

Delayed cerebral oxidative glucose metabolism after traumatic brain injury in young rats

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Abstract

Traumatic brain injury (TBI) results in a cerebral metabolic crisis that contributes to poor neurologic outcome. The aim of this study was to characterize changes in oxidative glucose metabolism in early periods after injury in the brains of immature animals. At 5 h after controlled cortical impact TBI or sham surgery to the left cortex, 21–22 day old rats were injected intraperitoneally with [1,6-¹³C]glucose and brains removed 15, 30 and 60 min later and studied by ex vivo ¹³C-NMR spectroscopy. Oxidative metabolism, determined by incorporation of ¹³C into glutamate, glutamine and GABA over 15–60 min, was significantly delayed in both hemispheres of brain from TBI rats. The most striking delay was in labeling of the C4 position of glutamate from neuronal metabolism of glucose in the injured, ipsilateral hemisphere which peaked at 60 min, compared with the contralateral and sham-operated brains, where metabolism peaked at 30 and 15 min, respectively. Our findings indicate that (i) neuronal-specific oxidative metabolism of glucose at 5–6 h after TBI is delayed

in both injured and contralateral sides compared with sham brain; (ii) labeling from metabolism of glucose via the pyruvate carboxylase pathway in astrocytes was also initially delayed in both sides of TBI brain compared with sham brain; (iii) despite this delayed incorporation, at 6 h after TBI, both sides of the brain showed apparent increased neuronal and glial metabolism, reflecting accumulation of labeled metabolites, suggesting impaired malate aspartate shuttle activity. The presence of delayed metabolism, followed by accumulation of labeled compounds is evidence of severe alterations in homeostasis that could impair mitochondrial metabolism in both ipsilateral and contralateral sides of brain after TBI. However, ongoing oxidative metabolism in mitochondria in injured brain suggests that there is a window of opportunity for therapeutic intervention up to at least 6 h after injury.

Keywords: ¹³C-NMR spectroscopy, GABA, glucose, glutamate, immature brain, metabolism, PC, PDHC, traumatic brain injury.

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Traumatic brain injury (TBI) is the leading cause of morbidity and mortality in children (Keenan and Bratton 2006). TBI is characterized by depressed cerebral metabolism, which is attributed to mitochondrial dysfunction induced by increased production of reactive oxygen species after trauma, alterations in intracellular calcium homeostasis and dissipation of transmembrane potential (Fiskum 2000; Verweij *et al.* 2007). Numerous adult animal and human investigations demonstrated that the decreased oxidative metabolism is non-ischemic, suggesting that it is due to mitochondrial alterations *per se*, and not solely a result of lack of oxygen and glucose supply because of decreased cerebral perfusion pressure to the injured tissue (Xiong *et al.* 1998; Soustiel and Sviri 2007; Marcoux *et al.* 2008). A decrease in oxidative metabolism after TBI results in a shift to anaerobic metabolism, tissue lactate accumulation, decreased pH and further mitochondrial impairment.

Mitochondria are important for energy production, maintaining redox state in brain, detoxification of reactive oxygen species and intracellular calcium homeostasis (Robertson *et al.* 2006a). Production of glutamate and γ -amino butyric acid (GABA), the major excitatory and inhibitory neurotransmitters in brain, respectively, involves oxidative metabolism via the tricarboxylic acid (TCA) cycle in neurons (Waagepetersen *et al.* 2000). Mitochondrial metabolism is

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Abbreviations used: ASP C3, [3-¹³C]aspartate; PC, pyruvate carboxylase; PDHC, pyruvate dehydrogenase complex; TBI, traumatic brain injury; TCA, tricarboxylic acid.

finely tuned to fluctuations in the concentrations of key metabolites in the intra- and extracellular milieu under normal conditions (McKenna 2007). However, following TBI mitochondria are injured and many of these normal functions are disrupted (Robertson *et al.* 2006a; Opii *et al.* 2007; Robertson *et al.* 2007; Casey *et al.* 2008).

Using ^{13}C -NMR spectroscopy Bartnik *et al.* (Bartnik *et al.* 2005) reported significant increases in brain lactate and glutamine production as early as 3.5 h after TBI in immobilized adult rats. Investigations on the effects of TBI on energy metabolism of the immature brain are limited, despite the prevalence of TBI in children. Nevertheless, brain mitochondrial respiration and pyruvate dehydrogenase complex activity levels are reduced within 4 h after TBI in immature rats (Robertson *et al.* 2007). A recent ^1H -NMR study reported increased lactate at 4 h, and decreased *N*-acetylaspartate at 24 h and 7 days after TBI providing further evidence of mitochondrial dysfunction after TBI in immature brain (Casey *et al.* 2008).

The aim of this study was to determine glycolytic and oxidative glucose metabolism and neurotransmitter synthesis using ^{13}C -NMR spectroscopy to further clarify the alterations in immature brain 5–6 h after TBI.

Materials and methods

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. Immature [postnatal day 21–22], male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were used in all studies. The animals were weaned away from the dam on postnatal day 19 and housed with littermates before surgery and after recovery from anesthesia.

TBI model

The injury paradigm for immature rats was previously described by Robertson *et al.* (Robertson *et al.* 2007). Anesthesia was induced in a plexiglass chamber with 4% isoflurane. The head was fixed in a stereotactic device and isoflurane (2–2.5%) with oxygen (30%) was administered via a nose cone for the duration of surgery. Rectal temperature was maintained at $37.0 \pm 0.5^\circ\text{C}$. A midline scalp incision was made, and a left parietal craniotomy performed. Traumatic brain injury was performed using the controlled cortical impact (CCI) device (Pittsburgh Precision Instruments, Pittsburgh, PA, USA) as previously described (Dixon *et al.* 1991; Robertson *et al.* 2006b). CCI injury was produced with a 6 mm metal impactor tip with a 1.5 mm depth of penetration, 5.5 ± 0.3 m/s velocity, and 50 ms duration of deformation. Following injury, the bone flap was replaced, craniotomy sealed, and the scalp incision closed with sutures. Isoflurane was discontinued and, after regaining consciousness, the rats were returned to their cages with littermates. Sham rats had identical surgeries, without CCI injury. Rats were not randomized, but treatment (TBI and sham) was alternated between rats on each day of study.

At 5 h following surgery rats were given i.p. injections of [1,6- ^{13}C]glucose (200 mg/kg; 99% ^{13}C enriched, Cambridge Isotope Laboratories, Woburn, MA, USA). Rats were killed by decapitation 15, 30 and 60 min after injection. Brains were rapidly dissected into injured (left, ipsi) and contralateral (right, contra) sides (cortex, including the immediate subcortical structures, i.e., hippocampus, thalamus, hypothalamus, parietal and motor areas) and snap-frozen in liquid nitrogen. The time from decapitation to freezing brain was <40 s, and the order of freezing left and right sides of brain was alternated to avoid any bias from freezing time. All samples were stored at -80°C until extraction.

Tissue extraction

Each frozen tissue sample was homogenized in 2 mL ice-cold 7% perchloric acid (PCA), and processed as described by Richards *et al.* (Richards *et al.* 2007). The neutralized, lyophilized extracts were stored at -20°C .

NMR spectroscopy

Lyophilized samples were reconstituted in deuterium oxide (D_2O) with 0.4% dioxane added as an internal standard for quantification, adjusted to pH 6.8–7.0. Proton-decoupled 125.5-MHz ^{13}C -NMR spectra were obtained at the NMR Center, University of Maryland Baltimore, on a Varian Inova 500 MHz spectrometer with a broad band detection probe. Spectra were acquired at 25°C using a 35° pulse angle, 25 KHz spectra width, and 64 K data points. Acquisition time and relaxation times were 1.3s and 4.3s, respectively. The number of scans was typically 11 000–13 000. Spectra were corrected for nuclear Overhauser effects (Richards *et al.* 2007). A line broadening of 5 Hz was used. Chemical shifts are reported relative to the dioxane peak at 67.4 ppm, and peak assignments were made by comparison spectra from ^{13}C standards and literature values.

Protein and amino acid quantification

The protein content of the pellets from the PCA extracts of the brains was determined using the Pierce BCA microreagent assay (Smith *et al.* 1985). Amino acids were determined by HPLC (Richards *et al.* 2007).

Statistical analyses

Data are expressed as mean \pm SEM for $n = 5$ for the 15 and 30 min post-injection groups, and $n = 7$ for the 60 min group. Data were analyzed by one-way ANOVA with $p < 0.05$ as the level of significance, using SigmaStat software (Systat Software Inc, Point Richmond, CA, USA).

Labeling from metabolism of [1,6- ^{13}C]glucose in brain

The labeling pattern of brain metabolites after infusion of [1,6- ^{13}C]glucose is the same as [1- ^{13}C]glucose, which has been documented in many studies (Haberg *et al.* 2001) (Fig. 1). [1,6- ^{13}C]glucose enters glycolysis forming [3- ^{13}C]pyruvate, which can be converted to [3- ^{13}C]lactate or be metabolized via the TCA cycle, giving rise to [4- ^{13}C] α -ketoglutarate in the first turn, which is converted to [4- ^{13}C]glutamate (GLU C4) in neurons and the corresponding [4- ^{13}C]glutamine (GLN C4) in astrocytes. GLN C3 and GLU C3 are formed in the next turn of the cycle, and because of randomization of the label in the symmetrical fumarate step some

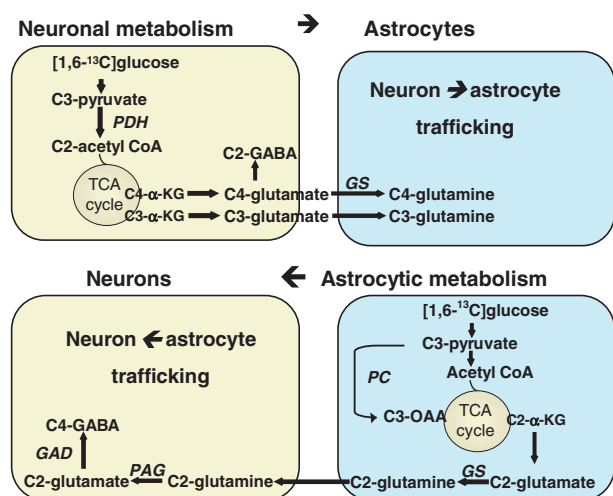


Fig. 1 Labeling and neuronal glial interactions from metabolism of [1,6-¹³C]glucose in rat brain. Details of the labeling are given in methods section. Abbreviations PDH, pyruvate dehydrogenase complex; PC, pyruvate carboxylase; GS, glutamine synthetase; PAG, phosphate-activated glutaminase.

labeling in GLU C2 and GLN C2 also occurs. GLU C4 can be converted directly to [2-¹³C]GABA (GABA C2) in neurons. [3-¹³C]Oxaloacetate formed in the first turn of the TCA cycle can be converted to [3-¹³C]aspartate (ASP C3).

In astrocytes, glucose metabolism occurs primarily via pyruvate carboxylase (PC) leading to formation of GLU C2 and GLN C2 in astrocytes. Labeling in GLU C3 can also occur from PC in astrocytes because of the subsequent equilibration of oxaloacetate with fumarate (Hassel *et al.* 1995). Via the activity of phosphate-activated glutaminase, GLU C2 can be synthesized in neurons from astrocytic GLN C2. These precursors can be converted to GABA C4 in neurons. Labeling in GABA C3 reflects both neuronal metabolism of GLU C3 as well as extensive metabolism of the glial-derived precursors.

Cycling ratios, which show the relative amount of labeling from the second turn/first turn of the TCA cycle, were calculated for glutamate glutamine and GABA using the amount in GLU C3/GLU C4, GLN C3/GLN C4 and GABA C3/GABA C2 (Qu *et al.* 2003).

Results

Swelling of tissue from the ipsi side of TBI brain was apparent at 5–6 h after injury. Hematomas were not usually seen. Incorporation of label from the metabolism of [1,6-¹³C]glucose into metabolites in the ipsi and contra sides of brain of TBI rats was compared at 15, 30 and 60 min after injection of the labeled ¹³C-precursor to determine the extent and pathways of glucose metabolism. These time points corresponded to 5 h 15 min, 5 h 30 min and 6 h after the TBI injury. Since sham-operated controls underwent craniotomy and skull closure in the ipsi side, metabolism was determined separately in both sides of brains from sham-operated rats.

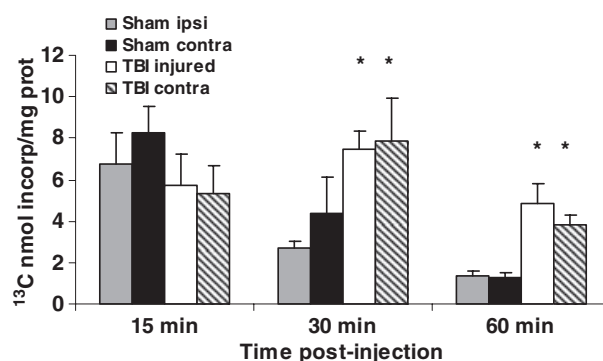


Fig. 2 Delayed increase in formation [3-¹³C]lactate (LAC C3) in ipsi and contra sides of brain from young TBI rats at 15, 30 and 60 min after i.p. injection of [1,6-¹³C]glucose. These time points represent 5 h 15 min, 5 h 30 min, and 6 h after the TBI injury. Spectra we obtained on PCA extracts of brain as described in methods. Values are mean \pm SE of data for nmol ¹³C incorporated/mg protein, from 6 TBI and 5 sham rats at each time point. Data were analyzed using one-way ANOVA. * p < 0.05 significant difference between ipsi and contra sides of TBI and sham brain.

Incorporation of ¹³C into lactate (LAC) after TBI

At 15 min after i.p. injection of [1,6-¹³C]glucose the concentration of unmetabolized brain glucose, and incorporation of label into LAC C3 was not different in TBI and sham rats suggesting that glucose delivery to the brain 5 h 15 min post-injury was essentially preserved (Fig. 2). Initial labeling of LAC C3 was comparable with values of 5.95 ± 0.47 reported for adult rat brain (Hassel *et al.* 1995; Qu *et al.* 2003). There was a significantly increased labeling of LAC C3 in both ipsi and contra sides of TBI rats at 30 min and 60 min (p < 0.05) compared with shams.

Incorporation of ¹³C into metabolites in 21–22 day old rat brain

In sham rat brain label from metabolism of [1,6-¹³C]glucose was incorporated into glutamate, glutamine, GABA, aspartate, and lactate. Incorporation of label in sham brain was highest at 15 min post-injection, consistent with reports that metabolism of ¹³C-glucose in brain peaks at 15 min after injection of the precursor in adult mice and rats (Hassel and Sonnewald 1995). The incorporation of label into GLU C4 and GLN C4 in the contra side of sham rat brain (essentially normal side; Fig 3a and b) was comparable to values reported for GLU C4 [4.88 (calculated from concentration and percent enrichment) and 4.18], and GLN C4 (0.99 and 1.0) in normal mouse and rat brain (Hassel *et al.* 1995; Qu *et al.* 2003). In the contra side of brain of sham rats labeling of GLU C3 was comparable with that of GLU C4 at 15 and 30 min. This is in contrast to reports of higher labeling in GLU C4 than GLU C3 in adult mouse and rat brain (Hassel *et al.* 1995; Qu *et al.* 2003). Labeling of GLU C2 and GLN C2 (Fig 4) via the PC pathway in astrocytes was higher in immature brain than

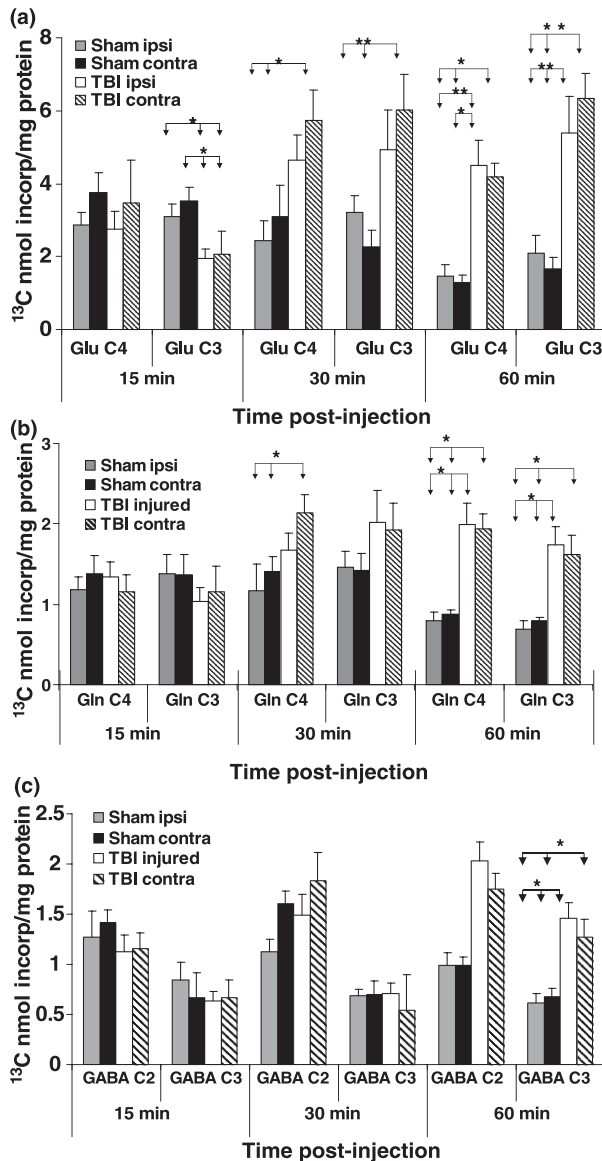


Fig. 3 Delayed increase in labeling of (a) [4- ^{13}C]glutamate (GLU C4) and [3- ^{13}C]glutamate (GLU C3), (b) [4- ^{13}C]glutamine (GLN C4) and [3- ^{13}C]glutamine (GLN C3) and (c) [2- ^{13}C]GABA (GABA C2) and [3- ^{13}C]GABA (GABA C3) in ipsi and contra sides of the brain from young TBI rats at 15, 30 and 60 min after i.p. injection of [1,6- ^{13}C]glucose. These time points represent 5 h 15 min, 5 h 30 min and 6 h post-TBI injury. Values are mean \pm SE of data for nmol ^{13}C incorporated/mg protein from six TBI and five sham rats at each time point. Data were analyzed using one-way ANOVA. * p < 0.05, ** p < 0.01 significant difference between ipsi and contra sides of TBI and sham brain as indicated by the horizontal lines over the bars.

values reported for adult brain (GLU C2, 2.96; GLN C2, 0.7 recalculated from (Hassel *et al.* 1995; Qu *et al.* 2003)). Indeed, the high labeling in GLU C3 noted above may also reflect increased formation from the PC pathway. Incorporation of label from glucose metabolism into GABA C2 was

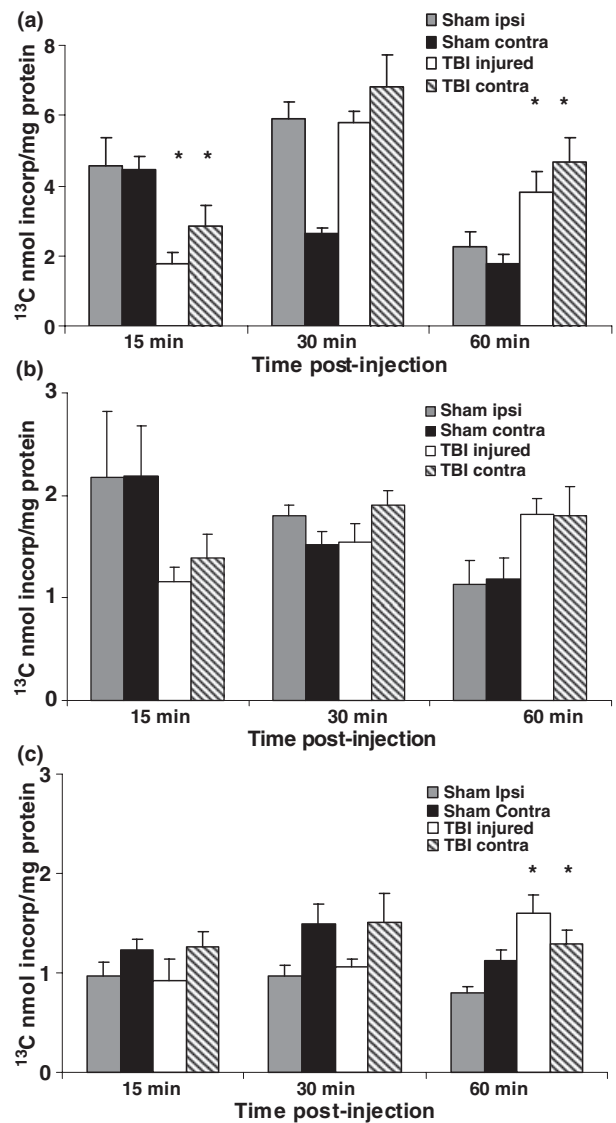


Fig. 4 Formation of (a) [2- ^{13}C]glutamate (GLU C2) and (b) [2- ^{13}C]glutamine (GLN C2) via the anaplerotic pyruvate carboxylase pathway, in ipsi and contra sides of the brain from young TBI rats at 15, 30 and 60 min after i.p. injection of [1,6- ^{13}C]glucose. These time points represent 5 h 15 min, 5 h 30 min and 6 h post TBI injury. Values are mean \pm SE of data for nmol ^{13}C incorporated/mg protein from 6 TBI and 5 sham rats at each time point. Data were analyzed using one-way ANOVA. * p < 0.05 significant difference between ipsi and contra sides of TBI and sham brain.

also comparable with values of 1.04 and 0.85 for adult mouse and rat brain, respectively (Hassel *et al.* 1995; Qu *et al.* 2003). Consistent with adult brain, labeling was higher in GABA C2 > GABA C4 > GABA C3 (Figs 3c and 4).

Incorporation of ^{13}C into GLU and GLN in brain after TBI

At 15 min after injection there was no difference in labeling of GLU C4 in the ipsi and contra sides of brain from TBI rats

and sham-operated controls as shown in Fig. 3a. However, labeling of GLU C3 was significantly decreased ($p < 0.05$) in both ipsi and contra sides of brain from TBI rats. The significantly decreased cycling ratio ($p < 0.03$, supporting information Table S1) for GLU at 15 min indicated the decreased TCA cycle activity in ipsi side of TBI brain. At 30 min, labeling of GLU C4 and C3 was significantly higher in both sides from TBI brain compared with shams. At 60 min (6 h after TBI), labeling of GLU C4 and C3 was increased more than two-fold in both sides of TBI brain compared with shams.

In sham and TBI rat brain 15 min after injection of [1,6- ^{13}C]glucose there were no differences in labeling of GLN C4 and GLN C3, which are formed from the GLU C4 and GLU C3 synthesized in neurons (Fig. 3b). At 30 min, labeling of GLN C4 was significantly increased only in the contra side, and there was a trend towards an increase in GLN C3 in both sides of TBI rat brain. At 60 min, labeling of GLN C4 and GLN C3 was increased more than 2-fold in both ipsi and contra sides of TBI brain compared with shams, in parallel with the increase in labeling of the precursors GLU C4 and GLU C3 formed in neurons.

Incorporation of ^{13}C into GABA after TBI

At 15 min and 30 min after injection of [1,6- ^{13}C]glucose there were no significant differences in labeling of GABA C2, which is formed directly from the GLU C4 derived from metabolism of glucose in neurons (Fig. 3c). However, at 60 min (6 h after TBI), there was significantly more labeling of GABA C2 in both ipsi and contra sides in TBI rats compared with shams, that paralleled the increased GLU C4. GABA C3 reflects labeling from both neuronally derived (GLU C3, GLN C3) and astrocytic precursors (GLU C2, GLN C2). At 15 and 30 min, labeling of GABA C3 was not different from shams; however, at 60 min there was significantly ($p < 0.05$) increased GABA C3 in both sides of brain from TBI rats.

Alterations in metabolism via the pyruvate carboxylase pathway

At 15 min and 30 min after injection of [1,6- ^{13}C]glucose there was a tendency towards decreased GLN C2 formed from metabolism of glucose via the pyruvate carboxylase (PC) pathway in astrocytes (Fig. 4). The corresponding GLU C2 formed from GLN C2 was significantly decreased at 15 min. At 60 min labeling of GLU C2 was significantly increased in both ipsi and contra side of TBI brains compared with shams. At 60 min, in ipsi TBI brain there was significantly increased GABA C4 (Fig. 4c), that is derived from the GLU C2 and GLN C2 formed via PC in astrocytes.

Incorporation of ^{13}C into aspartate

There was no significant difference in labeling of ASP C3 formed primarily from metabolism via the first turn of the

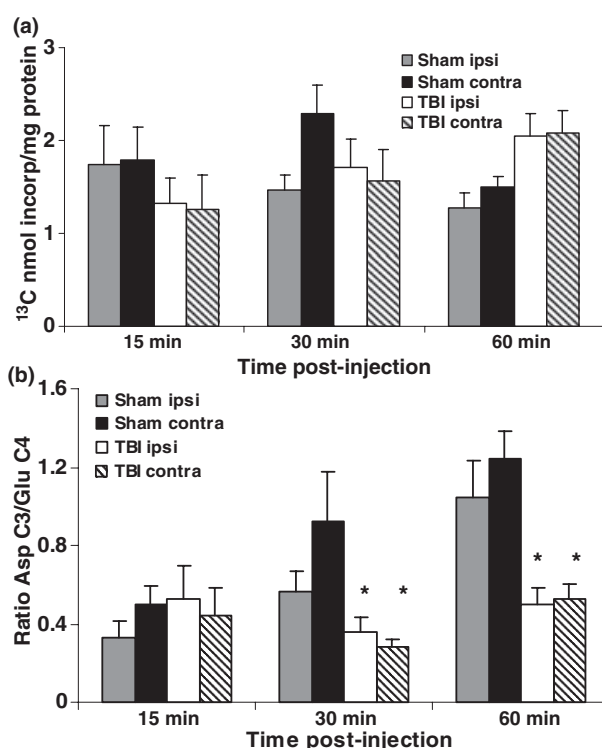


Fig. 5 TBI leads to decrease of Asp C3/GLU C4 in both ipsi and contra hemispheres of TBI rats. (a) There was no significant difference in ^{13}C incorporation into [3- ^{13}C]aspartate (ASP C3) between ipsi and contra sides of the brain from young TBI rats at 15, 30 and 60 min after i.p. injection of [1,6- ^{13}C]glucose. These time points represent 5 h 15 min, 5 h 30 min and 6 h post-TBI injury. Values are mean \pm SE of data for nmol ^{13}C incorporated/mg protein from 6 TBI and 5 sham rats at each time point. Data were analyzed using one-way ANOVA. (b) Ratio of Asp C3/GLU C4 decreased at 30 and 60 min post injection. Values are mean \pm SE of data from six TBI and five sham rats at each time point. Data were analyzed using one-way ANOVA. * $p < 0.05$ significant difference between ipsi and contra sides of TBI and sham brain.

TCA cycle in neurons (Fig. 5a). However, the ratio of ASP C3/GLU C4 was significantly decreased ($p < 0.05$) at 30 and 60 min in both ipsi and contra sides of TBI brain compared with shams (Fig. 5b).

Discussion

This is, to our knowledge, the first study that determined oxidative glucose metabolism in immature brain following TBI. Multiple animal and clinical studies have provided evidence of decreased oxidative metabolism accompanied by rise in lactate following TBI in adults (Siesjo 1984; Schroder *et al.* 1996). In those studies, the decreased oxidative metabolism was attributed to hypotension after the brain injury and decreased oxygen and glucose supply. However, in immature brain the hypoperfusion state is

short-lived and rapidly converts to prolonged hyperemia (Biagas *et al.* 1996). The results of the present study showing comparable levels of unmetabolized glucose and labeling in LAC C3 and GLU C4 in the ipsi and contra sides of TBI brain and shams at 15 min post injection suggests that there was adequate blood supply 5–6 h after moderate TBI in young rats.

A number of animal and clinical studies have implicated glutamate excitotoxicity in the pathophysiology of TBI (Bullock *et al.* 1998; Zhong *et al.* 2006). Although these studies using brain microdialysis probes demonstrated a surge in glutamate in the interstitial fluid within the first hours after TBI, the mechanism of this rise in glutamate is not completely clear. Up to 20-fold increase in glutamate and smaller alterations in aspartate within 15–30 min after TBI have been reported (Zhong *et al.* 2006). Cerebral ischemia, which can occur as a secondary injury following TBI, is also associated with glutamate rise and accompanying mitochondrial impairment (Globus *et al.* 1995; Takeuchi *et al.* 2008); however, the mechanisms underlying these changes are not fully understood. During energy failure glutamate can be released from neurons or from astrocytes by vesicular release or reversal of transporters (Montana *et al.* 2004; Haydon and Carmignoto 2006). Although we found no difference in total brain glutamate concentration at any of the time points after TBI (Supporting information Fig. S1), measurements of total amount in brain extracts cannot distinguish between intra- and extracellular glutamate. However, results of the present study showed an initial decrease in labeling of GLU C3, and decreased GLU cycling ratio, both of which are consistent with impaired oxidative metabolism. Our findings are consistent with Robertson *et al.* (Robertson *et al.* 2007) who found a lower ipsi/contra ratio of pyruvate dehydrogenase complex (PDHC) activity 4 h after TBI in immature rat brain compared with shams. They also reported elevated state 4 mitochondrial respiration rates and uncoupling of respiration indicating a loss of mitochondrial integrity at 1 h after TBI. Impairment of PDHC after TBI in immature rats may be prolonged for weeks (Kochanek *et al.* 2006). The decreased formation of GLU C2 in the present study is evidence of impaired astrocyte mitochondrial function. Several other recent studies attribute the metabolic changes in adult TBI to altered mitochondrial function (Lifshitz *et al.* 2003; Opii *et al.* 2007; Pandya *et al.* 2007). Since the results of Robertson *et al.* (Robertson *et al.* 2007) were obtained with the same immature TBI model used in the present study, it is likely that mitochondrial dysfunction is responsible for the observed delayed glucose metabolism.

Although labeling of ASP C3 was not changed, the increased incorporation of ^{13}C into GLU C4 led to a significantly decreased ratio of ASP C3/GLU C4 at 30 and 60 min after glucose injection (5½ h and 6 h after TBI). Aspartate is formed from the TCA cycle intermediate oxaloacetate, and labeling in ASP C3 reflects primarily

formation from neuronal metabolism (Oz *et al.* 2004). The decreased ASP/GLU ratio could be due, in part, to decreased activity of α -ketoglutarate dehydrogenase, an enzyme that is subject to damage by oxidative stress (Starkov *et al.* 2004). Intramitochondrial aspartate has a key role in the malate–aspartate shuttle, the most important mechanism for transferring reducing equivalents from the cytosol to the mitochondria in brain (McKenna *et al.* 2006). Oxidative metabolism of glutamate requires entry into the mitochondria via the aspartate–glutamate carrier, which occurs as an electrogenic exchange with intramitochondrial aspartate (McKenna *et al.* 2006; McKenna 2007; Satrustegui *et al.* 2007; Xu *et al.* 2007). Thus, an imbalance in ASP/GLU could limit entry of glutamate into mitochondria and subsequent conversion to α -ketoglutarate. The apparent hypermetabolism and increased GLU C4 and GLU C3 at 30 and 60 min in the present study may reflect, in part, accumulation of glutamate subsequent to impaired entry into mitochondria and/or subsequent oxidative metabolism that would normally provide energy when entry of pyruvate into the TCA cycle is impaired (McKenna *et al.* 2006; McKenna 2007).

Astrocytes have a number of crucial roles in brain including uptake and disposal of glutamate from the synaptic cleft, maintaining the pH and appropriate concentrations of K^+ and other ions in the interstitial milieu and providing neurons with energy substrates. Metabolism of glucose via the anaplerotic PC pathway in astrocytes fulfills the crucial role of carboxylating pyruvate to form oxaloacetate, thereby adding net carbons to the astrocytic TCA cycle. This serves to ultimately replenish the neuronal TCA cycle which is continuously depleted by synthesis of neurotransmitters (Oz *et al.* 2004). Thus, glutamine labeled in C2 position from metabolism via PC in astrocytes, is released, taken up by neurons and used as a substrate for synthesis of GLU C2 and GABA C4, and replenishing neuronal TCA cycle intermediates. Metabolism via PC is important in developing brain and hypomyelination and lactic acidosis are found in clinical PC deficiency (Higgins *et al.* 1994). Data from the present study show an initial decrease in labeling of GLU C2, followed by increased labeling at 5½–6 h after TBI in young rats. The significantly decreased GLU C2 may reflect energy impairment in astrocytes since label can be ‘trapped’ in GLU C2 if there is insufficient ATP for glutamine formation via glutamine synthetase in astrocytes. Since synthesis of GLN and GLU via the PC pathway in astrocytes provides important substrates for replenishing the neuronal TCA cycle, even transient impairment of this process and the associated glial \rightarrow neuronal trafficking could lead to ongoing alterations in metabolism after TBI in developing brain.

Our findings differ to a large extent with those reported by Bartnik *et al.* (Bartnik *et al.* 2005) who showed that 3.5 and 24 h after CCI TBI in adult rats, entry of [1,2- ^{13}C]glucose into the TCA cycle was not inhibited and oxidative

metabolism in glutamatergic neurons was maintained. The same investigators reported that labeling of GLU C3 and GLU C2 (via PC) were not altered, indicating intact function of the TCA cycle in both injured and contralateral mitochondria in adult rats brain at 3.5 and 24 h after TBI. In contrast, the results of the present study showed that 5–6 h post TBI there were profound alterations in both ipsi and contra sides of brain, with an initial decrease, and subsequently apparent increased glucose metabolism in neurons and via the PC pathway in astrocytes. There are several explanations for the differences between the present study and those reported in adult brain (Bartnik *et al.* 2005). First, in the present study, metabolism was determined in immature brain between 5 h 15 min and 6 h after TBI injury. Clinically, the first 6 h after severe pediatric TBI may represent a critical window for improving the outcome (Samant *et al.* 2008). Robertson *et al.* (Robertson *et al.* 2007), demonstrated that young animals have abnormal mitochondrial function early (< 4 h), thus it is important to determine oxidative metabolism that occurs via the TCA cycle after these early abnormal states of the mitochondria. Second, while direct evidence for alterations in mitochondrial energy metabolism after TBI in the developing brain is limited, pre-clinical investigations showed that alterations in cerebral glucose metabolism are characterized by early (first 30 min) increased cerebral metabolic rates and secondary metabolic depression 1–3 days after TBI (Thomas *et al.* 2000). The pattern of alterations in developing brain is different from adult rats, which show an acute increase in cerebral glucose metabolism for hours (Yoshino *et al.* 1991), followed by a subsequent metabolic depression and decreased cytochrome oxidase activity for 10 days after injury (Hovda *et al.* 1991). The increased labeling of GLN reported 3.5 h after injury by Bartnik *et al.* (Bartnik *et al.* 2005) is consistent with the hypermetabolism reported by Hovda *et al.* (Hovda *et al.* 1991) and suggests intact, overactive glial metabolism early after injury in adult brain. In contrast, results from the present study show that the metabolic alterations in immature brain differ from those in adult brain, particularly with respect to the impaired glutamine formation and metabolism via PC in astrocytes. The decreased labeling of GLU C3 and GLU C2 at 15 min after glucose administration demonstrates that significantly decreased neuronal and glial metabolism occurs early after TBI in young rats, followed by an apparent hypermetabolism in both neuronal and glial pathways at 5 ½ to 6 h after injury. However, an important conclusion drawn from the present study is that 5–6 h after moderate–severe TBI in young rats there is still considerable metabolism of glucose via the TCA cycle in brain. Our results suggest that the altered metabolism is due to a combination of early impairment in the TCA cycle, specifically at the level of PDHC and possibly at α -ketoglutarate dehydrogenase, coupled with impairment of malate–aspartate shuttle activity

and utilization of glutamate and glutamine that leads to accumulation of labeled metabolites. The temporal duration of these metabolic alterations in immature brain needs to be studied further. It is important to note that any long-lasting metabolic alterations superimposed on the intense biosynthetic requirements of the developing brain could contribute significantly to poor outcome.

Overall, the present studies show that severe alterations in cerebral metabolism occur from 5–6 h after TBI in immature brain. ^{13}C -NMR spectroscopy is a valuable tool for determining the extent of metabolic changes as well as for identifying the specific pathways altered in neuronal and glial metabolism. Further experiments determining the specific alterations in key enzymes after TBI are warranted and may lead to development of new targeted therapeutic interventions to improve both neuronal and glial cerebral oxidative metabolism after pediatric TBI.

Acknowledgements

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Supporting information

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

Fig. S1 Total amino acid concentration of brains of 21–22-day-old sham operated and TBI rats.

Table S1 Metabolite cycling ratios.

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Conflicts of interest

All authors declare no conflicts of interests.

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