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# Branched-chain amino acids influence the immune properties of microglial cells and their responsiveness to pro-inflammatory signals

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ABSTRACT

The branched-chain amino acids (BCAAs) valine, leucine and isoleucine are essential amino acids involved in several important brain functions. Although commonly used as nutritional supplements, excessive intake of BCAAs might favour the establishment of neurotoxic conditions as indicated by the severe neurological symptoms characterising inherited disorders of BCAA catabolism such as maple syrup urine disease (MSUD). Recent evidence indicates that BCAAs induce excitotoxicity through mechanisms that require the presence of astrocytes. In the present study, we evaluated the effects of BCAAs on microglia, the main immune cells of the brain. As an experimental model we used primary microglial cells harvested from mixed glial cultures that had been kept in normal or high BCAA medium (H-BCAA). We show that H-BCAA microglial cells exhibit a peculiar phenotype characterized by a partial skewing toward the M2 state, with enhanced IL-10 expression and phagocytic activity but also increased free radical generation and decreased neuroprotective functions. We suggest that such an intermediate M1/M2 phenotype might result in a less efficient microglial response, which would promote the establishment of a low grade chronic inflammation and increase the likelihood of neurodegeneration. Although based on in vitro evidence, our study adds on to an increasing literature indicating that the increasing use of dietary integrators might deserve consideration for the possible drawbacks. In addition to excitotoxicity, the altered immune profile of microglia might represent a further mechanism by which BCAAs might turn into toxicants and facilitate neurodegeneration.



## 1. Introduction

Valine, leucine and isoleucine are essential amino acids that share side chain structure features to which the collective term branched-chain amino acids (BCAAs) refers to. Unlike most of amino acids, only a minor fraction of the dietary BCAAs is metabolized by the liver; the largest part of BCAAs enters the systemic circulation to reach the main sites of BCAA metabolism, namely skeletal muscles, adipose tissue and brain [1,2].

It has been estimated that in healthy subjects about 10% of infused BCAAs reach the brain where they participate to several important biochemical processes such as protein synthesis, energy production and glutamate synthesis [1–3].

Although commonly used as nutritional supplements to improve mental and physical performance [4,5], excessive intake of BCAAs might favour the establishment of neurotoxic conditions and exert negative consequences on brain functions. Neurotoxic effects of BCAAs or their catabolic products are supported by the severe neurological symptoms characterising inherited disorders of BCAA catabolism such as maple syrup urine disease (MSUD), in which levels of BCAAs, as well as their branched-chain keto-acids, increases up to about 30 folds in blood, urine and cerebrospinal fluid, as compared to control subjects, [6–8]. The negative impact of high BCAA levels on neurons is further supported by recent experimental evidence indicating that BCAAs are neurotoxic per se and enhance excitotoxicity in cortical neuronal cultures through mechanisms that require the presence of astrocytes and involve NMDA receptor activation [9]. In addition, hyperexcitability of cortical neurons by BCAAs, which could facilitate excitotoxic events, has been demonstrated by electrophysiological recordings in both cultured neurons and in motor cortex slices from mice fed with BCAA-enriched diet [10].

*Abbreviations:* 15-F2t-IsoP, 15-$F_{2t}$-isoprostane; ADP, adenosine 5 -diphosphate; Arg-1, arginase-1; BCAAs, branched-chain amino acids; BME, basal Eagle's medium; IGF-1, insulin-like growth factor-1; IL, interleukin; iNOS, inducible nitric oxide synthase; KPBS, potassium-PBS; LPS, lipopolysaccharide; mMP, mitochondrial membrane potential; MRC-1, mannose receptor; MSUD, maple syrup urine disease; mTOR, mammalian target of rapamycin; NO, nitric oxide; PBS, phosphate-buffered saline; PFA, paraformaldehyde; TMRE, tetramethylrhodamine ethyl ester perchlorate; TNF-α, tumor necrosis factor-α; UDP, uridine 5 -diphosphate

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Besides neurons and astrocytes, high BCAA levels could influence the functional activities of other types of brain cells, including microglia, the main macrophage population of brain parenchyma. Microglial cells actively survey the brain parenchyma [11] to readily respond to signals released from damaged cells or pathogens. This physiological protective function, if not properly regulated, can turn into an aberrant microglial activation and contribute to neurotoxicity. As described for peripheral macrophages, the functions acquired by microglia during the process of activation can either promote or attenuate the inflammatory response, and though not yet fully defined, the repertoire of microglial phenotypes is likely to encompass the full spectrum ranging from the exemplified classically activated (pro- inflammatory, M1), to the alternatively activated (anti-inflammatory, M2) phenotypes [12]. The correct balance between pro- and anti-inflammatory activities is critical for preserving tissue homeostasis and several local cues can disrupt it, thus promoting neuronal impairment. BCAAs have been described to influence immune functions and in particular to be required for lymphocyte responsiveness and supporting of other immune cell functions [13] but, to the best of our knowledge, no studies have been devoted to brain immune response.

In the present study, we evaluated the effects of BCAAs on microglial reactivity through the analysis of several genes and markers associated with either M1 or M2 phenotypes. As an experimental model we used primary microglial cells harvested from mixed glial cultures that had been cultivated in normal or high BCAA medium (H-BCAA) for several days. We also compared specific functional properties of normal and H-BCAA microglial cultures, including migration, phagocytosis and reaction to a typical pro-inflammatory challenge such as bacterial endotoxin (lipopolysaccharide, LPS). We show that H-BCAAs influence microglial gene expression profile and immune properties, leading to an intermediate phenotype characterized by a partial skewing toward the M2 state, increased free radical generation and phagocytic activity, and altered responsiveness to LPS.

## 2. Materials and methods

### 2.1. Reagents

All cell culture reagents were from Invitrogen (Grand Island, NY, U.S.A) and virtually endotoxin free (less then 10E.U./ml as determined by the manufacturer). LPS (from Escherichia Coli, serotype 026:B6), ADP, UDP and BCAAs were obtained from Sigma (Saint Louis, MO, USA).

### 2.2. Cell cultures

Mixed primary glial cultures were obtained from the cerebral cortex of 1-day-old rats, as previously described [14] and in accordance with the European Communities Council Directive N. 86/609/EEC. Mixed primary glial cultures were maintained for 4 days in Basal Eagle's Medium (BME), supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine and 100 μg/ml gentamicin (control medium). Culture medium was then replaced by fresh control medium or high BCAA medium (H-BCAA), consisting in control medium supplemented with 10 mM valine, leucine and isoleucine (1:1:1) or additioned with 1 mM BCAAs when indicated. After 6 days, microglial cells were harvested by mild shaking and resuspended in control medium or H-BCAA and plated on uncoated plastic wells at a density of $1.5\times10^5$ cells/cm$^2$. Cells were allowed to adhere for 20 min and then washed to remove non-adhering cells. The cultures were maintained in control medium or H-BCAA for 24 h before further analysis and throughout the entire experimental period. Cell viability was greater than 95% in both culture conditions, as tested by Trypan Blue exclusion or MTT assay (see below).

### 2.3. Actin staining and immunocytochemistry

Purified microglia were plated on coverslips at a density of $1.5\times10^5$ cell/cm$^2$ and cultured for 24 h in control medium or 10 mM H-BCAA. Cells were then fixed with 4% PFA and pre-incubated in T-PBS (0.025% Triton in PBS) plus 5% BSA for 30 min, followed by incubation with rabbit anti-Ionized calcium binding adaptor molecule 1 (Iba1) antibody (Wako; 1:1000) in T-PBS plus 5% BSA for 1 h RT. After rinsing in T-PBS, cells were incubated with N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phallacidin (NBD-PhallAcidin, Molecular Probes; 100 U/ml, 1:30) to selectively label F-actin, and Cy3 donkey-anti-rabbit (Jackson Immunoresearch; 1:200) in PBS, 1 h at RT. Coverslips were then mounted with DAKO fluorescent mounting medium.

### 2.4. Reverse transcription and real time PCR

Total RNA (1 μg) from each sample was transcribed into cDNA using the RT-PCR Superscript III kit (Invitrogen, Eugene, OR, U.S.A.) according to the manufacturer's instructions. Real-time PCR was performed on the reverse transcription (RT) products with SensiMix SYBR Kit (Bioline, UK), or (for the arginase-1 mRNA expression) with SensiMix II probe Kit (Bioline, UK), following the manufacturer's instructions, using an ABI Prism 7000 Sequence Detection System (Applied Biosystems Inc, Foster City, CA, U.S.A.). Primer sequences (from MWG Operon, Ebersberg, Germany) and accession numbers are listed in Table 1. Annealing temperature was 60 °C for all the primer pairs listed. All samples were run in triplicate, and each well of PCR contained 20 μl as a final volume of reaction, including 2 μl of cDNA corresponding to 20 ng of total RNA, 750nM of each primer and 10 μl of PCR master mix. Thermal cycling conditions were as follow: 1 cycle at 95 °C for 10 min; 40 cycles 95 °C for 15 s and 60 °C for 1 min. Expression levels of genes of interest were compared between control unstimulated cultures and H-BCAA, LPS or LPS/H-BCAA cultures using the Relative Quantification (ΔΔCt) Study of Applied Biosystems 7000 System SDS Software. Hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as internal control gene and amplification specificity was checked using a melting curve, following the manufacturer's instructions. The reference group used is indicated in the Results section, for each study.

**Table 1**
Primers used for real time PCR analyses.

| Gene | Primers sequence | Acc. number |
|---|---|---|
| HPRT | Forward 5 -CTC ATG GAC TGA TTA TGG ACA GGA C-3<br>Reverse 5 -GCA GGT CAG CAA AGA ACT TAT AGC C-3<br>Product size:123 bp | S79292 |
| IL-1β | Forward 5 - CAC CTC TCA AGC AGA GCA CAG-3<br>Reverse 5 -GGG TTC CAT GGT GAA GTC AAC-3<br>Product size 79 bp | M98820 |
| iNOS | Forward 5 -GCC ACC TCG GAT ATC TCT TG-3<br>Reverse 5 -TCT GGG TCC TCT GGT CAA AC-3<br>Product size 81 bp | NM_0126113 |
| TNF-α | Forward 5 -AAAGGGCTCCCTCTCATCAGT-3<br>Reverse 5 -TCTGCTTGGTGGTTTGCTACGA-3<br>Product size 109 bp | NM_012675.3 |
| IL-10 | Forward 5 -GCCAAGCCTTGTCAGAAATGA-3<br>Reverse 5 -TTTCTGGGGCCATGGTTCTCT-3<br>Product size 73 bp | NM_012854.2 |
| Arg-1 | Forward 5 -ATATCTGCCAAGGACATCGTG-3<br>Reverse 5 -AGGTCTCTTCCATCACTTTGC-3<br>Probe: 5 -CAATGACTGAAGTGGACAAGCTGGGA-3<br>Product size 141 bp | N0_17134 |
| MRC-1 | Forward 5 -TGG ACT AAG CCA AGG GGC AA-3<br>Reverse 5 -CAG GAG CAG GGG GAG TCT CA-3<br>Product size 121 bp | NM_001106123 |