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Seizure termination by acidosis depends on ASIC1a

Adam E Ziemann[1,2,9], Mikael K Schnizler[3,9], Gregory W Albert[4], Meryl A Severson[4], Matthew A Howard III[4,5], Michael J Welsh[1–3,5,6] & John A Wemmie[5,7,8]

Most seizures stop spontaneously; however, the molecular mechanisms that terminate seizures remain unknown. Observations that seizures reduced brain pH and that acidosis inhibited seizures indicate that acidosis halts epileptic activity. Because acid-sensing ion channel 1a (ASIC1a) is exquisitely sensitive to extracellular pH and regulates neuron excitability, we hypothesized that acidosis might activate ASIC1a, which would 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 seizure progression. CO_2 inhalation, long known to lower brain pH and inhibit seizures, required ASIC1a to interrupt tonic-clonic seizures. Acidosis activated inhibitory interneurons through ASIC1a, suggesting that ASIC1a might limit seizures by increasing inhibitory tone. Our results identify ASIC1a as an important element in seizure termination when brain pH falls and suggest both a molecular mechanism for how the brain stops seizures and new therapeutic strategies.

Investigators have discovered many gene disruptions and mutations that predispose humans and mice to seizures[1]. Numerous ion channels and other proteins that prevent seizure onset or initiation have been identified. In contrast, 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 occurs in status epilepticus, which can damage the brain and is often fatal.

Multiple mechanisms might halt seizures. One possibility is that seizures deplete factors that are required for neuron firing. Supporting this theory, seizures can reduce oxygen, glucose and metabolic substrates that are required for neurotransmission[4,5]. However, the function of these factors remains uncertain. Although some studies have suggested that depleting oxygen, glutamate and ATP can interrupt seizure-like activity[6,7], other results suggest that 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 (refs. 10,11) through lactic acid production, CO_2 accumulation and other mechanisms[12]. Acidosis was first implicated in seizure inhibition in 1929, when it was discovered that hypercarbic acidosis eliminated seizure discharges in patients with epilepsy[13], a finding that was verified by others[14,15]. Similarly, acidosis interrupts seizure-like epileptiform activity in brain slices[16]. Notably, some anticonvulsants, such as acetazolamide, reduce extracellular pH in the brain, suggesting that acidosis might contribute to their antiepileptic effects.

The method by which acidosis inhibits seizures probably involves multiple mechanisms. For example, extracellular acidosis inhibits NMDA receptors[17], and NMDA receptor antagonists attenuate acid's effect on epileptiform activity in brain slices[16]. A reduced extracellular pH also inhibits voltage-gated Na^+ and Ca^{2+} channels and modulates $GABA_A$ receptors[18]. 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 slices[19].

The ability of extracellular acidosis to activate the ASICs suggests that these proteins might also mediate the effects of pH on seizures. ASICs are proton-gated members of the degenerin/epithelial Na^+ channel family[20]. At least three ASICs, ASIC1a, ASIC2a and ASIC2b, which form homo- and heteromultimeric channels, are widely expressed in the CNS[20–25]. ASIC1a homomeric channels are activated by protons and conduct Na^+ and Ca^{2+} with a half maximal effective concentration of ~6.8 (refs. 23,26,27). In CNS neurons, ASIC1a is required to generate a current response to pH values between 7.2 and 5.0 (refs. 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. A previous study showing that inhibitory interneurons had larger H^+-gated currents than did excitatory neurons[32] suggests that ASICs might dampen excitability under some conditions.

On the basis of 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.

[1]Medical Scientist Training Program, [2]Departments of Molecular Physiology and Biophysics, [3]Internal Medicine, [4]Neurosurgery, [5]Neuroscience Program, [6]Howard Hughes Medical Institute, [7]Department of Psychiatry, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242, USA. [8]Department of Veterans Affairs Medical Center, Iowa City, Iowa 52242, USA. [9]These authors contributed equally to this work. Correspondence should be addressed to M.J.W. (michael-welsh@uiowa.edu) or J.A.W. (john-wemmie@uiowa.edu).

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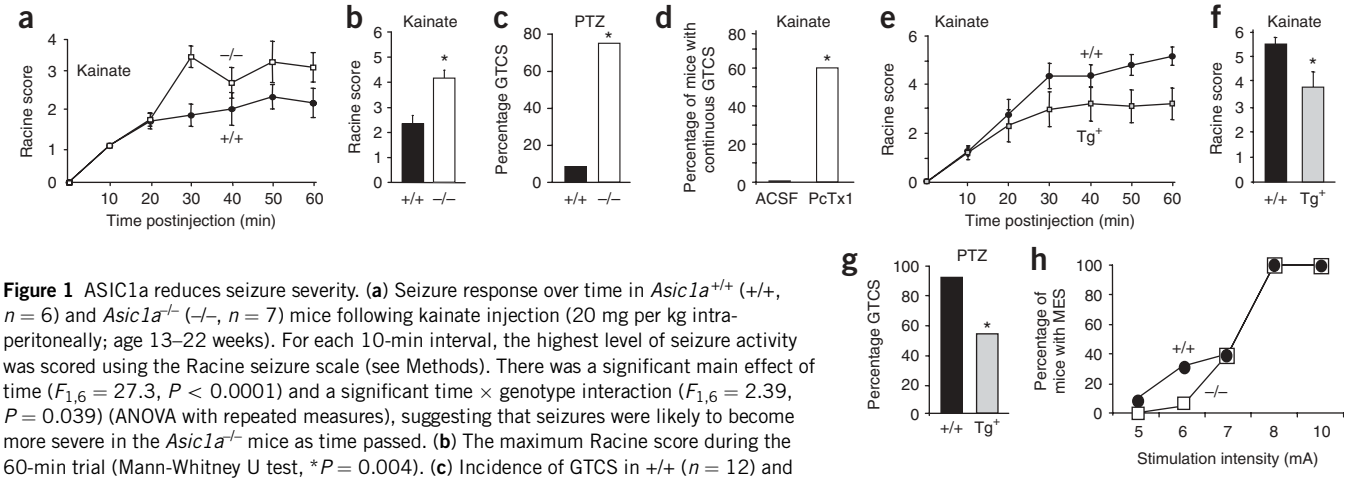

Figure 1 ASIC1a reduces seizure severity. (**a**) Seizure response over time in *Asic1a*$^{+/+}$ (+/+, $n = 6$) and *Asic1a*$^{-/-}$ (–/–, $n = 7$) mice following kainate injection (20 mg per kg intraperitoneally; age 13–22 weeks). For each 10-min interval, the highest level of seizure activity was scored 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 × genotype interaction ($F_{1,6} = 2.39$, $P = 0.039$) (ANOVA with repeated measures), suggesting that seizures were likely to become more severe in the *Asic1a*$^{-/-}$ mice as time passed. (**b**) The maximum Racine score during the 60-min trial (Mann-Whitney U test, $^*P = 0.004$). (**c**) Incidence of GTCS in +/+ ($n = 12$) and –/– ($n = 8$) mice following PTZ injection (50 mg per kg intraperitoneally; 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 μL of ACSF (ICV) or PcTx1 (9 ng μL^{-1}). 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 +/+ ($n = 11$) and ASIC1a-overexpressing transgenic (Tg$^+$, $n = 9$) mice following intraperitoneal injection of 30 mg per kg of kainate (age 31–36 weeks). With time, seizures were less severe in mice Tg$^+$ (ANOVA with repeated measures, $F_{1,3.35} = 3.295$, $P = 0.022$). (**f**) The maximum Racine score during the 60-min trial (Mann-Whitney U test, $^*P = 0.041$). (**g**) Incidence of GTCS in +/+ ($n = 13$) and Tg$^+$ ($n = 13$) mice following PTZ injection (65 mg per kg intraperitoneally; age 25–41 weeks, Fisher's exact test, $^*P = 0.037$). (**h**) Occurrence of MES in +/+ and –/– mice in response to electrical stimulation. ASIC1a disruption did not significantly alter MES threshold (+/+, $n = 50$, CD_{50} 95% confidence interval = 6.24–7.95; –/–, $n = 46$, CD_{50} 95% confidence interval = 6.71–7.76). The sample sizes and current intensities were 5 mA, +/+ ($n = 11$) versus –/– ($n = 7$); 6 mA, +/+ ($n = 22$) versus –/– ($n = 21$); 7 mA, +/+ ($n = 10$) versus –/– ($n = 10$); 8 mA, +/+ ($n = 4$) versus –/– ($n = 4$); 10 mA, +/+ ($n = 3$) versus –/– ($n = 4$); age 8–22 weeks.

RESULTS

ASIC1a disruption increases seizure severity

To learn whether ASIC1a affects seizure severity, we injected wild-type and *Asic1a*$^{-/-}$ (also known as *Accn2*$^{-/-}$) mice with kainate, a chemoconvulsant that activates glutamate receptors. During the first 20 min after injection, mice of both genotypes had similar seizures that affected the head or forelimbs (**Fig. 1a**). With time, however, 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 have been described previously by others[34,35]. The majority of *Asic1a*$^{-/-}$ mice developed GTCS, whereas wild-type mice were less likely to suffer from GTCS (**Fig. 1c**). Thus, with two different chemoconvulsants, the 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]; this form of PcTx1 delivery blocks the effects of ASIC1a on ischemic stroke[29] and fear[37]. 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 that we observed in *Asic1a*$^{-/-}$ mice.

Our 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

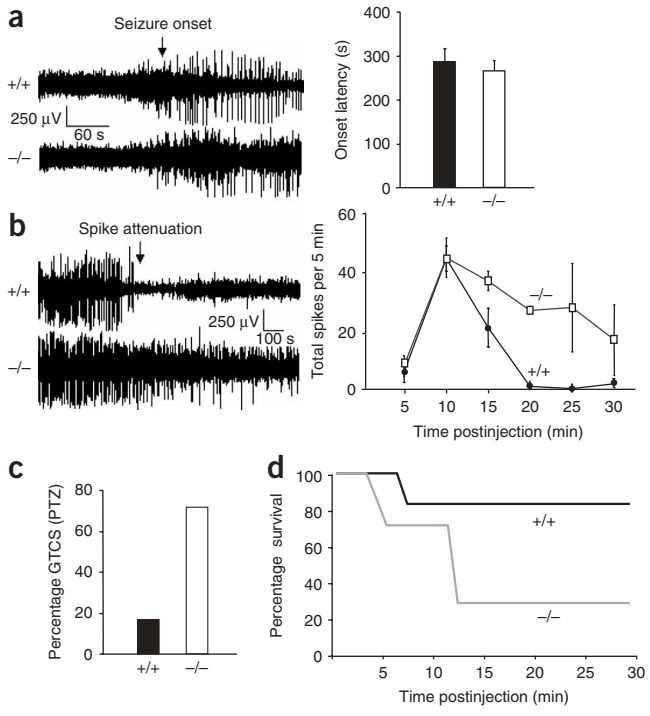

Figure 2 ASIC1a disruption increases seizure duration and progression. (**a**) Representative EEG tracings and quantification of time from PTZ injection (50 mg per kg intraperitoneally) until first seizure spikes in +/+ and –/– mice (+/+, $n = 6$; –/–, $n = 7$; age 18–22 weeks, unpaired *t*-test: $t(11) = 0.544$, $P = 0.597$). (**b**) Representative EEG tracings and total number of seizure spikes per 5-min 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 × genotype interaction ($F_{1,6} = 32.9$, $P < 0.001$), suggesting that –/– mice had prolonged seizure activity as time elapsed. (**c**) Incidence of GTCS following PTZ injection (50 mg per kg intraperitoneally; +/+, $n = 6$; –/–, $n = 7$; Fisher's exact test, $P = 0.078$). (**d**) Survival over time (+/+, $n = 6$; –/–, $n = 7$; Mantel-Cox Log Rank, $P = 0.025$).

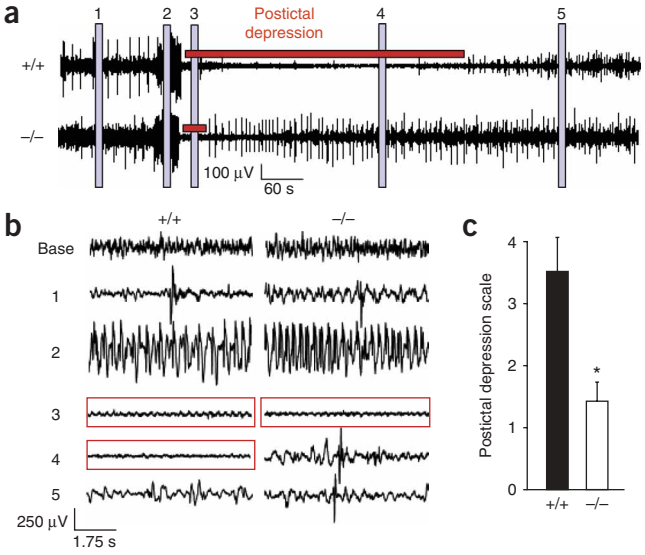

Figure 3 ASIC1a disruption reduces postictal depression. (**a**,**b**) Representative EEG tracings from a +/+ and a –/– mouse approximately 5 min after PTZ injection (50 mg per kg intraperitoneally). Five 5-s intervals are denoted by vertical bars. These are shown below in rows using an expanded time scale (**b**). EEG tracings before PTZ injection (base), initial spike-wave activity (1) and seizures associated with forelimb clonus (2) were similar in both genotypes. Immediately following seizures (3), however, mice entered a period of postictal depression (red boxes). Postictal depression quickly reverted to seizure activity in –/– mice (4 and 5). (**c**) Quantification of postictal depression as scored using the postictal depression scale (see Methods) (+/+, $n = 6$; –/–, $n = 7$, age 18–22 weeks, Mann-Whitney U test, $^*P = 0.011$).

CNS neurons have larger amplitude acid-evoked currents than wild-type littermates[38]. 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 results suggest that there is a relationship between the level of ASIC1a expression and the degree of seizure protection.

ASIC1a shortens seizure duration

The effect of ASIC1a that we observed 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 test[39]. 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, suggests that ASIC1a is not important in determining seizure threshold.

To further assess how ASIC1a reduces seizure severity, we examined PTZ-evoked seizures using electroencephalography (EEG) to observe epileptiform discharges while simultaneously monitoring seizures behaviorally. We reasoned that if ASIC1a inhibited the initiation of seizures, then disrupting ASIC1a would accelerate the onset of EEG spike activity and enhance the initial seizure severity. On the other hand, if ASIC1a enhanced seizure termination, then disrupting ASIC1a would prolong EEG spike activity and increase the probability that seizures would 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, both wild-type and *Asic1a*$^{-/-}$ mice had seizures of similar severity in the first 10 min following PTZ injection, which were characterized by myoclonic jerks (data not shown) and a similar number of EEG spike discharges (**Fig. 2b**). As the seizures continued, however, 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**). Because we could only measure EEG spike activity in surviving animals, however, we probably underestimated 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 postictal depression, has been suggested to result from the factors that

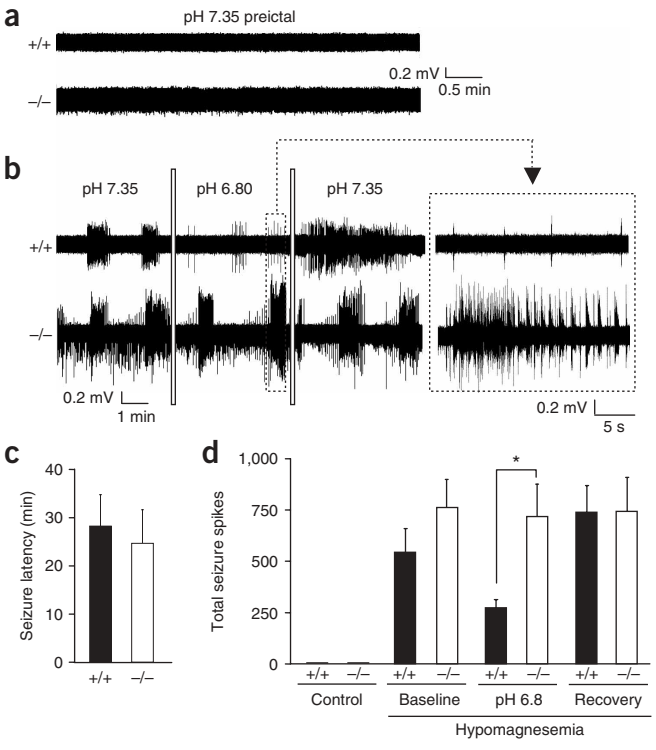

Figure 4 ASIC1a mediates the antiepileptic effects of acid in hippocampal slices. (**a**) Representative CA3 extracellular recording from +/+ and –/– slices before ictal activity caused by hypomagnesemia (nominal Mg^{2+}). (**b**) Representative CA3 recordings from +/+ and –/– slices demonstrating ictal 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 Mg^{2+} (+/+, $n = 7$; –/– $n = 5$; $t(10) = 0.366$, $P = 0.722$). (**d**) Total number of seizure spikes over 4.5 min before induction of ictal activity (control), and then in the presence of nominal Mg^{2+} at pH 7.35 (baseline), pH 6.8 and after return to pH 7.35 (recovery) (+/+, $n = 9$; –/– $n = 8$). In +/+ mice, an ANOVA revealed a significant effect of pH ($F_2 = 7.124$, $P = 0.006$); however, this was not the case in the –/– mice ($F_2 = 0.104$, $P = 0.902$). A within-subjects comparison revealed a significant pH × genotype interaction ($F_2 = 3.78$, $P = 0.034$). At pH 6.8, the spike number was significantly greater in the –/– mice (unpaired *t*-test: $t(15) = -2.88$, $^*P = 0.006$), whereas at baseline and during recovery, the +/+ and –/– mice did not significantly differ (unpaired *t*-test, $P = 0.238$ and 0.581, respectively).

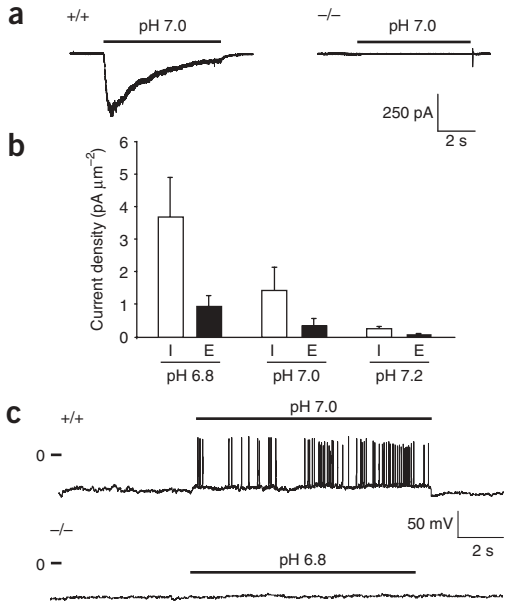

Figure 5 Inhibitory interneurons have prominent ASIC1a currents. (**a**) Representative traces of acid-evoked current in interneurons from the hippocampus of +/+ and –/– mice. Application of pH 7.0 evoked an inward current in +/+ but not –/– neurons. (**b**) From +/+ mice, inhibitory neurons (I) had 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 a +/+ inhibitory neuron. –/– 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 +/+ inhibitory neurons.

cause seizure termination[3,9,40]. In contrast with 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 postictal depression ($P = 0.011$; **Fig. 3c**). This loss of an EEG pattern that is 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 activity[16]. In this model, low pH is known to inhibit seizure-like activity in the hippocampus[16] and ASIC1a is expressed in hippocampal neurons[28].

Before inducing seizures, we observed no epileptiform activity in slices of either genotype (**Fig. 4a**). At pH 7.35, both genotypes showed a similar latency to onset of epileptiform activity (**Fig. 4b**,**c**) and had an equivalent number of epileptiform spikes (**Fig. 4b**,**d**). When we reduced the pH to 6.8, however, 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 is important in limiting epileptiform activity. Therefore, we examined the effects of physiologically relevant reductions in pH on acutely dissociated hippocampal interneurons, as well as on excitatory pyramidal neurons. Neurons were identified on the basis of their location (lacunosum moleculare versus CA1), size, morphology and firing pattern[32,41].

These experiments resulted in four important findings. First, reducing extracellular pH activated inward current in wild-type, but not *Asic1a*$^{-/-}$ interneurons (**Fig. 5a**), a result that is consistent with previous studies that showed that disrupting *Asic1a* eliminated currents evoked by pH reductions to as low as 5.0 (refs. 24,28,29). Second, 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 that we measured (see below). Third, 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 neurons[32]. Finally, when we reduced extracellular pH, we stimulated action potential firing in inhibitory neurons; a pH of 6.8 induced firing in 78% of inhibitory neurons ($n = 9$) and a pH of 7.0 induced firing in 80% of the interneurons ($n = 5$) (**Fig. 5c**). These results suggest that the

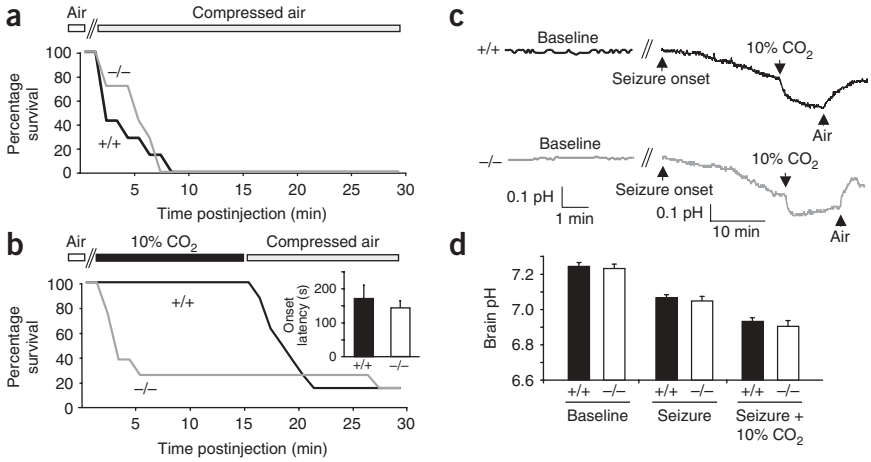

Figure 6 ASIC1a mediates the seizure-terminating effects of 10% CO_2. (**a**) Kaplan-Meier survival analysis of +/+ ($n = 7$) and –/– ($n = 7$) mice in response to PTZ injection (90 mg per kg intraperitoneally) while breathing compressed air (age 13–16 weeks). Both +/+ and –/– mice had the same survival rate (Mantel-Cox Log Rank, $P = 0.582$). (**b**) In a parallel experiment, we administered 10% CO_2 at the onset of GTCS. The onset latency and time of CO_2 administration was similar between genotypes (inset, unpaired *t*-test, $t(14) = 0.663$, $P = 0.518$). The chamber was perfused with CO_2 until minute 15 and was then switched to compressed air for the duration of the trial (+/+, $n = 8$; –/–, $n = 8$; age 13–16 weeks). In CO_2, the likelihood of survival was significantly greater in the +/+ 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 before injection (+/+, $n = 5$; –/–, $n = 5$; $t(4) = 0.283$, $P = 0.784$), during seizures (+/+, $n = 5$; –/–, $n = 5$; $t(4) = 0.505$, $P = 0.627$) or during CO_2 inhalation (+/+, $n = 4$; –/–, $n = 4$; $t(3) = 0.670$, $P = 0.528$) (age 13–15 weeks).

Asic1a$^{-/-}$ animals may lack a source of inhibitory tone during central acidosis and, as a result, fail to inhibit seizure activity.

CO_2 inhalation requires ASIC1a to interrupt seizures

Almost 80 years ago, it was reported that inhaling CO_2 inhibited seizures in humans[13]. Subsequent studies have demonstrated that CO_2 reduces cortical pH in seconds of inhalation[42,43] and that breathing CO_2 increases brain acidosis during a PTZ-evoked seizure[11]. 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% CO_2 immediately after the onset of tonic-clonic seizures (**Fig. 6b**). Notably, 10% CO_2 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 CO_2 back to air at 15 min. They then rapidly died.

To verify that brain pH drops *in vivo* during seizures and CO_2 inhalation, we implanted a fiber optic pH sensor into the lateral cerebral ventricle of wild-type and *Asic1a*$^{-/-}$ mice. Generalized seizures caused the brain pH to fall (pH ~7.05) (**Fig. 6c,d**). CO_2 inhalation rapidly and reversibly lowered the pH even further in the seizing mice (pH ~6.9). Notably, 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 antiepileptic 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 of our findings support the key features of this model. We found that seizures lowered brain pH, as has been 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 Mg^{2+} 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 is part of a feedback inhibition system that limits seizure severity.

Our 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, seizures were less likely to progress to tonic-clonic seizures and death in wild-type mice. Third, ASIC1a increased postictal 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 a seizure has proven difficult[44]. Even less is known about the mechanisms that stop seizures. Our discovery that reducing pH to values that occur during seizures evoked ASIC currents and triggered action potential firing in inhibitory neurons suggests that ASICs might trigger inhibitory neuron activity to terminate seizures. There are several types of inhibitory neurons, however, 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 by depolarization blockade. In addition, although we focused on the hippocampus, ASIC channels are also expressed in many other 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 studies of how the 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 that ASIC1a is involved in mouse models of ischemic stroke[29], neurodegeneration[45] and psychiatric disease[37]. 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 lactate[46]. 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.

METHODS

Mice. We used age- and gender-matched wild-type, *Asic1a*$^{-/-}$, and *Asic1a*$^{Tg+}$ mice on a congenic C57/Bl6 background[28,38]. The care of the mice met the standards set forth by the US National Institutes of Health and our procedures were approved by the University of Iowa Animal Care and Use Committee.

Convulsants. Kainate or pentylenetetrazole (PTZ) (Sigma-Aldrich) were injected into the peritoneum following suspension in phosphate-buffered saline (Gibco) and titration to pH 7.4 with 0.1 N NaOH (ref. 35).

Behavioral assays. To score the effects of ASIC1a on seizure severity in response to kainate, we used the Racine[47] seizure scale: no response (0), staring and reduced locomotion (1), activation of extensors and rigidity (2), repetitive head and limb movements (3), sustained rearing with clonus (4), loss of posture (5), and status epilepticus and death (6). To assess the effects of ASIC1a on seizure severity in response to PTZ, we scored the incidence of GTCS, which were identified by generalized clonus followed by tonic hind limb extension[34]. 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 per kg of body weight) that would evoke GTCS 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 per kg) that evoked GTCS in a large percentage of the control, wild-type mice. A trained observer that was 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). We injected 5 μL of PcTx1-containing venom (SpiderPharm) (9 ng μL^{-1}) in artificial cerebral spinal fluid (ACSF; 124 mM NaCl, 3 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$ and 26 mM $NaHCO_3$) or ACSF alone 3–5 d later into wild-type mice using a 10-μL Hamilton syringe connected to a 30-gauge injector (over 10 s). We injected mice 2 h later with kainate (20 mg per kg, intraperitoneal). 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 previously[39] in *Asic1a*$^{+/+}$ and *Asic1a*$^{-/-}$ mice. Electroshock was delivered (0.2 s, 60 Hz, maximal voltage of 500 V) using the Rodent Shocker (type 221, Harvard Apparatus) with ear electrodes moistened with saline. The occurrence of generalized seizures with sustained hind-limb extension was assessed[39].

EEG recordings and analysis. We stereotactically implanted two 3.2-mm stainless steel screws (Stoelting) 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 intraperitoneal injection of PTZ (50 mg per 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 was 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 that was blinded to genotype. The 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.

Postictal depression was defined as a low-amplitude, slow-wave EEG signal without seizure spikes occurring after a seizure[40]. The duration of postictal 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 100 μV[40]. On the basis of seizure severity and the longest observed period of postictal suppression, each mouse was scored and separated into one of five categories: no postictal depression and lethal seizures (1), no postictal depression with persistent seizure activity (2), depression <60 s (3), depression $= 60$–180 s (4), and depression > 180 s (5).

Slice recordings and analysis. Horizontal hippocampal slices (400 μm) were prepared from 14–24-d-old *Asic1a*$^{+/+}$ and *Asic1a*$^{-/-}$ mice as described previously[28,48]. Prior to sectioning, the mice were transcardially perfused with a high-Mg^{2+}/low-Ca^{2+} solution chilled to 4 °C (4.9 mM $MgSO_4$, 0.5 mM $CaCl_2$, 126 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 27.7 mM $NaHCO_3$, 10 mM dextrose, 1.1 mM $MgCl_2$, pH 7.35, bubbled with 95% O_2/5% CO_2). After sectioning, slices were incubated in ACSF for at least 1 h before testing (126 mM NaCl, 5 mM KCl, 1.8 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 27.7 mM $NaHCO_3$, 10 mM dextrose and 1.6 mM $CaCl_2$). Standard extracellular field-potential recording techniques were carried out in a submerged chamber perfused with ACSF (flow rate of 4 ml min^{-1}, 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, we replaced normal ACSF with ACSF that lacked $MgSO_4$. The latency to onset of epileptiform activity was defined as the time elapsed between switching to nominal Mg^{2+} ACSF and the first epileptiform spike. After scoring the latency to epileptiform activity and recording 5 min of seizure activity, we reduced the pH to 6.8 by lowering the $NaHCO_3$ concentration to 11.4 mM and increasing the sodium gluconate concentration to 16.3 mM to maintain osmolarity. We measured the pH in the recording chamber both before and during infusion of ACSF (pH 6.8). The effects of low pH were then recorded for 5 min, and the 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 previously described method[49]. 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 the pH to 6.8. The 30 s that were 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-d-old *Asic1a*$^{+/+}$ and *Asic1a*$^{-/-}$ mice as described previously[50]. Mice were anesthetized (isoflurane) and decapitated, and we cut 500-μM coronal sections with a vibratome in ice-cold PIPES buffered saline (115 mM NaCl, 5 mM KCl, 20 mM PIPES, 1 mM $CaCl_2$, 4 mM $MgCl_2$ and D-glucose 25, pH 7.0 with NaOH) in the presence of 100% O_2. CA1 and the lacunosum moleculare layer of the hippocampus were removed by microdissection and were trypsin digested (15 mg) for 30 min at 30 °C in 20 ml of PIPES saline. Tissue was washed three times in PIPES saline and triturated in 0.5 ml of PIPES saline with Pasteur pipettes of decreasing apertures to dissociate neurons. Neurons were then diluted in 8 ml of Dulbecco's modified Eagle's medium with 25 mM HEPES, 25 mM glucose (Gibco) and 5% horse serum (vol/vol) 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 in 1 to 5 h as previously described[37]. In brief, neurons were superfused in bath solutions containing 145 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 10 mM 10 2-(4-morpholino)-ethanesulfonic acid, and the pH was adjusted with tetramethylammonium hydroxyl. Pipettes (3–5 MΩ polished glass pipettes, Drummond Scientific, 100 μl) contained 5 mM NaCl, 90 mM potassium gluconate, 15 mM KCl, 1 mM $MgCl_2$, 10 mM EGTA, 60 mM HEPES and 3 mM Na_2ATP, adjusted to pH 7.3 with KOH. Extracellular pH was switched with a Rapid Solution Changer (RSC-200, Biologic). Membrane potential was maintained at –70 mV in voltage-clamp mode. Holding voltage was adjusted to –77 ± 2 mV in current clamp. 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 antiepileptic effects of CO_2 *Asic1a*$^{+/+}$ and *Asic1a*$^{-/-}$ mice were injected intraperitoneally with 90 mg per kg of PTZ. After the onset of generalized clonic seizures, compressed air or 10% CO_2 (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, the surviving mice were killed.

Measuring brain pH. Age (13–15 weeks) and gender-matched *Asic1a*$^{+/+}$ and *Asic1a*$^{-/-}$ mice were anesthetized with ketamine/xylazine. We placed a fiber optic pH sensor (pHOptica) in the left lateral ventricle (coordinates listed above) 60 min after sedation. 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 intraperitoneally. We found that, as a result of the anesthesia, a high dose (120 mg per 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, we injected an additional 60 mg per kg 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 per kg, *Asic1a*$^{-/-}$ = 216 ± 14.7 mg per kg, $P = 0.37$).

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% CO_2 for at least 5 min. Following CO_2 administration, air was administered for an additional 10 min. We assessed the baseline brain pH, minimum pH during seizure and minimum pH during CO_2 administration.

Statistics. Values are expressed as mean ± s.e.m. Where indicated, analyses of significance were carried out 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 $\log_{10}$ 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. We used the Kaplan-Meier analysis and Mantel-Cox log rank to assess survival. Probit

analysis with 95% confidence intervals was used to calculate the convulsive dose that caused seizures in 50% of mice in threshold experiments. $P < 0.05$ was considered statistically significant (Microsoft Excel, SPSS).

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AUTHOR CONTRIBUTIONS

A.E.Z. helped to generate the hypotheses for ASIC1a involvement in seizures, carried out the slice and *in vivo* electrophysiology/behavioral experiments, assessed seizure threshold, measured brain pH in response to seizures and CO_2, assisted in studies using acutely dissociated neurons, wrote initial manuscript drafts and worked closely with other authors in editing the figures and manuscript. M.K.S. initiated, conceived, performed and interpreted electrophysiological experiments in dissociated neurons and assisted in preparation of the manuscript. M.A.S. helped to carry out and interpret the EEG experiments. G.W.A. assisted with seizure threshold studies. M.A.H. provided EEG equipment and helped with EEG interpretation. M.J.W. provided important overall direction and initiation for the project, contributed to experimental design and data interpretation, provided funding and wrote the manuscript. J.A.W. initiated development of the project, led experimental direction, interpreted data, carried out pilot experiments, provided funding and wrote the manuscript.

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