High-resolution detection of ^{13}C multiplets from the conscious mouse brain by ex vivo NMR spectroscopy

Isaac Marin-Valencia[1], **Levi B. Good**[1], **Qian Ma**[1], **F. Mark Jeffrey**[2,3], **Craig R. Malloy**[2,3,4], and **Juan M. Pascual**[1,5,6]

[1]Rare Brain Disorders Clinic and Research Laboratory, Department of Neurology and Neurotherapeutics, The University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75390

[2]Department of Radiology, The University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75390

[3]Advanced Imaging Research Center, The University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75390

[4]Department of Internal Medicine, The University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75390

[5]Department of Physiology, The University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75390

[6]Department of Pediatrics, The University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75390

Abstract

Glucose readily supplies the brain with the majority of carbon needed to sustain neurotransmitter production and utilization., The rate of brain glucose metabolism can be computed using ^{13}C nuclear magnetic resonance (NMR) spectroscopy by detecting changes in ^{13}C contents of products generated by cerebral metabolism. As previously observed, scalar coupling between adjacent ^{13}C carbons (multiplets) can provide additional information to ^{13}C contents for the computation of metabolic rates. Most NMR studies have been conducted in large animals (often under anesthesia) because the mass of the target organ is a limiting factor for NMR. Yet, despite the challengingly small size of the mouse brain, NMR studies are highly desirable because the mouse constitutes a common animal model for human neurological disorders. We have developed a method for the *ex vivo* resolution of NMR multiplets arising from the brain of an awake mouse after the infusion of $[1,6-^{13}C_2]$glucose. NMR spectra obtained by this method display favorable signal-to-noise ratios. With this protocol, the ^{13}C multiplets of glutamate, glutamine, GABA and aspartate achieved steady state after 150 min. The method enables the accurate resolution of multiplets over time in the awake mouse brain. We anticipate that this method can be broadly applicable to compute brain fluxes in normal and transgenic mouse models of neurological disorders.

Correspondence: Juan M. Pascual, MD, PhD, Rare Brain Disorders Clinic and Research Laboratory, Department of Neurology and Neurotherapeutics, The University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Texas 75390-8813. USA, Tel.: 214-648-5818, Fax: 214-645-6238, Juan.Pascual@UTSouthwestwern.edu .
The first two authors are equal contributors.

INTRODUCTION

The majority of biological processes of man and animals are intimately coupled to the supply of carbon and energy derived from glycolysis and the tricarboxylic acid (TCA) cycle. In the brain, glucose metabolism provides both carbon and energy to fuel neural signaling operating via neurotransmitter fluxes and consequent changes in cell membrane potential (Schurr, 2002). Differences in brain functional states are thus dependent on changes in metabolic flux, whereas brain disorders inexorably alter brain function. Neurological disorders can thus be characterized by understanding the relation between fuel supply, neurotransmitter synthesis, and resting metabolic rates. However, achieving this goal *in vivo* is difficult.

The measurement of relative or absolute metabolic fluxes in any tissue requires information about the fate of a carbon tracer and suitable mathematical models for analysis of the data. Because of the complexity of carbon metabolism in the brain, various tracer methods have been introduced. Positron emission tomography, ^{14}C tracer methods, and ^{13}C studies with detection by mass spectrometry generally provide limited information about the fate of the tracer, and some are impractical in the mouse. Compared to these alternative methods, ^{13}C NMR spectroscopy, in principle, offers far greater information about the fate of a carbon tracer because of information encoded in the chemical shift and in ^{13}C-^{13}C spin coupled multiplets. The ability to measure brain TCA cycle and neurotransmitter flux rates in the mouse, a common animal model for human neurological disorders would be an important advance. However, detection of ^{13}C multiplets has proven elusive because of the small mass of the brain, the generally low levels of ^{13}C enrichment achievable in vivo, the low sensitivity of ^{13}C (compared to ^{1}H) for NMR detection, and uncertainty about conditions required to achieve isotopic and metabolic steady state after infusion of the ^{13}C-labeled substrate. In the anesthetized mouse brain (175 µl at most), ^{13}C NMR spectroscopy is practicable but suboptimal: glutamate and glutamine are detectable in vivo with a temporal resolution of 8.6 min, but accurate multiplet detection is not yet feasible (Nabuurs et al., 2008).

The purpose of this report is to describe a technique to obtain high-quality ^{13}C NMR spectra from extracts of the brain from conscious mice at different points during infusion of ^{13}C - enriched glucose. This method enables quantitation of ^{13}C-^{13}C spin coupled multiplets over time in multiple neurotransmitters and other key intermediates including glutamate, glutamine, γ-aminobutiric acid (GABA) and aspartate. The ^{13}C multiplets of each cerebral isotopomer achieved steady state after 150 min infusion. The evolution of the fractional amount of multiplets of interrelated molecules (i.e., glutamate and glutamine) was similar to each other. In combination with modeling, this method may allow the computation of brain metabolic fluxes in the normal and the diseased mouse brain.

MATERIALS AND METHODS

^{13}C infusions

This study was performed under Institutional Animal Care and Use Committee of UT Southwestern Medical Center at Dallas guidelines. Female C57BL/6J mice were used for the study (n=7, weight: 23.7 ± 2.6 g, age: 8.6 ± 1.3 m; all ranges and errors hereafter are expressed as standard deviation unless otherwise indicated). The right jugular vein was aseptically cannulated under intraperitoneal anesthesia provided by ketamine (100mg/kg) and xylazine (10mg/kg). Outer catheter diameter was 0.025 mm (Braintree Scientific, Braintree, MA). The catheters were filled with glycerol and heparin (500 U/ml) to prevent clotting. After cannulation, the animals were individually housed under standard animal care conditions with *ad libitum* access to water. Seven days post-cannulation, the mice were

habituated and confined to a cylindrical Lucite cage to prevent ambulation and the catheter purged with 2 µL saline solution. $[1,6\text{-}^{13}C_2]$glucose $(1\text{-}^{13}C$, 99% enrichment; $6\text{-}^{13}C$, 97% enrichment, Cambridge Isotope Laboratories, Cambridge, MA) was administered as a bolus containing 0.4mg/g (in 0.2 ml of saline) infused over 30 sec, followed by continuous infusion of 0.012 mg/g/min (in 0.375 ml of saline) at 150 µl/hr during increasing periods of time (one per animal) at room temperature in an unperturbed environment. Infusion time periods were: 20, 30, 50, 75, 150 and 300 min. Animals were decapitated at the end of infusion and forebrains were rapidly removed (in less than 15 sec) after blood collection, weighed (forebrain weight: 314.03 ± 14 mg), frozen in liquid nitrogen, and stored at $-85°C$. Blood was simultaneously collected from the cervical stump, weighed (190 ± 10 µL), frozen in liquid nitrogen, and stored at $-85°C$.

Preparation of tissue extracts

A modified version of perchloric acid extraction protocol was applied (Walton et al., 2003). In brief, frozen brain or blood samples were finely grounded in a mortar under liquid nitrogen. Perchloric acid (4%; 1:4 w/v) was added to each sample, followed by centrifugation at 47800 g for 15 min. The supernatant was transferred to a new tube where chloroform / tri-n-octylamine (78% / 22%; v/v) was added in 1:2 volumetric ratio to a pH of 6. The samples were centrifuged at 3300 g for 15 min, the aqueous phase removed and transferred to a microfuge tube and then lyophilized. 200 µL of deuterium oxide (99.96%, Cambridge Isotope Laboratories) was added to each sample and the pH adjusted to 7.0 with 2–3 µL of 1 M sodium deuteroxide (99.5%, Cambridge Isotope Laboratories). The pH-neutral samples were then centrifuged at 18,400 g for 1 min and the supernatant removed and placed into a 3-mm NMR tube for subsequent NMR analysis. In addition NMR study of blood samples, total blood glucose was measured by enzyme , Sigma, St Louis, MO).

NMR Spectroscopy

Proton decoupled ^{13}C spectra were acquired on a 600 MHz Oxford magnet and Varian VNMRS Direct Drive console using a 3 mm broadband probe (Varian Inc., Palo Alto, CA) at room temperature. Proton decoupling was performed at 2.3 kHz using a Waltz-16 sequence. ^{13}C NMR spectroscopy parameters included a 45° flip angle per transient, a relaxation delay of 1.5 s, an acquisition time of 1.5 s, and a spectral width of 36.7 kHz. Samples were spun at 20 Hz and 25°C. A heteronuclear 2H lock was used to compensate for magnet drift during data acquisition. To achieve adequate signal-to-noise in brain spectra, the number of scans acquired for short infusions (20–75 min) were typically 10,000 to 12,000 and for long infusions (150–300 min) were between 4,000 and 6,000. Proton spectra were similarly acquired to characterize the fractional enrichment of the α-anomer of the C1 proton resonance of D-glucose in blood. ^1H NMR parameters included a 45° flip angle per transient, a relaxation delay of 1 s, an acquisition time of 2 s, and a spectral width of 20 kHz. Samples were spun and the temperature maintained as above. A heteronuclear 2H lock was also used. For proton NMR in blood samples, the number of scans acquired to achive favorable signal-to-noise was 120.

NMR spectrum analysis

NMR spectral analyses were performed with ACD/Spec Manager 11.0 software (Advanced Chemistry Development, Inc., Toronto ON, Canada). Time-series free induction decays were zero-filled and windowed with an exponential weighting function prior to Fourier transformation for analysis of spectral contents. Metabolite peaks were then identified based on chemical shift position referenced to the glutamate C4 singlet at 34.2 ppm. Each peak was then fitted with a Gauss-Lorentz function and the area measurements for each fitted resonance peak and their multiplets estimated. For each isotopomer, multiplet areas were defined as a fraction of the total atomic resonance area, here called multiplet fractional

amount (Malloy, 1998). For example, the fractional amount of glutamate C4 singlet (C4S) represents the area of C4S relative to the total area of glutamate C4; the fractional amount of the doublet 34 (C4D34) refers to area of C4D34 due to J_{34} (coupling between ^{13}C carbons in positions 3 and 4 of glutamate) relative to the total area of glutamate C4; the same contention applies to the doublet 45 (D45) and the quartet (Q) of glutamate C4. By convention, the sum of all multiplets arising from a given isotopomer equals unity; in this case glutamate C4S + C4D34 + C4D45 + C4Q = 1. The ^{13}C enrichment of glucose in blood was computed by ^{1}H-NMR as previously described (Dobbins and Malloy, 2003). The portion of blood glucose that included ^{13}C at the carbon 1 position was defined as the area of the ^{13}C doublets, predominantly derived from ^{13}C coupling between carbon 1 and 6, relative to the total ^{1}H resonance area (^{13}C + ^{12}C).

RESULTS

Animals tolerated and recovered from cannulation uneventfully and did not exhibit abnormal behavior during the infusion. All brain-derived NMR spectra were resolved with favorable signal-to-noise ratios. As shown by others for the microwave-fixed cerebral cortex (Walls et al., 2011), the brief time of dissection (<15 s) does not significantly impact metabolite abundance in the brain relative to other methods of brain fixation. The resonances ascribed to cerebral glutamate and glutamine in C1, C2, C3 and C4, together with aspartate C2 and C3, GABA C2, C3, C4, N-acetyl-aspartate C2, C3 and C6, lactate C2 and C3 and taurine C1 and C2 were readily identified. As expected from previous ^{13}C-glucose studies in brain extracts (Alexander et al., 2005; Hassel et al., 1997), glutamate, glutamine and lactate constituted the largest resonances in the NMR spectrum consistent both with the preference of neurons and glia to produce glutamate and glutamine from glucose, respectively, and also with the high glycolytic capacity of brain cells, which are able to robustly metabolize glucose to produce lactate (Escartin et al., 2006; Hassel et al., 1995; Sonnewald et al., 1994; Tansey et al., 1991).

Figure 1 illustrates the distribution of ^{13}C-label into intermediates of [1,6-$^{13}C_2$]glucose metabolism. [3-^{13}C]pyruvate generated *via* glycolysis and converted into [2-^{13}C]acetyl-CoA by pyruvate dehydrogenase. The condensation of [2-^{13}C]acetyl-CoA with non-labeled oxaloacetate yields [4-^{13}C]citrate, [4-^{13}C]ketoglutarate and, consequently, [4-^{13}C]glutamate in the first cycle ("turn") of the TCA cycle, which appears in the ^{13}C spectrum as a singlet glutamate C4 resonance (Figure 1). [2-^{13}C]oxaloacetate produced in the first turn of the TCA cycle, can coalesce with another [2-^{13}C]-acetyl-CoA molecule, yielding the first TCA cycle intermediate containing two adjacent ^{13}C carbons, [2,3-$^{13}C_2$]citrate. This molecule is subsequently converted into [3,4-$^{13}C_2$]glutamate, which appears as a doublet (D34) surrounding the glutamate C4 singlet (Figure 1 and 2). A similar progression of enrichment can be expected for glutamate-derived molecules such as glutamine and GABA (Figure 2). As shown in Figure 3, the multiplets arising from all detected isotopomers gradually increased in signal area as the infusion progressed, reflecting the distribution of ^{13}C amount the TCA cycle intermediates as the cycle turnover continues. Of note, the amplitude of the singlet glutamate C4 resonance decreased beyond 75 min of infusion (Figure 3). This phenomenon is probably the consequence of progressive enrichment of [2-^{13}C]acetyl-CoA and [2-^{13}C]oxaloacetate, with longer infusions resulting in reduced formation of [4-^{13}C]citrate and [4-^{13}C]glutamate. As previously noted (Malloy, 1998; Robitaille et al., 1993; Weiss et al., 1995), the intensity of multiplet resonances also depends on metabolite pool sizes, TCA cycle flux and on other metabolic exchanges. In glutamate C3 and glutamine C3, the area of the doublet (quantified as the sum of doublet 23 and 34, as they share the same J-J coupling constant 34 Hz) and the triplet evolve proportionally over time (Figure 4). The singlet C3 resonance appears to increase along the infusion time course probably due to the parallel raise of the triplet, which overlaps with the singlet resonance.

Importantly, the relative areas of multiplet isotopomers were approximately constant after 150 min of infusion (Figure 3 and 4). At this point, the distribution of ^{13}C among TCA cycle-derived intermediates reached steady state and the relative abundance of ^{13}C multiplets no longer varied significantly. As stated earlier, there was a close correlation between a progressive decrease observed in the resonance areas attributed to singlet isotopomers and a simultaneous increase in the doublet, triplet, and quartet resonances detectable for each molecule. For example, Figure 2 illustrates a typical spectrum in which all isotopomers are identified after 75 min of infusion. Taurine labeled in carbons 1 and 2 manifested a modest signal in the NMR spectrum, probably deriving from naturally abundant ^{13}C. Moreover, resonances from N-acetylaspartate in carbons 2, 3 and 6 were represented only as singlets, owing to the slow rate of ^{13}C-label incorporation into both aspartyl (NAA C2 and C3) and acetyl (NAA C6) groups (Choi and Gruetter, 2004).

The mean blood glucose concentration at the end of the infusions was 7.5 ± 3.45 mM (averaging all infusion time courses) (normal range: 6.5–7 mM (Ayala et al., 2006)). The enrichment of blood ^{13}C-glucose detected by ^{1}H-NMR rose above natural abundance (1.1 %) to 21.2% at 20 min of infusion and progressively increased as follows: 40.88% at 30 min, 27.8% at 50 min, 33.6% at 75 min, 51.3% at 150 min, 37.4% at 300 min.

DISCUSSION

We have for the first time, illustrated the dynamic evolution of multiplet isotopomers in the awake mouse brain by *ex vivo* ^{13}C NMR spectroscopy. Because of the superior resolution of the ^{13}C spectra, the signals arising from glutamate, glutamine, aspartate and GABA were not only detected separately, but were also discerned as multiplets due to the coupling of adjacent ^{13}C carbons within each molecule, which were precisely resolved using conventional NMR equipment. This method overcomes the common technical challenges posed by the mouse brain, namely the low sensitivity of ^{13}C-NMR techniques to resolve ^{13}C isotopomers and the notable effect of anesthesia on brain metabolism, while also allowing for the determination of isotopomer steady state commonly assumed in intraperitoneal injection approaches (Walls et al., 2011). We have shown that an accurate resolution of ^{13}C multiplets in the awake mouse brain is feasible and potentially valuable to determine rates of substrate uptake and of major brain metabolic pathways with further assistance from metabolic modeling. Importantly, in contrast with other studies performed in rats (Patel et al., 2004; Sibson et al., 1998; Sibson et al., 2001), the concentration of ^{13}C label infused avoided or minimized hyperglycemia (the maximum blood glucose level reached was 11 mM), preventing potential systemic metabolic confounders when excessive, supraphysiological glucose concentrations are reached.

Kinetic studies designed to measure rates of biochemical reactions within a metabolic network rely upon the analysis of multiple ^{13}C spectra. Steady-state isotopomer analysis can also allow for the determination of relative flux and labeling patterns of metabolic pathways, such as anaplerosis (Malloy, 1998). For instance, in the heart (mostly considered as a single-type cell organ for the purposes of NMR analysis), a comprehensive isotopomer analysis of glutamate C4 under steady-state ^{13}C enrichment conditions allowed the determination of carbon flux through the oxidative and anaplerotic pathways (Malloy et al., 1987). Moreover, a similar analysis of glutamate C4 – in this case not restricted to isotopic steady-state conditions- can be applied to estimate the enrichment of ^{13}C-acetyl-CoA in the intact tissue, a valuable parameter that provides information about substrate oxidation and competition in cells (Malloy et al., 1990). In the brain, the same analysis can be developed to compute relative rates of anaplerosis and ^{13}C enrichment of acetyl-CoA, with the caveats imposed by a multi-compartment system in which the rate of anaplerosis may differ between

compartments, and in which the pool of ^{13}Cacetyl-CoA may substantially diverge, depending of the availability and of substrate preference in each cell type considered.

As illustrated in Figure 2, multiplets were well resolved in all isotopomers, with glutamate exhibiting the highest resolvability of multiplets in all carbon positions relative to other isotopomers. Two potential reasons explain this finding: 1) glutamate is highly abundant in the brain (~ 12 µmol/g of wet tissue) relative to other ^{13}C-labeled amino acids (Chang et al., 1981), 2) and glutamate and α-ketoglutarate are in rapid exchange relative to neuronal and glial TCA cycle flux, resulting in the early appearance of glutamate in the ^{13}C spectrum relative to other isotopomers (Oz et al., 2004; Yang et al., 2009). On the other hand, the ^{13}C label incorporation (detected as ^{13}C multiplets) was found to be roughly proportional for interrelated molecules such as glutamate, glutamine and GABA. Overall, brain metabolic steady state was reached approximately at 150 min of infusion, which was significantly earlier than that previously observed in anesthetized mice (~250 min after infusion of larger amount of $[1,6-^{13}C_2]$glucose than the present protocol (bolus: 0.130 mg/g, infusion: 0.016 mg/g/min; (Nabuurs et al., 2008)). As noted in earlier studies of the rat brain, a likely explanation for this phenomenon is that anesthesia can limit the interpretation of metabolic fluxes as it significantly impacts the rate of certain key fluxes, such as the rate of the glutamate-glutamine cycle (V_{nt}), which is notably higher in conscious rats (0.57 ± 0.21 µmol/g/min) (Oz et al., 2004) relative to rats anesthetized with α-chloralose (0.2 ± 0.08 µmol/g/min) (Sibson et al., 2001) or pentobarbital (0.04 ± 0.01 µmol/g/min) (Choi et al., 2002).

Previous *in vivo* NMR approaches to mouse brain metabolism were limited because of the low ^{13}C spectral resolution leading to a substantial overlap between adjacent isotopomers, i.e. glutamate and glutamine C3, and because animals were subjected to anesthesia that interferes, in all likelihood, with brain metabolism (Nabuurs et al., 2008; Peled-Kamar et al., 1998). The studies cited have analyzed the appearance of ^{13}C in TCA cycle-related metabolites (for example, glutamate and glutamine) by measuring the ^{13}C contents of individual carbons identified in the NMR spectra. However, we have shown that multiplets can provide additional information (superior to ^{13}C contents alone) for the computation of metabolic flux rates and the ^{13}C enrichment of TCA cycle substrates (Jeffrey et al., 1999). For instance, the estimation of the oxygen consumption in the rat heart is significantly improved when multiplets are added to the kinetic analysis of NMR data, detecting 60% changes in oxygen utilization depending upon experimental conditions (Jeffrey et al., 1999). This feature of the method can simplify the characterization of different physiological and pathological conditions associated with abnormal oxygen consumption and metabolic rate. On the other hand, the presented approach will potentially benefit from innovative ^{13}C-enhancing techniques such as cryogenically cooled probes and hyperpolarization (Kovacs et al., 2005). Cryogenically cooled probes typically enable a 3–4-fold enhancement of the detection sensitivity in high-resolution NMR compared to conventional probes (Kovacs et al., 2005). Additionally, methods that strengthen the polarization of nuclear spins (termed hyperpolarization methods) such as dynamic nuclear polarization (DNP) can increase the ^{13}C sensitivity by a factor of up to 10,000 (Ardenkjaer-Larsen et al., 2003). We anticipate that these techniques may potentially improve even more the quality of the *ex vivo* ^{13}C spectra and will enhance the resolution of *in vivo* ^{13}C multiplets in the mouse brain.

In summary, the detection and analysis of time-dependent *ex vivo* ^{13}C NMR spectroscopy data arising from awake mouse brain metabolism can be enhanced to include information-rich multiple resonances. In this work, we have presented a method for the generation of high-quality ^{13}C spectra from the mouse brain that permitted accurate resolution of multiplets in glutamate, glutamine, GABA and aspartate. The results demonstrate that, overall, all detected TCA cycle-derived isotopomers manifest a similar evolution of

multiplets reaching steady state at 150 min with a quasi-physiological $[1,6-^{13}C_2]$glucose infusion. We anticipate that this method will be useful for the estimation of the rates of major metabolic pathways in the normal awake mouse brain and also in transgenic mouse models of human neurological diseases.

Highlights

We have developed a method for the *ex vivo* resolution of NMR multiplets arising from the awake mouse brain after the infusion of $[1,6-^{13}C_2]$glucose. (Most NMR studies have been conducted in large animals (often under anesthesia) because the weight of the target organ is a limiting factor for NMR).

NMR spectra obtained by this method display favorable signal-to-noise ratios that lend themselves to precise analysis.

The method enables the accurate resolution of multiplets over time in the awake mouse brain.

We anticipate that this method will be broadly applicable to compute brain fluxes in normal and transgenic mouse models of neurological disorders.

Abbreviations

NMR   nuclear magnetic resonance

TCA   tricarboxylic acid

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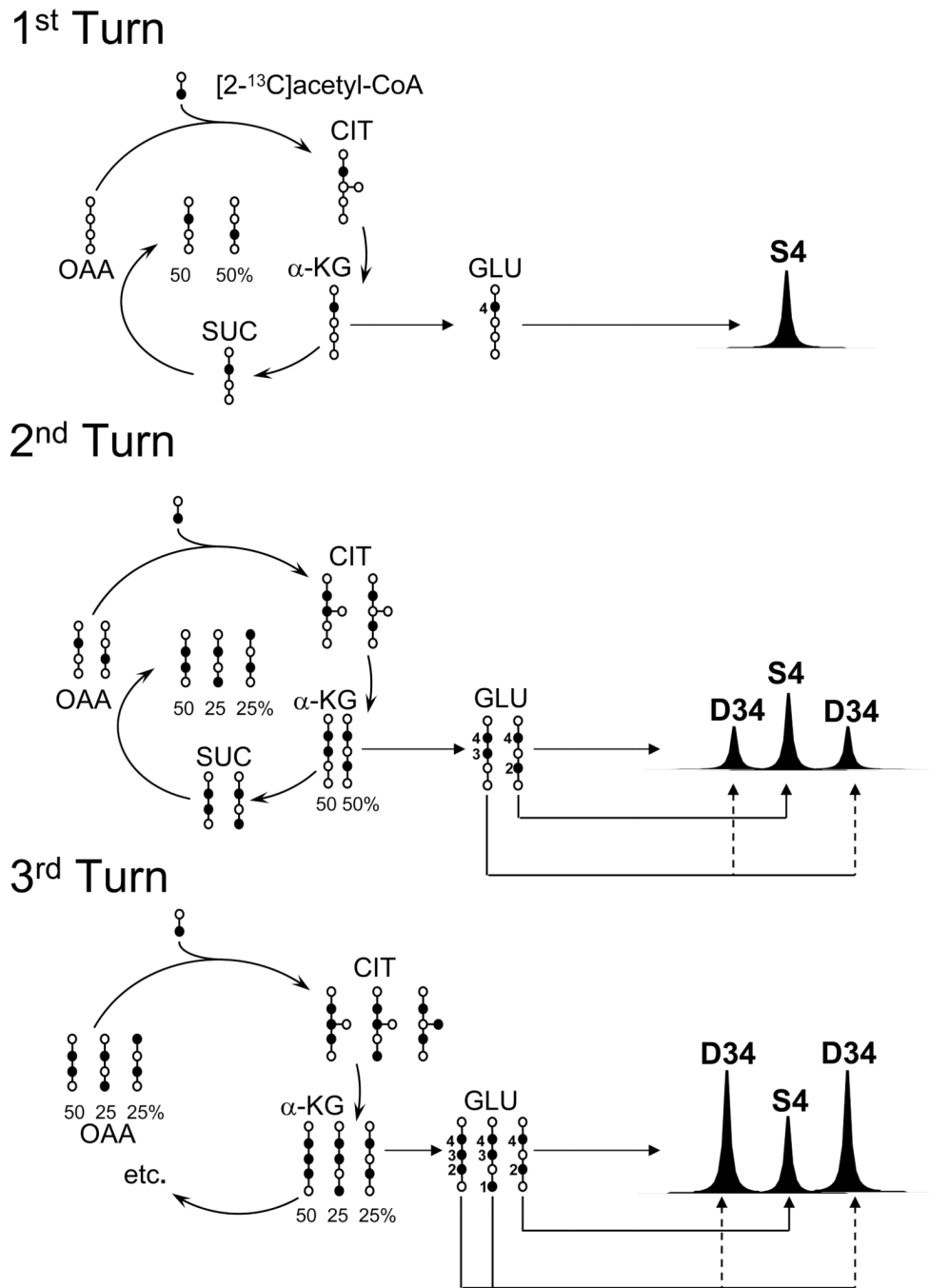
FIGURE 1. Evolution of glutamate labeling in the mouse brain TCA cycle
Schematic of the key reactions of the TCA cycle and the consequent ^{13}C-labeling and spectral pattern of brain glutamate (GLU) labeled in position 4 after the administration of [1,6-^{13}C$_2$]glucose. The intermediates citrate (CIT), α-ketoglutarate (α-KG), succinate (SUC) and oxaloacetate (OAA), are numbered from bottom (carbon 1) to top. This model assumes that the only source of ^{13}C for the TCA cycle is acetyl-CoA and that 100% of the acetyl-CoA is [2-^{13}C]acetyl-CoA. The resulting labeling pattern of glutamate via TCA cycle intermediate metabolism and the predicted ^{13}C NMR spectrum of glutamate arising from each "turn" of the cycle (number to the left) are shown. Note that carbons 4 and 5 of

glutamate are derived directly from acetyl-CoA and carbons 1, 2 and 3 originate from oxaloacetate.
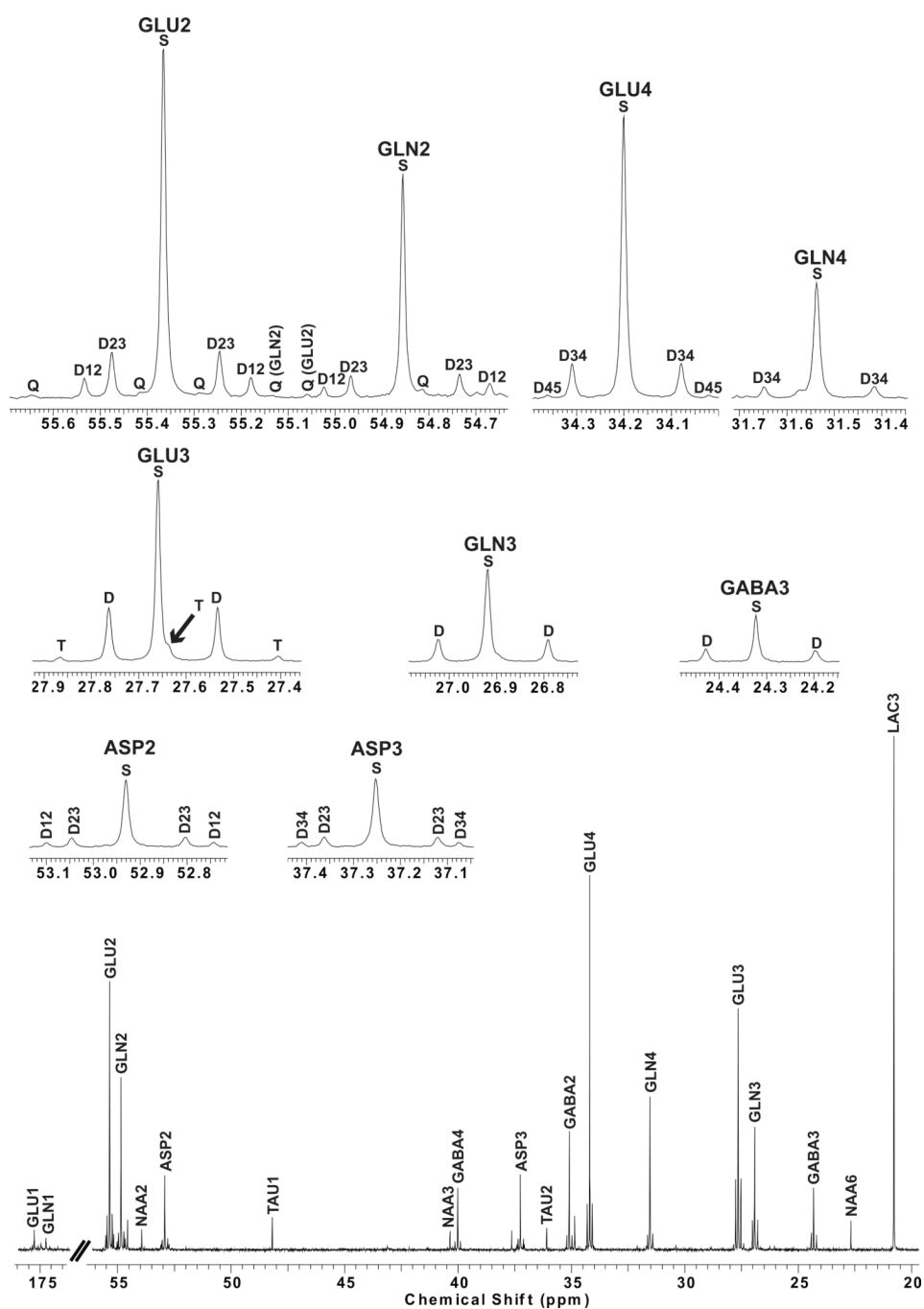
FIGURE 2. ^{13}C-NMR spectrum of the mouse forebrain at 75 min of infusion
Insets display the multiplets of some isotopomers detected in the ^{13}C spectrum. GLU: glutamate, GLN: glutamine, ASP: aspartate, NAA: n-acetylaspartate, TAU: taurine. C#: carbon labeled in position #. Sx: singlet, Dxx: doublet, T: triplet, Q: quartet.
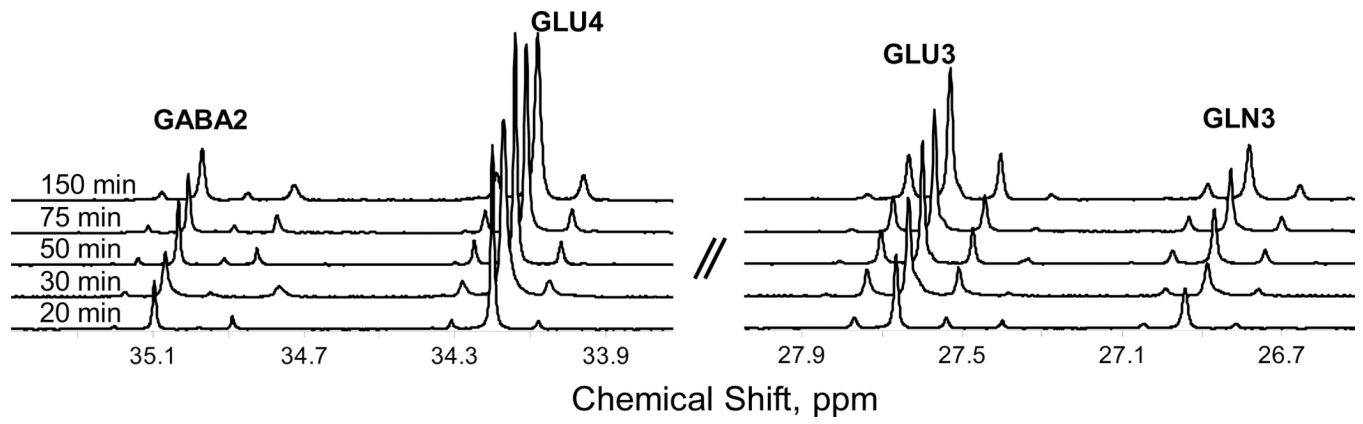
FIGURE 3. Evolution of ^{13}C multiplets of GABA C2, glutamate C3, C4 and glutamine C4 over time
The largest resonance achievable for each isotopomer is reached at 150 min of ^{13}C-glucose infusion initiation.
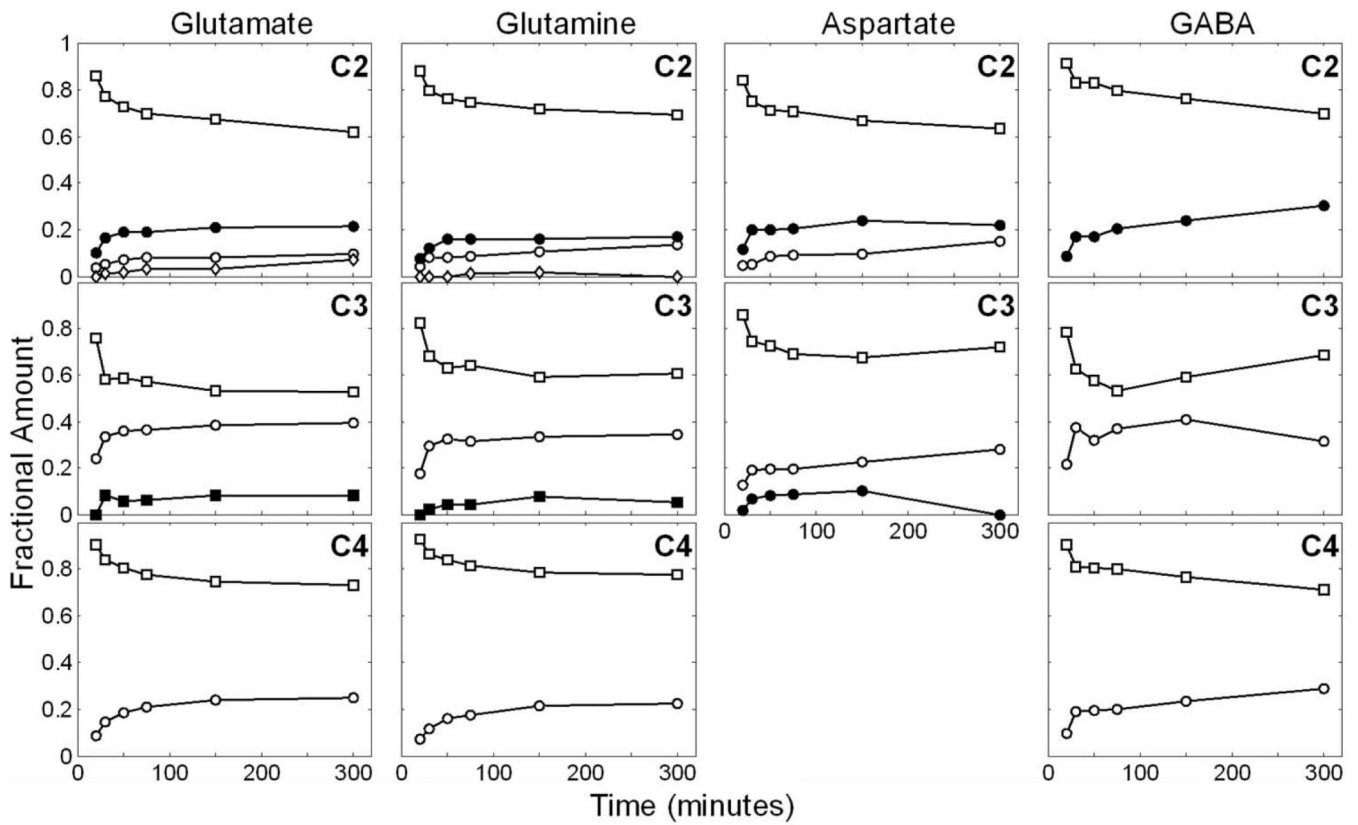
FIGURE 4. Time course of ^{13}C multiplets of glutamate, glutamine, GABA and aspartate in all carbon positions as detected by ^{13}C NMR spectroscopy
The multiplet fractional amount is calculated by the area of each multiplet relative to the total resonance area of the isotopomer as described in the methods section. Overall, the fractional amount of each multiplet in each isotopomer reaches steady state at 150 min with the present infusion protocol, implying brain metabolic equilibrium. Open square: singlet; C2: open circle: doublet12, closed circle: doublet23, diamond: quartet; C3: open circle: doublet, closed square: triplet. C4: open circle: doublet34. For aspartate C3: open circle: doublet23, closed circle: doublet34.