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Cerebral glutamine metabolism under hyperammonemia determined *in vivo* by localized ^1H and ^{15}N NMR spectroscopy

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Brain glutamine synthetase (GS) is an integral part of the glutamate–glutamine cycle and occurs in the glial compartment. *In vivo* Magnetic Resonance Spectroscopy (MRS) allows noninvasive measurements of the concentrations and synthesis rates of metabolites. ^{15}N MRS is an alternative approach to ^{13}C MRS. Incorporation of labeled ^{15}N from ammonia in cerebral glutamine allows to measure several metabolic reactions related to nitrogen metabolism, including the glutamate–glutamine cycle. To measure ^{15}N incorporation into the position 5N of glutamine and position 2N of glutamate and glutamine, we developed a novel ^{15}N pulse sequence to simultaneously detect, for the first time, $[5\text{-}^{15}\text{N}]\text{Gln}$ and $[2\text{-}^{15}\text{N}]\text{Gln} + \text{Glu}$ *in vivo* in the rat brain. In addition, we also measured for the first time in the same experiment localized ^1H spectra for a direct measurement of the net glutamine accumulation. Mathematical modeling of ^1H and ^{15}N MRS data allowed to reduce the number of assumptions and provided reliable determination of GS ($0.30 \pm 0.050 \mu\text{mol/g}$ per minute), apparent neurotransmission ($0.26 \pm 0.030 \mu\text{mol/g}$ per minute), glutamate dehydrogenase ($0.029 \pm 0.002 \mu\text{mol/g}$ per minute), and net glutamine accumulation ($0.033 \pm 0.001 \mu\text{mol/g}$ per minute). These results showed an increase of GS and net glutamine accumulation under hyperammonemia, supporting the concept of their implication in cerebral ammonia detoxification.

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Keywords: ^{15}N and ^1H MRS; glutamate dehydrogenase; glutamine synthetase; hyperammonemia; net glutamine accumulation

Introduction

In brain, glutamine synthetase (GS) activity is predominantly localized in the astrocytes (Martinez-Hernandez *et al*, 1977; Norenberg and Martinez-Hernandez, 1979). It is responsible for the synthesis of glutamine (Gln) from glutamate (Glu) cleared from the synaptic cleft, thus having a major role in

neurotransmission. This glutamine is then shuttled to neurons where it can be deaminated, completing the so-called glutamate–glutamine cycle (Berl *et al*, 1968; Clarke *et al*, 1970; Gruetter, 2004; McKenna, 2007; Shank and Aprison, 1981; Zwingmann and Leibfritz, 2003). In addition, in conditions of hyperammonemia, GS is used for detoxification of brain ammonia (Cooper and Plum, 1987; Kanamori *et al*, 1993; Kanamori and Ross, 1993). In this process, glutamine is accumulated in the brain, while glutamate concentration remains constant. Thus, glutamine accumulation requires *de-novo* synthesis from glucose, with involvement of both glutamate dehydrogenase (GDH) and aspartate aminotransferase (Berl *et al*, 1962; Shen *et al*, 1998).

Because brain GS is an integral part of the glutamate–glutamine cycle (Berl *et al*, 1968; Clarke *et al*, 1970; Shank and Aprison, 1981) and is also functioning as a detoxification pathway for cerebral ammonia (Cooper and Plum, 1987; Kanamori and

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Ross, 1993; Kanamori *et al*, 1993), developments of new methods for the noninvasive measurement of the GS activity became important. Several *in vitro* studies were performed to measure the GS activity in rat brain homogenates (Butterworth *et al*, 1988) or in cultured rat and mouse brain astrocytes (Yudkoff *et al*, 1986). However, knowledge of *in vivo* GS activity is necessary because glial–neuronal interaction has a crucial role in the glutamate–glutamine cycle (Erecinska and Silver, 1990). ^{13}N -labeled ammonia has been used to study the cerebral uptake and metabolism of ammonia in conscious rat (Cooper *et al*, 1979). However, the rate of GS was not determined, because the isotope enrichment of the precursor ammonia and the physical size of the compartment were not known (Cooper *et al*, 1979; Kanamori and Ross, 1993). A potential drawback of ^{13}N is its short half-life (10 minutes) which limits its usefulness to experiments of <1 hour (Cooper *et al*, 1979). *In vivo* Magnetic Resonance Spectroscopy (MRS) is a useful technique for the continuous and noninvasive measurement of the concentrations and synthesis rates of individual metabolites within precisely defined areas in the brain. In particular, *in vivo* noninvasive ^{13}C MRS has been used to study metabolic fluxes, enzymes activities in the living brain of animals and humans (Duarte *et al*, 2011; Gruetter *et al*, 2001). ^{15}N MRS is an alternative approach to ^{13}C MRS in studying glutamate–glutamine metabolism and can in principle provide a more direct interpretation than ^{13}C studies. The wide chemical shift dispersion of ^{15}N provides resolution of important metabolites in glutamate–glutamine cycle. Sparse *in vivo* ^{15}N studies and utilization of different protocols for infusion of ^{15}N -enriched substrates lead to a diversity of results and limited their integration on mathematical models of compartmentalized brain intermediary metabolism.

From these previous studies (Kanamori *et al*, 1993, 1996; Kanamori and Ross, 1993; Shen *et al*, 1998), discrepancies have been noticed concerning the GS flux (V_{syn}) and the net glutamine accumulation (also called *de-novo* glutamine synthesis rate; Shen *et al*, 1998). These divergences could be intrinsically bound to the employment of different experimental protocols and assumptions used to calculate the metabolic fluxes. Therefore, there is a need to acquire additional experimental data during the same experiment, to minimize the number of assumptions to be done on mathematical modeling.

A few past *in vivo* MRS studies under ammonia infusion used either *in vivo* ^1H (Fitzpatrick *et al*, 1989) or unlocalized ^{15}N spectroscopy (Kanamori *et al*, 1993, 1996; Kanamori and Ross, 1993; Shen *et al*, 1998) but never combined and used *in vitro* brain extracts (biochemical assays or high-resolution nuclear magnetic resonance (NMR)) for absolute quantification. These studies mainly focused on the incorporation of ^{15}N of ammonia into $[5\text{-}^{15}\text{N}]\text{Gln}$ and measured the apparent GS flux (Kanamori *et al*, 1993, 1996; Kanamori and Ross, 1993; Shen *et al*, 1998).

The incorporation of ^{15}N into $[2\text{-}^{15}\text{N}]\text{Gln} + \text{Glu}$ may provide further insight into the net flux through GDH (V_{GDH}) (Kanamori and Ross, 1995). However, due to the large chemical shift dispersion between $[5\text{-}^{15}\text{N}]\text{Gln}$ and $[2\text{-}^{15}\text{N}]\text{Gln} + \text{Glu}$, V_{syn} and V_{GDH} were never simultaneously determined. Kanamori and Ross (1995) measured $[2\text{-}^{15}\text{N}]\text{Gln} + \text{Glu}$ (but not $[5\text{-}^{15}\text{N}]\text{Gln}$) during ^{15}N -enriched ammonia infusion and estimated V_{GDH} .

In this context, we used a new acquisition strategy combined with an appropriate mathematical model of compartmentalized cerebral glutamine metabolism and determined, in the same experiment, an important number of metabolic fluxes with minimal assumptions. For that purpose, we developed a new ^{15}N pulse sequence to simultaneously detect, for the first time $[5\text{-}^{15}\text{N}]\text{Gln}$ and $[2\text{-}^{15}\text{N}]\text{Gln} + \text{Glu}$ *in vivo*. Together with *in vivo* localized ^{15}N spectra, we additionally measured for the first time in the same experiment localized ^1H spectra for a direct measurement of the net glutamine accumulation. From our knowledge, the combination of localized ^{15}N MRS and ^1H MRS in interleaved mode has not been reported previously under ^{15}N ammonia infusion. Consequently, ^1H MRS allowed to follow up the total Gln accumulation during ammonia infusion and thereby to directly measure the net glutamine accumulation without any assumptions. Mathematical modeling of ^1H and ^{15}N MRS data provided reliable estimations of V_{syn} , the apparent neurotransmission rate V_{nt} , V_{GDH} , and the net glutamine accumulation ($V_{\text{syn}} - V_{\text{nt}}$) under ^{15}N -labeled ammonia infusion in the rat brain. Moreover, we performed a direct *in vivo* absolute quantification of total Gln, $[5\text{-}^{15}\text{N}]\text{Gln}$ and $[2\text{-}^{15}\text{N}]\text{Glu} + \text{Gln}$ in the same experiment.

Materials and methods

Animal Preparation

In vivo ^{15}N and ^1H MRS experiments were performed on male Sprague-Dawley rats (six animals, 300 to 350 g), which were fasted overnight (12 hours) with free access to water before the experiment. Rats were anesthetized using isoflurane (Attane, Minrad, NY, USA), intubated and mechanically ventilated (2% isoflurane in O_2 gas). Two femoral veins were cannulated for infusion of α -chloralose (Fisher Scientific, Pittsburgh, PA, USA) and ^{15}N ammonium chloride (99% enriched, Cambridge Isotopes, Andover, MA, USA), and one cannulated artery allowed regular blood sampling for monitoring blood gases, pH and plasma ammonia and urea levels. After the surgery, anesthesia was switched to α -chloralose given as an 80 mg/kg bolus and continuous infusion at 26.7 mg/kg per hour. Afterwards, the head was fixed in a stereotaxic system (bite bar and a pair of ear bars) and the animal was placed in an in-house-built holder. After giving a bolus (0.25 mmol/320 g of body weight) >1 minute (Kanamori *et al*, 1993), ^{15}N ammonium chloride was then continuously infused at 4.5 mmol/h per kilogram. Arterial blood was sampled every hour to

monitor blood gases (PaO_2 , PaCO_2) and pH using an AVL Compact 3 blood gas analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Plasma ammonia and urea concentrations were measured using an Analox GM7 analyzer (Analox Instruments, London, UK) and a Reflotron Plus analyzer (Roche Diagnostics GmbH), respectively. Respiration rate and volume were adjusted to maintain pH and blood gases within normal physiological ranges ($\text{pH} = 7.41 \pm 0.04$; $\text{PaCO}_2 = 40.0 \pm 1.8$ mm Hg, O_2 saturation $> 99.8\%$). In addition, sodium bicarbonate (7%, Sigma-Aldrich, Steinheim, Germany) diluted in Dulbecco's Phosphate Buffered Saline (dPBS, Sigma-Aldrich, 70% solution) was infused continuously at an adjustable rate to maintain arterial pH in the normal physiological range. The total amount of blood draw from each animal was $< 10\%$ of the total blood volume of each rat. During the MRS experiments, respiration rate, heart rate, and blood pressure were monitored by a small-animal monitor system (SA Instruments, New York, NY, USA). Body temperature was measured with a rectal thermosensor and maintained at $38.0 \pm 0.5^\circ\text{C}$ by warm water circulation.

All animal experiments were conducted according to federal and local ethical guidelines and the protocols were approved by the local regulatory body of the Canton Vaud, Switzerland (Service de la consommation et des affaires vétérinaires, Affaires vétérinaires, Canton de Vaud, Suisse).

Magnetic Resonance Spectroscopy Measurements

All ^1H and ^{15}N MRS data were acquired interleaved on a Varian Direct Drive (Palo Alto, CA, USA) console interfaced to an actively shielded 9.4 T magnet (Magnex Scientific, Oxford, UK) with a 31-cm horizontal bore. The magnet was equipped with 12 cm inner-diameter actively shielded gradient sets giving a maximum gradient of 400 mT/m in 120 μs . A home-built 14 mm-diameter ^1H quadrature surface coil combined with a single 5-loop 10 mm ^{15}N coil was used as transceiver. The static field homogeneity was adjusted using first- and second-order shims using an echo-planar version of FASTMAP (Gruetter and Tkac, 2000). Localizer images were obtained in the coronal planes using a multislice fast spin echo protocol with echo time (TE)/repetition time (TR) = 60/5,000 ms, a slice thickness of 1 mm and an in-plane resolution of 94 μm .

^1H spectra were obtained using the ultra-short TE (TE/TR = 2.8/4,000 ms, 4,096 complex data points, SW (spectral width) = 7 kHz, 160 averages) SPin ECho, full Intensity Acquired Localized technique (SPECIAL) (Mlynarik *et al*, 2006, 2008). The voxel of interest (VOI) for ^1H MRS was placed in the center of the brain ($5 \times 7 \times 7 \text{ mm}^3$). The reproducibility of the voxel placement was based on anatomical landmarks using the localizer images. After first- and second-order shimming, the typical linewidth of water resonance at TE = 2.8 ms was 16 Hz. The water signal was suppressed by a series of seven 25 ms asymmetric variable power radio frequency (RF) pulses with optimized relaxation delays, i.e., VAPOR (Tkac *et al*, 1999). The water suppression pulses were interleaved with three modules of

outer volume saturation (OVS) as described previously (Tkac *et al*, 1999). To compensate for the magnetic field drift, spectra were collected in blocks of 16 averages, which were stored separately in the memory and corrected for the relative shift in frequency and potential variations in phase before summation.

^{15}N spectra were acquired using a new pulse sequence based on the previously published Single-shot Inversion Recovery based Non-Echo (SIRENE) sequence (Choi *et al*, 2000). Briefly, in the conventional SIRENE sequence adiabatic pulses (HS8 modulation) are applied to invert the Z magnetization in two x-slices adjacent to the VOI. Then, the magnetization in these two slices follows an inversion recovery trajectory and is minimized by adjusting the inversion time. Residual magnetization outside the VOI was further suppressed by using six OVS pulses applied with a nominal 90° flip angle. The thickness of the upper and lower saturated y-slices was different to adapt the geometry of the rat head. An adiabatic half passage (AHP) pulse was then applied for excitation. In addition, bilevel WALTZ-16 was applied on the ^1H channel for Nuclear Overhauser Effect (NOE) and decoupling during acquisition. Due to the large chemical shift difference between $[5\text{-}^{15}\text{N}]\text{Gln}$ and $[2\text{-}^{15}\text{N}]\text{Glu} + \text{Gln}$ (~ 70 p.p.m.) in ^{15}N spectra, when using the conventional sequence with the carrier frequency placed on the $[5\text{-}^{15}\text{N}]\text{Gln}$, the second signal of interest ($[2\text{-}^{15}\text{N}]\text{Glu} + \text{Gln}$) was only partially excited by the AHP RF pulse. Consequently, conventional SIRENE sequence was modified in our study for ^{15}N nuclei by adding a second AHP RF pulse. The two ^{15}N signals of interest were acquired separately in an interleaved mode using two adiabatic excitation pulses with opposite frequency modulations. Figure 1A shows the evolution of the Z magnetization after the first AHP RF pulse with the carrier frequency placed on the $[5\text{-}^{15}\text{N}]\text{Gln}$. As can be seen,

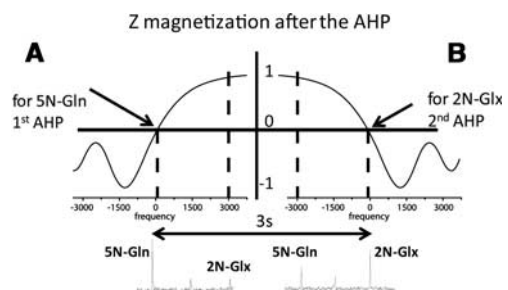


Figure 1 Description of the Z magnetization after the two AHP RF pulses with opposite frequency modulations used interleaved in the new sequence. **(A)** The evolution of the Z magnetization after the first AHP RF pulse with the carrier frequency placed on the $[5\text{-}^{15}\text{N}]\text{Gln}$. As can be seen, after the AHP RF pulse, $[5\text{-}^{15}\text{N}]\text{Gln}$ was fully excited (no longitudinal magnetization left), but the reduction of the Z magnetization of $[2\text{-}^{15}\text{N}]\text{Glu} + \text{Gln}$ was $< 10\%$; **(B)** After 3 seconds, the second AHP RF pulse was applied with the carrier frequency on $[2\text{-}^{15}\text{N}]\text{Glu} + \text{Gln}$ and flipped the magnetization of $[2\text{-}^{15}\text{N}]\text{Glu} + \text{Gln}$ into the transverse plane, whereas the Z magnetization of $[5\text{-}^{15}\text{N}]\text{Gln}$ was only slightly reduced by this pulse. $[5\text{-}^{15}\text{N}]\text{Gln}$ and $[2\text{-}^{15}\text{N}]\text{Glu} + \text{Gln}$ correspond to 5N Gln and 2N Gln in the Figure, respectively. AHP, adiabatic half passage; RF, radio frequency.

after the AHP RF pulse, [5-¹⁵N]Gln was fully excited (no longitudinal magnetization left), but the reduction of the Z magnetization of [2-¹⁵N]Glu+Gln was <10%. After 3 seconds, the second AHP RF pulse was applied with the carrier frequency on [2-¹⁵N]Glu+Gln (Figure 1B). Similarly, the second AHP RF pulse flipped the magnetization of [2-¹⁵N]Glu+Gln into the transverse plane, whereas the Z magnetization of [5-¹⁵N]Gln was only slightly reduced by this pulse. This interleaved excitation scheme allows to selectively excite the two resonances of interest in the same effective TR. In addition, in our new sequence the adiabatic pulses (HS8 modulation) used to invert the Z magnetization in two x-slices adjacent to the VOI were eliminated; and consequently, the localization was performed by using only the OVS modules. Radio frequency power for excitation, NOE, and decoupling was carefully calibrated using a small sphere (3 mm diameter) containing 99% ¹⁵N-enriched glycine (Gly, Cambridge Isotopes) placed at the center of the ¹⁵N linear coil as an external reference as described previously (Gruetter *et al*, 1994, 2003). These calibrations were used to establish minimal power necessary for decoupling. Unlocalized ¹⁵N spectra were acquired in the first hours of infusion (256 scans, TR=6 seconds), followed by a localized spectrum (VOI=5 × 7 × 7 mm³, 256 scans) used for quantification. The robustness of localization was verified from the absence of the Gly signal in the *in vivo* spectra.

¹⁵N-enriched compounds are characterized by a relative long T₁ relaxation time (longitudinal relaxation time). To optimize the TR in the ¹⁵N experiments, the *in vitro* T₁ relaxation time of [2-¹⁵N]Glu was measured using an adiabatic inversion recovery experiment. The inversion time was varied in the range from 0.1 to 20 seconds. To minimize effects of partial saturation, spectra acquired with different inversion time were collected with the same relaxation delay. The T₁ relaxation curves were fitted with nonweighted two-parameter single exponential functions based on the Levenberg-Marquardt algorithm, fitting M(0) and T₁. For each fit, the correlation coefficients reflecting the quality of the least squares fit versus the original data were computed. A typical standard error of the fitted T₁ was ~5%. The *in vitro* T₁ relaxation time of [2-¹⁵N]Glu was 4.0 ± 0.3 seconds.

Brain Extraction and *In Vitro* Nuclear Magnetic Resonance Spectroscopy

At the end of each MRS experiment, the animals were killed by focused microwave irradiation within 2 seconds (4 kW, Gerling Applied Engineering, Modesto, CA, USA), which inactivates brain enzymes before extraction (Morgenthaler *et al*, 2006). The dissected brain was immediately frozen in liquid nitrogen and then stored at -80°C until extraction. The frozen brain was powdered in liquid nitrogen with a mortar and pestle and the water-soluble metabolites were extracted with 0.9 mol/L perchloric acid, as previously described (Duarte *et al*, 2007). After vigorous mixing, the preparation was centrifuged at 21,000 g for 15 minutes at 4°C. The supernatant was then

neutralized with potassium hydroxide (KOH), filtered (Ultrafree-CL filters; Millipore Corporation, Bedford, MA, USA) to remove high molecular weight molecules and dried in a concentrator (SpeedVac Concentrator DNA 120; Thermo Electron Corporation, Milford, MA, USA, PLACE). Afterwards, extracts were resuspended in 500 μL D₂O (99.9% atom D, Sigma-Aldrich, Steinheim, Germany). The pD of the solution was adjusted to ~9 to resolve the [2-¹⁵N]Gln peak from the [2-¹⁵N]Glu peak in high-resolution ¹⁵N spectra. The brain extract measurements were performed on a 600-MHz vertical-bore DMX-600 spectrometer (Bruker, Fallanden, Switzerland). One-dimensional pulse-and-acquire ¹⁵N spectra were measured with NOE, under fully relaxed conditions (TR=100 seconds) and with ¹H decoupling to measure the [2-¹⁵N]Gln and [2-¹⁵N]Glu peaks and the corresponding fractional enrichment (FE) ratio ($[(2-^{15}\text{N})\text{Gln}]/[(2-^{15}\text{N})\text{Glu}]$).

Ammonia concentration in the brain extracts was measured with the Analox GM7 Analyzer as described above for plasma samples.

Spectra Quantification

¹H spectra were quantified using LCModel (<http://s-provencher.com/pages/lcmodel.shtml>) combined with a quantum mechanics simulated basis-set of metabolites based on the density-matrix formalism (Mulkern and Bowers, 1994), using published values of J-coupling constants and chemical shifts (Govindaraju *et al*, 2000), and containing the spectrum of macromolecules measured *in vivo* as described previously (Mlynarik *et al*, 2008; Pfeuffer *et al*, 1999). Absolute quantification was based on the water signal as an internal reference. The Cramér-Rao lower bounds were used as reliability measure of the metabolite concentration estimates.

In vivo and *in vitro* ¹⁵N spectra were analyzed using the jMru software (<http://www.mru.uab.es/mru/>). The signals were fitted using AMARES (Advanced Method for Accurate, Robust, and Efficient Spectral fitting) (Vanhamme *et al*, 1997). For each *in vivo* signal, two Lorentzian spectral components were selected to fit the major contributions of the metabolites ([5-¹⁵N]Gln and [2-¹⁵N]Gln+Glu), the zero-order phase was estimated and the first-order phase was fixed to zero. The precision of the amplitude estimates was assessed using the Cramér-Rao lower bounds. Absolute quantification was performed using an external reference method described previously for ¹³C data (Gruetter *et al*, 2003). Briefly, the amplitudes of the localized *in vivo* signals were compared with those obtained under identical experimental conditions from a phantom solution containing ¹⁵N uniformly labeled glutamine and glutamate. The effect of variable coil loading on sensitivity was assessed by measuring *in vivo* and in the phantom the signal of the sphere placed at the center of the ¹⁵N coil containing an aqueous solution of ¹⁵N-labeled Gly. Both measurements of coil loading effects on sensitivity were within 10% to 15% of each other. The NOE and T₁ effects were corrected *in vivo* and *in vitro* by performing each time two different acquisitions: one performed with NOE using the parameters

of the *in vivo* experiment and the second using fully relaxed conditions in absence of NOE (Gruetter *et al*, 1994, 2003).

Metabolic Modeling

The neuroglial two-compartment metabolic model used in the present study is shown in Figure 2. Ammonia ($\text{NH}_3 + \text{NH}_4^+$) passes the blood–brain barrier and enters glial cells at a rate V_{in} . In astrocytes, ammonia and glutamate are converted to glutamine through GS, thereby labeling the Gln at the position 5N (Figure 2A). Gln is released by the astrocytes and transported into the neurons where it is converted to glutamate by glutaminase, which completes the glutamate–glutamine cycle (Figure 2A). For the labeling of $[2\text{-}^{15}\text{N}]\text{Glu} + \text{Gln}$ (Figure 2B), ammonia is incorporated in glial glutamate through GDH, then glutamate is converted to glutamine by GS. Glutamine is transported into neurons and converted to glutamate by neuronal glutaminase. Since most ammonia is detoxified by the GS reaction (Du Ruisseau *et al*, 1957), which is restricted to astrocytes (Martinez-Hernandez *et al*, 1977; Norenberg and

Martinez-Hernandez, 1979), and little by GDH (Cooper and Plum, 1987), in the present study we assumed that the astrocyte rather than the neuron is uniquely responsible for ammonia detoxification in the brain.

In neurons, the exchange of ^{15}N from glutamate in interaction with the tricarboxylic acid cycle happens through transamination reactions, while GDH is essentially taking place in the astrocytes (Aoki *et al*, 1987; Zaganas *et al*, 2001). We modeled the exchange of ^{15}N from the position 2 of neuronal glutamate to the position 2 of aspartate through transamination, assuming a neuronal aspartate pool of $2\ \mu\text{mol/g}$, as measured by ^1H NMR spectroscopy and in agreement with previous reports (Kunz *et al*, 2010; Xin *et al*, 2010). This interaction has the role of a transient dilution of the neuronal $[2\text{-}^{15}\text{N}]\text{Glu}$ pool. We further varied the exchange flux V_x between glutamate and the neuronal tricarboxylic acid cycle through transamination from 0 to $1\ \mu\text{mol/g}$ per minute, which represents a typical range for this chemical exchange in the anesthetized rat brain (Duarte *et al*, 2011). Varying V_x in this range did not affect the labeling curves of $[2\text{-}^{15}\text{N}]\text{Glx}$ significantly and had very low impact on the other determined metabolic fluxes. The model was therefore simplified and the exchange with the neuronal tricarboxylic acid cycle was neglected. We failed to observe any other amino acids to become enriched. A substantial enrichment of aspartate that occurs at $\sim 2\ \text{mmol/L}$ (almost exclusively in neurons) would be observable in the ^{15}N spectra.

The time courses of total Gln measured by ^1H MRS, $[5\text{-}^{15}\text{N}]\text{Gln}$, and $[2\text{-}^{15}\text{N}]\text{Glu} + \text{Gln}$ were used as experimental data to which the model was fitted as follows. The differential equations derived for $[\text{Gln}]$, $[5\text{-}^{15}\text{N}]\text{Gln}$, and $[2\text{-}^{15}\text{N}]\text{Glu} + \text{Gln}$ (see Appendix) were fitted simultaneously using SAAM II environment (The SAAM Institute, Seattle, WA, USA). Since no direct measurement of the dynamic labeling of brain ammonia was possible, V_{in} was assumed $0.23\ \mu\text{mol/g}$ per minute (Cooper *et al*, 1979; Cooper and Plum, 1987; Shen *et al*, 1998). In addition, we assumed a negligible efflux from the Gln pool compared with V_{syn} (Kanamori and Ross, 1993; Kanamori *et al*, 1996; Shen *et al*, 1998). For the fit of $[2\text{-}^{15}\text{N}]\text{Glu} + \text{Gln}$, we assumed that V_{GDH} was mainly in the glial compartment. This assumption was verified by measuring the FE ratio ($([2\text{-}^{15}\text{N}]\text{Gln})\text{FE}/([2\text{-}^{15}\text{N}]\text{Glu})\text{FE}$) in high-resolution ^{15}N brain extract spectra at a pH of ~ 9 . The time evolution of the total Gln, $[5\text{-}^{15}\text{N}]\text{Gln}$, and $[2\text{-}^{15}\text{N}]\text{Glu} + \text{Gln}$ was fitted simultaneously. The linear fit of the time evolution of the total Gln provided a measure of the net glutamine accumulation ($V_{syn} - V_{nt}$) as well as the initial Gln concentration ($\text{Gln}(0)$). In addition, from the fit of the time evolution of $[5\text{-}^{15}\text{N}]\text{Gln}$ we obtained V_{syn} and from these two fluxes ($V_{syn} - V_{nt}$ and V_{syn}) the neurotransmission rate (V_{nt}) was calculated. Finally, using our model the fit of $[2\text{-}^{15}\text{N}]\text{Glu} + \text{Gln}$ enabled the measurement of V_{GDH} (see Appendix, equations (18), (19) and (23)). Results were generally presented as mean \pm s.d. ($n=6$).

In addition, using the values obtained for the different fluxes, the FEs of $[2\text{-}^{15}\text{N}]\text{Glu}$ and $[2\text{-}^{15}\text{N}]\text{Gln}$ were simulated for the end of the experiment and their ratio was compared with the high-resolution NMR measurements obtained on brain extracts.

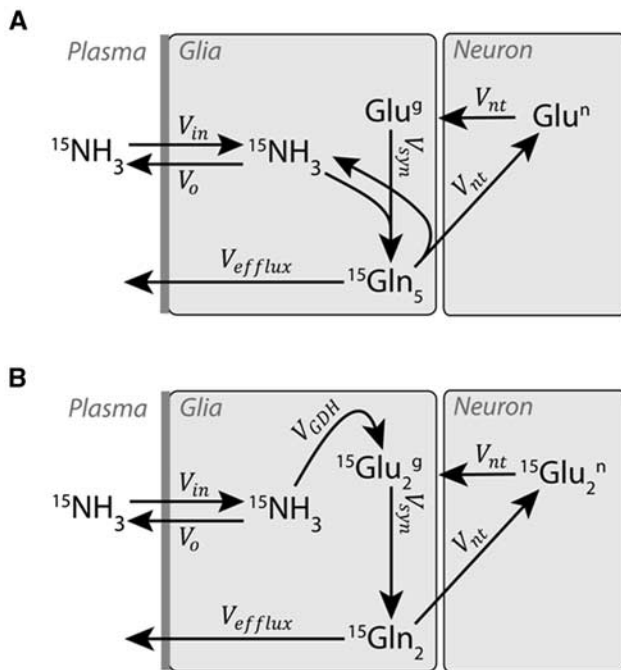


Figure 2 Neuroglial two-compartment metabolic model: **(A)** for $[5\text{-}^{15}\text{N}]\text{Gln}$: ammonia ($\text{NH}_3 + \text{NH}_4^+$) passes the blood–brain barrier (BBB) and enters glial cells at a rate V_{in} . In astrocytes, ammonia and glutamate are converted to glutamine through glutamine synthetase, thereby labeling the Gln at the position 5N. Gln is released by the astrocytes and transported into the neurons where it is converted to glutamate by glutaminase, which completes the glutamate–glutamine cycle; **(B)** for $[2\text{-}^{15}\text{N}]\text{Glu} + \text{Gln}$: ammonia is incorporated in glutamate through glutamate dehydrogenase, then glutamate is converted to glutamine by glutamine synthetase. Glutamine is transported into neurons and converted to glutamate by neuronal glutaminase. Glial glutaminase was neglected.

Results

During the *in vivo* experiments, the physiological variables were maintained within normal physiology (pH = 7.41 ± 0.04 ; PaCO₂ = 40.0 ± 1.8 mm Hg, O₂ saturation >99.8%). The plasma ammonia concentration increased from 0.08 ± 0.02 to 0.95 ± 0.08 mmol/L and remained approximately constant during the experiment. Plasma urea concentration was 7.6 ± 0.8 mmol/L before the ammonium chloride infusion, increased linearly on infusion of ammonium chloride and reached a final concentration of 29.9 ± 10.8 mmol/L. The brain ammonia concentration measured at the end of each experiment in brain extracts was 3.8 ± 0.8 μ mol/g in agreement with previous measurements (Kanamori *et al*, 1993, 1996; Kanamori and Ross, 1993). Because ammonia diffuses across the blood–brain barrier mainly as neutral NH₃ and brain pH (7.1) is lower than the blood pH (7.4), brain ammonia level at equilibrium is normally 1.5 to 3 times that of the blood, which is typically maintained under hyperammonemia (Cooper and Plum, 1987). At the end of the infusion period, we determined a brain ammonia/blood ammonia ratio of 3.8 ± 0.8 (mean \pm s.d.) that was in the range of previous reports (Cooper and Plum, 1987).

In general, ¹H spectra exhibited excellent signal-to-noise ratio allowing easy separation of Gln from Glu. The increase in the total Gln pool at different time points during infusion was thus apparent in the ¹H

spectra (Figure 3). The total Gln (0) concentration (before starting the infusion) was 2.3 ± 0.4 μ mol/g, which increased to 17.7 ± 4.0 μ mol/g at the end of the infusion. As can be seen in Figures 3B and 3C, the increase of total Gln started immediately after the infusion and continued to increase linearly over time. The total concentrations of all other metabolites remained within <10% fluctuation in standard deviation of the mean concentration over the ammonium chloride infusion (i.e., the *N*-acetyl-aspartate concentration before the ammonium chloride infusion was 7.1 ± 0.5 μ mol/g and at the end of infusion was 7.6 ± 0.5 μ mol/g). Although lactate concentration was observed to correlate with the total Gln increase ($R^2 = 0.90$), it never exceeded the physiological range of 2.5 to 3 μ mol/g.

To compare the new acquisition sequence with the conventional SIRENE sequence, Figure 4A shows a series of *in vivo* unlocalized ¹⁵N spectra acquired in the rat brain during ammonium chloride infusion using the new pulse sequence. It can be observed that the [5-¹⁵N]Gln peak (−271 p.p.m.) was detected within 25 minutes, whereas the [2-¹⁵N]Gln + Glu peak (−342 p.p.m.) was detected during the second scan (i.e., after 25 minutes of ammonium chloride infusion). With the conventional pulse sequence (Figure 4B), the [5-¹⁵N]Gln peak was also visible in the first and all subsequent scans; however, the [2-¹⁵N]Gln + Glu peak started to appear only from the fourth scan. Compared with the conventional SIRENE sequence, the new pulse sequence using the

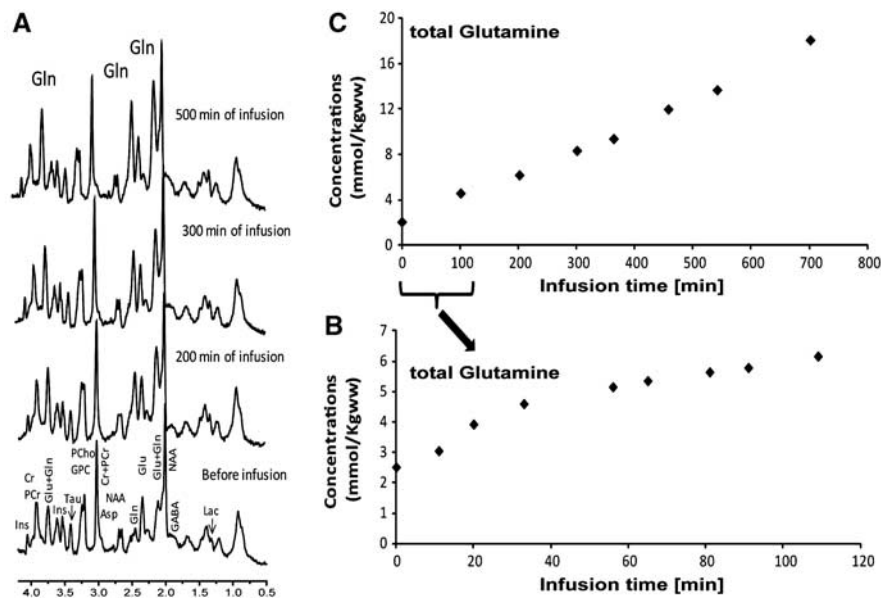


Figure 3 (A) Representative *in vivo* ¹H spectra acquired at 9.4 T in the rat brain during ammonium chloride infusion (TE/TR = 2.8/4,000 ms, 160 averages, voxel size of $5 \times 7 \times 7$ mm³); (B) Detailed time course of total Gln acquired *in vivo* in the first 2 hours of ¹⁵N ammonium chloride infusion; (C) The time course of total Gln acquired *in vivo* in one rat more than ~12 hours of ¹⁵N ammonium chloride infusion. Phosphocholine (PChol), glycerophosphocholine (GPC), *N*-acetyl-aspartate (NAA), lactate (Lac), creatine (Cr), phosphocreatine (PCr), taurine (Tau), myo-inositol (Ins), glutamate (Glu), glutamine (Gln), aspartate (Asp), γ -aminobutyrate (GABA). TE, echo time; TR, repetition time.

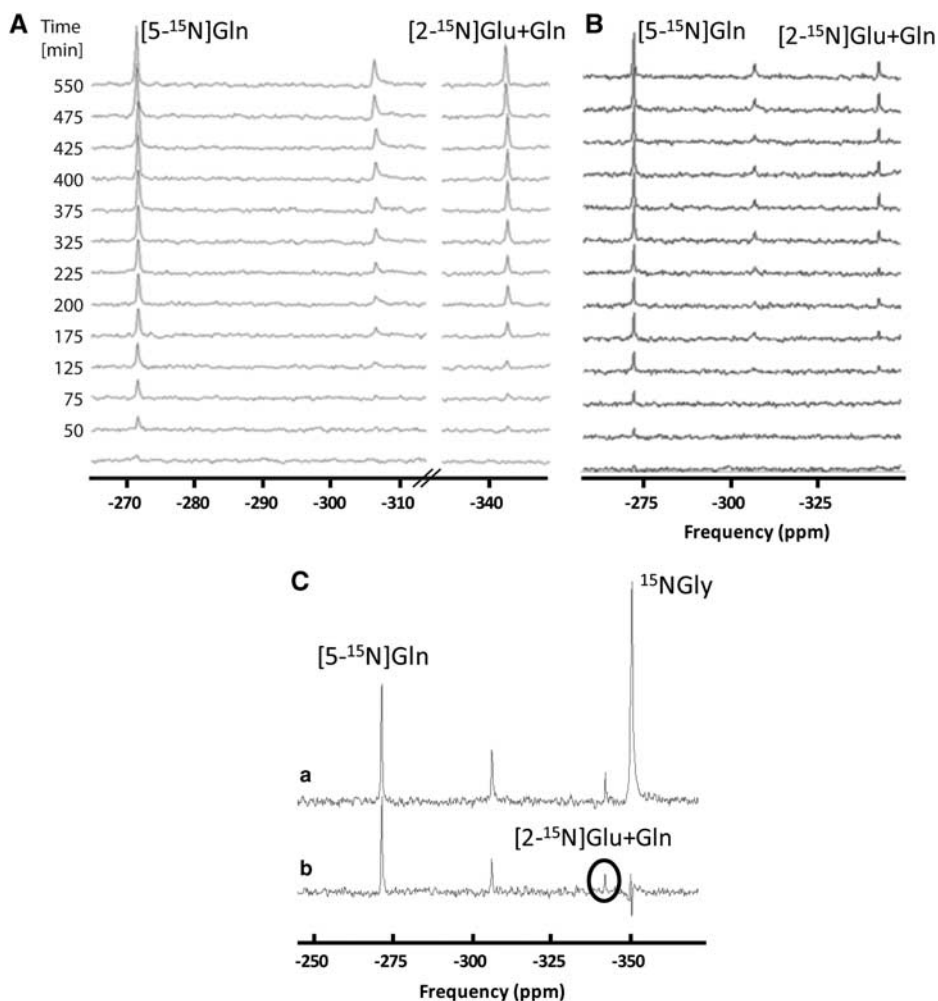


Figure 4 (A) A representative series of *in vivo* unlocalized ¹⁵N spectra acquired using the new sequence with two AHP RF pulses with opposite frequency modulations at 9.4 T in a rat brain at different time points (two spectra were acquired per TR); (B) Representative spectra acquired using the conventional sequence (one spectrum was acquired per TR); (C) Localized *in vivo* ¹⁵N spectrum acquired in a rat brain (a) during ammonium chloride infusion versus an unlocalized spectrum acquired in the same rat (b) at 25 minutes of interval. The performance of the OVS-based localization is proved by the suppression of the ¹⁵N Gly signal, which originates from the sphere placed in the center of the ¹⁵N coil. The ¹⁵N chemical shifts were referenced to nitromethane. The unmarked resonance in the figures represents the urea signal. AHP, adiabatic half passage; RF, radio frequency; TR, repetition time.

two AHP RF pulses allowed the detection of [2-¹⁵N]Gln + Glu peak ~50 minutes earlier due to a better excitation and consequently an increased signal-to-noise ratio.

To assess the performance of the OVS localization technique, Figure 4C shows an example of a localized (a) *in vivo* ¹⁵N spectrum versus an unlocalized one (b) acquired at 25 minutes of interval in the same rat during ammonium chloride infusion. As can be seen from the Figure, the performance of the OVS-based localization is proved by the suppression of the ¹⁵N Gly signal, which originates from the sphere placed in the center of the ¹⁵N coil. In addition, by comparing the two resonances of [5-¹⁵N]Gln we can notice that there are very small differences, suggesting that almost the entire [5-¹⁵N]Gln signal comes from the brain and not from surrounding tissues.

Using the external reference method described previously for ¹³C data (Gruetter *et al*, 2003), we were able to perform a direct *in-vivo* absolute quantification for [5-¹⁵N]Gln and [2-¹⁵N]Gln + Glu. Consequently, at the end of infusion absolute concentrations of [5-¹⁵N]Gln and [2-¹⁵N]Gln + Glu were 13.3 ± 2.9 and 9.6 ± 3.5 $\mu\text{mol/g}$, respectively, in accordance with reported *in vitro* brain extracts determinations (biochemical assays or high-resolution NMR; Kanamori *et al*, 1993, 1996; Kanamori and Ross, 1993; Shen *et al*, 1998).

The time courses of total Gln, [5-¹⁵N]Gln, and [2-¹⁵N]Gln + Glu were highly reproducible in all six rats. The application of the model to the *in vivo* data showed an excellent fit (Figure 5). Using the model presented (Figure 2), we obtained from the ¹H data a net Gln accumulation ($V_{\text{syn}} - V_{\text{nt}}$) of 0.033 ± 0.001 $\mu\text{mol/g}$ per minute (Figure 5A). By

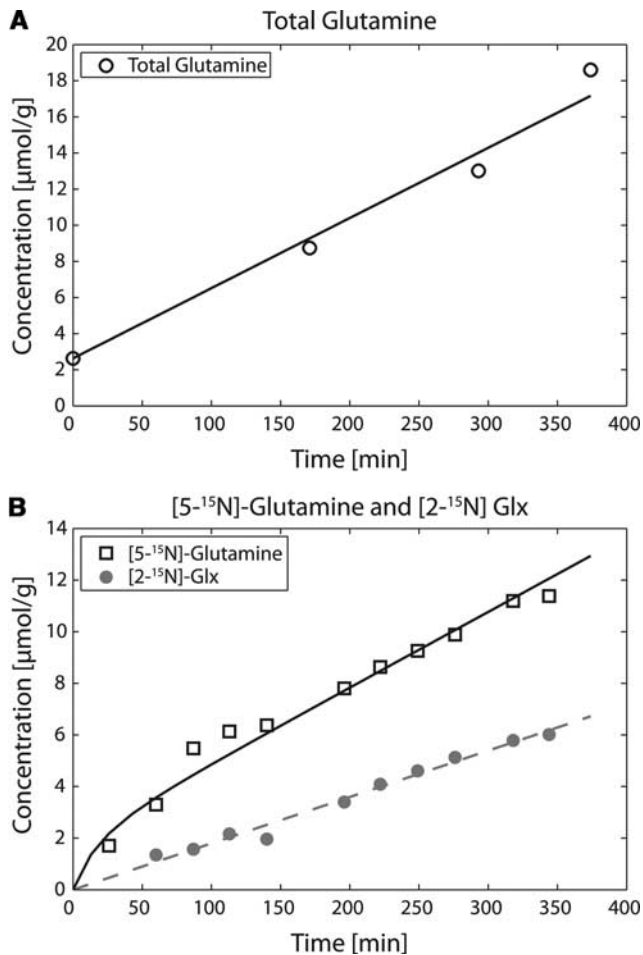


Figure 5 The time courses and corresponding fits of total Gln (A), [5-¹⁵N]Gln and [2-¹⁵N]Gln + Glu (B) from one rat (duration of ammonium chloride infusion ~7 hours). [2-¹⁵N]Gln + Glu corresponds to [2-¹⁵N]Glx in the Figure.

fitting *in vivo* [5-¹⁵N]Gln and [2-¹⁵N]Glu + Glu time courses (Figure 5B), V_{syn} was $0.30 \pm 0.050 \mu\text{mol/g}$ per minute, V_{GDH} was $0.029 \pm 0.002 \mu\text{mol/g}$ per minute, and the plasma NH_3 FE was $91 \pm 5\%$. Finally, the apparent neurotransmission rate, V_{nt} , was $0.26 \pm 0.030 \mu\text{mol/g}$ per minute.

To determine the predominant cellular localization of GDH, we measured the ratio between the FEs of [2-¹⁵N]Gln and [2-¹⁵N]Glu in brain extracts. From high-resolution measurements on brain extracts, we calculated $([2-^{15}\text{N}]\text{Gln})\text{FE}/([2-^{15}\text{N}]\text{Glu})\text{FE} = 1.48 \pm 0.04$ which was in agreement with the simulations done for the FEs of [2-¹⁵N]Glu and [2-¹⁵N]Gln based on the values obtained for the different fluxes.

Discussion

In the present study, we directly and simultaneously measured for the first time V_{syn} , V_{nt} , V_{GDH} , and the net Gln accumulation under ¹⁵N-labeled ammonium chloride infusion in the rat brain. This was possible

by using a new acquisition strategy: *in vivo* localized ¹⁵N MRS interleaved with *in vivo* ¹H MRS. In this context, from the modeling point of view this new acquisition strategy required fewer assumptions to determine metabolic rates.

By acquiring *in vivo* ¹H spectra, we followed the total Gln accumulation during ammonium chloride infusion. Unlike to previous studies, we directly measured for the first time the net Gln accumulation without any assumptions. Previous measurements of net Gln accumulation (i.e., *de-novo* glutamine synthesis; Shen *et al*, 1998) assumed that the increase of glutamine pool size during hyperammonemia is attributed to increased pyruvate carboxylase and GDH activities. Moreover, these studies assumed that pyruvate carboxylase is equal to GDH under hyperammonemia and that together these fluxes account for the net transport of ammonia.

In addition to the acquisition of *in vivo* ¹H spectra, we now simultaneously measured, for the first time, *in vivo* [5-¹⁵N]Gln and [2-¹⁵N]Gln + Glu time courses during infusion of ¹⁵N-enriched ammonium chloride. The new ¹⁵N pulse sequence proposed in the present study, which uses the two AHP RF pulses, allowed the detection of [2-¹⁵N]Gln + Glu peak ~50 minutes earlier compared with the conventional SIRENE sequence, due to a better excitation and consequently an increased signal-to-noise ratio. Moreover, by acquiring localized ¹⁵N spectra and by using the external reference method, we performed a direct *in vivo* absolute quantification for [5-¹⁵N]Gln and [2-¹⁵N]Gln + Glu. Finally, we directly quantified total Gln, [5-¹⁵N]Gln, and [2-¹⁵N]Glu + Glu in the same experiment *in vivo* without the need of *in vitro* brain extracts.

The linear and continuous increase of total Gln during the period of ammonium chloride infusion observed in our *in vivo* ¹H MRS data implies increased anaplerosis (Berl *et al*, 1962; Shen *et al*, 1998; Zwingmann, 2007), which appears to be coupled to the ammonia detoxification pathway. As can be seen in Figures 3B and 3C, the increase of total Gln started immediately after the infusion and was linear over the infusion time, in contrast to previous studies (Rothman *et al*, 1999; Shen *et al*, 1998), suggesting that no delay in Gln accumulation was observed. The total Gln concentration measured at the end of the infusion is comparable to previous studies based on *in vitro* brain extract determinations (Kanamori *et al*, 1993, 1996; Kanamori and Ross, 1993; Shen *et al*, 1998). Using our metabolic model, Gln(0) (initial total glutamine concentration measured under standard physiological conditions) was also fitted and the obtained value ($2.4 \pm 0.3 \mu\text{mol/g}$) is in agreement with the measured concentration of $2.3 \pm 0.4 \mu\text{mol/g}$. Contrary to previous studies under ammonia infusion, we continuously quantified the total Gln increase over time, which allowed us to directly measure net Gln accumulation without any assumptions. The obtained $V_{\text{syn}} - V_{\text{nt}}$ of $0.033 \pm 0.001 \mu\text{mol/g}$ per minute was twofold lower than

the previously reported value under hyperammonemia measured indirectly by extrapolation using ^{15}N MRS (Shen *et al*, 1998). The main source of discrepancy between our study and the previous one may be related to the fact that the previous study required modeling extrapolation to estimate net Gln accumulation.

The pyruvate carboxylation flux measured using ^{13}C MRS under normoammonemic conditions is well known to be high in the conscious rat (Oz *et al*, 2004) and nearly absent under isoelectricity (Choi *et al*, 2002; Sibson *et al*, 1998), whereas under light α -chloralose anesthesia was measured to be $0.07\ \mu\text{mol/g}$ per minute (Duarte *et al*, 2011). In the present study, the measurement of the pyruvate carboxylation flux gave a lower limit, which is likely to be higher due to pyruvate recycling and glutamine efflux. Consequently, the *de-novo* glutamine synthesis measured in our study under hyperammonemia might be assumed to be equal to the sum of the pyruvate carboxylation flux measured under normoammonemic conditions (Duarte *et al*, 2011) and the net Gln accumulation measured in our study under hyperammonemia, i.e., $0.07 + 0.033 = 0.103\ \mu\text{mol/g}$ per minute. This estimated value under hyperammonemia for the *de-novo* glutamine synthesis is similar to previously reported unidirectional measurements under hyperammonemia using ^{13}C MRS (Sibson *et al*, 2001) and indicated a significant increase in anaplerotic flux during hyperammonemia supporting the hypothesis that the anaplerotic flux is coupled to nitrogen removal from the brain (ammonia detoxification) under hyperammonemia conditions (Berl *et al*, 1962; Sibson *et al*, 2001; Zwingmann, 2007).

The incorporation of ^{15}N of ammonia into $[5\text{-}^{15}\text{N}]\text{Gln}$ gave a V_{syn} of $0.30 \pm 0.050\ \mu\text{mol/g}$ per minute, which was higher than previous published data under hyperammonemia ranging from 0.06 to $0.20\ \mu\text{mol/g}$ per minute obtained when using ^{15}N MRS (Kanamori *et al*, 1993, 1996; Kanamori and Ross, 1993; Shen *et al*, 1998) and lower than 0.29 to $0.43\ \mu\text{mol/g}$ per minute (Sibson *et al*, 1997, 2001) obtained when using ^{13}C MRS. As discussed previously, the main source of discrepancy between our study and the previous ones might be related to the increased number of assumptions used for the modeling of the previous studies. Together with V_{syn} , we also fitted the plasma NH_3 FE which was $91 \pm 5\%$, consistent with experimental measurements (Shen *et al*, 1998). The apparent GS rate obtained in the present study under hyperammonemia conditions $0.30 \pm 0.050\ \mu\text{mol/g}$ per minute (see Results) was higher than the value of $0.18\ \mu\text{mol/g}$ per minute reported under normoammonemic conditions using ^{13}C MRS (Duarte *et al*, 2011), supporting the role of GS as a detoxification pathway for ammonia in the brain.

From the values of $V_{\text{syn}} - V_{\text{nt}}$ of $0.033 \pm 0.001\ \mu\text{mol/g}$ per minute and V_{syn} of $0.30 \pm 0.050\ \mu\text{mol/min}$ per gram, we obtained a V_{nt} of $0.26 \pm 0.030\ \mu\text{mol/g}$ per

minute. Our results (see Results) indicate that under hyperammonemia conditions the neurotransmission rate represents $\sim 80\%$ of the flux through the GS which is comparable to the values reported previously using ^{15}N MRS or ^{13}C MRS (Shen *et al*, 1998; Sibson *et al*, 1997, 2001). Since in the present study, we are assuming a negligible efflux from the Gln pool compared with V_{syn} based on previous reports (Kanamori and Ross, 1993; Kanamori *et al*, 1996; Shen *et al*, 1998), the obtained neurotransmission rate V_{nt} represents an upper limit. By considering a Gln efflux of $0.07\ \mu\text{mol/g}$ per minute (Duarte *et al*, 2011), we obtain a lower limit for V_{nt} of $0.19\ \mu\text{mol/g}$ per minute which is similar with the value reported under normoammonemic conditions using ^{13}C MRS (Duarte *et al*, 2011).

Although $[2\text{-}^{15}\text{N}]\text{Glu}$ and $[2\text{-}^{15}\text{N}]\text{Gln}$ cannot be separated in ^{15}N spectra *in vivo*, the application of the model (Figure 2B; equations (18), (19) and (23) from Appendix) provided a V_{GDH} with a high precision. Consequently, the incorporation of ^{15}N into $[2\text{-}^{15}\text{N}]\text{Gln} + \text{Glu}$ alone, provided a V_{GDH} of $0.029 \pm 0.002\ \mu\text{mol/g}$ per minute, where V_{syn} and V_{nt} needed to model the exchange between $[2\text{-}^{15}\text{N}]\text{Glu}$ and $[2\text{-}^{15}\text{N}]\text{Gln}$ were obtained from the simultaneous fit of the position 5N and total Gln. To maintain a constant Glu concentration with increasing Gln, glutamate needs to be synthesized from tricarboxylic acid intermediates. To determine the predominant cellular localization of GDH, we performed high-resolution measurements on brain extracts and consequently measured the ratio between the FEs of $[2\text{-}^{15}\text{N}]\text{Glu}$ and $[2\text{-}^{15}\text{N}]\text{Gln}$ (see Results). From these measurements, we obtained $([2\text{-}^{15}\text{N}]\text{Gln})\text{FE} > ([2\text{-}^{15}\text{N}]\text{Glu})\text{FE}$ which showed that net glutamate synthesis results from higher GDH activity in the glial compartment. Note that previous studies assumed that this replenishment takes place primarily in the glial compartment in hyperammonemic conditions (Shen *et al*, 1998). The high glial GDH flux obtained in our study is in agreement with higher specific activity in astrocytes relative to neurons (Schmitt and Kugler, 1999; Zaganas *et al*, 2001). In the presented model, a unidirectional flux was assumed for GDH in the astrocytes. When including a reversible flux for the GDH reaction, the reversible component of GDH was always converging to zero in the fitting process. In addition, our results also showed that under hyperammonemia $V_{\text{GDH}} \approx (V_{\text{syn}} - V_{\text{nt}})$, meaning that the rate of breakdown of Glu to glial 2-oxoglutarate was negligible. To our knowledge, there was only one previous study which measured V_{GDH} under ^{15}N ammonia infusion (Kanamori and Ross, 1995) reporting a V_{GDH} value of 0.018 to $0.020\ \mu\text{mol/min}$ per gram for an infusion rate of $3.3\ \text{mmol/kg}$ per hour, which is consistent with our results considering that our infusion rate was higher ($4.5\ \text{mmol/kg}$ per hour) and thus likely to stimulate glutamine synthesis.

The incorporation of ^{15}N of ammonia takes place mainly into $[5\text{-}^{15}\text{N}]\text{Gln}$ through V_{syn} and into

[2-¹⁵N]Glu + Gln through V_{GDH} . Since V_{GDH} obtained in our study represents only 10% from the obtained V_{syn} , it supports the concept of GS as a major detoxification pathway for ammonia in the brain.

We conclude that it is feasible to combine localized *in vivo* ¹⁵N with ¹H MRS and with an appropriate mathematical model to directly, simultaneously and reliably detect for the first time along with V_{syn} , the net glutamine accumulation, V_{nt} and V_{GDH} under ammonia infusion in the *in vivo* rat brain. From the modeling point of view, this new acquisition strategy required fewer assumptions to determine metabolic rates. In addition, this new strategy allowed a robust absolute quantification of total Gln, [5-¹⁵N]Gln and [2-¹⁵N]Gln + Glu in the same experiment. Our results showed an increase of GS and net glutamine accumulation under hyperammonemia, supporting the concept of their implication in cerebral ammonia detoxification. In addition, under hyperammonemia conditions the neurotransmission rate represents ~80% of the flux through the GS. The higher ([2-¹⁵N]Gln)FE compared with ([2-¹⁵N]Glu)FE showed that net glutamate synthesis results from higher GDH activity in the glial compartment. Our results also showed that under hyperammonemia the rate of breakdown of Glu to glial 2-oxoglutarate was negligible because $V_{\text{GDH}} \approx (V_{\text{syn}} - V_{\text{nt}})$. Finally, the role of GS as an important detoxification pathway for ammonia in the brain was also supported by the fact that, in our study, the obtained V_{GDH} was smaller than V_{syn} .

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Appendix

Derivation of the Differential Equations for Brain Metabolism During [¹⁵N]Ammonia Infusion

(A) Modeling of the Total Glutamine Concentration (¹H MRS Measurement)

In the neuroglial two-compartment metabolic model (Figure 2), metabolic steady-state was assumed for the metabolic fluxes, i.e., the fluxes V are assumed to be constant throughout the duration of the experiment.

Applying mass balance, we obtained:

$$\frac{d[\text{Glu}]}{dt} = \frac{d[\text{Glu}^n]}{dt} + \frac{d[\text{Glu}^g]}{dt} = V_{nt} - V_{nt} + V_{nt} + V_g - V_{syn} = V_{nt} + V_g - V_{syn} \quad (1)$$

$$\frac{d[\text{Gln}]}{dt} = V_{syn} - V_{nt} - V_{efflux} \quad (2)$$

However, as confirmed by the ¹H NMR spectra, not all the concentrations of the metabolites of interest were constant over time in conditions of hyperammonemia. The concentration of glutamate was stable, while the concentration of glutamine increased linearly over time. The constant glutamate concentration gave the following relationship between the fluxes:

$$\frac{d[\text{Glu}]}{dt} = 0 \Rightarrow V_{syn} = V_{nt} + V_g \quad (3)$$

Assuming constant metabolic fluxes, the differential equation for the total glutamine concentration can be solved:

$$[\text{Gln}](t) = [\text{Gln}](0) + (V_{syn} - V_{nt} - V_{efflux})t \quad (4)$$

It was reported (Kanamori and Ross, 1993; Kanamori *et al*, 1996; Shen *et al*, 1998) that V_{efflux} was small compared with V_{syn} and equation 4 was therefore simplified to:

$$[\text{Gln}](t) = [\text{Gln}](0) + (V_{syn} - V_{nt})t \quad (5)$$

Where $[\text{Gln}](0)$ is the initial total glutamine concentration measured under standard physiological conditions, i.e., before starting the infusion of ammonia.

(B) Modeling of the [^{5-¹⁵N]} Glutamine Concentration

We assumed metabolic steady-state conditions for the fluxes and involved chemical pools, except for glutamine. The labeling equation for brain ammonia was given by:

$$\frac{d[\text{NH}_3]_{\text{brain}}}{dt} = V_{in} + V_{nt} - (V_o + V_{syn} + V_{GDH}) = 0 \quad (6)$$

$$\Rightarrow V_o = V_{in} + V_{nt} - V_{syn} - V_{GDH}$$

$$\begin{aligned} \frac{d^{15}\text{NH}_3_{\text{brain}}}{dt} &= V_{in} \frac{^{15}\text{NH}_3_{\text{plasma}}}{[\text{NH}_3]_{\text{plasma}}} + V_{nt} \frac{[5-^{15}\text{N}]\text{Gln}}{[\text{Gln}]} \\ &\quad - (V_o + V_{syn} + V_{GDH}) \frac{^{15}\text{NH}_3_{\text{brain}}}{[\text{NH}_3]_{\text{brain}}} \end{aligned} \quad (7)$$

The isotopic enrichment of ammonia in plasma ($\text{FE}_{\text{plasma}}$) was assumed to be constant during the infusion period, which was valid and consistent with relatively low ammonia levels before infusion (0.08 ± 0.02 mmol). Using the result of equation (6), equation (7) can be rewritten:

$$\frac{d^{15}\text{NH}_3_{\text{brain}}}{dt} = V_{in} \text{FE}_{\text{plasma}} + V_{nt} \frac{[5-^{15}\text{N}]\text{Gln}}{[\text{Gln}]} - (V_{in} + V_{nt}) \frac{^{15}\text{NH}_3_{\text{brain}}}{[\text{NH}_3]_{\text{brain}}} \quad (8)$$

The labeling of [^{5-¹⁵N]} glutamine was thus given by:

$$\frac{d[5-^{15}\text{N}]\text{Gln}}{dt} = V_{syn} \frac{^{15}\text{NH}_3_{\text{brain}}}{[\text{NH}_3]_{\text{brain}}} - V_{nt} \frac{[5-^{15}\text{N}]\text{Gln}}{[\text{Gln}]} \quad (9)$$

The first term on the right of equation (9) can be isolated from the labeling equation of brain ammonia (equation (8)). Equation (9) is then rewritten as:

$$\begin{aligned} \frac{d[5-^{15}\text{N}]\text{Gln}}{dt} &= V_{syn} \left(\frac{V_{in}}{(V_{in} + V_{nt})} \text{FE}_{\text{plasma}} + \frac{V_{nt}}{(V_{in} + V_{nt})} \frac{[5-^{15}\text{N}]\text{Gln}}{[\text{Gln}]} \right. \\ &\quad \left. - \frac{1}{(V_{in} + V_{nt})} \frac{d^{15}\text{NH}_3_{\text{brain}}}{dt} \right) - V_{nt} \frac{[5-^{15}\text{N}]\text{Gln}}{[\text{Gln}]} \end{aligned} \quad (10)$$

$$\begin{aligned} \frac{d[5-^{15}\text{N}]\text{Gln}}{dt} + \frac{V_{syn}}{(V_{in} + V_{nt})} \frac{d^{15}\text{NH}_3_{\text{brain}}}{dt} \\ = V_{syn} \left(\frac{V_{in}}{(V_{in} + V_{nt})} \text{FE}_{\text{plasma}} + \frac{V_{nt}}{(V_{in} + V_{nt})} \frac{[5-^{15}\text{N}]\text{Gln}}{[\text{Gln}]} \right) - V_{nt} \frac{[5-^{15}\text{N}]\text{Gln}}{[\text{Gln}]} \end{aligned} \quad (11)$$

Changes in $\text{NH}_3_{\text{brain}}$ were assumed to be small in comparison with the glutamine pool ('small pool approximation'; Duarte *et al*, 2011; Uffmann and Gruetter, 2007), i.e.:

$$\frac{d[5-^{15}\text{N}]\text{Gln}}{dt} \gg \frac{d^{15}\text{NH}_3_{\text{brain}}}{dt} \quad (12)$$

Equation (11) is then simplified to:

$$\frac{d[5-^{15}\text{N}]\text{Gln}}{dt} = \frac{V_{syn} V_{in}}{(V_{in} + V_{nt})} \text{FE}_{\text{plasma}} + \frac{V_{syn} V_{nt}}{(V_{in} + V_{nt})} \frac{[5-^{15}\text{N}]\text{Gln}}{[\text{Gln}]} - V_{nt} \frac{[5-^{15}\text{N}]\text{Gln}}{[\text{Gln}]} \quad (13)$$

In equation (13), not only the ¹⁵N-labeled metabolite concentrations are changing over time, but also $[\text{Gln}]$ was increasing, as described in part (A) of this appendix. Using equation (5), equation (13) can be rewritten more explicitly:

$$\frac{d[5-^{15}\text{N}]\text{Gln}}{dt} = \frac{V_{\text{syn}} V_{\text{in}}}{(V_{\text{in}} + V_{\text{nt}})} \text{FE}_{\text{plasma}} + \frac{V_{\text{syn}} V_{\text{nt}}}{(V_{\text{in}} + V_{\text{nt}})} \frac{[5-^{15}\text{N}]\text{Gln}}{[\text{Gln}](0) + (V_{\text{syn}} - V_{\text{nt}})t} - V_{\text{nt}} \frac{[5-^{15}\text{N}]\text{Gln}}{[\text{Gln}](0) + (V_{\text{syn}} - V_{\text{nt}})t} \quad (14)$$

$$\frac{d[5-^{15}\text{N}]\text{Gln}}{dt} = \frac{V_{\text{syn}} V_{\text{in}}}{(V_{\text{in}} + V_{\text{nt}})} \text{FE}_{\text{plasma}} - V_{\text{nt}} \left[\frac{(V_{\text{in}} + V_{\text{nt}}) - V_{\text{syn}}}{(V_{\text{in}} + V_{\text{nt}})} \right] \frac{[5-^{15}\text{N}]\text{Gln}}{[\text{Gln}](0) + (V_{\text{syn}} - V_{\text{nt}})t} \quad (15)$$

From the equation (15), the initial slope of the $[5-^{15}\text{N}]\text{Gln}$ labeling curve can be derived:

$$\lim_{t \rightarrow 0} \left(\frac{d[5-^{15}\text{N}]\text{Gln}}{dt} \right) = \frac{V_{\text{syn}} V_{\text{in}}}{(V_{\text{in}} + V_{\text{nt}})} \text{FE}_{\text{plasma}}, \quad (16)$$

since $[5-^{15}\text{N}]\text{Gln}(0) = 0$

(C) Modeling of the $[2-^{15}\text{N}]$ Glutamine and Glutamate Concentrations

From the model presented in Figure 2B, the rate of labeling of $[2-^{15}\text{N}]\text{Glu}^g$, $[2-^{15}\text{N}]\text{Glu}^n$, $[2-^{15}\text{N}]\text{Gln}$ was expressed as:

$$\frac{d[2-^{15}\text{N}]\text{Glu}^g}{dt} = V_{\text{GDH}} \frac{^{15}\text{NH}_3_{\text{brain}}}{[\text{NH}_3]_{\text{brain}}} - V_{\text{syn}} \frac{[2-^{15}\text{N}]\text{Glu}^g}{[\text{Glu}^g]} + V_{\text{nt}} \frac{[2-^{15}\text{N}]\text{Glu}^n}{[\text{Glu}^n]} \quad (17)$$

$$\frac{d[2-^{15}\text{N}]\text{Glu}^n}{dt} = V_{\text{nt}} \frac{[2-^{15}\text{N}]\text{Gln}}{[\text{Gln}]} - V_{\text{nt}} \frac{[2-^{15}\text{N}]\text{Glu}^n}{[\text{Glu}^n]} \quad (18)$$

$$\frac{d[2-^{15}\text{N}]\text{Gln}}{dt} = V_{\text{syn}} \frac{[2-^{15}\text{N}]\text{Glu}^g}{[\text{Glu}^g]} - V_{\text{nt}} \frac{[2-^{15}\text{N}]\text{Gln}}{[\text{Gln}]} \quad (19)$$

Again, $[\text{Gln}]$ is not a constant pool in the case of hyperammonemia and has to be included in the

modeling. The ‘small pool approximation’ (Duarte *et al*, 2011; Uffmann and Gruetter, 2007) was assumed for $^{15}\text{NH}_3_{\text{brain}}$. Using equation (8), the isotopic enrichment of brain ammonia can be isolated and injected in equation (17):

$$\frac{d[2-^{15}\text{N}]\text{Glu}^g}{dt} = V_{\text{GDH}} \left(\frac{V_{\text{in}}}{(V_{\text{in}} + V_{\text{nt}})} \text{FE}_{\text{plasma}} + \frac{V_{\text{nt}}}{(V_{\text{in}} + V_{\text{nt}})} \frac{[5-^{15}\text{N}]\text{Gln}}{[\text{Gln}]} - \frac{1}{(V_{\text{in}} + V_{\text{nt}})} \frac{d^{15}\text{NH}_3_{\text{brain}}}{dt} \right) - V_{\text{syn}} \frac{[2-^{15}\text{N}]\text{Glu}^g}{[\text{Glu}^g]} + V_{\text{nt}} \frac{[2-^{15}\text{N}]\text{Glu}^n}{[\text{Glu}^n]} \quad (20)$$

$$\begin{aligned} \frac{d[2-^{15}\text{N}]\text{Glu}^g}{dt} &+ V_{\text{GDH}} \frac{1}{(V_{\text{in}} + V_{\text{nt}})} \frac{d^{15}\text{NH}_3_{\text{brain}}}{dt} \\ &= V_{\text{GDH}} \left(\frac{V_{\text{in}}}{(V_{\text{in}} + V_{\text{nt}})} \text{FE}_{\text{plasma}} + \frac{V_{\text{nt}}}{(V_{\text{in}} + V_{\text{nt}})} \frac{[5-^{15}\text{N}]\text{Gln}}{[\text{Gln}]} \right) \\ &- V_{\text{syn}} \frac{[2-^{15}\text{N}]\text{Glu}^g}{[\text{Glu}^g]} + V_{\text{nt}} \frac{[2-^{15}\text{N}]\text{Glu}^n}{[\text{Glu}^n]} \end{aligned} \quad (21)$$

The ‘small pool approximation’ was given by the following expression:

$$\frac{d[2-^{15}\text{N}]\text{Glu}^g}{dt} \gg \frac{d^{15}\text{NH}_3_{\text{brain}}}{dt} \quad (22)$$

Finally, the labeling equation of glial glutamate (equation (17)) can be directly written in terms of plasma ammonia enrichment as:

$$\begin{aligned} \frac{d[2-^{15}\text{N}]\text{Glu}^g}{dt} &= V_{\text{GDH}} \left(\frac{V_{\text{in}}}{(V_{\text{in}} + V_{\text{nt}})} \text{FE}_{\text{plasma}} + \frac{V_{\text{nt}}}{(V_{\text{in}} + V_{\text{nt}})} \frac{[5-^{15}\text{N}]\text{Gln}}{[\text{Gln}]} \right) \\ &- V_{\text{syn}} \frac{[2-^{15}\text{N}]\text{Glu}^g}{[\text{Glu}^g]} + V_{\text{nt}} \frac{[2-^{15}\text{N}]\text{Glu}^n}{[\text{Glu}^n]} \end{aligned} \quad (23)$$

Equations (18), (19) and (23) are a set of three coupled differential equations, also coupled to the $[5-^{15}\text{N}]\text{Gln}$ enrichment. The measured $[2-^{15}\text{N}]\text{Glu} + \text{Gln}$ was then fitted to the sum of the numerical solution of these equations.