Dependence of *in vivo* glutamine synthetase activity on ammonia concentration in rat brain studied by ^1H – ^{15}N heteronuclear multiple-quantum coherence-transfer NMR

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The dependence of the *in vivo* rate of glutamine synthesis on the substrate ammonia concentration was studied in rat brain by ^1H – ^{15}N heteronuclear multiple-quantum coherence-transfer NMR in combination with biochemical techniques. *In vivo* rates were measured at various steady-state blood and brain ammonia concentrations within the ranges 0.4–0.55 $\mu\text{mol/g}$ and 0.86–0.98 $\mu\text{mol/g}$ respectively, after low-rate intravenous $^{15}\text{NH}_4^+$ infusion to ^{15}N -enrich brain glutamine, followed by $^{14}\text{NH}_4^+$ infusion (isotope chase). The rate of glutamine synthesis at steady state was determined from the change in brain [5- ^{15}N]glutamine levels during isotope chase, observed selectively through the amide proton by NMR, and ^{15}N enrichments of brain glutamine and of blood and brain ammonia measured by gas chromatography–MS. The *in vivo* rate (v) was 3.3–4.5 $\mu\text{mol/h}$ per g of brain at blood ammonia concentrations (s) of

0.40–0.55 $\mu\text{mol/g}$. A linear increase of $1/v$ with $1/s$ permitted estimation of the *in vivo* glutamine synthetase (GS) activity at a physiological blood ammonia concentration to be 0.4–2.1 $\mu\text{mol/h}$ per g. The observed ammonia-dependence strongly suggests that, under physiological conditions, *in vivo* GS activity is kinetically limited by sub-optimal *in situ* concentrations of ammonia as well as of glutamate and ATP. Comparison of the observed *in vivo* GS activity with the reported *in vivo* rates of glutaminase and of γ -aminobutyrate (GABA) synthesis suggests that, under mildly hyperammonaemic conditions, glutamine is synthesized at a sufficiently high rate to serve as a precursor of GABA, but glutaminase-catalysed hydrolysis of glutamine is too slow to be the sole provider of glutamate used for GABA synthesis.

INTRODUCTION

Glutamine synthetase (GS; EC 6.3.1.2) catalyses the reaction:

In the brain, GS is localized in astrocytes [1] and oligodendrocytes [2] and is one of the key enzymes in the glutamine/glutamate cycle [3–5]. Glutamine is an important precursor of the neurotransmitter pools of glutamate [6–8] and possibly of γ -aminobutyrate (GABA) [8–11]. Hence the rate of glutamine synthesis in intact brain can indirectly control the turnover rates of these neurotransmitters.

GS also regulates the level of ammonia, a potential neurotoxin, in the brain. In hepatic encephalopathy (HE), a metabolic disease of the brain caused by dysfunction of the liver, decreased ammonia removal in the liver results in hyperammonaemia and diffusion of neurotoxic levels of ammonia into the brain. While disturbance of ammonia metabolism is clearly implicated in the pathogenesis of HE [12], the mechanism of ammonia neurotoxicity [13,14] and the capacity of GS to remove excess ammonia [15] have been the subject of considerable debate. Hence measurement of *in vivo* GS activities under various degrees of hyperammonaemia and clarification of factors that regulate the rate can contribute to a better understanding of the role of this enzyme in HE.

The dependence of the rate of glutamine synthesis on the blood or brain ammonia concentration has not, to our knowledge, been measured in the intact brain. Although studies with

cultured astrocytes have provided useful information [5,16–18], it is also important to measure the rate in an intact brain where influx of blood-borne ammonia by diffusion is facilitated by the brain–blood pH gradient [19]. In an *in vivo* study using the radioisotope ^{15}N , the proportion of the ‘small-pool’ ammonia converted into glutamine in a given time was found to be smaller in brains from hyperammonaemic rats than in normal brains [20,21]. However, the rate of glutamine synthesis was not determined because isotopic enrichment of the precursor ammonia and the physical size of the compartment were not known.

NMR is a useful technique for the non-invasive monitoring of metabolite flux in animals and humans [22]. Our previous work showed that direct ^{15}N -NMR is useful for measuring the *in vivo* rate of glutamine synthesis at steady state in the brains of hyperammonaemic rats at blood and brain ammonia concentrations of 0.62 and 2.9 $\mu\text{mol/g}$ respectively [23]. Physiological blood and brain ammonia levels in the rat are 0.15 ± 0.1 and 0.22 ± 0.07 mM respectively [19]. For kinetic studies at these lower tissue ammonia levels, a more sensitive method for the detection of brain [5- ^{15}N]glutamine is required. We have recently shown that brain [5- ^{15}N]glutamine can be selectively observed through the ^{15}N -bound amide protons by ^1H – ^{15}N heteronuclear multiple-quantum coherence (HMQC)-transfer NMR in anaesthetized, spontaneously breathing rats after low-rate $^{15}\text{NH}_4^+$ infusion [24]. We report here the measurement of *in vivo* rates of glutamine synthesis in rat brain, measured by the isotope chase method at steady-state blood and brain ammonia concentrations in the ranges 0.40–0.55 and 0.8–1.0 $\mu\text{mol/g}$ respectively. The

Abbreviations used: GS, glutamine synthetase; GABA, γ -aminobutyrate; HE, hepatic encephalopathy; HMQC, heteronuclear multiple-quantum coherence; GC-MS, gas chromatography–MS; STEAM, stimulated echo acquisition mode; DSS, 2,2-dimethylsilapentane-5-sulphonic acid; TAB-glutamate, *N*-trifluoroacetyl-*n*-dibutylglutamate; PCS, portacaval-shunt; GAD, glutamate decarboxylase; PAG, phosphate-activated glutaminase; PET, positron emission tomography.

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dependence of the *in vivo* activity on the blood ammonia level was found to follow Michaelis–Menten kinetics, permitting estimation of *in vivo* GS activity at a physiological blood ammonia concentration.

EXPERIMENTAL

Animal preparation and ammonia infusion

Male Wistar rats weighing 250–300 g were used. Rats were anaesthetized with an intraperitoneal injection of sodium pentobarbital (Nembutal; 40 mg/kg body weight), and prepared for ammonia infusion through the femoral vein as described previously [25]. Three ammonia infusion protocols were used. In experiments designed to quantify brain [5-¹⁵N]glutamine from its observed *in vivo* NMR peak intensity, ¹⁵NH₄Cl (> 97% enriched in ¹⁵N from Cambridge Isotopes) in a 1 M aqueous solution at pH 7.4 was infused at a constant rate in each rat, but at rates ranging from 2.3 to 4.5 mmol/h per kg body wt. in 10 rats to attain brain [5-¹⁵N]glutamine levels of 2.0–7.7 μmol/g in < 4 h of infusion. For the kinetic study of the rate of glutamine synthesis by the isotope chase method, one group of rats (Group I; *n* = 8) was given a ¹⁵NH₄Cl (1 M) infusion at a rate of 2.3 ± 0.04 mmol/h per kg body wt. for 3.1 ± 0.18 h followed by ¹⁴NH₄Cl infusion at the same rate for an additional 3.2 h (chase period). In Group II (*n* = 7), ¹⁵NH₄Cl (2 M) infusion at a rate of 3.3 ± 0.07 mmol/h per kg for 4.4 ± 0.12 h was followed by ¹⁴NH₄⁺ infusion for 3.2 h. In this group, the ammonia concentration was increased to 2 M to keep the infusate volume below 1 ml/h.

Kinetic analysis

The rate of glutamine synthesis at steady state was determined by the isotope chase method as described in detail previously [23]. ¹⁵NH₄⁺ infusion was performed until brain ammonia, glutamate and glutamine as well as blood ammonia reached steady state and brain glutamine 5-N was 35–42% ¹⁵N-enriched. This was followed by ¹⁴NH₄⁺ infusion at the same rate (3.2 h chase period). At steady state, the rate of glutamine synthesis, *v*₁, is equal to the rate of glutamine utilization, *v*₂, and is given by:

$$v_1 = v_2 = (dB^*/dt)/(s_A - s_B) \quad (1)$$

where *B*^{*} is the brain [5-¹⁵N]glutamine concentration at time *t*, *s*_A is the ¹⁵N enrichment of precursor ammonia and *s*_B is the ¹⁵N enrichment of brain glutamine. During isotope chase, *B*^{*}, *s*_A and *s*_B change while the total (¹⁴N + ¹⁵N) concentrations of the metabolites involved in the GS reaction remain constant. The rate of change of the brain [5-¹⁵N]glutamine concentration during chase was measured *in vivo* by NMR. ¹⁵N enrichment of brain glutamine was determined from the brain [5-¹⁵N]glutamine concentration observed by NMR and the total steady-state brain glutamine concentration measured *in vitro* in a perchloric acid extract of the brain. ¹⁵N enrichments of blood and brain ammonia were determined by gas chromatography–MS (GC–MS).

In vivo NMR

In vivo ¹H–¹⁵N HMQC spectra were obtained at 200 MHz for ¹H on a CSI-II spectrometer from General Electric Instruments (Fremont, CA, U.S.A.) equipped with a 4.7 T, 25 cm-bore Oxford magnet. The probe, consisting of separate, orthogonal resonators for ¹H (observe) and ¹⁵N (decouple), has been described in detail [24].

The anaesthetized rat, prepared for intravenous ¹⁵NH₄⁺ infusion, was placed in the magnet with its head (secured in a specifically designed holder) inside the observe coil. After starting

the ¹⁵NH₄Cl infusion, the head was imaged by the spin-echo method to determine the three-dimensional co-ordinates of the brain. Shimming was then performed on a 14 mm³ voxel of the brain by the stimulated echo acquisition mode (STEAM) sequence to a brain water linewidth of 30 ± 5 Hz. *In vivo* ¹H–¹⁵N HMQC spectra were then acquired to monitor changes in the brain [5-¹⁵N]glutamine concentration. The pulse sequence and the acquisition parameters for water-suppressed, ¹⁵N-decoupled, phase-cycled ¹H–¹⁵N HMQC have been described in detail [24]. ¹H chemical shifts are reported in p.p.m. from 2,2-dimethylsilapentane-5-sulphonic acid (DSS). Assignment of ¹⁵N-bound protons selectively observed *in vivo* was made as follows. The amide proton (H₂) of [5-¹⁵N]glutamine in water at pH 7.1 had a chemical shift of 2.08 p.p.m. from water and 6.9 p.p.m. from DSS [24]. In the *in vivo* HMQC spectra, the peak observed at 2.07 ± 0.02 p.p.m. (*n* = 9) from the brain water (as observed by STEAM) was therefore assigned to brain [5-¹⁵N]glutamine amide proton H₂.

For quantification of brain [5-¹⁵N]glutamine from its observed peak intensity, brain [5-¹⁵N]glutamine was monitored *in vivo* until its peak intensity reached a steady level. The peak intensity (measured as the integrated peak area) acquired in the last 30 min of the *in vivo* experiment was then calibrated against the actual concentration of [5-¹⁵N]glutamine found in the extract of the same brain after the rat was killed in 10 rats to determine how the observed *in vivo* peak intensity varied with the quantity of brain [5-¹⁵N]glutamine. For the kinetic study of the rate of glutamine synthesis, ¹⁵NH₄⁺ infusion was performed for 3–4 h until the brain [5-¹⁵N]glutamine level reached 3.1–3.6 μmol/g, followed by isotope chase at steady state. During the chase, an *in vivo* spectrum was acquired for 22–30 min (1000–1400 scans) before restarting acquisition, to monitor changes in brain [5-¹⁵N]glutamine levels. Within each acquisition block, the free induction decay signal was stored cumulatively every 200 scans (4.7 min). This permitted subsequent selection of any time interval within the acquisition block to detect the short-term changes in brain [5-¹⁵N]glutamine level.

After the *in vivo* experiment, the rat was killed and the brain was frozen immediately in liquid nitrogen for preparation of a perchloric acid extract. The [5-¹⁵N]glutamine concentration in the brain extract was measured by direct ¹⁵N-NMR as described in detail previously [23].

In vitro metabolite assays

Brain ammonia, glutamate and total (¹⁵N + ¹⁴N) glutamine at the end of the *in vivo* experiment were assayed in perchloric acid extracts of the brain. The concentrations of these brain metabolites at earlier time points were assayed in the brain extracts of rats given parallel ammonia infusions outside the magnet and killed at the times indicated. Arterial blood samples (0.3 ± 0.05 ml) for measurements of ammonia concentration and ¹⁵N enrichment were periodically collected from the femoral artery (cannulated with PE10 polyethylene tubing) by free flow at a rate of 60 μl/min. The blood sample was immediately mixed with 0.5 ml of 20% perchloric acid at 0 °C for deproteinization, followed by centrifugation (37000 *g*) and storage in liquid nitrogen until analysis. Enzymic assays for ammonia, glutamine and glutamate were performed according to published procedures [26–28].

GC–MS analyses of ¹⁵N enrichment of brain and blood ammonia

To determine ¹⁵N enrichment, ammonia in brain and blood extracts was purified by elution through cation-exchange resin and converted into glutamate by the method of Yudkoff et al.

[29], with the modification described previously [23]. The method has been shown to achieve complete separation of brain ammonia from glutamate prior to enzymic conversion of ammonia into glutamate [23]. The ammonia-derived glutamate was derivatized to *N*-trifluoroacetyl-*n*-dibutylglutamate (TAB-glutamate) by the method of Gehrke et al. [30]. Isotopic abundance was analysed by GC-MS on a Hewlett-Packard 5989A GC/MS/IR instrument using the selected ion monitor mode, as described in detail previously [23]. Three pairs of ions were chosen to ensure specificity of the analysis; 198, 199; 254, 255; 152, 153. Enrichments of TAB-glutamate were measured using the most intense pair of ions, i.e. 198, 199.

RESULTS

Steady-state metabolite levels

Figure 1(a) shows the changes in blood and brain ammonia concentrations in rats given an ammonia infusion at the rate of 2.3 mmol/h per kg body wt. (Group I). Before infusion, the arterial blood ammonia level was $0.170 \pm 0.018 \mu\text{mol/g}$ ($n = 3$), in accord with reported values ($0.15 \pm 0.10 \text{ mM}$ [19]). After 0.25 h of infusion, blood ammonia reached steady state at $0.395 \pm 0.005 \mu\text{mol/g}$, a level 2.3 times higher than that in the normal rat. Brain ammonia, which is present at a concentration of $0.18 \pm 0.06 \mu\text{mol/g}$ in the normal rat [19], increased to a steady-state value of $0.86 \pm 0.12 \mu\text{mol/g}$ after 0.5 h of infusion. The brain/blood ammonia concentration ratio was 2.2, within the equilibrium ratio of 1.5–3.0 expected from the brain/blood pH gradient (0.3 pH unit) [19]. After the initial rise, brain ammonia was at steady state for at least several hours, suggesting that blood-borne ammonia diffusing into brain was effectively incorporated into glutamine by GS.

Figure 1(b) shows the progressive increase in brain [$5\text{-}^{15}\text{N}$]glutamine during the first 2.4 h of $^{15}\text{NH}_4^+$ infusion. Total brain glutamine ($^{14}\text{N} + ^{15}\text{N}$), which is $5.3 \pm 0.7 \mu\text{mol/g}$ in normal rats [14,31], increased for about 2 h and reached steady state around 2.5 h (Group I). In rats given an ammonia infusion at a higher rate of 3.3 mmol/h per kg (Group II), brain glutamine had attained a steady-state level by 4 h. Hence, for *in vivo* NMR experiments, the isotope chase was started after $3.1 \pm 0.2 \text{ h}$ (Group I) or $4.4 \pm 0.12 \text{ h}$ (Group II) to ensure steady-state conditions. Table 1 lists the pre- and post-chase levels of blood ammonia, brain ammonia, glutamate and glutamine for Groups I and II. Clearly, the metabolites involved in the GS reaction were at steady state during the chase period.

Changes in brain [$5\text{-}^{15}\text{N}$]glutamine during the chase

Figure 2 shows an *in vivo* $^1\text{H}\text{-}^{15}\text{N}$ HMQC spectrum, acquired in 13 min with ^{15}N decoupling, from the brain region of an anaesthetized, spontaneously breathing rat, after 3.1 h of $^{15}\text{NH}_4^+$ infusion at the rate of 2.3 mmol/h per kg body wt. The peak at 6.9 p.p.m. can be assigned to the upfield amide proton (H_α) of brain [$5\text{-}^{15}\text{N}$]glutamine (structure shown in the inset of Figure 2) [24]. We have previously shown that, even without spatially localized acquisition, the observed [$5\text{-}^{15}\text{N}$]glutamine arises almost exclusively from the brain [32] and that, in $^1\text{H}\text{-}^{15}\text{N}$ HMQC experiments, spatially localized shimming on brain water minimizes any contribution from other tissues to the observed amide proton signal [24]. Proton peaks of brain metabolites not coupled to ^{15}N in the 1–4 p.p.m. region are completely suppressed by phase-cycling, while the residual water peak is observed at 4.7–5.0 p.p.m.

To quantify brain [$5\text{-}^{15}\text{N}$]glutamine from the observed peak intensity, the *in vivo* H^α peak intensity acquired in the last 30 min

Figure 1 Blood ammonia and brain ammonia and glutamine levels during ammonia infusion

(a) Arterial blood (●) and brain (□) ammonia levels ($\mu\text{mol/g}$) in rats given an ammonia infusion at the rate of 2.3 mmol/h per kg body wt. (Group I). The values are means $\pm$ S.E.M. for 2–3 rats. (b) Brain [$^{14}\text{N} + ^{15}\text{N}$]glutamine (●) and [$5\text{-}^{15}\text{N}$]glutamine (▲) levels in Group I rats given an $^{15}\text{NH}_4^+$ infusion followed by an $^{14}\text{NH}_4^+$ infusion (chase). Also shown are brain [$^{14}\text{N} + ^{15}\text{N}$]glutamine levels (□) observed just before and during the chase in rats given an ammonia infusion at the rate of 3.3 mmol/h per kg body wt. (Group II). For changes in brain [$5\text{-}^{15}\text{N}$]glutamine during the chase ($t > 3 \text{ h}$), see Figure 5.

of the *in vivo* experiment was plotted against the actual concentration of [$5\text{-}^{15}\text{N}$]glutamine found in the extract of the same brain for 10 rats (Figure 3). The *in vivo* peak intensity increased linearly with brain [$5\text{-}^{15}\text{N}$]glutamine concentration. Reproducibility in the low concentration range (2–3 $\mu\text{mol/g}$; $n = 6$) used in this study is remarkably good. Hence the equation of the least-squares line through the plots was used to convert the observed *in vivo* peak intensities into brain [$5\text{-}^{15}\text{N}$]glutamine concentrations in subsequent time course experiments.

Figure 4 shows representative *in vivo* $^1\text{H}\text{-}^{15}\text{N}$ HMQC spectra obtained from rats given an ammonia infusion at the rate of 2.3 mmol/h per kg body wt. and examined at $t = 0.4, 1.25, 1.9$

Table 1 Tissue levels and ^{15}N enrichments of cerebral metabolites and blood ammonia in rats given an $^{15}\text{NH}_4^+$ infusion followed by $^{14}\text{NH}_4^+$

Tissue levels of metabolites, measured in extracts after 3–4 h of $^{15}\text{NH}_4^+$ infusion and after a subsequent 3.2 h of isotope chase by $^{14}\text{NH}_4^+$ infusion, are shown as pre-chase and post-chase levels respectively in $\mu\text{mol/g}$ of tissue (means $\pm$ S.E.M.) with the numbers of rats in parentheses. ^{15}N enrichment, defined as $([^{15}\text{N}]/\text{metabolite concentration})/(\text{total } [^{15}\text{N} + ^{14}\text{N}]/\text{metabolite concentration})$, was measured at the indicated time after the start of chase ($t = 0$) by NMR *in vivo* (glutamine) or by GC-MS in extracts (ammonia).

Metabolite	NH_3 infusion rate (mmol/h per kg)	Tissue level ($\mu\text{mol/g}$)		^{15}N enrichment		
		Pre-chase	Post-chase	$t = 1.5 \pm 0.05$ h	$t = 2.0 \pm 0.05$ h	$t = 2.5$ h
Brain ammonia	2.3	0.86 ± 0.12 (4)	0.85 ± 0.10 (11)	0.122 ± 0.022 (2)	0.09 (1)	0.085 ± 0.005 (2)
	3.3	0.96 ± 0.09 (4)	0.99 ± 0.12 (8)	0.18 ± 0.03 (2)	0.20 ± 0.02 (2)	—
Brain glutamate	2.3	8.6 ± 0.40 (4)	9.2 ± 0.64 (5)	—	—	—
	3.3	9.2 ± 0.69 (4)	9.2 ± 0.64 (5)	—	—	—
Brain glutamine	2.3	8.5 ± 0.96 (4)	8.9 ± 0.37 (11)	0.264 ± 0.019 (8)	0.226 ± 0.017 (8)	—
	3.3	9.8 ± 0.86 (6)	10.3 ± 0.46 (8)	0.261 ± 0.019 (4)	0.226 ± 0.016 (4)	—
Blood ammonia	2.3	0.40 ± 0.018 (8)	0.39 ± 0.048 (4)	0.053 ± 0.009 (3)	0.058 ± 0.009 (2)	—
	3.3	0.56 ± 0.052 (3)	0.54 ± 0.028 (6)	0.115 ± 0.015 (2)	0.09 (2)	—

Figure 2 *In vivo* ^1H – ^{15}N HMQC spectrum of brain $[5\text{-}^{15}\text{N}]$ glutamine

A typical ^1H – ^{15}N HMQC spectrum of brain $[5\text{-}^{15}\text{N}]$ glutamine in an anaesthetized, $^{15}\text{NH}_4^+$ -infused and spontaneously breathing rat, acquired in 13 min. The ^{15}N -decoupled amide proton (H^2) of brain $[5\text{-}^{15}\text{N}]$ glutamine is selectively observed at 6.9 p.p.m. (see inset for structure). All protons not coupled to ^{15}N are cancelled by phase cycling. The peak at 4.7–5.0 p.p.m. is residual water. The peaks at 5.6 and 6.0 p.p.m. arise from $[^{15}\text{N}]$ urea protons in the brain [24].

and 3.1 h after the start of isotope chase. A progressive decrease in the peak intensity of the brain $[5\text{-}^{15}\text{N}]$ glutamine H^2 peak was observed. Figure 5(a) shows the change in brain $[5\text{-}^{15}\text{N}]$ glutamine concentration (means $\pm$ S.E.M. for 8 rats) during the chase, determined from the observed *in vivo* H^2 peak intensities. The equation of the best-fit exponential curve is shown in the caption. Figure 5(b) shows the exponential decrease of the brain $[5\text{-}^{15}\text{N}]$ glutamine concentration during chase in rats ($n = 7$) given an ammonia infusion at the higher rate of 3.3 mmol/h per kg body wt. Comparison with Figure 5(a) shows that the

Figure 3 *In vivo* peak intensity of the brain $[5\text{-}^{15}\text{N}]$ glutamine amide proton as a function of concentration

The *in vivo* peak intensity of the amide proton (H^2 in Figure 2) of brain $[5\text{-}^{15}\text{N}]$ glutamine observed *in vivo* by NMR is plotted against the actual concentration of $[5\text{-}^{15}\text{N}]$ glutamine found in the extract of the same brain for 10 rats. The *in vivo* peak intensity increased linearly with concentration. The equation of the least-squares line through the plots was used to convert the observed *in vivo* peak intensities to brain $[5\text{-}^{15}\text{N}]$ glutamine concentrations in subsequent time course experiments. For the peak intensity unit, the peak area of the amide proton H^2 of 10 μmol of $[5\text{-}^{15}\text{N}]$ glutamine in water at pH 7.1, after HMQC acquisition for 384 scans, was set as 1000.

brain $[5\text{-}^{15}\text{N}]$ glutamine level at the start of the chase was slightly higher at this infusion rate.

Calculation of the rate of glutamine synthesis

The *in vivo* rate of glutamine synthesis in the brain at steady state was calculated from eqn. (1) using the experimentally determined values of $\text{d}B^*/\text{d}t$, s_A and s_B . Table 1 lists the ^{15}N enrichments of precursor (blood and brain) ammonia, s_A , at $t = 1.5$ and 2 h after the start of chase, as determined by GC-MS. Also shown are ^{15}N enrichments of brain glutamine, calculated from the brain $[5\text{-}^{15}\text{N}]$ glutamine level determined by NMR (Figure 5) and the total $[^{15}\text{N} + ^{14}\text{N}]$ glutamine concentration measured at 1.5–2.0 h after the start of the chase (Figure 1b). All time periods in the following description refer to the exact time after the start of the chase.

Figure 4 *In vivo* ^1H – ^{15}N HMQC spectra of brain $[5\text{-}^{15}\text{N}]$ glutamine during isotope chase

After $^{15}\text{NH}_4^+$ infusion at a rate of 2.3 mmol/h per kg, chase was started at $t = 0$. Spectra acquired at $t = 0.4, 1.25, 1.9$ and 3.1 h show a progressive decrease in the brain $[5\text{-}^{15}\text{N}]$ glutamine amide proton peak intensity. The indicated time is at the centre of a 22 min acquisition period for each spectrum.

In Group I rats, the equation of the best-fit exponential curve for the change in brain $[5\text{-}^{15}\text{N}]$ glutamine was $B^* = 3.6e^{-0.29t}$ (see Figure 5a caption). Hence, at $t = 2.05$ h, $B^* = 1.99 \mu\text{mol/g}$ and $dB^*/dt = -0.29 \times 1.99 = -0.576 \mu\text{mol/h}$ per g. The total brain glutamine concentration was $8.8 \pm 0.65 \mu\text{mol/g}$ (Figure 1b; $n = 8$) and hence $s_B = 0.226 \pm 0.017$. As shown in Table 1, $s_A = 0.058 \pm 0.009$ for blood ammonia and 0.09 for brain ammonia. Substituting these values into eqn. (1), we obtain $v = (dB^*/dt)/(s_A - s_B) = 3.4 \pm 0.34 \mu\text{mol/h}$ per g if the ^{15}N enrichment of ammonia at the site of GS in astrocytes is equal to that of blood ammonia (Assumption I), and $v = 4.1 \pm 0.5 \mu\text{mol/h}$ per g if it is equal to that of brain ammonia (Assumption II). Using experimental values obtained at $t = 1.5$ h, i.e. $B^* = 2.3 \mu\text{mol/g}$, $dB^*/dt = -0.667 \mu\text{mol/h}$ per g, $s_A = 0.053 \pm 0.009$ (blood) or 0.122 ± 0.022 (brain) and $s_B = 0.264 \pm 0.019$, we obtain very similar rates of $3.2 \pm 0.4 \mu\text{mol/h}$ per g (Assumption I) and $4.7 \pm 1.3 \mu\text{mol/h}$ per g (Assumption II).

In Group II rats, the equation of the best-fit exponential curve was $B^* = 3.81e^{-0.283t}$. Hence, at $t = 1.96$ h, $B^* = 2.19 \mu\text{mol/g}$ and $dB^*/dt = -0.619 \mu\text{mol/h}$ per g. With a total brain glu-

Figure 5 Changes in the brain $[5\text{-}^{15}\text{N}]$ glutamine concentration observed *in vivo* during isotope chase

(a) Group I rats. After 3.1 h of $^{15}\text{NH}_4^+$ infusion at a rate of 2.3 mmol/h per kg body wt., chase by ^{14}N was started at $t = 0$. The cerebral $[5\text{-}^{15}\text{N}]$ glutamine concentration (mean $\pm$ S.E.M.; $n = 8$) was calculated from the observed *in vivo* peak intensities as described in the text. The equation of the best-fit exponential curve was $B^* = 3.6e^{-0.29t}$ ($r = 0.98$). (b) Group II rats, given an $^{15}\text{NH}_4^+$ infusion at the rate of 3.3 mmol/h per kg body wt., followed by chase starting at $t = 0$. The equation of the best-fit curve for the exponential decay of cerebral $[5\text{-}^{15}\text{N}]$ glutamine (mean $\pm$ S.E.M.; $n = 7$) was $B^* = 3.81e^{-0.283t}$ ($r = 0.99$).

tamine level of $9.67 \pm 0.70 \mu\text{mol/g}$ (Figure 1b; $n = 4$), and hence $s_B = 0.226 \pm 0.016$, and $s_A = 0.09$, we obtain $v = 4.5 \pm 0.5 \mu\text{mol/h}$ per g for the *in vivo* rate with Assumption I. With Assumption II, the data at $t = 1.46$ h (i.e. $B^* = 2.52 \mu\text{mol/g}$, $dB^*/dt = -0.713 \mu\text{mol/h}$ per g, $s_B = 0.261 \pm 0.019$ and $s_A = 0.18 \pm 0.03$) give $v = 8.8 \pm 2.3 \mu\text{mol/h}$ per g for the rate of glutamine synthesis.

Dependence of the *in vivo* GS activity on ammonia concentration

Table 2 summarizes the *in vivo* rates of glutamine synthesis at various steady-state concentrations of blood ammonia, brain ammonia and glutamine, determined in this work and in our previous publication [23]. The *in vivo* rates decrease as blood and brain ammonia concentrations decrease. The *in vivo* rates calculated on the assumption that the ^{15}N enrichment of substrate

Table 2 *In vivo* rates of glutamine synthesis in rat brain at various blood and brain ammonia levels

In vivo rates of glutamine synthesis at steady state were determined from the rate of change in brain [$5\text{-}^{15}\text{N}$]glutamine observed by NMR, and ^{15}N enrichments of precursor ammonia and of brain glutamine as described in the text. The *in vivo* rate is calculated on the assumption that the ^{15}N enrichment of the substrate ammonia for the GS reaction is (I) equal to that of blood ammonia, and (II) equal to that of whole-brain ammonia. Steady-state tissue levels of metabolites are expressed as $\mu\text{mol/g}$ of tissue (means $\pm$ S.E.M.) with the numbers of rats in parentheses.

<i>In vivo</i> rate of glutamine synthesis (μmol/h per g)		Steady-state tissue levels (μmol/g)				Ref.
		Blood	Brain			
I	II	Ammonia	Ammonia	Glutamate	Glutamine	
3.3 ± 0.3	4.4 ± 1.3	0.40 ± 0.018 (12)	0.86 ± 0.080 (15)	8.9 ± 0.39 (9)	8.8 ± 0.36 (15)	This work
4.5 ± 0.5	8.8 ± 2.3	0.55 ± 0.024 (9)	0.975 ± 0.12 (12)	9.2 ± 0.69 (9)	10.1 ± 0.45 (14)	This work
4.8 ± 1.1	13.0 ± 3.9	0.63 ± 0.026 (4)	2.9 ± 0.4 (9)	9.4 ± 0.8 (7)	14.4 ± 1.5 (9)	[23]

Figure 6 Dependence of the *in vivo* GS activity on the blood ammonia concentration

Shown is a plot of $1/v$ versus $1/s$, where v is the *in vivo* GS activity determined on the assumption that the ^{15}N enrichment of substrate ammonia is equal to that of blood ammonia, and s is the blood ammonia concentration, converted from $\mu\text{mol/g}$ in Table 2 to mM on the usual assumption that 80% of tissue weight is water. The value of v was determined for $n = 7\text{--}8$ (Figure 5 and Table 1) or $n = 5$ [23]. The vertical error bar shows the uncertainty in the calculated value of $1/v$ due to the S.E.M. in the observed values of s_b and s_A , as described in the text. The least-squares fit to the data is given by $1/v = a \times (1/s) + b$ ($r = 0.997$), with $a = 0.118 \pm 0.0087$ and $b = 0.054 \pm 0.014$. The numbers after the $\pm$ sign are the S.E.M.s. The linear change of $1/v$ versus $1/s$ permitted estimation of the *in vivo* rate at physiological blood ammonia levels, as described in the text.

ammonia for the GS reaction is equal to that of blood ammonia are consistently lower than those based on the assumption that the enrichment is equal to that of brain ammonia. The actual rate is probably closer to the lower estimate because GS is localized in astrocytic processes which interpose between cerebral capillaries and neurons and act as a metabolic trap for blood-borne ammonia diffusing into the brain [19].

The dependence of the *in vivo* GS activity (v) on the substrate ammonia concentration (s) can be determined from a plot of $1/v$ versus $1/s$. Figure 6 plots $1/v$ versus $1/s$, where v is the *in vivo* GS activity determined on the assumption that the ^{15}N enrichment of ammonia at the site of GS is equal to that of blood ammonia, and s is the blood ammonia concentration (converted from

$\mu\text{mol/g}$ in Table 2 to mM on the usual assumption that 80% of tissue weight is water; hence a blood ammonia concentration of $0.4 \mu\text{mol/g}$ corresponds to $s = 0.5 \text{ mM}$). A linear increase of $1/v$ with $1/s$ is observed. This suggests that the ammonia concentration at the site of GS in astrocytes is proportional to the steady-state blood ammonia concentration and that the dependence of the *in vivo* GS activity on the ammonia concentration follows Michaelis–Menten kinetics. The equation of the least-squares line through the plots was:

$$1/v = (0.118 \pm 0.0087) \times 1/s + (0.054 \pm 0.014) \quad (2)$$

where the number after the $\pm$ sign is the S.E.M. This equation permits estimation of the *in vivo* GS activity at a physiological arterial blood ammonia concentration, $0.15 \pm 0.10 \text{ mM}$ [19]. Substitution of $s = 0.15 \text{ mM}$ into eqn. (2) yields $1/v = 0.84 \pm 0.087 \mu\text{mol}^{-1} \cdot \text{g} \cdot \text{h}$, and hence $v = 1.2 \pm 0.12 \mu\text{mol/h}$ per g, with a 95% confidence level. When the uncertainty in the reported physiological blood ammonia level, $0.15 \pm 0.10 \text{ mM}$, is taken into account (by substitution into eqn. 2), a reasonable range for the estimated *in vivo* rate under physiological conditions is $0.4\text{--}2.1 \mu\text{mol/h}$ per g.

With v representing the *in vivo* GS activity, determined on the assumption that the ^{15}N enrichment of substrate ammonia is equal to that of brain ammonia, and s representing whole-brain ammonia, a plot of $1/v$ versus $1/s$ was non-linear (result not shown). A probable reason is that, since astrocytes and neurons represent 25% and 45% respectively of the brain cell population [33], ammonia measured in the whole brain mainly represents neuronal ammonia. Ammonia which escapes the action of GS and diffuses into neurons, as well as endogenous ammonia generated by catabolic pathways, tend to accumulate in neurons because glutamate dehydrogenase, the only ammonia-assimilating enzyme in neurons, has a low affinity for ammonia [19]. Thus the whole-brain ammonia level is likely to be higher than, and not necessarily proportional to, the ammonia concentration at the site of GS in astrocytes.

DISCUSSION

Our results show that the *in vivo* GS activity in rat brain decreases with decreasing blood ammonia concentration in accordance with Michaelis–Menten kinetics, and is $3.3\text{--}4.8 \mu\text{mol/h}$ per g at blood ammonia concentrations of $0.40\text{--}0.64 \mu\text{mol/g}$. The estimated *in vivo* rate at a physiological blood ammonia level was $0.4\text{--}2.1 \mu\text{mol/h}$ per g. Evidence showing that the pentobarbital anaesthesia used in this study had little, if any, effect on the rate of glutamine synthesis has been described previously [23]. It is to be noted that the decrease in

brain [5-¹⁵N]glutamine observed *in vivo* during the isotope chase (Figure 5) represents flux of ¹⁵N into glutamine-utilizing pathways within the brain. Release of brain [5-¹⁵N]glutamine into the circulation, which would result in overestimation of the rate, is expected to be negligible because (a) no efflux of brain glutamine was observed even at brain glutamine levels of 14–17 $\mu\text{mol/g}$ in acute hyperammonaemia [34], and (b) more recently, in porta-caval-shunted (PCS) rats, the rate of release of brain glutamine into the circulation was determined to be 4 nmol/min per brain (2 g), corresponding to a rate of 0.12 $\mu\text{mol/h}$ per g [35]. Hence, with the ¹⁵N enrichment of 0.2–0.3 for brain glutamine during the chase, the maximum possible rate of release of brain [5-¹⁵N]glutamine into the circulation is estimated to be 0.02–0.04 $\mu\text{mol/h}$ per g, which is negligible compared with the observed rate of decrease within the brain (Figure 5).

Previous studies of the rate of glutamine synthesis and its dependence on ammonia levels in animal brains were performed with cultured astrocytes [16–18]. While direct comparison with the rate observed in the intact brain is difficult, the report that the GS rate increases 2.6-fold on addition of 0.1 mM ammonia to the medium in rat brain astrocyte culture [18] suggests that, *in situ*, GS is not saturated with ammonia. Comparison with *in vivo* rates in the human brain should be informative because rat and human brain GS show > 90% identity in amino acid sequence [36,37] and their kinetic and regulatory properties, as well as their cellular compartmentation within the brain, are remarkably similar [1,38–40]. A recent study by Gruetter and co-workers [41,42] on the rates of tricarboxylic acid cycle and of glutamate/glutamine exchange in the human brain, measured *in vivo* by ¹³C-NMR after intravenous infusion of [1-¹³C]glucose, is intriguing in this respect. However, the large range of the reported rate of glutamine synthesis in the human brain precludes meaningful comparison with the rat brain data at present. Another interesting study is the measurement of the brain ammonia utilization rate by ¹⁵N positron emission tomography (PET) after intravenous injection of ¹⁵NH₃ [43]. The rate, estimated from the blood ¹⁵NH₃ clearance rate and the fraction of ¹⁵N observed in the brain relative to the whole body, was 32 $\mu\text{mol/min}$ per whole brain (corresponding to 1.4 $\mu\text{mol/h}$ per g of brain) in normal humans and 2.3 $\mu\text{mol/h}$ per g in HE patients with a blood ammonia concentration of 0.15 mM. While PET measures the total ¹⁵N activity (¹⁵NH₃ and its metabolites) in the brain, and not the rate of ¹⁵NH₃ incorporation into specific metabolites, the reported ammonia utilization rate can be regarded as the maximum possible rate of ammonia incorporation into brain glutamine if blood-borne ammonia is the major source of ammonia for the GS reaction. Thus our study and reports from other laboratories, taken together, strongly suggest that the *in vivo* rate of glutamine synthesis in mammalian brain at steady state is of the order of 1 $\mu\text{mol/h}$ per g and that the rate increases with increasing blood ammonia concentration.

Factors regulating *in vivo* GS activity

The low level of *in vivo* GS activity, compared with the *in vitro* activity measured at enzyme-saturating concentrations of substrates (275–350 $\mu\text{mol/h}$ per g [31,44]) can most reasonably be attributed to low astrocytic glutamate levels and to subsaturating *in situ* concentrations of ATP and metal ion cofactors (Mg²⁺ or Mn²⁺), as discussed in detail previously [23]. Furthermore, the progressive decrease of *in vivo* GS activity with decreases in blood and brain ammonia levels observed in the present study strongly suggests that, in normal brain, *in vivo* GS activity is kinetically limited by low *in situ* ammonia levels relative to the reported K_m of GS for ammonia, i.e. 0.3 mM [39]. (Rat brain and

liver GS genes are virtually identical [36,37], and hence kinetic and regulatory properties described for the liver enzyme [38,39,45] most probably apply to brain GS.)

It has been suggested on the basis of ¹⁵N tracer studies in normal and PCS rats that the brain has little capacity to buffer against excessive ammonia influx [21]. This raised the question of whether the increased brain glutamine that accompanies PCS is the result of increased flux through the residual capacity of the enzyme or due to reduced glutamine degradation. This question had not been definitively answered previously [15]. The results of our work clearly show that (a) *in vivo* GS activity increases with increasing blood ammonia levels, and (b) increased brain glutamine, at a blood ammonia level similar to that in PCS rats, is mainly due to increased flux through GS (Figure 1b).

As shown in Figure 1(b) and Table 2, brain glutamine reached steady-state levels of 8.8, 10.1 and 14.4 $\mu\text{mol/g}$ at blood ammonia levels of 0.4, 0.55 and 0.63 $\mu\text{mol/g}$ respectively, after 3–4 h of ammonia infusion. Hence, when brain glutamine reaches this level, the rates of glutamine synthesis and utilization become equal, either because the rate of glutamine synthesis decreases and/or the rate of glutamine utilization increases. Product inhibition of GS by glutamine *in vitro* is observed with Mn²⁺, but not Mg²⁺, as cofactor, with 30% inhibition at 20 mM glutamine [39]. The effectiveness of glutamine as an inhibitor is further enhanced when the glutamate concentration is decreased from saturating levels to levels at or near the apparent K_m [45]. Astrocytic glutamine and glutamate levels in rats studied here may well be within this range. Immunocytochemical evidence shows that the glutamate/glutamine ratio is about 0.2 in astrocytes and 2.0–4.5 in excitatory nerve terminals [46]. Hence, at the whole-brain levels shown in Table 2, a reasonable estimate for astrocytic glutamate is 3 ± 1 $\mu\text{mol/g}$ (lower than the reported K_m of 5 mM for glutamate [39] and 15–26 $\mu\text{mol/g}$ for glutamine). Hence an intriguing possibility exists that, in hyperammonaemia, astrocytic glutamine may rise to level high enough to partially inhibit GS if rat brain GS is a Mn²⁺ enzyme, as has been claimed for the ovine brain GS [47,48]. Such regulation of GS activity through product inhibition, which is enhanced at low glutamate levels, would provide an important mechanism for controlling both glutamate and glutamine levels in astrocytes. Astrocytes would then be protected against excessive loss of glutamate as well as overproduction of glutamine. It is worth noting that the higher levels of brain glutamine, i.e. 19–20 $\mu\text{mol/g}$, often observed in acute hyperammonaemia occur when the blood ammonia level is also rising rapidly due to hepatic artery ligation, urease injection, etc. [14,34], and represent non-steady-states. To investigate possible product inhibition of GS activity *in vivo*, an experimental model of steady-state hyperammonaemia combined with isotope chase, such as that presented in this study, is expected to be useful.

Glutamine as a precursor of GABA

If glutamine is the predominant precursor not only of the neurotransmitter pool of glutamate but also of the neuronal pool of glutamate that is converted into GABA by glutamate decarboxylase (GAD), the *in vivo* rate of glutamine synthesis in astrocytes must be faster than the rate of GABA synthesis in GABAergic nerve terminals in order to maintain steady state. The rate of GABA synthesis in rat brain, measured from the rate of its accumulation on inhibition of GABA transaminase, is estimated to be 2.6 ± 1.5 $\mu\text{mol/h}$ per g [10,49–51]. The effect of hyperammonaemia on the rate of GABA synthesis is not known, but hyperammonaemia causes no change in the GABA concentration [14]. The *in vivo* rate of glutamine synthesis under mild

hyperammonaemia observed in this study, 3.3–8.8 $\mu\text{mol/g}$ per h at blood ammonia level of 0.40–0.55 $\mu\text{mol/g}$ (Table 2), is higher than the rate of GABA synthesis. This suggests that, under hyperammonaemia, GS can produce sufficient glutamine to serve as a precursor (via glutamate) of GABA. Glutamine is converted into glutamate by phosphate-activated glutaminase (PAG), as well as by amidotransferase reactions in which glutamine amide N serves as a nitrogen donor to purine and pyrimidine with the concomitant release of glutamate. While the *in vivo* PAG activity in hyperammonaemic rat brain, 0.9–1.3 $\mu\text{mol/g}$ per h [52], is lower than the rate of GABA synthesis and suggests that PAG-catalysed hydrolysis of glutamine cannot be the sole provider of glutamate used for GABA synthesis, amidotransferase reactions, present in rat brain [53,54], may provide an additional pool of glutamate. Further studies are required for comparisons of *in vivo* GS and GAD activities in normal brain at steady state, since the *in vivo* GS activity under physiological conditions, presented in this study, is an estimate. Furthermore, a recent study showed that the activity of GAD₆₇ (one of two molecular forms of GAD) in rat brain decreases substantially when GABA accumulates in the presence of the GABA transaminase inhibitor γ -vinyl-GABA [55], suggesting that measurement of *in vivo* GAD activity at a physiological GABA concentration is desirable.

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