Published in final edited form as:

Neurochem Res. 2014 March ; 39(3): 460–470. doi:10.1007/s11064-013-1227-5.

The Discovery of Human of *GLUD2* Glutamate Dehydrogenase and its Implications for Cell Function in Health and Disease

Pullanipally Shashidharan^{1,*} and Andreas Plaitakis²

¹Department of Neurology, Icahn School of Medicine at Mount Sinai, New York, NY 10029

²Department of Neurology, School of Health Sciences, Faculty of Medicine, University of Crete, Heraklion, Crete 71003, Greece

Abstract

While the evolutionary changes that led to traits unique to humans remain unclear, there is increasing evidence that enrichment of the human genome through DNA duplication processes may have contributed to traits such as bipedal locomotion, higher cognitive abilities and language. Among the genes that arose through duplication in primates during the period of increased brain development was *GLUD2*, which encodes the hGDH2 isoform of glutamate dehydrogenase expressed in neural and other tissues. GDH is an enzyme central to the metabolism of glutamate, the main excitatory neurotransmitter in mammalian brain involved in a multitude of CNS functions, including cognitive processes. In nerve tissue GDH is expressed in astrocytes that wrap excitatory synapses, where it is thought to play a role in the metabolic fate of glutamate removed from the synaptic cleft during excitatory transmission. Expression of GDH rises sharply during postnatal brain development, coinciding with nerve terminal sprouting and synaptogenesis. Compared to the original hGDH1 (encoded by the *GLUD1* gene), which is potently inhibited by GTP generated by the Krebs cycle, hGDH2 can function independently of this energy switch. In addition, hGDH2 can operate efficiently in the relatively acidic environment that prevails in astrocytes following glutamate uptake. This adaptation is thought to provide a biological advantage by enabling enhanced enzyme catalysis under intense excitatory neurotransmission. While the novel protein may help astrocytes to handle increased loads of transmitter glutamate, dissociation of hGDH2 from GTP control may render humans vulnerable to deregulation of this enzyme's function. Here we will retrace the cloning and characterization of the novel *GLUD2* gene and the potential implications of this discovery in the understanding of mechanisms that permitted the brain and other organs that express hGDH2 to fine-tune their functions in order to meet new challenging demands. In addition, the potential role of gain-of-function of hGDH2 variants in human neurodegenerative processes will be considered.

Introduction

Glutamate dehydrogenase (GDH) (E.C. 1.4.1.3) catalyses the reversible inter-conversion of glutamate to α -ketoglutarate and ammonia using NADP(H) and/or NAD(H) as cofactors. This enzyme is expressed by all living organisms, providing an important link between amino acid and carbohydrate metabolism. In prokaryotes and protists, GDH is thought to operate primarily in the amination direction producing glutamate needed for the synthesis of proteins and other compounds. These organisms express different proteins with GDH catalytic activity specific for NAD(H) or NAD(P). On the other hand, mammalian GDH utilizes both co-factors and thought to operate predominantly in the oxidative deamination

*Address Correspondence to: Dr. P. Shashidharan, Department of Neurology, Box 1137, Icahn School of Medicine at Mount Sinai, One Gustave L Levy Place, New York, NY 10029, USA, pullani.shashi@mssm.edu.

direction. Glutamate oxidation by GDH in mammalian tissues is linked to Krebs cycle anaplerotic mechanisms, energy production and ammonia homeostasis. Mammalian GDH is a hexameric molecule composed of six identical subunits, each of which has a molecular mass of ~56 kDa and consists of a polypeptide chain of 505 amino acids. Its activity is allosterically regulated with GTP and ADP serving as its main endogenous negative and positive modulators, respectively. Until recently, all mammals were thought to possess a single functional GDH-specific gene highly conserved during evolution through strong purifying selection. As described below, our work led to cloning and characterization of the second GDH-specific gene in the human that shows a distinct tissue expression profile and regulatory properties.

Evidence for multiplicity of human GDH

About three decades ago, when we started our work on human GDH, the mammalian enzyme had already been extensively studied. Its catalytic mechanisms and allosteric regulation had been the subject of excellent analyses [1,2]. Moreover, the enzyme had been purified from several mammalian tissues (mostly from the liver, where it accounts for about 1% of the total protein) and directly sequenced [3]. The overwhelming evidence stemming from those investigations, pointed towards a single housekeeping GDH that has been remarkably conserved during mammalian evolution. Contrary to this belief, we obtained evidence for multiplicity of the human enzyme by showing that GDH in human tissues exists in particulate-bound and readily solubilized isoforms, differing in their thermal stability and regulatory properties [4]. The two isoforms, named *heat-stable* and *heat-labile* GDH, were differently altered in leukocytes of patients with late-onset neurologic disorders characterized clinically by a combination of extrapyramidal deficits (parkinsonism), cerebellar dysfunction and other features, but without progressive autonomic failure [4]. However, no such abnormalities were detected in the leukocytes of patients with dominantly inherited Spinocerebellar Atrophy (SCA), Friedreich's Ataxia, Amyotrophic Lateral Sclerosis (ALS), typical Parkinson's Disease (PD), Progressive Supranuclear Palsy or Huntington's Disease [4].

In light of these findings, we sought to determine whether electrophoretically distinct GDH isoforms are present in human nerve tissue. For this, GDH activities were purified to homogeneity from human brain using a combination of ammonium sulfate fractionation, hydrophobic interaction and GTP affinity chromatography and analyzed by non-equilibrium pH gradient gel electrophoresis [5]. We studied postmortem cerebellar tissue obtained from three control subjects and seven patients with well-characterized chronic degenerative neurological disorders. Of the latter, two had PD, two ALS, two dominantly inherited SCA1 and one dominantly inherited SCA7 (based on the present classification criteria).

Two-dimensional gel electrophoresis of the purified enzymes revealed that brain GDH in non-neurologic controls was composed of four major isoproteins (designated GDH isoprotein 1,2,3 and 4), differing in their electric charge and slightly in their molecular weight. In contrast, pulse-chase labeling studies using HepG2 and U373 cultured cells revealed that these cells synthesized a single GDH isoprotein (not modified post-translationally) that co-migrated with GDH isoprotein 2 of the human brain [5]. While the origin of the four GDH isoforms of human brain remained unclear, cloning of the *GLUD1* gene (see below) permitted the detection of four different sizes of mRNAs in human tissues, including the brain [6]. Sequencing of a *GLUD1* gene cDNA predicted a 53-amino acid mitochondrial targeting signal, the amino-terminal residues of which were Ala-Arg-Arg-His-Tyr [6]. In light of this, it was hypothesized that cleavage at the Ala (-5) residue and subsequent slow removal of Arg (-4) and Arg (-3) residues could generate acidic GDH isoprotein species [5]. However, following the cloning of the two human GDH-specific

genes, we obtained recombinant hGDH1 and hGDH2 isoproteins by expressing the *GLUD1* and *GLUD2* cDNAs in Sf9 insect cells, and sequenced their N-terminus domains. These studies revealed that the leader peptide in both human isoproteins were cleaved between Tyr(-1) and Ser(+1) [7]. Subsequent studies that used molecular biological methods to follow the subcellular distribution of hGDH1 and hGDH2 in cultured cells revealed that both isoenzymes were localized primarily to the mitochondria and that the deletion of 53 amino acid-long N-terminal sequence prevented the enzymes from entering these organelles, thus confirming the role of this sequence as mitochondrial target signal [8]. These studies also revealed that both human GDHs localized partly to the endoplasmic reticulum (ER) [8], in accordance with previous studies showing that GDH activity was recovered from ER fractions obtained from various mammalian tissues, including the brain.

While the electrophoretic pattern of GDH purified from the cerebellum of patients with PD, ALS and SCA1 was similar to that of non-neurologic controls that of the patient with SCA7 showed a marked reduction in GDH isoprotein 1 [5]. Functional studies revealed that this patient's brain GDH displayed altered kinetics (K_m for the enzyme's substrates) as compared to controls or to patients with other neurologic patients. Moreover, glutamate levels were reduced markedly in the patient's cerebellum ($2.17 \mu\text{mol/mg}$ wet tissue) as compared to non-neurologic controls (8.75 ± 1.72 ; range: 6.75 - $11.20 \mu\text{mol/mg}$ wet tissue; $N=7$) with aspartate showing lesser decreases. In contrast, the levels of GABA, taurine and glutamine were within the normal range. These findings suggest that the abnormal electrophoretic pattern of this patient's brain GDH was associated with altered catalytic function, resulting in depletion of brain glutamate pools. Also, as aspartate in brain is primarily derived from glutamate and oxaloacetate by transamination, the decrease in the aspartate levels could be secondary to the severe glutamate reduction [5].

The reasons for the marked reduction in GDH isoprotein 1 remains unclear, although defective synthesis or post-translational modification were implicated [5]. The latter possibility seems more likely at the present, given that SCA7 proved to be a (CAG) n expansion disorder [9]. In this regard, sequestration of GDH to aggregates formed by the TXN-7 mutant polyglutamine may prevent the normal processing and function of the enzyme. By analogy, mutant huntingtin is shown to form a ternary complex with GAPDH and ubiquitin-E3-ligase, which enhances the nuclear translocation and cytotoxicity of the polyglutamine [10]. As such, our observations, though limited to single SCA7 case, may provide important clues for understanding the disease's pathophysiology. Obviously, data on additional SCA-7 patients are needed to see whether the abnormalities in brain GDH polymorphism and in glutamate metabolism, detected in our patient, occur consistently in SCA7 or other forms of polyglutamine disorders.

Cloning of the *GLUD1* gene

Parallel to our work on human GDH protein, we initiated a collaborative effort with Dr. N. Moschonas at the Institute of Molecular Biology and Biotechnology (IMBB) of Crete to clone the gene that encodes the human enzyme. These efforts were met with success as screening of a cDNA library derived from human liver, using synthetic oligonucleotides corresponding to amino acid sequences of human GDH led to the isolation of a series of cDNA clones specific for this enzyme [6]. Sequencing of an isolated full-length cDNA clone revealed that it specified a single 558-amino acid long protein that included the 53 amino acid N-terminal cleavable peptide described above [6]. Northern blot analysis of RNA from human, monkey and rabbit showed that GDH mRNAs of four different sizes were present in all tissues analyzed but at different ratios, while Southern blots of human genomic DNA revealed evidence for the presence of multiple GDH-specific genes. Subsequent studies indeed led to the cloning and characterization of several truncated

GLUD1 genes localized to the 10th human chromosome near the *GLUD1* gene locus [11, 12]. Concurrent efforts by Nakatani, et al., [13] also led to the isolation of GDH-specific cDNA clones from a fibroblast cDNA library. The authors provided a full sequence of the *GLUD1* gene and also identified the same 53 amino acid-long cleavable N-terminal sequence as described above.

Cloning of the *GLUD2* gene

Southern blot analysis of human genomic DNA revealed evidence for additional GDH-specific genes located on the 10th chromosome, all these proved to be non-functional pseudogenes [11, 12]. Thus, molecular proof for the presence of more than one functional *GLUD* genes and their products in the human was lacking. In our further attempts to detect structurally distinct GDH isoproteins, particulate-bound and readily solubilized GDH activities were purified from human brain (using the method described above) and studied by peptide mapping. While the electrophoretic pattern of peptides generated by trypsin digestion of the two purified proteins was distinct, sequencing of these peptides produced disappointing results as all amino acid sequences obtained corresponded to those predicted by the cloned *GLUD1* gene [6]. The reasons for the inability to detect hGDH2-specific sequences in human brain were not entirely clear. However, after the *GLUD2* gene was cloned we realized that the hGDH2 does not bind to GTP whereas hGDH1 does (see below). As such, failure to detect hGDH2 in fractions eluted from the GTP-Sepharose column used for purifying the enzymes from human brain tissue may be due to its inability to bind to this column.

It was against this background that we intensified our efforts to clone the *heat-labile* GDH. Initial screening of cDNA libraries constructed from human tissues led again to the isolation of cDNA clones specific for *GLUD1*. These results were consistent with data from Public access EST libraries derived from human tissues, which showed that these are highly enriched in *GLUD1*. Following the cloning of the *GLUD2* gene [14] (see below), however, a few EST clones specific for this gene derived from human brain (hippocampus), testis, embryonic tissue and various tumors have been deposited in these databases.

The breakthrough came when we screened a cDNA library constructed from human retina. This led to the isolation of a full-length cDNA clone [14] encoding a protein distinct from that specified by the *GLUD1* gene. The novel gene was subsequently named *GLUD2* and localized to the human chromosome X [14]. It specified a human GDH isoenzyme (named hGDH2) that is highly homologous to hGDH1, with the two proteins sharing in their mature forms all but 15 of their 505 amino acids. However, their N-terminal mitochondrial signal peptide (53 amino acid-long in both) showed a greater of amino acid sequence divergence [14]. In this regard, Rosso, et al., [15] found evidence that leader peptide of hGDH2 provided the enzyme with an enhanced mitochondrial targeting specificity. Analysis of mRNA isolated from human tissues revealed that the novel gene was expressed by human retina, brain and testis but not by the human liver [14]. More recently, Tomita et al. [16] have used a brain cDNA library to isolate a *GLUD2* clone, thus verifying the existence of mature full-length *GLUD2* mRNAs in human brain. To identify the gene encoding our cDNA we collaborated with the IMBB of Crete team that had isolated several GDH-specific genomic clones [11,12]. These efforts revealed that our *GLUD2* cDNA corresponded to an intronless gene that may have arisen from retro position of the *GLUD1* gene to the human X chromosome[14].

Characterization of the *GLUD2* GDH

The next challenge was to demonstrate the functionality of the *GLUD2* gene. Initially, *GLUD1* and *GLUD2* were translated *in vitro* using the rabbit reticulocyte lysate system [14], that produced proteins with a molecular weight of about 62 KD corresponding to the full length human GDHs that retains the mitochondrial signal peptide. Interestingly, the *GLUD2*-derived isoenzyme migrated on SDS-PAGE slightly higher than the *GLUD1*-isoprotein [14]. Subsequent studies on recombinant human GDHs, obtained by expression in eukaryotic systems [17,18] or even in *E. Coli* [19] revealed that this migration pattern is one of the intrinsic properties of hGDH2 that resulted from substitution of Ser for Arg443 [17,18]. Following the successful *in vitro* translation, we sought to obtain a functional recombinant hGDH2 protein. Initial efforts to express the *GLUD2* cDNA in the Sf9 cells (of the insect of *Spodoptera frugiperda*) using the baculovirus expression system proved highly successful, yielding a recombinant hGDH2 isoprotein with catalytic properties comparable to those of the endogenous mature GDH purified from human brain [14].

Following cloning of the *GLUD2* gene, we have undertaken extensive investigations in order to characterize the expression profile and the functional properties of hGDH2 as well as its role in the biology of human tissues. Initial functional studies performed on recombinant hGDH1 and hGDH2 obtained by expression of the corresponding *GLUD1* and *GLUD2* cDNAs in Sf9 cells, revealed that while hGDH1 was *heat-stable*, hGDH2 was *heat-labile* [7], thus confirming our original observations suggesting that human GDH exists in two isoforms differing in their thermal stability [4]. Subsequent studies revealed that the sensitivity of hGDH2 to thermal inactivation results from substitution of Ser for Arg443 [17, 19], a fundamental evolutionary change that equipped hGDH2 with unique functional properties as described below.

Expression of human *GLUD* genes in Sf9 or Sf21 cells using the baculovirus expression system proved highly reproducible, yielding over the years high quality recombinant wild-type hGDH1 and hGDH2 and their mutants. This has facilitated structure/function analyses of these proteins. Amino acid sequencing of the N-terminus of recombinant hGDH1 and hGDH2, obtained by expression of the corresponding cDNAs in Sf9 cells, revealed that both had been cleaved at the predicted cleavage site [7]. These data suggest that the expressed proteins were processed in the insect cells in manner similar to that in human tissues. That the obtained recombinant human GDHs are identical to the endogenous tissue enzymes has been confirmed by functional studies showing that recombinant hGDH1 had the catalytic and regulatory properties of GDH purified from human liver that expresses only the hGDH1[14].

Direct comparison of hGDH1 and hGDH2 obtained by expression of the corresponding cDNAs in Sf9 cells revealed that while both proteins showed comparable catalytic activities when fully activated by 1.0 mM ADP, hGDH2 displayed only 5% of its maximal activity in the absence of this activator. Under these conditions, the housekeeping hGDH1 displayed approximately 35%-40% of its maximal activity. Low physiological levels of ADP (0.05-0.25 mM) induced a concentration-dependent enhancement of enzyme activity that was proportionally greater for hGDH2 (by 550-1,300%) than for hGDH1 by 120-150%. These results suggested that allosteric modulation by of hGDH2 activity by rising ADP levels may be of importance for regulating brain glutamate fluxes *in vivo* under changing energy demands [7,20].

L-leucine, at 1.0 mM, also induced a proportionally greater activation of hGDH2 (by 1,600%) than of hGDH1 (by 75%). Whereas L-leucine, at levels similar to those found in mammalian tissues (about 0.10-0.15 mM) had little effect on either human isoenzyme, the

low concentrations of ADP (0.01-0.05 mM) permitted activation by physiologically relevant levels of L-leucine. While activation of hGDH1 by L-leucine can stimulate insulin secretion by the pancreatic beta-cells [2, 22], the physiological implications of hGDH2 activation by rising L-leucine levels in nerve tissue function remain unclear. There is however, evidence that L-leucine levels increase in brain during ischemia and that L-leucine enhances glutamate oxidation by brain synaptosomes [23]. As ADP and L-leucine act synergistically to enhance GDH activity, this amplification may permit enhanced glutamate metabolism in response to small-scale energy changes.

As magnesium ions are known to interact with mammalian GDH [24], we tested their effect on recombinant human GDHs [7]. Results revealed that magnesium chloride (at 1.0-2.0 mM) inhibited hGDH1 (by 45-64%), but ADP reversed this inhibition. While magnesium chloride had no effect on the basal activity of hGDH2, it modified the ADP activation curve of hGDH2, rendering it sigmoidal instead of hyperbolic (no Mg^{2+}) [7]. As the levels of Mg^{2+} in the mitochondrial matrix are about 1.0 mM [25] and those of ADP vary between 0.05->1.0 mM (depending on the rate of oxidative phosphorylation) [26], modulation of enzyme activity by magnesium may keep hGDH2 relatively idle when high rates of phosphorylation prevail (low ADP levels), while allowing full enzyme recruitment under decreased cellular energy charge (high ADP levels). Magnesium ions are known to bind at an internal binding site of the NMDA receptor creating a voltage-dependent block [27, 28]. While this Mg^{2+} effect may play a role in modulating excitatory transmission at the glutamate receptor level, interaction with human GDHs may provide parallel mechanism by which of Mg^{2+} affects the metabolism of transmitter glutamate. As such, the implications of these findings in nerve tissue physiology in health and disease remains to be further elucidated.

It is well established that mammalian GDH is powerfully inhibited by GTP and activated by ADP [1,2]. There is evidence that control of enzyme activity by GTP generated by the Krebs cycle is essential for regulating the flux of glutamate through this pathway. Thus, attenuation of GDH inhibition by GTP due to decreased Krebs cycle rate (low cellular energy charge) may permit glutamate to fuel the Krebs cycle, thus replenishing cellular energy stores. Initial studies carried out in crude extracts of Sf9 cells revealed that, while GTP powerfully inhibited hGDH1 ($IC_{50} = 0.20 \mu M$), hGDH2 was insensitive to it [20]. Further studies on recombinant hGDH1 and hGDH2 purified to homogeneity confirmed this differential sensitivity [17]. These observations showing the hGDH2 has dissociated its function from GTP control may have important implications for the role of this novel human enzyme in cell biology as discussed below.

Origin and adaption of the *GLUD2* gene

As indicated above, there is molecular evidence that the *GLUD1* gene was retroposed to the X chromosome, where it gave rise to the *GLUD2* gene [14]. Burki and Kaessmann [29] presented evidence that the retroposition event might have occurred rather recently in human evolution (<23 million years ago). The authors showed that while the *GLUD2* gene is present in the humans and the great apes, it does not exist in old world monkeys, suggesting that retroposition occurred after the separation of the human from the African green monkey lineage and before the separation of the human from the gibbon lineage [29]. As such, *GLUD2* is one of the youngest human genes [30]. Following its retroposition to the X-chromosome, the *GLUD2* gene evolved via random mutations and natural selection acquiring novel properties. To delineate the molecular basis of the functional differences between hGDH1 and hGDH2, site-directed mutagenesis studies were undertaken [18, 31]. Of these, substitution of Ala for Gly456 provided resistance to GTP [31], thus dissociating hGDH2 function from the control of this energy switch. Another evolutionary change that had major functional consequence was substitution of Ser for Arg443Ser [18]. It markedly

decreased basal activity while permitting full restoration of enzyme function by ADP and L-leucine [17,18]. In addition, the Arg443Ser change made hGDH2 sensitive to thermal inactivation and to inhibition by estrogens [32] and neuroleptic agents [33]. The two major amino acid replacements (Arg443Ser and Gly456Ala) occurred soon after the birth of the *GLUD2* gene, along with the Glu34Lys, Asp142Glu, Ser174Asn and Asn498Ser changes [29]; these are thought to be under positive natural selection. Also, the Ser331Thr, Met370Leu and Arg470His mutations that occurred just before the separation of the human from the orangutan lineage (Fig. 1) may have been positively selected [29]. While the Arg443Ser and Gly456Ala mutations resulted in major functional changes, these are not sufficient to explain all hGDH2 properties. As such, additional amino acid replacements in *GLUD2* that occurred later on during the human evolution must have provided further functional refinement. This challenging issue remains to be further elucidated.

The genesis and evolutionary adaptation of the *GLUD2* gene coincides with the time period of increase in primate brain size and complexity [29]. During the same period, similar evolutionary changes to those of the *GLUD2* gene occurred in many other genes involved in brain size determination, neurotransmission, vision, memory and neural cell metabolism. Of the brain-expressed genes that were positively selected in the ape stem period of human ancestry (25-6 million years ago), those encoding mitochondrial proteins involved in aerobic energy metabolism were particularly favored [34-36]. This has been explained by the high energy demands of the developing nerve system during gestation with fetal brain thought to consume about 65% of the total's body metabolic energy [37]. Given the dual role of GDH in transmitter glutamate and energy metabolism, the newly acquired *GLUD2* gene may have contributed to increased human brain capabilities as suggested by Burki and Kaessmann [29]. While a large number of new human genes emerged after a burst of retropositions in primates [36] many of those followed a reverse course (from the X-chromosome to an autosome), probably to maintain important functions during spermatogenesis in males. On the other hand, the X-linked *GLUD2* gene protein encodes hGDH2, which is a mitochondrial protein not expressed in spermatids (haploid cells) nor in spermatozoa that contain practically no mitochondria [38]. Instead, in human testis hGDH2 is expressed in Sertoli cells that line the somniferous tubules and that support and nourish sperm cells, providing them with lactate and other substances [38].

Expression of *GLUD2* in human tissues

While the cellular and subcellular distribution of GDH in rat CNS has been studied extensively, studies in human brain are largely lacking. Using IHC methods Aoki et al, [39] showed that in rat brain GDH is mainly expressed in astrocytes, with GDH-specific staining following the pattern of brain glutamatergic pathways. These results along with data showing that GDH is enriched in synaptic mitochondria and that its activity increases markedly during brain development in parallel with synaptogenesis [40] support the thesis that GDH plays a role in neurotransmission mechanisms. Because the antibodies used in previous ICH investigations on mammalian brain were raised against the readily available bovine liver GDH, these antibodies cross-react with GDHs from different species, including human hGDH1 and hGDH2 [38]. In light of this, efforts were undertaken to obtain antibodies specific for each of the human GDH isoenzymes, although their high amino acid sequence similarity made this undertaking challenging. Initial efforts to immunize rabbits against a 12-amino acid-long peptide (PTAEFQDSISGA; residues 436-447 of the mature hGDH2) containing the R443S evolutionary replacement that alters the conformation of the protein were met with success [38]. Indeed, the obtained antibody reacted with high affinity with the wild-type hGDH2, but not the wild-type hGDH1. The antibody did not bind to the Ser443Arg-hGDH2 mutant and showed decreased affinity for other hGDH2 mutants carrying mutations of residues within the above 12 amino acid-long peptide (used to raise

the antibody). On the other hand, substitution of residues outside of this epitope did not affect binding of the antibody to the hGDH2 mutants. Remarkably while the antibody did not bind in Western blots to the wild-type hGDH1, it recognized the single Arg443Ser hGDH1 mutant, thus confirming the specificity of the antibody for this hGDH2 epitope [38].

Development of this antibody was a major step for elucidating the cellular and subcellular distribution of hGDH2 isoprotein in human tissues, including the brain. As detailed in the paper by Spanaki et al., in this issue, IHC studies in human brain revealed that hGDH2 is expressed in astrocytes in the gray and white matter of human cortex [39]. In these cells, the hGDH2-specific staining was observed in the cytoplasm and along the proximal and distal processes within coarse structures that resemble mitochondria. A similar punctuate staining was also observed in the wall of cerebral vessels, probably representing astrocytic end-feet that embrace the cerebral vasculature. On the other hand, neurons showed faint diffuse cytoplasmic staining, although, occasionally a superimposed punctuate intense staining was also observed. No expression of hGDH2 was found in oligodendrocytes or in myelinated fibers. In addition to human brain, hGDH2 was also expressed by the Sertoli cells of human testis and the cells that line the proximal convoluted tubules of human kidney [38, 41, 42]. More recent studies have led to the realization that expression of hGDH2 in human tissues is more widespread than initially appreciated (Spanaki, et al., unpublished data).

Putative Role of hGDH2 in Cell Function

While mammalian GDH has been studied for over half century, its exact role in cell biology has not been fully understood. As detailed in the paper by Spanaki et al., in this issue, the cellular distribution of GDH in various mammalian tissues is heterogeneous and that glutamate flux through GDH is linked to cell signalling mechanisms that may be tissue-specific [43]. To keep this flux under tight control the mammalian GDH is subject to complex allosteric regulation with GTP and ADP serving as the main negative and positive modulators as noted above. Regulation of GDH activity by these endogenous effectors permits glutamate to fuel the Krebs cycle according to the cellular need for ATP [1]. Evidence to show that hGDH1 catalytic function boosts cellular energy charge were obtained for the pancreatic beta cells, which are known to release insulin in response to increased ATP levels [43]. Consistent with this thesis are observations in children with mutations in the *GLUD1* gene that attenuate GTP inhibition. In these patients, the overactive GDH leads to hypoglycemia due inappropriate insulin release through enhanced synthesis of ATP [44].

Sensitivity of hGDH1 to GTP is essential for regulating glutamate flux according to cellular energy needs, as the GTP pool that interacts with hGDH1 is derived from the Krebs cycle with each turn of cycle generating one GTP molecule. As such, when the rate of Krebs cycle decreases (due to limited availability of substrates or other factors) the resultant attenuation of GTP inhibition may permit hGDH1 to convert glutamate to α -ketoglutarate, which can then replenish the Krebs cycle restoring its function as noted above. While these considerations are applicable to hGDH1, the discovery of hGDH2 and the realization that the isoenzyme is regulated by distinct molecular mechanisms have introduced new complexities. Specifically, dissociation of hGDH2 from GTP control implies that enzyme function is no longer linked to the rate of the Krebs cycle (and consequently to GTP formation). While hGDH2 shows low basal activity when assayed under standard conditions, its specific activity increased linearly ($r=0.94$) with increasing enzyme concentrations (0.5-6.5 $\mu\text{g/ml}$) [17]. As the enzyme can attain very high levels (up to 10 mg/ml) in mitochondrial matrix [45], hGDH2 may be active in the matrix environment in the absence of allosteric activator and even when Krebs cycle function generates GTP levels sufficient to inhibit hGDH1.

In the human nerve tissue hGDH2 is predominantly expressed in astrocytes, the cells involved in many aspects of the glutamate recycling that occurs in excitatory synapses. In the *tripartite* glutamatergic synapse the astrocytic processes that wrap nerve endings are packed with mitochondria, a finding indicative of the importance of energy metabolism in neurotransmission processes [46]. Astrocytes are largely responsible for the removal and metabolism of transmitter glutamate, which they can convert to glutamine by Glutamine Synthetase (GS) or to α -ketoglutarate via the action of GDH or aspartate amino transferase (AAT). While glutamine is exported to neurons to be used as precursor of transmitter glutamate (glutamate-glutamine cycle), α -ketoglutarate generated in the mitochondrial matrix via GDH can enter the Krebs cycle being eventually metabolized to CO_2 and H_2O [47]. However, complete oxidation of glutamate requires pyruvate recycling [48]. In this regard, α -ketoglutarate that enters the Krebs cycle can generate malate and/or oxaloacetate that may be decarboxylated to form pyruvate. The latter can in turn become a substrate for pyruvate dehydrogenase or be reduced in the cytosol to lactate and be released in the extracellular space [49]. In addition, astrocytes have been assigned many other roles, including modulation of excitatory transmission and of synaptogenesis [50]. It has also been suggested that hormonal regulation the the tripartite synapse may enhance the ability of humans to respond to changes in the external and internal environment [51]. This is of particular interest in light of data showing that hGDH2 is sensitive to modulation by steroid hormones [32].

Synaptic glutamate is transported inside the astrocytes via two classes of membrane-bound excitatory amino-acid transporters (EAAT1 and EAAT2 in the human). It is an energy intense process that increases the overall ATP consumption by two- to three-fold [52]. Glutamate uptake is Na^+ -coupled with one glutamate being co-transported with three Na^+ and one proton in exchange with one K^+ [53]. Co-transport of one proton with each cycle of glutamate transport has been shown to lead to significant cellular acidification [54]. Azarias et al [55] found that primary cultures of cortical astrocytes, obtained from newborn mice, showed significant mitochondrial matrix acidification upon glutamate transport. In light of these findings, the lower optimal pH of hGDH2 may provide a biological advantage by enabling the enzyme to efficiently metabolize transmitter glutamate under the acidic conditions that prevail in the mitochondrial matrix following glutamate uptake. However, Azarias et al., [55] reported that glutamate transport was associated with decreased mitochondrial respiration, probably to protect the cells from mROS production [55,56]. As such, while hGDH2 properties may help human astrocytes to handle an increased load of transmitter glutamate, a fine line may exist between the physiological role of hGDH2 and its potential to induce cell damage as discussed below.

Putative role hGDH2 deregulation in human degenerations

We have originally reported that abnormalities in glutamate metabolism occur in patients with parkinsonism/cerebellar syndromes [57] and in patients affected by typical ALS [58]. Others [59] have also reported similar abnormalities in PD patients. In light of these findings, we searched for sequence alterations in the *GLUD1* and *GLUD2* genes in 281 patients with PD and 80 patients Amyotrophic Lateral Sclerosis (ALS). These investigations [60] revealed that a T1492G rare variant, which results in the substitution of Ala for Ser445 in the regulatory domain of hGDH2, interacted significantly with age at onset in the PD, but not in ALS patients. An expanded study that included 584 PD patients from Greece and 224 PD patients from Central California revealed that in the Greek cohort, G hemizygotes developed PD at a younger age (54.6 ± 11.1 yrs) than G/T heterozygotes (67.7 ± 8.1 yrs, $P=0.001$), T hemizygotes (64.0 ± 10.7 yrs, $P=0.003$) or T/T homozygotes (62.9 ± 11.0 yrs, $P=0.011$). Similarly, in the American cohort, patients hemizygous (N=224) for the G allele also developed PD 6 to 13 years earlier than subjects with other genotypes, with this

difference being significant when G hemizygotes (mean age at PD onset: 61.3 ± 15.7 yrs) were compared with G/T heterozygotes (mean age at PD onset: 74.4 ± 5.8 yrs; $P=0.049$). (60). In contrast, no such age differences were found in G heterozygous PD females. The frequency of T1492G polymorphism was rare, with the G allele being present in 3.6% PD patients in the Greek cohort (N=584) and in 4.3% in the American PD group (N=224). These frequencies were not significantly different than those of the control sample from each population, suggesting that, while the presence of this polymorphism accelerates the onset of PD, it may not be sufficient to cause PD in non-susceptible individuals [60].

The detected T1492G change results in substitution of Ala for Ser-445, which is located in small descending helix of the “antenna” that undergoes marked conformational changes during catalysis [61]. The “antenna”, a 48-amino acid protrusion in the regulatory domain of GDH is thought to play a role in mechanisms of allostery, although this structure is not directly involved in the binding of the two main endogenous modulators (GTP or ADP) of the enzyme. Functional analyses of the Ala445-hGDH2 variant obtained in recombinant form revealed that its specific activity was substantially greater than that of the wild-type hGDH2, with this difference becoming greater by increasing enzyme concentration during the assay (Figure 2 [60]). Substitution of Ala for Ser445 is thought to stabilize the small α -helix of the antenna, as alanine is considered to be better α -helix formers than serine. Stabilization of the alpha helix would favour an open mouth conformation, thereby counteracting the capacity of the evolutionary Arg443Ser change of hGDH2 to minimize basal activity. The deregulated activity of the Ala445-hGDH2 variant was resistant to GTP inhibition and could be further enhanced by ADP and L-leucine [60]. However, similarly to the wild-type hGDH2, the overactive Ala445-hGDH2 variant was also sensitive to inhibition by estrogens and is thought to protect female PD patients who were heterozygous for the T1492G change. [60]

PD is characterized by degeneration of the dopaminergic (DAergic) neurons located in the substantia nigra (SN) pars compacta of the midbrain. The resulting loss of the DAergic terminals in the neostriatum is thought to be responsible for the main neurologic manifestations of the disorder. While the aetiology of this rather selective DAergic neuronal loss that occurs in the common sporadic PD cases remains unknown, rare genetic defects causing familial PD are shown to alter mitophagy, a form of autophagy linked to energy sensing [62]. Mitophagy is thought to protect the cells from disordered metabolism and the release of pro-apoptotic agents. The importance of GDH in these processes is underscored by recent observations showing that glutamate flux through this enzyme mediates cell signalling by modulating the mTORC1 pathway that leads to autophagy [63]. As DAergic neurons are vulnerable to energy perturbations, our findings that a gain-of-function for hGDH2 variant accelerates PD onset have connected altered glutamate/energy metabolism with cellular processes implicated in PD pathogenesis.

Some of the properties acquired by hGDH2, such as resistance of hGDH2 GTP inhibition and ability to operate efficiently at a relatively acidic cellular environment, may represent an adaptation to conditions that prevail in astrocytes during excitatory neurotransmission. As described above, glutamate transport in mouse astrocytic cultures induced significant mitochondrial matrix acidification that in turn decreased respiration and ROS formation [55]. As mROS is a by-product of normal mitochondrial respiration, with 0.2–2% of consumed oxygen shown to be transformed into mROS from complexes I and III of the mitochondrial respiratory chain [56], decreased mitochondrial oxidative metabolism may protect the cell from ROS production.

While the ability of hGDH2 to function efficiently in an acidic environment may enable human astrocytes to handle increased loads of transmitter glutamate under physiological

conditions, increased glutamate oxidation by the overactive Ala-445-hGDH2 variant may inappropriately boost mitochondrial oxidative metabolism with resultant increased mROS formation. Previous observations on the rotenone PD model have shown that mitochondria exposed to this neurotoxin generate increased ROS upon glutamate oxidation [64]. While normal mitochondria may be protected from deregulated hGDH2 action through safeguard systems, dysfunctional mitochondria that may accumulate in PD brain due to impaired mitophagy may be particularly vulnerable to enhanced mitochondrial glutamate oxidation.

Perspective

As discussed in this review, the enhanced capability of hGDH2 to metabolize glutamate under conditions that prevail in astrocytes during excitatory neurotransmission may have played a role in brain evolution and the expanded cognitive abilities of the humans. It is known that the adult nerve tissue has the highest energy demands and that this demand is increased in the developing brain, as the growth of axonal and dendritic arborization and the formation of elaborate synaptic connections are energy requiring processes. Moreover, the intense excitatory neurotransmission associated with motor, sensory and cognitive processes augment the energy demands of tripartite glutamatergic synapses. Under these conditions, the properties acquired by hGDH2 during its evolution may help astrocytes to handle increased loads of transmitter glutamate. However, given that hGDH2 is not subject to GTP control, humans by acquiring this isoenzyme may have become vulnerable to deregulation of this enzyme's function. Specifically, mutations that enhance the catalytic function of hGDH2 may alter a fine line that exists between its physiological role and its potential to induce cell damage. Given the dynamic nature of the catalytic function of GDH and the availability of agents capable of regulating this pathway, understanding of these mechanisms may provide the tools for modifying the natural history of PD and perhaps other human degenerations.

Acknowledgments

The work was supported by a grant from NINDS, NIH NS43038 (PS) and by THALIS – UOC No. 377226 grant (AP) “Mitochondrial dysfunction in neurodegenerative diseases” from the European Union (European Social Fund – ESF) and from Greek national funds through the Operational Program “Education and Lifelong Learning” of the National Strategic Reference Framework (NSRF) - Research Funding Program.

References

- Engel PC. A marriage full of surprises: forty-five years living with glutamate dehydrogenase. *Neurochem Int.* 2011; 59:489–494. [PubMed: 21419817]
- Frieden C. A lifetime of kinetics. *J Biol Chem.* 2008; 283:19873–19878. [PubMed: 18442970]
- Julliard JH, Smith EL. Partial amino acid sequence of the glutamate dehydrogenase of human liver and a revision of the sequence of the bovine enzyme. *J Biol Chem.* 1979; 254:3427–3438. [PubMed: 429360]
- Plaitakis A, Berl S, Yahr MD. Neurological disorders associated with deficiency of glutamate dehydrogenase. *Ann Neurol.* 1984; 15:144–153. [PubMed: 6703655]
- Hussain MM, Zannis VI, Plaitakis A. Characterization of glutamate dehydrogenase isoproteins purified from the cerebellum of normal subjects and patients with degenerative neurological disorders, and from human neoplastic cell lines. *J Biol Chem.* 1989; 264:20730–20735. [PubMed: 2573605]
- Mavrothalassitis G, Tzimagiorgis G, Mitsialis A, Zannis V, Plaitakis A, Papamatheakis J, Moschonas N. Isolation and characterization of cDNA clones encoding human liver glutamate dehydrogenase: evidence for a small gene family. *Proc Natl Acad Sci U S A.* 1988; 85:3494–3498. [PubMed: 3368458]

7. Shashidharan P, Clarke DD, Ahmed N, Moschonas N, Plaitakis A. Nerve tissue-specific human glutamate dehydrogenase that is thermolabile and highly regulated by ADP. *J Neurochem.* 1997; 68:1804–1811. [PubMed: 9109504]
8. Mastorodemos V, Kotzamani D, Zaganas I, Arianoglou G, Latsoudis H, Plaitakis A. Human GLUD1 and GLUD2 glutamate dehydrogenase localize to mitochondria and endoplasmic reticulum. *Biochem Cell Biol.* 2009; 87:505–516. [PubMed: 19448744]
9. Lindblad K, Savontaus ML, Stevanin G, Holmberg M, Digre K, Zander C, Ehrsson H, David G, Benomar A, Nikoskelainen E, Trottier Y, Holmgren G, Ptacek LJ, Antinen A, Brice A, Schalling M. An expanded CAG repeat sequence in spinocerebellar ataxia type 7. *Genome Res.* 1996; 6:965–971. [PubMed: 8908515]
10. Bae BI, Hara MR, Cascio MB, Wellington CL, Hayden MR, Ross CA, Ha HC, Li XJ, Snyder SH, Sawa A. Mutant huntingtin: nuclear translocation and cytotoxicity mediated by GAPDH. *Proc Natl Acad Sci U S A.* 2006; 103:3405–3409. [PubMed: 16492755]
11. Michaelidis TM, Tzimagiorgis G, Moschonas NK, Papamatheakis J. The human glutamate dehydrogenase gene family: gene organization and structural characterization. *Genomics.* 1993; 16:150–160. [PubMed: 8486350]
12. Tzimagiorgis G, Leversha MA, Chroniary K, Goulielmos G, Sargent CA, Ferguson-Smith M, Moschonas NK. Structure and expression analysis of a member of the human glutamate dehydrogenase (GLUD) gene family mapped to chromosome 10p11.2. *Hum Genet.* 1993; 91:433–438. [PubMed: 8314555]
13. Nakatani Y, Schneider M, Banner C, Freese E. Complete nucleotide sequence of human glutamate dehydrogenase cDNA. *Nucleic Acids Res.* 1988; 16:6237. [PubMed: 3399399]
14. Shashidharan P, Michaelidis TM, Robakis NK, Kresoali A, Papamatheakis J, Plaitakis A. Novel human glutamate dehydrogenase expressed in neural and testicular tissues and encoded by an X-linked intronless gene. *J Biol Chem.* 1994; 269:16971–16976. [PubMed: 8207021]
15. Rosso L, Marques AC, Reichert AS, Kaessmann H. Mitochondrial targeting adaptation of the hominoid-specific glutamate dehydrogenase driven by positive Darwinian selection. *PLoS Genet.* 2008; 4:e1000150. [PubMed: 18688271]
16. Tomita T, Kuzuyama T, Nishiyama M. Structural basis for leucine-induced allosteric activation of glutamate dehydrogenase. *J Biol Chem.* 2011; 286:37406–37413. [PubMed: 21900230]
17. Kanavouras K, Mastorodemos V, Borompokas N, Spanaki C, Plaitakis A. Properties and molecular evolution of human GLUD2 (neural and testicular tissue-specific) glutamate dehydrogenase. *J Neurosci Res.* 2007; 85:1101–1109. [PubMed: 17253646]
18. Zaganas I, Spanaki C, Karpusas M, Plaitakis A. 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.* 2002; 277:46552–46558. [PubMed: 12324473]
19. Yang SJ, Huh JW, Hong HN, Kim TU, Cho SW. Important role of Ser443 in different thermal stability of human glutamate dehydrogenase isozymes. *FEBS Lett.* 2004; 562:59–64. [PubMed: 15044002]
20. Plaitakis A, Metaxari M, Shashidharan P. Nerve tissue-specific (GLUD2) and housekeeping (GLUD1) human glutamate dehydrogenases are regulated by distinct allosteric mechanisms: implications for biologic function. *J Neurochem.* 2000; 75:1862–1869. [PubMed: 11032875]
21. Sener A, Malaisse WJ. L-leucine and a nonmetabolized analogue activate pancreatic islet glutamate dehydrogenase. *Nature.* 1980; 288:187–189. [PubMed: 7001252]
22. Panten U, Zielmann S, Langer J, Zunkler BJ, Lenzen S. Regulation of insulin secretion by energy metabolism in pancreatic B-cell mitochondria. Studies with a non-metabolizable leucine analogue. *Biochem J.* 1984; 219:189–196. [PubMed: 6372787]
23. Erecinska M, Nelson D. Activation of glutamate dehydrogenase by leucine and its nonmetabolizable analogue in rat brain synaptosomes. *J Neurochem.* 1990; 54:1335–1343. [PubMed: 1968960]
24. Fahien LA, Teller JK, Macdonald MJ, Fahien CM. Regulation of glutamate dehydrogenase by Mg²⁺ and magnification of leucine activation by Mg²⁺ *Mol Pharmacol.* 1990; 37:943–949. [PubMed: 2359406]

25. Taylor JS, Vigneron DB, Murphy-Boesch J, Nelson SJ, Kessler HB, Coia L, Curran W, Brown TR. Free magnesium levels in normal human brain and brain tumors: ³¹P chemical-shift imaging measurements at 1.5 T. *Proc Natl Acad Sci U S A*. 1991; 88:6810–6814. [PubMed: 1650484]
26. Gabriel JL, Zervos PR, Plaut GW. Activity of purified NAD-specific isocitrate dehydrogenase at modulator and substrate concentrations approximating conditions in mitochondria. *Metabolism*. 1986; 35:661–667. [PubMed: 3724458]
27. Qian A, Johnson JW. Permeant ion effects on external Mg²⁺ block of NR1/2D NMDA receptors. *J Neurosci*. 2006; 26:10899–10910. [PubMed: 17050728]
28. Kupper J, Ascher P, Neyton J. Internal Mg²⁺ block of recombinant NMDA channels mutated within the selectivity filter and expressed in *Xenopus* oocytes. *J Physiol*. 1998; 507(Pt 1):1–12. [PubMed: 9490808]
29. Burki F, Kaessmann H. Birth and adaptive evolution of a hominoid gene that supports high neurotransmitter flux. *Nat Genet*. 2004; 36:1061–1063. [PubMed: 15378063]
30. Varki A. How to make an ape brain. *Nat Genet*. 2004; 36:1034–1036. [PubMed: 15454937]
31. Zaganas I, Plaitakis A. 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*. 2002; 277:26422–26428. [PubMed: 11950837]
32. Borompokas N, Papachatzaki MM, Kanavouras K, Mastorodemos V, Zaganas I, Spanaki C, Plaitakis A. Estrogen modification of human glutamate dehydrogenases is linked to enzyme activation state. *J Biol Chem*. 2010; 285:31380–31387. [PubMed: 20628048]
33. Plaitakis A, Latsoudis H, Spanaki C. The human GLUD2 glutamate dehydrogenase and its regulation in health and disease. *Neurochem Int*. 2011; 59:495–509. [PubMed: 21420458]
34. Goodman M, Sterner KN, Islam M, Uddin M, Sherwood CC, Hof PR, Hou ZC, Lipovich L, Jia H, Grossman LI, Wildman DE. Phylogenomic analyses reveal convergent patterns of adaptive evolution in elephant and human ancestries. *Proc Natl Acad Sci U S A*. 2009; 106:20824–20829. [PubMed: 19926857]
35. Uddin M, Goodman M, Erez O, Romero R, Liu G, Islam M, Opazo JC, Sherwood CC, Grossman LI, Wildman DE. Distinct genomic signatures of adaptation in pre- and postnatal environments during human evolution. *Proc Natl Acad Sci U S A*. 2008; 105:3215–3220. [PubMed: 18305157]
36. Goodman M, Sterner KN. Colloquium paper: phylogenomic evidence of adaptive evolution in the ancestry of humans. *Proc Natl Acad Sci U S A*. 2010; 107(Suppl 2):8918–8923. [PubMed: 20445097]
37. Marques AC, Dupanloup I, Vinckenbosch N, Reymond A, Kaessmann H. Emergence of young human genes after a burst of retroposition in primates. *PLoS Biol*. 2005; 3:e357. [PubMed: 16201836]
38. Spanaki C, Zaganas I, Kleopa KA, Plaitakis A. Human GLUD2 glutamate dehydrogenase is expressed in neural and testicular supporting cells. *J Biol Chem*. 2010; 285:16748–16756. [PubMed: 20194501]
39. Zaganas I, Spanaki C, Plaitakis A. Expression of human GLUD2 glutamate dehydrogenase in human tissues: functional implications. *Neurochem Int*. 2012; 61:455–462. [PubMed: 22709674]
40. Aoki C, Milner TA, Berger SB, Sheu KF, Blass JP, Pickel VM. Glial glutamate dehydrogenase: ultrastructural localization and regional distribution in relation to the mitochondrial enzyme, cytochrome oxidase. *J Neurosci Res*. 1987; 18:305–318. [PubMed: 2826798]
41. Rothe F, Schmidt W, Wolf G. Postnatal changes in the activity of glutamate dehydrogenase and aspartate aminotransferase in the rat nervous system with special reference to the glutamate transmitter metabolism. *Brain Res*. 1983; 313:67–74. [PubMed: 6661665]
42. Spanaki C, Plaitakis A. The role of glutamate dehydrogenase in mammalian ammonia metabolism. *Neurotox Res*. 2012; 21:117–127. [PubMed: 22038055]
43. Sener A, Malaisse-Lagae F, Malaisse WJ. Stimulation of pancreatic islet metabolism and insulin release by a nonmetabolizable amino acid. *Proc Natl Acad Sci U S A*. 1981; 78:5460–5464. [PubMed: 6117857]
44. Stanley CA, Lieu YK, Hsu BY, Burlina AB, Greenberg CR, Hopwood NJ, Perlman K, Rich BH, Zammarchi E, Poncz M. Hyperinsulinism and hyperammonemia in infants with regulatory

- mutations of the glutamate dehydrogenase gene. *N Engl J Med*. 1998; 338:1352–1357. [PubMed: 9571255]
45. Rothe F, Wolf G, Schunzel G. Immunohistochemical demonstration of glutamate dehydrogenase in the postnatally developing rat hippocampal formation and cerebellar cortex: comparison to activity staining. *Neuroscience*. 1990; 39:419–429. [PubMed: 1708115]
 46. Rothe F, Brosz M, Storm-Mathisen J. Quantitative ultrastructural localization of glutamate dehydrogenase in the rat cerebellar cortex. *Neuroscience*. 1995; 64:iii–xvi. [PubMed: 7753371]
 47. Grosche J, Matyash V, Moller T, Verkhratsky A, Reichenbach A, Kettenmann H. Microdomains for neuron-glia interaction: parallel fiber signaling to Bergmann glial cells. *Nat Neurosci*. 1999; 2:139–143. [PubMed: 10195197]
 48. Cerdan S, Kunnecke B, Seelig J. Cerebral metabolism of [1,2-13C]acetate as detected by in vivo and *in vitro* 13C NMR. *J Biol Chem*. 1990; 265:12916–12926. [PubMed: 1973931]
 49. Hassel B, Sonnewald U. Glial formation of pyruvate and lactate from TCA cycle intermediates: implications for the inactivation of transmitter amino acids? *J Neurochem*. 1995; 65:2227–2234. [PubMed: 7595511]
 50. Slezak M, Pfrieder FW. New roles for astrocytes: regulation of CNS synaptogenesis. *Trends Neurosci*. 2003; 26:531–535. [PubMed: 14522145]
 51. Garcia-Segura LM, McCarthy MM. Minireview: Role of glia in neuroendocrine function. *Endocrinology*. 2004; 145:1082–1086. [PubMed: 14670989]
 52. Chatton JY, Marquet P, Magistretti PJ. A quantitative analysis of L-glutamate-regulated Na⁺ dynamics in mouse cortical astrocytes: implications for cellular bioenergetics. *Eur J Neurosci*. 2000; 12:3843–3853. [PubMed: 11069579]
 53. Levy LM, Warr O, Attwell D. Stoichiometry of the glial glutamate transporter GLT-1 expressed inducibly in a Chinese hamster ovary cell line selected for low endogenous Na⁺-dependent glutamate uptake. *J Neurosci*. 1998; 18:9620–9628. [PubMed: 9822723]
 54. Amato A, Ballerini L, Attwell D. Intracellular pH changes produced by glutamate uptake in rat hippocampal slices. *J Neurophysiol*. 1994; 72:1686–1696. [PubMed: 7529821]
 55. Azarias G, Perreten H, Lengacher S, Poburko D, Demaurex N, Magistretti PJ, Chatton JY. Glutamate transport decreases mitochondrial pH and modulates oxidative metabolism in astrocytes. *J Neurosci*. 2011; 31:3550–3559. [PubMed: 21389211]
 56. Balaban RS, Nemoto S, Finkel T. Mitochondria, oxidants, and aging. *Cell*. 2005; 120:483–495. [PubMed: 15734681]
 57. Plaitakis A, Berl S, Yahr MD. Abnormal glutamate metabolism in an adult-onset degenerative neurological disorder. *Science*. 1982; 216:193–196. [PubMed: 6121377]
 58. Plaitakis A, Carosio JT. Abnormal glutamate metabolism in amyotrophic lateral sclerosis. *Ann Neurol*. 1987; 22:575–579. [PubMed: 2892463]
 59. Iwasaki Y, Ikeda K, Shiojima T, Kinoshita M. Increased plasma concentrations of aspartate, glutamate and glycine in Parkinson's disease. *Neurosci Lett*. 1992; 145:175–177. [PubMed: 1361223]
 60. Plaitakis A, Latsoudis H, Kanavouras K, Ritz B, Bronstein JM, Skoula I, Mastorodemos V, Papapetropoulos S, Borompokas N, Zaganas I, Xiomerisiou G, Hadjigeorgiou GM, Spanaki C. Gain-of-function variant in GLUD2 glutamate dehydrogenase modifies Parkinson's disease onset. *Eur J Hum Genet*. 2010; 18:336–341. [PubMed: 19826450]
 61. Smith TJ, Schmidt T, Fang J, Wu J, Siuzdak G, Stanley CA. The structure of apo human glutamate dehydrogenase details subunit communication and allostery. *J Mol Biol*. 2002; 318:765–777. [PubMed: 12054821]
 62. Egan DF, Shackelford DB, Mihaylova MM, Gelino S, Kohnz RA, Mair W, Vasquez DS, Joshi A, Gwinn DM, Taylor R, Asara JM, Fitzpatrick J, Dillin A, Viollet B, Kundu M, Hansen M, Shaw RJ. Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. *Science*. 2011; 331:456–461. [PubMed: 21205641]
 63. Duran RV, Oppliger W, Robitaille AM, Heiserich L, Skendaj R, Gottlieb E, Hall MN. Glutaminolysis activates Rag-mTORC1 signaling. *Mol Cell*. 2012; 47:349–358. [PubMed: 22749528]

64. Panov A, Dikalov S, Shalbuyeva N, Taylor G, Sherer T, Greenamyre JT. Rotenone model of Parkinson disease: multiple brain mitochondria dysfunctions after short term systemic rotenone intoxication. *J Biol Chem*. 2005; 280:42026–42035. [PubMed: 16243845]

Fig. 1. Amino acid changes acquired by *GLUD2* and their effect on enzyme function

The phylogenetic tree shown here, depicting the evolution of the *GLUD2* gene in primates, including internal nodes (A–F) and approximate divergence times (Mya: millions in years) was modified from Burki and Kaessmann [29]. The effect of evolutionary mutations in enzyme function has been studied in single hGDH1 mutants as previously described [33]. This Figure is from Plaitakis, et al., *Neurochem Intern*, 59:495-509 2011 [33]

Fig. 2. Basal-specific activities of purified recombinant wild-type hGDH1 and hGDH2 and the Ala445-hGDH2 variant

Assays were performed at 25° C in the absence of allosteric effectors as described (60). Each point represents the mean $\pm$ SEM (bar) of three to five experimental determinations. The slope ($\pm$ SE) of each regression line is given below as the rate of change of specific activity (in mmol/min/mg) per mg increase in enzyme concentration. The R correlation coefficient and the P-value (probability that R is zero) of the linear regression are given in parentheses. Slope for Ala445-hGDH2: 6.06 ± 0.52 ($R=0.986, P=0.0003$). Slope for hGDH2: 1.56 ± 0.23 ($R=0.940, P=0.0005$). Slope for hGDH1: 0.76 ± 0.57 ($R= -0.556, P=0.251$). This Figure is from Plaitakis, et al., *Eur J Hum Genet*,18:336-341 [60]