

NMDAR-Mediated Hippocampal Neuronal Death is Exacerbated by Activities of ASIC1a

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Abstract NMDARs and ASIC1a both exist in central synapses and mediate important physiological and pathological conditions, but the functional relationship between them is unclear. Here we report several novel findings that may shed light on the functional relationship between these two ion channels in the excitatory postsynaptic membrane of mouse hippocampus. Firstly, NMDAR activation induced by either NMDA or OGD led to increased $[Ca^{2+}]_i$ and greater apoptotic and necrotic cell deaths in cultured hippocampal neurons; these cell deaths were prevented by application of NMDAR antagonists. Secondly, ASIC1a activation induced by pH 6.0 extracellular solution (ECS) showed similar increases in apoptotic and necrotic cell

deaths; these cell deaths were prevented by ASIC1a antagonists, and also by NMDAR antagonists. Since increased $[Ca^{2+}]_i$ leads to increased cell deaths and since NMDAR exhibits much greater calcium permeability than ASIC1a, these data suggest that ASIC1a-induced neuronal death is mediated through activation of NMDARs. Thirdly, treatment of hippocampal cultures with both NMDA and acidic ECS induced greater degrees of cell deaths than either NMDA or acidic ECS treatment alone. These results suggest that ASIC1a activation up-regulates NMDAR function. Additional data supporting the functional relationship between ASIC1a and NMDAR are found in our electrophysiology experiments in hippocampal slices, where stimulation of ASIC1a induced a marked increase in NMDAR EPSC amplitude, and inhibition of ASIC1a resulted in a decrease in NMDAR EPSC amplitude. In summary, we present evidence that ASIC1a activity facilitates NMDAR function and exacerbates NMDAR-mediated neuronal death in pathological conditions. These findings are invaluable to the search for novel therapeutic targets in the treatment of brain ischemia.

Su Gao and Yang Yu have contributed equally to this work.

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Keywords NMDA receptor · Acid-sensing ion channel 1a · Whole-cell patch-clamping recording · Oxygen-glucose deprivation · Neurotoxicity · Neuroprotection

Abbreviations

NMDAR	<i>N</i> -methyl <i>D</i> -aspartate receptor
ASIC1a	Acid-sensing ion channel 1a
ACSF	Artificial cerebral spinal fluid
OGD	Oxygen-glucose deprivation
AMPA receptor	α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

GABA _A receptor	γ-Aminobutyric acid A-type receptor
VGCC	Voltage-gated calcium channels
PCs	Pyramidal cells
Mg ²⁺	Magnesium ion
[Ca ²⁺] _i	Intracellular calcium concentration
NBQX	1,2,3,4-Tetrahydro-6-nitro-2,3-dioxbenzo[f]quinoxaline-7-sulfonamide
APV	2-Amino-5-phosphonopentanoic acid
PcTX1	Psalmotoxin 1
ECS	Extracellular solution

Introduction

Ischemia-induced neuronal death is a major problem associated with stroke. Among various theories on the molecular mechanism of ischemic neuronal damage, hypotheses involving the overactivation of NMDARs have attracted more attention because of the unique properties of NMDARs. NMDAR activities are associated with various neuronal processes ranging from learning and memory to neuronal degeneration. Activation of NMDARs requires both glutamate and glycine binding to their respective sites, as well as membrane depolarization to remove the Mg²⁺ blockade (Mayer et al. 1984; Johnson and Ascher 1987). Once activated, NMDARs are highly permeable to Ca²⁺. It is this high Ca²⁺ permeability that makes them the ideal mediator of extracellular Ca²⁺ influx, which in turn activates Ca²⁺-dependent enzymes, including nNOS, protease, protein kinase, and DNase in neurons and damage cells (Schanne et al. 1979; Choi 1985; Mayer and Westbrook 1987; Lipton and Rosenberg 1994; Lee et al. 1999; Arundine and Tymianski 2004; Makhro et al. 2010; Szydłowska and Tymianski 2010). In addition, the function of NMDARs can be modified by many extracellular molecules, such as protons (H⁺), Zn²⁺, and polyamine (MacDermott et al. 1986; Mayer and Vyklícký 1989; Rock and Macdonald 1992; Williams 1997; Cull-Candy et al. 2001).

Recent studies have shown that the activation of homomeric acid-sensing ion channel 1a (ASIC1a) could also lead to Ca²⁺ influx into neurons and cause cell damage in cultured cortical and hippocampal neurons (Xiong et al. 2004; Gao et al. 2005).

Homotrimeric ASIC1a channels are a proton-gated and Ca²⁺-permeable ion channel that conducts Na⁺ and Ca²⁺ (Waldmann et al. 1997; Xiong et al. 2004; Yermolaieva et al. 2004). They are widely expressed in peripheral and central nervous systems (CNS) in mammals (Waldmann et al. 1997; Waldmann and Lazdunski 1998; Waldmann et al. 1999; Alvarez de la Rosa et al. 2002, 2003). ASIC1a channels are

present at excitatory postsynaptic synaptic terminals (Wemmie et al. 2003, 2006; Zha et al. 2006), which could be activated by sudden pH drops from 7.4 to 6.9 and below, and show a half-maximal activation at pH 6.2 (Waldmann et al. 1997). After activation, ASIC1a rapidly goes into desensitization state (Waldmann et al. 1997; Xiong et al. 2004; Wemmie et al. 2006). In physiological condition, ASIC1a plays an important role in long-term potentiation (Wemmie et al. 2002) and dendritic structural plasticity in hippocampal neurons (Xiong et al. 2004; Zha et al. 2006). Behavior studies showed that disrupting ASIC1a impaired spatial learning, eye-blinking conditioning, and fear conditioning of the mouse (Wemmie et al. 2002, 2003). Conversely, over-expression of ASIC1a in transgenic mice increased fear conditioning (Wemmie et al. 2004).

In pathological conditions, such as brain ischemia, activation of ASIC1a induced Ca²⁺ overload in the neuron and caused cell death (Xiong et al. 2004; Gao et al. 2005). Pharmacologically blocking ASIC1a channels can also reduce ischemic neuronal death (Xiong et al. 2004; Gao et al. 2005).

Since NMDARs and ASIC1a show functional similarity in physiological and ischemic conditions, and since glutamate transmitter release is always accompanied by proton release from the presynaptic terminal, these two receptors may be co-activated (Krishtal et al. 1987; Zha et al. 2006). We ask whether the co-activation of these two ion channels in the postsynaptic site is obligatory or not. Since NMDAR is the main player in central synaptic transmission, completely blocking NMDARs is harmful for normal synaptic function, causes chronic neuronal degeneration, and does not benefit neuronal survival in brain ischemia (Hardingham and Bading 2003). Thus, we decided to examine whether inhibition of ASIC1a affects NMDAR function, whether inhibition of ASIC1a can prevent ischemic cell death, and what the underlying mechanism is.

Our previous electrophysiology studies in mouse brain slices have shown that electrical stimulation of Shaffer collateral fibers evoked excitatory postsynaptic currents in physiological pH (7.4) in low Mg²⁺ (0.1 mM) extracellular solution (ECS) under the blockade of AMPA, GABA_A, and glycine receptors. In this condition, NMDAR activity should be mostly facilitated. We found that the excitatory currents were completely blocked by NMDAR antagonists, which suggests that the currents were mediated by NMDARs. Another set of patch-clamp recording data showed that NMDAR currents were distinctly suppressed when ASIC1a antagonists were added to the perfusion solution (pH 7.4), suggesting that NMDARs, not ASIC1a, are the main player in mediating excitatory synaptic transmission in the hippocampus.

In this study, we found that treatment of cultured hippocampal neurons with NMDA-induced neuronal deaths at physiological pH (7.4). Interestingly, inhibition of ASIC1a

greatly reduced NMDA-induced neuronal death. However, stimulation of ASIC1a in pH 6.0 ECS greatly enhanced NMDA-induced neuronal death. We then further examined the involvement of ASIC1a in the NMDAR-mediated $[Ca^{2+}]_i$ increase and apoptotic effector enzyme activation.

Materials and Methods

Primary Culture of Hippocampal Neurons

Dissociated cultures of hippocampal neurons were prepared from postnatal 0–1 day mice. All animal care and experimental procedures were complied with local and internal guidelines on ethical use of animals and were approved by the University animal welfare committee. The tissue was isolated by a standard enzyme treatment protocol. Briefly, shredded tissue was treated with 0.125 % trypsin (T1426, Sigma-Aldrich, USA) and incubated for 25 min at 37 °C. After centrifuging the mixture, the supernatant was discarded. The neurons were plated onto coverslips coated with poly-L-lysine (D0301, Sigma-Aldrich, USA) and then grown in DMEM with/F12 and 10 % fetal calf serum (21103-049, Gibco, USA) for the first 24 h. The cultures were maintained at 37 °C in a 5 % CO₂ humidified atmosphere. The culture medium was replaced by neurobasal containing 2 % B27 on the second day. The culture medium was replaced every 3 days. Glial growth was suppressed by addition of 5-fluoro-2-deoxyuridine (10 μM) and uridine (10 μM) (Sigma-Aldrich, USA). Mature neurons (12–16 DIV) were used for experiments. To induce neuronal injury, hippocampal cultures were washed three times in Mg²⁺-free ECS containing (in mM): 140 NaCl, 5 KCl, 2 CaCl₂, 20 HEPES, and 3 glucose, with pH 7.4 and osmolarity 310–320 mOsm.

NMDA Treatment

To examine NMDA's effect on cultured hippocampal neurons, specific blockade of synaptic AMPARs, glycine receptors, GABA_A receptors, and voltage-gated calcium channels (VGCCs) were achieved by adding NBQX (5 μM), strychnine (1 μM), bicuculline (10 μM), and nifedipine (10 μM) into the Mg²⁺-free ECS. The cultures were then exposed to Mg²⁺-free ECS containing NMDA (100 μM) and glycine (10 μM) for 25 min at room temperature (RT). Following a thorough wash with 1.3 mM Mg²⁺ and 15 mM glucose ECS (normal ECS), the cultures were returned to the normal culture medium and continued to grow for 24 h.

Acidic ECS Treatment

To test the effect of acidic ECS (pH 6.0) on cultured hippocampal neurons, the cultures were washed 3 times with

Mg²⁺-free ECS, followed by exposure to Mg²⁺-free acidic ECS for 25 min at room temperature. The treatment was terminated by washing the cultures with normal ECS. The cultures were then returned to the normal medium and continued to grow for 24 h.

Oxygen-Glucose-Deprivation

To test the effect of OGD on cultured hippocampal neurons, the cultures were washed three times with Mg²⁺- and glucose-free ECS (deoxygenated in the anaerobic container for 30 min before use), and then the cultures were then transferred to an anaerobic container in Mg²⁺ and glucose-free ECS saturated by 5 % CO₂/95 % N₂ gases. The cultures remained in this anoxic condition for 90 min at 37 °C. OGD treatment was terminated by removing the cultures out of the container. The cultures were washed three times with normal ECS and were then returned to the normal culture medium, allowing to grow for 24 h. NBQX (5 μM), bicuculline (10 μM), strychnine (1 μM), and nifedipine (10 μM) were contained in the ECS to block AMPARs, GABA_A receptors, glycine receptors, and VGCCs, respectively. Specific inhibitors of ASIC1a or NMDARs, such as amiloride and PcTX1, or Ketamine and APV were added to the ECS as needed.

Cell Viability Assay

Apoptotic neuronal death was determined by visualizing neurons stained with Hoechst-33342 (C1022, Beyotime Institute of Biotechnology, China). For visualizing apoptotic neurons, Hoechst-33342 (5 μg/ml) was added to the culture medium 24 h after treatments and incubated at 37 °C for 25 min. Images were taken with a fluorescence microscope (IX71SIF-3, Olympus, Japan). Cells with condensed or fragmented chromatin morphology were considered as apoptotic. These observations were quantified by double-blind counting of apoptotic and total neurons in each visual field and expressed as percentage. Necrotic cell death was quantified by measuring lactate dehydrogenase (LDH) released into the culture medium 24 h after treatments using a Cyto Tox 96 assay kit (Promega, Madison, WI). The absorbance readings were measured using a microplate reader (MULTISKAN MK3, Thermo Scientific, USA).

Ca²⁺ Imaging

The cultured hippocampal neurons grown on 8 × 8 mm glass coverslips were transferred to 12-well plates, washed twice with normal ECS, and then incubated with Fluo-4 AM (4 μM; F312, Dojindo Laboratories, Japan) in normal ECS for 60 min at 37 °C in the dark. The cultures were

washed again and equilibrated for 30 min at room temperature. The cultures were then washed in modified ECS (Mg^{2+} -free, 3 mM glucose, 2.9 mM Ca^{2+}). After this, the coverslip was transferred into a container on the inverted confocal microscope (LEICA TCS SPE, Germany), and was incubated with modified ECS containing NBQX, bicuculline, strychnine, and nifedipine to block AMPARs, glycine receptors, $GABA_A$ receptors, and VGCCs, respectively, for 15 min at room temperature. Before scanning the images, the container was placed on the stage of the confocal microscope equipped with a 63 \times oil-immersion objective lens and set up in xyt mode with a laser wavelength of $Ex = 488$ nm and $Em = 530$ nm. The resolution of frame scanned was set to 512 \times 512 pixels.

Five minute baseline images followed by 30-min experimental treatment images were taken. The intensities of fluorescence were analyzed with the dynamic intensity analysis module of LAS AF. For normalizing the variations of Ca^{2+} loading, we used relative fluorescence intensity $\Delta F_i/F_0$ to express the changes in the intracellular Ca^{2+} concentration (fold of Ca^{2+} increase). F_i is the averaged fluorescence intensity of ROI from each frame at the given time, and F_0 is the averaged 5-min baseline fluorescence intensity from the same ROI immediately prior to experimental treatments. Background fluorescence (from a region within the same field lacking cells) was subtracted from ROI averages (Feldman et al. 2008).

Electrophysiology

Preparation of brain slices: Prior to decapitation, postnatal 5 to 6-week C57/BL6J mice were anesthetized with isoflurane. The brain was removed and placed in oxygenated (95 % O_2 & 5 % CO_2) ACSF at 4 °C containing (mM) 126 NaCl, 2.5 KCl, 1 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4 , 2 $CaCl_2$, and 10 glucose. The osmolarity of the ACSF was adjusted to 300 mOsm and the pH to 7.2–7.4. Acute coronal brain slices (300 μ m) containing the hippocampus were obtained with a vibrating microtome (Leica VT 1000S, Nassloch, Germany). The slices were then transferred to a chamber containing oxygenated ACSF and maintained at room temperature for at least 1 h prior to patch-clamp voltage recordings. Postsynaptic responses were evoked by electrical stimulation of the Schaffer collaterals (SCs) with a bipolar microelectrode positioned in the stratum radiatum of hippocampal slices. Stimulation was delivered every 12 s with the pulse duration of 100 μ s. NMDAR-mediated excitatory postsynaptic currents (NMDAR EPSCs) were recorded from CA1 pyramidal neurons in voltage-clamp mode. The neuronal membrane potential was held at -30 mV. The recording electrode was filled with intracellular solution containing (in mM) 130 Cs^+ -methanesulfonate, 10 HEPES, 2 $MgCl_2$, 2 ATP-Mg, 0.5 GTP, 1 lignocaine (lidocaine) *N*-ethyl bromide

(QX-314), and 2 EGTA. The osmolarity was adjusted to 280 mOsm and the pH to 7.2–7.4. NMDAR EPSCs were pharmacologically isolated by perfusion of the brain slices with low Mg^{2+} ACSF ($MgCl_2$ 0.1 mM; $CaCl_2$ 2.9 mM) containing CNQX (5 μ M), bicuculline (10 μ M), and strychnine (1 μ M), respectively, to block synaptic AMPAR, $GABA_A$ receptor, and glycine receptor responses.

Western Blot

Hippocampal cultures that were treated by NMDA for 30 min at room temperature were lysed in cell lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 % Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1 % Na_3VO_4 , 0.5 μ g/ml leupeptin, and 1 mM phenylmethanesulfonyl fluoride). Protein samples (30 μ g) were run in sodium dodecyl sulfate–polyacrylamide gels (12 %) for 1.5 h using Tris–glycine running buffer. Gels were then transferred onto a PVDF membrane in transfer buffer containing 25 mM Tris-base, 0.2 M glycine, and 20 % (v/v) methanol for 1 h at 70 V. The membrane was blocked with 5 % non-fat milk in 1 \times TBS, 0.1 % Tween-20 at 25 °C for 1 h and subsequently incubated overnight at 4 °C with primary antibodies for cleaved caspase-3 at Asp175 (rabbit anti-mouse, 1:1000, Cell Signaling Technology, USA) and β -tubulin (rabbit anti-mouse, 1:1000, Cell Signaling Technology, USA). After washing with TBST, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (Goat anti-rabbit IgG, ZSGB-BIO, China) for 1 h, and protein signals were detected with a chemiluminescence system (Clinx Science Instruments). Quantification of protein levels was achieved by densitometry analysis using Image J software and expressed as the ratio of the values of the detected protein band to the β -tubulin band.

Hippocampal slices were transferred from ice-cold oxygenated ACSF to room-temperature oxygenated ACSF after slicing, and were incubated for 1.5 h to allow recovery. The slices were then incubated in low glucose (3 mM) and Mg^{2+} -free D-hanks solution (pH 7.4) containing bicuculline (10 μ M), NBQX (5 μ M), strychnine (1 μ M), and nifedipine (10 μ M) for 15 min at room temperature to block $GABA_A$ receptors, AMPARs, glycine receptors, and VGCCs, respectively. The pH value of the incubation solution was then dropped to 6.0 for 30 min to activate ASIC1a under continuous blockade of $GABA_A$ receptors, AMPARs, glycine receptors, and VGCCs. The slices were put into pre-cold lysis buffer immediately after the acidic solution treatment. The extracted protein samples (30 μ g) from the slices were run in sodium dodecyl sulfate–polyacrylamide gels (8 %) for 1.5 h using Tris–glycine running buffer. Gels were then transferred onto a PVDF membrane in transfer buffer containing 25 mM Tris-base, 0.2 M glycine, and 20 % (v/v) methanol for 2.5 h at 70 V. The membrane was blocked with

5 % non-fat milk in $1 \times$ TBS, 0.1 % Tween-20 at room temperature (RT) for 1 h and subsequently incubated overnight at 4°C with primary antibodies for probing phosphorylated NR2B (p-NR2B) (rabbit anti-mouse 1:1000, Cell Signaling Technology, USA). After washing, the membrane was incubated with secondary antibody (Goat anti-rabbit IgG, 1:5000, ZSGB-BIO, China) for 1 h at RT. The protein signals were detected and quantification of protein levels was achieved.

Data Analysis

The data are presented as mean \pm SEM. ANOVA analysis was used for comparison among multiple groups, followed by the post hoc analysis for comparison between two groups. Student's *t* test was also used. Statistical significance was defined as $p \leq 0.05$.

Results

To explore the effect of activation of either NMDARs or ASIC1a alone, and the activation of both ASIC1a and NMDARs on neuronal damage, we used, respectively, NMDA, pH 6.0 acidic ECS, and OGD to challenge the hippocampal cultures. The parameters measured included neuronal viability, intracellular Ca^{2+} concentration increase, and apoptosis-related caspase-3 levels. Interestingly, we found that NMDARs played a pivotal role in neuronal death induced by activation of either NMDARs or ASIC1a, and even more so with activation of both.

Overactivation of NMDARs Induces Neuronal Death

It is documented that in brain ischemia, the ensuing neuronal death is due to mass glutamate transmitter release and overstimulation of NMDARs (Hardingham and Bading 2003). Using Hoechst-33342 staining, we observed that NMDA treatment of hippocampal cultures induced a $54 \pm 4\%$ neuron death with the characteristic apoptotic morphological changes (Fig. 1a, b), including cell shrinkage, nuclear condensation, and fragmentation. This NMDA-induced apoptotic cell death was effectively prevented by pre-inhibition of NMDARs with their specific antagonists APV or Ketamine (Fig. 1a, b).

LDH released into extracellular environment has been a useful indicator for evaluating cell necrosis (Xiong et al. 2004). NMDA treatment of hippocampal cultures caused a fourfold increase of LDH concentration in the extracellular medium (Fig. 1c). This increase in LDH levels was effectively prevented by blocking NMDARs prior to NMDA treatment. These data suggest that overstimulation of

NMDARs caused the cultured neurons to undergo both apoptotic and necrotic death.

Overactivation of ASIC1a also Induces Neuron Death

Excess glutamate release and acidosis often occur concurrently in brain ischemia. We treated the cultures with pH 6.0 ECS to mimic brain tissue acidosis under the blockade of AMPARs, glycine receptors, GABA_A receptors, and VGCCs, respectively. We found that acidic ECS induced an increase in apoptotic neuron death ($32 \pm 4\%$) (Fig. 2a, b), and also led to a significant increase in LDH release (\sim twofold) (Fig. 2c). The apoptotic cell death and LDH release evoked by acidic ECS were all prevented by the non-selective ASIC1a blocker amiloride or specific ASIC1a blocker PcTX1. These data suggest that overstimulation of ASIC1a induced both apoptotic and necrotic neuronal deaths in cultured hippocampal neurons.

Stimulation of ASIC1a Enhances NMDAR-Mediated Neuronal Death

The release of glutamate transmitter is commonly accompanied by the release of H^+ during synaptic activity (Krishtal et al. 1987; Wemmie et al. 2003, 2006). Our electrophysiology data from mouse brain slices showed that stimulation of ASIC1a by pH 6.0 acidic ECS enhanced synaptic NMDAR activity in the early phase (within the first 2 min after the onset of pH 6.0 ECS perfusion), but exhibited an inhibitory effect on NMDAR activity thereafter (Fig. 3a–c). The observation that H^+ inhibited NMDAR EPSCs was made by Traynelis two decades ago (Traynelis and Cull-Candy 1990, 1991). However, H^+ -enhancing NMDAR activity was observed for the first time. The most exciting finding was that H^+ made NMDAR EPSCs rise time much faster and the decay time much slower (Fig. 3d–f). These data suggest that ASIC1a activity facilitates NMDAR channel open and hinder the channel close.

The early phase of ASIC1a activation played a crucial role in the facilitation of NMDAR-mediated neuronal death, and the facilitation of the channel opening as well (Fig. 3g, h). NMDA induced a $54 \pm 4\%$ neuronal death in normal physiological pH (7.3–7.4), whereas NMDA with acidic ECS at the pH 6.0 induced a much higher rate of apoptotic cell death ($67 \pm 2.42\%$) and necrotic cell death (LDH release increased by more than sixfold).

Stimulation of ASIC1a Up-Regulates NMDAR Activity

To test the hypothesis that ASIC1a activation enhances NMDAR activity, we tried to inhibit ASIC1a activation by

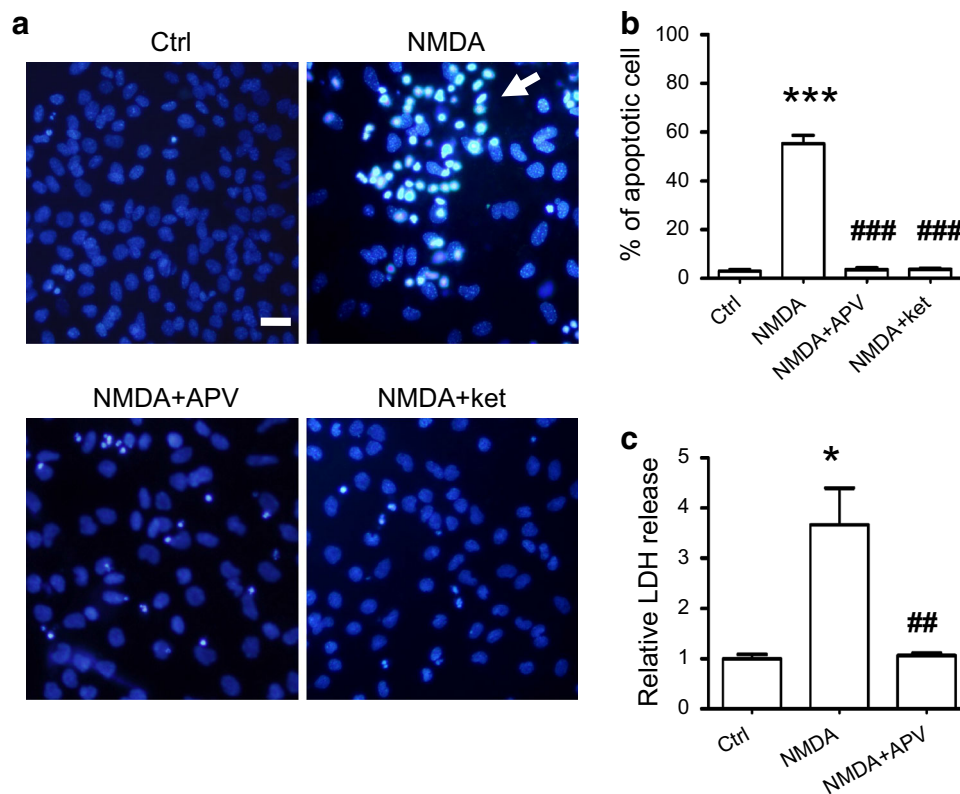


Fig. 1 Overstimulation of NMDAR induces neuronal death. **a** Hippocampal cultures (16 DIV) stained with Hoechst-33342 did not show any obvious apoptotic cell death (*upper left panel*). NMDA treatment induced a significant increase in apoptotic neuronal death as evidenced by typical morphological features of nuclear condensation and DNA fragmentation (*upper right panel*). NMDAR antagonist APV (100 μ M) or ketamine (30 μ M) prevented NMDA-induced cell deaths (*lower left and right panels*). Scale bar is 20 μ m. **b** Quantitative analysis of apoptotic death of cultured hippocampal neurons is shown in the histogram. There was a small percentage (3 %) of cell death in

control cultures ($n = 7$), whereas NMDA exposure led to a 54 % cell death rate ($n = 8$). Antagonizing NMDARs markedly reduced neuronal death to ~ 6 % ($n = 6$). $***p < 0.005$ compared with control; $###p < 0.005$ compared with NMDA treatment. **c** LDH release in cultures representing the extent of necrotic neuronal death was measured and is shown in the histogram. LDH release was fourfold higher in the NMDA-treated cultures ($n = 5$) than in the control ($n = 5$). LDH release was greatly reduced by blocking NMDARs with APV ($n = 3$). $*p < 0.05$ compared with the control; $###p < 0.01$ compared with NMDA treatment

adding amiloride or PcTX1 prior to treating the cultures with NMDA or acidic ECS. We found that the apoptotic cell death was greatly reduced to 4.1 ± 0.8 or 4.23 ± 1.7 %, and that the necrotic cell death (as indicated by LDH release) was markedly reduced as well (Fig. 4a–c) to a level comparable to the control group. These data clearly show that activation of ASIC1a greatly enhanced NMDA-induced cell death.

In another experiment, we used NMDAR antagonist to block NMDARs and treated the culture with acidic ECS. Interestingly, we found that both apoptotic and necrotic cell deaths were distinctly reduced (7 ± 0.92 %) (Fig. 4a, d, e).

Since the phosphorylation of NR2B subunits of the NMDAR was well known to correlate with cell death (Tu et al. 2010; Lai et al. 2014), we examined whether activation of ASIC1a resulted in an increase in NR2B phosphorylation. We incubated mouse hippocampal slices with normal ECS, acidic ECS, and acidic ECS containing APV (50 μ M),

respectively. We found that the slices incubated in acidic ECS had a dramatic increase in NR2B phosphorylation compared to those slices incubated in normal ECS and in acidic ECS containing APV (supplementary Fig. 1a, b).

Together, these data suggest that activation of ASIC1a up-regulated NMDAR activity and exacerbated NMDAR-mediated cell death in acidic conditions, which is consistent with previous reports that ASIC1a channels conduct negligible Ca^{2+} entry to the cells (Samways et al. 2009). We speculate that ASIC1a potentiates and/or facilitates NMDAR function in both physiological and pathological conditions. This speculation is supported by our electrophysiology experimental results from brain slices (Fig. 4f), which showed a great reduction of NMDAR EPSC amplitudes after pharmacological inhibition of ASIC1a channel activity by PcTX1 (25 nM). It is also supported by Western blot analysis results, which showed that acidic ECS treatment of the hippocampal slices induced a marked

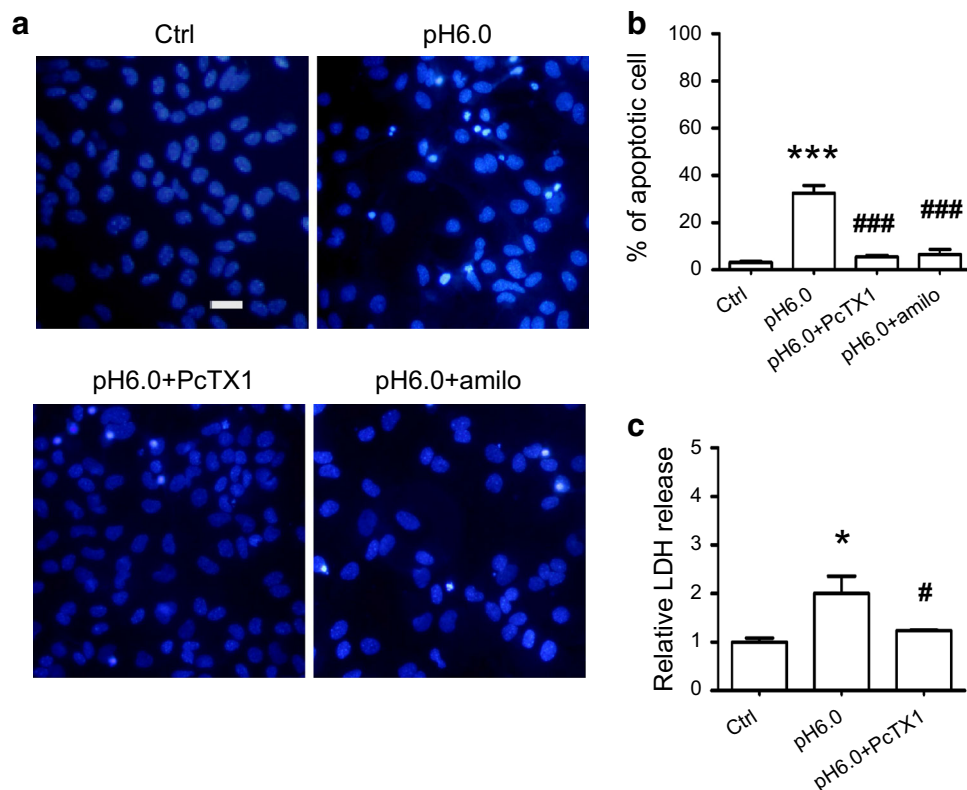


Fig. 2 Acidic ECS induces neuronal death. **a** Representative images of hippocampal neuronal cultures showing that acidic ECS (pH 6.0) treatment induced more apoptotic cell deaths (*upper right panel*) than control (*upper left panel*). Blocking ASIC1a by pre-treatment with its antagonists PcTX1 (50 nM) or amiloride (100 μ M) effectively prevented acid-induced apoptotic cell deaths (*lower left and right panels*). Scale bar is 20 μ m. **b** Quantitative analysis of apoptotic death of cultured hippocampal neurons is shown in the histogram. Acidic ECS induced \sim 32 % apoptotic cell death ($n = 4$). Blocking ASIC1a

by pre-treatment with PcTX1 ($n = 3$) or amiloride ($n = 3$) greatly reduced neuronal deaths to 5.5 and 6.6 %, respectively. $***p < 0.005$ compared with the control ($n = 7$); $###p < 0.005$ compared with pH 6.0 treated cultures. **c** The LDH release in acidic ECS-treated cultures is twofold higher ($n = 3$) than in control cultures ($n = 5$). Inhibition of ASIC1a reduced LDH release to 1.23 times that of control ($n = 3$). $*p < 0.05$ compared with the control; $#p < 0.05$ compared with pH 6.0 treated cultures

increase in NR2B phosphorylation (supplementary Fig. 1). These data provide strong support for the idea that ASIC1a activity caused neuronal death through up-regulation of NMDARs. Without activation of NMDARs, ASIC1a alone could not induce significant neuronal death.

Stimulation of ASIC1a Facilitates NMDAR-Mediated Ca^{2+} Increase

NMDARs are the main Ca^{2+} permeable ion channels that mediate Ca^{2+} overload and result in neuronal death in brain ischemia. Using NMDA to challenge hippocampal cultures caused a great intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) increase in neurons when leaving both NMDARs and ASIC1a channels unblocked (Fig. 5a, c, f). It was logical to see that the $[\text{Ca}^{2+}]_i$ increase was prevented after blocking NMDARs with ketamine, since NMDARs are the main Ca^{2+} permeable ion channels in the neurons (Mayer and Westbrook 1987; Iino et al. 1990). After inhibition of

ASIC1a with amiloride or PcTX1, NMDA could not induce any appreciable $[\text{Ca}^{2+}]_i$ increase, although NMDARs were left unblocked (Fig. 5b, c). Using pH 6.0 ECS to challenge hippocampal cultures caused a small amount of $[\text{Ca}^{2+}]_i$ increase in neurons when both NMDARs and ASIC1a channels were left unblocked (Fig. 5d); however, even this small amount of $[\text{Ca}^{2+}]_i$ increases was likely mediated by NMDARs because after blocking the NMDARs, stimulation of ASIC1a alone did not induce any appreciable $[\text{Ca}^{2+}]_i$ increase (Fig. 5d). Thus, ASIC1a channel activity plays a powerful role in facilitating NMDAR-mediated intracellular Ca^{2+} overload and neuronal death, especially in conditions of tissue acidification (Fig. 5e). We also calculated the total $[\text{Ca}^{2+}]_i$ accumulation in the neurons by measuring the area underneath the recorded curve and above the baseline. We found that $[\text{Ca}^{2+}]_i$ accumulation in the cell was 6.5-fold greater after challenge with NMDA than after challenge with pH 6.0 (Fig. 5f); $[\text{Ca}^{2+}]_i$ accumulation in the neurons was

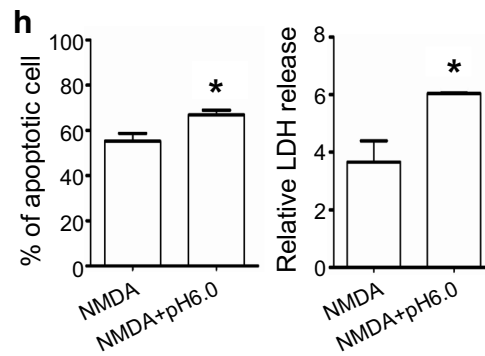
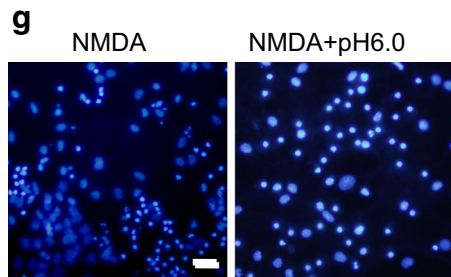
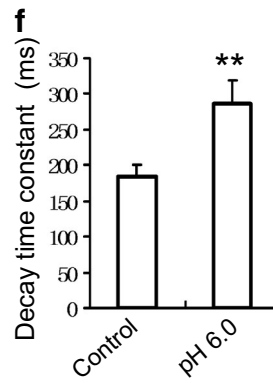
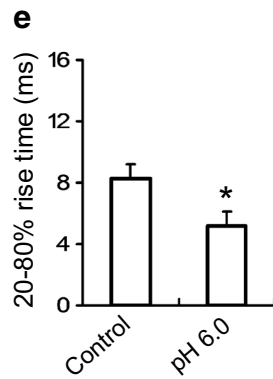
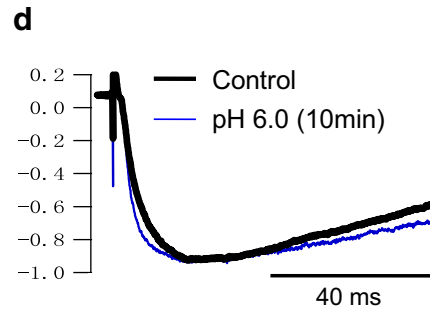
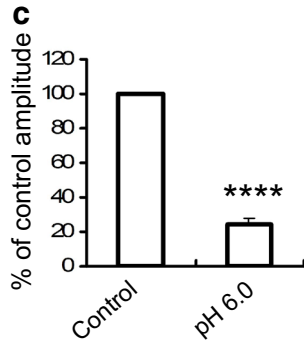
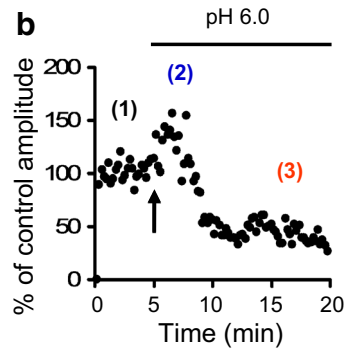
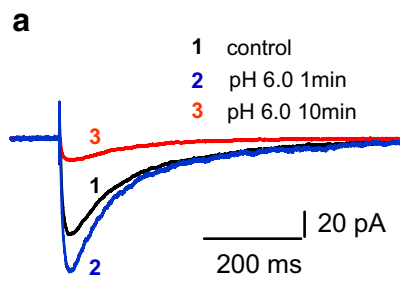


Fig. 3 Stimulation of ASIC1a enhances NMDAR activity and exacerbates NMDAR-mediated neuronal death. **a** Whole-cell voltage-clamp recordings from mouse hippocampal slices showing the amplitudes of NMDAR EPSCs in normal ACSF, in the first 2 min after onset of pH 6.0 ACSF perfusion, and in the prolonged pH 6.0 ACSF perfusion phase, as indicated by normalized traces 1, 2, and 3. **b** Dynamic changes of the amplitudes of NMDAR EPSCs in brain slices before and during pH 6.0 ACSF perfusion. (1) The 5-min baseline recording of the control NMDAR EPSC amplitudes; (2) the amplitudes of NMDAR EPSCs transiently increased at the onset of pH 6.0 ACSF perfusion; (3) the amplitudes of NMDAR EPSCs decreased in the prolonged pH 6.0 ACSF perfusion phase. *Arrow* indicates the onset of pH 6.0 ACSF perfusion. **c** Summarized histogram showing that the NMDAR EPSC amplitudes were greatly suppressed by prolonged perfusion of brain slices in pH 6.0 ACSF ($n = 5$). $****p < 0.001$. **d** Normalized traces showing that the perfusion of brain slices in pH 6.0 ACSF caused the rise time of NMDAR EPSCs to become faster and their decay time slower. **e** The 20–80 % rise time of NMDAR EPSCs in brain slices was significantly reduced by pH 6.0 ACSF perfusion compared to that of the control. $*p < 0.05$. **f** The decay time constant of NMDAR EPSCs in brain slices was significantly prolonged by pH 6.0 ACSF perfusion. $**p < 0.01$. **g** NMDA treatment-induced apoptotic neuronal death (*left panel*) was exacerbated by lowering the ACSF pH to 6.0 (*right panel*). Scale bar is 20 μm . **h** Histogram showing that the percentage of apoptotic neuronal death (*left panel*) induced by NMDA treatment ($n = 8$) was greatly enhanced by lowering the ACSF pH to 6.0 ($n = 7$). $*p < 0.05$. The LDH release induced by NMDA treatment (*right panel*) was markedly increased by lowering the ACSF pH to 6.0. $*p < 0.05$

dramatically increased to tenfold after challenge with both NMDA and acidic ECS compared to challenge with pH 6.0 alone.

These data again suggest that direct activation of NMDARs allowed much more Ca^{2+} to enter neurons than the activation of ASIC1a that indirectly up-regulates NMDAR activity. Concurrent activation of both NMDARs and ASIC1a synergistically enhanced NMDAR-mediated intracellular Ca^{2+} overload in the cells and exacerbated neuronal death.

In some experiments we tried to determine if NMDAR activation could induce Ca^{2+} release from intracellular stores; to do so we used Ca^{2+} -free ECS before and during NMDA treatment of the culture. As expected, we did not see any detectable $[\text{Ca}^{2+}]_i$ concentration increase (data not shown). These results were consistent with the cell viability assay described above and further confirmed that the increased $[\text{Ca}^{2+}]_i$ in the neuron is through NMDAR channels, not released from intracellular stores in our experimental conditions.

Stimulation of ASIC1a Enhances NMDAR-Associated Cleaved Caspase-3

A growing body of evidences has shown that activation of NMDARs induces remarkable intracellular Ca^{2+}

concentration increases in neurons, and activation of ASIC1a channels at the same time enhances NMDAR-mediated $[\text{Ca}^{2+}]_i$ increase (see Fig. 5). The excessive Ca^{2+} in the cytosol could activate Ca^{2+} -dependent proteolytic enzymes, subsequently inducing necrotic cell death by degradation of intracellular proteins. Increased $[\text{Ca}^{2+}]_i$ could also cause mitochondrial dysfunction, including loss of mitochondrial membrane potential, increased mitochondrial membrane permeability through opening mitochondrial permeability transition pore (MPTP), and leakage of apoptotic effectors such as SMACs (small mitochondria-derived activator of caspases) and cytochrome c (Mattson and Chan 2003; Halestrap et al. 2004). Those changes in mitochondria subsequently cause the activation of a group of caspases through a cascade of reactions (Fesik and Shi 2001), eventually producing a cleaved caspase-3 (the activate form of caspase-3), which is one of the main effector enzymes that carry out the cell death program. Although there exists a caspase-independent apoptotic cell death, but stroke-induced apoptosis occurs predominantly in neurons and is caspase-dependent (Broughton et al. 2009).

To investigate further the NMDAR-mediated intracellular Ca^{2+} increase that resulted in apoptotic neuronal death in hippocampal cultures, we measured the production of cleaved caspase-3 caused by excessive $[\text{Ca}^{2+}]_i$ through performing Western blot analysis using a specific antibody to probe the cleaved caspase-3. We found that NMDA treatment in pH 7.4 ECS caused a greater increase in the production of cleaved caspase-3 in cultures compared to that of control cultures (no NMDA treatment; Fig. 6a, b). This increase in cleaved caspase-3 production was markedly reduced by pre-incubation of the cultures with NMDAR antagonist ketamine. As predicted, this increase in cleaved caspase-3 production was also remarkably reduced by pre-blocking ASIC1a channels with PcTX1 (Fig. 6a, b). This set of data is consistent with the results of Ca^{2+} imaging; it is also consistent with the results of cell viability assays and electrophysiology experiments. In contrast, activation of ASIC1a channel by pH 6.0 ECS greatly enhanced the increase in cleaved caspase-3 product (Fig. 6a, d). This ASIC1a-induced increase in cleaved caspase-3 is most likely mediated through NMDAR since this increase was remarkably suppressed by blocking NMDARs (Fig. 6a, c). Together, this set of experimental results clearly demonstrated that inhibition of ASIC1a channel activity suppressed NMDAR-associated cleaved caspase-3 production, and activation of ASIC1a channel activity enhanced NMDAR-associated cleaved caspase-3 production. These data provide further supportive evidence that ASIC1a up-regulates NMDAR function in cultured hippocampal neurons.

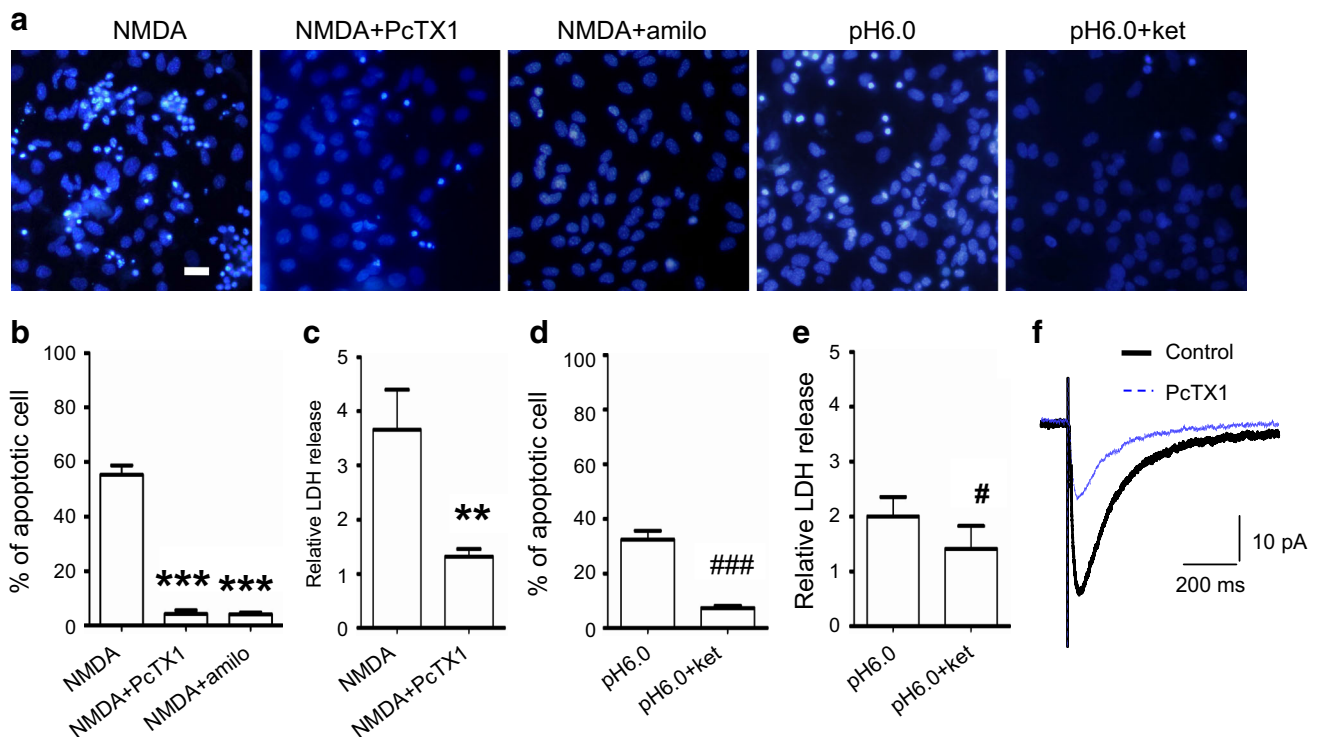


Fig. 4 Antagonizing ASIC1a suppresses NMDA-induced neuronal death. **a** Representative images showing that NMDA treatment-induced apoptotic neuronal death was prevented by antagonizing ASIC1a with PcTX1 or amiloride. However, the pH 6.0-induced cell death was also prevented by blocking NMDAR with Ketamine. Scale bar is 20 μm . **b** Quantitative analysis showing that pre-treatment with either PcTX1 or amiloride effectively prevented NMDA treatment-induced apoptotic cell deaths. $***p < 0.005$. **c** LDH release induced by NMDA (3.7-fold of the control; $n = 5$) was greatly reduced by pre-treatment with PcTX1 (1.3-fold of the control; $n = 6$). $**p < 0.01$ compared with NMDA-treated cultures. **d** Quantitative

analysis of apoptosis showing that pH 6.0 induced 32 % ($n = 4$) apoptotic cell death, which was lowered to 7.4 % ($n = 4$) by NMDAR blocker ketamine. $###p < 0.005$ compared with pH 6.0-treated cultures. **e** LDH release induced by pH 6.0 treatment was twofold ($n = 4$) that of the control ($n = 6$), whereas this increase in LDH release was brought down to 1.4-fold ($n = 3$) that of the control by blocking NMDARs with ketamine. $#p < 0.05$ compared with pH 6.0 treatment. **f** Whole-cell voltage-clamp recordings in brain slices showing that NMDAR EPSCs were markedly suppressed by PcTX1 (25 nM), which suggests that antagonizing ASIC1a prevented neuronal apoptosis via suppression of NMDAR activity

Stimulation of ASIC1a Exacerbates NMDAR-Mediated Neuronal Death in OGD

Since excessive release of glutamate and acidosis is the central features of brain ischemia, we tried to determine whether ASIC1a channel activity exacerbates NMDAR-induced neuronal death in OGD condition (an accepted in vitro model for ischemia) (Xiong et al. 2004; Gao et al. 2005; Liu et al. 2007). We first verified if NMDAR mediated neuronal death by exposing cultures to OGD condition. Indeed, OGD treatment induced a huge increase in neuronal death which was demonstrated by cell viability assays and LDH measurements. This increase in neuronal death was prevented by pre-treatment of cultures with ketamine, a NMDAR blocker (Fig. 7a–c). This data confirmed that OGD induced increased glutamate release and caused neuronal death through overactivation of

NMDARs. Next we tested if inhibition of ASIC1a activity could also prevent neuronal death—as we had observed in previous experiments—by treating the cultures with both NMDA and acidic ECS. As predicted, we did observe a great reduction in neuronal deaths in cultures when ASIC1a activity was inhibited by PcTX1. These results once again suggest that NMDAR-mediated neuronal death and ASIC1a activity up-regulated NMDAR activity in OGD condition.

Discussion

Neurotransmitters glutamate and H^+ are simultaneously released during excitatory synaptic transmission (DeVries 2001; Traynelis and Chesler 2001). The release of H^+ from presynaptic terminals may bring about a rapid pH drop at

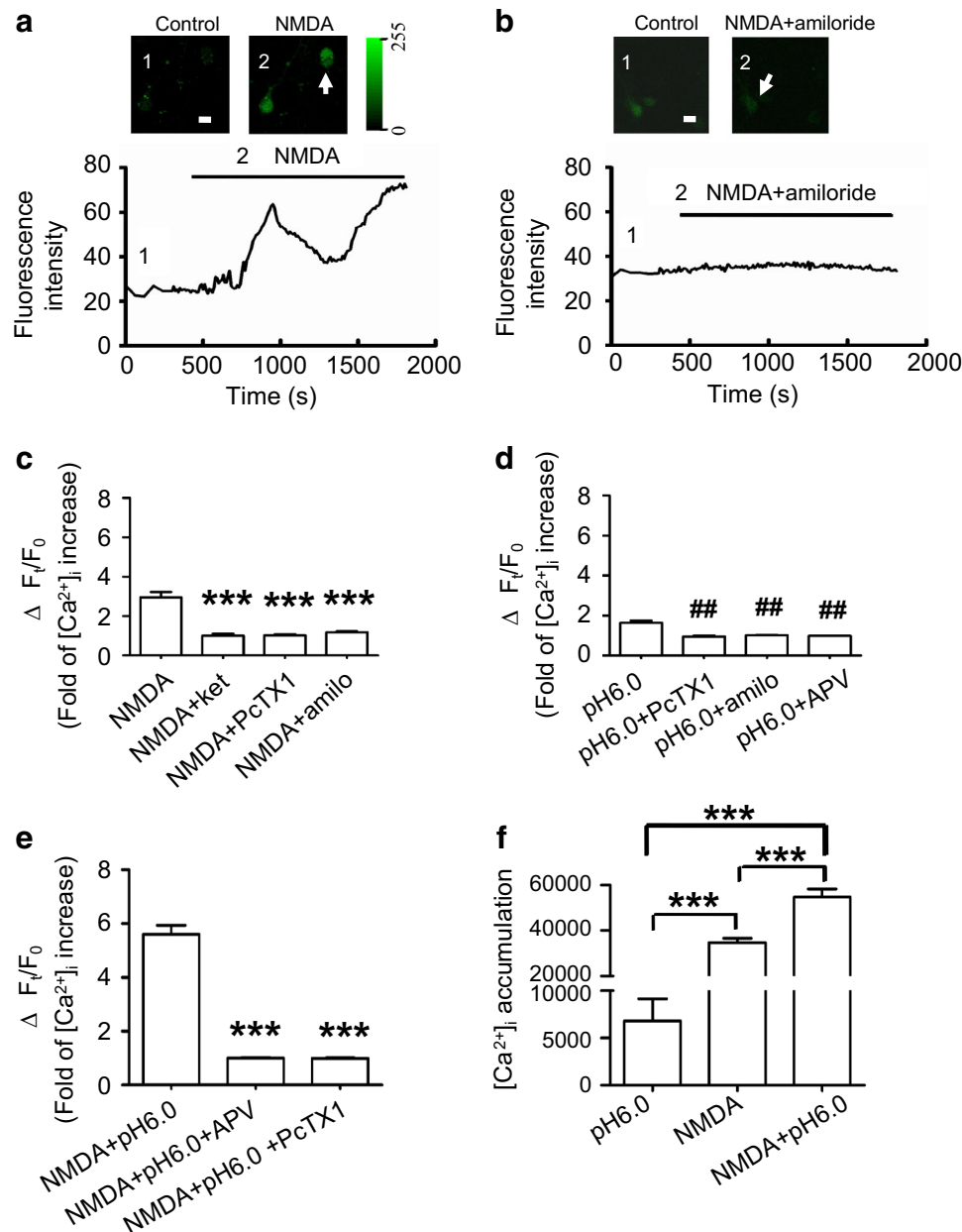


Fig. 5 ASIC1a activity facilitates NMDAR-mediated intracellular Ca^{2+} increase in the neuron. **a** Representative images showing that over-stimulation of NMDARs evoked a sustained intracellular Ca^{2+} increase in cultured hippocampal neurons under the condition of blockade of AMPARs, $GABA_A$ receptors, glycine receptors, and VGCCs (inserted images on the *top left* and Ca^{2+} increase on the *top right*), as reflected by changes in fluorescence intensity within neurons over time (*bottom trace*). Scale bar is 10 μm . **b** NMDA could not induce an intracellular Ca^{2+} increase in the neuron after pre-inhibition of ASIC1a with amiloride (inserted images on the *top left*, *top right* and the *trace at the bottom*). Scale bar is 10 μm . **c** Summarized histogram showing that NMDA induced a threefold intracellular Ca^{2+} increase ($n = 12$) compared to control. Pre-treatment with ketamine prevented this NMDA-induced Ca^{2+} increase ($n = 7$). This NMDA-induced Ca^{2+} increase was also prevented by pre-treatment with PcTX1 (onefold; $n = 21$) and amiloride (1.7-fold; $n = 12$), respectively. $***p < 0.005$ compared with NMDA treatment. **d** Summarized data showing that pH 6.0 treatment alone induced a 1.8-fold intracellular Ca^{2+} increase ($n = 29$). This effect was inhibited by

antagonists PcTX1 ($n = 25$) and amiloride ($n = 6$), as well as the NMDAR antagonist APV ($n = 10$). $^{##}p < 0.01$ compared with pH 6.0 treatment. **e** Treatment of cultures with NMDA plus pH 6.0 induced a much higher increase in intracellular Ca^{2+} (5.6-fold; $n = 13$) compared to the treatment with NMDA or pH 6.0 ECS alone (see **c**, **d**). The enhanced intracellular Ca^{2+} increase was prevented by blocking either NMDARs with APV ($n = 9$) or ASIC1a with PcTX1 ($n = 7$). $***p < 0.005$ compared with NMDA plus pH 6.0 ECS treatment. **f** Intracellular Ca^{2+} accumulation was measured and calculated by computing the area between the baseline and the fluorescence intensity traces for 25 min recordings. The area reflecting Ca^{2+} accumulation induced by NMDA treatment was 35,567 ($n = 29$), by pH 6.0 treatment was 5500 ($n = 12$), and by NMDA plus pH 6.0 treatment was 55,732 ($n = 13$). NMDA treatment alone induced a 6.5-fold higher intracellular Ca^{2+} accumulation in cultured neurons than pH 6.0 treatment alone ($***p < 0.005$), but NMDA plus pH 6.0 ECS treatment dramatically increased the intracellular Ca^{2+} accumulation to 1.57-fold that of NMDA treatment alone ($***p < 0.005$) and 10.1-fold that of pH 6.0 ECS treatment alone ($^{###}p < 0.005$)

synaptic cleft and activate ASIC1a (Waldmann et al. 1997; Xiong et al. 2004). On the other hand, glutamate molecules bind to and activate NMDARs, resulting in cell survival or cell death depending on the local environment and the amount of glutamate released (Hardingham and Bading 2003). NMDARs are highly Ca^{2+} permeable ion channels (Choi 1985, 1987; Mayer and Westbrook 1987) which can mediate large amounts of extracellular Ca^{2+} influx to neurons when they are overactivated (Manev et al. 1989; Marcoux et al. 1990). Ca^{2+} accumulates in neurons due to the inactivation of ATP-dependent ion pumps (Hardingham and Bading 2003; Brookes et al. 2004). Overstimulation of NMDARs induces excessive Ca^{2+} accumulation in neurons, which in turn triggers many downstream neurotoxic cascades. These include the uncoupling of mitochondrial electron transfer from ATP synthesis, as well as the activation and overstimulation of enzymes such as calpains and other proteases, protein kinases, nitric oxide synthase (NOS), calcineurins, and endonucleases (Brookes et al. 2004). Alterations in activity of these enzymes can lead to increased production of toxic reactive oxygen species (ROS) such as nitric oxide (NO), superoxide and hydrogen peroxide, proteins, and DNA degradation, leading to alterations in the organization of the cytoskeleton and activation of genetic signals that trigger the cell death.

In our cell viability assay experiments, we reproduced previous findings that overstimulation of NMDARs with agonist NMDA induced necrotic and apoptotic death in cultured hippocampal neurons. The NMDA-induced cell death was exacerbated when ECS's pH dropped to 6.0, which would have activated ASIC1a in the postsynaptic membrane (Krishtal et al. 1987). In addition, reducing ECS pH to 6.0 alone without adding extra NMDA to the medium also induced necrotic and apoptotic neuronal death, which is consistent with previous reports that activation of ASIC1a with acidic ECS or ischemic condition resulted in neuronal death (Xiong et al. 2004; Gao et al. 2005). Our most exciting finding is that the NMDAR-mediated neuronal death can be prevented or greatly reduced by specifically blocking ASIC1a.

When using calcium imaging to study the relationship between receptor channel activity and the pattern of neuronal Ca^{2+} influx, we found that stimulation of ASIC1a alone induced only a small intracellular Ca^{2+} increase, whereas stimulation of NMDAR channels alone evoked a 6.5-fold greater Ca^{2+} influx into neurons. Moreover, activation of both NMDAR and ASIC1a evoked a huge intracellular Ca^{2+} increase (tenfold). Most interestingly, this increase in $[\text{Ca}^{2+}]_i$ was greatly suppressed by inhibition of ASIC1a. These Ca^{2+} imaging data further support our cell viability assay finding that neuronal death due to NMDA-evoked intracellular Ca^{2+} increase can be prevented by blocking ASIC1a.

ASIC1a is Required for NMDAR Function

The release of H^+ from presynaptic terminals brings about a pH drop at synaptic cleft and activates ASIC1a (Krishtal et al. 1987; Waldmann et al. 1997; Xiong et al. 2004). The data from our patch-clamp recordings in hippocampal brain slices showed that acidic pH enhanced NMDAR EPSC amplitudes shortly after the onset of pH drop in ECS, whereas the NMDAR EPSC amplitudes were greatly reduced after keeping perfusion of the brain slice in acidic pH. However, 20–80 % rise time of NMDAR EPSCs was always reduced no matter the EPSC amplitude increased or decreased induced by acidic ECS perfusion. These data suggest that ASIC1a activity evoked by acidic ECS do enhance NMDAR function through affecting NMDAR channel open. It might be the desensitization feature of ASIC1a and the inhibitory property of H^+ on NMDAR EPSC that made NMDAR EPSC amplitude decrease after prolonged acidic ECS perfusion understandable.

Other evidences were when inhibition of ASIC1a greatly suppressed NMDAR EPSC amplitude and impeded NMDAR channel open at physiological pH (data in separate paper). Moreover, at basic pH ECS (pH 8.0), the NMDAR EPSC amplitude was greatly increased, but the 20–80 % rise time was markedly increased which means that NMDAR channel open was slowed down. These data again confirmed that ASIC1a activity plays a key role in facilitating NMDAR channel open.

In other set of experiments involving hippocampal cultures, we found that prolonged exposure to NMDA resulted in a great amount of neuronal death at pH 7.4, whereas exposure to pH 6.0 ECS alone without addition of NMDA resulted in a comparable amount of neuronal death. However, inhibition of ASIC1a abolished the NMDA-induced neuronal death. These data strongly suggest that direct overstimulation of NMDARs is responsible for the neuronal death. We hypothesized that activating ASIC1a through increasing the concentration of H^+ in ECS can indirectly activate NMDARs and cause neuronal damage.

Taken together, our data strongly suggest that NMDAR-mediated synaptic transmission is the primary excitatory pathway, and that ASIC1a activity is essential for modulating the efficiency of this transmission process. In other words, ASIC1a plays an important role in maintaining the synaptic functional homeostasis in physiological condition.

ASIC1a Exacerbate NMDAR-Mediated Neuronal Death

Overstimulation of NMDARs causes the necrotic and apoptotic neuronal deaths. Acute Na^+ influx mediates toxic neuronal swelling and largely contributes to necrotic neuronal death (Choi 1987). However, Ca^{2+} overload

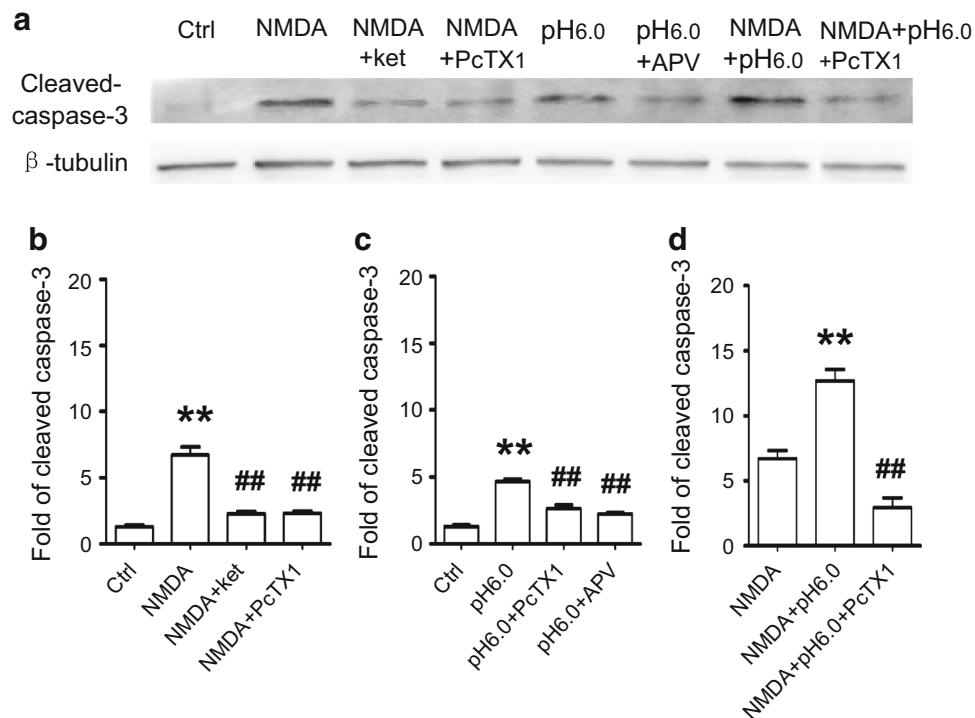


Fig. 6 NMDA-induced cleaved caspase-3 increase is enhanced by acidic ECS. **a** Antibody specifically recognizing cleaved caspase-3 (activated caspase-3, ~17 kD) was used to probe the activated form of caspase-3. β -tubulin served as a loading control for various treatments. The protein bands show that NMDA treatment of hippocampal cultures induced a dramatic increase in cleaved caspase-3 production. Ketamine (30 μ M) suppressed the NMDA-induced cleaved caspase-3 production, and PcTX1 (50 nM) also markedly suppressed cleaved caspase-3 production. Acidic ECS (pH 6.0) treatment also induced cleaved caspase-3 production. This increase was inhibited by NMDAR antagonist APV (100 μ M). NMDA plus pH 6.0 treatment of the cultures induced a dramatic increase in cleaved caspase-3, and the increase was significantly reduced by antagonizing ASIC1a. **b–d** Quantitative analysis of the protein band density is shown in these histograms. **b** NMDA treatment of hippocampal neuronal cultures induced a dramatic 6.7-

fold increase ($n = 4$) in cleaved caspase-3 compared to control (** $p < 0.01$). Blocking NMDARs with ketamine or PcTX1 distinctly reduced cleaved caspase-3 production to 2.29-fold ($n = 4$) and 2.32-fold ($n = 4$) that of the control. Either ketamine or PcTX1 significantly reduced the NMDA-induced cleaved caspase-3 production. ## $p < 0.01$. **c** pH 6.0 treatment of cultures increased the cleaved caspase-3 level to 4.7-fold that of the control (** $p < 0.01$). This large increase was lowered to twofold ($n = 4$) and 1.72-fold ($n = 4$) that of control, respectively, by either PcTX1 or APV. ## $p < 0.01$ compared with pH 6.0 ECS treatment. **d** NMDA plus pH 6.0 treatment of cultures induced a dramatic increase in cleaved caspase-3 level to 12.7-fold ($n = 4$) that of the control. This increase was distinctly higher than that of NMDA treatment alone (** $p < 0.01$). However, pre-treatment with PcTX1 effectively lowered this increase to only 2.93-fold of control ($n = 4$) (## $p < 0.01$)

mediated by NMDARs is the primary factor that causes cell death (Choi 1985, 1987). This is because Ca^{2+} entry through NMDARs is tightly linked to the overactivation of NOS, which is the most cytotoxic signaling pathway downstream from NMDARs (Sattler et al. 1999; Lau and Tymianski 2010). High $[\text{Ca}^{2+}]_i$ is deleterious to mitochondrial function because the mitochondria is the primary intracellular buffer for $[\text{Ca}^{2+}]_i$. Mitochondrial dysfunction leads to not only apoptotic neuronal death due to the release of apoptotic inducers, but also reduces ATP production and causes necrotic cell death (Szydłowska and Tymianski 2010).

ASIC1a channel is activated by a sudden pH drop from 7.4 to 6.8 and below (Waldmann et al. 1997; Yermolaieva et al. 2004; Baron et al. 2008), and then rapidly undergoes a desensitization stage (Waldmann et al. 1997; Diochot et al.

2007). The activation phase of ASIC1a may be most crucial for initiation or enhancing NMDAR function. Our electrophysiology data showed that acidic stimulation of ASIC1a immediately increased not only NMDAR EPSC amplitudes, but also reduced their rise time as described above, which is linked to NMDAR channel open kinetics (Erreger et al. 2005) and the conductance (Erreger et al. 2005). In addition, our Western blot results added more evidence that activation of ASIC1a markedly increased NMDAR's NR2B subunit phosphorylation. In contrast, blocking ASIC1a not only reduced the amplitudes of NMDAR EPSCs, but also increased their rise time. These electrophysiological data revealed for the first time the functional relationship between NMDAR and ASIC1a at the glutamatergic postsynaptic membrane. It is now easier to understand why NMDAR-mediated neuronal death is exacerbated in acidic ECS.

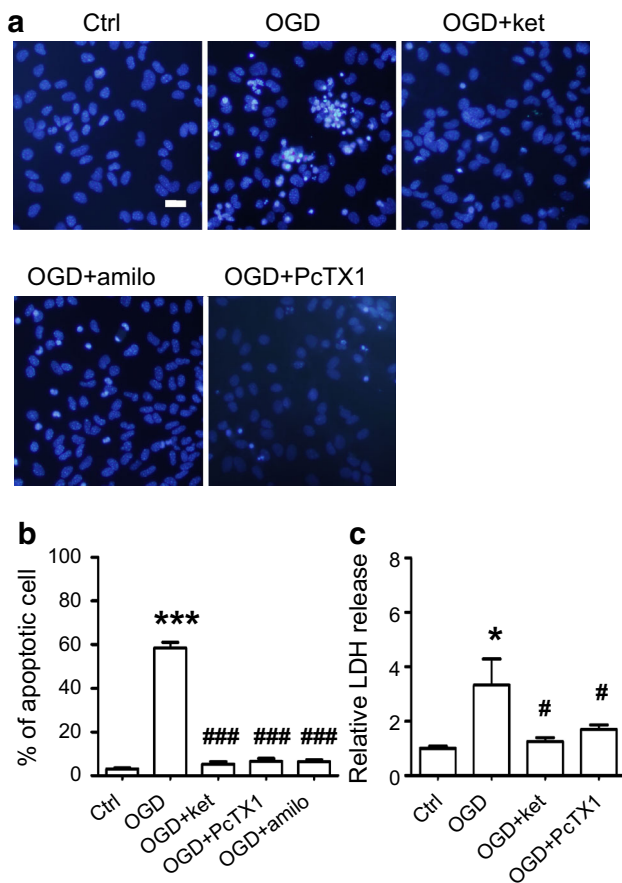


Fig. 7 Inhibition of either NMDAR or ASIC1a protected neurons from OGD-evoked cell death. **a** Representative images of Hoechst-33342 stained neurons showing that OGD induced typical apoptotic cell death (*upper middle panel*). Cell death was largely prevented by inhibition of NMDARs with ketamine (*upper right panel*), or by inhibition of ASIC1a with amiloride (*lower left panel*) or PcTX1 (*lower right panel*). Scale bar is 20 μ m. **b** Summarized data showing that OGD treatment of hippocampal neuronal cultures induced a large amount of apoptotic cell death (58.4 %; $n = 4$), which is much higher than that of control (3.1 %; $n = 7$; $***p < 0.005$). However, when NMDARs were blocked with ketamine, this OGD-evoked cell death was reduced to 5.3 % ($n = 4$; $####p < 0.005$). When ASIC1a was blocked with PcTX1 or amiloride, the cell death rate was reduced to 6.6 % ($n = 4$) and 6.5 % ($n = 5$), respectively. ASIC1a antagonists demonstrated very effective neuro-protective properties in the OGD treatment of hippocampal cultures ($####p < 0.005$). **c** LDH release is also measured in this set of experiments. Consistent with the data on apoptotic cell death, OGD induced high levels of LDH release (3.3-fold; $n = 4$) compared to control ($*p < 0.05$). As expected, NMDAR antagonists and ASIC1a antagonists suppressed LDH release in OGD-treated cultures to 1.3-fold ($n = 4$) and 1.7-fold ($n = 6$) that of control, respectively. $#p < 0.05$ compared with OGD treatment

It seems that NMDAR and ASIC1a do not induce cell death through parallel and separate pathways, because ASIC1a activation-induced cell death could be prevented by blocking either NMDARs or ASIC1a. Zha and co-workers proposed that ASIC1a induced intracellular Ca^{2+} influx largely through VGCCs (Zha et al. 2006; Samways et al. 2009). However, our data suggest that ASIC1a

induces $[Ca^{2+}]_i$ increase through activating NMDARs because we already blocked VGCCs in our experiments. It is also not very likely that ASIC1a itself mediated the large Ca^{2+} influx, since ASIC1a has negligible permeability to Ca^{2+} (Samways et al. 2009). This is illustrated in our experiments where, after blocking NMDARs, stimulation of ASIC1a could not induce either increased NR2B phosphorylation or greater neuronal deaths.

To determine whether the ASIC1a activation-induced $[Ca^{2+}]_i$ overload and neuronal death is mediated through NMDAR channels, the activity of major downstream proteins in NMDAR-related intracellular signaling pathway needed to be further examined; for example, nNOS activation or CaMKII phosphorylation.

ASIC1a Facilitates NMDAR-Mediated Ca^{2+} Entering Neurons

Previous measurements estimate the Ca^{2+} permeability of ASIC1a ($P_{Ca/Na}$) to 0.02–0.4 (Kovalchuk Yu et al. 1990; Bassler et al. 2001; Wu et al. 2004; Neaga et al. 2005), which is higher than values obtained for other members of the ASICs. However, this Ca^{2+} permeability is several hundred times less than that of NMDARs (Mayer and Westbrook 1987). It is therefore debatable whether direct Ca^{2+} entry through these channels makes a substantial and direct contribution to $[Ca^{2+}]_i$ homeostasis. Zha and co-workers reported a very small fraction of Ca^{2+} influx directly through ASIC1a receptors, most $[Ca^{2+}]_i$ got into neurons was secondary through VGCCs (Zha et al. 2006). We need to point out here that when we measured $[Ca^{2+}]_i$ changes, VGCCs were blocked by containing nifedipine in ECS. Samways and colleagues (2009) showed that native and recombinant homomeric ASIC1a did not show a measurable Ca^{2+} permeability. Most recent studies suggest that ASIC1a is not a primary mediator of $[Ca^{2+}]_i$ increase in physiological or pathological conditions (Samways et al. 2009).

Our experimental findings strongly suggest that ASIC1a channel activation can directly influence the activity of NMDARs, thereby altering their Ca^{2+} permeability and indirectly inducing $[Ca^{2+}]_i$ increases. Our novel findings may be helpful for explaining why loss of ASIC1a disrupts hippocampal-dependent LTP and spatial memory in the Morris water maze (Wemmie et al. 2002, 2003), why ASIC1a affects the density of dendritic spines development (Zha et al. 2006), and how ASIC1a causes neuronal death through disrupting $[Ca^{2+}]_i$ homeostasis in brain ischemia (Xiong et al. 2004; Gao et al. 2005; Sherwood et al. 2011). The key to answering all these questions is the discovery of the close association between ASIC1a and NMDAR channel function. Although further studies need to be conducted to ascertain whether ASIC1a and NMDAR interact directly at the cell membrane level or through

intracellular protein–protein interactions, but the most important conclusion from our study is that inhibition of ASIC1a can effectively inhibit NMDAR-mediated Ca^{2+} increase without fully suppressing NMDAR-mediated synaptic transmission.

Since neurons exposure to acidic ECS alone evoked a small amount of intracellular Ca^{2+} increased than exposure to NMDA alone, we theorize that in normal physiological condition, the activity of neurons in the neuronal network causes just enough glutamate release to meet the need for activation of NMDARs in normal physiological synaptic transmission. Extracellular pH drop may up-regulate NMDAR function by exciting ASIC1a, but the total number of activated NMDARs is not changed because there is no increased glutamate release; on the contrary, the released glutamate might be decreased (Traynelis and Cull-Candy 1990). The integrated output may be a slightly increase in intracellular Ca^{2+} as we observed in our experiments. However, adding NMDA to the medium directly activated more NMDARs and tipped off the balance toward inducing more Ca^{2+} influx into neurons. Thus, NMDA stimulation in an acidic environment can cause a much higher intracellular Ca^{2+} accumulation than does either stimulation alone. ASIC1a behaves like the gating controller for NMDARs, thereby influencing NMDAR-mediated Ca^{2+} influx.

Therapeutic Benefit of NMDAR-Associated Disease

Almost all NMDAR antagonists, except memantine (Areosa et al. 2005), have failed to pass clinical trials because of their poor therapeutic efficiency and poor tolerability (Ikonomidou et al. 1989; Ikonomidou and Turski 2002). This is understandable because NMDAR signaling plays important physiological roles in normal CNS synaptic transmission, as well as crucial adaptive processes such as synaptic plasticity, neuronal survival, and resistance to trauma (Forrest et al. 1994; Linden 1994; Kohara et al. 2001). The reason that memantine has shown the best clinical effect is because it is a low affinity and voltage-dependent unbinding NMDAR channel blocker that spares much of the synaptic NMDAR activity, but inhibits a large part of pathological chronic NMDAR activation (Parsons et al. 1999; Hardingham and Bading 2003; Areosa et al. 2005). Because brain ischemia is always accompanied by local acidosis, antagonists of ASIC1a may be ideal therapeutic agents for neuronal protection as they will not completely disrupt NMDAR-mediated synaptic transmission. When ASIC1a activity is inhibited, the CNS activates a compensate mechanism to maintain normal synaptic NMDAR function, thus minimizing disruption to the physiological function of NMDARs (Zha et al. 2006).

Therefore, ASIC1a may be an attractive therapeutic target for the treatment of brain ischemia.

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Conflict of interest We have no conflict of interest.

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