Neurochem Res (2015) 40:2544–2556
DOI 10.1007/s11064-015-1667-1

ORIGINAL PAPER

Critical Evaluation of the Changes in Glutamine Synthetase Activity in Models of Cerebral Stroke

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Received: 13 March 2015 / Revised: 14 May 2015 / Accepted: 20 May 2015 / Published online: 2 August 2015

Abstract The following article addresses some seemingly paradoxical observations concerning cerebral glutamine synthetase in ischemia–reperfusion injury. In the brain, this enzyme is predominantly found in astrocytes and catalyzes part of the glutamine-glutamate cycle. Glutamine synthetase is also thought to be especially sensitive to inactivation by the oxygen- and nitrogen-centered radicals generated during strokes. Despite this apparent sensitivity, glutamine synthetase specific activity is elevated in the affected tissues during reperfusion. Given the central role of the glutamine-glutamate cycle in the brain, we sought to resolve these conflicting observations with the view of providing an alternative perspective for therapeutic intervention in stroke.

Keywords Brain · Glutamine synthetase · Ischemia–reperfusion · Oxidative stress · Stroke

Abbreviations

EPR	Electron paramagnetic resonance
GABA	γ-Aminobutyrate
MSO	L-Methionine-*S*,*R*-sulfoximine

Special Issue: In Honor of Dr. Gerald Dienel.

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Introduction

We are honored to contribute an article to the special volume dedicated to the retirement of Gerald Dienel. One of us (AJLC) has known Gerry for over 40 years as a friend, mentor and scientific colleague. Gerry is an outstanding neurochemist and has been a source of inspiration to many in the field of neurochemistry, particularly those interested in brain energy metabolism, the biology of astrocytes and trafficking of metabolites between astrocytes and neurons.

Structure and Enzymology of the Mammalian Glutamine Synthetase

Human glutamine synthetase (glutamate ammonia ligase) is encoded by a single gene on chromosome 1 known as *GLUL* [1]. An additional four *GLUL*-like genes are present in the human genome [1] but it is not known whether these genes are transcribed or translated. The expression of glutamine synthetase in the central nervous system was thought to be restricted to astrocytes [2]. Bernstein et al. [3], however, identified glutamine synthetase in white and grey matter astrocytes, oligodendrocytes, ependymal cells, and some neurons of human brain. The genes responsible for the glutamine synthetase in some of these cells may differ from *GLUL*, given that the epigenetic processes underlying cellular differentiation may favor the expression of different genes. To date, two forms of glutamine synthetase have been isolated from the human brain that differed in size. One protein has a molecular mass of 44 kDa and is the likely product of the *GLUL* gene [4]. The other protein has a molecular mass of 54 kDa and is named glutamine synthetase-like protein [5]. Canine brain also

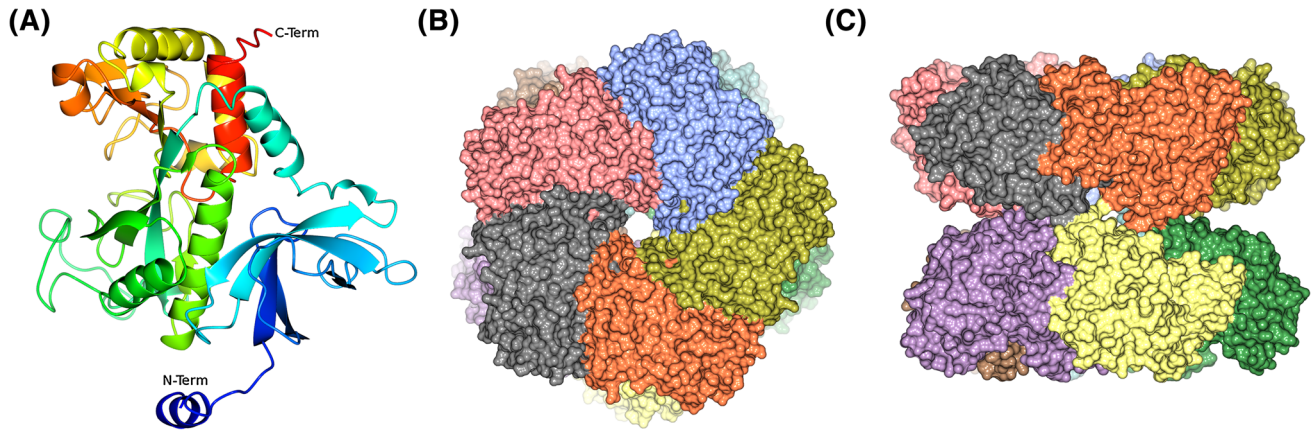


Fig. 1 Structure of mammalian glutamine synthetase. **a** Depicts the ribbon diagram of the canine glutamine synthetase colored from *blue* to *red* beginning from the N-terminus. The *blue-cyan* unit corresponds to the N-terminal β-grasp domain, while the remainder represents the C-terminal catalytic domain. The overall structure of the active enzyme (a decamer of two stacked pentamers) is shown in two orientations and as surface plots in (**b**) and (**c**). The *top-down view* (**b**) reveals that the decamer is formed via a crystallographic twofold axis that places the second pentamer ring behind the first. In this panel, the individual monomers are distinguished by color. Active sites are formed between the subunits and oriented such that the active sites in the second pentamer are placed between those in the first and face in the opposite direction. The *side view* (**c**) illustrates the pentamer–pentamer contacts, which are weaker than those holding the subunits in the pentamer together. This figure is based on the studies of Krajewski et al. [8] and was created using CCP4mg [105]

contains two forms of glutamine synthetase, although in this case, the larger form of the enzyme represents a splice variant [6]. The canine glutamine synthetase gene contains an additional exon in the first intron of the *GLUL* gene (relative to the glutamine synthetase genes in other mammals [7]) that is variably excised [6]. Despite the possible variations, most discussion of mammalian glutamine synthetase—including the current discussion—focuses on the product of the *GLUL* gene.

The human *GLUL* gene encodes a 373 amino acid protein with three domains as shown in Fig. 1a: an N-terminal meander (residues 3–24), a β-grasp domain (residues 25–112), and a catalytic domain (residues 113–373) [8]. Five such monomers are conjoined in a ring such that the β-grasp domain of one subunit aligns with the catalytic domain of the adjacent subunit to form a funnel-shaped cavity (Fig. 1b). The ring structure thus optimizes the orientation of the five active sites toward its substrates (Fig. 1b). Two such pentameric rings are stacked on top of each other, such that the orientation of each ring is opposite the other to comprise the native decameric glutamine synthetase (Fig. 1b, c). The individual rings are stabilized by contacts between adjacent β-grasp and catalytic domains as well as contacts between the N-terminal meanders, which project into the core of the pentamer (Fig. 1b, c). Five loops comprising residues 150–156 within the catalytic domain of each subunit interact with the corresponding loops in the neighboring ring to stabilize the decamer. All of the known eukaryote glutamine synthetases exhibit a similar organization of stacked and oppositely-oriented concentric rings, although the number of subunits varies among species [8–11]. The number of stacked rings may also vary [8, 11–14], but the physiological relevance of the stacking remains unknown.

Glutamine synthetase catalyzes the ATP-dependent condensation of ammonia[1] and glutamate to glutamine:

$$L-Glutamate+\mathrm{NH_4^+}+\mathrm{ATP}\leftrightarrows L-Glutamine+\mathrm{ADP}+\mathrm{P_i} \quad (1)$$

This reaction begins with the binding of ATP to a hydrophobic pocket bounded by Trp130, Arg262, Tyr336, and Pro208 at the top of the catalytic funnel in the structure of the human enzyme (Fig. 2) [8]. Figures 1 and 2 were obtained with glutamine synthetase complexed to phosphorylated L-methionine-*S*,*R*-sulfoximine (MSO) [15], which facilitated the crystallization of the enzyme [8]. MSO is an inhibitor of glutamine synthetase that competes with glutamate for binding at the active site and upon phosphorylation irreversibly inactivates the enzyme [15]. ATP binds by alignment of the adenosine moiety with the side chains of Trp130 and Arg262 to form an aromatic–arginine planar stacking configuration. Hydrogen bonding between the hydroxyl of Ser257 and N1 and N6 of the adenine moiety further stabilizes the nucleotide within the binding pocket. These events serve to project the triphosphate chain into the reaction funnel such that the terminal

[1] Under normal intracellular physiological conditions (pH 7.2–7.4) ammonia exists predominantly (~99 %) as the conjugate acid, ammonium (NH_4^+). Even so, the term ammonia is used throughout the text to indicate the sum of NH_3 plus NH_4^+ for the sake of convenience.

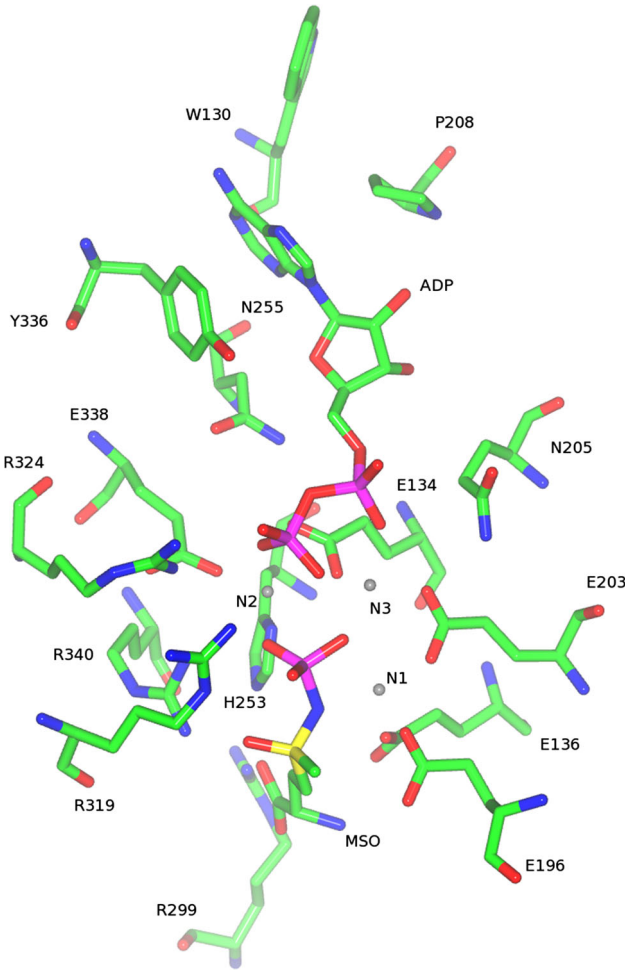


Fig. 2 Active site of the human glutamine synthetase. Shown is the active site of the human glutamine synthetase with bound ADP, MSO, and manganese ions (n1, n2, and n3). Conserved hydrophobic residues interacting with the adenine ring of ADP are shown with ball-and-stick representations. This figure is based on the studies of Krajewski et al. [8] and was created using CCP4mg [105]

phosphate of ATP juxtaposes the terminal carboxyl group of glutamate.

The binding of ATP is accompanied by two simultaneous events: the importation of three manganous (Mn^{2+}) ions and the *cis*-isomerization of Pro208 in the binding pocket. Catalysis requires the presence of three manganous ions within the active site [8]. Prior to binding ATP the mammalian glutamine synthetase contains one magnesium ion at the n1 position (Fig. 2). Thus, the manganous ions probably enter the active site as ATP chelates. The stability constants of ATP-Mg and ATP-Mn are 4.00 and 3.98, respectively [16] and consequently both types of chelates exist in cells. Taken together, these observations suggest that ATP-Mn binds to glutamine synthetase and that the three associated manganous ions complex with the enzyme at the n1, n2 and n3 sites by interacting with Glu196, Glu136, Glu338 and Glu308 (Fig. 2). Complexing the manganous ions in this manner serves to position the terminal phosphate group to undergo a nucleophilic attach by the terminal carboxyl group of glutamate. The alternative possibility that the complexing of the nucleotide-bound Mn^{2+} by glutamine synthetase lessens the charge on the phosphate groups thereby priming the terminal phosphate for a nucleophilic attack by the terminal carboxyl group of glutamate is negated by the studies of Admiraal and Herschlag [17].

As discussed in our review of the activation of transglutaminase 2 [18], *cis*-proline isomerization can produce profound conformational changes in proteins. *Cis*-isomerization of Pro208 (i.e., isomerization from *cis* to *trans*) in the catalytic domain of glutamine synthetase, however, does not appear to be responsible for the major motions reported for this enzyme. Pro208 is situated such that the *cis*-isomerization could theoretically act to restrict the departure of ATP or ADP from the catalytic domain. If this were the case, then the isomerization of proline from the *cis* to *trans* configuration might regulate the ejection of ADP from the active site. This hypothesis might also account for the faster rates of reactions noted for the prokaryote forms of this enzyme relative to the eukaryotic forms. The order with which ATP and ammonia are released from glutamine synthetase remains a topic of debate for the eukaryotic forms of this enzyme. Pro208 is conserved in eukaryotes and *cis*-isomerizations are sufficiently slow to be the rate-limiting step in the overall reaction sequence [19]. In contrast, bacterial glutamine synthetases have a phenylalanine in place of Pro208 and therefore the release of ADP from the active site would not be limited by a *cis–trans* isomerization reaction.

The binding of ATP to mammalian glutamine synthetase causes significant conformational changes involving residues 311–337 of the catalytic domain and residues 63–77 of the β-grasp domain of the adjacent subunit. These movements serve to create a binding pocket for ammonia between Glu305 in the active site and Asp 63 of β-grasp domain [20] and to close the active site. Closure of the active site constrains glutamate to its binding site in an orientation that juxtaposes the γ carboxyl of this amino acid with the terminal phosphate of the bound ATP and enables phosphate transfer between these groups. The resulting acylphosphate intermediate then undergoes nucleophilic attack by the bound ammonia to generate glutamine.

Nitration of Glutamine Synthetase

Tyrosyl nitration is one of the major biological effects resulting from the formation of nitric oxide ($^{\cdot}NO$) and is produced in sufficient amounts to act as an index for the production of this gas. The major pathway for the nitration

of tyrosyl residues by $^{\cdot}NO$ begins with a reaction with superoxide ($O_2^{\cdot-}$) to generate peroxynitrite ($ONOO^-$):

$$O_2^{\cdot-} + {}^{\cdot}NO \rightarrow ONOO^- \quad (2)$$

This remarkable chemistry occurs despite the dismutation of $O_2^{\cdot-}$ by superoxide dismutase at a bimolecular rate of 1–2 $\times$ 10^9 M^{-1} s^{-1} [21]:

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 \quad (3)$$

The reaction of $^{\cdot}NO$ and $O_2^{\cdot-}$, however, is 10 times faster with a second-order rate constant of $\sim 10^{10}$ M^{-1} s^{-1} [22] and ensures the generation of appreciable amounts of peroxynitrite under physiological conditions. Peroxynitrite then reacts with carbon dioxide to produce a nitrosoperoxocarboxylate adduct ($ONOOCO_2^-$) that rapidly decomposes to nitric dioxide ($^{\cdot}NO_2$) and carbonate radicals ($CO_3^{\cdot-}$) [23–25]:

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \rightarrow {}^{\cdot}NO_2 + CO_3^{\cdot-} \quad (4)$$

These radicals act together to nitrate tyrosyl residues [26]. This modification proceeds with the conversion of tyrosyl residues to a radical by $CO_3^{\cdot-}$ followed by combination with $^{\cdot}NO_2$ to form 3-nitrotyrosine:

$$TyrH + CO_3^{\cdot-} \rightarrow Tyr^{\cdot} + HCO_3^- \quad (5)$$

$$Tyr^{\cdot} + {}^{\cdot}NO_2 \rightarrow Tyr - NO_2 \quad (6)$$

Although there are other sources of $^{\cdot}NO_2$ and $CO_3^{\cdot-}$, the abundance of CO_2 in cells and the rate constant of 5.8 $\times$ 10^4 M^{-1} s^{-1} for reaction 5 ensures that this reaction is the major source of these radicals.

Another route for the formation of $^{\cdot}NO_2$ deserves mention in light of the fact that manganese is bound to the active site of glutamine synthetase. Peroxynitrite reacts with transition metals in proteins such as the manganese in Mn-superoxide dismutase [27–29]. The metals in these sites act as Lewis acids and form Lewis adducts upon reaction with peroxynitrite [22]. Such adducts typically undergo homolysis to yield $^{\cdot}NO_2$ and the corresponding oxyradical-metal complex, which then rearranges to an oxo-metal complex [22, 30]:

$$\begin{aligned} ONOO^- + Me^nX &\rightarrow ONOO - Me^nX \\ &\rightarrow {}^{\cdot}NO_2 + {}^{\cdot}O - Me^nX \\ &\rightarrow {}^{\cdot}NO_2 + O = Me^{n+1}X \end{aligned} \quad (7)$$

Glutamine synthetase is regulated by $^{\cdot}NO$ in a site-specific and reversible manner. The generation of $^{\cdot}NO$ by either pharmacological means [31–35] or the applications of cytokines [36, 37], results in the inhibition of glutamine synthetase activity in vivo. This inhibition is prevented by blockade of cellular nitric oxide synthase activity [31–33, 35–37], except in the case of benzodiazepine-treated astrocytes [34]. Interestingly, the addition of nitric oxide synthetase inhibitors alone increases glutamine synthetase activity and suggests the activity of this enzyme is controlled tonically by $^{\cdot}NO$ [32, 35]. Benzodiazepines also inhibit nitric oxide synthases, presumably by competing with L-arginine for binding to these enzymes [38]. Thus, nitric oxide synthase inhibition may account for the increased glutamine synthetase activity in astrocytes treated with benzodiazepines and classical nitric oxide synthase inhibitors [34]; the glutamine synthetase in these cells appears to be either insensitive to further inhibition of nitric oxide synthases or act synergistically with the additional inhibitors. $^{\cdot}NO$-generating compounds also inhibit purified glutamine synthetase [36] consistent with the above in vivo observations.

$^{\cdot}NO$ inhibits glutamine synthetase by promoting the nitration of Tyr336 [36, 37] as described by reactions 2–7. Glutamine synthetase is significantly nitrated by 5 μM peroxynitrite, a concentration likely to be physiologically relevant [39]. The same amount of peroxynitrite does not nitrate glyceraldehyde-3-phosphate dehydrogenase or inhibit the activity of this enzyme [40]. Inactivation of glyceraldehyde-3-phosphate dehydrogenase only occurs at peroxynitrite concentrations of 100 μM and higher [40]. Glyceraldehyde-3-phosphate dehydrogenase is a sensitive and major target of cellular oxidants, including those derived from $^{\cdot}NO$ [41]. The greater susceptibility of glutamine synthetase to the actions of peroxynitrite, as compared to that of glyceraldehyde-3-phosphate dehydrogenase, indicates that the former enzyme is exquisitely sensitive to tyrosyl nitration.

In vitro studies indicate that the nitration of Tyr336 residue of glutamine synthestase is reversible. Proteinaceous extracts from various tissues contain a putative 'denitrase' activity capable of removing nitrite from the nitrotyrosyl residues of a variety of proteins [42–47] including the nitrated Tyr336 on glutamine synthetase [39]. Denitrase activity has been attributed to the E3 component of the α-ketoglutarate dehydrogenase complex [48], glutathione *S*-transferase, and superoxide dismutase 1 [47]. This activity, however, has not been purified to homogeneity from any of the 'denitrating' extracts and thus the identity of the actual denitrase(s) remains unknown. Nonetheless, the existence of denitrase(s) coupled with the demonstration of catalyzed removal of the nitro group from nitrated Tyr336 on glutamine synthetase [39] lends support to the idea that this enzyme is regulated under physiological conditions by $^{\cdot}NO$.

Görg et al. [39] hypothesized that the nitration of Tyr336 of glutamine synthetase[2] inhibits this enzyme by preventing the binding of purines. This hypothesis is supported by the structural analysis of glutamine synthetase.

[2] Görg et al. [39] refers to this residue as Tyr335.

Tyr336 together with Trp130, Arg262, and Pro208 form a hydrophobic pocket that accommodates the adenine ring of ATP and ADP [8]. As shown in Fig. 2, Tyr336 interacts with the adenosine ring by way of aromatic planar stacking. Nitration of the phenolic ring of Tyr336 would prevent the formation of such a stack and thus interfere with the binding of ATP or ADP.

The above observations suggest an interesting possibility for the loss of glutamine synthetase activity in neurodegenerative diseases: namely, the inactivation of denitrases. Such inactivation would ensure that Tyr336 remains nitrated and glutamine synthetase inhibited. Loss of denitrase activity is thought to account, in part, for the accumulation of nitrated proteins in the lungs of patients with chronic obstructive pulmonary disease [46]. Similarly, Shi et al. [48] argued that if the α-ketoglutarate dehydrogenase E3 subunit is a significant denitrase, then the loss of α-ketoglutarate dehydrogenase complex activity might explain the surfeit of nitrated proteins in the Alzheimer Disease brain [49–51]. In support of this argument, α-ketoglutarate dehydrogenase complex activity is significantly diminished in the brains of AD patients [52]. The α-ketoglutarate dehydrogenase complex, however, is restricted to mitochondria and the loss of the E3 denitrase activity is unlikely to influence the nitration of cytosolic enzymes such as glutamine synthetase. Nonetheless, the loss of cellular denitrase activity may lead to sustained nitration of glutamine synthetase in the brain. This is a distinct possibility given the extent to which cytoplasmic proteins are nitrated in the astrocytes of Alzheimer Disease brains [51].

Exposure of purified glutamine synthetase to supraphysiological amounts of peroxynitrite results in *S*-nitrosylation and carbonylation of the enzyme and a profound loss of activity [39]. These oxidative changes also promote the degradation of glutamine synthetase by the 20S proteasome [39], consistent with earlier reports of the enhanced proteolysis of this enzyme following its oxidation [53, 54]. In contrast, nitration of Tyr336 alone did not promote the proteolytic degradation of glutamine synthetase [39].

Site-Specific Inactivation of Glutamine Synthetase by Hydroxyl Radicals

Mammalian glutamine synthetase is remarkably resistant to oxidation by hydrogen peroxide [55, 56]. The combination of reduced iron and hydrogen peroxide, however, causes a profound loss of activity [55, 56]. Similar observations were made with cultured astrocytes [56]. The glutamine synthetase activity in these cells was not affected by incubation in the presence of up to 10^{-4} M hydrogen peroxide. Moreover, the inactivation of the cellular enzyme by 10^{-3} M hydrogen peroxide required an intracellular source of ferrous iron. Studies with the *E.coli* glutamine synthetase indicate that this enzyme is inactivated by the combination of iron and hydrogen peroxide in a site-specific manner by the generation of hydroxyl radical through Fenton chemistry:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^{-} \quad (8)$$

The reactivity of this particular radical is diffusion limited and therefore rarely reacts in a specific manner. In the case of glutamine synthetase, the specificity is conferred by the binding of ferrous iron to one of the metal binding sites in the active site. The bound and reduced iron can then reduce hydrogen peroxide to hydroxyl ion and radical, with the latter oxidizing the histidinyl and argininyl residues that bind the iron [57–59].

Glutamine Synthetase and Stroke

The role of glutamine synthetase in stroke is the subject of numerous conflicting reports. One of the earliest changes that occur during cerebral ischemia is an increase in the extracellular amounts of glutamate. This was first demonstrated in a landmark study by Benveniste et al. [60] using rats and later confirmed in another remarkable study utilizing human tissue [61]. The human study exploited the temporary ischemia that occurs during the resection of the temporal lobe for intractable epilepsy [61]. In rats, a 10-min period of ischemia produced an eightfold increase in extracellular glutamate [60]. The increase in humans was an astonishing 100-fold change [61]. This magnitude is remarkable even taking into account the fact that the resected tissue originated in brains of epileptics [61]. These increases in glutamate are excitotoxic and are thought to contribute to the damage due to ischemia.

The increases in extracellular glutamate were also accompanied by decreases in the extracellular amounts of glutamine [60, 61]. This is to be expected as the neurons replenish the glutamate released during neurotransmission with the hydrolysis of glutamine as catalyzed by glutaminase [62]. The decrement in extracellular glutamine, however, did not match the increases in extracellular glutamate [60, 61]. One explanation for this difference is an inhibition of glutamine synthetase. This possibility was corroborated, in part, by the research of Kranjc et al. [63] (Table 1). These researchers induced hypoxia by exposing rats to 8 % O_2 for 3 h, which led to an approximate 20 % reduction of glutamine synthetase specific activity in the striatum, cortex, and hippocampus [63] (Table 1), the regions most affected by stroke. Swamy et al. [64] also reported an approximate 30 % loss of cortical glutamine synthetase specific activity following 5 min of anoxia

Table 1 Glutamine synthetase levels in mammalian stroke models

Species	Vessels occluded	Ischemia (min)	Hypoxia (min)	Anoxia (min)	Reperfusion (h)	Striatum	Cortex	Hippocampus	a
Rat	4. Vertebral & carotid arteries	30	-	-	3	Activity EM	Activity	Activity	[72]
					24	Activity EM	Activity	Activity	
	2: Middle cerebral arteries	30	-	-	2	Activity	Activity	Activity	[73]
	1: Middle cerebral artery	180	-	-	3	IMH	IMH		[74]
		120	-	-	72	Activity	Activity	Activity	[75]
		180	-	-	1	Activity	Activity		[76]
		60	-	-	24	Activity WB	Activity WB		[77]
		120	-	-	72	Activity	Activity	Activity	[78]
		120	-	-	70	Activity	Activity	Activity	[79]
	None	-	180	-	0 to 0.25	Activity mRNA	Activity mRNA	Activity mRNA	[63]
					1 to 3	Activity mRNA	Activity mRNA	Activity mRNA	
					6	Activity mRNA	Activity mRNA	Activity mRNA	
					24	Activity mRNA	Activity mRNA	Activity mRNA	
		-	-	5	0		Activity		[64]
					5		Activity		
	Pial vessels	1440			0		Activity mRNA		[80]
Gerbil	2: Middle cerebral arteries	10	-	-	1 to 3		Activity		[81]
					24		Activity		
Piglet	None	-	30	-	1 to 72		IMH	IMH	[82]

Shown are the effects of various stroke models on glutamine synthetase specific activity, amounts, and where measured: mRNA. The amounts of glutamine were determined by specific activity measurements (reported as activity in the table), electron microscopy (EM), Western blotting (WB), and immunohistochemistry (IMH). No change, increases or decreases are indicated by blue, green and red lettering, respectively

[a] References

(Table 1). In both of these studies, the cause for the reductions of specific activity was not ascertained. Free iron chelators mitigate the damage due to ischemia–reperfusion injury, which suggest that this metal is liberated during ischemia [65, 66]. The availability of free iron during ischemia further suggests that the hydroxyl radical is produced during oxygen starvation in the brain and may inactivate glutamine synthetase.

Despite the considerable interest in stroke, little direct data exist for the formation of nitrogen- or oxygen-centered radicals during ischemia or reperfusion. Electron paramagnetic spin (EPR) studies of rats, undergoing either four- [67] or two-vessel [68] occlusions indicated little production of ·NO even after 2 h of ischemia [68]. The lack of measurable ·NO during ischemia is explicable. Oxygen and ·NO compete for binding to the a3 iron of mitochondrial complex IV [69]. This complex normally catalyzes the simultaneous four electron reduction of oxygen to water: the final step of oxidative phosphorylation. The absence of oxygen prevents the reduction of oxygen to water, but not the oxidation of substrates by the tricarboxylic acid cycle. Consequently, the mitochondrial electron transport chain becomes highly charged with reducing equivalents. At the same time, the a3 iron of complex IV primarily binds ·NO. Reperfusion of hypoxic cells by oxygen causes the displacement of the ·NO and the spurious reduction of the incoming oxygen to superoxide by the highly charged electron transport chain. Thus, during reperfusion, both superoxide and ·NO are available in close proximity for the production of peroxynitrite. The relatively small number of proteins that undergo tyrosyl nitration during experimental hypoxia-reoxygenation [70] is probably a function of the chemical environment/reactivity of the tyrosyl residue.

In contrast to the situation with ·NO, EPR studies indicate significant hydroxyl radial production during the ischemia produced in the rat four-vessel occlusion model [71] and this production may account for the decrements of glutamine synthetase activity during cerebral oxygen starvation reported by Krajnc et al. [63] and Swamy et al. [64]. This hypothesis, however, remains to be confirmed.

With few exceptions, glutamine synthetase activity is either restored or increased following ischemia and during

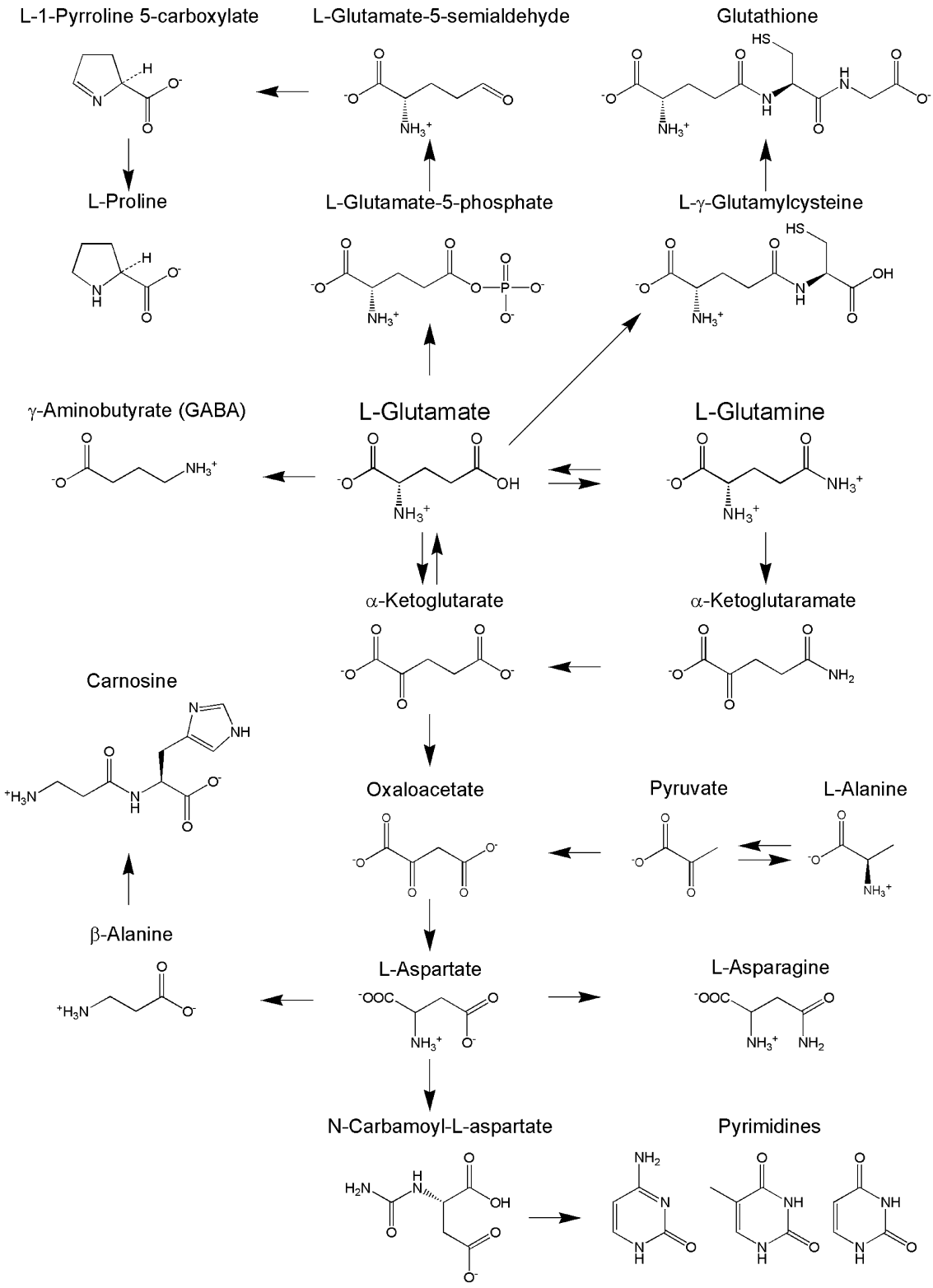
reperfusion [63, 64, 72–82] (Table 1). These changes occurred notwithstanding the production of both the ·NO and hydroxyl radicals as measured directly by EPR [67, 68] and indirectly by other measures [64, 73, 75, 76, 78–81]. The initial rise in ·NO is probably due to the displacement of this molecule from cytochrome *c* oxidase, while the later elevation is due to the activation of ·NO synthase by the increases in cytosolic calcium elicited by glutamate acting via the NMDA and AMPA receptors [83–87]. As noted above, reperfusion also generates superoxide [68]. The application of superoxide dismutase during reperfusion increases the EPR signal due to ·NO [68] by preventing reaction 2. Thus, reperfusion is replete with the reactive species that have been shown to inactivate glutamine synthetase.

After the initial losses of activity following hypoxia, glutamine synthetase activities increase to greater than control levels in the striatum, within an hour of reperfusion, and to control levels in the cortex and hippocampus 3 h after the resumption of blood flow [63] (Table 1). Similar changes were reported in a gerbil model of ischemia–reperfusion except that the restoration of glutamine synthetase activity took greater than 3 h (Table 1, [81]). The difference in recovery time could reflect the differences in species used (rat vs. gerbil) as well as the severity and length of oxygen deprivation (hypoxia for 180 min due to the inhalation of 8 % O_2 versus ischemia due to bicarotid occlusion for 10 min). As noted earlier, five min of anoxia resulted in an approximate 30 % loss of cortical glutamine synthetase specific activity, which was paralleled by similar losses of activity in the cerebellum and brain stem [64]. These activities continued to decline during reperfusion in contrast to most of the studies shown in Table 1. This difference may be attributed to the use of anoxia, which while brief profoundly affected the cerebellum and brain stem: two brain regions not adversely damaged by ischemia–reperfusion or hypoxia-reperfusion (see references in Table 1). The extent of damage may also account for the loss of glutamine synthetase immunoreactivity following ischemia–reperfusion in piglet brains; holes were evident in the histological sections presented in this study and may reflect cerebral vascular differences in response to anoxia between pigs and rats [88]. In general, though, reperfusion in the rodent models of frontal cerebral ischemia is not accompanied by a sustained loss of glutamine synthetase activity.

The above conclusion is consistent with the earliest experimental investigations of the specific activity of glutamine synthetase in ischemia–reperfusion injury in rats and the clinical observations of infants who had died of strokes [89]. Petito et al. [72] observed increased specific activity in the cortex, striatum, and hippocampus at three and 24 h following ischemia (Table 1). The increases in glutamine synthetase specific activity described by Petito et al. [72] were substantiated by other studies of ischemia–reperfusion [74–76, 78, 79] and hypoxia reperfusion in rats [63]. These increases were accompanied by the redistribution of the glutamine synthetase immunoreactivity to the astrocytic processes [72, 74]. Neurons abut astrocytic processes. The mobilization of glutamine synthetase may therefore serve to position the enzyme near the affected nerves.

The mechanisms which cause the increases in glutamine synthetase specific activity in the ischemia–reperfusion paradigms are not known. Elevations in glutamine synthetase mRNA, were observed in the striatum, cortex, and hippocampus following hypoxia and reperfusion (Table 1, [63]). Glucocorticoids promote the transcription of *GLUL* in astrocytes [90–92] and the levels of these hormones

Fig. 3 Major metabolic fate of the L-glutamate and L-glutamine carbon skeletons. This schematic shows some of the major metabolic pathways for the metabolism of L-glutamate and L-glutamine carbon skeletons in mammalian organs with particularly emphasis on the brain. These pathways are likely to be affected by changes in glutamine synthetase activity. L-Glutamate is converted to L-glutamine and GABA by glutamine synthetase and glutamate decarboxylase, respectively. L-Glutamate may also be converted to α-ketoglutarate by the glutamate dehydrogenase reaction or by transamination with, for example, oxaloacetate or pyruvate. The products of the two transamination reactions are L-alanine and L-aspartate, respectively. Finally, L-glutamate is also a precursor for L-proline. L-Glutamine may be converted back to L-glutamate by the glutaminase reaction. In order to prevent futile cycling in the brain these two enzymes are localized preferentially to astrocytes and neurons, respectively, and are components of the glutamine cycle (see the text). L-Glutamine may also undergo transamination to α-ketoglutaramate, a reaction catalyzed by at least two glutamine transaminases. α-Ketoglutaramate is hydrolyzed to α-ketoglutarate by ω-amidase. Note that two carbons from pyruvate can enter the TCA cycle to contribute to the carbon skeletons of oxaloacetate and α-ketoglutarate. α-Ketoglutarate carbon may be converted to oxaloacetate carbon through the TCA cycle. The brain contains considerable pyruvate carboxylase activity, so that pyruvate may also be converted to oxaloacetate anaplerotically. Transamination of oxaloacetate with α-ketoglutarate yields L-aspartate, which is a precursor of pyrimidine carbon and nitrogen. The conversion of L-aspartate to β-alanine is catalyzed by L-aspartate 1-decarboxylase, an enzyme present in bacteria and fruit flies but not in mammalian brain. β-Alanine can, however, be formed in mammalian tissues by the action of β-ureidopropionase on β-ureidopropionate (a metabolite of uracil). β-Alanine and its metabolite carnosine (β-alanine L-histidine) are well represented in brain. Nitrogen (N) transfer reactions involving glutamine amide are not shown. However, these are important in ammonia production catalyzed by, for example, glutamate dehydrogenase, glutaminase and ω-amidase, and in supplying N to the following metabolites: CMP (one N), AMP (two Ns), GMP (three Ns), asparagine, NAD^+, and glucosamine 6-phosphate. Aspartate N is incorporated into pyrimidines (as noted above) and also into purines. The de novo biosynthetic routes for purines and pyrimidines are important in liver, whereas the salvage pathways are important in brain. Note that for simplicity only the major products of the pathways depicted in this figure are shown—cofactors and some additional products are omitted.


L-1-Pyrroline 5-carboxylate
L-Glutamate-5-semialdehyde
Glutathione
L-Proline
L-Glutamate-5-phosphate
L-γ-Glutamylcysteine
γ-Aminobutyrate (GABA)
L-Glutamate
L-Glutamine
α-Ketoglutarate
α-Ketoglutaramate
Carnosine
Oxaloacetate
Pyruvate
L-Alanine
β-Alanine
L-Aspartate
L-Asparagine
N-Carbamoyl-L-aspartate
Pyrimidines

increase in stroke [93, 94]. Increased transcription of *GLUL* due to glucocorticoids occurs over several hours [90, 92] and this observation suggests that the elevations in glutamine synthetase mRNA results from an, as yet unidentified, stabilization of this message. Changes in transcription alone would not account for increased glutamine synthetase activity following oxygen deprivation in the brain. Lin and Dunn [95] estimated the half-life of glutamine synthetase in the rat brain to be 3.8 days and therefore the mRNA increases must be accompanied by comparable changes in translation. It may be that during oxygen starvation and reperfusion, the net synthesis of glutamine synthetase is accelerated as an emergency measure to cope with attendant increase in astrocytic glutamate. The activities of the bacterial and plant glutamine synthetase are increased by adenylation and phosphorylation, respectively [9, 96]. No such post-translational modifications, though, have been reported for the mammalian enzymes [97].

One mechanism for increasing the net protein synthesis is to slow proteolysis. The degradation of glutamine synthetase is, however, stimulated by oxidation of its residues [98, 99], and in particular, nitration of the tyrosyl residues [39]. This observation coupled with the known sensitivity of glutamine synthetase to oxidative inactivation lead to the conclusion that glutamine synthetase is protected from the oxidative stress during stroke. The protection of glutamine synthesis may reflect the superior defenses of the astrocytes, as compared to neurons, against damaging levels of oxygen-and nitrogen-centered reactive species or the activity of denitrases as discussed earlier. Peinado et al. [70] reported increased tyrosyl nitration of cortical glutamine synthetase following hypoxia and reperfusion. The identity of the nitrated tyrosyl residues was not established and therefore may not include Tyr336, which when nitrated inactivates glutamine synthetase [39]. Human glutamine synthetase consists of 373 amino acids of which 15 are tyrosyl residues. It was also not established which of the pools of glutamine synthetase—astrocytic or neuronal—was nitrated.

Cellular measures to preserve astrocytic glutamine synthetase are understandable given the central role played by glutamine in metabolism and cellular repair (Fig. 3). Demonstrating the roles of glutamine and glutamine synthease in stroke, though, remain to be established, as do the mechanisms regulating this activity in the brain. The prevalence of cerebral strokes in aging human populations highlights the need for better strategies for the treatment of this pathology. Cerebral glutamine synthetase specific activity is increased in a significant number of stroke studies (Table 1). Treatments that lowered the clinical correlates of stroke also normalize the increases in glutamine synthetase activity [73, 75, 78, 79]. Thus, it is possible that increased activity contributes to glutamate excitotoxicity by supplying glutamine to neurons for eventual release as glutamate. This idea was the basis for the use of a glutamine synthetase inhibitor—MSO—to treat the G93A superoxide dismutase 1 murine model of amyotrophic lateral sclerosis [100–102]. The administration of MSO decreased both the contents of glutamate and glutamine in the G93A superoxide dismutase 1 mice [102]. As we have discussed in early work, MSO may be safe to use in humans [15, 103, 104]. These observations suggest that the inhibition of glutamine synthetase by compounds such as MSO may provide avenues for the treatment of cerebral strokes.

Conclusions

In the brain, glutamine synthetase is predominantly located in the astrocytes. The enzyme is crucial for maintenance of cerebral nitrogen homeostasis, detoxification of cerebral ammonia, and effective recycling of the neurotransmitters glutamate and GABA between neurons and astrocytes. Many studies on the level of cerebral glutamine synthetase in various animal models of ischemia–reperfusion (and in a few cases anoxia- or hypoxia-reperfusion) have been reported. Despite the variety of models used in these studies and the disparate times at which the analysis of brain specimens were carried out, most studies have reported an increase in the specific activity of cerebral glutamine synthetase at time of sacrifice (Table 1). This finding is remarkable given the fact that glutamine synthetase is well documented to be especially sensitive to inactivation by reactive oxygen- or nitrogen-centered radicals and that the turnover time of glutamine synthetase in the normal brain is longer than the reperfusion times (except perhaps in the study by Lee et al. [82]). The mechanism by which ischemia–reperfusion results in increased levels of active cerebral glutamine synthetase must await further study. Nevertheless, an increase in the specific activity of cerebral glutamine synthetase has clinical implications. An overproduction of glutamine in astrocytes is a contributing factor to the neurotoxicity associated with hyperammonemia [104] and may contribute to the production of excess excitatory glutamate in a mouse model of amyotrophic lateral sclerosis [100–102]. It is interesting that a potent inhibitor of glutamine synthetase, namely MSO, is neuroprotective in animal models of hyperammonemia [103, 104] and in a mouse model of amyotrophic lateral sclerosis [100–102]. We hypothesize that cerebral ischemia–reperfusion injury may result, at least in part, from overproduction of glutamine, which in turn may result in increased levels of excitatory glutamate. Based on this hypothesis, we further suggest that, as noted for animal models of hyperammonemia and amyotrophic lateral

sclerosis, prior treatment with MSO may be beneficial in animal models of stroke.

Acknowledgments Part of the work described in this review was supported by NIH grant DK 16739 (AJLC) and the Theresa Patnode Santmann Foundation (TMJ).

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