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

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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 infusion 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.

1. 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 1H) for NMR detection, and uncertainty about conditions required

Abbreviations: NMR, nuclear magnetic resonance; TCA, tricarboxylic acid.

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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.

2. Materials and methods

2.1. ^{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 (100 mg/kg) and xylazine (10 mg/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-$^{13}C_2$]glucose (1-^{13}C, 99% enrichment; 6-^{13}C, 97% enrichment, Cambridge Isotope Laboratories, Cambridge, MA) was administered as a bolus containing 0.4 mg/g (in 0.2 ml of saline) infused over 30 s, followed by continuous infusion of 0.012 mg/g/min (in 0.375 ml of saline) at 150 μl/h 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 s) 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.

2.2. 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 47,800 × 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 spectrophotometric assay (GAHK20-1KT kit, Sigma, St Louis, MO).

2.3. 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). 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 10,000–12,000 and for long infusions (150–300 min) were between 4000 and 6000. Proton spectra were similarly acquired to characterize the fractional enrichment of the α-anomer of the C1 proton resonance of D-glucose in blood. ^{1}H 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 achieve favorable signal-to-noise was 120.

2.4. 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 (Sherry and 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).

3. 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