The turnover of the H3 deuterons from (2-\textsuperscript{13}C) glutamate and (2-\textsuperscript{13}C) glutamine reveals subcellular trafficking in the brain of partially deuterated rats

Sebastián Cerdán,* Alejandra Sierra,* Luis L. Fonseca,† Paloma Ballesteros‡ and Tiago B. Rodrigues*§

*Instituto de Investigaciones Biomédicas “Alberto Sols” CSIC/UAM, Madrid, Spain
†Instituto de Tecnología Química e Biológica (ITQB), Oeiras, Portugal
‡Laboratorio de Síntesis Orgánica e Imagen Molecular, Instituto Universitario de Investigación, UNED, Madrid, Spain
§ Faculdade de Ciências e Tecnologia, Centro de Neurociências e Biologia Celular, Universidade de Coimbra, Coimbra, Portugal

A plethora of dynamic \textsuperscript{13}C NMR approaches have been proposed in the last decades to monitor cerebral metabolism \textit{in vivo} and \textit{in vitro} through the exchange of the naturally occurring \textsuperscript{12}C of cerebral metabolites by the \textsuperscript{13}C derived from appropriate precursors (Cruz and Cerdan 1999; Garcia-Espinosa \textit{et al}. 2004; Shulman and Rothman 2004; Rodrigues \textit{et al}. 2007). Normally, (1-\textsuperscript{13}C) glucose or (2-\textsuperscript{13}C) acetate precursors were routinely used to investigate the neuronal or glial metabolism, respectively (Garcia-Espinosa \textit{et al}. 2003; Rodrigues and Cerdan 2005; Ramirez \textit{et al}. 2007). Mathematical analysis of \textsuperscript{13}C turnover curves, as monitored by \textsuperscript{13}C NMR, provided quantitative values for the cerebral tricarboxylic acid (TCA) cycle flux and its neuronal or glial components, as well as for the glutamate-glutamine or GABA cycles among other processes (Mason \textit{et al}. 1992a, 1995; Künnecke \textit{et al}. 1993; Shen \textit{et al}. 1999; Gruetter \textit{et al}. 2001; Lebon \textit{et al}. 2002; Rodrigues and Cerdan 2007). This progress led \textsuperscript{13}C NMR approaches to become a well-established tool in the investigation of cerebral energetics and

Abstract
We investigated by \textsuperscript{13}C NMR the turnover of the H3 deuterons of (2-\textsuperscript{13}C) glutamate and (2-\textsuperscript{13}C) glutamine in the brain of partially deuterated rats. Adult animals (150–200 g) fed ad \textit{libitum} received 50% \textsuperscript{2}H\textsubscript{2}O or tap water 9 days before infusing (1-\textsuperscript{13}C) glucose or (2-\textsuperscript{13}C) acetate for 5, 10, 15, 30, 60, or 90 min. The brains were then funnel-frozen and acid extracts were prepared and analyzed by high-resolution \textsuperscript{13}C NMR. The deuteration of one or the two H3 hydrogens of (2-\textsuperscript{13}C) glutamate or glutamine resulted in single (\textit{\textsuperscript{0.07 ppm}) or double (\textit{\textsuperscript{0.14 ppm}}) isotopic shifts upfield of the corresponding C2 perprotonated resonance, demonstrating two sequential deuteration steps. The faster monodeuteration generated 3\textit{R} or 3\textit{S} (2-\textsuperscript{13}C, 3-\textsuperscript{2}H) glutamate or glutamine through the alternate activities of cerebral aconitase or isocitrate dehydrogenase, respectively. The slower process produced bideuterated (2-\textsuperscript{13}C, 3,3’-\textsuperscript{2}H\textsubscript{2}) glutamate or glutamine through the consecutive activity of both enzymes. The kinetics of deuteration was fitted to a Michaelis–Menten model including the apparent \(K_m\) and \(V_{max}\) values for the observed deuterations. Our results revealed different kinetic constants for the alternate and consecutive deuterations, suggesting that these processes were caused by the different cytosolic or mitochondrial isoforms of aconitase and isocitrate dehydrogenase, respectively. The deuterations of (2-\textsuperscript{13}C) glutamate or glutamine followed also different kinetics from (1-\textsuperscript{13}C) glucose or (2-\textsuperscript{13}C) acetate, revealing distinct deuteration environments in the neuronal or glial compartments.

Keywords: \textsuperscript{13}C NMR, aconitase, deuterated rats, isocitrate dehydrogenase, subcellular compartmentation.


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Address correspondence and reprint requests to Sebastián Cerdán, Laboratory for Imaging and Spectroscopy by Magnetic Resonance LISMAR, Instituto de Investigaciones Biomédicas “Alberto Sols” Calle, Arturo Duperier 4, Madrid E-28029, Spain. E-mail: scerdan@iib.uam.es

\textbf{Abbreviations used:} AC, aconitase; Gln, glutamine; Glu, glutamate; Glx, glutamate and/or glutamine and/or \(\alpha\)-ketoglutarate; ICDH, isocitrate dehydrogenase; \(K_m^\prime\), apparent \(K_m\) \textit{under in vivo} conditions; TCA, tricarboxylic acid; \(V_{max}^\prime\), apparent \(V_{max}\) \textit{under in vivo} conditions.

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metabolism (Sonnewald et al. 1996; Gruetter 2002; Rodrigues and Cerdan 2005).

Classical $^{13}$C NMR approaches involved necessarily, however, relatively long acquisitions to accumulate sufficient signal-to-noise in order to be able to observe appropriately the $^{13}$C resonances, both in vivo and in vitro. This limitation does not allow time resolution of metabolic processes occurring in a faster time-scale than the $^{13}$C NMR acquisitions. More importantly, $^{13}$C NMR is not able to distinguish directly between metabolic processes occurring without a change in the distribution of $^{13}$C label within the same metabolite, as it happens in many exchange or transport reactions as well as in some rapid enzymatic processes such as hydration–dehydration reactions.

To overcome these limitations, we proposed earlier the use of a double $^2$H and $^{13}$C-labeling technique, capable of following metabolic processes under the faster time-scale of hydrogen turnover and of discriminating between different deuterated isotopomers of the same $^{13}$C isotopomer (Garcia-Martin et al. 2001). We first implemented this method in the perfused mouse liver metabolizing (3-$^{13}$C) alanine and 50% $^2$H$_2$O. By monitoring the exchange of the H3 hydrogens of (2-$^{13}$C) glutamate, it was possible to follow its subcellular trafficking through the cytosolic and mitochondrial compartments, a process not detectable previously by $^{13}$C NMR (Garcia-Martin et al. 2002; Cerdan et al. 2003).

The pattern of $^{13}$C and $^2$H labeling in the cerebral TCA cycle intermediates during the simultaneous metabolism of (1-$^{13}$C) glucose or (2-$^{13}$C) acetate in the presence of $^2$H$_2$O is illustrated in Fig. S1. The present work focuses on the turnover of the H3 hydrogens of (2-$^{13}$C) glutamate and glutamine in the adult rat brain of deuterated animals, metabolizing (1-$^{13}$C) glucose or (2-$^{13}$C) acetate. H3 turnover studies revealed the existence of two consecutive deuteration processes involving first, the alternate deuteration of the H3$_{proH}$ or H3$_{proS}$ hydrogens and second, the consecutive bidexteration of both H3 hydrogens of the same (2-$^{13}$C) glutamate or glutamine isotopomer. Our results indicated that the alternate and consecutive deuterations were described by different kinetic constants, as expected from the cytosolic and mitochondrial isoforms of aconitase (AC) and isocitrate dehydrogenase (ICDH), thus providing insight into the subcellular trafficking of cerebral (2-$^{13}$C) glutamate and glutamine in the neuronal and glial compartments. Preliminary accounts of this work have been published (Sierra et al. 2004, 2005).

**Materials and methods**

**Preparation of partially deuterated animals and infusion conditions**

The experimental protocols used in this study were approved by appropriate institutional review committees and met the guidelines of the responsible governmental agency, all efforts being made to minimize animal suffering. Experiments were carried out in well-nourished adult male Wistar rats (150–180 g, n = 62), fed with a standard laboratory rat chow. Nine days before killing, drinking water of half of the animals was replaced by water containing 50% (vol/vol) $^2$H$_2$O (deuterated animals, n = 36), while the other remained drinking tap water (control animals, n = 36). All animals were placed in metabolic cages in a humidity- and temperature-controlled room on a 12-hour light/dark cycle and were allowed to drink and eat ad libitum during the 9 days of deuteration. The weight of the animals, the amounts of water and food consumed, and the urine released were determined (Fig. S2). Drinking water containing 50% $^2$H$_2$O (vol/vol) was prepared and used daily.

The degree of deuteration achieved by the animals during $^2$H$_2$O pre-treatment was determined in 20 μL urine samples obtained daily from each metabolic cage by attenuated total reflection Fourier transform infrared spectroscopy using a Bruker Spectrometer IFS28 (Bruker, Ettlingen, Germany). Spectral processing was carried out using the Bruker OPUS software. Basically, the areas under the peaks at 2500 and 3300 per cm reflecting the deuterated water vibrations were determined and compared with model solutions containing known concentrations of deuterium oxide. A time-dependent increase in the relative urine deuteration was observed (Fig. S2) and fitted non-linearly to a single increasing exponential to determine the achieved rate constant and steady-state value of deuteration (SigmaPlot; Systat Software Inc., San Jose, CA, USA).

Before the infusion of $^{13}$C, rats were deeply anesthetized with a 10 : 1 mixture of ketamine and xylazine (0.1 mL/100 g body weight). Body temperature was monitored with a rectal probe (Panlab, Barcelona, Spain) and maintained at approximately 37°C using a thermostatic blanket and a temperature-regulated circulating water bath. The right jugular vein was dissected and cannulated, and solutions of 0.2 M (1-$^{13}$C) glucose (pH 7.2; 8 μmol/min/100 g) or 0.6 M (2-$^{13}$C) acetate (pH 7.2; 24 μmol/min/100 g) were infused for increasing periods of 5, 10, 15, 30, 60, and 90 min, using a programmable infusion pump (Cole-Parmer Instrument Company, Vernon Hills, IL, USA). At the end of the infusions, the heads of the animals were funnel-frozen with liquid N$_2$ and perchloric acid extracts of cerebral tissue were prepared prior to high-resolution $^{13}$C NMR analysis (Chapa et al. 1995, 2000; Cruz and Cerdan 1999).

**$^{13}$C NMR spectroscopy**

High-resolution proton-decoupled $^{13}$C NMR spectra of rat brain extracts were obtained at 11.9 T (125.13 MHz, 25°C, pH 7.2) with a Bruker AVANCE 500 WB NMR spectrometer using a commercial triple resonance probe (5 mm) optimized for direct $^{13}$C NMR detection. Acquisition conditions were 60° pulse width, 30 kHz spectral width, 1.7 s acquisition time, 64k words data table, and 6.0 s recycling time. Proton decoupling was gated only during acquisition using a broadband composite pulse decoupling sequence that removed the scalar $^{13}$C–$^1$H multiplets, maintaining $^{13}$C–$^{13}$C and $^{13}$C–$^2$H couplings and minimizing the nuclear Overhauser enhancement contributions. Chemical shifts were calibrated with an external reference of dioxane (10% vol/vol, 67.4 ppm). The signal-to-noise ratio was enhanced by exponential multiplication with a line broadening of 1 Hz. Spectral deconvolution and multiplet structures were analyzed using a PC-based (Intel Centrino Platform) NMR program, NUTS™ (Acorn, Freemont, CA, USA). Resonance
assignments were based on literature values and on the addition of internal standards (Cerdan et al. 1990).

High-resolution $^{13}$C NMR is well suited to detect the deuteration patterns in $^{13}$C-labeled metabolites (Garcia-Martin et al. 2002; Rodrigues et al. 2005). This happens because the presence of one or more $^2$H atoms bound vicinally (two sigma bonds) or gernially (one sigma bond) to the observed $^{13}$C resulted in the appearance of characteristic $^2$H-induced isotopic shifts ($\Delta_1$ or $\Delta_2$ for one or two sigma bond distances to the observed $^{13}$C) and $^{13}$C-$^2$H couplings (Hansen 1988). In the case of (2-$^{13}$C) glutamate and (2-$^{13}$C) glutamine, the substitution of the H3 hydrogens by one or two deuterons caused the formation of two different singlets, shifted $\sim 0.07$ and $\sim 0.14$ ppm, upfield of the corresponding C2 perprotonated resonance, respectively. Thus, the analysis of the isotopically shifted and non-shifted singlets of glutamate and glutamine C2 resonances allowed for the determination of the number of deuteration replacements in the vicinal H3 hydrogens (Moldes et al. 1994; Chapa et al. 2000; Garcia-Martin et al. 2001, 2002).

Mathematical model of H3 deuteration

The pathways of $^{13}$C-labeling and deuteration of $\alpha$-ketoglutarate in the cerebral TCA cycle during the metabolism of (1-$^{13}$C) glucose or (2-$^{13}$C) acetate in the presence of $^2$H$_2$O are illustrated in Fig. S1. In this study we focused specifically in the dynamics of deuteration replacements in the H3$_{pro}$ and H3$_{pde}$ hydrogens of (2-$^{13}$C) $\alpha$-ketoglutarate, glutamate, and glutamine (Glx). These constitute the simplest deuteration pattern of (2-$^{13}$C) Glx isotopomers detectable by $^{13}$C NMR, as H3 monodeuterations or bideuterations occurred in clearly resolved singlets, shifted $\sim 0.07$ or $\sim 0.14$ ppm from the corresponding C2 perprotonated resonance, respectively.

We implemented a minimal model of dynamic H3 deuterations of the (2-$^{13}$C) isotopomers of Glx (Fig. 1). The model considered a constant input flux of (2-$^{13}$C) Glx perprotonated isotopomers from the upstream reactions of the TCA cycle and associated anaplerotic processes (TCA$_1$ cycle), the alternative ($\phi_1$ or $\phi_2$) or sequential ($\phi_3$ and $\phi_4$) deuterations of one or both H3 hydrogens, and the output fluxes of monodeuterated ($\phi_2$) or bideuterated ($\phi_4$) isotopomers. These were thought to release Glx molecules leaving the TCA cycle through the glutamine cycle ($\phi_2$) or continuing downstream in its oxidative decarboxylation reactions ($\phi_4$). The input flux was assumed to be constant as expected from a steady-state supply of (2-$^{13}$C) Glx intermediates from upstream TCA cycle reactions while the outputs were assumed to follow first order kinetics ($\phi_2$: $k_{Glx/Gln}$; and $\phi_4$: $k_{TCA2}$). Michaelis–Menten kinetics were assumed for the alternative (2-$^{13}$C, 3R-$^2$H) or (2-$^{13}$C, 3R-$^2$H) ($\phi_{tor}$ or 3; $K_{m1}$ or 3; $V_{max1}$ or 3; $K_r$) and consecutive ($\phi_2$ or 4; $K_{m2}$ or 4; $V_{max2}$ or 4) deuterations. The following equations were used:

$$\frac{\partial[(2-13C)\text{Glx}]}{\partial t} = F_{TCA1} - \phi_1 - \phi_3, \quad [1]$$

$$\frac{\partial[(2-13C, 3R-2^2H)\text{Glx}]}{\partial t} = \phi_1 - \phi_4 - \phi_5, \quad [2]$$

$$\frac{\partial[(2-13C, 3S-2^2H)\text{Glx}]}{\partial t} = \phi_3 - \phi_4, \quad [3]$$

where

$$\phi_1 = \frac{\left[(2-13C)\text{Glx}\right] \cdot V_{max1}}{\left[(2-13C)\text{Glx}\right] + K_{m1}}, \quad [5]$$

$$\phi_2 = \frac{\left[(2-13C, 3R-2^2H)\text{Glx}\right] \cdot V_{max2}}{\left[(2-13C, 3R-2^2H)\text{Glx}\right] + K_{m2}}. \quad [6]$$

Fig. 1. Mathematical model of deuteration of the H3R and H3S hydrogens of (2-$^{13}$C) isotopomers of $\alpha$-ketoglutarate, glutamate, or glutamine (Glx). Perprotonated (2-$^{13}$C) Glx isotopomers originating from upstream activity of the tricarboxylic acid (TCA) cycle (orange) may become monodeuterated in the H3R or H3S positions through fluxes $\phi_1$ (dark green) or $\phi_3$ (magenta). Bideuterated isotopomers are produced after a new turn of the cycle through fluxes $\phi_2$ (gray) or $\phi_4$ (dark blue). Output of monodeuterated or bideuterated isotopomers occurs through fluxes $\phi_5$ (light blue) and $\phi_6$ (light green). Aconitase (AC) and isocitrate dehydrogenase (ICDH) are known to catalyze the H3R and H3S deuterations of (2-$^{13}$C) Glx by deuterating its precursors citrate and isocitrate, respectively (see Fig. S1). AC and ICDH present kinetically and structurally different cytosolic (AC$_{cyt}$ and ICDH$_{cyt}$) and mitochondrial (AC$_{mit}$ and ICDH$_{mit}$) isoforms. $\phi_1$ and $\phi_3$ are proposed to involve the cytosolic isoenzymes catalyzing fast cytosolic monodeuterations. These occur on already available perprotonated citrate and isocitrate molecules originating alternatively 3R or 3S (2-$^{13}$C) Glx without the need to undergo TCA cycle turnover. Notably, the formation of one monodeuterated H3 Glx stereoisomer appears to inhibit the formation of the other. $\phi_2$ and $\phi_4$ represent slower mitochondrial bideuterations occurring during the de novo synthesis of (2-$^{13}$C) Glx in the TCA cycle. The dotted lines ($\phi_2$ and $\phi_4$) indicate that bideuterated (2-$^{13}$C) Glx isotopomers are not produced directly from monodeuterated Glx, but require de novo resynthesis in a new turn of the cycle (see Fig. S1).
In equations [1–10], \( F_{\text{TCA1}} \) represents the constant input flux of perprotonated (2-13C) isotopomers from upstream reactions of the TCA cycle; Glx represents \( \alpha \)-ketoglutarate, glutamate or glutamine; \( V_{\text{max,1'}} \) and \( K_{\text{m1'}} \) are the apparent Michaelis–Menten kinetic constants associated with the fluxes \( \varphi_{4,4'} \); \( K_i \) is the inhibition constant associated with the uncompetitive inhibition of flux \( \varphi_3 \) by (2-13C, 3R-2H) Glx; and \( k_{\text{TCA2}}, k_{\text{Glu/Gln}} \) are the first order kinetic constants of removal of (2-13C, 3R-2H) Glx or (2-13C, 3,3-2H2) Glx, respectively.

Mathematical modeling and fittings were performed using MATLAB\textsuperscript{\textregistered} V6.5 (The MathWorks Inc., Natick, MA, USA). A multiple objective function was used to simultaneously determine the three sets of parameters that fitted the three individual experimental replicates, while making sure that the parameter set obtained by averaging these three sets also fitted the mean experimental values. To this end a weighted sum of the squared differences between experimental and simulated data points was used:

\[
\text{Total_error} = \left( E_1 - M(P_1) \right)^2 + \left( E_2 - M(P_2) \right)^2 + \left( E_3 - M(P_3) \right)^2 + 6 \times \left( E_M - M(P_M) \right)^2,
\]

where \( M \) is the Model, \( E_j \) the experimental results for each one of the three replicates, \( E_M = (E_1 + E_2 + E_3)/3 \), their mean value and \( P_M = (P_1 + P_2 + P_3)/3 \), the mean parameter set. The final regression was carried out by minimization of the Total_error by changing the three sets of 12 parameters.

### Results

\[ ^{13} \text{C NMR of the (2-13C) glutamate and (2-13C) glutamine region in the brain of deuterated and non-deuterated animals} \]

Figure 2 compares expanded regions from \( ^{13} \text{C} \) NMR spectra containing the C2 resonances from (2-13C) glutamate and (2-13C) glutamine in brain extracts from control (Fig. 2a and b) and deuterated (Fig. 2c and d) animals infused for 90 min with (1-13C) glucose (Fig. 2a and c) or (2-13C) acetate (Fig. 2b and d). In the control animals, infusion of (1-13C) glucose resulted in the appearance of singlets derived from the perprotonated (2-13C) glutamate (resonance 1) and (2-13C) glutamine (resonance 4) isotopomers and doublets derived from the contiguously \( ^{13} \text{C} \)-labeled (2,3-13C2) glutamate (resonances a) isotopomers. Similar spectra were obtained after (2-13C) acetate infusion in control animals, with the additional appearance of the doublets derived from (1,2-13C2) glutamate (resonances b) or (2,3-13C2)-glutamine (resonances c) and (1,2,3-13C3) glutamine (resonances d). The spectra obtained from brain extracts of deuterated animals infused with (1-13C) glucose or (2-13C) acetate increased in complexity (Fig. 2c and d), demonstrating that deuterons were effectively incorporated into these metabolites. The C2 resonances of cerebral glutamate and glutamine in deuterated animals showed, in addition to the unshifted resonance from the perprotonated isotopomers (resonances 1, 4), the isotopically shifted resonances from the H3 monodeuterated isotopomers (ss, resonances 2 and 5, \( A_2 = -0.07 \text{ ppm} \)) and the doubly shifted resonance from the H3 bideuterated isotopomers (dds, resonances 3 and 6, \( A_2 = -0.14 \text{ ppm} \)). Interestingly, the 3\( R \) and 3\( S \) (2-13C, 3-2H) glutamate and glutamine isotopomers showed slightly different isotopic shifts in the corresponding C2 resonance, but their small chemical shift difference did not allow the resolution of both components at this field. They could be demonstrated, however, at an 18.8 T field (results not shown).

### Turnover of the H3 hydrogens of cerebral (2-13C) glutamate and (2-13C) glutamine during (1-13C) glucose or (2-13C) acetate infusions

Figure 3 shows the dynamics of specific (\( ^{13} \text{C} \), 2\( \text{H} \)) isotopomers of (2-13C) glutamate and (2-13C) glutamine in brain extracts of deuterated rats during (1-13C) glucose or (2-13C) acetate infusions. The time courses reflected the relative contributions of each observable isotopomer, expressed as the fractional contribution of the corresponding singlet to the area of the combined C2 singlet resonance containing the

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**Materials**

(1-13C)-D-glucose (99% \( ^{13} \text{C} \)) and (2-13C) acetate (99% \( ^{13} \text{C} \)) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). \( ^2 \text{H}_2 \text{O} \) (99.9% \( ^2 \text{H} \)) was obtained from Apollo Scientific (Cheshire, UK). Ketamine hydrochloride (Ketolar\textsuperscript{\textregistered}) and xylazine (Rompun\textsuperscript{\textregistered}) were purchased from Parke–Davis SL (Madrid, Spain) and Bayer AG (Leverkusen, Germany), respectively. The rest of the reagents were of the highest purity available commercially from Sigma (Madrid, Spain).
perprotonated and deuterated resonances. It should be mentioned here that the deuteration reactions detected occurred in a much faster time-scale than the $^{13}$C replacement of the corresponding C2 carbons (see Fig. S3 for $^{13}$C2 turnover curves).

Fig. 2 Expanded regions containing the $^{13}$C resonances from (2-$^{13}$C) glutamate and (2-$^{13}$C) glutamine in brain extracts of control (a and b) or deuterated (c and d) animals infused with (1-$^{13}$C) glucose (a and c) or (2-$^{13}$C) acetate (b and d) for 90 min. 1: (2-$^{13}$C) glutamate; 2: (2-$^{13}$C, 3-$^{2}$H) glutamate; 3: (2-$^{13}$C, 3,3-$^{2}$H$_2$) glutamate; 4: (2-$^{13}$C) glutamine; 5: (2-$^{13}$C, 3-$^{2}$H) glutamine; 6: (2-$^{13}$C, 3,3-$^{2}$H$_2$) glutamine; a: (2,3-$^{13}$C$_2$) glutamate; b: (1,2-$^{13}$C$_2$) glutamate; c: (2,3-$^{13}$C$_2$) glutamine; d: (1,2-$^{13}$C$_2$) glutamine.

Fig. 3 Time course of perprotonated, monodeuterated, and bideuterated H3 isotopomers of cerebral (2-$^{13}$C) glutamate (a, c) and (2-$^{13}$C) glutamine (b, d) during (1-$^{13}$C) glucose (a, b) or (2-$^{13}$C) acetate (c, d) infusions in partially deuterated rats. The results are represented as mean ± SD of three independent experiments. The dots indicate relative concentrations of (2-$^{13}$C) isotopomers (empty), (2-$^{13}$C, 3-$^{2}$H) isotopomers (gray), and (2-$^{13}$C, 3,3-$^{2}$H$_2$) isotopomers (black). Lines indicate the best fit of the data to the model of Fig. 1. Dotted lines represent the calculated contributions of the H3R or H3S (2-$^{13}$C) isotopomers. Note that the time course of deuteration is much faster than the $^{13}$C turnover of the C2 carbon of glutamate or glutamine (Fig. S3).

The fastest processes observed were the formation of perprotonated (2-$^{13}$C) glutamate and (2-$^{13}$C) glutamine (empty dots). Monodeuterated isotopomers of (2-$^{13}$C, 3-$^{2}$H) glutamate and glutamine were produced subsequently (filled gray dots). The formation of 3R or 3S diastereoisomers could...
not be resolved experimentally, the observed kinetics reflecting the added contributions of both H3 diastereoisomers. The fact that the combined H3 monodeuteration observed [mainly during the (2-13C) acetate infusions] showed a rapid burst followed later by a decrease, suggested that one of the H3 diastereoisomers was formed rapidly and transiently, its formation being inhibited later by the dominant accumulation of the other. Finally, the bideuterated isotopomers in H3 (2-13C, 3,3-2H2) glutamate and (2-13C, 3,3-2H2) glutamine (filled black dots), were the slowest deuterated species to be produced.

To obtain a quantitative interpretation of the kinetics of 2H incorporation, we fitted the experimental data to the model of (2-13C) glutamate or glutamine deuteration described in incorporation, we fitted the experimental data to the model of Table 1. Fitted values for the parameters are found between the calculated and experimentally determined values of deuteration, providing a measure of confidence in the model used. Fitted values for the parameters are summarized in Table 1.

In all cases, H3 monodeuteration (Km1’-Vmax1’, Km3’-Vmax3’) or bideuteration (Km2’-Vmax2’, Km4’-Vmax4’) processes were characterized by different apparent Km’ and Vmax’ values. This finding was consistent with the presence in brain of two different cytosolic and mitochondrial isoenzymes of AC and ICDH. The maximal deuteration rates (Vmax’ values) obtained, provided information on the relative capacities of the different routes of deuteration while corresponding Km’s revealed the affinity of the different processes. Vmax1’ values were larger than Vmax3’, revealing a larger capacity of monodeuteration through pathway 1. Similarly, Vmax2’ values were larger than Vmax4’, implying a larger capacity of bideuteration through pathway 2. Km2’ and Km4’ were normally smaller than Km1’ or Km3’, suggesting a higher affinity for the second deuteration. Nevertheless, deuteration of (2-13C) glutamine during (2-13C) acetate infusions depicted values of Km2’ larger than Km1’, indicating less affinity for the second deuteration in this case. Notably, the kinetic parameters determined for the monodeuterations or bideuterations of (2-13C) glutamate and (2-13C) glutamine were different during the infusions with (1-13C) glucose or (2-13C) acetate, suggesting different deuteration kinetics for the neuronal or glial isoenzymes.

Figure 4 shows the time evolution of the different fluxes considered in the model of Fig. 1, expressed as a percentage of the input fluxes of (2-13C) glutamate or (2-13C) glutamine during (1-13C) glucose or (2-13C) acetate metabolism. The fluxes maintain the same color code as in Fig. 1. The formation of (2-13C, 3R-2H) glutamate and its efflux from the cycle accounted for 98% or 95% of the input flux of (2-13C) glutamate production flux during the metabolism of (2-13C) acetate (Fig. 4b) or (1-13C) glucose (Fig. 4a),

| Table 1 Parameter values obtained from non-linear fittings of the kinetics of deuteration of (2-13C) glutamate and (2-13C) glutamine during infusions of (1-13C) glucose or (2-13C) acetate |
|----------------|----------------|----------------|
| Metabolite observed | (1-13C) Glucose | (2-13C) Acetate |
|                | (2-13C) Glutamate | (2-13C) Glutamine | (2-13C) Glutamate | (2-13C) Glutamine |
| F<sub>TCA1</sub> | 0.43 ± 0.04 | 0.54 ± 0.04 | 0.46 ± 0.05 | 0.50 ± 0.02 |
| V<sub>max1’</sub> | 1.84 ± 0.24 | 3.56 ± 0.26 | 3.40 ± 0.26 | 2.03 ± 0.06 |
| K<sub>m1’</sub> | 2.27 ± 0.03 | 4.00 ± 0.26 | 4.28 ± 0.28 | 1.95 ± 0.09 |
| V<sub>max2’</sub> | 0.61 ± 0.20 | 1.27 ± 0.23 | 0.00016 ± 0.00002 | 0.00130 ± 0.0005 |
| K<sub>m2’</sub> | 0.27 ± 0.019 | 0.048 ± 0.32 | 2.76 ± 0.39 | 4.71 ± 1.13 |
| V<sub>max3’</sub> | 0.004 ± 0.001 | 0.0012 ± 0.0005 | 0.06 ± 0.046 | 0.047 ± 0.024 |
| K<sub>m3’</sub> | 0.56 ± 0.27 | 0.41 ± 0.08 | 0.62 ± 0.09 | 0.83 ± 0.13 |
| V<sub>max4’</sub> | 0.24 ± 0.04 | 0.29 ± 0.09 | 0.0015 ± 0.0017 | 0.0009 ± 0.0002 |
| K<sub>’</sub> | 0.44 ± 0.29 | 0.27 ± 0.09 | 0.350 ± 0.120 | 0.053 ± 0.005 |
| k<sub>TCA1’</sub> | 0.009 ± 0.02 | 0.015 ± 0.006 | 0.520 ± 0.200 | 0.182 ± 0.079 |
| k<sub>Glu/Gln’</sub> | 1.80 ± 0.38 | 2.30 ± 0.57 | 1.87 ± 0.04 | 1.92 ± 0.15 |
| k<sub>Glu/Gln’</sub> | 0.27 ± 0.10 | 0.81 ± 0.30 | 0.0016 ± 0.0009 | 0.0007 ± 0.0002 |

TCA, tricarboxylic acid; Gin, glutamine; Glu, glutamate; K<sub>m’</sub>, apparent Km under in vivo conditions; V<sub>max’</sub>, apparent V<sub>max</sub> under in vivo conditions. Results are given as mean ± SD of at least three animals. K<sub>m’</sub> and K<sub>’</sub> values are given in mM, V<sub>max’</sub> in µmol/min/g, F<sub>TCA1</sub> in µmol/min/g, k<sub>TCA1’</sub> and k<sub>Glu/Gln’</sub> in per minute. All values refer to apparent kinetic constants.
Fig. 4 Kinetics of the deuteration fluxes \( \phi_1 \rightarrow \phi_6 \) during infusions of \((1-{13C})\) glucose or \((2-{13C})\) acetate in partially deuterated rats. Fluxes \( \phi_1 \rightarrow \phi_6 \) are those indicated in Fig. 1 with the same color code. Relative contributions of \( \phi_1 \rightarrow \phi_6 \) are expressed as a percentage of the constant influx of \((2-{13C})\) perprotonated isotopomer. Insets show expansions of the first 20 min of infusion. Note that the largest changes in flux occur in the first 10 min.

Discussion

The present study investigates the exchange of the two H3 hydrogens of cerebral \((2-{13C})\) glutamate and \((2-{13C})\) glutamine by deuterons of heavy water in partially deuterated rats metabolizing \((1-{13C})\) glucose or \((2-{13C})\) acetate. These H3 deuterations arise from the stereospecific hydration–dehydration reactions catalyzed by cerebral AC and ICDHs on the glutamate precursors citrate and isocitrate, respectively. The formation of \((2-{13C}, 3S^-2H)\) glutamate accounted for the rest, being mainly used as a precursor for the TCA cycle which generated most of the \((2-{13C}, 3,3^-2H)\) glutamate isotopomers. The production of \((2-{13C}, 3^-2H)\) glutamine followed a similar pattern. \((2,{13C}, 3R^-2H)\) glutamine formation reached 80% or 98% of the \((2,{13C})\) glutamine input during the metabolism of \((1-{13C})\) glucose (Fig. 4c) or \((2-{13C})\) acetate (Fig. 4d), becoming the main precursor for the output of monodeuterated isotopomers leaving the cycle. The production of \((2-{13C}, 3S^-2H)\) glutamine accounted for the remaining 20% and 2% of the \((2-{13C})\) glutamine input, becoming the main contributor to its terminal oxidation in the glial TCA cycle and the production of bideuterated glutamine isotopomers.

Aconitase and ICDH are present in the brain as two different isoenzymes located in cytosol and mitochondria. In addition, ICDH presents two isoforms operating with NAD+ or NADP+ as cofactors.

During the cerebral metabolism of \((1-{13C})\) glucose or \((2-{13C})\) acetate, the glutamate precursors, \((2-{13C})\) citrate and \((2-{13C})\) isocitrate, become available in the cytosol and mitochondria of neural cells. In partially deuterated animals, the hydration-dehydration reactions end up incorporating \(^{2}H\) into these precursors which will become the H3R and/or H3S deuterons of \((2-{13C})\) glutamate or \((2-{13C})\) glutamine, respectively. The \(^{13}C\) NMR approach described here has made it possible for the first time to our knowledge to monitor the kinetics of these hydration-dehydration processes in the brain allowing for the evaluation of the corresponding kinetic constants.

The monodeuterations processes occur first, almost immediately after the formation of perprotonated \((2-{13C})\) glutamate or glutamine. The two possible monodeuterations originating from the 3R or 3S \((2-{13C}, 3^-2H)\) isotopomers depict different kinetic constants, as expected from the different kinetic properties of AC or ICDH. The bideuteration processes occurring later, depict also different kinetic properties, as it would be predicted for the operation of the cytosolic and mitochondrial isoforms of both enzymes. Summarizing, the different kinetic constants determined for the H3 mono- and bideuterations by \(^{13}C\) NMR indicate that these hydration-dehydration reactions cannot be catalyzed by the same AC or ICDH isoforms operating in a single compartment. The observed deuterations must therefore proceed in different compartments as illustrated in Fig. 5. The faster monodeuterations were thought to occur for cytosolic \((2-{13C})\) citrate and \((2-{13C})\) isocitrate precursors of \((2-{13C})\) glutamate or glutamine. The slower bideuterations may then proceed in the mitochondrial space during the de novo formation of \((2-{13C})\) glutamate or glutamine in the first turn of the TCA cycle and associated anaplerotic reactions. This is so because the cytosolic monodeuteration of one of the pre-existing 3R...
Fig. 5 The turnover of the H3 hydrogens of cerebral glutamate and glutamine reveals subcellular compartmentation in the neuronal and glial compartments of rat brain. A fast deuterium exchange occurs during the metabolism of (1-13C) glucose or (2-13C) acetate in the TCA cycle. A similar subcellular trafficking of (2-13C) glutamate or (2-13C) glutamine are different either from (1-13C) glucose or (2-13C) acetate, catalyzed by cytosolic aconitase or isocitrate dehydrogenase (NADP+), respectively. A slower double exchange of the H3proR and H3proS occurs only in newly formed (2-13C) glutamate molecules in the TCA cycle. The kinetics of deuteration in (2-13C) glutamate or (2-13C) glutamine are different either from (1-13C) glucose or (2-13C) acetate, revealing different kinetic deuteration environments in the neuronal or glial compartments. Deuterons are indicated by black triangles. 13C atoms are shown as dark gray circles.

or 3S (2-13C, 3-2H) glutamate or glutamine appears to inhibit the incorporation of a second deuteron in the opposite H3 position of the same molecule. The turnover of any (2-13C) isotope would then start with the rapid formation of the perprotonated molecule, followed by the slower production of the monodeuterated cytosolic isotope, its degradation in the mitochondrial TCA cycle and finally, the formation of a new molecule of doubly deuterated (2-13C) isotope also in the TCA cycle. A similar subcellular trafficking of (2-13C) glutamate in the perfused mouse liver metabolizing (3-13C) glutamate–glutamine cycle. In contrast, the H3S isotope of the same molecule normally amenable to classical13C turnover studies and that it is possible to follow the time-dependent deuteration of specific hydrogens within the same 13C isotope molecule. This enables us to trace the short-term history of a 13C isotope in the faster time-scale of deuteration turnover. The proposed approach may be extended to other 13C-labeled precursors and 13C isotope molecules, opening a new time-scale in the investigation of cerebral metabolism and compartmentation.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

- **Figure S1** The turnover of carbon and hydrogen in the tricarboxylic acid cycle.
- **Figure S2** Physiological parameters evolution in control and deuterated animals during the deuteration process.
- **Figure S3** Kinetics of \(^{13}\)C incorporation in the C2 carbons of cerebral glutamate (a and c) and glutamine (b and d) during infusions of (1-\(^{13}\)C) glucose (a and b) or (2-\(^{13}\)C) acetate (c and d) in normal and deuterated rats as observed by \(^{13}\)C NMR.

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**Conflicts of interest**

All authors declare no conflicts of interests.

**References**

Cerdan S., Kunnecke B. and Seelig J. (1990) Cerebral metabolism of [1,2-\(^{13}\)C\(^2\)] acetate as detected by in vivo and in vitro \(^{13}\)C NMR. *J. Biol. Chem.* 265, 12916–12926.


Künnecke B., Cerdan S. and Seelig J. (1993) Cerebral metabolism of \([1,2,13\)C\(^2\)] glucose and \([U-^{13}\)C\(^3\)]-3-hydroxybutyrate in rat brain as detected by \(^{13}\)C NMR spectroscopy. *NMR Biomed.* 6, 264–277.


Moldes M., Cerdan S., Erhardt P. and Seelig J. (1994) \(^{1}\)H-\(^{2}\)H exchange in the perfused rat liver metabolizing \([3-13\)C\(^2\)]alanine and \(^{2}\)H\(^2\)O as detected by multinuclear NMR spectroscopy. *NMR Biomed.* 7, 249–262.


Rodrigues T. B., Granado N., Ortiz O., Cerdan S. and Moratalla R. (2007) Metabolic interactions between glutamatergic and dopa-


