

Leucine-nitrogen metabolism in the brain of conscious rats: its role as a nitrogen carrier in glutamate synthesis in glial and neuronal metabolic compartments

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Abstract

The source of nitrogen (N) for the de novo synthesis of brain glutamate, glutamine and GABA remains controversial. Because leucine is readily transported into the brain and the brain contains high activities of branched-chain aminotransferase (BCAT), we hypothesized that leucine is the predominant N-precursor for brain glutamate synthesis. Conscious and unstressed rats administered with [U-¹³C] and/or [¹⁵N]leucine as additions to the diet were killed at 0–9 h of continuous feeding. Plasma and brain leucine equilibrated rapidly and the brain leucine-N turnover was more than 100%/min. The isotopic dilution of [U-¹³C]leucine (brain/plasma ratio 0.61 ± 0.06) and [¹⁵N]leucine (0.23 ± 0.06) differed markedly, suggesting that 15% of cerebral leucine-N turnover derived from proteolysis and 62% from leucine

synthesis via reverse transamination. The rate of glutamate synthesis from leucine was 5 μmol/g/h and at least 50% of glutamate-N originally derived from leucine. The enrichment of [5-¹⁵N]glutamine was higher than [¹⁵N]ammonia in the brain, indicating glial ammonia generation from leucine via glutamate. The enrichment of [¹⁵N]GABA, [¹⁵N]aspartate, [¹⁵N]glutamate greater than [2-¹⁵N]glutamine suggests direct incorporation of leucine-N into both glial and neuronal glutamate. These findings provide a new insight for the role of leucine as N-carrier from the plasma pool and within the cerebral compartments.

Keywords: branched-chain aminotransferase, gas chromatography/mass spectrometry, glia, leucine/α-ketoisocaproate shuttle, α-ketoglutarate, neuron.

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Glutamate and its metabolic product GABA are, respectively, major excitatory and inhibitory neurotransmitters. Because there is virtually no entry of plasma glutamate into the brain (Oldendorf 1971), the brain glutamate pool derives almost exclusively from synthesis de novo. Although it is well established that brain glutamate biosynthesis utilizes plasma glucose as the main carbon precursor (Shank and Campbell 1983), the main source of brain glutamate-N remains controversial.

The general consensus with regard to the origin of neuronal glutamate is that it proceeds via the initial synthesis of glutamine in the glia and that this is transported to the neurons where its deamidation releases glutamate for storage or further metabolism to GABA (Rothman 2001). Following synaptic excitation, a portion of the glutamate and GABA released from the neuron is taken up by glial cells and

recycled to glutamine synthesis (de Barry *et al.* 1983). Accordingly, the rate of glutamate-glutamine cycle (Sibson *et al.* 1997) exceeds the total N-influx predicted from the influx of individual amino acids across the blood–brain

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¹This paper is dedicated with affection to the memory of one of its co-authors, Peter J. Reeds.

Abbreviations used: AUC, area under the curve; BCAT, branched-chain aminotransferase; C, carbon; GC/MS, gas chromatography/mass spectrometry; GDH, glutamate dehydrogenase; α-KG, α-ketoglutarate; α-KIC, α-ketoisocaproate; MPE, mol percent excess; N, nitrogen; PCA, perchloric acid; TTR, tracer/tracee ratio.

barrier (Smith *et al.* 1987). Although the glutamate/glutamine cycle could conserve the N-moieties of both amino acids, there is, in fact, a necessity for the continual replenishment of the glial N-pool because the brain releases glutamine to the jugular vein (Grill *et al.* 1992). On the basis of the incorporation of glucose carbon (-C) into cerebral glutamine, Lapidot and Gopher (1994) estimated that the anaplerotic pyruvate carboxylase pathway accounts for 34% of the brain glutamine synthesis, indicating the significance of *de novo* synthesis of glutamate in the glutamate-glutamine cycle. There are two possible pathways for the incorporation of nitrogen (N) into glutamate: reductive amination via glutamate dehydrogenase (GDH; EC 1.4.1.2) or transamination. Thus, both ammonia and amino acids could act as potential N donors, although the uptake of each is limited and perhaps regulated by the transporter across the blood-brain barrier (Smith *et al.* 1987).

Neutral amino acids are transported across the blood-brain barrier by a common L-system transporter (Preston *et al.* 1989). Leucine has one of the highest affinities for this transporter (Smith *et al.* 1987), and its uptake by the brain is greater than any other amino acid (Grill *et al.* 1992). The brain not only contains abundant branched-chain amino transferase (BCAT; EC 2.6.1.42), but also expresses a brain-specific cytoplasmic form in addition to the ubiquitous mitochondrial BCAT (Hall *et al.* 1993; Bixel *et al.* 1997). Therefore, leucine is an attractive candidate as a major N-precursor for the synthesis of brain glutamate. Recently, Kanamori *et al.* (1998) have shown that leucine-N enters brain glutamate in rats infused intravenously with [¹⁵N]leucine. While this investigation established the potential central metabolic role of leucine, the experimental conditions involved anesthetized rats, a condition that likely affects metabolic activity in the brain. Moreover, the rate of tracer infusion was sufficiently large that the circulating and brain leucine concentrations increased to very high and non-physiological values. The first objective of the current study was to determine the contribution of leucine-N to the synthesis of brain glutamate under physiological and non-stressed conditions.

Because the synthesis of glutamine involves the prior synthesis of glutamate, the overall pathway requires an additional supply of nitrogen. Although the pathway of glutamine synthesis via glial glutamine synthetase (EC 6.3.1.2) from glutamate and free ammonia is well established (Martinez-Hernandez *et al.* 1977), the source of the ammonia remains poorly characterized. Ammonia generated via neuronal glutaminase in the glutamate-glutamine cycle could contribute to the amide-synthesis of glutamine to some extent (Rothman 2001). However, this must be an insufficient source because of the net release of glutamine from the brain (Grill *et al.* 1992). In addition, it has also been shown that under hyperammonemic conditions, plasma ammonia can supply a portion of the amide-N of glutamine (Farrow *et al.*

1990). From the perspective of the present study, it is important that studies with cerebellar explants (Yudkoff *et al.* 1983) have demonstrated the considerable incorporation of leucine-N into the amide-N of glutamine. Thus, a second objective of the study was to quantify the potential role of leucine as a nitrogen source for the synthesis of ammonia and hence, glutamine amide-N.

Transamination via BCAT is readily reversible (Hall *et al.* 1993) and the direction of the reaction is highly dependent on the relative concentrations of the four reactants [leucine, glutamate, α -ketoglutarate (α -KG) and α -ketoisocaproate (α -KIC)]. It is generally believed that BCAT catalyzes forward transamination (i.e. glutamate synthesis) in the glia (Yudkoff 1997; Kanamori *et al.* 1998; Hutson *et al.* 2001). However, in the presence of added α -KIC, BCAT also catalyzes leucine synthesis in cultured glial and neuronal cells as well as in neuronal synaptosomes (Yudkoff *et al.* 1990, 1994a, 1996; Hutson *et al.* 1998). Since measurable quantities of α -KIC can be found in the brain (Keen *et al.* 1989; Matsuo *et al.* 1993), reverse transamination via BCAT may be of metabolic significance at this site. From these findings, Yudkoff *et al.* (1996) have proposed that there is a neuronal-glia leucine/ α -KIC shuttle that parallels the glutamate/glutamine shuttle and allows the transfer of nitrogen from the neuron to the glia. However, although the evidence favors its existence, we know of no *in vivo* studies that have investigated whether reverse leucine transamination from glutamate does occur in the brain. The third objective of the current study was to attempt both to quantify the flux of nitrogen to leucine via reverse transamination in the brain, and to assess the entry of leucine-N into the neuronal metabolic pool.

For the above objectives, leucine-N and carbon (C) metabolism was studied using conscious rats fed with the diet containing [¹⁵N] and/or [U-¹³C]leucine.

Experimental procedures

Animals

The study received prior approval from the Animal Protocol Review Committee of Baylor College of Medicine. Housing and care of the animals conformed to USDA guidelines. Outbred male weanling Wistar rats purchased from Harlan (Indianapolis, IN, USA) were maintained under a reversed light-dark cycle (light on from 21:00 to 09:00 h) and at 25°C. The animals were housed individually in stainless steel cages and had free access to tap water. The rats were fed only during the dark period (09:00–21:00 h) with a purified diet [AIN-93G (Reeves *et al.* 1993)] containing crystalline amino acids (alanine: 4.0, arginine: 6.2, aspartate: 4.0, asparagine monohydrate: 4.5, cysteine hydrochloride monohydrate: 5.8, glutamate: 40.0, glycine: 6.0, histidine: 3.3, isoleucine: 8.6, leucine: 13.0, lysine hydrochloride: 13.7, methionine: 8.1, phenylalanine: 8.8, proline: 4.0, serine: 4.0 g/kg diet; Ajinomoto Co., Inc., Tokyo, Japan). The amino acid composition met the recommendation of the National Research Council (Gahl *et al.* 1991). The rats were adapted in these

conditions for more than 2 weeks. An experimental diet in which natural leucine was replaced with either [^{15}N]leucine [$> 98\%$ (w/w), Cambridge Isotope; Woburn, MA, USA (experiment 1; 30 animals)] or a mixture of [^{15}N]leucine [78.4–80.0% (w/w)] and [^{13}C]leucine [19.4–19.6% (w/w) (experiment 2: 18 animals)] was prepared. When the animals attained a mean body weight of 177.0 ± 7.0 g, they were offered each hour a limited amount of the labeled diet (9.05 g/kg body weight/h; approximately 90% of *ad lib.* intake). All animals started to eat each meal immediately after giving it, and consumed it completely. Stomachs of all the animals were full 1 h after the last meal. The measured rate of [^{15}N]leucine intake was 844 ± 26 $\mu\text{mol/kg}$ body weight/h in experiment 1. In experiment 2, the rate of [^{15}N]leucine intake was 677 ± 8 $\mu\text{mol/kg}$ body weight/h ($n = 12$), and that of [^{13}C]leucine was 163 ± 2 $\mu\text{mol/kg}$ body weight/h ($n = 18$).

Animals anesthetized with pentobarbital sodium (30 mg/kg) were decapitated at 0, 0.5, 1, 2, 3, 6 or 9 h, and mixed trunk blood and the brain were isolated. Blood was taken into a heparinized funnel and centrifuged to collect plasma. The plasma was frozen at -80°C . Brain was harvested in toto after removing the skull using scissors, and then clamped between two flat metal plates cooled in liquid nitrogen. This enabled the brain to be frozen within 30 s of decapitation and to a thickness of less than 0.5 mm. Frozen brain specimens were powdered in liquid nitrogen and stored at -80°C before the analysis.

Sample analysis

Aliquots of plasma were mixed with an equal volume of methionine sulfone solution and centrifuged at 10 000 g for 2 h at 4°C through a 10 kDa cut-off filter. Aliquots of frozen brain powder were deproteinized by 0.5 M perchloric acid (PCA) solution containing methionine sulfone, and neutralized with K_2CO_3 . The plasma filtrate and the brain extract were dried, derivatized by phenylisothiocyanate (Cohen *et al.* 1986) and analyzed by an HPLC system equipped with a Pico-Tag column (Waters, Milford, MA, USA). Amino acids were quantified using methionine sulfone as an internal standard.

Isotopic enrichments of plasma and brain amino acids were analyzed by GC/MS. PCA extracts of plasma and brain specimens were neutralized with K_2CO_3 , centrifuged, and the supernatant fluid brought to pH 4–6 with 1 M acetic acid. The specimens were applied to a 1 mL bed volume cation exchange column (Dowex 50Wx8, H^+ form; Bio-Rad, Hercules, CA, USA). The amino acids were eluted with 3 M ammonia solution and dried under vacuum. The dried samples were dissolved in water, adjusted to pH 8 by adding ammonia solution and applied to an anion exchange column (Dowex 1x8, Cl^- form; Bio-Rad). Neutral amino acids including glutamine were collected as the pass through fraction, and glutamate and aspartate were eluted with 1 M acetic acid according to the methods described previously (Prusiner and Milner 1970). The specimens were dried and the *n*-propyl esters of the heptafluorobutyramides were prepared as reported previously (Jahoor *et al.* 1994). Briefly, the carboxyl moieties were esterified by 1-propanol at 80°C for 30 min in the presence of acetyl chloride, and the amino and amide moieties were derivatized with heptafluorobutyric anhydride at 60°C for 20 min. For analysis of the glutamine fraction, the esterification step was performed at room temperature overnight to minimize deamidation of glutamine (Reeds *et al.* 1996). Each amino

acid was separated by gas chromatography (5989B, Hewlett Packard, Palo Alto, CA, USA) equipped with an HP-5 column (Hewlett Packard), and the isotopic distribution was analyzed with mass spectrometry (5890 Series II, Hewlett Packard). Negative ions generated by methane chemical ionization were analyzed by selected ion monitoring. The monitored ions were: glutamate (407, 408), aspartate (393, 394), glutamine (346–348), GABA (321, 322), leucine (349–355), isoleucine (349, 350), valine (335, 336), alanine (307, 308), glycine (293, 294), phenylalanine (383, 384), methionine (367, 368), tyrosine (311, 312) and serine (519, 520). For the determination of [$2\text{-}^{15}\text{N}$]glutamine enrichment, its deamidated product, the same derivative as that of glutamate, was analyzed by monitoring ions at m/z 407 and 408.

Plasma and brain ammonia were quantified enzymatically (van Anken and Schiphorst 1974). ^{15}N -enrichment of plasma and brain ammonia was analyzed after their conversion to norvaline by reaction with 2-oxovaleric acid and glutamate dehydrogenase as reported by Nieto *et al.* (1996). Brain ammonia was partially purified and concentrated before the conversion. Briefly, the neutralized brain PCA extract was applied to a 1 mL column of Dowex 1x8 (OH^- form) and the pass through fraction was collected, brought to pH 3–5, and dried under vacuum. Norvaline that was generated from 2-oxovaleric acid and ammonia was purified by cation exchange column (Dowex 50Wx8, H^+ form) and dried. Its tertiary butyldimethylsilyl derivative was analyzed by GC/MS. Selected ions ($m/z = 186$ and 187) generated by electron impact ionization were measured for determination of ^{15}N -enrichment.

Calculations

After adjustment for the baseline enrichment of the $[M + 1]$ signal of natural standards, the isotopic enrichment of ^{15}N -containing compounds was expressed as mol percent excess (MPE, mol%). This was calculated from the tracer/tracee ratio (TTR, mol%) as $MPE = TTR/(TTR + 1)$. [^{15}N]amino acid content (nmol/g tissue) was calculated from the product of the brain amino acid content ($\mu\text{mol/g}$ tissue) and the corresponding ^{15}N -enrichment. Throughout this paper, 'g' in 'g' denotes g of tissue wet weight. All curve fitting was carried out using the numerical routines in SAAMII (SAAM Institute, Seattle, WA, USA).

Leucine kinetics

$$\text{plasma leucine flux}(Q_{\text{plasma}}, \mu\text{mol/g/h}) = \frac{\text{Rate of tracer input}}{\text{Steady state isotopic enrichment}} \quad (1)$$

in which the rate of tracer input was taken as the rate at which the leucine tracers were fed to the animals. The steady state isotopic enrichment was the asymptotic value calculated by fitting the relationship between the isotopic enrichment of plasma leucine ($E_{\text{leu, plasma}}(t)$) and time (Fig. 2) to the equation:

$$E_{\text{leu, plasma}}(t) = A \times (1 - e^{-k \times t}) \quad (2)$$

A (the steady state isotopic enrichment) in eqn 2 was calculated separately for [^{13}C] and [^{15}N] leucine tracers to calculate $Q_{\text{C, plasma}}$ and $Q_{\text{N, plasma}}$, the flux (turnover) of leucine carbon ($-\text{C}$) or $-\text{N}$, respectively, in plasma.

$$Q_{C,plasma} = I_d + P_{plasma} \quad (3)$$

$$Q_{N,plasma} = I_d + P_{plasma} + R_{tr,plasma} \quad (4)$$

where I_d , P_{plasma} and $R_{tr,plasma}$ are the rate of leucine uptake from the diet, the rate of leucine appearance from the tissue via proteolysis and the rate of leucine-N appearance via leucine formation by transamination of α -KIC, respectively. Equations 3 and 4 enabled the rate of leucine-N appearance via leucine formation by transamination of α -KIC to be calculated from the relative fluxes of leucine-C and N:

$$R_{tr,plasma}(\mu\text{mol/g/h}) = Q_{N,plasma} - Q_{C,plasma} \quad (5)$$

$$\begin{aligned} R_{tr,plasma}/Q_{N,plasma}(\text{fraction of plasma leucine-N}) \\ = \frac{Q_{N,plasma} - Q_{C,plasma}}{Q_{N,plasma}} \end{aligned} \quad (6)$$

Similarly, leucine flux in the brain (Q_{brain}) is the function of the leucine influx (I_f) from plasma to brain, and the steady state enrichment ratio of brain to plasma (SER).

$$Q_{brain}(\mu\text{mol/g/h}) = \frac{I_f}{SER} \quad (7)$$

Leucine formation via reverse transamination in the brain ($R_{tr,brain}$) is the function of leucine-C flux ($Q_{C,brain}$) and leucine-N flux ($Q_{N,brain}$) in the brain as is eqn 5

$$R_{tr,brain}(\mu\text{mol/g/h}) = Q_{N,brain} - Q_{C,brain} \quad (8)$$

Therefore, contribution of leucine formation via reverse transamination to the brain leucine-N flux ($R_{tr,brain}/Q_{N,brain}$) was calculated from the steady state enrichment ratios (brain/plasma) of [U - ^{13}C]leucine (SER_C) and [^{15}N]leucine (SER_N).

$$R_{tr,brain}/Q_{N,brain} = \frac{Q_{N,brain} - Q_{C,brain}}{Q_{N,brain}} = 1 - \frac{SER_N}{SER_C} \quad (9)$$

Contribution of leucine influx from the plasma pool to brain leucine-N flux ($I_f/Q_{N,brain}$), contribution of leucine appearance via proteolysis in the brain ($P_{brain}/Q_{N,brain}$, fraction of leucine-N flux) was calculated from SER_C and SER_N .

$$I_f/Q_{N,brain} = SER_N \quad (10)$$

$$P_{brain}/Q_{N,brain} = \frac{SER_N}{SER_C} - SER_N \quad (11)$$

As with the plasma data (eqn 2), the steady state isotopic enrichments were the asymptotes of the relationship between the enrichment of brain [U - ^{13}C] and [^{15}N]leucine and time (Fig. 1).

On the assumption that the ^{15}N in the brain derived exclusively from leucine (Table 2), an estimate of the apparent uptake of plasma leucine by the brain ($I_{f,ap}(t)$) can be calculated at any time t from the sequestration of ^{15}N in the brain ($\Sigma^{15}\text{N}(t)$), and the area under the curve (AUC) of the ^{15}N -enrichment of plasma leucine ($AUCE_{leu,plasma}(t)$).

$$I_{f,ap}(t)(\mu\text{mol/g/h}) = \frac{\Sigma^{15}\text{N}(t)}{AUCE_{leu,plasma}(t)} \quad (12)$$

in which $\Sigma^{15}\text{N}(t)$ is the sum of the ^{15}N ($\mu\text{mol/g}$) contained in brain leucine, glutamate, glutamine, aspartate, alanine, GABA, glycine,

serine and NH_3 . This provides a minimum estimate of leucine uptake at time t , because there is undoubtedly simultaneous release of ^{15}N from the brain along with the uptake of [^{15}N]leucine by the brain, and as a result there is an exponential fall in the apparent rate of leucine uptake with time (Fig. 2). Real rate of leucine uptake from the plasma pool (I_f) was therefore calculated from the intercept ($= B$) at time zero as predicted from the relationship.

$$I_{f,ap}(t)(\mu\text{mol/g/h}) = B \times e^{-t \times t} \quad (13)$$

Using the estimated I_f , brain leucine-C and -N fluxes were calculated by eqn 7, and then $R_{tr,brain}$ and P_{brain} were calculated by eqns 9 and 11, respectively.

Glutamate kinetics

Because of the known substrate specificity of BCAT, it is reasonable to assume that the large majority of newly transported leucine is transaminated specifically to glutamate, as opposed to aspartate and alanine. On the assumption that subsequent glutamine, aspartate, alanine and GABA ^{15}N -labeling derives from glutamate, the apparent rate of transamination from leucine to α -KG at time t ($F_{tr,ap}(t)$, $\mu\text{mol/g/h}$) can be calculated from the ^{15}N -labeling of these products of glutamate metabolism. Similarly to eqn 12,

$$F_{tr,ap}(t)(\mu\text{mol/g/h}) = \frac{\Sigma G^{15}\text{N}(t)}{AUCE_{leu,brain}(t)} \quad (14)$$

in which $\Sigma G^{15}\text{N}(t)$ is the sum of ^{15}N contained in glutamate, glutamine (amino N), aspartate, GABA and alanine in the brain, $AUCE_{leu,brain}(t)$ ($\text{mol}\% \times \text{h}$) is AUC of the isotopic enrichment of brain [^{15}N]leucine, and t is in h. This relationship was also exponential so that the rate of forward transamination (F_{tr}) was estimated from the intercept at time zero as is the rate of leucine uptake by the brain (eqn 13).

Statistics

All data are presented as means \pm standard deviations (SD). Statistical significance between time points (i.e. between animals) was determined either by Dunnett's test or by Tukey's honestly significant difference (HSD) test following an ANOVA for multiple comparisons. Within animal comparisons among amino acids were assessed with paired t -tests. A p (two-tailed)-value of less than 0.05 was considered statistically significant.

Results

Plasma and brain amino acid concentrations

Despite marked changes in the plasma, the concentrations of amino acids in the brain were almost constant (Table 1). The steady state of brain concentration also applied to both GABA and ammonia.

Kinetics of U- ^{13}C - and ^{15}N -labeling in plasma and brain leucine

The isotopic enrichment of plasma [U - ^{13}C]leucine (Fig. 1a) and [^{15}N]leucine (Fig. 1b) increased gradually and reached a plateau 6 h after the start of the tracer feeding. The steady

Table 1 Time course of amino acid concentrations in plasma and brain

	Time of feeding (h)				Time of feeding (h)			
	0	3	6	9	0	3	6	9 ^a
Brain ($\mu\text{mol/g}$)					Plasma ($\mu\text{mol/mL}$)			
Leucine	0.07 \pm 0.01	0.08 \pm 0.01	0.08 \pm 0.02	0.09 \pm 0.01	0.06 \pm 0.01	0.14 \pm 0.02**	0.17 \pm 0.04**	0.21 \pm 0.03**
Isoleucine	0.03 \pm 0.01	0.04 \pm 0.00	0.04 \pm 0.00	0.04 \pm 0.01*	0.05 \pm 0.01	0.12 \pm 0.02**	0.15 \pm 0.04**	0.16 \pm 0.04**
Valine	0.09 \pm 0.01	0.09 \pm 0.01	0.09 \pm 0.00	0.10 \pm 0.01*	0.12 \pm 0.03	0.27 \pm 0.05**	0.30 \pm 0.13**	0.40 \pm 0.09**
Glutamate	10.48 \pm 1.24	11.03 \pm 1.18	10.95 \pm 1.28	11.06 \pm 0.97	0.20 \pm 0.02	0.13 \pm 0.03**	0.12 \pm 0.04**	0.12 \pm 0.03**
Glutamine	4.53 \pm 0.51	5.11 \pm 0.26	5.29 \pm 0.37**	5.39 \pm 0.49**	0.59 \pm 0.09	0.80 \pm 0.10**	0.76 \pm 0.09**	0.74 \pm 0.05*
GABA	2.82 \pm 0.61	2.84 \pm 0.61	2.89 \pm 0.58	3.06 \pm 0.68	–	–	–	–
Aspartate	1.51 \pm 0.10	1.72 \pm 0.13	1.65 \pm 0.17	1.68 \pm 0.15	0.01 \pm 0.00	0.01 \pm 0.00	0.01 \pm 0.00	0.01 \pm 0.00
Alanine	0.52 \pm 0.05	0.55 \pm 0.03	0.54 \pm 0.05	0.54 \pm 0.02	0.37 \pm 0.03	0.86 \pm 0.14**	0.79 \pm 0.12**	0.84 \pm 0.12**

Amino acid concentration in plasma and brain before ($t = 0$ h) and during feeding. All data are expressed as mean \pm SD ($n = 6$). * $p < 0.05$, ** $p < 0.01$ versus before the feeding ($t = 0$ h) (Dunnett's test).

^aOne of the plasma samples was missed because of failure.

state enrichments were 10.0 mol% for [U-¹³C]leucine and 30.9 mol% for plasma [¹⁵N]leucine. The calculated plasma leucine-N flux (2.7 mmol/kg body weight/h) exceeded that of leucine-C (1.5 mmol/kg body weight/h), suggesting that more than 40% of the plasma leucine-N derived from the reverse transamination (i.e. amination of α -KIC). The enrichments in both labeled forms of brain leucine paralleled that of the plasma, and although complete isotopic equilibrium with the plasma pool was achieved within 30 min of the start of isotope administration (see insets in Fig. 1), the isotopic enrichments of both leucine tracers were lower in the brain than in the plasma. The brain to plasma ratio of [U-¹³C]leucine enrichment, 0.61 ± 0.06 , indicates that 39% of the leucine-C flux derived from proteolysis. However, the brain to plasma ratio of [¹⁵N]leucine enrichment was only 0.23 ± 0.06 , so that the brain leucine-N flux was 2.6-fold of leucine-C flux [i.e. $(1/0.23)/(1/0.61)$], indicating that a large part (62%) of leucine-N derived from the reverse transamination of α -KIC in the brain.

¹⁵N uptake by the brain

As expected from the role of leucine as a nitrogen donor at many sites within the body, other plasma amino acids were rapidly and significantly labeled with ¹⁵N. Because of this, we regarded it as essential to estimate the degree to which other amino acids could have contributed to the brain ¹⁵N and hence, glutamine and glutamate synthesis. The potential contribution of labeled amino acids in the plasma is shown in Table 2. Leucine transport accounted for approximately 90% of the brain ¹⁵N uptake over the course of the period of labeling; the remaining ¹⁵N was accounted for by the uptake of [¹⁵N]isoleucine and [¹⁵N]valine (117 nmol ¹⁵N/g brain at 9 h, 5.5% of total ¹⁵N uptake), and [¹⁵N]glutamine (97 nmol ¹⁵N/g brain at 9 h, 4.5% of total ¹⁵N uptake).

By dividing the accumulated ¹⁵N in the brain at each time point (see Table 3) by the AUC of the plasma, [¹⁵N]leucine

isotopic enrichment allows an estimate of the apparent rate of leucine uptake from the plasma pool. Excluding the calculated uptake of isoleucine, valine and glutamine, the apparent ¹⁵N uptake was $1.18 \pm 0.11 \mu\text{mol } ^{15}\text{N/g brain}$ over the whole 9 h period, yielding an apparent rate of leucine uptake of $0.52 \pm 0.04 \mu\text{mol/g/h}$. However, it is clear from Fig. 2 that the rate fell with time, presumably reflecting a progressively increasing loss of ¹⁵N from the brain free amino acid pool. The exponential relationship between apparent rate of leucine uptake and time (Fig. 2) predicted a real rate of leucine uptake of $1.3 \mu\text{mol/g/h}$ as a y-intercept, a rate that is comparable with the value ($0.9 \mu\text{mol/g/h}$) estimated by Smith *et al.* (1987). Using this estimate of the uptake of leucine, the data on the labeling of brain [U-¹³C]leucine suggest that the leucine-C flux was $2.2 \mu\text{mol/g/h}$ and the entry of leucine from proteolysis within the brain was $0.9 \mu\text{mol/g/h}$. On the basis of the relative labeling of [U-¹³C] and [¹⁵N]leucine, we estimate that the leucine-N flux was $5.8 \mu\text{mol/g/h}$ (a fractional turnover rate of 120%/min), the difference reflecting leucine synthesis from α -KIC via reverse transamination ($3.6 \mu\text{mol/g/h}$). Under the assumption that protein pool size is constant in the brain during the experiment, 15% of brain leucine-N was utilized for protein synthesis and the other 85% ($5.0 \mu\text{mol/g/h}$) for the forward transamination mainly to α -KG.

Glutamate, aspartate and GABA and glutamine (amino-N) labeling

The time course of the labeling of the amino-N of brain glutamine, glutamate, GABA and aspartate is shown in Table 4. Although the isotopic enrichment of [¹⁵N]glutamate was close to that of [2-¹⁵N]glutamine, it was in fact $2.2 \pm 2.7\%$ more enriched with ¹⁵N than glutamine amino-N ($p < 0.05$, paired *t*-test, $n = 30$). The isotopic enrichments of [¹⁵N]aspartate and [¹⁵N]GABA were also $14.4 \pm 9.7\%$ ($p < 0.00001$, $n = 30$) and $11.0 \pm 11.2\%$ ($p < 0.0001$,

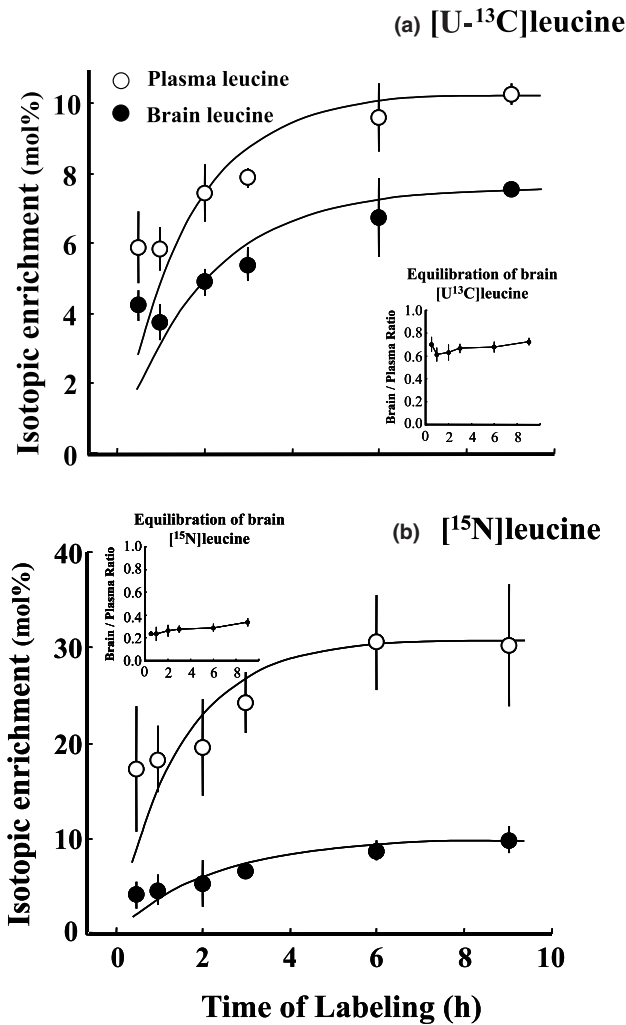


Fig. 1 Time course of leucine labeling. The diet containing [¹⁵N]leucine or [U-¹³C]leucine was fed to the rat for 0.5–9 h after 12 h of fasting. U-¹³C- and ¹⁵N-enrichment of plasma leucine (open circles) and brain leucine (closed circles) were plotted. Insets show the time course of the isotopic enrichment ratio of brain to plasma leucine. (a) [U-¹³C]leucine [dietary enrichment: 19.4–19.6% (w/w) of total leucine, *n* = 2–6]. (b) [¹⁵N]leucine [dietary enrichment: > 98% (w/w), *n* = 6 at 1–9 h or 78.4–80.0% (w/w), *n* = 3 at 0.5 h, the data of the latter were used after the correction by the dietary enrichment]. All data are expressed as mean ± SD. U-¹³C- and ¹⁵N-enrichments of brain leucine were significantly lower than those of plasma leucine at any time points (*p* < 0.01, paired *t*-test). Brain to plasma ratios of [¹⁵N]leucine enrichment were significantly lower than those of [U-¹³C]leucine enrichment at any time points (*p* < 0.01, Tukey-HSD test). No statistical difference was detected either among the ratios of [U-¹³C]leucine or among those of [¹⁵N]leucine (Tukey-HSD test), except the ratios of [¹⁵N]leucine between 1 and 9 h of feeding.

n = 30) higher than that of [2-¹⁵N]glutamine. Moreover, aspartate and GABA were more enriched with ¹⁵N than glutamate (*p* < 0.00001 and *p* < 0.001, *n* = 30, respectively). Thus, the ¹⁵N-enrichment of the pool of glutamate that had

Table 2 Potential contribution of [¹⁵N]amino acid in the plasma to ¹⁵N uptake by the brain

	Influx ^a (μmol/g/h)	Predicted ¹⁵ N-uptake by the brain (nmol ¹⁵ N/g brain)		
		0–3 h	0–6 h	0–9 h
Leucine	0.85	423	1119	1892
Isoleucine	0.17	17	44	76
Valine	0.13	8	23	41
Glutamine	0.24	13	47	97
Methionine	0.10	1	4	8
Phenylalanine	0.64	3	8	14
Tyrosine	0.46	1	3	7

Cumulative ¹⁵N uptake by the brain via each amino acid (*U(t)*, μmol/g tissue) was calculated according to the equation *U(t)* = *f_{in}* × *B(t)*, in which, *f_{in}* (μmol/g/h) is the influx of each amino acid from the plasma to the brain, and *B(t)* (mol% × h) is AUC of the ¹⁵N-enrichment of the corresponding amino acid in the plasma from 0 to *t* h in the rat fed with [¹⁵N]leucine.

^aThe amino acid influx measured in *in situ* rat brain by Smith *et al.* (1987) was used as *f_{in}* for the calculation.

Table 3 [¹⁵N]amino acid content in the brain in the rat fed with [¹⁵N]leucine diet

(nmol/g)	Time of [¹⁵ N]leucine labeling (h)		
	3	6	9
Leucine	5.2 ± 0.5	7.4 ± 2.0	8.5 ± 1.7
Isoleucine	1.1 ± 0.2	1.5 ± 0.3	2.4 ± 0.6
Valine	2.1 ± 0.3	3.3 ± 0.2	5.1 ± 0.9
Glutamate	245.8 ± 22.4	400.6 ± 23.0	562.3 ± 40.8
Glutamine	166.8 ± 13.6	295.5 ± 13.6	426.2 ± 27.9
GABA	69.2 ± 17.3	112.4 ± 23.6	170.0 ± 45.2
Aspartate	43.2 ± 3.4	65.3 ± 5.9	90.6 ± 6.8
Alanine	11.9 ± 1.8	17.6 ± 2.3	25.5 ± 2.1
Serine	5.6 ± 0.4	8.7 ± 1.3	13.7 ± 1.9
Glycine	3.4 ± 0.7	7.8 ± 1.4	13.4 ± 2.5
Ammonia	2.9 ± 0.8	6.2 ± 1.5	10.5 ± 3.9
Total	557.2 ± 47.7	926.2 ± 49.7	1328.2 ± 122.6

Cerebral ¹⁵N-content of each amino acid and ammonia in the rat fed with [¹⁵N]leucine diet. Total is the sum of the ¹⁵N-content in the 10 kinds of amino acids and ammonia shown in the Table. [¹⁵N]glutamine includes both [2-¹⁵N] and [5-¹⁵N]glutamine. All data are expressed as mean ± SD (*n* = 6).

been utilized for aspartate and GABA synthesis was greater than that of the bulk pool of glutamate, implying that there were at least two sources of glutamate labeling in the brain.

Contribution of leucine-N to brain amino-N metabolism

Table 3 shows the distribution of ¹⁵N among other amino acids in the brain. Glutamate (43 ± 3%), glutamine

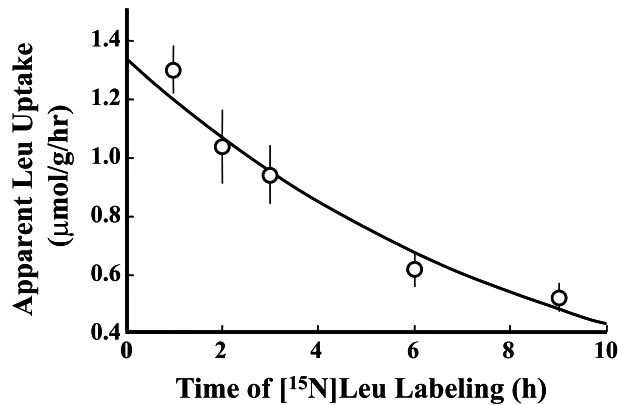


Fig. 2 Estimation of the rate of leucine uptake by the brain. Apparent rate of ^{15}N -uptake ($\mu\text{mol/g/h}$) calculated as described in the Experimental procedures decreases exponentially ($1.34 \times e^{-0.11 \times t}$) during the ^{15}N leucine labeling. The y-intercept at 0 h predicted an estimate of the real rate of leucine uptake of $1.3 \mu\text{mol/g/h}$.

($31 \pm 2\%$), GABA ($12 \pm 3\%$), aspartate ($7 \pm 0.7\%$) and alanine ($2 \pm 0.3\%$) collectively accounted for 95% of the total quantity of ^{15}N in the acid soluble pool, and brain ^{15}N leucine accounted for less than 1% of the total. The quantity of ^{15}N among the other individual amino acids and ammonia was maintained within 2% of the total ^{15}N in the brain throughout the experiment.

Glutamate synthesis

Because of the substrate specificity of BCAT (Hall *et al.* 1993), it is likely that the initial transamination of leucine

generates glutamate, and that this glutamate then acts as the common precursor for ^{15}N entry into the other products (i.e. glutamine, GABA, aspartate and alanine). With this assumption, the quantity of ^{15}N sequestered in brain glutamate, glutamine (amino-N), aspartate, alanine and GABA was divided by the AUC of the isotopic enrichment of brain leucine to calculate the apparent rate at time t , and then to estimate its real rate of transamination of leucine to glutamate. The rate of forward transamination so estimated was $5.4 \mu\text{mol/g/h}$, which was close to that calculated from the kinetics of $[\text{U-}^{13}\text{C}]$ and ^{15}N leucine labeling ($5.0 \mu\text{mol/g/h}$). The ^{15}N -enrichment ratio of glutamate to brain leucine rose progressively to 0.52 ± 0.05 by 9 h of ^{15}N leucine feeding (Fig. 3), indicating that more than 50% of glutamate-N derived originally from leucine.

Leucine-N entry into ammonia and amide-N of glutamine in the brain

Both brain ammonia and brain glutamine (amide-N) became progressively more enriched with ^{15}N as the experiment proceeded (Table 5 and Fig. 3) so that by 9 h, the isotopic enrichment of brain $^{15}\text{NH}_3$ was 30% of that of brain ^{15}N leucine and significantly higher than the isotopic enrichment of $^{15}\text{NH}_3$ in plasma. The ratio of the isotopic enrichments of cerebral $[\text{5-}^{15}\text{N}]$ glutamine to plasma $^{15}\text{NH}_3$ also rose over the time of the study from 0.62 at 1 h to 1.45 at 9 h. The ^{15}N -enrichment in amide-N of glutamine was consistently (28%) higher than that of cerebral ammonia, indicating a certain pool of ammonia more enriched with ^{15}N than the mixed ammonia pool of the brain.

Table 4 Comparison of isotopic enrichments of brain GABA, aspartate and serine with glutamate and amino-N of glutamine

Isotopic enrichment (%)	Time of feeding (h)					<i>p</i>
	1	2	3	6	9	
^{15}N glutamate	0.78 ± 0.15	1.50 ± 0.08	2.23 ± 0.12	$3.68 \pm 0.28^*$	$5.10 \pm 0.27^*$	$< 0.05^a$
$[2\text{-}^{15}\text{N}]$ glutamine	0.75 ± 0.06 (1.00 ± 0.23)	1.50 ± 0.12 (1.01 ± 0.08)	2.26 ± 0.12 (1.01 ± 0.02)	3.58 ± 0.25 (0.97 ± 0.02) [#]	4.89 ± 0.20 (0.96 ± 0.03) [#]	– $< 0.05^b$
^{15}N GABA	$0.88 \pm 0.07^*$ (1.16 ± 0.23)	1.62 ± 0.16 (1.08 ± 0.13)	2.43 ± 0.25 (1.09 ± 0.11)	3.89 ± 0.17 (1.06 ± 0.10)	$5.50 \pm 0.43^{**}$ (1.08 ± 0.09)	$< 0.0001^a$ $< 0.001^b$
^{15}N aspartate	$0.94 \pm 0.04^{**}$ (1.24 ± 0.22) [#]	$1.69 \pm 0.14^*$ (1.13 ± 0.05) ^{##}	$2.52 \pm 0.17^{**}$ (1.13 ± 0.03) ^{###}	$3.97 \pm 0.33^{**}$ (1.08 ± 0.03) ^{###}	$5.41 \pm 0.26^{**}$ (1.06 ± 0.02) ^{###}	$< 0.00001^a$ $< 0.00001^b$
^{15}N alanine	0.74 ± 0.15 (1.00 ± 0.39)	1.40 ± 0.20 (0.94 ± 0.17)	2.18 ± 0.26 (0.98 ± 0.15)	$3.26 \pm 0.20^*$ (0.89 ± 0.08) [#]	4.77 ± 0.38 (0.93 ± 0.08)	$< 0.05^a$ $< 0.01^b$
^{15}N serine	$0.21 \pm 0.07^{**}$ (0.29 ± 0.12) ^{##}	$0.46 \pm 0.06^*$ (0.31 ± 0.05) ^{###}	$0.72 \pm 0.07^{**}$ (0.32 ± 0.01) ^{###}	$1.15 \pm 0.11^{**}$ (0.31 ± 0.03) ^{###}	$1.79 \pm 0.07^{**}$ (0.35 ± 0.02) ^{###}	$< 0.000001^a$ $< 0.000001^b$

^{15}N -enrichments of brain glutamate, amino-N of glutamine, GABA, aspartate, alanine and serine in the rats fed with the ^{15}N leucine diet. All data are expressed as mean \pm SD ($n = 6$). The values in the parentheses are isotopic enrichment ratios to ^{15}N glutamate.

* $p < 0.05$, ** $p < 0.01$ versus $[2\text{-}^{15}\text{N}]$ glutamine. # $p < 0.05$, ## $p < 0.01$ versus ^{15}N glutamate (paired *t*-test).

^aStatistical differences from $[2\text{-}^{15}\text{N}]$ glutamine (all data ($n = 30$) at 1–9 h of the feeding were compared by paired *t*-test).

^bStatistical differences from ^{15}N glutamate (paired *t*-test, $n = 30$).

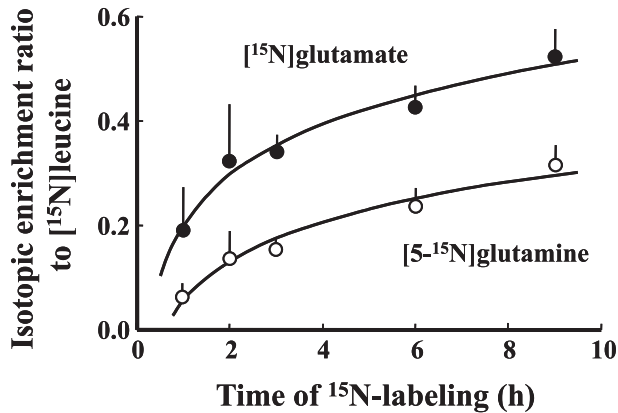


Fig. 3 Contribution of cerebral leucine-N to glutamate-N and amide-N of glutamine. Isotopic enrichment ratio of brain [¹⁵N]glutamate (closed circles) and [5-¹⁵N]glutamine (open circles) to brain [¹⁵N]leucine was taken in each animal fed with the [¹⁵N]leucine diet for 1–9 h ($n = 6$). The ratio of [¹⁵N]glutamate and [5-¹⁵N]glutamine reached 0.52 ± 0.05 and 0.31 ± 0.04 , respectively, at 9 h of ¹⁵N-labeling. All data are expressed as mean \pm SD.

Discussion

A previous study using parenterally nourished and anesthetized rats demonstrated the facility with which leucine-N enters the brain glutamate pool (Kanamori *et al.* 1998), and the present study confirms this general observation. However, in this previous investigation the brain leucine concentration was elevated more than twofold by the leucine infusion, which could conceivably have altered nitrogen metabolism in the brain. In the present study, [¹⁵N]leucine was administered via the diet and the conditions of the experiment were deliberately designed to achieve a constant, and physiologically normal, brain leucine concentration in

non-anesthetized and unstressed animals. Under these conditions, the rate of incorporation of leucine-N into brain glutamate-N was $5 \mu\text{mol/g/h}$, and at least 50% of the brain glutamate-N had originally derived from leucine.

The degree to which tracer [U-¹³C]leucine was diluted in the brain ($61 \pm 6\%$ of plasma leucine isotopic enrichment) indicated that 39% of the brain leucine-C derived from proteolysis. However, the isotopic dilution of [¹⁵N]leucine was much greater than that of [U-¹³C]leucine, indicating that a large proportion of leucine-N derived from the reverse transamination (i.e. amination of α -KIC) as observed in previous *in vitro* studies (Yudkoff *et al.* 1994a, 1996; Hutson *et al.* 1998). The results indicated that 23% of brain leucine-N derived from plasma pool, 15% from proteolysis and the other 62% from the reverse transamination. The rate of leucine uptake by the brain ($1.3 \mu\text{mol/g/h}$) was much lower than the rate of forward transamination of leucine ($5.4 \mu\text{mol/g/h}$), also suggesting the significance of cerebral leucine synthesis from α -KIC via the reverse transamination.

While these results largely confirmed previous work, there were, however, a number of results that challenged some current assumptions about nitrogen metabolism in the brain. First, the amide-N of brain glutamine was rapidly labeled with ¹⁵N. The uptake of plasma [¹⁵N]glutamine could have contributed no more than 5% of total ¹⁵N uptake by the brain (Table 2), and the predicted uptake of plasma [5-¹⁵N]glutamine could have contributed less than 2.5% of brain [5-¹⁵N]glutamine (data not shown). Thus, most of the label must have been incorporated via glutamine synthesis within the brain. This raised the question of the source of labeled ammonia for the synthesis of glutamine. Labeling of the amide-N of brain glutamine has been shown in hyperammonemic rats infused with [¹⁵N]ammonia (Farrow *et al.* 1990) and in normal rats infused with a small amount of [¹³N]ammonia (Cooper *et al.* 1979), indicating the entry into

Table 5 Isotopic enrichment of amide-N of glutamine and ammonia in plasma and brain

	Time of feeding (h)					
	0	1	2	3	6	9 ^a
Ammonia ($\mu\text{mol/mL}$)						
Plasma	0.12 ± 0.03	0.15 ± 0.04	0.17 ± 0.05	0.14 ± 0.04	0.17 ± 0.05	0.13 ± 0.05
Brain	$0.36 \pm 0.15^{**}$	$0.36 \pm 0.05^{**}$	$0.34 \pm 0.07^{**}$	$0.35 \pm 0.07^{**}$	$0.37 \pm 0.10^{**}$	$0.40 \pm 0.12^*$
[¹⁵ N]ammonia (%)						
Plasma	–	0.41 ± 0.28	0.67 ± 0.24	0.90 ± 0.19	1.37 ± 0.21	2.09 ± 0.41
Brain	–	$0.17 \pm 0.07^*$	0.43 ± 0.12	0.83 ± 0.12	$1.69 \pm 0.13^*$	$2.65 \pm 0.20^*$
[5- ¹⁵ N]glutamine (%)						
Plasma	–	0.26 ± 0.09	0.64 ± 0.07	1.12 ± 0.24	1.80 ± 0.27	2.94 ± 0.19
Brain	–	0.25 ± 0.10	$0.61 \pm 0.10^{\#}$	$1.00 \pm 0.11^{\#}$	$2.03 \pm 0.36^{\#}$	$3.04 \pm 0.20^{\#\#}$

Concentrations of ammonia, ¹⁵N-enrichment of ammonia and amide-N of glutamine both in plasma and brain in the rat fed with [¹⁵N]leucine diet. All data are expressed as mean \pm SD ($n = 6$). * $p < 0.05$, ** $p < 0.01$, versus plasma, # $p < 0.05$, ## $p < 0.01$ versus [¹⁵N]ammonia (%) in the brain (paired *t*-test).

^aOne of the plasma samples was missed because of failure.

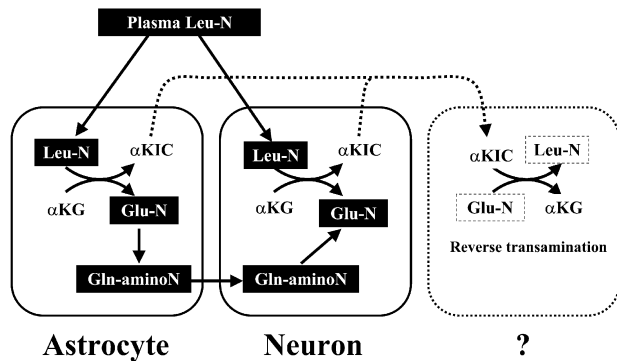


Fig. 4 Leucine nitrogen metabolism in the brain. ^{15}N -distribution in the brain in the present study suggested the incorporation of plasma-born leucine-N directly into the neuronal glutamate-N. Collectively with the existing prediction that glial BCAT dominantly catalyzes forward transamination (i.e. glutamate synthesis), it can be assumed that leucine-N enters into neuronal glutamate-N both directly via neuronal BCAT and indirectly from glutamine synthesized in glia. This raises the question of the site of the reverse transamination via BCAT, since much greater leucine-N flux than leucine-C flux in the brain indicates bidirectional transamination via BCAT.

brain of plasma ammonia for the amide-N synthesis. However, it seemed unlikely to us that plasma ammonia was the major precursor of the amide-N of brain glutamine because the brain ammonia concentration was considerably higher than that of the plasma, and because ammonia crosses the blood–brain barrier presumably via passive diffusion. Indeed, brain ammonia was more enriched with ^{15}N than plasma ammonia at 6–9 h of [^{15}N]leucine labeling. In addition, the ^{15}N -enrichment of the amide-N of brain glutamine was higher than that of the bulk pool of brain ammonia, suggesting that ammonia generation for glial glutamine synthesis is not only compartmentalized but that leucine-N is an important precursor for generation of this ammonia pool. Accordingly, the product to precursor relationship (Fig. 3) between leucine and glutamine showed that leucine supplied at least 30% of the amide-N of brain glutamine. This estimate is at least twice the value derived from studies using organ culture (Yudkoff *et al.* 1983). The most plausible pathway for the transfer of leucine-N to the amide-N of glutamine is the oxidative deamination of glutamate via the glutamate dehydrogenase (GDH) reaction. Although it has been shown that GDH is expressed at high levels in the brain and that brain GDH can catalyze glutamate synthesis from ammonia and α -KG under hyperammonemic conditions (Farrow *et al.* 1990), whether GDH functions in the direction of glutamate or ammonia generation under physiological conditions has not been clarified. Further study will be needed to elucidate the pathway for the ammonia generation including that via the purine-nucleotide cycle (Yudkoff *et al.* 1986).

The existence of a high rate of bi-directional transamination of leucine supports previous proposals of a pathway of

N-recycling via the leucine/ α -KIC shuttle in the brain (Yudkoff 1997; Hutson *et al.* 2001). On the basis of *in vitro* experiments, Yudkoff *et al.* (1996) concluded that the predominant direction of transamination via BCAT is toward leucine synthesis in neuronal synaptosomes. However, two observations from the present study indicate the possibility that neuronal BCAT catalyzes glutamate synthesis as well as glial BCAT. First, while the administration of either [^{14}C]HCO₃ (Waelsch *et al.* 1964) or [^{13}C]acetate (Cerdan *et al.* 1990) leads to a higher carbon isotopic enrichment in glutamine than in glutamate, this was not so for the ^{15}N -labeling in the present experiment in which isotopic enrichments of glutamate were almost the same as those of the amino-N of glutamine. Second, the ^{15}N -enrichments of both GABA and aspartate, glutamate metabolites that are largely confined to the neuronal compartments, were higher than the enrichment either glutamate or amino-N of glutamine. These results strongly suggest the direct entry of leucine-N into glutamate within the neuronal compartment. Here, the direct entry of leucine-N into neuronal glutamate does not necessarily mean *de novo* synthesis of glutamate, but would be only a transamination from leucine to α -KG, since *de novo* synthesis of glutamate takes place only in glia (Van den Berg 1972; Shank *et al.* 1985). Indeed, the well known fact that carbon skeletons of glucose enter glutamate more intensively than glutamine (Berl and Clarke 1969) indicates quantitative significance of conversion of α -KG to glutamate in the large glutamate compartment, i.e. in neurons.

It has been predicted that glial BCAT predominantly catalyzes forward transamination (i.e. glutamate synthesis), because synthesis of glutamine in glia requires *de novo* synthesis of glutamate and hence, nitrogen supply (Yudkoff 1997; Hutson *et al.* 2001). Anatomical access of glia to blood capillaries also supports this prediction, since glutamate synthesis via BCAT depends on the concentration of its substrates, and since blood leucine is taken up into the brain. Collectively with the previous observations (above), putative leucine-N metabolism in the brain is illustrated in Fig. 4. In the model, leucine-N enters into neuronal glutamate-N both directly via neuronal BCAT and indirectly from glutamine synthesized in glia.

Then, where is the site of the reverse transamination via BCAT? Bi-directional transamination could take place in a single compartment if the transamination reactions were near equilibrium. Our preliminary observations indicate that bi-directional transamination is not an intrinsic characteristic of BCAT; despite the higher BCAT activity of kidney (Suryawan *et al.* 1998), there were no differences between leucine-C and -N fluxes in the kidney (data not shown). Thus, it is reasonable to assume individual compartments for both forward and reverse transamination. As one of the examples, the mitochondrial compartment in neurons could be proposed, since the mitochondrial inner membrane limits the transport of amino acids and their metabolites. However,

Bixel *et al.* (2001) have shown that neuronal cultures express BCATc but not BCATm. Further study would be needed to elucidate the site of the reverse transamination, including the physiological meaning of nitrogen metabolism via a leucine/ α -KIC shuttle in the brain.

In the present study, the amino-N of glutamine, aspartate-N, GABA-N, alanine-N and serine-N equilibrated very quickly with brain glutamate-N (Table 4), indicating considerably rapid metabolism of cerebral glutamate-N, which agrees with previous observations by Mason *et al.* (1995). According to their estimation, the rate of glutamate-N turnover was as fast as 3400 $\mu\text{mol/g/h}$. Thus, the contribution of leucine-N to glutamate synthesis was calculated as only 0.15% [= 5 ($\mu\text{mol/g/h}$)/3400 ($\mu\text{mol/g/h}$)] of glutamate-N turnover, while the ^{15}N -enrichment ratio of glutamate to leucine indicated that more than 50% of glutamate-N derived originally from leucine-N (Fig. 3). The much higher value in the latter estimation indicates that a large proportion of ^{15}N incorporated into glutamate in the first pass was metabolized to the other amino acids and recycled again, which agrees with previous findings indicating glutamate-N recycling in the brain via the glutamate-glutamine cycle (Rothman 2001), the GABA-shunt (Walsh and Clark 1976) and the aspartate/malate shuttle (Yudkoff *et al.* 1994b). Therefore, the ^{15}N -enrichment ratio of glutamate to leucine suggests the importance of leucine as an N-carrier from plasma, although transamination between glutamate and leucine is only a minor portion of total glutamate-N metabolism in the brain.

In conclusion, the present study investigated leucine-N metabolism in the brain using conscious and unstressed rats. Leucine-N was incorporated into glutamate directly both in glial and neuronal metabolic pools via BCAT. The glutamate-N was utilized for both the amino and the amide-N of glutamine in the glia, or incorporated in GABA and aspartate in the neurons. The rate of glutamate synthesis from leucine was much higher than the rate of uptake of leucine from the plasma pool, indicating that a large proportion of cerebral leucine-N was derived from reverse transamination via BCAT. These suggest a critical role of leucine not only as an N-precursor for the synthesis of cerebral glutamate but also as an N-carrier within the brain.

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References

- van Anken H. C. and Schiphorst M. E. (1974) A kinetic determination of ammonia in plasma. *Clin. Chim. Acta* **56**, 151–157.
- de Barry J., Vincendon G. and Gombos G. (1983) Uptake and metabolism of L-[^3H]glutamate and L-[^3H]glutamine in adult rat cerebellar slices. *Neurochem. Res.* **8**, 1321–1335.
- Berl S. and Clarke D. D. (1969) Compartmentation of amino acid metabolism, in *Handbook of Neurochemistry* (A. Lajtha, ed.), Vol. 2, pp. 447–472. Plenum Press, New York.
- Bixel M. G., Hutson S. M. and Hamprecht B. (1997) Cellular distribution of branched-chain amino acid aminotransferase isoenzymes among rat brain glial cells in culture. *J. Histochem. Cytochem.* **45**, 685–694.
- Bixel M., Shimomura Y., Hutson S. and Hamprecht B. (2001) Distribution of key enzymes of branched-chain amino acid metabolism in glial and neuronal cells in culture. *J. Histochem. Cytochem.* **49**, 407–418.
- Cerdan S., Kunnecke B. and Seelig J. (1990) Cerebral metabolism of [$1,2\text{-}^{13}\text{C}_2$]acetate as detected by *in vivo* and *in vitro* ^{13}C NMR. *J. Biol. Chem.* **265**, 12916–12926.
- Cohen S. A., Bidlingmeyer B. A. and Tarvin T. L. (1986) PITC derivatives in amino acid analysis. *Nature* **320**, 769–770.
- Cooper A. J., McDonald J. M., Gelbard A. S., Gledhill R. F. and Duffy T. E. (1979) The metabolic fate of ^{13}N -labeled ammonia in rat brain. *J. Biol. Chem.* **254**, 4982–4992.
- Farrow N. A., Kanamori K., Ross B. D. and Parivar F. (1990) A ^{15}N -n.m.r. study of cerebral, hepatic and renal nitrogen metabolism in hyperammonaemic rats. *Biochem. J.* **270**, 473–481.
- Gahl M. J., Finke M. D., Crenshaw T. D. and Benevenga N. J. (1991) Use of a four-parameter logistic equation to evaluate the response of growing rats to ten levels of each indispensable amino acid. *J. Nutr.* **121**, 1720–1729.
- Grill V., Bjorkman O., Gutniak M. and Lindqvist M. (1992) Brain uptake and release of amino acids in nondiabetic and insulin-dependent diabetic subjects: important role of glutamine release for nitrogen balance. *Metabolism* **41**, 28–32.
- Hall T. R., Wallin R., Reinhart G. D. and Hutson S. M. (1993) Branched chain aminotransferase isoenzymes. Purification and characterization of the rat brain isoenzyme. *J. Biol. Chem.* **268**, 3092–3098.
- Hutson S. M., Berkich D., Drown P., Xu B., Aschner M. and LaNoue K. F. (1998) Role of branched-chain aminotransferase isoenzymes and gabapentin in neurotransmitter metabolism. *J. Neurochem.* **71**, 863–874.
- Hutson S. M., Lieth E. and LaNoue K. F. (2001) Function of leucine in excitatory neurotransmitter metabolism in the central nervous system. *J. Nutr.* **131**, 846S–850S.
- Jahoor F., Burrin D. G., Reeds P. J. and Frazer M. (1994) Measurement of plasma protein synthesis rate in infant pig: an investigation of alternative tracer approaches. *Am. J. Physiol.* **267**, R221–R227.
- Kanamori K., Ross B. D. and Kondrat R. W. (1998) Rate of glutamate synthesis from leucine in rat brain measured *in vivo* by ^{15}N NMR. *J. Neurochem.* **70**, 1304–1315.
- Keen R. E., Barrio J. R., Huang S. C., Hawkins R. A. and Phelps M. E. (1989) *In vivo* cerebral protein synthesis rates with leucyl-transfer RNA used as a precursor pool: determination of biochemical parameters to structure tracer kinetic models for positron emission tomography. *J. Cereb. Blood Flow Metab.* **9**, 429–445.

- Lapidot A. and Gopher A. (1994) Cerebral metabolic compartmentation. Estimation of glucose flux via pyruvate carboxylase/pyruvate dehydrogenase by ^{13}C NMR isotopomer analysis of D-[U- ^{13}C]glucose metabolites. *J. Biol. Chem.* **269**, 27198–27208.
- Martinez-Hernandez A., Bell K. P. and Norenberg M. D. (1977) Glutamine synthetase: glial localization in brain. *Science* **195**, 1356–1358.
- Mason G. F., Gruetter R., Rothman D. L., Behar K. L., Shulman R. G. and Novotny E. J. (1995) Simultaneous determination of the rates of the TCA cycle, glucose utilization, alpha-ketoglutarate/glutamate exchange, and glutamine synthesis in human brain by NMR. *J. Cereb. Blood Flow Metab.* **15**, 12–25.
- Matsuo Y., Yagi M. and Walser M. (1993) Arteriovenous differences and tissue concentrations of branched-chain ketoacids. *J. Lab. Clin. Med.* **121**, 779–784.
- Nieto R., Calder A. G., Anderson S. E. and Lobley G. E. (1996) Method for the determination of ^{15}N enrichment in biological samples by gas chromatography/electron impact ionization mass spectrometry. *J. Mass Spectrom.* **31**, 289–294.
- Oldendorf W. H. (1971) Brain uptake of radiolabeled amino acids, amines, and hexoses after arterial injection. *Am. J. Physiol.* **221**, 1629–1639.
- Preston J. E., Segal M. B., Walley G. J. and Zlokovic B. V. (1989) Neutral amino acid uptake by the isolated perfused sheep choroid plexus. *J. Physiol. (Lond.)* **408**, 31–43.
- Prusiner S. and Milner L. (1970) A rapid radioactive assay for glutamine synthetase, glutaminase, asparagine synthetase, and asparaginase. *Anal. Biochem.* **37**, 429–438.
- Reeds P. J., Burrin D. G., Jahoor F., Wykes L., Henry J. and Frazer E. M. (1996) Enteral glutamate is almost completely metabolized in first pass by the gastrointestinal tract of infant pigs. *Am. J. Physiol.* **270**, E413–E418.
- Reeves P. G., Nielsen F. H. and Fahey G. C. Jr (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.* **123**, 1939–1951.
- Rothman D. L. (2001) Studies of metabolic compartmentation and glucose transport using in vivo MRS. *NMR Biomed.* **14**, 149–160.
- Shank R. P. and Campbell G. L. (1983) Glutamate, in *Handbook of Neurochemistry* (Lajtha A., ed.), Vol. 3, pp. 381–404. Plenum Press, New York.
- Shank R. P., Bennett G. S., Freytag S. O. and Campbell G. L. (1985) Pyruvate carboxylase: an astrocyte-specific enzyme implicated in the replenishment of amino acid neurotransmitter pools. *Brain Res.* **329**, 364–367.
- Sibson N. R., Dhankhar A., Mason G. F., Behar K. L., Rothman D. L. and Shulman R. G. (1997) In vivo ^{13}C NMR measurements of cerebral glutamine synthesis as evidence for glutamate-glutamine cycling. *Proc. Natl Acad. Sci. USA* **94**, 2699–2704.
- Smith Q. R., Momma S., Aoyagi M. and Rapoport S. I. (1987) Kinetics of neutral amino acid transport across the blood-brain barrier. *J. Neurochem.* **49**, 1651–1658.
- Suryawan A., Hawes J. W., Harris R. A., Shimomura Y., Jenkins A. E. and Hutson S. M. (1998) A molecular model of human branched-chain amino acid metabolism. *Am. J. Clin. Nutr.* **68**, 72–81.
- Van den Berg C. J. (1972) A model of compartmentation in mouse brain based on glucose and acetate metabolism, in *Metabolic Compartmentation in The Brain. Proceedings of a Symposium on Metabolic Compartmentation at the Rockefeller Foundation, Bellagio, Italy, July 11–16, 1971* (Balazs R. and Cremer J. E., eds), pp. 138–166. Jon Wiley & Sons, New York.
- Waelsch H., Berl S., Rossi C. A., Clarke D. P. and Purpura D. P. (1964) Quantitative aspects of CO_2 fixation in mammalian brain in vivo. *J. Neurochem.* **11**, 717–728.
- Walsh J. M. and Clark J. B. (1976) Studies on the control of 4-aminobutyrate metabolism in 'synaptosomal' and free rat brain mitochondria. *Biochem. J.* **160**, 147–157.
- Yudkoff M. (1997) Brain metabolism of branched-chain amino acids. *Glia* **21**, 92–98.
- Yudkoff M., Nissim I., Kim S., Pleasure D., Hummeler K. and Segal S. (1983) [^{15}N]leucine as a source of [^{15}N]glutamate in organotypic cerebellar explants. *Biochem. Biophys. Res. Commun.* **115**, 174–179.
- Yudkoff M., Nissim I., Hummeler K., Medow M. and Pleasure D. (1986) Utilization of [^{15}N]glutamate by cultured astrocytes. *Biochem. J.* **234**, 185–192.
- Yudkoff M., Nissim I. and Hertz L. (1990) Precursors of glutamic acid nitrogen in primary neuronal cultures: studies with ^{15}N . *Neurochem. Res.* **15**, 1191–1196.
- Yudkoff M., Daikhin Y., Lin Z. P., Nissim I., Stern J. and Pleasure D. (1994a) Interrelationships of leucine and glutamate metabolism in cultured astrocytes. *J. Neurochem.* **62**, 1192–1202.
- Yudkoff M., Nelson D., Daikhin Y. and Erecinska M. (1994b) Tricarboxylic acid cycle in rat brain synaptosomes. Fluxes and interactions with aspartate aminotransferase and malate/aspartate shuttle. *J. Biol. Chem.* **269**, 27414–27420.
- Yudkoff M., Daikhin Y., Nelson D., Nissim I. and Erecinska M. (1996) Neuronal metabolism of branched-chain amino acids: flux through the aminotransferase pathway in synaptosomes. *J. Neurochem.* **66**, 2136–2145.