Divergent Induction of Branched-Chain Aminotransferases and Phosphorylation of Branched Chain Keto-Acid Dehydrogenase Is a Potential Mechanism Coupling Branched-Chain Keto-Acid–Mediated-Astrocyte Activation to Branched-Chain Amino Acid Depletion-Mediated Cognitive Deficit after Traumatic Brain Injury

Guoqiang Xing,^{1,*} Ming Ren,^{2,*} and Ajay Verma³

Abstract

Deficient branched-chain amino acids (BCAAs) are implicated in cognitive dysfunction after traumatic brain injury (TBI). The mechanism remains unknown. BCAAs are catabolized by neuron-specific cytosolic and astrocyte-specific mitochondrial branched-chain aminotransferases (BCATc, BCATm) to generate glutamate and branched-chain keto-acids (BCKAs) that are metabolized by the mitochondrial branched-chain keto-acid dehydrogenase (BCKD) whose activity is regulated by its phosphorylation state. BCKD phosphorylation by BCKD kinase (BCKDK) inactivates BCKD and cause neurocognitive dysfunction, whereas dephosphorylation by specific phosphatase restores BCKD activity. Real-time polymerase chain reaction showed rapidly and significantly decreased BCATc messenger RNA (mRNA) levels, but significantly increased BCATm mRNA level post-CCI (controlled cortical impact). BCKD and BCKDK mRNA decreased significantly immediately after CCI-induced TBI (CCI) in the rat. Phosphorylated BCKD proteins (pBCKD) increased significantly in the ipsilateral-CCI hemisphere. Immunohistochemistry revealed significantly increased pBCKD proteins in ipsilateral astrocytes post-CCI. BCKD protein expression is higher in primarily cultured cortical neurons than in astrocytes, whereas pBCKD protein level is higher in astrocytes than in cortical neurons. Transforming growth factor beta treatment (10 μ g/mL for 48 h) significantly increased pBCKD protein expression in astrocytes, whereas glutamate treatment (25 μ M for 24 h) significantly decreased pBCKD protein in neurons. Because increased pBCKD would lead to increased BCKA accumulation, BCKA-mediated astrocyte activation, cell death, and cognitive dysfunction as found in maple syrup urine disease; thus, TBI may potentially induce cognitive deficit through diverting BCAA from glutamate production in neurons to BCKA production in astrocytes through the pBCKD-dependent mechanism.

Keywords: astrocyte; branched-chain amino acid; branched-chain aminotransferases; branched-chain keto-acid dehydrogenase; catabolism; traumatic brain injury

Introduction

TRAUMATIC BRAIN INJURY (TBI) is a leading cause of mortality and morbidity in children and young people under 44 years of age in the United States.^{1,2} TBI is a complex and diffuse injury with multi-modes of lesions, including focal cerebral contusion, parenchymal laceration, hemorrhages (subdural, epidural, and/or subarachnoid), diffuse axonal injury, diffuse hypoxic-ischemic

insults, cerebral swelling, increased intracranial pressure, and brain compression and herniation.^{3–6} Adjacent brain tissues that are not destroyed immediately after the primary injury often experience subacute injury or delayed death caused by secondarily generated autodestructive factors.^{7–11}

Secondary brain injury post-TBI is currently the main target for therapeutic intervention.^{12,13} Glutamate-mediated excitotoxicity and dysfunctional energy metabolism are intertwined major contributors

¹Department of Radiology and Imaging, Institute of Rehabilitation and Development of Brain Function, The Second Clinical Medical College of North Sichuan Medical College, Nanchong Central Hospital, Nanchong, China.

²Department of Neurology, Xuanwu Hospital, Capital Medical University, Beijing, China.

³United Neuroscience, Dublin, Ireland.

*These authors contributed equally to this work.

of secondary brain injury.^{14–17} Metabolic routes for bioenergetic fuel selection changes dramatically after brain insults.^{18,19}

There appears to be increased demand and/or deficiency of branched-chain amino acids (BCAAs) leucine, isoleucine, and valine for the recovery of TBI given that dietary supplement has neuro-protective effects in TBI.^{20,21} Although the underlying mechanism is unknown, BCAAs may play multiple roles in functional recovery post-TBI, including protein/enzyme synthesis through mammalian target of rapamycin (mTOR) activation, transamination reactions that buffer glutamate neurotoxicity, and serving as a secondary fuel source.^{22–25} BCAAs are essential amino acids that play important roles in protein synthesis, gluconeogenesis, and energy metabolism.²⁶ BCAAs are hydrophobic amino acids that play a critical role in determining the globular protein structures and the interaction of the transmembrane domains of membranous proteins with phospholipid bilayers.²⁷ BCAAs act as essential signals that control messenger RNA (mRNA) translation.²⁸ The BCAA/glutamate cycle, under physiological conditions, is responsible for maintaining a steady supply of glutamate, the major excitatory neurotransmitter for glutamatergic neurons for interneuronal communication and brain function and the substrate for the inhibitory neurotransmitter, γ -aminobutyric acid (GABA).^{29–35}

There are two steps of BCAA catabolism in the brain. The first reversible reaction of brain BCAA catabolism is catalyzed by neuron-specific cytosolic and astrocyte-specific mitochondrial branched-chain aminotransferases (BCATc, BCATm), transferring the α -amino acids of BCAA to α -ketoglutarate (α -KG) to form glutamate and respective branched-chain α -keto acids (BCKAs: α -ketoisocaproate [KIC], α -keto- β -methylvalerate [KMV], or α -ketoisovalerate [KIV]).^{36,37} The BCKA dehydrogenase (BCKD) complex catalyzes the irreversible oxidative decarboxylation of BCKAs to produce branched-chain acyl-CoA intermediates, which then follow separate catabolic pathways.²⁶

The glutamine-glutamate cycle is an astrocytic-neuronal pathway transferring precursors for neurotransmitters glutamate and GABA from astrocytes to neurons. The astrocyte-specific BCATm isoenzyme is responsible for initiating BCAA catabolism in astrocytes,³² transferring the α -amino groups of BCAAs to α -KG to produce glutamate. Glutamate is then converted to glutamine by glutamine synthetase in glia, and released to adjacent neurons where glutaminase converts glutamine back to glutamate, thus completing the glutamate/glutamine cycle. Glutamate dehydrogenase in neurons can also fix ammonia to α -KG to produce glutamate. Glutamate released from the neuron into synapses for neurotransmission is taken up by astrocytes to terminate the neurotransmission. Some of the glutamate is oxidized ($\sim 25\%$) during this process and must be replaced by anaplerotic mechanisms involving pyruvate carboxylase.³²

BCKAs are metabolized as bioenergetic fuels via the mitochondrial BCKD complex. Brain BCKD activity is dynamically regulated by the balance between its phosphorylation and dephosphorylation cycle. Phosphorylation of BCKD by BCKD kinase (BCKDK) inactivates BCKD, whereas dephosphorylation of BCKD by BCKD phosphatase restores its activity.²⁷ Although altered metabolic routes and selection of alternative bioenergetic fuel post-TBI have been reported,³⁸ so far the partition of BCAA between different metabolic pathways in different neural cell types post-TBI remains poorly understood.

Recent studies indicate a critical role of BCKAs in astrocytic morphology and function and, possibly, in the pathogenesis of the neurological dysfunction and brain damage found in patients with TBI and maple syrup urine disease (MSUD) given that BCKAs

accumulating in MSUD induce oxidative stress, s100b release from glial cells, morphological alterations and cell death in cultured astrocytes from rat cerebral cortex, and induce reorganization of phosphorylated glial fibrillary acidic protein (GFAP) in C6-glioma cells.^{39–42} BCKAs also induce apoptosis in neural cells without mitochondrial membrane depolarization or cytochrome c release.⁴³

In this study, we investigated the expression of BCATs, BCKD, and BCKDK mRNA and/or proteins in a rat model of controlled cortical impact (CCI), and in an *in vitro* model of cortical neuron/astrocytes insults. The results indicate that the divergent patterns of BCATs, BCKD, and BCKDK expression are potential biomarkers of altered BCAA/BCKA catabolism and clinical prognosis post-TBI. Figure 1 is presented to help the readers to understand the questions examined in this study.

Methods

Animals and controlled cortical impact-induced traumatic brain injury

Experimental procedure was conducted as recently reported.¹⁸ In brief, 48 male Sprague–Dawley rats (170–200 g; Taconic Farms, Germantown, NY) were randomly assigned to three different groups: 1) naïve control; 2) craniotomy (sham CCI); and 3) CCI. Four animals per group were sacrificed at 4 h, 24 h, 3 days, and 7 days post-CCI. For the CCI group, animals were initially anesthetized with 4% isoflurane in O₂ to a surgical plane in a vented anesthesia chamber connected to an isoflurane scrubber. Rats were mounted in the injury device, secured by ear bars and incisor bar, and spontaneously anesthetized with a 2% isoflurane in O₂ by blow-by nose cone connected to a charcoal canister passive isoflurane scavenger. Animal body temperature was maintained at the temperature range between 35°C and 37°C during the surgery by a warming lamp. An incision and a 10-mm craniotomy were made over the left primary and secondary motor cortex (bregma 3.70 mm, interaural 12.70 mm). After removal of the bone flap, cortical injury was induced with a CCI device,⁴⁴ with a penetration depth of 1.5 mm, velocity of 5 m/s, and duration of 50 msec. The bone flap was replaced and sealed with dental cement, and the scalp incision was closed with staples. Animals were observed after the surgery until fully recovered. All CCI and craniotomy animals recovered from isoflurane anesthesia and became mobile within 5 mins after isoflurane discontinuation. Although most CCI animals resumed some exploratory behavior 30 min after CCI, their motor activity (open-field and rotarod performance) was significantly reduced immediately after the surgery, but recovered to baseline within 3 days post-CCI (data not shown). No additional post-operative pain medicine was given to the animals with CCI or craniotomy. Animals were sacrificed and brain samples were collected at 4 h, 24 h, 3 days, and 7 days post-CCI ($N=4$ /group/time). Coronal sections of the hemisphere corresponding to the CCI epicenter and the corresponding contralateral area were separated, rapidly frozen in pre-cooled isopentane (on dry ice), and stored at -80°C for mRNA (rostral half section) and protein analysis (caudate half section). For immunohistochemistry, animals were perfused with 100 mL of normal saline and then 500 mL of 4% paraformaldehyde (PFA) as reported.⁴⁵ All animal procedures were approved by the institutional animal care and use committee of the Uniformed Services University of the Health Sciences.

Primary cortical neuronal and astrocyte cultures

Embryos from pregnant rats (E-18) were used for the culture of cortical neurons. Pregnant rats were euthanized with CO₂, and the embryos were removed immediately. Cortices were dissected carefully and cut into small pieces, and 0.5 mL of 2.5% trypsin in Hank's balanced salt solution (HBSS) was added and incubated

FIG. 1. Our proposed model depicting the coordination of BCAA metabolism between the putative neuron: astrocyte and microglia post-injury. In the case of TBI, BCAA metabolism may diminish in neurons through the reduced BCATc activity, and the predominant glutamate/glutamine cycle between neurons and astrocytes may shut down (causing cognitive deficit) in favor of an increased BCKA production in astrocytes/glia (by the BCKD kinase and phosphatase-regulated BCKD/pBCKD recycling mechanism) to accelerate the removal of damaged cells and debris and neurorepair through astroglia activation/phagocytosis. akt, protein kinase B; BCAA, branched-chain amino acid; BCATc, cytosolic-specific branched-chain aminotransferase; BCATm, astrocyte-specific mitochondrial branched-chain aminotransferase; BCKA, branched-chain keto-acid; BCKDK, branched-chain-keto-acid dehydrogenase kinase; Glu, Glutamate; Gln, glutamine; Gln-S, glutamine synthase; Glutms, glutaminase; GFAP, glial fibrillary acidic protein; IL, interleukin; α -KG, α -ketoglutarate; mTOR, mammalian target of rapamycin; NF κ B, nuclear factor kappa B; pBCKD, phosphorylated BCKD; ROS, reactive oxygen species; TBI, traumatic brain injury; TNF- α , tumor necrosis factor alpha. Color image is available online at www.liebertpub.com/neu

15 min at 37°C. Tissue was then washed three times with Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Tissue was triturated in three cycles with 20 triturations each using a plastic serological pipette to dissociate cells from tissue. Neurons were plated in poly-D-lysine-coated six-well plates or 10-cm dishes at a density of 6×10^6 in DMEM. Neurons were adjusted with DMEM to the density and then plated. The medium was changed into neurobasal medium with 5% B27 the following day and every 3 days thereafter. Cells were studied on day 11.

Primary culture of astrocytes was prepared from cortices of postnatal day 3 Sprague-Dawley rat pups. Pups were anesthetized by hypothermia and rapidly decapitated; the cranium was cut away and the brain was removed. Brain cortices were dissected and chopped to fine pieces in HBSS with penicillin/streptomycin, then trypsinized with 2.5% trypsin and 0.001% DNase for 25 min at 37°C. The trypsin reaction was inhibited with DMEM containing 10% FBS, 1% penicillin-streptomycin, and 50 μ g/mL of gentamicin. Tissue was triturated in three cycles with 20 triturations each using a plastic serological pipette to dissociate the cells from the tissue. Cells were passed through a cell strainer and then plated in DMEM containing 10% FBS, 1% penicillin-streptomycin, and 50 μ g/mL of gentamicin to a density of 40,000 cells/cm², counted with a hemocytometer. Media was changed every 3 days, and cells were allowed to grow to 95% confluence.

Because a high level of glutamate is a chemical stress to neurons, and transforming growth factor beta (TGF- β) is a stimulus for astrocytes,^{46,47} they were selected to challenge the appropriate neural cells in our *in vitro* study.

RNA extraction and real-time quantitative polymerase chain reaction

Frozen brain tissues were homogenized and total RNA was extracted using the RNeasy kit (Qiagen, Hilden, Germany). One

microgram of total RNA was reverse transcribed (RT) into first-strand complementary DNA (cDNA) in a total volume of 20 μ L using the RETROscript reverse transcriptase kit and oligo dT primers (Ambion, Austin, TX). Quantification of mRNA expression was performed in triplicate using a SYBR Green SuperMix (BioRad, Hercules, CA) in a two-step polymerase chain reaction (PCR) reaction procedure, performed on the MyiQ Single Color Real-Time PCR Detection System (BioRad). One microliter cDNA from the reverse transcription (RT) reaction was used as the template for quantitative real-time PCR (qPCR) reaction with a final PCR reaction volume of 25 μ L, with the 5' and 3' gene-specific PCR primer concentrations at 200 nM each. Real-time PCR primers were designed using Primer3 software (Whitehead Institute, MIT, Cambridge, MA) according to the coding sequences of each gene. After initial denaturation at 95°C for 3 min, 40 cycles of primer annealing and elongation were conducted at 60°C for 45 sec, followed by denaturation at 95°C for 10 sec. Fluorescent emission data were captured, and mRNA levels were quantified using the threshold cycle value (Ct). To compensate for variations in input RNA amounts and efficiency of RT, qPCR data for mRNA for each sample were normalized by reference to the data obtained for housekeeping beta-actin (GenBank #BC063166) determined from the same sample. Fold change in mRNA expression was calculated using the equation: fold change = $2^{-\Delta\Delta C_t}$, where ΔC_t = target gene Ct - house-keeping gene (β -actin) Ct, and $\Delta\Delta C_t$ is ΔC_t control - ΔC_t CCI-TBI (or fold change) = $2^{(\Delta C_t \text{ control} - \Delta C_t \text{ CCI-TBI})}$.

Immunohistochemistry

Perfusion-fixed (4% PFA) brains were cut into coronal sections of 50- μ m thickness with a cryostat, and sections were rinsed with 1% Triton X-100 in phosphate-buffered saline (PBS) for 30 min. Endogenous peroxidase and peroxidase-like activity were blocked by incubating the sections in 0.36% H₂O₂ for 30 min. For

immunostaining, brain sections were incubated at 4°C overnight with a goat polyclonal immunoglobulin G (IgG) against pBCKD (generous gift from Dr. C.J. Lynch, 1:500). After three 10-min washes with PBS, brain slices were incubated at 4°C overnight with biotin-conjugated anti-goat IgG (1:400; Santa Cruz Biotechnology, Santa Cruz, CA) and then horseradish peroxidase (HRP)-conjugated streptavidin (1:500; Upstate Biotechnology, Lake Placid, NY), respectively. Brain sections were rinsed three times in PBS for 10 min. Finally, sections were incubated with 0.05% 3,3'-diaminobenzidine and 2.5% nickel ammonium sulfate. The omission of the first antibody was used as the negative control.

Western blotting

Brain tissues samples from the cortex around the CCI territory, the corresponding area in the contralateral side, and sham-operated and normal rats were homogenized and sonicated for 40 sec in a lysis buffer as previously described.^{18,45} Protein concentrations were determined and aliquots of 10 µg of the total proteins were separated by electrophoresis on sodium dodecyl sulfate/polyacrylamide gels (10%). Proteins were subsequently transferred to a polyvinylidene difluoride membrane, which was then incubated with the monoclonal antibodies to BCKD (generous gift from C.J. Lynch, 1:500), pBCKD (generous gift from C.J. Lynch, 1:500), or BCKDK (generous gift

from R. Harris, 1:200) at 4°C overnight, respectively. Membranes were washed three times with PBS and then incubated overnight at 4°C with an HRP-conjugated secondary antibody. Immunoreactivity was detected by enhanced chemiluminescent autoradiography. Bands of western blotting were scanned and quantified with NIH IMAGE 1.61 software (version 1.61; NIH, Bethesda, MD).

Statistical analysis

Data of real-time qPCR of mRNA expression level and western blotting of protein levels were expressed as mean ± standard deviation. Effects of CCI/craniotomy treatment on gene/protein expression were analyzed using analysis of variance followed by post-hoc test. A difference with a *p* value less than 0.05 was considered statistically significant.

Results

BCATc, BCATm, BCKD, and BCKDK messenger RNA expression

Because BCAAs are first catabolized by BCATc and BCATm to generate glutamate and BCKAs that are further metabolized by the mitochondrial BCKD, a change in BCATs could reflect the

FIG. 2. qPCR data showed that compared to that of naïve controls, the mRNA expression of (A) BCATc, (B) BCATm, (C) BCKDα, and (D) BCKDK were significantly altered in the ipsilateral CCI, contralateral CCI, and craniotomy compared at 4 h, 24 h, 3 days, and 7 days after CCI-TBI in rats. N, naïve; S, sham (craniotomy); C, contralateral CCI; I, ipsilateral CCI; **p* < 0.05 versus naïve control; ***p* < 0.01 versus naïve control. BCATc, cytosolic-specific branched-chain aminotransferase; BCATm, astrocyte-specific mitochondrial branched-chain aminotransferase; BCKA, branched-chain keto-acid; BCKDα, branched-chain-keto-acid dehydrogenase alpha; BCKDK, branched-chain-keto-acid dehydrogenase kinase; CCI, controlled cortical impact; mRNA, messenger RNA; qPCR, quantitative polymerase chain reaction.

utilization of BCAA in neurons or astrocytes. In this study, a significant decrease of BCATc mRNA expression levels was found at 4 h, 24 h, and 7 days post-CCI in the ipsilateral CCI of the rat brain. In the contralateral CCI, BCATc mRNA levels decreased at 4 h, 24 h, and 3 days post-CCI and then returned to baseline at 7 days post-CCI. In the sham-operated TBI (craniotomy), BCATc mRNA decreased significantly at 24 h and 3 days post-CCI (Fig. 2A). In contrast to BCATc, BCATm mRNA expression increased significantly in the contralateral CCI 3 days post-TBI and in the ipsilateral CCI 7 days post-TBI (Fig. 2B). These results suggest an immediate and persistent reduction of BCAA catabolism in neurons, but a gradual increase of BCKA/BCAA catabolism in astrocytes after CCI-TBI.

Brain BCKD mRNA levels decreased significantly in the craniotomy, contralateral CCI, and ipsilateral CCI at 4 and 24 h post-TBI, and the decrease remained significant in the contralateral CCI 3 days post-CCI (Fig. 2C). BCKDK mRNA level decreased significantly in the craniotomy, contralateral, and ipsilateral CCI at 4 and 24 h post-TBI and in the contralateral CCI 3 days post-TBI (Fig. 2D). These results suggest that CCI-TBI caused rapid reduction in BCKD-mediated BCKA metabolism could be potentially regulated by a reduction or increase in BCKDK expression. A

reduction in BCKD expression or increase in BCKDK expression (thus increased phosphorylation of BCKD) could inhibit BCKD-mediated BCKA/BCAA catabolism.

Western blotting of BCKD and pBCKD

Western blotting showed no change in BCKD protein levels or treatment differences in BCKD protein levels between treatment groups throughout the observation (Fig. 2A–H). pBCKD protein levels, however, increased transiently in the craniotomy, contralateral CCI, and ipsilateral CCI at 1, 3, and 7 days post-CCI, resulting in greatly increased pBCKD:BCKD ratio in the injured brain (Fig. 3A–H). pBCKD protein levels returned to baseline 7 days post-CCI (Fig. 3G,H). As discussed above, a reduction in BCKD expression or increase in phosphorylation of BCKD could inhibit BCKD-mediated BCKA/BCAA catabolism. These results of increased pBCKD:BCKD ratio would suggest an overall reduction of brain BCKA/BCAA catabolism post-TBI.

Similar to that of BCKDK mRNA expression, BCKDK protein levels decreased significantly in the craniotomy, contralateral- and ipsilateral-CCI 4 h post-CCI (Fig. 4A,B), recovered in the ipsilateral-CCI 24 h post-CCI (Fig. 4C,D), and bounded to higher levels in

FIG. 3. Western blotting showed no changes in BCKD protein levels or treatment differences in BCKD protein levels between treatment groups throughout the observation (A, C, E, and G). pBCKD protein levels, however, increased transiently in the craniotomy, contralateral CCI, and ipsilateral CCI at 1, 3, and 7 days post-CCI (A, C, E, and G), resulting in greatly increased pBCKD:BCKD ratio in the injured brain (B, D, F, and H). pBCKD protein levels returned to baseline 7 days post-CCI (G, H). As discussed above, a reduction in BCKD expression or increase in phosphorylation of BCKD could inhibit BCKD-mediated BCKA/BCAA catabolism. These results of increased pBCKD:BCKD ratio would suggest an overall reduction of brain BCKA/BCAA catabolism post-TBI.

FIG. 4. CCI-induced changes in protein expression of BCKD kinase in rat brain tissue extract at 4 h, 24 h, 3 days, and 7 days post-CCI (A–H). Quantified results of western blotting of BCKDK protein levels expressed as means $\pm$ SEM of percentage of normal control, which were normalized to β -actin. N, naïve; Sham, craniotomy; Contra, contralateral CCI; Ipsi, ipsilateral CCI; * $p < 0.05$ versus naïve control; * $p < 0.01$ versus naïve control; # $p < 0.05$; ## $p < 0.01$ compared to sham-operated control; \$ $p < 0.05$ compared with the contralateral side. BCKDK, branched-chain-keto-acid dehydrogenase kinase; CCI, controlled cortical impact; SEM, standard error of the mean.

the craniotomy, contralateral-, and ipsilateral-CCI, with the ipsilateral CCI showing the greatest increase 72 h post-CCI (Fig. 4E,F). BCKDK protein levels decreased in the contralateral- and ipsilateral-CCI 7 days post-CCI, (Fig. 3G,H). The immediate decrease, but subsequent increase, in BCKDK protein expression would predict a biphasic phosphorylation/activity of BCKD post-TBI: immediate increase followed by a long-term decrease in BCKD-mediated BCKA/BCAA catabolism.

Immunohistochemistry of pBCKD

To determine the potential cellular location of phosphorylated BCKD, immunohistochemistry was conducted. Immunostaining revealed low levels of pBCKD protein expression in cortical neurons of naïve control and of contralateral CCI. However, a robust and predominant increase in pBCKD protein expression was found in the astrocytes of ipsilateral CCI 24 and 72 h post-CCI (Fig. 5A–G). These results suggest a shift of predominant inhibition of BCKD activity in neurons toward a predominant inhibition of BCKD activity in astrocytes after CCI-TBI.

Different expression and induction of BCKD and pBCKD proteins in cultured cortical neurons and astrocytes

To further confirm/determine whether there is a cell-type-specific expression/induction of BCKD and pBCKD proteins under baseline and challenged conditions, expression of BCKD and pBCKD proteins was determined in primarily cultured rat cortical neurons and astrocytes, using semiquantitative western blotting. Basal levels of BCKD protein expression were found significantly higher in neurons than in astrocytes, whereas the basal levels of pBCKD proteins were significantly higher in astrocytes than in neurons (Fig. 6A–D). However, after the neurons were challenged with 25 μ M of glutamate for 48 h, there was a marked decrease of pBCKD protein levels in cortical neurons, whereas protein levels of BCKD remained unchanged (Fig. 7A–D). In contrast, there was a marked increase in pBCKD protein levels in astrocytes after the challenge of 10 ng of TGF- β for 48 h with no significant change found in protein levels of BCKD (Fig. 8A–D). These results suggest a predominant BCKD-mediated BCKA metabolism in neurons and a more inhibited BCKA metabolism in astrocytes attributed to a

FIG. 5. Immunostaining of phosphorylation of BCKD in reactive astrocytes at 24 and 72 h post-CCI. (A) Immunostaining staining (darkened cells) shows that pBCKD is mainly present in astrocytes in the area around the traumatic lesion 72 h post-CCI. (B) Immunostaining shows that pBCKD expression (arrowed dark staining) is increased in the ipsilateral astrocyte compared to that in contralateral neurons 24 h post-CCI (C). Perivascular astrocytes staining of pBCKD in contralateral cortex versus ipsilateral cortex 72 h post-CCI (D and E). Immunostaining of pBCKD expressed in contralateral hippocampus (F) and ipsilateral hippocampus (G) 72 hours post-CCI. The results show increased pBCKD staining primarily in ipsilateral astrocytes. BCKD, branched-chain-keto-acid dehydrogenase; CCI, controlled cortical impact; pBCKD, phosphorylated BCKD. Color image is available online at www.liebertpub.com/neu

greater difference in pBCKD:BCKD ratio under baseline conditions. Metabolic challenges further tilted the unbalanced partition of BCKA metabolism between the neurons and astrocytes through reducing the phosphorylation status of BCKD in neurons (attributed to the reduced BCKDK expression by approximately 3-fold) and increasing the phosphorylation status of BCKD in astrocytes (attributed to the increased BCKDK expression by >2-fold).

Discussion

In this study, CCI-TBI induced divergent expression of BCATs and phosphorylation of BCKD, the key enzymes involved in BCAA catabolism in the rat brain. CCI-TBI caused rapid and persistent reduction in neuron-specific cytosol BCATc mRNA expression, but an increase in astrocyte-specific mitochondrial BCATm mRNA expression from days 3 to 7 after CCI-TBI. The divergent expression of BCATc and BCATm mRNA could indicate different BCAAs catabolism/utilization between neurons and astrocytes after CCI-TBI.³⁷ Specifically, the reduced neuron-specific BCATc expression could predict reduced BCAA catabolism in neurons and the increased astrocytes-specific BCATm mRNA expression could indicate enhanced BCAA catabolism in astrocytes after CCI-TBI.³⁷ It is known that the BCATm isoenzyme is primarily responsible for initiating mitochondrial BCAA catabolism in astrocytes.³²

BCATc and BCATm catalyze the first reversible reaction in the catabolic pathway of BCAA by transferring the α -amino acids of BCAA to α -KG to form glutamate and respective BCKAs (KIC, KMC, or KIV).^{36,37} The BCKA dehydrogenase complex (BCKD) catalyzes the irreversible oxidative decarboxylation of BCKAs to produce branched-chain acyl-CoA intermediates, which then follow separate catabolic pathways.²⁶

Cole and colleagues showed that BCATm is exclusively expressed in astrocytes in white matter and in neuropil whereas the branched-chain α -keto acid dehydrogenase enzyme complex (BCKD) is primarily expressed in neurons.⁴⁸ These results suggest that BCKAs produced in astrocytes must be exported to neurons in order to be irreversibly oxidatively decarboxylated by BCKD. Because BCKAs, but not BCAAs, can stimulate glutamate transamination to α -KG and thus the irreversible decarboxylation of glutamate to pyruvate and lactate, thereby promoting glutamate oxidative breakdown,³⁵ the increased BCATm expression in astrocytes and potentially increased BCKA availability to neurons could have buffered glutamate toxicity post-TBI. The BCKAs produced from BCAAs in astrocytes are transferred to the neurons where they can be transaminated back to BCAAs by BCATc. In this process, the BCKAs receive an α -amino group from glutamate, and the glutamate is converted to α -KG. Thus, BCATc has a dual role for adjusting glutamate and BCAA levels in neurons.³²

FIG. 6. pBCKD and BCKD protein expression in normalized primarily cultured cortical neurons and astrocytes as determined by western blotting (A). pBCKD protein level was higher in astrocytes than in neurons (expressed as means $\pm$ SEM of percentage of neuron; B); baseline BCKD protein level was lower in astrocytes than in neurons (expressed as % of neuron; C); and (D) the ratio of pBCKD:BCKD was significantly higher in astrocytes than in cortical neurons (% of neurons). * $p < 0.05$; ** $p < 0.01$, astrocytes versus neuron. BCKD, branched-chain-keto-acid dehydrogenase; pBCKD, phosphorylated BCKD; SEM, standard error of the mean.

Rate of oxidative decarboxylation of BCAA-derived BCKA is tightly controlled by the activity or phosphorylation state of BCKD, which is further controlled by the expression/activity of BCKDK that can phosphorylate and inhibit BCKD.⁴⁹ In this study, BCKD protein level remained constant throughout the study. However, levels of phosphorylated BCKD (pBCKD) proteins increased significantly after CCI that persisted for at least 3 days. This increased pBCKD expression would indicate a significantly reduced oxida-

tive decarboxylation of BCKA/BCAA (in astrocytes only; see discussion below) in the early phase of CCI. Interestingly, immunohistochemistry staining showed that the increased pBCKD expression was primarily present in astrocytes of the ipsilateral CCI (Fig. 7). Considering that brain BCKD expression was ubiquitously expressed in neurons, astrocytes, and glia,³⁷ this selective phosphorylation and thus inhibition of BCKD in astrocytes, but not in neurons, could prevent potential BCKD-mediated oxidative

FIG. 7. Glutamate (Gluta) treatment (25 μ M, 24 h) for 24 h significantly suppressed BCKD phosphorylation (pBCKD) levels and pBCKD:BCKD ratio in primarily cultured cortical neurons (A, B, and D). BCKD protein levels were not affected by the treatment (A, C, and D). * $p < 0.05$; ** $p < 0.01$ compared to normal control level. BCKD, branched-chain-keto-acid dehydrogenase.

FIG. 8. TGF-beta treatment (10 ng, 48 h) significantly enhanced BCKD phosphorylation and the pBCKD:BCKD protein ratio in primarily cultured astrocytes (**A**, **B**, and **D**). BCKD protein levels, however, were not affected by the TGF-beta treatment (**C**). Means $\pm$ SEM of the percentage of normal astrocyte; * p < 0.05 versus normal control level. BCKD, branched-chain-keto-acid dehydrogenase; pBCKD, phosphorylated BCKD; SEM, standard error of the mean; TGF, transforming growth factor.

decarboxylation of BCKA in astrocytes, but not in neurons, after brain injury. Indeed, *in vitro* neuroexcitatory insult study of the primary cortical neurons with glutamate toxicity markedly decreased the expression levels of pBCKD protein, whereas metabolically challenging astrocytes with TGF- β significantly increased pBCKD protein levels in astrocytes (Fig. 8). These results indicate that BCKD phosphorylation could be an intrinsic mechanism of shunting astrocytes-derived BCKA to neurons for oxidative carboxylation after injury or metabolic challenges. Further, BCKD kinase appears to have played a key role in controlling BCKD phosphorylation/activity and BCKA shunting in this regard.

Recent studies indicate a critical role of BCKAs in astrocytic function and possibly in the pathogenesis of the neurological dysfunction and brain damage found in patients with TBI and MSUD given that BCKAs accumulating in MSUD induce oxidative stress, s100b release from glial cells, morphological alterations and cell death in cultured astrocytes from rat cerebral cortex, and induce reorganization of phosphorylated GFAP in C6-glioma cells.^{39–42} BCKAs also induce apoptosis in neural cells without mitochondrial membrane depolarization or cytochrome c release.⁴³

BCKAs are powerful morphogens in astrocytes and glia. It has been reported that when exposed to various concentrations of BCKA, cultured neonate rat cortical astrocytes showed reorganization of actin and GFAP cytoskeleton and changed their usual astrocyte polygonal morphology to the appearance of fusiform or process-bearing cells.⁴⁰ Further, longer exposures to the BCKA elicited cell death at all concentrations studied, attaining massive death at the highest concentrations.⁴⁰ BCKA increased the phosphorylation of serine-containing proteins (including GFAP and intermediate filament [IF]) in C6-glioma cells.³⁹ These suggest that BCKA-induced GFAP and IF network reorganization could be involved in TBI-induced secondary injury.

In this study, we used the whole hemisphere sections that included the cerebral cortex, subcortical regions, and hippocampus for western blotting and qPCR analysis. Although this approach could not link any region-specific and/or cell-type-specific alter-

ations of the key enzymes involved in BCAA catabolism to specific biological functions, such as inflammatory and cognitive dysfunction post-TBI, our other data showed the co-occurrence of CCI-TBI-induced dysregulations of energy metabolism,⁵⁰ hyaluronic acid metabolism,⁵¹ inflammation, damaged vasculature, blood extravasation, and trapped erythrocytes/thrombosis in these regions post-TBI, as reflected in GFAP mRNA expression (the methodology is in a previous work⁵⁰) and direct visual inspection of the damaged brain tissue (Fig. 9) and neuronal apoptosis in ipsilateral hippocampal cornu amonis (CA)1–3 and dentate gyrus (DG) regions, as reflected in the Fluoro-Jade-B staining (Chemicon International, Inc., Temecula, CA; Fig. 10).

Thus, TBI may alter the balance of BCAA availability and catabolism between astrocytes and neurons by depleting the glutamate production in neurons by simultaneously inhibiting BCATc activity and enhancing BCKD activity (reduced pBCKD expression) and by increasing BCKA production in astrocytes by enhancing BCATm activity and inhibiting BCKD activity (increased pBCKD expression). This shunting of neuronal BCAA utilization toward a predominant BCKA production in astrocytes would likely promote BCKA-induced astrocyte morphological transformation and microglia activation required for tissue repair and rapid removal of damaged neural cells and tissue debris from the brain. If the predominant GFAP mRNA induction in the hippocampus and cortical and subcortical region is an indication of the BCKA-mediated astrocyte activation, but depletion of neuronal BCAA and increased neuronal apoptosis, as shown by the Jade-B labeling, that would predict cognitive deficits associated with hippocampal neuronal degeneration.

Indeed, a recent study showed that dietary BCAA supplements may reverse mild TBI-induced cognitive deficit in mice with several key findings²¹: 1) Dietary BCAA supplement restored TBI-induced BCAA deficits in the ipsilateral hippocampus; 2) dietary BCAA intervention completely restored injury-induced hippocampal-dependent cognitive impairment; 3) dietary BCAA amelioration of injury-induced cognitive impairment is directly

FIG. 9. *In situ* hybridization shows the progressive induction of GFAP mRNA expression in rat brain at 4 h (top line) and 24 h after CCI-TBI (bottom line). 1) Left column, high GFAP mRNA expression (black color) in the DG and hippocampal CA regions indicates astrocyte activation/proliferation; 2) right column, visual inspection of the frozen sections of the injured rat brain. CA, cornu amonis; CCI, controlled cortical impact; DG, dentate gyrus GFAP, glial fibrillary acidic protein; mRNA, messenger RNA; TBI, traumatic brain injury. Color image is available online at www.liebertpub.com/neu

correlated with restoration of net synaptic efficacy; 4) hippocampal area CA1 slices derived from TBI mice show decreased net synaptic efficacy; BCAA treatment had no effect on the excitability in any region in slices from sham animals; 5) glutamate production from leucine is significantly impaired after mild brain injury; 6)

BCATc protein decreased significantly in hippocampal area CA1. No change in BCATm protein level was found 7 days after TBI. BCKD protein was reduced in DG, but no change in CA. Glutamate dehydrogenase, which deaminates glutamate to form α -KG for entry into the tricarboxylic acid cycle, upregulated in the DG of TBI

FIG. 10. Hippocampal neuron plays an important role in learning and memory, and they are vulnerable to various types of insults. By using a Fluoro-Jade-B technique (Chemicon International, Inc., Temecula, CA), we detected neuronal apoptosis in CCI-TBI rats. Fluoro-JadeB is a polyanionic fluorescein derivative that sensitively and specifically binds to degenerating neurons. Our data show that CCI-TBI induced prominent cell death in the adjacent cortical, subcortical regions. Neuronal apoptosis is specially high in the hippocampal dentate gyrus (DG) and the CA3 regions. CA, cornu amonis; CCI, controlled cortical impact; TBI, traumatic brain injury. Color image is available online at www.liebertpub.com/neu

animals; 7) BCKAs did not restore network excitability; and 8) BCAA-mediated glutamate synthesis occurs primarily in the astrocyte and astrocytic glutamine is transported to neurons and converted to glutamate by glutaminase, which is solely expressed in neurons. That study also showed that BCAAs restore altered net synaptic efficacy by a pathway independent of this glutamate/glutamine cycle.

In this study, the BCKDK mRNA levels decreased rapidly at 4 h post-CCI, but bounced back at 3 and 7 days post-CCI (Fig. 1), whereas BCKDK protein expression level also showed a down-up-down swing pattern, that is, BCKDK protein reduced rapidly and significantly 4 h post-CCI, bounced back to significantly higher levels 3 days post CCI, and decreased again to a significantly low level after 7 days of CCI (Fig. 3). Although the mechanism controlling BCKDK expression remains to be fully understood, previous work by Harris and colleagues⁴⁹ has established that BCKDK is allosterically inhibited by α -KG (and to a lesser degree by other BCKAs), the alpha-keto acid produced by leucine transamination, whereas depletion of dietary protein increased BCKDK expression.⁴⁹ Brain BCKDK expression/activity is likely the result of a combined action of multiple hormonal and metabolic factors that needs further investigation. For example, thyroid hormone induces, whereas glucocorticoids and peroxisome proliferator-activated receptor alpha ligands repress, BCKDK expression.^{52,53}

Interestingly, thyroid hormone deficiency is found to be one of the most common permanent pituitary lesions in children with TBI.⁵⁴ Further, low thyroid hormone in patients with TBI, hydrocephalus, aneurysmal subarachnoid hemorrhage and brain tumor is associated with greater disease severity, complicated clinical course, poor prognosis, and greater mortality and handicap rate.⁵⁵ TBI in rats is also associated with a reduction in serum thyroxine (T4) and triiodothyronine (T3) and in brain expression of genes important for thyroid hormone action (MCT8 and Dio2 mRNA).⁵⁶ Thyroid hormone treatment activates neuroprotective pathways in both *in vivo* and *in vitro* models of neuronal injury,⁵⁶ although the underlying mechanism remains unknown.⁵⁵ These suggest that thyroid hormone-mediated BCKDK expression and thus BCKD phosphorylation and BCKA catabolism could play a key role in TBI prognosis.

Deficiency in BCAAs and in BCKCK/BCKD-mediated BCKA shunting has been implicated in TBI-induced dysregulated neurotransmission, neurotoxicity, energy metabolism, and cognitive function. Decreased blood BCAAs levels and their metabolites levels were reported in patients with severe TBI⁵⁷ and in those with mild TBI.⁵⁸ Dietary supplement of BCAAs improved the cognitive function in patients with severe TBI^{20,59} and in an animal model of TBI.²¹ Dietary supplement of BCAAs also increased mitochondrial biogenesis and enhanced neuroprotection against reactive oxygen species, physical endurance, and life span in mice.^{60,61}

Our study provides the direct evidence of a coordinated action of divergent BCAT expression and BCKD phosphorylation between neurons and astrocytes under different conditions in shunting BCKA/BCAA catabolism. Although the biological significance of this remains to be understood, it is probable that the rapidly decreased BCATc expression in neurons subsequent to insult would spare neuronal BCAAs from further metabolic consumption as the fuel, whereas the decreased phosphorylation of BCKD (thus increased BCKD activity) in neurons would favor the oxidative carboxylation of astrocyte-derived BCKA in neurons. The opposite situation could be true for BCKA catabolism in astrocytes, that is, after brain injury, the enhanced BCATm activity and BCKD phosphorylation (thus inhibited BCKD activity) in astrocytes could help to generate more BCKAs that can only be metabolized by

active BCKD in neurons to meet increased energy demands or for other vital functions, such as glutamate detoxification. It is known that BCKAs can promote glutamate oxidative breakdown.³⁰ The increased availability of BCKAs to neurons would not only help to shunt more glutamate toward oxidation for energy utilization in intact neurons, but perhaps also increased apoptosis and removal of injured neurons. A higher intracellular level of BCKAs may also help morphological transformation of astrocytes to better perform debris removal and tissue repair/neuroregeneration functions.

It is noted that the BCATm mRNA level was increased 7 days after TBI in our study, but no change in BCATm protein level was found in another study.²¹ Although we do not know the answer yet, the discrepancy between the two studies may reflect several possibilities: 1) Our CCI-TBI rat model was a more severe TBI model whereas their mouse lateral fluid percussion injury model was a mild-moderate TBI model; 2) we used the whole-hemisphere sections instead of their specific hippocampal CA1 and DG regions; 3) we measured mRNA level using qPCR, which is more quantitative than western blotting; 4) we used isoflurane as the only anesthesia whereas the other study used a combination of ketamine (2.6 mg/kg), xylazine (0.16 mg/kg), and isoflurane for anesthesia.

Isoflurane has been shown to have neuroprotective effects compared to other types of anesthesia of diazepam, fentanyl, ketamine, morphine, pentobarbital, and propofol in a rat CCI-TBI model.^{62,63} These studies showed that TBI rats treated with isoflurane had the best cognitive recovery and hippocampal neuronal survival, whereas rats treated with ketamine had the worst hippocampal neuronal death. Morphine or propofol produced the poorest motor function on immediate post-trauma days. Multiple mechanisms may underlie isoflurane-induced neuroprotection, including activation of glycine and GABA receptors, antagonism of ionic channels, and alteration of the function and activity of other cellular proteins.⁶⁴ Therefore, it is possible that isoflurane-mediated neuroprotection may have affected the potential differences between the CCI-TBI rats and sham-operated rats.

Although craniotomy was often regarded as sham-operated control of CCI, it has now been proven to be a kind of TBI.⁶⁵ It is not surprising to observe that the expression of BCATs, pBCKD, and BCKDK were also altered in the craniotomy-operated brain as in the CCI brain.

Conclusion

Our TBI model and cell injury studies of the key enzymes involved in BCAA catabolism suggests that, under physiological conditions, neurons (and less so astrocytes) appear to be the major sites for BCAA catabolism to generate glutamate for neurotransmission and BCKAs for oxidative decarboxylation by BCKD in neurons. Post-injury, however, BCAA catabolism is severely compromised in neurons primarily attributed to diminished BCATc expression, whereas BCKA accumulation may occur in astrocytes attributed to increased BCKDK expression and increased phosphorylation of BCKD that prevent BCKAs' oxidative decarboxylation in astrocytes, and increased BCATm expression that would promote BCAA conversion to BCKA. Further studies should determine whether the increased BCKA production in astrocytes is involved in oxidative stress, morphological transformation, and activation of astrocytes in post-injury neural apoptosis and regeneration.

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No competing financial interests exists.

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Address correspondence to:
 Ming Ren, MD, PhD
 Department of Neurology
 Xuanwu Hospital
 Capital Medical University
 Beijing
 China

E-mail: mren201017@hotmail.com

Guoqiang Xing, PhD
 Department of Radiology and Imaging
 Institute of Rehabilitation and Development of Brain Function
 The Second Clinical Medical College
 of North Sichuan Medical College
 Nanchong Central Hospital
 Nanchong 637000
 China

E-mail: gxing99@yahoo.com