Corrections

NEUROSCIENCE

The authors note that the author contributions footnote should be revised to reflect the following. Itzhak Nissim should be credited with designing the research and writing the paper. He should not be credited with performing the research. The corrected author contributions footnote appears below.

Author contributions: J.T.C., A.V., I.N. and A.S.C. designed research; J.T.C., C.M.M., S.K., and J.A.E. performed research; J.T.C. and A.S.C. analyzed data; and J.T.C., I.N., and A.S.C. wrote the paper.

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Dietary branched chain amino acids ameliorate injury-induced cognitive impairment

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Neurological dysfunction caused by traumatic brain injury results in profound changes in net synaptic efficacy, leading to impaired cognition. Because excitability is directly controlled by the balance of excitatory and inhibitory activity, underlying mechanisms causing these changes were investigated using lateral fluid percussion brain injury in mice. Although injury-induced shifts in net synaptic efficacy were not accompanied by changes in hippocampal glutamate and GABA levels, significant reductions were seen in the concentration of branched chain amino acids (BCAAs), which are key precursors to de novo glutamate synthesis. Dietary consumption of BCAAs restored hippocampal BCAA concentrations to normal, reversed injury-induced shifts in net synaptic efficacy, and led to reinstatement of cognitive performance after concussive brain injury. All brain-injured mice that consumed BCAAs demonstrated cognitive improvement with a simultaneous restoration in net synaptic efficacy. Posttraumatic changes in the expression of cytosolic branched chain aminotransferase, branched chain dehydrogenase, glutamate dehydrogenase, and glutamic acid decarboxylase support a perturbation of BCAA and neurotransmitter metabolism. Ex vivo application of BCAAs to hippocampal slices from injured animals restored posttraumatic regional shifts in net synaptic efficacy as measured by field excitatory postsynaptic potentials. These results suggest that dietary BCAA intervention could promote cognitive improvement by restoring hippocampal function after a traumatic brain injury.

branched chain amino acids | cognitive impairment | hippocampus | traumatic brain injury

Every 23 s in the United States an individual experiences a traumatic brain injury (TBI), with a fatality occurring once every 7 min (1). TBI causes serious neurological pathologies culminating in cognitive impairment (2). To date, treatment has typically targeted physical manifestations of the injury, e.g., alleviating increased intracranial pressure, without addressing the underlying mechanisms contributing to injury-associated impairments. Current research has begun to focus on fundamental changes in brain activity with the intention of developing efficacious therapies for mild to moderate TBI patients.

The limbic hippocampus, a brain structure implicated in higher learning and memory, is often damaged in TBI. Lateral fluid percussion injury (LFPI) reproducibly damages this structure, and causes cognitive impairment (3, 4) and regional shifts in network excitability in both area CA1 (decreased net synaptic efficacy) and dentate gyrus (increased net synaptic efficacy) (4–7).

At present, it is thought that astrocytes remove released glutamate from the synaptic cleft and amidade this glutamate to form glutamine for return to the neuron. Because this biological process, known as the glutamate-glutamine cycle, is inefficient, de novo synthesis of glutamate is required to maintain synaptic neurotransmitter pools. Branched chain amino acids (BCAAs) are key amino acids involved in de novo glutamate synthesis (Fig. S1), as ≈50% of brain glutamate contains BCAA-derived nitrogen (8). Previous studies have indicated an essential role for BCAA transamination in the synthesis of glutamate and subsequently GABA. Furthermore, de novo glutamate synthesis contributes ≈40% of the releasable synaptic glutamate. In addition to their conventional synaptic function, glutamate and GABA are integral in cellular metabolism and are major sources of TCA cycle intermediates. Although BCAA administration has demonstrated therapeutic benefits in treating taurine dyskinesia (9), chronic hepatic encephalopathy (10–12), and sepsis (13) the underlying mechanisms remain unknown. Moreover, despite their important role in glutamate and GABA synthesis, no study has mechanistically investigated the efficacy of dietary BCAAs in promoting recovery from TBI. Because alterations in excitation and inhibition directly affect hippocampal function, we hypothesized that dietary BCAAs could restore cognitive performance by ameliorating posttraumatic hippocampal impairment.

Results

For all studies, adult male C57BL/J6 mice received either LFPI or sham injury (4) and were allowed to recover for 7 days. The rationale for selecting 7 days after injury for extensive study is 2-fold: first, this time point is within the clinically relevant “therapeutic time window” (14); and second, results should not be complicated by acute transient alterations induced by brain injury that subside by 48 h. Furthermore, all comparisons were made between ipsilateral slices from sham and LFPI mice, as LFPI also effects the contralateral side (15). Because changes in excitatory and inhibitory transmitter distribution and concentration could explain previously demonstrated changes in net synaptic efficacy after LFPI (16), we quantified the concentration of amino acids in homogenates from the hippocampal region by HPLC. Surprisingly, brain injury did not alter the concentration of glutamate and GABA. This result may reflect an inability to isolate the synaptic pools of glutamate and GABA from the significantly larger metabolic pools that are insular with virtually no cross talk with synaptic pools (17). Interestingly, of the 18 amino acids quantified, the concentrations of only the three branched chain amino acids (BCAAs) were significantly altered after injury (Table 1). Seven days after LFPI, in the ipsilateral hippocampus, the BCAAs (valine, isoleucine, and leucine) were reduced by 50.8%, 21.1%, and 52.3%, respectively. As BCAAs are integral in glutamate and subsequent GABA synthesis, this
Table 1. Brain injury causes significant reduction in BCAA concentration in hippocampus

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Sham (nmol/mg protein)</th>
<th>FPI (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valine</td>
<td>16.33 ± 1.64</td>
<td>8.04 ± 1.35*</td>
</tr>
<tr>
<td>Leucine</td>
<td>15.03 ± 1.92</td>
<td>7.17 ± 1.48*</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>10.19 ± 0.51</td>
<td>8.04 ± 0.96*</td>
</tr>
<tr>
<td>Threonine</td>
<td>2.58 ± 1.30</td>
<td>3.91 ± 0.95</td>
</tr>
<tr>
<td>Citrulline</td>
<td>0.18 ± 0.12</td>
<td>0.41 ± 0.91</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.87 ± 0.26</td>
<td>1.07 ± 0.79</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.08 ± 0.01</td>
<td>0.05 ± 0.02</td>
</tr>
</tbody>
</table>

Seven days after a fluid percussion injury, the hippocampal formation was removed and analyzed via HPLC to determine concentrations of 18 amino acids. Of the amino acids analyzed, the concentrations of three (valine, leucine, and isoleucine) were significantly altered (P < 0.05) after injury. For both sham and injury, n = 6 hippocampi from six mice, and represent means ± SEM.

*Denotes significance at P < 0.05 confidence level compared with sham values.

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Depletion may be caused by alterations in the underlying mechanism that maintains synaptic pools of these neurotransmitters, thus ultimately impairing hippocampal function.

Having observed this significant alteration in the hippocampal amino acid profile, the feasibility of using dietary intervention to restore BCAA concentrations to normal was investigated. Therefore, 2 days after the injury procedure, both LFPI- and sham-injured mice received water that was either untreated (control) or contained a mixture of all three BCAs (100 μM leucine, isoleucine, and valine, denoted here as LIV). After consuming the treatment for 5 days (starting at 2 days after injury and continuing until 7 days after injury), BCAA levels in the ipsilateral hippocampus were evaluated. Treatment with BCAs in the drinking water restored BCAA concentration to levels not significantly different from sham animals (Fig. 1A). Furthermore, neither injury nor BCAA treatment after injury significantly altered the remaining tested amino acids, such as glutamine and alanine (Fig. 1B). Dietary intervention did not raise BCAA levels in sham LIV above those seen in the hippocampi from sham animals. Furthermore, consumption of BCAs did not alter the blood BCAA concentration within injury models, although injured mice alone and undergoing dietary intervention had lower circulating BCAA levels than did sham animals. Sham mice had a total BCAA (i.e., combined leucine, isoleucine, and valine) circulating concentration of 558.75 ± 25.76 nmol/mL, compared with 533.49 ± 37.37 nmol/mL in sham-LIV mice. In LFPI mice, the total BCAA concentration of 509.01 ± 39.44 was not significantly different (P > 0.05) from that seen in LFPI-LIV mice (483.05 ± 19.55). Body weight was not affected by either injury or dietary intervention. Mice were randomly selected from a group of mice having the same initial body weights and assigned to one of four combinations of injury status and dietary intervention. Seven days after injury, and 5 days after consumption of the dietary treatment, no significant difference (P > 0.05) was observed in the body weights of the mice (sham, 23.17 ± 0.43; sham-LIV, 22.65 ± 0.40; LFPI, 21.95 ± 0.48; LFPI-LIV, 22.7 ± 0.60 g).

Given that dietary intervention restored BCAA concentrations in the ipsilateral hippocampus, we hypothesized that injury-induced cognitive impairment would be diminished or ameliorated. Therefore, an additional set of LIV animals were prepared and subjected to hippocampal-dependent cognitive assessment with both anterograde and retrograde fear conditioning. Conditioned fear response (CFR) uses a single trial, is robust, and can distinguish problems in acquisition, consolidation, and retrieval that are difficult to differentiate with the Morris water maze. Furthermore, previous reports have documented complications using the MWM with mice (18). In addition, CFR has been used to investigate cognitive impairment in mice in a variety of paradigms, including traumatic brain injury (3), cannabinoi administration to transgenic mice (19), and the role of GABA receptors in cognition (20). In the retrograde task, animals were trained before injury. The aversive stimuli in sham animals resulted in a freezing percentage over 5 min, which is significantly (P < 0.05) higher than that observed in the LFPI mice (Fig. 1C, insets). Mice that consumed the LIV treatment for 5 days after injury did not display any reduction in cognitive performance after injury. In fact, the freezing percentage was no different (P > 0.05) from that observed in sham mice (Fig. 1C and insets). Similarly, when animals were tested in the anterograde task, the animals were trained after injury. There was a significant improvement in performance in LFPI animals that consumed the LIV treatment when compared with LFPI mice on water alone (P < 0.05, Fig. 1D and insets). Therefore, dietary intervention appears to completely restore injury-induced hippocampal-dependent cognitive impairment.

To substantiate that cognitive reinstatement was associated with restoration of net synaptic efficacy, brain slices were generated from the animals used for cognitive assessment and regional I/O curves were generated. Slices from LIV animals demonstrated a return to net synaptic efficacy levels not significantly different from those obtained in slices from sham animals in both area CA1 (Fig. 1E and insets) and DG (Fig. 1F and insets). Injury-induced regional alterations in net synaptic efficacy are caused in part by changes in inhibitory transmission, as previously demonstrated by Witgen et al. (4). That is, diminished miniature inhibitory synaptic currents (mIPSCS) in DG together with augmented mIPSC amplitude in area CA1 in slices from LFPI animals. No animal demonstrated improved cognition without a simultaneous restoration of net synaptic efficacy to levels seen in sham animals. To demonstrate that dietary intervention was BCAA specific, another set of injured animals were generated and placed on phenylalanine dietary intervention. Phenylalanine, like the BCAs, is a large neutral amino acid.

These animals showed no cognitive improvement or restoration of network excitability in either hippocampal subregion (Fig. 1 D–F and insets). This supports the contention that dietary amelioration of injury-induced cognitive impairment is directly correlated with restoration of net synaptic efficacy.

To begin to elucidate the potential mechanism(s) by which dietary BCAs restore cognitive function and net synaptic efficacy, hippocampal slices from both sham and LFPI mice were subjected to ex vivo application of BCAs, gas chromatography–mass spectroscopy (GC-MS) analysis, and Western blot quantification of key enzymes. Seven days after either LFPI or sham procedure, extracellular input/output (I/O) curves were generated in hippocampal slices from the two populations (Fig. 2). Initially a baseline I/O curve in normal artificial cerebrospinal fluid (aCSF) was conducted in the subregion of interest (area CA1 or DG) and then a second I/O curve generated after a 20-min superfusion in modified aCSF containing a combination of valine, isoleucine, and leucine (100 μM each). Plotting the linear slope of the extracellular field excitatory postsynaptic potential (EPPSP) at increasing stimuli substantiated that in slices derived from injured mice, area CA1 shows decreased net synaptic efficacy, whereas net synaptic efficacy in DG is significantly increased when compared with that in slices from sham animals (P < 0.05; Fig. 2 A and B) (4). However, a 20-min application of a mixture containing the three BCAs completely reversed these shifts in net synaptic efficacy, and restored network excitability to levels obtained in slices derived from sham animals (P > 0.05; Fig. 2 A and B) in both subregions. BCAA treatment had no effect (P > 0.05) on the excitability in any region in slices from sham animals. In addition, for each treatment, the fiber volley was quantified. Neither injury nor application of BCAs to...
Consumption of BCAAs restored cognitive performance by correcting injury-induced alterations in net synaptic efficacy. (A) Consumption of 100 mM each of leucine, isoleucine, and valine (LIV) for 5 days restored hippocampal BCAA levels. (B) Consumption of the LIV treatment significantly increased both alanine and glutamine concentrations \((P < 0.05)\). Both retrograde (G) and an anterograde (D) contextual fear conditioning tests demonstrated significant cognitive impairment \((P < 0.05)\) after LFPI. This was completely reversed by the consumption of the LIV treatment. In contrast, phenylalanine consumption \((100 \text{ mM}; \text{LFPI-Phe})\) had no effect on cognitive performance \((P > 0.05)\). Bars indicate the average percentage of “fear behaviors” per 5 min. Insets show a representative sample of the percentage of “fear behaviors” on a per-minute basis. In area CA1 (E) and dentate gyrus (F), consumption of the LIV treatment restored net synaptic efficacy \((P < 0.05)\), whereas consumption of phenylalanine \((100 \text{ mM})\) had no effect \((P > 0.05)\). Insets depict representative waveforms collected at maximal stimulation. Scale bar, 0.5 mV/10 ms. (A and B) \(n = 6\) samples. (C) Sham, LFPI and LFPI-LIV had \(n = 12, 11,\) and 9, respectively. (D) Sham, LFPI, LFPI-LIV, and LFPI-Phe had \(n = 14, 11, 10,\) and 10, respectively. (E and F) \(n = 8\) samples. For all panels, data are means \(\pm\) SEM. *Means differ significantly from sham values \((P < 0.05)\). †Means of data collected from LFPI-Phe mice differ significantly from sham values \((P < 0.05)\).
Branched chain amino acids restore net synaptic efficacy. In both area CA1 (A) and the dentate gyrus (B), input/output curves demonstrate that FPI significantly alters net synaptic efficacy. BCAA administration (100 μM each of valine, isoleucine, and leucine) completely restores net synaptic efficacy. Insets depict representative waveforms at maximal stimuli. Scale bar, 0.5 mV/10 ms. In area CA1 (C) and dentate gyrus (D), individual application of leucine, isoleucine, and valine (100 μM) completely restored net synaptic efficacy when compared with either slices from sham or LFPI animals alone. For each panel, n = 8, and data are means ± SEM. *Significance when compared with sham values (P < 0.05).

F and G). However, only AAT2 was significantly reduced (P < 0.05) in area CA1 (Fig. 3G). The reduction in these enzymes, coupled with the decreased conversion of 15N-leucine to 15N-glutamate, likely accounts for the significant decrease in 15N-aspartate synthesis after injury.

Discussion

The key finding in this study is that dietary delivery of BCAs ameliorates hippocampal-dependent cognitive dysfunction together with a restoration of net synaptic efficacy after concussive brain injury. Specifically, in every animal, cognitive improvement occurred only in conjunction with restored net synaptic efficacy, i.e., administration of BCAs to severely brain damaged patients has previously shown moderately beneficial effects demonstrated by a slight improvement assessed with both the Glasgow Coma Scale and the Disability Rating Scale (cognitive ability for "feeding," "toileting" and "grooming" reflecting levels of disability). Unfortunately, no specific cognitive testing or underlying mechanism was pursued in these studies (10–12, 21, 22).

Traumatic brain injury causes cognitive impairment and altered ipsilateral net synaptic efficacy. Specifically, an overall decrease in area CA1 net synaptic efficacy and an overall increase in DG net synaptic efficacy has been previously observed (4, 16) and replicated here. We hypothesized that injury-induced alterations in concentrations of the excitatory neurotransmitter glutamate and the inhibitory neurotransmitter GABA contribute to changes in net synaptic efficacy. However, HPLC analysis surprisingly showed no alterations in the overall concentration of these amino acids in hippocampal homogenates. Instead, the concentrations of BCAs showed a significant reduction in the ipsilateral, but not the contralateral, hippocampus of injured animals. This is probably due to the intrinsic role of BCAs in maintaining glutamate and thus GABA stores, which likely are under a heightened posttraumatic demand for use by distinct metabolic pathways (e.g., neurotransmission, energy production). Neurons contain multiple "pools" of both glutamate and GABA. The largest pool is the "metabolic pool," which is approximately 1,000-fold higher than the synaptic pool, and is thought to be insular from the synaptic pool (23). However, because of greatly increased energy demand (24–26), released glutamate could be catabolized in the astrocyte for entry into the TCA cycle. Alternatively, glutamate that is returned to the neuron could be deaminated to form glutamine that is destined for energy production instead of simply replenishing the synaptic pool of glutamate. We speculate that an increased demand for de novo glutamate synthesis toward maintaining the synaptic glutamate pool significantly alters the normal ratio (17:1) of BCAA transamination:BCAA oxidation and leads to a decline in hippocampal BCAA concentration after injury. This would cause irreversible catabolism of BCAs as a byproduct of increasing demand for glutamate synthesis, thus accounting for their decreased concentration after injury.

The complete restoration of net synaptic efficacy after both the ex vivo and in vivo delivery of BCAs suggests that BCAA supplementation may relieve a local metabolic stress in the hippocampus and improve synthesis, buffering, and maintenance of the synaptic glutamate and GABA pool. The mechanism underlying...
the observed changes in the BCAA metabolic enzyme levels and the reduced $^{15}$N-leucine labeling rates remains to be fully elucidated. However, these novel injury-associated observations are likely to contribute to the impaired capacity for buffering synaptic glutamate and GABA pools. The transamination of $\alpha$-ketoglutarate to glutamate using BCAA-derived nitrogen occurs despite the diminution of BCATc expression. However, as BCATc is found primarily in the synaptic terminals (27), and it is not the rate-limiting step in BCAA metabolism, it is ideally positioned to use exogenous BCAAs for the synthesis of glutamate.

Ex vivo BCAA supplementation restored net synaptic efficacy even though glutaminase was inhibited. This suggests that the restorative effect is likely to be independent of the glutamate:glutamine cycle. However, as BCATc is found primarily in the synaptic terminals (27), and it is not the rate-limiting step in BCAA metabolism, it is ideally positioned to use exogenous BCAAs for the synthesis of glutamate.

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We propose that concussive brain injury induced regional molecular alterations in BCAA metabolic pathways in turn stress the homeostasis of regional excitatory and inhibitory neurotransmitter pools to produce opposing regional shifts in network excitability in the hippocampus. Specifically, these alterations cause a significant impairment in the hippocampal de novo synthesis capacity for glutamate and GABA using BCAA-derived amino groups. Furthermore, the restoration of leucine, isoleucine, and valine in the ipsilateral injured hippocampus to levels seen in sham animals can be achieved through dietary intervention. This BCAA refurbishing may be sufficient to restore glutamate and GABA pools, to reset net synaptic efficacy, and to eradicate injury-induced cognitive impairment. Indeed, in no

Fig. 3. Brain injury leads to diminished BCAA transamination and alterations in BCAA and glutamate metabolizing enzymes. (A) After incubation with $^{15}$N-leucine (100 μM), LFPI significantly reduced the production of $^{15}$N-labeled GABA, aspartate, and glutamate. n = 6, with bars representing means ± SEM. (B) Densitometry analysis of the expression of cytosolic branched chain aminotransferase (BCATc; 49 kDa) reveals a significant reduction ($P < 0.05$) in area CA1 of injured animals. (C) Branched chain keto acid dehydrogenase (BCKD; 51 kDa) expression significantly decreased ($P < 0.05$) in the dentate gyrus of injured animals. (D) Glutamate dehydrogenase (GDH; 61 kDa) expression significantly increased ($P < 0.05$) in the dentate gyrus of injured mice. (E) Glutamic acid decarboxylase (GAD; 65 kDa) was significantly reduced ($P < 0.05$) in area CA1. (F) Aspartate aminotransferase 1 (AAT1; 46 kDa) was significantly reduced ($P < 0.05$) in the dentate gyrus. (G) Expression of aspartate aminotransferase 2 (AAT2; 47 kDa) was significantly reduced ($P < 0.05$) in both the dentate gyrus and area CA1 from injured mice. For each Western blot, n = 4 samples, with densitometry representing means ± SEM *Denotes significance at the $P < 0.05$ confidence level when compared to sham values.
animal was cognitive improvement seen without an accompanying restoration in net synaptic efficacy. Although these results show tremendous promise for clinical applications, further study needs to be conducted to determine the relative permanence of the changes mediated by BCAA application. In addition, it will be important to determine the effect of chronic BCAA treatment on improving delayed effects of TBI, which can take months or years to manifest themselves. Presently, no effective interventions are available to reverse or diminish the significant consequences of brain injury, i.e., cognitive impairment. This study demonstrates that dietary intervention with branched chain amino acids ameliorate cognitive impairment together with restoring net synaptic efficacy after a traumatic brain injury.

Methods

Animals. All experiments were performed on 5–7 week old, 20–25 g, C57BL/J6 mice (Jackson Laboratory). All procedures were approved by the Children’s Hospital of Philadelphia Institution for Animal Care and Use Committee in accordance with international guidelines on the ethical use of animals. The fluid percussion brain injury (FPI) protocol was carried out over 2 days as detailed in SI Methods.

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