Published in final edited form as:

ACS Chem Neurosci. 2017 June 21; 8(6): 1204–1212. doi:10.1021/acscchemneuro.6b00355.

Acidotoxicity via ASIC1a Mediates Cell Death during Oxygen Glucose Deprivation and Abolishes Excitotoxicity

Saurav Bhowmick^{†,‡,iD}, Jeanette T. Moore[‡], Daniel L. Kirschner[†], Mary C. Curry[†], Emily G. Westbrook[†], Brian T. Rasley[†], and Kelly L. Drew^{*,†,‡}

[†]Department of Chemistry and Biochemistry, University of Alaska Fairbanks, Fairbanks, Alaska 99775, United States

[‡]Institute of Arctic Biology, University of Alaska Fairbanks, Fairbanks, Alaska 99775, United States

Abstract

Ischemic reperfusion (I/R) injury is associated with a complex and multifactorial cascade of events involving excitotoxicity, acidotoxicity, and ionic imbalance. While it is known that acidosis occurs concomitantly with glutamate-mediated excitotoxicity during brain ischemia, it remains elusive how acidosis-mediated acidotoxicity interacts with glutamate-mediated excitotoxicity. Here, we investigated the effect of acidosis on glutamate-mediated excitotoxicity in acute hippocampal slices. We tested the hypothesis that mild acidosis protects against I/R injury via modulation of NMDAR, but produces injury via activation of acid sensing ion channels (ASIC1a). Using a novel microperfusion approach, we monitored time course of injury in acutely prepared, adult hippocampal slices. We varied the duration of insult to delay the return to preinsult conditions to determine if injury was caused by the primary insult or by the modeled reperfusion phase. We also manipulated pH in presence and absence of oxygen glucose deprivation (OGD). The role of ASIC1a and NMDAR was deciphered by treating the slices with and without an ASIC or NMDAR antagonist. Our results show that injury due to OGD or low pH occurs during the insult rather than the modeled reperfusion phase. Injury mediated by low pH or low pH OGD requires ASIC1a and is independent of NMDAR activation. These findings point to ASIC1a as a mediator of ischemic cell death caused by stroke and cardiac arrest.

Graphical abstract

*Corresponding Author. Mailing address: Biochemistry and Neuroscience Program, Institute of Arctic Biology, University of Alaska Fairbanks, PO Box 757000, Fairbanks, AK 99775-7000, USA. kdrew@alaska.edu. Telephone: 907-474-7190.

ORCID

Saurav Bhowmick: 0000-0001-9069-4728

Author Contributions

S.B., J.T.M., and K.L.D. contributed to development of study design, performance of research, writing of manuscript. D.L.K., M.C.C., E.G.W., and B.T.R. contributed to performance of research. K.L.D. contributed to financial support. All authors revised the manuscript for important intellectual content and approved the final version.

The authors declare no competing financial interest.

Keywords

Brain Ischemia; excitotoxicity; glutamate; pH; oxygen-glucose deprivation and reperfusion; acute hippocampal slice

INTRODUCTION

Ischemic reperfusion (I/R) injury associated with stroke and cardiac arrest can lead to severe morbidity and has a mortality rate between 30 and 100%.¹ Several clinical trials aimed at NMDA (*N*-methyl-D-aspartic acid) mediated excitotoxicity have failed.^{2,3} Acidosis exacerbates ischemic brain injury due to a drop in tissue pH to 6.5–6.0.^{4,5} However, an intriguing debate has emerged regarding the influence of mild acidosis on cell damage/protection during cerebral I/R injury. One line of evidence suggests that mild acidosis is neuroprotective^{6,7} and the neuroprotection is due to modulation of the NMDA receptor mediated ischemic cascade.^{8–10} On the contrary, several other works suggest that acidosis activates acid sensing ion channels (ASICs) which results in intracellular Ca^{2+} overload causing acidotoxicity independent of NMDA mediated excitotoxicity.^{11,12}

Although previous in vitro and in vivo studies show that brain ischemia triggers excessive glutamate release,^{13,14} activation of NMDA receptors,¹⁵ and concomitant tissue acidosis,¹⁶ it remains elusive how the acidotic component interacts with glutamate-mediated excitotoxicity. Challenges in deciphering the interaction between these two mechanisms are overcome in the current study by use of a novel in vitro microperfusion technique in which pH is manipulated independently from oxygen glucose deprivation (OGD).

The present study tested the hypothesis that low pH during OGD blocks NMDA mediated excitotoxicity but produces acidotoxicity via ASIC1a. We utilized acute rat hippocampal slices to ascertain the time course of injury from OGD with and without acidosis as well as acidosis alone. Cell death subsequent to these manipulations was assessed by lactate dehydrogenase (LDH) release.¹⁷

RESULTS AND DISCUSSION

OGD Injury via Microperfusion Approach Resembles Similar Disruption in Energy Homeostasis and Excitatory Neurotransmitter Efflux That Mimics in Vivo I/R Injury

During cerebral ischemia in vivo, acidotoxicity and excitotoxicity occur concomitantly and it is unclear how these two mechanisms interact. Moreover, it is not possible to manipulate acidosis to decipher how it interacts with excitotoxicity. Traditional in vitro slice preparations do not produce the same changes in the extracellular milieu as what occurs in vivo because of rapid washing with a highly diluted bath solution.¹⁸ The finding of this study involves using a novel microperfusion technique that allows for time-resolved study of cell death and manipulation of pH with and without OGD thus allowing for study of the interaction between acidosis and OGD-induced injury. The advantages of this model include the opportunity for temporal resolution of injury as well as overflow of metabolites and neurotransmitters and to manipulate each of the components of the ischemic cascade to better understand interactions. With the microperfusion approach we measured only LDH release as a marker of cell death. Cytosolic lactate dehydrogenase release following injury is due to membrane disruption that correlates with cell death. In the present study, for the first time, we have monitored continuous LDH release in an acute slice preparation where it allows for observation of the time course of membrane disruption. To validate the technique, we investigated the effect of in vitro ischemia-like condition (OGD) exposure on energy homeostasis¹⁹ and excitotoxic neurotransmitter efflux,¹³ a hallmark of ischemic injury in acute hippocampal slices from rat. Results show that exposure of acute hippocampal slices to 30 min OGD insult resulted in a significant depletion in ATP levels. Slices treated with OGD showed a significant drop in ATP when compared to control slices. Slices with OGD showed no signs of ATP restoration even after 3 h of reperfusion (Figure 1A). This indicates that energy homeostasis is highly dysregulated due to OGD exposure. To further validate the disruption of energy homeostasis in correlation to adenosine, we investigated the level of adenosine efflux upon OGD exposure. Slices exposed to 30 min of OGD showed significant efflux in extracellular adenosine when compared to the control slices. The adenosine level returned to baseline within 15 min of the onset of reperfusion (Figure 1B). From our results, we conclude that when acute hippocampal slices are subjected to 30 min of OGD insult, they release extracellular adenosine produced from rapid degradation of ATP due to ischemic-like conditions.

We next investigated the effect of OGD exposure on excitotoxic neurotransmitter efflux such as glutamate and aspartate. Consistent with previous in vivo studies,^{14,20} perfused slices exposed to OGD injury for 30 min released glutamate (62-fold increase over basal level) (Figure 2A). Similarly, aspartate efflux also increased during OGD (11.4-fold increase over basal level) (Figure 2B). After the onset of reperfusion, the glutamate levels returned to baseline levels within 60 min, aspartate returned to baseline levels within 45 min.

The observations reported herein validate the microperfusion approach. Dysregulation in energy homeostasis and excessive release of excitatory neurotransmitters are decisive for the development of excitotoxicity during cerebral ischemia. With our approach, when hippocampal slices are subjected to OGD, tissue ATP declines and adenosine, glutamate,

and aspartate are released into the extracellular space. These observations are in accord with previous in vitro and in vivo studies that supports similar energy dysregulation^{21,22} and excitatory neurotransmitter release during cerebral ischemia in vivo and OGD in vitro.^{13,14,23–25}

OGD or Acidosis Causes Injury during Insult Rather than during Reperfusion

How much injury occurs during the ischemic and reperfusion phases continues to be controversial in both experimental animal and clinical studies.²⁶ Intrinsic to ischemia reperfusion injury is the concept that harmful events are occurring during both ischemia and reperfusion.^{27,28} With the microperfusion approach, we monitored the timing of injury to decipher whether cell death was due to “insult” (OGD or low pH) or to “reperfusion”. In these experiments, reperfusion was modeled by returning oxygen and glucose or by returning pH to 7.3. We varied the duration of insult between 15, 30, and 45 min with the expectation that delayed return of oxygen and glucose or delayed return to pH 7.3 would delay LDH release if injury was caused by the modeled reperfusion phase. Delaying onset of modeled reperfusion (for 15, 30, or 45 min) or the return to pH 7.4 from pH 6.4 (for 15, 30, or 45 min) did not delay the peak time of LDH release. However, the magnitude of injury was increased (Figure 3). This indicates that injury was caused by the primary OGD or low pH insult and not by modeled reperfusion.

One study suggests that low pH causes detrimental effect during reperfusion.²⁹ However, extracellular pH decreases immediately³⁰ after the onset of ischemia and is restored to the normal physiological range upon reoxygenation.³¹ Given this time course of acidosis, a sustained severe acidosis during the reperfusion phase is unlikely. When acidosis was prolonged during the low pH insult phase, injury increased in magnitude but was not offset by the time of reperfusion. A similar observation was made with OGD insult. Thus, with regard to timing of acidosis, our experimental setup mimicked the in vivo scenario and shows that injury associated with OGD or low pH occurs during insult rather than during reperfusion.

Low pH Delays OGD Injury and Shows Injury Independent of OGD Insult

Buffering of the OGD solution during in vitro experiments eliminates the acidotic component that occurs during cerebral ischemia in vivo. To study the interaction between acidosis and OGD on cellular injury, the pH of the OGD solution was adjusted from 7.3 to 6.4. OGD at pH 6.4 decreased and delayed LDH release as compared to OGD at physiological pH 7.3. Results show that OGD with low pH shows similar time course of injury as observed with low pH alone. With similar time course of injury, no significant statistical differences were observed at any given time point between OGD with low pH and low pH alone (Figure 4A). This suggests that when low pH is introduced along with OGD, low pH overrides OGD mediated injury. When the total injury represented by the area under the curve was considered, LDH released by pH 6.4 was not different from LDH released by OGD, pH 7.3 or by OGD pH 6.4 (Figure 4B). This indicates that low pH delays OGD injury, but on its own causes injury that is as deleterious as OGD.

Injury Caused by OGD Insult Is Due to NMDAR Activation Whereas Low pH Injury Is ASIC1a Mediated

We next ask whether the delay in injury caused by acidosis is due to modulation of NMDAR. While application of NMDAR antagonist (MK-801) decreased LDH release in groups subjected to OGD, pH 7.3 (Figure 5A), no significant effect on LDH release was observed in slices subjected to acidosis, pH 6.4 (Figure 5B) or OGD with acidosis, pH 6.4 (Figure 5C). This suggests that OGD injury at physiological pH involves NMDAR activation whereas injury associated with acidosis alone or acidosis in combination with OGD is independent of NMDAR mediated excitotoxicity.

Because acidosis contributes to cell death independent of OGD (Figure 4), we next explored the role of ASICs. We expected that targeting the ASIC1a with specific and nonspecific blockers would attenuate pH 6.4-mediated cell death. As illustrated in Figure 6A and B, continuous perfusion of 100 μ M amiloride (nonspecific ASIC blocker which acts by blocking the channel pore) during acidosis, pH 6.4 and OGD pH 6.4 tended to decrease LDH release; however, it failed to produce a statistically significant effect. Next, we tested the effect of Psalmotoxin (PcTx), a specific blocker of Ca^{2+} -permeable homomeric ASIC1a. Application of PcTx (50 nM) blocked aCSF pH 6.4 and OGD pH 6.4-induced LDH release (Figure 6C,D). This result supports the involvement of ASIC1a in low pH mediated injury.

Results reported herein demonstrate that delayed injury was solely due to ASIC1a activation since NMDAR blockade had no effect on OGD-induced cell death in the presence of proton load. No decrease in LDH release with NMDAR blockade in the presence of proton load could also be due to high concentration of MK-801 (100 μ M). The higher concentration might have compromised the specificity of MK-801 on the NMDAR favoring nonspecific binding that masked the neuroprotective effect of NMDAR blockade. Since, with the same concentration of MK-801, we observed decreased LDH release in groups subjected to OGD at pH 7.3, we believe that the nonspecificity associated with high concentration of MK-801 did not confound the results of our study. Blocking the NMDAR with MK-801 will block the NR2B-NMDARs/CaMKII cascade described previously to enhance ASIC1a-mediated current.³² The present work cannot rule out a NR2B-specific pathway; however, injury caused by low pH alone or with OGD did not depend on a NR2B/CaMKII pathway since MK-801 blocks all NMDARs including those that contain NR2B.

Preclinical in vivo studies and clinical trials have also failed to show neuroprotective effects of MK-801.^{33–35} The interpretation that low pH abolishes NMDAR mediated excitotoxicity contrasts with other studies where authors have suggested that OGD-induced cell death is due to NMDAR, but delayed by pH dependent inhibition of NMDA.^{7,8,36} Here we show that delayed injury is solely due to ASIC1a, that is unmasked by complete attenuation of excitotoxicity.

This interpretation is consistent with prior work showing that mild acidosis during brain ischemia protects against excitotoxic injury by modulating glutamate-mediated excitotoxicity.^{6,7} These results also support prior work showing that increased load of proton due to acidosis^{4,37} activates a distinct family of ligand-gated channels, the acid-sensing ion channels, ASICs (ENaC/Deg superfamily)^{38–40} and mediates injury unrelated to NMDAR.

The role of ASIC1a activation during acidotoxicity has been previously confirmed in in vivo studies. Furthermore, ASIC1a knockout or pharmacological blockade of ASIC1a attenuates injury associated with brain ischemia. Our present results with selective antagonism of ASIC1a with PcTX1 are consistent with the role of ASIC1a in ischemic damage as previously reported. The less robust ability of amiloride to attenuate the effect of low pH could be due to dose. The IC₅₀ of amiloride to inhibit ASICs is between 5 and 100 μ M.⁴¹ In this dose range, amiloride is not specific for ASIC1a. For example, at 100 μ M, the concentration used in this study, amiloride enhances the sustained current evoked in cells transfected with ASIC3.^{42,43} At slightly higher concentrations, amiloride affects other targets including other ion channels (e.g., epithelial Na⁺ channel and calcium channels) and exchangers (e.g., Na⁺/H⁺ and Na⁺/Ca²⁺).^{44,45} Thus, it was not feasible to test a higher dose of amiloride without confounding results by nonspecific effects. For this reason, we tested the specific ASIC1a blocker PcTX1. This specific blocker of ASIC1a completely abolished LDH release caused by both low pH alone and low pH with OGD.

Both activation of NMDAR and ASIC1a causes high influx of intercellular Ca²⁺ that causes cell damage. Previous studies provide strong evidence that ASIC1a-mediated increase in Ca²⁺ influx could contribute to Ca²⁺ overload, an important mechanism of cell damage and death in ischemia.^{11,12} Others have shown that Ca²⁺ is not required for ASIC1a-mediated cell death.⁴⁶ Our study of the interaction of OGD and pH was enabled through the use of a novel microperfusion technique. This in vitro preparation allowed for independent manipulation of OGD and pH and for time-resolved assessment of cell death. The approach was validated by demonstrating that it mimics an in vivo cerebral ischemia scenario in terms of dysregulation in energy homeostasis and excessive release of excitatory neurotransmitters, hallmarks of ischemic injury. With the microperfusion approach, we measured only LDH release as a marker of cell death throughout the slice and cannot distinguish between sub regions of the hippocampus or between cell type. LDH release is a standard approach to monitor cell death.⁴⁷ LDH is released from the cytosol due to membrane disruption and not due to metabolic changes related to OGD. In the present study, for the first time we have monitored continuous LDH release in an acute slice preparation where it allows for observation of the time course of membrane disruption from the entire slice. We interpret membrane disruption as an early indicator of cell death. Other markers of cell death such as propidium iodide (PI) staining produce high background due to damage of the cells at the surface during acute slice preparation. Results here, based on time-resolved release of LDH, are consistent with prior work from this laboratory, based on PI staining in acutely prepared or short-term cultured slices.^{48,49} Although slices are incubated until high baseline levels of LDH reach a stable minimum before insult is added, there is a possibility that acutely injured cells on the surface of the slice are affected more than cells at the center of the slice. Nonetheless, the novel microperfusion method developed in our laboratory⁵⁰ for study of cell death in acute, adult slices overcomes the limitations of methods used in prior studies where high baseline PI staining could have compromised interpretation.^{48,49} The microperfusion approach minimizes background cell death caused by trauma at the surface of the slice by capturing LDH release from the entire slice. The microperfusion approach also monitors the time course of injury as well as the overflow of metabolites and neurotransmitters. Despite these advantages over other in vitro approaches to monitor cell

death, we acknowledge that the slice preparation differs from the *in vivo* scenario.^{51,52} However, the microperfusion approach gives superior control over the extracellular environment that could not be achieved *in vivo*. Here we show that LDH release during OGD or low pH insult reaches a maximum peak and then subsides. We suggest that the decay in the LDH signal peak is not a consequence of dilution because flow rate and sampling rate are constant throughout the experiment. Furthermore, the waning in LDH release is not due to depletion of LDH since no difference in total LDH content measured in homogenized slices at the end of the experiment was observed in slices subjected to aCSF, OGD, or low pH treatment (data not shown). Further study will be required to understand why LDH release subsides over time.

Previous *in vitro* studies performed in cell culture or in cultured postnatal brain tissue^{8,9,29,53} suggest 4 h of OGD is necessary to induce cell death. By contrast, our study shows that 30 min of insult is sufficient to cause immediate membrane disruption marked by LDH release. It is to be noted that there is a difference in how each model responds to a commonly studied ischemic-like paradigm, oxygen-glucose deprivation. Previous studies show that acute slice viability is affected by OGD lasting as little as 10 min,^{54,55} whereas organotypic hippocampal slice cultures tolerate a much longer duration of challenge.^{53,56,57} We chose to use acute slices, because it allows for study of cell death in adult tissue not possible with organotypic hippocampal slice culture. Organotypic cultures differ from adult brain slices in several ways such as influence of age (adult vs neonatal) which may influence the mechanisms of resistance to injury, proportion of neurons to glia, synaptic connections between neurons, neural circuitry that influences metabolic demand of the tissue, and maturity of the neurons studied.^{58–60} Thus, study in cultured cells or cultured postnatal tissue may not reflect mechanisms operative in adult brain tissue.

CONCLUSION

This paper reports several important findings using a novel microperfusion technique that for the first time provide time-resolved observations of excitotoxicity and acidotoxicity in an *in vitro* model of cerebral ischemia/reperfusion in adult hippocampus. First, our results demonstrate that injury associated with acidosis (pH 6.4) or OGD (pH 7.3) occurs during insult rather than during the reperfusion phase. Second, we show that acidosis combined with OGD delays OGD injury. Finally, we show that when OGD is combined with acidosis as occurs *in vivo*, acidotoxicity mediated via ASIC1a occurs but low pH abolishes NMDAR mediated excitotoxicity.

In conclusion, we show that acidosis eliminates the NMDAR contribution to OGD-induced cell death but causes injury through an ASIC1a mediated mechanism. Although this study resolves controversy surrounding the interplay between NMDA and ASIC1a, the translational potential of ASIC1a may be limited. Acidosis may have longer-term effects not addressed here. Pignataro et al. observed protective effects when an ASIC1a antagonist was applied as late as 5 h after occlusion *in vivo*.⁶¹ Other mechanisms such as uncoupling of NADPH oxidase from NMDAR activation by intracellular acidosis⁶² or acidosis mediated reorganization of mitochondrial efficiency⁶³ could also contribute to longer-lasting intercellular acidosis mediated protective effects. Identification and understanding of the

interaction between acidotoxicity and NMDAR mediated excitotoxicity point toward a better mechanistic approach in design of an effective therapeutic strategy for brain ischemia caused by stroke and cardiac arrest.

METHODS

Animals

Adult Male Sprague–Dawley rats (Simonsen laboratories, Gilroy, CA; age 3–4 months, 250–400 g) were used for all experiments. The animals used in this study were housed in University of Alaska Fairbanks Animal Resource Center (ARC), fed ad libitum, and kept under a 12:12 light/dark cycle with lights on at 0700 and off at 1900. During housing, animals were monitored once daily for health status. No adverse events were observed during housing. All experimental procedures were approved by the University of Alaska Fairbanks Institutional Animal Care and use Committee (IACUC), and performed in accordance with the strict guidelines of the eighth edition of the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Research was conducted in accordance with ARRIVE guidelines for reporting animal research.⁶⁴

Acute Hippocampus Slices Preparation

Slices were prepared between 0800 and 1030. Animals were anesthetized using 5% (v/v) isoflurane with medical grade O₂ at a constant flow rate of 1.5 L/min. Once unresponsive, the animals were euthanized via rapid decapitation and their brains quickly removed. The whole brain was then placed in ice chilled, oxygenated HEPES buffered artificial cerebral spinal fluid (HEPES-aCSF) containing 120 mM NaCl, 20 mM NaHCO₃, 6.68 mM HEPES acid, 3.3 mM HEPES sodium salt, 5.0 mM KCl, 2.0 mM MgSO₄ (pH 7.3–7.4) to attenuate edema during the course of slicing and incubation. Rapidly dissected hippocampi were embedded in 2.5% agar. Approximately 10 mm was discarded from both ends, and 400 µm thick slices from each hippocampus were cut at approximately 2 °C in oxygenated HEPES-aCSF using a Vibratome 1000plus sectioning system (The Vibratome Company, St. Louis, MO). The slices were then transferred to a brain slice keeper (Scientific Systems Design Inc., Mississauga, Ontario, CA) and allowed to recover for 1–1.5 h at room temperature in HEPES-aCSF bubbled continuously with 95% O₂/5% CO₂ before transferring to experimental conditions as indicated.

Microperfusion Chamber

To address the time course of injury, treatment was applied using an in vitro microperfusion technique described previously.⁵⁰ Briefly, after 1–1.5 h recovery as described above, individual slices were transferred gently to microperfusion chambers and lids sealed. The 4–8 parallel chambers were perfused with artificial cerebrospinal fluid (aCSF), pH 7.3 containing 120 mM NaCl, 45 mM NaHCO₃, 10 mM glucose, 3.3 mM KCl, 1.2 mM NaH₂PO₄, 2.4 mM MgSO₄, 1.8 mM CaCl₂ bubbled with 95% O₂/5% CO₂. Sealed chambers were submerged in a water bath at 36 °C (±0.2 °C) and perfused with aCSF at a flow rate of 7 µL/min. Sampling began 75 min after submerging the sealed chambers to allow adequate time for stabilization of neurochemical efflux. At 15 min intervals, perfusates (7 µL/min × 15 min = 105 µL) were collected and analyzed as indicated. The volume of each chamber

was estimated at 35 μL based on dimensions of $9 \times 5 \times 0.7 \text{ mm}^3$ ($L \times W \times H$) with 0.3 mm deep microchannels. Approximately half of this volume was displaced by the 400 μm thick slice.

Treatment/Injury Conditions

To model ischemia-induced alterations in the ionic microenvironment, acute hippocampal slices were exposed to 30 min of one of the four treatments: (1) aCSF, pH 7.3, (2) OGD, pH 7.3 (glucose-oxygen free aCSF), (3) aCSF, pH 6.4 (120 mM NaCl, 7.03 mM NaHCO_3 , 10 mM glucose, 3.3 mM KCl, 1.2 mM NaH_2PO_4 , 2.4 mM MgSO_4 1.8 mM CaCl_2 , and (4) OGD, pH 6.4 (glucose-oxygen free low pH aCSF). Acidosis was induced with a pH of 6.4 based on previous studies that suggest hippocampal homomeric ASIC1a peak current showed a high sensitivity to pH 6.4 ($\text{pH}_{0.5} = 6.4$ and $nH = 1.65$ for ASIC1a).⁶⁵ Ischemia-induced alteration was made by switching the solution (from aCSF pH 7.3 to OGD pH 7.3, aCSF, pH 6.4 and OGD, pH 6.4) 8 min prior to the start of insult, the time required to completely replace the solution in the chamber at a flow rate of 7 $\mu\text{L}/\text{min}$. All aCSF solutions were equilibrated with 95% O_2 and 5% CO_2 whereas the OGD solutions were equilibrated with 5% CO_2 and 95% N_2 , for a minimum of 1 h until pH stabilized in the desired range. The osmolarity of these solutions was between 290 and 300 mOsm. The PO_2 in OGD solution varied from 0 to 2.9 mmHg with an average for six determinations of 1.1 mmHg as measured using a miniature Clark-style electrode (Instech Laboratories, Plymouth Meeting, PA). Collected fractions were analyzed for cell death (LDH release) on the day of collection. Remaining volume was kept at -80°C for subsequent analysis.

For evaluating the effect of NMDAR and ASIC1a on OGD or acidosis mediated cellular injury in acute hippocampal slices, the respective inhibitors were added throughout the experiment (during pretreatment, the insult phase and reperfusion). MK-801 (noncompetitive NMDAR antagonist, 100 μM , Sigma-Aldrich, St Louis, MO), Amiloride (nonspecific ASIC inhibitor, 100 μM , Cayman chemical, Ann Arbor, MI), and Psalmotoxin (PcTX1, specific ASIC1a inhibitor, 50 nM, Abcam, Cambridge, MA) were used for NMDAR and ASIC manipulations.

Assessment of Cellular Injury: LDH Measurement

Cell injury was assessed by the measurement of LDH release.¹⁷ About half (50 μL) of each 15 min sample was transferred to 384-well plates and mixed with 50 μL of reaction solution provided by the LDH assay kit (Cayman chemical, Ann Arbor, MI). Optical density was measured at 490 nm 60 min later, using a microplate reader (Epoch Microplate Spectrophotometer, BioTek, Winooski, VT). Background absorbance at 620 nm was subtracted. The LDH release was quantified as an arbitrary unit per mg of protein (Bio-Rad protein assay kit, Hercules, CA) and represented as a fraction of baseline LDH release. The maximal releasable LDH was also obtained by treating the slices with 1% NP-40 lysis buffer (50 mM Tris-HCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Igepal, 150 mM NaCl, 0.05% Triton X-100) at the end of each experiment.

Determination of Glutamate and Aspartate

Glutamate and aspartate were analyzed by capillary electrophoresis. Perfusates were derivatized as described previously^{50,66} to form highly fluorescent cyanobenz[*f*]isoindole amino acids (CBI-amino acids). Briefly, 2 μ L of thawed perfusate was reacted with 2 μ L of 2 mM naphthalene-2,3-dicarboxaldehyde (NDA) in methanol and 2 μ L of NaCN (5.5 mM) in 60 mM sodium tetraborate. Cyanide solution contained 3 μ M D-amino adipic acid as internal standard. Samples were reacted at room temperature for approximately 20 min prior to analysis. CE-LIF was performed on a custom in-house-built instrument using a 420 nm diode laser, 490 nm bandpass filter, and PMT for LIF detection. Samples were injected onto a bare fused silica capillary of dimensions 25 cm $\times$ 25 μ m (22.5 cm to detection window) for 1–3 s at 380-mbar vacuum and separated by using positive polarity (20 kV). Separation buffer optimized for analysis of L-glutamate and L-aspartate is a 50 mM sodium tetraborate background electrolyte (BGE) adjusted to pH 9.3. BGE additionally contained 1.0 mM β -cyclodextrin to decrease sample run time for glutamate and aspartate analysis. The addition of β -cyclodextrin allowed for baseline resolution of glutamate, aspartate, and the internal standard in less than 2 min. PeakFit software (SeaSolve Software Inc., Framingham, MA) was used to process raw data and quantify peak areas in all experiments based on a Gaussian peak shape for each analyte. Linear calibration curves were constructed for glutamate and aspartate as a function of concentration versus the peak area ratio (analytes area/internal standard area). Efflux of analytes was quantified as a concentration of glutamate or aspartate normalized to total protein for the slice used in the efflux experiment and represented as a fraction of baseline glutamate or aspartate release.

Determination of ATP and Adenosine

ATP was determined after bath application of OGD or normoxic conditions as described previously.⁴⁸ In brief, slices were incubated in either aCSF or OGD solution and collected at the time point indicated. From each treatment conditions, three hippocampal slices were transferred to a flat-bottomed microfuge tube containing 200 μ L of ice-cold 5% trichloroacetic acid, sonicated (8 pulses), and centrifuged at 16 000*g* for 1 min at 4 $^{\circ}$ C. Supernatant was then collected and assayed for ATP using ENLITEN ATP assay (Promega, Madison, WI). The pellets were further reconstituted with 200 μ L of 1 M NaOH, and protein concentration was analyzed (Bio-Rad protein assay kit, Hercules, CA). ATP level was represented as a concentration of ATP normalized to total protein for the slices.

Adenosine level was determined using HPLC as previously reported with slight modifications. In brief, 2 mM acetic acid (pH 4.0) in 7% acetonitrile constitute the mobile phase having a flow rate of 20 μ L/min produced by a PrimeLine pump (ASI, Analytical Scientific Instruments, El Sobrante, CA). The process of separation was achieved by a BAS microbore column (MF-8949; 1 $\times$ 100 mm and 1 $\times$ 14 mm guard column, with C18 packing of 3 μ m particle size, BASi, West Lafayette, IN), which was attached directly to the injector (Rheodyne 9125, Rohnert Park, CA) and to the UV detector (Waters 2487 UV detector, outfitted with a Waters microbore cell kit, Waters Corporation, Milford, MA). Adenosine was detected at a wavelength of 258 nm. Chromatographic data were recorded with a Peak Simple Chromatography data system (SRI, model 203, SRI Instruments, Torrance, CA) and the peak areas of microperfusion samples were compared to the peak areas of adenosine

standards (10 μ L of 50 nM) for quantification. The detection limit of the assay was 33 fmol (based on a signal-to-noise ratio of 3:1). Concentration is expressed as nM and retention time for adenosine was between 11.0 and 11.3 min.

Statistical Analysis

A priori power analysis (using G*Power software) was performed to estimate sample size needed to yield 80% power for detecting a therapeutically significant effect of treatment. No animal was excluded from the study. Slices from animals were randomly assigned to treatment and control groups without prior knowledge to the treatment conditions. Data processing and statistical analyses were performed using Microsoft Office Excel 2010 and GraphPad Prism 6. Data were analyzed with one-way or two-way ANOVA with repeated measures. Significant main effects or interactions were followed by *t* tests with Bonferroni's correction. Data are expressed as mean $\pm$ SEM, and $p < 0.05$ was considered as statistically significant.

Acknowledgments

The authors thank Dr. Tom Kuhn for helpful discussions and Ian Herriott, technician, UAF DNA core facility for assisting with the microplate reader.

Funding

Research reported in this publication was supported by the US Army Medical research and Material Command, No 0517800; the National Institute of Neurological Disorder and Stroke, Nos. NS041069-06 and R15NS070779; Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under Grant Number P20GM103395. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

1. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Huffman MD, Judd SE, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Mackey RH, Magid DJ, Marcus GM, Marelli A, Matchar DB, McGuire DK, Mohler ER 3rd, Moy CS, Mussolino ME, Neumar RW, Nichol G, Pandey DK, Paynter NP, Reeves MJ, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Wong ND, Woo D, Turner MB. Heart disease and stroke statistics—2014 update: a report from the American Heart Association. *Circulation*. 2014; 129:e28–e292. [PubMed: 24352519]
2. Lipton SA. Failures and successes of NMDA receptor antagonists: molecular basis for the use of open-channel blockers like memantine in the treatment of acute and chronic neurologic insults. *NeuroRx*. 2004; 1:101–110. [PubMed: 15717010]
3. Hoyte L, Barber PA, Buchan AM, Hill MD. The rise and fall of NMDA antagonists for ischemic stroke. *Curr. Mol. Med*. 2004; 4:131–136. [PubMed: 15032709]
4. Rehncrona S, Rosen I, Siesjo BK. Brain lactic acidosis and ischemic cell damage: 1. Biochemistry and neurophysiology. *J. Cereb. Blood Flow Metab*. 1981; 1:297–311. [PubMed: 7328145]
5. Nedergaard M, Goldman SA, Desai S, Pulsinelli WA. Acid-induced death in neurons and glia. *J. Neurosci*. 1991; 11:2489–2497. [PubMed: 1869926]
6. Tombaugh GC, Sapolsky RM. Mild acidosis protects hippocampal neurons from injury induced by oxygen and glucose deprivation. *Brain Res*. 1990; 506:343–345. [PubMed: 2154291]
7. Kaku DA, Giffard RG, Choi DW. Neuroprotective effects of glutamate antagonists and extracellular acidity. *Science*. 1993; 260:1516–1518. [PubMed: 8389056]
8. Giffard RG, Monyer H, Christine CW, Choi DW. Acidosis reduces NMDA receptor activation, glutamate neurotoxicity, and oxygen-glucose deprivation neuronal injury in cortical cultures. *Brain Res*. 1990; 506:339–342. [PubMed: 1967968]

9. Tang CM, Dichter M, Morad M. Modulation of the N-methyl-D-aspartate channel by extracellular H⁺ + Proc. Natl. Acad. Sci. U. S. A. 1990; 87:6445–6449. [PubMed: 1696732]
10. Traynelis SF, Cull-Candy SG. Proton inhibition of N-methyl-D-aspartate receptors in cerebellar neurons. *Nature*. 1990; 345:347–350. [PubMed: 1692970]
11. Xiong ZG, Zhu XM, Chu XP, Minami M, Hey J, Wei WL, MacDonald JF, Wemmie JA, Price MP, Welsh MJ, Simon RP. Neuroprotection in ischemia: blocking calcium-permeable acid-sensing ion channels. *Cell*. 2004; 118:687–698. [PubMed: 15369669]
12. Yermolaieva O, Leonard AS, Schnizler MK, Abboud FM, Welsh MJ. Extracellular acidosis increases neuronal cell calcium by activating acid-sensing ion channel 1a. *Proc. Natl. Acad. Sci. U. S. A.* 2004; 101:6752–6757. [PubMed: 15082829]
13. Benveniste H, Drejer J, Schousboe A, Diemer NH. Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *J. Neurochem.* 1984; 43:1369–1374. [PubMed: 6149259]
14. Mitani A, Andou Y, Matsuda S, Arai T, Sakanaka M, Kataoka K. Origin of ischemia-induced glutamate efflux in the CA1 field of the gerbil hippocampus: an in vivo brain microdialysis study. *J. Neurochem.* 1994; 63:2152–2164. [PubMed: 7964735]
15. Choi DW. Excitotoxic cell death. *J. Neurobiol.* 1992; 23:1261–1276. [PubMed: 1361523]
16. Siemkowicz E, Hansen AJ. Brain extracellular ion composition and EEG activity following 10 minutes ischemia in normo- and hyperglycemic rats. *Stroke*. 1981; 12:236–240. [PubMed: 7233472]
17. Koh JY, Choi DW. Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *J. Neurosci. Methods*. 1987; 20:83–90. [PubMed: 2884353]
18. Yao H, Sun X, Gu X, Wang J, Haddad GG. Cell death in an ischemic infarct rim model. *J. Neurochem.* 2007; 103:1644–1653. [PubMed: 17727626]
19. Dale N, Frenguelli BG. Release of adenosine and ATP during ischemia and epilepsy. *Current neuropharmacology*. 2009; 7:160–179. [PubMed: 20190959]
20. Cui Y, Zhang L, Utsunomiya K, Yanase H, Mitani A, Kataoka K. Ischemia-induced glutamate release in the dentate gyrus. A microdialysis study in the gerbil. *Neurosci. Lett.* 1999; 271:191–194. [PubMed: 10507701]
21. Latini S, Pedata F. Adenosine in the central nervous system: release mechanisms and extracellular concentrations. *J. Neurochem.* 2001; 79:463–484. [PubMed: 11701750]
22. Dunwiddie TV, Diao L, Proctor WR. Adenine nucleotides undergo rapid, quantitative conversion to adenosine in the extracellular space in rat hippocampus. *J. Neurosci.* 1997; 17:7673–7682. [PubMed: 9315889]
23. Hagberg H, Lehmann A, Sandberg M, Nystrom B, Jacobson I, Hamberger A. Ischemia-induced shift of inhibitory and excitatory amino acids from intra- to extracellular compartments. *J. Cereb. Blood Flow Metab.* 1985; 5:413–419. [PubMed: 4030918]
24. Butcher SP, Bullock R, Graham DI, McCulloch J. Correlation between amino acid release and neuropathologic outcome in rat brain following middle cerebral artery occlusion. *Stroke*. 1990; 21:1727–1733. [PubMed: 2264080]
25. Shimizu H, Graham SH, Chang LH, Mintoovitch J, James TL, Faden AI, Weinstein PR. Relationship between extracellular neurotransmitter amino acids and energy metabolism during cerebral ischemia in rats monitored by microdialysis and in vivo magnetic resonance spectroscopy. *Brain Res.* 1993; 605:33–42. [PubMed: 8096789]
26. Kloner RA. Does reperfusion injury exist in humans? *J. Am. Coll. Cardiol.* 1993; 21:537–545. [PubMed: 8426022]
27. Jaeschke H. Kupffer cell-induced oxidant stress during hepatic ischemia-reperfusion: does the controversy continue? *Hepatology*. 1999; 30:1527–1528. [PubMed: 10573534]
28. Kalogeris T, Baines CP, Krenz M, Korthuis RJ. Cell biology of ischemia/reperfusion injury. *Int. Rev. Cell Mol. Biol.* 2012; 298:229–317. [PubMed: 22878108]
29. Gu L, Liu X, Yang Y, Luo D, Zheng X. ASICs aggravate acidosis-induced injuries during ischemic reperfusion. *Neurosci. Lett.* 2010; 479:63–68. [PubMed: 20478356]

30. Simon RP, Benowitz N, Hedlund R, Copeland J. Influence of the blood-brain pH gradient on brain phenobarbital uptake during status epilepticus. *J. Pharmacol. Exp. Ther.* 1985; 234:830–835. [PubMed: 4032292]
31. Regli L, Anderson RE, Meyer FB. Effects of intermittent reperfusion on brain pH, rCBF, and NADH during rabbit focal cerebral ischemia. *Stroke.* 1995; 26:1444–1451. discussion 1451–1442. [PubMed: 7631351]
32. Gao J, Duan B, Wang DG, Deng XH, Zhang GY, Xu L, Xu TL. Coupling between NMDA receptor and acid-sensing ion channel contributes to ischemic neuronal death. *Neuron.* 2005; 48:635–646. [PubMed: 16301179]
33. Ellison G. The N-methyl-D-aspartate antagonists phencyclidine, ketamine and dizocilpine as both behavioral and anatomical models of the dementias. *Brain Res. Rev.* 1995; 20:250–267. [PubMed: 7795658]
34. LeBlanc MH, Vig V, Smith B, Parker CC, Evans OB, Smith EE. MK-801 does not protect against hypoxic-ischemic brain injury in piglets. *Stroke.* 1991; 22:1270–1275. [PubMed: 1926238]
35. Horvath ZC, Czopf J, Buzsaki G. MK-801-induced neuronal damage in rats. *Brain Res.* 1997; 753:181–195. [PubMed: 9125402]
36. Sapolsky RM, Trafton J, Tombaugh GC. Excitotoxic neuron death, acidotic endangerment, and the paradox of acidotic protection. *Adv. Neurol.* 1996; 71:237–244. discussion 244–235. [PubMed: 8790802]
37. Kalimo H, Rehnström S, Söderfeldt B. The role of lactic acidosis in the ischemic nerve cell injury. *Acta Neuropathol., Suppl.* 1981; 7:20–22. [PubMed: 6939234]
38. Waldmann R, Champigny G, Bassilana F, Heurteaux C, Lazdunski M. A proton-gated cation channel involved in acid-sensing. *Nature.* 1997; 386:173–177. [PubMed: 9062189]
39. Baron A, Waldmann R, Lazdunski M. ASIC-like, proton-activated currents in rat hippocampal neurons. *J. Physiol.* 2002; 539:485–494. [PubMed: 11882680]
40. Alvarez de la Rosa D, Krueger SR, Kolar A, Shao D, Fitzsimonds RM, Canessa CM. Distribution, subcellular localization and ontogeny of ASIC1 in the mammalian central nervous system. *J. Physiol.* 2003; 546:77–87. [PubMed: 12509480]
41. Baron A, Lingueglia E. Pharmacology of acid-sensing ion channels - Physiological and therapeutical perspectives. *Neuropharmacology.* 2015; 94:19–35. [PubMed: 25613302]
42. Yagi J, Wenk HN, Naves LA, McCleskey EW. Sustained currents through ASIC3 ion channels at the modest pH changes that occur during myocardial ischemia. *Circ. Res.* 2006; 99:501–509. [PubMed: 16873722]
43. Li WG, Yu Y, Huang C, Cao H, Xu TL. Nonproton ligand sensing domain is required for paradoxical stimulation of acid-sensing ion channel 3 (ASIC3) channels by amiloride. *J. Biol. Chem.* 2011; 286:42635–42646. [PubMed: 21998313]
44. Frelin C, Barbry P, Vigne P, Chassande O, Cragoe EJ Jr, Lazdunski M. Amiloride and its analogs as tools to inhibit Na⁺ transport via the Na⁺ channel, the Na⁺/H⁺ antiporter and the Na⁺/Ca²⁺ exchanger. *Biochimie.* 1988; 70:1285–1290. [PubMed: 2852509]
45. Kleyman TR, Cragoe EJ Jr. Amiloride and its analogs as tools in the study of ion transport. *J. Membr. Biol.* 1988; 105:1–21. [PubMed: 2852254]
46. Wang YZ, Wang JJ, Huang Y, Liu F, Zeng WZ, Li Y, Xiong ZG, Zhu MX, Xu TL. Tissue acidosis induces neuronal necroptosis via ASIC1a channel independent of its ionic conduction. *eLife.* 2015; 4:e05682. [PubMed: 26523449]
47. Chan FK, Moriwaki K, De Rosa MJ. Detection of necrosis by release of lactate dehydrogenase activity. *Methods Mol. Biol.* 2013; 979:65–70. [PubMed: 23397389]
48. Christian SL, Ross AP, Zhao HW, Kristenson HJ, Zhan X, Rasley BT, Bickler PE, Drew KL. Arctic ground squirrel (*Spermophilus parryi*) hippocampal neurons tolerate prolonged oxygen-glucose deprivation and maintain baseline ERK1/2 and JNK activation despite drastic ATP loss. *J. Cereb. Blood Flow Metab.* 2008; 28:1307–1319. [PubMed: 18398417]
49. Ross AP, Christian SL, Zhao HW, Drew KL. Persistent tolerance to oxygen and nutrient deprivation and N-methyl-D-aspartate in cultured hippocampal slices from hibernating Arctic ground squirrel. *J. Cereb. Blood Flow Metab.* 2006; 26:1148–1156. [PubMed: 16395285]

50. Kirschner DL, Wilson AL, Drew KL, Green TK. Simultaneous efflux of endogenous D-ser and L-glu from single acute hippocampus slices during oxygen glucose deprivation. *J. Neurosci. Res.* 2009; 87:2812–2820. [PubMed: 19437552]
51. Cho S, Wood A, Bowlby MR. Brain slices as models for neurodegenerative disease and screening platforms to identify novel therapeutics. *Current neuropharmacology.* 2007; 5:19–33. [PubMed: 18615151]
52. Saeidnia S, Manayi A, Abdollahi M. From in vitro Experiments to in vivo and Clinical Studies; Pros and Cons. *Curr. Drug Discovery Technol.* 2016; 12:218–224.
53. Lushnikova IV, Voronin KY, Malyarevskyy PY, Skibo GG. Morphological and functional changes in rat hippocampal slice cultures after short-term oxygen-glucose deprivation. *J. Cell. Mol. Med.* 2004; 8:241–248. [PubMed: 15256072]
54. Small DL, Monette R, Mealing G, Buchan AM, Morley P. Neuroprotective effects of omega-Aga-IVA against in vitro ischaemia in the rat hippocampal slice. *NeuroReport.* 1995; 6:1617–1620. [PubMed: 8527726]
55. Taylor CP, Weber ML, Gaughan CL, Lehning EJ, LoPachin RM. Oxygen/glucose deprivation in hippocampal slices: altered intraneuronal elemental composition predicts structural and functional damage. *J. Neurosci.* 1999; 19:619–629. [PubMed: 9880582]
56. Sullivan BL, Leu D, Taylor DM, Fahlman CS, Bickler PE. Isoflurane prevents delayed cell death in an organotypic slice culture model of cerebral ischemia. *Anesthesiology.* 2002; 96:189–195. [PubMed: 11753020]
57. McManus T, Sadgrove M, Pringle AK, Chad JE, Sundstrom LE. Intraischemic hypothermia reduces free radical production and protects against ischaemic insults in cultured hippocampal slices. *J. Neurochem.* 2004; 91:327–336. [PubMed: 15447666]
58. Lein PJ, Barnhart CD, Pessah IN. Acute hippocampal slice preparation and hippocampal slice cultures. *Methods Mol. Biol.* 2011; 758:115–134. [PubMed: 21815062]
59. De Simoni A, Griesinger CB, Edwards FA. Development of rat CA1 neurones in acute versus organotypic slices: role of experience in synaptic morphology and activity. *J. Physiol.* 2003; 550:135–147. [PubMed: 12879864]
60. Lossi L, Alasia S, Salio C, Merighi A. Cell death and proliferation in acute slices and organotypic cultures of mammalian CNS. *Prog. Neurobiol.* 2009; 88:221–245. [PubMed: 19552996]
61. Pignataro G, Simon RP, Xiong ZG. Prolonged activation of ASIC1a and the time window for neuroprotection in cerebral ischaemia. *Brain.* 2006; 130:151–158. [PubMed: 17114797]
62. Lam TI, Brennan-Minnella AM, Won SJ, Shen Y, Hefner C, Shi Y, Sun D, Swanson RA. Intracellular pH reduction prevents excitotoxic and ischemic neuronal death by inhibiting NADPH oxidase. *Proc. Natl. Acad. Sci. U. S. A.* 2013; 110:E4362–4368. [PubMed: 24163350]
63. Khacho M, Tarabay M, Patten D, Khacho P, MacLaurin JG, Guadagno J, Bergeron R, Cregan SP, Harper ME, Park DS, Slack RS. Acidosis overrides oxygen deprivation to maintain mitochondrial function and cell survival. *Nat. Commun.* 2014; 5:3550. [PubMed: 24686499]
64. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. *J. Pharmacol. Pharmacother.* 2010; 1:94–99. [PubMed: 21350617]
65. Baron A, Schaefer L, Lingueglia E, Champigny G, Lazdunski M. Zn²⁺ and H⁺ are coactivators of acid-sensing ion channels. *J. Biol. Chem.* 2001; 276:35361–35367. [PubMed: 11457851]
66. Kirschner DL, Jaramillo M, Green TK. Enantioseparation and stacking of Cyanobenz[f]isindole-amino acids by reverse polarity capillary electrophoresis and sulfated beta-cyclodextrin. *Anal. Chem.* 2007; 79:736–743. [PubMed: 17222044]
67. Drew, KL., Zuckerman, JA., Shenk, PE., Bogren, LK., Jinka, TR., Moore, JT. Hibernation: A Natural Model of Tolerance to Cerebral Ischemia/Reperfusion. In: Gidday, MJ. Perez-Pinzon, AM., Zhang, HJ., editors. *Innate Tolerance in the CNS: Translational Neuroprotection by Pre- and Post-Conditioning.* Springer; New York: 2013. p. 37-50.

Figure 1.

Integrity of acute slices is validated from observation that OGD injury causes disruption in energy homeostasis. (A) Levels of whole tissue ATP were determined over a course of time using bath application in aCSF slices or slices treated with OGD. Gray bar indicates 30 min insult period. $*p < 0.05$ OGD, $n = 4$ versus aCSF, $n = 4$. Triplicate slices were pooled per treatment per time point, $n = 4$ animals. (B) Level of adenosine efflux monitored in slices subjected to 30 min aCSF or OGD using the microperfusion approach. Gray bar indicates 30 min insult period. $*p < 0.05$ OGD vs aCSF (aCSF, $n = 7$ slices, 4 animals; OGD, $n = 7$ slices, 4 animals).

Figure 2.

Oxygen glucose deprivation triggers efflux of excitatory amino acids similar to in vivo brain ischemia. Time-dependent efflux in rat hippocampal slices induced by 30 min OGD insult.

(A, B) Glutamate and aspartate efflux in hippocampal slices during OGD insult ($n = 17$ slices from 6 animals) and during aCSF (aCSF, pH = 7.3–7.4, $n = 16$ slices from 6 animals). Gray bar indicates insult period. Data shown are normalized to baseline (glutamate ($\mu\text{M}/\text{mg}$ protein): aCSF = 3.7; OGD = 4.7; aspartate: aCSF = 1.2 ; OGD = 1.9); $*p < 0.05$ for OGD versus aCSF. (Data shown in Figure 2A was published previously.⁶⁷)

Figure 3.

OGD or low pH acidosis causes injury during insult rather than during reperfusion. Time course of LDH release induced by (A) OGD, pH 7.3 insult for 15 min ($n = 4$ slices), 30 min ($n = 5$ slices), or 45 min ($n = 5$ slices) followed by reperfusion (aCSF, pH = 7.3–7.4); Significant differences are represented as ^a $p < 0.05$ versus OGD, pH 7.3 for 15 min, ^b $p < 0.05$ versus OGD, pH 7.3 for 30 min. (B) aCSF, pH 6.4 insult for 15 min ($n = 3$ slices), 30 min ($n = 3$ slices) or 45 min ($n = 3$ slices) followed by reperfusion (aCSF, pH = 7.3). Significant differences are represented as ^a $p < 0.05$ versus aCSF, pH 6.4 for 15 min, ^b $p < 0.05$ versus aCSF, pH 6.4 for 30 min. Gray bar indicates insult period. “I” indicates insult

phase ($I_{15} = 15$ min, $I_{30} = 30$ min, $I_{45} = 45$ min). All data were normalized to baseline measured as LDH release per mg of protein (15 min OGD, pH 7.3 = 0.315, 30 min OGD, pH 7.3 = 0.250, 45 min OGD, pH 7.3 = 0.359; 15 min aCSF, pH 6.4 = 0.163, 30 min aCSF, pH 6.4 = 0.169, 45 min aCSF, pH 6.4 = 0.162).

Figure 4.

Acidosis (pH 6.4) delays OGD induced injury but produces injury independent of OGD. (A) Time course of LDH release as a marker of cell death in acute hippocampal slices exposed to OGD, pH 7.3 ($n = 23$ slices from 23 animals); OGD, pH 6.4 ($n = 17$ slices from 9 animals) or aCSF, pH 6.4 ($n = 28$ slices from 14 animals). Gray bar indicates insult period. Data were normalized to baseline measured as LDH release per mg of protein (aCSF, pH 7.3 = 0.276; OGD, pH 7.3 = 0.324; aCSF, pH 6.4 = 0.174; OGD, pH = 0.244). Note that acidosis (pH 6.4) during OGD delays OGD induced cell death. ^a $p < 0.05$ versus aCSF (pH 7.3), ^b $p < 0.05$ versus aCSF (pH 6.4), ^c $p < 0.05$ versus OGD (pH 6.4). (B) Total LDH release

represented by area under the curve. Note that the overall injury caused by pH 6.4 is as large as OGD or OGD with pH 6.4. $*p < 0.05$ versus aCSF, pH 7.3 ($n = 19$).

Figure 5.

OGD-induced cell death is due to NMDAR activation whereas low pH injury is independent of an NMDAR-mediated cytotoxic cascade. (A) Slices were incubated with 100 μ M MK-801 followed by 30 min OGD, pH 7.3 ($n = 6$ slices, 3 animals) insult and LDH release was compared to OGD, pH 7.3 ($n = 23$ slices, 23 animals). (B,C) Slices preincubated with 100 μ M MK-801, exposed to either aCSF pH 6.4 ($n = 12$ slices, 3 animals) or OGD pH 6.4 ($n = 12$ slices, 4 animals) and LDH release was compared to aCSF pH 6.4 ($n = 28$ slices, 14 animals) or OGD pH 6.4 ($n = 17$ slices, 9 animals). Gray bar indicates insult period and dotted line indicates presence of MK-801. Data normalized to baseline measured as LDH

release per mg of protein (aCSF, pH 7.3 = 0.276; OGD, pH 7.3 = 0.324; OGD + MK-801 = 0.677). * $p < 0.05$ versus OGD, pH 7.3; + $p < 0.05$ versus aCSF, pH 7.3.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6.

ASIC1a is partly responsible for mediating low pH mediated injury. LDH measurement in response to aCSF pH 6.4 ($n = 28$) and during OGD pH 6.4 ($n = 17$) in the presence of (A) aCSF pH 6.4 + 100 μ M AMI ($n = 20$ slices, 7 animals), (B) OGD pH 6.4 + 100 μ M AMI ($n = 9$ slices, 5 animals), (C) aCSF pH 6.4 + 50 nM PcTx ($n = 12$ slices, 3 animals), and (D) OGD pH 6.4 + 50 nM PcTx ($n = 12$ slices, 3 animals). Gray bar indicates insult period, and dotted line indicates presence of AMI or PcTx. Note that blocking ASIC1a channels with PcTx1 significantly attenuated LDH release in both aCSF pH 6.4 and OGD pH 6.4 groups. Data were normalized to baseline measured as LDH release per milligram of protein (aCSF pH 6.4 = 0.174; OGD pH 6.4 = 0.244; aCSF pH 6.4 + AMI = 0.361, aCSF pH 6.4 + PcTx = 0.482, OGD pH 6.4 + AMI = 0.398, OGD pH 6.4 + PcTx = 0.299). Significant differences indicated as * $p < 0.05$ versus aCSF pH 6.4 + PcTx; # $p < 0.05$ versus OGD pH 6.4 + PcTx.