Parallel Changes in Intracellular Water Volume and pH Induced by NH3/NH4+ Exposure in Single Neuroblastoma Cells

Victor M. Blanco
Martín S. Márquez
Francisco J. Alvarez-Leefmans

Wright State University - Main Campus, francisco.alvarez-leefmans@wright.edu

Follow this and additional works at: https://corescholar.libraries.wright.edu/ptox

Part of the Chemicals and Drugs Commons

Repository Citation
Blanco, V. M., Márquez, M. S., & Alvarez-Leefmans, F. J. (2013). Parallel Changes in Intracellular Water Volume and pH Induced by NH3/NH4+ Exposure in Single Neuroblastoma Cells. Cellular Physiology and Biochemistry, 32 (S1), 57-76.
https://corescholar.libraries.wright.edu/ptox/84

This Article is brought to you for free and open access by the Pharmacology and Toxicology at CORE Scholar. It has been accepted for inclusion in Pharmacology and Toxicology Faculty Publications by an authorized administrator of CORE Scholar. For more information, please contact library-corescholar@wright.edu.
Parallel Changes in Intracellular Water Volume and pH Induced by NH₃/NH₄⁺ Exposure in Single Neuroblastoma Cells

Víctor M. Blanco⁎  Martín S. Máquez⁎  Francisco J. Alvarez-Leefmans

Department of Pharmacology & Toxicology, Wright State University, Boonshoft School of Medicine, Dayton, OH, USA; ⁎Present address: The Vontz Center for Molecular Studies, Division of Hematology/Oncology, Department of Internal Medicine, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA; ⁎Present address: Division of Neuroscience, Instituto Nacional de Psiquiatría “Ramón de la Fuente”, México DF, México

Key Words
Ammonium • Ammonia • Isosmotic cell swelling • Isosmotic cell shrinkage • Isosmotic regulatory volume decrease • Isosmotic regulatory volume increase • Hyperammonemia • Brain edema

Abstract

Background: Increased blood levels of ammonia (NH₃) and ammonium (NH₄⁺), i.e. hyperammonemia, leads to cellular brain edema in humans with acute liver failure. The pathophysiology of this edema is poorly understood. This is partly due to incomplete understanding of the osmotic effects of the pair NH₃/NH₄⁺ at the cellular and molecular levels. Cell exposure to solutions containing NH₃/NH₄⁺ elicits changes in intracellular pH (pHᵢ), which can in turn affect cell water volume (CWV) by activating transport mechanisms that produce net gain or loss of solutes and water. The occurrence of CWV changes caused by NH₃/NH₄⁺ has long been suspected, but the mechanisms, magnitude and kinetics of these changes remain unknown. Methods: Using fluorescence imaging microscopy we measured, in real time, parallel changes in pHᵢ and CWV caused by brief exposure to NH₃/NH₄⁺ of single cells (N1E-115 neuroblastoma or NG-108 neuroblastoma X glioma) loaded with the fluorescent indicator BCECF. Changes in CWV were measured by exciting BCECF at its intracellular isosbestic wavelength (~438 nm), and pHᵢ was measured ratiometrically. Results: Brief exposure to isosmotic solutions (i.e. having the same osmolality as that of control solutions) containing NH₄Cl (0.5-30 mM) resulted in a rapid, dose-dependent swelling, followed by isosmotic regulatory volume decrease (iRVD). NH₄Cl solutions in which either extracellular [NH₃] or [NH₄⁺] was kept constant while the other was changed by varying the pH of the solution, demonstrated that [NH₄⁺]ᵢ rather than [NH₃]ᵢ is the main determinant of the NH₄Cl-induced swelling. The iRVD response was sensitive to the anion channel blocker NPPB, and partly dependent on external Ca²⁺. Upon removal of NH₄Cl, cells shrank and displayed isosmotic regulatory volume increase (iRVI). Regulatory volume responses could not be activated by comparable CWV changes.
produced by anisosmotic solutions, suggesting that membrane stretch or contraction by themselves are not sufficient to trigger these responses. Inhibition of glutamine synthetase partially blocked the NH₄Cl-induced swelling. **Conclusions:** A quantitative description of the osmotic changes produced by exposure to NH₄⁺/NH₄⁺ in single neurons and glial cells shows that ~35 to 45% of the initial cell swelling can be explained by intracellular accumulation of NH₄⁺ due to rapid permeation and protonation of NH₃. Another ~23% of the swelling can be accounted for by rapid glutamate accumulation. The results are discussed in terms of basic cell physiology and their potential relevance to the pathophysiology of hyperammonemic cellular brain edema.

**Introduction**

The transport of the neutral base ammonia (NH₃) and its conjugated acid, ammonium (NH₄⁺) across cell membranes is of high biological significance. In mammals, besides its importance in nitrogen metabolism, transport of NH₃ and NH₄⁺ plays a key role in the regulation of systemic pH [1, 2]. Increased blood levels of NH₃ and NH₄⁺ are implicated in the pathogenesis of various neurological disorders, collectively termed hyperammonemic encephalopathies [3, 4]. Hyperammonemia produces cellular brain edema, which is a leading cause of mortality in humans with acute liver failure [5-9]. The pathophysiology of this edema is poorly understood [4, 10]. This is partly due to our lack of understanding of the osmotic effects of the pair NH₃/NH₄⁺ at the cellular and molecular levels, particularly in neurons and glial cells. Cell exposure to NH₃/NH₄⁺ produces changes in intracellular pH (pHᵢ) that are determined by the relative permeability of the plasma membrane to NH₃ and NH₄⁺, and the specific pH regulating transport systems present in each cell type. These changes in pHᵢ and their underlying mechanisms have been extensively studied and are well understood [11-13], but relatively little is known about how NH₃/NH₄⁺ and the pHᵢ changes it produces affect cell osmotic control, a basic problem of cell physiology that has been addressed but not solved for more than a half a century. Osmotic changes in cell volume are expected to occur upon changes in pHᵢ because regulation of these two functional processes often involves common membrane ion transporters and channels.

With a few notable exceptions [14-16], most cells are highly permeable to NH₃ and to a variable extent to NH₄⁺. Early investigations addressing the cell membrane permeability to weak acids and bases correctly attributed the alkalization observed in plant and animal cells exposed to ammonium salts to rapid permeation of the non-electrolyte NH₃ [17, 18]. Inside the cells, protonation of NH₃ determines an increase in pHᵢ that will proceed until NH₃ achieves equilibrium across the plasma membrane. During this process, NH₄⁺ is generated and can be accumulated intracellularly in millimolar concentrations [11, 19, 20]. Thus, a net intracellular accumulation of osmotically active particles (NH₄⁺) proceeds concomitantly with the change in pHᵢ. This accumulation may be enhanced by direct NH₄⁺ influx via membrane channels or transporters. Thus, exposure to NH₃/NH₄⁺ results in an increase in intracellular osmotic pressure leading to water influx and cell swelling. Unless counteracted by volume regulatory mechanisms, the osmotic swelling can culminate in cell lysis. A similar reasoning was invoked several decades ago to explain the osmotic effects of ammonium salts on erythrocytes and sea urchin eggs [21, 22]. Remarkably, apart from these early studies, few recent investigations address the biophysical mechanisms and kinetics of the osmotic effects of short-term exposure to NH₄⁺/NH₄⁺ of animal cells [23, 24].

In the present study, we used a method to measure, in real-time (resolution of <1 s), parallel changes in cell water volume (CWV) and pHᵢ in response to brief exposure to solutions containing NH₃/NH₄⁺, in single cells loaded with the fluorescent dye BCECF [25]. As model systems, we used two murine cell lines, N1E-115 neuroblastoma and NG-108 neuroblastoma X glioma. The cells were briefly exposed to isosmotic solutions containing various concentrations of NH₄Cl (0.5–20 mM). With BCECF, changes in CWV are assessed from the emitted fluorescence upon exciting the fluorophore at its pH-insensitive intracellular
isobestic wavelength (~438 nm), while changes in pH are assessed ratiometrically. In addition to the expected changes in pH, exposure to NH₄Cl-containing solutions isomotic with extracellular media induced a highly reproducible pattern of CWV changes consisting of a rapid cell swelling followed by regulatory volume decrease (isosmotic RVD, or iRVD). Removal of the NH₄Cl solution caused cell shrinkage, after which CWV was restored by regulatory volume increase (isosmotic RVI, or iRVI). Experiments in which either [NH₄⁺] or [NH₄⁺] were kept fixed showed that the initial increase in both pH and CWV depended on the external [NH₄⁺], and not on external [NH₄⁺]. Thus, it is concluded that a rapid entry and protonation of NH₄ leads to a rapid increase in intracellular NH₄⁺ concentration ([NH₄⁺]), exerting an increase in intracellular osmotic pressure that results in cell swelling. From the concomitant pH measurements and using the Henderson-Hasselbalch formalism, changes in [NH₄⁺] were quantified and their relative contribution to the raise in intracellular osmolality and cell water volume were modeled. It was found that about 35 to 45 % of the initial cell swelling could be explained by intracellular accumulation of NH₄⁺. Interestingly, after the initial peak swelling caused by NH₄⁺/NH₄⁺ application, in spite of a gradual, sustained increase in [NH₄⁺] (and hence intracellular osmolality) now caused by slow NH₄⁺ influx, the cells displayed isosmotic RVD (iRVD). These results provide clear evidence that neuronal or glial swelling can result from direct osmotic effects of intracellular NH₄⁺ accumulation, and establish for the first time that initial NH₄⁺ accumulation is due primarily to NH₄⁺ influx followed by delayed NH₄⁺ influx. Further, the present study establishes the validity of this in vitro model to study, at the single cell level, the consequences of NH₄⁺/NH₄⁺ on cell volume and its interdependence with pH. The results are discussed in terms of their potential relevance to the pathophysiology of hyperammonemic cellular brain edema.

Materials and Methods

Cell Culture

N1E-115 mouse neuroblastoma [26] and NG108-15 mouse neuroblastoma x rat glioma [27] cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM, Gibco-Invitrogen No. 12430) supplemented with 10% fetal calf serum (FCS, HyClone), 1% hypoxanthine-aminopterin-thymidine (HAT; Sigma-Aldrich) and 1% L-glutamine (Gibco-Invitrogen), in a 5% CO₂/95% air atmosphere. N1E-115 cells are an adrenergic clone of mouse neuroblastoma C1300 cells and possess acetylcholinesterase and tyrosine hydroxylase activity [26]. NG108-15 are a fusion of the mouse neuroblastoma clone N18TG-2 and the rat glioma clone C6 BV-1, express characteristics of both glial and neuronal cells and have both cholinergic and GABAergic properties [28]. Cells from passages 16-25 were plated on 25-mm-diameter glass coverslips previously treated with poly-D-lysine (BD Biosciences). Differentiation was induced 24 h after plating by supplying the cells with a differentiation medium composed of 98% DMEM, 2% FCS, 1% HAT, 1% L-glutamine, 1 mM theophylline, and 10 μM prostaglandin E1 [29]. Theophylline and prostaglandin E1 came from Sigma-Aldrich. Cells were used for experiments 3 to 7 days after the differentiation treatment.

Solutions

The control isosmotic solution (ISO) contained (in mM): 120 NaCl, 5.5 KCl, 2.5 CaCl₂, 1.25 MgCl₂, 20 HEPES and 10 D-glucose. The final osmolality was adjusted with sucrose (~32 mM) to ~312 mosmol/kg water; to match the osmolality of the differentiation media. Anisomotic calibration solutions were prepared by sucrose addition or removal to keep the ionic concentrations constant and at the value of the control ISO; they were expressed as percentage decrement or increment with respect to the control ISO (i.e., ± 10% with respect to the osmolality of the ISO). NH₄Cl-containing solutions (0.5 to 30 mM) were prepared by equimolar replacement of NaCl by NH₄Cl in the ISO. The zero-Ca²⁺ solution was made by substituting CaCl₂ for MgCl₂ and adding 1 mM EGTA. The pH of all these solutions was adjusted to 7.3 with NaOH. The isosmotic solutions with fixed [NH₄⁺] (0.23 mM) and various [NH₄⁺] (4.77, 9.77, 19.77 and 29.77 mM) were made from solutions containing 5, 10, 20 and 30 mM NH₄Cl in which the pH was adjusted to 7.91, 7.61, 7.31 and 7.13, respectively. The solutions having fixed [NH₄⁺] (10 mM) and various [NH₄⁺] (0.05, 0.11, 0.22 and 0.33 mM) were prepared from solutions with 10.05, 10.11, 10.22 and 10.33 mM NH₄Cl, and the pH was set to 7.0, 7.3, 7.6 and 7.77,
respectively. 5-nitro-2-(3-phenylpropylamine) benzoic acid (NPPB) from Sigma-Aldrich was dissolved in DMSO. The final concentration of DMSO in the experimental solutions was ≤ 0.1%. Experimental solutions were perfused into the recording chamber at a rate of 6.0 ml/min using an electric valve-controlled system (Warner Instruments). The fluid at the recording area of the chamber was exchanged by 90% in 12.6 ± 0.6 s (n = 6).

**Dye Loading**

A coverslip with the cells attached and equilibrated with ISO was mounted in an imaging chamber (Warner Instruments RC-21BRW) and placed on the stage of an epifluorescence inverted microscope (Olympus IX81). After recording the background fluorescence from each cell, they were loaded with ISO containing the acetoxymethyl (AM) esters of either BCECF (5 μM) or calcine (2 μM; both from Invitrogen-Molecular Probes) dissolved in DMSO. Dye loading progress was monitored from each of the cells in the field of view until reaching the desired levels of fluorescence (~ 10-30 min). The loading solution was washed-out with ISO and the cells were equilibrated for ~15-30 min in this solution before starting recording. For measurement of changes in intracellular free-Ca²⁺ concentration, cells were loaded by incubation for ~30 min with ISO containing 4 μM Fura-2-AM (TEF Labs) and 10% (wt/wt) pluronic F-127 (Invitrogen-Molecular Probes) dissolved in DMSO.

**Fluorescence Measurements**

Total fluorescence from a small circular digital pinhole region placed at the image plane of each fluorophore-loaded single cell was measured with an imaging system described in detail elsewhere [25]. Each pinhole region was placed at the soma of each neuron. The size of each pinhole region was 3 to 10% of the total area of the soma at the image plane. The imaging system included an epifluorescence inverted microscope equipped with a 40 X oil immersion objective lens (NA = 1.35; Olympus America, Melville, NY). The excitation light coming from a 75W xenon arc lamp passed through the input slit of a monochromator (Optoscan, Cairn Research Limited, Faversham, UK) that allowed independent slit width control for each wavelength. BCECF was excited with light pulses (40-80 ms duration) at its pH-sensitive wavelength (495 ± 2-3 nm), and at 438 ± 2-4 nm, its intracellular isosbestic wavelength (IW). The interval between these two excitation wavelengths was 15 ms. Calcine was excited at 495 ± 2 nm and Fura-2 at 340 ± 3 nm and 380 ± 3 nm. Emitted fluorescence (detected at 535 nm for BCECF and calcine, and at 510 nm for Fura-2) was monitored on-line using a cooled CCD camera (ORCA-ER C4742-95, Hamamatsu). MetaFluor imaging software (Molecular Devices, Sunnyvale, CA) was used for image acquisition, digital pinhole size and positioning and fluorescence recording. Fluorescence was sampled at 0.2 Hz. All experiments were done at room temperature (21-24°C).

**Cell Water Volume Measurements**

The validation of the method and procedures for measuring cell water volume (CWV) changes using fluorescent dyes has been explained in detail elsewhere [25, 30]. In brief, CWV changes (V/V₀) are computed from monitored changes in relative fluorescence (F/F₀) resulting from exciting BCECF at the intracellular IW (~438 nm) or calcine at 495 nm, according to the following equation:

\[
\frac{[F/F₀ - F_{bgd}]}{1 - F_{bgd}} = V/V₀
\]  

(Eq 1)

where F₀ is the fluorescence from a pinhole region of the cell equilibrated with an ISO control solution at t = 0; F is the fluorescence of the same region at t = t; F_{bgd} is the fraction of intracellularly trapped and osmotically-insensitive fluorescence of the dye-loaded cell (see below); V₀ is the baseline CWV in ISO (V₀ = 1) and V is the CWV at time t.

One of the advantages of this method is that the fluorescence signals can be calibrated for defined changes in extracellular osmolality in each individual cell, so that each cell is used as its own osmometer. Accordingly, osmotic calibrations were done by exposing each cell to two pulses of anisomotic solutions (5 to 7 min duration) having nominal osmolalities of ± 10% with respect to the ISO. Within this range of osmolalities, the cells do not show any signs of regulatory responses and exhibit osmometric behavior. An example of the changes in BCECF relative fluorescence in response to pulses of anisomotic calibration
Fig. 1. Calibration of fluorescence signals to measure CWV changes in single cells. (A) Changes in BCECF relative fluorescence \( F_r/F_0 \) recorded by exciting the dye at its intracellular isosbestic wavelength (438 ± 2.5 nm) in response to two pulses of calibration solutions (± 10% anisomotic), as indicated by the bars at the bottom of panel C. (B) Plot of the reciprocal of the apparent steady state changes in fluorescence \( F_{\text{recip}}/F_r \) as a function of the reciprocal of the relative osmotic pressure of the medium \( (\pi_r/\pi_i) \) for the calibration pulses shown in A. The \( y \) intercept of the linear regression fitted to the observed data points (solid line) corresponds to the fraction of intracellularly trapped and osmotically-insensitive fluorescence \( F_{\text{trapped}} \) that in this case was 0.58. Subtraction of \( F_{\text{trapped}} \) yields the dotted line indicating ideal osmometric behavior. (C) Changes in CWV \( (V_r/V_i) \) resulting from transformation of the fluorescence signals shown in A, using Eq. 1. (D) Plot of the apparent steady-state changes in \( V_r/V_i \) as a function of \( \pi_r/\pi_i \). The solid line fitted to the data points has a slope \( = 1 \) which corresponds to ideal osmometric behavior.

solutions when exciting the dye at its intracellular IW (438 ± 2.5 nm) is shown in Fig. 1 A. Relative fluorescence is expressed as \( F_r/F_p \), where \( F_p \) is the fluorescence measured at the pinhole region in an ISO control solution having osmotic pressure \( \pi_p \) and \( F_r \) is the fluorescence of the same region upon exposure to a calibration solution having osmotic pressure \( \pi_r \). Plotting the reciprocal of the apparent steady state changes in fluorescence \( F_{\text{recip}}/F_r \) for each calibration pulse as a function of the reciprocal of the relative osmotic pressure of the medium \( (\pi_r/\pi_i) \) yields a linear relationship (Fig. 1 B), indicating that the changes in fluorescence reflect changes in intracellular concentration of BCECF that in turn reflect changes in CWV. Before subtraction of \( F_{\text{trapped}} \) the slope of the regression line fitted to the data points (solid line in Fig. 1B) is always less than 1, the slope expected for ideal osmometric behavior (dotted line Fig. 1B). \( F_{\text{trapped}} \) was estimated for each cell from the \( y \) intercept of plots of \( F_{\text{recip}}/F_r \) versus \( \pi_r/\pi_i \), like the one shown in Fig. 1B. Subtraction of \( F_{\text{trapped}} \) yields the dotted line shown in Fig. 1B, indicating ideal osmometric behavior. The nature of \( F_{\text{trapped}} \) as well as alternative methods to determine its value, has been extensively discussed in previous publications [25, 30, 31]. Changes in CWV \( (V_r/V_i) \) resulting from transformation of the fluorescence signals shown in Fig. 1A, using Eq 1 are shown in Fig. 1C. A plot of the apparent steady state changes in \( V_r/V_i \) as a function of \( \pi_r/\pi_i \) is shown in Fig. 1D. The solid line fitted to the data points has a slope \( = 1 \), which corresponds to ideal osmometric behavior, as discussed in detail in a previous publication [25].

Analysis of Cell Water Volume Changes

When drift of the fluorescence signal occurred as a result of dye leakage and/or photobleaching, it was corrected by fitting a linear regression to the base line and multiplying the slope of this regression line by the time at which each data point was sampled. This process yields point by point drift values that in turn are subtracted from the fluorescence record yielding the corrected trace. The nomenclature used to refer to the measured variables of CWV changes in response to transient exposure to isosmotic solutions containing NH₄Cl is shown in Fig. 3A. The extent of the iRVD response was measured 4 min after its onset (f in Fig. 3A) and represents the percentage of cell volume recovery from the initial normalized peak swelling (100%), according to the following expression:
\[
% \text{iRVD} = \left(\frac{\text{peak } V_x / V_o - \text{reg } V_x / V_o}{\text{peak } V_x / V_o - 1}\right) \times 100
\]  
(Eq. 2)

where ‘peak \( V_x / V_o \)’ is the peak swelling value and ‘reg \( V_x / V_o \)’ is the regulated relative volume 4 min after the onset of the iRVD response.

**Measurement of Intracellular pH**

Intracellular pH changes measured in parallel with CWV were estimated from the ratio of emitted fluorescence upon excitation of BCECF at 495 and 438 nm, using the high K⁺/-nigericin in vivo calibration technique as described by Boyarski’s group [32, 33]. Only cells having an initial pH between 6.8 and 7.4 were analyzed. Fig 3B illustrates the nomenclature used to refer to the measured variables of the pH transient in response to NH₄Cl exposure and removal.

**Statistical analyses**

Two-tailed t-test analysis was used to compare differences between means. A p value of <0.05 was considered as significant. Results are expressed as the mean ± SEM (standard error of the mean).

**Results**

**Parallel Changes in Cell Water Volume and Intracellular pH Induced by NH₄⁺/NH₄Cl in Neuroblastoma Cells**

Changes in CWV and pH produced by brief (7 to 20 min) exposure to isosmotic solutions containing NH₄Cl (0.5-30 mM) were recorded simultaneously in single neuroblastoma cells loaded with BCECF. Fig 2 shows a representative example of these responses. The upper panel (A) shows the CWV changes (\( V_x / V_o \); black trace), and the lower panel (B) shows the concomitant pH transients produced by exposure and removal of an isosmotic solution that, in this case, contained 20 mM NH₄Cl. Upon exposure to the NH₄Cl solution the cell swelled (phase 1v) and underwent regulatory volume decrease (phase 2v). Since the latter response occurs in isosmotic media, we denote it as “isosmotic regulatory volume decrease” (iRVD) to distinguish it from the much studied “regulatory volume decrease” (RVD) elicited by exposure to hyposmotic solutions. Upon removal of the NH₄Cl solution the cell shrank (phase 3v) and slowly recovered its initial volume (phase 4v). We call this response “isosmotic regulatory volume increase” (IRVI) to distinguish it from the RVI that occurs during exposure to a hyperosmotic solution or the RVI that occurs after a cell that was challenged with an hyposmotic solution is placed back into isosmotic solution.

Concomitant with the changes in CWV, the cell responded to the NH₄Cl pulse with the pH transients shown in Fig. 2B. These are the pH changes expected for a cell permeable to both NH₄⁺ and NH₄⁺ [11, 13]. They consisted of an initial rapid alkalinization (phase 1p) followed by a slow decrease in pH (phase 2p) known as plateau acidification. Removal of the NH₄Cl pulse resulted in a marked and rapid acidification (phase 3p) followed by a relatively slow pH recovery (phase 4p). The inset in Fig. 2A shows the initial changes in CWV and pH upon exposure to the NH₄Cl pulse, displayed at a faster time base. As expected, the changes in pH precede those in CWV.

The results obtained from 60 N1E-115 cells exposed to 20 mM NH₄Cl are summarized in Table 1. The changes in CWV and pH were quantified following the nomenclature described in Fig. 3. In these experiments, the NH₄Cl pulse had an average duration of 7.45 ± 0.4 min. The number of observations for some parameters describing the late parts of the response (e.g. phases 3v and 4v) is less than 60 because upon removal of the NH₄Cl pulse a small number of cells (7% of the population) returned to their initial volume without presenting shrinkage. For the cells that shrunk (i.e. 93% of the population), the latency of the peak shrinkage (\( g \) in Fig. 3A) and the time course of the subsequent iRVI were variable, and in a few occasions, iRVI was absent.

The basic pattern of CWV changes described above was observed also in NG108 cells (n=13), a hybrid of neuroblastoma and glioma (see below). Further, the basic pattern of CWV
Fig. 2. Changes in CWV and pH recorded simultaneously in a single BCECF-loaded N1E-115 cell exposed to an isosmotic solution containing NH₄Cl. (A) Changes in CWV (V/V₀, black trace). Upon exposure to the NH₄Cl solution (20 mM), the cell swelled to a maximum of 8.3% above its initial volume (phase 1v) in ~21 s, at an initial rate of ~56% min⁻¹. The cell remained swollen at its maximum value for ~120 s and then displayed isosmotic regulatory volume decrease (iRVD; phase 2v) at an initial rate of ~8.2% min⁻¹. The extent of cell volume recovery, measured 4 min after the onset of iRVD as defined by Eq. 2, was 36.7%. Upon termination of the NH₄Cl pulse and readministration of the isosmotic control solution, CWV decreased (phase 3v) to a maximum of ~3% below the baseline in ~30 s. This cell shrinkage was followed by isosmotic regulatory volume increase (iRVI; phase 4v) that proceeded at an initial rate of ~3.2% min⁻¹. Inset in (A) shows the initial changes in CWV (dark blue trace) and pH (orange trace) at an expanded time base. The green trace superimposed on the CWV transient in (A) corresponds to the [NH₄⁺], calculated from Eq. 3 using the pH data recorded during phase 2p in panel B. The values of [NH₄⁺] were corrected for the recorded changes in CWV for each instant. The red trace corresponds to the “predicted” changes in CWV (V/V₀) calculated from the increase in intracellular osmolality resulting from NH₄⁺ accumulation (Eq. 4) assuming osmometric behavior (i.e., lack of regulatory responses). (B) pH changes occurring simultaneously with those in CWV. The initial basal pH was 7.1. Upon exposure to NH₄Cl there was a rapid alkalization (phase 1p) that peaked at pH 7.53, followed by a recovery phase (plateau acidification or phase 2p) that proceeded at an initial rate of ~0.022 pH units min⁻¹. Upon NH₄Cl removal, there was a rapid decrease in pH that peaked at 6.8 (phase 3p) followed by a recovery phase (phase 4p) at an initial rate of 0.02 pH units min⁻¹.

Changes in cells loaded with BCECF could also be reproduced in cells loaded with calcine (Fig. 4), a fluorescent dye that has spectral properties different from those of BCECF and is well characterized as an independent CWV marker [25, 30, 31]. These results demonstrate that the observed CWV changes were not dye- or cell line type-dependent.

**Mechanisms of the Cell Swelling Induced by NH₄⁺/NH₄⁺**

The initial pH increase and the subsequent time course of the pH response upon NH₄Cl exposure and removal indicate that N1E-115 and NG 108 cells, like most animal cell types, have a higher permeability to NH₃ than to NH₄⁺ [12, 34, 35]. Consequently, when they are challenged with NH₄Cl, NH₃ rapidly enters through the plasma membrane and combines with intracellular protons, forming NH₄⁺ and shifting the pH in the alkaline direction. Since NH₄⁺, but not NH₃, is the osmotically active species, we hypothesize that the net gain in osmotically active particles represented by NH₄⁺ accumulation increases the intracellular osmolality, thereby inducing net osmotic water influx and cell swelling. To test this hypothesis, we calculated the magnitude and time course of intracellular NH₄⁺ accumulation during the NH₄Cl pulse. To this end, we measured for each individual cell the values of pH at the peak alkalization and throughout the plateau acidification. At the peak alkalization and during the slowly developing plateau phase, NH₃ is expected to be at or near equilibrium.
Table 1. CWV and pH Changes in N1E-115 Cells Exposed to 20 mM NH₄Cl. All values are means ± SEM. The nomenclature for CWV (a - i) and pH parameters is as in Fig. 3.

| Parameter | n  |
|-----------|----|
| a, peak swelling, (%) | 14 ± 1 | 60 |
| b, initial rate of swelling, (% min⁻¹) | 63 ± 5 | 60 |
| c, time to peak swelling, (s) | 36 ± 2 | 60 |
| d, latency of iRVD onset, (s) | 69 ± 5 | 60 |
| e, initial rate of iRVD, (% min⁻¹) | -9.2 ± 1 | 60 |
| f, extent of iRVD at t = 4 min after onset, (%) | 34 ± 3 | 60 |
| g, time to peak shrinkage upon NH₄Cl removal, (s) | 75 ± 8 | 56 |
| h, peak shrinkage, (%) | -7 ± 0.6 | 56 |
| i, initial rate of iRVI, (% min⁻¹) | 12.3 ± 2 | 54 |
| pH₁₋₀ | 7.15 ± 0.02 | 60 |
| pH₁₋₁ | 7.55 ± 0.03 | 60 |
| ΔpH₁ (pH₁₋₁ - pH₁₋₀) | 0.39 ± 0.01 | 60 |
| pH₅₋₂ | 7.41 ± 0.02 | 59 |
| pH₅₋₃ | 6.82 ± 0.02 | 59 |
| pH₅₋₄ | 7.05 ± 0.05 | 60 |

- dpH₁/dt, initial rate of plateau acidification (pH₁ units·min⁻¹) = -0.024 ± 0.001 | 60 |

Fig. 3. Schematic drawing illustrating measured variables of CWV and pH changes elicited by NH₄Cl exposure and removal. (A) CWV changes. Ordinate: relative cell volume (V/V₀). Abscissa: time. (a) Maximum (peak) swelling, (b) initial rate of swelling, (c) time to peak swelling, (d) latency of iRVD onset, (e) initial rate of iRVD, (f) extent of iRVD at t = 4 min, (g) time to peak shrinkage upon NH₄Cl removal, (h) peak shrinkage, (i) initial rate of iRVI. (B) Points at which pH₁ was measured: t = 0, basal; t = 1, at peak alkalization; t = 2, before NH₄Cl removal; t = 3, at peak acidification; t = 4, after recovery from acidification; -dpH₁/dt, initial rate of plateau acidification.

Across the plasma membrane, and [NH₄⁺], can be calculated using the following expression, derived from the Henderson-Hasselbalch equation:

\[
[NH₄⁺] = [NH₄⁺]₀ \times 10^{pH₀ - pH₁}
\]  

(Eq. 3)

where [NH₄⁺] is the intracellular NH₄⁺ concentration and [NH₄⁺]₀ is the extracellular NH₄⁺ concentration, which for a 20 mM NH₄Cl solution at 25°C has a value of 19.78 mM.
Fig. 4. Changes in CWV produced by NH$_3$ /NH$_4^+$ in a single N1E-115 cell loaded with calcein. The cell was exposed to a pulse (7 min) of an isosmotic solution containing 20 mM NH$_4$Cl. The observed CWV changes had the same basic pattern observed in cells loaded with BCECF.

Fig. 5. Peak cell swelling amplitude as a function of [NH$_4^+$], and basal pH, in response to NH$_3$ /NH$_4^+$ exposure. Peak increase in cell volume ($V/V_0$) plotted as a function of the corresponding [NH$_4^+$], (A) and initial (t=0) basal pH (B) in response to a 20 mM NH$_4$Cl pulse. Each point represents a single cell (n=60). The correlation coefficient of the regression lines was 0.78 ($p<0.0001$) in A, and 0.79 ($p<0.0001$) in B.

(pK =9.25; pH$_i$ = 7.3). The values of [NH$_4^+$] were corrected point by point for the measured changes in CWV, to take into account intracellular dilution caused by such changes. For the cell in Fig. 2, the estimated [NH$_4^+$], with respect to time is shown in the green trace in (A). At the peak alkalization, [NH$_4^+$], reached ~12.5 mM, and thereafter it continued increasing gradually throughout the duration of the NH$_4$Cl exposure, reaching a value of ~15.7 mM just before the end of the NH$_4$Cl pulse (i.e., at t ≈ 7 min). The [NH$_4^+$] at the peak swelling is likely to result mostly from rapid entry of NH$_3$ and formation of NH$_4^+$. The continued buildup of [NH$_4^+$], during NH$_4$Cl exposure is probably due to influx of NH$_4^+$ as suggested by the plateau acidification of the pH$_i$ response. To evaluate if the increase in [NH$_4^+$] could explain the magnitude of the observed peak swelling, we calculated the change in CWV that would result from such accumulation, according to the following equation:

$$V/V_0 = (\text{mosmol/kg water})_{t>0}/(\text{mosmol/kg water})_{t=0} \quad (\text{Eq. 4})$$

where $V_t$ and $V_0$ have been defined, and the intracellular osmolality is expressed in mosmol/kg water. For this calculation, it is assumed that prior to NH$_4$Cl exposure the cell is in osmotic equilibrium with the extracellular solution, and hence the intracellular and extracellular osmolalities are equal (311 mosmol/kg water in this case). For the cell in Fig. 2, a net increase in intracellular osmolality resulting from accumulation of 12.5 mosmol/kg water should produce a peak swelling of ~4% ($V/V_0 = (311 +12.5) / 311$). However, the observed peak swelling was 8.3%, thus ~48% of the peak swelling can be explained, in this example, by intracellular accumulation of NH$_4^+$. We also calculated the theoretical time course of the CWV changes that would occur if there were no regulatory volume adjustments, using Eq. 4. This is shown in Fig. 2A ($V/V_0$, red trace). Note that under these assumptions, CWV progressively increases in parallel with the net gain in intracellular NH$_4^+$. However, the cell responds with IRVD, thereby counteracting the progressive increase in intracellular osmolality and the volume expansion that would otherwise occur.
In the 60 N1E-115 cells analyzed, the mean [NH₄⁺] estimated at the peak alkalinization was 14.2 ± 0.8 mM. The predicted mean peak swelling for this [NH₄⁺] was 4.6 ± 0.3 % (range 1.7 to 9.6%). The observed mean peak swelling (a in Fig. 3) was 14 ± 1% (range 3 to 29%, Table 1). Thus, ~35% of the peak swelling can be explained by intracellular accumulation of NH₄⁺. Similarly, in NG-108 cells the peak swelling was 15.1±1.7 %, and the predicted volume 5.9±0.8%. Thus, ~46% of the peak swelling amplitude can be accounted for intracellular NH₄⁺ accumulation. Although NH₄⁺ accumulation by itself cannot account for the observed swelling, the strong correlation (r = 0.78; p < 0.0001) between the magnitude of the observed cell swelling and the [NH₄⁺] (Fig. 5A) is consistent with the hypothesis that NH₄⁺ accumulation is a significant determinant of the increase in CWV. Coherent with this hypothesis is also the observation that the peak swelling amplitude was inversely related to the initial pH_i (i.e. pH_i,0), as shown in Fig. 5B. This was expected since lowering the pH_i favors the protonation of NH₄⁺.

The above mentioned results indicate that in addition to NH₄⁺, other osmolytes, likely derived from metabolic reactions that utilize NH₄⁺, contribute to the initial cell swelling. We addressed the question of whether one of these osmolytes could be glutamine, a main product of the detoxification of NH₄⁺/NH₄⁺ that has been found to accumulate in the brain during hyperammonemia [36]. First, we confirmed the presence of glutamine synthetase (GS) in N1E-115 and NG108 cells by Western blot analysis (data not shown). Next, N1E-115 cells were exposed to control and test pulses with 20 mM NH₄Cl, in the absence and in the presence of the GS inhibitor methionine sulfoxime (MSO), respectively. Peak swelling amplitude was 23% lower in the presence of MSO (n=6; p<0.05), suggesting that glutamine synthesis contributes to the initial volume change mediated by NH₄⁺/NH₄⁺ (data not shown). These results indicate that in addition to NH₄⁺, glutamine accumulation partly contributes to the initial increase in CWV mediated by NH₄⁺/NH₄⁺.

The Amplitude of the Peak Swelling Varies with the External Concentration of NH₄Cl

It has long been known that the amplitude of the intracellular alkalinization produced by NH₄Cl exposure depends on the external concentration of this salt, and that this effect is due to rapid NH₄⁻ permeation and its subsequent intracellular protonation [34]. Our findings are consistent with the hypothesis that an important part of the initial swelling induced by NH₄Cl is due to rapid NH₄⁻ permeation and intracellular accumulation of the osmotically active species NH₄⁺. Accordingly, the peak amplitude of the swelling and the magnitude of the initial intracellular alkalinization (ΔpH_i = pH_i,0 - pH_i,0) should be a function of the extracellular concentration of NH₄Cl ([NH₄Cl]₀). Fig. 6A shows the relationship between the observed (filled circles) and predicted (open circles) values of the peak swelling amplitude as a function of [NH₄Cl]₀. The inset shows typical CWV traces from a cell exposed to two pulses of low [NH₄Cl]₀ (0.5 and 1 mM, respectively). The latter solutions have NH₄⁺ concentrations similar to those present in acute liver failure, as well as in some congenital hyperammonemias [6, 8]. As predicted by our model (Fig. 2), the amplitude of the peak swelling increased with [NH₄Cl]₀. Consistent with the results presented in the previous section, there was a significant difference between predicted and observed values of the peak swelling amplitudes for the entire range of [NH₄Cl]₀ tested. Also, as predicted, both the ΔpH_i and the [NH₄⁺] were a function of [NH₄Cl]₀ (Fig 6B).

The External Concentration of Ammonia (NH₄⁺) but not Ammonium (NH₄⁺) Determines the Peak Amplitude of Both Cell Swelling and Intracellular Alkalinization

The results so far presented are consistent with intracellular NH₄⁺ accumulation being a major determinant of the NH₄⁺-induced cell swelling. Given that N1E-115 and NG108 cells are permeable to both NH₄⁺ and NH₄⁺, accumulation of intracellular NH₄⁺ could result not only from protonation of entering NH₄⁺, but also from direct NH₄⁺ influx. In NH₄⁺-containing solutions, NH₄⁺ is in chemical equilibrium with NH₄⁺, and the relative concentration of each species is determined by the pH of the solution. To investigate the relative contribution of each of these species to the magnitude of the peak swelling, we tested the effects on CWV
Fig. 6. Peak cell swelling amplitude, [NH₄⁺], and ΔpH in cells exposed to various NH₄Cl concentrations. (A) Observed (filled circles) and predicted (open circles) mean peak increase in CVW (V/Vₒ), plotted as a function of external [NH₄Cl]. The predicted peak increase in CVW (peak cell swelling) was calculated from the increase in intracellular osmolality that would result from [NH₄⁺] estimated from pH values concomitant with the peak cell swelling, applying Eqs. 3 and 4. Inset shows that volume responses could already be elicited with low NH₄Cl concentrations, i.e. 0.5 mM and 1 mM, respectively. (B) Magnitude of the initial change in pH, ΔpHₒ (filled circles) and [NH₄⁺] (open circles) corresponding to peak alkalinization and peak cell swelling values plotted as a function of external [NH₄Cl]. Symbols are mean ± SEM. The number of cells analyzed for each [NH₄Cl] is shown in parentheses in A. *, p < 0.05; ***, p < 0.001.

Fig. 7. Influence of external [NH₄⁺] and [NH₄⁺] on CVW and pHₒ. (A) Changes in CVW (V/Vₒ; top) and pHₒ (bottom) recorded in a single BCECF-loaded cell exposed to two NH₄Cl pulses (P₁ and P₂) in which [NH₄⁺]ₒ was kept constant (10 mM), while [NH₄⁺]ₒ was varied from 0.11 mM (P₁) to 0.33 mM (P₂). Note that the amplitude of both the cell swelling and ΔpHₒ increased with [NH₄⁺]ₒ. (B) Changes in CVW (V/Vₒ) and pHₒ produced by two NH₄Cl pulses in which [NH₄⁺]ₒ was kept constant (0.23 mM), while [NH₄⁺]ₒ was varied from 19.77 (P₁) to 4.77 mM (P₂). Note that the amplitude of both the swelling and the pH change was similar in both pulses despite a 4-fold difference in [NH₄⁺]ₒ.

and pHₒ of isosmotic solutions containing NH₄Cl in which either [NH₄⁺]ₒ or [NH₄⁺]ₒ was kept constant while the other was changed by varying the pH of the solution. Fig. 7A shows an example of changes in CVW (V/Vₒ; upper traces) and pHₒ (lower traces) elicited in a single cell by two consecutive NH₄Cl pulses (P₁ and P₂) in which [NH₄⁺]ₒ was varied from 0.11 mM (P₁) to 0.33 mM (P₂), while [NH₄⁺]ₒ was kept constant (10 mM). The amplitude of both the initial swelling and the intracellular alkalinization were ostensibly larger in P₂, i.e. the pulse in which [NH₄⁺]ₒ was higher. Fig. 7B shows CVW and pHₒ changes recorded from a
Fig. 8. Peak swelling and ΔpH as a function of external [NH₄⁺] and [NH₄⁺]. Data was collected from experiments like those shown in Fig. 7. (A) Amplitude of the peak swelling (V/V₀) and (C) ΔpH, plotted as a function of [NH₄⁺]₀ keeping [NH₄⁺]₀ constant (10 mM). (B) Amplitude of the peak swelling and (D) ΔpH, plotted as a function of [NH₄⁺], keeping [NH₄⁺]₀ constant (0.23 mM). Symbols are mean ± SEM. The number of cells for each data point is shown in parentheses. Lines are linear regressions.

single cell exposed to two NH₄Cl pulses in which the [NH₄⁺]₀ was now fixed (0.23 mM), and [NH₄⁺]₀ varied from 19.77 mM (P₁) to 4.77 mM (P₂). Note that although the [NH₄⁺]₀ in P₁ was ~4 times higher than that in P₂, the amplitude of the swelling and the pH increase were similar for both pulses.

Data obtained from experiments like those shown in Fig. 7 are summarized in Fig. 8. Note that both peak swelling and ΔpH increased with [NH₄⁺]₀ (Figs. 8A and C) when [NH₄⁺]₀ was kept constant (10 mM). In contrast, in cells exposed to solutions in which [NH₄⁺]₀ was kept constant (0.23 mM) but [NH₄⁺]₀ varied, the amplitude of the cell volume and the ΔpH did not change significantly with [NH₄⁺]₀ (Figs. 8B and D). These results demonstrate that [NH₄⁺]₀ rather than [NH₄⁺]₀ is the main determinant of the NH₄Cl-induced swelling.

Membrane Permeability of N1E-115 cells to NH₄⁺

The permeability to NH₄⁺ (P_NH₄⁺) of mammalian neuronal membranes has not yet been reported. We calculated the P_NH₄⁺ of N1E-115 cells from the transmembrane flux of NH₄⁺ (J_NH₄⁺) upon NH₄Cl exposure, defined as

\[ J_{\text{NH}_4} = \frac{dpH_i}{dt} \times \beta_i \times \frac{V}{S} \]  
(Eq. 5)

where \( \frac{dpH_i}{dt} \) is the initial rate of intracellular alkalinization, \( \beta_i \) is the intrinsic buffering power of the cells (in mM pH units⁻¹) and \( \frac{V}{S} \) is their volume to surface ratio. \( \frac{dpH_i}{dt} \) was measured in cells exposed to 0.5 to 5 mM NH₄Cl, having a basal pH of ~6.7 to 7.0. Since the pK for NH₄⁺/NH₃⁺ at 25°C is ~9.25, less than 1% of the NH₃⁺ entering the cells will release their protons at this pH_i range, and hence the initial rate of the intracellular alkalinization will not be substantially affected by concurrent NH₃⁺-born protons. In this group of cells, cell volume (V) and membrane surface area (S) were measured from images obtained using differential interference optics. Since the cells were attached to coverslips, we considered them as hemispheres, with \( S = 3 \pi r^2 \) and \( V = \frac{4}{3} \pi r^3 \). The mean radius of cell’s soma was 16 ± 0.4 μm (n = 26), and \( \frac{V}{S} \approx 3.6 \) μm. \( \beta_i \) is defined as \( [\text{NH}_4^+ + \text{NH}_3^+] / \Delta \text{pH} \) and was calculated from the "off" pH_i response measured upon removal of NH₄Cl pulses in two sets of experiments. The first set included short (~2 min) NH₄Cl pulses (0.5-20 mM) in which pH_i returned to the baseline, i.e. there was no rebound acidification. In the second group, NH₄Cl (20 mM) exposure and removal were done in isosmotic Na+-free solution, a maneuver that blocked pH_i...
Fig. 9. Intrinsic buffering power and transmembrane flux of NH$_3$ in N1E-115 cells. (A) Intrinsic buffering power ($\beta_i$) as a function of pH$_i$, $\beta_i$ was estimated from $\Delta$pH$_i$ measured upon removal of short (~2 min) NH$_4$Cl (0.5-20 mM) pulses (open circles), or long (~7 min) NH$_4$Cl (20 mM) pulses in Na$^+$-free solutions (filled circles). (B) Transmembrane flux of NH$_3$ ($I_{NH3}$) as a function of the external [NH$_3$], calculated using Eq. 5. The slope of the best-fit line corresponds to the apparent membrane permeability to NH$_3$ (3.8 x 10^{-4} cm$^2$s$^{-1}$).

recovery (not shown) by inhibition of Na$^+/H^+$ exchange [37, 38]. Blocking Na$^+/H^+$ exchange permits a more accurate estimate of $\beta_i$; activation of this antipporter upon cell acidification in the “off” response decreases the amplitude of the acidification and causes overestimations of $\beta_i$ [39]. Fig. 9A shows a plot of $\beta_i$ as a function of pH$_i$, the latter being the midpoint of $\Delta$pH$_i$ for the “off” response. As in other cell types [39-41], $\beta_i$ was inversely related to pH$_i$. The slope of a linear regression (solid line) fitting the data points was -39.8 mM pH$_i$ unit$^{-1}$. The $\beta_i$ used for calculation of $I_{NH3}$ was obtained for each cell by linear interpolation from the data shown in Fig. 9A.

Figure 9B shows $I_{NH3}$ plotted as a function of [NH$_3$]$_o$. The slope of the straight line represents the approximate $P_{NH3}$ of these cells, which was 38 ± 2 μm$^{-1}$s$^{-1}$ (n = 28), a value that falls within the range reported for other animal cell membranes [35].

**Isosmotic Regulatory Volume Decrease**

The mechanisms mediating volume regulation in anisosmotic media have been extensively studied in various cell types. In contrast, little is known about regulatory volume responses in isosmotic media. Given that the osmolality of the extracellular fluids is tightly regulated and maintained constant within narrow limits, isosmotic volume regulatory responses are likely to predominate under physiological conditions and under pathological conditions that do not seriously compromise renal function. We observed two isosmotic regulatory responses during exposure and removal of NH$_4$Cl, iRVD and iRVI, respectively (Fig. 2, phases 2v and 4v). A salient feature of these regulatory volume responses is that they occurred upon relatively small or moderate changes in CWV elicited in isosmotic media containing NH$_4^+/NH_4^+$, but not upon changes in CWV of similar amplitude produced by exposure to anisosmotic solutions (Fig. 10A). This suggests that the mechanism(s) that trigger volume regulatory responses in isosmotic and anisosmotic conditions are different, and demonstrates that changes in membrane mechanical tension (i.e., expansion or contraction) are not by themselves a sufficient condition for volume regulation to ensue.

We began to study the mechanisms underlying the iRVD response triggered during NH$_4^+/NH_4^+$ exposure. Anion channels are known to play a central role in the RVD produced by relatively large cell expansions elicited by hypsomotic media (~40%) in different cell types [42-44]. To determine if anion channels are also involved in iRVD elicited by NH$_4$Cl exposure, we evaluated the effects of NPPB (10 μM), an anion channel blocker known to inhibit anisosmotic RVD. NPPB inhibited or even reversed the regulatory response (Figs. 10B and C). These results suggest that activation of NPPB-sensitive anion channels is a key component of iRVD elicited during NH$_4^+/NH_4^+$ exposure.
Fig. 10. Properties of iRVD elicited during exposure to NH₄⁺/NH₄⁺. (A) Superimposed traces of CWV (V/V₀) and pH elicited in the same cell by exposure to anisosmotic (~±11 %) solutions (gray traces) or to 20 mM NH₄Cl (black trace). Note that changes in cell volume produced by the anisosmotic solutions were neither followed by regulatory responses nor were they accompanied by changes in pH. In contrast, the NH₄Cl solution and its removal elicited isosmotic regulatory volume responses (iRVD and iRVI respectively) and the characteristic changes in pH. (B) Effect of NPPB on iRVD: superimposed traces of V/V₀ elicited by exposure to NH₄Cl (20 mM) in the absence (gray trace) and in the presence (black trace) of NPPB (10 μM). Note that in the presence of NPPB the regulatory response was abolished and the cell continued increasing its volume during the duration of the NH₄Cl pulse. (C) Percent inhibition of iRVD produced by NPPB (10 μM) in cells exposed to either 20 or 5 mM NH₄Cl pulses. The number of cells analyzed in each case is shown in parentheses. ***, p < 0.001. (D) Extent of iRVD measured during exposure to a first (control) pulse of 20 mM NH₄Cl and a second (zero Ca²⁺) pulse of 20 mM NH₄⁺-gluconate. (E) Superimposed traces of V/V₀ in a cell exposed to NH₄Cl in the presence (control) and in the absence of external Ca²⁺. (F) Effects of removal of external Ca²⁺ on the initial rate (−dCWV/dt) and extent of iRVD. The number of cells analyzed is shown in parentheses. ***, p < 0.001. (G) Changes in fura-2 fluorescence in a single cell exposed to NH₄Cl in the presence and in the absence of external Ca²⁺. Exposure to NH₄Cl induced a transient increase in intracellular Ca²⁺ that was attenuated in the absence of external Ca²⁺.

Among the intracellular anions that could be exiting the cells through NPPB-sensitive channels during iRVD are Cl⁻ and organic osmolytes. As shown in Fig. 10 D, the extent of iRVD measured during exposure to Cl⁻-free, NH₄⁺-gluconate pulses (23.7 ± 5.6%) was in fact slightly larger, but not significantly different, than the control response (17.6 ± 3.2%; n = 8). This suggests that osmolytes other than Cl⁻, probably organic anions, contribute to this regulatory response.

It has been previously shown that an increase in intracellular Ca²⁺ occurs in N1E-115 cells upon exposure to large (~40%) hypotonic solutions. This increase, however, was not necessary for RVD to occur [45]. To investigate the possible Ca²⁺ dependency of iRVD, we exposed the cells to isosmotic solutions containing NH₄Cl (20 mM) in the presence (control pulse) and in the virtual absence of external Ca²⁺ (test pulse). Both the initial rate (−dCWV/ dt) and the extent of iRVD were significantly reduced in Ca²⁺-free-NH₄Cl solutions (Fig. 10EF). This effect was independent of the order in which the control and the test pulses were applied. Similar results were obtained in cells loaded with calcine (not shown). In parallel experiments, changes in intracellular Ca²⁺ concentration ([Ca²⁺]) were recorded in cells loaded with Fura-2 and exposed to NH₄Cl solutions with or without Ca²⁺. NH₄Cl exposure
consistently elicited an increase in [Ca\(^{2+}\)], that was attenuated in the virtual absence of external Ca\(^{2+}\) (Fig. 10G). These results suggest that unlike anisosmotic RVD, the iRVD elicited by NH\(_4\)/NH\(_4\)^+ depends, to a large extent, on the presence of external Ca\(^{2+}\).

**Discussion**

Classical observations on hemolysis of erythrocytes produced by ammonium salts, and theoretical considerations on the relative permeability and equilibrium distribution of NH\(_3\) and NH\(_4\)^+ across cell membranes, led Merkel H. Jacobs (1884–1974) to propose several decades ago, that exposure to isomotic solutions containing the pair NH\(_3\)/NH\(_4\)^+ should result in cell swelling [18, 21, 22]. In spite of its fundamental importance for cell biophysics of pH and volume regulation, and its possible clinical implications in the pathophysiology of hyperammonemnic disorders, this hypothesis has remained largely untested. The present paper provides a kinetic description, at the single cell level, of the changes in cell water volume associated with exposure and removal of NH\(_3\)/NH\(_4\)^+ and their mechanistic relationship with concurrent pH\(_i\) changes. The method used here allows real time recording of parallel changes in pH\(_i\) and CWV in single cells, with time resolution of <1 second and sensitivity to osmotic changes of ~1% [25]. Sudden exposure of neuroblastoma cells (N1E-115 and NG-108) to isomotic solutions containing the pair NH\(_3\)/NH\(_4\)^+ (NH\(_4\)Cl concentrations ranging from 0.5 to 20 mM) produces a rapid cell swelling followed by iRVD. Removal of NH\(_4\)Cl and readmission of the isomotic control solution causes cell shrinkage, followed by iRVI. These volume changes are accompanied by the changes in pH\(_i\) expected for a cell significantly more permeable to NH\(_3\) than to NH\(_4\)^+, i.e. an initial alkalinization followed by a plateau acidification, and upon removal of the NH\(_4\)Cl pulse, a rebound acidification followed by pH\(_i\) recovery [11, 13].

**Mechanisms of Cell Swelling Elicited by Isomotic Solutions Containing NH\(_3\)/NH\(_4\)^+**

In isomotic media, any increase in cell volume results from a primary increase in intracellular osmolyte contents. The present results suggest that an increase in intracellular osmolality brought about by rapid intracellular NH\(_4\)^+ accumulation is an important determinant of the initial cell swelling observed upon exposure to NH\(_3\)/NH\(_4\)^+-containing solutions. The NH\(_4\)^+ accumulation could result from protonation of entering NH\(_3\) from direct influx of NH\(_4\)^+ via channels or transporters, or from a combination of these processes. Our results show that, like most cell types [35], the permeability of N1E-115- and NG108-cells to NH\(_3\) is much higher than that to NH\(_4\). Thus, exposure to NH\(_3\)/NH\(_4\)^+ produces an intracellular alkalinization due to rapid permeation of NH\(_3\) followed by its intracellular protonation. Consequently, osmotically active NH\(_4\)^+ is formed and accumulated in the cytosol, thus increasing intracellular osmotic pressure and promoting water influx and cell swelling. By varying the concentration of either NH\(_3\) or NH\(_4\)^+ in the isomotic solution, while keeping the concentration of the remaining species constant, we demonstrated that intracellular NH\(_4\)^+ generation due to rapid NH\(_3\) influx and protonation, rather than direct influx of NH\(_4\), is the main determinant of the observed cell swelling. After both pH\(_i\) and swelling peaks are reached, a gradual acidification ensues, due in part to slow entry of NH\(_4\)^+. In spite of this progressive NH\(_4\)^+ accumulation, cells decrease their volume by activation of compensatory mechanisms i.e., iRVD.

The magnitude of the NH\(_4\)^+ accumulation during the peak swelling accounted for ~35% of its measured amplitude (~46% in NG108 cells). Since NH\(_4\)^+ accumulation imposes a net gain in intracellular cations, an equimolar increase in intracellular negative charges must occur to preserve macroscopic electroneutrality [46]. Such an increase in intracellular negative charges, however, is unlikely to promote a further rise in intracellular osmolality and cell volume, as it should result primarily from deprotonation of intrinsic fixed and mobile buffers [47, 48], whose concentration is expected to remain unaltered. In other words, as NH\(_3\) is protonated, NH\(_4\)^+ is formed, cytoplasmic pH rises, and the net charge on the cytoplasmic
buffers becomes more negative, exactly balancing the positive charge on the \( \text{NH}_4^+ \) that is formed. Part of the initial swelling, however, could also result from \( \text{Cl}^- \) influx, driven by the \( \text{NH}_4^+ \)-induced depolarization and/or via cotransport of \( \text{NH}_4^+ \) through the \( \text{K}^- \) binding site of the Na\(^+-\text{K}^-\)2Cl\(^-\)cotransporter 1, i.e. NKCC1 [49] or that of the K\(^+-\text{Cl}^-\)cotransporters, e.g. KCC2 [50]. Cl\(^-\) influx via channels is unlikely inasmuch as N1E-115 cells are derived from sympathetic ganglion neurons, in which the equilibrium potential for \( \text{Cl}^- \) (\( E_{\text{Cl}} \)) is 10 to 20 mV more positive than the resting membrane potential (\( E_m \)), under basal conditions [51] and hence the driving force for \( \text{Cl}^- \) is outward. Consistent with this hypothesis, we observed no significant differences in the magnitude of the initial swelling upon replacement of external \( \text{Cl}^- \) with gluconate. However, experiments performed in the presence of 10 \( \mu \text{M} \) bumetanide to block NKCC1 in cells challenged with 20 mM \( \text{NH}_4\text{Cl} \) produced a slight but significant increase in the peak intracellular alkalization (\( \Delta \text{pH} \) control 0.37 \( \pm \)0.4 to 0.43 \( \pm \)0.05 n= 9, p<0.001). Likewise, the initial rate of plateau acidification decreased in the presence of bumetanide from -0.023\( \pm \)0.003 to -0.016\( \pm \)0.002 pH units/min (p<0.0001). These observations suggest that part of the \( \text{NH}_4^+ \) entry may be mediated by NKCC1. Expression of NKCC1 was ascertained in these cells by Western blot and immunolabeling (data not shown). Interestingly, changes in CWV were unaffected by bumetanide, suggesting that \( \text{NH}_4^+ \) influx mediated by NKCC1 may be balanced by efflux of osmolytes involved in iRVD. These results are in agreement with recent observations using 3-D image reconstructions of rat astrocytes, showing that bumetanide has no measurable effect on \( \text{NH}_4\text{Cl} \)-induced swelling [52].

As mentioned above, another potential \( \text{NH}_4^+ \) influx pathway is \( \text{K}^-\text{Cl}^- \) cotransport; \( \text{NH}_4^+ \) is a transport substrate of KCC2 when this cotransporter is exogenously expressed in mutant MDCK-LK-C1 cells challenged with 30 mM \( \text{NH}_4\text{Cl} \) pulses [50]. This transport pathway, however, can be ruled out in our experiments; Western blot analysis revealed that N1E-115 cells do not express KCC2 (not shown). Nevertheless, \( \text{NH}_4^+ \) influx could occur through isoforms of \( \text{K}^-\text{Cl}^- \) cotransporter other than KCC2. However, we found that variations in external \( [\text{NH}_4^+] \) in the range between \( \sim 5 \) to \( \sim 20 \text{ mM} \) while keeping constant extracellular \( [\text{NH}_3] \) did not affect the extent of cell swelling induced by \( \text{NH}_4\text{Cl} \), which is rather a function of the external \( [\text{NH}_3] \). Thus, any potential effects on cell volume due to \( \text{NH}_4^+ \) entry via \( \text{K}^-\text{Cl}^- \) cotransport are negligible in our experimental models. Moreover, furosemide, at a concentration expected to block all known isoforms of \( \text{K}^-\text{Cl}^- \) cotransporters (1mM), had similar effects to those of bumetanide (not shown).

We hypothesized that part of the unaccounted fraction of the swelling induced by \( \text{NH}_3/\text{NH}_4^+ \) was due to accumulation of organic osmolytes. The neutral aminoacid glutamine, a main product of the detoxification of \( \text{NH}_3/\text{NH}_4^+ \), has been implicated in brain cell edema in animal and \textit{in vitro} models of liver failure [9, 36, 52], and could be one of the osmolytes that accumulates besides \( \text{NH}_4^+ \). The enzyme glutamine synthetase (GS) catalyzes the production of glutamine from glutamate and \( \text{NH}_3 \). The presence of GS in murine neuroblastoma cells N2a has been reported [53, 54]. Using Western blot analyses we demonstrated the expression of this enzyme in both N1E-115 and NG108 cells (data not shown). In the presence of the GS inhibitor MSO, the amplitude of the peak swelling induced by 20 mM \( \text{NH}_4\text{Cl} \) was reduced by \( \sim 23\% \). Taken together these results suggest that a significant fraction of the swelling induced by \( \text{NH}_3/\text{NH}_4^+ \) is due to intracellular accumulation of glutamine.

The results discussed so far suggest that intracellular accumulation of \( \text{NH}_4^+ \) and glutamine can explain \( \sim 60 \) to 70\% of the swelling; the question arises as to which are the other osmolytes that are accumulated. Besides glutamine, other organic osmolytes like lactate, which has been shown to accumulate in neuroblastoma cells exposed to \( \text{NH}_4\text{Cl} \) [55], could also contribute to some of the swelling. Further, a number of metabolic pathways that utilize \( \text{NH}_4^+ \) as a nitrogen donor are likely to intervene in the generation of organic anions in neuroblastoma cells. For instance, glutamate and aspartate can be produced by activation of glutamate dehydrogenase and subsequent glutamate transamination. In future studies, measurement of metabolite levels during exposure to \( \text{NH}_3/\text{NH}_4^+ \) should provide a better understanding of the resulting alterations in intracellular osmolality due to metabolic factors.
Isosmotic Volume Regulatory Responses in the Continuous Presence of NH$_3$/NH$_4^+$

The mechanisms by which animal cells respond to alterations in their water volume in *isosmotic* media are poorly understood. Another novel observation reported here is the occurrence of *isosmotic* regulatory volume decrease (iRVD) and *isosmotic* regulatory volume increase (iRVII) following exposure and removal of *isosmotic* solutions containing NH$_3$/NH$_4^+$.

A noteworthy characteristic of these responses is that they are triggered by increments and reductions in CWV as small as ~3%. The observation that CWV changes of similar magnitude elicited by hypo- or hyper-osmotic solutions, that is, *anisosmotic* solutions, do not result in regulatory responses suggests that the mechanisms sensing cell size are different in each case, and stresses the fact that volume expansion or contraction are not sufficient to trigger regulatory volume responses.

We began characterizing the mechanisms underlying iRVD and found that NPPB (10 μM) powerfully inhibited this response, suggesting that anion channels are importantly involved. We also show here that the iRVD was strongly attenuated by removal of extracellular Ca$^{2+}$. This effect indicates that in N1E-115 cells, RVD and iRVD entail different mechanisms, as hypotonic RVD has been shown to be Ca$^{2+}$-independent [45]. Although the molecular identity of the anion channels mediating iRVD remains unknown, a likely candidate is the volume-sensitive organic osmolyte/anion channel (VSOAC) that was described in N1E-115 cells [56]. In a recent study performed in cultured rat astrocytes, it was found that exposure to NH$_3$Cl (5mM) produces a progressive increase in cell volume without regulatory volume responses [52]. The lack of iRVD in their study could be due to a difference in cell type and/or insufficient time and amplitude resolution of the method they used to measure cell volume changes.

Relevance for the Pathophysiology of Hyperammonemnic Brain Edema

Brain edema is a dreaded complication of hyperammonemic syndromes and an important cause of death in acute liver failure [6, 57]. It has been proposed that this edema results from astrocyte swelling secondary to accumulation of glutamine, derived from metabolism of NH$_3$ and glutamate [58, 59]. However, direct proof of a causal relationship between glutamine accumulation and development of brain edema is lacking [60], and long term exposure of cultured astrocytes to NH$_3$Cl results in cell swelling without a concurrent increase in glutamine levels [61]. Other studies, however, show that glutamine and molecular mechanisms involved in NH$_3$-induced reactive oxygen and nitrogen species (RNS) formation are related to astrocyte swelling [52]. Although the specific mechanism of cellular and brain edema remain elusive, numerous cell culture studies in glial cells have implicated mitochondrial dysfunction and oxidative stress, leading to nuclear factor kappaB activation and nitric oxide production, as key features of the cell swelling resulting from chronic NH$_3$/NH$_4^+$ exposure [52, 62]. Surprisingly, neither in astrocytes nor in neurons the possibility that intracellular accumulation of NH$_3$ may contribute to the brain edema resulting from hyperammonemia has been hitherto tested. Our results show that upon exposure to NH$_3$/NH$_4^+$, NH$_4^+$ accumulate rapidly inside the cells and contribute to significant cell swelling.

Conflict of Interests

The authors declare that there are no conflicts of interest.

Acknowledgments

We are grateful to Dr. Robert Putnam for valuable discussions and to Mr. José J. Herrera for skillful technical assistance. This research was supported by the National Institute of Neurological Disorders and Stroke grant NS29227 to FJ, A.-L.
References

1. Good DW, Kneppe MA: Ammonia transport in the mammalian kidney. Am J Physiol 1985;248:F459-471.
2. Weiner ID, Verlander JW: Role of nh3 and nh4+ transporters in renal acid-base transport. Am J Physiol Renal Physiol 2013;300:F11-23.
3. Conn HO, Bircher J: Hepatic encephalopathy: Syndromes and therapies. Bloomington, Ill., Medi-Ed Press, 1994.
4. Cichoz-Lach H, Michalak A: Current pathogenetic aspects of hepatic encephalopathy and noncirrhotic hyperammonemic encephalopathy. World J Gastroenterol 2013;19:26-34.
5. Cooper AJ, Plum F: Biochemistry and physiology of brain ammonia. Physiol Rev 1987;67:440-519.
6. Felipo V, Butterworth RF: Neurobiology of ammonia. Prog Neurobiol 2002;67:259-279.
7. Vaquer J, Chung C, Blei AT: Brain edema in acute liver failure. A window to the pathogenesis of hepatic encephalopathy. Ann Hepatol 2003;2:12-22.
8. Clemmesen JO, Larsen FS, Kondrup J, Hansen BA, Ott P: Cerebral herniation in patients with acute liver failure is correlated with arterial ammonia concentration. Hepatology 1999;29:648-653.
9. Blei AT: Brain edema in acute liver failure. Crit Care Clin 2008;24:99-114, ix.
10. Noreen MR, Rama Rao KV, Jayakumar AR: Signaling factors in the mechanism of ammonia neurotoxicity. Metab Brain Dis 2009;24:103-117.
11. Boron WF, De Weer P: Intracellular pH transients in squid giant axons caused by CO3-, NH4+, and metabolic inhibitors. J Gen Physiol 1976;67:91-112.
12. Thomas RC: Experimental displacement of intracellular pH and the mechanism of its subsequent recovery. J Physiol 1984;354:3P-22P.
13. Putnam RW, Roos A: Intracellular pH; in Hoffman J, Jamieson J (eds): Handbook of physiology section 14 cell physiology. New York, Oxford University Press, 1997, pp 389-440.
14. Singh SK, Binder HJ, Geibel JP, Boron WF: An apical permeability barrier to NH3/NH4+ in isolated, perfused colonic crypts. Proc Natl Acad Sci USA 1995;92:11573-11577.
15. Kikeri D, Sun A, Zeidel ML, Hebert SC: Cell membranes impermeable to NH3. Nature 1989;339:478-480.
16. Waissbren SJ, Geibel JP, Modlin IM, Boron WF: Unusual permeability properties of gastric gland cells. Nature 1994;368:332-335.
17. De Vries H: Sur la perméabilité du protoplasme des bettaves rouges. Arch Neér Sci 1871;6:117-126.
18. Jacobs MH: The influence of ammonium salts on cell reaction. J Gen Physiol 1922;5:181-188.
19. Fresser F, Moser H, Mair N: Intra- and extracellular use and evaluation of ammonium-selective microelectrodes. J Exp Biol 1991;157:227-241.
20. Luo C, Clark JW Jr, Heming TA, Bidani A: A macrophage cell model for pH and volume regulation. J Theor Biol 2006;238:449-463.
21. Jacobs MH, Parpart AK: Osmotic properties of the erythrocyte. X. On the permeability of the erythrocyte to ammonia and the ammonium ion. J Cell Comp Physiol 1938;11:175-192.
22. Jacobs MH: Some aspects of cell permeability to weak electrolytes. Cold Spring Harbor Symposia on Quantitative Biology 1940;8:30-39.
23. Zeuthen T, Wu B, Pavlovic-Djuranyov S, Holm LM, Uzcategui NL, Duszenko M, Kun JR, Schultz JE, Beitz E: Ammonia permeability of the aquaglyceroporins from plasmolysis falciparum, toxoplasma gondii and trypanosoma brucei. Mol Microbiol 2006;61:1598-1608.
24. Holm LM, Jahn TP, Moller AL, Schjoerring JK, Ferri D, Klaerke DA, Zeuthen T: Nh3 and nh4+ permeability in aquaporin-expressing xenopus oocytes. Pflugers Arch 2005;450:415-428.
25. Alvarez-Leefmans FJ, Herrera-Perez JJ, Marquez MS, Blanco VM: Simultaneous measurement of water volume and pH in single cells using BCECF and fluorescence imaging microscopy. Biophys J 2006;90:608-618.
26. Amano T, Richelson E, Nirenberg M: Neurotransmitter synthesis by neuroblastoma clones (neuroblast differentiation-cell culture-choline acetyltransferase-acetylcholinesterase-tyrosine hydroxylase-axons-dendrites). Proc Natl Acad Sci USA 1972;69:258-263.
27. Hamprecht B: Structural, electrophysiological, biochemical, and pharmacological properties of neurobiota-glioma cell hybrids in cell culture. Int Rev Cytol 1977;49:99-170.
28. Sears CD, Singer HS: The identification and characterization of a gabaergic system in the cholinergic neuroblastoma x glioma hybrid clone ng108-15. Brain Res 1988;448:373-376.
29 Kasai H, Neher E: Dihydropyridine-sensitive and omega-conotoxin-sensitive calcium channels in a mammalian neuroblastoma-glioma cell line. J Physiol 1992;448:161-188.
30 Alvarez-Leefmans FJ, Altamirano J, Crowe WE: Use of ion-selective microelectrodes and fluorescent probes to measure cell volume. Meth Neurosci 1995;27:361-391.
31 Crowe WE, Altamirano J, Huerto L, Alvarez-Leefmans FJ: Volume changes in single N1E-115 neuroblastoma cells measured with a fluorescent probe. Neuroscience 1995;69:283-296.
32 Boyarsky G, Hassen C, Clyne LA: Inadequacy of high K+/nigericin for calibrating BCECF II. Intracellular pH dependence of the correction. Am J Physiol 1996;271:C1146-1156.
33 Boyarsky G, Hassen C, Clyne LA: Inadequacy of high K+/nigericin for calibrating BCECF I. Estimating steady-state intracellular pH. Am J Physiol 1996;271:C1131-1145.
34 Roos A, Boron WF: Intracellular pH. Physiol Rev 1981;61:296-434.
35 Marzagatti P, Coles JA: Ammonium in nervous tissue: Transport across cell membranes, fluxes from neurons to glial cells, and role in signalling. Prog Neurobiol 2001;64:157-183.
36 Blei AT: Brain edema in acute liver failure: Can it be prevented? Can it be treated? J Hepatol 2007;46:564-569.
37 Tolkovsky AM, Richards CD: Na+/H+ exchange is the major mechanism of pH regulation in cultured sympathetic neurons: Measurements in single cell bodies and neurites using a fluorescent pH indicator. Neuroscience 1987;22:1093-1102.
38 Bevensee MO, Cummins TR, Haddad GG, Boron WF, Boyarsky G: pH regulation in single CA1 neurons acutely isolated from the hippocampi of immature and mature rats. J Physiol 1996;494:315-328.
39 Vaughan-Jones RD, Wu ML: pH dependence of intrinsic H+ buffering power in the sheep cardiac purkinje fibre. J Physiol 1990;425:429-448.
40 Szatkowski MS, Thomas RC: The intrinsic intracellular H+ buffering power of snail neurones. J Physiol 1989;409:89-101.
41 Bevensee MO, Bashi E, Schluke WR, Boyarsky G, Boron WF: Shrinkage-induced activation of Na+/H+ exchange in rat renal mesangial cells. Am J Physiol 1999;276:C674-683.
42 Sardini A: Cell volume homeostasis: The role of volume-sensitive chloride channels; in Bittar EE, Pusch M (eds): Advances in Molecular and Cell Biology. Burlington, Elsevier, 2007, vol 38, pp 199-214.
43 Hoffmann EK, Lambert IH, Pedersen SF: Physiology of cell volume regulation in vertebrates. Physiol Rev 2009;89:193-277.
44 Furst J, Gschwentner M, Ritter M, Botta G, Jakob M, Mayer M, Garavaglia L, Bazzini C, Rodighiero S, Meyer G, Eichmuller S, Woll E, Paulmichl M: Molecular and functional aspects of anionic channels activated during regulatory volume decrease in mammalian cells. Pflugers Arch 2002;444:1-25.
45 Altamirano J, Bredwick MS, Alvarez-Leefmans FJ: Regulatory volume decrease and intracellular Ca2+ in murine neuroblastoma cells studied with fluorescent probes. J Gen Physiol 1998;112:145-160.
46 Fowler RH, Guggenheim EA: Statistical thermodynamics; a version of statistical mechanics for students of physics and chemistry. New York, Cambridge, Eng, The Macmillan company University Press, 1965.
47 Swietach P, Vaughan-Jones RD: Spatial regulation of intracellular pH in the ventricular myocyte. Ann N Y Acad Sci 2005;1047:271-282.
48 Al-Baldawi NF, Abercrombie RF: Cytoplasmic hydrogen ion diffusion coefficient. Biophys J 1992;61:1470-1479.
49 Nagaraj TN, Brooke N: Intracellular acidification induced by passive and active transport of ammonium ions in astrocytes. Am J Physiol 1998;274:C883-891.
50 Williams JK, Payne JA: Cation transport by the neuronal K+-Cl- cotransporter KCC2: Thermodynamics and kinetics of alternate transport modes. Am J Physiol Cell Physiol 2004;287:C919-931.
51 Ballanyi K, Grafe P: An intracellular analysis of gamma-aminobutyric-acid-associated ion movements in rat sympathetic neurones. J Physiol 1985;365:41-58.
52 Lachmann V, Gorg B, Bidmon HJ, Keitel V, Hausinger D: Precipitants of hepatic encephalopathy induce rapid astrocyte swelling in an oxidative stress dependent manner. Arch Biochem Biophys 2013:536:143-151.
53 Sandrasag A, Patejunas G, Young AP: Multiple mechanisms by which glutamine synthetase levels are controlled in murine tissue culture cells. Arch Biochem Biophys 1988;266:522-531.
54 Lacoste I, Chaudhary KD, Lapointe J: Derepression of the glutamine synthetase in neuroblastoma cells at low concentrations of glutamine. J Neurochem 1982;39:78-85.
55 Haghighat N, McCandless DW, Geraminegad P: The effect of ammonium chloride on metabolism of primary neurons and neuroblastoma cells in vitro. Metab Brain Dis 2000;15:151-162.
56 Bond T, Basavappa S, Christensen M, Strange K: ATP dependence of the ICl swell channel varies with rate of cell swelling. Evidence for two modes of channel activation. J Gen Physiol 1999;113:441-456.
57 Bhatia V, Singh R, Acharya SK: Predictive value of arterial ammonia for complications and outcome in acute liver failure. Gut 2006;55:98-104.
58 Albrecht J, Dolinska M: Glutamine as a pathogenic factor in hepatic encephalopathy. J Neurosci Res 2001;65:1-5.
59 Brusilow SW, Traystman R: Hepatic encephalopathy. N Engl J Med 1986;314:786-787; author reply 787.
60 Zwingmann C, Butterworth R: An update on the role of brain glutamine synthesis and its relation to cell-specific energy metabolism in the hyperammonemic brain: Further studies using NMR spectroscopy. Neurochem Int 2005;47:19-30.
61 Jayakumar AR, Rao KV, Murthy Ch R, Noreenb RG, MD: Glutamine in the mechanism of ammonia-induced astrocyte swelling. Neurochem Int 2006;48:623-628.
62 Sinke AP, Jayakumar AR, Panickar K S, Moriyma M, Reddy PV, Noreenberg MD: Nifkappab in the mechanism of ammonia-induced astrocyte swelling in culture. J Neurochem 2008;106:2302-2311.