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# Acute Ethanol Exposure Promotes Autophagy-Lysosome Pathway-Dependent ASIC1a Protein Degradation and Protects Against Acidosis-Induced Neurotoxicity

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## Abstract

Tissue acidosis is a common feature of brain ischemia which causes neuronal injury. Activation of acid-sensing ion channel 1a (ASIC1a) plays an important role in acidosis-mediated neurotoxicity. Acute ethanol administration has been shown to provide neuroprotective effects during ischemic stroke, but the precise mechanisms have yet to be determined. In this study, we investigated the effect of ethanol on the activity/expression of ASIC1a channels and acidosis-induced neurotoxicity. We showed that acute treatment of neuronal cells with ethanol for more than 3 h could reduce ASIC1a protein expression, ASIC currents, and acid-induced $[Ca^{2+}]_i$ elevation. We further demonstrated that ethanol-induced reduction of ASIC1a expression is mediated by autophagy-lysosome pathway (ALP)-dependent protein degradation. Finally, we showed that ethanol protected neuronal cells against acidosis-induced cytotoxicity, which effect was mimicked by autophagy activator rapamycin and abolished by autophagy inhibitor CQ. Together, these results indicate that moderate acute ethanol exposure can promote autophagy-lysosome pathway-dependent ASIC1a protein degradation and protect against acidosis-induced neurotoxicity.

**Keywords** ASICs · Ethanol · Neurotoxicity · Autophagy · Ischemia · Neuroprotection

## Introduction

Alcohol (ethanol) is one of the most widely consumed beverages in the world. While alcohol abuse and addiction is a serious problem, a number of studies have shown that light to moderate alcohol intake is associated with a decreased risk of ischemic stroke [1–4]. Numerous studies published over the past several decades also demonstrated that acute low to moderate ethanol administration before ischemia may reduce neuronal death and protect against the harmful effects of subsequent ischemia/reperfusion (I/R) [4–9]. Furthermore, intoxicating concentrations of ethanol have been shown to inhibit NMDA receptor-mediated excitotoxicity in neuronal cultures [10, 11].

Local tissue acidosis is a common feature of brain ischemia, which plays an important role in ischemic brain injury [12]. The mechanism underlying acidosis-mediated neuronal injury remained elusive until the discovery of acid-sensing ion channels (ASICs). ASICs are proton-gated cation channels belonging to the epithelial sodium channel/Degenerin superfamily [13, 14]. So far, at least six ASIC subunit proteins (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4) encoded by four separate genes (ACCN1, ACCN2, ACCN3, and ACCN4) have been identified in mammalian central and peripheral nervous systems [15]. In the central neurons, ASIC1a and ASIC2a are the major functional ASIC subunits [16–18]. Recent studies by our own and others have demonstrated that pharmacological inhibition or genetic deletion of ASIC1a resulted in a significant reduction in acidosis-mediated and ischemic brain injury, suggesting that ASIC1a is a potential therapeutic target for brain ischemia [18–23].

It has been shown that various effects of ethanol on the nervous system may be caused by its actions on ion channels [24]. For example, ethanol not only affects voltage-gated calcium channels and sodium channels, but also glutamate

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receptor-gated channels and GABA receptor-gated channels [24]. However, the role of ethanol on ASIC1a channels remains unclear. Accordingly, we explored the effects of ethanol on ASIC1a currents, protein expression, acid-mediated $[Ca^{2+}]_i$ accumulation, and neuronal injury, using the whole-cell patch-clamp technique, biochemical/molecular biological analysis, fluorescent $Ca^{2+}$ imaging, and cell injury assays. We, for the first time, demonstrate that ethanol inhibits ASIC1a currents, ASIC1a-mediated $[Ca^{2+}]_i$ accumulation, and neuronal cell death through autophagy-dependent ASIC1a protein degradation.

## Material and Methods

### Cell Culture

Cortical neuronal culture using embryos from pregnant (E16) Swiss mice (Charles River Laboratories) was performed as described previously [25, 26]. The experimental procedure was approved by the Institutional Animal Care and Use Committee of Morehouse School of Medicine. Briefly, mice were anesthetized with isoflurane followed by cervical dislocation. Brains of fetuses were removed rapidly and placed in $Ca^{2+}$/$Mg^{2+}$-free cold phosphate-buffered saline (PBS). Cortical tissues were dissected under a dissection microscope and incubated with 0.05% trypsin-EDTA for 10 min at 37 °C, followed by trituration using fire-polished glass pipettes. Cells were counted and plated in poly-L-ornithine-coated 35-mm culture dishes ($1 \times 10^6$ cells per dish) or 24-well plates ($2 \times 10^5$ cells per well) and cultured with Neurobasal medium (Invitrogen) supplemented with B-27 (Invitrogen) and glutamine, in a humidified 5% $CO_2$ atmosphere incubator at 37 °C. The medium was changed twice per week, and neurons were used for all the assays between 12 and 16 days after plating. NS20Y cells, derived from the mouse neuroblastoma, were cultured in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% fetal bovine serum and antibiotics [27].

### Electrophysiology

ASIC currents were recorded using the whole cell patch clamp technique as described previously [21]. Patch pipettes were pulled by a two-step puller (PP83; Narishige, Tokyo, Japan) from thin-walled borosilicate glass (1.5-mm diameter, WPI, Sarasota, FL, USA). The pipettes had a resistance of 3–4 MΩ after filling with intracellular solution (in mM): 140 CsF, 10 HEPES, 11 EGTA, 2 tetraethylammonium chloride, 1 $CaCl_2$, 2 $MgCl_2$, and 4 MgATP, pH 7.3 adjusted with CsOH, and 290 to 300 mOsm adjusted with sucrose. Extracellular solution contained (in mM): 140 NaCl, 5.4 KCl, 2 CaCl2, 1 MgCl2, 10 Glucose, and 10 HEPES, pH 7.4 adjusted with NaOH/HCl; 320–330 mOsm. PcTX1 (Peptide International) was dissolved in $ddH_2O$ at 20 μM, while amiloride was dissolved in dimethyl sulfoxide (DMSO) at 100 mM before adding to extracellular solutions to obtain final working concentrations. Unless otherwise specified, all chemicals were purchased from Sigma (St. Louis, MO, USA). Whole cell patch clamp recordings were performed using an Axopatch 200B amplifier and Digi data 1320 DAC unit. Unless otherwise stated, cells were clamped at a holding potential of − 60 mV. ASIC currents were elicited by pH drops from 7.4 to 6.0. Currents were activated at least 1.5 min apart to achieve a complete recovery from desensitization. A multi-barrel perfusion system (SF-77 Warner Instruments, Hamden, CT) was used to obtain rapid changes of extracellular solutions. Voltage-gated $Na^+$ current was induced by step depolarization of the membrane potential from − 80 to − 20 mV. The data were analyzed by pClamp 9.0 and Prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

### Lactate Dehydrogenase (LDH) Assay

Cytotoxicity was measured by LDH assay, which was performed as described previously [18, 28]. After 6 h exposure to ethanol, NS20Y cells were incubated in extracellular solution (pH 7.4 or pH 6.0) for 3 h and followed by a wash and incubation in normal culture medium for 21 h. Cultured mouse cortical neurons were treated with the indicated solutions for 1 h and followed by 23 h incubation in normal medium. At the end of the experiments, 50 μl medium was transferred from each well and placed into 96-well plate for the measurement of LDH release. Cells were then incubated with Triton X-100 (final concentration 0.5%) for 30 min to obtain the maximal releasable LDH. Fifty microliters assay reagent from cytotoxicity detection kit (Roche Diagnostics) was added to each well and mixed in the dark for 30 min. The absorbance at 492 and 620 nm was detected by spectrometer (Molecular devices), and the values of the absorbance at 492 nm were subtracted by those at 620 nm to yield the values of LDH release.

### MTT Assay

NS20Y cells were plated in 96-well plates overnight and then incubated with fresh medium containing ethanol, PcTX1, or vehicle for various lengths of time. After the treatments, cell viabilities were measured using Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific). The absorbance values at 570 nm were detected by spectrometer (Molecular devices).

### Western Blotting Analysis

Protein isolation and Western blot analysis were performed as described previously [27, 29]. Briefly, cells were treated with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1%

Triton X-100, protease inhibitor, and phosphatase inhibitor cocktail). After centrifugation (at 13,000 rpm) for 20 min at 4 °C, the supernatant was collected and mixed with Laemmli sample buffer, and then boiled for 10 min. Protein concentration was detected by the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Proteins were separated by 10% SDS-polyacrylamide gels transferred to PVDF membranes. After blocking, blots were probed with antibodies against ASIC1a (rabbit anti-mouse/human, 1:1000; Gift from Dr. Xiang-Ming Zha, University of South Alabama, Mobile, AL, USA), phospho-ERK or total-ERK (Cell Signaling, 1:1000), and β-actin (Abcam, 1:1000) followed by HRP-conjugated secondary antibodies (Thermo Fisher Scientific, 1:1000). The signals were visualized by an ECL kit (Millipore). The intensity of the target protein bands was quantified by ImageJ software (NIH, Bethesda, MD).

### Immunofluorescence Staining

Neurons were cultured on coverslips (Bellco Biotechnology) for 14 days and then treated with ethanol (100 mM) for 6 h. The neurons were then fixed with acetone at − 20 °C for 15 min and permeabilized with 0.5% Triton X-100 for 10 min. After being blocked with 5% bovine serum albumin, the slides were incubated with anti-ASIC1a antibody at 4 °C overnight. The cells were then washed three times and incubated with secondary antibodies at room temperature for 1 h. After counterstained with DAPI, the samples were observed with a fluorescence microscope.

### Quantitative Real-Time PCR

Quantitative real-time PCR was performed as described in our previous studies [30]. Total RNAs were isolated by TRIzol reagent (Invitrogen), and cDNAs were synthesized using the iScript Select cDNA synthesis kit (Bio-Rad Laboratories) in accordance with the manufacturer's protocol. Quantitative real-time PCR was performed with iQ SYBR® Green supermix (Bio-Rad) in C1000™ Thermal cycler (Bio-Rad), according to the manufacturer's instructions. The PCR amplification cycles consisted of denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 5 s, and annealing/extension at 61 °C for 10 s, followed by the detection of melt curve from 65 to 95 °C. Primers were designed based on GenBank sequences and synthesized by Invitrogen: for ASIC1a, forward, 5′-GGCCAACTTCCGTAGCTTCA-3′ and reverse, 5′-ATGCCCTGCTCTGTCGTAGAA-3′; for β-actin, forward, 5′-AGCCATGTACGTAGCCATC-3′ and reverse, 5′-CTCTCAGCTGTGGTGGTGAAC-3′. β-Actin was used as an endogenous control, and ΔΔCt values were calculated after β-actin normalization. Relative levels of target mRNAs were calculated as $2^{-\Delta\Delta Ct}$.

### Fluorescein Diacetate/Propidium Iodide (FDA/PI) Staining

Cells were seeded on coverslips. After overnight, cells were treated with different conditions for 3 h and then incubated in normal medium for 21 h. For staining of live and dead cells, cultures were incubated with normal medium containing FDA (3 μM) and PI (5 μM) for 5 min. Live (FDA-positive) and dead (PI-positive) cells were observed and counted with a fluorescent microscope (Zeiss Axio Observer Z1) at excitation/emission wavelengths of 470 nm/535 nm for FDA and 585 nm/615 nm for PI.

### $Ca^{2+}$ Imaging

The intracellular $Ca^{2+}$ level of mouse cortical neurons was imaged using Fura-2 AM as described previously [18, 31]. Cortical neurons grown on poly-L-ornithine-coated 25 mm diameter glass coverslips were washed three times with ECF and incubated with 5 μmol/L fura-2 AM for 30 min at room temperature. Neurons were then washed three times and incubated in normal ECF for another 30 min. Coverslips with Fura-2 loaded cells were transferred to a perfusion chamber on an inverted microscope (Nikon TE300, Nikon, Melville, NY, USA). Neurons were illuminated using a xenon lamp (75 W) and observed with a ×40 oil-immersion objective lens. Digitized images were acquired, stored, and analyzed by Axon Imaging Workbench software (AIW, Axon Instruments). The shutter and filter wheel (Lambda 10–3, Sutter Instrument, Novato, CA, USA) were controlled by AIW to allow timed illumination of cells at either 340- or 380-nm excitation wavelengths. Images were acquired at an emission wavelength of 510 nm. Ratio images of 340 nm/380 nm were analyzed via averaging pixel ratio values in circumscribed regions of cells within the field of view.

### Statistical Analysis

All data are expressed as mean ± SEM. Groups were compared using one-way analysis of variance followed by Dunnett's test or unpaired Student's $t$ test as appropriate. $p < 0.05$ was regarded as statistically significant.

## Results

### Brief Ethanol Exposure Does Not Affect ASIC Currents but Inhibits Voltage-Gated $Na^+$ Currents

To know whether ethanol has any effect on ASIC-mediated responses, we first determined whether brief application of ethanol influences ASIC currents in cultured NS20Y cells. ASIC currents were activated by decreasing extracellular pH

from 7.4 to 6.0 [18]. As shown in Fig. 1a, b, changing extracellular pH from 7.4 to 6.0 induced large transient inward currents. Amiloride (100 μM), a commonly used nonspecific inhibitor of ASICs [27, 32, 33], potently inhibited the acid-induced currents. Perfusion of synthetic psalmotoxin-1 (PcTX1, 20 nM for 10 min), a spider toxin from the venom of tarantula *Psalmopoeus cambridgei* that specifically blocks the current mediated by homomeric ASIC1a and heteromeric ASIC1a/2b channels [32, 34], also significantly reduced the amplitude of ASIC currents in NS20Y cells (Fig. 1b). To examine the acute effect of ethanol on ASIC currents, we added ethanol (100 mM) to both pH 7.4 and pH 6.0 extracellular solutions for up to 10 min. As shown in Fig. 1c, d, f, application of ethanol did not alter the amplitude of ASIC currents in NS20Y cells. Similar to NS20Y cells, application of 100 mM ethanol for 10 min did not affect the ASIC currents in cultured mouse cortical neurons (Fig. 1e, g).

Brief exposure to ethanol has been shown previously to inhibit the voltage-gated $Na^+$ currents [35]. As a positive control, we examined the inhibition of voltage-gated $Na^+$ current by ethanol in cultured mouse cortical neurons. We compared tetrodotoxin (TTX)-sensitive $Na^+$ currents recorded in the absence and presence of ethanol (100 mM). We found that the amplitude of the $Na^+$ currents was significantly suppressed by ethanol at pH 7.4, with a 27.33 ± 2.03% inhibition observed at − 20 mV (Fig. 1h, $n = 6$). Similar to the $Na^+$ currents recorded at pH 7.4, ethanol also reduced the amplitude of the $Na^+$ currents recorded at low pH (pH 6.0, Fig. 1i). Consistent with our previous results [36], we showed that lowering pH itself can inhibit the inward $Na^+$ current (Fig. 1j). These data suggested that brief ethanol exposure (i.e., for 10 min) has an acute inhibitory effect on voltage-gated $Na^+$ currents but has no effect on ASIC currents.

## Ethanol Treatment for Several Hours Suppresses ASIC Currents and Acid-Induced $[Ca^{2+}]_i$ Elevation

Since a brief (i.e., 10 min) ethanol exposure has no effect on ASIC currents both in NS20Y cells and cultured mouse cortical neurons, we explored whether ethanol has any effect on ASICs if pretreating the cells for longer duration (e.g., 1 to 24 h). As shown in Fig. 2a, b, pretreatment of NS20Y cells with ethanol (100 mM) for > 1 h produced a time-dependent inhibition of ASIC currents. The maximal inhibition of ASIC currents was achieved with a 6-h exposure to ethanol (71.83 ± 4.07% reduction in the peak amplitude, $n = 31$). To confirm whether longer duration of ethanol exposure also produces an inhibition of ASIC currents in cortical neurons, we compared ASIC currents recorded in control group with that treated with ethanol for 30 min or 6 h. We found that treatment with alcohol for 30 min did not suppress ASIC currents (Fig. 2c, d, $n = 6$). However, the amplitude of ASIC currents was significantly suppressed (by 30.01 ± 7.98%) in neurons pretreated with ethanol for 6 h (Fig. 2e, f, $n = 22$ or 14). Next, we tested the effect of ethanol on ASIC1a-mediated $[Ca^{2+}]_i$ elevation in cortical neurons. Similar to its effect on ASIC currents, ethanol exposure for 6 h significantly inhibited acid-induced $[Ca^{2+}]_i$ increase (Fig. 2g, $n = 16$ or 11). Consistent with our previous results [18, 31], acid-induced increase of $[Ca^{2+}]_i$ in neurons was dramatically reduced by ASIC inhibitors amiloride and PcTX1 (data not shown). Together, these findings strongly suggested that treatment of neurons with ethanol for several hours can inhibit the activity/function of ASIC1a channels.

## Acute Ethanol Exposure Decreases ASIC1a Protein Expression in NS20Y Cells and Cortical Neurons

To investigate whether the inhibition of ASIC currents by ethanol is mediated by a change in ASIC1a protein expression, NS20Y cells and cortical neurons were treated with 100 mM ethanol for different durations followed by a measurement of ASIC1a protein expression with Western blot. As shown in Fig. 3a, b, ethanol treatment for 3 and 6 h caused a significant decrease in ASIC1a protein level in NS20Y cells (ASIC1a protein level was reduced by 55.08 ± 9.68 and 63.01 ± 8.79% after 3 and 6 h ethanol treatment, $n = 6$). Interestingly, 24 h of ethanol treatment did not produce a significant reduction of ASIC1a protein level. In contrast to the decrease in ASIC1a protein, however, ethanol treatment has no effect on the level of ASIC1a mRNA (Fig. 3c, $n = 5$). Similar to NS20Y cells, treatment of neurons with 100 mM ethanol for 6 h significantly decreased the ASIC1a protein expression (Fig. 3d, e).

## Ethanol Promotes ASIC1a Protein Degradation Through Autophagy-Lysosome Pathway (ALP)

Protein degradation and clearance of damaged organelles are crucial for cellular physiology, as abnormal intracellular or extracellular protein accumulation has been shown to have pathological repercussions [37, 38]. Our data shows that acute ethanol does not affect the mRNA of ASIC1a but reduces its protein level (Fig. 3a–e), indicating that ethanol may affect ASIC1a protein degradation. To characterize ASIC1a protein degradation, we treated NS20Y cells with cycloheximide for 0, 2, 4, or 6 h to block the new protein synthesis. The effect of ethanol on ASIC1a protein expression was then examined by Western blotting in the absence and presence of ethanol treatment. As shown in Fig. 4a, with the blockade of protein synthesis, the level of ASIC1a protein decreased clearly with time regardless of ethanol treatment. However, it decreased at a much faster rate in ethanol-treated cells. After a 6 h treatment, only 14.15 ± 2.58% of the initial amount of ASIC1a protein remained in ethanol-treated cells, whereas 42.61 ± 2.87% was still present in sham-treated cells (Fig. 4b, $n = 6$). These findings suggested that ethanol increases ASIC1a protein degradation.

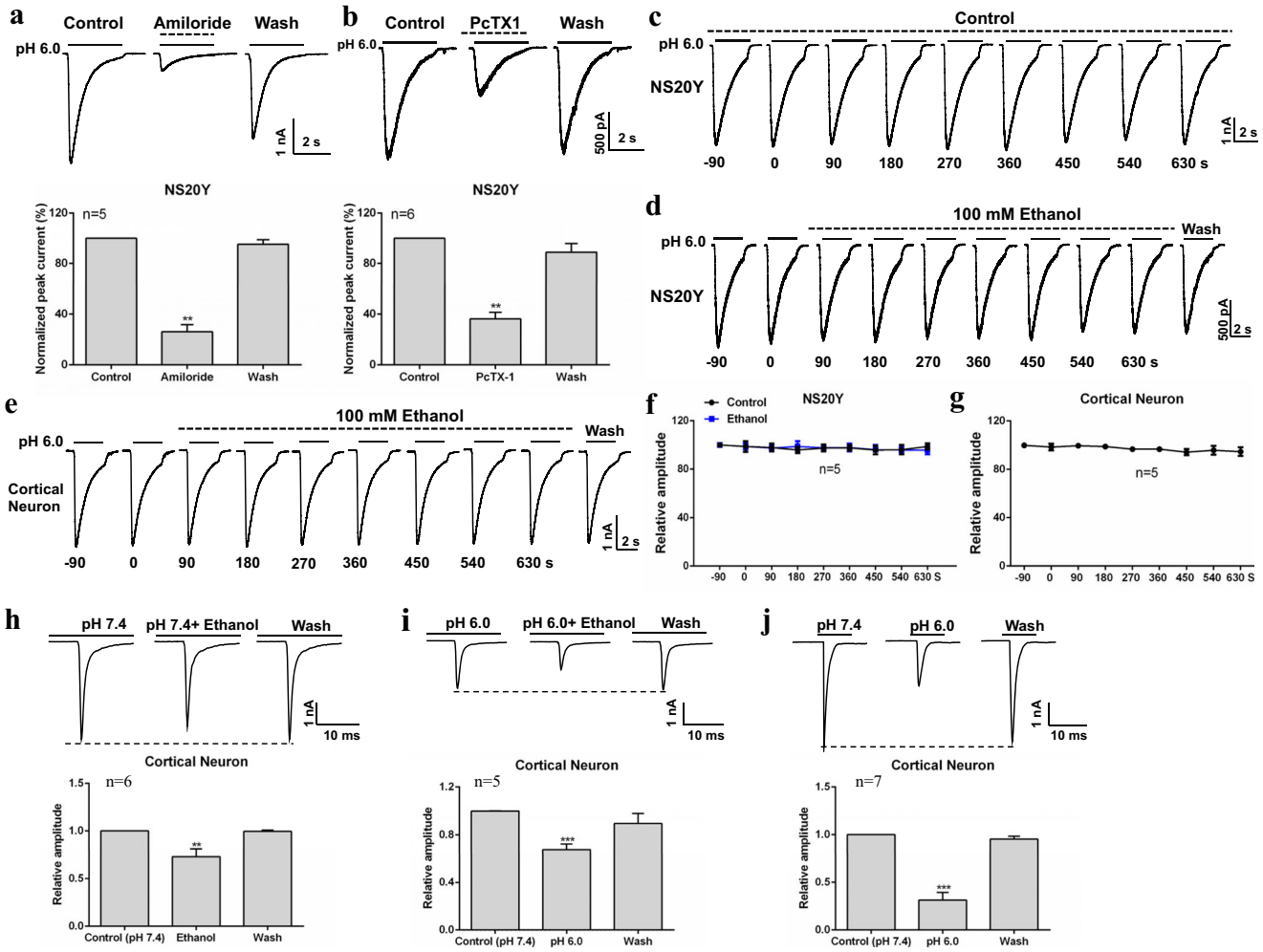


**Fig. 1** Brief ethanol exposure does not affect ASIC currents but significantly inhibits voltage-gated $Na^+$ currents. ASIC currents and voltage-gated $Na^+$ currents were examined using whole cell patch-clamp recording. **a**, **b** Amiloride and PcTX1 inhibited ASIC currents in NS20Y cells. **c**–**e** The ASIC currents were recorded in the presence of ethanol (100 mM) for 10 min in NS20Y cells and primary cultures of mouse cortical neurons. **f**–**g** Quantification of relative amplitude of ASIC currents ($n = 5$). **h**–**i** The voltage-gated $Na^+$ currents in mouse cortical neurons were evaluated in normal solution (pH 7.4) and acidic solution (pH 6.0) ($n = 6$ or 5). **j** The voltage-gated $Na^+$ currents were observed in normal (pH 7.4) and acidic solution (pH 6.0) without ethanol ($n = 7$). Data are expressed as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control

The major protein degradation pathways include ubiquitin-proteasome system (UPS), the autophagy–lysosomal pathway (ALP), and $Ca^{2+}$-activated proteases (calpain) systems [39–41]. To investigate the potential mechanism of ethanol-mediated increase of ASIC1a protein degradation, NS20Y cells were cultured for 6 h in the presence of MG-132 (10 μM) to inhibit proteasomes, chloroquine (CQ; 50 μM) to inhibit lysosomes, or *N*-[*N*-(*N*-acetyl-L-leucyl)L-leucyl]-L-norleucine (ALLN;100 μM) to inhibit the $Ca^{2+}$-activated protease calpain [38]. Figure 4c shows that inhibiting lysosomal functions and autophagy by 50 μM CQ reduced ethanol-induced ASIC1a degradation in NS20Y cells. In contrast, there was no clear effect on ethanol-induced ASIC1a degradation by proteasomes inhibitor MG-132 and calpain inhibitor ALLN (Fig. 4d, e, $n = 6$). In addition, inhibiting ALP by using 3-MA (5 mM) also attenuated ethanol-induced ASIC1a degradation (Fig. 4f, $n = 6$). Taken together, these findings indicated that ALP, but not UPS and $Ca^{2+}$-activated protease pathways, likely participates in ethanol-induced ASIC1a protein degradation.

## PKA but Not ERK Signaling Was Involved in Ethanol-Induced Change of ASIC1a Protein Expression

ERK signaling pathway is involved in ethanol-mediated change of some protein expression [42–44]. To determine whether ERK signaling was required for the decrease of ASIC1a protein by ethanol, we examined the change of phosphorylated form of ERK using phospho-specific antibodies and MEK1/2-specific inhibitor (U0126; 10 μM) which selectively blocks this signaling pathway. After treatment with ethanol, there was a rapid decrease in p-ERK1/2 protein level without a change in the level of total ERK1/2

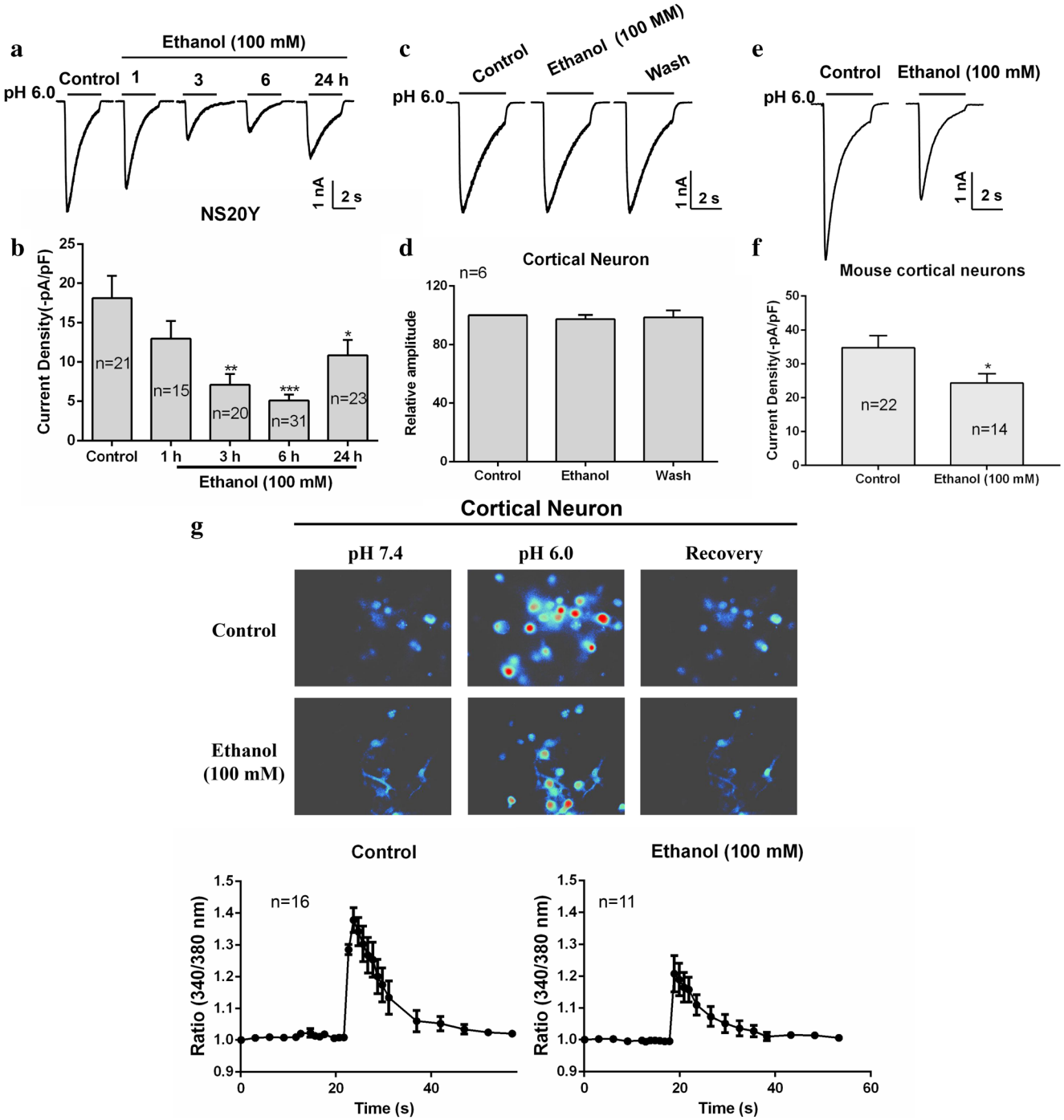


**Fig. 2** Ethanol suppresses ASIC currents and acid-induced $[Ca^{2+}]_i$ elevation. **a**–**f** ASIC currents were recorded at various time points after ethanol treatment in NS20Y cells (1, 3, 6, and 24 h) and cultured mouse cortical neurons (30 min and 24 h). **g** The acid-activated $[Ca^{2+}]_i$ elevation in cortical neurons was measured after ethanol treatment (6 h). Data are expressed as mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control

(Fig. 5a, $n = 5$). As shown in Fig. 5b, treatment of NS20Y cells with U0126 alone did not change ASIC1a protein expression and did not affect ethanol-induced reduction of ASIC1a protein level, suggesting that ERK pathway is not involved in ethanol-mediated change of ASIC1a protein expression.

cAMP-dependent protein kinase A (PKA) is a negative regulator of autophagy [45]. To know whether PKA signaling was involved in ethanol-mediated reduction of ASIC1a expression, we tested the effect of PKA inhibitors (H-89, 10 μM; KT 5720, 1 μM) and activator (Forskolin, 10 μM; cpt-cAMP, 200 μM) on ethanol-induced reduction of ASIC1a

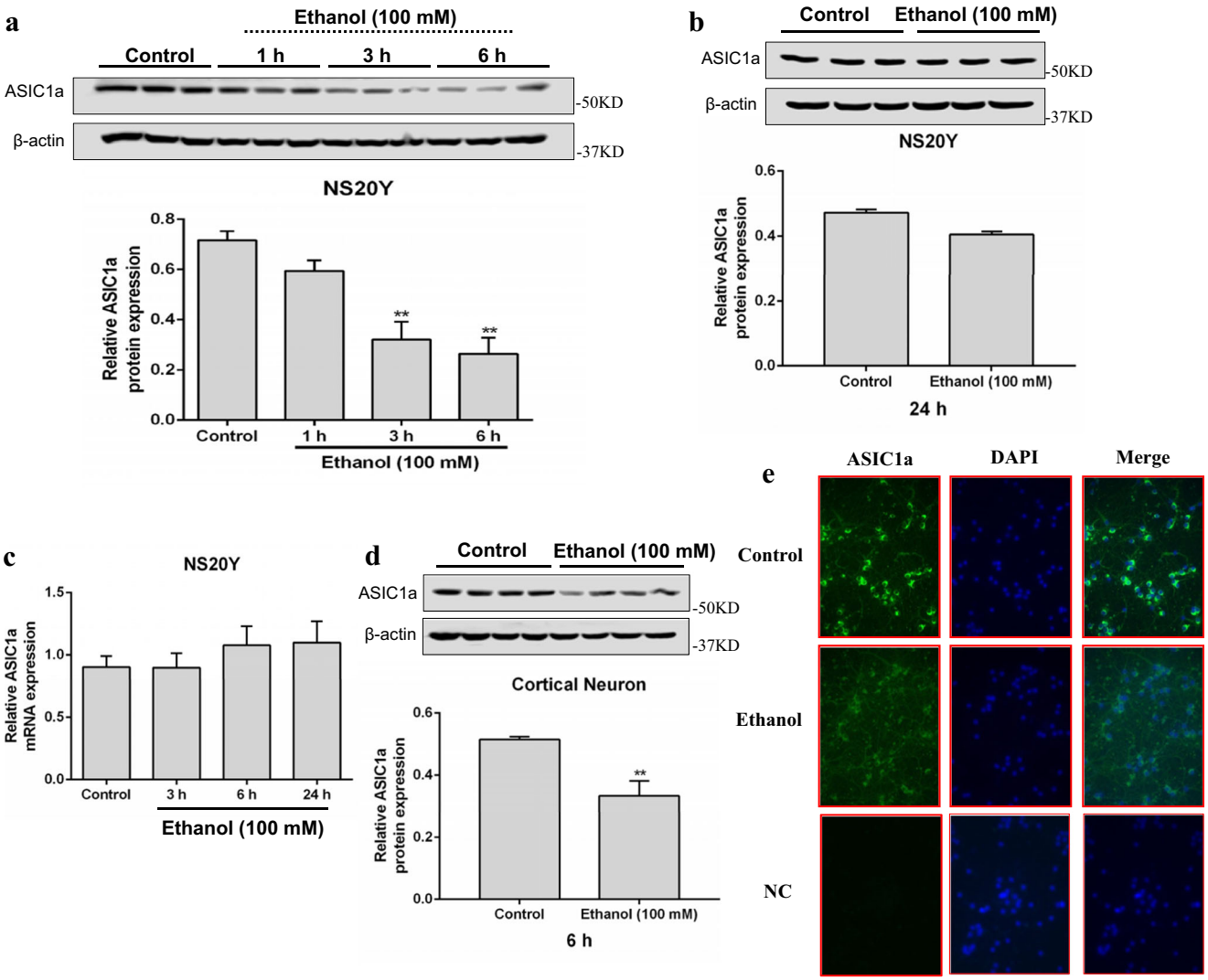


**Fig. 3** Ethanol downregulates ASIC1a protein expression in NS20Y cells and cortical neurons in a time-dependent manner. **a**, **b** The levels of ASIC1a protein expression were detected at various time points after 100 mM ethanol (1, 3, 6, and 24 h) in NS20Y cells ($n = 6$). **c** NS20Y cells were treated with ethanol for different times (3, 6, 24 h) followed by detecting mRNA expression with qRT-PCR ($n = 5$). **d**, **e** Ethanol-induced change in ASIC1a protein expression in cortical neurons was analyzed by Western blot and immunofluorescence staining ($n = 7$). Representative images from each group are presented (×200 magnification). NC negative control. Data are expressed as mean ± SEM. $^{**}P < 0.01$ versus control

protein expression in NS20Y cells. As shown in Fig. 5c, d, pretreatment with forskolin or cpt-cAMP significantly attenuated ethanol's effect on ASIC1a protein expression. In contrast, pretreatment with KT5720 or H-89 enhanced reduction of ASIC1a by ethanol (Fig. 5e, f, $n = 6$). These results, combined with the finding that ERK inhibition does not affect the ethanol-induced change in ASIC1a protein expression, suggested that PKA but not ERK pathway was involved in ethanol-induced inhibition of ASIC1a protein expression.

## PKA Activation Suppresses Ethanol-Induced Autophagy in NS20Y Cells

Autophagy is a conserved degradative process that plays an important role in the regulation of cellular homeostasis [46]. Because the autophagy inhibitor CQ inhibited ethanol-induced ASIC1a protein degradation, we further determined whether ethanol can indeed induce autophagy in our experimental system. Microtubule-associated protein 1A light chain 3 (LC3), a homolog of yeast autophagy-related gene 8 (Atg8), can be localized to autophagic membranes and is associated with the dynamic process of autophagosome formation [47]. When autophagy is activated, cytosolic LC3-I is conjugated to phosphatidylethanolamine (PE) to become LC3-II. Thus, LC3 conversion (from LC3-I to LC3-II) and lysosomal degradation of LC3-II reflect the progression of autophagy, while detecting LC3 by immunoblot analysis is often used to monitor the autophagic activity [48]. In addition, p62, also called sequestosome 1 (SQSTM1), is degraded by autophagy and serves as another marker of autophagic flux [48]. As an autophagy inhibitor, CQ acts by inhibiting

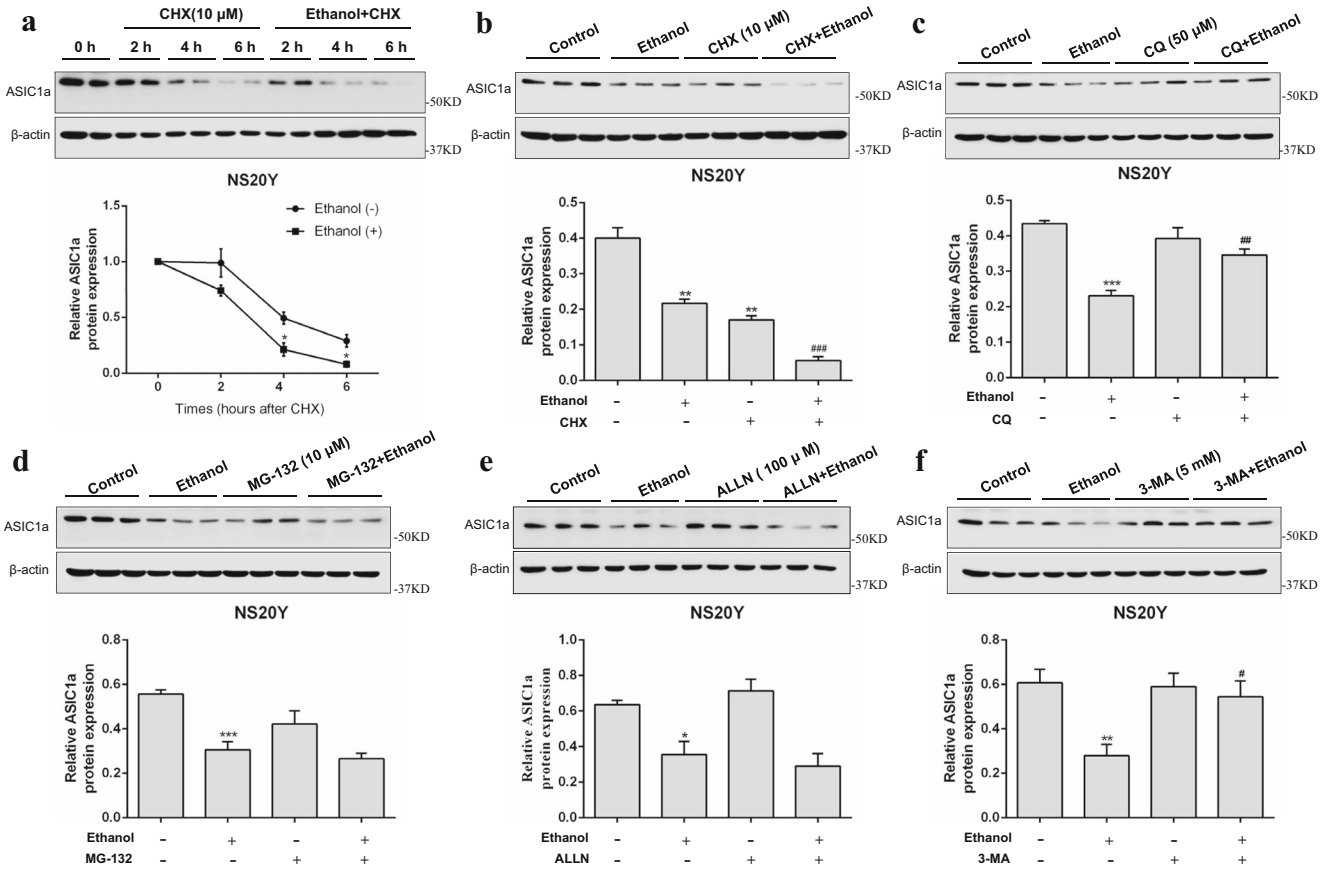


**Fig. 4** Ethanol accelerates ASIC1a protein degradation through autophagy-lysosome pathway. **a** NS20Y cells were treated by cycloheximide (CHX) with or without ethanol treatment for 0–6 h ($n = 6$). *$P <$ 0.05 versus CHX (4 h) or CHX (6 h). **b** Ethanol-induced change in ASIC1a protein expression was assessed after 6 h CHX treatment in NS20Y cells ($n = 6$). **c**–**f** The ASIC1a protein expression was analyzed by Western blot after treatment with lysosomal inhibitor CQ, MG-132, ALLN, or 3-MA with or without ethanol ($n = 6$). Data are expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus control. $^{\#}P <$ 0.05, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus ethanol

lysosomal activity, which is the last step in the autophagic degradation process [49]. Inhibition of lysosomal degradation by CQ can result in accumulation of LC3-II [50]. To further determine the involvement of autophagy in ethanol-mediated degradation of ASIC1a, the levels of LC3 and p62/SQSTM1 were examined by Western blotting after 6 h of ethanol treatment. As shown in Fig. 6a, incubation of NS20Y cells with 100 mM ethanol caused an increase in LC3B-II, whereas the level of SQSTM1/p62 was decreased, consistent with an increased activity of autophagy. As expected, inhibition of lysosomal degradation function by CQ (50 μM) resulted in an increase of LC3B-II, and the relative increase of LC3B-II was further enhanced by ethanol (Fig. 6b, $n = 5$). These data suggested that ethanol could enhance autophagy in NS20Y cells.

It is well known that PKA pathway is a negative regulator of autophagy [51]. Consistent with an involvement of PKA pathway, we found that inhibition of PKA with H-89 enhanced ethanol-induced accumulation of LC3B-II, while activation of PKA with forskolin inhibited ethanol-induced accumulation of LC3B-II (Fig. 6c, d, $n = 5$).

## Acute Ethanol Exposure Attenuates Acidosis-Induced Cytotoxicity

Activation of ASIC1a plays an important role in acidosis-mediated neuronal injury [18]. Since acute ethanol treatment reduces ASIC1a protein expression, we examined the potential effect of ethanol treatment on acid-induced toxicity of cultured mouse cortical neurons and NS20Y cells. MTT assay showed that cell viability was not affected by 6 h ethanol treatment alone at 6.25–100 mM (Fig. 7a, $n = 6$). Compared to cells treated at pH 7.4, 3 h acid incubation (pH 6.0) decreased cell viability which was inhibited by ASIC1a inhibitor psalmotoxin (PcTX1) (Fig. 7b, $n = 6$), confirming that activation of ASIC1a channels largely mediates acidosis-induced cytotoxicity [18, 31]. Consistent with its inhibition of ASIC1a protein expression, ethanol pretreatment (100 mM, 6 h) significantly attenuated acidosis-induced decrease in cell viability (Fig. 7b). In addition to cell viability assay, we analyzed acid-mediated cell injury and the effect of ethanol treatment by measuring LDH release [18, 52] in cultured neurons

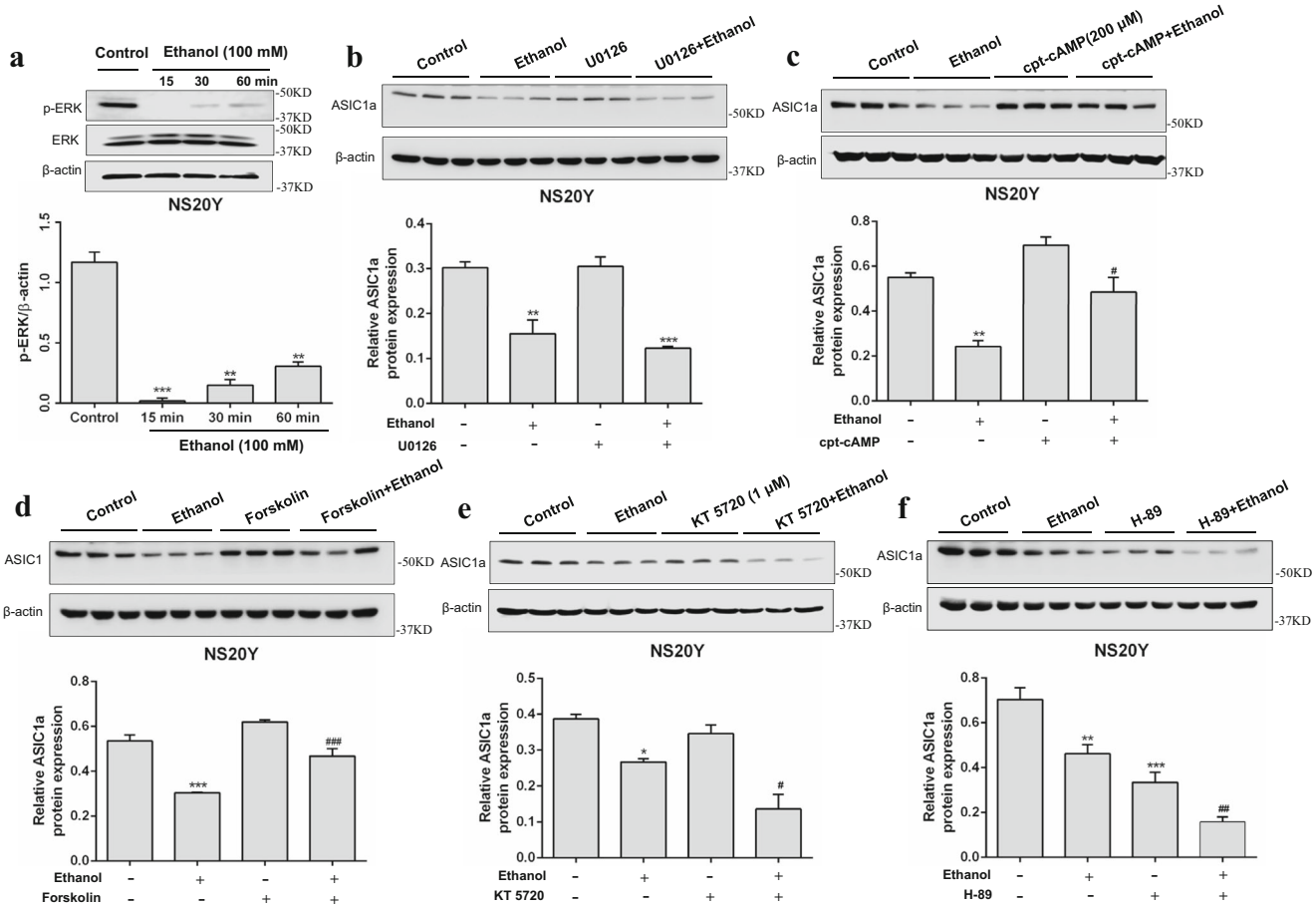


**Fig. 5** PKA is involved in ethanol inhibition of ASIC1a protein expression. **a** NS20Y cells were treated without or with ethanol for 15–60 min, and the levels of phosphorylated and total ERK were measured by Western blot ($n = 5$). **b** Ethanol-induced change of ASIC1a protein expression was assessed after pretreatment with ERK inhibitor U0126 in NS20Y cells ($n = 6$). **c**, **d** Ethanol-induced change in ASIC1a protein expression was measured after pretreatment of PKA activators (cpt-cAMP, forskolin) in NS20Y cells ($n = 8$). **e**, **f** Ethanol-induced change in ASIC1a protein expression was measured after pretreatment of PKA inhibitors (KT5720, H-89) in NS20Y cells ($n = 6$). Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus ethanol, forskolin, KT5720, or H-89

and NS20Y cells. As shown in Fig. 7c, d, 3 h acid incubation dramatically increased LDH release in both cells. As expected, addition of PcTX1 largely attenuated acidosis-induced cell injury (Fig. 7b–f, $n = 6$ or 8). Like PcTX1, treatment of cells with ethanol also reduced LDH release. To provide more evidence that ethanol reduces acidosis-mediated cell injury, we also stained live and dead cells with fluorescent dyes FDA and PI, respectively [28]. As shown in Fig. 7e–h, PI-positive cells were increased in pH 6.0 group and were reduced by ethanol pretreatment in both cortical neurons and NS20Y cells, providing further evidence that ethanol has a protective effect against acidosis-mediated cell injury. Combined treatment of ethanol and PcTX1 did not produce additional protection beyond PcTX1 alone, suggesting that ethanol treatment could attenuate acidosis-induced, ASIC1a-mediated cell death.

Finally, we determined whether the agents that activate or inhibit autophagy have any effect on acid-induced cytotoxicity and whether they influence ethanol's effect. Similar to ethanol, treatment of cells with rapamycin (Rapa), an autophagy activator [38], increased cell viability and reduced acid-induced LDH release in NS20Y cells and cortical neurons. Addition of ethanol with Rapa did not produce significant change in cell viability or LDH release compared to Rapa alone (Fig. 8a, b, $n = 8$ or 9). Treatment of cells with CQ, an autophagy inhibitor, did not affect the cell viability or LDH release; however, it eliminated the protective effect of ethanol on acid-induced cytotoxicity (Fig. 8a–e, $n = 8$ or 9) in NS20Y cells and cortical neurons. Collectively, these results indicated that ethanol protected acid-induced cytotoxicity by accelerating ASIC1a protein degradation in an autophagy-dependent manner.

## Discussion

In the present study, we explored the effect of ethanol on the activity/expression of ASIC1a channels and acid-induced neurotoxicity. In addition, we investigated whether ALP was involved in ethanol-induced change of ASIC1a protein

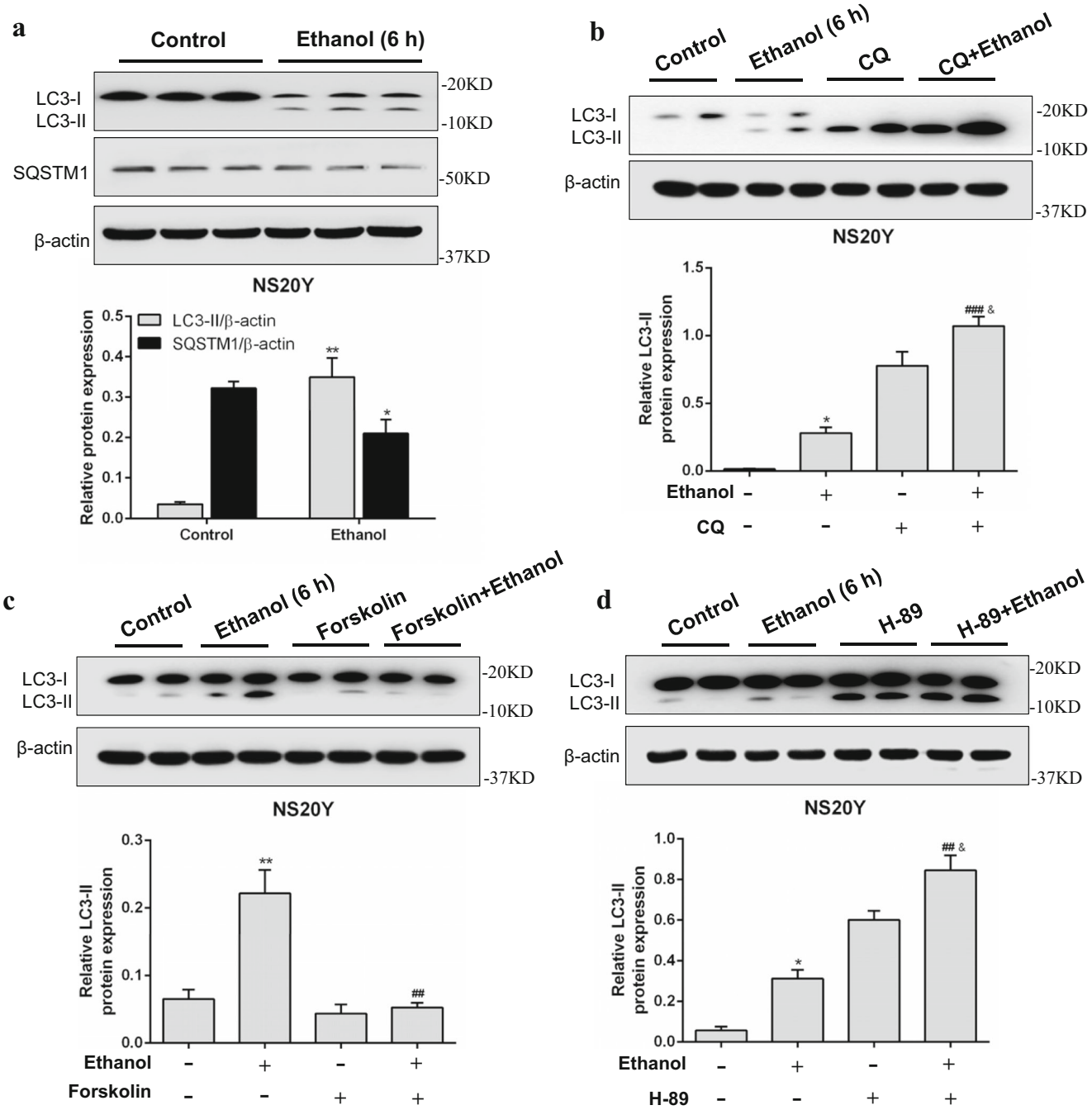


**Fig. 6** PKA activation prevents ethanol-induced autophagy in NS20Y cells. **a** NS20Y cells were treated with ethanol for 6 h, and the levels of LC3-II and SQSTM1 were measured by Western blot ($n = 6$). **b** The levels of LC3-II were determined after treatment of autophagy inhibitor CQ in NS20Y cells ($n = 5$). **c**, **d** The levels of LC3-II were detected after treatment with PKA activator (forskolin) and inhibitor (H-89) in NS20Y cells ($n = 5$). Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus ethanol. $^{\&}P < 0.05$, versus CQ or H-89

expression and function. The results showed that brief ethanol treatment (less than 60 min) has no effect on ASICs in NS20Y cells and cortical neurons. However, it could reduce ASIC1a protein expression and ASIC currents if pretreated for more than 3 h. In addition to ASIC currents, pretreatment of ethanol also suppressed acid-induced $[Ca^{2+}]_i$ elevation in neurons. We further demonstrated that ethanol inhibition of ASIC1a protein expression was mediated by ALP-dependent protein degradation. Moreover, PKA but not ERK pathway was involved in ethanol-mediated reduction of ASIC1a protein expression. Finally, we showed that ethanol protected NS20Y cells and cortical neurons against acid-induced cytotoxicity, which effect was abolished by autophagy inhibitors (e.g., CQ) and enhanced by autophagy activator rapamycin. These

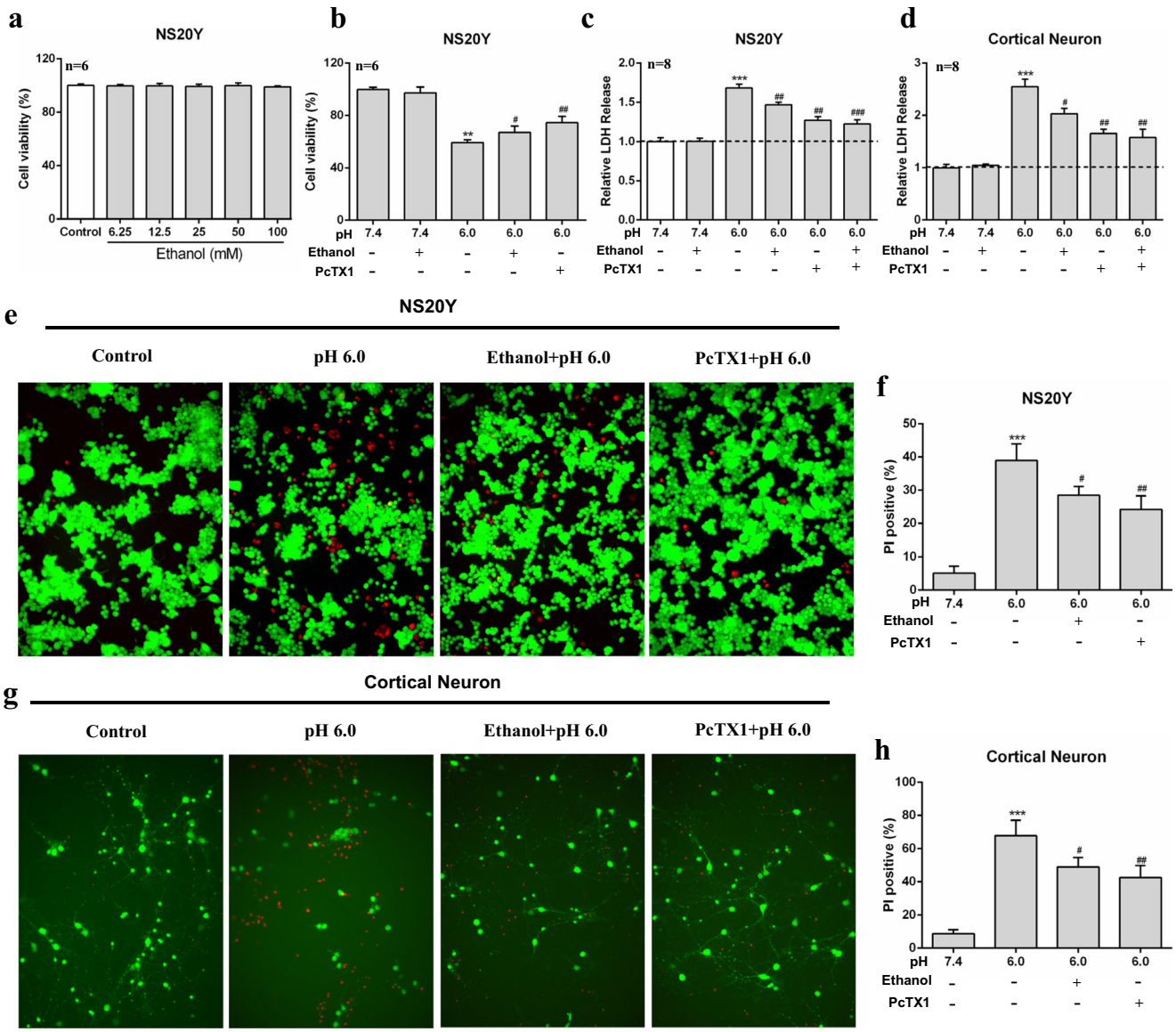


**Fig. 7** Ethanol attenuates acidosis-induced cytotoxicity in NS20Y cells and mouse cortical neurons. **a**–**d** Cell viability and cytotoxicity were assessed with the MTT and LDH assays, respectively, in NS20Y cells and primary cultures of mouse cortical neurons. **e**, **g** Representative images of acid-induced injury with fluorescein diacetate (FDA) staining of cell bodies of alive neurons and propidium iodide (PI) staining of nuclei of dead cells (×200 magnification). **f**, **h** Summary data showing the ratio of dead to alive cells. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control or pH 7.4. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus pH 6.0

results indicated that ethanol can inhibit ASIC1a channel function via autophagy-lysosome-dependent ASIC1a protein degradation.

ASICs are a family of proton-gated cation channels widely distributed in neurons of peripheral sensory and central nervous systems. Among them, ASIC1a channel has received much attention, due to its high $H^+$ sensitivity and permeability to both $Ca^{2+}$ and $Na^+$. Activation of ASIC1a by acid plays indispensable roles in pathophysiological processes such as brain ischemia, traumatic brain injury, and multiple sclerosis [18, 53, 54]. Results of our previous studies have shown that activation of ASIC1a is largely responsible for glutamate-independent, acidosis-mediated, ischemic brain injury [18, 31]. Many reports have shown that high concentrations of ethanol not only affected voltage-gated ion channels but also ligand-gated ion channels [24]. In addition, moderate ethanol exposure has been shown to protect neurons against NMDA receptor-mediated excitotoxicity [10, 11]. However, the role of ethanol in modulating the activity/function of ASIC1a channels remains unknown. Our present results show that brief ethanol exposure has no effect on ASIC1a current, but hours of ethanol exposure could reduce ASIC1a current and ASIC1a-mediated $Ca^{2+}$ influx. In contrast to ASIC1a, brief ethanol exposure has a clear inhibitory effect on TTX-

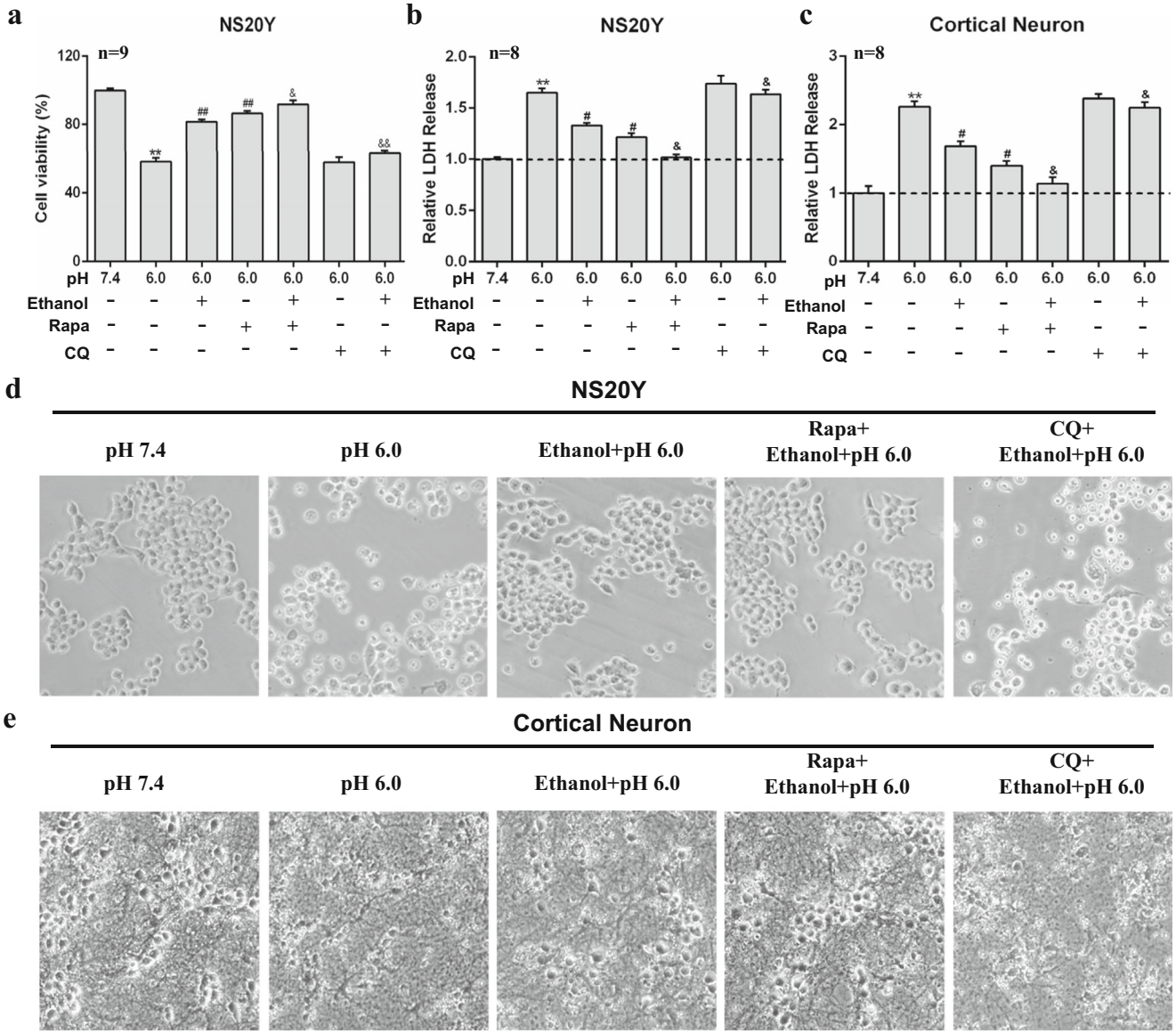


**Fig. 8** Ethanol attenuates acidosis-induced cytotoxicity via autophagy-dependent ASIC1a protein degradation. **a**–**c** Cell viability and cytotoxicity were assessed with the MTT and LDH assays, respectively, in NS20Y cells and primary cultures of mouse cortical neurons. **d**, **e** Representative phase-contrast images showing NS20Y cells and cultured mouse cortical neurons taken after treatment with the indicated solutions (×200 magnification). Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control or pH 7.4. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus pH 6.0. $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ versus pH 6.0 + ethanol

sensitive $Na^+$ currents. The inhibitory effect of ethanol on $Na^+$ current occurs at both normal and low pH, indicating that the lack of effect by brief ethanol exposure on ASIC1a current is not a cause of low pH.

Several studies have suggested the benefits of moderate alcohol intake in reducing heart disease and neurodegeneration [2, 7, 55]. For example, moderate ethanol consumption has been shown to exhibit neuroprotection by preconditioning the brain against ischemic stroke [1, 4, 8, 56]. Patients with severe brain damage have been shown to have higher survival rate with positive blood alcohol levels (BAL) [1]. These findings indicate that moderate consumption of ethanol has a neuroprotective role against brain damage. However, the potential neuroprotective role of moderate ethanol exposure in acidosis-induced neurotoxicity remains unclear. For in vitro studies, a concentration range of 10–100 mM ethanol is considered physiological [57–59]. The length of acute ethanol treatment ranges from seconds to hours, and it is currently accepted that ethanol exposure for up to 24 h is considered as an acute exposure [59]. Consistent with previous report [60, 61], the concentration of ethanol used in our studies (100 mM) did not change the viability of NS20Y cells after 6 h treatment, suggesting that ethanol itself has no toxic effect in this in vitro model (Fig. 7a). However, ethanol pretreatment could inhibit

acid-induced decrease of cell viability and increase of LDH release (Fig. 7b–h). Consistent with a reduction of ASIC1a activity/expression by ethanol, relative protection against acid-induced injury by PcTX1 is higher in the absence of ethanol than that in the presence of ethanol. Together, these findings indicated that ethanol can protect against acid-induced cytotoxicity through a reduction of ASIC1a channel function. Future studies will determine whether moderate alcohol exposure reduces ASIC1a expression in vivo and improves neuronal survival and the related neuropathologic changes against acidosis-induced neurotoxicity.

Regulation of protein degradation is important for maintaining proper cellular signaling and normal growth of all cells. Protein degradation in eukaryotic cells is mainly mediated by ubiquitin-proteasome system (UPS) and autophagy-lysosome pathway (ALP) [40, 41]. In general, proteasomes can degrade individual cellular proteins in a highly targeted fashion through UPS, while lysosomes non-selectively degrade random cytoplasmic components and organelle engulfment via autophagy [40]. Both UPS and autophagy are key factors to control protein quantity and quality. Recently, there have been some reports showing that amiloride-sensitive epithelial sodium channels (ENaC) could be degraded by UPS [62]. Our present study demonstrates that ethanol could induce ASIC1a protein degradation. Lysosomes inhibitor (CQ) and autophagy inhibitor (3-MA) abolish this effect of ethanol, while the inhibitors of proteasomes (MG-132) and $Ca^{2+}$-activated protease calpain (ALLN) have no effect on ethanol-induced ASIC1a protein degradation. Taken together, these findings indicated that ethanol could promote ASIC1a protein degradation via autophagy.

Autophagy has recently received increasing attention because it plays an important role in the pathogenesis of many diseases including stroke. It can be stimulated by various forms of cellular stress such as nutrient or growth factor deprivation, oxidative stress, and hypoxia [63]. Several studies have shown that acute ethanol treatment could increase the expression of autophagy-related genes [63, 64]. Consistent with previous studies, our results also demonstrated that ethanol treatment for several hours led to an increase in the LC3-II expression and a decrease in the SQSTM1/p62, suggesting that acute ethanol could induce autophagy in neuronal cells.

cAMP-activated protein kinase A (PKA) signaling controls multiple cellular homeostasis and processes. Recent studies have shown that PKA pathway acts as a novel negative regulator of autophagy [51, 65]. Our present results also showed that the effect of ethanol was attenuated in the presence of PKA activator but enhanced in the presence of PKA inhibitor, suggesting that PKA pathway is involved in ethanol-induced degradation of ASIC1a protein. Despite the frequent use of H-89 as a blocker of PKA, it is not a specific blocker of PKA. It has been shown that H-89 inhibits at least eight other kinases [66]. It is therefore likely that PKA-independent pathways may have partially contributed to its effects in the present study. Thus, while recognizing its PKA inhibiting property, it is important that H-89 should be used in conjunction with other PKA inhibitors, such as KT 5720. KT5720 is a potent, specific, and cell-permeable inhibitor of protein kinase A (Ki = 56 nM). We found that both H-89 and KT5720 enhanced ethanol-reduced ASIC1a protein expression. However, H-89 but not KT5720 treatment alone had an effect on the expression of ASIC1a. It is likely that the effect of H-89 alone on ASIC1a expression might be mediated by a PKA-independent pathway. In addition, our results showed that the protective effect of ethanol on acid-induced cytotoxicity could be abolished by autophagy inhibitors and enhanced by autophagy activator. Taken together, these findings suggest that ethanol can alleviate acid-induced cytotoxicity by stimulating autophagy-dependent ASIC1a degradation.

These results highlight a novel mechanism underlying the protective effect of ethanol against acidosis-induced neurotoxicity and provide potential future therapeutic strategies for stroke treatment.

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## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

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