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Seizure Termination by Acidosis Depends on ASIC1a

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SUMMARY

Most seizures stop spontaneously. However, the molecular mechanisms remain unknown. Earlier observations that seizures reduce brain pH and that acidosis inhibits seizures indicated that acidosis halts epileptic activity. Because acid-sensing ion channel-1a (ASIC1a) shows exquisite sensitivity to extracellular pH and regulates neuron excitability, we hypothesized that acidosis might activate ASIC1a to terminate seizures. Disrupting mouse ASIC1a increased the severity of chemoconvulsant-induced seizures, whereas overexpressing ASIC1a had the opposite effect. ASIC1a did not affect seizure threshold or onset, but shortened seizure duration and prevented progression. CO₂ inhalation, long known to lower brain pH and inhibit seizures, also required ASIC1a to interrupt tonic-clonic seizures. Acidosis activated inhibitory interneurons through ASIC1a, suggesting that ASIC1a might limit seizures by increasing inhibitory tone. These findings identify ASIC1a as a key element in

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AUTHOR CONTRIBUTIONS

AEZ played a key role in generating the hypotheses for ASIC1a involvement in seizures, performed slice and *in vivo* electrophysiology/behavioral experiments, assessed seizure threshold, measured brain pH in response to seizures and CO₂, assisted in studies using acutely dissociated neurons, wrote initial manuscript drafts and worked closely with other authors in editing the figures and manuscript. MKS initiated, conceived, performed and interpreted electrophysiological experiments in dissociated neurons and assisted in preparation of the manuscript. MAS helped perform and interpret EEG experiments. GWA assisted with seizure threshold studies. MAH provided EEG equipment and helped with EEG interpretation. MJW provided important overall direction and initiation for the project, contributed to experimental design and data interpretation, provided funding, and wrote the manuscript. JAW initiated development of the project, led experimental direction, interpreted data, performed pilot experiments, provided funding, and wrote the manuscript.

seizure termination when brain pH falls. The results suggest a molecular mechanism for how the brain stops seizures and suggest new therapeutic strategies.

Keywords

ASIC1a; seizure; termination; acidosis; epilepsy; pH

INTRODUCTION

Investigators have discovered many gene disruptions and mutations that predispose humans and mice to seizures¹. That work has identified numerous ion channels and other proteins that prevent seizure onset or initiation. In contrast, surprisingly little is known about how the brain limits seizure duration and terminates seizures^{2,3}. This is despite the consequences of a failure to stop seizures, as in status epilepticus, which can damage the brain and is often fatal.

Multiple mechanisms might stop seizures. One possibility is that seizures deplete factors required for neuron firing. Supporting this possibility, seizures can reduce oxygen, glucose, and metabolic substrates required for neurotransmission^{4,5}. However, the role these factors play remains uncertain. While some studies suggest that depleting oxygen, glutamate and ATP can interrupt seizure-like activity^{6,7}, other work suggests reduced levels of these factors might initiate and worsen seizures^{8,9}. Furthermore, in status epilepticus, seizures can persist for hours, suggesting that prolonged seizures do not exhaust the fuel that sustains them.

Another possibility is that seizures produce inhibitory factors that block continued seizure activity. Protons are an inhibitor that accumulates during seizures. Seizures can reduce brain pH from ~7.35 to 6.8^{10,11} through lactic acid production, CO₂ accumulation, and other mechanisms¹². Acidosis was first implicated in seizure inhibition in 1929 when Lennox discovered that hypercarbic acidosis eliminated seizure discharges in patients with epilepsy¹³, a finding verified by others^{14,15}. Similarly, in brain slices acidosis interrupts seizure-like epileptiform activity¹⁶. Interestingly, some anticonvulsants, such as acetazolamide, reduce extracellular pH in the brain, suggesting that acidosis might contribute to their antiepileptic effects.

How acidosis inhibits seizures likely involves multiple mechanisms. For example, extracellular acidosis inhibits NMDA receptors¹⁷, and NMDA receptor antagonists attenuated acid's effect on epileptiform activity in brain slices¹⁶. A reduced extracellular pH also inhibits voltage-gated Na⁺ and Ca²⁺ channels and modulates GABA_A receptors¹⁸. Recent data also suggest that extracellular acidosis increases the concentration of extracellular adenosine, which activates adenosine (A1) receptors and ATP (P2X and P2Y) receptors to reduce seizure-like activity in brain slices¹⁹.

The ability of extracellular acidosis to activate the acid-sensing ion channels (ASICs) suggests these proteins might also mediate effects of pH on seizures. ASICs are proton-gated members of the degenerin/epithelial Na⁺ channel family²⁰. At least three ASICs, ASIC1a, -2a, and -2b, which form homo- and heteromultimeric channels, are widely expressed in the central nervous system (CNS)^{20–25}. ASIC1a homomeric channels are activated by protons and conduct Na⁺ and Ca²⁺ with an EC₅₀ of ~6.8^{23,26,27}. In CNS neurons, ASIC1a is required to generate a current response to pH values between 7.2 and 5.0^{24,28,29}, and extracellular acidosis activates ASICs to initiate neuron firing^{30,31}. Thus ASIC1a may be critical for mediating the brain's response to acidosis. An earlier study showing that inhibitory interneurons had larger H⁺-gated currents than excitatory neurons³², suggested that ASICs might dampen excitability under some conditions.

Based on the known fall in extracellular pH during seizures, the ability of acidosis to stop seizures, and the pH-sensitivity of ASIC1a, we hypothesized that ASIC1a might contribute to seizure termination and thereby reduce seizure severity.

RESULTS

ASIC1a disruption increases seizure severity

To learn whether ASIC1a affects seizure severity, we injected wild-type and *ASIC1a*^{-/-} mice with kainate, a chemoconvulsant that activates glutamate receptors. During the first 20 min after injection, mice of both genotypes had similar seizures affecting the head or forelimbs (Fig. 1a). However with time, the *ASIC1a*^{-/-} mice developed more severe seizures (Fig. 1a, b). We also administered pentylenetetrazole (PTZ), a chemoconvulsant that may have multiple targets^{33, 34}, and quantified the percentage of mice that developed generalized tonic-clonic seizures (GTCS), as described previously by others^{34, 35}. The majority of *ASIC1a*^{-/-} mice developed GTCS, whereas wild-type mice were less likely to have GTCS (Fig. 1c). Thus, with two different chemoconvulsants, loss of ASIC1a increased seizure severity.

We also acutely inhibited ASIC1a in wild-type mice with an intracerebroventricular injection of the ASIC1a antagonist psalmotoxin 1 (PcTx1)³⁶; such PcTx1 delivery blocks ASIC1a effects on ischemic stroke²⁹ and fear³⁷. PcTx1 increased the incidence of continuous GTCS following kainate injection (Fig. 1d). Similar effects on seizure severity with both *ASIC1a* gene disruption and pharmacological blockade suggest that developmental abnormalities were not responsible for the effects in *ASIC1a*^{-/-} mice.

ASIC1a overexpression reduces seizure severity

Finding that ASIC1a disruption enhanced seizure severity suggested that overexpressing the channel might have the opposite effect. To test this prediction, we studied transgenic mice overexpressing ASIC1a via a pan-neuronal synapsin 1 promoter (*ASIC1a*^{Tg+})³⁸. In these mice, ASIC1a expression is increased throughout the brain, and CNS neurons have larger amplitude acid-evoked currents than wild-type littermates³⁸. Because we hypothesized that mice overexpressing ASIC1a would have less severe seizures, we injected *ASIC1a*^{Tg+} and wild-type mice with kainate and PTZ doses that were higher than those used in the earlier studies. ASIC1a overexpression reduced seizure severity following kainate injection (Fig. 1e, f) and reduced the incidence of GTCS after PTZ injection (Fig. 1g). Coupled with our experiments in *ASIC1a*^{-/-} mice, these findings suggest a relationship between the level of ASIC1a expression and the degree of seizure protection.

ASIC1a disruption does not alter seizure threshold

The effect of ASIC1a on seizure severity raised the question of whether it might also affect seizure onset. We tested this possibility using a common method of threshold analysis, the maximal electroconvulsive seizure threshold test³⁹. ASIC1a disruption did not reduce the amount of electrical current necessary to evoke a stereotypic seizure response (Fig. 1h). This result and our subsequent studies suggest that ASIC1a does not play a prominent role in determining seizure threshold.

ASIC1a shortens seizure duration

To further assess how ASIC1a reduces seizure severity, we examined PTZ-evoked seizures using electroencephalography (EEG) to examine epileptiform discharges while simultaneously monitoring seizures behaviorally. We reasoned that if ASIC1a inhibited the initiation of seizures, then disrupting ASIC1a would accelerate onset of EEG spike activity and enhance initial seizure severity. On the other hand, if ASIC1a enhanced seizure termination, then

disrupting ASIC1a would prolong EEG spike activity and increase the likelihood that seizures progress to GTCS or death.

Consistent with a normal seizure threshold, we found that the latency to EEG spike activity was the same in *ASIC1a*^{-/-} and wild-type mice (Fig. 2a). In addition, within the first 10 min following PTZ injection, both wild-type and *ASIC1a*^{-/-} mice had seizures of similar severity characterized by myoclonic jerks (not shown) and a similar number of EEG spike discharges (Fig. 2b). However as the seizures continued, differences between the two genotypes became apparent. In wild-type mice, EEG spikes decreased precipitously following the 10-min time point (Fig. 2b). In contrast, most *ASIC1a*^{-/-} mice progressed to tonic-clonic seizures and death (Fig. 2c, d). Surviving *ASIC1a*^{-/-} mice continued to have more seizure activity than wild-type mice (Fig. 2b). However, because we could only measure EEG spike activity in surviving animals, we likely underestimate the deficit in seizure termination in *ASIC1a*^{-/-} mice.

In wild-type mice, seizures were often followed by a suppression of spike discharges (Fig. 3a). This low-amplitude EEG pattern, called post-ictal depression, has been suggested to result from the factors that cause seizure termination^{3, 9, 40}. In contrast to wild-type mice, *ASIC1a*^{-/-} mice had only brief periods of EEG depression that were interrupted by seizure spikes (Fig. 3a, b). ASIC1a disruption significantly reduced post-ictal depression (Fig. 3c). This loss of an EEG pattern associated with seizure termination is consistent with the prolonged seizure activity and the increased severity observed in *ASIC1a*^{-/-} mice.

Termination of seizure-like activity by low pH depends on ASIC1a

We hypothesized that a reduced pH, which is known to occur during seizures, would terminate seizures through ASIC1a. We tested this hypothesis using a hippocampal slice model in which hypomagnesemia induces epileptiform activity¹⁶. In this model, low pH is known to inhibit seizure-like activity in the hippocampus¹⁶, and ASIC1a is expressed in hippocampal neurons²⁸. Before inducing seizures, we saw no epileptiform activity in slices of either genotype (Fig. 4a). At pH 7.35, both genotypes showed a similar latency to the onset of epileptiform activity (Fig. 4b, c), and had an equivalent number of epileptiform spikes (Fig. 4b, d). However, when we reduced pH to 6.8, seizure activity decreased in wild-type slices, but not in slices from *ASIC1a*^{-/-} mice. These data suggest that ASIC1a expression is required for the antiepileptic effects of low pH.

Inhibitory interneurons have ASIC currents

One potential mechanism by which acidosis-induced ASIC currents could inhibit epileptiform discharges would be through their activation of inhibitory interneurons, a cell population that plays a critical role in limiting epileptiform activity. Therefore, we examined the effects of physiologically relevant reductions in pH on acutely dissociated hippocampal interneurons, as well as excitatory pyramidal neurons. Neurons were identified based on their location (lacunosum moleculare vs. CA1), size, morphology, and firing pattern^{32, 41}. There were four main findings. a) Reducing extracellular pH activated inward current in wild-type, but not *ASIC1a*^{-/-} interneurons (Fig. 5a), a result that agrees with previous studies showing that disrupting *ASIC1a* eliminated currents evoked by pH reductions to as low as 5.0^{24, 28, 29}. b) Reducing pH from 7.4 to values of 7.2, 7.0, and 6.8, evoked ASIC currents (Fig. 5b). These pH values are in the range reported in seizures^{10, 11} and in the range we measured (see below). c) We found that interneurons had larger H⁺-gated current densities than pyramidal neurons (Fig. 5b). Inhibitory interneurons in the rat are also reported to possess larger acid-evoked currents than excitatory neurons³². d) When we reduced extracellular pH, we stimulated action potential firing in inhibitory neurons; pH 6.8 induced firing in 78% of inhibitory neurons (n = 9), and pH 7.0 induced firing in 80% of the interneurons (n = 5) (Fig. 5c shows an example).

These results suggest that the *ASIC1a*^{-/-} animals may lack a source of inhibitory tone during central acidosis, and as a result they fail to inhibit seizure activity.

CO₂ inhalation requires ASIC1a to interrupt seizures

Almost 80 years ago, Lennox reported that inhaling CO₂ inhibited seizures in humans¹³. Subsequent studies demonstrated that CO₂ reduces cortical pH within seconds of inhalation^{42, 43}, and that breathing CO₂ increases brain acidosis during a PTZ-evoked seizure¹¹. Thus, we reasoned that inducing hypercarbic acidosis would provide an *in vivo* test of whether ASIC1a was required for the antiepileptic effects of acidosis. We administered a high dose of PTZ to evoke lethal seizures in animals of both genotypes (Fig. 6a). In separate groups of animals, we switched from air to 10% CO₂ immediately after the onset of tonic-clonic seizures (Fig. 6b). Remarkably, 10% CO₂ prevented lethal seizures in wild-type mice, but had little effect in *ASIC1a*^{-/-} mice; in these mice, seizures continued to progress rapidly to death. All of the *ASIC1a*^{+/-} mice survived until we switched from CO₂ back to air at minute 15. They then rapidly died.

To verify that brain pH drops *in vivo* during seizures and CO₂ inhalation, we implanted a fiber optic pH sensor into the lateral cerebral ventricle of wild-type and *ASIC1a*^{-/-} mice. Generalized seizures caused brain pH to fall (pH ~7.05) (Fig. 6c, d). CO₂ inhalation rapidly and reversibly lowered pH even further in the seizing mice (pH ~6.9). Importantly, these are pH levels that elicit robust ASIC1a currents and firing in inhibitory neurons. Brain pH fell to similar levels in mice of both genotypes. Together, these results suggest that ASIC1a also mediates the anti-epileptic effects of low pH *in vivo*.

DISCUSSION

Our findings suggest a model in which seizure termination depends on ASIC1a. Seizures reduce extracellular pH^{10, 11}. Extracellular acidosis, in turn, activates ASIC1a, which terminates seizure activity. Several experiments support the key features of this model. We found that seizures lowered brain pH, as previously reported^{10, 11}. In addition to the acidosis generated by seizures, we also directly lowered extracellular pH *in vitro* and tested hypercarbic acidosis *in vivo*; both stopped seizure activity in an ASIC1a-dependent manner. We obtained similar results using three different chemoconvulsants (kainate, PTZ, and a reduced Mg²⁺ concentration). Finally, disrupting the *ASIC1a* gene or pharmacologically inhibiting ASIC1a increased seizure severity, whereas overexpressing ASIC1a had the opposite effect. These findings suggest that ASIC1a forms part of a feedback inhibition system that limits seizure severity.

The data indicate that ASIC1a reduced seizures by enhancing their termination. First, the duration of seizure activity measured by EEG was shorter in wild-type than *ASIC1a*^{-/-} mice. Second, in wild-type mice, seizures were less likely to progress to tonic-clonic seizures and death. Third, ASIC1a increased post-ictal depression of spike wave discharges, which coincides with and is thought to result from endogenous termination mechanisms^{3, 9, 40}. Fourth, ASIC1a disruption did not affect seizure threshold, the latency to seizure onset, or initial seizure severity, suggesting that ASIC1a did not contribute to seizure initiation.

Predicting how ion channel dysfunction precipitates a phenomenon as complex as seizures has proven difficult⁴⁴. Even less is known about the mechanisms that stop seizures. Our findings that reducing pH to values that occur during seizures evoked ASIC currents and triggered action potential firing in inhibitory neurons suggest that ASICs might trigger inhibitory neuron activity to terminate seizures. However, there are several types of inhibitory neurons and their relative importance in seizure termination and the contribution of ASIC currents to their *in vivo* activity remain uncertain. Moreover, ASIC channels are also expressed in excitatory

pyramidal neurons, where they could contribute either to increased activity or reduced activity, perhaps through depolarization blockade. In addition, while we focused on hippocampus, ASIC channels are also expressed in many different brain regions^{20, 21, 37}, and it is possible that ASIC activation of inhibitory neurons in other regions might also contribute to seizure termination. Thus, understanding how ASIC channels influence neuronal circuits to stop seizures suffers from the same complexity that hinders knowledge of how dysfunction of other channels starts seizures. Nevertheless, by identifying ASIC1a as a molecule involved in seizure termination, this work provides an important beginning for further investigation.

In addition to providing a foothold for understanding seizure termination, the ability of ASIC1a to stop seizures may have implications for human seizure disorders and treatment. Recent studies have suggested a role for ASIC1a in mouse models of ischemic stroke²⁹, neurodegeneration⁴⁵, and psychiatric disease³⁷. Whether ASIC channels are protective or damaging may depend on the magnitude and duration of acidosis, the location of ASIC activation, and the presence of factors that modulate ASIC function, such as lactate⁴⁶. However, the findings described here suggest a new, protective function for ASIC1a in brain physiology. Thus, agents that potentiate ASIC1a activity might reduce seizure severity or duration and possibly prevent status epilepticus.

MATERIAL AND METHODS

Mice

We used age and gender-matched wild-type, *ASIC1a*^{-/-}, and *ASIC1a*-overexpressing transgenic (*ASIC1a*^{Tg+}) mice on a congenic C57/Bl6 background^{28, 38}. Care of the mice met the standards set forth by the National Institutes of Health and the procedures were approved by the University of Iowa Animal Care and Use Committee.

Convulsants

Kainate or pentylenetetrazole (PTZ) (Sigma-Aldrich, Saint Louis MO) were injected into the peritoneum (IP) following suspension in phosphate buffered saline (Gibco, Carlsbad CA) and titration to pH 7.4 with 0.1 N NaOH³⁵.

Behavioral Assays

To score the effects of ASIC1a on seizure severity in response to kainate we used the Racine⁴⁷ seizure scale: (0) No response, (1) staring/reduced locomotion, (2) activation of extensors/rigidity, (3) repetitive head and limb movements, (4) sustained rearing with clonus, (5) loss of posture, (6) status epilepticus/death. To assess the effects of ASIC1a on seizure severity in response to PTZ we scored the incidence of generalized tonic-clonic seizures (GTCS), which were identified by generalized clonus followed by tonic hind limb extension³⁴. We chose different kainate and PTZ doses to test specific hypotheses, to decrease the overall number of animals required, and to avoid ceiling and floor effects. For example, to test the hypothesis that the *ASIC1a*^{-/-} mice have more severe seizures than controls, we used a PTZ dose (50 mg/kg) that would evoke generalized, tonic-clonic seizures in a small percentage of the control wild-type mice. The opposite was true for the experiments with the *ASIC1a* transgenic mice; to efficiently test the hypothesis that transgenics have less severe seizures, we used a PTZ dose (65 mg/kg) that evoked generalized, tonic-clonic seizures in a large percentage of the control, wild-type mice. A trained observer blinded to genotype scored seizure severity.

PcTx1 administration

Left-lateral, intracerebroventricular (ICV) guide cannulae were implanted in anesthetized mice (relative to bregma: anteroposterior −0.3 mm, lateral −1.0 mm, ventral −3.0 mm). Three to five days later, we injected 5 μ L of PcTx1-containing venom (SpiderPharm, Yarnell, AZ) (9 ng/ μ L) in ACSF (in mM: NaCl 124, KCl 3, NaH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 2, NaHCO₃ 26) or ACSF alone into wild-type mice using a 10 μ L-Hamilton syringe connected to a 30-gauge injector (over 10 s). Two hours later, mice were injected with kainate (20 mg/kg, IP). Following convulsant injection, mouse behavior was scored as above. Continuous, tonic-clonic seizures were identified by tonus and clonus in all four limbs with loss of posture lasting greater than 60 s. Cannula placement was verified by methylene blue injection after euthanasia.

Testing maximal electroconvulsive seizure (MES) threshold

Seizure threshold in response to an electrical stimulus was determined as described previously³⁹ in *ASIC1a*^{+/+} and *ASIC1a*^{−/−} mice. Electroshock was delivered (0.2 s, 60 Hz, maximal voltage 500 V) using the Rodent Shocker-type 221 (Harvard Apparatus, Holliston, MA) with ear electrodes moistened with saline. The occurrence of generalized seizures with sustained hind limb extension was assessed³⁹.

EEG Recordings and Analysis

Two, 3.2 mm stainless steel screws (Stoelting, Wood Dale IL) were stereotactically implanted under ketamine/xylazine anesthesia above the left frontal lobe and cerebellum; these electrodes served as an epidural recording and reference/ground electrodes respectively (frontal: anteroposterior + 1.5 mm, lateral −1.5 mm; cerebellum reference: anteroposterior −6.0 mm). Mice recovered from surgery for at least 1 week, and EEG activity was recorded by tethered connecting leads from freely moving mice in a sound-attenuated chamber. EEG was recorded at baseline and in response to a single IP injection of PTZ (50 mg/kg) in *ASIC1a*^{+/+} and *ASIC1a*^{−/−} mice. During the 30 min following injection, tonic-clonic and lethal seizures were identified behaviorally and electrographically by simultaneous video and EEG monitoring. EEG was captured using a TDT MEDUSA preamplifier and base-station and recorded at a sampling rate of 508.6 Hz with TDT OpenX software with high and low pass filters at 2 Hz and 70 Hz respectively. EEG recordings were analyzed using Origin 7.5 software by an experimenter blinded to genotype. Latency to seizure onset was defined as the time from injection to first seizure spike. Seizure spikes were detected using the peak analysis function of Origin v7.5. Major seizure events and sharply delimited seizure spikes exceeding twice the baseline amplitude were scored.

Post-ictal depression was defined as a low-amplitude, slow-wave EEG signal without seizure spikes occurring after a seizure⁴⁰. The duration of post-ictal depression was defined from its onset following a seizure until the resumption of seizure spikes or return of the EEG signal to an amplitude exceeding 2 mV⁴⁰. Based on seizure severity and the longest observed period of post-ictal suppression, each mouse was scored and separated into one of 5 categories: (1) no post-ictal depression and lethal seizures, (2) no post-ictal depression with persistent seizure activity, (3) depression < 60 seconds, (4) depression = 60–180 seconds, (5) depression > 180 seconds.

Slice recordings and analysis

Horizontal hippocampal slices (400 μ m) were prepared from 14 to 24-day-old *ASIC1a*^{+/+} and *ASIC1a*^{−/−} mice similar to methods described^{28, 48}. Prior to sectioning, the mice were transcardially perfused with a high Mg²⁺/low Ca²⁺ solution chilled to 4 °C (in mM): 4.9 MgSO₄, 0.5 CaCl₂, 126 NaCl, 5 KCl, 1.25 NaH₂PO₄, 27.7 NaHCO₃, 10 dextrose, 1.1 MgCl₂, pH 7.35 bubbled with 95% O₂/5% CO₂. After sectioning, slices were incubated in

artificial cerebral spinal fluid (ACSF) for at least 1 hour prior to testing: 126 NaCl, 5 KCl, 1.8 MgSO₄, 1.25 NaH₂PO₄, 27.7 NaHCO₃, 10 dextrose, and 1.6 CaCl₂. Standard extracellular field potential recording techniques were performed in a submerged chamber perfused with ACSF (flow-rate 4 ml/min, 33 ± 0.5 °C). Field-potentials were recorded in the proximal CA3 hippocampal field with ACSF-filled glass pipettes (< 5 MΩ). To evoke seizure activity, normal ACSF was replaced with ACSF minus MgSO₄. Latency to onset of epileptiform activity was defined as the time elapsed between switching to nominal Mg²⁺ ACSF until the first epileptiform spike. After scoring the latency to epileptiform activity and recording 5 min of seizure activity, pH was reduced to 6.8 by lowering NaHCO₃ concentration to 11.4 mM, and increasing sodium gluconate to 16.3 mM to maintain osmolarity. pH in the recording chamber was measured both prior to and during infusion of pH 6.8 ACSF. The effects of low pH were then recorded for 5 min, and pH was then switched back to 7.35. Slices that failed to develop ictal discharges were excluded (40% of *ASIC1a*^{+/+} slices, n = 15, and 38% of *ASIC1a*^{-/-} slices, n = 13). To quantify epileptiform activity, we used a method similar to that described by others⁴⁹. Using the threshold function in Clampfit v9.2 we quantified the total number of seizure spikes during three 4.5-min time windows occurring immediately before, during, and after dropping pH to 6.8. The 30 seconds required to completely change the bath solution to pH 6.8 were excluded from the analysis. A threshold was chosen for each slice that would detect seizure spikes, and not single-unit activity. The average thresholds were similar between genotypes (*ASIC1a*^{+/+} = 0.355 ± 0.032 mV; *ASIC1a*^{-/-} = 0.305 ± 0.04 mV). When slices were challenged repeatedly with low pH, the trials were pooled to calculate a mean number of discharges for each condition.

Whole cell electrophysiology

Acutely dissociated neurons were isolated from age 8–12 day old *ASIC1a*^{+/+} and *ASIC1a*^{-/-} mice according to an established protocol⁵⁰. Mice were anesthetized (isoflurane), decapitated, and 500 μm coronal sections were cut with a vibratome in ice-cold PIPES buffered saline (115 mM NaCl, 5 mM KCl, 20 mM PIPES, 1 mM CaCl₂, 4 mM MgCl₂, D-glucose 25, pH 7.0 with NaOH) in the presence of 100% O₂. CA1 and the lacunosum moleculare layer (LM) of the hippocampus were removed by microdissection and trypsin digested (15 mg) for 30 min at 30 °C in 20 ml PIPES saline. Tissue was washed three times in PIPES saline and triturated in 0.5 ml PIPES saline with Pasteur pipettes of decreasing apertures to dissociate neurons. Neurons were then diluted in 8 ml Dulbecco's modified Eagle's medium with 25 mM HEPES, 25 mM glucose (Gibco), 5% horse serum and placed on 10 mm glass cover slips (poly-D-lysine/laminin, BD Biosciences) in 24 well plates at 37 °C. Neurons were studied in voltage-clamp and current-clamp modes within 1 to 5 h as previously described³⁷. In brief, neurons were superfused in bath solutions containing (in mM) 145 NaCl, 5.4 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, 10 MES and pH was adjusted with TMA·OH. Pipettes (3–5 MΩ polished glass pipettes (Drummond Scientific, 100 μl)) contained (in mM) 5 NaCl, 90 K-gluconate, 15 KCl, 1 MgCl₂, 10 EGTA, 60 HEPES, and 3 Na₂ATP, adjusted to pH 7.3 with KOH. Extracellular pH was switched with a Rapid Solution Changer (RSC-200; Biologic, Grenoble, France). In voltage-clamp mode, membrane potential was maintained at -70 mV. In current-clamp, holding voltage was adjusted to -77 ± 2 mV. Inhibitory neurons were identified by: location (lacunosum moleculare layer microdissection), round morphology, and size (4.4 ± 0.3 μm)^{32, 41}. Excitatory, pyramidal neurons were identified by: location (CA1 microdissection), pyramidal morphology, spike frequency adaptation in response to current injection, and size (8.3 ± 0.3 μm).

Testing the anti-epileptic effects of CO₂

ASIC1a^{+/+} and *ASIC1a*^{-/-} mice were injected IP with 90 mg/kg PTZ. After the onset of generalized-clonic seizures, compressed air or 10% CO₂ (in air) was rapidly administered in an airtight Plexiglas chamber for 15 min. Generalized clonic-seizures were identified

behaviorally by clonus in all four limbs. The percentage of surviving mice was plotted during each min of the trial. After 30 min, surviving mice were euthanized.

Measuring brain pH

Age (13–15 weeks) and gender-matched *ASIC1a*^{+/+} and *ASIC1a*^{-/-} mice were anesthetized with ketamine/xylazine. 60 min after sedation, a fiber optic pH sensor (pHOptica, Sarasota, FL) was placed in the left lateral ventricle (coordinates above). The sensor was calibrated at 35 °C and pH values were calculated using pHOptica-v1.0 software, taking care to input the mouse core temperature under anesthesia. Following 5 min of baseline pH measurement, we injected PTZ (IP). We found that due to the anesthesia, a high dose (120 mg/kg) of convulsant was required to achieve an approximate level of seizure activity seen in unanesthetized mice. In the event that generalized seizure activity did not occur, an additional 60 mg/kg was injected every 20 min until generalized seizures began. The total amount of convulsant administered did not significantly differ between two genotypes (*ASIC1a*^{+/+} = 252 ± 34.9 mg/kg, *ASIC1a*^{-/-} = 216 ± 14.7 mg/kg).

During pH measurements, mice were continuously exposed to compressed air. Following the onset of generalized seizure activity, brain pH dropped to a stable level below 7.1 (see results). At that time, we administered 10% CO₂ for at least 5 min. Following CO₂ administration, air was administered for an additional 10 min. The baseline brain pH, minimum pH during seizure, and minimum pH during CO₂ administration were assessed.

Statistics

Values are expressed as mean ± s.e.m. Where indicated, analyses of significance were performed using the unpaired t-test or ANOVA to compare two groups at multiple time points or pH values. For ANOVA, current density data were transformed to log₁₀ values. The Mann-Whitney U-test (Wilcoxon rank sum) was used to compare two groups of ordinal variables. The Fisher's exact test was used to compare two groups of two categorical variables. Kaplan-Meier analysis and Mantel-Cox log rank were used to assess survival. Probit analysis with 95% confidence intervals was used to calculate the CD50 in threshold experiments. P-values less than 0.05 were considered statistically significant (Microsoft Excel, SPSS).

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Figure 1.

ASIC1a reduces seizure severity. (a) Seizure response over time in *ASIC1a*^{+/+} and *ASIC1a*^{-/-} mice following kainate injection (20 mg/kg IP) (+/+, n = 6; -/-, n = 7; age 13–22 weeks). For each ten-minute interval, the highest level of seizure activity was scored using the Racine seizure scale (see methods). There was a significant main effect of time ($F(1, 6) = 27.3$, $p < 0.0001$) and a significant time $\times$ genotype interaction ($F(1, 6) = 2.39$, $p = 0.039$) (ANOVA with repeated measures), suggesting that as time passes seizures were likely to become more severe in the *ASIC1a*^{-/-} mice. (b) Maximum Racine score during the 60 minute trial (Mann–Whitney U test; * $p = 0.004$). (c) Incidence of generalized tonic–clonic seizures (GTCS) in +/+ and -/- mice following PTZ injection (50 mg/kg IP) (+/+, n = 12; -/-, n = 8, age 18–22

weeks; Fisher's exact test; $*p = 0.004$). (d) Incidence of continuous, tonic-clonic seizures in wild-type mice injected with 5 mL ACSF (ICV) or PcTx1 (9 ng/mL). Treatment with PcTx1 significantly increased the incidence of sustained seizures (ACSF, $n = 10$; PcTx1, $n = 12$, age 9–11 weeks; Fisher's exact test $*p = 0.005$). (e) Seizure response over time in *ASIC1a*^{+/+} (WT) and *ASIC1a*-overexpressing-transgenic (Tg+) mice following 30 mg/kg IP kainate (WT, $n = 11$; Tg+, $n = 9$, age 31–36 weeks). With time, seizures were less severe in mice overexpressing *ASIC1a* (ANOVA with repeated measures; $F(1, 3.35) = 3.295$, $p = 0.022$). (f) Maximum Racine score during the 60 minute trial (Mann-Whitney U test; $*p = 0.041$). (g) Incidence of generalized tonic-clonic seizures (GTCS) in WT and Tg+ mice following PTZ injection (65 mg/kg IP) (WT, $n = 13$; Tg+, $n = 13$, age 25–41 weeks; Fisher's exact test; $*p = 0.037$). (h) Occurrence of maximal electroconvulsive seizures (MES) in *ASIC1a*^{+/+} and *ASIC1a*^{-/-} mice in response to electrical stimulation. *ASIC1a* disruption did not significantly alter MES threshold (+/+, $n = 50$, CD50 95% confidence interval = 6.24–7.95; -/-, $n = 46$, CD50 95% confidence interval = 6.71–7.76). The sample sizes and current intensities were: 5 mA, *ASIC1a*^{+/+} ($n = 11$) vs. *ASIC1a*^{-/-} ($n = 7$); 6 mA, *ASIC1a*^{+/+} ($n = 22$) vs. *ASIC1a*^{-/-} ($n = 21$); 7 mA, *ASIC1a*^{+/+} ($n = 10$) vs. *ASIC1a*^{-/-} ($n = 10$); 8 mA, *ASIC1a*^{+/+} ($n = 4$) vs. *ASIC1a*^{-/-} ($n = 4$); 10 mA, *ASIC1a*^{+/+} ($n = 3$) vs. *ASIC1a*^{-/-} ($n = 4$), age 8–22 weeks.

Figure 2.

ASIC1a disruption increases seizure duration and progression. (a) Representative EEG tracings and quantification of time from PTZ injection (50 mg/kg IP) until first seizure spikes in *ASIC1a*^{+/+} and *ASIC1a*^{-/-} mice ($+/+$, $n = 6$; $-/-$, $n = 7$, age 18–22 weeks; unpaired t-test: $t(11) = 0.544$, $p = 0.597$). (b) Representative EEG tracings and total number of seizure spikes per five minute interval in surviving mice ($+/+$, $n = 6$; $-/-$, $n = 7$). Spike number varied significantly with time (Mixed model analysis; $F(1, 5) = 23.5$, $p < 0.001$), and there was a significant time $\times$ genotype interaction ($F(1,6) = 32.9$, $p < 0.001$), suggesting that *ASIC1a*^{-/-} mice had prolonged seizure activity as time elapsed. (c) Incidence of generalized tonic-clonic seizures (GTCS) following PTZ injection (50 mg/kg IP) ($+/+$, $n = 6$; $-/-$, $n = 7$; Fisher's exact test; $p = 0.078$). (d) Survival over time ($+/+$, $n = 6$; $-/-$, $n = 7$; Mantel-Cox Log Rank, $p = 0.025$).

Figure 3.

ASIC1a disruption reduces post-ictal depression. (a) Representative EEG tracings from an $ASIC1a^{+/+}$ and $ASIC1a^{-/-}$ mouse approximately 5 min. following PTZ injection (50 mg/kg IP). Five, 5-second intervals are denoted by blue vertical bars. These are shown below (b) in rows using an expanded time scale. EEG tracings prior to PTZ injection (base), initial spike-wave activity (1), and seizures associated with forelimb clonus (2) were similar in both genotypes. However, immediately following seizures (3), mice entered a period of post-ictal depression (red boxes). Post-ictal depression quickly reverted to seizure activity in $ASIC1a^{-/-}$ mice (4, 5). (c) Quantification of post-ictal depression as scored using the post-ictal depression scale (see methods) ($+/+$, $n = 6$; $-/-$, $n = 7$, age 18–22 weeks; Mann–Whitney U test, $*p = 0.011$).

Figure 4.

ASIC1a mediates the antiepileptic effects of acid in hippocampal slices. (a) Representative CA3 extracellular recording from *ASIC1a*^{+/+} and *ASIC1a*^{-/-} slices prior to ictal activity caused by hypomagnesemia (nominal Mg²⁺). (b) Representative CA3 recordings from *ASIC1a*^{+/+} and *ASIC1a*^{-/-} slices demonstrating ictal activity before, during, and after pH 6.8 application. A seizure-like discharge during pH 6.8 is expanded in the inset. (c) Latency to seizure onset was recorded in CA3 in response to 0 Mg²⁺ (+/+, n = 7; -/- n = 5; t(10) = 0.366, p = 0.722). (d) Total number of seizure spikes over 4.5 min. before induction of ictal activity (control), and then in the presence of nominal Mg²⁺ at pH 7.35 (baseline), pH 6.8, and after return to pH 7.35 (recovery) (+/+, n = 9; -/- n = 8). In *ASIC1a*^{+/+} mice an ANOVA revealed a significant effect of pH (F(2) = 7.124, p = 0.006), however this was not the case in the *ASIC1a*^{-/-} mice (F(2) = 0.104, p = 0.902). A within-subjects comparison revealed a significant pH x genotype interaction (F(2) = 3.78, p = 0.034). At pH 6.8, the spike number was significantly greater in the *ASIC1a*^{-/-} mice (unpaired t-test: t(15) = -2.88, *p = 0.006), whereas at baseline and during recovery, the *ASIC1a*^{+/+} and *ASIC1a*^{-/-} mice did not significantly differ (unpaired t-test, p = 0.238 and 0.581 respectively).

Figure 5.

Inhibitory interneurons have prominent ASIC1a currents. (a) Representative traces of acid-evoked current in interneurons from the hippocampus of *ASIC1a*^{+/+} and *ASIC1a*^{-/-} mice. Application of pH 7.0 evoked an inward current in *+/+*, but not *-/-* neurons. (b) From *ASIC1a*^{+/+} mice, inhibitory neurons (I) had larger acid-evoked current density than excitatory pyramidal neurons (E) at pH 6.8–7.2 (inhibitory, *n* = 30; excitatory, *n* = 18; *F*(1, 42) = 10.5, *p* < 0.01). (c) Current-clamp recording demonstrating acid-evoked firing in an *ASIC1a*^{+/+} inhibitory neuron. *ASIC1a*^{-/-} neurons did not respond to even greater reductions in pH. pH 6.8 stimulated firing in 78% (*n* = 9) and pH 7 stimulated firing in 80% (*n* = 5) of *ASIC1a*^{+/+} inhibitory neurons.

Figure 6.

ASIC1a mediates the seizure-terminating effects of 10% CO₂. (a) Kaplan–Meier survival analysis of *ASIC1a*^{+/+} and *ASIC1a*^{-/-} mice in response to PTZ injection (90 mg/kg IP) while breathing compressed air (+/+, n = 7; -/-, n = 7, age 13–16 weeks). Both +/+ and -/- mice had the same survival rate (Mantel–Cox Log Rank, p = 0.582). (b) In a parallel experiment, 10% CO₂ was administered at the onset of generalized tonic–clonic seizures. The onset latency and time of CO₂ administration was similar between genotypes (inset) (unpaired t-test, t(14) = 0.663, p = 0.518). The chamber was perfused with CO₂ until minute 15, and then switched to compressed air for the duration of the trial (+/+, n = 8; -/-, n = 8, age 13–16 weeks). In CO₂, the likelihood of survival was significantly greater in the *ASIC1a*^{+/+} mice (Mantel–Cox

Log Rank, $p = 0.002$). (c) Representative pH tracings from a $+/+$ and $-/-$ mouse brain before PTZ injection, during seizure, and during seizure and 10% CO_2 inhalation. (d) ASIC1a expression did not significantly alter brain pH prior to injection ($p = 0.784$), during seizures ($p = 0.627$), or during CO_2 inhalation ($p = 0.528$) (pre-injection: $+/+$, $n = 5$; $-/-$, $n = 5$, age 13–15 weeks; $t(4) = 0.283$; seizure: $+/+$, $n = 5$; $-/-$, $n = 5$; $t(4) = 0.505$; CO_2 : $+/+$, $n = 4$; $-/-$, $n = 4$; $t(3) = 0.670$).