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The First *in Vivo* Observation of ^{13}C–^{15}N Coupling in Mammalian Brain

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[5-^{13}C,^{15}N]Glutamine, with $^1J(^{13}C–^{15}N)$ of 16 Hz, was observed *in vivo* in the brain of spontaneously breathing rats by ^{13}C MRS at 4.7 T. The brain [5-^{13}C]glutamine peak consisted of the doublet from [5-^{13}C,^{15}N]glutamine and the center [5-^{13}C,^{14}N]glutamine peak, resulting in an apparent triplet with a separation of 8 Hz. The time course of formation of brain [5-^{13}C,^{15}N]glutamine was monitored *in vivo* with a time resolution of 20–35 min. This [5-^{13}C,^{15}N]glutamine was formed by glial uptake of released neurotransmitter [5-^{13}C]glutamate and its reaction with $^{15}NH_3$ catalyzed by the glia-specific glutamine synthetase. The neurotransmitter glutamate C5 was *selectively* ^{13}C-enriched by intravenous [2,5-^{13}C]glucose infusion to ^{13}C-label whole-brain glutamate C5, followed by [^{12}C]glucose infusion to chase ^{13}C from the small and rapidly turning-over glial glutamate pool, leaving ^{13}C mainly in the neurotransmitter [5-^{13}C]glutamate pool, which is sequestered in vesicles until release. Hence, the observed [5-^{13}C,^{15}N]glutamine arises from a coupling between ^{13}C of neuronal origin and ^{15}N of glial origin. Measurement of the rate of brain [5-^{13}C,^{15}N]glutamine formation provides a novel noninvasive method of studying the kinetics of neurotransmitter uptake into glia *in vivo*, a process that is crucial for protecting the brain from glutamate excitotoxicity.

Key Words: **^{13}C NMR; *in vivo*; ^{13}C–^{15}N coupling; glutamate; glutamine.**

INTRODUCTION

Glutamate (GLU) is a major excitatory neurotransmitter and is also a key component of intermediary metabolism. The neurotransmitter pool of GLU is sequestered in presynaptic vesicles (Fig. 1) and constitutes a separate pool from the glial and neuronal cytoplasmic and mitochondrial pools that undergo metabolic transformation (*1*). The neurotransmitter glutamate (GLU), after release into synaptic fluid and binding to the receptor, is taken up into astrocytes by excitatory amino acid transporters and metabolized to glutamine by astrocyte-specific glutamine synthetase. This process is crucial for maintaining a low extracellular concentration of GLU to protect the brain from its excitotoxicity (*2*). To study the kinetics of the uptake process *in vivo*, we have attempted to *selectively* ^{13}C-enrich the *neurotransmitter* GLU in rat brain. First, whole-brain GLU C5 was ^{13}C-enriched by intravenous infusion of [2,5-^{13}C]glucose. Then [^{12}C]glucose was infused to chase ^{13}C from the small and rapidly turning-over astrocyte GLU pool (∼1–2 micromol/g vs 10 micromol/g for the whole brain (*1, 3*)). This leaves ^{13}C mainly in the sequestered neurotransmitter GLU pool and the neuronal cytoplasmic pool which replenishes this vesicular pool. Subsequent uptake of the released neurotransmitter [5-^{13}C]GLU into astrocytes and metabolism to [5-^{13}C,^{15}N]GLN by reaction with $^{15}NH_3$ was observed *in vivo* by ^{13}C MRS. This [5-^{13}C,^{15}N]GLN is derived from ^{13}C of neuronal origin and ^{15}N of astrocyte origin, and the rate of its formation depends on the rate of neurotransmitter uptake into glia.

In animal brain, ^{13}C and ^{15}N MRS has been useful for measuring metabolic fluxes that contribute to the synthesis or utilization of GLU. ^{15}N MRS has permitted measurement of glutamine synthetase and glutaminase activities *in vivo* (*4, 5*), while ^{13}C MRS has been used to measure the overall GLN–GLU cycle rate through observation of their ^{13}C enrichments at C4, C3, and C2 during [1-^{13}C]glucose infusion (*6*). A recent ^{13}C MRS study demonstrates resolution of $^1J(^{13}C–^{13}C)$ of 34 Hz in C4 and C3 resonances of GLU–GLN *in vivo* (*7*). When the infusate is [2,5-^{13}C]glucose, acetyl-CoA ^{13}C-enriched in C1 enters the tricarboxylic acid (TCA) cycle and labels α-ketoglutarate C5 which, in turn, labels GLU C5 (Fig. 1). During further passage through the cycle, the label at α-ketoglutarate C5 is transferred to oxaloacetate C1 and C4. The former is removed as CO_2/HCO_3^- during the second turn of the cycle, while the latter is retained as α-ketoglutarate C1 (and GLU C1), with an enrichment one-half of that at C5 (not shown in Fig. 1; see for example Ref. (*8*)). *In vivo* observation of ^{13}C incorporation into C5 and C1 of GLU–GLN has been reported after a bolus injection of [2-^{13}C]glucose in monkey brain (*9*). However, to the best of our knowledge, *selective* ^{13}C enrichment of neurotransmitter GLU C5 and *in vivo* observation of the heteronuclear ^{13}C–^{15}N coupling in a mammalian brain metabolite have not been attempted previously.

In vitro, $^1J(^{13}C–^{15}N)$ of GLN has been observed in brain extracts. Natural-abundance ^{13}C NMR was used to measure $^1J(^{13}C–^{15}N)$ of 16.2 Hz in GLN, ^{15}N-enriched in 5-N by $^{15}NH_4Cl$ infusion, in rabbit brain extracts (*10*). Observation of [5-^{13}C,^{15}N]GLN in rat brain extracts after i.v. infusion of

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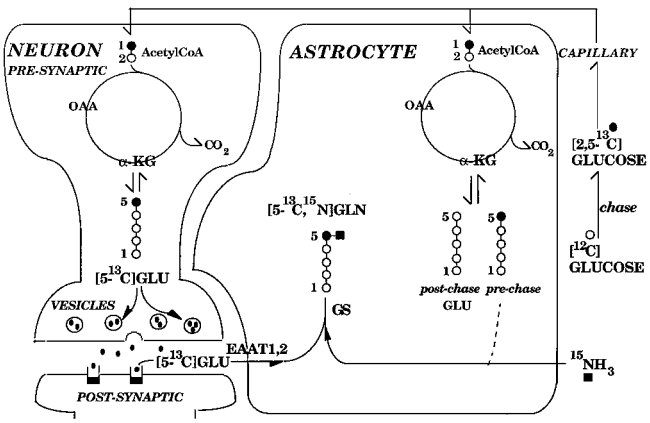

FIG. 1. A schematic diagram showing ^{13}C labeling of brain GLU C5 from infused [2,5-^{13}C]glucose and GLU transport from neuron to astrocyte. Abbreviations: α-KG, α-ketoglutarate; OAA, oxaloacetate; GS, glutamine synthetase; EAAT, excitatory amino acid transporter.

$^{15}NH_4Cl$ and [1,2-$^{13}C_2$]acetate has also been reported (*11*). It is interesting to note that this [5-^{13}C,^{15}N]GLN was formed by two glia-specific pathways, acetyl-CoA synthetase, which ^{13}C labels *glial* GLU from acetate, and glutamine synthetase, which catalyzes the reaction of [^{13}C]GLU with $^{15}NH_3$. By contrast, we infused [2,5-^{13}C]glucose, which labels neuronal and glial GLU C5, then removed ^{13}C from astrocyte GLU C5 before $^{15}NH_4Ac$ infusion. Hence, [5-^{13}C,^{15}N]GLN in our isotope-chase experiment arises from a coupling between ^{13}C of *neuronal* origin and ^{15}N of astrocyte origin. To maximize the ^{13}C enrichment of brain GLU C5, we have used the double-labeled [2,5-^{13}C]glucose as infusate. We report here *in vivo* observation of [5-^{13}C,^{15}N]GLN, formed mainly by glial uptake of selectively ^{13}C-enriched neurotransmitter GLU [5-^{13}C], in the brain of spontaneously breathing rats.

METHODS

Animal Preparation and Infusion Protocol

Male Wistar rats (250 $\pm$ 10 g) were prepared for intravenous infusion through the femoral vein and allowed to recover for 24 h. This minimized the duration of anaesthesia on the day of the MRS experiment and optimized physiological condition and brain glucose utilization under light anaesthesia by i.p. pentobarbital ($\sim$3.5 mg/kg wt every 15–20 min).

Glucose and ammonia were infused according to published procedures for achieving steady-state concentrations rapidly in the plasma (*12, 13*). Four infusion protocols, with the described objectives, were used in 16-h fasted rats.

Group I. Optimization of *in vivo* observation of carbonyl carbons and examination of the time course of brain GLU C5 enrichment. [2,5-^{13}C]Glucose (Isotec. Miamisburg, OH) was given, per 250 g body wt, as a bolus injection of 225 micromol followed by 150 micromol given in exponentially decreasing quantities over the next 8 min. Subsequently, a constant infusion rate of 1.0 mmol/h was used for 2–3.6 h.

Group II. Optimization of *in vivo* acquisition and quantitation of brain [5-^{13}C,^{15}N]GLN. $^{15}NH_4Ac$ (Cambridge Isotopes) was given, 20 min before [2,5-^{13}C]glucose infusion, as a bolus injection of 15 micromol followed by 720 micromol in exponentially decreasing quantities over 18 min. Subsequently, the infusion rate was 1.4 mmol/h for 2 h and then decreased to 1.26 mmol/h to maintain steady-state blood ammonia concentration. In this group, [2,5-^{13}C]glucose infusion rate was reduced to 0.9 mmol/h after 1.7 h to maintain blood glucose at steady state.

Group III. Optimization of the ^{12}C chase protocol. The *single-labeled* [2-^{13}C]glucose (Cambridge Isotopes) was infused for 2 h, followed by [^{12}C]glucose infusion for 0.3–0.46 h to examine the time course of decrease in brain [2-^{13}C]glucose and [5-^{13}C]GLU.

Group IV. *In vivo* observation of brain [5-^{13}C,^{15}N]GLN arising mainly from neurotransmitter [5-^{13}C]GLU. The *double-labeled* [2,5-^{13}C]glucose infusion for 2 h followed by 0.35 h (optimized duration) of [^{12}C]glucose infusion to chase ^{13}C from astrocyte GLU C5; then coinfusion of [^{12}C]glucose and $^{15}NH_4Ac$ (1.6 h) for formation of brain [5-^{13}C,^{15}N]GLN arising mainly from neurotransmitter [5-^{13}C]GLU. The $^{15}NH_4Ac$ dose was 710 micromol in the first 18 min followed by a constant infusion rate of 1.7 mmol/h. The [^{12}C]Glucose infusion rate was 1.0 mmol/h for 0.65 h, then reduced to $\sim$0.8 mmol/h to maintain steady-state blood glucose concentration.

The protocol was approved by the Institutional Animal Care and Use Committee and in conformance with the U.S. Public Health Service's *Guide for the Care and Use of Laboratory Animals.*

Optimization of Chase Time

The chase time required to replace astrocyte [5-^{13}C]GLU with ^{12}C was estimated from (a) experimental observation of the time course of decrease in brain [2-^{13}C]glucose and [5-^{13}C]GLU and (b) the following biochemical and kinetic considerations. *Glial* GLU pool, approximately 1–2 micromol/g (*1, 3*), is much smaller than whole-brain GLU pool (10 micromol/g), and is in rapid exchange with α-ketoglutarate (Fig. 1). α-Ketoglutarate C5, ^{13}C-labeled in the first turn of the TCA cycle, is removed as $^{13}CO_2/H^{13}CO_3^-$ during the second turn of the cycle. The reported whole-brain TCA cycle rate in rat is 0.46–1.59 micromol/g/min (*14, 15*). The lowest *glial* TCA cycle rate reported for *normal rat* brain is 0.4 micromol/g/min (*16*). This rate was derived from ^{13}C isotopomer analyses of brain metabolites after i.v. infusion of [1,2-^{13}C]glucose and [1,2-^{13}C]acetate. Because the TCA cycle rate is much slower than the GLU–α-ketoglutarate exchange rate (*14*), the rate-limiting step in removing *glial* GLU 5-^{13}C as $^{13}CO_2$ by chase with ^{12}C is the TCA cycle rate. (The *glial* TCA cycle rate reported for *hyperammonemic* rat brain is discussed later.) For *human* brain, lower *glial* TCA cycle rates (*17, 18*) and an α-ketoglutarate–glutamate exchange rate comparable to

the *neuronal* TCA cycle rate (*18*) have been reported recently. However, due to the difference in species as well as in the assumptions made in the kinetic analyses, we focus on published reports on rodent brain.

In normal rodent brain, the following reported observation strongly suggests that isotope label incorporation from acetyl-CoA to glutamate can proceed as rapidly in glia as in the whole brain. The ^{13}C enrichment of brain GLU measured 15 min after an intravenous injection of [2-^{13}C]acetate (which is metabolized only in glia) was compared with that observed after an injection of [1-^{13}C]glucose (metabolized in both glia and neurons) (*19*). With [2-^{13}C]acetate, the ^{13}C enrichment in whole-brain GLU C4 was 2.78%. After correction for the difference in GLU pools (~2 micromol/g in glia vs 10 micromol/g in the whole brain), this corresponds to a ^{13}C enrichment of $2.78/0.2 = 13.9\%$ of *glial* GLU. With equimolar [1-^{13}C]glucose injection, the ^{13}C enrichment of brain GLU C4 was 5.9%, which, after correction for the twofold label dilution from [1-^{13}C]glucose to [2-^{13}C]acetyl CoA, corresponds to an enrichment of 11.8%. Comparison of the two values (13.9 and 11.8%) strongly suggests that ^{13}C incorporation from acetyl- CoA to GLU C4 through the first half of the TCA cycle and the α-ketoglutarate–glutamate exchange can proceed as rapidly in the glia as in the whole-brain in normal rodents. Another study reports that, on injection of [1-^{14}C]acetate, specific activities of rat brain GLU and aspartate (ASP) were 1.17 and 0.086, respectively, after 2 min, and nearly equal at 1.23 and 1.1 after 5 min in normal rat (*20*). On the reasonable assumption that GLU/ASP concentration ratios are the same in glia and the whole brain, the rapid equilibration of specific activities strongly suggests that the rate of α-ketoglutarate–glutamate exchange catalyzed by aspartate transaminase

$$\alpha\text{-ketoglutarate} + \text{ASP} \rightleftharpoons \text{oxaloacetate} + \text{GLU}$$

is rapid in the glia of normal rat brain.

On the basis of this evidence, the time needed to chase ^{13}C from *glial* GLU C5 in rat brain was calculated as follows. Let P_{12} be the *glial* [5-^{12}C]GLU concentration, and P_{tot} the total glial GLU concentration ([5-^{12}C] + [5-^{13}C]). Let k be the rate constant for the rate-limiting step in replacing ^{13}C with ^{12}C. Then P_{12}/P_{tot} at time t can be estimated from

$$[P_{12}]/[P_{\text{tot}}] = E\left(1 - e^{-kt/[P_{\text{tot}}]}\right), \qquad [1]$$

where E is the fractional $[^{12}C]/[^{13}C + {}^{12}C]$ value for the substrate. Rearranging for t and substituting $[P_{\text{tot}}] = 2$ micromol/g (the upper limit for the estimated astrocyte GLU pool) and $k = 1.59$ micromol/g/min (the maximum whole-brain TCA cycle rate in rat (*14*)), we obtain

$$\begin{aligned} t &= -\ln[1 - (P_{12}/P_{\text{tot}})/E] \times ([P_{\text{tot}}]/k) \\ &= -1.26 \times \ln[1 - (P_{12}/P_{\text{tot}})/E]. \end{aligned} \qquad [2]$$

Substituting $k = 0.4$ micromol/g/min (the minimum *glial* TCA cycle rate reported for *normal rat brain* (*16*), we obtain

$$t = -5.0 \times \ln[1 - (P_{12}/P_{\text{tot}})/E]. \qquad [3]$$

If P_{tot} is 1 micromol/g (the lower limit for the estimated *glial* GLU pool in normal brain),

$$t = -2.5 \times \ln[1 - (P_{12}/P_{\text{tot}})/E]. \qquad [4]$$

In *hyperammonemic* rat brain, a rate of 0.096 micromol/min/g has been reported for *glial* TCA pathway from oxaloacetate to α-ketoglutarate (Fig. 1), which includes the decarboxylation of α-ketoglutarate C5 labeled in the first turn, on the assumption that *glial* GLU concentration is 0.2 micromol/g (*21*). Substituting $k = 0.096$ micromol/g/min and $P_{\text{tot}} = 0.2$ micromol/g, we obtain

$$t = -2.0 \times \ln[1 - (P_{12}/P_{\text{tot}})/E], \qquad [5]$$

which is within the range calculated for normal brain. Hence, the time t required to attain a target value of P_{12}/P_{tot} (the fraction of ^{12}C in *glial* GLU C5) can be calculated for a known value of E.

In Vivo ^{13}C MRS

Localized *in vivo* ^{13}C spectra were taken on Bruker-GE CSI-II spectrometer at 4.7 T. The probe (USA Instruments, Aurora, IN) consisted of a ^{13}C surface coil (25 mm diameter) and a saddle-type ^{1}H coil for shimming and decoupling. The rat, with the scalp retracted to minimize lipid signal (*22*), was placed with the skull 2 mm below the surface coil. Brain water was shimmed to a $\upsilon_{1/2}$ of ~20 Hz over a $16(x) \times 10(y) \times 18(z)$ mm^3 voxel using the STEAM sequence, and the $\upsilon_{1/2}$ checked every 40–120 min to ensure B_0 homogeneity during the *in vivo* experiments. The acquisition parameters were optimized in the following sequence. To resolve the GLN C5 "triplet" separated by 8 Hz (consisting of the center peak from [5-^{13}C,^{14}N]GLN and the flanking doublet from [5-^{13}C,^{15}N]GLN), it was necessary to acquire for 450 ms to achieve a spectral resolution of 2.25 Hz/data point. The relaxation delay was set at 3500 ms to minimize average nominal decoupler power output (20 W during acquisition and 10 W during delay) and tissue power absorption. To optimize sensitivity under these conditions for carbonyl carbons that have long T_1, the pulse width was varied. A 25-μs pulse resulted in an S/N ratio per unit time for brain [5-^{13}C]GLU *in vivo* better than that of a 40-μs pulse. Proton decoupling by WALTZ 16, using the bilevel decoupler power, removed $^{1}J(^{13}C$–$^{1}H)$ coupling from [2,5-^{13}C]glucose and C2, C3, and C4 of GLU/GLN and maximized their nuclear Overhauser enhancement (NOE) *in vitro*. To remove the two-bond coupling of [5-^{13}C]GLN with the amide protons ($J \sim 2.5$ Hz) and with the C-4 protons ($J \sim 7$ Hz), the decoupler frequency was set halfway between

these protons at 4.3 ppm with a 1000-Hz bandwidth; this resulted in minimum ^{13}C $\upsilon_{1/2}$ for [5-^{13}C,^{15}N]GLN *in vitro*.

In vivo ^{13}C spectra were acquired in 600-scan blocks; within each block, FID was stored cumulatively every 100 scans (6 min) for subsequent analysis at higher time resolution. The reported infusion time is at the center of the acquisition block. The FID spectrum acquired before [^{13}C]glucose infusion (preinfusion spectrum) was subtracted from post-infusion spectra before Fourier transformation, to remove the contribution from natural-abundance ^{13}C signals. ^{13}C peak assignments were based on published results (*23, 24*).

For quantitation, the *in vivo* peak area of a ^{13}C-labeled metabolite observed in the final acquisition block (300–600 scans) was divided by the actual quantity (micromol) of the ^{13}C-labeled metabolite measured in the perchloric acid extract of the same brain, to calculate the *in vivo* peak area/micromol/g of brain per unit acquisition time. This calibration factor was then used to convert the observed *in vivo* peak area during time course experiments to the metabolite concentration in micromol/g of brain. For partially overlapping triplet peaks of [5-^{13}C]GLN, the area under each peak was determined as follows. The spectral form of a triplet was assumed to be a sum of three Lorentzian-shaped peaks, 8 Hz apart. The amplitude and the width of each Lorentzian peak was determined by a least-squares fit to the observed spectrum, and the area computed by integration of each Lorentzian peak with frequency.

In Vitro Metabolite Assay

After the *in vivo* experiment, the brain was rapidly (within 15 s) frozen in liquid nitrogen (*25*) for preparation of a perchloric acid extract. For measurement of blood glucose and ammonia, serial arterial blood samples were taken in parallel bench-top infusion experiments and deproteinized (*26*). Glucose and ammonia in tissue extracts were assayed by enzymatic methods (*27, 28*). GLU and GLN in brain extracts were assayed after precolumn derivatization with *ortho*-phthaldehyde (OPA) and 2-mercaptoethanol, separation by reverse-phase HPLC (Beckman Instruments), and fluorometric detection (Jasco, FP920 fluorometer), according to published procedure (*29*), with the following modification in the chromatographic program. Elution with 25% methanol and 75% aqueous sodium phosphate buffer (50 mM, pH 5.29) for 10 min, followed by a linear increase of methanol to 84% in 36 min, achieved complete separation of the OPA derivatives of GLU and GLN from those of other common amino acids. ^{13}C and ^{15}N enrichments of brain and blood metabolites were determined from the total ($^{13}C + {}^{12}C$, or $^{15}N + {}^{14}N$) metabolite concentration and the concentration of ^{13}C- or ^{15}N-labeled metabolite measured by NMR. ^{15}N NMR spectra of tissue extracts were obtained as described previously (*30*) with the following modifications. The acquisition time was 450 ms, the relaxation delay 3500 ms, and the pulse flip angle was 55° for analysis of brain [5-^{15}N]GLN and 34° for blood $^{15}NH_4^+$, with on resonance-proton decoupling for the latter.

RESULTS AND DISCUSSION

^{13}C Enrichment of Whole-Brain GLU C5

Figure 2 shows an *in vivo* ^{13}C spectrum (after subtraction of the preinfusion spectrum) of rat brain after 1.5 h of [2,5-^{13}C]glucose infusion. ^{13}C incorporation into brain GLU C5 (182.0 ppm), GLN C5 (178.4 ppm), GLU–GLN (abbreviated to GLX) C1 (175.3–174.8 ppm), and $H^{13}CO_3^-$ (161 ppm) is observed. Contribution from natural-abundance ^{13}C was eliminated as shown by the clean baseline at 30 ppm where the methylene carbons of lipid resonate. Brain [2,5-^{13}C]glucose shows well-resolved peaks for C5 β (77 ppm) and C2 β (75.2 ppm) from which the time course of its increase could be monitored *in vivo*. As shown in Fig. 3A, brain [2,5-^{13}C]glucose reached near steady state after ~0.5 h of infusion. The total ($^{12}C + {}^{13}C$) brain glucose concentration at $t = 2$ h was 2.5 ± 0.1 micromol/g, with a ^{13}C enrichment of 0.48, as measured in the brain extract at end point (Table 1). This brain glucose concentration is only slightly higher than that (1.4–2.1 micromol/g) reported for normal fed rats (*31, 32*). Figure 3B shows blood glucose concentration during 3.5 h of infusion. From the preinfusion level of 4.3 micromol/g (fasted), blood glucose rose to 9.8 ± 0.5 micromol/g within 9 min and was maintained at 9–10 micromol/g for 3.5 h with a ^{13}C enrichment of 0.53 ±0.01.

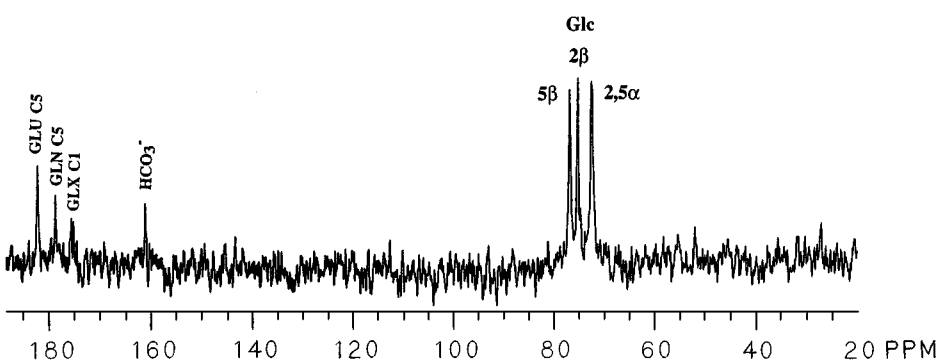

FIG. 2. An *in vivo* ^{13}C spectrum of rat brain after 1.5 h of [2,5-^{13}C]glucose infusion.

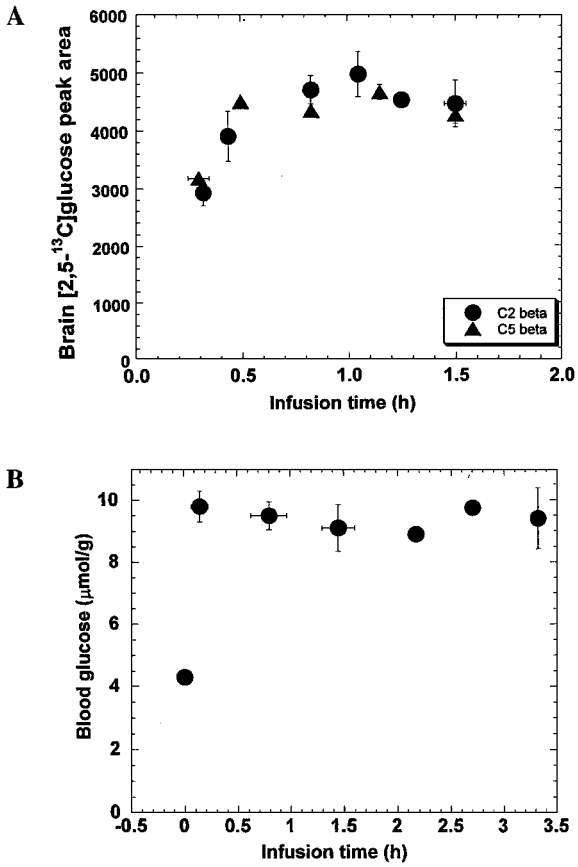

FIG. 3. (A) Changes in the *in vivo* peak areas of brain glucose C5 β anomer and C2 β anomer, shown as the mean $\pm$ sem for each peak, during i.v. infusion of [2,5-^{13}C]glucose ($n = 4$) or [2-^{13}C] glucose ($n = 6$; Group III). (B) Arterial blood glucose ($^{13}C + {}^{12}C$) concentration (mean $\pm$ sem for $n = 2$–6) during 3.5 h infusion.

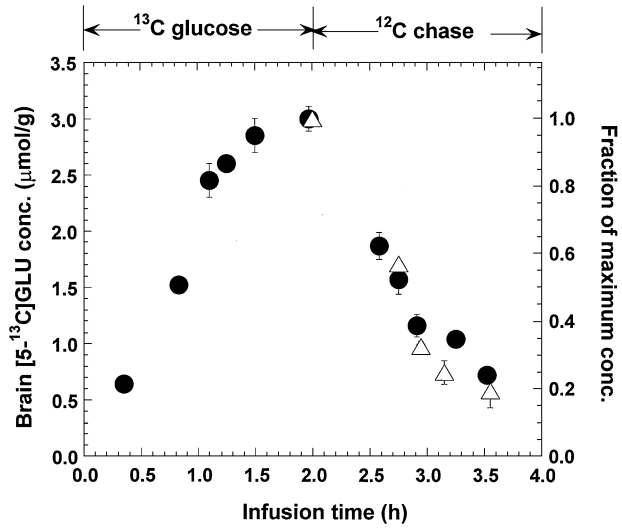

FIG. 4. Changes in brain [5-^{13}C]GLU *concentration* (●; left ordinate) ($n = 4$) during [2,5-^{13}C]glucose infusion ($t = 0$–2 h) followed by chase with [^{12}C]glucose (Group IV). For the chase period, brain [5-^{13}C]GLU decrease in [2-^{13}C]glucose-infused rats (Group III; $n = 6$), expressed as a *fraction* (△; right ordinate) of maximum prechase concentration, is also shown for comparison.

This blood glucose concentration is only slightly higher than that in fed rats (7–8 micromol/g (*22*)), indicating that our rats were only slightly hyperglycemic. Figure 4 shows the time course of increase in brain [5-^{13}C]GLU observed *in vivo* during 2 h of [2,5-^{13}C]glucose infusion. By $t = 1.1$ h, brain [5-^{13}C]GLU concentration reached 2.45 ± 0.15 micromol/g. Further gradual increase to 3.0 ± 0.11micromol/g, with a fractional ^{13}C enrichment of 0.35 ± 0.01 was observed by $t = 2$ h. Because longer infusion (3.6 h) resulted in only a modest increase in ^{13}C enrichment to 0.45 (see Table 1, not shown in Fig. 4), [2,5-^{13}C]glucose infusion to ^{13}C-enrich whole-brain GLU C5 was performed only for 2 h, in experiments involving isotope chase (Fig. 4).

[5-^{13}C,^{15}N]GLN Derived from Whole-Brain [5-^{13}C]GLU

Figure 5A shows an *in vivo* ^{13}C spectrum (carbonyl region) of rat brain after coinfusion of [2,5-^{13}C]glucose and $^{15}NH_4Ac$ (Group II). GLN C5 peak is elevated and the C1 peaks of GLU (175.3 ppm) and GLN (174.8 ppm) are more clearly observed than in Fig. 2. Figure 5B shows an expanded plot of the *in vivo* GLN C5 peak after 1.1 h of infusion. The doublet arising from brain [5-^{13}C,^{15}N]GLN with $^1J(^{13}C–^{15}N) = 16$ Hz flanks the center peak from [5-^{13}C,^{14}N]GLN, resulting in an apparent triplet with 8 Hz separation. This is the first *in vivo* observation of ^{13}C–^{15}N coupling in a mammalian brain metabolite. Figure 5C

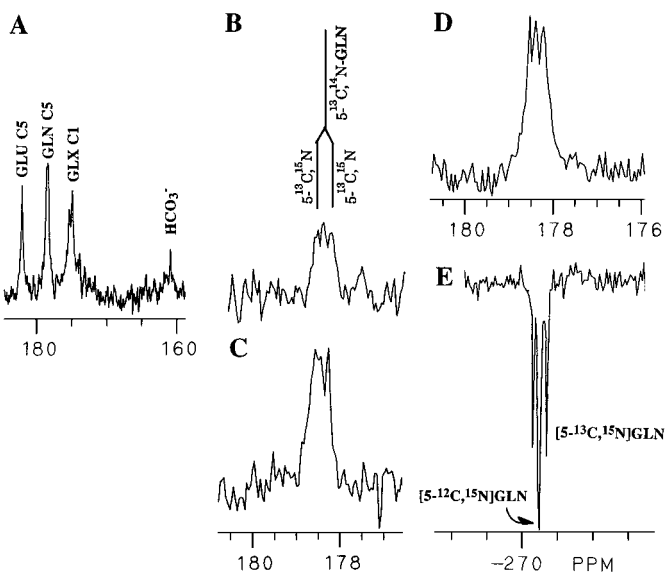

FIG. 5. (A–C) *In vivo* ^{13}C spectra of rat brain during coinfusion of [2,5-^{13}C] glucose and $^{15}NH_4Ac$ acquired in 33–39 min. (A) Carbonyl region after 3.8 h of infusion. (B) An expanded plot of the GLN C5 peak after 1.1 h and (C) 3.1 h of infusion. (B) and (C) were processed with a line broadening of 2 Hz. (D, E) *In vitro* spectra of the brain extract at endpoint. (D) A ^{13}C spectrum of the GLN C5 region. (E) An ^{15}N spectrum of the [5-^{15}N]GLN region. In the proton-decoupled, NOE-enhanced spectrum, the peaks are inverted due to the negative gyromagnetic ratio of ^{15}N.

TABLE 1

The Concentrations and Isotopic Enrichments of Brain and Blood Metabolites after I.V. Infusion of [2,5-^{13}C]Glucose, ^{12}C-Glucose, or $^{15}NH_4Ac$ for the Indicated Time

Metabolite	Conc. (μmol/g)	Fractional enrichment ^{13}C	Fractional enrichment ^{15}N	Conc. (μmol/g)	Fractional enrichment ^{13}C	Fractional enrichment ^{15}N
	[2,5-^{13}C]Glc infusion: $t = 2$ h				$t = 3.6$ h	
Blood glucose	9.5 ± 0.3	0.53 ± 0.01		9.4 ± 0.24		
Brain glucose	2.5 ± 0.1	0.48		2.6 ± 0.08	0.53 ± 0.03	
Brain GLU						
Group I						
Total	8.5			8.8		
5-^{13}C	3.0 ± 0.11	0.35 ± 0.01		4.0	0.45	
1-^{13}C	1.15 ± 0.05	0.13 ± 0.05		1.7	0.19	
Brain GLN						
Group I						
Total	5.8 ± 0.1					
5-^{13}C	1.2 ± 0.12	0.21 ± 0.02				
	[2,5-^{13}C]Glc + $^{15}NH_4Ac$: $t = 2.2$ h				$t = 4.1$ h	
Group II						
Total	15.5			17.8		
5-^{13}C total	3.9 ± 0.2	0.25 ± 0.02		4.58 ± 0.23	0.26	
5-^{13}C,^{15}N	2.5	0.16	0.16	3.1	0.18	0.18
5-^{13}C,^{14}N	1.4 ± 0.1	0.09 ± 0.001		1.48	0.08	
5-^{12}C,^{15}N	5.7		0.37	5.7		0.32
5-^{15}N total	8.2		0.53	8.8		0.5
1-^{13}C	1.2 ± 0.3	0.08 ± 0.02		1.48 ± 0.083	0.083	
	[2,5-^{13}C]Glc infusion: $t = 2$ h			[^{12}C]Glc 1.5 h + $^{15}NH_4Ac$ 1.15 h		
Group IV						
Total	5.8 ± 0.1			11.2 ± 0.6		
5-^{13}C total	0.95 ± 0.05	0.15 ± 0.01		1.65 ± 0.02	0.15 ± 0.01	
5-^{13}C,^{15}N				1.17 ± 0.1	0.10 ± 0.01	0.10 ± 0.01
5-^{13}C,^{14}N				0.48 ± 0.05	0.04 ± 0.004	
5-^{12}C,^{15}N				4.4 ± 0.4		0.39 ± 0.04
5-^{15}N total				5.6 ± 0.7		0.50 ± 0.02
	Glc + $^{15}NH_4Ac$: $t = 0.2$–2 h				$t = 3.5$ h	
Blood NH_3						
Group II	0.85 ± 0.02			0.86 ± 0.01		
				Post-chase $^{15}NH_4Ac$: $t = 0.2$–2 h		
Group IV				1.0 ± 0.03		0.87 ± 0.01
Brain NH_3						
Group II	1.1					
Group IV				1.3 ± 0.02		

Note. The values are mean ± sem for $n = 2$–5, except in exploratory long-infusion (3.6–4.1 h) experiments performed on a single animal.

shows the corresponding *in vivo* spectrum after 3.1 h of infusion. The S/N ratio of the [5-^{13}C]GLN triplet is high, but only the high-field component of the [5-^{13}C,^{15}N]GLN doublet is resolved from the center [5-^{13}C,^{14}N]GLN peak. This is mainly due to partial loss of resolution when sensitivity is enhanced. However, a slight asymmetry of the apparent triplet is expected due to the ^{15}N isotope effect on ^{13}C chemical shift which, *in vitro*, caused an upfield shift of 0.016 ppm of the [5-^{13}C,^{15}N]GLN doublet relative to the [5-^{13}C,^{14}N]GLN peak, resulting in an asymmetric triplet as first reported by Lapidot and Gopher (*10*). The linewidth of the high-field component of brain [5-^{13}C,^{15}N]GLN observed *in vivo*, measured without application of an exponential filter, was 5 Hz. The singlet peak for brain [5-^{13}C]GLU also had a $\upsilon_{1/2}$ of 5 Hz *in vivo*. This is the ^{13}C linewidth expected from the observed ^{1}H linewidth of 20 Hz for brain water and $\gamma(^{1}H)/\gamma(^{13}C)$ of 4. The result strongly suggests that other possible causes of line broadening, such as incomplete removal of long-range ^{13}C–^{1}H couplings, have been eliminated under our experimental conditions. Figure 5D shows a ^{13}C spectrum, and Fig. 5E an ^{15}N spectrum of the brain extract prepared after the *in vivo* experiment. In the ^{15}N spectrum, the doublet from [5-^{13}C,^{15}N]GLN flanks the center peak from [5-^{12}C,^{15}N]GLN.

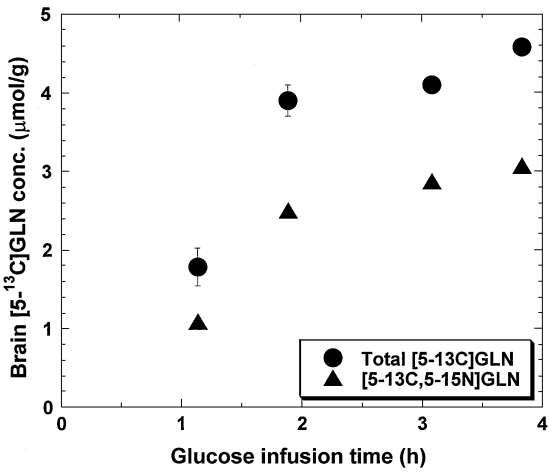

FIG. 6. The time course of increase in brain [5-^{13}C,^{15}N]GLN (▲) and total [5-^{13}C]GLN ($^{14}N + {}^{15}N$) (●) during coinfusion of [2,5-^{13}C]glucose and $^{15}NH_4Ac$. The error bar shows the range for $n = 2$.

The concentrations and ^{13}C and ^{15}N enrichments of brain GLN are listed in Table 1 (Group II). The time course of increase in brain [5-^{13}C,^{15}N]GLN and in total ($^{14}N + {}^{15}N$)[5-^{13}C]GLN, as observed *in vivo*, are shown in Fig. 6. This [5-^{13}C,^{15}N]GLN was formed by reaction of $^{15}NH_3$ with [5-^{13}C]GLU labeled within glia, as well as with [5-^{13}C]GLU labeled in neurons, released as neurotransmitter and taken up into astrocytes.

Selective ^{13}C Enrichment of Neurotransmitter GLU C5

In rats given [2-^{13}C]glucose infusion for 2 h followed by chase (Group III), brain [2-^{13}C]glucose lost 90% of the *in vivo* ^{13}C signal (2 β peak) intensity by 18 min of chase (see Fig. 7B for result obtained subsequently with [2,5-^{13}C]glucose infusion). Because the fractional ^{13}C enrichment of brain glucose was 0.48 before the chase, the fraction of ^{12}C in brain glucose C 2β after the 18-min chase is $[1 - 0.48 \times 0.1] = 0.95$. It is then reasonable to assume that C1 of acetyl-CoA entering the TCA cycle and C5 of α-ketoglutarate that exchanges with astrocyte GLU (Fig. 1) also have $E = 0.95$ in Eq. [1]. Substituting this into Eq. [2] on the reasonable assumption that the upper limit for *glial* GLU pool is 2 micromol/g, we obtain $t = 3$ min for the time needed to raise P_{12}/P_{tot} (the fraction of ^{12}C in astrocyte GLU C5) to 0.88. Substitution in Eq. [3] yields $t = 13$ min. If the *glial* GLU pool is 1 micromol/g (*3*) and the *glial* TCA cycle rate is the minimum value reported for normal rat brain, 0.4 micromol/g/min (*16*), we obtain $t = 6.5$ min from Eq. [4]. Hence, if we add a chase time of 3–13 min to the initial 18-min chase determined experimentally above, we can expect the fraction of ^{12}C in astrocyte GLU C5 to be >0.88 at the end of the chase. The observed whole-brain [5-^{13}C]GLU will then represent mainly neuronal GLU, which consists of the metabolically inactive vesicular neurotransmitter pool and the cytoplasmic pool that replenishes the neurotransmitter pool. Accordingly, a total chase time of 21 min (0.35 h) was used in subsequent experiments before starting coinfusion of $^{15}NH_4Ac$ and [^{12}C]glucose for formation and *in vivo* observation of [5-^{13}C,^{15}N]GLN arising from *glial* uptake of released neurotransmitter [5-^{13}C]GLU. The longer total chase time of $18 + 13 = 31$ min was not used for the following reasons. After the start of $^{15}NH_4Ac$ and [^{12}C]glucose coinfusion, a further increase in the fraction of ^{12}C in astrocyte GLU C5 is expected to occur in the 12-min interval that is needed for the ^{15}N enrichment of blood ammonia to reach steady state (see below). Furthermore, the quantity of [5-^{15}N]GLN formed in the first 8 min of $^{15}NH_4Ac$ infusion (which is close to the difference between the shorter chase time of 21 min and the longer one of 31 min) was only 4% of the total [5-^{15}N]GLN formed in the chase experiment, as determined in the parallel bench-top infusion experiment. Hence, any additional [5-^{13}C,^{15}N]GLN that may arise from trace *glial* [5-^{13}C]GLU as a result of using the shorter chase time would only be $0.12 \times (4\%) = 0.48\%$ of the total [5-^{13}C,^{15}N]GLN formed, which is negligible.

Figure 7A is a typical prechase *in vivo* spectrum after 2 h of [2,5-^{13}C]glucose infusion (Group IV). Figure 7B shows the *in vivo* spectrum after 0.35 h of chase with [^{12}C]glucose; 90% of brain [2,5-^{13}C]glucose and ~30% of whole-brain [5-^{13}C]GLU were replaced by ^{12}C. The latter observation is consistent with the loss of ^{13}C from astrocyte GLU (10–20% of whole-brain GLU) and a part of the neuronal metabolic pool of GLU that exchanges with α-ketoglutarate. The time course of a decrease in brain [5-^{13}C]GLU observed *in vivo* during the chase is shown in Fig. 4 (filled circle for Group IV). Also shown is the time course of a decrease in brain [5-^{13}C]GLU from [2-^{13}C]glucose-infused rats (open triangle; Group III), expressed as a fraction of the maximum prechase concentration (right ordinate). When the infusate is single-labeled [2-^{13}C]glucose, the mean prechase brain [5-^{13}C]GLU *concentration* after a 2-h infusion was 1.6 ± 0.08 micromol/g, which, as expected, was approximately one-half of that (3.0 micromol/g) achieved with double-labeled glucose infusion, but the time courses of decrease expressed as a *fraction* of the prechase maximum concentration are very similar in the two groups, demonstrating the reproducibility of the effects of the chase.

The $H^{13}CO_3^-$ peak (161 ppm) in the prechase brain (Fig. 7A) is produced from [5-^{13}C]α-ketoglutarate, via oxaloacetate C1, during passage through the TCA cycle. During chase by ^{12}C, [5-^{13}C]GLU that exchanges with α-ketoglutarate also produces $^{13}CO_2/H^{13}CO_3^-$, but in decreasing quantity as ^{12}C replaces ^{13}C. The observed decrease in the $H^{13}CO_3^-$ peak after 0.35 h of chase (Fig. 7B) is consistent with the loss of [5-^{13}C]GLU from the smaller *glial* pool and part of the larger neuronal *metabolic* pool. After 0.95 h of chase, the $H^{13}CO_3^-$ peak is undetectable (Fig. 7C), while [5-^{13}C]GLU and [5-^{13}C,^{15}N]GLN are clearly observed (Fig. 7C). This decrease in $H^{13}CO_3^-$ beyond the limit of detection is consistent with the removal of ^{13}C from the TCA cycle intermediates in the glia and the neuronal metabolic compartment. The observed residual [5-^{13}C]GLU is likely to represent mainly the sequestered vesicular neurotransmitter

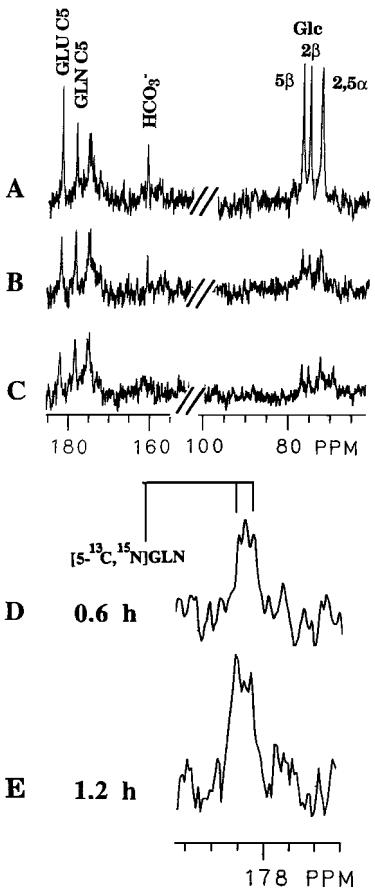

FIG. 7. *In vivo* brain ^{13}C spectra of [2,5-^{13}C]glucose-infused rat before (A) and after (B–E) chase with [^{12}C]glucose for selective ^{13}C enrichment of neurotransmitter GLU C5: (A) After 2 h of [2,5-^{13}C]glucose infusion, (B) after 0.35 h of chase with [^{12}C]glucose, (C) after 0.96 h of chase, (D) expanded plot of GLN C5 peak, acquired in 20 min after 0.6 h of $^{15}NH_4Ac$ coinfusion with [^{12}C]glucose, showing formation of [5-^{13}C,^{15}N]GLN arising from *glial* uptake of neurotransmitter [5-^{13}C]GLU, and (E) a corresponding spectrum after 1.2 h of infusion.

pool that does not exchange with α-ketoglutarate to produce $H^{13}CO_3^-$.

Additional evidence for removal of ^{13}C from *glial* precursor of GLU C5 by the chase was obtained as follows. If *glial* pyruvate C2 had residual ^{13}C in the post-chase period, the label would be partly transferred to C1 of acetyl-CoA by pyruvate dehydrogenase and hence to GLU C5, and partly, by the *glial*-specific pyruvate carboxylase, to oxaloacetate C2. This will label α-ketoglutarate at C3 and, through further metabolism in the TCA cycle, at C2 as well, resulting in the labeling of GLU and GLN at C3 and C2. The C2 and C3 carbons of α-ketoglutarate are retained during passage through the TCA cycle, unlike C5 and C1, which are removed as CO_2. Hence, the total ^{13}C in GLU–GLN C2 + C3 in the post-chase brain is expected to be *higher* than that in the prechase brain, *if* ^{13}C continued to flow into *glial* pyruvate C2 from brain glucose after the end of the 0.35-h chase. Because NH_4Ac infusion (performed in the post-chase period) is known to stimulate the pyruvate-carboxylase pathway (*21* and references cited therein), control experiments to examine prechase brain were performed both with and without NH_4Ac infusion. Table 2 shows the concentrations of [2-^{13}C]GLU, [3-^{13}C]GLU, [2-^{13}C]GLN, and [3-^{13}C]GLN, as well as their sum, in the pre- and post-chase brain extracts (Group III rats, see legend for details). The total ^{13}C in the post-chase brain with NH_4Ac infusion was 28% lower than that observed in the corresponding prechase brain with NH_4Ac infusion, and 6% lower than that in prechase brain without NH_4Ac infusion. The result is consistent with an effective removal of ^{13}C from *glial* pyruvate C2 by the 0.35-h chase.

Brain [5-^{13}C,^{15}N]GLN Derived Mainly from Neurotransmitter [5-^{13}C]GLU

On start of $^{15}NH_4Ac$ infusion, blood NH_3 reached a steady-state concentration of 1.0 ± 0.03 micromol/g with an ^{15}N enrichment of 0.87 ± 0.01 within 12 min (Table 1, Group IV). Figure 7D shows a post-chase *in vivo* ^{13}C spectrum (GLN C5 region) of rat brain, acquired in 20 min, after 0.6 h of $^{15}NH_4Ac$ infusion. The [5-^{13}C,^{15}N]GLN doublet flanking the center [5-^{13}C,^{14}N]GLN peak is clearly observed. A corresponding *in vivo* spectrum obtained after 1.2 h (Fig. 7E) shows an increase in the peak area of the [5-^{13}C,^{15}N]GLN doublet. The time courses of increase in brain [5-^{13}C,^{15}N]GLN and total ($^{14}N + {}^{15}N$)[5-^{13}C]GLN are shown in Fig. 8. The concentration and isotopic enrichments at endpoint are listed in Table 1 (Group IV). The

TABLE 2
Concentrations of Brain GLU and GLN ^{13}C-Enriched at C2 and C3 Carbons, Measured in Brain Extracts after I.V. Infusion of [2-^{13}C]Glucose, with or without Chase by ^{12}C and NH_4Ac Infusion

	Concentration (μmol/g)				
	[3-^{13}C]GLU	[2-^{13}C]GLU	[3-^{13}C]GLN	[2-^{13}C]GLN	Total
Normal pre-chase brain[a] ($n = 2$)	0.155 ± 0.005	0.17 ± 0.01	0.17 ± 0.01	0.125 ± 0.015	0.62 ± 0.01
Hyperammonemic pre-chase brain[b] ($n = 2$)	0.165 ± 0.025	0.14 ± 0.02	0.38 ± 0.07	0.16 ± 0.01	0.80 ± 0.1
Hyperammonemic post-chase brain[c] ($n = 4$)	0.10 ± 0.01	0.09 ± 0.01	0.21 ± 0.02	0.17 ± 0.009	0.58 ± 0.01

[a] [2-^{13}C]Glucose infusion for $t = 0$–2 h.
[b] The same as in footnote *a* but NH_4Ac infused during $t = 1$–2 h.
[c] [2-^{13}C]Glucose infusion for $t = 0$–2 h, followed by [^{12}C]glucose infusion during $t = 2$–3.6 h and NH_4Ac coinfused during 2.35–3.6 h.

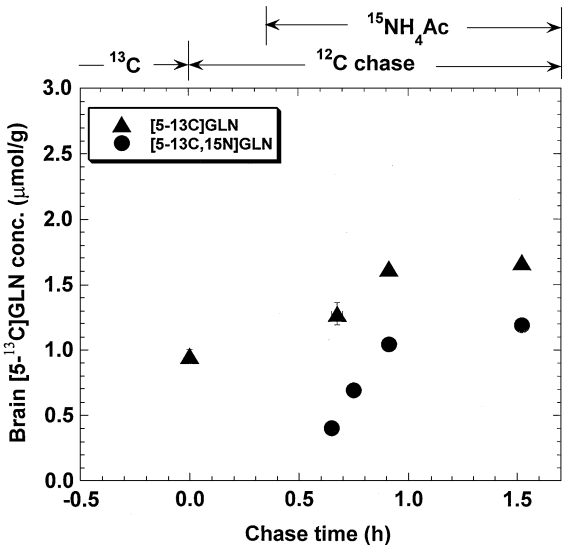

FIG. 8. The time course of increase in [5-^{13}C,^{15}N]GLN (●) and total [5-^{13}C]GLN ($^{15}N + {}^{14}N$) (▲), shown as the mean ± sem for $n = 2$, after ^{12}C chase to selectively ^{13}C-enrich neurotransmitter GLU C5.

result demonstrates that [5-^{13}C,^{15}N]GLN, arising mainly from glial uptake of released neurotransmitter [5-^{13}C]GLU, can be observed *in vivo* in the brain of spontaneously breathing rats.

In conclusion, we report the first *in vivo* observation of [5-^{13}C,^{15}N]GLN, with $^1J(^{13}C–^{15}N)$ of 16 Hz, in mammalian brain. This metabolite arises mainly from glial uptake of neurotransmitter [5-^{13}C]GLU, which was selectively ^{13}C-enriched by intravenous infusion of [2,5-^{13}C]glucose followed by isotope chase. Hence, measurement of the rate of formation of this metabolite provides a novel, noninvasive method of studying the kinetics of neurotransmitter uptake *in vivo*. This kinetic study is now in progress in our laboratory. The work reported here strongly suggests that *in vivo* MRS monitoring of heteronuclear coupling in a brain metabolite that was formed from two precursors that were isotopically enriched in different brain compartments can be a powerful tool for studying the kinetics of their intercompartmental flux in the intact brain. Although sensitivity enhancement resulted in partial loss of resolution for brain [5-^{13}C]GLN "triplet" in some of our *in vivo* spectra, the observation that the linewidths of the carbonyl carbons of GLU and GLN *in vivo* were mainly limited by B_0 inhomogeneity in the brain is encouraging. In the brains of larger species, improved shimming, and hence B_0 homogeneity, may permit higher spectral resolution of the J-coupled resonances. At higher B_0 fields, improved sensitivity and temporal resolution may permit kinetic studies of transport processes faster than that addressed in our study.

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