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Review

Brain glutaminases

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Abstract

Glutaminase is considered as the main glutamate producer enzyme in brain. Consequently, the enzyme is essential for both glutamatergic and gabaergic transmissions. Glutamine-derived glutamate and ammonia, the products of glutaminase reaction, fulfill crucial roles in energy metabolism and in the biosynthesis of basic metabolites, such as GABA, proteins and glutathione. However, glutamate and ammonia are also hazardous compounds and danger lurks in their generation beyond normal physiological thresholds; hence, glutaminase activity must be carefully regulated in the mammalian brain. The differential distribution and regulation of glutaminase are key factors to modulate the metabolism of glutamate and glutamine in brain. The discovery of novel isoenzymes, protein interacting partners and subcellular localizations indicate new functions for brain glutaminase. In this short review, we summarize recent findings that point consistently towards glutaminase as a multifaceted protein able to perform different tasks. Finally, we will highlight the involvement of glutaminase in pathological states and its consideration as a potential therapeutic target.

Keywords: astrocytes; glutamate; glutamine; glutaminase-interacting proteins; neurones.

Introduction

Glutaminase (GA, EC 3.5.1.2) catalyzes the hydrolytic deamidation of glutamine (Gln) giving rise to stoichiometric amounts of glutamate (Glu) and ammonium ions. The enzyme is widely distributed in mammalian tissues where it fulfills essential tasks related to tissue-specific function (1). In brain, the biosynthesis of transmitter Glu from Gln through GA is considered as the prevailing pathway for excitatory Glu production (2), although transamination of α-ketoglutarate involving tricarboxylic acid cycle (TCA) reactions has also been reported to contribute to generation of neurotransmitter Glu (3). In addition to the transmitter pool of Glu in excitatory synapses, a metabolic pool also exists as this amino acid is a primary metabolic fuel for brain cells (4) and a precursor of many other important metabolites (5). In fact, the mitochondrial breakdown of glutamine to pyruvate, known as glutaminolysis, is initiated by GA and has been largely recognized as a fundamental process for energy supply in the bioenergetics of many normal and transformed cell types (6).

The concentration of free Glu in mammalian brain is higher than in any other organ (7). Glu is found in higher concentrations than any other free amino acid in the central nervous system (8); however, it is a nonessential amino acid that crosses the blood-brain barrier relatively poorly (9) and hence it must be synthesized in the brain. In the tripartite synapsis (presynaptic neurone, postsynaptic neurone and astrocytes), the balance between glutamate release and reuptake is termed 'glutamate homeostasis', which modulates synaptic activity and plasticity by controlling the activation of ionotropic and metabotropic Glu receptors (10). GA is a central player for Glu homeostasis in brain as the most important presynaptic source of releasable Glu. Nevertheless, most studies related to brain Glu homeostasis have investigated Glu receptors and transporters, which clear the Glu released in the synaptic cleft lowering its otherwise excitotoxic concentration. Thus, considerably less focus has been given to presynaptic mechanisms of glutamate generation, particularly to the regulation of GA expression.

Brain Gln homeostasis is inextricably linked to Glu homeostasis: a vivid example is the Glu/Gln cycle between neurones and astrocytes (11). This Glu/Gln shuttle has been postulated for neurotransmitter generation and recycling: given the lack of quantitatively important anaplerotic enzymes for *de novo* synthesis of Glu in neurones, this amino acid must be supplied by astrocytes to prevent depletion of the Glu pool. To fulfill this goal, synaptic Glu is mostly removed by glial cells and converted into Gln by glutamine synthetase (GS, EC 6.3.1.2), an enzyme expressed in astrocytes but absent in neurones (12). Gln is then transported out of the glial cells and into nerve terminals where it is metabolized back to Glu by GA, an enzyme thought to be exclusively located in neurones (13). GA plays a central role in this neurotransmitter recycling scheme, as the main Gln-utilizing enzyme of the brain (14).

The discovery of novel isoforms, extramitochondrial locations and protein interacting partners for GA in the brain of mammals opens new perspectives on its role in cerebral function. In this review, we will discuss recent findings that

point to GA as a multifunctional protein with the ability to perform different tasks, some of them beyond their classical role restricted to neuronal presynaptic biosynthesis of Glu. Finally, we will also highlight studies providing experimental support for the functional implication of GA in important neurological disorders and diseases.

Glutaminase isoenzymes in mammalian brain

The GA protein family members are encoded by two paralogous genes, *Gls* and *Gls2*, presumably derived by gene duplication of a common ancestor (15, 16). In humans, *Gls* is located in chromosome 2 and encodes GA isozymes classically referred to as kidney type (K-type or K), whereas the *Gls2* gene is located in chromosome 12 and codes for liver type (L-type or L) isozymes (17). Orthologous genes have been described in rat (15) and mouse (18). Two isoforms derived from each GA gene have been identified thus far (Figure 1). The transcripts known as KGA and GAC arise by alternative splicing of the *Gls* gene: KGA mRNA is formed by joining exons 1–14 and 16–19, whereas the alternative spliced transcript GAC uses only the first 15 exons, omitting exons 16–19 (19, 20) (Figure 1). In human tissues, GAC mRNA is expressed predominantly in cardiac muscle and pancreas, appreciably in placenta, kidney and lung, but not in brain and liver (19). KGA mRNA was found to be ubiquitous in most nonhepatic human tissues (17). KGA cDNAs have been cloned from human (21) and rat brain (22).

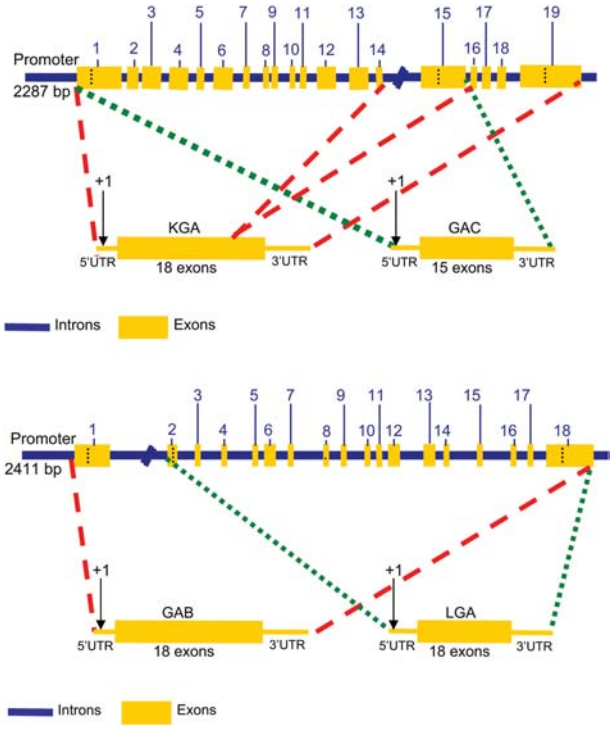

Figure 1 Human glutaminase genes and mRNA transcripts. (Top panel) Human glutaminase *Gls* gene and alternative transcripts KGA and GAC. (Bottom panel) Human glutaminase *Gls2* gene and transcripts GAB and LGA. Each gene is shown with introns depicted as solid blue lines and exons as numbered yellow boxes. The promoter regions are also indicated on the 5′-end of each gene. Dashed red lines indicate the exons forming KGA and GAB mRNA transcripts, whereas dotted green lines comprise exons involved in the generation of transcripts GAC and LGA. The transcription start site is marked by an arrow and numbered as +1. (For further information see text.)

The mammalian *Gls*2 gene is split into 18 exons (16) (Figure 1). Two L-type transcripts have been identified from the *Gls2* gene: the canonical long transcript termed GAB, formed by joining the full 18 exons of the gene (16), and the short transcript LGA that lacks exon 1 and was originally identified in rat liver (23) (Figure 1). Human GAB transcript was isolated as a cDNA clone from ZR75 breast cancer cells encoding a protein of 602 amino acids, which is 67 amino acids longer than rat liver LGA protein (24) (Figure 1). Experimental evidence supporting GAB as a novel L-type GA isoform, different from the classical LGA liver isozyme, has recently been published (25). L-type transcripts derived from the *Gls*2 gene were originally thought to be present in adult liver tissue and absent in extrahepatic tissues (23, 26). This restricted pattern of expression was generally accepted until recently, when results from our laboratory demonstrated L-type GA expression in extrahepatic tissues such as brain, pancreas and breast cancer cells (24).

In mammalian brain, two GA isoforms with different kinetic and regulatory properties are expressed (27) (Figure 2). Northern analysis of GA transcripts indicated simultaneous expression of L-type and K-type mRNAs in human brain. Both isozymes are ubiquitously expressed in brain regions with the strongest signal appearing in cerebral cortex (17, 24). Expression of K- and L-type transcripts was also demonstrated in brain of other mammalian species such as cow, mouse, rabbit and rat. The coexpression was verified at the protein level by biochemical and immunological approaches (27). The presence of multiple GA transcripts and proteins, even in a single cell type, could be more frequent than previously believed (28, 29).

GA isozymes differ in molecular structures as well as in kinetic, immunological and regulatory properties (1, 26). The distinct kinetic behavior has been a hallmark frequently used to distinguish between GA isoforms. The main kinetic differences have been observed in the dependence of the activator inorganic phosphate (Pi) – low for L-type, high for K-type –, the relative affinity for the substrate Gln – higher in K- than in L-types –, and the inhibitory effect of Glu, a unique characteristic reported only for K-type isozymes (6, 26). In brain, a wide variety of endogenous and exogenous effectors can modulate GA activity [see Refs. (4, 30) for a detailed list of compounds]. Ca^{2+} activates GA in mitochondria, brain synaptosomes, brain slices and homogenates, but does not act on purified enzyme, indicating that its effect is indirect (4, 30).

Phosphate is the most prominent stimulator of GA. Purified brain KGA is an allosteric enzyme highly sensitive to changes in the level of Pi (30, 31). Main effectors of GA

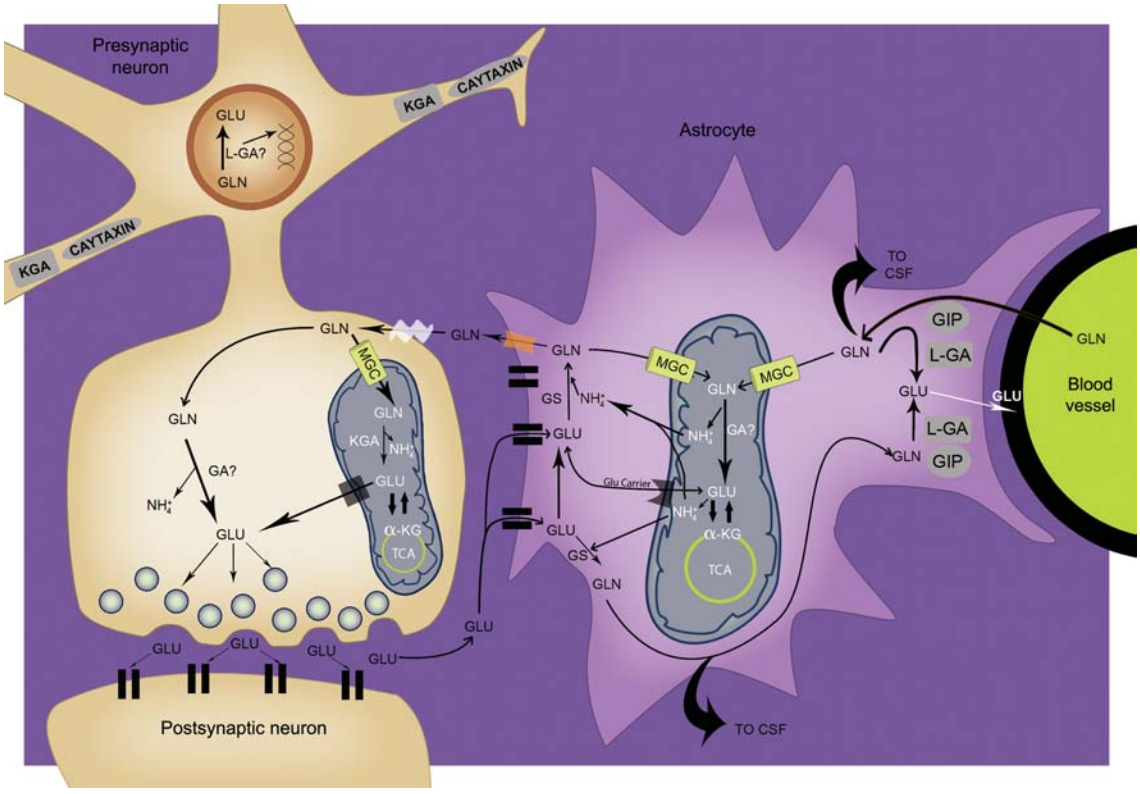

Figure 2 Schematic illustration of the Glu/Gln cycle between neurones and astrocytes at the tripartite synapsis.
This is a simplified model highlighting the main functions of GA isozymes, even if not enough evidence exists for a particular task. In the latter case, a question mark denotes 'not enough evidence'. For example, in neuronal nuclei the presence of L-type GA isoform has been demonstrated; however, the nuclear function of this GA has not been fully ascertained. Two alternative functions appear: regulation of the Gln/Glu levels or transcriptional regulation (arrow pointing toward DNA). The relative contribution of K-type and L-type isoforms in the synthesis of the neurotransmitter Glu pool remains to be clarified. In neuronal body, Gln is converted to Glu by mitochondrial KGA, whereas the existence of a cytosolic GA, which could contribute to the Glu transmitter pool, has not yet been confirmed. Synaptic Glu is primarily taken up by astrocytes, mostly converted to Gln by GS in the cytosol, and then cycled back to neurones where GA regenerates the transmitter Glu. Exogenous Glu can be transported to glial mitochondria and converted to α-KG by GDH or transamination, followed by oxidation into the TCA cycle. Astroglial Gln can be exchanged with blood. Some Gln can be oxidatively degraded by astrocytic GA and TCA cycle after being transported into mitochondria through the uncharacterized MGC; the ammonium generated might be channeled to cytosolic Gln synthesis. Experimental evidence supports L-type GA expression in astrocyte (evidence is lacking for KGA). L-type GA and GIP can interact *in vivo* and astrocytes are a likely anatomic substrate for their coupling in brain. The colocalization of GIP and L-type GA in astrocytes, particularly in perivascular end feet surrounding capillaries, might be related to the regulation of the vascular tone. The detailed anatomy of synapses, astrocytes and blood vessels is not portrayed. MGC, Mitochondrial Gln Carrier; CSF, cerebrospinal fluid; L-GA: L-type GA (GAB or LGA isoforms); KGA, K-type long GA isoform; α-KG, α-ketoglutarate.

activity are, in fact, compounds that alter Pi activation (30). Whether Pi is the true physiological stimulator of GA in brain remains to be determined, but considering its brain concentration and the fact that it can be rapidly altered during neuronal activity, its candidature as an important physiological regulator of brain GA *in vivo* has been postulated (4). Main inhibitors of brain GA include Glu, ammonium ions, protons, cAMP and cGMP (4, 30). Glutamate is a competitive inhibitor and the relative concentrations of Gln and Glu in glutamatergic terminals (30) suggest that GA can be strongly inhibited in nerve cells as in their terminals, in agreement with early experiments done with synaptosomes (4).

Despite the fact that protein levels of GA isozymes have not yet been quantified, we found clear protein segregation in rat and monkey brain. In both species, K-type GA protein was detected in mitochondria, in agreement with previous biochemical and immunocytochemical studies (32, 33), whereas L-type GA protein was mostly localized in neuronal nuclei (27). For the first time, an extramitochondrial localization for a mammalian GA enzyme was reported, because they were considered to be exclusively mitochondrial enzymes (6, 26). L-type GA was mostly concentrated in neuronal nuclei, although a minor cytoplasmic immunolabeling was also detected. Many neuronal cells, but not all, expressed nuclear L-type GA protein (27). Furthermore, the nuclear GA was catalytically active although showing kinetic characteristics atypical for L-type isozymes (27). The novel L-type GAB isozyme shows mixed kinetic characteristics of K- and L-type isoforms (34) and seems the most plausible

candidate for brain nuclear GA (25). Importantly, human recombinant GAB colocalizes in mitochondria and nuclei of Sf9 cells, reinforcing the view that this isoform could be targeted to different subcellular locations, including the cell nuclei (34).

Finally, it is important to note that presence of brain GA in the cytoplasm has also been suggested by subcellular fractionation and immunocytochemistry studies (32, 35, 36). Nevertheless, the relevance of extramitochondrial GA to Gln-Glu metabolism requires further investigation and is presently unknown (Figure 2). Of interest, interaction of GA isoforms with protein interacting partners recently discovered in brain could allow targeting of GA to different subcellular locations (see section 'Other GA functions in mammalian brain' and Figure 2).

Function of GA in glutamatergic neurotransmission

The importance of GA in glutamatergic synaptic function has been largely recognized. Physiological, biochemical, immunological and nuclear magnetic resonance (NMR) spectroscopic data indicate that neurotransmitter Glu is mainly generated through GA reaction (37), although the relative contribution of each GA isoform to the transmitter pool is presently unknown. A knockout (KO) mice model for the *Gls* gene has recently been generated (38). Mice lacking the *Gls* gene die shortly after birth as a result of altered functioning of key glutamatergic neural networks, stressing the importance of K-type GA (KGA and/or GAC isozymes) in glutamatergic transmission. Although neurones lacking K-type GA showed a more rapid decay of excitatory postsynaptic currents (EPSCs), there was persistence of glutamatergic activity in null mutant mice and the miniature EPSC amplitude was not reduced in cultured $Gls^{-/-}$ cortical neurones, suggesting that there were adequate levels of intrasynaptic Glu under conditions of basal activity (38). The authors concluded that persistence of glutamatergic transmission in null mutants can be accounted for by upregulation of other Glu-synthetic pathways, such as transamination reactions, or by direct neuronal Glu reuptake. In addition, L-type GA isozymes, coded by the *Gls2* gene, should also be considered as suitable candidates for Glu biosynthesis in these null mutants for K-type GA. Even though the amounts of transmitter Glu generated by different GA isoforms in normal and pathological situations are unknown, recent studies in mammalian brain are clarifying the isozyme-specific pattern of GA expression and the relative abundance of GA isozymes, as a prerequisite to infer their respective roles in cerebral function (27, 39).

The homeostasis of Glu and Gln in brain should be carefully regulated owing to the toxic effects elicited by an excess of Glu. The Glu/Gln cycle between neurones and astrocytes is a central pathway for neurotransmitter recycling and to deal with Glu toxicity (Figure 2). *In vivo* ^{13}C-NMR kinetic studies along with metabolic models to distinguish the Glu/Gln cycle from other sources of isotopic Gln labelling, particularly Gln synthesis by glial anaplerosis, have demonstrated that the Glu/Gln cycle between astrocytes and neurones is the major pathway for neuronal Glu repletion in rat and human cerebral cortex (40, 41). Furthermore, it was concluded from these studies that the rate of the Glu/Gln cycle is very high and similar in magnitude to the rate of glucose oxidation, supporting the model proposed by Magistretti et al. which couples neuronal activity to glucose utilization and where glial glycolitic ATP represents the major source of energy for neurotransmission (42, 43).

The Glu/Gln cycle is a key mechanism for homeostatic control of these amino acids, although its entirety has been questioned. For example, it has become clear that part of the recycled Glu is oxidatively degraded in astrocytic mitochondria by the TCA cycle and, thus, Glu consumed must be restored by net synthesis from glucose (*de novo* synthesis through pyruvate carboxylase, an enzyme exclusively expressed in astrocytes) (44–46). Experimental data *in vivo* also showed that flux through GS in the intact brain is even higher than the Glu supply from the Glu/Gln cycle, and therefore net synthesis of Glu in astrocytes is required (40, 47–49). Another unexplained issue in the functional recycling of Glu between neurones and astrocytes is the source of the ammonium needed to sustain the highly active Gln synthesis through GS in astrocytes. Three variants of the Glu/Gln cycle have been proposed to explain the intercellular flux of ammonium from presynaptic neurones to astrocytes: ammonia diffusion, a nitrogen shuttle based on branched-chain amino acids/branched-chain keto acids interchange, and an alanine-lactate nitrogen cycle (45, 50). Recently, three alternative cycles for functional glutamate trafficking have been proposed by linking glucose catabolism to Glu recycling (51). Each of these cycles is based on the transport of a TCA cycle intermediate (α-ketoglutarate, malate or citrate) along with one Gln from astrocytes to presynaptic neurones, and their exchange by two Glu taken up by astrocytes from the synaptic cleft. Although these novel cycles are presented as energetically more efficient (less dependent on ATP) and they incorporate inherent mechanisms for nitrogen transport from neurones to astrocytes, the question of which is the functional cycle for Glu recycling and its possible variants under different physiological conditions still remains unanswered (51).

A subject highly related to the Glu/Gln cycle, and matter of great controversy, is whether or not Glu can be formed from Gln in astrocytes. A GA activity in astrocytes could fit well with the Glu/Gln cycle, because it would endorse astrocytes with an endogenous mitochondrial source of both Glu and ammonium. This endogenous, non-synaptic, Gln-derived Glu could be converted through glutamate dehydrogenase (GDH) or transamination to α-ketoglutarate, and then proceeds via TCA cycle to fulfill astrocytic energetic needs (Figure 2). Thus, astrocytes will not significantly deplete synaptic Glu stores saving most of it for Gln synthesis in the cytosol; such mechanisms might be particularly relevant in periods of great synaptic activity. In addition, the ammonium generated in mitochondria by this glutaminolytic process could be channeled to Gln synthesis in cytosol, providing an

additional source of nitrogen needed to recycle Glu in Gln through GS.

As mentioned previously, *in vivo* kinetics studies with ^{13}C-NMR spectroscopy in cerebral cortex have estimated that approximately 10–30% of neuronal Glu repletion is contributed by glial anaplerosis to replace the Glu oxidatively degraded by astrocytes (41, 52). However, the hypothesis of an active GA in astrocytes could raise the question of which is the metabolite preferentially oxidized *in vivo* by glial mitochondria: Gln or Glu. In other words, which metabolite escapes from the Glu/Gln cycle to fuel glial mitochondria and to account for the estimated 10–30% of Glu that must be anaplerotically restored (Figure 2). *In vitro* cultures of astrocytes have the ability to oxidize both Gln and Glu, although notable differences have been found in the metabolism of endogenous – generated from Gln through GA – versus exogenous Glu. In cultured astrocytes, Gln oxidation proceeds mostly by transamination through aspartate aminotransferase (46, 53), or to some extent through branched-chain amino acid transaminase (45), whereas Glu is oxidized primarily through GDH and at a much higher rate than Gln (54).

Before mitochondrial oxidation, Glu and Gln need to be transported into the mitochondria (Figure 2). It has been shown that brain astrocytes do not express the aspartate-glutamate carrier (AGC) (55, 56), which forces exogenous Glu to entry into astrocytes uniquely by the Glu/hydroxyl carrier. Results obtained from transgenic mice KO for AGC underscored the importance of this carrier for Glu transport in brain and skeletal muscle mitochondria (57). Evidence indicates that no other Glu carrier can substitute for AGC in those tissues [(58) and references therein]. In contrast, Gln can be actively concentrated into astrocyte mitochondria by using a high-affinity mitochondrial Gln carrier (59) (Figure 2). Despite the fact that mitochondrial Gln carriers have not been characterized at the molecular level in any mammalian tissue, studies with isolated mitochondria have shown that Gln transport is mostly saturable, concentrative, osmosensitive, largely stereospecific and stimulated by ammonia in nonsynaptic mitochondria (60). Transported Gln will then be released into the mitochondrial matrix near the GA catalytic site, although some controversy still exists about the submitochondrial location of GA [see Ref. (61) and references therein for a more detailed discussion on this issue]. The generation of Glu into the matrix facilitates their further catabolism for bioenergetics or biosynthetic purposes. Thus, Gln-derived Glu is generated into the mitochondrial matrix without the need of an additional carrier, as required for exogenous Glu.

Another problem facing cytosolic Glu to be preferentially transported into astrocytic mitochondria instead of Gln is the fast induction of GS protein in active synapse, which can rapidly convert the captured synaptic Glu into Gln. In fact, it has been shown that after reaching a steady state in the astrocytes, the flux of Glu to the TCA cycle became negligible as compared with the flux through GS (62). Actually, these authors conclude that astrocytes adapted to a constant supply of Glu by increasing Glu uptake and GS activity, in such a way that Glu was converted almost exclusively through GS. However, other studies on Glu metabolism in astrocytes using short incubation times do not allow for GS induction, as in the case of a prolonged and permanent challenge with Glu. Under these conditions, conversion of Glu to α-ketoglutarate and further oxidative catabolism is the prevalent pathway, instead of synthesis of Gln through GS (62).

The existence of GA activity *in vivo* in astrocytes has not yet been confirmed. Primary cultures of astrocytes displayed strong GA activity (63–65) and GA mRNA transcripts (66), but these *in vitro* results have been questioned claiming that GA could be induced by culture conditions (4). By contrast, contradictory results appear in the literature about the expression of GA in astrocytes: immunohistochemical studies have shown expression of GA protein and GA activity in rat brain astrocytes (32, 67); however, no GA was found in astrocytes from rat cerebellum via post-embedding immunocytochemistry with colloidal gold (33). Recently, we have found L-type GA protein in rat brain astrocytes from the cerebral cortex by immunocytochemical analyses (68). This L-type GA immunolabel does not necessarily reflect enzymatic activity. Further study is needed to ascertain if this GA is catalytically active and, if so, its function in astrocytes (see next section). Nevertheless, available experimental data predict an *in vivo* GA activity considerably lower in astrocytes as compared with that shown by neurones. Finally, it should be emphasized that the existence of astrocytic GA might have physiological significance mostly confined to the mitochondria; a cytosolic activity seems unlikely taking into account the strong inhibition that synaptic Glu can exert. In addition, the simultaneous operation of GA and GS in the cytosol will give rise to a futile cycle with high energy expenditure in the form of ATP.

Other GA functions in mammalian brain

In the search for discovering new physiological functions for brain GA, proteomics approaches, such as two-hybrid genetic screenings and immunoprecipitations coupled to mass spectrometry (MS) analysis, have proven to be very useful strategies for isolation of potential protein interacting partners of GA. A yeast two-hybrid genetic assay was performed by screening a human brain cDNA library with the C-terminal region of human GAB. Two PDZ domain-containing proteins were isolated: alpha-syntrophin (SNT) and Glutaminase-Interacting Protein (GIP) (69). Human GAB has been endowed with consensus protein motifs and domains that might support its role as a multifunctional protein (1). In the C-terminal region, for example, the last four amino acids, ESMV, matches the consensus sequence required for interactions with PDZ proteins (70). The binding of key C-terminal amino acid residues of GAB with the PDZ domain of GIP and SNT was shown to be a highly specific interaction by both *in vivo* yeast genetic screenings and *in vitro* pull-down assays (69).

The physiological relevance of the GA-GIP interaction was first assessed by studies of protein localization in rat and monkey brain. A purified anti-GIP antibody prominently immunostained brain regions such as cerebral cortex, hippocampus, striatum and olfactory bulb, whereas cerebellum, thalamus and medulla oblongata were more lightly labeled (68). The cellular distribution of GIP showed the presence of this protein in both neurones and glial cells, throughout the different brain regions analyzed, with a cytosolic and mitochondrial subcellular localization. The results revealed that all GIP-positive glial cells were astrocytes. The immunoreactivity pattern for the GIP antigen was found in both astroglial cell bodies and processes, including the perivascular end feet. In other glial cells, such as microglia and oligodendrocytes, GIP labeling was absent (68).

Immunocytochemical studies for GIP and L-type GA were performed aiming to ascertain whether both proteins might be interacting partners *in vivo*. In astrocytes, GIP colocalizes with GA: the presence of L-type GA in GIP-immunopositive astrocytes was demonstrated by double GIP-GA immunofluorescence confocal experiments in cerebral cortex from rat brain. Double-label studies revealed that both proteins colocalize in astrocytes cell bodies and processes, including their perivascular end feet (68) (Figure 2). The presence of GIP in astrocytes was also confirmed by electron microscopic (EM) immunocytochemistry, which revealed immunoreactive astrocytic end feet surrounding endothelial cells (68). These results strengthen the argument that GA and GIP can interact *in vivo* and point to astrocytes as a likely anatomic substrate for their coupling in brain.

The hypothesis of brain GA being regulated by PDZ proteins is appealing because it can provide a mechanism for both control of glutamate synthesis and targeting of cerebral GA to concrete cellular compartments. In fact, GIP has been shown to inhibit L-type GA activity in crude extracts of rat liver (71). Interestingly, a role in the targeting of PDZ protein interaction partners to concrete subcellular localizations, including cell nucleus, has also been reported (72). Therefore, we cannot exclude that GIP could be involved in the targeting of L-type GA to neuronal nuclei. A potential nuclear function for GA could be the regulation of Gln/Glu levels (27), taking into account that Gln is a signal molecule involved in gene expression (73, 74). Therefore, the significance of its nuclear localization could be as simple as being an enzyme controlling *in situ* the Gln levels in the nucleoplasm and thus being indirectly involved in the expression of Gln-regulated genes (Figure 2). Alternatively, GA can act as a transcriptional coregulator (27). In this regard, a recent study has revealed that overexpression of the human GAB cDNA in T98 glioblastoma cell lines induced a marked change in the transcriptome of the cell correlated with a reversion of the transformed phenotype (75). Human malignant gliomas have been shown to express K-type GAs (KGA and GAC isoforms) but show a negligible expression of L-type GA (76). Taking into account its presence in the nuclei of the cell, it has been speculated that GAB overexpression could contribute to the altering of transcriptional programming of glioma cells yielding a less malignant and more differentiated phenotype (75), but the concrete molecular mechanisms underlying this phenotypical change have not been ascertained.

The colocalization of GIP and L-type GA in astrocytes, particularly in perivascular end feet surrounding capillaries, might be related to the regulation of the vascular tone (Figure 2). Interestingly, a perivascular and pial localization of GA in rat brain was previously reported (77), and we found that GIP and L-type GA colocalize in perivascular end feet. Thus, the targeting of GA to specific populations of astrocyte processes surrounding blood vessels might be a plausible mechanism implicated in the regulation of the vascular function, considering the key role of astrocytes in cerebrovascular regulation (78). The fact that glutamate is a vasoactive compound (79) and the existence of glutamate receptors in perivascular glia and vascular endothelial cells (80, 81) would implicate GIP-GA interaction in regulating the vascular tone (Figure 2).

A recent study has reported the first interacting partner for K-type GA: the brain-specific protein BNIP-H (for BNIP-2 homology) or caytaxin, a protein exclusively expressed in neural tissues and encoded by a gene associated with human cerebellar Cayman ataxia (82). This protein contains a novel protein-protein interaction domain known as the BNIP-2 and Cdc42GAP homology (BCH) domain (83). The regional distribution of caytaxin in mouse brain broadly matched the pattern of expression previously known for KGA. With regard to subcellular distribution, caytaxin relocalized KGA from cell body to neurite terminals. After cotransfection of PC12 cells with KGA and full-length caytaxin, KGA was specifically redistributed by caytaxin to neurite terminals independently of and away from mitochondria (Figure 2). Thus, the neuronal trafficking of KGA was specifically ascribed to its interaction with caytaxin (82). Furthermore, caytaxin could have functions other than just promoting intracellular relocalization of KGA. The same authors demonstrate that caytaxin reduces the steady-state levels of Glu by inhibiting KGA activity. Therefore, the interaction KGA-caytaxin seems relevant for regulating the homeostasis of Glu synthesis important for proper neurotransmission and/or neuronal cell growth (Figure 2). For example, the absence of functional caytaxin would increase Glu levels in the cell bodies of neurones leading to neurotoxicity and/or abnormal neuronal growth.

Involvement of GA in brain pathological states

There is compelling experimental evidence pointing toward GA as an important pharmacological target for a variety of brain disorders and diseases. Toxic levels of Glu leading to neuronal death, a process known as receptor-mediated excitotoxicity, contribute to the brain damage observed in stroke, cardiac arrest, seizures and trauma (84). Elevated extracellular concentrations of the excitatory transmitter Glu are an important cause of neuronal damage and death after hypoxic-ischemic insult, although the sources of increased Glu release responsible for these pathological effects are incompletely

described. In this regard, GA reaction has been demonstrated to be an essential pathway to generate excitotoxic Glu after neuronal death (85). The authors demonstrated that the GA present in fragments of damaged neurones is sufficient to cause neuronal death in *in vitro* models of neuronal injury. This finding is consistent with the delayed increase in extracellular Glu observed in brain ischemia and with the fact that GA remains active in the ischemic periphery hours after onset of focal ischemia (86). Therefore, selective reagents that can inhibit the more accessible GA of damaged neurones without affecting the enzyme in undamaged cells could prove to be clinically useful following ischemic insults (85).

Glu toxicity also appears in traumatic brain injury (TBI). Brain trauma leads to an excitotoxic cascade involving Glu and other excitatory amino acids giving rise to neuronal death in the tissue surrounding the original injury site. By using cerebral microdialysis extracellular Glu was measured in 165 patients with TBI during a 120-h monitoring period (87). The authors showed that Glu levels were correlated with mortality and 6-month functional outcome: high Glu levels were predictive of poor outcome, whereas a delayed Glu increase carried the worst prognosis. Therefore, drugs which stop the release of excitatory amino acids or which block them could reduce brain damage and potentiate neuronal survival. However, the use of anti-Glu therapy in patients with stroke and TBI has not yet demonstrated its efficacy in humans and additional studies are worthwhile (88). Adaptation of the Glu/Gln cycle has been proposed as a potential mechanism to avoid neuronal death after brain injury. In this regard, a graded reduction of hippocampal GA activity paralleled by increases in GS activity were found in two *in vivo* models of severe and mild brain injury (89). The authors pointed out that this graded modification of the Glu/Gln cycle allowed to increase the net Gln output while reducing the Glu excitotoxicity and could be a key factor in apoptotic and necrotic neuronal demise.

Excessive glutamatergic transmission has been assumed to be one causal factor of epilepsy. The extracellular level of Glu is considerably increased in human epileptic hippocampus (90). However, to assign the sources of such heightened Glu concentration has been somehow puzzling owing to the epileptogenic hippocampus loss of many glutamatergic neurones (91). To explain this altered Glu homeostasis in epilepsy, changes in the enzymes GS and GA, as well as in Glu receptors, have been postulated. Thus, patients with mesial temporal lobe epilepsy (MTLE) showed a severe deficiency in astrocytic hippocampal GS (92), although this decrease was not observed neither in human epileptic neocortex (93) nor in kainate models of temporal lobe epilepsy (94). The deficiency in GS has been postulated as a mechanism to explain the pathologically enhanced Glu levels by diminishing the conversion of Glu to Gln (91). An increased expression of mitochondrial GA in hippocampal neurones in human MTLE has been recently reported (95). This upregulation of GA is a likely source of exocytotic Glu, although direct evidence linking GA overexpression and loss of Glu homeostasis causing epilepsy is still lacking (95). The contribution of astrocytes to epileptogenesis has been highlighted lately: astrocytic Glu release plays an essential role in seizure activity and epileptic disorders whereby an astrocytic basis of epilepsy has been proposed recently (96).

Dysregulation of glutamatergic neurotransmission has become recognized as a key component in the pathophysiology of schizophrenia (97). The so-called Glu hypothesis of schizophrenia emerged from seminal studies showing that phencyclidine (PCP, 'angel dust'), an antagonist of *N*-methyl-D-aspartate (NMDA) Glu receptors, induces many of the symptoms of schizophrenia (98, 99). The hypothesis states that there is a hypofunction of Glu and NMDA receptors in schizophrenic brains leading to excess Glu release. An acute increase in Glu levels in prefrontal cortex (PFC) and hippocampus has been found in schizophrenia (100). Accordingly, enhanced KGA mRNA levels in the brain thalamus and a four-fold increase in GA activity in PFC have been reported in schizophrenic patients compared to control subjects (101–103), which suggest involvement of GA in the excess of Glu release. In contrast, a recent study employing qPCR and microarrays found a significant downregulation of the *Gls* gene in PFC of postmortem samples from subjects with schizophrenia (104).

In light of the above presented data, novel therapeutic approaches have been developed aiming to achieve a presynaptic reduction of Glu neurotransmission. One preclinical study reported that activation of group II metabotropic Glu receptors (mGluR2/3) could reverse the PCP effects by attenuating Glu release (105). The mGluR2/3 agonist LY2140023 is currently being evaluated as an antipsychotic drug in clinical trials (106). A genetic association between GA genes (*Gls* and *Gls2*) and schizophrenia has not been found (107); hence, GA is unlikely to be a causal factor for this neurological disorder, although typical antipsychotics can cause decreases in GA activity (108). Another appealing approach to reduce presynaptic glutamatergic transmission targets GA for antipsychotic drug research (109). These authors have shown that hyperfunction of hippocampal CA1 region is linked to the pathogenesis of schizophrenia. Therefore, they postulate that a reduction of Glu in hippocampus after GA inhibition could be an effective therapeutic strategy in schizophrenia. As a proof of principle, the researchers employed transgenic heterozygous mutant mice partially lacking the *Gls* gene and showing 50% global reduction in GA activity (110). The mice displayed hippocampal hypometabolism mainly in the CA1 region (the inverse pattern observed in schizophrenic patients) and were less sensitive to pro-psychotic drugs. The results underscore the relevance of GA in schizophrenia and open new directions for its pharmacotherapy (110).

Alterations in the Glu/Gln cycle between neurones and astrocytes have been implicated in a variety of brain dysfunctions and neurological diseases, but only those in which GA has been clearly involved will be mentioned. Thus, the presence of heightened brain Gln level owing to a decrease in both neuronal-glial Glu/Gln cycling and GA activity has been postulated as a neurochemical marker in Huntington's disease (111). Moreover, elevation in whole brain Gln is also a hallmark in patients with chronic hepatic encephal-

opathy (HE) (112). Ammonia neurotoxicity is a major factor in the pathogenesis of HE. Recent thoughts in this field point to Gln-derived ammonia within mitochondria as the main cause of astrocyte dysfunction leading to brain edema associated with hepatic failure (60). Astrocyte swelling induced by ammonia exposure is a key feature of acute HE (113). Ammonia has been shown to generate free radicals *in vivo* and in cultured astrocytes (114). One consequence of oxidative stress is the induction of the mitochondrial permeability transition (MPT), leading to a collapse of the inner mitochondrial membrane, mitochondrial dysfunction and enhanced free radical production. In addition, ammonia has been shown to induce the MPT in cultured astrocytes (115).

Glutamine has been implicated in the mechanism of ammonia neurotoxicity. Administration of methionine sulfoximine (MSO), an inhibitor of GS, prevented cerebral Gln accumulation and the increase of brain water (116). Furthermore, astrocyte swelling, ammonia-induced MPT and free radical production can be completely blocked by MSO [(115, 117) and references therein], which suggest that Gln can mediate ammonia toxicity to the brain. Similar and convergent results were also obtained by inhibiting the mitochondrial transport of Gln or by blocking the mitochondrial Gln catabolism with the GA inhibitor 6-diazo-5-oxo-norleucine (60). A 'Trojan horse' hypothesis has been formulated to explain why Gln is capable of reproducing many of the toxic effects of ammonia on astrocytes (112). Mitochondrial Gln behaves as a 'Trojan horse' and ends up causing neurotoxicity through deamidation by GA, thereby generating very high levels of ammonia in mitochondria. The hypothesis needs the existence of astrocytic GA activity *in vivo*, a strongly questioned issue and matter of debate and controversy for many years, as already discussed. Nevertheless, the important physiological and therapeutic implications demand further investigations to clarify this issue.

Expert opinion

Based on novel and growing evidence, as summarized above, the roles of GA in cerebral function are multiple and extensive. In the past years, significant progress has been made in this field stressing the relevance of GA in brain and its prospect as a therapeutic target. Nowadays, the pattern of GA expression has been shown to be considerably more complex: the old concept of only one type of GA expressed in mammalian brain (KGA) has to be discarded in the light of overwhelming evidence demonstrating that at least two GA isoenzymes are expressed. The concept of an exclusively mitochondrial location for GA was also demonstrated to be incorrect as new subcellular locations (e.g., nucleus, cytosol) have been described for these proteins. Furthermore, the concept of GA as exclusive neuron-specific enzymes has been challenged by recent findings reporting expression of GA in astrocytes. To reach their final destinations in brain cells, GA can interact with newly discovered scaffold proteins, such as GIP, SNT and caytaxin. Such arrangements could provide the molecular basis for selective and regulated targeting to concrete cellular locations. The interactome of brain GA is beginning to be uncovered. These protein interacting partners fulfill their function not only by helping GA to traffic in brain, but also by regulation of GA activity and control of Glu synthesis. Nevertheless, the physiological relevance of the interaction between GA and protein binding partners, as well as their implications in neurological disorders, still remain to be elucidated.

These unanticipated findings open a new avenue of research on how GA could affect the homeostasis of Glu/Gln in tripartite synapsis. Emerging roles for GA could include transcriptional control, neuronal growth and differentiation and cerebrovascular regulation, in addition to their classical role in glutamatergic transmission. GA can now be envisioned as multifunctional 'moonlighting' proteins that can be reused for different tasks. The coexpression of at least two GAs in mammalian brain is so far unexplained. Glu synthesis must be a process finely tuned because of its harmful potential; otherwise, alterations of Glu levels could give rise to pathological situations and neurological disorders. This, in turn, implies that sophisticated mechanisms of control and regulation of biosynthetic enzymes responsible of Glu generation should be operative. The existence of several GA isoforms in brain could represent the biochemical basis to achieve this fine tuning under different physiological situations.

The implementation of state-of-the-art proteomics methodologies will be invaluable to unravel the network of interacting protein partners for GA in brain. The combination of sophisticated NMR and MS techniques (with enhanced resolution power to perform *in vivo* measurements), metabolic modeling and classical biochemical, immunological and enzymology studies will bring significant advances into the determination of *in vivo* rates of metabolism and neurotransmitter trafficking. Crystallization of isolated mammalian GA and solving their tridimensional structure will give clues to the rational design of specific inhibitors that might be relevant as therapeutic drugs. Most of the currently employed GA inhibitors are somewhat unspecific and usually inhibit other amidotransferase enzymes, avoiding to withdrawn clear-cut conclusions from kinetic and metabolic studies.

Outlook

There are several key issues about GA function in brain that remain unresolved. However, considering the current pace of research we can expect essential progress towards elucidation of the GA role in Glu homeostasis and glutamatergic transmission. Given our new knowledge about the presence of GA in astrocytes, it is expected that in the next years we can determine the function of GA in glial cells in physiological conditions and whether astrocytic GA contribute to the changes in brain function under pathological conditions. Further studies will undoubtedly reveal insights into the nuclear role of L-type GA; particularly, the role of GA and/or Gln in the regulation of gene expression. We expect that the function of KGA in neuronal growth and differentiation,

after intracellular trafficking by interaction with caytaxin, can be fully addressed in the future as well. The design of *in vivo* experiments with isotopic labeling will help to discriminate between variants of the Glu/Gln cycle, identifying the cycle that is operative in synaptic function under normal physiological conditions.

The selective KO of specific genes involved in the glutamatergic transmission will yield useful cellular and animal models to identify the function of each gene product. Moreover, these models will offer essential information regarding the involvement of gene products in pathological states. Future analysis of inherited defects of glutaminolytic enzymes, molecular characterization of mitochondrial glutamine carriers and structure-function studies on GA will provide further insights into the specific roles of GA in mammalian brain. The knowledge derived from such studies will uncover the physiological relevance of brain GA, giving further rationale for its consideration as a potential therapeutic target.

Highlights

Key conclusions:

- At least two different GA isozymes are expressed in the brain of mammals: KGA and L-type GA (GAB/LGA). There are marked kinetic, regulatory and molecular differences between both GA isozymes.
- GAB is a novel L-type GA isoform different from the classical LGA liver isoform.
- There is clear protein segregation in neurones: KGA is confined to mitochondria whereas GAB/LGA appears mostly in cell nuclei.
- L-type GA protein is present in astrocytes.
- Scaffold proteins interact with brain GA through a high-affinity binding.

Main questions that remain unanswered:

- The source of neurotransmitter Glu. What is the relative contribution of each GA isoform to the transmitter pool of Glu? What fraction of Glu released from neurones is taken up into glia and what fraction of this is then cycled back to neurones as Gln?
- Characterization of new functions of L-type GA in neurones and of KGA in neurites. Is L-type GA active in astrocytes? Does a cytosolic pool of GA exist?
- Isolation and molecular characterization of the mitochondrial Gln carriers.
- The role of scaffold proteins in GA selective targeting and regulation of activity, as well as their involvements in neurological disorders.
- Which factors regulate GA activity *in vivo*, and hence Glu supply, during neuronal stimulation?

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