Chronic continuous hypoxia decreases the expression of SLC4A7 (NBCn1) and SLC4A10 (NCBE) in mouse brain

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1Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut; 2Department of Physiology, Emory University, Atlanta, Georgia; 3Departments of Pediatrics and 4Neuroscience, University of California San Diego, La Jolla, California; and 5The Rady Children’s Hospital, San Diego, California

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Chen L-M, Choi I, Haddad GG, Boron WF. Chronic continuous hypoxia decreases the expression of SLC4A7 (NBCn1) and SLC4A10 (NCBE) in mouse brain. Am J Physiol Regul Integr Comp Physiol 293: R2412–R2420, 2007. First published October 10, 2007; doi:10.1152/ajpregu.00497.2007.—In the mammalian CNS, hypoxia causes a wide range of physiological effects, and these effects often depend on the stage of development. Among the effects are alterations in pH homeostasis. Na+/HCO3−coupled HCO3− transporters can play critical roles in intracellular pH regulation and several, such as NCBE and NBCn1, are expressed abundantly in the central nervous system. In the present study, we examined the effect of chronic continuous hypoxia on the expression of two electroneutral Na-coupled HCO3− transporters, SLC4a7 (NBCn1) and SLC4a10 (NCBE), in mouse brain, the first such study on any acid-base transporter. We placed the mice in normobaric chambers and either maintained normoxia (21% O2; 21% O2) for a duration of either 14 days or 28 days, starting from ages of either postnatal age 2 days (P2) or P90. We assessed protein abundance by Western blot analysis, loading equal amounts of total protein for each condition. In most cases, hypoxia reduced NBCn1 levels by 20–50%, and NCBE levels by 15–40% in cerebral cortex, subcortex, cerebellum, and hippocampus, both after 14 and 28 days, and in both pups and adults. We hypothesize that these decreases, which are out of proportion to the expected overall decreases in brain protein levels, may especially be important for reducing energy consumption.

electroneutral; bicarbonate transporter; SLC4; central nervous system

HYPOXIA. A LOW-OXYGEN LEVEL in tissue, may be either continuous or intermittent. Chronic continuous hypoxia (CHH) may occur during normal events, such as embryonic development and ascent to altitude, and in pathological states, such as pulmonary disease (i.e., decreased O2 uptake), anemia (i.e., decreased O2 content in blood), ischemia (i.e., decreased blood flow to tissues), and cancer (i.e., increased O2 utilization by tissues). Chronic intermittent hypoxia (CIH) occurs during obstructive sleep apnea. Due to its very high energy demands, the mammalian brain is particularly sensitive to hypoxia.

Mammals immediately respond to hypoxemia by increasing first alveolar ventilation and then heart rate. The central nervous system (CNS) also rapidly responds with several metabolic adaptations (3, 39, 40, 44, 54, 74, 78). Over a longer period of time, the acclimation to hypoxemia includes both functional and structural changes in many tissues, including the CNS (for reviews, see Refs. 35, 39, and 74). Structural changes in response to chronic hypoxia include a decrease in body mass and an increase in capillary density, which in the brain can amount to a doubling over a period of ~4 wk (7, 41) (for reviews, see Refs. 39 and 74). The angiogenic response is under the control of hypoxia-inducible factor-1 (for reviews, see Refs. 29, 33, and 64) and angiopoietin-2 (39, 48).

A systemic consequence of the hypoxia-induced hyperventilation is a fall in the arterial CO2 partial pressure (respiratory alkalosis) and a compensatory fall in plasma HCO3− concentration. At the level of brain tissue, hypoxia induces complex changes in extra- as well as intracellular pH (pHi; for a review, see Ref. 76). Electrode measurements show that ischemia in intact brain (69) can lead to a large fall of extracellular pH and a large rise in extracellular CO2 concentration. Similarly, anoxia in hippocampal slices causes a fall in extracellular pH (36). Regarding pH, at first glance, it appears that acute hypoxia produces variable effects, sometimes a fall in pH (43, 58, 69), sometimes a rise (19, 44, 75), and sometimes a fall followed by a rise (22, 43, 65). However, it is likely that the intrinsic effect of acute hypoxia on neuronal pH is reproducible but depends in important ways on species (i.e., rat vs. mouse), cell type (e.g., brainstem vs. hippocampus), preparation (i.e., slices vs. acutely dissociated cells vs. cells in primary culture), temperature (i.e., room temperature vs. 37°C), and especially on the buffer (i.e., HCO3−-free vs. CO2/HCO3−; see Ref. 75), and the presence vs. the absence of glucose (22, 43).

Underlying at least part of the above hypoxia-induced changes in pH are almost certainly changes in acid-base transporters that play critical roles in the regulation of the pH of cells and of brain extracellular fluid and cerebrospinal fluid. These transporters include: 1) several members of the SLC9 family of Na+/H+ exchangers (NHE; for reviews, see Refs. 47 and 66), and 2) several members of the SLC4 family of HCO3− transporters (59). The latter includes the Na+−independent Cl−/HCO3− anion exchangers (AE1–3), of which AE2 (42) and AE3 (34, 37) are expressed in brain, and five characterized Na+/HCO3− cotransporters (NCTs), all of which are expressed in the brain. The NCTs include two electrogenic Na+/H+/CO2 cotransporters-NBCC1 (4, 26, 60) and NBCC2 (11, 62, 68), which may not play a major role in neuronal pH regulation, as well as three electroneutral members, the electroneutral Na+/HCO3− cotransporter NBCn1 (8, 16, 18, 52, 55), the Na+/HCO3−-driven Cl−/HCO3− exchanger NDCBE (27), and NCBE (13, 25, 51, 70). The electroneutral NCTs function as acid extruders (i.e., mediating a net uptake of HCO3− and raising pH) and play key roles in pH regulation in at least hippocam-
pal pyramidal neurons (63). Immunocytochemistry studies demonstrate that NBCn1 and NCBE are localized to the basolateral membrane of choroid-plexus epithelial cells (11, 13, 51). In cultured hippocampal neurons, both NBCn1 (18) and NCBE (13) are present on the somatodendritic plasma membranes.

CIH differs from CCH in several important ways (for a review, see Ref. 46). For a given intensity and duration, CIH produces greater effects on transcriptional activation. Unlike CCH, CIH signals via increased reactive oxygen species and other pathways not used during CCH. CIH is also distinctive in being associated with a reversible deterioration in memory and motor function (2), as well as with changes in lipids, insulin, and other metabolic pathways (for a review, see Ref. 50). Douglas et al. (23) examined the effect of CIH on the expressions of several different acid-base transporters, including NHE1, NHE3, NHE4, NBCe1, and AE3. They found that CIH decreased the protein expression of NHE1, NHE3, and NBCe1 in mouse brain. In the present study, we examined the effect of CCH (14- or 28-days duration) using two newly developed antibodies, on the expression of NBCn1 and NCBE in the CNS of both neonate pups and adult mice. To our knowledge, this is the first study of the effects of CCH on the expression of any acid-base transporter. We found that hypoxia generally reduced the protein levels of both NBCn1 (by 20–50%) and NCBE (by 15–40%) compared with normoxic mice.

MATERIALS AND METHODS

Antibodies

The polyclonal antibody against NBCn1 antibody is a new antibody that we generated by immunizing a rabbit with a maltose binding protein (MBP)-fusion protein including an 87-residue peptide (KREL ... ETSL) that corresponds to most of the predicted cytoplasmic COOH terminus of rat (r) NBCn1-B (16). Note that NBCn1-B, as well as NBCn1-A, lack the 36-residue cassette that is present in NBCn1-D (16).

For characterization of the new NBCn1 antibody, we fused enhanced green fluorescent protein (EGFP) to rNBCn1-B (GenBank accession no. NM_058211) and human (h) NDCBE-B (Gene Bank accession no. NM_004585) as described previously (13). We also used hNDCBE-A (GenBank accession no. AAY79176) and hNCBE-B (GenBank accession no. AY376402). We made cRNA, prepared and injected oocytes with the cRNA, and incubated the oocytes at 18°C for 4–5 days, and then made oocyte membrane protein preparations as described previously (13). The polyclonal antibody against NCBE was previously described (13).

Chronic Exposure of Mice to Hypoxia

CD1 mice from Charles River Laboratories (Raleigh, NC) were subjected to either normoxia or chronic hypoxia according to protocols approved by the Animal Care and Use Committees at Albert Einstein College of Medicine and University of California San Diego. As described previously (38), we used a computer-controlled system to control the inspired O2 concentration in normobaric chambers (OxyCycler; Reming Bioinstruments, Redfield, NY). In each experiment, we randomly assigned animals to normoxic and hypoxic groups. The mice entered the chamber at the postnatal age of 2 days (P2) in the case of pups, or at the age of P90 in the case of adults. We housed one group of mice under conditions of normoxia and simultaneously housed the other paired, age-matched group under conditions of CCH (11% O2). The treatments lasted for 14 or 28 days. The mice were then anaesthetized with halothane by inhalation and killed. The brains were dissected into four regions: cerebral cortex, subcor- tex, cerebellum, and hippocampus, according to the definition previously stated (23). Tissues were dissected and immediately frozen in liquid nitrogen and then stored at –80°C until they were processed for preparation of membrane proteins.

Preparation of Membrane Proteins

For brain analyses, we pooled frozen mouse brain tissue from animals (8 pups or 5 adult mice) of the same group that together had been treated under hypoxic conditions for either 14 or 28 days. We similarly pooled tissue from paired animals that together had simultaneously been treated under normoxic conditions and performed the following protocol simultaneously for each of the two pools of tissue. For the purposes of statistics, we count each such pool or group as one experiment. Each pool of frozen tissue was placed in ice-cold Na+-phosphate buffer (in mM: 7.5 NaH2PO4, 250 sucrose, 5 EDTA, 5 EGTA, pH 7.0) containing 1% protease inhibitor cocktail for mam-malian tissues (cat. no. P8340; Sigma-Aldrich, St. Louis, MO) and was homogenized by 10 strokes of a Teflon pestle probe on a homogenizer (Glas-Col, Terre Haute, IN) rotating at 5,000 rpm. To remove cellular debris, we then centrifuged the homogenate at 3,000 g at 4°C for 15 min. We then subjected the supernatant to ultracentrifugation at 100,000 g at 4°C × 1 h. The pellet was resuspended in protein-suspension buffer (in mM: 20 Tris-HCl, 5 EDTA, pH 8.0) containing 5% SDS. We measured the total protein concentration with the bicinchoninic acid protein assay reagent (cat. nos. 23228 and 23224; Pierce, Rockford, IL) following the manufacturer’s protocol and stored the membrane protein preparations in aliquots at –80°C until used.

Western Blot Analysis

Membrane proteins were mixed with 2× SDS-sample loading buffer containing 6 M of urea and were denatured for 8 min at 95°C. The proteins (25 μg/lane) were then separated on SDS-polyacrylamide gel and transferred onto Immuno-Blot polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The blots were blocked in TBST (in mM: 10 Tris-HCl, 150 NaCl, 0.1% Tween 20, pH 7.5) containing 5% Nestle powdered milk at 4°C overnight. We then incubated the blots with primary antibody in 1% powdered milk in TBST at room temperature for 2 h and then washed them five times for 6 min with TBST. Afterward, we incubated the blots with secondary antibody in TBST containing 1% powdered milk at room temperature for 1 h and then washed them five times for 6 min with TBST. We performed chemiluminescent detection with ECL Plus Western blot analysis reagents (Amersham Biosciences) prior to X-ray film exposure.

Deglycosylation Reaction

We mixed 80 μg of a mouse brain membrane protein preparation with 10× denaturing buffer and then boiled the mixture for 10 min at 98°C. We then added 2 μl PNGase F (500 U/μl, New England BioLabs, Ipswich, MA) and incubated the mixture for 1 h at 37°C as previously described (13).

Statistics Analysis

An unpaired two-tailed Student’s t-test was performed to examine the difference in body weight or hematocrit between the normoxic and corresponding hypoxic mice. A paired two-tailed Student’s t-test was applied to analyze the density data of Western blots of paired groups of mice subjected simultaneously to normoxia or hypoxia. To compare the values of NBCn1 (or NCBE) in different regions, we performed a one-way ANOVA and a Student-Newman-Keuls Multi- ple Comparison test, using KaleidaGraph (Version 4, Synergy Software). Data are presented as means ± SE. The difference between means was considered statistically significant at P < 0.05.
RESULTS

Effect of Continuous Chronic Hypoxia on Body Weight and Hematocrit

Figure 1 summarizes the effect of CCH on body weight and hematocrit. Hypoxia caused body weight (1A) to decrease by 10–35%, the effect being greatest on a percentage basis in pups subjected to hypoxia for 14 days (a 35% decrease). Similar results have been observed by colleagues (23, 24, 32). Conversely, hypoxia caused the hematocrit (Fig. 1B) to increase by 16–40%, the effect being least on a percentage basis for pups at 14 days. Under hypoxic conditions, the other three groups increased their hematocrits to ~70%. Similar results have been observed by colleagues (24, 32). These results confirm that the animals exhibited hallmarks of CCH.

It is interesting that it was the pups treated for 14 days that exhibited both the greatest decrease in body weight and the smallest increase in hematocrit. In early life, mice, like humans, experience a physiological anemia, which may reflect hemolysis and iron deficiency, as well as reduced sensitivity to hypoxia. These effects could account for the blunted hematocrit response in the pups at 14 days.

Characterization of the New NBCn1 Antibody

As outlined in MATERIALS AND METHODS, we generated a new rabbit polyclonal antibody against the COOH terminus of rat NBCn1. We validated the new antibody by Western blot analysis. Fig. 2A shows a Western blot of the immunogen, which has a predicted molecular mass of 52.6 kDa. Fig. 2, B and C shows Western blots of membrane preparations from oocytes injected with either H2O or cRNA encoding full-length versions of four closely related SLC4 members: NCBE-B, NBCn1-B-EGFP, NDCBE-A-EGFP, or NDCBE-B-EGFP. These data show that the new NBCn1 antibody recognizes NBCn1-B-EGFP, but does not cross-react with NCBE-B, NDCBE-A-EGFP, nor NDCBE-B-EGFP.

Fig. 3A shows a Western blot of membrane protein preparations from the cerebral cortex and hippocampus of mouse brain. The antibody recognizes a band at molecular mass ~150 kDa; the predicted unglycosylated molecular mass of the various NBCn1 splice variants is from 134.4 (NBCn1-C) to 139.9 (NBCn1-D) kDa. When we preabsorbed the primary antibody with the immunogen, the 150-kDa bands disappeared (Fig. 3B). Deglycosylation treatment with PNGase F reduced the molecular mass of NBCn1 to ~130 kDa (Fig. 3C). Thus, all detectable NBCn1 expressed in mouse brain is N-glycosylated.

Effects of CCH on the Expression of NBCn1 and NCBE

Hypoxia pups of 14-days duration. Figure 4A, top shows typical Western blot analysis and Figure 4A, bottom shows the mean densitometric data for NBCn1 in four brain regions:
cerebral cortex (CX), subcortex (SCX), cerebellum (CB), and hippocampus (HC) of mouse pups subjected to normoxia or chronic hypoxia for 14 days. As noted in the presentation of Fig. 3A, the antibody recognized a band at ~150 kDa. Hypoxia caused a significant decrease in the levels of NBCn1 protein in all four regions: by 28% in CX, 31% in SCX, 39% in CB, and 27% in HC.

Fig. 4B shows analogous Western blot analysis and densitometric data for NCBE obtained from the same mice as in Fig. 4A. The antibody recognized a band at ~150 kDa. As was the case for NBCn1, hypoxia of 14-days duration caused a decrease in the expression of NCBE protein in all four brain regions: by 32% in CX, 34% in SCX, 26% in CB, and 46% in HC.

Hypoxia pups of 28-days duration. Fig. 5A shows typical Western blot analysis and mean densitometric data for NBCn1 in four brain regions of mouse pups subjected to normoxia or chronic hypoxia for 28 days. Hypoxia caused a significant decrease in the expression of NBCn1 in all four brain regions: by 29% in CX, 35% in CB, 42% in SCX, and by 31% in HC. Fig. 5B shows analogous Western blot analysis and densitometric data for NCBE, obtained from the same mice as in Fig. 5A. In addition to the band at ~150 kDa, the antibody recognized a fainter band in SCX and CB at ~127 kDa (predicted unglycosylated molecular mass for NCBE splice variants: 122.5–128.3 kDa). As noted previously, this band may correspond to a protein that has only a core glycosylation (13). Our densitometry data in Fig. 5B include both bands. As was the case for NBCn1, hypoxia of 28-days duration caused a significant decrease in the levels of NCBE protein: by 16% in CX, 29% in CB, and 38% in HC. The difference in means was not statistically significant in SCX. A separate analysis shows that hypoxia did not have a significant effect on the distribution of NCBE protein between the 150- and 127-kDa bands.

Hypoxia adults of 14-days duration. Fig. 6A shows a typical Western blot analysis and mean densitometric data for NBCn1 in four brain regions of adult mice (P90 at the start of the challenge) subjected to normoxia or chronic hypoxia for 14 days. Hypoxia caused a significant decrease in levels of NBCn1 protein in all four brain regions: by 42% in CX, 44% in SCX, 42% in CB, and 19% in HC.

Fig. 6B shows analogous Western blot analysis and densitometric data for NCBE, obtained from the same mice as in Fig. 6A. As was the case for NBCn1, hypoxia of 14-days
Indeed, pH changes in the CNS can affect a wide variety of events, including enzyme activity, signal transduction, synaptic transmission, and neuronal excitability (20). Like other cells, those of the CNS employ mechanisms for the homeostasis of intra- as well as extracellular pH, including intrinsic cytosolic buffering of H\(^{+}\), sequestering of H\(^{+}\) into intracellular organelles, and transport of acid-base equivalents across the cell membrane (for reviews, see Refs. 14, 15, 20, and 61).

**NBCn1.** The protein is expressed in embryonic and adult hippocampal neurons, and some NBCn1 colocalizes with the postsynaptic density marker PSD-95 (18). Because NBCn1 is associated with a Na\(^{+}\) conductance (16), it may not only regulate pH\(_{i}\) but also modulate membrane potential and excitability. In mice, knockout of NBCn1 leads to blindness and deafness (8), similar to the symptoms of Usher Syndrome 2B. Indeed, this genetic disease maps to a genetic locus that is virtually identical to that of the SLC4A7 gene (30). Immunocytochemistry demonstrates that NBCn1 is present at the basolateral membrane of the choroid-plexus epithelium where this transporter is probably responsible for DIDS-insensitive Na\(^{+}\)-coupled HCO\(_{3}^{-}\) transport (11). Cultured brain endothelial cells also express high levels of NBCn1 mRNA, and NBCn1 may contribute significantly to HCO\(_{3}^{-}\) transport by these cells (67). Finally, because NBCn1 appears to play an important role in pH\(_{i}\) regulation in vascular smooth muscle cells elsewhere in

**Distribution of NBCn1 and NCBE in the Brain**

The homeostasis of pH in the CNS, both in the cells and the brain extracellular fluid, is critically important (14, 15, 20).
numbers come from the raw densitometry data that are the changes as a function of time under normoxic conditions. The fractional distribution of NBCn1 among four brain regions—cerebral cortex, subcortex, cerebellum, and hippocampus—summarizes how NBCn1 contribution varied across these regions. The relative abundance of NBCn1 in SCX was relatively stable. The relatively abundant expression of NCBE in CX and HC suggests it might play important roles in those regions.

**Differences in NBCn1 vs. NCBE expression patterns.** Figs. 2, B and C show that our new NBCn1 antibody is specific for the body (6), it is reasonable to postulate that it plays a similar role in the CNS as well.

**NCBE.** Like NBCn1, NCBE is present at the basolateral membrane of the choroid-plexus epithelial cells (11, 13, 51) where the two transporters may play important roles in the production of cerebrospinal fluid (51). Also like NBCn1, NCBE is present in cultured brain endothelial cells (67). By in situ hybridization, NCBE is expressed in hippocampal CA1–3 regions as well as the dentate gyrus, cerebellar Purkinje cells, and astrocytes (25). By Western blot analysis Chen LM, et al. (13) found that NCBE is strongly expressed in cerebral cortex, hippocampus, and cerebellum, both in crudely dissected tissue samples like those in the present study and microdissected samples like those in the present study and microdissected studies verified by the absence of AQPI protein to be virtually free of contamination from the choroid plexus. Thus, the dominant NCBE signals in the Western blots of the present study almost certainly originate outside of the choroid plexus. Finally, immunocytochemistry studies have demonstrated that NCBE is expressed on the plasma membrane of cultured hippocampal neurons (13).

**Time course of NBCn1 expression.** Fig. 8A summarizes how the fractional distribution of NBCn1 among four brain regions changes as a function of time under normoxic conditions. The numbers come from the raw densitometry data that are the basis for Figs. 4A (P16), 5A (P30), 6A (P104), and 7A (P118). Because it is not possible to compare raw densitometry data from one time point to another (because they are derived from different gels), at each time point we summed the densities for the four brain regions and then determined the fraction of 100% that each region contributed. In P16 pups, NBCn1 is most highly expressed in CX and HC. With increasing age, the NBCn1 in HC tends to represent an increasing fraction of the total NBCn1 in the four brain regions, while NBCn1 in CX tends to represent a decreasing fraction. At P118, the HC contained twice as much NBCn1 (per mg total protein) as any of the other regions. The relative abundance of NBCn1 in SCX and CB was relatively stable. The relatively abundant expression of NBCn1 in HC suggests it might play important roles in corresponding HC.

**Time course of NCBE expression.** Fig. 8B is comparable to Fig. 8A except for NCBE. The numbers come from the raw densitometry data that are the basis for Figs. 4B (P16), 5B (P30), 6B (P104), and 7B (P118). In P16 pups, NCBE is most highly expressed in CX and HC. With increasing age, the relative abundance in the four brain regions does not change significantly. The relatively abundant expression of NCBE in CX and HC suggests it might play important roles in those regions.
Bicarbonate Transporters

Effect of Hypoxia on the Expression of Bicarbonate Transporters

Hypoxia generally leads to suppression of pathways both of ATP utilization and of ATP synthesis (31). An early example of how a decrease in intracellular ATP concentration ([ATP]) can reduce the activity of a transporter that depends on the Na⁺ gradient—not ATP per se, for its energy is the blockade by low [ATP] of Na⁺-driven Cl/HCO₃ exchanger of the squid giant axon (10). In mammalian cells, AMP-activated protein kinase, which can be switched on by stresses such as hypoxia and ischemia, appears to be a critical fuel sensor for regulating cellular pathways of energy consumption and production (28, 56).

Hypoxia suppresses both protein synthesis, mediated in part a rapid downregulation of mRNA translation (for a review, see Ref. 72), and protein degradation (31). However, hypoxia leads to an increase in protein synthesis in certain “rescue” pathways (31), such as angiogenesis (for reviews, see Refs. 29, 33, and 64).

Hypoxia generally decreases the expression of transporters and ion channels. At the protein level, hypoxia downregulates the Na⁺/Ca⁺ exchanger-1 in cortical astrocytes (1), the α-subunits of voltage-gated K⁺ channels in pulmonary arterial smooth muscle cells (71) and in freshwater turtle brain (53), and NHE1 and NHE3, as well as NBCe1, in mouse brain (23). In turtles subjected to anoxia for 3–21 days at 3°C, protein levels of the NR1 subunit of the NMDA receptor decrease markedly (5). In developing mouse brain, intermittent hypoxia decreases levels of certain voltage-gated Na⁺ channels after 2 wk, but increases the levels after 4 wk (77).

On the mRNA level, hypoxia downregulates the ENaC epithelial Na⁺ channel and the Na⁺-K⁺ pump in cultured alveolar epithelial cells (49; for a review, see Ref. 17). Chronic hypoxia induces a decrease in GLUT1 expression in the adult rat brain but an increase in the fetal as well as in the developing brain (73). Chronic hypoxia also raises the mRNA and channel currents of T-type voltage-gated Ca²⁺ channels but not of other types of Ca²⁺ channels in pheochromocytoma-derived PC12 cells (21).

In the present study, we examined the effect of CCH of two durations (14 and 28 days) on the expression of two electro-neutral NCBTs (i.e., NBCn1 and NCBE) in four brain regions (i.e., CX, SCX, CB, and HC) of mice of two ages (i.e., pups and adults). Hypoxia caused a significant decrease in the abundance of both NBCn1 and NCBE protein for both ages of animals subjected to hypoxia for both durations in at least three of the four mouse-brain regions examined. Only in two cases, both involving the SCX, did the decrease fail to reach statistical significance, once for NBCn1 in pups subjected to 28 days of hypoxia (P = 0.08) and once for NCBE in adults subjected to 14 days of hypoxia (P = 0.055). Note that we loaded equal amounts of protein onto each lane of our gels for Western blot analysis. Thus, if hypoxia led to a fall in total brain protein in each of our conditions (31), as one might surmise from the decrease in body weight, the fall in NBCn1 and NCBE levels were out of proportion to the fall in overall protein levels.

Note that at this stage we do not know the extent to which the decreases in NBCn1 and NCBE protein levels are general or restricted to particular cell types. For example, because hypoxia increases brain capillarity (7, 41), and the capillary endothelia contain both NBCn1 and NCBE (67), hypoxia might raise overall NBCn1 and NCBE protein levels in capillaries. However, this effect, if it occurs, must be overwhelmed by decreases in other cell types, most likely neurons and/or astrocytes. As we discussed above, both NBCn1 (18) and NCBE (13) are expressed in neurons. Moreover, at least in the case of NCBE, hippocampal astrocytes do not express detectable levels of protein (13). Thus, it is reasonable to hypothesize that CCH decreases the expression of NCBE, and perhaps NBCn1 as well, in at least some, though perhaps not all, neurons.

We also note that we do not know the extent to which the changes we observed reflect pathology per se (i.e., an unavoidable consequence of low O₂ concentration) vs. a prosurvival adaptation [i.e., a programmed response that, in principle, one ought to be able to block (23)].

In principle, CCH could modulate 1) levels of mRNA, 2) levels of protein, 3) fraction of the protein that is actually in the plasma membrane, and 4) the activity of individual protein molecules in the plasma membrane. In the present study, we find that CCH substantially decreases brain protein levels of NBCn1 and NCBE. However, even if CCH in fact reduces overall NBCn1 and NCBE activity, the result would not necessarily be a decrease in steady-state pHᵢ, which depends on balance between acid-extrusion and acid-loading processes (9). For example, if CCH produced similar reductions in overall acid extrusion and overall acid loading, steady-state pHᵢ would not change. Indeed, Musch et al. (45) found that chronic hypoxia caused no change in brain pH (presumably representing some average pHᵢ of neurons and astrocytes). In fact, if CCH inhibited acid loading more than acid extrusion, steady-state pHᵢ could even rise, as Rios et al. (57) reported for pulmonary arterial smooth muscle cells.
We emphasize that monitoring steady-state values of pH_i would not tell the whole story of pH regulation. For example, even if steady-state pH_i were stable in the face of depressed acid-extruding activity, we would predict that the cell would recover more slowly from acute intracellular acid and alkali loads (9), perhaps making the cell more susceptible to the consequences of wide swings in pH_i.

Conclusions and Hypotheses

We find that CCH of both 14- and 28-days duration leads to decreased protein levels (except in two cases of near statistical significance in SCX) of two important electroneutral bicarbonate transporters, NBCn1 (SLC4A7) and NCBE (SLC4A10), in each of several brain areas of both pups and adults mice. These decreases, which are out of proportion to a likely overall decrease in brain protein levels, could reduce energy consumption by at least three mechanisms. First, if the decreases reflect a reduction in NBCn1 and NCBE synthesis, this effect would reduce energy consumption. Second, the reduced expression of NBCn1 and NCBE might reduce the load of Na^+ pump and further lowering ATP consumption. Third, decreases in NBCn1 and NCBE expression could contribute to shifts in brain intra- or extracellular pH that could reduce neuronal excitability and thus reduce energy consumption even more. Although reduced NBCn1 and NCBE expression could well have a negative impact on pH_i regulation, an important housekeeping function in which the cell invests considerable resources during times of normoxia, compromised pH_i regulation may be worth the price if the concomitant advantages of reduced energy consumption promote cell survival.

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