

## Metabolism of acetyl-L-carnitine for energy and neurotransmitter synthesis in the immature rat brain

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### Abstract

Acetyl-L-carnitine (ALCAR) is an endogenous metabolic intermediate that facilitates the influx and efflux of acetyl groups across the mitochondrial inner membrane. Exogenously administered ALCAR has been used as a nutritional supplement and also as an experimental drug with reported neuroprotective properties and effects on brain metabolism. The aim of this study was to determine oxidative metabolism of ALCAR in the immature rat forebrain. Metabolism was studied in 21–22 day-old rat brain at 15, 60 and 120 min after an intraperitoneal injection of [2-<sup>13</sup>C]acetyl-L-carnitine. The amount, pattern, and fractional enrichment of <sup>13</sup>C-labeled metabolites were determined by *ex vivo* <sup>13</sup>C-NMR spectroscopy. Metabolism of the acetyl moiety from [2-<sup>13</sup>C]ALCAR via the tricarboxylic acid cycle led to incorporation of label into the C4, C3 and C2 positions of glutamate (GLU), glutamine (GLN) and GABA. Labeling patterns indicated that [2-<sup>13</sup>C]ALCAR was metabolized by both neurons and glia; however, the percent enrichment was higher in GLN and GABA than in GLU, demonstrating high metabolism in astrocytes and GABAergic neurons. Incorporation of label into the C3 position of alanine, both C3 and C2 posi-

tions of lactate, and the C1 and C5 positions of glutamate and glutamine demonstrated that [2-<sup>13</sup>C]ALCAR was actively metabolized via the pyruvate recycling pathway. The enrichment of metabolites with <sup>13</sup>C from metabolism of ALCAR was highest in alanine C3 (11%) and lactate C3 (10%), with considerable enrichment in GABA C4 (8%), GLN C3 (~4%) and GLN C5 (5%). Overall, our <sup>13</sup>C-NMR studies reveal that the acetyl moiety of ALCAR is metabolized for energy in both astrocytes and neurons and the label incorporated into the neurotransmitters glutamate and GABA. Cycling ratios showed prolonged cycling of carbon from the acetyl moiety of ALCAR in the tricarboxylic acid cycle. Labeling of compounds formed from metabolism of [2-<sup>13</sup>C]ALCAR via the pyruvate recycling pathway was higher than values reported for other precursors and may reflect high activity of this pathway in the developing brain. This is, to our knowledge, the first study to determine the extent and pathways of ALCAR metabolism for energy and neurotransmitter biosynthesis in the brain.

**Keywords:** acetyl-L-carnitine, GABA, glutamate, glutamine, metabolism, pyruvate recycling.

*J. Neurochem.* (2010) **114**, 820–831.

Acetyl-L-carnitine (ALCAR) is an endogenous metabolic intermediate present in the brain and involved in trans-mitochondrial membrane trafficking of acetyl units for catabolic and anabolic metabolism (Dolezal and Tucek 1981), and buffering the pool of acetyl CoA (Miecz *et al.* 2008; Jones *et al.* 2010). ALCAR is synthesized intramitochondrially in many tissues including brain, liver, heart, kidney and muscle by transferring acetyl groups to carnitine (Farrell *et al.* 1986; Rebouche 2004; Jones *et al.* 2010). Serum levels of ALCAR increase during exercise or starvation (Stephens *et al.* 2007; Jones *et al.* 2010).

Received December 27, 2009; revised manuscript received April 7, 2010; accepted May 5, 2010.

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*Abbreviations used:*  $\alpha$ -KG,  $\alpha$ -ketoglutarate; ALCAR, acetyl-L-carnitine; ASP, aspartate; GLN, glutamine; GLU, glutamate; LAC, lactate; OAA, oxaloacetate; OCTN, organic cation/carnitine transporter; PCA, perchloric acid; TCA, tricarboxylic acid.

Carnitine and acyl carnitines, including ALCAR, cross the blood-brain barrier primarily via the high affinity, Na<sup>+</sup> dependent organic cation/carnitine transporter OCTN2 (SLC22A5) and to a lesser extent via the B(0,+)-amino acid transporter (Inano *et al.* 2003; Nalecz *et al.* 2004; Miecz *et al.* 2008). Recent studies have demonstrated that the high affinity OCTN2 and OCTN3 transporters are present on both astrocytes and neurons in suckling and adult brain (Januszewicz *et al.* 2009, 2010).

Acetyl-L-carnitine has been studied by many groups as numerous reports in the literature show neuroprotective effects of exogenous ALCAR in a variety of neurological disorders (Rosenthal *et al.*, 1992; Miljkovic-Lolic *et al.*, 1997; Ishii *et al.* 2000; Virmani *et al.* 2001; Calabrese *et al.* 2005, 2008; Zanelli *et al.* 2005). Exogenously administered ALCAR is rapidly removed from plasma and taken up by many tissues including kidney, gut, heart, brain and liver (Rebouche 2004). ALCAR was taken up in brain within 15 s following jugular artery injection in rats (Kido *et al.* 2001). In positron emission tomography studies a single injection of [1-<sup>11</sup>C] or [2-<sup>11</sup>C]ALCAR was taken up into primate brain within 5 min and a high concentration remained in the cortex 60 min later (Kuratsune *et al.* 1997). Within brain cells ALCAR can be converted via the reversible enzyme carnitine acetyltransferase (EC 2.3.1.7) into acetyl CoA and carnitine without ATP utilization (Ramsay *et al.* 2001). Thus, ALCAR can serve as a reservoir of the acetyl groups which can be utilized in many pathways including synthesis of lipids, glycogen and acetylcholine (Dolezal and Tucek 1981; Farrell *et al.* 1986; Aureli *et al.* 1998; Ricciolini *et al.* 1998). Ricciolini *et al.* (1998) demonstrated that [1-<sup>14</sup>C]acetyl-L-carnitine injected directly into the lateral ventricle was primarily expired as CO<sub>2</sub> and also rapidly incorporated into saturated, monounsaturated and polyunsaturated fatty acids in brain. ALCAR also can supply acetyl groups for the production of the neurotransmitter acetylcholine (Dolezal and Tucek 1981; Ricny *et al.* 1992).

A number of studies have shown that intravenous ALCAR modulates brain energy metabolism leading to increased cerebral glucose metabolism in adult rats within 10–30 min of administration (Ori *et al.* 2002; Freo *et al.* 2009), and decreased brain lactate after ischemia (Rosenthal *et al.*, 1992; Zanelli *et al.* 2005). Although investigators have proposed that ALCAR stimulates cerebral energy metabolism by providing acetyl CoA as an oxidizable substrate to the tricarboxylic acid (TCA) cycle (Kuratsune *et al.* 1997; Zanelli *et al.* 2005), direct evidence of the metabolism of ALCAR for energy and neurotransmitter synthesis has not been reported. The present study tested the hypothesis that the immature brain is capable of utilizing the acetyl moiety of exogenous [2-<sup>13</sup>C]acetyl-L-carnitine for both energy metabolism and neurotransmitter biosynthesis.

## Materials and Methods

### Chemicals

[2-<sup>13</sup>C]acetyl-L-carnitine 99% <sup>13</sup>C enriched, was synthesized by Isotec (Sigma, St Louis, MO, USA), and generously provided by Sigma Tau, SpA (Pomezia, Italy). The <sup>13</sup>C-ALCAR was dissolved in bicarbonate at a concentration of 30 mg/mL and adjusted to pH 7.4.

### Animals

This study was approved by the University of Maryland, Baltimore, Animal Care and Use Committee. All care and handling of rats were in compliance with the National Institutes of Health guidelines. Male Sprague–Dawley rats were obtained with the dam from Charles River Laboratories (Wilmington, MA, USA). Immature male rat pups 21–22 days old, weighing 50–60 g were used in all studies.

Rats were given intraperitoneal (i.p.) injections of [2-<sup>13</sup>C]acetyl-L-carnitine (100 mg/kg). Animals were killed at 15, 60 and 120 min after injection of <sup>13</sup>C-ALCAR. Brains were rapidly removed, and the forebrain snap-frozen in liquid nitrogen. Blood samples (400 µL of trunk blood) were obtained from some rats at each time point and also at 5 and 30 min; the plasma was obtained by centrifugation. All samples were stored at –80°C until further processing.

### Tissue extraction

Each frozen tissue sample was homogenized in ice-cold 7% perchloric acid (PCA) using a dounce homogenizer (Fisher, Fairlawn, NJ, USA) and extracted as described by Richards *et al.* (2007). The supernatants were combined and neutralized with KOH, centrifuged, lyophilized and stored at –20°C. Lyophilized samples were reconstituted in 800 µL of deuterium oxide (D<sub>2</sub>O) with 0.4% dioxane added as an internal standard for quantification. Samples were adjusted to pH 6.8–7.0, and 0.65 mL was transferred to a 5 mm NMR tube (Wilmad, Buena, NJ, USA).

### NMR spectroscopy

Proton-decoupled 125.5-MHz <sup>13</sup>C-NMR spectra were obtained at the NMR Center, University of Maryland, Baltimore, using a Varian Inova 500 MHz spectrometer with a broad band detection probe. <sup>13</sup>C-NMR spectra were acquired at ambient temperature using a 35° pulse angle, 25 KHz spectra width, and 64 K data points as described by Richards *et al.* (2007). An acquisition time of 1.3 s and a relaxation delay of 4.3 s were used. The number of scans was typically 11 000–13 000. Plasma samples were run as described by Melo *et al.* (2005); however, 55 000 scans were obtained so that <sup>13</sup>C-ALCAR or any other <sup>13</sup>C-labeled compounds would be detected. All spectra were corrected for nuclear Overhauser effects by using correction factors obtained by comparing peak intensities from spectra collected with decoupling throughout the experiment to spectra decoupled only during acquisition as described by Richards *et al.* (2007). A line broadening of 5 Hz was used. Dioxane was used as an internal concentration standard for quantification. Chemical shifts are reported relative to the dioxane peak at 67.4 ppm, and peak assignments were made by comparison to <sup>13</sup>C-labeled standards and to the literature values.

### Protein, amino acid and lactate quantification

The protein content of the pellets from the PCA extracts of the brains was determined using the Pierce BCA microreagent assay (Pierce, Rockford, IL, USA) (Smith *et al.* 1985). Amino acids were determined with a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge, UK). Lactate was determined with a lactate oxidase assay as described in earlier studies (Richards *et al.* 2007).

### Calculations of percent enrichment

The total concentration of lactate or specific amino acids, and the amount of  $^{13}\text{C}$ -labeling (corrected for natural abundance) was used to calculate percent enrichment for each isotope isomer of lactate, glutamate, glutamine, GABA or alanine according to the following equation:

$$\% \text{ Enrichment} = \frac{(\text{amount of } ^{13}\text{C}\text{-labeled of a given isotopomer} - 1.1\% \text{ of total amount})}{\text{total amount of compound (unlabeled } (^{12}\text{C}) + ^{13}\text{C labeled})} * 100$$

It should be noted that this formula actually calculates the 'excess enrichment' as enrichment from natural abundance is subtracted. However, we refer to this value as percent enrichment to be consistent with the other study to which our data are compared (Hassel *et al.* 1995).

### Statistical analyses

Data are expressed as mean  $\pm$  SEM for  $n = 5$  brains at 15 min,  $n = 6$  at 60 min, and  $n = 8$  at 120 min. Data were analyzed by one-way ANOVA with  $p < 0.05$  as the level of significance, and all pair wise multiple comparisons tested with Tukey's *post hoc* test using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

## Results

Plasma was extracted at 5, 15, 30, 60 and 120 min after intraperitoneal injection to determine how much  $[2\text{-}^{13}\text{C}]\text{acetyl-L-carnitine}$  remained in plasma, and if metabolism in peripheral tissues led to labeling of any metabolites. There were only very low amounts of labeled glucose and myoinositol detectable by NMR in plasma at any time after injection of the  $[2\text{-}^{13}\text{C}]\text{acetyl-L-carnitine}$  (Figure S1). The enrichment of glucose C4 in plasma was 0.16, 0.15, 0.14, and 0.20% at 5, 15, 60 and 120 min, respectively. Enrichment of plasma glucose C1 was 0 at 5 min and less than 0.01% at all other time points. There was no quantifiable enrichment in lactate or alanine in plasma at any time point. These findings are consistent with results from a recent pharmacokinetic study demonstrating that the label from exogenously administered  $^{11}\text{C}$ -acetyl-L-carnitine was rapidly removed from plasma and taken up into brain and other tissue (Kuratsune *et al.* 1997).

### Amino acid concentrations in brain

The concentrations of the amino acids glutamate, glutamine, GABA, aspartate and alanine in 21–22 day-old rat brain

were not different at 15, 60 and 120 min after i.p. injection of ALCAR (Table S1). The concentrations of all amino acids were comparable to values recently reported for 21–22 day-old rat brain (Scafidi *et al.* 2009). The percent enrichment of all isotopomers (isotope isomers) of compounds labeled from metabolism of  $[2\text{-}^{13}\text{C}]\text{acetyl-L-carnitine}$  was calculated from the nmol  $^{13}\text{C}$  incorporated/mg protein and the amino acid concentration data.

### Incorporation of label from $[2\text{-}^{13}\text{C}]\text{acetyl-L-carnitine}$ into metabolites in the immature rat brain

Label from the metabolism of  $[2\text{-}^{13}\text{C}]\text{acetyl-L-carnitine}$  was incorporated into glutamate, glutamine, GABA, aspartate,

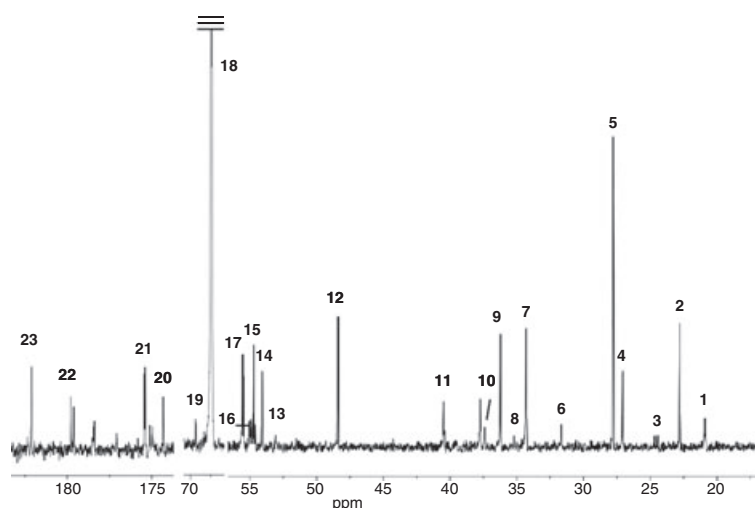
lactate and alanine in 21–22 day-old rat brain. A typical  $^{13}\text{C}$ -NMR spectrum of metabolites in a perchloric acid extract of brain is shown in Fig. 1. At 15 min after i.p. injection of ALCAR incorporation of label into metabolites was observed in the spectra and there was low but detectable enrichment of all amino acids and lactate. In contrast to the labeling with glucose which peaks at 15 min and then declines, the incorporation of label from  $[2\text{-}^{13}\text{C}]\text{acetyl-L-carnitine}$  continued to increase over time, with the highest incorporation in many metabolites found at 120 min as described in detail below.

### Labeling pattern from metabolism of $[2\text{-}^{13}\text{C}]\text{acetyl-L-carnitine}$ via the TCA cycle

The labeling pattern from the metabolism of  $[2\text{-}^{13}\text{C}]\text{acetyl-L-carnitine}$  is shown in Fig. 2 and the metabolism is described in detail in the figure legend. Briefly, in the mitochondria  $[2\text{-}^{13}\text{C}]\text{acetyl-L-carnitine}$  ( $[2\text{-}^{13}\text{C}]\text{ALCAR}$ ) is converted to  $[2\text{-}^{13}\text{C}]\text{acetyl CoA}$ , which condenses with unlabeled oxaloacetate to form citrate which is metabolized via the TCA cycle, giving rise to  $[4\text{-}^{13}\text{C}]\alpha\text{-ketoglutarate}$  in the first turn. The  $[4\text{-}^{13}\text{C}]\text{glutamate}$  formed from  $\alpha\text{-ketoglutarate}$  can be converted to glutamine, and also to  $[2\text{-}^{13}\text{C}]\text{GABA}$ .  $[4\text{-}^{13}\text{C}]\alpha\text{-ketoglutarate}$  can remain in the TCA cycle leading to formation of  $[3\text{-}^{13}\text{C}]\text{malate}$  and  $[3\text{-}^{13}\text{C}]\text{oxaloacetate}$ , which can be subsequently converted to  $[3\text{-}^{13}\text{C}]\text{aspartate}$ . Labeling from randomization and from subsequent turns of the TCA cycle are described in the legend for Fig. 2.

### Labeling pattern from recycling of carbons from metabolism of $[2\text{-}^{13}\text{C}]\text{acetyl-L-carnitine}$ via the pyruvate recycling pathway

As  $^{13}\text{C}$  from  $[2\text{-}^{13}\text{C}]\text{ALCAR}$  enters the TCA cycle directly at the level of acetyl CoA,  $^{13}\text{C}$ -labeling in other metabolites that eventually generate acetyl CoA (i.e. pyruvate, lactate and



**Fig. 1** A typical  $^{13}\text{C}$ -NMR spectrum of metabolites in brain 120 min after intraperitoneal injection of  $[2\text{-}^{13}\text{C}]$ acetyl-L-carnitine. The numbers for labeled peaks correspond to: 1, Lactate C3; 2, *N*-acetyl aspartate C6; 3, GABA C3; 4, glutamine C3; 5, glutamate C3; 6, glutamine C4; 7, glutamate C4; 8, GABA C2; 9, taurine C2; 10, aspartate C3; 11, GABA C4; 12, taurine C1; 13, aspartate C2; 14,

*N*-acetyl aspartate C2; 15, creatine C4; 16, glutamine C2; 17, glutamate C2; 18, dioxane (internal standard); 19, lactate C2; 20, glutamine C1; 21, glutamate C1; 22, glutamine C5; 23, glutamate C5. Alanine C3 was observed in some, but not all spectra. Spectrum was obtained as described in methods. Details of the labeling are given in results section.

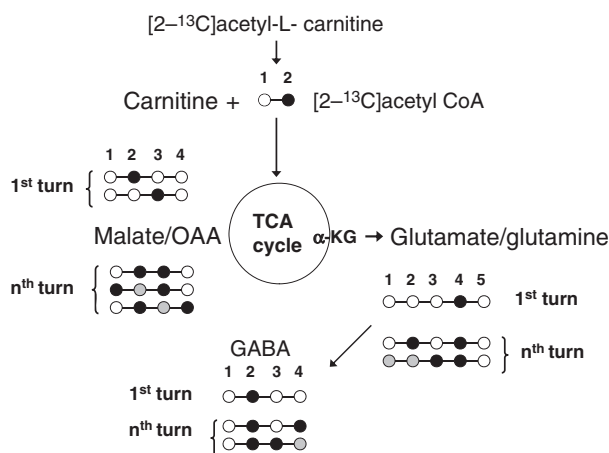
alanine), can only be formed from further metabolism of the labeled TCA cycle intermediates via the pyruvate recycling pathway, as shown in Fig. 3. This pathway involves the conversion of either  $^{13}\text{C}$ -malate to pyruvate via malic enzyme, or conversion of  $^{13}\text{C}$ -oxaloacetate to pyruvate via the combined action of phosphoenolpyruvate carboxykinase and pyruvate kinase (Cerdan *et al.* 1990; Kunnecke *et al.* 1993; Sonnewald *et al.* 1996; Cruz *et al.* 1998; Haberg *et al.* 1998). Pyruvate formed from these TCA cycle intermediates can be converted to lactate or alanine labeled in the C2 and C3 positions (Fig. 3). The pyruvate formed from these TCA cycle intermediates can also re-enter the TCA cycle as acetyl CoA labeled in the C1 or C2 position. Metabolism of  $[2\text{-}^{13}\text{C}]$ acetyl CoA formed via pyruvate recycling can not be distinguished from the acetyl CoA formed directly from metabolism of  $[2\text{-}^{13}\text{C}]$ ALCAR. However, the  $[1\text{-}^{13}\text{C}]$ acetyl CoA combines with oxaloacetate to form citrate and leads to formation of  $[5\text{-}^{13}\text{C}]$ glutamate,  $[5\text{-}^{13}\text{C}]$ glutamine and also  $[1\text{-}^{13}\text{C}]$ glutamate,  $[1\text{-}^{13}\text{C}]$ glutamine (Haberg *et al.* 1998; Olstad *et al.* 2007).

#### Temporal course of $[2\text{-}^{13}\text{C}]$ acetyl-L-carnitine metabolism in brain

At 15 min post-injection label was detected in  $[4\text{-}^{13}\text{C}]$ glutamate (GLU C4),  $[3\text{-}^{13}\text{C}]$ glutamate (GLU C3), and  $[2\text{-}^{13}\text{C}]$ glutamate (GLU C2) which are labeled in the first turn (C4) and later turns (C3 and C2) of the TCA cycle.  $[3\text{-}^{13}\text{C}]$ Glutamate was higher than  $[4\text{-}^{13}\text{C}]$ glutamate indicating that metabolism had occurred rapidly and multiple turns of the TCA cycle had taken place at 15 min after injection

(Table 1). Incorporation of label into GLU C4 increased over time and was significantly higher at 120 min compared to 15 min. Labeling in GLU C3 tended to increase over time from 15 to 120 min after injection of  $[2\text{-}^{13}\text{C}]$ ALCAR, but was not significantly different from the 15 min time point. This indicates that labeled carbon from metabolism of  $[2\text{-}^{13}\text{C}]$ ALCAR continued to enter the TCA cycle over the 2-h period. The fractional enrichment of GLU C4, C3 and C2 in brain was low at 15 min after i.p. injection of  $[2\text{-}^{13}\text{C}]$ ALCAR, but increased with time (Fig. 4). The enrichment of these glutamate isotopomers labeled via the TCA cycle ranged from  $\sim 0.2$  to 1.7% at 15, 60 and 120 min after i.p. injection of  $[2\text{-}^{13}\text{C}]$ ALCAR (Fig. 4).

At 15 min post-injection label was detected in  $[4\text{-}^{13}\text{C}]$ glutamine (GLN C4),  $[3\text{-}^{13}\text{C}]$ glutamine (GLN C3), and  $[2\text{-}^{13}\text{C}]$ glutamine (GLN C2) in brain (Table 1). These isotopomers can be formed directly from metabolism in the first turn (C4) and later turns (C3 and C2) of the TCA cycle in astrocytes. Alternatively, the labeled glutamine can be synthesized from the corresponding glutamate isotopomers (GLU C4, C3 and C2) that were synthesized in neurons, released, taken up by astrocytes and metabolized via glutamine synthetase. Incorporation of label into GLN C4 and GLN C3 increased over time and was significantly higher at 120 min than at 15 and 60 min. In contrast, incorporation of label into  $[2\text{-}^{13}\text{C}]$ glutamine was not significantly higher at 60 and 120 min. The absolute amount of label (nmol/mg protein) incorporated into the C4 and C3 positions of glutamate was higher than the labeling in the C4 and C3 positions of glutamine at corresponding time intervals (15, 60



**Fig. 2** Labeling pattern obtained from the metabolism of [2-<sup>13</sup>C]acetyl-L-carnitine via the tricarboxylic acid (TCA) cycle in rat brain. Labeling pattern from the first and *n*<sup>th</sup> turns of the cycle are shown. Black circles indicate carbons labeled from metabolism in the first turn of the TCA cycle; grey circles are labeled from metabolism in the second and later turns of the TCA cycle. Open circles are carbons that are not labeled from the metabolism of [2-<sup>13</sup>C]ALCAR via the TCA cycle. [2-<sup>13</sup>C]acetyl-L-carnitine is converted to [2-<sup>13</sup>C]acetyl CoA which condenses with unlabeled oxaloacetate to form labeled citrate which is metabolized via the TCA cycle, giving rise to [4-<sup>13</sup>C]α-ketoglutarate (α-KG) in the first turn. [4-<sup>13</sup>C]α-ketoglutarate can be converted to [4-<sup>13</sup>C]glutamate (GLU C4), which can be converted to [2-<sup>13</sup>C]GABA in GABAergic neurons. In astrocytes, [4-<sup>13</sup>C]glutamate can be converted to [4-<sup>13</sup>C]glutamine by the enzyme glutamine synthetase. [4-<sup>13</sup>C]α-ketoglutarate can remain in the TCA cycle leading to formation of [3-<sup>13</sup>C]fumarate, which is a symmetrical molecule so randomization of the label occurs leading to some labeling in the two position as well. The same labeling pattern occurs in malate. [3-<sup>13</sup>C]Oxaloacetate and [2-<sup>13</sup>C]oxaloacetate formed in the first turn of the TCA cycle can be converted to [3-<sup>13</sup>C]aspartate (ASP C3) and also [2-<sup>13</sup>C]aspartate (ASP C2). GLN C3 and GLU C3 are formed after metabolism in the second turn of the cycle, and ASP C2 can also be formed. Abbreviations: GLU, glutamate; GLN, glutamine; OAA, oxaloacetate.

and 120 min). However, enrichment of GLN C3 and C2 occurred more rapidly than enrichment in glutamate, and tended to be higher ranging from ~0.3 to 4% (Fig. 4). Enrichment in GLN C3 was considerably higher than enrichment of the corresponding glutamate GLU C3 at 120 min. Enrichment in both GLN C4 and C3 were higher at 120 min than at 15 min after injection of [2-<sup>13</sup>C]ALCAR (Fig. 4).

Label from the metabolism of [2-<sup>13</sup>C]ALCAR was incorporated into [2-<sup>13</sup>C]GABA, [3-<sup>13</sup>C]GABA and [4-<sup>13</sup>C]GABA at 15, 60 and 120 min after injection (Table 1). Labeling in the C4 position of GABA increased significantly over time compared with labeling at 15 min. Labeling in the C3 position of GABA tended to increase over the 2-h period, but was not significantly different than the 15

and 60 min time points. Enrichment of GABA C2 and C3 ranged from 1 to 5% at 60 and 120 min (Fig. 5). In contrast, the percent enrichment in GABA C4 (labeled from later turns of the TCA cycle when [2-<sup>13</sup>C]ALCAR is the precursor), although variable, was particularly high reaching 8% at 120 min suggesting active metabolism in GABAergic neurons (Fig. 5).

### Cycling of <sup>13</sup>C-labeled glutamate, glutamine and GABA in the TCA cycle

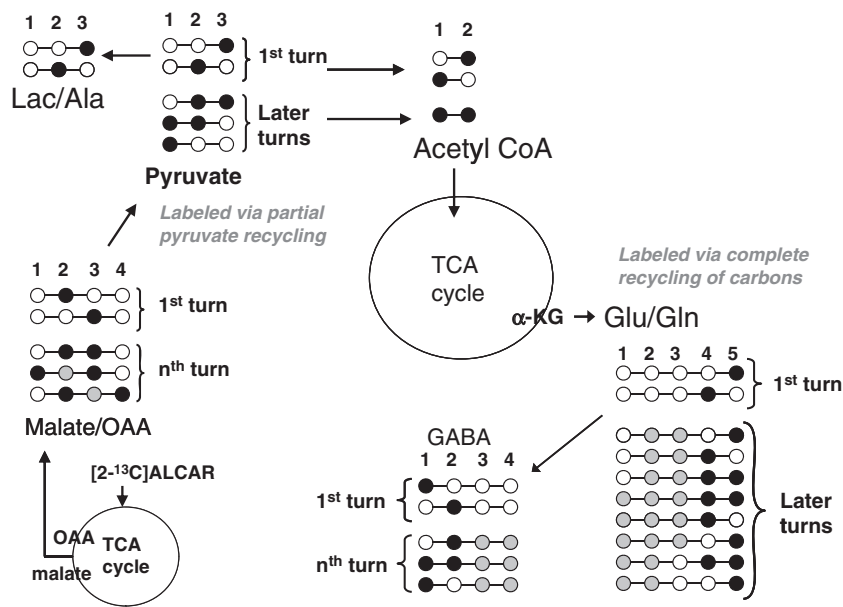
Cycling ratios, which show the relative amount of labeling from the second turn/labeling in the first turn of the TCA cycle, were calculated for glutamate, glutamine and GABA using the amount of label in GLU C3/GLU C4, GLN C3/GLN C4 and GABA C3/GABA C2 (Qu *et al.* 2003). The cycling ratio for glutamate tended to be higher at 15 min (1.46 ± 0.13) but was not significantly different than the ratios at 60 or 120 min (Table S2). The cycling ratio for glutamine was ~1.5–1.6 at all time points, suggesting that labeled glutamine was not lost from the TCA cycle over time. The cycling ratio for GABA was more variable than the ratios for GLU and GLN. However, the ratio for GABA was ~1.8–2.8 at all time points, suggesting that label was not lost from GABA over 120 min.

### Incorporation of label from [2-<sup>13</sup>C]ALCAR metabolism into aspartate

Label from the metabolism of [2-<sup>13</sup>C]ALCAR was incorporated into [3-<sup>13</sup>C]aspartate and [2-<sup>13</sup>C]aspartate (Table 1). The C3 and C2 isotopomers of aspartate are formed from metabolism in the first and second turn of the TCA cycle, respectively as shown in Fig. 2. Incorporation of label into [3-<sup>13</sup>C]aspartate was higher than incorporation into [2-<sup>13</sup>C]aspartate at all time points. There was no significant difference in the amount of label incorporated in aspartate C3 over time; however, the amount of label incorporated into aspartate C2 was increased at 120 min compared with 15 min (*p* < 0.05). This may be due in part to the cycling of label from glutamate metabolism in the TCA cycle as well as labeling from isomerization of labeling in the symmetrical molecule fumarate. The percent enrichment of aspartate C3 in brain ranged from ~1 to 4% after i.p. injection of [2-<sup>13</sup>C]ALCAR (Fig. 4).

### Pyruvate recycling of <sup>13</sup>C-labeled metabolites from ALCAR metabolism

In the present study metabolism of [2-<sup>13</sup>C]ALCAR led to formation of [2-<sup>13</sup>C]lactate, [3-<sup>13</sup>C]lactate and [3-<sup>13</sup>C]alanine (ALA C3) at 15, 60 and 120 min (Table 2). As noted above, when [2-<sup>13</sup>C]ALCAR is the precursor, <sup>13</sup>C-lactate and <sup>13</sup>C-alanine can only be labeled when metabolites formed in the TCA cycle are further metabolized via the pyruvate recycling pathway (Fig. 3). Incorporation of label into <sup>13</sup>C-lactate and <sup>13</sup>C-alanine was detected at all time points, although labeling



**Fig. 3** Labeling pattern obtained from the metabolism of [2-<sup>13</sup>C]acetyl-L-carnitine (ALCAR) via the pyruvate recycling pathway in brain. The initial labeling of OAA and malate from metabolism of [2-<sup>13</sup>C]ALCAR in the tricarboxylic acid (TCA) cycle is shown in the bottom left section of the figure. The [3-<sup>13</sup>C]malate, [2-<sup>13</sup>C]malate and [3-<sup>13</sup>C]oxaloacetate and [2-<sup>13</sup>C]oxaloacetate formed from metabolism of labeled ALCAR in the TCA cycle are converted to pyruvate via either malic enzyme or the combined action of pyruvate kinase and PEPCK, respectively. The labeled pyruvate formed can be converted to lactate and/or alanine labeled in the C1, C2 or C3 positions ('partial' recycling pathway). If

'complete' recycling occurs the labeled pyruvate formed is converted to acetyl CoA labeled in the C1 position which condenses with citrate in the TCA cycle and gives rise to [5-<sup>13</sup>C]α-ketoglutarate and subsequently GLU C5 and GLN C5. [1-<sup>13</sup>C]α-ketoglutarate and glutamate (GLU) C1 and glutamine (GLN) C1 is labeled from further metabolism of the [5-<sup>13</sup>C]α-ketoglutarate formed from recycling via subsequent turns of the TCA cycle. Although, pyruvate recycling can give rise to acetyl CoA labeled in the C2 position, it is not possible to distinguish this from the [2-<sup>13</sup>C]acetyl CoA formed directly from [2-<sup>13</sup>C]ALCAR.

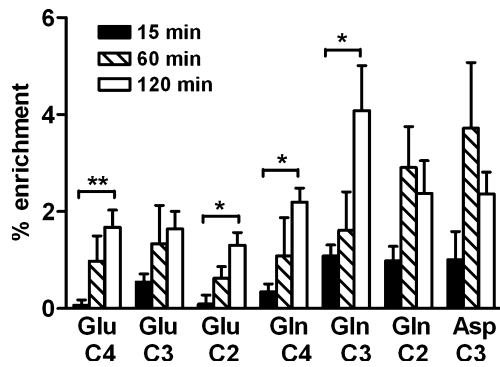
**Table 1** Metabolites labeled via the tricarboxylic acid cycle in extracts of brain from 21–22 day-old rats after injection of [2-<sup>13</sup>C]acetyl-L-carnitine

	nmol <sup>13</sup> C incorporated/mg protein <sup>#</sup>		
	15 min	60 min	120 min
[4- <sup>13</sup> C]Glutamate	0.378 ± 0.033**	0.605 ± 0.056	1.045 ± 0.158
[3- <sup>13</sup> C]Glutamate	0.544 ± 0.042	0.665 ± 0.105	1.010 ± 0.141
[2- <sup>13</sup> C]Glutamate	0.389 ± 0.067**	0.513 ± 0.075*	0.912 ± 0.171
[4- <sup>13</sup> C]Glutamine	0.180 ± 0.021**	0.212 ± 0.053**	0.477 ± 0.080
[3- <sup>13</sup> C]Glutamine	0.278 ± 0.022	0.305 ± 0.070	0.685 ± 0.091
[2- <sup>13</sup> C]Glutamine	0.263 ± 0.035	0.535 ± 0.168	0.471 ± 0.090
[3- <sup>13</sup> C]Aspartate	0.222 ± 0.059	0.288 ± 0.142	0.486 ± 0.104
[2- <sup>13</sup> C]Aspartate	0.042 ± 0.009**	0.076 ± 0.007	0.134 ± 0.029
[2- <sup>13</sup> C]GABA	0.110 ± 0.021	0.177 ± 0.085	0.141 ± 0.034
[3- <sup>13</sup> C]GABA	0.222 ± 0.053	0.231 ± 0.065	0.371 ± 0.083
[4- <sup>13</sup> C]GABA	0.127 ± 0.025***	0.230 ± 0.051***	0.582 ± 0.093

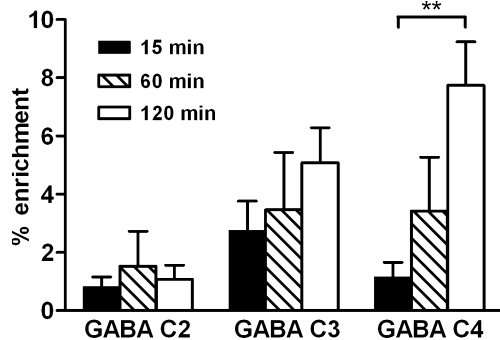
<sup>#</sup>Data were obtained from peak integration of the <sup>13</sup>C-NMR spectra as described in Methods. Percent enrichment was calculated from <sup>13</sup>C labeling and total concentration as described in Methods. Values are mean ± SE of data from *n* = 5 brains at 15 min, *n* = 6 at 60 min, and *n* = 8 at 120 min. Data were analyzed using one-way ANOVA. Asterisks indicate values that are significantly different from 120 min time point, \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001.

in alanine was not detectable in all samples. Labeling in [3-<sup>13</sup>C]lactate (LAC C3) was higher at 120 min than at 15 min (*p* < 0.01) and at 60 min (*p* < 0.05). Incorporation of label into [2-<sup>13</sup>C]lactate (LAC C2) was comparable to the labeling of lactate C3 at 15 min and 60 min. The percent enrichment of LAC C3 was ~ 4–5% at 15 min and 60 min, but increased to ~ 10% at 120 min (Fig. 6). Enrichment in LAC C2 did not change with time and was considerably lower than LAC C3 at 120 min. The absolute amount of label incorporated into ALA C3 was lower than LAC C3 at all time points. Although the percent enrichment of ALA C3 was low (~ 2%) at 15 min, it increased significantly over time and at 60 and 120 min the enrichment in alanine was ~ 10–11% (Fig. 6).

Metabolism of [2-<sup>13</sup>C]acetyl-L-carnitine also led to formation of [1-<sup>13</sup>C]glutamate and [5-<sup>13</sup>C]glutamate and the corresponding [1-<sup>13</sup>C]glutamine and [5-<sup>13</sup>C]glutamine at 15, 60 and 120 min (Table 2). Labeling in the C1 and C5 positions of glutamate and glutamine is further evidence of metabolism via the pyruvate recycling pathway because with [2-<sup>13</sup>C]ALCAR, this is the only pathway for such labeling to occur (Fig. 3). Incorporation of label into the C1 position of glutamate was higher than in the C5 position at all time points. Labeling in GLU C1 and GLU C5 increased with



**Fig. 4** Percent enrichment of glutamate, glutamine and aspartate isotopomers labeled from the metabolism of [2-<sup>13</sup>C]acetyl-L-carnitine in the tricarboxylic acid cycle in 21–22 day-old rat brain. [4-<sup>13</sup>C]glutamate (GLU C4), [3-<sup>13</sup>C]glutamate (GLU C3), and [2-<sup>13</sup>C]glutamate (GLU C2) are formed from metabolism in the first, second and third turns of the tricarboxylic acid cycle respectively. [4-<sup>13</sup>C]glutamine (GLN C4), [3-<sup>13</sup>C]glutamine (GLN C3), and [2-<sup>13</sup>C]glutamine (GLN C2) are formed from the corresponding glutamate isotopomers. [2-<sup>13</sup>C]acetyl-L-carnitine was injected intraperitoneally and rats were killed at 15, 60 or 120 min after injection. Spectra were obtained on perchloric acid extracts of brain as described in methods. Values are mean ± SE of data from  $n = 5$  brains at 15 min,  $n = 6$  at 60min, and  $n = 8$  at 120 min. Data were analyzed using one-way ANOVA and Tukey's *post hoc* test. Asterisks indicate significant difference between time points, \* $p < 0.05$ , \*\* $p < 0.01$ .

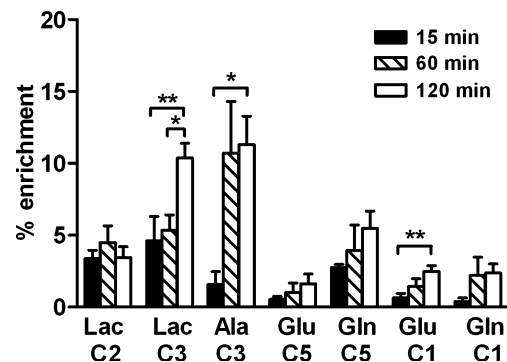


**Fig. 5** Percent enrichment of isotopomers of GABA labeled from metabolism of [2-<sup>13</sup>C]acetyl-L-carnitine in 21–22 day-old rat brain at 15, 60 and 120 min after i.p. injection. [2-<sup>13</sup>C]GABA (GABA C2), [3-<sup>13</sup>C]GABA (GABA C3), and [4-<sup>13</sup>C]GABA (GABA C4) are formed via the enzyme glutamic acid decarboxylase from GLU C4, GLU C3 and GLU C2, respectively. Labeled carbon from glutamine can also be readily converted to glutamate and incorporated into GABA. Spectra were obtained on perchloric acid extracts of brain as described in methods. Values are mean ± SE of data from  $n = 5$  brains at 15 min,  $n = 6$  at 60 min, and  $n = 8$  at 120 min. Data were analyzed using one-way ANOVA and Tukey's *post hoc* test. Asterisks indicate significant difference between time points, \*\* $p < 0.01$ .

**Table 2** Metabolites labeled via the pyruvate recycling pathway in extracts of brain from 21–22 day-old rats after injection of [2-<sup>13</sup>C]acetyl-L-carnitine

	nmol <sup>13</sup> C incorporated/mg protein <sup>#</sup>		
	15 min	60 min	120 min
[2- <sup>13</sup> C]Lactate	0.194 ± 0.019	0.360 ± 0.119	0.369 ± 0.098
[3- <sup>13</sup> C]Lactate	0.218 ± 0.053**	0.403 ± 0.101*	0.997 ± 0.179
[3- <sup>13</sup> C]Alanine	0.103 ± 0.024	0.183 ± 0.060	0.258 ± 0.052
[5- <sup>13</sup> C]Glutamate	0.545 ± 0.063*	0.581 ± 0.078*	0.970 ± 0.182
[5- <sup>13</sup> C]Glutamine	0.507 ± 0.037	0.569 ± 0.125	0.983 ± 0.242
[1- <sup>13</sup> C]Glutamate	0.579 ± 0.096**	0.781 ± 0.074*	1.417 ± 0.260
[1- <sup>13</sup> C]Glutamine	0.190 ± 0.036*	0.319 ± 0.086	0.490 ± 0.105

<sup>#</sup>Data were obtained from peak integration of the <sup>13</sup>C-NMR spectra as described in Methods. Percent enrichment was calculated from <sup>13</sup>C labeling and total concentration as described in Methods. Values are mean ± SE of data from  $n = 5$  brains at 15 min,  $n = 6$  at 60 min, and  $n = 8$  at 120 min. Data were analyzed using one-way ANOVA. Asterisks indicate values that are significantly different from 120 min time point, \* $p < 0.05$ , \*\* $p < 0.01$ .



**Fig. 6** Percent enrichment of compounds labeled from the metabolism of [2-<sup>13</sup>C]acetyl-L-carnitine via the pyruvate recycling pathway in brain. Labeling in lactate C2 and C3, alanine C3 and the C1 and C5 positions of glutamate and glutamine occurs only from pyruvate recycling as shown in Fig. 3. Spectra were obtained on perchloric acid extracts of brain as described in methods. Values are mean ± SE of data from  $n = 5$  brains at 15 min,  $n = 6$  at 60min, and  $n = 8$  at 120 min. Data were analyzed using one-way ANOVA and Tukey's *post hoc* test. Asterisks indicate significant difference between time points, \* $p < 0.05$ , \*\* $p < 0.001$ .

time and was significantly higher at 120 min than at 15 min or 60 min (Table 2). Labeling in GLN C1 tended to increase with time and was higher at 120 min than at 15 min ( $p < 0.05$ ). In contrast to the pattern seen with glutamate, the labeling in GLN C5 was higher than in GLN C1 at all time points. The percent enrichment in GLU C1, GLN C1 and GLU C5 was comparable; however, enrichment in GLN C5 was higher than GLN C1 and GLU C1 and C5 at all time points (Fig. 6).

## Discussion

### Rapid metabolism of ALCAR

In the present study label from [2-<sup>13</sup>C]ALCAR was incorporated into the major neurotransmitters glutamate and GABA in the brains of immature rats within 15 min after i.p. injection. This is consistent with a positron emission tomography study of <sup>11</sup>C-acetyl-L-carnitine that demonstrated rapid clearance of ALCAR from blood and very active uptake into primate brain (Kuratsune *et al.* 1997). That study also showed that the peak labeling in plasma was at 3 min, and rapidly decreased by ~70% by 10 min after infusion. Earlier reports demonstrated that following systemic injection of <sup>14</sup>C-ALCAR, the label was rapidly removed from plasma and taken up into the liver, heart, brain, kidney and skeletal muscle (Farrell *et al.* 1986; Aureli *et al.* 1999; Rebouche 2004). In the present study, following i.p. administration, no compounds labeled from the peripheral metabolism of <sup>13</sup>C-ALCAR were observed in plasma at any time point studied (5, 15, 30, 60 and 120 min), suggesting that the <sup>13</sup>C-amino acids and lactate detected in the PCA extracts of forebrain were from synthesis within brain tissue.

### Metabolism of [2-<sup>13</sup>C]acetyl-L-carnitine in neurons and astrocytes

Studies from the Nalecz laboratory recently demonstrated that the high affinity Na<sup>+</sup>-dependent carnitine/acetyl-L-carnitine transporter OCTN2 is abundant on astrocyte endfeet surrounding the endothelial cells of the blood brain barrier (Miecz *et al.* 2008; Januszewicz *et al.* 2009). This group also recently reported that both OCTN2 and the sodium-independent high affinity transporter OCTN3 are present on neurons in brain (Januszewicz *et al.* 2010). Data from the present study demonstrate that [2-<sup>13</sup>C]ALCAR is metabolized in brain, and the label is rapidly enriched in the C4, C3 and C2 positions of glutamate, glutamine and GABA which increased over time. The higher level of OCTN2 on mitochondria in developing brain, compared with adult brain, would facilitate delivery of ALCAR for metabolism via mitochondrial pathways (Kaminska *et al.* 1993). The percent enrichment of glutamine isotopomers was higher than enrichment in glutamate suggesting active uptake and metabolism of ALCAR in astrocytes in developing brain.

In the present study both the incorporation of label and enrichment were considerably higher in the C3 position of aspartate compared with aspartate C2 at all times studied. This finding may suggest incomplete randomization of the carbons in the symmetrical fumarate molecule in the latter part of the TCA cycle. The possibility that some of the pyruvate C3 formed via the pyruvate recycling pathway re-entered the TCA cycle via pyruvate carboxylase leading to higher enrichment in oxaloacetate C3 and thus aspartate C3

can not be ruled out. However, electron microscopy studies of aspartate localization in brain show far more immunogold labeled aspartate particles in neuronal elements, particularly in presynaptic glutamatergic and GABAergic nerve terminals, than in astrocytes suggesting that aspartate synthesis occurs primarily in neurons *in vivo* (Gundersen *et al.* 2001). The differential labeling of aspartate isotopomers may reflect compartmentation of metabolism in brain.

A number of studies from other groups support the concept that ALCAR either provides energy or interacts with energy metabolism as administration led to increased ATP and phosphocreatine levels (Aureli *et al.* 1994), increased glycogen (Aureli *et al.* 1998), and prevented lactic acidosis after ischemia (Rosenthal *et al.* 1992; Aureli *et al.* 1994; Zanelli *et al.* 2005).

### Metabolism of [2-<sup>13</sup>C]ALCAR in GABAergic neurons

Results from the current study show that the percent enrichment of GABA C4 and GABA C3 was considerably higher than enrichment in GLU C2 and GLU C3 at all time points studied. It is well established that the carbon skeleton of glutamine is preferentially incorporated into GABA (Sonnewald *et al.* 1993; Waagepetersen *et al.* 1999; Sonnewald and McKenna 2002). The high percent enrichment in GABA may arise from trafficking and further metabolism of the glutamine labeled from [2-<sup>13</sup>C]ALCAR in astrocytes. However, the finding that the enrichment in GABA C3 and GABA C4 was generally higher than enrichment of glutamine cannot rule out the possibility of selective uptake and direct metabolism of <sup>13</sup>C-ALCAR in GABAergic neurons. A number of studies have reported data suggesting that carnitine and/or acetyl-L-carnitine are taken up into brain (Burlina *et al.* 1989), synaptosomes (Hannuniemi and Kontro 1988), or cortical neurons (Virmani *et al.* 1994, 1995), in part, by either the GABA transporter, or by a mechanism that can also transport GABA because of the structural similarity of these compounds. More recent molecular studies support the concept of distinct transporters since uptake of acetyl-L-carnitine via OCTN2 was not inhibited by GABA (Wawrzencyk *et al.* 2001; Januszewicz *et al.* 2009, 2010), and GABA transporters expressed in *Xenopus laevis* oocytes did not transport carnitine (Wawrzencyk *et al.* 2001). However, it should be noted that the relative amount of the high affinity carnitine transporters OCTN2 and OCTN3 on glutamatergic and GABAergic neurons has not been reported but warrants further investigation.

The high percent enrichment of GABA from metabolism of [2-<sup>13</sup>C]ALCAR was particularly striking and demonstrates prolonged and active incorporation of label from this precursor in GABAergic neurons. GABA can be synthesized directly from glutamate via glutamic acid decarboxylase, or can be formed from astrocyte derived glutamine after extensive cycling of the carbons in the neuronal TCA cycle

(Waagepetersen *et al.* 1999). In the present study it can not be determined if the high enrichment in GABA C4 is from neuronal or glial precursors because the metabolism of [2-<sup>13</sup>C]ALCAR via the TCA cycle would lead to the same labeling pattern in both astrocytes and neurons. Additionally, the enrichment of GLU C2 and GLN C2, which give rise to labeling in GABA C4, is comparable at both 60 and 120 min. At 120 min 13.9% of the total GABA pool was enriched with <sup>13</sup>C from metabolism of ALCAR compared with 8.7% of the total glutamate pool and 16.5% of the total glutamine pool.

### Extensive and prolonged cycling of the carbon from [2-<sup>13</sup>C]acetyl-L-carnitine in the TCA cycle

In the present study metabolism of [2-<sup>13</sup>C]ALCAR in rat brain led to labeling patterns and cycling ratios that are different from ratios reported for either glucose or acetate metabolism. Hassel *et al.* (1995) reported the time course of labeling from the metabolism of [1-<sup>13</sup>C]glucose and [2-<sup>13</sup>C]acetate in brain of adult mice at 5, 15 and 30 min after injection or infusion of the precursors. They determined that higher labeling was evident at 15 min than at 5 min (Hassel *et al.* 1995). Both Hassel and our group have reported that when [1-<sup>13</sup>C]glucose or [1,6-<sup>13</sup>C]glucose is given as a precursor labeling in glutamate C4 is highest at 15 min and declines at later time points in both rat and mouse brain (Hassel *et al.* 1995; Scafidi *et al.* 2009). The cycling ratio GLU C3/C4 reported by Hassel *et al.* (1995) for mouse brain was  $0.38 \pm 0.02$  at 15 min and  $0.63 \pm 0.09$  at 30 min after a single intravenous injection, and similar ratios for glutamine cycling, indicating a relative loss of label from the C4 position with subsequent turns of the TCA cycle. In the present study, the cycling ratio for glutamate C3/C4 from ALCAR metabolism was unexpectedly high, with a ratio of  $1.46 \pm 0.13$  at 15 min. This ratio from [2-<sup>13</sup>C]ALCAR was higher than the ratios of  $0.97 \pm 0.11$ , and  $0.81 \pm 0.10$  that we recently reported for glutamate cycling from [1,6-<sup>13</sup>C]glucose metabolism 15 and 30 min in 21-day-old rat brain (Scafidi *et al.* 2009).

The pattern of metabolism and high cycling ratios in the present study are also different than those reported for acetate which is selectively taken up and rapidly metabolized by astrocytes in brain (Hassel *et al.* 1995; Taylor *et al.* 1996; Bachelard 1998; Waniewski and Martin 1998, 2004). Cycling ratios reported for metabolism of [2-<sup>13</sup>C]acetate at 15 and 30 min in adult mouse brain were  $0.13 \pm 0.02$  and  $0.33 \pm 0.15$  for GLN C3/GLN C4 and  $0.27 \pm 0.05$  and  $0.55 \pm 0.01$  for GLU C3/GLU C4 (Hassel *et al.* 1995). In the present study the cycling ratios of glutamine and GABA were higher than for glutamate and remained essentially constant from 15 to 120 min.

It is interesting to note that an earlier pharmacokinetic study in primates reported that the labeled carbon from the acetyl moiety of [2-<sup>11</sup>C]acetyl-L-carnitine remained in brain

considerably longer than the label from [1-<sup>11</sup>C]acetyl-L-carnitine (Kuratsune *et al.* 1997). The authors speculated that the more rapid loss of label from the C1 position of the acetyl moiety reflected metabolism for energy and subsequent loss of <sup>11</sup>CO<sub>2</sub>, whereas the prolonged retention of the label from the C2 position of the acetyl moiety reflected incorporation of the carbon into other compounds in brain including the neurotransmitters acetylcholine, glutamate and GABA. Indeed, the data from the present study clearly demonstrate that the label from the C2 position of the acetyl group from ALCAR is incorporated into neurotransmitters and other brain metabolites for a prolonged period of time (120 min). Our data are consistent with the report by Aureli *et al.* (1998) that a single dose of ALCAR injected 20 min prior to injection of <sup>13</sup>C-glucose, had a significant and prolonged effect on brain glucose metabolism leading to decreased overall labeling of amino acids at 15, 30, 45 and 60 min, and increased glycogen content in brain at 60 min. Kido *et al.* (2001) reported that that labeling from <sup>3</sup>H-ALCAR was still detected at 4 h following a single injection in adult mouse brain. However, we found no labeled neurotransmitters were detected at 360 min after ALCAR injection (data not shown). In contrast to the prolonged length of time that the label from ALCAR remained in brain, when a low infusion rate of labeled acetate was used (0.5 g/kg/h), brain acetate decreased to undetectable levels in the *in vivo* spectra within 50 min (Deelchand *et al.* 2009).

Ricciolini *et al.* (1998) demonstrated that 60% of [1-<sup>14</sup>C]acetyl-L-carnitine injected directly into the lateral ventricle of adult rat brain was expired as CO<sub>2</sub> and at 1 h 1.95% was incorporated into brain lipids. Labeling in brain lipids decreased over time (at 3, 6 and 22 h) suggesting turnover and reutilization of carbons (Ricciolini *et al.* 1998). Thus the continued increase in labeling of metabolites in the present study may reflect, in part, incorporation of acetate provided by the oxidation of labeled fatty acids. Indeed, it is relevant to note that Ebert *et al.* (2003) determined that when <sup>13</sup>C-octanoate was infused in adult rats, oxidation of octanoate contributed ~ 20% of the total brain oxidative metabolism.

### Metabolism of [2-<sup>13</sup>C]acetyl-L-carnitine via the pyruvate recycling pathway

Metabolism via the pyruvate recycling pathway in brain *in vivo* was first reported by Cerdan and coworkers in 1990 (Cerdan *et al.* 1990; Kunnecke *et al.* 1993). This pathway uses the TCA cycle intermediates malate or oxaloacetate to form pyruvate which can be converted to lactate and alanine, or can re-enter the TCA cycle providing additional substrate for metabolism leading to formation of glutamate and glutamine. This pathway is considered to be neuroprotective as the pyruvate formed from TCA cycle intermediates can re-enter the TCA cycle condensing with oxaloacetate to form citrate thereby providing substrate for energy metabolism when the formation of pyruvate from glucose metabolism is

low (Cerdan *et al.* 1990; Kunnecke *et al.* 1993). Although this pathway was first reported to be neuronal (Cerdan *et al.* 1990; Kunnecke *et al.* 1993), both the presence and cellular localization of this pathway has been elusive and controversial (Sonnewald *et al.* 1996; Cruz *et al.* 1998; Haberg *et al.* 1998; Pascual *et al.* 1998; Cruz and Cerdan 1999; McKenna *et al.* 2000; Waagepetersen *et al.* 2002). Pyruvate recycling from metabolism of [U-<sup>13</sup>C]glutamate in primary cultures of mouse astrocytes was reported by Sonnewald and co-workers (Sonnewald *et al.* 1996; Waagepetersen *et al.* 2002). More recently, evidence of pyruvate recycling from metabolism of [U-<sup>13</sup>C]glutamate was detected in cerebellar granule cells (Olstad *et al.* 2007). Recycling in brain was higher with 3-hydroxybutyrate and acetate as substrates compared with that observed with glucose (Cerdan *et al.* 1990; Kunnecke *et al.* 1993; Haberg *et al.* 1998; Cruz and Cerdan 1999). A number of groups have reported that enzymes involved in the pyruvate recycling pathway are localized in synaptic terminals (Cruz *et al.* 1998; McKenna *et al.* 2000).

In the present study the incorporation of label into the C2 and C3 positions of lactate confirms that [2-<sup>13</sup>C]ALCAR was metabolized via the pyruvate recycling pathway. Incorporation of label into the C1 and C5 positions of glutamate and glutamine demonstrates that complete recycling occurred with re-entry of the labeled pyruvate formed into the TCA cycle. Our data suggest that this pathway is active in both astrocytes and neurons. However, the high enrichment in glutamine C5 and alanine C3 (which is synthesized at a higher rate in cultured astrocytes than neurons) (Westergaard *et al.* 1993; Bouzier-Sore *et al.* 2006) could be interpreted as compartmentation of this pathway more in astrocytes than in neurons. The high percent enrichment of alanine C3 and lactate C3 labeled via the pyruvate recycling pathway reflects very active metabolism of ALCAR via this pathway in developing brain. Indeed, the metabolism of [2-<sup>13</sup>C]ALCAR via the pyruvate recycling pathway is considerably higher than that reported for any other substrate.

### Relevance of ALCAR metabolism to neuroprotection

In the present study <sup>13</sup>C-ALCAR metabolism was determined in brain of normal 21–22 day-old rats. A large number of both clinical and animal studies provide evidence that acetyl-L-carnitine is neuroprotective in adult brain (Zanelli *et al.* 2005; Calabrese *et al.* 2008), however the effect of the ALCAR on developing brain has not been previously explored. The beneficial effect of carnitine and L-carnitine supplementation of children with metabolic disorders is well established and is presumably due to facilitation of free fatty acid transport and metabolism (Stanley 2004; Virmani and Binienda 2004). Although L-carnitine has been shown to protect developing brain from cell injury due to hypoxia-ischemia (Wainwright *et al.* 2003, 2006) and subsequent to anesthesia (Zou *et al.* 2008), the protective effect of ALCAR in immature brain has not been reported. We propose that

ALCAR may be a more effective neuroprotective agent than L-carnitine because it provides *both* carnitine to facilitate fatty acid transport, and an acetyl CoA moiety that can be used for energy by mitochondria.

Our <sup>13</sup>C-NMR studies demonstrate that, like substrates including glucose, acetate, and 3-hydroxybutyrate, the acetyl moiety of <sup>13</sup>C-ALCAR is used for oxidative energy production and incorporated into the carbon skeleton of the neurotransmitters glutamate and GABA. In contrast to other substrates, metabolism of <sup>13</sup>C-ALCAR led to prolonged cycling of carbon from the acetyl moiety in the TCA cycle, which was particularly evident in glutamine and GABA cycling ratios. Our study also revealed particularly active metabolism of carbon from <sup>13</sup>C-ALCAR via the pyruvate recycling pathway. Overall, the data provide evidence of considerable compartmentation of ALCAR metabolism in developing brain which may be due in part to a differential distribution and function of the OCTN2 and OCTN3 transporters in brain cells.

### Acknowledgement

We thank Jennifer Racz for obtaining the blood samples and determining the glucose concentration in plasma. This work was supported in part by NIH Grant P01 HD16596 and the Passano Foundation.

### Supporting information

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

**Figure S1.** <sup>13</sup>C-NMR spectra of metabolites in perchloric acid extracts of plasma at specific times after i.p. injection of [2-<sup>13</sup>C]acetyl-L-carnitine.

**Table S1.** Total amino acid concentration of rat brain after i.p. injection of [2-<sup>13</sup>C]ALCAR.

**Table S2.** Cycling ratios of glutamate, glutamine and GABA labeled from metabolism of [2-<sup>13</sup>C]ALCAR in 21–22 day-old rat brain.

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