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Metabolic fate of a high concentration of glutamine and glutamate in rat brain slices: A ^{13}C NMR study

Maha El Hage [a,b,*], Agnès Conjard-Duplany [a], Gabriel Baverel [b], Guy Martin [a]

[a] Institut National de la Santé et de la Recherche Médicale, Unit # 820 (Métabolomique et Maladies Métaboliques), 69372 Lyon Cedex 08, France
[b] Metabolys, Inc., Faculté de Médecine R.T.H. Laennec, 69372 Lyon Cedex 08, France

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ABSTRACT

This study was performed to analyze the metabolic fate of a high concentration (5 mM) of glutamine and glutamate in rat brain slices and the participation of these amino acids in the glutamine–glutamate cycle. For this, brain slices were incubated for 60 min with [3-^{13}C]glutamine or [3-^{13}C]glutamate. Tissue plus medium extracts were analyzed by enzymatic and ^{13}C NMR measurements and fluxes through pathways of glutamine and glutamate metabolism were calculated. We demonstrate that both substrates were utilized and oxidized at high rates by rat brain slices and served as precursors of neurotransmitters, tricarboxylic acid (TCA) cycle intermediates and alanine. In order to determine the participation of glutamine synthetase in the appearance of new glutamine molecules with glutamine as substrate, brain slices were incubated with [3-^{13}C]glutamine in the presence of methionine sulfoximine, a specific inhibitor of glutamine synthetase. Our results indicate that 36.5% of the new glutamine appeared was glutamine synthetase-dependent and 63.5% was formed from endogenous substrates. Flux through glutamic acid decarboxylase was higher with glutamine than with glutamate as substrate whereas fluxes from α-ketoglutarate to glutamate and through glutamine synthetase, malic enzyme, pyruvate dehydrogenase, pyruvate carboxylase and citrate synthase were in the same range with both substrates.

1. Introduction

Glutamate has a central role in brain metabolism. It is the major excitatory neurotransmitter and a precursor of GABA, the main inhibitory neurotransmitter in the central nervous system. The intra-neuronal concentration of glutamate is high (approximately 10 mM) but its extracellular concentration needs to be kept low (<5–10 μM) both to maximize the signal-to-noise ratio upon depolarization and to prevent toxicity caused by excessive excitation of glutamate receptors (Lucas and Newhouse, 1957; Olney, 1969; Yudkoff, 1997). For this, glutamate must be rapidly removed from the extracellular space. Glutamate uptake is the main mechanism responsible for maintenance of low extracellular concentrations of glutamate. This uptake is accomplished by several types of specific transporter proteins, and uptake into astrocytes through EAAT1 and EAAT2 is more important than reuptake into neurons (Danbolt, 2001; Schousboe et al., 2003). In astrocytes, glutamate may have several metabolic fates: direct amidation to glutamine (Yu et al., 1982; Yudkoff et al., 1986), oxidation in the TCA cycle (McKenna et al., 1996; Sonnewald et al., 1993) or incorporation into proteins or glutathione (Dringen, 2000). To compensate for the flow of glutamate from the neuronal to the astrocytic compartment, a flow of glutamine occurs in the opposite direction; this phenomenon is known as the glutamate–glutamine cycle (Berl and Clarke, 1983; Van den Berg and Garfinkel, 1971). Glutamine transport from astrocytes to the extracellular fluid is mediated by a N-system transporter SN1 (Bröer and Brookes, 2001; Chaudhry et al., 1999) and sodium-coupled amino acid transporter (SAT) is responsible for glutamine uptake into neurons (Reimer et al., 2000; Varoqui et al., 2000). In neurons, glutamine is first converted to glutamate via phosphate-activated glutaminase (Kvamme et al., 2000) completing the glutamate–glutamine cycle. Glutamine also functions as a precursor of GABA (Reubi et al., 1978; Westergaard et al., 1995) thereby expanding the glutamate–glutamine cycle to a GABA–glutamate–glutamine cycle (Waagepetersen et al., 2007). Many studies have demonstrated that astrocyte-derived glutamine can also enter the neuronal tricarboxylic acid (TCA) cycle, acting in this way as an anaplerotic substrate by providing oxaloacetate which is a substrate for the entry of acetyl-CoA into the TCA cycle and as a mitochondrial energy substrate producing 2 molecules of CO_2 (Zwingmann and Leibfritz, 2007).

Both glutamate and glutamine are available as substrates in the extracellular fluid and can be used as alternative fuels by the brain

* Corresponding author at: INSERM Unit # 820, Laennec Faculty of Medicine, 7-11 rue G. Paradin, 69372 Lyon Cedex 08, France. Tel.: +33 478778666; fax: +33 478778739.
E-mail address: maha-elhage@hotmail.com (M. El Hage).

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in vivo (Kanamori et al., 2002; Kanamori and Ross, 2006; Lajtha, 1958; Pascual et al., 1998) and in vitro by neurons (Olstad et al., 2007; Peng et al., 2007; Westergaard et al., 1995). Glutamate has also been shown to be utilized by astrocytes (Sonnewald et al., 1993; Yu et al., 1982; Yudkoff et al., 1986). Brain slices, which retain the in vivo structure and heterogeneity of the tissue and maintain the cell–cell interactions occurring in vivo, were also found to metabolize glutamate (De Barry et al., 1983; Haslam and Krebs, 1963; Jones and Bachelard, 1999; Okamoto and Quastel, 1972) and glutamine (De Barry et al., 1983).

Given the renewed interest in the use of the latter cellular brain model, we have re-examined its metabolism of both glutamine and glutamate. For this, we have incubated rat brain slices with [3-^{13}C]glutamate or [3-^{13}C]glutamine as substrate and used our cellular metabolomic approach that combines the measurement of substrate uptake and product formation by both enzymatic and ^{13}C NMR techniques and mathematical models of metabolic pathways (Martin et al., 1997).

2. Experimental procedure

2.1. Reagents

Enzymes, coenzymes were supplied by Roche Molecular biochemicals (Meylan, France). The [3-^{13}C]glutamine and [3-^{13}C]glutamate (isotopic enrichment of 99%) were obtained from Euriso-Top (St. Aubin, France). Other reagents used were from Sigma Chemicals (St. Louis, MO).

2.2. Animals

All experiments were approved by the Institutional Animal Care and Use Committee of the Lyon 1 University. Male Wistar rats weighing 300–320 g were obtained from Charles River (Saint Germain sur l'Arbresle, France) and were fed on a standard diet.

2.3. Preparation of brain slices and incubation

Each rat was anaesthetized with intraperitoneal Nembutal (35 mg/kg body weight). The skin was removed from the skull. The skull was opened by introducing one blade of scissors into the foramen magnum and by cutting the skull along its caudal to rostral axis. Then, the brain was quickly extracted and the two hemispheres were separated. All the steps of preparation were performed in ice-cold oxygenated Krebs–Henseleit buffer. Then, each hemisphere was rapidly cut into 250 μm-thick transverse slices with a McIlwain Tissue Chopper.

All slices obtained from each complete hemisphere were incubated with shaking (80 cycles/min) at 37 °C in 100 ml Erlenmeyer flasks containing an atmosphere of O_2/CO_2 (19:1) and 8 ml of Krebs–Henseleit buffer. Brain slices were incubated for 60 min in the presence of the substrate present at a 5 mM concentration. Incubation was stopped by the addition of perchloric acid (2% (v/v) final concentration) to each flask.

After removal of the denaturated protein by centrifugation, the supernatant was neutralized with a mixture of 20% (w/v) KOH and 1% (v/v) H_3PO_4 (8 M) for metabolite determination and NMR spectroscopy. The pellet of brain slices was dried for 48 h at 60 °C and then for 1 week at 37 °C for determination of their dry weight.

2.4. Analytical methods

2.4.1. Metabolite assays

Glutamine, glutamate, alanine, lactate, pyruvate, aspartate, glucose, α-ketoglutarate and GABA were determined according to the methods of Passonneau and Lowry (1993). Ammonia was determined as described by Kun and Kearney (1974).

2.4.2. ^{13}C NMR techniques

NMR measurements were performed and data were recorded as indicated previously (Chauvin et al., 1994, 1997) at 125.17 MHz on a Bruker AM-500 WB spectrometer using a 5 mm broadband probe thermostatically maintained at 8 ± 0.5 °C. In brief, magnet homogeneity was adjusted using the deuterium lock signal. In order to obtain absolute quantitative results, special care was taken in data acquisition.

Metabolites were dissolved in D_2O and acquisition parameters were as follows: spectral width, 25,000 Hz; tilt angle, 90°; data size, 32K; repetition time, 50 s; 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. 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 (Horthwarth and Lilley, 1978).

2.4.3. Calculations and statistical analysis

Net substrate utilization and product formation were calculated as the difference between the total flasks 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 μmole of metabolite removed or produced per g of brain tissue dry weight per hour.

We used mathematical models derived from models previously validated and published for glutamate (Chauvin et al., 1997; Martin et al., 1997). The models, which are based on the incorporation of ^{13}C into various metabolites and combined with the distribution of the enzymes in the neuronal and glial cells are briefly described in the supplementary data section. They allow the calculation of reaction rates of the labeled substrate uptake, and fluxes through, phosphate activated glutaminase, glutamine synthetase, α-ketoglutarate dehydrogenase, glutamate dehydrogenase, succinate dehydrogenase, malate dehydrogenase, malic enzyme, aspartate aminotransferase, pyruvate kinase, alanine aminotransferase, lactate dehydrogenase, pyruvate dehydrogenase, pyruvate carboxylase and citrate synthase.

The $^{13}CO_2$ production was calculated as the difference between the removal of the labeled substrate and the sum of the labeled non volatile products calculated from the ^{13}C NMR spectra. The results were analyzed by the Student's t test for unpaired data or by analysis of variance (ANOVA) for repeated measurements, followed by the Student–Newman–Keuls test. A p value <0.05 was considered to be statistically significant.

3. Results

3.1. Metabolism of [3-^{13}C]glutamine and [3-^{13}C]glutamate in rat brain slices

Table 1 shows that, at an equimolar concentration (5 mM) and when determined enzymatically, the apparent rate of glutamate utilization was approximately 3 times higher than the apparent rate of glutamine utilization. Note that, in the presence of both glutamine and glutamate, the lactate present at zero-time was also metabolized by the slices (−16.9 ± 2.9 and −19.4 ± 1.2 μmol/g dry weight/h, respectively). With both glutamine and glutamate as substrate, no net GABA production was observed; rather, a small net utilization of the GABA present at zero-time occurred.

When glutamine was the substrate, very little glutamate accumulated (Table 1). By contrast, when glutamate was the substrate, a significant amount of glutamine was synthesized. Irrespective of the availability of glutamate, the amount of alanine synthesized in the presence of glutamine and glutamate was small.

Table 1
Metabolism of [3-^{13}C]glutamine and [3-^{13}C]glutamate in rat brain slices (enzymatic data).

Experimental condition	Metabolite removal (−) or production (μmol/g dry weight/h)					
	Alanine	Glutamate	Glutamine	Aspartate	GABA	NH_4^+
5 mM [3-^{13}C]glutamine (n = 4)	3.7 ± 0.2	2.6 ± 0.7	−36.8 ± 7.0	16.6 ± 2.9	−0.4 ± 1.1	68.1 ± 6.0
5 mM [3-^{13}C]glutamate (n = 5)	4.6 ± 0.2[a]	−107.5 ± 4.5	20.2 ± 2.3	34.5 ± 0.5[a]	−0.1 ± 0.7	11.4 ± 1.4[a]
No added substrate (n = 4)	1.6 ± 0.0	−7.4 ± 0.7	−0.8 ± 1.0	10.7 ± 0.4	−1.6 ± 0.3	33.0 ± 0.4

Brain slices were incubated for 1 h with 5 mM [3-^{13}C]glutamine or [3-^{13}C]glutamate. The slice dry weight was 262 ± 13 mg under the condition with no added substrate, 231 ± 10 mg with [3-^{13}C]glutamine and 243 ± 17 mg with [3-^{13}C]glutamate as substrate. Results (μmol/g dry weight/h) are presented as means ± S.E.M. for 4 or 5 experiments (n = 4 or 5). Statistical difference was measured by the unpaired Student's t test and $p < 0.05$ was considered statistically significant.

[a] Different from results obtained with [3-^{13}C]glutamine.

The corresponding ^{13}C NMR data are reported in Table 2.