

Apoptotic signaling pathways induced by acute administration of branched-chain amino acids in an animal model of maple syrup urine disease

Thais C. Vilela^{1,2,3} · Giselli Scaini^{1,2,3} · Camila B. Furlanetto^{1,2,3} ·
Matheus A. B. Pasquali⁴ · João Paulo A. Santos⁴ · Daniel P. Gelain⁴ ·
José Cláudio F. Moreira⁴ · Patrícia F. Schuck⁵ · Gustavo C. Ferreira⁶ ·
Emilio L. Streck^{1,2,3}

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Abstract Maple Syrup Urine Disease (MSUD) is an inborn error of metabolism caused by a deficiency of the branched-chain α -keto acid dehydrogenase complex activity. This blockage leads to accumulation of the branched-chain amino acids leucine, isoleucine and valine, as well as their corresponding α -keto acids and α -hydroxy acids. The affected patients present severe neurological symptoms, such as coma and seizures, as well as edema and cerebral atrophy. Considering that the mechanisms of the neurological symptoms presented by MSUD patients are still poorly understood, in this study, protein levels of apoptotic factors are measured, such as Bcl-2, Bcl-xL, Bax, caspase-3 and -8 in hippocampus and cerebral cortex of rats submitted to acute administration of

branched-chain amino acids during their development. The results in this study demonstrated that BCAA acute exposure during the early postnatal period did not significantly change Bcl-2, Bcl-xL, Bax and caspase-8 protein levels. However, the Bax/Bcl-2 ratio and procaspase-3 protein levels were decreased in hippocampus. On the other hand, acute administration of BCAA in 30-day-old rats increase in Bax/Bcl-2 ratio followed by an increased caspase-3 activity in cerebral cortex, whereas BCAA induces apoptosis in hippocampus through activation and cleavage of caspase-3 and -8 without changing the Bax/Bcl-2 ratio. In conclusion, the results suggest that apoptosis could be of pivotal importance in the developmental neurotoxic effects of BCAA. In addition, the current studies also suggest that multiple mechanisms may be involved in BCAA-induced apoptosis in the cerebral cortex and hippocampus.

Thais C. Vilela and Giselli Scaini contributed equally to this work.

✉ Emilio L. Streck
emiliostreck@gmail.com

- ¹ Laboratório de Bioenergética, Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense, Av. Universitária, 1105, Criciúma 88806-000, SC, Brazil
- ² Instituto Nacional de Ciência e Tecnologia Translacional em Medicina (INCT-TM), Porto Alegre, RS, Brazil
- ³ Núcleo de Excelência em Neurociências Aplicadas de Santa Catarina (NENASC), Florianópolis, Santa Catarina, Brazil
- ⁴ Centro de Estudos em Estresse Oxidativo, Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
- ⁵ Laboratório de Erros Inatos do Metabolismo, Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil
- ⁶ Laboratório de Neuroquímica, Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

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Introduction

Maple syrup urine disease (MSUD; branched-chain keto aciduria) is an autosomal recessive disorder caused by the deficiency of branched chain α -ketoacid dehydrogenase complex (BCKAD, E.C. 1.2.4.4) activity. The metabolic defect leads to accumulation of the branched-chain amino acids (BCAA) leucine (Leu), isoleucine (Ile) and valine (Val) and the corresponding branched-chain α -ketoacids (BCKA), α -ketoisocaproic acid (KIC), α -keto- β -methylvaleric acid (KMV) and α -ketoisovaleric acid (KIV) in tissues and body fluids (Chuang and Shih 2001; Treacy et al. 1992).

MSUD presents as heterogeneous clinical and molecular phenotypes, characterized by ketoacidosis, hypoglycemia, poor feeding, apnea, ataxia, convulsions, coma, psychomotor delay and mental retardation, as well as generalized edema in the central nervous system (CNS), atrophy of the cerebral hemispheres, white matter spongy degeneration and delayed myelination (Chuang and Shih 2001; Schonberger et al. 2004). The mechanisms of brain damage in MSUD are still poorly understood; it has been postulated that the metabolites accumulating in MSUD may affect energy metabolism in rat brain (Howell and Lee 1963; Land et al. 1976; Pilla et al. 2003; Ribeiro et al. 2008; Sgaravatti et al. 2003) and may cause significant alterations of the concentrations of the neurotransmitters glutamate, aspartate and aminobutyric in the brain (Dodd et al. 1992; Prensky and Moser 1966; Tavares et al. 2000; Yudkoff et al. 1994). The brain injury in this disorder may also be related to reduction of brain uptake of essential amino acids (Araujo et al. 2001), apoptosis of neural cells (Jouvet et al. 1998, 2000a, b) oxidative stress (Barschak et al. 2008a, b, 2009; Bridi et al. 2003, 2005; Fontella et al. 2002; Mescka et al. 2011) and DNA damage (Mescka et al. 2014, 2015; Scaini et al. 2012).

Studies have also reported altered apoptotic factors and their mediated responses in MSUD. Changes include DNA damage, mitochondrial dysfunction and enhanced oxidative stress, as described above. Additionally, magnetic resonance imaging studies in children with MSUD have confirmed both white matter and neuronal injury, including extensive brain edema and pathological changes in the basal ganglia (Brismar et al. 1990; Steinlin et al. 1998). At the histopathological level, deficiencies in myelination of major tracts in the pons and spinal cord, widespread areas of spongy change in the white matter, focal areas of astrocytosis, and binucleated neurons have also been reported (Langenbeck 1984). These studies implicate the involvement of cell death in the pathophysiology of neurological dysfunction in MSUD. To further clarify the possible involvement of apoptosis in MSUD, protein levels of apoptotic factors are measured, such as Bcl-2, Bcl-xL, Bax, caspase-3 and -8 in hippocampus and cerebral cortex of rats submitted to acute administration of BCAA during their development. For this purpose, a chemically-induced model of MSUD, which produce high sustained levels of BCAA that are similar to those found in plasma of MSUD patients, is used.

Materials and methods

Animals

Male Wistar rats at 10 and 30 days old (weighing 20–25 g, and 80–100 g, respectively) were obtained from the Central Animal House of the Universidade do Extremo Sul

Catarinense. Ten-day-old rats were left with their dams until the day of the experiment, and the 30-day-old rats were weaned at 21 days of life. All rats were caged in groups of 5 with free access to food and water and were maintained on a 12-h light/dark cycle (lights on 7:00 am) at a temperature of 23 ± 1 °C. All experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior recommendations for animal care, with the approval of the Ethics Committee of the Universidade do Extremo Sul Catarinense (protocol number 60/2010).

Acute administration of BCAA pool

The animals received three subcutaneous administrations of BCAA pool (15.8 $\mu\text{L/g}$ body weight at 1-h intervals) containing 190 mmol/L leucine, 59 mmol/L isoleucine, and 69 mmol/L valine in saline solution (0.85 % NaCl) or saline alone (control group). The BCAA pool and saline solution were given to rats on postnatal day (PD) 10 or PD 30 ($n = 6$). One hour after the last injection, the animals were sacrificed by decapitation, the brain was rapidly removed, and the hippocampus and cerebral cortex were collected. The choice of doses of BCAA and age of animals were based on a previous study (Bridi et al. 2006) showing that the administration of BCAA pool to rats (doses and ages similar to those used in the present study) resulted in increased levels of leucine, isoleucine and valine in blood and brain, mimicking the main biochemical finding observed in MSUD patients during crises.

Immunoblotting

To perform the immunoblot experiments, the samples were first homogenized in Laemmli-sample buffer (62.5 mM Tris-HCl, pH 6.8, 1 % (w/v) SDS, 10 % (v/v) glycerol). Equal amounts of protein (30 $\mu\text{g/well}$) were fractionated by SDS-PAGE and electro-blotted onto nitrocellulose membranes. The protein loading and electro-blotting efficiency were verified with Ponceau S staining. The membranes were blocked in Tween-Tris buffered saline (TTBS: 100 mM Tris-HCl, pH 7.5, containing 0.9 % NaCl and 0.1 % Tween-20) containing 5 % albumin. The membranes were incubated overnight at 4 °C with a rabbit polyclonal antibody against Bcl-2 Antibody (Cell Signaling - 2876), Bcl-xL (Cell Signaling - 2762), Bax (Cell Signaling - 2772), caspase-3 and -8 (Cell Signaling - 9662, 4927, respectively). The primary antibody was then removed and the membranes were washed 4 times for 15 min. After washing, an anti-rabbit IgG peroxidase linked secondary antibody was incubated with the membranes for 1 h (1:10000 dilution) and the membranes were washed again. Finally, the immunoreactivity was detected using an enhanced chemiluminescence ECL Plus kit. After

exposure, the membranes were then stripped and incubated with a mouse monoclonal antibody to β -Actin (sigma - A2228) in the presence of 5 % milk. An anti-mouse IgG peroxidase linked secondary antibody was incubated with the membranes for 1 h (1:10000 dilution) and the membranes were washed again. The immunoreactivity was detected using an enhanced chemiluminescence ECL Plus kit. Densitometry was performed using Image J v.1.34 software. SeeBlue® Plus2 Prestained Standard (Invitrogen) was used as a molecular weight marker to make sure that the correct bands were analyzed for Bcl-2, Bcl-xL, Bax, caspase-3, caspase-8 and β -Actin.

Caspase activity measurement

The caspase-3 and -8 activities in the hippocampus and cerebral cortex [homogenised in lysis buffer (50 mM HEPES, pH 7.5, 0.1 % CHAPS, 2 mM dithiothreitol, 0.1 % Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μ g/ml leu-peptin, and 2 μ g/ml pepstatin)] were determined

by monitoring proteolysis of the appropriate fluorochrome substrates. DEVD-AMC and Z-IETD-AFC served as substrates employed to detect caspase-3 and -8 enzyme activities, respectively. Briefly, 50 μ g of tissue homogenate protein was added to a buffer containing 100 μ l of caspase buffer with 50 μ M of appropriate caspase-specific substrate (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 1 h at 37 °C. Fluorescence was read on a SpectraMax® M2 Microplate Reader (Molecular Devices, Sunnyvale, CA) at an excitation of 355 nM and an emission of 460 nM.

Statistical analysis

Results are presented as the mean \pm standard deviation. All assays were performed in duplicate, and the mean was used for statistical analysis. Tests for determination of normality and equal variances were performed to examine whether the data qualified for parametric statistical tests. The data were normally distributed (Shapiro–Wilk, $P > 0.05$) with equal variances among samples (equal variances test, $P > 0.05$). Thus,

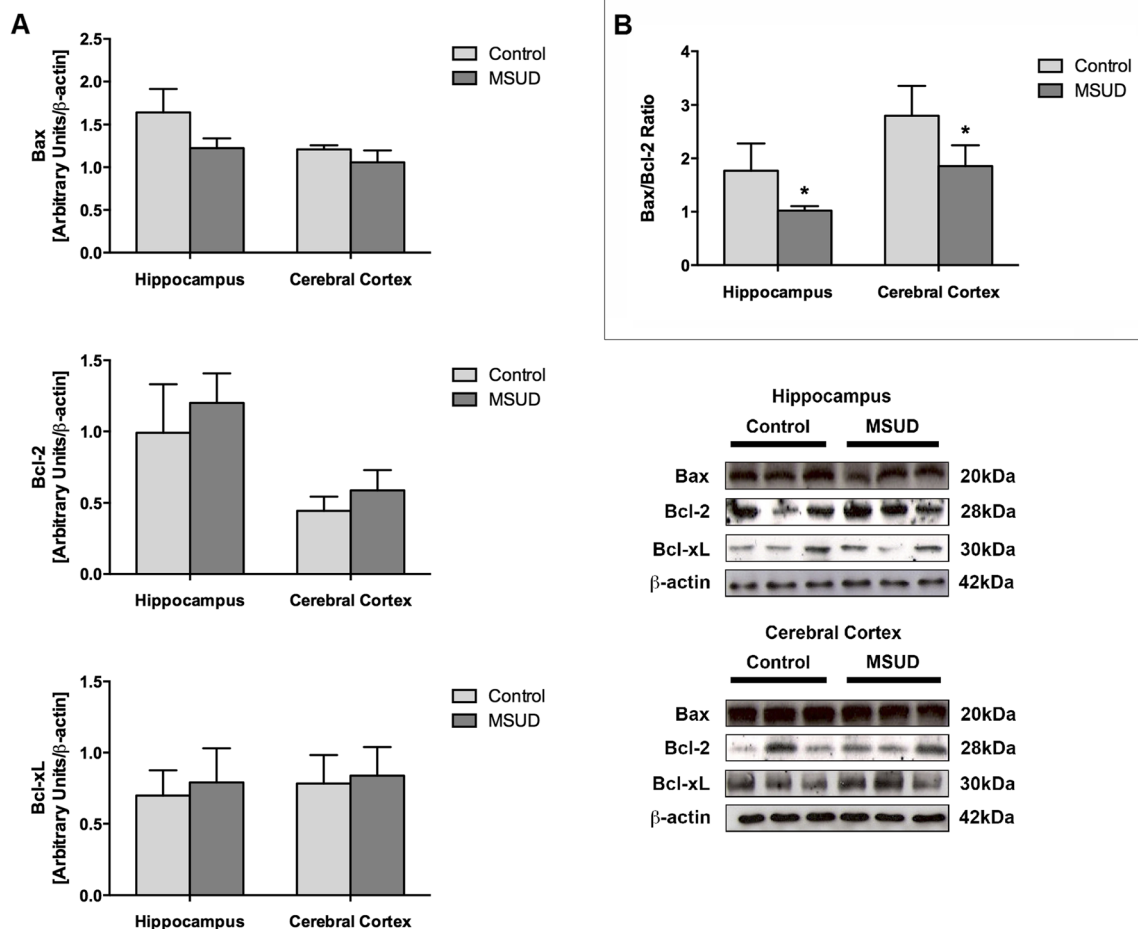


Fig. 1 Effect of acute administration of branched-chain amino acids on Bax, Bcl-2 and Bcl-xL (a) and Bax/Bcl-2 ratio (b) in hippocampus and cerebral cortex of 10-day-old rats. Data are ratios of optical density of

Bcl-2, Bcl-xL and Bax to β -actin, expressed as arbitrary units/ β -actin. Data are expressed as mean \pm standard deviation for 5–6 animals per group. Different from control, * $p < 0.05$ (Student's t test)

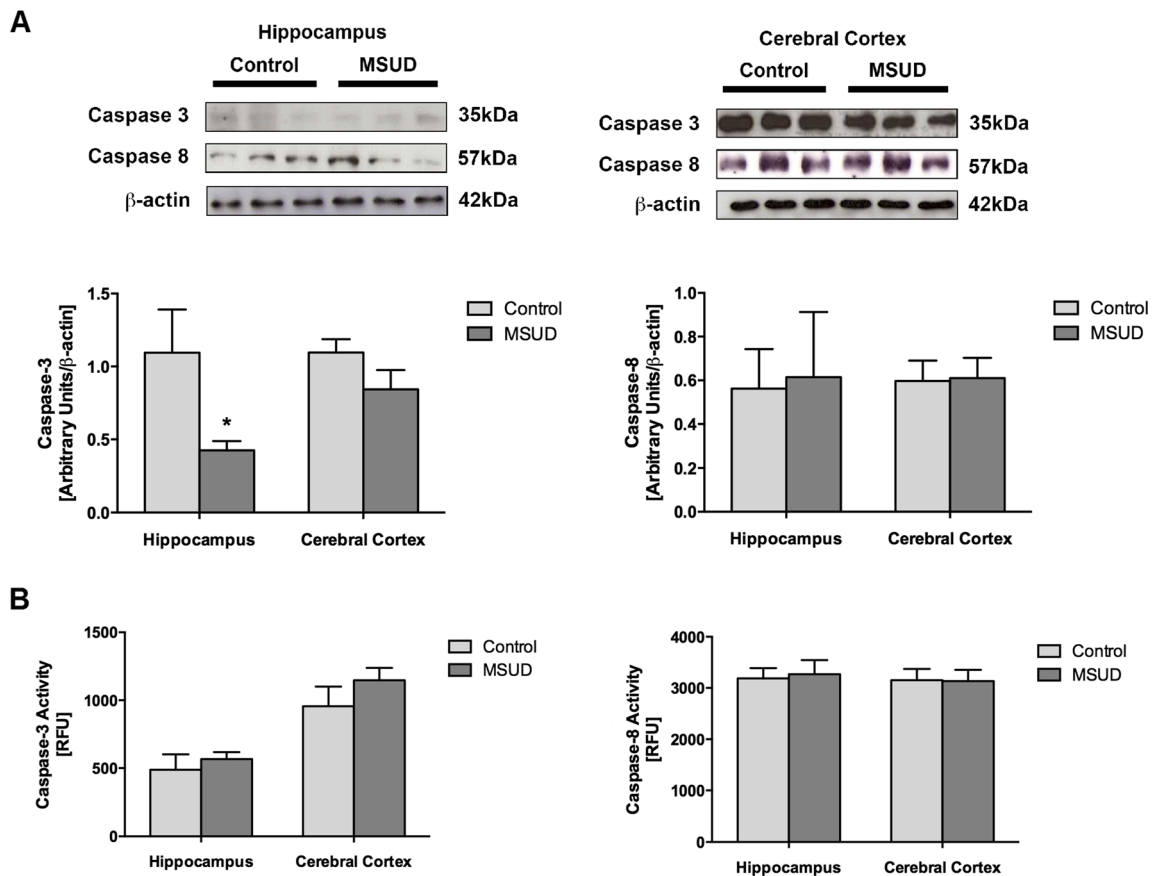


Fig. 2 Effect of acute administration of branched-chain amino acids on protein level of procaspase-3 and -8 (a), and enzyme activity levels for caspase-3 and -8 (b) in hippocampus and cerebral cortex of 10-day-old

rats. Data are expressed as mean \pm standard deviation for 5–6 animals per group. Different from control, * $p < 0.05$ (Student's *t* test)

Student's *t* test was used for the comparison of two means. Differences between the groups were considered to be significant at $p < 0.05$. All analyses were carried out on an IBM-compatible PC computer using the Statistical Package for the Social Sciences software (Armonk, New York, USA).

Results

To determine whether BCAA leads to changes in Bcl-2 family protein levels in hippocampus and cerebral cortex after acute administration of BCAA in 10 and 30-day-old rats, the Bcl-xL, Bcl-2 and Bax protein levels were examined. Figure 1a shows that there was no significant difference in protein level of pro-apoptotic factor Bax and anti-apoptotic factors Bcl-2 and Bcl-xL among groups in the hippocampus and cerebral cortex after acute administration of BCAA in 10-day-old rats. In addition, the Bax/Bcl-2 ratio was lower in hippocampus and cerebral cortex in BCAA group when compared with control (Fig. 1b). On the other hand, the acute administration of BCAA in 10-day-old rats decreased protein levels of procaspase-3 without altering the activity of caspase-3 in hippocampus,

but not in cerebral cortex (Fig. 2a). Moreover, BCAA administration did not change protein levels of procaspase-8 and caspase-8 activity in hippocampus and cerebral cortex of 10-day-old rats (Fig. 2b).

According to the data presented in Fig. 3a, the protein levels of Bax were increased significantly in the cerebral cortex of 30-day-old rats after acute administration of BCAA, while protein levels of Bcl-2 and Bcl-xL were not affected. On the other hand, in the hippocampus of 30-day-old rats the acute administration of BCAA increased the protein levels of Bcl-2 and Bcl-xL, and did not affect the protein levels of Bax. Additionally, the BCAA administration increased significantly the Bax/Bcl-2 ratio in cerebral cortex when compared with control (Fig. 3b).

Western blot analysis showed that the levels of the procaspase-3 and -8 were significantly decreased in hippocampus of 30-day-old rats after acute administration of BCAA. However, the activity of caspase-3 and -8 were increased significantly (Fig. 4a). Moreover, a significant increase in the levels of procaspase-3 accompanied by an increase in caspase-3 activity was found in cerebral cortex of 30-day-old rats submitted to acute administration of BCAA (Fig. 4b).

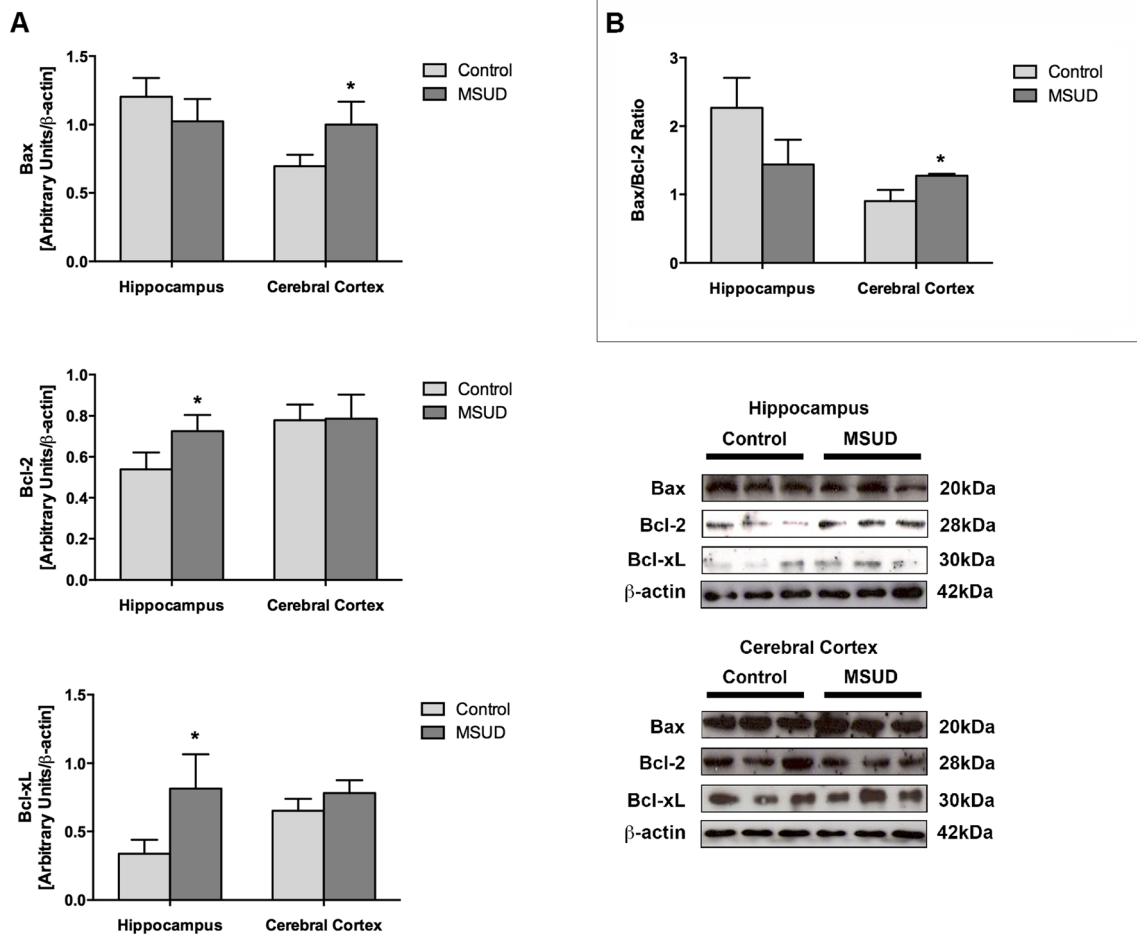


Fig. 3 Effect of acute administration of branched-chain amino acids on Bax, Bcl-2 and Bcl-xL (a) and Bax/Bcl-2 ratio (b) in hippocampus and cerebral cortex of 30-day-old rats. Data are ratios of optical density of

Bcl-2, Bcl-xL and Bax to β -actin, expressed as arbitrary units/ β -actin. Data are expressed as mean \pm standard deviation for 5–6 animals per group. Different from control, * $p < 0.05$ (Student's t test)

Discussion

Acute neurological deterioration in children is often associated with increased plasma and cerebrospinal fluid concentrations of BCAA and BCKA (Levin et al. 1993; Riviello et al. 1991). In neurological disorders, cell death occurs by necrosis or apoptosis depending on the specific disease or insult (Friedlander 2003; Kanazawa 2001; Vajda 2002). The pathway for these processes can vary and the same players may participate in both pathways. Neurological sequelae are common in MSUD patients. Brain imaging methods have structurally defined the pathophysiology seen in persons with uncontrolled MSUD, but not the cellular events that cause the dysmorphology (Brismar et al. 1990; Holmes et al. 1997; Korein et al. 1994; Ong et al. 1998; Riviello et al. 1991; Uziel et al. 1988).

In this study, it was demonstrated that BCAA acute exposure during the early postnatal period (postnatal day 10) did not significantly change Bcl-2, Bcl-xL, Bax and procaspase-8 protein levels and caspase-8 activity. On the other hand, the procaspase-3 protein levels were decreased without altering

the activity of caspase-3 in hippocampus. In addition, BCAA administration in infant rats decreased Bax/Bcl-2 ratios in hippocampus and cerebral cortex. The results suggest that acute administration of BCAA during early postnatal period does not induce apoptosis by mechanisms evaluated in this study, but a possibility that other pathway may lead to cell death at this stage of development cannot be ruled out. Corroborating this hypothesis, Jouvet et al. (2000b) showed that direct intracerebral injection of KIC leads to neuronal apoptosis in the hippocampus of neonatal rats in a dose-dependent manner.

Briefly, the proapoptotic Bax and antiapoptotic Bcl-2 are membrane-bound pore-forming proteins that interact through heterodimerization. Together, they regulate the mitochondrial transmembrane passage of cytochrome c, which in turn activates caspase proteins. The Bax/Bcl-2 ratio appears more important than the individual Bax or Bcl-2 levels on determining a cell vulnerability to apoptosis; high Bax/Bcl-2 ratios lead to greater apoptotic activity (Oltvai et al. 1993). Neuronal apoptosis in the CNS is significantly lower than normal in Bax-deleted mice and in Bcl-2-overexpressing mice, while in Bcl-

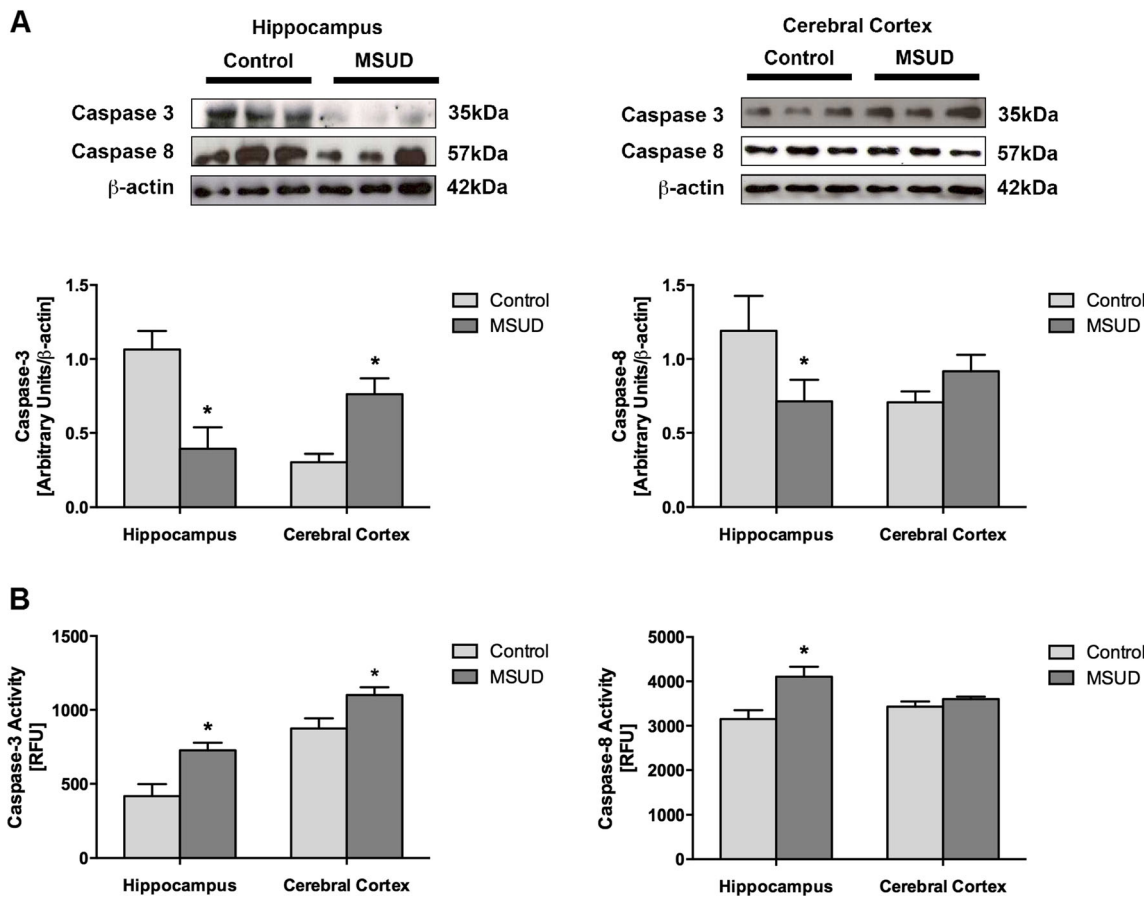


Fig. 4 Effect of acute administration of branched-chain amino acids on protein level of procaspase-3 and -8 (a), and enzyme activity levels for caspase-3 and -8 (b) in hippocampus and cerebral cortex of 30-day-old. Data are ratios of optical density of caspase-3 and caspase-8 to β -actin,

expressed as arbitrary units/ β -actin. Data are expressed as mean \pm standard deviation for 5–6 animals per group. Different from control, * $p < 0.05$ (Student's t test)

2-deleted mice and in Bax-overexpressing mice, apoptosis is greater than normal (Vekrellis et al. 1997). Furthermore, the ratio between Bax/Bcl-2 appears to be essential in deciding the survival of a cell (Korsmeyer 1999; White 1996). In this study we showed that BCAA administration in 30-day-old rats induces increase in Bax/Bcl-2 ratio followed by an increased caspase-3 activity in cerebral cortex, suggesting that apoptosis is activated after BCAA exposure in 30-day-old rats and may contribute to secondary cell injury and cell death. The increased activities of caspase-3 induced by BCAA suggests that BCAA-induced apoptosis in the cerebral cortex was likely mediated by mitochondria/cytochrome c pathway.

On the other hand, our results shown that BCAA induces apoptosis in hippocampus through activation and cleavage of caspase-3 and -8 without changing the Bax levels. Caspase-8 may be involved in death receptor-mediated apoptosis pathway (Ashkenazi and Dixit 1998). Following trimerization of the Fas receptor, procaspase-8, one of the most proximal members of caspases, is recruited to the Fas receptor–FADD complex and is physically associated with Fas-Associated protein with Death Domain (FADD) via a death effector

domain (DED) homology interaction (Chinnaiyan and Dixit 1997), resulting in the activation of procaspase-8 to activated caspase-8 and finally to the activation of caspase-3. In this line, we suggest that BCAA induces apoptosis in hippocampus through extrinsic (death receptor) pathways. Supporting this hypothesis Juvet et al. (2000b) showed that apoptosis induced by BCAA was associated with a reduction in cell respiration but without impairment of respiratory chain function, without early changes in mitochondrial membrane potential and without cytochrome c release into the cytosol.

Based on the results, we suggest that BCAA can induce apoptosis in the cerebral cortex and hippocampus by multiple mechanisms, through activation of both intrinsic and extrinsic pathways, and these mechanisms can be negatively regulated by antiapoptotic proteins Bcl-2 and Bcl-xL. However, the acute administration of BCAA during early postnatal period does not induce apoptosis by mechanisms evaluated in this study. This raises questions regarding the potential age-related differences in apoptotic response to BCAA. Further study will be required to identify the apoptotic pathways and mediators, which cause the resistance to BCAA-induced

apoptosis in neonate rats. In conclusion, these results suggest that during crises of metabolic decompensation, as observed in untreated MSDU patients, when brain is exposed to high concentrations of BCAA (at millimolar concentrations) and the metabolites, various deleterious mechanisms might be triggered, including apoptosis. These observations may explain, at least in part, the neurological sequelae associated with high plasma concentrations of MSUD metabolites.

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