. B1, Sample images showing sections of injured cords that were double stained by TUNEL (red) and Hoechst 33342 (blue). A large number of TUNEL-positive cells appeared in saline treatment sections at 24 hours after SCI. In contrast, ASIC1a antisense (AS) and PcTx1 venom significantly decreased number of TUNEL-positive cells. Images for IS and amiloride treatment groups are not shown. B2, Histogram summarizing the number of TUNEL-positive cells in rats treated with saline control, AS, IS, PcTx1 venom, and Amiloride at 24 hours after injury. Quantification was performed from 9 sections of 3 rats per group, and was analyzed in 5 regions for each section. Values (mean ± SE) were normalized to the saline control, *P < 0.05, compared with saline control (by ANOVA with Fisher’s PLSD). Scale bars: without Inset panels, 100 μm; Inset panels, 50 μm; B1, 100 μm.

Furthermore, we observed spinal cord damage in transverse sections after delivery of ASIC1a antagonists or antisense by labeling of neuronal marker NeuN. As shown in Figure 6 and Supplemental Digital Content 2 (available at: http://links.lww.com/SLA/A143), treatment with either ASIC1a antagonists PcTx1 venom, amiloride or ASIC1a antisense reduced the size of tissue damage and prevented the decrease of NeuN-positive cells after SCI, compared with saline control. These results provide direct evidence that suppression of ASIC1a confers neuroprotection, suggesting that increased activity of ASIC1a contributes to SCI-induced cell death and tissue damage.

To provide further evidence for the involvement of ASIC1a in spinal neuronal death, we tested whether acidosis was sufficient to induce spinal neuronal death via ASIC1a. Alive/dead staining with fluorescein diacetate (FDA, green) and propidium iodide (PI, red) showed marked increase of cell death by 1 hour acid (pH 6.0) incubation in cultured spinal neurons (Fig. 7A). Treatment with ASIC1a antagonist PcTx1 venom significantly attenuated acid-induced neuronal death (Fig. 7A and B). Moreover, cotreatment of acid with NaCN largely exacerbated acid-induced neuronal death and PcTx1 venom exerted similar inhibitory role (Fig. 7A and B). Collectively, these data suggest that increased activity of ASIC1a by secondary injury-induced acidosis may play a crucial role in mediating neuronal death after SCI.

Suppressing ASIC1a Promotes Functional Recovery After SCI

To examine whether inhibition or down-regulation of ASIC1a could promote the recovery of neurological function in the animal model of weight-drop SCI, we first investigated the effect of PcTx1 venom and amiloride on hindlimb locomotor deficits in injured animals by the test of Basso-Beattie-Bresnahan (BBB) rating scale.39 The locomotor function was lost immediately after injury, and no obvious recovery was observed up to 3 days after SCI (Fig. 8A). Compared with control rats injected with saline, intrathecal injection of PcTx1 venom (10 μL, 0.5 mg/ml total protein, ~25 μg/kg; twice daily) or amiloride (10 μL, 53.2 μg/mL, ~0.5 μg/kg; twice daily)
increased the locomotor score 3 days after injury (Fig. 8A). Furthermore, intrathecal injection of specific antisense oligonucleotide (AS) targeting to ASIC1a decreased ASIC1a expression (Fig. 8C and D) and enhanced the locomotor recovery of SCI rats (Fig. 8B). These results suggest that suppressing ASIC1a may be a potentially therapeutic strategy for SCI treatment.

DISCUSSION

In this study, we demonstrated that the expression and function of ASIC1a were markedly increased in the injured spinal neurons after SCI. Importantly, suppressing ASIC1a in vivo was shown to markedly attenuate spinal cell damage and promoted functional recovery of injured animals. Thus, this study reveals a mechanism underlying spinal cord secondary injury by which ASIC1a mediates acidotoxic cell death after SCI.

In the CNS, a stable extracellular and intracellular pH is important to maintain normal cellular function. In physiological conditions, extracellular pH is maintained at ~7.3 whereas intracellular pH is at ~7.0 through various H⁺ transporting mechanisms. The pH range of spinal cord interstitial fluid under physiological conditions is more acidic than cerebrospinal fluid (7.35–7.40), with a pH of 7.10–7.35, and pH in the dorsal spinal cord (7.10–7.20) is slightly more acidic than ventral spinal cord (7.25–7.35). However, the role of pH in spinal cord under pathological conditions, including SCI, remains unclear.

Increasing evidence suggests that falling pH may play a significant role in spinal cord after traumatic injury. Acidosis is shown to be induced by progressive ischemia or anoxia and the pH value is decreased by approximately 0.6 to 0.8 pH unit in the ischemic spinal cord. Acidosis is also induced in injured spinal cord through other secondary injury mechanisms: (1) accumulation of lactic acid, due to enhanced anaerobic glucose metabolism, triggers tissue acidosis; (2) excess release of glutamate and ATP induces H⁺ accumulation at the injury site and peri-injury regions after SCI; and (3) inflammation, a crucial episode after SCI, lowers pH value in the injured spinal cord. Taken together, these findings support the notion that acidosis is involved in secondary injury-mediated spinal cell death after SCI.

Our study showed that ASIC1a expression was dramatically increased in the peri-injury regions after SCI. Interestingly, this is opposite to ischemic stroke-induced brain injury, in which ASIC1a...
expression was not significantly altered. Thus, in different neurons ASIC1a may respond to different insults in a distinct manner to participate in the injured process. Of note, our data indicate that the ASIC1a expression is increased in the ventral horn (Fig. 2B1), a key component controlling voluntary movement of body and limbs. This is consistent with previous findings that the ventral horn motor neurons are selectively degenerated in a variety of spinal cord diseases including amyotrophic lateral sclerosis (ALS) and SCI. Excitotoxicity induced by excessive glutamate has been proposed to contribute to the death of ventral horn neurons, but clinical trials with the use of glutamate receptor antagonists failed to demonstrate efficacy. Because the neuroprotective effect of ASIC1a blockade is not mediated through inhibition of glutamate-induced excitotoxicity, suppressing ASIC1a may offer a new therapeutic strategy for SCI.

The critical evidence obtained in this study is that suppressing ASIC1a not only protects against spinal cell death but also promotes functional recovery of injured animals. Importantly, this study provides direct evidence that amiloride, widely used as diuretics in clinic, promotes the recovery of injured spinal cord in vivo. Thus, amiloride may be potentially used as a therapeutic agent for the treatment of SCI patients.

In conclusion, the study demonstrates for the first time that ASIC1a increased activity contributes to delayed cell death in injured spinal cord induced by the secondary damage of SCI. Suppressing ASIC1a attenuated the death of spinal cells and promotes the recovery of neurological function in SCI animals. Our results provide direct evidence suggesting that ASIC1a may be a potential therapeutic target for the development of novel strategy for the treatment of SCI patient.

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FIGURE 8. Suppressing ASIC1α promotes functional recovery in SCI animals. A, Functional recovery evaluated by the BBB scale after SCI. ASIC1α antagonists PcTx1 venom and amiloride improved locomotor function of injured rats at 3, 11, 17, 28, and 42 days after SCI. Data represent mean ± SE, n = 6 for Sham-operated, n = 12 for saline control, n = 13 for PcTx1 venom-treated rats, and n = 12 for amiloride-treated rats. *P < 0.05, PcTx1 venom group compared with saline control; #P < 0.05, amiloride group compared with saline control. ANOVA with Fisher’s PLSD. B, ASIC1α specific antisense oligos significantly improved locomotor function of injured rats. n = 6 for Sham-operated, n = 12 for saline control, n = 15 for antisense group, and n = 13 for sense group. *P < 0.05, compared with saline control or sense group. ANOVA with Fisher’s PLSD. C, Representative Western blots showing specific knockdown of ASIC1α by antisense. In contrast, treatment with IS (inverted sequence of ASIC1α antisense oligos) did not affect ASIC1α expression. β-Tubulin was used as the loading control. D, Summary data showing the knockdown of ASIC1α by antisense. After 4 days of AS or IS injection, the expression of ASIC1α was compared with saline control. The relative levels of ASIC1α protein expression were quantified by densitometry. Data (mean ± SE) were from 3 independent rats and normalized to the mean value for those from 3 rats in corresponding saline control. **P < 0.01, compared with saline control (Student’s unpaired t-test).

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