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Effects of Branched-Chain Amino Acid Supplementation on Spontaneous Seizures and Neuronal Viability in a Model of Mesial Temporal Lobe Epilepsy

Shaun E. Gruenbaum, MD, PhD[1], **Roni Dhaher, PhD**[2], **Amedeo Rapuano, BS**[3], **Hitten P. Zaveri, PhD**[4], **Amber Tang**[2], **Nihal de Lanerolle, PhD**[3], and **Tore Eid, MD, PhD**[2,3]

[1]Department of Anesthesiology, Yale School of Medicine, P.O. Box 208051, New Haven, CT 06520, United States

[2]Department of Laboratory Medicine, P.O. Box 208035, Yale School of Medicine, New Haven, CT 06520, United States

[3]Department of Neurosurgery, Yale School of Medicine, P.O. Box 208082, New Haven, CT 06520, United States

[4]Department of Neurology, P.O. Box 208018, Yale University School of Medicine, New Haven, CT 06520, United States

Abstract

Background—The essential branched-chain amino acids (BCAAs) leucine, isoleucine and valine have recently emerged as a potential novel treatment for medically-refractory epilepsy. Blood-derived BCAAs can readily enter the brain, where they contribute to glutamate biosynthesis and may either suppress or trigger acute seizures. However, the effects of BCAAs on chronic (i.e. spontaneous recurrent) seizures and epilepsy-associated neuron loss are incompletely understood.

Methods—Sixteen rats with mesial temporal lobe epilepsy (MTLE) were randomized into two groups that could drink, *ad libitum*, either a 4% solution of BCAAs in water (n = 8) or pure water (n = 8). The frequency and relative percent of convulsive and non-convulsive spontaneous seizures were monitored for a period of 21 days, and the brains were then harvested for immunohistochemical analysis.

Results—Although the frequency of convulsive and non-convulsive spontaneous recurrent seizures over a 3-week drinking/monitoring period were not different between the groups, there were differences in the relative percent of convulsive seizures in the first and third week of treatment. Moreover, the BCAA-treated rats had over 25% fewer neurons in the dentate hilus of the hippocampus compared with water-treated controls.

Conclusions—Acute BCAA supplementation reduces seizure propagation, while chronic oral supplementation with BCAAs worsens seizure propagation and causes neuron loss in rodents with MTLE. These findings raise the question of whether such supplementation has a similar effect in humans.

Corresponding Author: Tore Eid, Department of Laboratory Medicine, 330 Cedar St., P.O. Box 208035, Yale School of Medicine, New Haven, CT 06520, United States, tore.eid@yale.edu.

Keywords

branched-chain amino acids; glutamate; glutamine; glutamine synthetase; isoleucine; leucine; methionine sulfoximine; temporal lobe epilepsy; valine

Introduction

Epilepsy is a chronic and debilitating neurological disorder that affects approximately 1% of the population.[1] Up to 40% of patients with epilepsy continue to experience seizures despite optimal medical management, or suffer intolerable side effects to currently available antiepileptic agents.[2] Mesial temporal lobe epilepsy (MTLE), the most common form of focal epilepsy and most common intractable seizure disorder, is characterized by spontaneous recurrent seizures and an increased risk of comorbid conditions including anxiety, depression, cognitive impairment, and sudden death.[3–5] Patients with MTLE are often referred for surgical resection of the seizure focus. Such surgery necessitates coordinated medical care with a multidisciplinary team that includes neurosurgeons, neurointensivists, neurologists, and neuroanesthesiologists. Although surgery is effective in reducing seizures in approximately 80% of patients for up to 2 years,[6] up to 11% of these patients experience significant complications including neurocognitive and psychiatric sequelae, visual field deficits, infection, and delayed seizure recurrence.[7] More effective treatments with favorable side effect profiles are therefore urgently needed to reduce the frequency of seizures in patients with MTLE.

In recent years, the essential branched-chain amino acids (BCAAs) leucine, isoleucine, and valine have emerged as a potential novel treatment for medically-refractory epilepsy. Although several laboratory studies have demonstrated anticonvulsant effects of BCAAs,[8–13] nearly all investigations of BCAAs in epilepsy have been limited to acute seizures and seizure threshold after a single dose of the amino acids. Notably, no long-term studies have been carried out in MTLE. It is further unknown whether chronic, high-dose BCAA administration to patients with epilepsy can damage the brain. The latter is a concern because high levels of BCAAs and associated metabolites have been shown to cause neurodegeneration *in vitro*.[14]

Hence, the goal of the present study was to investigate the effects of chronic BCAA supplementation on recurrent seizures and neuron loss in a rodent model of MTLE. The model is created by inhibiting the enzyme glutamine synthetase (GS) in the right hippocampal formation in rats using a chronic intrahippocampal infusion of methionine sulfoximine (MSO).[15] Because GS is necessary for efficient glutamate metabolism and clearance in the brain, we hypothesized that chronic, oral supplementation with all three BCAAs would increase the frequency and tissue propagation of recurrent seizures and aggravate hippocampal neuron loss in the MSO model of MTLE.

Materials and Methods

Chemicals and animals

All chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise noted. A 4% BCAA solution was dissolved in water [1.38 g isoleucine, 1.38 g leucine, 1.24 g valine in 1000 mL water as described previously[12]] Male Sprague Dawley rats were obtained from Harlan (Indianapolis, IN.). Rats were individually housed and maintained in a temperature-controlled colony room (21°C–23°C) on a 12 h light–dark cycle. Rats were allowed free access to food (Teklad Global 18% Protein Rodent Diet, which contains 18 mcg/g leucine, 8 mcg/g isoleucine, and 9 mcg/g valine) and water, and were given at least 1 week of acclimation prior to the start of the experiment. All procedures were approved by the Institutional Animal Care and Use Committee at Yale University and were conducted in accordance with current guidelines.

Experimental design

The experimental protocol is summarized in Fig. 1. Sixteen rats (weight 455 – 615 g, ~16 weeks in age) were randomly separated into two groups: one group had unrestricted access to a 4% BCAA solution dissolved in water (n=8) and the other group to a plain water solution (n=8) *ad libitum*. Following 10 days of consumption, rats were implanted with the MSO pump, and the first set of venous blood samples were drawn. After surgery, rats were monitored for both convulsive and nonconvulsive spontaneous seizures over a period of 21 days. For the duration of the EEG recording period, MSO was continuously infused and rats were maintained on the BCAA or plain water solution. Water bottles were weighed daily to confirm consumption. After this monitoring period, rats were then perfused, during which time the second set of venous blood samples were drawn. The brains were then harvested for immunohistochemical analysis.

Surgery

Rats were anesthetized with 0.5 – 2% Isoflurane (Baxter, Deerfield, IL) in O_2 and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). A 30-gauge stainless steel cannula, with a length of 8.1 mm, attached to a plastic pedestal (Plastics One, Roanoke, VA) was stereotaxically lowered into the right dentate gyrus using the following coordinates, with bregma marking zero for the mediolateral (ML) and anteroposterior (AP) directions, and the top of the skull marking zero for the dorsoventral (DV) direction: AP = −2.6 mm, ML = 4.6 mm, DV = −8.1 mm. The cannula was lowered into the brain till the pedestal touched the skull. The pedestal was then glued to the skull with medical grade cyanoacrylate (Vetbond Tissue Adhesive, Butler Animal Health Company, Chicago, IL). The cannula and pedestal were connected via plastic tubing to a subcutaneously implanted Alzet osmotic pump (Model 2004, Durect Corp., Cupertino, CA.) which delivers a continuous flow of 0.25 μL/h for ~28 days. The pumps were filled with MSO (2.5 mg/mL; dissolved in Dulbecco's phosphate buffered saline (PBS) to achieve a delivery of 0.625 μg of MSO per h. This rate was used because previous studies demonstrated ipsilateral irreversible inhibition of GS without affecting glutathione levels.[15] Following placement of the cannula and pedestal, four stainless steel epidural screw electrodes (Plastics One, Roanoke, VA) were implanted to record cortical electroencephalogram (EEG) activity. Two stainless-steel screw electrodes

(one in each hemisphere) were positioned in the epidural space over the cortex. On the left side (contralateral to the injection), the electrode was placed in the skull above the dorsal anterior hippocampal formation (AP = −2.0 mm, ML = −2.5 mm). On the right side, the electrode was placed in the skull above the somatosensory cortex (AP = −6.25 mm, ML = 5.4 mm). One screw electrode was positioned in the epidural space (AP= 8.5 mm, ML= −2.2 mm) to serve as the reference. A fourth electrode, which was positioned in the skull above the cerebellum (AP = −10.0 mm, ML = 1.5 mm) served as the ground. Three additional stainless-steel screws were inserted into the skull to reduce the risk of head cap detachment. The female socket contacts on the ends of each electrode were inserted into a plastic pedestal (Plastics One), and the entire implant was secured by UV light cured acrylated urethane adhesive (Loctite 3106 Light Cure Adhesive, Henkel Corp., Rocky Hill, CT) to form a head cap.

Blood BCAA levels

Venous blood samples were collected in tubes containing lithium heparin anticoagulant at 2 time points during the experiment: during surgical implantation of the MSO pump at day 10 (drawn from the site of burr holes) and when the rats were sacrificed at 31 days (drawn from left ventricle of the heart). The blood was centrifuged at 12,000 g for 10 minutes at 4°C. The plasma was collected and stored at −80°C for later analysis. Concentrations of valine, leucine, and isoleucine were determined using the Ez:faast Free Amino Acid analysis kit (Phenomenex, Torrence, CA) followed by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC/MS/MS) using a Waters Acquity, Xevo TQS mass spectrometer (Waters, Milford, MA). Briefly, 6 μL of sample was extracted according to the kit procedure. The samples were dried in N_2 and reconstituted in 50 μL of 50% LC/MS grade methanol in water, and 5 μL of the sample was analyzed by UPLC/MS/MS. The sample was separated by UPLC using reverse-phase gradient elution at a flow rate of 0.5 mL/min. The separated sample was then subjected to MS/MS by multiple reaction monitoring. The area under the curve for each transition was determined, and the ratio of the area under the curve for each amino acid to its corresponding internal standard solution was used for relative concentration determinations. Absolute concentrations were calculated from a 5-point calibration curve, using standard solutions prepared from accurately weighed, analytical grade amino acids.

Video-intracranial EEG monitoring

After implantation of the MSO pump, rats were monitored for spontaneous seizures for 21 days. The experimental setup for recording video-EEG was adapted from Bertram et al.[16] The rats were placed individually in custom-made Plexiglas cages. A spring-covered, 6-channel cable was connected to the electrode pedestal on one end and to a commutator (Plastics One) on the other. A second cable connected the commutator to the digital EEG recording unit (CEEGraph Vision LTM, Natus/Bio-Logic Systems Corp., San Carlos, CA). Digital cameras with infrared light detection capability were used to record animal behavior (two cages per camera). The digital video signal was encoded and synchronized to the digital EEG signals. Seizures were identified by visual inspection of the EEG record. As detailed by Avoli et al.,[17] seizures were defined by EEG characteristics of the discharge. Specifically, seizures displayed distinct signal changes from background (interictal) activity.

Such signal changes included sustained rhythmic or spiking EEG patterns and a clear evolution of signal characteristics from onset to termination (Fig. 2).

Subclinical seizures were distinguished from clinical seizures by examination of the video record. The start and stop points of seizures were identified by the following commonly used method. By visual inspection of the EEG, we determined a point that was unequivocally within the seizure. Next, we moved backward in time to determine the seizure start time as the first point where the EEG was different from background activity and forward in time to establish the seizure end time. The temporal distribution and total number of seizures in all animals was determined by reviewing the entire video-EEG record. Behavioral seizures were graded on the Racine seizure scale,[18] and were subsequently classified as non-convulsive (subclinical to stage 2) or convulsive (stage 3–5) (Fig. 3). Rats were classified in this manner because we previously demonstrated a progressive change in regional involvement of the brain associated with behavioral severity, with the largest increase in signal power observed between Racine stage 2 and 3 seizures.[19]

Histology

Rats were anesthetized with 0.5 – 2% Isoflurane in O_2 and perfused transcardially with 0.9% NaCl followed by 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed and left in the same fixative at 4°C for 24 h and then transferred to phosphate buffered saline (PBS). The brains were stored at 4°C until being sectioned on a Vibratome at 50-μm thickness. Every fifth section through the hippocampus was mounted on gelatin-coated slides and stained with cresyl violet. Adjacent sections to the series above were stained with NeuN antibody (Millipore cat. # MAB377/lot # 2424507), diluted 1:10,000 in PB containing 0.3% Triton-X-100. The sections were processed using the avidin-biotin complex (ABC) kit (Vectastain, Burlingame, CA) with diaminobenzidine as the chromogen and mounted on a microscope slide for later analysis.

Our previous studies demonstrated a consistent and predictable pattern of seizure activity and neuronal loss when the MSO micro-infuser was localized to the hippocampal dentate gyrus.[20] Therefore, only rats in which the location of the micro-infuser was confirmed in dentate gyrus were included in the final analysis.

Neurons were counted in the hilus of dentate gyrus, where the extent of neuron loss correlates with seizure activity in this model.[20] Neuron counts were performed ipsilateral and contralateral to the infusion site using the optical fractionator method with StereoInvestigator, (Site Mark West, MBF Bioscience, Williston, VT). On the ipsilateral side, NeuN sections adjacent to the infusion site were counted (sections containing the infusion site were omitted). On the contralateral side, which did not have an infusion site, the series of sections stained with NeuN were counted. The sum of sections counted ranged from six to eight. After outlining the hilus, a 40 μm × 40 μm sector was randomly place by the software in the traced region and cells were counted within the sectors. The total area was calculated by multiplying the area of the sector by the number of sampling sites. The software randomly selected the number of sample sites to exclude bias, ranging from 60 – 110. Counts were calculated and expressed as neurons per 100 mm^3.

Statistics

Parametric data were compared with Student's t-test. Repeated measures ANOVA was used to compare the total frequency of non-convulsive (stage 1–2) and convulsive (stage 3–5) seizures over 21 days in 7-day bins (1–7, 8–14, 15–21). ANOVA was followed by a post hoc Fisher least significant difference (LSD) test. Chi-square was used to compare the relative percent of convulsive seizures over 21 days in 7-day bins. Statistical significance was defined as $p \leq 0.05$. Data is presented as mean ± standard error (SE). Statistical analysis was performed with Statistica 8.0 (Statsoft; Tulsa, OK).

Results

Chronic BCAA ingestion increased blood concentrations of all three BCAAs

Three rats in the BCAA-treated group were excluded because the location of the micro-infuser was outside the dentate gyrus. To assess the palatability and effectiveness of the drinking approach, we first determined fluid consumption and plasma BCAA levels in all rats. There was no difference in fluid consumption between the BCAA- and plain water-treated rats (Fig. 4A). Rats in the BCAA-treated group consumed on average 1.06 ± 0.031g (1.75 g per kg body wt.) of BCAAs per day. After 10 days of drinking, the plasma concentrations of BCAAs were 29 to 75% higher in the BCAA-treated versus plain water-treated animals (Fig. 4B). After 31 days of drinking, the plasma concentrations of BCAAs continued to remain higher (35 to 52%) in BCAA-treated animals (Fig. 4C). There were no differences in BCAA concentrations between 7 and 31 days.

Chronic BCAA ingestion did not alter the frequency of convulsive or non-convulsive recurrent seizures

We next analyzed the video-EEG records from all rats for the entire 3-week collection period. There was no significant difference in the seizure frequency between the BCAA- and plain water-treated rats during weeks 1, 2, or 3 of the collection period (Fig. 5). However, during the second week of collection, there was a nonsignificant trend ($p = 0.077$) towards fewer seizures in the BCAA-treated rats. There was no difference in the average number of non-convulsive (i.e. subclinical to Racine stage 2) or convulsive (Racine stage 3 to 5) seizures during weeks 1, 2, of 3 of analysis.

Chronic BCAA ingestion altered the relative percent of convulsive seizures during weeks 1 and 3 of treatment

Next, we analyzed the relative percent of convulsive (Racine stage 3 to 5) seizures during weeks 1, 2, and 3 of the EEG recording period (Fig. 6). In week 1, the BCAA-treated rats had a lower relative percent of convulsive seizures compared with water-treated rats (13.6% vs. 26.3%, respectively, $p < 0.001$). In week 2, there were no significant differences in the relative percent of convulsive seizures between the BCAA- and water-treated rats. In week 3, the BCAA-treated rats had a higher relative percent of convulsive seizures compared with water-treated rats (77.1% vs. 53.0%, $p < 0.05$).

Chronic BCAA ingestion aggravated the neuronal loss in the dentate hilus

Finally, we determined the density of NeuN-stained neurons in the hilus of the left and right dentate gyrus of BCAA- and plain water-treated rats (Fig. 7). BCAA-treated rats had 36% fewer neurons in the ipsilateral (right) dentate hilus compared with the plain water-treated group (575.1 ± 67.9 vs. 894.3 ± 59.5 cells per 100 mm^3, respectively, $p<0.0001$), and 29% fewer neurons in the contralateral (left) dentate hilus compared with the plain water-treated group (499.5 ± 58.3 vs. 707.7 ± 33.6 cells per 100 mm^3, respectively, $p<0.001$).

Discussion

To our knowledge, this is the first study of the effect of high-dose, oral BCAA supplementation on recurrent seizures and brain pathology. We demonstrated that chronic BCAA ingestion was ineffective in reducing the frequency of convulsive and non-convulsive recurrent spontaneous seizures. However, there were differences between groups with respect to the relative percent of convulsive seizures, which reflects the propagation of seizure activity from the hippocampus to neocortical areas, such as the motor cortex. While BCAAs initially reduced the propagation of seizures during the first week of treatment, the seizure propagation subsequently worsened with a longer duration of BCAA treatment. Lastly, chronic BCAA treatment resulted in increased loss of hippocampal hilar neurons.

BCAAs are known to play important roles in normal physiology, and are widely used by athletes to promote muscle growth and recovery.[21] BCAAs account for 35% of the essential amino acids in muscle[22] and stimulate muscle growth by activating the mechanistic target of rapamycin (mTOR) signaling pathway.[23] Moreover, diet-derived BCAAs contribute to approximately one-third of *de novo* synthesized glutamate in the rodent central nervous system (CNS).[24]

Ingested BCAAs undergo minimal first-pass metabolism, enter the systemic circulation and readily distribute to the CNS via several types of amino acid transporters, particularly the large neutral amino acid transporter 1 (LAT1)[25] and the solute carrier family 6 member 15 (SLC6A15).[26] Once in the CNS, each BCAA can donate its amine group to alpha-ketoglutarate, forming glutamate and the corresponding branched-chain keto acid, via the branched-chain aminotransferase (BCAT) reaction.[27] Diet-derived BCAAs in the blood can therefore modulate brain glutamate levels, which are normally under tight homeostatic control to facilitate efficient neurotransmission and prevent glutamate-mediated excitotoxicity.[28] It is well known that sustained high levels of extracellular glutamate in the brain lead to excessive stimulation of neuronal glutamate receptors and subsequent abnormal neurotransmission, increased excitability, seizures and neuronal loss.[29] Furthermore, the actions of glutamate and gamma-aminobutyric acid (GABA; a metabolite of glutamate) have been implicated in both the toxic and therapeutic effects of many general anesthetics.[30–32] Therefore, understanding the mechanisms of glutamate and GABA metabolism is important because it may lead to the development of targeted neuroprotective agents, antiepileptic therapies, and general anesthetics.

While some studies report neurotoxic and disease-promoting properties of BCAAs and their associated metabolites, others demonstrate protective effects. For example, perturbations in

the homeostasis of brain BCAAs have been implicated in the pathophysiology of several neurological disorders such as maple syrup urine disease-associated encephalopathy,[33] malignant gliomas,[34] autism spectrum disorders,[35] amyotrophic lateral sclerosis[36] and epilepsy.[8, 9, 13, 37] With respect to epilepsy, a small number of studies have shown that administration of BCAAs alone, or in combination with a ketogenic diet, reduce the frequency of seizures and increase the seizure threshold in children with epilepsy[8] as well as in the kainic acid[9] and pentylenetetrazole[13] models of acute convulsive seizures. However, in the Genetic Absence Epilepsy Rats from Strasbourg (GAERS) model of absence seizures, a single intraperitoneal dose of BCAAs increases the frequency of electrographic seizures.[37]

In the present study, BCAAs were ineffective in reducing the frequency of convulsive and non-convulsive recurrent seizures in the epileptic rats. This observation contrasts with previous studies in other animal models that show clear anticonvulsant effects of BCAAs.[8–13] Several animal models have been used to study the mechanisms of epileptogenesis and seizure generation in MTLE, in which acute seizures are induced by chemoconvulsants, electrical or sound stimuli, or traumatic brain injury. Although each model has unique neuropathological and behavioral characteristics[38], the MSO model of MTLE we developed is advantageous in that it recapitulates several important features of the human MTLE condition, making the model highly translatable. Some of these features include inhibition in GS in hippocampal astrocytes,[29, 39] an initial insult followed by recurrent spontaneous seizures that originate from the mesial temporal lobe,[15] loss of hippocampal neurons,[40, 41] worsening of the seizures as the disease progresses over time,[20] comorbid depressive features[42] and perturbations in the brain glutamate homeostasis.[43, 44]

Although the mechanisms by which BCAAs prevent seizures in other epilepsy models are poorly understood, an efficient metabolism and clearance of glutamate might be necessary. For example, administration of BCAAs has been shown to favor the synthesis of GABA over glutamate, by inhibiting the activities of glutamate dehydrogenase (GDH)[45, 46] and glutamate oxaloacetate transaminase (GOT).[47] Moreover, administration of BCAAs could potentially reduce neuronal glutamate concentrations via the glial-neuronal BCAA shuttle,[37] as follows. Blood-derived BCAAs taken up astrocytes may transaminate α-ketoglutarate, thereby producing glutamate and branched-chain ketoacids (BCKAs). The BCKAs are poorly oxidized in astrocytes and accumulate in the extracellular fluid.[48] Neurons take up the BCKAs, which are transaminated back to BCAAs in a reaction that consumes glutamate.[27, 37, 48] The glutamate produced in astrocytes from the BCAA transamination reaction are likely converted to glutamine via the GS reaction. The excess astroglial glutamine are then exported from the brain to the blood.[49]

However, when GS is deficient, such as in patients with MTLE and in the rodent model used here, the astrocyte glutamate excess caused by the BCAAs cannot be effectively eliminated from the brain.[29, 43, 50] It is well known that glutamate is a potent excitotoxin, and its accumulation in the extracellular compartment of the brain has deleterious effects on neuronal function and survival in laboratory animals.[51] As such, the accumulation of extracellular glutamate that results from the GS deficiency is thought to play a critical role in the pathophysiology of MTLE.[52] Moreover, slowed glutamate-glutamine cycling may further contribute to seizures by reducing neuronal glutamate available for GABA synthesis.

[53] While we did not directly measure glutamate levels in the brain in this experiment, it is well-known that BCAAs readily the blood brain barrier in both the healthy and injured brain.[54] Moreover, our unpublished microdialysis experiments with MSO-treated rats have demonstrated that BCAAs readily cross the blood brain barrier, where they are subsequently converted to glutamate.

Although generalized tonic-clonic seizures are not the typical primary manifestation of MTLE, these types of seizures can certainly be observed, and patients often experience secondarily generalized seizures.[55] Unlike the human condition, there is a rapid spread of seizure activity in the rodent MSO model, which might result in a larger fraction of generalized seizures in rats compared with humans. While the present study failed to demonstrate differences in seizure frequency in BCAA- vs. water-treated rats, the relative percent of convulsive seizures changed as the duration of treatment increased. This observation is important because convulsive seizures (compared with non-convulsive seizures) are known to reflect a spread of seizure activity from the hippocampal seizure focus to neocortical areas such as the motor cortex.[19] Here we demonstrated that during the early treatment period, BCAAs inhibited the propagation of seizures to the motor cortex, whereas with a longer duration of exposure, chronic BCAA treatment facilitated motor cortical propagation. This suggests that the effect of BCAA treatment on the propagation of seizure activity changes as the duration of exposure to BCAAs increases. Although the underlying mechanism for time-related difference in effect is unclear, its implications might significantly preclude the use of BCAAs as a chronic treatment for refractory epilepsy.

Hippocampal neurons, particularly cells in the dentate hilus, are often lost in patients with MTLE and animal models of the disease, including the model used here.[20, 56, 57] We have previously shown that rats treated with MSO exhibit various degrees of hippocampal neuron loss. Most animals demonstrate minimal or modest neuron loss throughout the hippocampus, with a minority (< 25%) displaying classic hippocampal sclerosis, defined as loss of CA1, CA3 and dentate neurons along with shrinkage of the hippocampal formation.[15, 41] There is considerable variability in neuron loss among patients with MTLE as well; however, up to one-third of all patients with surgically-treated MTLE exhibit classic hippocampal sclerosis, [58] which is higher than in the MSO model. The reason for the difference in prevalence of hippocampal sclerosis between the MSO and human MTLE is no understood, but might reflect a longer duration of the disease in the surgically resected patients with more time for the sclerosis to develop. Despite these differences, the overall patterns of neuropathology are very similar between our MSO model and humans with MTLE.

Our study is the first to show that high-dose, oral supplementation with BCAAs worsens the loss of neurons associated with MTLE. Although dentate granule cells are generally relatively resistant to seizures, hilar interneurons are known to be particularly vulnerable to degeneration in patients with epilepsy and in several animal models.[59, 60] Because it would be a formidable task to count all neuron populations, we chose a sentinel and particularly vulnerable neuronal population as a first screening to determine whether treatment with BCAAs enhances the neuron loss in epilepsy. Importantly, the BCAA-induced hilar neuron loss demonstrated in the present study highlights the need for follow up studies that

systematically investigate other neuronal populations, as well as the underlying mechanisms of neurodegeneration with chronic BCAA administration.

Several mechanisms may explain the neurotoxic effects of BCAAs. First, as described above, an excess of brain BCAAs in the setting of astroglial GS deficiency may lead to increased astroglial and extracellular glutamate, resulting in excitotoxic neuronal loss. Second, BCAAs, especially leucine, stimulate ammonia production in the brain by activating GDH, which results in glutamate oxidation in synaptosomes.[37, 61] Ammonia is neurotoxic, possibly via mechanisms that involve astrocyte swelling or shrinking, increased astrocyte calcium signaling, and reduced potassium clearance by saturating potassium uptake mechanisms.[62–64] There are several metabolic pathways through which ammonia is normally cleared from the brain, including the GS pathway in astrocytes.[65] Therefore, the combination of increased ammonia production via BCAAs and reduced ammonia clearance via GS inhibition may account for the enhanced neuron loss observed in BCAA-treated rats. Third, BCAAs compete with entry of other large amino acids, resulting in amino acid deficiency and altered protein synthesis in the brain.[66] Some of these amino acids, including phenylalanine and tyrosine, are precursors for neurotransmitter synthesis, and competition for transport into the brain might therefore interfere with neurotransmitter synthesis.[66] Fourth, accumulation of BCAAs can stimulate lipid peroxidation and increase the production and accumulation of free radicals, resulting in oxidant cortical injury.[67]

The BCAA-potentiated neuronal loss is of significant interest not only with respect to epilepsy, but also in the developing brain. Several *in vivo* animal studies have demonstrated that general anesthetics are toxic to the developing brain,[68] especially the hippocampus,[69] and alterations in glutamate metabolism are thought to play an important mechanistic role.[70, 71] To better understand the underlying metabolic pathways implicated in anesthesia-induced neurotoxicity, recent studies have used cerebral metabolomics profiling to characterize chemical changes in the rodent[72] and human[73] brain during exposure to volatile and intravenous anesthetics. Interestingly, prolonged exposure to volatile anesthetics has been shown to significantly alter amino acid metabolism in the developing brain,[74] suggesting a possible mechanistic link between amino acid metabolism and anesthesia-induced neurotoxicity. In this way, the developing brain might be particularly susceptible to the neurotoxic effects of a BCAA-rich diet, and potentiated by prolonged or repeated exposure to inhaled general anesthetics. This possibility is intriguing and should be further evaluated in future studies.

Moreover, although not specifically investigated in the present study, BCAA-induced neuron loss might have important implications for healthy individuals who use BCAAs as nutritional supplements. In our study, BCAA-treated rats consumed on average approximately 1.75 g/kg body wt. of BCAAs per day. While it is unclear precisely how this dose translates in humans, BCAA supplementation in high-performance athletes is highly variable and often well exceeds 1.0 g/kg per body wt. per day.[75] It is currently unknown whether high-dose BCAA supplementation affects brain physiology, cognition or behavior in healthy individuals. Similarly, it is unknown whether BCAAs can lead to neuron loss in the absence of epilepsy. Further studies are needed to assess these possibilities.

In summary, we demonstrated for the first time the effects of chronic BCAA ingestion on spontaneous seizures in a translationally-relevant model of MTLE. Chronic BCAA ingestion aggravated hilar neuron loss, but had no significant effect on the frequency of convulsive or non-convulsive spontaneous seizures in GS-inhibited, epileptic rats. Although the cortical spread of seizure activity was inhibited in the first week of BCAA supplementation, a longer duration of treatment facilitated the spread of seizures and aggravated neuron loss. These findings suggest that GS function may be necessary for the previously demonstrated anticonvulsant effects of BCAAs, and that chronic BCAA supplementation may facilitate seizure spread and aggravate neuron loss in a setting of MTLE.

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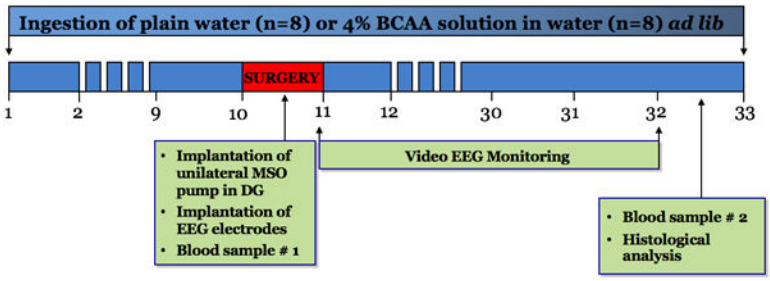


Figure 1. Experimental Protocol

Sixteen rats were randomly separated to receive either unrestricted access to a 4% BCAA solution dissolved in water (n=8) or a plain water solution (n=8) *ad libitum*. Following 10 days of consumption, rats were implanted with the MSO pump, and seizure activity was monitored thereafter for a period of 21 days. For the duration of the EEG recording period, MSO was continuously infused and rats were maintained on the BCAA or plain water solution. After this monitoring period, rats were perfused and the brains were harvested for immunohistochemical analysis. BCAA concentrations were analyzed in venous blood samples that were drawn at two time points during the experiment. Three rats from the BCAA group were excluded from the final analysis after postmortem histological analysis confirmed that the MSO pump was outside of dentate gyrus.

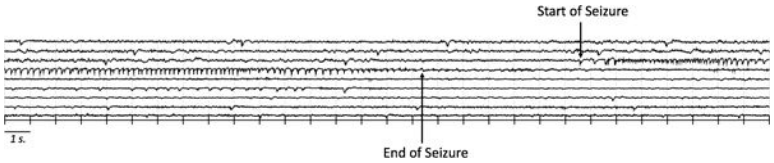


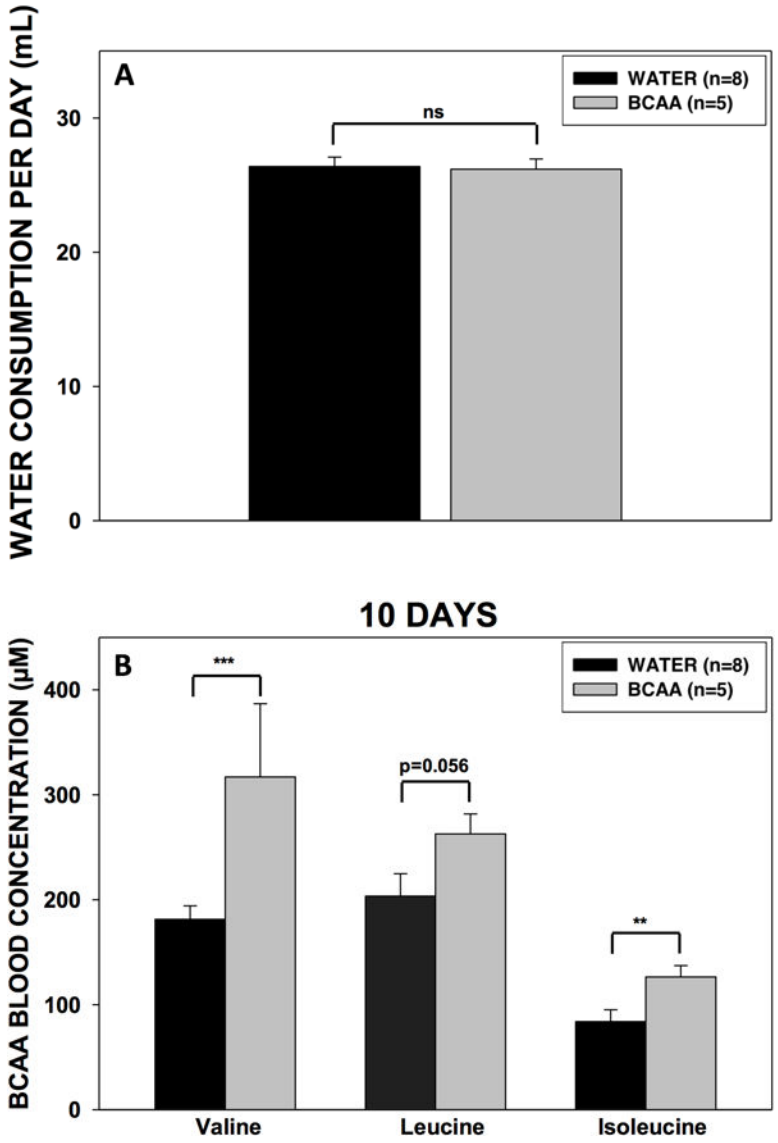
Figure 2. Two hundred-seventy seconds of continuous EEG recording from a screw electrode over the cortex in a rat chronically infused with MSO in the dentate gyrus

A seizure approximately 24 seconds in duration is identified by arrows indicating the start and end of the seizure. The seizures were like those previously described.[20, 41]

RACINE SCALE	ASSOCIATED BEHAVIOR	
Subclinical	Electrographical seizure with no associated behavior	Non-convulsive
1	Freezing	
2	Head nodding	
3	Forelimb clonus	Convulsive
4	Rearing with forelimb clonus	
5	Rearing and falling with forelimb clonus	

Figure 3. Behavioral seizures were graded on the Racine seizure scale

Seizure activity was monitored over 21 days with synchronized video and EEG. Seizures were identified by visual inspection of the EEG record, and behavioral seizures were graded from subclinical through stage 5 as previously described by Racine.[18] Seizures were subsequently classified as non-convulsive (subclinical to stage 2) or convulsive (stage 3–5).



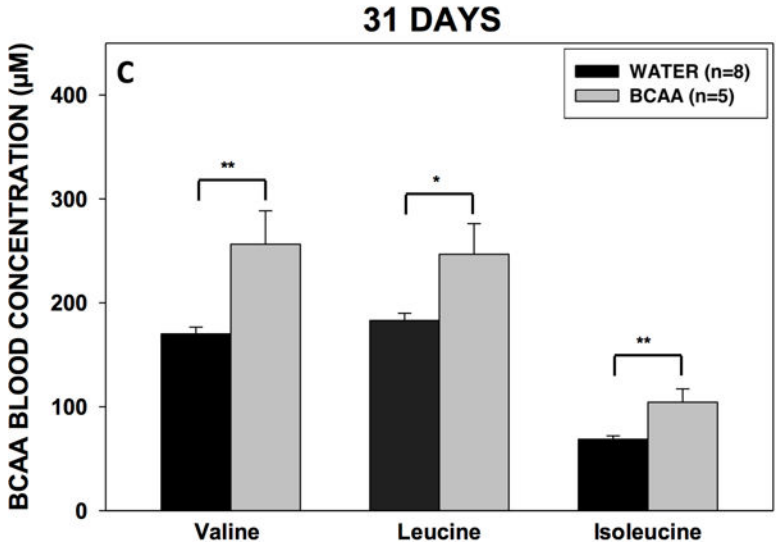


Figure 4. Volume consumption and blood BCAA concentrations

(A) Rats drank either plain water (n=8) or a solution of 4% BCAAs dissolved in water (n=5) *ad libitum* for 31 days. The water bottles were weighed daily for the duration of the experiment. There were no differences in water consumption between BCAA- and water-treated animals. Data is presented as mean water consumption (mL) per day ± SEM. Blood samples were drawn from the scalp during surgery for placement of methionine sulfoximine pump at 10 days (B) after drinking, and again from the heart during cardiac perfusion at 31 days (C) after drinking. Samples were analyzed for BCAAs using tandem mass spectrometry. At both time points, concentrations of BCAAs were higher in the rats that drank the BCAA solution compared with the rats that drank plain water. Data is presented as mean concentrations of branched-chain amino acids ± SEM. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

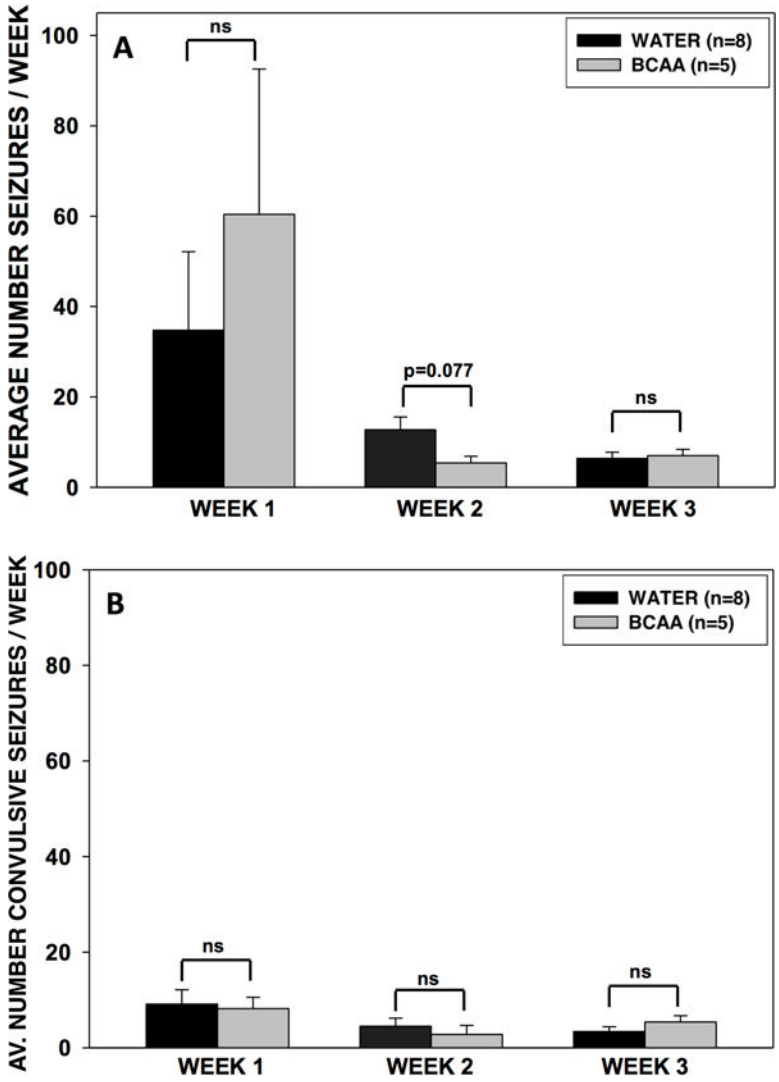


Figure 5. Frequency and severity of recurrent seizures in BCAA- and water-treated animals

After 10 days of drinking either plain water or a solution of 4% BCAAs dissolved in water, rats were implanted with an osmotic pump through which MSO was chronically infused at a rate of 0.625 μg per hour in the right dentate gyrus. Rats were then subjected to 3 weeks of video-EEG analysis, whereby the frequency and severity (Racine scale 1–5) of seizures were recorded. (A) There were no differences in the total average number of seizures per day during weeks 1, 2, or 3 of EEG analysis. During week 2, there was a nonsignificant trend ($p = 0.077$) towards fewer seizures in rats who drank branched-chain amino acid solution. (B) There were no differences in the average number of non-convulsive (subclinical – Racine stage 2) or convulsive (Racine stage 3 – 5) seizures during weeks 1, 2, of 3 of EEG analysis. Data is presented as mean number of seizures per day ± SEM.

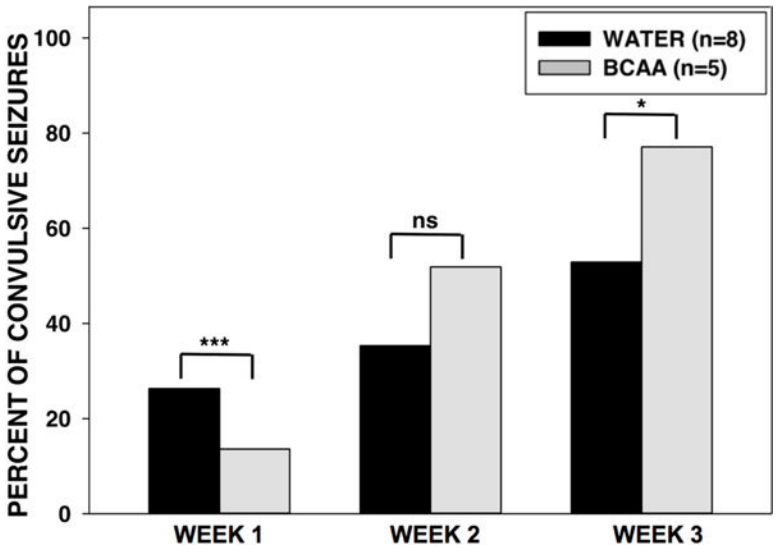
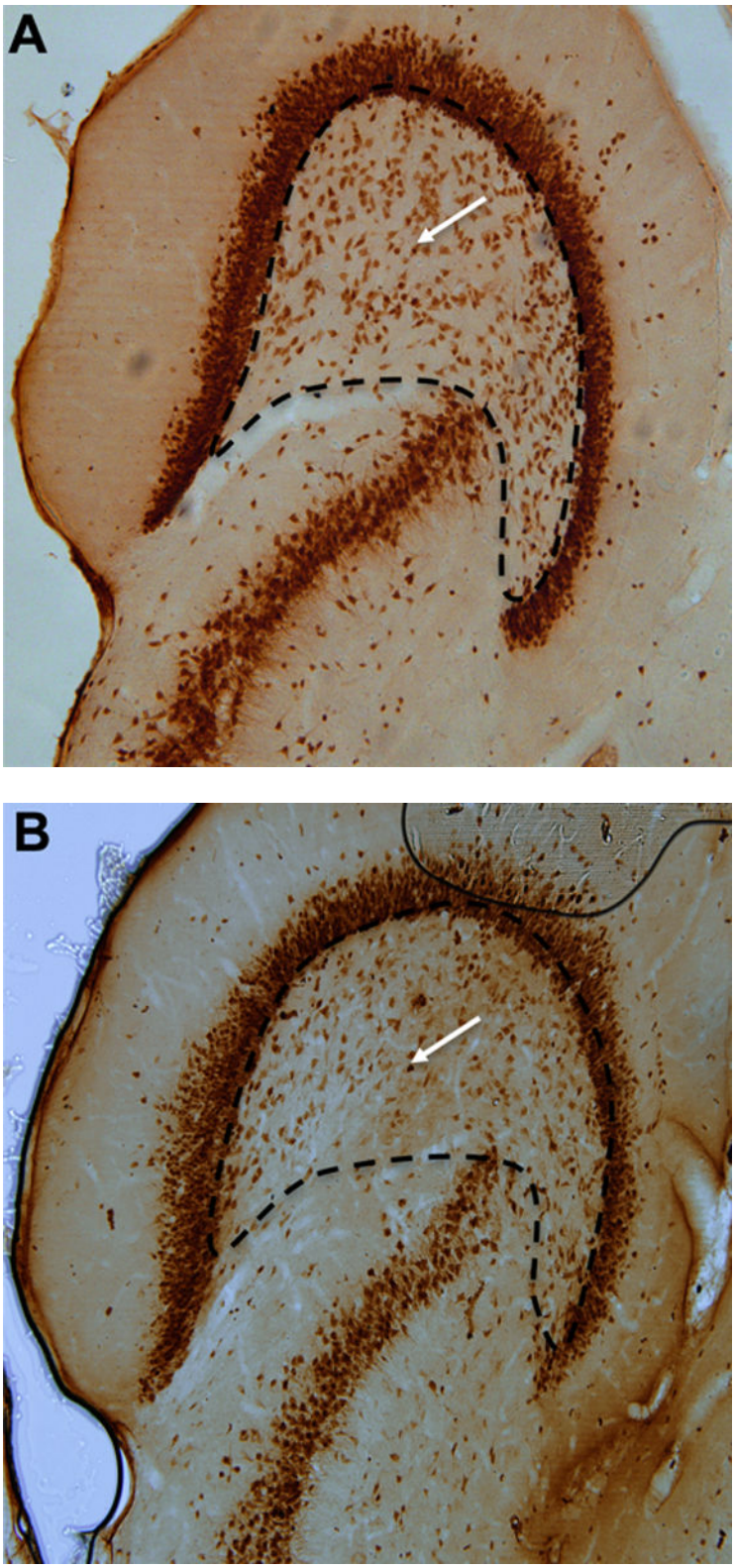


Figure 6. Relative percent of convulsive in BCAA- and water-treated animals

The relative percent of convulsive (Racine stage 3 to 5) seizures during weeks 1, 2, and 3 of the EEG recording period was analyzed. In the first week of the recording period, the BCAA-treated rats had a lower relative percent of convulsive seizures compared with water-treated rats ($p < 0.001$). In week 2, there were no significant differences in the relative percent of convulsive seizures between the BCAA- and water-treated rats, whereas in week 3 the BCAA-treated rats had a higher relative percent of convulsive seizures ($p < 0.05$). Data is presented as percent of convulsive seizures. ***$p < 0.001$, *$p < 0.05$.



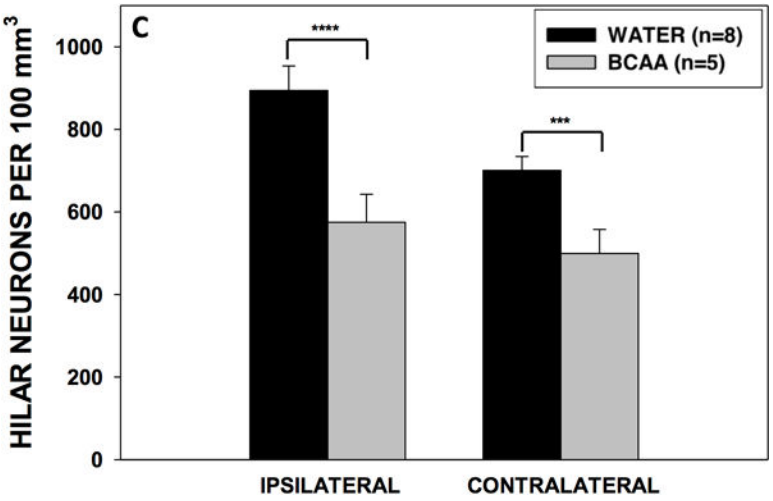


Figure 7. Hippocampal neuron counts

(A) Section stained with NeuN demonstrating neurons within the dentate hilus (borders outlined with dashed lines) in a representative control and (B) branched-chain amino acid (BCAA) rat. The white arrows point to a stained neuron. (C) Rats in the branched-chain amino acid vs. control group had 36% fewer neurons in the dentate hilus of the hemisphere ipsilateral ($p<0.0001$) to the methionine sulfoximine infusion and 29% fewer neurons contralateral ($p<0.001$) to the infusion. Data is presented as mean number of cells per 100 mm^3 ± SEM. ****$p < 0.0001$, ***$p < 0.001$