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ORIGINAL PAPER

Transgenic Mice Carrying *GLUD2* as a Tool for Studying the Expressional and the Functional Adaptation of this Positive Selected Gene in Human Brain Evolution

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Abstract

Human evolution is characterized by brain expansion and up-regulation of genes involved in energy metabolism and synaptic transmission, including the glutamate signaling pathway. Glutamate is the excitatory transmitter of neural circuits sub-serving cognitive functions, with glutamate-modulation of synaptic plasticity being central to learning and memory. *GLUD2* is a novel positively-selected human gene involved in glutamatergic transmission and energy metabolism that underwent rapid evolutionary adaptation concomitantly with prefrontal cortex enlargement. Two evolutionary replacements (Gly456Ala and Arg443Ser) made hGDH2 resistant to GTP inhibition and allowed distinct regulation, enabling enhanced enzyme function under high glutamatergic system demands. *GLUD2* adaptation may have contributed to unique human traits, but evidence for this is lacking. *GLUD2* arose through retro-positioning of a processed *GLUD1* mRNA to the X chromosome, a DNA replication mechanism that typically generates pseudogenes. However, by finding a suitable promoter, *GLUD2* is thought to have gained expression in nerve and other tissues, where it adapted to their particular needs. Here we generated *GLUD2* transgenic (Tg) mice by inserting in their genome a segment of the human X chromosome, containing the *GLUD2* gene and its putative promoter. Double IF studies of Tg mouse brain revealed that the human gene is expressed in the host mouse brain in a pattern similar to that observed in human brain, thus providing credence to the above hypothesis. This expressional adaptation may have conferred novel role(s) on *GLUD2* in human brain. Previous observations, also in *GLUD2* Tg mice, generated and studied independently, showed that the non-redundant function of hGDH2 is markedly activated during early post-natal brain development, contributing to developmental changes in prefrontal cortex similar to those attributed to human divergence. Hence, *GLUD2* adaptation may have influenced the evolutionary course taken by the human brain, but understanding the mechanism(s) involved remains challenging.

Keywords *GLUD2* Transgenic mice · *GLUD2* Adaptation · Brain hGDH2 expression · Human evolution

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Abbreviations

BAC	Bacterial artificial chromosome
EDTA	Ethylenediaminetetraacetic acid
FITC	Fluorescein isothiocyanate
GFAP	Glial fibrillary acidic protein
hGDH1	Human glutamate dehydrogenase isoenzyme
hGDH2	Human glutamate dehydrogenase isoenzyme 2
IDH1	Isocitrate dehydrogenase 1
IF	Immunofluorescence
mGDH1	Mouse glutamate dehydrogenase 1
MTS	Mitochondrial targeting sequence
Tg	Transgenic
Wt	Wild-type
WB	Western blot
TCA	Tricarboxylic acid cycle

Introduction

Human evolution is characterized by brain expansion and acquisition of unique traits, including language function, symbolic thought and other complex cognitive processes. There is disproportional enlargement of the prefrontal cortex (PFC), the excitatory pyramidal neurons of which exhibit increased dendritic branching and synaptic spine density [1]. In addition, human PFC displays increased neuropil space (to accommodate synapses and dendritic processes) and a high density of glial cells relative to neurons [2]. This adaptation is thought to reflect increased metabolic demands arising from the high neuronal activity that characterizes the human brain [2–4]. To meet these demands, a rapid metabolic evolution has taken place [3, 4], with large-scale mRNA analyses of human brain having shown up-regulation of genes involved in synaptic transmission and energy metabolism [5].

While the underlying molecular-genetic mechanisms that drive these adaptations remain unclear, glutamatergic transmission is a major player with glutamate release and recycling thought to consume about 60–80% of energy supplied by glucose [3, 4]. Energy demands are even greater in the developing nervous system, with fetal brain consuming about 65% of the body's total metabolic energy [3]. Glutamate, by activating a subclass of glutamate receptors and calcium influx, can induce long-lasting modification of synapses associated with structural plasticity, including increased dendritic spine density [6]. This ability of synapses to change their strength, known as "synaptic plasticity", is thought to represent the "cellular correlate of long-term memory" [7]. Such experience-dependent plasticity may promote the sculpting of neural circuits in response to environmental influences [8, 9], thus contributing to a synergy between nature and nurture, processes of fundamental importance in human brain evolution [8, 9].

In support of these considerations, comparative studies on the brain of primates have shown that the glutamatergic signaling pathway is the primary target of human brain evolution [10]. Specifically, there is up-regulation of genes encoding proteins involved both in pre-synaptic glutamatergic mechanisms (glutamate storage and release) and in post-synaptic excitatory transmission (glutamate receptors, synaptic scaffolding proteins and calcium signaling proteins) [10]. However, the genetic determinants of this up-regulation remain largely unknown, with DNA analyses providing no evidence for accelerated evolution of genes and their regulatory elements that encode the above proteins [11].

While it has proven difficult to assign evolutionary genomic changes to specific human traits, several genes expressed in human brain underwent positive Darwinian selection, a process indicative of adaptive DNA sequence evolution [12]. Such positive selection is known to generate new phenotypes from existing ones [13] and can lead to the development of new genes in an environment shaped by social and cultural influences [9]. To this end, a number of genes involved in brain size, neurotransmission, vision, memory and neural cell metabolism are shown to have evolved under positive Darwinian selection [12, 14, 15].

GLUD2 is a novel gene [16] that evolved in the human lineage under positive selection and that underwent rapid evolutionary adaptation, concomitantly with pre-frontal cortex expansion [15]. It encodes the hGDH2 isoform of glutamate dehydrogenase, an enzyme involved both in glutamatergic transmission and in energy metabolism, two processes pivotal to human brain evolution. There is tantalizing evidence that birth and adaptation of *GLUD2* may have contributed to human brain development and perhaps to acquisition of cognitive capabilities [15, 17] possibly by enhancing glutamatergic transmission and the bioenergetic and biosynthetic function of the TCA cycle [17].

GLUD2 was born through gene duplication, a process thought to have played a major role in the evolution of eukaryotes [13]. Thus, duplicated genes often undergo positive selection, which as noted above, can lead to sequence, function and/or expression divergence [18]. Whereas new genes typically arise through genomic duplication or gene conversion events [19], *GLUD2* was born through retro-positioning of a processed *GLUD1* mRNA, a process known to generate pseudogenes [19]. It has been hypothesized, however, that, by finding a suitable promoter, *GLUD2* gained expression in nerve and other tissues where it adapted to their specific metabolic needs [15, 19]. To test this hypothesis, we generated *GLUD2* transgenic (Tg) mice by inserting in their genome a 176.6 Kb segment of human X chromosome, containing the *GLUD2* gene and its putative regulatory elements (including its promoter). Using antibodies that specifically recognize hGDH2 (encoded by the *GLUD2* gene) or GDH1 (encoded by the endogenous mouse Glud1 gene), we studied the expressional pattern of the human gene in the host brain. Results obtained were then compared with those derived from the study of human brain, as described below.

Materials and Methods

Reagents

A human BAC clone (RP11-610G22) was obtained from ImaGenes, GmbH and used for constructing the *GLUD2* transgenic mice. Nitrocellulose membrane (Porablot NCP) was from Macherey–Nagel. The anti-hGDH1-specific antibody was obtained from Aviva Systems Biology. The

anti-hGDH2-specific antibody was raised in rabbits as previously described [20]. Other primary antibodies used included mouse raised anti-NEUN (Millipore 1:400) and anti-GFAP (Sigma-Aldrich; 1:2000) antibodies. 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).

The Anti-hGDH1 and Anti-hGDH2 Antibodies

For specifically detecting the hGDH2 protein, we used a polyclonal antibody raised in rabbits using a 12-amino acid long hGDH2-specific peptide containing the Arg443Ser evolutionary replacement that induces drastic changes in the enzyme's behavior, including its migration in SDS-PAGE [20]. The antibody has been previously characterized [20]. It specifically recognizes hGDH2 without interacting with hGDH1 [20]. For detecting the endogenous mouse GDH1 we used a polyclonal antibody raised in rabbits (Aviva Systems Biology) against a 50 amino acid-long hGDH1-specific peptide that contains three amino acid residues evolutionary replaced in hGDH2 [21]. Characterization of this antibody showed that it specifically recognizes hGDH1 without interacting with hGDH2 [21].

Generation of *GLUD2* Transgenic Mice

The above BAC clone, containing a segment of the human X chromosome that encompasses the *GLUD2* gene and 40 kb of upstream and 135 kb of downstream DNA sequences, was used to construct the transgenic mice. A *Not*I fragment of 176,610 bp was isolated from the above BAC clone and microinjected into the pronuclei of fertilized (C57BL/6J × CBA/J) F2 oocytes as previously described [22, 23]. Microinjections and embryo implantations were carried out by the Transgenics & Gene Targeting Facility at the Biomedical Sciences Research Center 'Alexander Fleming'. The resulting offspring, first obtained in May 2013, were genotyped by PCR using the primers described below.

DNA Analysis

Mouse tail DNA was extracted using the phenol/chloroform method. Founders were identified by PCR analysis using primers specific for coding sequences of the *GLUD2* gene (F: 5′-TGAATGCTGGAGGAGT GACA-3′and R: 5′-TGG ATTGACTTGTTGAGAATGG-3′). Founders were crossed with C57BL/6 mice. F1 offspring were genotyped to identify germ line transmission using the same method. Transgenic offspring, used to maintain the transgenic line, were bred under specific pathogen-free conditions in the animal facility at the Institute of Molecular Biology and Biotechnology (IMBB) of FORTH, Crete, Greece. The presence of the transgene was monitored throughout the course of the study by PCR from tail genomic DNA using the above primers. To produce heterozygous transgenic lines, *GLUD2* Tg mice were crossed with wild-type C57BL/6 animals. The control mice that were used in our experiments were wild-type littermates of the *GLUD2* Tg animals. The animals were housed 4 mice per cage in standard cages, with a sawdust bedding, at constant temperature (23 + 2 °C), humidity (55 + 5%) and under normal 12 h light/dark cycle (lights on from 7:00 to 19:00). Food and water were available ad libitum.

All experimental procedures were approved by the local ethics committee for animal experiments and met the governmental guidelines. All efforts were made to minimize the number of animals and their suffering. Two *GLUD2* Tg strains (lines Tg13 and Tg32) showing comparable levels of hGDH2 expression in the brain were used and studied in parallel.

Western Blot Analyses

Crude Brain Extract Preparation

About 50–100 mg of nerve tissue from different CNS regions (for olfactory bulbs about 20 mg) of young adult Tg and Wt mice was dissected and homogenized (glass to glass) in 10 mM Tris–HCl (pH 7.4) containing 0.1 mM EDTA, 0.5 M NaCl, 1% Triton X-100, and protease inhibitors. Crude extracts obtained by centrifugation (11,000×*g* for 10 min) of the cell lysates were used.

Western Blots

Tissue extracts, obtained as described above, were run on an 8.5% SDS-PAGE gel. Proteins were transferred on a nitrocellulose membrane and incubated with the primary anti-hGDH1 or the primary anti-hGDH2 specific antibody described above. Proteins bands were visualised with the use of the ChemiLucent Detection System kit (Chemicon).

Immunofluorescence Studies

Brain Slice Preparation of Tg Mice

All animals were deeply anesthetized using pentobarbital sodium (60 mg/kg, i.p) and then perfused with 30 ml PBS followed by 30 ml 4% paraformaldehyde (PFA). After successful perfusion, the brains were removed immediately and fixed in 4% PFA for 40 min. Upon fixation, tissues were sucrose cryoprotected (30% sucrose in phosphate buffer, pH 7.4) and embedded in gelatin (7.5% gelatin/15% sucrose in phosphate buffer, pH 7.4) and then were snap-frozen

by exposure to isopentane. Coronal serial sections were obtained via cryotome and transferred to gelatin coated glass slides (or in positively charged SuperFrost slides, ThermoFischer Scientific).

Preparation of Human Brain Slices

Postmortem human brain samples from donors, not suffering from a neurologic disorder while alive, were provided to us by the UK Multiple Sclerosis Society Tissue Bank as previously described [21] (http://www.ukmstissuebank.imperial.ac.uk). These were collected after informed donor consent approved by the UK Ethics Committee (08/MRE09/31). 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. Sequential cryostat sections of 10-µm thickness from frontal, parietal and temporal lobes were obtained and processed for immunofluorescence (IF).

Double Immunofluorescence Staining

Brain sections were fixed in acetone for 8 min. Non-specific binding sites were blocked at RT for 40 min in 5% BSA in 0.5% Triton x-100 PBS and then incubated at 4 °C for 18 h with rabbit primary antibodies recognizing either the hGDH1 or hGDH2 protein and with mouse primary antibodies for NEUN or GFAP. After three washes in PBS, incubation with fluorescence-labeled secondary antibodies was performed with biotinylated goat anti-rabbit secondary antibody followed by Streptavidin FITC or goat anti-mouse Alexa Fluor 555 secondary antibody. Nuclei were visualized with TOPRO. Images were obtained using a Leica Confocal microscope.

Results

Generation of *GLUD2* Transgenic Mice

Two lines of *GLUD2* transgenic mice (T13 and T32), constructed independently, were obtained. Similar breeding rates were observed between the Tg and control mice. Moreover, Tg mice from our two lines passed their developmental milestones at about the same time as their wild-type littermates. All studies described here were performed using male Tg mice and their male wild-type (Wt) littermates. No differences in physical health measures, food consumption, posture, physical condition of the fur and home cage behaviors, were detected between Wt and Tg animals. Also, irregular spontaneous behaviors such as excessive grooming, digging, rearing, or stereotypies were not different between the two groups.

Immunoblotting

Western blot analyses revealed that the anti-hGDH1 antibody recognized the endogenous mouse GDH1 (mGDH1) with the same affinity as with the hGDH1 (used as Standard), without recognizing the expressed hGDH2 (Fig. 1). These results are expected, as hGDH1 and mGDH1 are essentially identical, having been conserved via purifying selection. Conversely, our anti-hGDH2 antibody recognized the expressed hGDH2 protein without interacting with the endogenous mGDH1 enzyme. Immunoblots of brain extracts revealed that hGDH2 is expressed in all CNS regions of the Tg mice studied and that this expression did not affect the levels of the endogenous mouse GDH1 (Fig. 1, upper panel). Analyses of brain extracts from our two Tg lines by WB yielded similar results.

Double Immunofluorescence

IF experiments, using cerebral cortical tissue from the *GLUD2* Tg mice, revealed a dense hGDH2-specific expression in the neuropil, where it co-localized with GFAP (Fig. 2a–c). Specifically, hGDH2-specific punctate-like stain was detected in GFAP-positive astrocytes and their processes (Fig. 2a–c). As previously described for human brain [20, 21], the endogenous GDH1 mouse enzyme was also co-expressed in astrocytes (data not shown). Examination of human cortical sections by IF also revealed a dense hGDH2-specific expression in the neuropil, where it co-localized with GFAP (Fig. 2d–f). As shown in Fig. 2d–f, human astrocytes, labelled by the anti-hGDH2 and anti-GFAP antibodies are substantially larger and exhibit a much greater degree of arborisation than mouse astrocytes. In addition, the anti-hGDH2 antibody labelled a subpopulation of large cortical

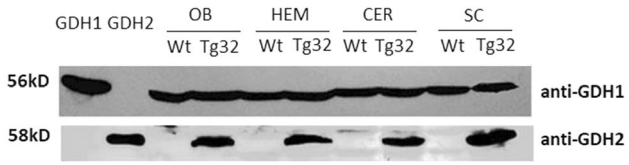

Fig. 1 *GLUD2* expression in the central nervous system of Tg mice. Immunoblots were performed in brain extracts from 6-month old male Tg32 and Wt mice. Tissue extracts (50 µg protein) from various CNS regions were run on a 8.5% SDS-PAGE gel, transferred to a nitrocellulose membrane and incubated with the primary anti-hGDH1 or anti-hGDH2-specific antibody (dilutions 1:5000 and 1:2000 respectively). Proteins bands were visualised with the use of the ChemiLucent Detection System kit (Chemicon), Recombinant human GDH1 and GDH2 were used as standards. *GLUD2* Tg mice show specific hGDH2 expression in spinal cord (SC), cerebellum (CER), cerebral hemispheres (HEM) and olfactory bulb (OB). The endogenous mouse GDH1 is detected at comparable levels in all brain regions of both Tg and Wt mice. WB analyses of the CNS from the second (T13) transgenic line yielded similar findings

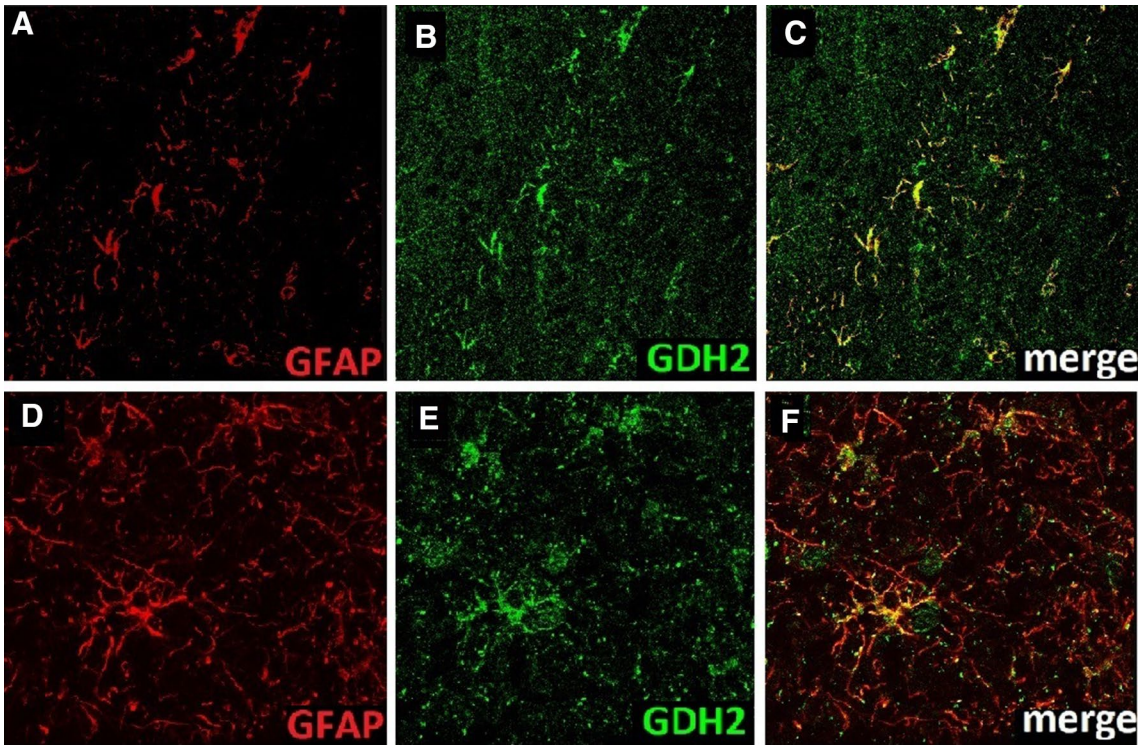

Fig. 2 hGDH2 expression in cerebral cortical astrocytes. IF images of snap frozen fixed brain sections from the cerebral cortex of a 6-month old male *GLUD2* Tg13 mouse (**a**–**c**) and of a neurologically normal human subject (**d**–**f**) immunostained with a mouse monoclonal antibody against GFAP (1:2000; red) and our rabbit anti-hGDH2 specific antibody (1:2000; green). In both tissues there is punctate-like GDH2-specific immunoreactivity in the perinuclear cytoplasm and along the proximal and distal processes of GFAP-positive astrocytes. Human astrocytes are larger showing more extensive arborization than mouse astrocytes. These results are consistent with previous observations on human brain [20, 21]. Similar results were obtained using brain tissue from the Tg32 line. Images obtained using a Leica confocal microscope (×40). (Color figure online)

neurons with pyramidal morphology, with hGDH2-positive "puncta" observed in the peripheral cytoplasm of these neurons (Fig. 3a–c, e, f). These structures are similar to hGDH2-positive "puncta" observed on the cytoplasmic membrane of human cortical neurons (Fig. 3g–i) and may represent clusters of hGDH2-positive mitochondria in presynaptic nerve terminals [21]. As shown in Fig. 3j–l, the periphery of a large hGDH2-positive neuron was delineated by GFAP-specific staining of astrocytic processes enwrapping excitatory synapses [21]. In this neuron, large "puncta" lie in close proximity (but do not co-localize with) to the GFAP positive structures on the cell membrane (Fig. 3j–l). In contrast, the hGDH1 antibody did not label any neurons of the host mouse brain. These results accord those obtained in human brain, which showed that cortical neurons are devoid of hGDH1-specific expression [21].

Implications of the Expressional *GLUD2* Adaptation

The present morphological studies on *GLUD2* Tg mice revealed that expression of hGDH2 in the host brain is very similar to that of human brain. This involves dense expression in the perikaryon of astrocytes and their processes and in the peripheral cytoplasm of large cortical neurons of pyramidal morphology. In these neurons, large hGDH2 "puncta" that may represent clusters of hGDH2-positive mitochondria of pre-synaptic nerve endings, were found on the cell membrane in close proximity to GFAP-labeled labeled astrocytic structures. In contrast, the endogenous GDH1 was expressed only in astrocytes. As shown here, human astrocytes are larger than the host mouse astrocytes and exhibit increased arborisation. These results accord observations by Oberheim et al. [24] showing that human protoplasmic astrocytes are 2.6-fold larger in diameter and extend tenfold more GFAP-positive primary processes than rodent astrocytes. There is increasing evidence that astrocytes are involved in synaptic transmission and plasticity, including LTP emergence and maintenance [25].

As we have obtained comparable morphological data on both of our Tg lines (T13 and T32), the present findings cannot be attributed to alterations of the mouse genome induced by the random (stochastic) insertion of the human DNA segment. Because our Tg mice were constructed using a segment of the human X chromosome that contains the *GLUD2*

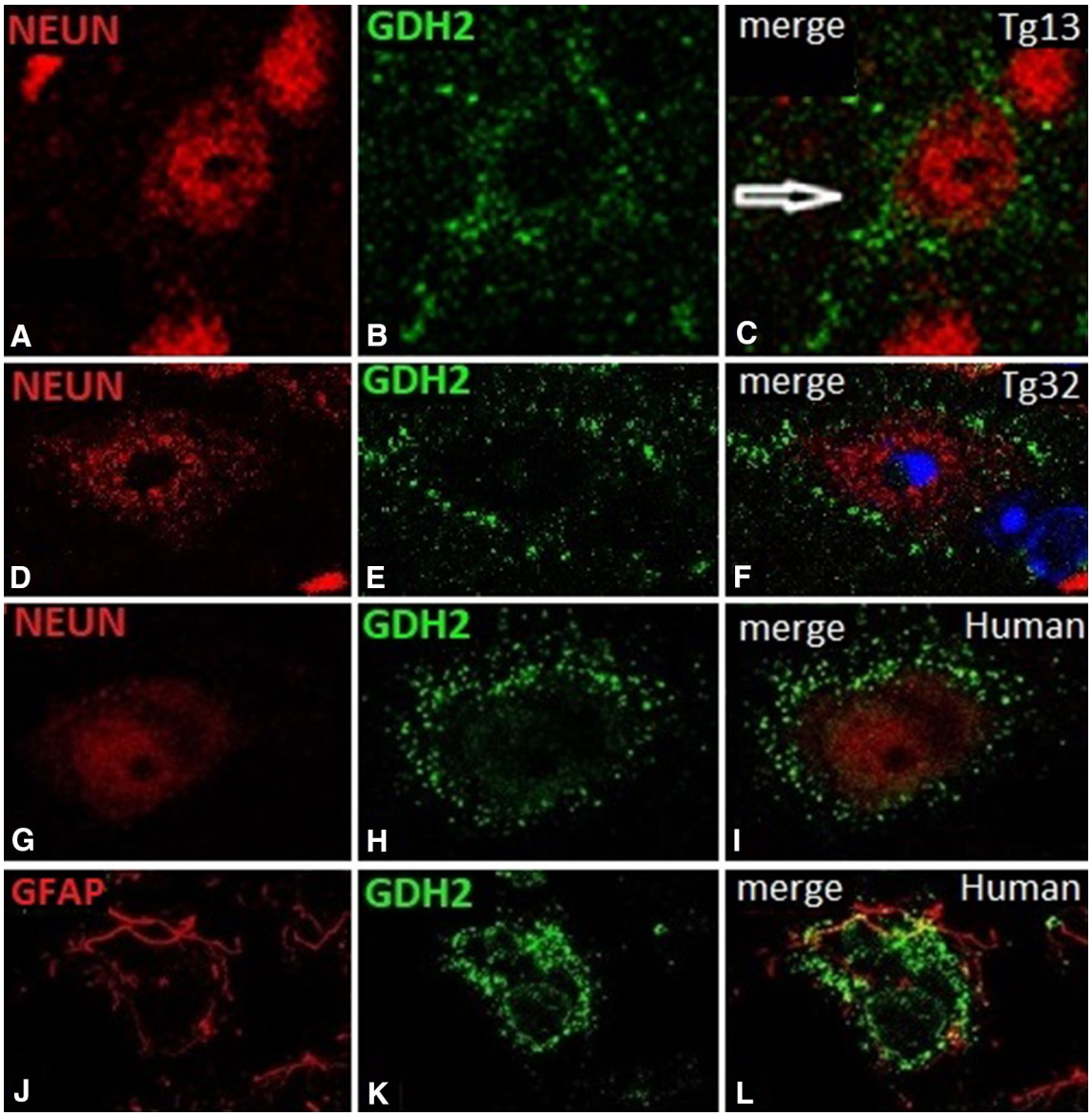

Fig. 3 hGDH2 expression in cerebral cortical neurons. IF images of snap frozen fixed cerebral cortical sections from a 6-month old male *GLUD2* Tg13 mouse (**a**–**c**) and a 6-month old male *GLUD2* Tg32 mouse (**d**–**f**) immunostained with a mouse monoclonal antibody against NeuN (1:400; red) and our rabbit antiserum against hGDH2 (1:2000; green). **g**–**l** IF images of unfixed human cerebral cortical sections from a neurologically normal male subject obtained using the same protocol. There is punctate hGDH2-specific immunoreactivity in the peripheral cytoplasm of large NEUN-positive neurons of Tg13 (arrow) and Tg32 mice cerebral cortex, and of human cerebral. Blue staining in C represents TOPRO labelled nuclei. Images obtained using a Leica Confocal microscope (×80). **j**–**l** Composite figure of superimposed consecutive confocal images of unfixed human cortex immunostained with a mouse antibody against GFAP (1:2000; red) and our rabbit antiserum against hGDH2 (1:2000; green). As shown here, the periphery of a large hGDH2-positive neuron is delineated by GFAP-positive astrocytic-end feet enwrapping excitatory synapses on the cell membrane. These lie in close proximity (but do not co-localize with) to large hGDH2-specific "puncta" that may represent clusters of hGDH2-positive mitochondria of presynaptic nerve terminals as previously described [21]. Figures **g**–**l** are reproduced with permission from [21]. (Color figure online)

gene and its regulatory elements, the observed cellular distribution of hGDH2 in the host brain represents an expressional adaptation driven by the gene's new promoter. Under the influence of this promoter, the duplicated *GLUD2* gene may have diversified its roles in human brain and perhaps in other tissues where is expressed. While the precise role(s) of hGDH2 in brain remain to be better understood, endowment of human cortical astrocytes and some of pyramidal neurons and their terminals with enhanced glutamate metabolizing capacity by hGDH2 expression is thought to strengthen cortical excitatory transmission [21]. Previous studies have shown that transgenic expression of GDH1 in the neurons of

Tg mice increases pre-synaptic glutamate release [26, 27]. In addition to this expressional diversification, *GLUD2* underwent rapid sequence adaptation acquiring novel properties that include resistance to GTP control and development of a novel mechanism for activity regulation. These major functional adaptations permit enhanced hGDH2 function in the nervous system, where GTP levels are greater than those found in other tissues. Given the observed close similarities between the animal and the human brain in hGDH2 expression, the *GLUD2* Tg mice are expected to serve as a useful model for studying the role of this novel, positively selected, gene in human evolution.

The thesis that *GLUD2* is a functional, non-redundant, human gene is supported not only by its unique expressional and functional profile, by also by extensive sequencing data involving > 1000 normal humans of diverse genetic background showing that the gene did not accumulate any disabling mutations, as typically seen in pseudogenes [28]. Also, highly consistent sequence data have been obtained in over a 1000 subjects of diverse genetic background affected by neurodegenerative disorders. However, a rare normal polymorphism (Leu445Ser) interacted significantly with age at disease onset in Parkinson's disease patients [28]. These results accord phylogenetic data showing that the structure of the human *GLUD2* gene has been preserved by purifying selection [15].

Functional and Molecular Adaptation of *GLUD2*

Functional Properties of the Ancestral Glutamate Dehydrogenase 1

Mammalian housekeeping GDH1 catalyzes the reversible conversion of glutamate to α-ketoglutarate and ammonia while reducing $NAD(P)^+$ to NAD(P)H. The enzyme shows dual co-enzyme specificity, being able to utilize either NAD^+ or $NADP^+$ for catabolic and synthetic reactions, respectively [17]. Also, mammalian GDH1 is subject to strong allosteric regulation as described below. Structurally, mammalian GDH1 is a hexameric molecule composed of six identical subunits, each of which consists of 505 amino acids (molecular mass: ~56 kDa). Three functional domains have been identified: the NAD^+ binding, the glutamate binding and the regulatory domain (Fig. 4a).

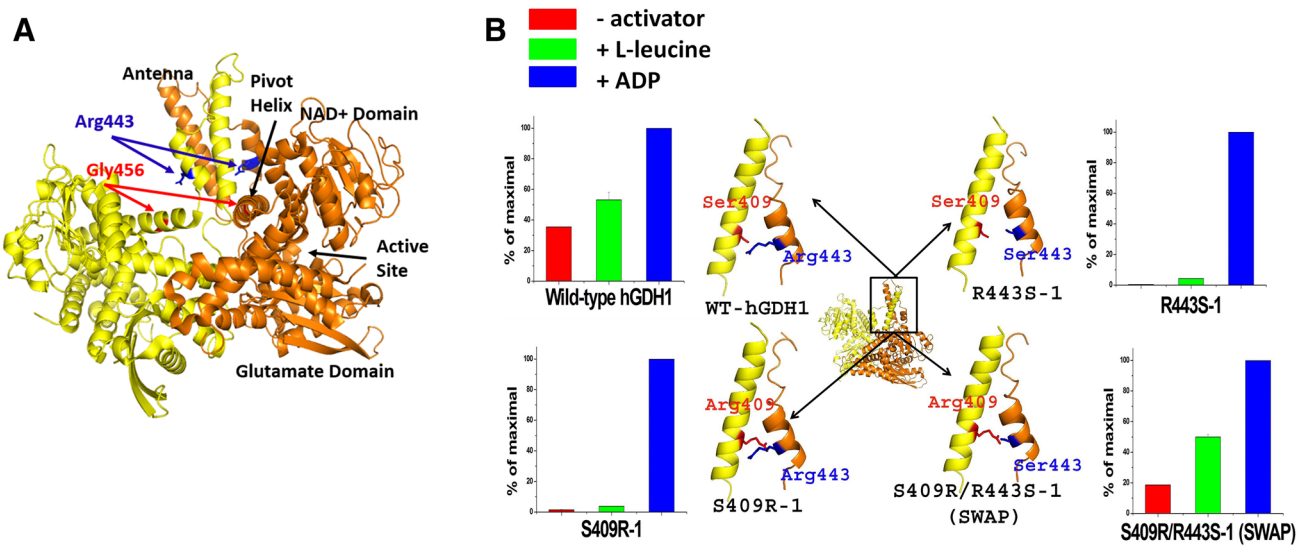

Fig. 4 Structural models and functional consequences of amino acid replacements in the regulatory domain of hGDH1. Shown in **a** is a cartoon diagram of human GDH in open conformation in the absence of ligands based on X-ray crystallographic data (PDB entry ILIF) showing the three functional domains of the enzyme. For simplicity only two of the six subunits (painted in different color). The regulatory domain consists of "antenna" and the "pivot helix". The antenna is composed of an ascending alpha-helix and a descending random coil that terminates in a small alpha-helix (**a**, **b**). The antennas of adjacent subunits are intertwined mediating allostery [29]. Interaction of Ser409 in the ascending alpha-helix with Arg443 in the small N terminal a-helix in the wild-type hGDH1 permits the enzyme to maintain basal activity (-activator) that is 30–40% of maximal and to be stimulated by L-leucine (4.5 mM) and ADP (1.0 mM) (**b**). The Ser409-Arg443 interaction is affected in the wild-type hGDH2 via the evolutionary substitution of Ser for Arg-443. This results in major functional consequences as described in the text. As shown in **b** replacement of Ser409 by Arg (S409R-1) results in loss of basal activity (-activator) and abrogation of L-leucine stimulation. Also, replacement of Arg443 by Ser (R443S-1) resulted in similar functional consequences, while a swap mutant (S409R-1/R443S-1) restored basal activity and regulation. Molecular dynamics simulation predicted that Ser409 and Arg443 (as in wild type hGDH1) come in close proximity in the open conformation and that introduction of Ser443 (as in the wild-type hGDH2) or of Arg409 (in the S409-R-1 mutant) causes them to separate with the swap mutant re-instating this proximity [30]. The cartoon diagrams were created using the PolyMOL Molecular Graphics System, Version 1.4, Schrodinger, L.L.C. Fig. 4b reproduced with permission from [30]. (Color figure online)

The latter consists of the pivot helix and the "antenna", a 48 amino acid-long structure composed of an ascending α-helix, a random coil and a small C-terminal α-helix (Fig. 4a, b). The antennas of adjacent subunits are intertwined with this subunit communication thought to mediate allosteric regulation [29]. Mastorodemos et al. [30] recently showed that interaction between Ser409 in the ascending α-helix of the antenna with Arg443 in the terminal small α-helix of the adjacent subunit is of importance for catalysis, regulation and pH dependency (Fig. 4b). It is of particular interest that this interaction was the target of the evolutionary adaptation of the *GLUD2* gene as described below.

There is increasing evidence that GDH1 functions both in the metabolism of neurotransmitter glutamate and in TCA cycle anaplerosis, as noted above. The neurotransmitter role is supported by immunohisto-chemical studies on rat brain showing that the enzyme is densely expressed in the mitochondria of astrocytes, the regional distribution of which corresponds to glutamatergic pathways [32]. In these glial cells, GDH1 attains very high levels (up to 10 mg/ml of mitochondrial matrix) [33], thus endowing these cells with high glutamate metabolizing capacity. The role of GDH1 in energy metabolism and in TCA anaplerosis is supported by several lines of evidence [34–38]. Specifically, astrocytes isolated from normal mice generated ATP on glutamate exposure in a GDH1-dependent manner [35], while astrocytes from mice with brain-specific deletion of Glud1 exhibited deficient glutamate oxidation and handling [36]. Also, siRNA knock down of GDH1 in cultured astrocytes was associated with a dysfunctional TCA cycle [37].

In spite of high GDH1 expression levels, early observations on cultured astrocytes showed little glutamate flux through this pathway [39], suggesting that the enzyme is highly regulated in vivo. In this regard, previous in vitro studies on GDH1, purified from various sources, had established that the enzyme is subject to strong allosteric regulation, with chemically diverse compounds (ADP, GTP, NADH, L-leucine, steroid hormones and neuroleptic agents) found to influence its velocity [17]. GTP, generated by TCA cycle, serves as the main negative modulator, whereas ADP as the main endogenous activator. As a result, GDH1 function is thought to depend primarily on the opposing actions of ADP and GTP. This energy sensing mechanism, permits glutamate flux through this pathway according to cellular energy requirements [17, 31]. Thus, under conditions of adequate energy charge, associated with low ADP levels, GTP generated by an active TCA cycle exerts a powerful inhibitory effect on GDH1. In contrast, when low energy states prevail, GTP levels decrease while those of ADP increase allowing glutamate to fuel the TCA cycle [31].

Evolutionary Adaptation of Glutamate Dehydrogenase 2

As noted above, after achieving expression in the nerve and other tissues, *GLUD2* evolved rapidly by adapting to the particular needs of these tissues. The cloning of *GLUD2* gene [16] and the ancestral housekeeping *GLUD1* gene [40], allowed us to study pure hGDH2 and hGDH1 enzymes obtained in recombinant form. When assayed in the presence of 1.0 mM ADP, both hGDH1 and hGDH2 showed comparable catalytic activities and kinetic properties (Vmax and K*m* for α-ketoglutarate, NADPH and ammonia) [41]. However, hGDH2 differed markedly from hGDH1 in its regulation profile. Thus, while hGDH1 is potently inhibited by GTP, hGDH2 is resistant to this compound [41]. By dissociating its function from GTP control, hGDH2 permits glutamate flux independently of the rate of GTP production by the TCA cycle. However, to prevent unregulated activity from perturbing cell metabolism, hGDH2 downregulated its basal activity while remaining remarkably responsive to activation by ADP/L-leucine. Thus, physiological levels of ADP (0.05–0.25 mM) induced a proportionally greater enhancement of hGDH2 (up to 1300%) than of hGDH1 (up to 150%) activity. Similarly, L-leucine activated hGDH2 by 1600% and hGDH1 by 75% [31]. While L-leucine concentrations required for this activation (5–10 mM) are substantially higher than those found in mammalian tissues, low concentrations of ADP (0.01–0.05 mM) permitted enzyme activation by physiologically relevant levels of L-leucine (synergistic effect) [31]. Also, hGDH2 is more sensitive than hGDH1 to inhibition by steroid hormones, spermidine and neuroleptic agents [42, 43]. In addition, hGDH2 shows a lower optimal pH than hGDH1 [41], an adaptation that may facilitate enzyme function under conditions of acidification that prevail in astrocytes following transmitter glutamate uptake [44].

To understand the role of these amino acid replacements in hGDH2 function, we carried out mutagenesis studies on the *GLUD1* gene at sites that underwent evolutionary change in *GLUD2*. As hGDH2 differs from hGDH2 primarily in its regulation profile, our initial efforts were concentrated on the regulatory domain of the protein. Two changes located in the "antenna" (M415L and R443) and another two in the "pivot helix" (G456A and R470V) were studied. Results revealed that substitution of Ala for Gly456 in hGDH1 made the enzyme markedly resistant to GTP inhibition without altering its catalytic properties [45]. On the other hand, replacement of Arg443 by Ser in hGDH1 diminished basal activity, abrogated activation by L-leucine and provided sensitivity to steroid hormones and neuroleptic agents [42, 43, 46]. In addition, the Arg443Ser mutation made hGDH1 thermo-sensitive, concentration-dependent and shifted its migration in SDS-PAGE and its optimal pH from 8.0 to 7.0

[46]. Molecular modeling suggested that Arg443Ser interrupts hydrogen bonds between the 443 residue (in the small helix of the descending part of the antenna) of one monomer and the 409 residue (in the ascending helix of the antenna) of the opposing monomer leading to closed enzyme conformation [30] (Fig. 4b). The Arg443Ser mutation, however, does not disrupt the tertiary structure of the protein as revealed by native gradient PAGE analyses, which showed that the molecular weight of the Arg443Ser mutant (360 kDa) is comparable to that of the wild-type hGDH1 and wild-type hGDH2 [47]. This molecular size is consistent with the hexameric structure of these proteins. Although the Arg443Ser substitution was a crucial event in *GLUD2* evolution, its introduction in hGDH1 as single mutant is too disruptive, rendering the mutant enzyme non-functional. Hence, other amino acid substitutions, acquired during hGDH2 evolution, must act in concert to moderate the functional consequences of Arg443Ser. Indeed, additional evolutionary substitutions, acquired by *GLUD2* evolution, are shown to affect hGDH2 function (Fig. 5), thus providing its unique functional properties.

In sharp contrast to the evolutionary adaptation of *GLUD2*, the housekeeping *GLUD1* gene has been conserved via strong purifying selection. Indeed, *GLUD1* accumulated no amino acid substitutions after the duplication event. As a result, alignment of the human *GLUD1* sequence with mouse, rat or bovine Glud1 sequences reveals less divergence than alignment of *GLUD1* with the *GLUD2* sequence.

Molecular Evolution of *GLUD2* in the Ape Lineages

Phylogenic evidence suggest that the Arg443Ser and Gly456Ala evolutionary mutations occurred soon after the birth of the *GLUD2* gene, along with the Glu34Lys, Asp142Glu, Ser174Asn and Asn498Ser changes (Fig. 5). These are thought to be under positive natural selection

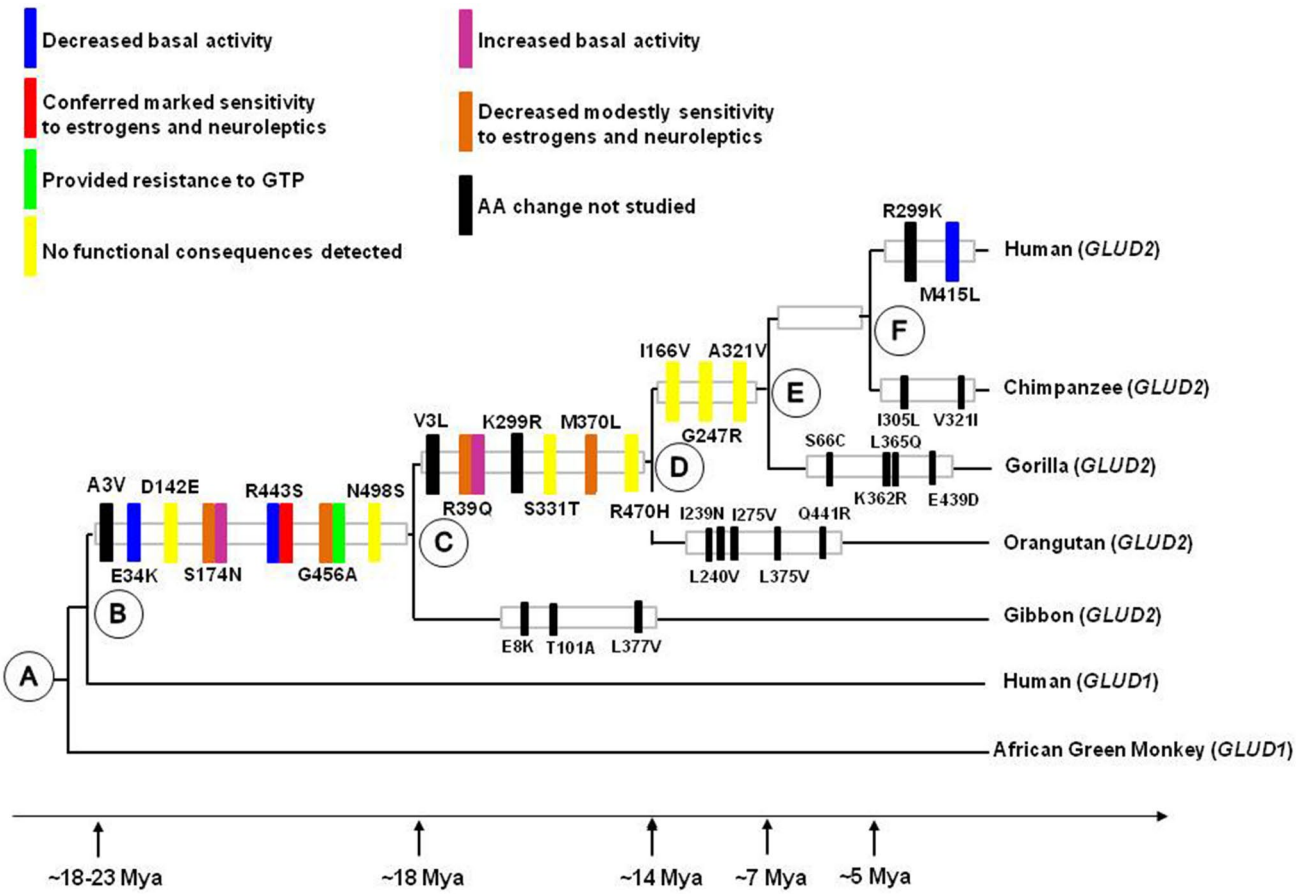

Fig. 5 Evolutionary amino acid substitutions acquired by hGDH2 and their functional consequences. Shown here is evolutionary adaptation of the *GLUD2* gene on the ape lineages. Mya: approximate divergence in millions of years. As shown here, evolutionary substitution of Ala for Gly456 made hGDH2 resistant to GTP, thus dissociating its function from this energy switch. On the other hand, evolutionary substitution of Ser for Arg443 diminished basal activity, abrogated L-leucine sensitivity and shifted the optimal pH from 8.0 to 7.0. The drastic effects of the Arg443Ser change are modified by the opposing action of the Arg39Gln, Ser174Asn Met370Leu and Gly456Ala evolutionary changes, thus providing the unique functional properties of the wild-type hGDH2. Reproduced with permission from [48]

[15]. Moreover, the Ser331Thr, Met370Leu and Arg470His mutations, which occurred just before the separation of the human from the orangutan lineage (Fig. 5), may have also been positively selected [15]. As shown in Fig. 5, five amino acid changes occurred in the orangutan lineage after its separation from the common ancestor of the gorilla, chimpanzee and human. Also, four amino acid changes occurred in the gorilla lineage after its split from the common ancestor of the humans and chimpanzees. Additionally, since the human and the chimpanzee lineages diverged about 4.6–6.2 million years ago, two amino acid changes occurred in the human lineage (one was a recurrent mutation) and two changes occurred in the chimpanzee lineage (Fig. 5). As a result, the chimpanzee GDH2 differs from the human by four amino acid residues, whereas the gorilla and orangutan GDH2 differ from the hGDH2 and chimpanzee GDH2 by 6 and 10 amino acid residues, respectively. Also, gorilla GDH2 differs from orangutan GDH2 by 12 amino acid changes. These phylogenetic data accord evidence that molecular evolution rates were slower in humans and chimpanzees than in gorillas and orangutans, probably due to longer generation times and improved mechanisms of DNA repair and genome integrity maintenance [8]. In addition, the cleavable mitochondrial targeting sequence (MTS) of hGDH2 evolved under positive selection acquiring a higher positive charge than that of hGDH1 [49]. Whether this enhanced mitochondrial targeting of hGDH2 facilitates the import of the protein into astrocytic mitochondria, the inner membrane potential of which is lower than that of hepatic cells [50], it remains to be further studied.

Putative Role of *GLUD2* in Human Brain Development

Whereas, humans and chimpanzees share 98% of their DNA sequence [51, 52], human brain is larger and shows a greater degree of complexity. Also, a substantial expansion of interneuronal connections and a formation of new neuronal circuits occur early in human post-natal life [1]. Phylogenetic data suggest that genesis and adaptation of the *GLUD2* gene coincided with the time period of increased structural and functional complexity of ape brain (Fig. 6), suggesting that the novel gene contributed to these processes [15]. That *GLUD2* may have helped the divergence of humans from the chimpanzees, is supported by data showing that human newborns have higher levels of expression of *GLUD2* than chimpanzee newborns [4]. Moreover glutamate levels are lower in the human brain than in non-human primates indicative of an increased glutamate turnover [4]. While the mechanisms underlying these changes have not been understood, endowment of human cortical astrocytes and neurons with *GLUD2* expression could have played a role by providing these cells with a non-redundant pathway for enhancing glutamatergic

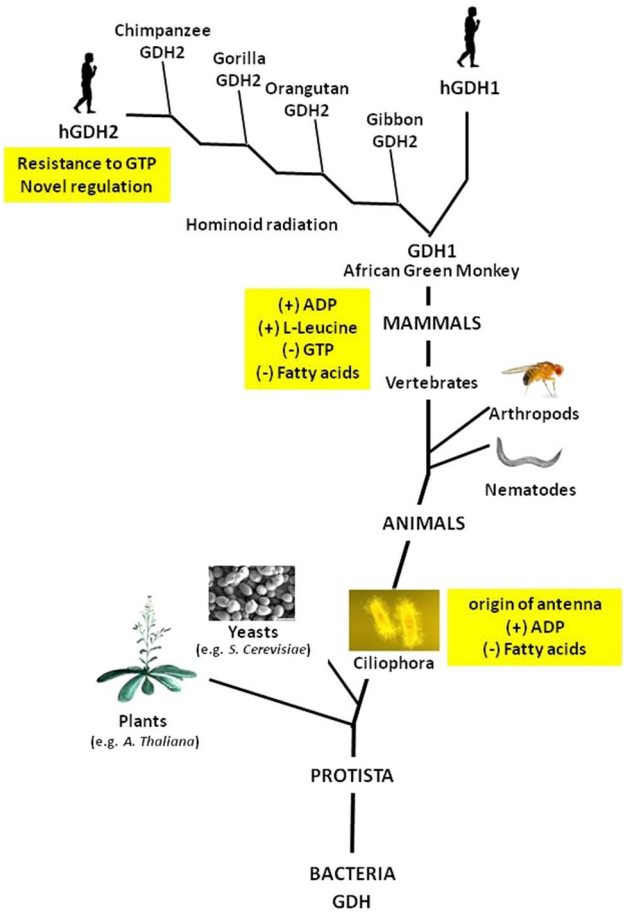

Fig. 6 Schematic representation of GDH evolution from prokaryotes to lower eukaryotes, animals and the great apes. Yellow boxes display the appearance of major allosteric characteristics. As shown here, the antenna first emerged in the Ciliophora, enabling enzyme activation by ADP and partial inhibition by fatty acids. However, the GDH of ciliates is not sensitive to activation by L-leucine or inhibition by GTP inhibition [54]. In subsequent evolutionary steps, GDH1 acquired additional properties linking enzyme function with energy metabolism [55]. The novel hGDH2 isoenzyme was born through retropositioning in the hominoid ancestor and evolved in the ape linages. It underwent rapid evolutionary adaptation concomitantly with brain expansion and acquisition of unique traits. It is likely that emergence and evolution of *GLUD2* resulted from selective pressures on the genome that probably relate to the increased glutamatergic transmission demands of the expanding ape brain. Evolution of GDH1 regulation in Ciliates and in Vertebrates has been described by Allen et al. [54] and Banerjee et al. [55] respectively. (Color figure online)

mechanisms and TCA cycle efficiency. Whether *GLUD2* adaption has contributed to the high neuronal activity and energy consumption that characterize the human brain [4] or even to cognitive human abilities remains to be further investigated. As *Glud1* is one of genes upregulated during memory formation in the rat [53], it is likely that emergence and evolution of *GLUD2* has resulted from selective pressures on the genome related to the expanding role of glutamatergic transmission in ape brain.

Evidence favoring a role of *GLUD2* in human brain development was recently provided by Li et al. [56] who generated independently *GLUD2* transgenic mice by inserting in their genome the above described BAC clone containing the *GLUD2* gene and its regulatory elements. Large scale analyses of polyA-plus RNA isolated from the brain of these mice revealed that the developmental expression trajectory of *GLUD2* in Tg mouse brain was distinct from that of *GLUD1*, but it was strikingly similar to the developmental expression trajectory of *GLUD2* in human PFC [56]. The differential effects of the *GLUD2* gene were mostly prominent during early post-natal development, affecting the expression of several genes, some of which encode transcription factors involved in neural development. A large divergence (between the *GLUD2* Tg and the wild-type mice) was detected in the developing PFC with these changes being similar to those that distinguish human from macaque brain [56]. Moreover, metabolomic analyses revealed that *GLUD2* expression affected pathways surrounding the TCA cycle. Li et al. [56] accordingly concluded that a non-redundant *GLUD2* metabolic function supports early brain development. The authors further hypothesized that, since hGDH2 function in isocitrate dehydrogenase 1 (IDH1) mutant glioma cells (see below) provides α-ketoglutarate for the oxidative generation of citrate through the TCA cycle, *GLUD2* may influence early brain development by promoting the synthesis of lipids [56].

Obviously, these important observations raise a number of issues: First, shunting of glutamate-derived carbons via *GLUD2 t*o citrate synthesis, as observed in IDH1 mutant glioma, represents a metabolic reprogramming of neoplastic cells (needed for their survival), necessitated by the inability of the mutant IDH1 to synthesize α-ketoglutarate from isocitrate [57]. Whether this pathway is activated in developing human brain expressing the wild-type (non-mutant) IDH it remains to be established. As shown in Fig. 7, oxidative deamination of glutamate α-ketoglutarate by GDH1/2 enhances the bioenergetic and biosynthetic function of the TCA cycle, leading to formation of several compounds of importance for cell growth. Moreover, dividing cells need to maintain high levels of α-ketoglutarate required by the nuclear α-ketoglutarate ketoglutarate-dependent dioxygenases [58]. Efforts to explain the observed biological effects of *GLUD2* on the basis of its metabolic functions should take into consideration the specificity of the *GLUD2* related changes for the prefrontal lobe.

Second, the high non-redundant activity of *GLUD2*, detected during the early post-natal period, coincides with

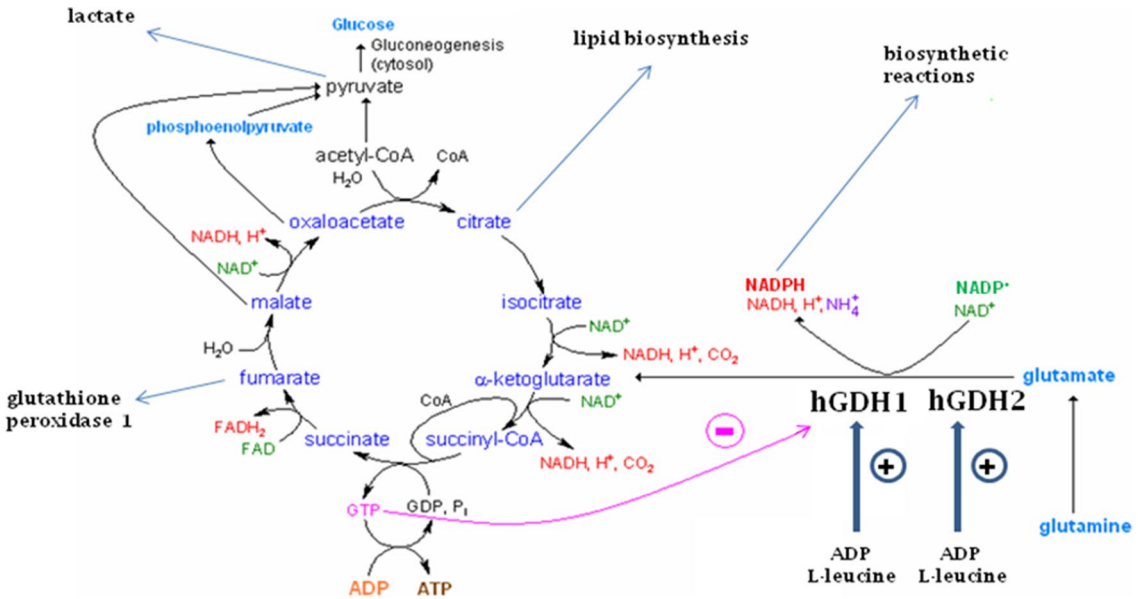

Fig. 7 The GDH pathway and TCA cycle function. Oxidative deamination of glutamate by hGDH1 and hGDH2 generates α-ketoglutarate, ammonia and NADH or NADPH. α-ketoglutarate enters the TCA cycle enhancing its bio-energetic and biosynthetic functions. GTP, synthesized at the succinyl-CoA/succinate step, serves as a potent inhibitor of hGDH1. This control links hGDH1 function to the rate of the TCA cycle. On the other hand, hGDH2 can operate independently of GTP control and therefore of TCA cycle rate. Both enzymes are activated by ADP and L-leucine (acting synergistically), with this activation being proportionally greater for hGDH2 than hGDH1 [41]. Fumarate, generated after succinate, can stimulate glutathione peroxidase 1, thus contributing to homeostasis against oxidative stress [59]. As shown here, α-ketoglutarate metabolism via the TCA cycle can boost lactate production [60]. Also, citrate, generated by the TCA cycle can support the biosynthesis of lipids. Lastly, NADPH resulting from glutamate oxidation can be used for various biosynthetic reactions requiring reducing equivalents [61]. There is evidence that glutamate flux through hGDH1/2 is used by different cells to serve some of their unique functions [61]. Reproduced from [17]

the burst of synaptogenesis and new nerve circuit formation, processes mediated by transmitter glutamate. Indeed previous studies have shown that NMDA glutamate receptor activation and Ca^{2+} influx is crucial to neurogenesis, survival of neurons, neuronal migration and synapse formation [27, 62–65]. Nissen et al. [37] grew astrocytes from the brain of 7 day old Tg mice, constructed by Li et al. [56], expressing *GLUD2* and found that these cells had an increased capacity for uptake and oxidative metabolism of glutamate, particularly during intense glutamatergic activity and glucose deprivation. It is well established that strong glutamatergic stimulation, induced by high-frequency stimuli, results in potentiation of synaptic responses or long-term potentiation (LTP) [6, 7]. As such, the ability of hGDH2 to support intense glutamatergic activity suggests an important role of the new enzyme in brain plasticity. Hence, given the complexities of the metabolic pathways served by *GLUD2* as well as the complexities of the developmental processes influenced by neurotransmitter glutamate, identifying the mechanisms by which a highly activated *GLUD2* promotes the early postnatal development and the human-specific differentiation of PFC represents challenging experimental question.

Evolutionary Adaptation *GLUD2* Confers hGDH2 a Non-redundant Glioma IDH1 Promoting Property

Since the pioneering work of Warburg demonstrating that cancer cells use alternate pathways to compensate for lack of nutrients or oxygen, metabolic reprogramming has been recognized as the defining characteristic of these cells. Thus, when glucose supply is limited, glutaminolysis is activated generating glutamate. This is turn is converted (either by GDH1/2 or by GOT1/2) to α-ketoglutarate which enters the TCA cycle providing anabolic carbons for the biosynthesis of amino acids, nucleotides and lipids, in addition to leading to ATP synthesis [57]. There is increasing evidence that oxidative deamination of glutamate by GDH (rather than transamination by GOT) is the main pathway for α-ketoglutarate production with up-regulation of hGDH1/2 expression shown to occur in various cancers, including gliomas [66]. Recently, somatic mutations in IDH1, an enzyme that inter-converts isocitrate to α-ketoglutarate, were identified in the vast majority (70–90%) of low grade glioma and secondary glioblastoma multiform [67]. The IDH mutants aberrantly reduce α-ketoglutarate to D-2-hydroxyglutarate, which accumulates at high concentrations in glioma cells acting as oncometabolite [67]. Because the IDH mutants cannot convert isocitrate to α-ketoglutarate, glioma cells upregulate alternative metabolic pathways, including hGDH1 and hGDH2 [66]. Chen et al. [68] showed that over expression of *GLUD2* (but not *GLUD1*) promoted tumor expansion in IDH1 mutant glioma. Labeling studies further revealed that glutamate flux through hGDH2 provides α-ketoglutarate for oxidative generation of citrate through the Krebs cycle [68]. More recently, Waitkus et al. [69] showed that hGDH2 expression non-redundantly increases several TCA cycle substrates (including α-ketoglutarate, citrate and aconitate) through glutamate-dependent anaplerosis [69]. Importantly, using site directed mutagenesis the authors showed that the two evolutionary substitutions (Arg443Ser and Gly456Ala) in the regulatory domain of hGDH2 that provide novel functional properties to hGDH2 (as described above) conferred the glioma-supporting ability of hGDH2 [69]. Specifically, when Ser443 and Ala456 were mutated back to Arg443 and Gly456, the effect on glioma growth was attenuated. As such, the ability of hGDH2 to supply TCA substrates in glioma cells, a process disrupted by IDH1 mutations, is conferred by the adaptive evolution of the allosteric domain of hGDH2. This property is not shared by hGDH1. These observations on a neoplastic system that requires a high glutamate flux confirms the above described model according to which, while hGDH1 function is subject to control by GTP generated by the TCA cycle, hGDH2 is freed of this control owing to the Arg443Ser and Gly456Ala evolutionary amino acid changes.

The Glutamate Dehydrogenase Pathway and Species Evolution

The GDHs of Prokaryotes and Lower Eukaryotes

The GDH pathway is present in all domains of life. Study of early life forms revealed that GDHs from different species are involved in two metabolic pathways: one in glutamate catabolism (generally NAD^+-dependent, EC 1.4.1.2) and another in ammonia assimilation (generally $NADP^+$-dependent, EC 3.4.1.4). In *Pseudomonas aeruginosa* and *Neurospora crassa* tetrameric ("catabolic") and hexameric ("anabolic") GDH forms exist [70] that acquired NAD^+ or $NADP^+$ specificity through independent evolutionary processes [71]. In the yeast *Saccharomyces cerevisiae*, two NADP-specific (ScGDH1 and ScGDH3) and one NAD^+-specific (sGDH2) isoforms, all of which are extra-mitochondrial, have been described [72]. The NAD^+-specific enzyme (sGDH2) functions in the oxidative deamination of glutamate, while the $NADP^+$-specific isoforms (ScGDH1 and ScGDH3) in glutamate synthesis under either fermentative or respiratory conditions [73]. The ScGDH3 and ScGDH1 genes arose from whole genome duplication and evolved under selective pressure to sustain glutamate production under different metabolic conditions [74]. In lower eukaryotes, cytosolic, nuclear and mitochondrial localizations have been reported [75]. In plants, the catabolic NAD^+-specific GDH isoform that localizes to the mitochondria predominates [76]. However, in the presence

of high ammonium concentrations, enzyme activity is detected in the cytosol. On the other hand, low activities of $NADP^+$-depended GDHs have been demonstrated in a range of higher plants, including *Arabidopsis* and *Oryza*. These $NADP^+$-specific GDHs localize to the chloroplasts, probably involved in the assimilation of the photo-respiratory NH3 into glutamate [77]. In prokaryotes and lower eukaryotes GDH activity is not regulated, with regulation being accomplished at the transcription level.

The Animal GDHs

In contrast to prokaryotes and lower eukaryotes in which GDH exists in distinct NAD^+ and $NADP^+$-specific forms, most animals possess a single GDH with dual co-enzyme specificity, the activity of which is subject to strong regulation as noted above. There is evidence that this regulation evolved concomitantly with the evolution of the antenna thus enabling control of GDH activity according to the cell's energy needs (Figs. 4, 6). The antenna first appeared in ciliates, permitting a primitive regulation of GDH in these protozoans by fatty acids and coinciding with a gradual transfer of fatty acid oxidation from peroxisomes to mitochondria [54, 55]. Control of enzyme activity by Palmitoyl CoA is retained in mammalian GDH [17]. Moreover, further evolution of the antenna equipped mammalian GDH1 with a sophisticated allosteric regulation profile as noted above. Interestingly, evolutionary adaptation of the novel hGDH2 isoenzyme that first appeared in the apes targeted the regulatory domain of the enzyme as described above. During animal evolution oxidative metabolism was transferred to the mitochondria with mammalian GDH having acquired a MTS needed for import of the enzyme into the mitochondrial matrix [78, 79]. The use of the yeast mitochondria import system, in conjunction with studies on mammalian cell lines, permitted detailed structure/function investigations of the MTS of hGDH1 and hGDH2. These studies revealed that the 53 amino acid long MTS represents a highly efficient mitochondrial import system. This is due to the positive charge and to a complex interplay between two amphipathic α helices predicted by these pre-sequences [79]. Although all mammals possess a single GDH (hGDH1 in the human) humans and other great apes have acquired through duplication a novel hGDH2 isoenzyme with distinct regulation and tissue expression profile as noted above. Phylogenetic evidence suggests that this duplication event occurred in the hominoid ancestor and as such is found in all members of the hominoid radiation (Figs. 5, 6). As described above, all mammals except for the apes possess a single GDH-specific gene (*GLUD1* in the human) that appears to suffice for their metabolic needs. Deletion of the single *Glud1* in mice was found to alter energy metabolism without affecting glutamatergic transmission, suggesting that in the absence of GDH1 alternative metabolic pathways or mechanisms compensate allowing glutamatergic transmission to proceed. This is consistent with the model described above that hGDH1 function is essentially controlled by GTP which acts as an energy switch.

Future Perspectives

As detailed here, *GLUD2* is a novel human gene that, driven by positive selection, underwent rapid evolutionary adaptation concomitantly with neocortical brain expansion. Its emergence may thus reflect selective pressures on the genome arising from increased primate brain demands in glutamatergic transmission and energy metabolism. As hGDH2 functions mainly during intense excitatory transmission (needed for LTP formation), the novel gene may have enabled the glutamatergic signaling pathway to amplify its role in synaptic plasticity and cognitive processes. These exciting possibilities and whether *GLUD2* evolution has provided humans with a genetic/molecular basis for expanding experience-dependent formation of new neural circuits remain to be further investigated. Additional morphological, neurochemical and electrophysiological investigations, using this Tg model, are expected to shed light on these important questions. A crucial experimental aim is to evaluate whether transgenic expression of the human gene modulates long-lasting modification of excitatory synapses (LTP) and structural plasticity (dendritic spine density). Moreover, the animal model can be used to test whether brain areas involved in cognitive functions are specifically targeted by *GLUD2* expression and whether this is associated with behavioral changes. Lastly, as there is increasing evidence that astrocytes can modulate LTP dynamics (formation and maintenance), the Tg model may prove useful to investigate whether adaptation in the astrocytic metabolism, induced by hGDH2 expression, affects synaptic transmission and plasticity. Understanding the precise role of the novel gene in human evolution and brain biology represents a challenging and exciting endeavor expected to yield useful information.

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References

1. Bianchi S, Stimpson CD, Bauernfeind AL, Schapiro SJ, Baze WB, McArthur MJ, Bronson E, Hopkins WD, Semendeferi K, Jacobs B, Hof PR, Sherwood CC (2013) Dendritic morphology

of pyramidal neurons in the chimpanzee neocortex: regional specializations and comparison to humans. Cereb Cortex 23(10):2429–2436
2. Sherwood CC, Stimpson CD, Raghanti MA, Wildman DE, Uddin M, Grossman LI, Goodman M, Redmond JC, Bonar CJ, Erwin JM, Hof PR (2006) Evolution of increased glia-neuron ratios in the human frontal cortex. Proc Natl Acad Sci USA 103(37):13606–13611
3. Grossman LI, Schmidt TR, Wildman DE, Goodman M (2001) Molecular evolution of aerobic energy metabolism in primates. Mol Phylogenet Evol 18(1):26–36
4. Fu X, Giavalisco P, Liu X, Catchpole G, Fu N, Ning ZB, Guo S, Yan Z, Somel M, Paabo S, Zeng R, Willmitzer L, Khaitovich P (2011) Rapid metabolic evolution in human prefrontal cortex. Proc Natl Acad Sci USA 108(15):6181–6186
5. Caceres M, Lachuer J, Zapala MA, Redmond JC, Kudo L, Geschwind DH, Lockhart DJ, Preuss TM, Barlow C (2003) Elevated gene expression levels distinguish human from non-human primate brains. Proc Natl Acad Sci USA 100(22):13030–13035
6. Malenka RC, Nicoll RA (1993) NMDA-receptor-dependent synaptic plasticity: multiple forms and mechanisms. Trends Neurosci 16(12):521–527
7. Lynch MA (2004) Long-term potentiation and memory. Physiol Rev 84(1):87–136
8. Goodman M, Sterner KN (2010) Colloquium paper: phylogenomic evidence of adaptive evolution in the ancestry of humans. Proc Natl Acad Sci USA 107(Suppl 2):8918–8923
9. Gomez-Robles A, Hopkins WD, Schapiro SJ, Sherwood CC (2015) Relaxed genetic control of cortical organization in human brains compared with chimpanzees. Proc Natl Acad Sci USA 112(48):14799–14804
10. Muntane G, Horvath JE, Hof PR, Ely JJ, Hopkins WD, Raghanti MA, Lewandowski AH, Wray GA, Sherwood CC (2015) Analysis of synaptic gene expression in the neocortex of primates reveals evolutionary changes in glutamatergic neurotransmission. Cereb Cortex 25(6):1596–1607
11. Varki A, Altheide TK (2005) Comparing the human and chimpanzee genomes: searching for needles in a haystack. Genome Res 15(12):1746–1758
12. Biswas S, Akey JM (2006) Genomic insights into positive selection. Trends Genet 22(8):437–446
13. O'Bleness M, Searles VB, Varki A, Gagneux P, Sikela JM (2012) Evolution of genetic and genomic features unique to the human lineage. Nat Rev Gen 13(12):853–866
14. Nielsen R, Bustamante C, Clark AG, Glanowski S, Sackton TB, Hubisz MJ, Fledel-Alon A, Tanenbaum DM, Civello D, White TJ, Adams JJS, Cargill MD M (2005) A scan for positively selected genes in the genomes of humans and chimpanzees. PLoS Biol 3(6):e170
15. Burki F, Kaessmann H (2004) Birth and adaptive evolution of a hominoid gene that supports high neurotransmitter flux. Nat Genet 36(10):1061–1063
16. Shashidharan P, Michaelidis TM, Robakis NK, Kresovali A, Papamatheakis J, Plaitakis A (1994) Novel human glutamate dehydrogenase expressed in neural and testicular tissues and encoded by an X-linked intronless gene. J Biol Chem 269(24):16971–16976
17. Plaitakis A, Kalef-Ezra E, Kotzamani D, Zaganas I, Spanaki C (2017) The glutamate dehydrogenase pathway and its roles in cell and tissue biology in health and disease. Biology 6(1):E11. https://doi.org/10.3390/biology6010011
18. Li WH, Yang J, Gu X (2005) Expression divergence between duplicate genes. Trends Gen 21(11):602–607. https://doi.org/10.1016/j.tig.2005.08.006
19. Varki A (2004) How to make an ape brain. Nat Gen 36(10):1034–1036
20. Spanaki C, Zaganas I, Kleopa KA, Plaitakis A (2010) Human GLUD2 glutamate dehydrogenase is expressed in neural and testicular supporting cells. J Biol Chem 285(22):16748–16756
21. Spanaki C, Kotzamani D, Kleopa K, Plaitakis A (2016) Evolution of GLUD2 glutamate dehydrogenase allows expression in human cortical neurons. Mol Neurobiol 53(8):5140–5148
22. Douni E, Alexiou M, Kollias G (2004) Genetic engineering in the mouse: tuning TNF/TNFR expression. Methods Mol Med 98:137–170
23. Rinotas V, Niti A, Dacquin R, Bonnet N, Stolina M, Han CY, Kostenuik P, Jurdic P, Ferrari S, Douni E (2014) Novel genetic models of osteoporosis by overexpression of human RANKL in transgenic mice. J Bone Miner Res 29(5):1158–1169
24. Oberheim NA, Takano T, Han X, He W, Lin JH, Wang F, Xu Q, Wyatt JD, Pilcher W, Ojemann JG, Ransom BR, Goldman SA, Nedergaard M (2009) Uniquely hominid features of adult human astrocytes. J Neurosci 29(10):3276–3287
25. Paixao S, Klein R (2010) Neuron-astrocyte communication and synaptic plasticity. Curr Opin Neurobiol 20(4):1–8
26. Bao X, Pal R, Hascup KN, Wang Y, Wang WT, Xu W, Hui D, Agbas A, Wang X, Michaelis ML, Choi IY, Belousov AB, Gerhardt GA, Michaelis EK (2009) Transgenic expression of Glud1 (glutamate dehydrogenase 1) in neurons: in vivo model of enhanced glutamate release, altered synaptic plasticity, and selective neuronal vulnerability. J Neurosci 29(44):13929–13944
27. Wang X, Patel ND, Hui D, Pal R, Hafez MM, Sayed-Ahmed MM, Al-Yahya AA, Michaelis EK (2014) Gene expression patterns in the hippocampus during the development and aging of Glud1 (glutamate dehydrogenase 1) transgenic and wild type mice. BMC Neurosci 15:37
28. Plaitakis A, Latsoudis H, Kanavouras K, Ritz B, Bronstein JM, Skoula I, Mastorodemos V, Papapetropoulos S, Borompokas N, Zaganas I, Xiromerisiou G, Hadjigeorgiou GM, Spanaki C (2010) Gain-of-function variant in GLUD2 glutamate dehydrogenase modifies Parkinson's disease onset. Eur J Hum Genet 18(3):336–341
29. Smith TJ, Schmidt T, Fang J, Wu J, Siuzdak G, Stanley CA (2002) The structure of apo human glutamate dehydrogenase details subunit communication and allostery. J Mol Biol 318(3):765–777
30. Mastorodemos V, Kanavouras K, Sundaram S, Providaki M, Petraki Z, Kokkinidis M, Zaganas I, Logothetis DE, Plaitakis A (2015) Side-chain interactions in the regulatory domain of human glutamate dehydrogenase determine basal activity and regulation. J Neurochem 133(1):73–82
31. Mastorodemos V, Zaganas I, Spanaki C, Bessa M, Plaitakis A (2005) Molecular basis of human glutamate dehydrogenase regulation under changing energy demands. J Neurosci Res 79(1–2):65–73
32. Aoki C, Milner TA, Berger SB, Sheu KF, Blass JP, Pickel VM (1987) Glial glutamate dehydrogenase: ultrastructural localization and regional distribution in relation to the mitochondrial enzyme, cytochrome oxidase. J Neurosci Res 18(2):305–318
33. Rothe F, Wolf G, Schunzel G (1990) Immunohistochemical demonstration of glutamate dehydrogenase in the postnatally developing rat hippocampal formation and cerebellar cortex: comparison to activity staining. Neuroscience 39(2):419–429
34. McKenna MC (2013) Glutamate pays its own way in astrocytes. Front Endocrinol 4:191
35. Karaca M, Frigerio F, Migrenne S, Martin-Levilain J, Skytt DM, Pajecka K, Martin-del-Rio R, Gruetter R, Tamarit-Rodriguez J, Waagepetersen HS, Magnan C, Maechler P (2015) GDH-dependent glutamate oxidation in the brain dictates peripheral energy substrate distribution. Cell Rep 13(2):365–375
36. Frigerio F, Karaca M, De Roo M, Mlynarik V, Skytt DM, Carobbio S, Pajecka K, Waagepetersen HS, Gruetter R, Muller D, Maechler P (2012) Deletion of glutamate dehydrogenase 1 (Glud1) in the

central nervous system affects glutamate handling without altering synaptic transmission. J Neurochem 123(3):342–348
37. Nissen JD, Pajecka K, Stridh MH, Skytt DM, Waagepetersen HS (2015) Dysfunctional TCA-cycle metabolism in glutamate dehydrogenase deficient astrocytes. Glia 63(12):2313–2326
38. Nissen JD, Lykke K, Bryk J, Stridh MH, Zaganas I, Skytt DM, Schousboe A, Bak LK, Enard W, Paabo S, Waagepetersen HS (2017) Expression of the human isoform of glutamate dehydrogenase, hGDH2, augments TCA cycle capacity and oxidative metabolism of glutamate during glucose deprivation in astrocytes. Glia 65(3):474–488
39. Farinelli SE, Nicklas WJ (1992) Glutamate metabolism in rat cortical astrocyte cultures. J Neurochem 58(5):1905–1915
40. Mavrothalassitis G, Tzimagiorgis G, Mitsialis A, Zannis V, Plaitakis A, Papamatheakis J, Moschonas N (1988) Isolation and characterization of cDNA clones encoding human liver glutamate dehydrogenase: evidence for a small gene family. Proc Natl Acad Sci USA 85(10):3494–3498
41. Kanavouras K, Mastorodemos V, Borompokas N, Spanaki C, Plaitakis A (2007) Properties and molecular evolution of human GLUD2 (neural and testicular tissue-specific) glutamate dehydrogenase. J Neurosci Res 85(15):3398–3406
42. Borompokas N, Papachatzaki MM, Kanavouras K, Mastorodemos V, Zaganas I, Spanaki C, Plaitakis A (2010) Estrogen modification of human glutamate dehydrogenases is linked to enzyme activation state. J Biol Chem 285(41):31380–31387
43. Spanaki C, Zaganas I, Kounoupa Z, Plaitakis A (2012) The complex regulation of human glud1 and glud2 glutamate dehydrogenases and its implications in nerve tissue biology. Neurochem Int 61(4):470–481
44. Azarias G, Perreten H, Lengacher S, Poburko D, Demaurex N, Magistretti PJ, Chatton JY (2011) Glutamate transport decreases mitochondrial pH and modulates oxidative metabolism in astrocytes. J Neurosci 31(10):3550–3559
45. Zaganas I, Plaitakis A (2002) Single amino acid substitution (G456A) in the vicinity of the GTP binding domain of human housekeeping glutamate dehydrogenase markedly attenuates GTP inhibition and abolishes the cooperative behavior of the enzyme. J Biol Chem 277(29):26422–26428
46. Zaganas I, Spanaki C, Karpusas M, Plaitakis A (2002) Substitution of Ser for Arg-443 in the regulatory domain of human housekeeping (GLUD1) glutamate dehydrogenase virtually abolishes basal activity and markedly alters the activation of the enzyme by ADP and L-leucine. J Biol Chem 277(48):46552–46558
47. Zaganas I, Kanavouras K, Mastorodemos V, Latsoudis H, Spanaki C, Plaitakis A (2009) The human GLUD2 glutamate dehydrogenase: localization and functional aspects. Neurochem Int 55(1–3):52–63
48. Plaitakis A, Latsoudis H, Spanaki C (2011) The human GLUD2 glutamate dehydrogenase and its regulation in health and disease. Neurochem Int 59(4):495–509
49. Rosso L, Marques AC, Reichert AS, Kaessmann H (2008) Mitochondrial targeting adaptation of the hominoid-specific glutamate dehydrogenase driven by positive Darwinian selection. PLoS Genet 4(8):e1000150. https://doi.org/10.1371/journal.pgen.1000150
50. Matthews GD, Gur N, Koopman WJ, Pines O, Vardimon L (2010) Weak mitochondrial targeting sequence determines tissue-specific subcellular localization of glutamine synthetase in liver and brain cells. J Cell Sci 123(Pt 3):351–359
51. Nowick K, Gernat T, Almaas E, Stubbs L (2009) Differences in human and chimpanzee gene expression patterns define an evolving network of transcription factors in brain. Proc Natl Acad Sci USA 106(52):22358–22363
52. Uddin M, Wildman DE, Liu G, Xu W, Johnson RM, Hof PR, Kapatos G, Grossman LI, Goodman M (2004) Sister grouping of chimpanzees and humans as revealed by genome-wide phylogenetic analysis of brain gene expression profiles. Proc Natl Acad Sci USA 101(9):2957–2962
53. Cavallaro S, Meiri N, Yi CL, Musco S, Ma W, Goldberg J, Alkon DL (1997) Late memory-related genes in the hippocampus revealed by RNA fingerprinting. Proc Natl Acad Sci USA 94(18):9669–9673
54. Allen A, Kwagh J, Fang J, Stanley CA, Smith TJ (2004) Evolution of glutamate dehydrogenase regulation of insulin homeostasis is an example of molecular exaptation. Biochemistry 43(45):14431–14443
55. Banerjee S, Schmidt T, Fang J, Stanley CA, Smith TJ (2003) Structural studies on ADP activation of mammalian glutamate dehydrogenase and the evolution of regulation. Biochemistry 42(12):3446–3456
56. Li Q, Guo S, Jiang X, Bryk J, Naumann R, Enard W, Tomita M, Sugimoto M, Khaitovich P, Paabo S (2016) Mice carrying a human GLUD2 gene recapitulate aspects of human transcriptome and metabolome development. Proc Natl Acad Sci USA 113(19):5358–5363. https://doi.org/10.1073/pnas.1519261113
57. Boroughs LK, DeBerardinis RJ (2015) Metabolic pathways promoting cancer cell survival and growth. Nat Cell Biol 17(4):351–359
58. Carey BW, Finley LW, Cross JR, Allis CD, Thompson CB (2015) Intracellular alpha-ketoglutarate maintains the pluripotency of embryonic stem cells. Nature 518(7539):413–416
59. Jin L, Li D, Alesi GN, Fan J, Kang HB, Lu Z, Boggon TJ, Jin P, Yi H, Wright ER, Duong D, Seyfried NT, Egnatchik R, DeBerardinis RJ, Magliocca KR, He C, Arellano ML, Khoury HJ, hin DM, Khuri FR, Kang S (2015) Glutamate dehydrogenase 1 signals through antioxidant glutathione peroxidase 1 to regulate redox homeostasis and tumor growth. Cancer Cell 27:257–270
60. Sonnewald U, Westergaard N, Petersen SB, Unsgard G, Schousboe A (1993) Metabolism of [U-13C] glutamate in astrocytes studied by 13C NMR spectroscopy: incorporation of more label into lactate than into glutamine demonstrates the importance of the tricarboxylic acid cycle. J Neurochem 61(3):1179–1182
61. Spanaki C, Kotzamani D, Plaitakis A (2017) Widening spectrum of cellular and subcellular expression of human GLUD1 and GLUD2 glutamate dehydrogenases suggests novel functions. Neurochem Res 42(1):92–107
62. Bhatt DH, Zhang S, Gan WB (2009) Dendritic spine dynamics. Annu Rev Physiol 71:261–282
63. Komuro H, Rakic P (1993) Modulation of neuronal migration by NMDA receptors. Science 260(5104):95–97
64. Mattson MP, Dou P, Kater SB (1988) Outgrowth-regulating actions of glutamate in isolated hippocampal pyramidal neurons. J Neurosci 8(6):2087–2100
65. Kwon HB, Sabatini BL (2011) Glutamate induces de novo growth of functional spines in developing cortex. Nature 474(7349):100–104
66. Yang C, Sudderth J, Dang T, Bachoo RM, McDonald JG, DeBerardinis RJ (2009) Glioblastoma cells require glutamate dehydrogenase to survive impairments of glucose metabolism or Akt signaling. Cancer Res 69(20):7986–7993
67. Waitkus MS, Diplas BH, Yan H (2016) Isocitrate dehydrogenase mutations in gliomas. Neuro-oncology 18(1):16–26
68. Chen R, Nishimura MC, Kharbanda S, Peale F, Deng Y, Daemen A, Forrest WF, Kwong M, Hedehus M, Hatzivassiliou G, Friedman LS, Phillips HS (2014) Hominoid-specific enzyme GLUD2 promotes growth of IDH1R132H glioma. Proc Natl Acad Sci 111(39):14217–14222
69. Waitkus MS, Pirozzi CJ, Moure CJ, Diplas BH, Hansen LJ, Carpenter AB, Yang R, Wang Z, Ingram BO, Karoly ED, Mohney RP, Spasojevic I, McLendon RE, Friedman HS, He Y, Bigner DD, Yan H (2018) Adaptive evolution of the GDH2 allosteric domain

promotes gliomagenesis by resolving IDH1(R132H)-induced metabolic liabilities. Cancer Res 78(1):36–50
70. Smits RA, van de Wijngaard WM, Stassen AP, van der Drift C (1984) Mutants of pseudomonas aeruginosa unable to inactivate allantoinase and NADP-dependent glutamate dehydrogenase. Arch Microbiol 140(1):40–43
71. Britton KL, Baker PJ, Rice DW, Stillman TJ (1992) Structural relationship between the hexameric and tetrameric family of glutamate dehydrogenases. Eur J Biochem 209(3):851–859
72. Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW, Weissman JS, O'Shea EK (2003) Global analysis of protein localization in budding yeast. Nature 425(6959):686–691
73. DeLuna A, Avendano A, Riego L, Gonzalez A (2001) NADP-glutamate dehydrogenase isoenzymes of *Saccharomyces cerevisiae*. Purification, kinetic properties, and physiological roles. J Biol Chem 276(47):43775–43783
74. Campero-Basaldua C, Quezada H, Riego-Ruiz L, Marquez D, Rojas E, Gonzalez J, El-Hafidi M, Gonzalez A (2017) Diversification of the kinetic properties of yeast NADP-glutamate-dehydrogenase isozymes proceeds independently of their evolutionary origin. Microbiol Open 6 (2):e00419
75. Duschak VG, Cazzulo JJ (1991) Subcellular localization of glutamate dehydrogenases and alanine aminotransferase in epimastigotes of *Trypanosoma cruzi*. FEMS Microbiol Lett 67(2):131–135
76. Labboun S, Terce-Laforgue T, Roscher A, Bedu M, Restivo FM, Velanis CN, Skopelitis DS, Moschou PN, Roubelakis-Angelakis KA, Suzuki A, Hirel B (2009) Resolving the role of plant glutamate dehydrogenase. I. In vivo real time nuclear magnetic resonance spectroscopy experiments. Plant Cell Physiol 50(10):1761–1773
77. Cammaerts D, Jacobs M (1985) A study of the role of glutamate dehydrogenase in the nitrogen metabolism of *Arabidopsis thaliana*. Planta 163(4):517–526
78. Kotzamani D, Plaitakis A (2012) Alpha helical structures in the leader sequence of human GLUD2 glutamate dehydrogenase responsible for mitochondrial import. Neurochem Int 61(4):463–469
79. Kalef-Ezra E, Kotzamani D, Zaganas I, Katrakili N, Plaitakis A, Tokatlidis K (2016) Import of a major mitochondrial enzyme depends on synergy between two distinct helices of its presequence. Biochem J 473(18):2813–2829