

Brain slices from glutaminase-deficient mice metabolize less glutamine: a cellular metabolomic study with carbon 13 NMR

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In the brain, glutaminase is considered to have a key role in the provision of glutamate, a major excitatory neurotransmitter. Brain slices obtained from wild-type (control) and glutaminase-deficient (GLS1 +/-) mice were incubated without glucose and with 5 or 1 mmol/L [3-¹³C]glutamine as substrate. At the end of the incubation, substrate removal and product formation were measured by both enzymatic and carbon 13 nuclear magnetic resonance (¹³C-NMR) techniques. Slices from GLS1 +/- mice consumed less [3-¹³C]glutamine and accumulated less [3-¹³C]glutamate. They also produced less ¹³CO₂ but accumulated amounts of ¹³C-aspartate and ¹³C-gamma-aminobutyric acid (GABA) that were similar to those found with brain slices from control mice. The newly formed glutamine observed in slices from control mice remained unchanged in slices from GLS1 +/- mice. As expected, flux through glutaminase in slices from GLS1 +/- mice was found diminished. Fluxes through all enzymes of the tricarboxylic acid cycle were also reduced in brain slices from GLS1 +/- mice except through malate dehydrogenase with 5 mmol/L [3-¹³C]glutamine. The latter diminutions are consistent with the decreases in the production of ¹³CO₂ also observed in the slices from these mice. It is concluded that the genetic approach used in this study confirms the key role of glutaminase for the provision of glutamate.

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Introduction

Glutamine is considered to be the preferred precursor of glutamate, the major excitatory neurotransmitter (Fonnum, 1984; Kvamme, 1998; Kvamme *et al*, 2000). The conversion of glutamine into glutamate is catalyzed by phosphate-activated glutaminase (PAG). It is generally believed that glutamate is mainly formed in neurons from which it is released into the synaptic space. Glutamate binds to postsynaptic receptors to facilitate the transmission of the nerve influx and then, astrocytes take up glutamate, which is known to be neurotoxic, and convert it into glutamine thanks to glutamine synthetase by binding to ammonia. The resulting glutamine is transferred to

the neurons closing the so-called glutamate-glutamine cycle (Berl and Clarke, 1983; Van den Berg and Garfinkel, 1971).

Phosphate-activated glutaminase is the most important glutamine-metabolizing enzyme for the synthesis of glutamate (Kvamme *et al*, 2000). This enzyme is regulated by a variety of activators (Pi, calcium, certain fatty acyl CoA) and inhibitors (proton, glutamate, ammonia, and SH reagents) (Kvamme *et al*, 2001). Phosphate-activated glutaminase has been shown to be active in neurons and, to a much lesser extent, in astrocytes (Hogstad *et al*, 1988; Kvamme *et al*, 2001). It has been shown that PAG is functionally localized in brain mitochondria (Bak *et al*, 2008; Roberg *et al*, 1995).

There are two genes encoding PAG. The first one (GLS1) codes for the brain/kidney PAG form; the second one (GLS2) encodes the liver isoform (Aledo *et al*, 2000; Chung-Bok *et al*, 1997; Shapiro *et al*, 1991). It has been shown that mice that are knocked out for GLS1 (GLS1^{-/-}) are not viable and die

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shortly after birth (Masson *et al*, 2006). This shows that PAG has a crucial role in brain function. In this study, we took advantage of the availability of glutaminase-deficient mice (GLS1+/-) that have been shown to survive and grow normally despite a decrease of ~50% of their cerebral PAG activity (Gaisler-Salomon *et al*, 2009). In these mice and in the appropriate controls, we studied the cerebral metabolism of glutamine by using our metabolomic approach that previously allowed us to unravel the metabolism of various physiological substrates in renal cells (Chauvin *et al*, 1994, 1997; Dugelay *et al*, 1999; Fouque *et al*, 1996; Vittorelli *et al*, 2005) and in rat brain slices (El Hage *et al*, 2011a,b,c).

Materials and methods

Reagents

Enzymes and coenzymes were supplied by Roche Molecular Biochemicals (Meylan, France). L-[3-¹³C]glutamine (isotopic enrichment of 99%) was obtained from Euriso-Top (St Aubin, France). Other reagents used were from Sigma Chemicals (St Louis, MO, USA).

Animals

All experiments were approved by the Institutional Animal Care and Use Committee of the Lyon 1 University. The wild-type (GLS1+/+) and glutaminase-deficient (GLS1+/-) mice were kindly provided by Dr J Masson (Masson *et al*, 2006). At the time of experiments, they weighed 33.8 ± 0.5 (*n*=8) and 32.9 ± 1.2 (*n*=8) grams, respectively, in the 5 mmol/L [3-¹³C]glutamine series; in the 1 mmol/L [3-¹³C]glutamine series, they weighed 32.5 ± 0.3 (*n*=8) and 31.7 ± 0.9 (*n*=8) grams, respectively. They had free access to a standard diet and to tap water.

Preparation of Brain Slices and Incubation

The mice were killed by cervical dislocation. The skin was removed from the skull, which was opened by introducing one blade of scissors into the foramen magnum and by cutting it along its caudal to rostral axis. Then, the brain was quickly dissected. All the steps of preparation were performed in ice-cold oxygenated Krebs–Henseleit buffer (Krebs and Henseleit, 1932). Then, the brain was rapidly cut into 250 μm-thick transverse slices with a McIlwain Tissue Chopper (The Vibratome Company, O'Fallon, MO, USA).

All slices obtained from each complete brain were incubated with shaking (80 cycles/min) at 37°C in 100 mL Erlenmeyer flasks containing an atmosphere of O₂/CO₂ (19:1) and 8 mL of Krebs–Henseleit buffer. Brain slices were incubated for 60 minutes in the absence of glucose and in the presence of glutamine at a 5 or 1 mmol/L concentration. The 5 mmol/L glutamine concentration was chosen to facilitate the observation of small carbon 13 nuclear magnetic resonance (¹³C-NMR) because ¹³C-NMR spectroscopy is a relatively insensitive technique. The 1 mmol/L glutamine concentration better mimicked the

in-vivo situation in which the circulating blood concentration is around 0.5 mmol/L (Hallemeesch *et al*, 2001). Incubation was stopped by the addition of perchloric acid (2% (v/v) final concentration) to each flask.

Zero-time samples were obtained by adding perchloric acid to the incubation medium before the freshly prepared slices.

After removal of the denaturated protein by centrifugation, the supernatant was neutralized with a mixture of 20% (w/v) KOH and 1% (v/v) H₃PO₄ (8 mol/L) for metabolite determination and NMR spectrometry. The pellet of brain slices was dried for 48 hours at 60°C and then for 1 week at 37°C for determination of their dry weight.

Analytical Methods

The concentrations of metabolites were measured by enzymatic methods whereas the ¹³C enrichment of the individual metabolite carbons was measured by ¹³C-NMR spectrometry.

Glutamine, glutamate, alanine, aspartate, and gamma-aminobutyric acid (GABA) were determined according to the methods of Passonneau and Lowry (1993).

¹³C-Nuclear Magnetic Resonance Techniques

The NMR measurements were performed and the data were recorded as indicated previously (Chauvin *et al*, 1994, 1997) at 125.17 MHz on a Bruker AM-500 WB spectrometer (Bruker Biospin, Wissembourg, France) using a 5-mm broadband probe thermostatically maintained at 8 ± 0.5°C. In brief, magnet homogeneity was adjusted using the deuterium lock signal. To obtain absolute quantitative results, special care was taken in data acquisition. Metabolites were dissolved in D₂O and acquisition parameters were as follows: spectral width, 25,000 Hz; tilt angle, 90°; data size, 32 K; repetition time, 50 seconds; number of scans, 420.

We used a standard (Waltz-16) pulse sequence for inverse-gated proton decoupling (Shaka *et al*, 1983). We did not use nuclear Overhauser enhancement during proton decoupling to avoid the use of corresponding correction factors. A 1-Hz line broadening was applied. [2-¹³C]glycine was used as internal standard. Chemical shifts were expressed as p.p.m. (parts per million) relative to tetramethylsilane. Assignments were made by comparing the chemical shifts obtained with those given in the literature (Howarth and Lilley, 1978). We used a Lorentzian deconvolution (line shape fitting) for spectra analysis included in Bruker Topspin 2.1 (Bruker Biospin, Wissembourg, France).

Calculations and Statistical Analysis

Net substrate utilization and product formation were calculated as the difference between the total flask contents (tissue plus medium) at the start (zero-time flasks) and after the period of incubation. The net metabolic rates, reported as means ± s.e.m., are expressed in μmoles of metabolite removed or produced per gram of brain tissue dry weight per hour.

We used a mathematical model derived from a model previously validated and published for glutamine (Chauvin

et al, 1997; El Hage *et al*, 2011b). The model, which is based on the incorporation of ^{13}C into various metabolites and combined with the distribution of the enzymes in the neuronal and glial cells, allows the calculation of rates of the labeled substrate uptake, and fluxes through glutaminase, glutamine synthetase, glutamate decarboxylase, succinate dehydrogenase, malate dehydrogenase, malic enzyme, aspartate aminotransferase, pyruvate kinase, alanine aminotransferase, pyruvate dehydrogenase, pyruvate carboxylase, and citrate synthase.

The $^{13}\text{CO}_2$ production was calculated as the difference between the removal of the labeled substrate and the sum of the labeled nonvolatile products calculated from the ^{13}C NMR spectra. The results were analyzed by the Student's *t*-test for unpaired data. A *P* value <0.05 was considered to be statistically significant.

Results

Table 1 shows the results obtained when glutamine metabolism was studied by enzymatic methods. The removal of glutamine in brain slices from glutaminase-deficient mice was statistically different from that in brain slices from control animals only when glutamine was used at a near-physiological (1 mmol/L) concentration. The removal of glutamine was not accompanied by the accumulation of glutamate, but rather by the removal of part of the glutamate present at the start of the incubation period; this removal was higher in brain slices from glutaminase-deficient mice than in brain slices from control mice only with 5 mmol/L glutamine as substrate. The small amount of GABA present at the start of incubation was also partly removed at similar rates in brain slices from both glutaminase-deficient and control mice (Table 1). A small amount of alanine and a large amount of aspartate were found to accumulate in brain slices from both glutaminase-deficient and control mice; with 1 mmol/L glutamine as substrate,

less alanine accumulated in brain slices from glutaminase-deficient than from control animals.

Figure 1 shows representative ^{13}C -NMR spectra obtained after incubation of brain slices from a control (Figure 1A) and a glutaminase-deficient (Figure 1B) mice when 5 mmol/L [3- ^{13}C]glutamine was the substrate. It can be seen that all significant peaks could be assigned and corresponded to carbons of the products determined by enzymatic methods. From these spectra and those obtained with 1 mmol/L [3- ^{13}C]glutamine as substrate (not shown), it was possible to calculate the amounts of labeled products after correction for the 1.1% ^{13}C natural abundance.

Table 2 reports the results obtained when the metabolism of glutamine was studied by ^{13}C NMR spectrometry. With both 1 and 5 mmol/L [3- ^{13}C]glutamine as substrate, the removal of [3- ^{13}C]glutamine measured was much greater than that measured enzymatically (see Table 1) in brain slices from both control and glutaminase-deficient mice. This indicates that the removal of [3- ^{13}C]glutamine was partially masked by the concomitant appearance of newly formed unlabeled glutamine. This newly formed glutamine could be calculated as the difference between the removal of [3- ^{13}C]glutamine and the removal of glutamine measured enzymatically; the corresponding values are 38.8 ± 6.5 and 28.1 ± 4.0 (not significant) $\mu\text{mol/g}$ dry weight per hour in brain slices from control and glutaminase-deficient mice, respectively, with 5 mmol/L [3- ^{13}C]glutamine; with 1 mmol/L [3- ^{13}C]glutamine, they were 13.2 ± 2.7 and 17.9 ± 2.0 (not significant) $\mu\text{mol/g}$ dry weight per hour. Table 2 also shows that the removal of [3- ^{13}C]glutamine was significantly lower in brain slices from glutaminase-deficient mice than in those from control mice with both 1 and 5 mmol/L [3- ^{13}C]glutamine as substrate.

The lower removal of [3- ^{13}C]glutamine in brain slices from glutaminase-deficient mice was associated with a lower accumulation of [3- ^{13}C]glutamate,

Table 1 Metabolism of 5 and 1 mmol/L [3- ^{13}C]glutamine in brain slices of wild-type (WT) and glutaminase-deficient (GLS1 +/-) mice (Enzymatic data)

Experimental condition	Metabolite removal (–) or production (in $\mu\text{mol/g}$ dry weight per hour)				
	Alanine	Glutamate	Glutamine	Aspartate	GABA
5 mmol/L [3-^{13}C]glutamine					
WT mice (<i>n</i> = 8)	2.0 ± 0.2	-6.5 ± 1.5	-57.1 ± 4.4	29.3 ± 1.9	-2.0 ± 0.2
GLS1 +/- mice (<i>n</i> = 8)	2.0 ± 0.3	-11.6 ± 0.9^a	-47.2 ± 3.6	27.2 ± 2.2	-2.4 ± 0.3
1 mmol/L [3-^{13}C]glutamine					
WT mice (<i>n</i> = 8)	0.9 ± 0.1	-26.3 ± 1.1	-17.2 ± 2.1	22.6 ± 0.8	-3.3 ± 0.2
GLS1 +/- mice (<i>n</i> = 8)	0.2 ± 0.2^a	-25.9 ± 0.5	-4.9 ± 1.5^a	20.8 ± 0.5	-3.1 ± 0.1

GABA, gamma-aminobutyric acid.

Brain slices were incubated for 1 hour with 5 or 1 mmol/L [3- ^{13}C]glutamine. The slice dry weight was 122 ± 3 mg (WT mice) and 119 ± 5 mg (GLS1 +/- mice) with 5 mmol/L [3- ^{13}C]glutamine, and 123 ± 4 mg (WT mice) and 130 ± 2 mg (GLS1 +/- mice) with 1 mmol/L [3- ^{13}C]glutamine. Results (in $\mu\text{mol/g}$ dry weight per hour) are presented as mean values \pm s.e.m. for eight experiments (*n* = 8). Statistical difference was measured by the unpaired Student's *t*-test and *P* < 0.05 was considered statistically significant. The corresponding ^{13}C NMR data are reported in Table 2.

^aDifferent from WT mice.

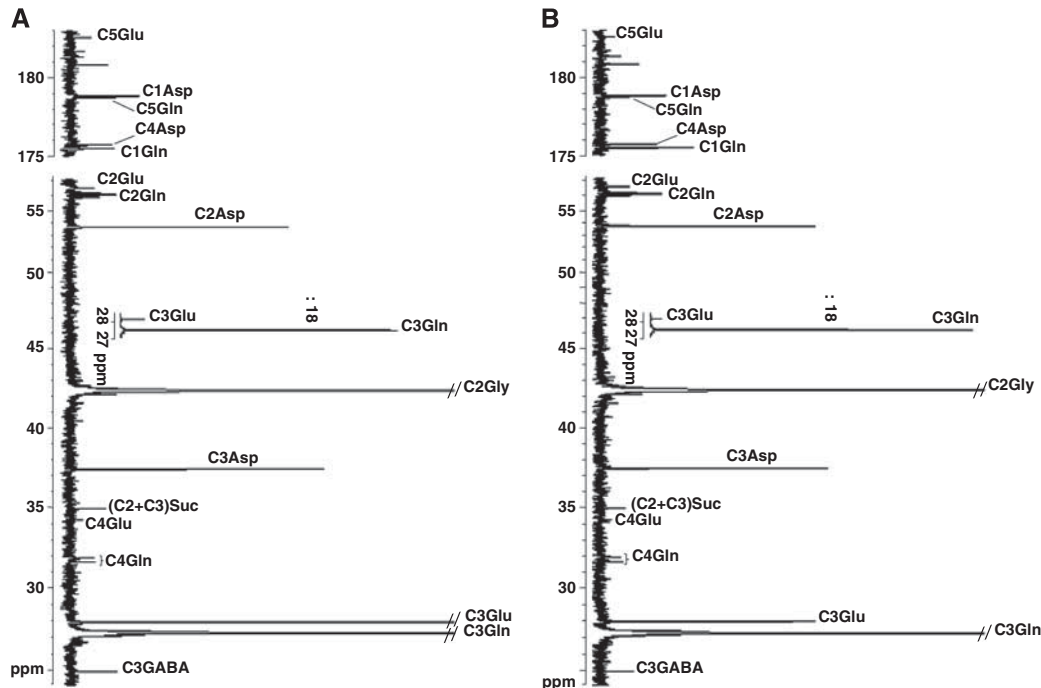


Figure 1 ^{13}C -NMR spectra of neutralized HClO_4 extracts obtained from rat brain slices incubated for 1 hour with 5 mmol/L $[3\text{-}^{13}\text{C}]$ glutamine from a control (**A**) and a glutaminase-deficient mice (**B**). For each peak, the corresponding ^{13}C metabolite is indicated. The chemical shifts, in parts per million (p.p.m.), are referred to tetramethylsilane. The peaks of C3 glutamate and of C3 glutamine divided by 18 are also shown. Glu, glutamate; Asp, aspartate; Gln, glutamine; Gly, glycine; GABA, gamma-aminobutyric acid; Suc, succinate.

the carbon product of the glutaminase reaction. Interestingly, glutamate and glutamine were found to be labeled on carbons other than their carbon 3, which means that resynthesis of both glutamate and glutamine occurred during the metabolism of the removed $[3\text{-}^{13}\text{C}]$ glutamine. As expected, because their labeling was already low with 5 mmol/L $[3\text{-}^{13}\text{C}]$ glutamine, the labeling of the C4 and the C5 of glutamate and that of the C2+C3 of succinate could not be detected with 1 mmol/L $[3\text{-}^{13}\text{C}]$ glutamine as substrate.

Aspartate labeling explained $\sim 20\%$ and 30% of the fate of the carbon 3 of the $[3\text{-}^{13}\text{C}]$ glutamine metabolized with 5 and 1 mmol/L substrate concentration, respectively. It was not modified in brain slices from glutaminase-deficient mice when compared with that in brain slices from control mice. Such labeling occurred mainly and in equal amounts on its inner carbons (the C2 and the C3) and, to a much lesser extent, on its outer carbons (the C1 and the C4). Gamma-aminobutyric acid was found to be slightly labeled on its carbon 3 in brain slices from both control and glutaminase-deficient mice whereas alanine, whose formation requires malic enzyme or phosphoenolpyruvate carboxylase activity, was not found to be labeled.

The production of $^{13}\text{CO}_2$ (calculated as the difference between the $[3\text{-}^{13}\text{C}]$ glutamine removed and the sum of the ^{13}C resonances found in the spectra), which was lower in brain slices from glutaminase-deficient mice than in brain slices from control mice,

was the major fate of the C3 of the $[3\text{-}^{13}\text{C}]$ glutamine metabolized. Indeed, with 5 mmol/L $[3\text{-}^{13}\text{C}]$ glutamine, $^{13}\text{CO}_2$ represented 62.2% and 61.3% of the C3 of the $[3\text{-}^{13}\text{C}]$ glutamine metabolized in brain slices from control and glutaminase-deficient mice, respectively; with 1 mmol/L $[3\text{-}^{13}\text{C}]$ glutamine, the corresponding values were 56.9% and 58.3%, respectively.

Table 3 shows the fluxes through enzymes of glutamine metabolism whose scheme is presented in Figure 2. Only fluxes through glutaminase, succinate dehydrogenase, and citrate synthase (and through other enzymes of the tricarboxylic acid cycle, i.e., aconitase, isocitrate dehydrogenase, α -ketoglutarate dehydrogenase, and malate dehydrogenase only with 1 mmol/L $[3\text{-}^{13}\text{C}]$ glutamine) were statistically diminished in brain slices from glutaminase-deficient mice when compared with brain slices from control mice with both 5 and 1 mmol/L $[3\text{-}^{13}\text{C}]$ glutamine as substrate.

Discussion

Characteristics of Glutamine Metabolism in Brain Slices from Glutaminase-Deficient Mice

Glutamine Utilization: This study establishes that, in brain slices from glutaminase-deficient (GLS1+/-) mice, which contain $\sim 50\%$ of the glutaminase activity of control mice, there was a statistically significant diminution of glutamine utilization as

Table 2 Metabolism of 5 and 1 mmol/L [$3\text{-}^{13}\text{C}$]glutamine in brain slices of wild-type (WT) and glutaminase-deficient (GLS1 +/-) mice (nuclear magnetic resonance (NMR) data)

Experimental condition	Metabolite removal (–) or production (in $\mu\text{mol/g}$ dry weight per hour)												
	Glutamate				Glutamine		Aspartate				Succinate	GABA	$^{13}\text{CO}_2$
	C-2	C-3	C-4	C-5	C-2	C-3	C-1	C-2	C-3	C-4	C-2+C-3	C-3	
5 mmol/L [$3\text{-}^{13}\text{C}$]glutamine													
WT mice ($n=8$)	0.9 ± 0.2	9.4 ± 1.4	0.1 ± 0.05	0.2 ± 0.1	1.6 ± 0.2	–95.8 ± 5.7	2.4 ± 0.2	8.9 ± 0.8	8.7 ± 0.7	1.9 ± 0.2	0.5 ± 0.2	1.6 ± 0.1	59.6 ± 4.5
GLS1 +/- mice ($n=8$)	0.8 ± 0.2	5.2 ± 0.7 ^a	0.1 ± 0.05	0.2 ± 0.1	1.5 ± 0.2	–75.3 ± 4.7 ^a	2.5 ± 0.2	7.7 ± 0.6	7.5 ± 0.6	1.7 ± 0.2	0.6 ± 0.2	1.3 ± 0.1	46.2 ± 3.9 ^a
1 mmol/L [$3\text{-}^{13}\text{C}$]glutamine													
WT mice ($n=8$)	0.4 ± 0.1	2.2 ± 0.3	—	—	0.3 ± 0.1	–30.4 ± 0.8	0.9 ± 0.1	3.9 ± 0.2	3.8 ± 0.2	0.7 ± 0.1	—	0.9 ± 0.1	17.3 ± 0.7
GLS1 +/- mice ($n=8$)	0.2 ± 0.1	0.9 ± 0.1 ^a	—	—	0.2 ± 0.1	–22.8 ± 0.9 ^a	0.8 ± 0.1	3.2 ± 0.2	3.0 ± 0.2	0.6 ± 0.1	—	0.7 ± 0.1	13.2 ± 0.9 ^a

Brain slices were incubated for 1 hour with 5 or 1 mmol/L [$3\text{-}^{13}\text{C}$]glutamine. The slice dry weight was 122 ± 3 mg (WT mice) and 119 ± 5 mg (GLS1 +/- mice) with 5 mmol/L [$3\text{-}^{13}\text{C}$]glutamine, and 123 ± 4 mg (WT mice) and 130 ± 2 mg (GLS1 +/- mice) with 1 mmol/L [$3\text{-}^{13}\text{C}$]glutamine. Results (in $\mu\text{mol/g}$ dry weight/hour) are presented as mean values \pm s.e.m. for eight experiments ($n=8$). Statistical difference was measured by the unpaired Student's t -test and $P < 0.05$ was considered statistically significant. The corresponding enzymatic data are reported in Table 1.

^aDifferent from WT mice.

Table 3 Fluxes through pathways of 5 and 1 mmol/L [$3\text{-}^{13}\text{C}$]glutamine metabolism in brain slices of wild-type (WT) and glutaminase-deficient (GLS1 +/-) mice

Experimental condition	Flux (μmol of C3 units/g dry weight per hour)														
	PAG	GS	Uni Glu \rightarrow α KG	Uni α KG \rightarrow Glu	GAD	SucDH	MDH	ME	AspAT	PK	AlaAT	PDH	PC	CS	
5 mmol/L [$3\text{-}^{13}\text{C}$]glutamine															
WT mice ($n=8$)	101.1 ± 6.2	14.0 ± 2.3	104.4 ± 5.6	15.9 ± 2.3	5.1 ± 0.5	257.6 ± 12.1	154.7 ± 18.7	102.9 ± 11.6	29.3 ± 1.9	5.8 ± 0.2	2.0 ± 0.2	85.6 ± 4.1	36.5 ± 13.0	162.0 ± 8.4	
GLS1 +/- mice ($n=8$)	80.3 ± 5.6 ^a	10.1 ± 1.4	89.4 ± 5.5	11.9 ± 1.6	4.4 ± 0.3	223.4 ± 9.6 ^a	119.0 ± 20.5	104.4 ± 17.3	27.2 ± 2.2	6.0 ± 0.3	2.0 ± 0.3	77.5 ± 3.6	47.3 ± 17.6	139.2 ± 5.8 ^a	
1 mmol/L [$3\text{-}^{13}\text{C}$]glutamine															
WT mice ($n=8$)	40.7 ± 1.0	4.7 ± 1.6	65.3 ± 1.9	7.2 ± 0.6	4.2 ± 0.4	182.2 ± 8.6	110.9 ± 4.7	71.3 ± 3.9	22.6 ± 0.8	5.7 ± 0.2	0.9 ± 0.1	61.6 ± 3.4	28.3 ± 1.2	116.6 ± 6.4	
GLS1 +/- mice ($n=8$)	32.4 ± 1.3 ^a	6.4 ± 1.4	57.0 ± 1.2 ^a	8.4 ± 1.0	3.3 ± 0.7	153.4 ± 5.2 ^a	91.9 ± 2.5 ^a	61.5 ± 2.7	20.8 ± 0.5	5.4 ± 0.1	0.2 ± 0.2 ^a	54.8 ± 2.0	27.2 ± 0.7	98.4 ± 3.6 ^a	

PAG, phosphate-activated glutaminase; GS, glutamine synthetase; Uni Glu \rightarrow α KG, unidirectional flux from glutamate to α -ketoglutarate; Uni α KG \rightarrow Glu, unidirectional flux from α -ketoglutarate to glutamate; GAD, glutamic acid decarboxylase; SucDH, succinate dehydrogenase; MDH, malate dehydrogenase; ME, malic enzyme; AspAT, aspartate aminotransferase; PK, pyruvate kinase; AlaAT, alanine aminotransferase; PDH, pyruvate dehydrogenase; PC, pyruvate carboxylase; CS, citrate synthase.

Brain slices were incubated for 1 hour with 5 or 1 mmol/L [$3\text{-}^{13}\text{C}$]glutamine. Results (in μmol of C3 units/g dry weight per hour) are presented as means \pm s.e.m. for eight experiments. Statistical difference was measured by the unpaired Student's t -test and $P < 0.05$ was considered statistically significant.

^aDifferent from WT mice.

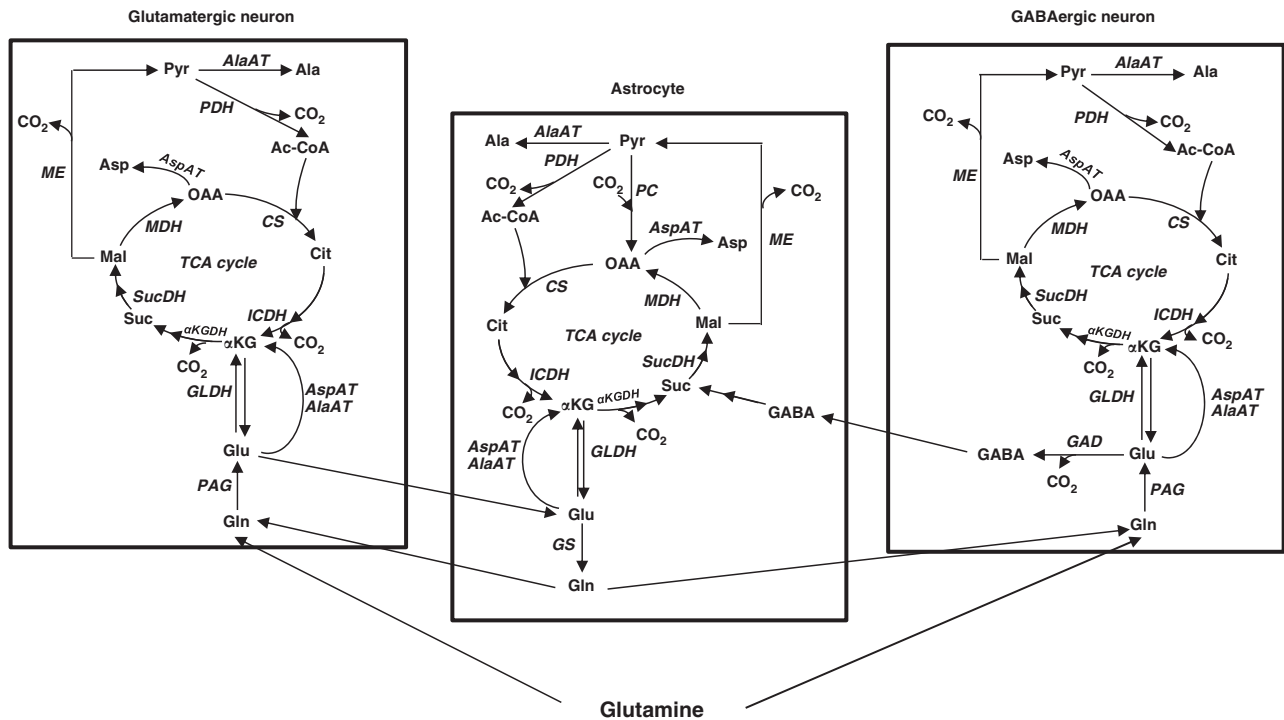


Figure 2 Scheme of glutamine metabolism in the brain. PAG, phosphate-activated glutaminase; GS, glutamine synthetase; GLDH, glutamate dehydrogenase; AlaAT, alanine aminotransferase; AspAT, aspartate aminotransferase; GAD, glutamic acid decarboxylase; GABA-T, gamma aminobutyric acid aminotransferase; α KGDH, α -ketoglutarate dehydrogenase; SucDH, succinate dehydrogenase; MDH, malate dehydrogenase; ME, malic enzyme; PDH, pyruvate dehydrogenase; PC, pyruvate carboxylase; CS, citrate synthase; ICDH, isocitrate dehydrogenase; TCA cycle, tricarboxylic acid cycle; Gln, glutamine; Glu, glutamate; α KG, α -ketoglutarate; Suc, succinate; Mal, malate; OAA, oxaloacetate; Cit, citrate; Asp, aspartate; Pyr, pyruvate; Ala, alanine; Ac-CoA, acetyl coenzyme A; GABA, gamma-aminobutyric acid.

demonstrated by the NMR measurements. However, there was not a good correlation between the reduction of the glutaminase activity and that of glutamine utilization, which was decreased by only 21.4%.

It is noteworthy that, taking a dry weight/protein ratio equal to 1.3 (Antal *et al*, 1984), in both control and glutaminase-deficient mice, flux through glutaminase represented only 0.7% and 1.1% (with 5 mmol/L [$3\text{-}^{13}\text{C}$]glutamine) and 0.3% and 0.4% (with 1 mmol/L [$3\text{-}^{13}\text{C}$]glutamine) of the corresponding maximal glutaminase activity measured in brain homogenates of these mice (Gaisler-Salomon *et al*, 2009). Thus, despite this, our study shows that a reduction of the maximal activity of glutaminase in the brain is accompanied by a reduction of flux through glutaminase.

Glutamate Accumulation: Our study also shows that the decrease in [$3\text{-}^{13}\text{C}$]glutamine utilization was accompanied by a reduction of [$3\text{-}^{13}\text{C}$]glutamate accumulation. This observation is in agreement with the view that glutamine is a major precursor of glutamate in the brain; such a view was put forward by other authors, who used 6-diazo-5-oxo-L-norleucine, a chemical inhibitor of glutaminase (Conti and Minelli, 1994). This finding is also in agreement with a diminution of the cerebral glutamate concentration

observed *ex vivo* in glutaminase-deficient mice (Gaisler-Salomon *et al*, 2009).

Glutamine Synthesis: Interestingly, the decrease in [$3\text{-}^{13}\text{C}$]glutamate accumulation was not associated with a decrease in flux through glutamine synthetase or in glutamine synthesis. This suggests that there was an unchanged availability of glutamate as suggested by an increased utilization of the glutamate present at the beginning of the incubation period in brain slices from glutaminase-deficient mice with 5 mmol/L [$3\text{-}^{13}\text{C}$]glutamine (see Table 1). This illustrates the fact that mouse brain slices need and avidly consume glutamate. This fact is further illustrated by the results obtained with 1 mmol/L [$3\text{-}^{13}\text{C}$]glutamine as substrate; indeed, under this condition, the utilization of the glutamate present at the beginning of incubation was high.

It is also interesting to note that, like rat brain slices metabolizing glutamine (El Hage *et al*, 2011b), mouse brain slices concomitantly degraded and synthesized glutamine (see Tables 1 and 2 and the corresponding comments in Results section).

Gamma-Aminobutyric Acid and Aspartate Production: The fact that glutamate and GABA labeling were observed despite a net utilization of these two amino

acids when measured enzymatically simply means that part of the glutamate and GABA present at the beginning of the incubation was replaced by newly formed ^{13}C -glutamate and ^{13}C -GABA.

It is interesting to mention that, despite a diminution of the formation of glutamate from glutamine in brain slices from glutaminase-deficient mice, there were no changes in the accumulation of ^{13}C -labeled aspartate and GABA. This suggests that the preservation of the production of these two important neurotransmitters represents a priority for the brain. It is of interest to mention here that cultured neurons from GLS1 $^{-/-}$ mice released GABA like neurons from normal mice (Masson *et al*, 2006). Note that the C1 and C4 of aspartate became labeled after the formation of the C1 and C4 of oxaloacetate resulting from a complete tricarboxylic acid cycle turn involving the formation of [1- ^{13}C]succinyl-coenzyme A synthesized from [2- ^{13}C] α -ketoglutarate.

Role of Malic Enzyme and Pyruvate Recycling: It is of interest to mention that malic enzyme was involved in the formation of pyruvate in the present experiments. By contrast, it is unlikely that phosphoenolpyruvate carboxylase, whose activity is very low in the brain (Wiese *et al*, 1991), was involved in such formation. Note that malic enzyme has been described as a carboxylating enzyme (Hassel and Brathe, 2000) but that this has been shown to be incorrect by Waagepetersen *et al* (2001). The existence of pyruvate recycling, judged by the labeling of the C4 and C5 of glutamate, was observed with 5 mmol/L but not with 1 mmol/L [3- ^{13}C]glutamine as substrate.

CO₂ Production: The fact that the $^{13}\text{CO}_2$ produced from [3- ^{13}C]glutamine in brain slices from glutaminase-deficient mice was decreased raises the question of the enzyme(s) whose diminished flux was responsible for such a decrease. Among the enzymes whose operation leads to the release of CO₂ (malic enzyme, pyruvate dehydrogenase, isocitrate dehydrogenase, and α -ketoglutarate dehydrogenase) only isocitrate dehydrogenase and α -ketoglutarate dehydrogenase could be involved because fluxes through citrate synthase (which is equal to flux through isocitrate dehydrogenase) and flux through succinate dehydrogenase (which is virtually equal to flux through α -ketoglutarate dehydrogenase), but not fluxes through malic enzyme and pyruvate dehydrogenase, were reduced in brain slices from glutaminase-deficient mice (see Table 3). It should be mentioned here that the release of $^{13}\text{CO}_2$ from [3- ^{13}C]glutamine could occur only during the second passage through isocitrate dehydrogenase and the third passage through α -ketoglutarate dehydrogenase after the transfer of the carbon number 3 of glutamine to the external carbons of either malate or oxaloacetate and their metabolism through the tricarboxylic acid cycle.

Limitations of the Results Obtained

It should be underlined that the results presented are those obtained after 60 minutes of incubation and that the combination of these results with the model developed and used in the present study provides only mean rates over the incubation period used. In this respect, it is possible and likely that certain product accumulations and labelings reached steady states before others. Further studies would allow to examine this question but it should be underlined that time course experiments are difficult to perform because of the small amount of cerebral tissue available from each mouse and because of the limited number of genetically modified mice available. Another limitation is that, with 1 mmol/L [3- ^{13}C]glutamine as substrate, the ^{13}C resonances obtained and measured are much smaller and therefore much less reliable than those obtained with 5 mmol/L [3- ^{13}C]glutamine as substrate.

It should also be emphasized that, although we cannot assign fluxes through all enzymes to a specific compartment, we believe it is of interest to know these fluxes in the brain considered as a single compartment, which contains functional astrocytes, glutamatergic and GABAergic neurons.

It is also important to underline that our slices were incubated with [3- ^{13}C]glutamine as substrate but in the absence of glucose. Although we did not incubate mouse brain slices with [3- ^{13}C]glutamine plus glucose, we did so with rat brain slices (unpublished results). In the latter experiments, we observed that the addition of 5 mmol/L glucose to the incubation medium containing 5 mmol/L [3- ^{13}C]glutamine did not significantly modify glutamine utilization nor fluxes through glutaminase and glutamine synthetase. As expected, glucose addition greatly diminished aspartate accumulation because the oxaloacetate available was diverted from aspartate to citrate synthesis as a result of an increased formation of acetyl-coA in the presence of glucose. It is therefore very likely that the high rate of aspartate production observed in the present study was due to the absence of glucose in the incubation medium.

Functional Consequences of a Decreased Glutaminase Activity

This study shows that, *in vitro*, a reduction of ~50% of the glutaminase activity is accompanied by a more moderate diminution of glutamine utilization and flux through glutaminase. As expected, this effect was associated with a diminution of the accumulation of glutamate but not of the other major neurotransmitters aspartate and GABA.

It is important to mention here that a relationship has recently been shown between the genetic variation in GLS1 and the brain glutamine-to-glutamate ratio measured *in vivo* (Ongur *et al*, 2011); although the activity of glutaminase was not measured in the latter study, this suggests that the glutaminase

activity should be taken into consideration to understand better not only the pathophysiology of psychiatric disorders in which the glutamatergic system is implicated but also the action of therapeutics aimed at treating these disorders.

Studies by other authors have shown that a complete deficit of glutaminase leads to mice which die shortly after birth (Masson *et al*, 2006) whereas GLS1 +/- mice do not display any change in their growth, development, and behavior but have a hippocampal hypoactivity and are resistant to the effects of pro-psychotic challenges (Gaisler-Salomon *et al*, 2009).

Finally, one cannot rule out the possibility that major metabolic changes observed in the present study in GLS1 +/- mice are amplified in GLS1 -/- mice and contribute to their early death.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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