pH REGULATING TRANSPORTERS IN NEURONS FROM VARIOUS CHEMOSENSITIVE BRAINSTEM REGIONS IN NEONATAL RATS

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Running Title: pH Regulating Transporters in Rat Brainstem Neurons

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ABSTRACT

We studied the membrane transporters that mediate intracellular pH (pHi) recovery from acidification in brainstem neurons from chemosensitive regions of neonatal rats. Individual neurons within brainstem slices from the retrotrapezoid nucleus (RTN), the nucleus tractus solitarii (NTS) and the locus coeruleus (LC) were studied using a pH-sensitive fluorescent dye and fluorescence imaging microscopy. The rate of pHi recovery from an NH₄Cl-induced acidification was measured and the effects of inhibitors of various pH-regulating transporters determined. Hypercapnia (15% CO₂) resulted in a maintained acidification in neurons from all three regions. Recovery in RTN neurons was nearly entirely eliminated by amiloride, an inhibitor of Na⁺/H⁺ exchange (NHE). Recovery in RTN neurons was blocked about 50% by inhibitors of isoform 1 of NHE (NHE-1) but very little by an inhibitor of NHE-3 or by DIDS (an inhibitor of HCO₃-dependent transport). In NTS neurons, amiloride blocked over 80% of the recovery, which was also blocked about 65% by inhibitors of NHE-1 and 26% blocked by an inhibitor of NHE-3. Recovery in LC neurons, in contrast, was unaffected by amiloride or NHE isoforms, but was dependent on Na⁺ and increased by external HCO₃⁻. Based on these findings, pHi recovery from acidification appears to be largely mediated by NHE-1 in RTN neurons, by NHE-1 and NHE-3 in NTS neurons and by a Na- and HCO₃-dependent transporter in LC neurons. Thus, pHᵢ recovery is mediated by different pH-regulating transporters in neurons from different chemosensitive regions but recovery is suppressed by hypercapnia in all of the neurons.

Key Words: hypercapnia; locus coeruleus; Na/H exchange; HCO₃ transport; NTS; retrotrapezoid nucleus
INTRODUCTION

The response to hypercapnia of brainstem neurons located in various chemosensitive regions is of major interest in the study of the control of respiration. Chemosensitive neurons have been defined as neurons that respond to changes of CO₂/H⁺ and that are found in brainstem areas shown to alter ventilation when exposed to focal acidification (38). A maintained fall of intracellular pH (pHi), with no pHi recovery, has been shown to be an important component of the signaling pathway of hypercapnia in neurons from chemosensitive brainstem areas (17,19,38,42,51). However, these neurons do exhibit pHi recovery from acidification when external pH is held constant (17,19,42). This recovery can be mediated by several different membrane transport proteins including Na⁺/H⁺ exchangers (NHE) (33), Na-driven Cl⁻/HCO₃⁻ exchangers (NDCBE), and Na⁺-HCO₃⁻ cotransporters (NBC—both electrogenic and electrochemical) (12,37,45). To our knowledge, there is no evidence for any functional HCO₃⁻-dependent transporter in neurons from any central chemosensitive region.

Previous studies have suggested that NHE is the predominant transport protein mediating pHi recovery from acidification in brainstem neurons (32,42,53). Under conditions of extracellular acidification, as with hypercapnic acidosis, NHE is inhibited and chemosensitive neurons do not exhibit pHi recovery from acidification (41,42). Several isoforms of the NHE protein exist and are found to be active in different parts of the body. Of the nine isoforms, only five are thought to be present in rat brains (12). NHE-1 is the ubiquitous isoform and is very sensitive to amiloride inhibition (37) and is completely inhibited by cariporide (HOE642). NHE-2 and NHE-3 are found predominantly in the GI tract (3) and the kidney (37) but are also present in the brain. NHE-3 has a relatively specific inhibitor, S1611 (53,58). The isoform NHE-4 is
found in the hippocampus (7) and is thus not of interest in this study. NHE-5 is found in several regions of the rat brain, is closely related to NHE-3, and has no specific inhibitor (2).

Knowing that there are several isoforms of NHE present in the brain, the next issue to address is the question of which isoform is mediating pH\textsubscript{i} recovery in neurons from various chemosensitive brainstem regions. We have evidence that amiloride inhibits recovery from acidification in neurons from the nucleus of the solitary tract (NTS) and the ventrolateral medulla (VLM) (42). Studies have also been conducted in the VLM showing that NHE-3 mediates pH\textsubscript{i} regulation as well. Expression of mRNA for NHE-3 was found in some areas of the brain involved in respiratory control (23). When looking at cultured ventrolateral medullary neurons, exposure to NHE-3 specific inhibitors resulted in neuronal acidification and increased firing rate (58). In \textit{in vivo} studies, specific NHE-3 inhibitors have been shown to stimulate the central respiratory system (22) and studies in which the brain-permeant NHE-3 inhibitor, S8218, was applied to live rabbits showed that the drug caused an increase in ventilation (23). These studies suggest that NHE-3 is important in pH\textsubscript{i} regulation in neurons from chemosensitive brainstem regions.

The aim of our study was to determine which pH-regulating transporters mediate recovery from acidification in neurons from three chemosensitive regions of the brainstem: the retrotrapezoid nucleus (RTN), the NTS, and the locus coeruleus (LC). We particularly wanted to find evidence for any functional HCO\textsubscript{3}\textsuperscript{-}-dependent transporters and which isoforms of NHE are active in the neurons from the various regions. We found that NHE-1 predominates in NTS and RTN neurons, with a smaller possible contribution from NHE-3, while a Na- and HCO\textsubscript{3}\textsuperscript{-}-dependent transporter predominates in LC neurons.

A preliminary report of these data has been made (21).
MATERIALS AND METHODS

Solutions and materials

The normal solution, an artificial cerebrospinal fluid (aCSF), contained (in mM) 124 NaCl, 5 KCl, 2.4 CaCl₂, 1.3 MgSO₄, 1.24 KH₂PO₄, 26 NaHCO₃, and 10 glucose, and was equilibrated with 5% CO₂/balance O₂ (solution pH, pH₀, 7.45 at 37°C). NH₄Cl solutions of concentrations 40 and 75 mM were used for prepulse experiments (similar to previous values used in slices; 42,43). These solutions were prepared by substituting 40mM or 75mM NaCl for the equivalent concentration of NH₄Cl when preparing the aCSF solution. A 0 Na⁺ aCSF solution was prepared by isosmotically replacing all sodium with N-methyl-D-glucamine (NMDG). Solution was continuously bubbled with 5% CO₂, and NMDG powder was added to reach a pH of 7.45 at 37°C. HEPES-buffered saline was identical to aCSF except that Na-HEPES (N-2-hydroxyethylpiperazine-H'-2-ethanesulfonate) replaced NaHCO₃ isosmotically and the solution was equilibrated with 100% O₂ (41).

4,4’-diisothiocyanostilbene-2,2’-disulfonic acid (DIDS) was purchased from Sigma (St. Louis, MO) and a 250 mM stock was prepared with dimethyl sulfoxide (DMSO—vehicle had no effect on pH) and diluted to a final concentration of 500 µM. Amiloride was generously supplied by Merck, Sharp, and Dohme (Rahway, NJ) and a 0.5 M stock (made with DMSO) was diluted to a final concentration of 1-2 mM. The IC₅₀ values of amiloride for NHE1 is 1-10 µM and for NHE3 is 100-400 µM so amiloride should inhibit all NHE isoforms (25,46). Stock solutions (5mM concentration) were prepared in DMSO for cariporide (HOE 642) and for S1611. The cariporide and S1611 IC₅₀ values for NHE1 are 0.08 and 4.7 µM, respectively, and for NHE3 are 600-1000 and 0.69 µM, respectively (25,46). The stock solutions were diluted to 1 µM and 5 µM concentrations for each of the drugs which were generously supplied to us by
Sanofi-Aventis Pharmaceutical (Frankfurt am Main, Germany). Therefore, cariporide should offer a reasonable differentiation of NHE1 from NHE3 activity and if anything S1611 inhibition could be an overestimate of NHE3 activity (46). N-Ethyl-N-isopropyl-amiloride (EIPA) was made into a 200 mM stock with DMSO and was diluted to a final concentration of either 100 or 500 µM. The IC\textsubscript{50} values of EIPA for NHE1 and NHE3 were 0.01-0.02 and 2-3 µM, respectively (25). The drug was purchased from Sigma (St. Louis, MO). The pH\textsubscript{i} calibration solution contained (in mM) 104 KCl, 1.24 KH\textsubscript{2}PO\textsubscript{4}, 1.3 MgSO\textsubscript{4}, 2.4 CaCl\textsubscript{2}, 25 NMDG-HEPES, 25 K-HEPES, 10 glucose, and 0.016 nigericin titrated with either KOH or HCl to reach a pH of 7.2. Nigericin was purchased from Sigma (St. Louis, MO). A membrane permeable acetoxyethyl ester form of BCECF (2’,7’-bis(2-carboxyethyl)-5-(and 6)-carboxyfluorescein), BCECF-AM (Invitrogen/Molecular Probes, Eugene, OR), was diluted with DMSO to a concentration of 20 µM.

Preparation of brain slices

All procedures involving animals were reviewed and approved by the Wright State University Animal Care and Use Committee and are in agreement with its guidelines (AALAC no. A3632-01). The techniques for slice preparation, BCECF dye-loading and imaging of BCECF-loaded slices have been previously described (17,42,43,44). Briefly, brain slices were obtained from neonatal (P3 to P15) Sprague-Dawley rats of both sexes. Rats were sacrificed by decapitation after cold anesthesia. The brainstem was removed and then sliced (300-400 µm thick) using a vibratome (Pelco 101, series 1000, Pelco, St. Louis, MO). The vibratome was adjusted to slice at a speed of approximately 5 minutes per slice. Slices were cut into cold (5ºC) aCSF equilibrated with 5% CO\textsubscript{2}/ 95% O\textsubscript{2}.

Identification of different brainstem regions
Slices were taken from three different brainstem regions (Fig. 1). To study the LC, pontine slices were cut rostrally for 1-1.5 mm from the level of the seventh cranial nerve (red lines and upper trace in Fig. 1) (17). To study the RTN, slices were cut from the most caudal region of the facial nucleus for 600-900 μm rostrally (blue line and middle trace in Fig. 1) (44). To study the NTS, slices were cut up to 1.5 mm rostral from obex (green lines and lower trace in Fig. 1) (42,43). Neurons were studied from the regions indicated by the appropriate colors in the traces to the right in Fig. 1.

**BCECF dye-loading of slices**

Slices were allowed to recover from slice trauma for at least 1 hour in aCSF (room temperature) equilibrated with 5% CO₂/ 95% O₂. Slices were then transferred to a solution of aCSF containing 20 μM of the membrane permeable form of BCECF, BCECF-AM, and were incubated at 37°C for 30 minutes in the dark. Slices were then washed in dye-free aCSF solution at room temperature (equilibrated with 5% CO₂) until used for experiments.

**Imaging BCECF-loaded slices**

BCECF fluorescence in slices was studied using a technique modified from Ritucci et al. (43). Briefly, a 75-W xenon arc lamp was used with a Lambda 10-2 filter wheel (Sutter Instruments, Austin, TX) to excite the slices alternately at 500 and 440 nm (filters from Omega Optical, Brattleboro, VT). Emitted fluorescence was collected at 530 nm and sent to a GenIIsys image intensifier and a CCD72 camera (both from Dage-MTI, Michigan City, IN). The intensifier augmented the fluorescence images which were then acquired by a Gateway 2000 486/25C computer. Images were collected and processed using Metafluor software (Molecular Devices Corporation, Downingtown, PA), which also was used to control the filter wheel. Images acquired at 500 nm and 440 nm excitation were divided to obtain a fluorescence ratio.
(R_n), calculated as Fl_{500}/Fl_{440}. All images were displayed on a Sony RGB Trinitron super fine pitch monitor. By using the “Define Graph Regions” option in the MetaFluor software, individual neurons were selected from the R_n image for analysis. Dye-loaded slices were exposed to a high K^+/nigericin calibration solution (49) whose pH was 7.2, and a stable R_n-calib was obtained. All R_n values were normalized to this value for a given neuron (N_n = R_n/R_n-calib) and N_n values were converted to pH_i values using the equation of Ritucci et al (42) for NTS neurons, Filosa et al. (17) for LC neurons and Ritucci et al. (44) for RTN neurons. In all experiments the change in Fl_{440}, the excitation wavelength at which fluorescence is pH independent and based solely on the amount of dye in the neuron, was monitored over time to assess cell viability. The data from a neuron was only used if its Fl_{440} value fell by less than 1% min^{-1} (6).

**Experimental protocol**

In a typical experiment, BCECF-loaded slices were placed on the stage of an inverted Nikon Diaphot microscope. The microscope was positioned on a Micro-g air table (Technical Manufacturing, Peabody, MA) in order to minimize vibrations during experiments. Slices were immobilized by the use of grids made of nylon fibers attached to a U-shaped stainless steel bar. Slices were continuously perfused with aCSF at 3-4 ml/min using a gravity feed system. All experiments were performed at 37°C. Upon commencement of the experiment, images were acquired and the aCSF solution was superfused to wash cells until R_n stabilized. Images were collected every 30-60 seconds throughout all experiments. In between measurements, excitation light was blocked to prevent photo-bleaching of dye and photolytic damage to cells. Some neurons were acidified by exposure to hypercapnic acidotic solution (aCSF equilibrated with 15% CO_2, pH_0 ~ 7.00). Most neurons were acidified using the NH_4Cl prepulse technique (8).
with NH₄Cl exposures lasting for 15 ± 5 minutes (NH₄Cl exposure time and concentrations were varied in an attempt to obtain the same minimum pHᵢ with experimental solutions as seen with control exposures). Neurons acidified upon exposure to hypercapnic solutions and upon removal of the NH₄Cl and recovery from these acidifications were quantified by determining the slopes of the pHᵢ vs. time traces. Neurons were allowed to recover until Rᵢ values reached a plateau. To determine the effects of various treatments on pHᵢ recovery rates, the exposure to aCSF containing the appropriate inhibitor was initiated upon removal of NH₄Cl. After the neuronal pH acidified and a recovery slope was clear, the neurons were then exposed to aCSF without the inhibitor and recovery was followed until a plateau pHᵢ value was reached.

Data analysis and statistics

All values are reported as means ± 1 standard error of the mean (S.E.M.). The initial, maximum, minimum, and final pH values for each neuron were calculated by averaging 4-7 contiguous pHᵢ values at the appropriate part of the trace. All pHᵢ recovery slopes were estimated by least-squares regression lines fit to at least 10 points. In control experiments, least-squares regression lines were fitted from the point of minimum pH to the initial point of the pH plateau. In experimental trials, two recovery slopes were calculated. The first slope, representing the recovery of neurons exposed to the inhibitor or experimental drug, was fitted from the point of minimum pHᵢ to the last point of the exposure to the experimental solution. The second slope, representing recovery after cells were exposed to an inhibitor (when cells were returned to aCSF solution), was fitted from a point approximately 2 minutes after the return to normal solution to the initial point of the plateau.
All comparisons of three or more means were done with a one way analysis of variance (ANOVA). Multiple paired comparisons were performed using a Tukey-Kramer test. The level of significance was $P<0.05$. 
RESULTS

*Initial values of pHᵢ*

We determined the initial value for pHᵢ in aCSF equilibrated with 5% CO₂ for neurons from three brainstem regions. Initial pHᵢ varied and was 7.31 ± 0.003 (n=304), 7.43 ± 0.005 (n=266) and 7.34 ± 0.001 (n=349) for RTN, NTS and LC neurons, respectively. These values are somewhat more alkaline than pHᵢ values reported for neurons from another chemosensitive region, the medullary raphé (9). Differences in pHᵢ values measured in neurons from different regions have been discussed previously (43). The lower pHᵢ values in raphé neurons may reflect the fact that these neurons were studied in cell culture which has been shown previously to reduce the measured value of pHᵢ (39). Statistical analysis (one way ANOVA with Tukey-Kramer pair wise tests) showed that NTS neurons were significantly (P<0.001) more alkaline than RTN and LC neurons, as has previously been observed (17,42,43,44) and the small difference in pHᵢ between RTN and LC neurons was also significantly different (P<0.001).

*pHᵢ response to hypercapnia*

We measured the pHᵢ response to hypercapnia in neurons from the RTN, NTS, and LC. Neurons from the RTN had an initial pHᵢ of 7.29 ± 0.001 and acidified in response to hypercapnia (15% CO₂) to a minimum pHᵢ of 7.09 ± 0.007 (n=25) (Fig. 2A). RTN neurons did not recover from hypercapnia-induced acidification (-0.0017 ± 0.0007 pHᵢ/min) (n=25) (Fig. 2A, D white bar). Upon return to normocapnic (5% CO₂) aCSF, pHᵢ returned toward its original value (7.17 ± 0.036) (n=25) (Fig. 2A). In response to hypercapnia, neurons from the NTS acidified to a minimum pHᵢ of 7.22 ± 0.007 from an initial pHᵢ of 7.41 ± 0.009 (n=24) (Fig. 2B) and showed no recovery from acidification (-0.0008 ± 0.0010 pHᵢ/min) (n=24) (Fig. 2B, D white bar). After CO₂ levels were returned to normal, NTS neuron pHᵢ returned to 7.41 ± 0.010 (n=24)
Neurons from the LC had an initial pH$_i$ of 7.34 ± 0.002 and acidified in response to hypercapnia to a minimum pH$_i$ of 7.17 ± 0.004 (n=74) (Fig. 2C). LC neurons did not recover from hypercapnia-induced acidification (-0.0030 ± 0.0004 pH$_i$/min) (n=24) (Fig. 2C, D white bar). After CO$_2$ levels were returned to normal, LC neuron pH$_i$ returned to normal (7.34 ± 0.003) (n=24) (Fig. 2C). Thus, neurons from all three regions acidified in response to hypercapnia and this acidification was maintained in the maintained presence of hypercapnia.

**The NH$_4$Cl prepulse**

The purpose of these experiments was to determine which transport protein regulates pH$_i$ recovery from acidification in the neurons from three chemosensitive brainstem regions, the RTN, NTS, and LC. In all experiments, cells were exposed to an NH$_4$Cl prepulse. Experiments in the RTN began with cells having an initial pH of 7.29 ± 0.002 (n=26). Once the NH$_4$Cl was removed, RTN neurons acidified to a minimum pH of 7.04 ± 0.010 (n=26). RTN cells recovered from this acidification (in the presence of aCSF) at a rate of 0.0138 ± 0.0004 pH$_i$/min (n=26) (Fig. 2D black bar, Fig. 3A black line, Fig. 3B black bar). Cells in the NTS had an initial pH$_i$ value of 7.42 ± 0.017 and acidified after the NH$_4$Cl prepulse to a minimum pH$_i$ of 7.19 ± 0.011 (n=46). Recovery (in the presence of aCSF) proceeded at a rate of 0.0156 ± 0.0008 pH$_i$/min (n=46) (Fig. 2D black bar, Fig. 5A black line, Fig. 5B black bar). LC neurons acidified from an initial pH$_i$ value of 7.34 ± 0.0006 to a minimum pH$_i$ of 7.15 ± 0.0058 (n=52). Recovery occurred but at a significantly (P<0.001) slower rate of 0.0090 ± 0.0005 pH$_i$/min (n=52) (Fig. 2D black bar, Fig. 7A black line, Fig. 7B black bar). These data clearly show that the lack of pH$_i$ recovery from hypercapnia-induced acidification is not due to the lack of pH-regulating transporters, since neurons from all three brainstem regions exhibited pH$_i$ recovery from acidification induced by an NH$_4$Cl prepulse.
General transport inhibitors

To study the transmembrane transport systems that mediate pH\textsubscript{i} recovery in these neurons, we used both general and specific inhibitors of pH-regulating transporters. These pH-regulating transporters most likely include one or more isoforms of the sodium-hydrogen exchanger (NHE) and one or more of several HCO\textsubscript{3}-dependent exchangers (37). Tests were conducted to determine what type of exchange protein mediates pH\textsubscript{i} recovery in neurons from the RTN, NTS, and LC.

Na\textsuperscript{+}-free solution

Cells from all three regions were exposed to Na\textsuperscript{+}-free aCSF to see if the pH\textsubscript{i} recovery from NH\textsubscript{4}Cl-induced acidification was Na\textsuperscript{+} dependent. The recovery rate of RTN neurons for the duration of the exposure to Na\textsuperscript{+}-free solution was 0.0030 ± 0.0012 pH\textsubscript{i}/min (n=10) (Fig. 3A red line, Fig. 3B red bar). This represents an 86% reduction compared to the recovery upon re-addition of Na\textsuperscript{+} (P<0.001), which amounted to 0.0212 ± 0.0017 pH\textsubscript{i}/min (n=10) (Fig. 3A red line). Similar results were found for neurons from the other two regions. When NTS neurons were acidified, recovery rate in Na\textsuperscript{+}-free solution was 0.0050 ± 0.0022 pH\textsubscript{i}/min (n=12) (Fig. 5A red line, Fig. 5B red bar) and increased to a rate of 0.0259 ± 0.0043 pH\textsubscript{i}/min (n=12) upon re-addition of Na\textsuperscript{+} (Fig. 5A red line). Thus, Na\textsuperscript{+} free solutions inhibited recovery in NTS neurons by 81% compared to recovery upon re-addition of Na\textsuperscript{+} (P<0.001). LC neurons showed recovery at a rate of -0.0033 ± 0.0004 pH\textsubscript{i}/min (n=34) in Na\textsuperscript{+}-free solution (Fig. 7A red line, Fig. 7B red bar). The recovery rate increased to 0.0259 ± 0.0062 pH\textsubscript{i}/min (n=34) upon re-addition of Na\textsuperscript{+} (Fig. 7A red line). Therefore, pH\textsubscript{i} recovery from acidification in LC neurons was completely inhibited in Na\textsuperscript{+}-free solutions. These data show that pH\textsubscript{i} recovery from acidification is largely Na\textsuperscript{+}-dependent in neurons from all three brainstem regions. It is evident that NH\textsubscript{4}Cl results in a
larger acidification in the presence of Na\textsuperscript{+}-free solutions. This common observation is due to a larger acidification upon inhibition of pH-recovery mechanisms and the possible reversal of alkalinizing recovery systems, resulting in acidification of pH\textsubscript{i}. This greater acidification in Na\textsuperscript{+}-free solutions should result in greater pH\textsubscript{i} recovery so the inhibition of recovery by Na\textsuperscript{+}-free solutions is even more striking. Thus, the greater NH\textsubscript{4}Cl-induced acidification seen in Na\textsuperscript{+}-free solutions is further confirmation of the importance of Na\textsuperscript{+}-dependent transporters in neurons from the RTN, NTS and LC.

*Amiloride*

We next tested the ability of amiloride (1 mM), a known inhibitor of NHE, to inhibit pH\textsubscript{i} recovery in brainstem neurons. In RTN neurons, recovery rate was 0.0010 ± 0.0007 pH\textsubscript{i}/min (n=25) (Fig. 3A dark blue line, Fig. 3B dark blue bar) in the presence of amiloride (1 mM) and increased to 0.0152 ± 0.0018 pH\textsubscript{i}/min (n=25) upon removal of amiloride, indicating that amiloride inhibited recovery in RTN neurons by 93%. Similarly, amiloride inhibited recovery in NTS neurons, which had a recovery rate of 0.0063 ± 0.0006 pH\textsubscript{i}/min (n=42) (Fig. 5A dark blue line, Fig. 5B dark blue bar) in the presence of amiloride and 0.0140 ± 0.0011 pH\textsubscript{i}/min (n=42) after its removal, amounting to a 65% inhibition. In contrast, amiloride not only did not inhibit but increased recovery in LC neurons, which had a recovery rate of 0.0157 ± 0.0007 pH\textsubscript{i}/min (n=32) (Fig. 7A dark blue line, Fig. 7B dark blue bar) in the presence of amiloride and 0.0107 ± 0.0034 pH\textsubscript{i}/min (n=32) after its removal (Fig. 7A dark blue line). The increased recovery may be due to the fact that amiloride caused a larger NH\textsubscript{4}Cl-induced acidification (minimum pH\textsubscript{i} of 7.00 in the presence of amiloride vs. 7.15 in its absence) which should increase recovery rate. We do not know why amiloride enhances the cell acidification induced by the NH\textsubscript{4}Cl prepulse. In
summary, pH Recovery was largely inhibited by amiloride in RTN and NTS neurons but not inhibited by amiloride in LC neurons.

**DIDS**

Cells from all regions were exposed to DIDS, an inhibitor of HCO$_3$-dependent transporters (37). In RTN neurons, recovery rate was $0.0156 \pm 0.0018$ pH/$\text{min}$ (n=10) (Fig. 3A dark green line, Fig. 3B dark green bar) in the presence of DIDS (0.5 mM) and was $0.0102 \pm 0.0015$ pH/$\text{min}$ (n=10) upon removal of DIDS (Fig. 3A dark green line), indicating that DIDS did not inhibit recovery in RTN neurons. Similarly, DIDS did not inhibit recovery in NTS neurons, which had a recovery rate of $0.0115 \pm 0.0014$ pH/$\text{min}$ (n=12) (Fig. 5A dark green line, Fig. 5B dark green bar) in the presence of DIDS and $0.0116 \pm 0.0010$ pH/$\text{min}$ (n=12) after its removal (Fig. 5A dark green line). DIDS also did not inhibit recovery in LC neurons, which had a recovery rate of $0.0090 \pm 0.0007$ pH/$\text{min}$ (n=17) (Fig. 7A dark green line, Fig. 7B dark green bar) in the presence of DIDS and $0.0090 \pm 0.0005$ pH/$\text{min}$ (n=17) after its removal (Fig. 7A dark green line). The lack of an effect of DIDS on recovery in neurons from all three regions suggests that DIDS-inhibitable HCO$_3$-dependent transporters do not play a significant role in pH Recovery from acidification in RTN, NTS and LC neurons.

**NHE isoform inhibitors**

Our findings imply that NHE appears to be the predominant pH-regulating transporter mediating recovery from cell acidification, at least in RTN and NTS neurons. This raises the question of which isoform of NHE is active in pH$_i$ regulation in neurons from these brainstem regions. Three main isoforms of NHE are present in the rat brain: NHE-1, NHE-3, and NHE-5 (2,24,56,58), although there is some evidence for NHE-2 and NHE-4 expression in brainstem
Specific inhibitors are available for NHE-1 (25) and NHE-3 (23,46,58), thus our study focused on these isoforms.

**EIPA**

EIPA is an amiloride derivative and an inhibitor of NHE which is slightly more specific for the NHE-1 isoform (25). The effects of both 0.1 and 0.5 mM EIPA on the recovery from acidification were determined. Since the effects of EIPA were found not to be reversible, the inhibition of recovery was calculated using control recovery values (see “The NH₄Cl prepulse” section above). EIPA reduced recovery in RTN neurons to 0.0072 ± 0.0011 pHᵢ/min (n=8) and to 0.0048 ± 0.0008 pHᵢ/min (n=11) for 0.1 and 0.5 mM (Fig. 4A yellow line, Fig. 4B yellow bar), respectively. This amounts to 48 and 65% inhibition of recovery. For NTS neurons, recovery was reduced by EIPA to 0.0075 ± 0.0008 pHᵢ/min (n=48) and 0.0090 ± 0.0060 pHᵢ/min (n=25) (Fig. 6A yellow line, Fig. 6B yellow bar). This amounts to 50 and 42% inhibition of recovery. Finally, recovery of LC neurons in 0.1 mM EIPA was 0.010 ± 0.0008 pHᵢ/min (n=26) and in 0.5 mM EIPA was 0.0060 ± 0.0011 pHᵢ/min (n=23) (Fig. 8A yellow line, Fig. 8B yellow bar), amounting to 0 and 33% inhibition. These findings suggest that NHE-1 may account for about half of the recovery in RTN and NTS neurons, but a smaller fraction in LC neurons.

**Cariporide (HOE 642)**

Cariporide is a much more specific NHE-1 inhibitor than EIPA (25). Two concentrations of cariporide were tested in these experiments. At 1 μM, cariporide inhibited recovery only in NTS neurons, where recovery was 0.0075 ± 0.0006 pHᵢ/min (n=7) (Fig. 6B, white bar) in the presence and 0.0154 ± 0.0013 pHᵢ/min (n=7) in the absence of cariporide, an inhibition of 50%. At 5 μM, cariporide inhibited recovery in RTN and NTS neurons, but not in LC neurons. In RTN neurons, recovery was 0.0078 ± 0.0012 pHᵢ/min (n=58) (Fig. 4A light blue line, Fig. 4B
light blue bar) in the presence and $0.0131 \pm 0.0008$ pH$_i$/min (n=58) (Fig. 4A light blue line) upon removal of 5 μM cariporide, an inhibition of 40%. At 5 μM cariporide, recovery in NTS neurons was $0.0051 \pm 0.0007$ pH$_i$/min (n=27) (Fig. 6A light blue line, Fig. 6B light blue bar) which increased to $0.0194 \pm 0.0017$ pH$_i$/min (n=27) upon removal of cariporide (Fig. 6A light blue line), amounting to an inhibition of recovery by 74%. Finally, 5 μM cariporide did not inhibit recovery in LC neurons, which was $0.0110 \pm 0.0009$ pH$_i$/min (n=42) (Fig. 8A light blue line, Fig. 8B light blue bar) in the presence and $0.012 \pm 0.0016$ pH$_i$/min (n=42) in the absence (Fig. 8A light blue line) of cariporide. In general, the results with cariporide are similar to those with EIPA, with about 50% of the recovery inhibited in RTN and NTS neurons but very little in LC neurons.

S1611

S1611 is a reasonably specific inhibitor for NHE-3 (46). S1611 at 1 μM had no effect on pH$_i$ recovery in neurons from any of the three brainstem areas. At 5 μM, S1611 also had no effect on the recovery from acidification in RTN neurons (rate of $0.0194 \pm 0.0015$ pH$_i$/min in the presence and $0.0181 \pm 0.0019$ pH$_i$/min in the absence of S1611, n=28) (Fig. 4A light green line, Fig. 4B light green bar) and in LC neurons (rate of $0.0090 \pm 0.0005$ pH$_i$/min in the presence and $0.0090 \pm 0.0006$ pH$_i$/min in the absence of S1611, n=44) (Fig. 8A light green line, Fig. 8B light green bar). In NTS neurons, 5 μM S1611 caused an inhibition (26%) of pH$_i$ recovery (rate of $0.0109 \pm 0.0010$ pH$_i$/min in the presence and $0.0147 \pm 0.0007$ pH$_i$/min in the absence of S1611, n=38) (Fig. 6A light green line, Fig. 6B light green bar). Thus, we have some evidence that the NHE-3 isoform participates in pH$_i$ recovery in NTS neurons.

The neurons from the RTN, NTS and LC are heterogeneous in their properties (1,26,29) and thus it may be that a small pocket of neurons in each area express NHE-3. For instance, a
small group of neurons has been suggested to be responsible for chemosensitivity in the RTN (26). To determine if we have a small percentage of neurons containing NHE-3 in each area, we looked at the individual recovery of each neuron in the presence of 5 μM S1611. We used two criteria to define an inhibited neuron. The first criterion was that if the recovery in the presence of S1611 was less than 33% of the control recovery then recovery in that neuron was judged to be inhibited by S1611. The second criteria was that if the recovery in the presence of S1611 was less than the lowest recovery in control neurons then than neuron was judged to have recovery inhibited by S1611. For RTN neurons, the control recovery rate averaged 0.0138 pH/min and the range of recovery rates was 0.0110 to 0.0202 pH/min. Thus, a neuron was considered to be inhibited by S1611 if its recovery was below 0.0046 pH/min (criterion 1) or below 0.0110 pH/min (criterion 2). Only 1 out of 28 neurons (3.6%) met criterion 1 while 3 out of 28 (10.7%) met criterion 2. Thus, at most 5-10% of RTN neurons might contain significant amounts of NHE-3. NTS neurons exhibited control recovery rates of 0.0156 pH/min with a range of 0.0054 to 0.0279 pH/min, so recovery needed to be below 0.0051 (criterion 1) or 0.0054 (criterion 2) pH/min in the presence of S1611 to be considered inhibited. For both criteria, 7 out of 38 neurons (18.4%) appeared to contain NHE-3 activity. Finally, LC neurons had a control recovery rate of 0.009 pH/min with a range of 0.0043 to 0.0260 pH/min. Recovery needed to be below 0.003 (criterion 1) or 0.0043 (criterion 2) pH/min in the presence of S1611 to be considered inhibited. Only 1 out of 44 neurons (2.3%) met these criteria. Thus, at most 2-3% of LC neurons appear to contain NHE-3.

_HCO3-dependen recovery in LC neurons_

Our findings suggest that NHE does not play a major role in the pH recovery from acidification in LC neurons, yet no DIDS-inhibitable recovery was evident either. To see if
HCO$_3$-dependent transport could play a role in pHi regulation in LC neurons, we studied recovery in the presence and in the nominal absence of HCO$_3$. In aCSF, LC neurons exhibited brisk recovery from an NH$_4$Cl prepulse-induced intracellular acidification (Fig. 9A and B). Interestingly, the same experiment in HEPES-buffered medium showed a significantly slower pHi recovery (Fig. 9A and B), despite the fact that the total buffering power of these cells is much smaller and thus the rate of pHi recovery should be larger (37). We note that the recovery rate of these LC neurons in CO$_2$ is larger than reported earlier (compare control recovery in Fig. 7B with CO$_2$ recovery in Fig. 9B). The larger recovery in these experiments is most likely due to the greater NH$_4$Cl-induced acidification in the experiments in Fig. 9 (minimum pHi about 6.65) compared to the experiments in Fig. 7 (minimum pHi about 7.15). The findings of higher pHi recovery in the presence vs. the absence of CO$_2$ indicate that pHi recovery from acidification in LC neurons is faster in the presence of HCO$_3$, suggesting that a HCO$_3$-dependent transporter is important for pHi regulation in LC neurons.
DISCUSSION

There are several main conclusions from our data. 1) In neurons from the neonatal brainstem, recovery from acidification appears to be largely mediated by Na-dependent processes. 2) NHEs account for nearly all recovery from intracellular acidification in RTN and NTS neurons, with NHE-1 appearing to be predominant. 3) NHE-3 is present in at most a small percentage of brainstem neurons, but may account for up to 10% of recovery in RTN neurons and 20% of recovery in NTS neurons. 4) Recovery from acidification in LC neurons appears to be mediated largely by a DIDS-insensitive and Na- and HCO$_3^-$-dependent transporter. Despite the presence of different pH-regulating transporters in the different brainstem regions, pHi did not exhibit any recovery from hypercapnia-induced acidification in the neurons from all three regions.

Neuronal pH$_r$-regulating transporters

There are two main groups of pH-regulating transporters. One group is the SLC4 family of HCO$_3^-$-dependent transporters (45). The SLC4 family consists of a number of transporters including Cl$^-$/HCO$_3^-$ exchangers (AE), which normally mediate HCO$_3^-$ efflux in exchange for Cl$^-$ influx, thus acidifying cells in response to alkalinization. The clearest evidence for functional HCO$_3^-$-dependent transport in neurons is for AE, its activity having been demonstrated in cultured neurons from the cerebellum (18), the hippocampus (39) and the cortex (34) as well as medullary neurons within brainstem slices, including neurons from chemosensitive regions (41). In response to an acid load, there are many HCO$_3^-$-dependent transporters that can contribute to alkalinizing pH$_i$ recovery, including electrogenic Na$^+$-HCO$_3^-$ cotransporters (NBCe), electroneutral NBCs (NBCn1 and NBCn2), and electroneutral Na$^+$-driven Cl$^-$/HCO$_3^-$ exchanger (NDCBE) (45). NDCBE activity has been demonstrated in freshly dissociated hippocampal
neurons from the CA1 region (4,47), and cultured sympathetic (50) and cerebellar (36) neurons. While NBCe appears to be largely localized to glia (5), NBCn1 have been reported in rat hippocampal neurons (14) and, interestingly, it is largely insensitive to inhibition by DIDS (13). To date, however, no functional HCO3-dependent transporter, other than AE, has been shown to be active in neurons from putative chemosensitive regions.

The other main group of pH-regulating transporters is the SLC9 gene family of Na+/H+ exchangers (NHE) (33). In contrast to HCO3-dependent transporters, NHE isoforms appear to play a major role in neuronal pH, responses to acidification (37). Nine isoforms of NHE have been identified, but isoforms 6 through 9 are found exclusively within the cell (27). Of the 5 remaining isoforms, NHE-1 is ubiquitous, with a high level of mRNA expression throughout the brain and brainstem (24) and functional NHE-1 is likely, at least in brainstem (41). This isoform is characterized by a high sensitivity to inhibition by amiloride, EIPA, and cariporide but a much lower sensitivity to S1611 (25,46). NHE-2 mRNA has been detected at much lower levels than NHE-1 mRNA in the brain (24,52), but the expression of the protein has been found to be low in the brain and brainstem (59) suggesting that the NHE-2 protein is not crucial to pH regulation in the brainstem. NHE-3 mRNA has been detected at very low levels in the brainstem but appears to be present at somewhat greater levels in the cerebellum (24). Functional NHE-3 has been suggested to be present in a few neurons from the ventrolateral medulla (53,58). It is characterized by relatively low sensitivity to inhibition by amiloride and cariporide but higher sensitivity to inhibition by S1611 or related compounds (11,25,46). NHE-4 mRNA, but not protein, has been reported at low levels in the brain (24,35), although some expression of NHE-4 has been found in hippocampal neurons (7). NHE-4 protein has not been detected in the brainstem. NHE-4 is highly resistant to inhibition by amiloride and EIPA (25) and is not
activated by cell acidification (7). NHE-5 is unique among the NHEs because it is highly expressed in the rat brain, with little expression elsewhere (2). NHE-5 is structurally similar to NHE-3, and is similarly resistant to EIPA and amiloride but is not sensitive to S1611 (Dr. Klaus Wirth, personal communication).

In summary, since our study focuses on the mode of internal pH regulation in neurons from chemosensitive regions, we are mainly concerned with the NHE isoforms likely to be found in neurons from these regions. Of the five NHE isoforms that reside in the cell membrane, NHE-1, 3, and 5 are most likely to be present in the brainstem. We are also interested in any evidence for functional HCO₃⁻-dependent transport in neurons from putative chemosensitive brainstem regions.

**pH-regulating transporters in RTN, NTS and LC neurons**

While we recognize the limitations of purely pharmacological studies, we believe that our data comparing the responses of pHᵢ recovery among neurons from different brainstem areas with multiple NHE inhibitors does offer an initial indication of the pattern of the presence of pH-regulating transporters in the neurons from the RTN, NTS and LC. These patterns will ultimately have to be confirmed by molecular and immunocytochemical techniques.

In RTN neurons, recovery is nearly completely inhibited by Na-free solutions and by amiloride, but unaffected by DIDS (Table 1). These findings agree with earlier work on RTN neurons showing amiloride-sensitive pHᵢ recovery from acidification (32). Thus, the vast majority of recovery from acidification in RTN neurons appears to be mediated by NHE. Since EIPA and cariporide inhibit about half of this recovery, we assume that NHE-1 accounts for at least half of the pHᵢ recovery in RTN neurons. S1611 inhibits recovery in about 5-10% of these neurons indicating at most a small presence of NHE-3 in RTN neurons. The remainder of
recovery may possibly be due to NHE-5. These data indicate that recovery from acidification in RTN neurons is likely due to multiple NHE isoforms, with NHE-1 playing the predominant role.

Similarly, recovery from intracellular acidification in NTS neurons is largely inhibited by amiloride and Na-free solutions and unaffected by DIDS (Table 1), as previously reported (41,42). Since EIPA and cariporide inhibit this recovery between 50-74% (Table 1), NHE-1 probably accounts for nearly 2/3 of pH_i recovery in NTS neurons. Further, S1611 inhibits 26% of recovery while 18% of neurons have their recovery inhibited by S1611, suggesting that about 20% of NTS neurons contain NHE-3 which is active in pH_i recovery from acidification. Thus, NHE-1 and -3 account for nearly all pH_i recovery from acid loads in NTS neurons, with other isoforms (e.g. NHE-5) playing at most a small part.

The picture is quite different in LC neurons. While recovery is entirely inhibited by Na-free solutions and unaffected by DIDS, as in neurons from the other two brainstem regions, amiloride has no effect on pH_i recovery in LC neurons (Fig. 7; Table 1). The only NHE inhibitor that has any effect on pH_i recovery is EIPA, and this results in at most a small inhibition at very high EIPA concentrations. Finally, only 2% of LC neurons show inhibition with S1611. These data suggest that LC neurons, unlike NTS and RTN neurons, have relatively low NHE activity. The presence of non-NHE mediated recovery from acidification in LC neurons that is Na-dependent and DIDS-insensitive suggests the presence of HCO_3-dependent recovery, possibly NBCn1 (13). A strong HCO_3-dependence to this recovery is further supported by the fact that recovery from acidification is faster in the presence of CO_2/HCO_3 than in its absence in LC neurons (Fig. 9). Based on these findings, we propose that the majority of recovery of pH_i from acidification in LC neurons is mediated by NBCn1, although definitive proof for the presence of
this transporter in LC neurons will require the use of RT-PCR or the use of specific antibodies for NBCn1.

**Role of NHE-3 in central ventilatory control?**

Our studies suggest that NHE-3 is present in at most a few neurons from three different central chemosensitive regions, with the greatest presence being in NTS neurons. This would appear to be at odds with data suggesting that NHE-3 plays an important role in the control of breathing. In studies using organotypic cultures of VLM neurons, cariporide was applied to inhibit NHE-1 and no acidification or activation of neurons was observed (53,58). We have observed a similar lack of effect of an NHE-1 inhibitor, amiloride, on steady state pH, in NTS neurons (41). These data are consistent with the lack of a stimulating effect of cariporide on phrenic nerve activity in the working heart brainstem preparation (57). In contrast to these effects of NHE-1 inhibitors, the NHE-3 inhibitors S1611 and S3226 evoked changes in the neuronal pH and firing rate similar to those experienced during hypercapnia (53,58). Further, an *in vivo* study was performed that suggested that the level of expression of NHE-3 mRNA in the brainstem was inversely correlated with ventilation, suggesting that NHE-3 plays a role in the determination of central respiratory drive in rabbits (54,56). Expression levels of NHE-3 has also been shown to be altered in children that die of SIDS, compared to the expression level of children dying from non-SIDS related causes (55). A brain permeant inhibitor of NHE-3 increases respiration in adult rabbits (23). This latter study also found a few neurons within the brainstem that were immunoreactive for an NHE-3 antibody. Finally, infusion of an NHE-3 inhibitor into anesthetized rats resulted in increased ventilatory frequency (although neither tidal volume nor minute ventilation were increased) (40). This increase in respiratory frequency was accompanied by increased c-FOS staining in pre-Bötzinger complex and RTN neurons but not
NTS neurons (40). Together, these data suggest that NHE-3 plays a role in central ventilatory control.

We do not believe that our findings are at odds with these findings. First, we find relatively few neurons that show putative NHE-3 activity. This accords with the findings of low levels of expression of NHE-3 mRNA in rat brainstem (24), relatively few brainstem neurons found to be immunohistochemically positive for NHE-3 (23) and reports that NHE-3 is functionally present in chemosensitive neurons from the ventrolateral medulla (53). Thus, widespread NHE-3 activity in the brainstem would not be expected. It is believed that central chemosensitivity arises from a distributed network (31). The evidence that specific inhibitors of NHE-3 in the VLM results in increased ventilation is not in conflict with our findings. Further, NHE-3 likely also works on neurons from the pre-Bötzing complex (40), which are believed to be involved in respiratory rhythm generation (16) and to be chemosensitive (48). NHE-3 may be expressed on glial cells as well, which have been shown to affect ventilatory control (15,20). Kiwull-Schöne et al. (23) suggested that NHE-3 inhibitors may work by inhibiting NHE-3 in and altering the properties of other components of the complex respiratory network, and not simply by working on central chemosensitive neurons. Finally, inhibitors of NHE-3 seem in some cases to affect respiratory frequency more than tidal volume (40) and to cause a fraction of the ventilatory increase caused by systemic hypercapnia (23). These latter findings are consistent with NHE-3 inhibitors affecting at most a few chemosensitive sites, with NHE-3 activity not being involved in chemoreception in neurons from other sites. This is consistent with a distributed chemosensitive network (31).

Response of $pH_i$ to hypercapnia
Upon exposure to hypercapnia, the neurons from all three regions maintained a new, more acid, steady state pH. Without additional experiments, we cannot differentiate whether this was due to a complete inhibition of the pH-regulating transporters in these neurons or to a new balance between acid loading and extruding mechanisms. We have previously shown that in NTS neurons, a decrease of pHo to 7.0 was sufficient to inhibit NHE (41) and that this transporter was more sensitive to inhibition by pHo than the NHE in neurons from non-chemosensitive brainstem regions. However, it would be remarkable if hypercapnia fully inhibited the various NHEs in RTN and NTS neurons as well as the Na- and HCO3-dependent transporter from LC neurons. Also, the relatively small intracellular acidification seen in response to 15% CO2 implies that these neurons have a very high buffering power, which in fact has been shown for NTS neurons (41). Nevertheless, our data confirm previous findings that a maintained acidification in response to hypercapnic acidosis is invariably seen in a variety of CO2/H+-sensitive cells.

Perspective

Our data clearly indicate that pH-regulating transporters differ in neurons from different chemosensitive regions, with multiple NHE isoforms mediating recovery from acidification in RTN and NTS neurons but a Na- and HCO3-dependent transporter predominating in LC neurons (Table 1). These latter findings are the first demonstration of functional HCO3-dependent transport in neurons from central chemosensitive brainstem regions, although such transporters have been demonstrated in glomus cells of the carotid bodies (10), the peripheral chemoreceptors. It is noteworthy that despite the fact that the neurons from these different chemosensitive regions contain various pH-regulating transporters, no pHi recovery from acidification is observed during hypercapnic acidosis (Fig. 2). That hypercapnic acidosis results in a lack of pHi recovery in the
neurons from all of these brainstem regions suggests that changes of pH are an important part of the signaling mechanism in chemosensitive neurons (17,38).

A major question in the field of respiratory control is why there are so many central chemosensitive sites (30,31). It has been suggested that these sites may have arisen in a hierarchical fashion (28) as organisms passed through critical changes during phylogeny, such as emerging from an aqueous environment to become air breathing, the development of the ability to maintain a high, constant body temperature and the development of sleep. If respiratory control did indeed develop in a polyphyletic fashion, then it might be expected that neurons from different chemosensitive regions may have developed different cellular mechanisms of chemosensitivity, including differences in the regulation of pH$_i$. Thus, our finding of differences in the pH-regulating transporters in neurons from different chemosensitive regions may at least be consistent with a polyphyletic origin of multiple central chemoreceptive sites.

Finally, it is clear that the maintenance of acidification during hypercapnic acidosis requires inhibition of different pH-regulating transporters in neurons from different chemosensitive regions. If highly specific inhibitors could be found for each of these transporters, then it might be possible to use these inhibitors focally to study the role of each chemosensitive region in ventilatory control. This would allow us to start to address the question of why there are so many central chemosensitive regions and what the role of each region is in respiratory control.
ACKNOWLEDGEMENTS

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### Table 1

The % inhibition of mean pHᵢ recovery from NH₄Cl-induced acidification by various transport inhibitors

<table>
<thead>
<tr>
<th>Brainstem Area</th>
<th>DIDS (BDT)</th>
<th>0 Na⁺ (NDT)</th>
<th>AMIL (NHE)</th>
<th>EIPA (NHE-1)</th>
<th>CARIP (NHE-1)</th>
<th>S1611 (NHE-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retrotrapezoid Nucleus (RTN)</td>
<td>0</td>
<td>86</td>
<td>93</td>
<td>65</td>
<td>40</td>
<td>0 [5-10]*</td>
</tr>
<tr>
<td>Nucleus Tractus Solitarius (NTS)</td>
<td>0</td>
<td>81</td>
<td>65</td>
<td>50</td>
<td>74</td>
<td>26 [18]*</td>
</tr>
<tr>
<td>Locus Coeruleus (LC)</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0-33</td>
<td>0</td>
<td>0 [2]*</td>
</tr>
</tbody>
</table>

BDT  –  HCO₃-dependent transporters  
NDT  –  Na⁺-dependent transporters  
NHE  –  Na⁺/H⁺ exchangers  
NHE-1  –  Na⁺/H⁺ exchanger isoform 1  
NHE-3  –  Na⁺/H⁺ exchanger isoform 3  
[ ]* The percentage of individual neurons whose recovery is inhibited by S1611
FIGURE LEGENDS

Figure 1: Photograph of a dissected rat brain dorsal side up. The cerebellum has been removed so that the brainstem and proximal spinal cord are visible. The red lines delineate the area in the pons from which slices were taken to study the LC. The blue lines define the area spanning the pons and medulla from which the slices containing RTN were taken. The green lines show the area in the medulla from which slices were made to study the NTS. The tracings to the right show a drawing of a representative transverse slice indicating the location of the LC (top), RTN (middle), and NTS (bottom).

Figure 2: The effect of hypercapnia (15% CO₂) on pHᵢ in RTN, NTS and LC neurons. A. Hypercapnia results in a maintained acidification with no pHᵢ recovery in an RTN neuron. B. Hypercapnia results in a maintained acidification with no pHᵢ recovery in an NTS neuron. C. Hypercapnia results in a maintained acidification with no pHᵢ recovery in an LC neuron. D. The summary of the pHᵢ recovery from NH₄Cl-induced acidification (black bars) or during hypercapnia (white bars) in RTN, NTS and LC neurons. The height of a bar represents the mean recovery rate (pH unit/min) ± 1 S.E.M. The number in or above each bar represents the number of neurons. *** indicates that recovery from NH₄Cl-induced acidification is significantly different in LC neurons than the recovery in RTN and NTS neurons at P<0.001.

Figure 3: The effect of various drugs and ion substitution on pHᵢ recovery from an NH₄Cl prepulse-induced acidification in RTN neurons. A. The black line shows the pHᵢ response of an RTN neuron to an NH₄Cl prepulse (40 mM) including a control pHᵢ recovery in the presence of aCSF. The colored lines show the recovery of cells in the presence of 0.5 mM DIDS (green),
1 mM amiloride (AMIL) (blue), and 0 Na⁺ aCSF (NMDG substitution) (red). The arrows indicate the time at which the experimental solutions were replaced with aCSF. **B.** A summary of pHᵢ recovery in RTN neurons under various conditions. The height of a bar represents the mean recovery rate (pH unit/min) ± 1 S.E.M. The number in or above each bar represents the number of neurons. *** indicates that recovery is significantly different than control recovery at P<0.001.

**Figure 4:** The effect of the NHE-1 inhibitors (EIPA and cariporide) and an NHE-3 inhibitor (S1611) on pHᵢ recovery from an NH₄Cl prepulse-induced acidification in RTN neurons. **A.** The black line shows the pHᵢ response of an RTN neuron to an NH₄Cl prepulse (40 mM) including a control pHᵢ recovery in the presence of aCSF. The colored lines show the pHᵢ recovery of cells in the presence of 0.5 mM EIPA (yellow), 5 µM cariporide (CARIP) (light blue) or S1611 (light green). The arrows indicate the time at which inhibitors were replaced by aCSF. **B.** A summary of the effects of NHE inhibitors on pHᵢ recovery in RTN neurons. The height of a bar represents the mean recovery rate (pH unit/min) ± 1 S.E.M. The number in each bar represents the number of neurons. ** and *** indicates that recovery is significantly different than control recovery at P<0.01 and P<0.001, respectively.

**Figure 5:** The effect of various drugs and ion substitution on pHᵢ recovery from an NH₄Cl prepulse-induced acidification in NTS neurons. **A.** The black line shows the pHᵢ response of an NTS neuron to an NH₄Cl prepulse (40 mM) including a control pHᵢ recovery in the presence of aCSF. The colored lines show the recovery of cells in the presence of 0.5 mM DIDS (green), 1 mM amiloride (AMIL) (blue), and 0 Na⁺ aCSF (NMDG substitution) (red). The arrows
indicate the time at which the experimental solutions were replaced with aCSF.  **B.** A summary of pH$_i$ recovery in NTS neurons under various conditions. The height of a bar represents the mean recovery rate (pH unit/min) ± 1 S.E.M. The number in each bar represents the number of neurons. *** indicates that recovery is significantly different than control recovery at P<0.001.

**Figure 6:** The effect of the NHE-1 inhibitors (EIPA and cariporide) and an NHE-3 inhibitor (S1611) on pH$_i$ recovery from an NH$_4$Cl prepulse-induced acidification in NTS neurons.  **A.** The black line shows the pH$_i$ response of an NTS neuron to an NH$_4$Cl prepulse (40 mM) including a control pH$_i$ recovery in the presence of aCSF. The colored lines show the pH$_i$ recovery of cells in the presence of 0.5 mM EIPA (yellow), 5 µM cariporide (CARIP) (light blue) or S1611 (light green). The arrows indicate the time at which inhibitors were replaced by aCSF.  **B.** A summary of the effects of NHE inhibitors on pH$_i$ recovery in NTS neurons. The height of a bar represents the mean recovery rate (pH unit/min) ± 1 S.E.M. The number in each bar represents the number of neurons. *** indicates that recovery is significantly different than control recovery at P<0.001.

**Figure 7:** The effect of various drugs and ion substitution on pH$_i$ recovery from an NH$_4$Cl prepulse-induced acidification in LC neurons.  **A.** The black line shows the pH$_i$ response of an LC neuron to an NH$_4$Cl prepulse (40 mM) including a control pH$_i$ recovery in the presence of aCSF. The colored lines show the recovery of cells in the presence of 0.5 mM DIDS (green), 1 mM amiloride (AMIL) (blue), and 0 Na$^+$ aCSF (NMDG substitution) (red). The arrows indicate the times at which the experimental solutions were replaced with aCSF.  **B.** A summary of
pHi recovery in LC neurons under various conditions. The height of a bar represents the mean recovery rate (pH unit/min) ± 1 S.E.M. The number in each bar represents the number of neurons. *** indicates that recovery is significantly different than control recovery at P<0.001.

**Figure 8:** The effect of the NHE-1 inhibitors (EIPA and cariporide) and an NHE-3 inhibitor (S1611) on pHi recovery from an NH₄Cl prepulse-induced acidification in LC neurons. **A.** The black line shows the pHi response of an LC neuron to an NH₄Cl prepulse (40 mM) including a control pHi recovery in the presence of aCSF. The colored lines show the pHi recovery of cells in the presence of 0.5 mM EIPA (yellow), 5 µM cariporide (CARIP) (light blue) or S1611 (light green). The arrows indicate the times at which inhibitors were replaced by aCSF. **B.** A summary of the effects of NHE inhibitors on pHi recovery in LC neurons. The height of a bar represents the mean recovery rate (pH unit/min) ± 1 S.E.M. The number in each bar represents the number of neurons. * indicates that recovery is significantly different than control recovery at P<0.05.

**Figure 9:** The recovery of pHi from NH₄Cl-induced (40 mM) acidification in the absence and presence of CO₂ in LC neurons. **A.** The thin line shows the pHi response to an NH₄Cl-induced (40 mM) acidification in HEPES-buffered medium (nominal absence of CO₂/HCO₃). The thick line shows the pHi response to an NH₄Cl-induced acidification in CO₂/HCO₃-buffered medium (5% CO₂/26 mM HCO₃). Note that recovery is faster in CO₂/HCO₃-buffered medium even though total intracellular buffering power is higher in this medium. **B.** The mean recovery from NH₄Cl-induced (40 mM) acidification in the presence (black bar) and absence (gray bar) of CO₂ in LC neurons.
The height of a bar represents the mean recovery rate (pH unit/min) ± 1 S.E.M. The number in each bar represents the number of neurons. *** indicates that recovery is significantly different in CO₂- vs. HEPES-buffered medium at P<0.001.
**A.** RTN

- pH graph with a 15% CO₂ exposure indicated.

**B.** NTS

- pH graph showing a decrease in pH with 15% CO₂.

**C.** LC

- pH graph with a 15% CO₂ exposure indicated.

**D.** Recovery Rate (pH unit/min)

- Bar graph showing recovery rates for RTN, NTS, and LC with corresponding values:
  - RTN: 25
  - NTS: 46
  - LC: 52

- Significant difference indicated by ***.
A.

![Graph showing pH changes over time with NH4Cl, DIDS, AMIL, and 0 Na](image)

B.

![Bar chart showing recovery rates with NH4Cl, DIDS, and AMIL](image)
A. 

NH₄Cl

Time (min)

pHi

B. 

Recovery Rate (pH unit/min)

CONTROL

EIPA 0.5 mM

CARIP 1 μM

S1611 1 μM

S1611 5 μM

26

11

36

58

18

28
**NTS**

A.

![Graph showing pHi over time](image)

- **NH₄Cl**
- **DIDS**
- **0 Na⁺**
- **AMIL**

**Time (min)**

0 20 40 60

B.

![Bar chart showing recovery rates](image)

- **CONTROL**
- **DIDS**
- **AMIL**
- **EIPA**

**Recovery Rate (pH unit/min)**

- **0 Na⁺**: 12
- **AMIL**: 42
- **EIPA**: 48

**Note:** Significance levels indicated by ***.
A.

- **NH₄Cl**
- **CARIP**
- **S1611**
- **EIPA**

B.

- CONTROL
- EIPA 0.5 mM
- CARIP 1 μM
- CARIP 5 μM
- S1611 5 μM

**Recovery Rate (pH unit/min)**

- CONTROL: 0.016
- EIPA 0.5 mM: 0.014
- CARIP 1 μM: 0.012
- CARIP 5 μM: 0.016
- S1611 5 μM: 0.018

**Notes:**

- ***: Significant difference

**Values:**

- CONTROL: 46
- EIPA 0.5 mM: 25
- CARIP 1 μM: 7
- CARIP 5 μM: 21
- S1611 5 μM: 38
A. A graph showing the pH changes over time with treatments such as NH$_4$Cl, EIPA, CARIP, and S1611.

B. A bar chart comparing the recovery rates with different treatments. The rates are 52 for CONTROL, 23 for EIPA 0.5 mM, 42 for CARIP 5 μM, and 44 for S1611 5 μM.
A. 

[Graph showing pH dynamics over time with markers for NH₄Cl, CO₂, and HEPES]

B. 

[Bar graph showing recovery rates with markers for HEPES and CO₂, with values 20 and 10, respectively]

**Recovery Rate (pH unit/min)**