Mechanisms of \( \text{CO}_2/\text{H}^+ \) Sensitivity of Astrocytes

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Ventral regions of the medulla oblongata of the brainstem are populated by astrocytes sensitive to physiological changes in \( P_{\text{CO}_2}/[\text{H}^+] \). These astrocytes respond to decreases in pH with elevations in intracellular \( \text{Ca}^{2+} \) and facilitated exocytosis of ATP-containing vesicles. Released ATP propagates \( \text{Ca}^{2+} \) excitation among neighboring astrocytes and activates neurons of the brainstem respiratory network triggering adaptive increases in breathing. The mechanisms linking increases in extracellular and/or intracellular \( P_{\text{CO}_2}/[\text{H}^+] \) with \( \text{Ca}^{2+} \) responses in chemosensitive astrocytes remain unknown. Fluorescent imaging of changes in \([\text{Na}^+]_i\) and/or \([\text{Ca}^{2+}]_i\) in individual astrocytes was performed in organotypic brainstem slice cultures and acute brainstem slices of adult rats. It was found that astroglial \([\text{Ca}^{2+}]_i\) responses triggered by decreases in pH are preceded by \( \text{Na}^+ \) entry, markedly reduced by inhibition of \( \text{Na}^+/\text{HCO}_3^- \) cotransport (NBC) or \( \text{Na}^+/\text{Ca}^{2+} \) exchange (NCX), and abolished in \( \text{Na}^+ \)-free medium or by combined NBC/NCX blockade. Acidification-induced \([\text{Ca}^{2+}]_i\) responses were also dramatically reduced in brainstem astrocytes of mice deficient in the electrogenic \( \text{Na}^+/\text{HCO}_3^- \) cotransporter NBCe1. Sensitivity of astrocytes to changes in pH was not affected by inhibition of \( \text{Na}^+/\text{H}^+ \) exchange or blockade of phospholipase C. These results suggest that in pH-sensitive astrocytes, acidification activates NBCe1, which brings \( \text{Na}^+ \) inside the cell. Raising \([\text{Na}^+]_i\) activates NCX to operate in a reverse mode, leading to \( \text{Ca}^{2+} \) entry followed by activation of downstream signaling pathways. Coupled NBC and NCX activities are, therefore, suggested to be responsible for functional \( \text{CO}_2/\text{H}^+ \) sensitivity of astrocytes that contribute to homeostatic regulation of brain parenchymal pH and control of breathing.

Key words: acidosis; brainstem; breathing; chemosensitivity; hypercapnia; respiration

Significance Statement

Brainstem astrocytes detect physiological changes in pH, activate neurons of the neighboring respiratory network, and contribute to the development of adaptive respiratory responses to the increases in the level of blood and brain \( P_{\text{CO}_2}/[\text{H}^+] \). The mechanisms underlying astroglial pH sensitivity remained unknown and here we show that in brainstem astrocytes acidification activates \( \text{Na}^+/\text{HCO}_3^- \) cotransport, which brings \( \text{Na}^+ \) inside the cell. Raising \([\text{Na}^+]_i\) activates NCX to operate in a reverse mode leading to \( \text{Ca}^{2+} \) entry. This identifies a plausible mechanism of functional \( \text{CO}_2/\text{H}^+ \) sensitivity of brainstem astrocytes, which play an important role in homeostatic regulation of brain pH and control of breathing.

Introduction

Astrocytes are known to provide neuronal networks with essential structural and metabolic support. They also control the ionic environment of the neuropil, support synaptic transmission by supplying neurons with a renewable source of transmitters, and provide a neurovascular coupling interface by mediating cerebrovascular responses to heightened neuronal activity. (Haydon et al., 2015) This is done through the activity of 

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Recent evidence also suggests that astrocytes modulate the activities of CNS neuronal networks. Astrocytes are not electrically excitable, but display Ca\(^{2+}\) excitability and, via release of "gliotransmitters" (such as ATP/adenosine, N-serine, and others), are implicated in the control of neuronal excitability, synaptic transmission, plasticity, and information processing (Volterra and Meldolesi, 2005; Haydon and Carmignoto, 2006; Halassa and Haydon, 2010; Henneberger et al., 2010; Araque et al., 2014).

Brainstem regions adjacent to the ventral surface of the medulla oblongata are populated with pH-sensitive astrocytes, which play an important role in the operation of a fundamental homeostatic mechanism that maintains constant level of arterial pH (Volterra and Meldolesi, 2005; Haydon and Carmignoto, 2006; Halassa and Haydon, 2010; Henneberger et al., 2010; Araque et al., 2014).

The parambolic brainstem is the chemosensitive region of the brainstem respiratory network (Gourine et al., 2010). Brainstem astrocytes are chemosensitive and functionally specialized to detect physiological changes in P\(_{\text{CO}_2}/\text{H}^+\) by controlling the activity of the brainstem respiratory network (Gourine et al., 2010). Brainstem astrocytes are chemosensitive and functionally specialized to detect physiological changes in P\(_{\text{CO}_2}/\text{H}^+\) (Gourine et al., 2010; Kasymov et al., 2013). They respond to 0.2–0.4 unit decreases in pH with elevations in intracellular Ca\(^{2+}\) and increased rate of exocytosis of ATP-containing vesicles (Gourine et al., 2010; Kasymov et al., 2013). Released ATP propagates astroglial Ca\(^{2+}\) excitation and activates neurons of the brainstem respiratory networks triggering adaptive increases in breathing (Gourine et al., 2005, 2010). Astrogial dysfunction contributes to the disordered breathing patterns associated with respiratory network (Bergel et al., 2010). We hypothesized that acidification-induced Ca\(^{2+}\) responses in brainstem astrocytes are dependent on Na\(^+\)/HCO\(_3^-\) cotransporter (NBC) activity. Expression of high-affinity NBCs (such as the electrogenic Na\(^+\)/HCO\(_3^-\) cotransporter NBCe1) capable of fast HCO\(_3^-\) transport and effective cytosolic H\(^+\) buffering in astrocytes has been demonstrated recently (Theparambil et al., 2014; Theparambil and Deitmer, 2013). NBC activation and facilitated HCO\(_3^-\) entry may explain intracellular alkalization of astrocytes during hypercapnia observed in some earlier studies (Shrod and Putnam, 1994). We hypothesized that in chemosensitive astrocytes, acidification-induced NBC-mediated HCO\(_3^-\) and Na\(^+\) entry increases in [Ca\(^{2+}\)], by activation of the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) to operate in a reverse mode (Rojas et al., 2007).

### Materials and Methods

All experiments were performed in accordance with the European Commission Directive 2010/63/EU (European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes) and the UK Home Office (Scientific Procedures) Act (1986) with project approval from the Institutional Animal Care and Use Committee.

#### Experimental models

Organotypic brain slices. Rat pups [postnatal day (P) 8–P10 of either sex] were killed by halothane overdose. Brainstems were rapidly removed and bathed in ice-cold HBSS without Ca\(^{2+}\) with added 20 mM glucose (total 25.6 mM), 10 mM MgCl\(_2\), 1 mM HEPES, 1 mM kynurenic acid, 0.005% phenol red, 100 U ml\(^{-1}\) penicillin, and 0.1 mg ml\(^{-1}\) streptomycin. The medulla was isolated and a sequence of transverse slices (250 μm) was cut. Slices were inspected under a low-magnification dissecting microscope and 2–3 brainstem sections containing the facial nucleus (anatomical landmark for the chemosensitive region) were plated on Millicell-CM organotypic culture membrane inserts. Slices were cultured in medium containing 50% OptiMEM-1, 25% fetal bovine serum (FBS), 21.5% HBSS, 25 mM glucose, 100 U ml\(^{-1}\) penicillin, and 0.1 mg ml\(^{-1}\) streptomycin. After 3 d, the plating medium was removed and DMEM medium containing 10% FBS, 2 ml l-glutamine, 100 U ml\(^{-1}\) penicillin, and 0.1 mg ml\(^{-1}\) streptomycin was added and subsequently replaced twice a week. In some of the experiments, the astrocytes were targeted to express the (genetically encoded) Ca\(^{2+}\) indicator Case12 using an adenoviral vector (AVV) under the control of an enhanced glial fibrillary acidic protein (GFAP) promoter, as described previously (Guo et al., 2010). AVV-sGFAP-Case12 was added to the medium at the time of slice culture preparation at 5 × 10⁸–5 × 10¹⁰ transducing units ml\(^{-1}\). Experiments were performed after 7–10 d of incubation.

Acute brain slices. Astrocytes that reside at and near the ventral surface of the brainstem were targeted to express Ca\(^{2+}\) indicator Case12 following stereotaxic microinjections of AVV-sGFAP-Case12. Young male Sprague Dawley rats (100–150 g) were anesthetized with ketamine (60 mg kg\(^{-1}\), i.m.) and medetomidine (250 μg kg\(^{-1}\), i.m.) and placed in a stereotaxic frame. Adequate depth of surgical anesthesia was confirmed by the absence of a withdrawal response to a paw pinch. Two microinjections per side of AVV-sGFAP-Case12 (1 μl each) were delivered into the ventral regions of the medulla oblongata using the following coordinates from bregma: 11 and 12 mm caudal, 1.8 mm lateral, and 8.5–8.8 mm ventral. Anesthesia was reversed with atipamezole (1 mg kg\(^{-1}\), i.m.). For postoperative analgesia, rats received buprenorphine (0.05 mg kg\(^{-1}\), s.c.) for 3 d. After 7–10 d, the rats were terminally anesthetized with halothane overdose. Brains were removed and placed in ice-cold artificial CSF (aCSF) containing 124 mM NaCl, 26 mM NaHCO\(_3\), 3 mM KCl, 2 mM CaCl\(_2\), 1.25 mM Na\(_2\)HPO\(_4\), 1 mM MgSO\(_4\), and 10 mM glucose saturated with 95% O\(_2\)/5% CO\(_2\), pH 7.4, with an addition of 9 mM Mg\(^{2+}\).

### Cell culture

Primary astroglial cell cultures were prepared from the cortical and brainstem tissue of rat pups (P2–P3 of either sex) as well as the brainstems of wild-type (C57BL/6) and diabetic (db/db) mouse pups (P1–P4 of either sex) as described previously (Kasymov et al., 2013). The animals were killed by isoflurane overdose (rats) or rapid decapitation (mice), the brains were removed, and the ventral regions of the medulla oblongata were dissected out. Brainstem tissue cuts from 2–3 animals were used for each cell culture preparation. After isolation, the cells were plated on poly-D-lysine-coated glass coverslips and maintained at 37°C in a humidified atmosphere of 5% CO\(_2\) and 95% air for a ≥10 d before the experiments. Cultured rat astrocytes were transduced to express fluorescent proteins Case12 (cyclically permuted green fluorescent protein) or DsRed using adenoviral vectors AVV-sGFAP-Case12 and AVV-sGFAP-DsRed (5 × 10⁸–5 × 10¹⁰ transducing units ml\(^{-1}\)).

#### Imaging

Optical recordings were performed using Olympus FV1000 confocal microscope or an inverted epifluorescence Olympus microscope equipped with a cooled CCD camera (Retiga, QImaging), as described previously (Angelova et al., 2015; Turovsky et al., 2015). [Ca\(^{2+}\)] responses of individual astrocytes were visualized by recording changes in fluorescence of Case12 (Gourine et al., 2010; Guo et al., 2010) or conventional Ca\(^{2+}\) indicators Fura-2 (Invitrogen) and Fluoro-4 (Invitrogen). In the experiments using conventional Ca\(^{2+}\) indicators, chemosensitive brainstem...
astrocytes were identified by robust Ca$^{2+}$ responses to acidification and lack of Ca$^{2+}$ response to KCl (30 mM) application at the end of the recordings, as described previously (Turovsky et al., 2015). Recordings of acidification-induced [Ca$^{2+}$], responses in astrocytes transduced to express Case12 were performed both in organotypic and acute brainstem slices. For imaging, a section of the membrane with an organotypic slice or an acute slice was placed on an elevated grid in a flow chamber (volume, 2 ml). Recordings were made at 35–37°C in aCSF. The rate of perfusion was 4 ml min$^{-1}$. Images were obtained using a 40× water-immersion objective. The 488 nm argon laser line was used to excite Case12 fluorescence, which was measured using a 505–550 nm bandpass filter. Illumination intensity was kept to a minimum (0.5–0.7% of laser output).

For simultaneous recordings of changes in [Ca$^{2+}$], and [Na$^+$], organotypic brainstem slices were loaded with Fura-2 (5 μM; 40 min incubation; 37°C) and Sodium Green (10 μM, Invitrogen; 40 μM incubation; 37°C) with the addition of pluronic F-127 (0.005%). After incubation with the dyes, slices were washed three times before the experiment. Changes in [Ca$^{2+}$], and [Na$^+$], were monitored in individual cells using an inverted Olympus microscope with a 20× oil-immersion objective. Excitation light provided by a xenon arc lamp was passing sequentially through a monochromator at 340, 380, and 490 nm (Cairn Research); emitted fluorescence at 515 nm (Fura-2) and 530 nm (Sodium Green) was registered. All test drugs were applied 5–20 min before the experimental challenge.

In a separate experiment, changes in absolute [Na$^+$], in brainstem astrocytes were estimated following calibration of the Sodium Green signal. Organotypic brainstem slices were perfused with aCSF containing gramicidin D (3 μM), monensin (10 μM), and ouabain (100 μM) to equilibrate Na$^+$ across the cell membrane. Changes in Sodium Green fluorescence in response to increasing [Na$^+$] (0–100 mM) in the media were recorded and plotted as a function of [Na$^+$] to create the calibration curve. To record [Ca$^{2+}$], in cultured brainstem astrocytes of wild-type and NBCe1-deficient mice, the cells were loaded with Fluo-4 (5 μM; 15 min incubation; 20–22°C). Changes in [Ca$^{2+}$], were monitored using a confocal laser scanning microscope (LSM-700) with a 40× water-immersion objective. The 488 nm laser light was used to excite Fluo-4 fluorescence and emitted fluorescence was registered using a short-pass emission filter (SP 640).

**Drugs**

S0859 (10–30 μM; Sanofi-Aventis;  Ch‘en et al., 2008) and 4,4′-diisothiocyanato-2,2′-stibenedisulfonic acid (DIDS; 100 μM; Tocris Bioscience) were used to inhibit Na$^+$/HCO$_3^-$ cotransport. SN-6 (10 μM; Tocris Bioscience) was used to interfere with NCX. Cariporide (10 μM; Tocris Bioscience) and amiloride (0.5 mM; Tocris Bioscience) were used to block Na$^+$/H$^+$ exchange (NHE). Phospholipase C was inhibited by application of U73122 (10 μM; Tocris Bioscience).

**Isolation and purification of astrocytes**

Young adult male rats (∼100 g) and rat pups (P3 of either sex) were used to isolate cortical and brainstem astrocytes. For all the conditions (brain area/developmental stage), one biological replicate consisted of pooled cells from the cortices and the brainstems of 3–4 animals. The animals were humanely killed by isoflurane overdose and the brains were isolated. The cortices and the brainstems were dissected out and the meninges were removed. The tissue was enzymatically dissociated to make a suspension of individual cells as described previously (Zhang et al., 2014). Briefly, the tissue was incubated at 37°C for 40 min in 20 ml of HBSS containing 0.1% trypsin. After trypsin treatment, the tissue was washed three times with 10 ml of ice-cold HBSS containing FBS (10%) and trypsin inhibitor (1.0 mg ml$^{-1}$) and then mechanically dissociated by gentle sequential trituration using a 5 ml pipette. Samples were then diluted 10-fold, washed in HBSS, and passed through a 45 μm Nitex mesh to remove undissociated cell clumps before resuspension in 500 μl of PBS containing 0.5% BSA, 2 mM EDTA with the addition of 50 μl of myelin removal beads (Miltenyi Biotec). After 20 min of incubation at 4°C, cells were washed in PBS containing 2 mM EDTA and centrifuged for 10 min (1200 rpm). The cell pellet was resuspended in 500 μl of buffer and applied to a MACS column (Miltenyi Biotec). Magnetic labeled myelin was retained within the column. The second (positive) magnetic separation was then performed using astrocyte-specific anti-GLAST (anti-glutamate/aspartate transporter; ACSA-1) antibodies conjugated to the magnetic beads (Miltenyi Biotec). Anti-O4 selection using magnetic microbeads (Miltenyi Biotec) to separate O4+ immature oligodendrocytes from cell suspensions was also performed to remove ACSA-1-positive astrocytes contaminated by oligodendrocytes. Cell purity was assessed by anti-GLAST (ACSA-1) phycoerythrin antibody (Miltenyi Biotec) using flow cytometry (CyanADP Cytometer, Beckman Coulter). FACS analysis confirmed >95% purity of isolated astrocytes. Purified cells were harvested by centrifugation at 2000 × g for 5 min. The cell pellet was then used for RNA extraction.

Separately, astrocytes in cortical and brainstem cultures transduced with AVV-sGFAP-Case12 or AVV-sGFAP-DsRed were identified by green or red fluorescence and individually collected using patch pipettes (tip, ∼5 μm) made of borosilicate glass. One biological replicate consisted of 20–25 pooled cells from 3–4 cultures; two samples from each brain area were analyzed.

**RNA sequencing, read mapping, and expression level estimation**

Total RNA was isolated using the RNeasy Plus Micro Kit (Qiagen) following the manufacturer’s protocol and RNA quality was assessed using the RNA 6000 Pico Kit on a 2100 Bioanalyzer (Agilent Technologies). Samples containing 100 ng of total RNA were quantified via Nanodrop 2000 (Thermo Fisher Scientific) and used to prepare cDNA using the Ovation RNA-seq System v2 following the manufacturer’s protocols (Nugen).
The cDNA was then sonically fragmented with a Covaris S2 system (Covaris) to an average size of 400 bp. Sequencing-ready libraries were prepared using the TruSeq DNA Sample Prep Kit (Illumina). Final PCR-enriched fragments were quantified by qPCR using Kapa’s Library Quantification Kit (Kapa Biosystems) on the 7900HT (Applied Biosystems). Samples were then pooled before 100 bp paired-end sequencing on two lanes of a HiSeq 2000 (Illumina). Fastq conversion and demultiplexing was conducted with Casava 1.8.2 (Illumina). Reads were aligned to the rat reference genome RGCSC3.4.64 with TopHat 1.3.3. Cufflinks v1.0.2 was used to assemble and quantitate the transcriptome of each sample. A union set of transcripts in all samples was generated with Cuffcompare, and differential expression was assessed with Cuffdiff. Ex-enriched fragments were quantified by qPCR using Kapa's Library Quantification Kit (Kapa Biosystems) on the 7900HT (Applied Biosystems). Samples were then pooled before 100 bp paired-end sequencing on two lanes of a HiSeq 2000 (Illumina). Fastq conversion and demultiplexing was conducted with Casava 1.8.2 (Illumina). Reads were aligned to the rat reference genome RGCSC3.4.64 with TopHat 1.3.3. Cufflinks v1.0.2 was used to assemble and quantitate the transcriptome of each sample. A union set of transcripts in all samples was generated with Cuffcompare, and differential expression was assessed with Cuffdiff. Ex-green fluorescence ratio, 100 s

# Figure 1

(a) Facilitated Na\(^+\)/HCO\(_3^-\) cotransport and NCX reversal underlie chemosensory (Ca\(^{2+}\)) responses in brainstem astrocytes. Expanded representative recording illustrating time course of acidification-induced (Ca\(^{2+}\)) entry, which precedes Ca\(^{2+}\) response. Representative recording illustrating the effect of NBC blocker S0859 (10 \(\mu\)M) on Ca\(^{2+}\) responses of brainstem astrocytes induced by acidification. Averaged traces of (Ca\(^{2+}\)) and (Na\(^+\)), changes in 12 astrocytes recorded in an organotypic brainstem slice preparation are shown. Representative recording illustrating the effect of NCX inhibitor SN-6 (10 \(\mu\)M) on Ca\(^{2+}\) and Na\(^+\), changes in 17 astrocytes recorded in an organotypic brainstem slice are shown.

(b) Lack of an effect of NHE inhibitor cariporide (10 \(\mu\)M) on Ca\(^{2+}\) and Na\(^+\), changes in 18 astrocytes recorded in an organotypic brainstem slice preparation are shown. Averaged traces of (Ca\(^{2+}\)) and (Na\(^+\)), changes in 16 astrocytes recorded in an organotypic brainstem slice are shown.

(c) Summary data (expressed as the percentage of the initial response) of the pharmacology of acidification-induced Na\(^+\) and Ca\(^{2+}\) responses in astrocytes recorded in organotypic brainstem slices.

## Results

Brainstem regions adjacent to the ventral surface of the medulla oblongata are populated by astrocytes that respond to acidification (decreases in pH from 7.4 to 7.0 induced by an increase in PCO\(_2\) from 40 to ~80 mmHg in aCSF containing 26 mM HCO\(_3^-\)) with elevations in intracellular (Ca\(^{2+}\)) (Figs. 1–4). Robust Ca\(^{2+}\) responses to decreases in pH are observed both in cultured organotypic brainstem slices (Fig. 1) and acute brainstem slices (Fig. 4; Gourine et al., 2010). Simultaneous imaging of changes in (Ca\(^{2+}\)) and (Na\(^+\)) in individual astrocytes recorded in organotypic brainstem slices demonstrated that acidification-induced Ca\(^{2+}\) responses in chemosensitive cells are accompanied by Na\(^+\) entry (N = 39 cells; n = 5 slices; Fig. 1a). (Ca\(^{2+}\)) transients were not observed in brainstem astrocytes that were not responding to the decrease in pH with Na\(^+\) influx (n = 56 cells; n = 5 slices; Fig. 1b), suggesting that the mechanisms responsible for the increases in intracellular concentrations of both cations are coupled. Acidification-induced elevations in (Ca\(^{2+}\)), in pH-sensitive astrocytes were reversibly abolished (p < 0.001, F\(_{113}\) = 1298) in Na\(^{+}\)-free medium (replacement of Na\(^+\) with equimolar concen-
Acidic stimuli activate several parallel Na\(^{+}\)-dependent mechanisms responsible for intracellular pH regulation, including Na\(^{+}\)/H\(^{+}\) exchange and Na\(^{+}\)/HCO\(_3\) cotransport (Shrode and Putnam, 1994). It was noted that astrocytes display elevations in [Ca\(^{2+}\)]\(_i\), when aCSF is replaced with a Na\(^{+}\)-free medium (Fig. 1e) and that in response to acidification, Na\(^{+}\) entry precedes Ca\(^{2+}\) responses (by 12.7 ± 0.9 s, n = 19 slices; Fig. 2a). This suggested that acidification-induced Ca\(^{2+}\) responses in astrocytes might be driven by elevations in [Na\(^{+}\)], and reversal of NCX. Activation of either NHE, NBC, or both might be responsible for the increases in [Na\(^{+}\)], in response to decreased pH. Acidification-induced [Ca\(^{2+}\)]\(_i\), and [Na\(^{+}\)]\(_i\), responses in astrocytes were not affected by the NHE inhibitor cariporide (10 μM, n = 39 cells; n = 7 slices; Fig. 2a). However, Na\(^{+}\) entry induced by a decrease in pH was abolished and Ca\(^{2+}\) responses were markedly reduced in the presence of NBC inhibitor S0859 (10 μM; n = 32 cells; n = 6 slices; p < 0.001, F(1,71) = 2540; Fig. 2b), or anion exchange inhibitor DIDS (10 μM) which also interferes with NBC; n = 18 cells; n = 3 slices; p > 0.001, F(1,36) = 484; Fig. 2f). NCX inhibitor SN-6 (10 μM) reduced (n = 27 cells; n = 3 slices; p < 0.001, F(1,66) = 540; Fig. 2c), while combined NBC/NCX blockade [S0859 (10 μM)/SN-6 (10 μM)] reversibly abolished, astroglial [Na\(^{+}\)], and [Ca\(^{2+}\)]\(_i\), responses triggered by acidification (n = 41 cells; n = 6 slices; p < 0.001, F(1,80) = 9722; Fig. 2d).

In a separate experiment, absolute level of resting and peak acidification-induced increases in [Na\(^{+}\)]\(_i\), were determined (Fig. 3). There was no difference in resting level of [Na\(^{+}\)], between pH-sensitive and pH-insensitive astrocytes recorded in organotypic slices (11.8 ± 0.2 mM, n = 84 cells, 4 slices vs 12.1 ± 0.1 mM, n = 93, 3 slices, respectively; p = 0.28). In chemosensitive astrocytes, [Na\(^{+}\)], peaked at 19.7 ± 0.6 mM (n = 84, 4 slices) in response to acidification (Fig. 3c).

Chemosensory [Ca\(^{2+}\)]\(_i\), responses recorded in ventral brainstem astrocytes expressing Ca\(^{2+}\) indicator Case12 in acute slices of adult rats were reduced by S0859 (n = 53 cells; n = 5 slices), DIDS (n = 27 cells; n = 5 slices), and SN-6 (n = 25 cells; n = 3 slices), and were not affected by NHE blockade by amiloride (0.5 mM; n = 16 cells; n = 3 slices; p > 0.05) or inhibition of phospholipase C with U73122 (10 μM; n = 37 cells; n = 4 slices; p > 0.05; Fig. 4a). Astroglial [Ca\(^{2+}\)]\(_i\), responses triggered by acidification in acute slices of the rat brainstem were reversibly reduced by 86% in conditions of combined NBC/NCX blockade [S0859 (10 μM)/SN-6 (10 μM); n = 37 cells; n = 4 slices; p < 0.001, F(1,51) = 177; Fig. 4b,c].

To identify the transporter(s) responsible for the high sensitivity of brainstem astrocytes to decreases in pH, brainstem and cortical (not sensitive to changes in pH; Kasymov et al., 2013) astrocytes were isolated and differences in the expression of genes
encoding all known NBCs and NHEs were analyzed (Table 1). Only the expression of SLC4a4 gene encoding electrogenic Na\(^+\)/H\(^+\)/HCO\(_3\)\(^-\) cotransporter NBCe1 was found to be consistently higher in the brainstem (vs cortex) across different experimental conditions: in cultured astrocytes, in astrocytes acutely isolated from the brains of neonatal (P3) rats, and in astrocytes acutely isolated from the brains of young adult rats (Table 1). Brainstem astrocytes also showed higher expression of another notable astroglial gene, KCNJ10 (Table 1), which encodes the Kir4.1 subunit of inwardly rectifying K\(^+\)/H\(^+\) channels (potential significance of high Kir4.1 expression for astroglial chemosensitivity is discussed below).

These transcriptome data suggested that different levels of NBCe1 expression may underlie regional differences in pH sensitivity between astrocytes and that NBCe1 is the key transporter responsible for acidification-induced Na\(^+\) entry and Ca\(^{2+}\)/H\(^+\) responses in chemosensitive astrocytes. To test this hypothesis, [Ca\(^{2+}\)/H\(^+\)]\(_i\) responses induced by decreases in pH were next assessed in brainstem astrocytes of NBCe1-deficient mice. Cultured brainstem astrocytes of wild-type mice responded to CO\(_2\)-induced acidification with increased rate of fast Ca\(^{2+}\)/H\(^+\) oscillations (n = 28 cells, n = 4 cultures; Fig. 5a,b,c; Kasymov et al., 2013). These acidification-induced [Ca\(^{2+}\)/H\(^+\)]\(_i\) responses were markedly reduced in conditions of NBCe1 deficiency (n = 45 cells, n = 4 cultures; p < 0.001, F\(_{1,292}\) = 81; Fig. 5b,c,d). Amplitudes of [Ca\(^{2+}\)/H\(^+\)]\(_i\) elevations induced by ATP (100 \(\mu\)M) were similar in cultured astrocytes of wild-type and NBCe1 knock-out mice (Fig. 5a,d), indicating that reduced frequency of pH-evoked [Ca\(^{2+}\)/H\(^+\)]\(_i\) oscillations is not due to the effect of NBCe1 deficiency on cellular Ca\(^{2+}\)/H\(^+\) recruitment/handling mechanisms.

### Table 1. NBC, NHE, and inwardly rectifying K\(^+\) channel gene expression differences between cortical and brainstem astrocytes

| Gene  | Culture | Neomate | Adult |
|-------|---------|---------|-------|
|       | Brainstem | Cortex | Fold difference | Brainstem | Cortex | Fold difference | Brainstem | Cortex | Fold difference |
| NBCs  | SLC4a4   | 53.3 | 13.4 | 4.0 | 836.9 | 140.2 | 6.0 | 421.1 | 18.3 | 23.0 |
|       | SLC4a5   | 4.5 | 0 | — | 1.8 | 4.5 | 0.4 | 5.1 | 0.4 | 12.8 |
|       | SLC4a7   | 9.5 | 14.0 | 0.7 | 8.2 | 16.4 | 0.5 | 7.4 | 24.0 | 0.3 |
|       | SLC4a8   | 0 | 0 | — | 0 | 0 | — | 0 | 0 | — |
|       | SLC4a9   | 0 | 0 | — | 0 | 0 | — | 0 | 0 | — |
|       | SLC4a10  | 3.3 | 0 | — | 11.2 | 2.4 | 4.7 | 2.0 | 5.6 | 0.4 |
|       | SLC4a11  | 0 | 0 | — | 0 | 0 | — | 0 | 0 | — |
| NHEs  | SLC9A1   | 13.2 | 18.6 | 0.7 | 28.0 | 39.9 | 0.7 | 20.8 | 39.2 | 0.5 |
|       | SLC9A2   | 0 | 0 | — | 0.5 | 0 | — | 0 | 1.9 | — |
|       | SLC9A3   | 0.3 | 0 | — | 0 | 0.1 | — | 0 | 0 | — |
|       | SLC9A4   | 0 | 0 | — | 0 | 0 | — | 0 | 0 | — |
|       | SLC9A5   | 2.0 | 1.3 | 1.5 | 1.2 | 3.5 | 0.3 | 0.3 | 0 | — |
|       | SLC9B1   | 0 | 0 | — | 0 | 0 | — | 0 | 0 | — |

Data are presented in fragments per kilobase of transcript sequence per million mapped fragments.

**Discussion**

Intracellular Ca\(^{2+}\) governs key functions of astrocytes, including gliotransmitter release (Araque et al., 2014), lactate pro-
Indeed, in brainstem astrocytes, opening (that operates via modulation of the connexin-26 hemichannel (Wenker et al., 2010).彼ears to be independent of, and does not contribute to, potassium channels (like Kir4.1/Kir5.1) and certain DIDS-sensitive current in ventral brainstem astrocytes. The parambil et al., 2014). Results of the present study suggest that in pH-sensitive astrocytes, acidification activates NBCe1, which brings Na\(^+\) inside the cell. Raising [Na\(^+\)]\(_i\) activates NCX to operate in a reverse mode, leading to Ca\(^{2+}\) entry (Fig. 6) followed by activation of the downstream signaling pathways.

Several previous studies addressed the potential mechanisms underlying the sensitivity of brainstem astrocytes to changes in pH (Gourine et al., 2010; Wenker et al., 2010, 2012; Kasymov et al., 2013). Recordings of changes in membrane potential of brainstem astrocytes in acute slices of neonatal rats demonstrated moderate depolarizations (by 4–9 mV) in response to acidification (in HEPS-buffered solution from pH 7.5 to 6.9 or in ACSF saturated with 10–15% CO\(_2\), pH 6.8–7.1; Ritucci et al., 2005; Wenker et al., 2010). The pharmacological profile of the CO\(_2\)/H\(^+\)-sensitive current in ventral brainstem astrocytes suggested involvement of inward rectifying potassium channels (like Kir4.1/Kir5.1) and certain DIDS-sensitive NBCs (Wenker et al., 2010). These results, however, did not provide an answer on how changes in intracellular or extracellular P\(_{CO2}\)/H\(^+\) trigger Ca\(^{2+}\) responses, since astrocytes in situ do not express voltage-operated Ca\(^{2+}\) channels (Carmignoto et al., 1998). Indeed, in brainstem astrocytes, depolarization by >20 mV by current injections failed to trigger [Ca\(^{2+}\)]\(_i\) responses in the recorded and neighboring astrocytes (Gourine et al., 2010). The same study reported that acidification-induced Ca\(^{2+}\) responses in brainstem astrocytes are not affected in the presence of various pharmacological agents that interfere with several potential targets, including pH-sensitive K\(^+\), TRPV, and TRPP channels (Gourine et al., 2010).

There is evidence that brainstem astrocytes possess mechanism(s) of direct (i.e., independent of changes in [H\(^+\)], [HCO\(_3\)\(^-\)], and [Ca\(^{2+}\)]\(_i\)) CO\(_2\) sensing (Huckstep et al., 2010) that operates via modulation of the connexin-26 hemichannel opening (Meigh et al., 2013), leading to CO\(_2\)-dependent release of ATP (Huckstep et al., 2010). This mechanism appears to be independent of, and does not contribute to, astroglial pH sensing, since acidification-induced Ca\(^{2+}\) responses in brainstem astrocytes are either not affected or only partially reduced by pharmacological agents that block functional connexin/pannexin hemichannels (Gourine et al., 2010).

Hirata and Oku (2010) reported that acidification (pH 7.1)-induced Ca\(^{2+}\) responses in glia-rich brainstem cultures are not observed in HCO\(_3\)\(^-\)-free HEPES-buffered medium. The current study was further motivated by the data reported recently that suggested that NBCe1 expressed in astrocytes is a high-affinity HCO\(_3\) carrier capable of fast and effective cytosolic H\(^+\) buffering (Theparambil et al., 2014; Theparambil and Deitmer, 2015). This NBC is rapidly activated in response to acidic stimuli and requires only micromolar extracellular [HCO\(_3\)\(^-\)] to operate (Theparambil et al., 2014). We hypothesized that NBC activation by decreased pH, leading to rapid HCO\(_3\)\(^-\) and Na\(^+\) entry may trigger Ca\(^{2+}\) responses by activation of NCX to operate in a reverse mode (Rojas et al., 2007; Kirischuk et al., 2012; Parpura and Verkhratsky, 2012). The data obtained in the present study showing that acidification-induced [Ca\(^{2+}\)]\(_i\) responses in astrocytes are preceded by Na\(^+\) entry, significantly reduced by NBC blockade and abolished in Na\(^+\)-free medium or in conditions of NBCe1 deficiency, provide strong supporting evidence of the key role played by Na\(^+\)/HCO\(_3\)\(^-\) cotransport in the mechanisms underlying pH sensitivity of brainstem astrocytes. Reversal of NCX following Na\(^+\) entry appears to be responsible for subsequent increases in [Ca\(^{2+}\)]\(_i\). This conclusion is supported by the results of electrophysiological studies that demonstrated that the membrane potential of pH-responsive brainstem astrocytes in HCO\(_3\)\(^-\)-buffered medium is between ~75 and ~82 mV (Wenker et al., 2010), which is very close to the calculated reversal potential of NCX (~80 mV; Kirischuk et al., 2012) set by a relatively high (15–20 mm) [Na\(^+\)]\(_i\) in astrocytes (Kirischuk et al., 2012; Parpura and Verkhratsky, 2012). Indeed, in pH-sensitive brainstem astrocytes, the resting [Na\(^+\)]\(_i\) was found to be ~12 mS, increasing to ~20 mS during the chemosensory challenge.

Ventral regions of the brainstem are populated by functionally specialized astrocytes, which are different (in terms of their high pH-sensitivity) from the majority of astroglia residing in other parts of the CNS (Kasymov et al., 2013). What makes some astrocytes chemosensitive, i.e., capable of mounting Ca\(^{2+}\) responses to changes in pH? Higher expression of certain membrane channels, which maintain membrane potential at an appropriate level for NCX reversal, may determine astroglial pH sensitivity. Inwardly rectifying K\(^+\) channels containing the Kir4.1 subunit are believed to be largely responsible for establishing the resting membrane potential in astrocytes (Olsen and Sontheimer, 2008). Comparative analysis of brainstem and cortical astroglial transcriptomes performed in this study revealed higher expression of the Kir4.1 subunit in chemosensitive astrocytes. Interestingly, in mice, conditional deletion of the Kir4.1 subunit in astrocytes dramatically reduces central respiratory CO\(_2\) chemosensitivity (Hawkins et al., 2014), suggesting that in conditions of an established deletion of the key astroglial membrane channel, brainstem astrocytes are not able to sense changes in pH. It appears, however, that the differences between pH-sensitive and pH-insensitive astrocytes lay upstream from NCX reversal. Indeed, the results of this study showed that acidification-induced [Ca\(^{2+}\)]\(_i\) responses are triggered by Na\(^+\) entry, which is not observed in pH-insensitive astrocytes (although the resting [Na\(^+\)]\(_i\) was found to be similar in two astroglial populations). Therefore, we next hypothesized that differential expression and activities of certain NBCs underlie differences in pH-sensitivity between astrocytes.
[Ca\(^{2+}\)]\(_i\) responses induced by decreases in pH were found to be markedly reduced in brainstem astrocytes of NBCe1-deficient mice. NBCe1 is critically important for homeostasis as these knock-out animals do not survive beyond the third week of life. They display profound metabolic acidosis (Gawenis et al., 2007) and breathing deficit may contribute to this harmful phenotype. An extension of this study would require development of a novel transgenic mouse line allowing assessment of the respiratory activity following selective conditional deletion of NBCe1 in (brainstem) astrocytes.

Current models of central respiratory CO\(_2\) chemosensitivity (i.e., mechanisms that detect changes in brainstem parenchymal P\(_{\text{CO}_2}\)/[H\(^+\)] and trigger adaptive changes in ventilation) are centered at a group of specialized pH-sensitive neurons of the retrotrapezoid nucleus located near the ventral surface of the medulla oblongata (Guyenet, 2014). These chemosensitive neurons are proposed to play the key role, with neighboring pH-sensitive astrocytes providing an additional 20–30% of the chemosensory drive to breathe (Guyenet, 2014). However, there is evidence that the sensitivity of retrotrapezoid nucleus neurons to decreases in pH is, to a large extent, mediated by prior release of gliotransmitters(s), primarily ATP (Gourine et al., 2010). In addition, increases in ventilation are triggered when pH-evoked [Ca\(^{2+}\)]\(_i\) responses in ventral brainstem astrocytes are mimicked by optogenetic stimulation (Gourine et al., 2010), while in mice astrocyte-specific conditional deletion of certain genes (MeCP2, Kir4.1) is sufficient to dramatically impair ventilatory CO\(_2\) chemosensitivity (Hawkins et al., 2014; Garg et al., 2015). It appears, therefore, that intact pH-sensitive retrotrapezoid nucleus neurons (Guyenet, 2014) are not able to mount an appropriate ventilatory response when astroglial function and pH sensitivity are compromised. Together these lines of evidence support the idea of an important role played by astroglial pH sensitivity in the brain mechanisms linking changes in brainstem parenchymal P\(_{\text{CO}_2}\)/[H\(^+\)] and central respiratory drive. The data obtained in the present study suggest that NBCe1 and NCX activities underlie functional CO\(_2\)/[H\(^+\)] sensitivity of brainstem astrocytes that contribute to homeostatic regulation of brain parenchymal pH and control of breathing.

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