

Evolution of *GLUD2* Glutamate Dehydrogenase Allows Expression in Human Cortical Neurons

Cleanthe Spanaki^{1,3} · Dimitra Kotzamani¹ · Kleopas Kleopa² · Andreas Plaitakis¹

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Abstract Human hGDH2 arose via duplication in the apes and driven by positive selection acquired enhanced catalytic ability under conditions inhibitory to its precursor hGDH1 (common to all mammals). To explore the biological advantage provided by the novel enzyme, we studied, by immunohistochemistry (IHC) and immunofluorescence (IF), hGDH1 and hGDH2 expression in the human brain. Studies on human cortical tissue using anti-hGDH1-specific antibody revealed that hGDH1 was expressed in glial cells (astrocytes, oligodendrocytes, and oligodendrocyte precursors) with neurons being devoid of hGDH1 staining. In contrast, an hGDH2-specific antiserum labeled both astrocytes and neurons. Specifically, hGDH2 immunoreactivity was found in the cytoplasm of large neuronal cells within coarse structures resembling mitochondria. These were distributed either in the perikaryon or in the cell periphery. Double immunofluorescence (IF) suggested that the latter represented hGDH2-labeled mitochondria of presynaptic nerve terminals. Hence, hGDH2 evolution bestowed large human neurons with enhanced glutamate metabolizing capacity, thus strengthening cortical excitatory transmission.

Keywords hGDH1 · hGDH2 · Human brain · Neuron glial cells

✉ Cleanthe Spanaki
kliospanaki@gmail.com

¹ Department of Neurology, Faculty of Medicine, School of Health Sciences, University of Crete, Heraklion, Crete, Greece

² Neuroscience Laboratory and Neurology Clinics, The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus

³ Faculty of Medicine, School of Health Sciences, University of Crete, Voutes, 71003 Heraklion, Crete, Greece

Introduction

Enrichment of the human genome through DNA duplication may have played a key role in the development of traits unique to humans, including bipedal walking, cognitive function, and language. Such DNA duplication processes are shown to permit evolution of genes with new functions [1]. Glutamate dehydrogenase (GDH) (E.C. 1.4.1.3) is a mitochondrial enzyme central to the metabolism of glutamate, the main excitatory neurotransmitter in mammalian brain involved in a multitude of CNS functions, including motor, sensory, and cognitive processes [2, 3]. Expression of GDH rises sharply during postnatal brain development, coinciding with nerve terminal sprouting and synaptogenesis [4, 5]. Whereas GDH in almost all mammals is encoded by a single gene (*GLUD1* in the human) that is expressed widely (housekeeping), humans have also acquired a *GLUD2* gene with distinct tissue expression profile [6]. The *GLUD2* gene arose via duplication in the hominoid ancestor (<25 million years ago) and, driven by positive selection, it evolved on the line that descended to the human [7]. During this journey, the novel *GLUD2* acquired several evolutionary amino acid substitutions that provided unique functional properties [8]. While *GLUD2* may have contributed to human brain development [7, 9], the biological advantage provided to humans by the novel enzyme is not well understood.

GDH catalyzes the reversible oxidative deamination of glutamate to α -ketoglutarate that can be metabolized via the Krebs cycle leading to ATP synthesis and thus sparing glucose as energy source. Glutamate flux through GDH is thought to be important for astrocytes, the cells that remove and metabolize glutamate released at the nerve endings during excitatory transmission [10]. Compared to hGDH1 that is potently inhibited by GTP, generated via the Krebs cycle, hGDH2 has dissociated its function from this energy switch via the

evolutionary substitution of Ala for Gly456 [11]. Also, compared to hGDH1, which operates optimally at pH 8.00, hGDH2 is active at a wider pH range (7.25–8.00) [12]. This adaptation permits hGDH2 function under conditions inhibitory to its ancestor hGDH1 [8]. In addition, evolution of the *GLUD2* gene provided hGDH2 with enhanced mitochondrial targeting ability [13]. Previous immunohistochemical studies in rat brain have shown that GDH is mainly associated with astrocytes [14]. Aoki et al. [14], using a polyclonal antibody that was raised in rabbits against bovine liver GDH, performed a detailed mapping of GDH-positive astrocytes in rat brain and observed a regional distribution pattern that corresponded to glutamatergic pathways. These results, along with data showing that GDH is enriched in synaptic mitochondria [15], support the thesis that GDH is involved in glutamatergic transmission mechanisms [8].

Although hGDH1 and hGDH2 are highly homologous, sharing all but 15 of their 505 amino acid residues, we developed an antibody that specifically recognizes hGDH2 and used it to study human tissues [16]. Results showed that hGDH2 protein is expressed in the human brain, testis, and kidney [16–18] with the human liver being essentially devoid of hGDH2 [16]. These findings are consistent with mRNA data showing that the *GLUD2* gene is not expressed in human liver, which expresses only the *GLUD1* gene [6, 19]. However, there are limited data on the specific expression of hGDH1 in human tissues [19]. Previous immunohistochemistry (IHC) studies have used antibodies that cannot discriminate between hGDH1 and hGDH2 [14, 20]. We have recently characterized a polyclonal antibody raised in rabbits against a 50-amino acid-long segment of hGDH1 that contains three residues evolutionary replaced in hGDH2 and found that this antibody reacted specifically with recombinant hGDH1 without recognizing recombinant hGDH2 [19]. Using this hGDH1-specific antibody and the previously described hGDH2-specific antibody, we studied here by IHC and immunofluorescence (IF) human brain tissue. A variety of cellular and subcellular markers was used to perform double labeling experiments in an effort to better define the precise localization of human hGDH1 and hGDH2. Results are reported below.

Materials and Methods

Sf21 cells and the baculovirus expression vectors for the production of recombinant proteins were obtained from Invitrogen. The medium for the Sf21 insect cells and fetal calf serum was purchased from Invitrogen. Modified baculovirus (BaculoGold) was obtained from Pharmingen. NADPH and ADP were obtained from Roche Applied Science. For GDH purification, Phenyl-Sepharose HP was purchased from Amersham Biosciences, and Bio-Gel Hydroxyapatite HT from Bio-Rad. Nitrocellulose membrane (Porablot NCP)

was from Macherey-Nagel. A polyclonal anti-GDH antibody, raised in rabbit against full-length bovine GDH, was obtained from Biodesign International. A monoclonal antibody, raised in mice against a large N-terminal part of recombinant *GLUD1* protein (residues 129–178) with GST tag, was obtained from Sigma Aldrich (WH0002746M1). Lastly, a polyclonal antibody raised in rabbits against a 50-amino acid-long hGDH1-specific peptide that differs from the corresponding hGDH2-specific amino sequence at three amino acid residues was obtained from Aviva Systems Biology. Other primary antibodies used included NeuN (Millipore 1:400), glial fibrillary acidic protein (GFAP) (Sigma-Aldrich; 1:2000), lamin A/C (Abcam, 1:100), NG2, Connexin 47 (Zymed, 1:500), RIP (2',3'-cyclic nucleotide 3'-phosphodiesterase, a marker for early and mature oligodendrocytes, as well as for myelin sheaths) (Chemicon, 1:1200), HLA (Abcam, 1:50), Calnexin (Abcam, 1:100). Secondary antibodies used included fluorescein- and rhodamine-conjugated donkey cross-affinity purified secondary antibodies (Jackson ImmunoResearch, 1:100), biotinylated anti-rabbit and anti-mouse IgG (Vector Laboratories; 1:200), streptavidin/FITC (Dako; 1:800).

Production of Wild-type hGDH1 and hGDH2 Proteins

GLUD1 or *GLUD2* cDNAs were expressed in Sf21 cells using the baculovirus expression system, as described previously [6]. GDH activity contained in these cells was assayed in the direction of reductive amination of α -ketoglutarate, in the presence of NADPH, according to the previously described method [6]. As the endogenous GDH of the insect cells is NADH-specific [6], this method eliminated all background activity. The obtained recombinant wild-type hGDH1 and hGDH2 proteins were purified to homogeneity from the Sf21 cell extracts as previously described [12] and used for the present studies.

Characterization of the Anti-hGDH1 Antibody

The specificity of a monoclonal antibody raised in mice against the N-terminal part (residues 1–265) of recombinant hGDH1 (Sigma-Aldrich) and of a polyclonal antiserum raised in rabbits against a smaller informative segment (residues 129–178) of hGDH1 (Aviva Systems Biology) were initially tested by performing Western blot analyses of purified hGDH1 and hGDH2 (obtained in recombinant form as described above). Results revealed that only the second antibody that was raised against a small hGDH1-specific peptide (residues 129–178) recognized hGDH1 specifically without interacting with hGDH2 (Fig. 1) [19]. Hence, all subsequent studies were done using this polyclonal antiserum (identified here as anti-hGDH1-specific). For performing Western blots, tissue extracts and/or purified GDH preparations were run on an 8.5 % sodium dodecyl sulfate (SDS)-polyacrylamide gel

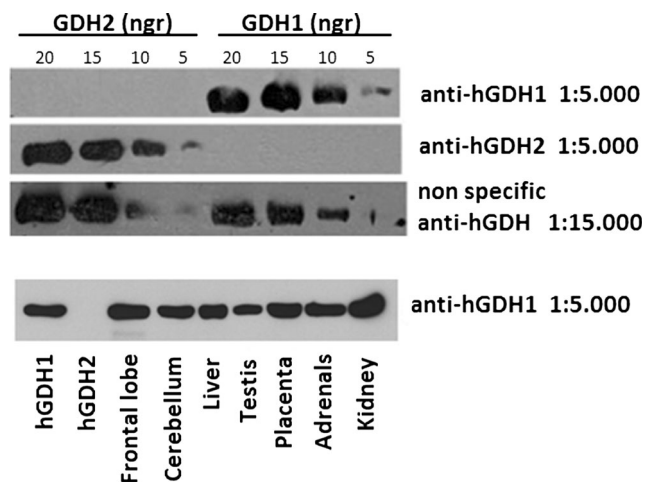


Fig. 1 Characterization of the anti-hGDH1-specific antibody. Western blot analyses showing that the anti-hGDH1 antibody reacts specifically with purified recombinant hGDH1, without recognizing the purified recombinant hGDH2. Conversely, the anti-hGDH2 antibody reacted with purified recombinant hGDH2, but not with the purified hGDH1. In contrast, the antibody raised against bovine liver GDH recognizes both human isoproteins (nonspecific anti-GDH). Western blot analyses of human tissue extracts using the anti-hGDH1 antibody reveal a single immune-reactive band that corresponds to purified recombinant hGDH1 in all tissues studied

and transferred to a nitrocellulose membrane. After blotting, the membranes were incubated either with the anti-hGDH1-specific or the anti-hGDH2-specific antibody. Protein bands were visualized using the ChemiLucent detection system kit (Chemicon). In addition, parallel studies were done using a commercially available antibody raised against the full-length bovine liver GDH (Bioscience International) that does not discriminate between hGDH1 and hGDH2 proteins. Characterization of the hGDH2-specific antibody in human tissues has been described previously [16].

Human Brain Samples

Postmortem human brain samples from ten brain donors, not suffering from a neurologic disorder while alive, were provided to us by the UK Multiple Sclerosis Society Tissue Bank (<http://www.ukmstissuebank.imperial.ac.uk>). These were collected after informed donor consent approved by the UK Ethics Committee (08/MRE09/31). The ages of these control individuals ranged from 35 to 92 years (mean 66 ± 16 years) and the postmortem intervals from 13 to 33 h (mean 24 ± 9 h). The regions of the human brain studied are shown in Table 1. Tissues had been frozen, unfixed or fixed (in 4 % PFA for a minimum of 4 h), cryo-protected in 30 % sucrose/PBS and stored at -80 °C. All cases were processed for immunofluorescence using the anti-hGDH1 and the anti-hGDH2 antibodies along with several cellular and subcellular markers. These included mouse antibodies against GFAP, NeuN, Connexin 47, HLA, RIP, NG2, calnexin, lamin A/C.

Table 1 Human cortical regions studied

	No. of individuals studied	
	hGDH1	hGDH2
Frontal lobe		
Superior frontal gyrus	4	4
Precentral gyrus and paracentral lobule	3	3
Middle frontal gyrus	1	1
Inferior rostral, straight, medial orbital gyri	2	2
Cingulate gyrus	3	3
Subcortical white matter	2	2
Parietal lobe		
Postcentral gyrus	2	2
Temporal lobe	hGDH1	hGDH2
Superior temporal gyrus	3	3
Medial temporal gyrus	3	3
Inferior temporal gyrus	3	3
Entorhinal cortex	2	2
Total number of individuals	10	10

Tissue Crude Extracts and Purified Enzyme Preparation

Human brain and liver tissues, obtained at autopsy after informed consent, were used to study the endogenous GDH protein. About 1.0–2.0 g of human cortical tissue from the frontal, parietal, and temporal lobes (Table 1) was homogenized (10–20 % *w/v* in glass to glass homogenizer) in 10 mM Tris-HCl (pH 7.4) containing 0.1 mM EDTA, 0.5 M NaCl, 1 % Triton X-100, and protease inhibitors. The homogenates were centrifuged (11,000g for 10 min at 5.0 °C), and the obtained extracts were used for enzyme assays and Western blot analyses. In addition, GDH contained in these extracts was purified using a combination of ammonium sulfate (30–55 %) precipitation, hydrophobic interaction (Phenyl-Sepharose column), and hydroxyapatite chromatography as previously described [12]. Fractions containing GDH activity were used for Western blot analyses.

Double Immunofluorescence of Unfixed Postmortem Human Brain

Sequential cryostat sections of 10- μ m thickness from three to four individuals for each brain area (frontal, parietal, temporal lobes; Table 1) were simultaneously stained for the two hGDH isoforms and for each one of the cellular and subcellular markers, using the following procedure: sections were permeabilized in cold methanol for 8 min, washed three times with phosphate buffered saline, blocked in 2 % normal goat serum for 45 min, and incubated overnight at 4 °C with two primary antibodies; the first was either the anti-hGDH2 antibody (1:2000) or the anti-hGDH1 antibody (1:2000), and the

second was one of the antibodies mentioned previously. Specifically, the brain sections were simultaneously incubated overnight at 4 °C with the two primary antibodies. After washing, sections treated with the anti-hGDH2 or anti-GDH1 specific antibody were incubated with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories BA-1000, 1:200) for 1 h at room temperature, followed by incubation with fluorescein isothiocyanate-conjugated streptavidin (1:800) and with TRITC-conjugated anti-mouse secondary antibody (1:100; Jackson ImmunoResearch Laboratories) for 1 h and counterstained with 4,6-diamidino-2-phenylindole. Only for GDH1 and connexin 47 double labeling, biotinylation was performed for connexin 47 detection, using a biotinylated anti-mouse secondary antibody (Vector Laboratories, 1:200), whereas hGDH1 staining was not enhanced (Fig. 3d). Finally, the sections were treated with an autofluorescence eliminator reagent (Millipore 2160) for 10 min and imaged as described above. Images were captured under a Zeiss fluorescence microscope with a digital camera using the Zeiss Axiovision software (Carl Zeiss MicroImaging), with comparable exposure times to allow comparison between hGDH1 and hGDH2 stainings. High-magnification single-scan images were obtained, sequentially for the different wavelengths, using a Leica Confocal microscope.

Results

Selection of Antibodies Specific for hGDH1 and hGDH2

To obtain an antibody specific for hGDH1, we tested initially a monoclonal antibody raised in mice against a full-length recombinant *GLUD1* protein with GST tag (see [Methods](#)). Western blot analyses revealed that this antibody reacted with equal affinity with recombinant hGDH1 and hGDH2 (data not shown). Similar results were obtained with the use of an antibody raised in rabbits against bovine liver GDH. Accordingly, this was used here as a rabbit non-discriminating GDH antibody (Fig. 1, nonspecific GDH antibody). We then tested a polyclonal antiserum raised in rabbits against a synthetic peptide that corresponds to residues 129–178 of hGDH1, containing three residues (142D, 166I, and 174S) evolutionary replaced in hGDH2 (142E, 166V, and 174N). Using purified recombinant human GDH isoproteins, we found that this antibody reacted with high affinity with hGDH1 without recognizing recombinant hGDH2 (Fig. 1, upper panel). Also, Western blot analyses of crude extracts of various human tissues, including the brain, revealed that the antibody recognized a single immunoreactive band that migrated on SDS-PAGE to the same level as the purified hGDH1 (Fig. 1, lower panel). For the specific detection of hGDH2, we used the previously described anti-hGDH2 antibody [16]. This was raised in

rabbits immunized with a 12-amino acid-long hGDH2-specific peptide that contained the R443S evolutionary change [16].

A Study of Human Cortical Tissue

Multiple regions of the human frontal, parietal, and temporal lobes were stained for either hGDH1 or hGDH2 (Table 1). A study of these cortical regions using the anti-hGDH1 antibody revealed that expression of the hGDH1 isoprotein was limited to glial cells, with neurons being essentially devoid of hGDH1-specific immunoreactivity. In contrast, the anti-hGDH2 antibody labeled most human cortical neurons in addition to staining human cortical astrocytes (Fig. 2). These results are detailed below.

Differential Expression of hGDH1 and hGDH2 in the Glial Cells of the Human Cortex

IF studies of human cerebral cortical tissue revealed that both human isoproteins were robustly expressed in gray and white matter GFAP-positive astrocytes (Fig. 2a–h). Specifically, a punctate hGDH1 and hGDH2-specific staining (consistent with mitochondrial localization) was detected in the perikaryon and in the proximal and distal processes of astrocytes (Fig. 2d, h). In addition, the anti-hGDH1 antibody labeled the nuclear membrane of a large number of non-neuronal cells (Figs. 2l and 3). A very small subpopulation of these cells exhibiting hGDH1 nuclear staining were astrocytes (Fig. 3b–c) whereas the majority were mature oligodendrocytes or oligodendrocyte precursors. Specifically, the anti-hGDH1-specific antibody labeled the nucleus of the vast majority of oligodendrocytes, where it co-localized with Connexin 47, a gap junction protein expressed by all mature oligodendrocytes [21] (Fig. 3d). Furthermore, oligodendrocyte precursor cells expressing the NG2 proteoglycan in their membrane were also stained by the anti-hGDH1 antibody (Fig. 3e–g). In these cells, hGDH1 was found to co-localize with lamin A/C, a nuclear membrane protein (Fig. 3h–j). In contrast, the anti-hGDH2 antibody did not stain the nucleus of glial cells. Additional double IF studies using the anti-hGDH1 and anti-hGDH2 antibodies and mouse antisera against RIP (a marker for myelin) and HLA (marker for microglia) showed that neither hGDH1 nor hGDH2 are expressed in oligodendrocytes myelin sheaths or in microglia (data not shown). These findings were consistently observed in cerebral cortical tissues obtained from different individuals and from different brain areas.

Expression of hGDH2 in Human Cortical Neurons

Double IF using either the anti-hGDH1 or the anti-hGDH2 antibody and an antiserum against NeuN (a neuronal marker)

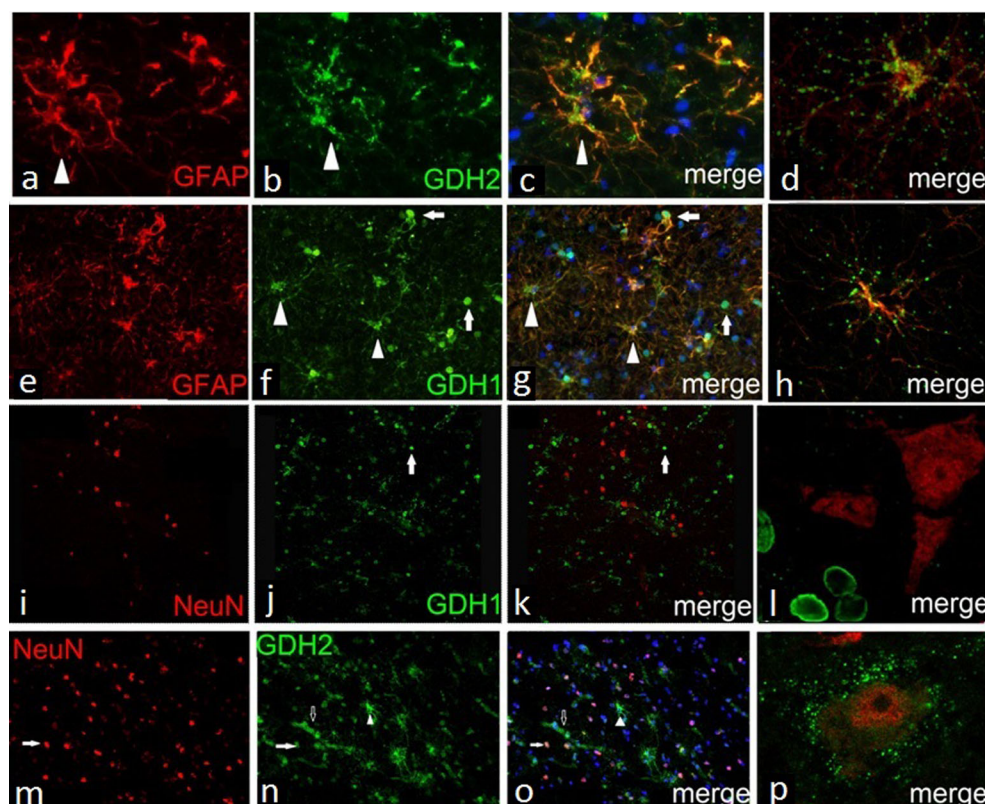


Fig. 2 Localization of hGDH1 and hGDH2 in human brain astrocytes and neurons. **a–h** Images of unfixed human cerebral cortex immunostained with a mouse monoclonal antibody against GFAP (red) and a rabbit antiserum against either hGDH2 (green) (**a–d**) or hGDH1 (green) (**e–h**). There is specific punctate-like immunoreactivity for hGDH1 and hGDH2 in the cytoplasm and along the proximal and distal processes of astrocytes as shown in the merged images of low (**white arrowheads**) (**c, g**) and high magnification (**d, h**). **i–p** Images of unfixed human cerebral cortex immunostained with a mouse monoclonal antibody against NeuN (red) (**i, m**) and a rabbit antiserum either against

hGDH1 (green) (**j–l**) or hGDH2 (green) (**n–p**). hGDH1-specific staining is detected in a large number of non-neuronal CNS cells (**white arrows**) (**k, l**) with neurons being devoid of hGDH1-specific immunoreactivity (**i–l**). In contrast, the majority of neurons labeled by NeuN (**m**) (**filled arrow**) stained positively for hGDH2 (**n, o**). Astrocytes (**white arrowhead**) and vessel walls (**empty arrow**) were also GDH2-positive (**p**). A high-magnification image showing hGDH2-specific dense “puncta” in the peripheral cytoplasm of a large neuron with pyramidal morphology. Blue staining (**c, g, o**) represent DAPI-labeled cell nuclei

revealed that most NeuN-positive cells of human cerebral cortex were stained with the anti-hGDH2 antibody (Fig. 2m–o). Using confocal microscopy, three different patterns of hGDH2-specific labeling of neurons emerged (Figs. 4, 5, and 7). The *first pattern*, observed in large neurons with sizable nuclei and prominent nucleoli (resembling pyramidal cells), was a characteristic constellation of large hGDH2-specific “puncta” that appeared in the periphery of these cells (Fig. 4a–c,d–f). Double IF using our anti-hGDH2-specific antibody and an antibody against GFAP revealed that the hGDH2-specific coarse puncta were on the cytoplasmic membrane of these large neurons as outlined by GFAP staining (Fig. 4g–i). Specifically, the hGDH2-specific puncta were in close proximity to GFAP-positive fine astrocytic feet that encircle synapses on cell membrane; as shown in Fig. 4(g–i), the hGDH2 puncta did not co-localize with GFAP. These results suggest that the observed dense puncta represent labeled mitochondria of presynaptic terminals. The *second pattern* consisted of hGDH2-specific

puncta dispersed throughout the cytoplasm of large cortical neurons, some of which had the morphology of pyramidal cells (Fig. 5). In the cytoplasm of these neurons, hGDH2-specific staining was detected within coarse structures resembling mitochondria also found along the axon or the main dendrite of the cell (Fig. 5). Additional double IF studies using the anti-hGDH2-specific antiserum and an antibody against calnexin (an endoplasmic reticulum (ER) marker) revealed that the two proteins do not co-localize in the ER (Fig. 6). Thus, in contrast to the hGDH2-positive puncta, the anti-calnexin antibody-labeled larger, net-forming, cytoplasmic structures, as expected for ER (Fig. 6). The *third pattern*, observed often in small cortical neurons with round dense nuclei, consisted of a circular delicate hGDH2-specific staining that corresponded to the nuclear membrane (Fig. 7). Indeed, double IF revealed that hGDH2 staining was associated with lamin A/C in these neurons (Fig. 7). These findings were again consistent among the different individuals studied.

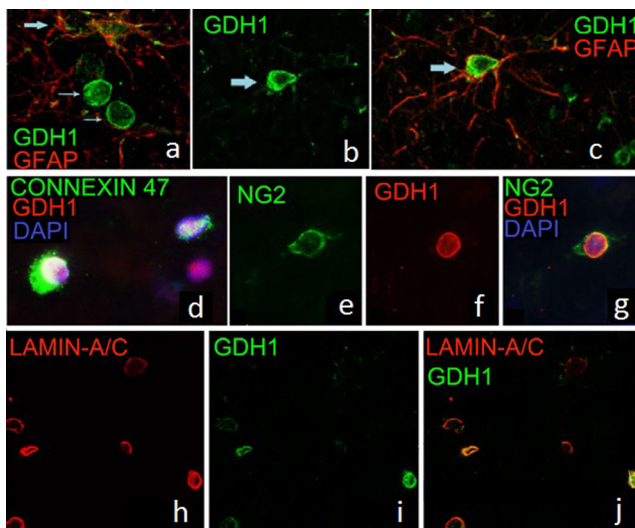


Fig. 3 Localization of hGDH1 to the nucleus of astrocytes, oligodendrocytes and oligodendrocyte precursors. **(a–c)** Images of unfixed human brain tissue immunostained with a rabbit antiserum against hGDH1 (green), and a mouse monoclonal antibody against GFAP (red) **(a)** Distinct hGDH1 staining patterns of astrocytes, involving proximal processes (thick arrow) and neighboring nuclei (thin arrows). **(b and c)** Prominent hGDH1-specific staining of the nucleus of a GFAP-positive astrocyte. **(d)** Double IF using anti-Connexin 47 (a marker of mature oligodendrocytes) (green) and anti-hGDH1 (red) antibodies, reveals hGDH1 specific staining in the nuclei of all Connexin 47 positive oligodendrocytes. **(e–g)** Double IF using the anti-hGDH1 antibody (red) and a mouse monoclonal anti-NG2 antibody (green) (marker of oligodendrocyte precursors) reveals complete co-localization of hGDH1 and NG2 in the nucleus of these cells. **(h–i)** Double IF using anti-Lamin A/C (red, **h**) and anti-hGDH1 (green, **i**) antibodies shows that the hGDH1-specific nuclear staining co-localizes with Lamin A/C on the nuclear membrane (**j**). Blue staining (**d** and **g**) represent DAPI-labeled cell nuclei

Discussion

Here, we studied the cellular and subcellular distribution of the human GDHs in a normal human brain and found that while hGDH1 expression was limited to glial cells, the hGDH2 isoenzyme was also expressed by a variety of human cortical neurons. Among the human cortical glial cells, hGDH1 was expressed in astrocytes, oligodendrocytes, and their precursors, with neurons being essentially devoid of hGDH1 immunoreactivity. In astrocytes, hGDH1 was associated mainly with mitochondria and less often with the nuclear membrane, whereas in oligodendrocytes and oligodendrocyte precursors, hGDH1 localized to the cell nucleus. While previous IHC studies in rat brain [14] failed to detect GDH-specific staining in the nucleus of glial cells, earlier biochemical investigations had shown that GDH activity can be recovered from the nuclear fraction of rat tissues [22]. The rather dense expression of hGDH1 in the nucleus of oligodendrocytes and oligodendrocyte precursors, as detected here, raises important questions regarding the putative function(s) of this protein in nuclear processes. In recent years, it has been realized that the

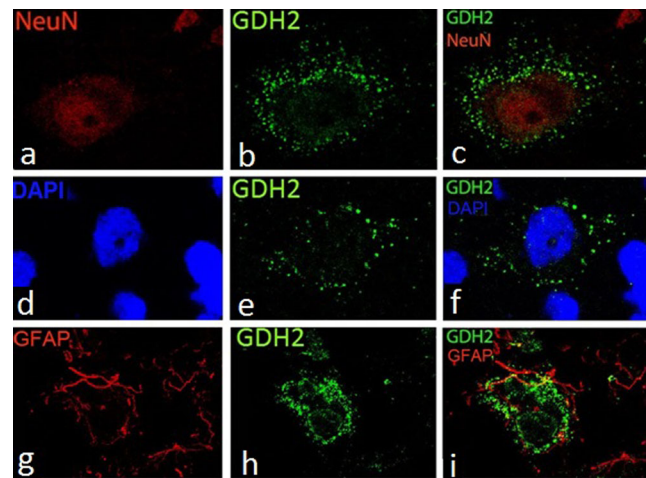


Fig. 4 Localization of hGDH2-positive “puncta” in the periphery of large human cortical neurons. **a–c** Images of unfixed human brain tissue immunostained with a mouse antibody against NeuN (red, **a**) and a rabbit antiserum against hGDH2 (green, **b**). Characteristic constellation of intense hGDH2-specific “puncta” is observed in the periphery of sizable frontal lobe neurons with large nuclei and prominent nucleoli. **d–f** Similar peripheral distributions of hGDH2 punctate staining in a large-size neuron from the human occipital lobe. Blue staining represents DAPI-labeled cell nuclei. **g–i** A composite figure consisting of superimposed consecutive confocal images of unfixed human brain frontal lobe cortex immunostained with a mouse antibody against GFAP (red, **g**) and a rabbit antiserum against hGDH2 (green, **h**). The GFAP-positive fine astrocytic processes that delineate the periphery of large neurons are in close proximity to (but do not co-localize with) hGDH2-specific large puncta on the cell membrane

GDH substrate α -ketoglutarate serves as a co-factor for specific dioxygenases (α -ketoglutarate-dependent dioxygenases) involved in DNA and histone demethylation processes and that proliferating embryonic stem cells maintain higher

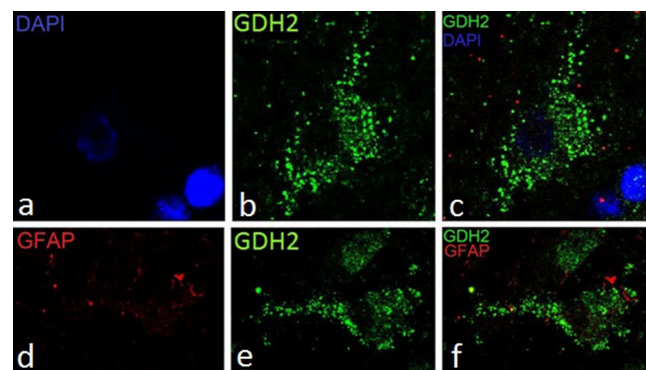


Fig. 5 Punctate hGDH2-specific staining within the cytoplasm of large human cortical neurons. Images of unfixed human brain tissue immunostained with a rabbit antiserum against hGDH2 (green) and counterstained with DAPI. **a–c** hGDH2-specific punctate staining within the cytoplasm of a large neuron from the occipital lobe. Blue staining represents DAPI-labeled cell nuclei. **d–f** Images of unfixed human brain frontal lobe cortex immunostained with a mouse antibody against GFAP (red, **d**) and a rabbit antiserum against hGDH2 (green, **e**). The hGDH2 punctate staining is observed in the cytoplasm and along the axon of this neuron (**f**). In contrast, GFAP staining is located in the periphery of the neuron (**f**)

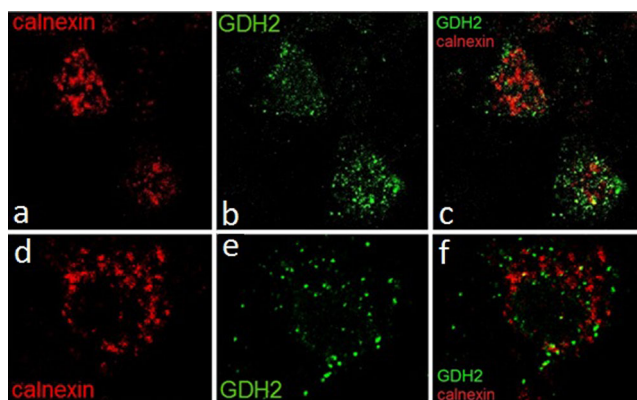


Fig. 6 hGDH2 does not co-localize with calnexin in human brain cells. Double IF images of unfixed human brain tissue (from the frontal lobe) immunostained with a mouse antibody against calnexin (red; **a, d**) and a rabbit antiserum against hGDH2 (green; **b, e**) reveals no co-localization of hGDH2 with calnexin (**c, f**), a marker for the endoplasmic reticulum

intracellular levels of α -ketoglutarate than more differentiated cells [23]. Hence, the expression of hGDH1 in oligodendrocyte precursors may enhance the production α -ketoglutarate needed for the differentiation of these cells into oligodendrocytes. Whereas, it remains unclear whether hGDH1 (a mitochondrial matrix enzyme) can function enzymatically in the nuclear environment, in which NAD levels are substantially lower than those present in the mitochondria [24], pyruvate dehydrogenase, which also uses NAD as a co-factor and which was previously thought to operate strictly within the mitochondrial matrix, was recently shown to translocate to cell nucleus to provide acetyl-CoA needed for histone acetylation [25]. On the other hand, a novel (non-metabolic) function of GDH in nuclear processes has been recently proposed. Thus, in nuclei isolated from chicken liver and brain, GDH was shown to act as H₃-specific protease involved in H₃ tail-clipping, a process that plays a role in the regulation of gene expression and chromatin dynamics [26, 27]. Hence, whether

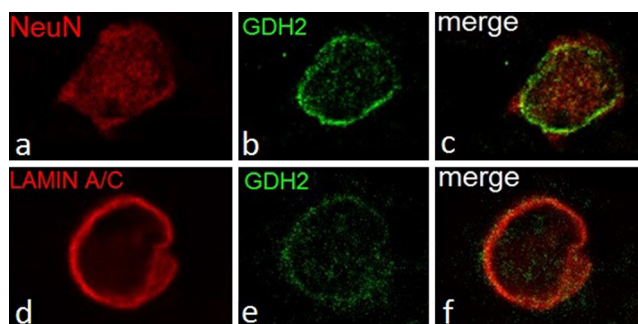


Fig. 7 Nuclear hGDH2-specific staining of small cortical neurons. Images of unfixed human brain tissue immunostained with a mouse antibody against NeuN (red, **a**) or against lamin A/C (red, **d**) and a rabbit antiserum against hGDH2 (green; **b, e**). **a–c** A delicate circular GDH2-specific staining is detected inside the NeuN-positive area in this small round frontal lobe neuron. **d–f** Association of the circular staining with the nuclear membrane is revealed by double immunostaining for hGDH2 and lamin A/C (a nuclear membrane protein)

hGDH1 in glial cell nuclei, as detected here, is important for α -ketoglutarate-dependent dioxygenases and/or is directly involved in chromatin dynamics need to be further explored.

Similarly to the anti-hGDH1 antibody, the anti-hGDH2 antiserum labeled most astrocytes of the human cerebral cortex; however, the hGDH2-specific staining was found exclusively in the cytoplasm within coarse structures consistent with mitochondria, as previously described [16]. Astrocytes are responsible for the removal and metabolism of neurotransmitter glutamate [10, 28]. Glutamate, taken up by astrocytes, is in part transported to the mitochondrial matrix where it is converted to α -ketoglutarate via AAT or GDH, with the latter pathway being activated under conditions of intense excitatory transmission [10]. Previous studies have shown that GDH is present in the synaptic mitochondria [15] and that the enzyme plays a role in synaptic glutamine oxidation [29]. As hGDH2 evolved to be also active at relatively low pH (7.25–7.50), co-expression of hGDH1 and hGDH2 in astrocytes, as found in this study, may enhance the ability of these cells to metabolize glutamate, particularly under conditions of intracellular acidification that prevails in astrocytes after glutamate uptake [30].

Whereas previous IHC studies in rat and human brain showed that GDH localizes mainly to glial cells [14, 16], the present study, using confocal microscopy, unexpectedly revealed that hGDH2 is also expressed in the neurons of the human cerebral cortex. Moreover, the pattern of this expression correlates with the morphological features of the neuronal cells. Thus, in small neurons with dark nuclei, hGDH2 localized to their nuclear membrane, whereas in large cortical neurons (some with pyramidal morphology), hGDH2 was found throughout their cytoplasm within coarse structures resembling mitochondria. In addition, in large cortical neurons (also resembling pyramidal cells), a characteristic constellation of intense hGDH2-positive puncta was detected in the peripheral cytoplasm. Double IF studies further suggested that these hGDH2-positive puncta are on the cell membrane within pre-synaptic boutons encircled by GFAP-positive astrocytic feet, thus suggesting that these puncta represent clusters of hGDH2-stained mitochondria of presynaptic terminals.

The expression of hGDH2 in human cortical neurons is expected to provide these cells with enhanced glutamate-metabolizing capacity, which may permit these high-energy demand cells to efficiently utilize glutamate for ATP production, a process shown to spare glucose as an energy source [31]. In this regard, the unique functional properties acquired by hGDH2, including the ability of the novel enzyme to operate at a wide pH range (7.25–8.0) and to remain functional when the Krebs cycle generates GTP quantities sufficient to completely inactivate hGDH1, may be of particular importance. Thus, because glutamate uptake by astrocytes is associated with intracellular acidification [30] and because neurons maintain a higher intracellular pH [30], evolution

equipped the new enzyme with the versatility needed for optimal function in both astrocytes and neurons. Most importantly, expression of hGDH2 in glutamatergic nerve endings may strengthen excitatory transmission by enhancing the formation of synaptic glutamate. Previous observations revealed that targeted expression of GDH in mouse cortical neurons (via a neuronal specific promoter) increased presynaptic glutamate release [32]. Hence, hGDH2 expression in large human cortical neurons may potentiate excitatory transmission, an energy-intensive process that requires the presence of mitochondria in the nerve terminals [33]. Such intense excitation is essential for long-term potentiation, a process involved in memory consolidation. GDH is indeed known to be upregulated during memory formation [34]. Moreover, in human subjects, fMR spectroscopy studies have shown that glutamate levels increase in the cortex during neuronal activation, a process thought to involve enhanced flux through GDH [35].

As compared to rat brain, the human prefrontal cortex has a much higher percentage of axonal boutons that contain mitochondria [36], which may represent an evolutionary adaptation to cover the high-energy demands that result from the excitatory processes of human cortical neurons. Moreover, recent observations have linked the number and morphology of mitochondria present in synaptic axonal boutons of the prefrontal cortex of primates with working memory performance [37]. In light of these considerations, expression of hGDH2 in human cortical neurons, a novel finding reported here, suggests that *GLUD2* evolution bestowed these neurons with augmented glutamate metabolizing capacity, which may in turn strengthen cortical excitatory transmission by enhancing the formation of synaptic glutamate. Whether this represents an important evolutionary event in human brain biology, it remains to be further explored.

GDH, glutamate dehydrogenase; hGDH1, human glutamate dehydrogenase encoded by the *GLUD1* gene; hGDH2, human glutamate dehydrogenase encoded by the *GLUD2* gene.

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