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# Upregulation of Acid-Sensing Ion Channel ASIC1a in Spinal Dorsal Horn Neurons Contributes to Inflammatory Pain Hypersensitivity

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Development of chronic pain involves alterations in peripheral nociceptors as well as elevated neuronal activity in multiple regions of the CNS. Previous pharmacological and behavioral studies suggest that peripheral acid-sensing ion channels (ASICs) contribute to pain sensation, and the expression of ASIC subunits is elevated in the rat spinal dorsal horn (SDH) in an inflammatory pain model. However, the cellular distribution and the functional consequence of increased ASIC subunit expression in the SDH remain unclear. Here, we identify the $Ca^{2+}$-permeable, homomeric ASIC1a channels as the predominant ASICs in rat SDH neurons and downregulation of ASIC1a by local rat spinal infusion with specific inhibitors or antisense oligonucleotides markedly attenuated complete Freund's adjuvant (CFA)-induced thermal and mechanical hypersensitivity. Moreover, *in vivo* electrophysiological recording showed that the elevated ASIC1a activity is required for two forms of central sensitization: C-fiber-induced "wind-up" and CFA-induced hypersensitivity of SDH nociceptive neurons. Together, our results reveal that increased ASIC activity in SDH neurons promotes pain by central sensitization. Specific blockade of $Ca^{2+}$-permeable ASIC1a channels thus may have antinociceptive effect by reducing or preventing the development of central sensitization induced by inflammation.

*Key words:* acid-sensing ion channel; spinal dorsal horn; calcium; sensitization; plasticity; inflammation; chronic pain

## Introduction

Inflammation and trauma lead to enhanced pain sensitivity (hyperalgesia), which is in part caused by altered plasticity in the spinal cord (Dubner and Ruda, 1992; Basbaum and Woolf, 1999; Salter, 2005; Hu et al., 2006; Ikeda et al., 2006; Wei et al., 2006). The molecular mechanism underlying this nociceptive plasticity is not fully understood. An important component in inflammation-induced hypersensitivity to thermal and mechanical stimuli (inflammatory pain hypersensitivity) is the amplification of pain-related information resulting from modulation and expression of specific ion channels in the spinal dorsal horn (SDH) (Woolf and Costigan, 1999; Ikeda et al., 2006).

The acid-sensing ion channels (ASICs) belong to the de-

generin/epithelial $Na^+$ channel superfamily. To date, at least six subunits of ASICs have been identified: 1a, 1b, 2a, 2b, 3, and 4 (Krishtal, 2003). Their location and properties make ASICs attractive candidates for the sensor of tissue acidosis associated with inflammation, ischemia, and cancer metastases (Wemmie et al., 2006). Indeed, elevation of ASIC subunit expression in the dorsal root ganglion (DRG) was observed during inflammation (Voilley et al., 2001) and could be induced by inflammatory mediators (Voilley et al., 2001; Mamet et al., 2002). Moreover, ASIC inhibitors attenuated peripheral acid-induced hyperalgesia in rats (Dube et al., 2005) and acid-elicited pain in human subjects (Ugawa et al., 2002; Jones et al., 2004), further implicating ASICs in pain sensation. However, previous genetic evidence suggests that deletion of *ASIC1* gene increases rather than decreases the sensitivity of the mice to painful stimuli (Mogil et al., 2005; Wemmie et al., 2006). This apparent discrepant result may be attributed to the compensatory expression of different ASIC subunits or other ion channels (Caterina et al., 2000; Bandell et al., 2004). Alternatively, in these complete ASIC1 knock-out mice, nociception may be affected at multiple levels in the CNS.

Peripheral inflammation induces persistent enhancement of nociceptive transmission in the spinal dorsal horn, a process related to central sensitization (Woolf and Costigan, 1999; Woolf and Salter, 2000). Central sensitization is a form of spinal plasticity that shares similar mechanisms with long-term potentiation

(LTP) of glutamatergic transmission in other brain regions and is generally considered to be a cellular basis of chronic pain (Ji et al., 2003; Salter, 2004). Deletion of *ASIC1* gene impairs hippocampal synaptic plasticity and spatial learning in mice (Wemmie et al., 2002), suggesting that ASICs may contribute to central sensitization and nociception. Because peripheral inflammation increases the expression of ASIC1a subunits in the rat spinal cord (Wu et al., 2004), ASIC1a may contribute to spinal processing of inflammation-related pain behaviors. To examine this possibility, we investigated the spinal distribution of the ASIC1a subunit and its *in vivo* functional presence and inflammation-induced changes in its expression in SDH neurons. Behavioral studies and *in vivo* electrophysiological measurements of nociceptive responses of SDH neurons were also performed to demonstrate that the enhanced ASIC1a channel activity underlies central inflammatory pain sensitization.

## Materials and Methods

*Animals.* The experimental protocols were approved by the Institutional Animal Care and Use Committee of Institute of Neuroscience, Shanghai, China. All behavioral measurements were done in awake, unrestrained Sprague Dawley rats (~200 g) of male. Complete Freund's adjuvant (CFA, 50% in saline, 150 $\mu$l) was injected into the plantar surface of the right hindpaw. For intrathecal injection, a polyethylene 10 catheter was implanted at L3–L5 spinal level after rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). In formalin test, a 2.5% formalin solution (50 $\mu$l) was injected into the subcutaneous space at the dorsal side of the right hindpaw.

*Behavioral test.* Animals were assigned in treatment groups in a blinded manner and pain response was measured in a blinded manner. Rats were habituated and basal pain sensitivity was tested before drug administration or surgery. Drugs, antisense oligonucleotide, and vehicle (saline) were administered intrathecally. Psalmotoxin 1 (PcTX1) was obtained as the venom of the South American tarantula *Psalmopoeus cambridgei* (Escoubas et al., 2000). Thermal paw withdrawal latency was measured using the Hargreaves radiant heat apparatus and averaged over five trials (Wu et al., 2004). Mechanical withdrawal threshold on the plantar surface of the hindpaw was measured with a set of von Frey hairs (North Coast Medical, Morgan Hill, CA) in the range of 0.4–26 gm (0.4, 1.2, 2, 4, 6, 8, 10, 15, 26 gm). A modification (the value of cutoff is 26 gm) of the "up–down" method was used to determine the value at which paw withdrawal occurred 50% of the time (Chaplan et al., 1994), interpreted to be the mechanical nociceptive threshold. The formalin-induced typical flinching behavior of the injected paw was counted at 2 min intervals for 60 min, starting immediately after formalin injection. At 0 min and 28 min, saline or PcTX1 was injected into the SDH intrathecally.

*Acute isolation of SDH neuron.* Dorsal horn neurons were mechanically dissociated from the rat according to the previous study by Wu et al. (2004). In brief, 2-week-old Sprague Dawley rats were killed by decapitation and a segment of lumbosacral (L4–S2) spinal cord was dissected out. Then, 400 $\mu$M transverse slices were sectioned with a vibratome tissue slicer (VT1000S; Leica, Wetzlar, Germany) in the incubation solution. A vibration-isolation system was used to mechanically dissociate SDH neurons from the spinal slices.

*Spinal neuron culture.* The SDH neurons from 15-d-old embryonic Sprague Dawley rats were isolated by a standard enzyme treatment protocol (Jiang et al., 2006). Briefly, SDH neurons were dissociated in Ca²⁺-free saline with sucrose (20 mM) and plated (1 × 10⁵ cell/ml) on poly-D-lysine (Sigma, St. Louis, MO)-coated cover glasses. The neurons were grown in DMEM (Invitrogen, Eugene, OR) with L-glutamine plus 10% fetal bovine serum (Invitrogen) and 10% F12 nutrient mixtures (Invitrogen). Neurobasal medium (2 ml; Invitrogen) with 2% B27 serum-free supplements (Invitrogen) was replaced 1 d after cell plating. The cultures were maintained at 37°C in a 5% CO₂ humidified atmosphere.

*SiRNA transfection.* After synthesizing four chemically modified oligonucleotides (5′ CUG AGC GUG UGC AGU ACU Adtdt 3′; 5′ GAU GAA UAU UUG CCU GUG Udtdt 3′; 5′ CUC AAU GAG UUC CGC UUU

Adtdt 3′; 5′ UUC GAC UCC UAC AGC AUC Adtdt 3′), we assessed the efficiency and specificity of siRNA oligos of ASIC1a with Western blotting and selected two sequences (5′ CUG AGC GUG UGC AGU ACU Adtdt 3′, named siRNA1; 5′ CUC AAU GAG UUC CGC UUU Adtdt 3′, named siRNA2) for additional experiments. A scrambled RNA (5′ UUC UCC GAA CGU GUC ACG Udtdt 3′) was used as control RNA. For siRNA electroporation (Gao et al., 2005), we used the rat Neuron Nucleofector kit (Amaxa, Koeln, Germany) according to the manufacturer's instructions. Transfected neurons were used for experiments 4 d after siRNA transfection.

*Antisense delivery.* The 18-mer antisense oligonucleotide (AS) was designed in the 5′ part of the coding sequence (5′ GTG CCC GCT TCA GAG ACA 3′), and the inverted sequence (IS) was used as a control (5′ ACA GAG ACT TCG CCC GTG 3′) (Ettaiche et al., 2006). Transfection of cultured SDH neurons with AS or IS was done with oligofectamine reagent (Invitrogen) using the protocol of the supplier, scaling up the given proportions for 35 mm dishes to keep 2.7 ng/$\mu$l oligonucleotide final concentration. Intrathecal treatment with both AS and IS was used at 45 $\mu$g in 10 $\mu$l of saline per rat, twice daily for 4 d. Intrathecal treatment with 10 $\mu$l of saline was used as vehicle control. In CFA groups, CFA was injected into the right hindpaw of rats on the third day of the 4 d antisense administration period. Rats of post-treatment groups were used after 4 d of cessation of antisense injection.

*Transfection of CHO cell.* All constructs were expressed in Chinese hamster ovary (CHO) cells. Transient transfection of CHO cells was performed as reported previously (Wu et al., 2004; Wang et al., 2006). Cotransfection with a green fluorescent protein (GFP) expression vector, pEGFP-N1, was used to enable identification of transfected cells for patch clamping by monitoring GFP fluorescence in some experiments. When two ASIC subunits were expressed, the plasmids were cotransfected in a ratio of 1:1. Electrophysiological recordings were performed 16–48 h after transfection.

*Whole-cell recordings.* Experiments were conducted with the Molecular Devices (Foster City, CA) system (Axoclamp 200B, Digidata 1320A, pClamp 9). Voltage-clamp experiments were performed as described previously (Wu et al., 2004). Patch pipettes (4–6 M$\Omega$) were filled with the following (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 either 10 mM HEPES, pH 6.0–7.4, or 10 mM 2-(N-morpholino)-ethanesulfonic acid (MES), pH <6.0. All drugs for electrophysiological experiments were purchased from Sigma except for PcTX1.

*Ca²⁺ imaging.* Intracellular Ca²⁺ imaging was performed as previously described (Gao et al., 2005; Wang et al., 2006). Primary cultured SDH neurons at 4 d *in vitro* (DIV) were incubated with 1 $\mu$M Fura-2-acetoxymethyl ester for 20 min at 37°C, washed three times, and incubated in standard extracellular solution for 30 min. Experiments were performed by using a 40× UV fluor oil-immersion objective lens, and 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 further analyzed by Origin 7.0.

*Immunohistochemistry and Western blotting.* Adult male Sprague Dawley rats (~200 g) 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 was removed and cryoprotected overnight in a 30% sucrose solution at 4°C. Transverse frozen sections (30 $\mu$m thick) were cut from the lumbar cord (L4–L5). Coimmunostaining was performed on parallel sections with antibodies against ASIC1a (1:200; Millipore, Temecula, CA), NeuN (1:200; Millipore), calcitonin gene-related peptide (CGRP; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), GFAP (1:50; Millipore), oligodendrocyte antibody [receptor-interacting protein (RIP); 1:5000; Millipore], or OX-42 (CD11b, microglial marker; 200; BD Biosciences, San Jose, CA). Secondary antibodies included goat anti-rabbit FITC and anti-mouse Rhodamine (1:70; Rockland, Gilbertsville, PA). Fluorescein-labeled *Griffonia simplicifolia* lectin

isolectin B4 (IB4; 1:100; Vector Laboratories, Burlingame, CA) was directly used for IB4 staining. In the Western blotting experiments, protein samples from cultured SDH neurons (see Fig. 1B, DIV 4; supplemental Fig. 2, DIV 13, available at www.jneurosci.org as supplemental material) or SDH homogenates (see Figs. 3, 4) were separated by SDS-PAGE gel and transferred to polyvinylidene difluoride filters. The filters were incubated overnight at 4°C with anti-ASIC1a, anti-ASIC2a (1:200; Alomone Labs Jerusalem, Israel), or anti-GAPDH (1:1000; KangChen, Shanghai, China) antibodies and finally visualized in ECL solution and exposed onto X-films for 1–10 min. Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD) was used for the analysis of optic density of ASIC1a in SDH neurons at the neuronal level. In brief, we first costained spinal cord section with antibodies against ASIC1a and NeuN. Then, we extracted the shape information of neurons from NeuN-positive staining and merged the ASIC1a staining with the extracted shape information of the neuron and quantified the fluorescence intensity of ASIC1a staining in each neuron in the SDH.

In vivo *single-unit recordings.* Recordings were performed as reported previously (Chen et al., 1998). In brief, the adult male Sprague Dawley rat (~200 g) was initially anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and maintained by tracheal intubation (1.1%; 2–2.5 cc tidal volume; 60–70 strokes per minute). A laminectomy was performed from T13–L1 vertebrae to expose the lumbar enlargement for spinal "wide dynamic range" (WDR) neuron recording. A paraffin pool was made with ambient skin flaps around the exposed incision area and filled with warm paraffin oil (37°C) to prevent drying of the exposed tissue. Core body temperature was monitored through a thermistor probe inserted into the rectum and maintained at 37.5 ± 0.5°C by means of a feedback-controlled heating pad under the ventral surface of the abdomen. Extracellular single-unit recordings were made from L4–L5 with glass capillary microelectrodes (10–15 MΩ, filled with 0.5 M sodium acetate). Explorations with microelectrodes were made in the dorsal horn using an electronically controlled microstepping manipulator in 2 μm steps. Electrical current pulse (electric stimulator isolator, SEN-3301; isolator, SS-202J; Nihon Kohden, Tokyo, Japan) at a consistent strength (100 μA, 50 μs, 1 Hz) was applied to the skin of the hindpaw ipsilateral to the recording site as a search stimulus to find WDR neurons. After successful identification of a single WDR unit (Chen et al., 1998), the unit responsiveness to 10 s mechanical stimuli was recorded. The mechanical stimuli were (1) brush, performed by stroking on the center of the cutaneous receptive field (cRF) at a frequency of twice per second with a hairy paint brush; (2) pressure, performed by picking up a fold of skin with flattened alligator clip to produce a consistent strength that was not painful when tested on the skin; and (3) noxious pinch, performed by pinching a fold of skin with a small serrated clip to produce a consistent strength, which was obviously painful when tested on the experimenter's skin. The force of pressure and pinch stimuli applied to the cRF was uniform although the precise strength of the stimulus was not available. Only WDR neurons without background discharges were studied further. To evoke "wind-up," 16 suprathreshold stimuli (at 1.5-fold of the threshold), with a 2 ms pulse width at 0.5 Hz, were applied to each WDR neuron. Threshold was taken as the current required to consistently evoke one or more spikes to one electrical current pulse. Control trials were performed at 10 min intervals and drug or saline was applied directly to the dorsal surface of the spinal cord in a volume of 10 μl after three stable control trials. To evaluate the effects of PcTX1 on wind-up and mechanical stimulation, the time point was measured at 15 min after the drug treatment. The original signals were filtered (20 Hz to 20 kHz) by an alternating current/direct current differential amplifier (A-M Systems, Sequim, WA) and simultaneously sampled with PowerLab/8SP (AD Instruments, Castle Hill, Australia) at 10 kHz. Chart 4.0 software (AD Instruments) was applied for recording and off-line analysis.

*Data analysis.* Results were expressed as the mean ± SE. Statistical comparisons were performed using unpaired or paired Student's $t$ tests or one-way ANOVA where significance was indicated by two-tailed $p$ values ≤0.05.

# Results

## Ca$^{2+}$-permeable ASIC1a channels are the major ASIC in SDH neurons

To understand the presence of ASIC1a-containing channels in SDH neurons, we examined the contribution of ASIC1a homomeric channels to acid-induced currents in these cells. We first tested the effect of PcTX1, a specific antagonist of homomeric ASIC1a channels. Consistent with a previous report (Escoubas et al., 2000), we found that in CHO cells heterologously expressing ASIC1a, 1b, and 2a, either alone or together, PcTX1 only blocked homomeric ASIC1a channel-mediated currents, without affecting homomeric ASIC2a channel- or heteromeric ASIC1a + 2a-mediated currents (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Interestingly, the same concentration of PcTX1 markedly enhanced the peak amplitude of ASIC1b channel-mediated currents (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) and prolonged the channel desensitization ($\tau = 1.4 \pm 0.1$ s in control currents vs $\tau = 3.9 \pm 0.5$ s in PcTX1-treated currents, $n = 6$), consistent with a previous report that PcTX1 promotes ASIC1b channel opening (Chen et al., 2006). In contrast, heat-inactivated (boiled) PcTX1 was ineffective in either reducing ASIC1a or enhancing ASIC1b channels (data not shown). Similar to its inhibition on recombinant ASIC1a channels in acutely dissociated SDH neurons, we found that PcTX1 (5 μg/ml) reduced the peak amplitude of acid-induced current to 14.1 ± 1.1% ($n = 8$) of the control, without affecting the currents elicited by NMDA, kainate, or GABA (Fig. 1A).

Additional experiments were performed to examine the effect of suppressing ASIC1a expression in cultured SDH neurons by RNA interference. Although we noted an apparent inconsistency of peak amplitude of acid-induced currents between acutely dissociated and cultured SDH neurons (804 ± 105 pA in acutely isolated neurons, $n = 8$, vs 458 ± 77 pA in cultured neurons, $n = 15$; $p < 0.05$) (Fig. 1A,C), the ASIC subunits were identical in the two groups, as reflected by RT-PCR and Western blotting measurements (data not shown). Out of four double-stranded siRNA sequences prepared against ASIC1a (see Materials and Methods), we found that two sequences were effective in reducing ASIC1a expression in cultured SDH neurons 4 d after transfection. The ASIC1a expression was reduced to 45.4 ± 4.5% (siRNA1) or 28.5 ± 14.1% (siRNA2) of that in cells transfected with the control RNA of a scrambled sequence (Fig. 1B, control). The expression of ASIC2a was not affected. Whole-cell recordings in neurons 4 d after transfection showed that ASIC1a silencing resulted in a marked reduction of the acid-induced current (at pH 6.0) from 47.3 ± 7.7 pA/pF (control RNA) to 20.6 ± 4.1 pA/pF (siRNA1) or 14.2 ± 1.9 pA/pF (siRNA2) (Fig. 1C). In these recordings, we found no evidence of ASIC2a-containing channels, which normally exhibit a transient component followed by a plateau phase after application of acidic solutions (Wu et al., 2004). Together, these results indicate that ASIC1a subunit is the major functional subunit underlying ASIC currents in SDH neurons.

Homomeric ASIC1a channels are permeable to Ca$^{2+}$ (Xiong et al., 2004; Yermolaieva et al., 2004; Gao et al., 2005; Zha et al., 2006). Using Fura-2 fluorescence ratio imaging of Ca$^{2+}$, we observed an elevation of intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) in response to acidic solution (pH 5.0) in cultured SDH neurons (Fig. 1D$_1$, top). This acid-induced Ca$^{2+}$ elevation was reversibly blocked by 100 μM amiloride, a nonspecific ASIC antagonist (Fig. 1D$_1$, second panel). Downregulating ASIC1a expression with ASIC1a-specific siRNA significantly reduced the acid-induced

$Ca^{2+}$ elevation without affecting NMDA receptor-mediated $Ca^{2+}$ elevation (Fig. $1D_1$, bottom two panels, $D_2$). This $Ca^{2+}$ elevation may be attributed to $Ca^{2+}$ influx through homomeric ASIC1a channels or other $Ca^{2+}$ channels as a result of ASIC-mediated depolarization. We found that acid-induced $Ca^{2+}$ elevation was indeed markedly reduced after the pharmacological blockade of voltage-gated $Na^+$ and $Ca^{2+}$ channels as well as glutamate receptors by a mixture of inhibitors including TTX, $CdCl_2$, D-APV, and CNQX (Fig. $1D_2$, referred to as "TCAC"). However, a residual $Ca^{2+}$ elevation remained even with the TCAC treatment, consistent with the presence of $Ca^{2+}$-permeable homomeric ASIC1a channels and the notion that these channels contribute to $Ca^{2+}$ signaling in SDH neurons. Silencing ASIC1a by siRNA resulted in a significantly further reduction of the residual $Ca^{2+}$ signal (Fig. $1D_2$).

## Blockade of spinal ASIC1a channel activity reduces nociceptive behaviors in inflamed rats

To address the role of $Ca^{2+}$-permeable ASIC1a channels of SDH neurons in nociception, we tested the effect of local blockade of homomeric ASIC1a channels with PcTX1 on thermal and mechanical hypersensitivity induced by subcutaneous injection of CFA into one of the rat hindpaws (Stein et al., 1988). The paw withdrawal latency (PWL) and the paw withdrawal threshold (PWT) in response to heat or mechanical stimulation were significantly decreased 1 d after CFA injection and the effect persisted for at least 3 d in CFA-treated rats, compared with that in control rats injected with saline (Fig. 2A, C). Intrathecal injection of PcTX1 (10 $\mu$l, 0.5 mg/ml total protein, ~25 $\mu$g/kg) 1 d after CFA injection significantly attenuated both thermal and mechanical hypersensitivity induced by the CFA treatment. This antihyperalgesic effect began 5 min after PcTX1 injection and decayed over the subsequent 20 min (Fig. 2B, D). The effect of PcTX1 was estimated by the percentage reduction of PWL, which showed a clear dose dependence (Fig. 2E). Intrathecal injection of amiloride (10 $\mu$l, 53.2 $\mu$g/ml, ~0.5 $\mu$g/kg), the nonspecific ASIC antagonist, also reversed CFA-induced thermal hypersensitivity with a similar time course (Fig. 2F). These effects of intrathecal PcTX1 and amiloride injections were not caused by drug-induced changes in locomotor functions, because placing, grasping, and righting reflexes of the injected rats were normal (supplemental Table 1, available at www.jneurosci.org as supplemental material). In addition, boiled PcTX1 was not antinociceptive when it was intrathecally injected ($n = 3$ rats) (data not shown).

To investigate whether ASIC1a subunit in the SDH plays an essential role in inflammatory pain hypersensitivity, we further performed antisense knockdown experiments. In cultured SDH neurons 10 d *in vitro*, treatment of the cells with an AS against
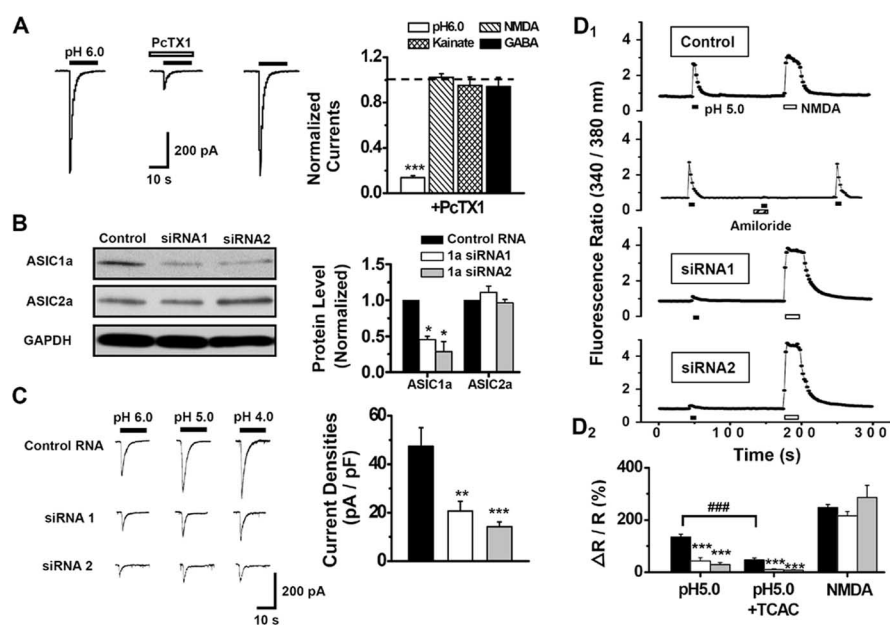


**Figure 1.** Blocking or silencing ASIC1a suppressed acid-induced currents and $Ca^{2+}$ elevation in SDH neurons. **A**, Representative traces showing the inhibitory effect of PcTX1 on ASIC currents induced by solution of pH 6.0 in acutely dissociated SDH neurons. Histograms on the right showed the effect of PcTX1 on membrane currents induced by perfusion with acid solution, pH 6.0 ($n = 8$), NMDA (20 $\mu$M; $n = 4$), kainate (20 $\mu$M; $n = 4$), or GABA (30 $\mu$M; $n = 10$), normalized to the mean value of the same cell before the perfusion. The dashed line indicates control currents without PcTX1 treatment. Data are shown as mean $\pm$ SE (***$p \leq 0.001$ compared with the currents of the corresponding group without PcTX1 treatment, Student's paired $t$ test). **B**, Representative Western blots of ASIC1a and ASIC2a showing specific knockdown of ASIC1a by two specific ASIC1a siRNA oligos. The relative levels of ASIC protein expression were quantified by densitometry. Data (mean $\pm$ SE; $n = 3$) were normalized by the mean value for control RNA. *$p \leq 0.05$ (Student's unpaired $t$ test). **C**, Suppression of acid-induced currents, pH 4.0 – 6.0, in cultured SDH neurons by downregulating ASIC1a expression with two different siRNA oligos. Shown on the right is a summary of data (mean $\pm$ SE; control RNA, $n = 15$; siRNA1, $n = 11$; siRNA2, $n = 15$). **$p \leq 0.01$; ***$p \leq 0.001$ (compared with the mean current density of the control RNA group, Student's unpaired $t$ test). **$D_1$**, Representative $Ca^{2+}$ imaging of acid-induced increase of $[Ca^{2+}]_i$, as indicated by the Furo-2 fluorescence ratio (at 340/380 nm). Data shown are $[Ca^{2+}]_i$ changes in response to brief periods of perfusion with acidic solution (pH 5.0; black bar), 20 $\mu$M NMDA in the presence of 3 $\mu$M glycine (pH 7.4 with $Mg^{2+}$-free; white bar), or 100 $\mu$M amiloride in acidic solution (pH 5.0; hatched bar), recorded in control or siRNA-transfected SDH neurons at DIV 4. **$D_2$**, Summary of experiments similar to those in **$D_1$**. Data represent percentage changes in the fluorescence ratio (340/380, $\Delta$R/R), observed in control RNA- and siRNA-transfected cells in response to acidic solution (pH 5.0) in the absence or presence of solution containing a mixture of channel inhibitors (TCAC; TTX, 1 $\mu$M; $CdCl_2$, 100 $\mu$M; D-APV, 20 $\mu$M; and CNQX, 10 $\mu$M), or NMDA (20 $\mu$M). ***$p \leq 0.001$ (Student's unpaired $t$ test) compared with the corresponding control RNA group; ###$p \leq 0.001$ (Student's unpaired $t$ test) compared with no TCAC-treated control RNA group. The black, white, and gray columns in **C** and **$D_2$** represent control RNA and two ASIC1a siRNA, respectively, as shown in **B**.

ASIC1a (Ettaiche et al., 2006) for 3 d significantly decreased the expression of ASIC1a to $31.5 \pm 7.3\%$ of the control, which was transfected with an oligonucleotide coding IS of the antisense (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). We then tested the effect of the two oligonucleotides in normal or inflamed rats. As indicated in Figure 3A, intrathecal injection of ASIC1a AS twice a day for 4 d reduced CFA-induced ASIC1a expression in the SDH homogenate (see Materials and Methods) to $54.4 \pm 7.2\%$ of the saline control. In contrast, IS treatment did not influence the ASIC1a expression ($104.0 \pm 4.1\%$ of the saline control). In parallel experiments, we found that intrathecal administration of ASIC1a AS significantly reduced the thermal and mechanical hypersensitivity in the CFA-inflamed rats, as indicated by higher values of PWL and PWT (Fig. 3B). Four days after cessation of antisense injections (posttreatment group), both the expression level of ASIC1a (Fig. 3A, top right) and the pain hypersensitivity (Fig. 3B) showed no significant difference between the AS and saline or IS control groups.
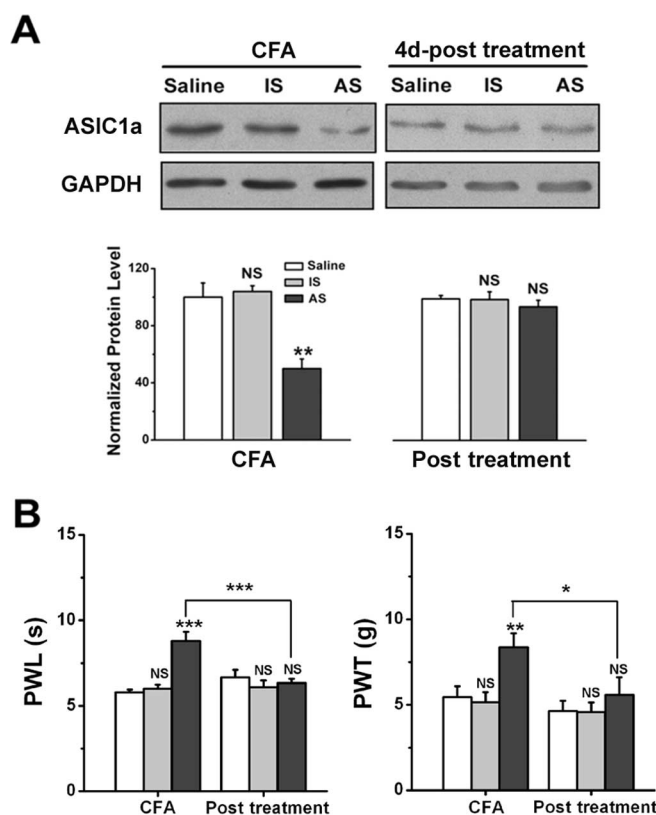
In contrast to that observed in inflamed rats, intrathecal injec-
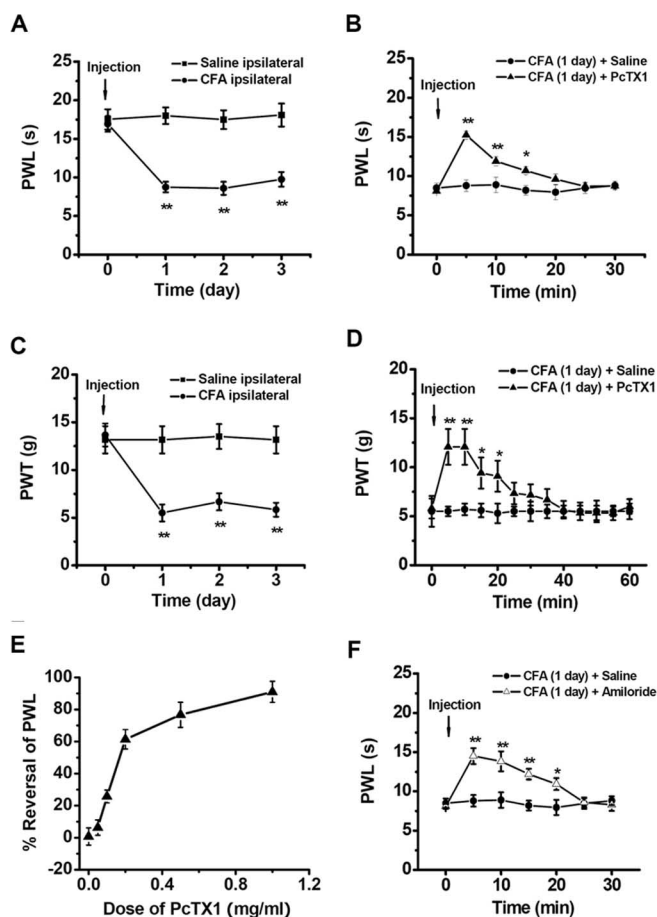
**Figure 2.** Intrathecal injection of ASIC antagonists attenuated peripheral inflammation-induced thermal and mechanical hypersensitivity. ***A***, Changes in PWL of the rat in response to heat stimuli after intraplantar injection with CFA or saline. Data represent mean ± SE (n = 6 for each group). **p ≤ 0.01 compared with saline injection on the same day (Student's unpaired t test). ***B***, Time course of the effect of intrathecal PcTX1 injection on the CFA-induced reduction of PWL one day after CFA injection. Injection of PcTX1 elevated PWL to a level close to the normal level (***A***, reversed the CFA effect) 5 min after the injection, and the effect decayed over the next 20 min. Data represent mean ± SE (n = 6 for each group). *p ≤ 0.05; **p ≤ 0.01 compared with intrathecal injection of saline (Student's unpaired t test). ***C, D***, Changes in PWT as tested by von Frey stimulation for mechanical hypersensitivity. Data were obtained by the same procedure as those in ***A*** and ***B*** (n = 6 each for CFA and saline groups). ***E***, Dose dependency of PcTX1 inhibition of CFA-induced reduction of PWL (n = 6 for each group). ***F***, Time course of the effect of intrathecal amiloride on the CFA-induced reduction of PWL. Data were obtained in the same manner as those in ***B*** (n = 6 for each group).

tion of PcTX1 did not affect either PWL or PWT in naive rats without CFA injection (Fig. 4A). Furthermore, although intrathecal injection of ASIC1a antisense oligonucleotide twice a day for 4 d significantly reduced ASIC1a expression in naive rats (to 46.2 ± 10.2% of the saline control) (Fig. 4B), their pain responses were not affected (Fig. 4C). These results suggest that ASIC1a channels specifically contribute to pathological but not physiological pain.

Additional support of spinal ASIC1a function in nociception came from the formalin test. Subcutaneous injection of formalin normally produces two distinct phases of spontaneous nociceptive behavioral responses (flinches of the affected paw) (Dickenson and Sullivan, 1987): an early/acute phase (0–5 min postinjection; phase 1) attributable to the direct irritant effect of the chemical, and a late/tonic phase (15–60 min; phase 2) that reflects a combination of ongoing sensory input and central sensitization. Intrathecal injection of PcTX1 (10 μl, 0.5 mg/ml total

protein, ∼25 μg/kg) immediately after formalin administration had no effect on the early phase of the formalin response, but the same PcTX1 injection reduced the second phase of formalin-induced pain behavior (Fig. 5A). This result indicates spinal sensitization caused by the activation of ASIC1a channel.

To address whether peripheral ASICs also play a role in CFA-induced pain hypersensitivity, we injected PcTX1 (10 μl, 0.5 mg/ml total protein, ∼25 μg/kg) subcutaneously into the hindpaw treated with CFA. Neither the normal baseline PWL (Fig. 5B, top two groups) nor the CFA-induced reduction in PWL (Fig. 5B, bottom two groups) was affected. Thus, ASIC1a channels appear to play a major role in central but not peripheral nociception, consistent with the previous observation that ASIC subunits other than ASIC1a may be involved in transducing nociceptive signals at the periphery (Sluka et al., 2003).

**Localization of ASIC1a in SDH neurons, but not central DRG nerve terminals**
The above studies showed that behavioral response to nociceptive stimuli in naive rats was not affected by intrathecal injection



**Figure 3.** Intrathecal injection of ASIC1a antisense oligonucleotide suppressed pain response of inflamed rats. ***A***, Western blot analysis indicated that delivery of a specific ASIC1a AS significantly reduced the expression of ASIC1a in the SDH compared with saline control groups in CFA-treated rats. In contrast, treatment with IS did not affect ASIC1a expression. After 4 d of cessation of AS or IS injection (4d-post treatment), the expression of ASIC1a was comparable with saline control. The relative levels of ASIC1a protein expression were quantified by densitometry. Each group of data (mean ± SE) was from three independent rats and was normalized by the mean value for those from three rats in corresponding saline group. **p ≤ 0.01 compared with each saline control; NS, No significant difference (Student's unpaired t test). ***B***, Behavioral changes parallel the ASIC1a protein reduction. Data represent the mean ± SE (AS group of CFA rats, n = 12; saline group of CFA rats, n = 12; antisense group of post-treatment rats, n = 11; saline group of post-treatment rats, n = 7). The bracket indicates significant difference from corresponding points. *p ≤ 0.05; ***p ≤ 0.001 (Student's unpaired t test). The white, light gray, and dark gray columns in ***B*** represent saline, IS, and AS, respectively, as shown in ***A***.

of PcTX1 and ASIC1a antisense oligonucleotide (Fig. 4). This suggests that ASIC1a channels in SDH neurons are not involved in physiological nociception. To determine how spinal ASIC1a channels may function in inflammatory pain, we examined the cellular distribution of ASIC1a in the SDH after peripheral CFA injection using the immunostaining method. The majority of SDH cells exhibiting ASIC1a staining were found to contain NeuN, a specific neuronal marker (Mullen et al., 1992), indicating that ASIC1a was mainly expressed in neurons (Fig. 6D, top). Moreover, an increased neuronal ASIC1a staining was observed in superficial layers of SDH ipsilateral to the inflammatory side, compared with that of contralateral SDH or the SDH of sham-operated rats without CFA injection (Fig. 6A–C). To test whether ASIC1a is expressed in the central terminal of DRG neurons, we costained ASIC1a with CGRP, a neuropeptide contained in the nerve terminals of a subpopulation of DRG neurons (Hokfelt et al., 1992). We found that the distribution of ASIC1a and CGRP was mostly nonoverlapping (Fig. 6D, bottom). Furthermore, ASIC1a did not colocalize with IB$_4$ (supplemental Fig. 3, available at www.jneurosci.org as supplemental material), a marker for nonpeptidergic small DRG neurons (Silverman and Kruger, 1990), consistent with the report that the IB4-positive DRG neurons express ASIC1a at a very low level (Poirot et al., 2006). In agreement with the previous report that ASICs were absent at central DRG terminals (Garcia-Anoveros et al., 2001), our result indicated that the elevated expression of ASIC1a was restricted to SDH neurons rather than the central terminals of DRG neurons, consistent with our behavioral finding of central, but not peripheral involvement of ASICs (Fig. 5A,B). Furthermore, ASIC1a was not colocalized with the astrocyte marker GFAP, the oligodendrocyte marker RIP, or the microglia marker OX-42 (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). Thus, we concluded that peripheral inflammation selectively increased the expression of ASIC1a in SDH neurons. Because the expression of ASIC1a is also upregulated in DRG after peripheral inflammation (Voilley et al., 2001), our finding suggests that ASIC upregulation in DRG serves other functions in the DRG cell body or peripheral terminals.

### Enhanced ASIC1a activity contributes to central sensitization

To address the relevance of ASIC1a in the development of inflammatory pain hypersensitivity, we next investigated the mechanism by which ASIC1a channels participate in nociceptive processing in SDH neurons. Two phenomena are known to reflect central sensitization underlying inflammatory pain: C-fiber stimulation-induced wind-up, a progressive increase in spike discharges, and CFA-induced hypersensitivity of SDH nociceptive neurons (Woolf and Salter, 2000; Salter, 2004). In vivo single-unit recordings were made from WDR SDH neurons, which are responsible for nociceptive transmission and spinal flexion reflex (Jiang et al., 1995). We found that the wind-up of single-unit activity evoked by prolonged repetitive C-fiber stimulation (0.5
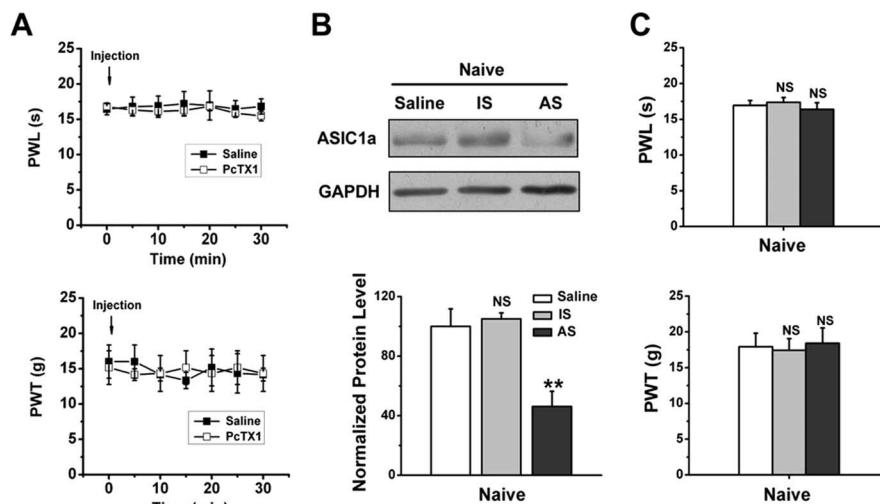


**Figure 4.** Central ASIC1a did not affect physiological nociceptive behavior. **A**, Blockade of ASIC1a did not affect normal thermal and mechanical nociception. Intrathecal injection of PcTX1 one day after intraplantar injection of saline did not alter normal thermal and mechanical nociceptive transmission. Data are shown as mean ± SE (n = 6 for each group) compared with the saline-treated group (one-way ANOVA test, p > 0.05). **B**, Western blot analysis indicated that delivery of specific ASIC1a AS significantly reduced the expression of ASIC1a in the SDH compared with the saline control groups in naive rats. In contrast, IS did not influence ASIC1a expression. Each group of data (mean ± SE) was from three independent rats and was normalized by the mean value for those from three rats in corresponding saline group. **p ≤ 0.01 compared with the saline control (Student's unpaired t test). **C**, Neither injection of AS of ASIC1a nor IS of ASIC1a altered normal thermal and mechanical nociceptive transmission (n = 11 in AS group; n = 9 in IS group; n = 11 in saline group, Student's unpaired t test). The white, light gray, and dark gray columns in **C** represent saline, IS, and AS, respectively, as shown in **B**.

Hz, 2 ms, 16 pulses), which mimics persistent C-fiber activation during inflammation, was mostly attenuated by infusion with PcTX1 (10 μl, 0.5 mg/ml total protein, ~25 μg/kg), whereas the same infusion with saline had no effect (n = 6 neurons in 6 rats) (Fig. 7A,B). Because the wind-up reflects activity-dependent synaptic integration in the spinal cord (Herrero et al., 2000; Woolf and Salter, 2000), these findings suggest that ASIC1a channels contribute to spinal neuron sensitization underlying inflammatory pain hypersensitivity.

The ASIC1a channels appeared to play a specific role in inflammation-induced nociception, because PcTX1 infusion of naive rats had no effect on WDR neuronal activity in response to non-noxious (brushing) and noxious (pressing and pinching) mechanical stimuli applied to peripheral receptive fields (Fig. 7C,D). This is consistent with the observed behavioral response to nociceptive stimuli in naive rats (Fig. 4). In contrast, in rats with CFA-induced inflammation, PcTX1 significantly attenuated the hyperactivity of WDR neurons in response to noxious mechanical stimuli, whereas the brush-evoked hyperactivity of WDR neurons was not significantly affected (p = 0.384; Post-PcTX1 vs Pre-PcTX1: 59.6 ± 18.6 vs 83.0 ± 17.3, in brushing-test group) (Fig. 7C,D). These results indicate that ASIC1a channels are not involved in normal sensory transmission in the spinal cord, but participate specifically in inflammatory pain hypersensitivity.

### Discussion

In the present study, we demonstrated that ASIC1a channels in SDH neurons are essential for inflammation-induced pain hypersensitivity and spinal neuron sensitization. This conclusion is based on three lines of evidence. First, SDH neurons express a high density of Ca$^{2+}$-permeable homomeric ASIC1a channels. Second, both genetic suppression and pharmacological blockade of homomeric ASIC1a channels attenuate inflammatory pain be-
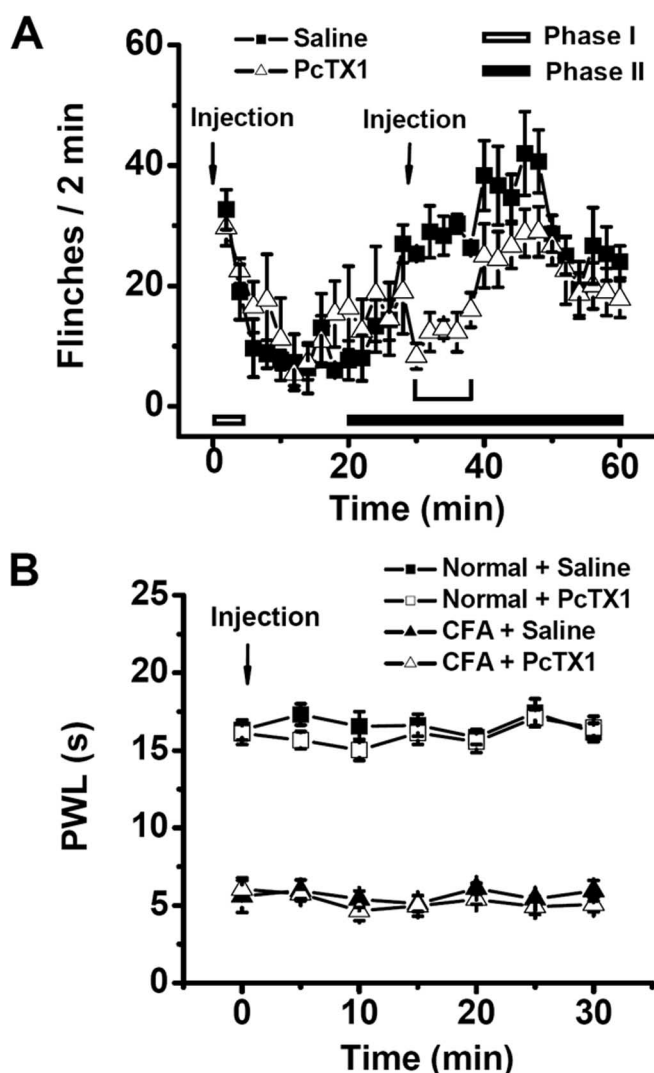
**Figure 5.** Central but not peripheral ASIC1a contributed to nociceptive transmission. *A*, Intrathecal injection of PcTX1 inhibited the late phase (phase 2) of flinch in the formalin test, but had no detectable effect in the early phase (phase 1). Data are shown as mean $\pm$ SE ($n = 6$ for each group). The bracket indicates significant difference from corresponding points in the saline group ( $p < 0.05$, Student's unpaired $t$ test). *B*, Intraplantar PcTX1 injection had no effect on CFA-induced reduction of PWL or on PWL of rats injected with saline instead of CFA (normal). Data are shown as mean $\pm$ SE ($n = 6$ for each group) compared with the corresponding intraplantar saline-treated group (one-way ANOVA test, $p > 0.05$ for normal and CFA groups).

haviors of rats. Third, peripheral inflammation upregulates the expression of ASIC1a protein in SDH neurons and preventing homomeric ASIC1a channel activity in the spinal cord reduces central sensitization, as reflected by the wind-up of SDH neuronal activity induced by C-fiber stimulation and the elevated firing of SDH neurons induced by peripheral inflammation. Spinal cord also expresses detectable levels of ASIC2a and 2b (Wu et al., 2004). Although changes in other types of ASICs may be induced by antisense suppression of ASIC1a expression, data from the acute blockade of ASIC1a channels with specific inhibitor PcTX1 establishes ASIC1a as a major contributor to inflammatory pain hypersensitivity.

### ASIC1a channels contribute specifically to pain hypersensitivity and spinal sensitization

We found that local blockade of ASIC1a channels had no effect on either the basal SDH neuronal activity or the nociceptive re-

sponse of normal rats. Thus, ASIC1a channels in SDH neurons are not involved in physiological nociception. This may be attributable to weak acidosis at synaptic cleft and/or low endogenous expression level of ASIC1a under normal physiological conditions. However, repetitive stimulation of the dorsal root evoked transient acidification in the dorsal horn by 0.25 pH units (Chvatal et al., 1988). The localized changes in synaptic cleft might be more pronounced because of the limited spatial and temporal resolution of pH microelectrodes in the measurement (Miesenbock et al., 1998). Thus, ASICs of SDH neurons might detect acidification associated with exacerbated synaptic activity under pathological conditions, such as inflammatory chronic pain.

Although synaptic transmission is primarily mediated by AMPA subtype of glutamate receptors, intense or sustained noxious stimuli that are associated with tissue damage activate NMDA receptors, allowing Ca²⁺ influx into the postsynaptic cells. This influx in turn activates Ca²⁺-sensitive intracellular signal cascades that lead to prolonged increases in the excitability of SDH neurons, a process known as central sensitization (Woolf and Salter, 2000). However, there is growing evidence for the involvement of NMDA receptor-independent mechanisms underlying the enhanced synaptic efficacy in SDH neurons after peripheral inflammation. In particular, Ca²⁺-permeable AMPA receptors in SDH neurons contribute to activity-induced sensitization in pain pathways (Gu et al., 1996; Hartmann et al., 2004). The present study further demonstrates that ASIC1a channels in SDH neurons directly contribute to both C-fiber stimulation-induced wind-up and inflammation-induced hyperactivity of SDH neurons, indicating that this additional type of Ca²⁺-permeable channels is also important determinants of activity-dependent modification of spinal processing of nociceptive signals and contribute to pain hypersensitivity. Unlike NMDA and AMPA receptors, which are involved in normal synaptic transmission, ASIC1a channels function specifically for spinal processing of nociceptive information during inflammatory chronic pain.

### How might ASICs modulate spinal nociceptive plasticity?
The functional significance of ASIC1a in synaptic plasticity has been examined previously in the context of LTP and synaptic modulation associated with activity-dependent learning (Wemmie et al., 2002, 2003, 2004). Previous evidence suggests that the molecular and cellular mechanisms involved in learning and memory are also operational in nociceptive hypersensitivity and chronic pain (Sandkuhler, 2000; Ji et al., 2003; Zhuo, 2007). Interestingly, analogous to a requirement for ASIC1a in hippocampal LTP (Wemmie et al., 2002), we found that Ca²⁺-permeable ASIC1a channels in SDH neurons are critical for wind-up and inflammation-induced hyperactivity of SDH neurons. In addition, repetitive stimulation of the dorsal root evoked transient acidification in the dorsal horn (Chvatal et al., 1988). Thus, together with their elevated expression, ASIC1a channels in SDH neurons are positioned to respond to acidification associated with the persistent synaptic activity after peripheral inflammation, resulting in enhanced Ca²⁺ signaling and increased firing of these neurons (Vukicevic and Kellenberger, 2004). This enhanced firing may in turn facilitate the activation of NMDA receptors, leading to the activation of downstream signaling molecules, such as Ca²⁺/calmodulin-stimulated adenylyl cyclases (Wei et al., 2002), mitogen-activated protein kinases (Ji et al., 1999), etc., and finally causing activity-dependent central sensitization. However, NMDA receptor activation can further sensitize the cells to acidic conditions by inducing ASIC1a phosphor-

ylation (Gao et al., 2005). Therefore, it is likely that ASIC1a channels may function together with NMDA receptors in activity-dependent central sensitization underlying pain hypersensitivity. Indeed, it has been reported that ASIC1a is preferentially targeted to neuronal dendrites and present in spines, where NMDA receptors are densely resident. We have not ruled out the possible role of ASIC1a modulation in inflammatory pain. However, regulation of the protein level may be more important in chronic pathological pain conditions. Even if the post-translational modulation of the ASICla channel is directly responsible for the behavioral effects, the upregulation of the ASIC1a protein will nevertheless enhance the overall effect. More importantly, previous study shows that overexpressing ASIC1a increases the number of spines (Zha et al., 2006). Thus, in the future, it would be interesting to examine whether the elevated expression of ASIC1a in SDH neurons after peripheral inflammation causes an altered dendritic spine density, a characteristic of several chronic brain diseases (Fiala et al., 2002).

Although ASIC1a channels appear to play a major role in central but not peripheral nociception, we should note that ASIC1a expression in DRG neurons is either upregulated after peripheral inflammation (Voilley et al., 2001; Mamet et al., 2002). The absence of ASIC1a in CGRP- and IB₄-containing DRG afferent terminals in the spinal cord does not necessarily imply that peripheral ASIC1a channels do not participate in pain sensation. It is possible that ASIC1a in DRG may function primarily in the peripheral terminals or in the cell body. Selective transport of ASIC2a (BNaC1α) toward DRG peripheral terminals was indeed observed (Garcia-Anoveros et al., 2001). Nevertheless, most previous studies on ASICs and pain regulation have so far focused on changes in subunit expression in DRG neurons (Garcia-Anoveros et al., 2001; Voilley et al., 2001; Chen et al., 2002; Mamet et al., 2002; Poirot et al., 2006). Our study indicates that inflammatory pain may result from changes in ASIC subunit expression in both DRG and higher-order spinal cord neurons. Supraspinal structures such as thalamus and cortex are also known to be involved in the chronic pain (Wu et al., 2005; Zhuo, 2007). It has been reported that ASICs are functionally expressed in hippocampus (Wemmie et al., 2002), amygdala, and cortex (Wemmie et al., 2003, 2004). However, it is still unknown whether suprospinal ASICs are engaged in the modulation of chronic pain hypersensitivity. Although we demonstrate that ASIC1a channels in SDH neurons contribute to inflammatory pain hypersensitivity, we cannot rule out the involvement of ASIC1a in neuropathic pain
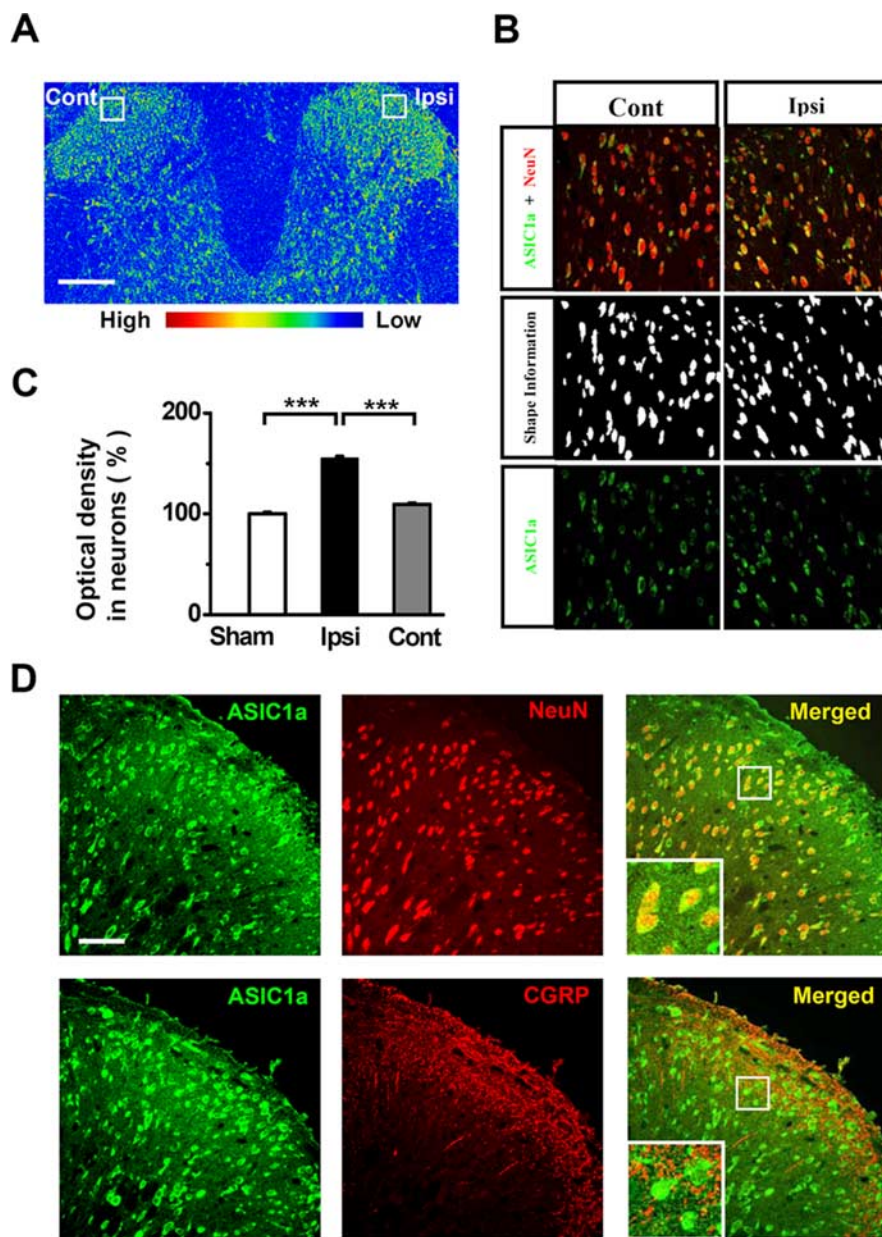


**Figure 6.** Increased ASIC1a expression in SDH neurons after peripheral inflammation. **A**, ASIC1a immunostaining in SDH contralateral (Cont) and ipsilateral (Ipsi) to the injection site 1 d after CFA injection, with the staining intensity coded by pseudo-colors. **B**, Illustration of the calculation of ASIC1a intensity in NeuN-positive SDH neurons (marked region in **A**). Shape information was extracted from NeuN staining. **C**, Average staining intensity of ASIC1a in SDH neurons, for ipsilateral and contralateral sides, normalized by the mean level from saline-injected rats (Sham). Data are shown as mean ± SE (Sham, $n = 1178$; Ipsi, $n = 968$; Cont, $n = 963$. Immunostainings were performed from nine sections of three sham or inflamed rats, respectively. ***$p \leq 0.001$ (Student's unpaired $t$ test). **D**, Coimmunostaining of ASIC1a with NeuN (a neuron-specific marker) or CGRP (a marker of primary afferents) in superficial layers of SDH. Insets, High magnification view of the area marked by the square. Scale bars: **A**, 200 $\mu$m; **D**, 50 $\mu$m.

models. In fact, previous evidence suggests a role of ASIC1a channels in neuropathic pain (Poirot et al., 2006; Mazzuca et al., 2007).

In summary, the present results suggest that enhanced Ca²⁺ signaling in spinal dorsal horn neurons after peripheral inflammation, through the upregulation of ASIC1a channels, may contribute to hypersensitivity of SDH neurons and inflammatory pain. Thus, specific blockade of Ca²⁺-permeable ASIC1a channels may have antinociceptive effect by reducing or preventing the development of spinal sensitization induced by inflammation.
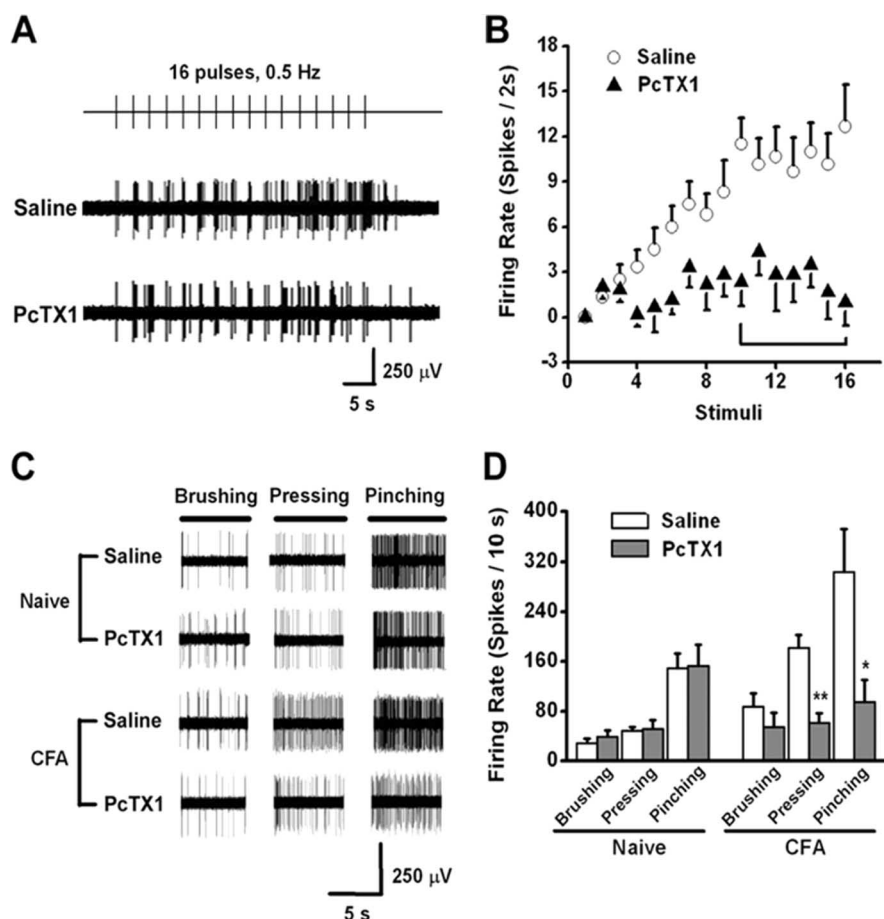
**Figure 7.** Blocking ASIC1a channels attenuated C-fiber-induced wind-up and CFA-induced hypersensitivity of SDH nociceptive neurons. ***A***, An example of wind-up in spinal WDR neurons after repetitive C-fiber stimulation (16 suprathreshold stimuli, 2 ms pulse width at 0.5 Hz) in normal rats. The wind-up was mostly abolished after infusion of PcTX1, but not saline, into the dorsal surface of spinal cord. The downward arrows in ***A*** indicate the starting time of electrical stimulation. ***B***, Summary of results from all experiments similar to that shown in ***A***. Data are shown as mean ± SE ($n = 6$ for each group). The bracket indicates significant difference from corresponding points in the saline group ( $p < 0.05$, Student's unpaired $t$ test). ***C***, Example recordings showing the effect of PcTX1 on the firing of WDR neurons in response to non-noxious (brushing) or noxious (pressing and pinching) mechanical stimulation applied to their cutaneous receptive fields in naive ($n = 5$) and CFA-treated rats ($n = 4$). ***D***, Summary of results from all experiments similar to that in ***C***. Data are shown as mean ± SE ($n = 4–5$). The CFA treatment significantly increased the firing rate for all stimuli ( $p < 0.05$) and PcTX1 significantly reduced the CFA effect (*$p < 0.05$; **$p ≤ 0.01$ compared with saline groups, Student's unpaired $t$ test).

Through membrane depolarization, activation of ASIC1a channels may also indirectly exerts its effect via relief of $Mg^{2+}$ blockade of NMDA receptors, which are essential for spinal nociceptive plasticity underlying chronic pain. Because blockers of NMDA receptors used for treatment of chronic pain have severe side effects (South et al., 2003), specific disruption of $Ca^{2+}$-permeable ASIC1a channels might offer an alternative therapeutic approach.

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