In vivo assessment of increased oxidation of branched-chain amino acids in glioblastoma

Eul Hyun Suh1, Edward P. Hackett1, R. Max Wynn2,3, David T. Chuang2,3, Bo Zhang4, Weibo Luo6,5, A. Dean Sherry1,6,7 & Jae Mo Park1,7,8

Altered branched-chain amino acids (BCAAs) metabolism is a distinctive feature of various cancers and plays an important role in sustaining tumor proliferation and aggressiveness. Due to the therapeutic and diagnostic potentials, the role of BCAA metabolism in cancer and the activities of associated enzymes remain unclear. Due to its pivotal role in BCAA metabolism and rapid cellular transport, hyperpolarized 13C-labeled α-ketosocaproate (KIC), the α-keto acid corresponding to leucine, can assess both BCAA aminotransferase (BCAT) and branched-chain α-keto acid dehydrogenase complex (BCKDC) activities via production of [1-13C]leucine or 13CO2 (and thus H13CO3−), respectively. Here, we investigated BCAA metabolism of F98 rat glioma model in vivo using hyperpolarized 13C-KIC. In tumor regions, we observed a decrease in 13C-leucine production from injected hyperpolarized 13C-KIC via BCAT compared to the contralateral normal-appearing brain, and an increase in H13CO3−, a catabolic product of KIC through the mitochondrial BCKDC. A parallel ex vivo 13C NMR isotopomer analysis following steady-state infusion of [U-13C]leucine to glioma-bearing rats verified the increased oxidation of leucine in glioma tissue. Both the in vivo hyperpolarized KIC imaging and the leucine infusion study indicate that KIC catabolism is upregulated through BCAT/BCKDC and further oxidized via the citric acid cycle in F98 glioma.

In tumors, energy dependence on substrates is significantly altered to compensate elevated biosynthesis and bioenergetics needed for tumor proliferation. For instance, most cancer cells rely on energy-inefficient aerobic glycolysis, known as the Warburg effect, to facilitate the uptake and incorporation of nutrients into the biomass for rapid proliferation. The biosynthetic demands in cancer also stimulate utilization of other nutrients such as glutamine1, acetate4,5, and branched-chain amino acids (BCAAs: leucine, valine, and isoleucine). In particular, BCAAs are reportedly essential for tumor proliferation6–8 and altered BCAA utilization has been demonstrated in multiple cancers9. Overexpressed branched-chain amino acid aminotransferase 1 (BCAT1), a cytosolic enzyme that transfers α-amino groups to α-ketoglutarate to yield the respective branched-chain α-keto acid (BCKA), has been suggested as a prognostic cancer indicator6,10–12. Moreover, a recent study showed increased expression of mitochondrial BCAT (BCAT2) in pancreatic adenocarcinoma13. Despite the emerging roles of BCAAs, however, the molecular mechanism of associated enzyme activities in cancer still remains elusive6,9,14.

In the brain, BCAAs serve as nitrogen donors in the glutamine/glutamate cycle as well as sources for energetic and biosynthetic demands15,16 (Fig. 1). As the first step of BCAA catabolism, BCAAs are transaminated to BCKAs by BCAT isoenzymes. Although the BCATs catalyze reversible transamination reaction between BCAA and BCKA, the catabolic pathway of BCAAs is the predominant direction in most cell types15. α-ketoglutarate is converted to glutamate via BCAT1 in neurons and via BCAT2 in astrocytes to yield the major excitatory neurotransmitter of central nervous system17. In the tripartite synapse, astrocytes convert excess glutamate to α-ketoglutarate by reanimating BCKAs to maintain the concentration of glutamate below 5–10 mM as a glutamate-buffering mechanism for brain nitrogen homeostasis. As a result of this regulatory mechanism, for instance, physiological

1Advanced Imaging Research Center, UT Southwestern Medical Center, Dallas, TX, USA. 2Department of Biochemistry, UT Southwestern Medical Center, Dallas, TX, USA. 3Department of Internal Medicine, UT Southwestern Medical Center, Dallas, TX, USA. 4Department of Pathology, UT Southwestern Medical Center, Dallas, TX, USA. 5Department of Pharmacology, UT Southwestern Medical Center, Dallas, TX, USA. 6Department of Chemistry and Biochemistry, UT Dallas, Richardson, TX, USA. 7Department of Radiology, UT Southwestern Medical Center, Dallas, TX, USA. 8Department of Electrical Engineering, UT Dallas, Richardson, TX, USA. Correspondence and requests for materials should be addressed to J.M.P. (email: jaemo.park@utsouthwestern.edu)
Concentration of $\alpha$-ketoisocaproic acid (KIC) is maintained at $0.7 \pm 0.1 \mu M$ in rat brain, which is significantly lower than KIC in rat blood plasma ($18.9 \pm 1.4 \mu M$). Subsequently, the BCKAs are converted to branched-chain acyl-CoAs via branched-chain $\alpha$-keto acid dehydrogenase complex (BCKDC) at an irreversible oxidative decarboxylation step. Thus, CO$_2$ produced in this step can serve as a direct indicator of BCAA oxidation. The ensuing enzymatic reactions are unique for each BCAA with end products from each degradative pathway eventually being oxidized through the citric acid cycle (CAC).

In brain malignancies (e.g., glioma), BCAA uptake via L–amino acid transporter-1 (LAT1) is enhanced compared to normal astrocytes and BCAT1 activity is augmented in primary gliomas with wild-type isocitrate dehydrogenase (IDH wt). In particular, the BCATs are suggested as cancer-specific biomarkers, but the directionality of the BCAT-mediated reaction and how the BCAT enzyme levels are associated with metabolic phenotypes in tumor have been addressed in different manners. In glioblastoma, BCAA catabolism is upregulated to support cell proliferation. In contrast, increased reverse direction was observed in non-small cell lung cancer for tissue protein synthesis and in leukemia for cancer progression. As the tumor metabolism is progressively remodeled by adapting to the surrounding tumor microenvironment, it is important to understand the environmental factors and tumor heterogeneities, and therefore necessary to assess comprehensive tumor characteristics.

Non-invasive assessment of in vivo metabolism is crucial to understand the altered BCAA metabolism and to identify potential imaging biomarkers in cancer. Dynamic nuclear polarization (DNP or ‘hyperpolarization’) in combination with a rapid dissolution and $^{13}$C MR spectroscopy allows in vivo investigation of multiple metabolic pathways by detecting metabolic flux of $^{13}$C labeled substrates. It has been a useful imaging tool for assessing cancer-specific enzyme-catalyzed metabolic phenotypes. In particular, hyperpolarized (HP) $[1-^{13}C]$KIC was used to assess BCAT-mediated leucine metabolism in the brain in vivo. HP $[1-^{13}C]$KIC has a relatively long longitudinal relaxation time ($T_1$), a key parameter that determines the length of the observable time-window for in vivo metabolism. KIC is rapidly transported into the cell via monocarboxylic transporter (MCT) and can assess both BCAT and BCKDC activities via production of $[1-^{13}C]$leucine or $^{13}$CO$_2$ (and thus $\text{H}^{13}\text{CO}_3^-$), respectively. Indeed, previous studies showed that the HP $[1-^{13}C]$KIC conversion to $[1-^{13}C]$leucine was correlated with BCAT activity in prostate cancer cells, lymphoma, and normal rat brain. In the prostate cancer cells, elevated BCAT levels resulted in increased production of HP $[1-^{13}C]$KIC and HP $[1-^{13}C]$KIC was observed to be converted to leucine in E14 lymphoma. Moreover, Butt, et al. demonstrated that $^{13}$C metabolic imaging using HP $^{13}$C KIC is a practical method to access the BCAT activity in vivo in rat brains.

We hypothesized that BCAA oxidation would be increased in glioma-bearing rats as a unique feature of tumor, which can be visualized by $^{13}$C MRI in combination with DNP. In this study, we investigated the increase of in vivo leucine oxidation in F98 glioma and the feasibility of HP $[1-^{13}C]$KIC as an imaging agent to measure altered BCAA metabolism in glioma by assessing BCAT- and BCKDC-catalyzed in vivo enzyme reactions. Moreover, $^{13}$C NMR isotope analysis was performed after a steady-state infusion of $[\text{U}-^{13}C]$leucine into glioma-bearing rats to elucidate the downstream metabolism of BCAA. These results were further evaluated by subsequent ex vivo tissue analysis of BCAT/BCKDC enzyme activities and its expression levels.

**Results and Discussion**

**GBM animal model.** We used F98 cell line for glioma model. It is histologically classified as an anaplastic glioma, which is pathologically undifferentiated malignant glioma, and the characteristics of tumor growth are similar with human glioblastoma multiforme (GBM) in overexpression of the epidermal growth factor receptor (EGFR). The growth of tumors was confirmed 15–18 days after the cell implantation using both $T_2$-weighted
and contrast-enhanced (CE) T₁-weighted proton MRI. The tumor regions identified by postmortem hematoxylin and eosin (H&E) stain was better matched to the hyperintense regions of the CE T₁-weighted-MRI rather than those of T₂-weighted images (Fig. S1).

In vivo ¹³C imaging of branched-chain amino acid metabolism in glioma using hyperpolarized [¹-¹³C]α-ketoisocaproate to assess BCAT/BCKDC activity of glioma. We first tested HP [¹-¹³C]KIC in F98 glioma cells in vitro. A time-resolved pulse-and-acquire ¹³C sequence with a non-selective radiofrequency (RF) pulse was used to monitor the injected [¹-¹³C]KIC and produced [¹-¹³C]leucine and H¹³CO₃⁻ peaks. The corresponding time courses of HP KIC and the products. (d–e) In vivo chemical shift imaging of a F98 glioma-bearing rat using HP [¹-¹³C]KIC and (f) the contrast-enhanced (CE) T₁-weighted MRI. Metabolite distributions of [¹-¹³C]leucine and H¹³CO₃⁻ in a tumor-bearing rat brain slice after an injection of HP [¹-¹³C]KIC. (g–h) The reconstructed spectra in the glioma (solid red) and the contralateral normal-appearing brain (NAB; dotted blue) and (i) in vivo metabolite ratio of [¹-¹³C]leucine to H¹³CO₃⁻.

Figure 2. (a) Metabolic pathway of hyperpolarized (HP) [¹-¹³C]KIC. ¹³C-labeled KIC and its metabolic products are highlighted in red. (b) An in vitro time-averaged spectrum from F98 cells showed the injected HP [¹-¹³C]KIC and produced [¹-¹³C]leucine and H¹³CO₃⁻ peaks. (c) The corresponding time courses of HP KIC and the products. (d–e) In vivo chemical shift imaging of a F98 glioma-bearing rat using HP [¹-¹³C]KIC and (f) the contrast-enhanced (CE) T₁-weighted MRI. Metabolite distributions of [¹-¹³C]leucine and H¹³CO₃⁻ in a tumor-bearing rat brain slice after an injection of HP [¹-¹³C]KIC. (g–h) The reconstructed spectra in the glioma (solid red) and the contralateral normal-appearing brain (NAB; dotted blue) and (i) in vivo metabolite ratio of [¹-¹³C]leucine to H¹³CO₃⁻.

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decarboxylated product of [1-13C]KIC, is irreversibly mediated by BCKDC and, therefore, indicates a pure metabolic catabolic flux. 13C-leucine, the transamination product of KIC was smaller in the tumor as compared to the contralateral NAB ([1-13C]leucine/total 13C = 0.0053 in tumor vs. 0.0081 in NAB, p < 0.05). The low 13C conversion from KIC to leucine in the tumor implies predominant net conversion of leucine to KIC or suppressed isotope exchange between KIC and leucine (e.g., depletion of intrinsic leucine pool). Conversely, more H13CO3− was detected in the tumor (H13CO3−/total 13C = 0.005, p < 0.05) compared to the NAB (0.0027), suggesting elevated KIC catabolism by BCKDC towards isovaleryl-CoA.

Increased of [U-13C]leucine oxidation in F98 glioma. BCAA can be partially oxidized for protein synthesis or can be fully oxidized as an energy source. Although major BCAA oxidative capacity is high in skeletal muscle or liver, brain has 20% proportion of the whole-body capacity to oxidize BCAA34 and this rate is greater than incorporation rate of BCAA into protein35. Previous studies have shown increased uptake of BCAA by C6 glioma cell19 and breast cancer cell lines (MDA-MB-231, MCF-7) via LAT135. To elucidate the metabolic fate of BCAA in glioma, leucine metabolism was further explored in F98 glioma using an ex vivo 13C NMR isotopomer analysis. The isotopomer analysis can track down multiple metabolic pathways by accessing the distribution of 13C isotopomers in tissue after infusion of 13C-labeled substrates. We evaluated downstream metabolism of leucine in brain tumor after a [U-13C]leucine infusion into F98 glioma-bearing rats. Oxidation of [U-13C]leucine in the glioma produced distinct labeling in several CAC-intermediates as downstream products. In particular, 13C-glutamate labeling patterns enable direct comparison of leucine utilization between glioma and NAB. The glutamate doublet in 13C NMR spectra were also analyzed to determine the quantity of 13C labeled acetyl-CoA entering the CAC. The protocol for 13C-leucine infusion study is described in Fig. 3a. Both glioma and contralateral NAB tissues were harvested and freeze-clamped after a steady-state infusion of [U-13C]-leucine. 13C NMR spectra from glioma and NAB tissue extracts were compared by examining the distribution of 13C isotopomers.
The data showed that [U-13C]leucine was converted to [U-13C]KIC and further catabolized to [1,2,3,4,5-13C5]isovaleryl-CoA and [1,2,3,4,5-13C5]HCO3− via mitochondrial BCKDC, followed by conversion to [2,3,4,5-13C4]acetoacetate and [1,2,3-13C]acetyl-CoA. Both [1,2,3-13C2] and [2-13C]acetyl-CoA are oxidized in the CAC and form various 13C isotopomers in 13C-glutamate. 13C NMR spectra reflected higher quantities of 13C-labeled leucine in glioma tissue versus NAB tissue (Fig. 3b). Glutamate derived from leucine was estimated from C4 doublet of [4,5-13C2]glutamate (23.1 ± 6.2% of total glutamate pool in glioma, 15.5 ± 5.9% in NAB, p < 0.05, Fig. 3d,e). Moreover, the multiplets appearing in the lactate C2 resonance were more abundant in the spectrum of glioma tissue, reflecting oxidation of leucine in the CAC followed by cycling of three carbon units out of the CAC and into pyruvate (pyruvate recycling) (Fig. 3c). Based on the 1H MR spectroscopy, leucine concentration in brain tissue was significantly higher in glioma (0.278 ± 0.035 μmol/g of wet tissue) than in contralateral NAB (0.075 ± 0.001 μmol/g). Lactate pool was also larger in the glioma (9.989 ± 1.413 μmol/g) compared to the NAB (7.639 ± 1.506 μmol/g) (Fig. S2). Together, these results indicate that leucine uptake and oxidation are increased in glioma. The total leucine plasma level was within the physiological condition during the infusion (91.7 ± 16 μmol/L in tumor-bearing rat)36. Increased catabolism of [U-13C]leucine in tumor tissue is consistent with the in vivo 13C imaging results using HP [1-13C]KIC in F98 glioma that showed higher decarboxylation product of HCO3− from [1-13C]KIC in the tumor compared to NAB.

**Ex vivo assay of BCAT and BCKDC activities in brain tissues.** To verify the results of the in vivo imaging and the steady-state infusion study, enzyme activities and expression levels of BCAT and BCKDC were measured in brain tissues. BCAT activity assays were performed as previously described using in vitro continuous spectrophotometric method. L-alanine aminotransferase and L-lactate dehydrogenase were used as coupled enzymes in this assay37. BCKDC subunit activity was assayed in the brain tissue according to the methods of radiocchemical assay by [1-14C]KIC substrate and measure the [14C]-decarboxylation by BCKDC38. The previous study reported that BCAT1 is a major isoenzyme in rat brain responsible for 60 - 70% of total BCAT activity39. This is congruent with our western blot analysis; The expression of BCAT1, rather than BCAT2, is relatively predominant in both NAB and glioma. While the total BCAT activities were comparable between glioma (3.34 ± 0.16 U/g of protein) and NAB (3.16 ± 0.05), the expression levels of isoenzyme BCAT1 and BCAT2 were significantly tumor-specific: lower BCAT1 and higher BCAT2 in glioma than in NAB (Fig. 4). The distinct levels of BCAT1 and 2 in glioma might be due to the characteristics of its cellular origin, glial cell30,31, as tumors conserve the metabolic phenotype of their tissue origin41. Indeed, BCAT2 is predominant in glial cells whereas BCAT1 is primarily in cortical neurons17,42,43. In contrast, both the activity and the expression level of the BCKDC-E1α subunit were at similar levels in glioma (3.80 ± 1.67 mmol CO2/min/g tissue) and NAB (4.41 ± 0.86) (Fig. 4). Increased expression level of BCAT2 and unaltered BCKDC level in glioma together suggests that BCAT2 is a key enzymatic step that regulates BCAA catabolism in glioma. Islam, M.M., et al.39 recently reported that BCAT2 forms a protein–protein complex with BCKDC such that the substrates like KIC are channeled to the adjacent E1 subunit of BCKDC for further catabolism. Such channeling of substrates could explain the enhanced H13CO3− production observed in glioma versus normal brain tissue.

**Outlook.** GBM is one of the most aggressive primary brain tumors. Nevertheless, diagnosis and prognosis is limited by poor understanding of molecular biomarker44 and biopsy is restricted if the tumor is inoperable. Although the role of BCAA in cancer needs further investigation, multiple enzymatic steps of BCAA metabolism can be important prognosis and diagnosis biomarkers in tumor. In particular, BCAT1 expression is dependent on the concentration of α-ketoglutarate substrate in glioma cell lines and could be suppressed by ectopic overexpression of mutant IDH1 isoform 1 (IDH1R132H) in immortalized human astrocytes, providing a link between IDH1 function and BCAT1 expression. This link is supported by IDH1 astrocytic gliomas being characterized by high BCAT1 expression. The overexpression of mutant IDH1 associated with reduced BCAT1 expression and reduced invasiveness and tumor growth provides a plausible rationale for the aggressiveness of primary GBM and the relatively less aggressive low grade or secondary gliomas. The HP KIC imaging method can assess activities of two key enzymes in BCAA catabolism, BCAT and BCKDC, and therefore potentially play a crucial role in tumor characterization, drug development and therapeutic decision.
Conclusion
HP [1-13C]KIC is a promising substrate to investigate in vivo brain tumor metabolism. KIC can be delivered to intracellular space via MCT and instantly converted [1-13C]leucine by BCAT to or to CO2 (and thus HCO3-) by BCKDC. The detection of BCKDC activity is crucial for assessing BCAA oxidation since it irreversibly catabolizes BCKAs and regulates the BCAA oxidation. Here we demonstrated the feasibility of HP [1-13C]KIC to assess BCAT/BCKDC activity in F98 glioma in vivo and investigated that leucine oxidation was increased in the tumor as a unique feature of glioma. In addition, we showed the increase of in vivo leucine oxidation in F98 glioma as a unique metabolic feature of brain tumor using steady-state infusion [U-13C]leucine. Both the in vivo HP KIC imaging and the leucine infusion study indicate that KIC catabolism is upregulated through BCAT/BCKDC and further oxidized via the CAC in F98 glioma. To our knowledge, this is the first report of HP [1-13C]KIC in vivo imaging in rat glioma model. Besides glioma and other cancer applications, we expect that this technique can be utilized to investigate BCAT/BCKDC-associated pathological features in multiple other diseases with altered BCAA metabolism such as traumatic brain injury.

Methods
GBM animal model and tumor implantation. F98 glioma cell line was used for both in vitro and in vivo studies. It is histologically classified as an anaplastic glioma, which is pathologically undifferentiated malignant glioma, and the characteristics of tumor growth are similar with human GBM in overexpression of the EGFR28. The F98 glioma cells (CRL-2397®) were obtained from ATCC (Manassas, VA, USA). The cells were cultured at 37°C in a monolayer using Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and streptomycin with 5% CO2. The cells were harvested at 85–90% confluency. Approximately 1 x 107 cells were prepared for each animal. After a Trypan blue exclusion test for cell viability, the cells were implanted into the right striatum of male Fisher rats (n = 12, body weight = 240 ± 10 g). The tumor implantation was performed at the Neuro–Moos Core Facility of the University of Texas Southwestern Medical Center. The growth of tumors was confirmed after 15–18 days of implantation by T2-weighted and CE T1-weighted proton MRI. The tumor regions identified by postmortem H&E stain was better matched to the hyperintense regions of the CE T1-weighted-MRI rather than those of T2-weighted images (Fig. S1).

When the tumors were established the animals were either injected with HP [1-13C]KIC for MRSI or infused with [U-13C]leucine for isotopomer analysis. Tumor volumes were estimated as 93.5 ± 10.3 mm3 by measuring hyperintense regions in a 2D T2-weighted MRI using OsiriX dicom viewer. Tumor sizes were further confirmed by a 2D CE T1-weighted MRI (50.0 ± 26.5 mm3). Immediately after the imaging or infusion studies, the glioma and NAB tissues were collected for enzyme assays, histology or isotopomer analysis. For comparison, healthy male Fisher rats (n = 3, body weight = 252 ± 10 g) were also examined. All the animal protocols were approved by the local Institutional Animal Care and Use Committee and all experiments were performed in accordance with relevant guidelines and regulations.

Hyperpolarization study of [1-13C] α-ketoisocaproic acid. [1-13C] α-ketoisocaproic acid (KIC) was prepared from [1-13C] α-ketoisocaproic acid sodium salt (Cambridge Isotope Laboratories, Andover, MA, USA) by dissolving in 1-M HCl (pH < 1) and extracting the aqueous layer with diethyl ether. The combined organic layer was dried over anhydrous sodium sulfate, filtered and evaporated to afford [1-13C]KIC (7 M, colorless oil, purity >95%, 94% yield)25. For hyperpolarization, a 70-μL sample of the [1-13C]KIC mixed with 11-mM trityl radical (OX063, Oxford Instruments Molecular Biotools Ltd, Oxfordshire, UK) was placed in a sample vial, which was then assembled with a research fluid path (GE Healthcare, Waukesha, WI, USA) containing 16 mL of dissolution media (0.1 g/L Na2EDTA) in the dissolution syringe. After placed in a SPINlab® polarizer (GE Healthcare), the sample was polarized at ~0.8 K of temperature in a 5 T magnet by irradiating microwaves at a frequency of 139.93 GHz. The polarized sample (3 - 4 hrs) was dissolved and mixed with neutralization media (0.72 M NaOH, 0.4 M Trizma and 0.1 g/L Na2EDTA), resulting in 6.5–7.0 mL of 80-mM [1-13C]KIC solution (pH 7.4–8.2).

Hyperpolarized 13C MRS study of F98 cell. HP 13C MR cell studies were performed on a clinical GE 3 T Discovery 750 W MR scanner (Ωinhom = 70 cm) and the GE SPINlab®. F98 cells were cultured as described above and collected (1.1 ± 0.1 x 106, n = 3) and washed with PBS twice. The cells suspended in 2 mL of DMEM media were placed in a Falcon50 mL centrifuge tube at 37°C, and 2 mL of HP [1-13C]KIC was injected into the tube within 15–18 sec following the dissolution. Dynamic and time-average 13C spectra were acquired using a custom-made 13C transmit/receive surface coil (Ωinner = 28 mm).

MR Imaging protocol. In vivo imaging studies were also carried out at the clinical GE 3 T MR scanner. For 1H imaging, a quadrature birdcage volume RF coil (Ωinhom = 80 mm) was used for both RF excitation and data acquisition. After locating the rat brain at the center of the birdcage coil using a 3-plane fast gradient-recalled echo (PGRE) sequence, high-resolution axial brain images were acquired using a dual-echo T2-weighted fast spin echo (FSE; echo time [TEs] = 11.3 msec/64.0 msec, TR = 5,000 msec, 7–13 slices, field of view [FOV] = 96 x 96 mm2, matrix size = 256 x 192, slice thickness = 2 mm, echo train length = 8). B0 field inhomogeneity over the brain region was minimized using a single voxel point-resolved spectroscopy (PRESS) sequence by adjusting shimming currents. For 13C MRSI, a 2D spiral chemical shift imaging pulse sequence described in previous study29 using the 13C surface coil with a variable flip angle scheme, θ = tan(i/x/4), n = 1, ..., 4, was used to acquire single time-point metabolite maps of rat brain (four spatial interleaves of spiral k-space readout, spectral bandwidth = 210.8 Hz, #echoes = 64, FOV = 50 x 50 mm2, matrix size = 16 x 16, slice thickness = 7.7 mm, acquisition time = 1.5 sec) following an intravenous injection of 80-mM HP [1-13C]KIC (injection-to-scan time = 25 sec, 1 mmol/kg body weight up to 4.0 mL). Finally, a CE T1-weighted spin echo (SE) images were...
acquired at the end of each imaging session (TE = 12 msec, TR = 700 msec, 96 × 96 mm², 7–13 slices, matrix size = 256 × 192, slice thickness = 2 mm).

**Image reconstruction.** The HP 13C MRSI were reconstructed for individual peak similarly as the described in previous study4. After a 5-Hz Gaussian line broadening and 4-fold zero-filling along the time domain, an inverse FFT was applied to the raw data. Prior to a spatial apodization (hanning function) and a zero-filling (4x) along each spatial domain, chemical shift artifact was removed by correcting the off-resonance and aliasing pattern of each peak (spectral tomosynthesis), followed by a gridding onto the Cartesian coordinate and a spatial 2D inverse FFT.

Metabolic maps of HP [1-13C]KIC, [1-13C]leucine, and H3CO2 were produced by integrating the signal around each peak in absorption mode. The metabolic images were normalized by the maximum signal. Regions of interest (ROIs) of tumor and NAB were drawn manually to calculate the signal intensities for the metabolites. All the 13C data were reconstructed and analyzed using MATLAB (Mathworks, Natick MA, USA).

**Infusion of [U-13C]leucine.** 13C isotope tracer infusion studies were performed as previously described4. After 15–18 days from the tumor implantation, rats were fasted (n = 3) for 15 hrs prior to leucine infusion (U-13C; 99%, Sigma-Aldrich Isotec). After a tail vein catheterization under anesthesia (<10 min), all the rats stayed awake during the infusion. Each infusion started with a bolus injection of [U-13C]leucine (0.25 mg/g per body weight in 4 mL saline solution over 1 min), continued by a slow infusion (0.0069 mg/g of body weight/min in saline solution at 4.8 mL/hr for 150 min). Following the completion of the infusion, each animal was fully anesthetized and 4–6 mL of blood sample was collected by cardiac puncture. After decapitation, the whole brain was harvested and sliced to 2-mm coronal sections using brain matrices (Ted Pella, Inc., Redding CA, USA). Tumor (112.2 ± 24 mg) and contralateral NAB tissues (92.0 ± 10.8 mg) were rapidly collected from the brain slices, immediately frozen by liquid nitrogen and stored at −80 °C. The quantification of 13C fractional enrichment on glutamate methods are in supporting material.

**Statistical analysis.** Statistical significance between tumor and contralateral NAB was evaluated by a paired t-test (α = 0.05, one-tailed) using GraphPad Prism version 7.0 (GraphPad Software, Inc., La Jolla CA, USA). Unpaired t-tests were used for comparison between tumor-bearing rats and healthy controls. All the data were presented as mean ± standard deviation.

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Author Contributions

E.H.S. and J.M.P. designed the research; E.H.S., E.P.H. and J.M.P. performed the hyperpolarized 13C imaging experiments; E.H.S. performed F98 cell culture; E.P.H. was responsible of tumor implantations and animal care; E.H.S. and E.P.H. performed steady-state infusion and tissue extractions; E.H.S., A.D.S. and J.M.P. analyzed 13C NMR spectra; E.H.S. analyzed tissue histology; E.H.S., R.M.W., and D.T.C. performed enzyme assays; B.Z. and W.L. performed Western blot analysis; E.H.S., R.M.W., D.T.C., W.L., A.D.S. and J.M.P. contributed on writing the manuscript.

Additional Information

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