Brain Alkalosis Causes Birth Asphyxia Seizures, Suggesting Therapeutic Strategy

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Objective: The mechanisms whereby birth asphyxia leads to generation of seizures remain unidentified. To study the possible role of brain pH changes, we used a rodent model that mimics the alterations in systemic CO2 and O2 levels during and after intrapartum birth asphyxia.

Methods: Neonatal rat pups were exposed for 1 hour to hypercapnia (20% CO2 in the inhaled gas), hypoxia (9% O2), or both (asphyxic conditions). CO2 levels of 10% and 5% were used for graded restoration of normocapnia. Seizures were characterized behaviorally and utilizing intracranial electroencephalography. Brain pH and oxygen were measured with intracortical microelectrodes, and blood pH, ionized calcium, carbon dioxide, oxygen, and lactate with a clinical device. The impact of the postexposure changes in brain pH on seizure burden was assessed during 2 hours after restoration of normoxia and normocapnia. N-methyl-isobutyl-amiloride, an inhibitor of Na+ /H+ exchange, was given intraperitoneally.

Results: Whereas hypercapnia or hypoxia alone did not result in an appreciable postexposure seizure burden, recovery from asphyxic conditions was followed by a large seizure burden that was tightly paralleled by a rise in brain pH, but no change in brain oxygenation. By graded restoration of normocapnia after asphyxia, the alkaline shift in brain pH and the seizure burden were strongly suppressed. The seizures were virtually blocked by preapplication of N-methyl-isobutyl-amiloride.

Interpretation: Our data indicate that brain alkalosis after recovery from birth asphyxia plays a key role in the triggering of seizures. We question the current practice of rapid restoration of normocapnia in the immediate postasphyxic period, and suggest a novel therapeutic strategy based on graded restoration of normocapnia.

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Treatment of seizures is a priority in hypoxic-ischemic encephalopathy of birth asphyxia,1,2 but neonatal seizures are notoriously difficult to suppress. Current anticonvulsant medication is largely ineffective,3 and it is therefore necessary to seek approaches tailored to neonatal physiology and pathophysiology. Asphyxia implies a local or global decrease in O2 and an accumulation of CO2 and other end products of energy metabolism, such as lactate. Intrapartum asphyxia is a persistence of this abnormal state, exposing the fetus or newborn to hypoxia and hypercapnia with significant acidosis.4 Profound metabolic acidosis (pH < 7.00) is a defining characteristic of perinatal asphyxia.5

An unexpected observation based on magnetic resonance spectroscopy in human postasphyctic neonatal encephalopathy (NE) was that, after a delay of 8 to 24 hours from birth, brain intracellular pH (pHi) was more alkaline than under control conditions.6 A subsequent study showed that the alkaline pH level after NE can persist for 2 weeks, and that a high magnitude and prolonged duration of the alkalosis are predictive for poor clinical outcome.7 These are interesting observations, because (1) it is generally known that pH plays a key role in modulating neuronal survival after trauma (where acidosis is generally protective8–10) and (2) the excitability of brain tissue is known to be highly sensitive to changes in pH, whereby acidosis suppresses neuronal activity, whereas alkalosis has an opposite effect.11–15

Resuscitation or spontaneous recovery of an asphyxiated neonate after breathing of normal (or...
oxygen-enriched) air leads to abrupt changes in the acid-base balance at the systemic level, as is clearly seen in measurements of blood gas parameters. However, it is not known whether the increase in brain pH that takes place during recovery from asphyxia plays a role in the triggering of seizures. Addressing this question would not only shed light on the basic mechanisms of generation of postasphyxic neonatal seizures, but would also provide further insight into putative therapeutic strategies based on controlling brain pH.

Unraveling the mechanistic links among brain trauma, pH changes, and induction of seizures is a challenging task for experimental work, calling for suitable experimental models. In the present study, we developed a novel animal model of birth asphyxia that is not based on structural damage of the brain and its vasculature. Instead, our model relies on exposing postnatal day (P) 6 rats to gas mixtures containing various levels of CO2 and O2. Brain pH is measured using an intracortical ion-sensitive microelectrode. We show here that, after inducing asphyxic conditions with a simultaneous decrease in O2 (to 9% in the inhaled gas) and increase in CO2 (20%), a fast restoration of normocapnia leads to a progressive brain alkalosis that is paralleled by pronounced seizure activity, as monitored behaviorally and with intracranial electroencephalographic (EEG) recordings. Achieving normocapnia after asphyxia in a graded manner by using 10% and 5% CO2 suppresses the alkaline shift in brain pH and, strikingly, leads to a virtual abolishment of seizures. These data suggest that (1) an increase in brain pH plays a causal role in the generation of acute postasphyxic neonatal seizures, and (2) to alleviate seizure burden in the clinic, normocapnic conditions should be established in a graded manner during spontaneous recovery or resuscitation.

Materials and Methods

Animals and Experimental Conditions

Male Wistar rat pups aged P6 (where P0 refers to the day of birth) were used. All experiments were approved by the Local Animal Ethics Committee of Helsinki University and the National Animal Ethics Committee in Finland. Four groups of pups (plus controls breathing room air only) were studied. None of the animals died during the experiments. The total number of animals used was 159. Three groups were exposed for 60 minutes to altered ambient levels of CO2 and O2 at constant experimental temperature (35°C) with appropriate adjustments of N2 to keep the partial pressure of CO2, O2, and N2 at 100% of atmospheric pressure, as follows (see inset in Fig 1A): (1) hypoxia was induced by a decrease of O2 to 9%, (2) hypercapnia by an elevation of CO2 to 20%, and (3) asphyxia by a simultaneous increase in CO2 (20%) and decrease in O2 (9%). After the 60 minute change in ambient gas concentrations, the above groups were immediately re-exposed to room air. In addition, a fourth experimental group was studied, which experienced the asphyxic conditions as described above, but re-establishment of normocapnia was made in a graded manner by lowering the CO2 levels first from 20% to 10% for 30 minutes, then to 5% for a further 30 minutes, and finally to virtually zero (room air).

In some experiments, N-methyl-isobutyl-amiloride (Sigma-Aldrich, St Louis, MO; 2.5mg/kg) was injected intraperitoneally 30 minutes before asphyxia. The mean weight of the pups was 15.2g, and none of them lost >0.1g during the experiments.

Behavioral Analysis of Seizures

Loss of righting reflex (LRR) occurs in seizures classified as tonic-clonic. After pilot experiments and comparisons between seizure scorings by experienced observers, we found that LRR was the most robust and reproducible indicator for a quantitative analysis of the total seizure burden. Although LRR is the most violent expression of neonatal seizures in rodents
and hence is given the highest score in a modified Racine scale.\(^{20,21}\) It is not equivalent to the electroencephalographic seizure burden, which would include less intense seizure activity. It is, however, a reliable tool for comparison of seizure burden between distinct experimental paradigms. The seizure burden was quantified, from video recordings in an experiment blind manner, as the cumulative number of LRRs during the 2-hour observation period after the termination of the 60-minute exposure to altered respiratory conditions (see above). Each incidence of LRR was given a score of 1. The score was averaged per pup, followed by calculation of cumulative seizure burden with data points given at 1-minute intervals. Seizure quantification based on LRRs were done in uninstrumented animals.

### Anesthesia

Anesthesia was induced in an acrylic box with isoflurane (3-4% 1-chloro-2,2,2-trifluoroethyl difluoromethyl ether, isoflurane, Baxter Medical, Deerfield, IL), and maintained at 1.5 to 3% with a mask using an Univentor 400 Anesthesia Unit (Agnthos, Stockholm, Sweden). During the operation, the pup was kept on a Supertech Thermal Pad set at 35°C on a Cunningham Mouse/Neonate Rat Sterotaxic Adaptor (Harvard Apparatus, Holliston, MA).

### Intracranial EEG Recordings

Electrodes were prepared as before\(^{17}\) with some modifications; epidural EEG was measured from parietal cortex in freely moving rat pups with simultaneous video recording. Alternating current recordings were made using a differential amplifier with a bandwidth of 0.07Hz to 2kHz. The sampling rate was 5kHz. A digital-to-analogue converter (DAC) board with 16-bit resolution (National Instruments, Austin, TX) and acquisition software based on LabView were used.

### pH-Sensitive Microelectrodes

pH-sensitive microelectrodes were prepared and calibrated as described by Voipio and Kaila.\(^{22}\) Tip diameters were 4-8\(\mu m\) for the ion-sensitive electrodes and 2-4\(\mu m\) for the reference electrodes. The microelectrodes had a resistance of 8 to 12GO (in calibration solution and brain tissue in a head-fixed configuration) and a slope of 55 to 60mV per pH unit. Electrometer amplifiers had an input bias current <10fA. Two burr holes were made, the first 1.6 to 2.0mm posterior to bregma and 1.6 to 2.0mm lateral to the midline, and the second 1.6mm posterior and lateral to the first. The pH-sensitive electrode was inserted through the anterior burr hole to a depth of about 500\(\mu m\), and the reference through the posterior burr hole to the same depth. A silver-silver chloride ground electrode was inserted subcutaneously above the scapula. DAC-board and acquisition software were the same as with the EEG recordings. Unpaired \(t\) test was used to compare means. Values are given as mean ± standard error of the mean in the text and figures. Recordings were started 15 minutes after recovery from anesthesia. The correlation between ApH and normalized seizure burden was calculated using Spearman rank order correlation coefficient, with \(p\) based on a 2-tailed test in accordance with the hypothesis of alkalosis-promoted seizures.

### Oxygen Microelectrodes

Modified Clark-type polarographic oxygen microelectrodes\(^{23,24}\) (10\(\mu m\), Unisense AS, Aarhus, Denmark) were connected to a high-impedance picoamperometer (Unisense AS) and calibrated in salines warmed to 35°C and equilibrated with 100% O\(_2\), air, and 100% N\(_2\). They were left in degassed water for 20 to 30 minutes before insertion into the brain to a depth of 500\(\mu m\).

### Blood Analysis

GEM 4000\(^{25}\) (Instrumentation Laboratory, Bedford, MA) was used in trunk blood analyses of pH, calcium, partial pressure of oxygen (pO\(_2\)), partial pressure of carbon dioxide (pCO\(_2\)), and lactate. One hundred microliters of arteriovenous blood was collected by rapid decapitation of the rat pups at 0, 30, 60, and 120 minutes. Statistical analysis was performed with R software\(^{26}\) using the NLME\(^{27}\) package. A model object was calculated using a linear model, and an analysis of variance table was extracted. In the case of interaction between treatment and time, the treatment effect was analyzed separately for each time point. Comparisons between individual pairs of treatments were done if the overall significance of the treatments yielded \(p < 0.05\). The GREGMISC\(^{26}\) package was used to fit these contrasts to a model not including the interaction. \(p\) values from multiple contrasts were adjusted using Holm’s procedure. For details, see Supporting Information Table 1.

### Results

#### Postasphyxia Seizures Are Induced during Fast Establishment of Normocapnic Conditions

Exposing the rat pups to the various gas mixtures for 60 minutes did not produce marked behavioral effects, apart from brief agitation on initiation of exposure. Seizures were never seen under these conditions. However, there were striking differences among the various groups in the responses that took place after the termination of the exposures. The start of this 2-hour recovery period is defined as time zero in the figures.

Figure 1A shows the cumulative seizure burden for all the paradigms. Control pups as well as pups exposed to hypoxia (trace a, \(n = 6\)) scored zero at all times. Notably, immediately after ending the hypoxic conditions (0 minutes), blood lactate levels increased from 1.0 ± 0.06 to 4.8 ± 0.8mM (\(n = 6\) for both groups; \(p < 0.01\)), demonstrating that the decrease in ambient O\(_2\) from room-air level (around 21%) to 9% was sufficient to shift the energy metabolism of the P6 pups toward a direction where anaerobic glycolysis plays a significant role, which is a hallmark of asphyxia.\(^{28}\)

The response seen after abrupt cessation of hypcapnia (trace b, \(n = 8\)) yielded an average cumulative seizure burden score of 1.4 LRRs/pup. In contrast, pups exposed to asphyxic conditions (combined hypcapnia and hypoxia) followed by immediate restoration of normocapnia (trace c, \(n = 6\)) showed a very high seizure...
burden, totaling 14.5 LRRs/pup. These results indicate that the recovery from asphyxic conditions strongly activates mechanisms that promote seizures.

Based on the above data, we hypothesized that the pronounced and progressive seizure burden seen after the establishment of control conditions in the asphyxic group might be caused by a fast increase in brain pH. Such an effect on acid-base regulation is expected to take place after the removal of excess ambient CO2 (20%) in combination with the removal of the metabolic acid load (ie, production of lactate) that was observed in pups exposed to 9% O2 (see above). Hence, we introduced yet another experimental paradigm, where the asphyxic conditions were followed by a graded restoration of the ambient CO2 level from 20% first to 10% (for 30 minutes) and thereafter to 5% (for 30 minutes), followed by room air (60 minutes). In this paradigm with graded restoration of normocapnia, a dramatic decrease (85%) in the cumulative postasphyxia seizure burden was observed (trace d, n = 6; a total of 2.2 LRRs/pup) when compared with the large seizure burden after abrupt restoration of normocapnia (trace c).

To gain more insight into the seizures, we made epidural EEG recordings from pups subjected to hypercapnia or asphyxia. In line with the extensive evidence demonstrating that an acidosis suppresses neuronal excitability (see Discussion), the baseline activity in the EEG of pups exposed to hypercapnic or asphyxic conditions was strongly suppressed (see Fig 1B), with the peak-to-peak amplitude falling from 10 to 15μV under control conditions to 5 to 7μV during hypercapnia or asphyxia (n = 6). In agreement with the behavioral data, no electrographic seizures were detected in the 2 of 2 pups recorded by EEG after exposure to hypercapnia. In contrast to this, in 4 of 4 pups with abrupt restoration of normocapnia after asphyxia, pronounced electrographic seizures developed, with a duration of 1 to 5 seconds, frequency of 12 to 14Hz, and amplitude of 50 to 200μV. Electrographic seizures were invariably detected during LRRs, except for those obvious cases where EEG recordings were dominated by movement artifacts.

**Postasphyxia Seizures Are Triggered by Brain Alkalosis**

The above data strongly suggest that postasphyxia seizures are triggered by a brain alkalosis, which would also explain the differences in seizure burden after abrupt
versus graded restoration of normocapnia. To directly test this idea, we performed measurements of intracortical pH using H\textsuperscript{+}-sensitive microelectrodes.

In the experimental group where asphyxic conditions were followed by an abrupt establishment of normocapnia (n = 6; Fig 2), the pre-exposure baseline pH was 7.19 ± 0.01. During asphyxic conditions, intracortical pH became rapidly acidotic, attaining a minimum of 6.77 ± 0.01, followed by a small recovery toward the end of the asphyxic conditions. Establishing the normoxic/normocapnic conditions induced a fast increase in the intracortical pH at a rate of 0.031/min during the first 5 minutes and 0.0054/min during the final 115 minutes.

In the experimental group where asphyxia was followed by graded restoration of normocapnia (n = 4; see Fig 2), the mean pre-exposure baseline pH was virtually identical to the above group, 7.20 ± 0.02. A key observation was that graded restoration of normocapnia greatly attenuated both the rate and amplitude of the postasphyxic alkalinization. Mean pH increased at a rate that was much smaller than what was seen with the abrupt restoration procedure, with an initial rate of 0.031/min and at 0.0054/min during the final 115 minutes. At the end of the observational period, brain pH was much more alkaline after the abrupt restoration paradigm (7.60 ± 0.01) when compared with graded restoration (7.34 ± 0.02; p < 0.0001).

The robust correlation between postasphyxic brain pH changes and seizure burden that is obvious in the above data is illustrated in Figure 3, where mean values of seizure burden (compare with Fig 1) are plotted as a function of deviations of brain pH from its control level (dotted lines in Fig 2C). The dependence of seizure burden on brain pH shows a correlation of 0.96 (p < 0.0001) and 0.99 (p < 0.0001) after graded and abrupt establishment of normocapnia, respectively. As a whole, these data clearly show that the magnitude of the alkaline shifts in the 2 experimental groups is a major factor determining the postasphyxia seizure burden. This conclusion gained further support from experiments that showed that LRRs evoked in the abrupt-restoration paradigm were terminated, and seizures were never observed, after application of 5% CO\textsubscript{2} in air (n = 6). Furthermore, \textit{N}-methyl-isobutyl-amiloride given intraperitoneally 30 minutes before asphyxia\textsuperscript{21} led to strong suppression (by 83%) of the cumulative seizure burden, to 2.5 LRRs/pup (n = 4).

**Postasphyxia Seizures Are Not Triggered by Reduced Brain Oxygenation**

To rule out the possibility that the alkalosis induced constriction of brain microvessels,\textsuperscript{29} thereby leading to hypoxic seizures,\textsuperscript{30} we measured intracortical pO\textsubscript{2} (Fig 4; n = 6). Under control conditions, the mean value was 10 ± 1.18mmHg and, during asphyxia, pO\textsubscript{2} fell by 5 to 9mmHg. Changes in pO\textsubscript{2} relative to control levels are shown in Figure 4B. After asphyxia (with abrupt restoration of normocapnia), a transient elevation in pO\textsubscript{2} was observed that is most likely attributable to a delay induced by readjustment of ventilation after the exposure to low pO\textsubscript{2} and elevated pCO\textsubscript{2}\textsuperscript{31,32}

**Changes in Brain pH Are Paralleled by Changes in Blood Parameters**

To facilitate comparisons between the present observations and clinical data, we examined blood pH in the
abrupt and graded restoration paradigms. The blood and brain pH data are plotted in Figure 5. Both the maximum acidosis (end of asphyxia) and alkalosis have significantly smaller amplitudes in the blood as compared to the brain. The maximum acidosis observed in blood immediately after asphyxia (0 minutes), 7.25 ± 0.02, is significantly lower than control pH, 7.42 ± 0.02, (p < 0.0001), and the maximum alkalosis, 7.49 ± 0.02 at 60 minutes, is significantly higher than control pH, (p = 0.0028). At 120 minutes, blood pH was 7.45 ± 0.02, which is significantly higher than normal, (p = 0.0214). However, it is important to note that the patterns of the pH changes in the brain and blood are qualitatively similar.

Statistical analysis of the blood parameters showed that treatment, time, and the interaction between treatment and time undergo statistically significant variation, except for pO2, where interaction yielded nonsignificant results, but treatment results were significant. The large decrease in blood ionized calcium due to abrupt restoration of normocapnia, which is also seen in asphyxiated babies,33 was suppressed by graded restoration of normocapnia (see Fig 5). In a manner consistent with the blood pH changes, pCO2 was higher during the graded restoration of normocapnia paradigm. Abrupt restoration of normocapnia resulted in a prolonged postasphyxic elevation in pO2. Detailed data with statistical comparisons among data points are provided in Supporting Information Table 1.

Discussion

Brain pH changes are known to be intimately involved in the modulation of neuronal excitability and in the control of cell death following trauma.10–14,28,34 The present rat pup model of neonatal birth asphyxia was designed to elucidate the role of the alkaline pH change in triggering seizures, in a manner where the immediate, confounding effects of neuronal damage on excitability are excluded. Our main observations include that (1) moderate hypoxic (9% O2) or hypercapnic (20% CO2) conditions do not lead to seizure generation. However, (2) exposure to combined hypoxia and hypercapnia (which mimics asphyxia) leads to pronounced postasphyxic seizures that show a steep dependence on brain pH, but not on brain pO2. Indeed, the most important observations in the present

FIGURE 5: Changes in brain pH and blood parameters (pH, ionized calcium, pCO2, and pO2) during and after asphyxia in the paradigms with abrupt and graded restoration of normocapnia. From top to bottom: Brain pH changes induced by asphyxia followed by abrupt establishment of normocapnia are paralleled by much smaller pH changes in arteriovenous blood samples. Data points show brain (dotted lines with green and red circles for abrupt and graded restoration of normocapnia, respectively) and blood (black, green, and red squares for control group, abrupt, and graded restoration of normocapnia, respectively) pH values. Bars indicate ± standard error of the mean. The large decrease in blood ionized calcium (iCalcium) due to abrupt restoration of normocapnia (green squares) is abolished by graded restoration of normocapnia (red squares). Graded restoration of normocapnia causes a gradual decrease in blood pCO2 levels. Abrupt restoration of normocapnia causes a prolonged postasphyxic elevation in blood pO2, n = 4 to 9 pups for each paradigm and time point.
study were that (3) the seizure burden was strictly related to postasphyctic brain alkalosis and (4) this alkalosis could be dramatically attenuated (by 85%) simply by controlling the rate of restoration of normocapnic conditions using a graded decrease in ambient CO₂.

The conclusion that the postasphyctic seizures were directly caused by cortical pH recovery and subsequent alkalosis is supported by (1) the much higher seizure burden seen on abrupt versus graded restoration of normocapnia (see Fig 3); (2) the rapid termination of seizures observed when applying 5% CO₂ as such; and (3) the dramatic reduction in seizures by the Na⁺/H⁺ exchange inhibitor N-methyl-isobutyl amiloride. Further support comes from our previous work, where injection of sodium bicarbonate in neonatal rat pups caused a rise in brain pH that was accompanied by seizure activity.

Our work shows that graded restoration of normocapnia greatly reduces abnormal neuronal activity, and a therapy that causes a significant reduction in seizure burden is expected to improve outcome after birth asphyxia. Extrapolating from our present results suggests that after birth asphyxia in human neonates, the high CO₂ level that is invariably observed in blood samples should not be corrected in an aggressive manner to establish fast normocapnia, because a pronounced alkaline rebound and consequent triggering of seizures is likely to occur. In light of the present data, it would be advisable to establish normocapnia in a graded manner. It is widely known that profound and acute changes in the arterial CO₂ partial pressure and acid-base balance occur in the presence of fetal acidemia, but the rationale behind current protocols of rapid correction is unclear. Moreover, brain alkalosis is associated with neuropathological outcome in human neonates, a conclusion that gains support from work on piglets. Another benefit of graded restoration of CO₂ in neonates and especially in preterm infants is that hypercapnia has been shown to protect ventilated newborns’ lungs from damage, probably by allowing the use of a low tidal volume strategy.

Optimum neuroprotection after NE may require the use of more than one therapy, such as hypothermia and application of xenon. Interestingly, in P6 rats after hypoxia-ischemia, under both normo- and hypothermic conditions, xenon was shown to cause CO₂ retention and a decrease in pH that is probably involved in the observed reduction of brain injury.

Regarding the molecular mechanisms of the delayed alkalosis seen after birth asphyxia, there is substantial evidence that stimulation of acid extrusion by the Na⁺/H⁺ exchange plays a key role. Interestingly, the inhibitor of the Na⁺/H⁺ exchanger, N-methyl-isobutyl-amiloride, has been shown to ameliorate brain injury when applied before hypoxic-ischemic conditions in neonatal mice. The brain is a multicompartment system and, under in vivo conditions, systemic regulation of pH by respiration and kidney-based mechanisms has a significant role in shaping the extracellular and intracellular pH. Hence, the most parsimonious way to explain the brain alkaloses in the present and previous studies is to postulate a net loss of acid from brain tissue that elevates both intra- and extracellular pH.

In agreement with such a unifying explanation, we found that applying N-methyl-isobutyl-amiloride before the start of the experimental asphyxia led to a dramatic decrease in the cumulative seizure burden.

In summary, our results show that birth asphyxia can lead to a severe alkalosis of brain tissue and subsequent seizures in the absence of experimentally induced brain lesion. The data also suggest a putative therapeutic strategy to reduce the fast, alkalosis-promoting loss of CO₂ that takes place during recovery from asphyxia. An analogous therapeutic strategy was proposed for suppression of febrile seizures, where brain alkalosis may play a crucial role. In practice, an easy way to block the postasphyxic seizures would be to apply air with CO₂ in declining steps during the initial phase of recovery, which is readily achieved using a mask or hood for ventilatory gas delivery. Obviously, such a graded restoration of normocapnia does not exclude a prompt restoration of normoxia.

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Potential Conflicts of Interest
Nothing to report.

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