Carbonic Anhydrase II in the Developing and Adult Human Brain

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**Abstract**

Carbonic anhydrase II (CA II) is one of 14 isozymes of carbonic anhydrases, zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide to bicarbonate. Mutations in CA II in humans lead to osteopetrosis with renal tubular acidosis and cerebral calcifications, a disorder often associated with mental retardation. Recently, new avenues in CA II research have opened as a result of discoveries that the enzyme increases bicarbonate and proton fluxes and may play an important role in brain tissue. In the human brain, CA II was localized to oligodendrocytes, myelin, and choroid plexus epithelium. Because this conclusion was based on a few fragmentary reports, we analyzed in more detail the expression of the enzyme in human telencephalon. By immunohistochemistry, CA II was found to be present not only in oligodendrocytes and choroid plexus epithelium (declining with aging in both these locations), but also in a subset of neurons mostly with GABAergic phenotype, in a few astrocytes, and transiently during brain development in the endothelial cells of microvessels. The enzyme also occurred in oligodendrocyte processes in contact with myelinating axons, myelin sheaths, and axolemma, but was either absent or appeared in minute amounts in compact myelin. These findings suggest the possible involvement of CA II in a wide spectrum of biologic processes in the developing and adult human brain and may contribute to better understanding of the pathogenesis of cerebral calcifications and mental retardation caused by CA II deficiency.

**Key Words:** Carbonic anhydrase II, GABAergic neurons, Human brain, Microvessels, Myelin, Oligodendrocytes.

**INTRODUCTION**

The carbonic anhydrases (CAs) represent a group of zinc metalloenzymes that occur in mammals in 14 different isoforms (1). They catalyze the reversible hydration of carbon dioxide to bicarbonate according to the equation

\[
\text{CO}_2 + \text{EZn}^{2+} + \text{OH}^- \rightleftharpoons \text{EZn}^{2+} - \text{HCO}_3^- \rightleftharpoons \text{EZn}^{2+} + \text{OH}^- + \text{H}^+
\]

that is followed by the transfer of a proton to regenerate the zinc-bound hydroxide:

\[
\text{EZn}^{2+} + \text{OH}_2^- \rightarrow \text{EZn}^{2+} - \text{OH}^- + \text{H}^+
\]

CAs are expressed abundantly by erythrocytes, in which they accelerate carbon dioxide hydration/dehydration between 13,000- and 25,000-fold, allowing efficient and rapid removal of carbon dioxide produced during oxidative respiration in the human body (2).

Recent studies demonstrated that at least 2 CA isozymes—CA II and CA IV—not only produce bicarbonate during hydration of carbon dioxide, but also increase bicarbonate fluxes by interacting with bicarbonate transporters of the Cl⁻/HCO_3⁻ anion exchangers family, some isoforms of Na⁺/HCO_3⁻ cotransporters, and some members of the SLC26 family (3). Furthermore, CA II also interacts with NHE1, an isoform of the mammalian Na⁺/H⁺ exchanger (NHE), which mediates the exchange of one extracellular sodium ion for one intracellular proton (4). Thus, because of their effect on generation and secretion of bicarbonate and H⁺, CAs also contribute to regulation of electrolyte and water balance, pH homeostasis, vasodilatation, and several metabolic pathways such as gluconeogenesis, lipogenesis, ureagenesis, bone resorption and calcification, tumorigenicity, and formation of cerebrospinal fluid and gastric acid. Inhibitors of CAs have already been used as antiglaucoma agents, diuretics, and hypoglycemic and anticonvulsive drugs and have potential application in the treatment of obesity and certain types of cancer (1, 5).

Isozymes of CAs are cytosolic (CA I–III, CA VII, CA XIII), membrane-bound (CA IV, CA IX, CA XII, CA XIV), mitochondrial (CA V), or secreted (CA VI). Some of these enzymes are widely distributed in various types of cells, tissues, and organs, whereas the others are restricted to specific types of cells (1, 6). Recent data indicate that several isoforms of CAs are expressed in the central nervous system (CNS); however, the role of individual isoforms in the function of the
Carbonic Anhydrase II in the Brain

MATERIALS AND METHODS

Human Subjects

Formalin-fixed and paraffin-embedded brain tissues from 2 embryos (6–7 wgs), 24 fetuses (11–40 wgs), and 30 neonates, children, and adults (ages 1 day to 83 years) were obtained from the Department of Developmental Neurobiology, the New York State Institute for Basic Research in Developmental Disabilities (IBR), Staten Island, New York; the Donald W. Reynolds Department of Geriatrics, University of Arkansas for Medical Sciences, College of Medicine, Little Rock, Arkansas; the Department of Neuropathology, the Institute of Psychiatry and Neurology, Warsaw, Poland; and the Department of Developmental Neuropathology, Medical Research Center, Polish Academy of Sciences, Warsaw, Poland.

Frozen brain tissues from 20 individuals (ages 17 wgs to 20 years) were obtained from the Brain Bank at IBR and the Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, Maryland. The study was approved by the IBR Institutional Review Board.

Immunohistochemistry and Immunofluorescence

Ten-micrometer-thick sections were deparaffinized in xylene, rehydrated in alcohol, and washed extensively with water. For antigen retrieval, sections were boiled 3–5 minutes in 10 mM citrate buffer (pH 6.0) in a microwave oven and then cooled for 20 minutes at room temperature. Endogenous peroxidase activity was blocked with 0.3% H2O2 in methanol for 20 minutes and then nonspecific binding sites were blocked with 10% fetal calf serum (FCS) in phosphate-buffered saline (PBS). Tissues were stained either with polyclonal antibodies (pAbs) to CA II-biotin conjugated (United States Biological, Swampscott, MA) diluted 1:2,000 (5 μg/mL) or with unconjugated pAbs to CA II (Rockland Immunologicals, Gilbertsville, PA) diluted 1:4,000 to 1:10,000 (1.0–2.5 μg/mL). Both these pAbs were raised in rabbits and highly purified from monospecific antisera by delipidation, fractionation, and ion exchange chromatography. Selected consecutive sections were also incubated with monoclonal antibodies (mAbs) to glutamic-acid decarboxylase 67 (GAD67), a marker for GABAergic interneurons (Sigma-Aldrich, St. Louis, MO), diluted 1:4,000. The incubation with primary antibodies diluted in 10% FCS in PBS lasted overnight at 4°C. Afterward, the sections were incubated with biotinylated species-specific secondary antibodies (Amersham Pharmacia, Piscataway, NJ) diluted 1:200 and then with extravidin–peroxidase-conjugated (Sigma), diluted 1:400, both for 1 hour at room temperature. Diaminobenzidine (0.5 mg/mL) in the presence of 0.03% hydrogen peroxide was used as a chromogen. Sections were lightly counterstained with hematoxylin. Control of the method included omission of the primary antibodies and preincubation of the primary antibodies with purified human CA II and CA I (Sigma) at various concentrations (2.5, 10, 16, and 50 μg/mL) in FCS/PBS for 4 hours at 4°C.

For double and triple immunolabeling, after antigen retrieval and blocking of nonspecific binding sites, as detailed previously, sections were incubated overnight at 4°C with pAbs to CA II and either mAbs to glial fibrillary acidic protein, an astrocyte marker, diluted 1:400 (Sigma); mAbs to 2′,3′-cyclic

CNS still awaits elucidation. A compelling line of evidence indicates that CAs may contribute significantly to signal processing, long-term synaptic transformation, and attentional gating of memory storage through the modulation of inotropic GABA–A receptor channels in the hippocampus (7).

CA II was the first isoform of CAs purified, initially from bovine (8) and then from human erythrocytes (9). It is one of the fastest enzymes identified to date, with a turnover rate of 108 s–1 at 37°C, and is widely distributed in various organs (6). The genetic defect of CA II is associated in humans with osteopetrosis with renal tubular acidosis and cerebral calcifications, a rare, autosomal-recessive disorder (10–12). Developmental delay with usually mild to moderate and, rarely, severe mental retardation occurs in approximately 90% of affected individuals (6, 13–15). Neuropathologic description of this syndrome is unavailable in the literature, and the cause of mental retardation in affected children is at present uncertain, although it appears not to be directly associated with cerebral calcifications (16). Interestingly, our proteomic studies showed increased expression of CA II in the brain of Ts65Dn mice, a mouse model of Down syndrome, as well as in the neocortex of the developing Down syndrome brain (Palminiello et al. unpublished observation), which points further to the possibility that CA II might be associated with cognitive processes in humans.

Although the distribution of CA II has been studied in the brain of various animal species, the results of these studies have not always been consistent. Choroid plexus epithelium and erythrocytes were invariably CA II-positive. However, in rodents, CA II was found by some researchers either exclusively in oligodendrocytes or in oligodendrocytes and myelin sheaths and was even regarded as an oligodendrocyte marker (17–21), whereas others have observed CA II also in some astrocytes (22–25), some microglia of young animals (26), or some neurons (27, 28). Astrocytes, not oligodendrocytes, were reported to express CA II in chick (29) and sheep brain (30). In chick brain, a limited population of neurons also was found to express CA II in chick (29) and sheep brain (30). In sections of adult cerebral cortex and cerebellum from 9 weeks, in a small number of oligodendrocytes in the pons by 17 weeks, and in the internal capsule by 20 weeks (32). In sections of adult cerebral cortex and cerebellum removed during glioma operations, CA II was found exclusively in oligodendrocytes and myelin sheaths (33, 34). However, analysis of various types of tumors, cerebral infarctions, and multiple sclerosis lesions demonstrated CA II (e.g., in reactive astrocytes, astrocytomas, glioblastomas, ganglio-gliomas, and some neurons in the areas of neoplasms [35], suggesting that other types of cells in the human CNS also may express CA II, at least under pathologic conditions.

In the present study, we analyzed the levels and immunolocalization of CA II in human telencephalon by using 2 highly specific and sensitive antibodies.
dinucleotide 3′-phosphodiesterase (CNPase), diluted 1:200 (Novus Biologicals, Littleton, CO), or mAbs to myelin basic protein (MBP) raised in rat (Sigma), diluted 1:200, both markers for oligodendrocytes and myelin; mAb SM31, a marker for phosphorylated neurofilaments (Sternberger Monoclonals, Berkeley, CA), diluted 1:3,000; or mAbs to GAD67, diluted 1:4,000 (Sigma), all diluted in 10% FCS in PBS. After several rinses in PBS, sections were incubated for 1 hour at room temperature with species-specific secondary antibodies conjugated with fluorescent dyes (Invitrogen, Carlsbad, CA): Alexa Fluor 488 (green), Alexa Fluor 555 (red), and for triple immunostaining, also with Alexa 633 (blue), diluted at 1:500. The primary antibodies were omitted as a control of the method. Sections were washed in PBS, mounted with mounting medium (Vectashield; Vector Laboratories, Burlingame, CA), and viewed with a Nikon C1 laser-scanning confocal system mounted on a Nikon 90i microscope. Z-stack major projections were generated by collecting images at 0.3-μm steps along the Z-axis. Optical images were processed by using Adobe Photoshop 6.
SDS-PAGE and Western Blotting

Brain tissue homogenates were prepared from the neocortex (the frontal inferior and temporal superior gyri) and the white matter (from the centrum semiovale of the frontal lobe adjacent to the corpus callosum or from the intermediate zone of the frontal lobe of fetal brains). Brain tissues were homogenized and then sonicated in a buffer containing 50 mM Tris (pH 7.4), 1% SDS, and protease inhibitor cocktail (Complete; Roche, Indianapolis, IN) supplemented with pepstatin A (Sigma). The protein content was measured by using a BCA method and BSA as a standard (Pierce Biotechnology, Rockford, IL). Twenty micrograms to 40 μg of protein per lane was loaded onto 10% Tris/Tricine gels, electrophoretically separated, electrotransferred onto nitrocellulose membranes, and developed by using chemiluminescence method, as described (36). Densitometry analysis of immunoblots was done by 1Dscan EX software (Scanalytics, Inc., Rockville, MD).

RESULTS

Immunoblot Analysis of Carbonic Anhydrase II Levels in Human Brain

Human CA II was visualized on immunoblots of brain tissue homogenates as a single band with an apparent molecular weight approximately 29 kDa, a molecular weight similar to that of the purified human CA II run in parallel as an internal standard, which confirmed the specificity of the antibodies we used (Fig. 1A, C, left panels). In homogenates of the temporal and frontal cortices and the white matter, CA II was already detectable in the earliest period of fetal life we examined, that is, at approximately 17 wgs. However, between 17 and 21 wgs, the protein was present in brain tissue in very small amounts and could be visualized only after longer exposure of the immunoblots, as shown in Figure 1B. The levels of CA II rose dramatically between 21 and 30 wgs in all brain regions studied. Although there were interindividual differences, CA II levels increased gradually in the temporal cortex up to adolescence, whereas they reached the highest values earlier, in young children, in the white matter and the frontal cortex and then decreased slightly (Fig. 1A, C, right panels).

Quantitation of CA II levels by comparing the optical density of the bands visualized on immunoblots of the white matter homogenates with the standard curve generated for human CA II run at 10, 25, 50, and 100 ng/lane on the same immunoblot showed that the levels of the enzyme rose from 8.5 ng/mg protein at 17 to 21 wgs to 3063.16 ng/mg protein at 1.8 year of age, i.e. more than 350-fold. Both pAbs to CA II we used showed identical staining patterns on immunoblots.

Carbonic Anhydrase II in the Epithelial Cells of the Choroid Plexus

Epithelial cells of the primitive choroid plexus were the first types of cells showing CA II immunoreactivity in human embryonic brain at 7 wgs (Fig. 2A). Strong labeling of the choroid plexus epithelial cells persisted throughout all periods of life (Fig. 2B), except for the oldest cases we

FIGURE 2. Carbonic anhydrase (CA) II immunoreactivity in choroid plexus epithelial cells and microvessels. (A–C) Immunoreactivity to CA II in epithelial cells of choroid plexus of an embryo at 7 weeks’ gestation (wgs) (A), a child at 3 years of age (B) and an elderly subject at 83 years of age (C). (D–F) Immunostaining of the endothelial cells of microvessels at 19 wgs (D, E) and 30 wgs (F). Original magnifications: (A–C) 400×; (D, F) 200×; (E) 600×.
examined (older than 75 years of age), which showed a distinct reduction of CA II immunoreactivity (Fig. 2C).

**Carbonic Anhydrase II Is Present in the Endothelial Cells of Microvessels in the Developing Brain**

Immunoreactivity to CA II appeared in primitive microvessels at approximately 11 wgs initially as weak labeling of a few endothelial cell columns in the cortical plate and germinal zones. In the next weeks of fetal life, the immunostaining became stronger and was visible in numerous microvessels penetrating the cortical plate (Fig. 2D) as well as in all other brain regions. The erythrocytes in the lumen of vessels became CA II-positive at approximately 26 wgs, later than the walls of primitive microvessels. Given that the reaction product was dispersed evenly in the cytoplasm of endothelial cells (Fig. 2E), distribution of CA II differs from that reported for CA IV, which was detected on the luminal surface of endothelial cells in the brain of adult mice and rats (18). In the later periods of fetal life, immunopositive microvessels were still numerous (Fig. 2F); however, after birth, the intensity of the staining and the number of immunoreactive microvessels declined. In young children, weak CA II immunoreactivity was seen in a few microvessels, and in adolescents, the microvessels were unstained.

The staining of microvessels was absent after the preabsorption of the antibody with an excess of human CA II (Fig. 3E, F), which suggests its specificity. Preincubation of the antibody with an excess of human CA I (another isoform abundant in red blood cells) did not affect the microvascular staining (not shown). The walls of larger vessels were not immunoreactive to CA II. In a few cases with anoxic/ischemic changes, some extracellular perivascular CA II deposits could be detected (not shown).

**Carbonic Anhydrase II Is Present in a Subset of Neuronal Population**

Beginning at approximately 26 wgs, immunoreactivity to CA II was found in a few migrating neurons in the intermediate zone and, from approximately 30 wgs, was also found in a few neurons in the grey matter. During fetal life and postnatally in immature, adult, and aging brains, immunopositive neurons were seen most often in the hippocampus (the end plate, stratum oriens, and pyramidal layer of the CA1 – CA3 sectors) (Fig. 3A – C) and rarely in the other brain regions, including the neocortex (Fig. 3D). Preincubation of pAbs to CA II with an excess of human CA II either significantly diminished (lower concentration of the antigen) or abolished (antigen at 16 and 50 μg/mL) the staining of neurons and other immunopositive structures (Fig. 3E, F).
By morphologic criteria, most of the CA II-positive neurons resembled large interneurons. Small immunopositive neurons were seen less often, mostly in the pyramidal layer of the hippocampal CA1 sector and in the neocortex. Double labeling with pAbs to CA II and mAbs to GAD67, a marker for GABAergic neurons (Fig. 3G–I), showed that most of the CA II-immunopositive neurons displayed GABAergic phenotype. To visualize the scale of this phenomenon, we counted CA II- and GAD67-positive neurons in the CA1 sector in 12 cases (ages 30 wgs to 83 years) with a comparable plane of the section through the hippocampus available. From 3% (fetal brains) to 10% to 20% (postnatal brain) of GAD67-positive interneurons demonstrated CA II immunoreactivity. However, the subcellular localization of both proteins most likely differs, as judged from the fact that CA II/GAD67-positive neurons showed poor overlapping of fluorophores used for their visualization by laser-scanning confocal microscopy (Fig. 3I). Some pyramidal neurons in the neocortex of children and adults and in the CA1 sector of the hippocampus of one infant and one adult with anoxic/ischemic changes also were CA II-positive (not shown).

Carbonic Anhydrase II in Oligodendrocytes and Their Processes

Immunoreactivity to CA II appeared in oligodendrocytes between 16 and 22 wgs, depending on the brain region studied. However, at that time, only a very few cells were immunopositive and the stain was limited to the cell cytoplasm. The staining of delicate oligodendrocyte processes appeared at approximately 24 to 26 wgs. A prominent increase in the number of immunopositive cells occurred at approximately 28 to 30 wgs (compare Fig. 2D with 2F), and subsequently the reaction product was visible also in delicate varicosities located alongside oligodendrocyte processes (Fig. 4A). Although some perineuronal (satellite) oligodendrocytes in grey matter areas also expressed CA II prenatally, their number increased gradually with aging, and they were most numerous in the brain tissue of the oldest individuals. In grey matter, strong immunostaining of the cell cytoplasm of oligodendrocytes and varicosities on their processes remained until the latest periods of observation, whereas it declined gradually in the white matter during adulthood and aging (Fig. 4B, C). Similar to what was observed in rats (37), not all oligodendrocytes expressed CA II in the human brain.

In the human brain, myelinogenesis starts during the late last trimester of fetal life or, in some regions, even postnatally (38). The most intensive formation of the myelin sheaths occurs during the first 2 postnatal years, but in some structures (e.g. intracortical axonal processes), it continues up to the third decade of life (39). One of the earliest protein markers of developing myelin is CNPase, which is present mostly in the noncompact regions of the myelin sheaths, including abaxonal and periaxonal loops, cytoplasmic incisures, paranodal loops, and lipid rafts (40), whereas MBP is one of the major protein components of the compact myelin (41).

Thus, to investigate the distribution of CA II in myelinating axons during the early stages of myelin formation,

FIGURE 4. Carbonic anhydrase (CA) II in oligodendrocytes and their processes. (A) Strong labeling of the cytoplasm of oligodendrocytes and delicate varicosities alongside oligodendrocyte processes in the thalamus at 34 weeks’ gestation (wgs). (B, C) Stronger CA II immunostaining of interfascicular oligodendrocytes and their processes in the white matter at 3 years of age (B) than at 57 years of age (C). (D, E) Different localization of CA II (left panels, red) and CNPase (middle panels, green) in myelinating fibers in the neocortex at 34 wgs (D) and at 7 months of age (E) as revealed on merged images generated by laser-scanning confocal microscope (right panels) and their colocalization at the periphery of focally distended oligodendrocyte processes contacting myelinating fibers (arrows). Original magnifications: (A–C) 400×; (D) 1,000×; (E) 600×.
using laser-scanning confocal microscope, we analyzed the brain tissues of fetuses and infants, double labeled by using pAbs to CA II and mAbs to CNPase. The distribution of CA II distinctly differed from that of CNPase (Fig. 4D, E). Although CNPase was seen in a thin coat enveloping the axons and in focal densities along their long axis, CA II was present predominantly in round and oval varicosities of various sizes contacting at more or less regular intervals CNPase-positive structures. However, both proteins often colocalized at the periphery of the lumen of varicosities on oligodendrocyte processes accompanying the myelinating axon (Fig. 4E, arrows).

Triple immunolabeling for CA II, MBP, and phosphorylated neurofilaments, followed by laser-scanning confocal microscope analyses of Z-stack projections, showed that the distribution of CA II and MBP also differs significantly (Fig. 5A–F). In delicate processes protruding from oligodendrocytes, MBP was present throughout their length, whereas CA II was mostly confined to their focal densities, forming small varicosities (Fig. 5A–C, arrows). In the immature brain, MBP was seen predominantly in the myelin sheath enveloping the axons, whereas CA II accumulated abundantly in varicosities contacting the myelinating axon but did not penetrate into the myelin sheaths. CA II-positive varicosities contacting axons were distinctly larger (some to a significant extent) (Fig. 5A, arrowhead) than those present on oligodendrocyte processes that did not form contacts with axons and were present alongside the majority of, but not all, myelinating axons. The close apposition of CA II-positive varicosities with the myelinating axons was especially striking in the white matter during the early postnatal periods of myelogenesis (Fig. 5D–F). CA II often accumulated at the periphery of the lumen of these varicosities, giving the appearance of ring-like structures (Fig. 5D, inset). These pictures suggested that in contrast to the usually uniform distribution of CA II in the cytoplasm of oligodendrocytes, the enzyme is positioned predominantly at or beneath the plasma membrane of varicosities formed at oligodendrocyte processes.

The number of CA II-positive varicosities forming physical contacts with the myelinated axons and the intensity of CA II immunoreactivity within them declined in the white matter after the most intense periods of myelin formation. CA II generally did not colocalize with MBP in myelin sheaths (Fig. 5G, H). Only very rarely and at short distances could colocalization of both proteins be found in

![Figure 5](http://jnen.oxfordjournals.org/)
the compact myelin in the white matter areas of the older individuals. However, a thin coat or small dots of CA II immunoreactivity were often visible beneath the myelin sheaths in contact with the axolemma (Fig. 5H, arrow), most likely corresponding to the innermost extension of the cytoplasm of myelinating oligodendrocyte processes forming a periaxial loop.

**Carbonic Anhydrase II in Other Types of Cells in the Human Brain**

Very few astrocytes showed CA II immunoreactivity. Immunopositive astrocytes were seen more often in brain tissues showing anoxic/ischemic changes; however, they stained weakly, especially in comparison with the robust immunostaining of oligodendrocytes. Only some reactive astrocytes found in small areas of incomplete necrosis in one older case were strongly labeled. Some macrophages in the periventricular leukomalacia that was present in one fetal brain also showed weak to moderate CA II immunoreactivity. Both pAbs to CA II we used showed identical staining patterns.

**DISCUSSION**

The presence of CA II in the endothelial cells of microvessels and in a subset of GABAergic interneurons that we detected in the human brain has not yet been reported in animals. We confirmed that in the human brain, oligodendrocytes express CA II as early as midgestation, whereas in the rodent brain, it is expressed postnatally (20, 22). We also showed that in the human brain, CA II is abundant in varicosities alongside long processes of oligodendrocytes contacting myelinating axons during the most intense periods of myelogenesis but is either absent or occurs in minute amounts in adult compact myelin. These observations suggest that CA II is involved in a wider spectrum of biologic processes in the human brain than was previously supposed and also shed new light on the pathogenesis of cerebral calcifications and mental retardation in individuals with CA II deficiency.

Mineral deposits that are formed in human brain under various pathologic conditions as well as during the aging process are stained with calcium stains and variably with stains for iron. They are usually associated with blood vessels (42). In subjects with CA II deficiency, cerebral calcifications are detectable by computed tomography scanning from the age of approximately 24 months in deep layers of the cortex adjacent to the white matter, mostly in the depths of the sulci rather than the gyri, as well as in the putamen and caudate, and less often and less intensely in the globus pallidus, the thalamus, and the cerebellar dentate nuclei (43). The mechanism of their formation is unclear and whether they represent a direct effect of CA II deficiency in the brain or a secondary complication (e.g. of systemic acidosis [12]) is unknown.

Based on our observation that CA II is present in microvessels in immature human brain, it is tempting to postulate that CA II assists in regulation of intracellular pH in proliferating and differentiating endothelial cells. Lack of this function in endothelial cells in individuals with CA II deficiency could create local conditions (i.e. intracellular and extracellular alkalinization) that favor precipitation of calcium salts. Once formed, small calcifications might act as seeds for further accretion of calcium salts. In support for this hypothesis, mutant mouse strain devoid of CA II does not develop intracerebral calcifications in the brain tissue, although these animals demonstrate renal abnormalities (44) and abundant calcium deposits in many internal organs, mostly in the media of small arteries (45). Thus, it appears that CA II is needed during the critical periods of vasculogenesis and formation of the blood–brain barrier in the human but not in the rodent brain.

It is well documented that CA II transcription can be regulated in both a developmental- (46) and cell type-dependent manner (47). Furthermore, the nucleotide sequences of 350 base pairs upstream from the translation initiation ATG codon have 2 sequences different between the mouse and the human, suggesting that some regulatory mechanisms involved in CA II expression may be different between human and rodent cells (48), which may explain temporal and spatial differences in CA II expression between human and mouse brain.

Maintaining intracellular pH at a neutral range is crucial for intracellular membrane trafficking, control of cell volume, initiation of cellular differentiation, and growth and regulation of metabolic pathways or intracellular messengers. Cells control this process as a result of the action of several plasma membrane H\(^+\) extrusion systems, including NHE, Na\(^+\)-dependent and Na\(^+\)-independent bicarbonate transporters of Cl\(^-\)/HCO\(_3^+\) exchange, and an ATP-dependent H\(^+\) pump (4). Recent studies demonstrated that CA II plays an important role in these processes. Binding to bicarbonate transporters localizes CA II to the cytosolic surface of the membrane, where the enzyme can either maximize the local concentration of bicarbonate during bicarbonate efflux or minimize its local concentration during the bicarbonate influx by its conversion to carbon dioxide (49). Binding of CA II to the C-terminal fragment of NHE1, the major protein with which neurons adjust their intracellular pH, allows for a greater transport rate of the H\(^+\) by the NHE1 (50). Furthermore, CO\(_2\)/HCO\(_3^+\) plays a key role in high-frequency stimulation-induced GABAergic depolarization of CA1 pyramidal neurons of the hippocampus (51, 52), thus activity that could contribute to shaping integrative functions and long-term plasticity, but also susceptibility to epileptiform activity. Our study suggests that CA II may be implicated in these processes in a subset of GABAergic interneurons in the developing and adult human brain, including the hippocampus, an area involved in learning and memory. Each GABAergic interneuron contacts numerous other neurons, for example, a single basket cell innervates over 1,500 pyramidal cells of hippocampal CA1 in the rat (53). Thus, even if only a small population of GABAergic interneurons expresses CA II, as our study documented, it may be functionally relevant, providing new insight into the pathomechanisms underlying mental retardation associated with CA II deficiency in humans.

For many decades, oligodendrocytes were regarded as cells whose function is limited to formation of myelin sheaths in the CNS. However, recent studies disclosed that oligodendrocytes also promote neuronal survival, increase axonal
stability, and induce local accumulation and phosphorylation of neurofilaments within the axons, thus increasing the caliber of axons (54–56). Oligodendrocytes also inhibit axonal outgrowth, but in the developing CNS, they may act as axon guidance molecules or may even promote axonal growth (57). Studies in mice lacking CNPase 1 (58, 59), mice with null mutations in proteolipid protein and its minor DM20 isoform, major proteins of compact myelin (60), and in MBP-TK and jimpy mice (61) emphasized the important role of myelin-associated oligodendrocyte proteins for organization of functional domains in myelinated axons, axonal survival, maintenance of nodal and paranodal regions, axon–glia interactions at nodes of Ranvier, and anterograde and retrograde axonal transport.

Marked upregulation of CA II in oligodendrocytes in various demyelinating conditions in humans (62, 63), and its appearance during remyelination periods in experimental animals in white matter tracts that are normally CA II-negative (64), support the suggestion that CA II might be involved in myelin formation/maintenance in the CNS. However, whereas some reports demonstrated the presence of CA II in the myelin sheaths per se (23, 33, 34), others documented the occurrence of the enzyme only in the cytoplasmic area of the myelin sheath (22) or in oligodendroglial processes and a layer of oligodendrocyte cytoplasm often coating the external surface of myelinated fibers (17, 19). Although either mild (65) or no major myelin abnormalities were reported in mice with CA II deficiency (66), detailed molecular composition of the myelin and functional organization of the myelinated axons in these animals have not yet been analyzed.

According to our data, CA II is not a significant component of compact myelin in the human brain. Instead, the enzyme accumulates abundantly in varicosities on oligodendrocyte processes closely associated with myelinated axons. These varicosities, according to earlier suggestions (67), may represent sites of extrasomatic CA II synthesis. Thus, it appears that in the human brain, CA II accumulates abundantly within focal indentions of oligodendrocyte processes when they envelop the axons during periods of intense myelin formation and persists in this location (although in lower amounts) when the formation of compact myelin is completed but myelin maintenance with constant myelin synthesis and turnover continues.

Interestingly, it was reported recently that CA II micro-injected into cultured oligodendrocytes was either freely diffused in the cytoplasm or associated with NHE in the perikaryon or Na⁺/HCO₃⁻ cotransporter in the cellular processes. Inhibition of CA II activity by ethoxyzolamide inhibited acidification of processes (68). Further studies are needed to determine whether a similar pattern of interactions and compartmentalization of CA II prevails in vivo, and thus whether CA II might function as a regulator of acidification of oligodendrocyte processes during axonal ensheathment and further maintenance of myelin sheaths. However, our observations demonstrating that in vivo the enzyme is distributed rather uniformly in the cytoplasm of oligodendrocytes but predominates at the periphery of varicosities associated with myelinated axons appear to support this possibility. The close association of CA II with the axolemma of myelinated axons in the human brain we described suggests that the enzyme may also be involved in interactions with myelinated axons.

Finally, we also observed a decline in CA II immunoreactivity in choroid plexus epithelium in the oldest individuals, similar to what was reported in rodents (69), which may contribute to lower production of cerebrospinal fluid in the aged human brain (70).

In summary, by unraveling previously unrecognized sites of CA II distribution in the developing and adult human brain, our study significantly widens the spectrum of biologic processes in which CA II potentially participates in the human CNS. Characterization of the localization of other CA isozymes in the human brain will allow us to determine in which cell types the function of CA II may be compensated for by one or more members of the growing family of CAs, thereby helping us to better understand the pathogenesis of CA II deficiency in humans.

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