RESEARCH ARTICLE

Complex behavioral and synaptic effects of dietary branched chain amino acids in a mouse model of amyotrophic lateral sclerosis

Aldina Venerosi[1], Alberto Martire[2], Angela Rungi[1], Massimo Pieri[3], Antonella Ferrante[2], Cristina Zona[3], Patrizia Popoli[2] and Gemma Calamandrei[1]

[1] Section of Neurotoxicology and Neuroendocrinology, Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Rome, Italy
[2] Section of Central Nervous System Pharmacology, Department of Therapeutic Research and Medicines Evaluation, Istituto Superiore di Sanità, Rome, Italy
[3] Department of Neuroscience, University of Rome ''Tor Vergata'', Rome, Italy

Received: June 30, 2010
Revised: October 13, 2010
Accepted: October 28, 2010

Scope: We hypothesized that chronic supplementation with branched chain amino acids (BCAAs) affects neurobehavioral development in vulnerable gene backgrounds.
Methods and results: A murine model of amyotrophic lateral sclerosis (ALS), G93A mice bearing the mutated human superoxide dismutase 1 (SOD1) gene, and control mice received from 4 to 16 wk of age dietary supplementation with BCAAs at doses comparable to human usage. Motor coordination, exploratory behaviors, pain threshold, synaptic activity and response to glutamatergic stimulation in primary motor cortex slices were evaluated between the 8th and 16th week. The glial glutamate transporter 1 (GLT-1) and metabotropic glutamate 5 receptor (mGlu5R) were analyzed by immunoblotting in cortex, hippocampus and striatum. BCAAs induced hyperactivity, decreased pain threshold in wild-type mice and exacerbated the motor deficits of G93A mice while counteracting their abnormal pain response. Electrophysiology on G93A brain slices showed impaired synaptic function, reduced toxicity of GLT-1 blocking and increased glutamate toxicity prevented by BCAAs. Immunoblotting indicated down-regulation of GLT-1 and mGlu5R in G93A, both effects counteracted by BCAAs.
Conclusion: These results, though not fully confirming a role of BCAAs in ALS-like etiology in the genetic model, clearly indicate that BCAAs' complex effects on central nervous system depend on gene background and raise alert over their spread use.

Keywords:
Amyotrophic lateral sclerosis / Branched chain amino acids / Central nervous system / Excitotoxicity / Transgenic mice

1 Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disease characterized by degeneration of neurons in the cortex, bulbus and spinal cord leading to progressive paralysis of bulbar, respiratory and limb muscles. The most common mutations found in familiar ALS (10% of total cases) involve the gene coding for the enzyme copper–zinc superoxide dismutase 1 (SOD1) that, however, explain only about 20% of familiar ALS cases and 2% of the sporadic form of this disease. This strongly supports the involvement of several genes and the possible role of environmental factors that might trigger the pathogenic

Correspondence: Dr. Gemma Calamandrei, Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Rome, Italy
E-mail: gemma.calamandrei@iss.it
Fax: +39-6-4957821

Abbreviations: ACSF, artificial cerebrospinal fluid; **ALS,** amyotrophic lateral sclerosis; **ANOVA,** mixed-model analysis of variance; **BCAA,** branched chain amino acids; **DHK,** dihydrokainic acid; **FPs,** extracellular field potentials; **G93A,** glycine/alanine substitution at codon 93 of SOD1; **GFAP,** glial fibrillary acidic protein; **GLT-1,** glutamate transporter 1; **mGlu5R,** metabotropic glutamate 5 receptor; **SOD1,** superoxide dismutase 1; **STD,** standard diet; **WT,** wild type

mechanisms in vulnerable individuals. So far, the transgenic mice carrying the human mutated *SOD1* gene with a glycine/alanine substitution at codon 93 (G93A) are the most widely used model for investigating the mechanism of ALS. The expression of the mutant human *SOD1* in mice leads to an ALS-like neurodegenerative pathology [1, 2]. Mutant *SOD1* seems to act at different levels, by converging molecular mechanisms that include enhanced free radical production, protein misfolding, excitotoxicity, mitochondrial dysfunction, neuroinflammation and apoptosis [1]. Glutamate-induced excitotoxicity is currently considered one of the main mechanisms involved in ALS and other neurodegenerative diseases [3, 4]. *SOD1* mutant mice and rats show selective impairment of the glutamate transporter 1 (GLT-1) subtype, the most important transporter that maintains extracellular glutamate concentration below neurotoxic levels, and altered expression of mGlu5 receptors [5].

Nutritional and exercise interventions have been assessed in pre-clinical and clinical trials with the hope of slowing the deterioration of neurons (reviewed in [6]). A line of investigation has verified the possibility that the branched chain amino acids (BCAAs) L-leucine, L-valine and L-isoleucine might be beneficial in ALS progression and motor symptoms. BCAAs, which make up about 40% of the free essential amino acids in blood plasma, play important roles in glyconeogenesis, particularly during exercise, and in the maintenance and growth of skeletal muscle, and are largely used as dietary integrators by professional and non-professional athletes.

However, studies did not yield favorable results with BCAAs. A large clinical trial was interrupted because there was an excess mortality in BCAA-treated ALS patients [7]. The results of this trial raised some concern on BCAAs' safety. Further to their role in muscle physiology, BCAAs have indeed significant biochemical and functional effects in the brain, and they are main nitrogen donors for glutamate synthesis [8]. It is thus possible that they might enhance excitotoxic mechanisms in CNS, as also suggested by a very recent study [9] that indicates that BCAAs significantly increase excitotoxicity in cultured rat cortical neurons. This is of particular concern since there is no good evidence for establishing BCAA tolerance levels in humans [10, 11] while their potential clinical use is currently proposed both in healthy individuals and in several metabolic and neurological human diseases (see [12] for a comprehensive review).

The role of gene–environment interaction in the etiology of sporadic ALS has received some support from the recent epidemiological findings reporting a tenfold increase in mortality rate for ALS among a specific subpopulation of athletes, the soccer players, both in Italy and US [13–15]. Among other factors, including recurring traumas and the use of anti-inflammatory drugs, high consumption of BCAA-containing dietary supplements has been suggested to play a role among susceptible individuals. Dietary integrators might have adverse impact in the case of gene mutations or variants reducing antioxidant defenses.

With this in mind, animal studies evaluating a specific vulnerability to dietary BCAAs in both normal and vulnerable gene backgrounds are needed to verify their potential toxicity. The present study investigates the hypothesis that chronic dietary supplementation with BCAAs could influence ALS-like symptoms in G93A transgenic model. Following BCAA supplementation from the first month of life, motor coordination, exploration and pain threshold were evaluated at 8, 9 and 12 wk of age. At the appearance of the frank neurological phenotype, mice were sacrificed and cortical synaptic transmission, electrophysiological response to glutamatergic stimulation and brain expression of the GLT-1 transporter analyzed (Fig. 1).

2 Materials and methods

2.1 Animals

B6SJL-TgN (*SOD1*-G93A)1Gur mice expressing the G93A mutant *SOD1* and B6SJL-TgN (*SOD1*)2Gur mice expressing

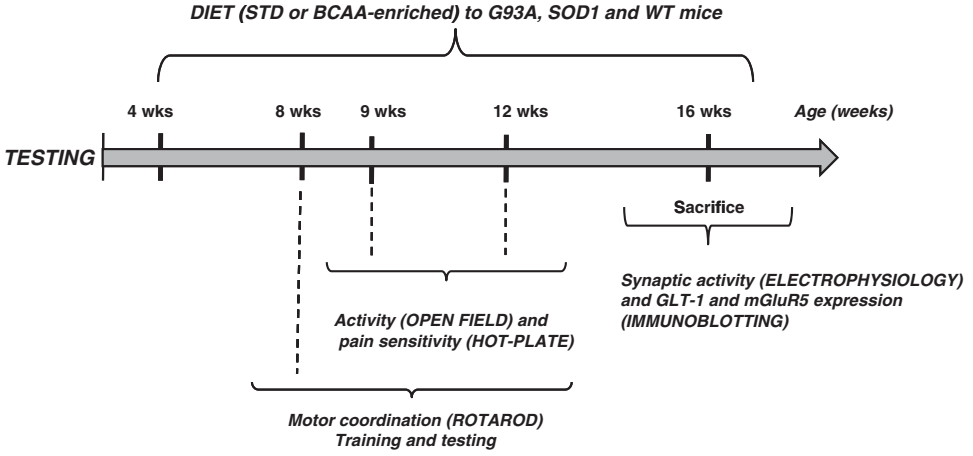

Figure 1. Experimental design of the study and temporal sequence of the different tests.

wild-type (WT) human *SOD1* constructed by Gurney *et al.* [2] were originally obtained from Jackson Laboratories (Bar Harbor, USA). They were genotyped by PCR analysis performed on DNA isolated from tail [16].

Standard (STD) or enriched BCAA diet (Mucedola, Italy) was administered from postnatal day 30 until sacrifice (16 wk of age). Food consumption was assessed once a week by weighing the residual pellets. The concentration of BCAAs in the diet was selected taking into account sport medicine indications [17], clinical protocols [7] and reported concentration of BCAAs in commercial products. The final concentration corresponded to rat NOAEL (2.5% wet/weight, 2:1:1 ratio for leucine, isoleucine and valine, respectively, see [18]), and resulted in a dose of 4 g/kg/day that is comparable to the maximum daily intake reported for professional and non-professional athletes.

A total of 14 WT male mice (7 STD diet and 7 BCAA diet), 10 SOD1 male mice (5 STD and 5 BCAA diet) and 9 SOD1-G93A (G93A) male mice (4 STD and 5 BCAA) were used in this study. The animals were kept in social condition (two/three animals in each cage) under standardized temperature, humidity and lighting conditions. Body weight was monitored throughout the entire experiment. Animal procedures were carried out according to the Italian legislation on animal experimentation (DL 116/92; personal license for animal experimentation to G. Calamandrei n° 224/2009-B).

2.2 Motor coordination – rotarod

Rotarod apparatus (Basile, Comerio, Italy) was used to measure fore- and hind-limb motor coordination and balance. Fourteen WT (7 STD and 7 BCAA diet), 10 SOD1 (5 STD and 5 BCAA diet) and 9 G93A mice (4 STD and 5 BCAA) underwent this test. During training, each mouse was placed on the rod at constant rotating speed of 20 rpm no longer than 60 s, and the latency to fall off recorded. Mice received four trials/day for three consecutive days, by which time a steady baseline level of performance was attained. Training was performed at 8 wk of age and testing at 9 and 12 wk. Testing consisted in ten trials, two for each of the five speed levels (12, 16, 24, 36, 40 rpm) as in [19].

2.3 Spontaneous motor activity and exploratory behavior – open-field

At 9 and 12 wk of age, after completion of the rotarod test, animals underwent a single 20-min open-field test. Thirteen WT (six STD and seven BCAA diet), ten SOD1 (five STD and five BCAA diet) and nine G93A mice (four STD and five BCAA) were individually placed in the center of a black plexiglas open-field arena (40 cm × 40 cm), with bottom subdivided by black lines into 5 cm × 5 cm.

Recordings were scored by an observer blind to genotype and diet received. A software package for collection and analysis of data (''The Observer'', Noldus, the Netherlands) was used to analyze frequency and duration of: crossing of the square limits with both forepaws; wall rearing, standing on the hind-limbs and touching the walls with the fore-limbs; open rearing, standing on the hind-limbs in the open; grooming (licking and rubbing the head or the whole body with both forepaws); immobility; jumping.

2.4 Pain threshold – hot-plate test

Thirteen WT (6 STD and 7 BCAA diet), 9 SOD1 (5 STD and 4 BCAA diet) and 9 G93A mice (4 STD and 5 BCAA) underwent this test at 9 wk of age, and at 12 wk 13 WT (6 STD and 7 BCAA diet), 10 SOD1 (5 STD and 5 BCAA diet) and 9 G93A mice (4 STD and 5 BCAA) subjects were used. Subjects were placed in the center of the apparatus (Model D837; Basile, Socrel, Italy), consisting of a hot metal plate set at 50 ± 1°C and enclosed by a 19-cm diameter Perspex cylinder. Behavioral analysis (cut-off time 60 s) included both nociceptive (latency of forepaw and hind paw licking) and exploratory/escape responses (frequency and duration of rearing and wall rearing).

2.5 Electrophysiological recordings

G93A mice in late symptomatic phase (16 wk), SOD1 and age-matched WT were used. Animals were decapitated, the brain was removed quickly from the skull and coronal slices (300-μm thick) were cut with a vibratome and maintained at room temperature (22–24°C) in artificial cerebrospinal fluid (ACSF), saturated with 95% O_2 and 5% CO_2. After incubation in ACSF for at least 1 h, a single slice was transferred to a submerged recording chamber and continuously superfused at 32–33°C with ACSF at a rate of 2.5–2.7 mL/min [20].

Extracellular field potentials (FPs) were recorded in layers II/III of the primary motor cortex and evoked by stimulation (one stimulus every 20 s) of the transition zone between layers III and V. Three consecutive responses were averaged, acquired and analyzed with LTP software [21]. FP amplitude values were normalized, taking as 100% the average of the values obtained over the 10-min period immediately before applying the test compound. In each experiment, the mean basal FP amplitude (*i.e.* the mean from values over the 10-min period immediately before drug application) was calculated, and the effects of the drugs are expressed as percentage variation. In all experiments, the selective GLT-1 blocker dihydrokainic acid (DHK 750 μM) or glutamate (12.5 mM) was applied to the slices over 10 and 5 min, respectively, and a reduction of at least 70% of basal FP amplitude was defined as FP disappearance (washout period 30 min).

2.6 Input/output plots

For each slice, single stimuli application was delivered every 20 s (square pulses with 100 μs duration at a frequency of 0.05 Hz) and three consecutive responses were averaged. Once the response was stable, the minimum stimulus intensity necessary to evoke an observable response was measured. An input–output curve was then obtained by recording averaged responses at ~8 μA increments, starting at threshold stimulation intensity (~25 μA) and ending at a maximum of 40 μA. Each point on the input–output curve represented the averaged responses collected after at least 5 min of recording.

2.7 Western blotting

A second group of mice (three mice for each genotype and condition) were sacrificed at 16 wk, and brain areas (hippocampus, cortex, striatum) and spinal cord were immediately dissected and conserved at −80°C. Tissues were homogenated on ice in RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS with protease inhibitors) at 12 000 rpm for 20 min at +4°C and supernatants were used for protein determination (Protein Assay Kit, BioRad, CA, USA).

Fifty micrograms of protein was diluted with 10× Laemmli sample buffer, separated by 8% SDS-PAGE and transferred to PVDF membranes (BioRad) by electroblotting overnight at +4°C. Membranes were incubated for 1 h in T-TBS (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.4) containing 5% non-fat milk and then overnight at +4°C with the primary antibodies, a guinea pig anti-GLT-1 (1:2000 dilution, Chemicon International by Millipore, USA), a rabbit anti-mGLUR5 (1:1000 dilution, Upstate Biotechnology, NY, USA), a rabbit anti-glial fibrillary acidic protein (GFAP; 1:1000, Chemicon International), a mouse anti-β-actin (1:3000 dilution, Chemicon International) diluted in T-TBS containing 5% non-fat milk. Immunoreactive bands were detected by horseradish peroxidase-conjugated secondary antibodies (Chemicon International) and revealed by enhanced chemiluminescent substrate (Thermo Scientific, Rockford, USA) onto X-ray films (GE Healthcare, UK). Densitometry analysis was conducted using the ImageJ64 program.

2.8 Statistical analysis

ANOVAs for repeated measures were used to analyze behavioral data. *Post hoc* comparisons were performed using Tukey's HSD test, which can be used in the absence of significant ANOVA results [22]. To analyze electrophysiology and Western blotting results unpaired Student's *t*-test was used.

3 Results

3.1 Diet consumption and body weight

At 2 months of age, G93A mice weighed significantly less than both WT and SOD1 mice [genotype $F(2, 31) = 5.38$, $p < 0.01$ and < 0.05 after *post hoc* comparisons] but only in the STD condition. G93A mice receiving BCAA diet attained comparable weight than control groups [genotype × diet $F(2, 31) = 4.38$, $p = 0.02$, G93A/STD *vs* BCAA, $p < 0.05$ after *post hoc*]. As for food consumption G93A mice showed a trend to higher intake, regardless of the diet condition (Table 1).

3.2 Rotarod test

Training (8 wk): All mice attained a stable level of performance within the 3 days of training at constant speed [$F(2,54) = 76,74$, $p < 0.001$], irrespective of the genotype (Fig. 2, upper panel). Training performances of G93A/BCAA were significantly worse than those of G93A/STD on Day 1 [genotype × diet × day $F(4,54) = 2.6$, $p < 0.05$: G93A-BCAA *vs* G93A-STD, $p < 0.05$; G93A-BCAA *vs* WT-BCAA, $p < 0.05$; G93-BCAA *vs* SOD1-BCAA, $p < 0.05$ after *post hoc* comparisons].

Test (9 wk): Performances were significantly affected by genotype [$F(2,27) = 4.25$, $p < 0.05$] but not by diet. A significant interaction between rotation speed and genotype was found [$F(8,108) = 2.33$, $p < 0.05$]. Latency to fall was significantly lower at 40 rpm in G93A in comparison to WT and SOD1 genotype ($p < 0.05$) irrespective of the diet received (Fig. 1b).

Test (12 wk): Performances were significantly affected by genotype [$F(2,27) = 4.5$, $p < 0.05$]. A significant interaction genotype × diet × rotation speed [$F(8,108) = 1.82$, $p = 0.08$] showed that under the STD condition G93A mice fell earlier

Table 1. Mean body weight (Day 56) and mean food consumption (g/day) recorded on Days 28, 42 and 56

Diet/Strain	Body weight			Food consumption		
	WT	SOD1	G93A	WT	SOD1	G93A
STD	28.62 ± 0.65	28.4 ± 0.68	23.25 ± 1.7*	3.13 ± 0.28	2.38 ± 0.24	4.43 ± 0.25
BCAA	27.60 ± 0.56	26.6 ± 0.68	27.0 ± 1.58	2.76 ± 0.15	2.24 ± 0.48	4.83 ± 0.48

BCAA diet started on Day 30.
*$p < 0.05$ indicates a significant difference in G93A *versus* WT and SOD1 mice.

from the rod than both WT and SOD1 mice at 12 rpm (p's < 0.05, Fig 2, lower panel, left box), whereas, after BCAA supplementation, G93A mice fell earlier than SOD1 at 36 and 40 rpm (p's < 0.05, Fig. 2, lower panel, medium and right boxes). Finally, at 40 rpm, G93A-BCAA had significantly worse performances than G93A-STD ($p < 0.05$, Fig. 2, lower panel, right box).

3.3 Open-field test

The observation of spontaneous behavior in the open-field at 9 wk revealed significant differences due to genotype, diet and their interaction (Fig. 3). ANOVA yielded a main effect of genotype [$F(2,26) = 8.16$, $p < 0.01$] and diet [$F(1,26) = 10.3$, $p < 0.01$] on crossing frequency. While G93A mice were significantly more active than WT mice ($p < 0.05$ after *post hoc* comparisons), BCAA diet enhanced motor activity in all genotypes. Wall-rearing frequency was significantly affected by genotype [$F(2,26) = 6.05$, $p < 0.01$], diet [$F(1,26) = 7.13$, $p < 0.05$] and their interaction [$F(2,26) = 6.39$, $p < 0.01$]: G93A-BCAA mice displayed significantly more wall rearing than WT and SOD1 mice exposed to the same diet ($p < 0.05$). Separate analysis of motor activity in the central or peripheral area of the arena (data not shown) indicated that G93A mice were more active than WT and SOD1 mice [center,

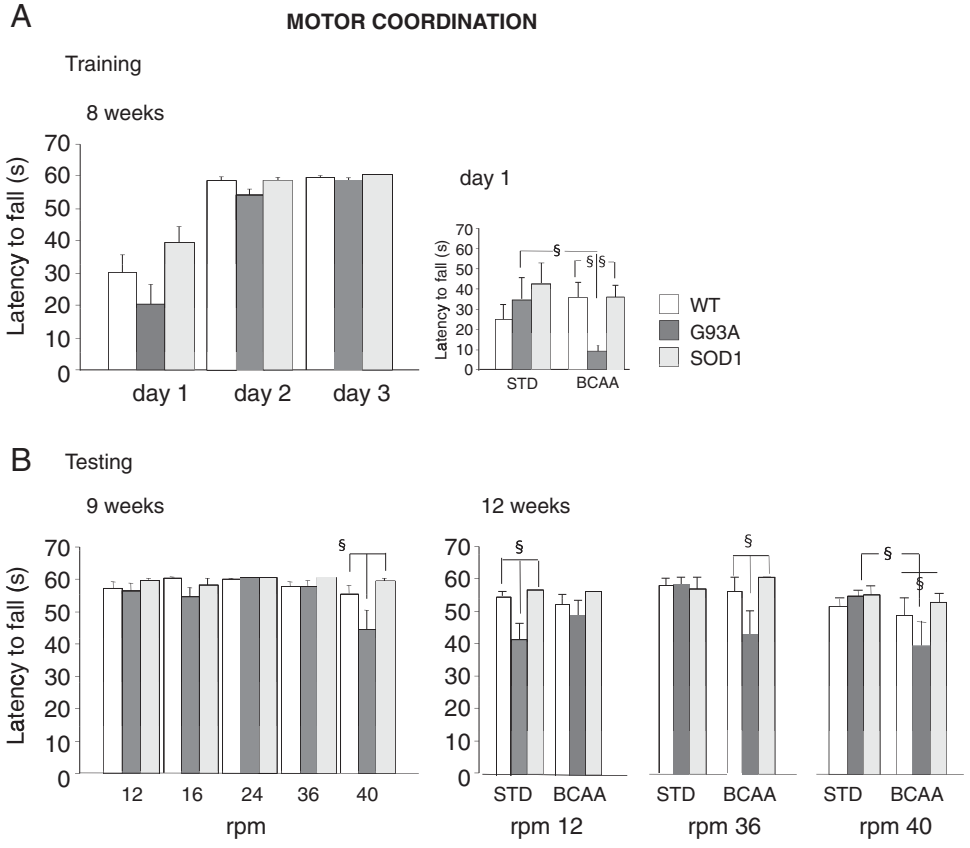

Figure 2. Motor coordination (means ± SEM) assessed in G93A, SOD1 and WT mice using a Rotarod apparatus. (A) Training session, latency to fall during a 3-day test (constant speed of 20 rpm); bordered graph: latency to fall on Day 1. $^{\S}p < 0.05$ after *post hoc* comparisons on genotype × diet interaction. (B) Testing session (top graphs) at 9 wk at five different speeds ranging from 12 to 40 rpm; §Significant difference G93A mice *versus* control groups after *post hoc* comparisons on significant genotype × speed interaction ($p \leq 0.05$). Bottom graphs: 12 wk of age, latency to fall at 12, 36 and 40 rpm, $^{\S}p < 0.05$ after *post hoc* comparisons on genotype × diet × speed interaction ($p \leq 0.05$).

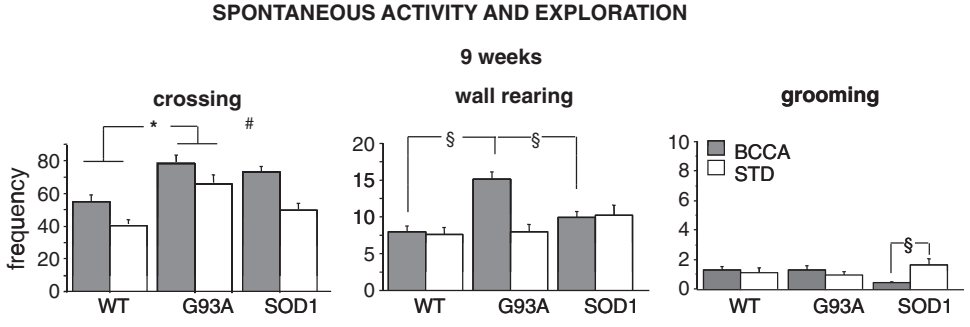

Figure 3. Frequency of crossing, wall rearing and grooming (mean ± SEM) recorded during a 20-min open-field test. *Significant effect of genotype ($p \leq 0.05$); $^{\#}$significant effect of diet ($p \leq 0.05$); §significant effect of genotype × diet interaction ($p \leq 0.05$).

$F(2,26) = 7.86$, $p < 0.01$; periphery, $F(2,26) = 6.97$, $p < 0.01$] in both areas. Grooming frequency was significantly affected by the interaction between genotype and diet [$F(4,52) = 2.95$, $p < 0.05$]: BCAA diet decreased grooming only in SOD1 genotype.

Unfortunately, at 12 wk of age video recordings of one SOD1-STD, one G93A-STD, one WT-STD and one WT-BCAA were lost for damaging of the tapes and this diminished the power of statistical analysis. Neither crossing nor wall-rearing frequency was significantly affected by genotype or diet, although the hyperactivity observed at 9 wk seemed to be present in G93A mice at 12 wk too (data not shown). BCAA diet significantly reduced rearing duration [$F(1,22) = 4.8$, $p < 0.05$].

3.4 Hot-plate test

At 9 wk, pain sensitivity was not affected by the genotype, either when considering latency to forepaw (Fig. 4, upper panel) or hindpaw licking. BCAA diet decreased latency to first licking of the forepaw [$F(1,25) = 5.012$ $p < 0.05$] and increased frequency of both fore- and hindpaw licking [$F(1,25) = 4.17$, $p = 0.05$ and $F(1,25) = 4.35$, $p < 0.05$, respectively]. These effects are consistent with a hyperalgesic effect of BCAA also potentially related to the diet-induced hyperactivity observed at this same age. When considering behaviors not directly related to pain sensitivity but describing the general response of the animals to the noxious stimulus, G93A mice showed higher wall-rearing frequency than WT and SOD1 [genotype $F(2,25) = 3.63$, $p < 0.05$], an effect that resulted significant only in the STD condition ($p < 0.05$ after *post hoc* comparisons performed on the significant interaction between diet and genotype).

At 12 wk of age (Fig. 4, lower panel), latency to forepaw licking was significantly affected by diet [$F(1,26) = 15.65$, $p < 0.01$], genotype [$F(2,26) = 3.67$, $p < 0.05$] and their interaction [$F(2,26) = 5.17$, $p < 0.05$]. G93A-STD mice had higher latency to pain response when compared either with WT-STD and SOD1-STD ($p < 0.05$, after *post hoc* comparisons), while under the BCCA condition G93A showed lower pain threshold than those fed STD diet. A trend to lower frequency of forepaw licking was shown by G93A mice [$F(2,26) = 3.13$, $p = 0.06$]. Genotype affected wall rearing [$F(2,26) = 3.67$, $p < 0.05$] as G93A performed this behavior first than the other two groups ($p < 0.05$, after *post hoc* comparisons). Finally, in agreement with results at 9 wk, genotype increased wall-rearing frequency, markedly elevated in G93A mice [$F(2,26) = 12{,}52$, $p < 0.01$] irrespective of diet.

3.5 Electrophysiology

All mice were sacrificed at about 16 wk of age, when ALS-like neurological symptoms (tremors and paralysis of one or more limbs) were detected.

Analysis of the FP amplitude indicated a significant impairment of synaptic function (Fig. 5A) in G93A-STD mice compared with age-matched WT-STD only at the higher stimulation intensity, 44 μA (0.3 ± 0.04 mV, $N = 10$ and 0.47 ± 0.04, $N = 11$, G93A-STD *vs* WT-STD mice, respectively, $p < 0.05$, Student's *t*-test, Fig. 5B), while basal synaptic transmission did not differ among genotypes in the BCAA condition (Fig. 5B).

In some control experiments, in order to confirm the glutamatergic nature of FPs recorded, the α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (50 μM, 10 min) was applied to slices, inducing the complete disappearance of synaptic transmission activity; on the contrary, the application of the *N*-methyl-D-aspartic acid receptor antagonist AP5 (50 μM, 10 min) induced only a slight and negligible reduction of FP amplitude (data not shown).

A lower vulnerability to the selective GLT-1 blocker DHK in the cortex of G93A-STD *versus* WT-STD mice was found. Specifically, in primary motor cortex slices obtained at 16 wk, 750 μM DHK induced an almost complete disappearance of the electrical response (Fig. 5C) followed by a recovery that, 30 min after washout, was significantly higher in G93A-STD than in WT-STD ($89.61 \pm 10.56\%$, $N = 8$ and $57.55 \pm 7.73\%$, $N = 8$, of pre-drug values, respectively; $p < 0.05$, to Student's *t*-test, Fig. 5C).

The decreased vulnerability to DHK in the cortex was maintained in G93A-BCAA with respect to WT-BCAA mice. As shown in Fig. 5D, 10 min of slice perfusion with DHK 750 μM induced a transient reduction of synaptic transmission followed by a recovery that was significantly higher in G93A-BCAA mice than in WT-BCAA mice after 30 min of washout ($115.1 \pm 11.8\%$, $N = 3$ and $67.05 \pm 12.42\%$, $N = 4$ of pre-drug values, respectively; $p < 0.05$, Student's *t*-test, Fig. 5D).

As for glutamate-induced toxicity, increased vulnerability was found in the cortex of G93A-STD *versus* WT-STD mice. In cortical slices, 12.5 mM glutamate induced a complete disappearance of the electrical response (Fig. 5E) followed by a recovery that, 30 min after washout, was significantly higher in WT-STD than in G93A-STD mice ($44.9 \pm 7.2\%$, $N = 3$ and, $19.7 \pm 5.2\%$, $N = 3$, of pre-drug values, respectively; $p < 0.05$, Student's *t*-test, Fig. 4E). The increased vulnerability to glutamate was no longer observed in G93A-BCAA diet with respect to WT-BCAA diet. As shown in Fig. 5F, 5 min of slice perfusion with 12.5 mM glutamate transiently reduced synaptic transmission, followed after 30 min of washout by similar recovery in G93A-BCAA and in WT- BCAA mice ($31.2 \pm 6.4\%$, $N = 3$, and $41.4 \pm 5.7\%$, $N = 3$ of pre-drug values, respectively, Fig. 5F). The BCAA diet did not influence at all the effects elicited by DHK or glutamate in slices from SOD1 mice (data not shown).

3.6 Western blotting

G93A-STD mice (Fig. 6, lane 3) presented a significant lower amount of GLT-1 in the cortex with respect to WT

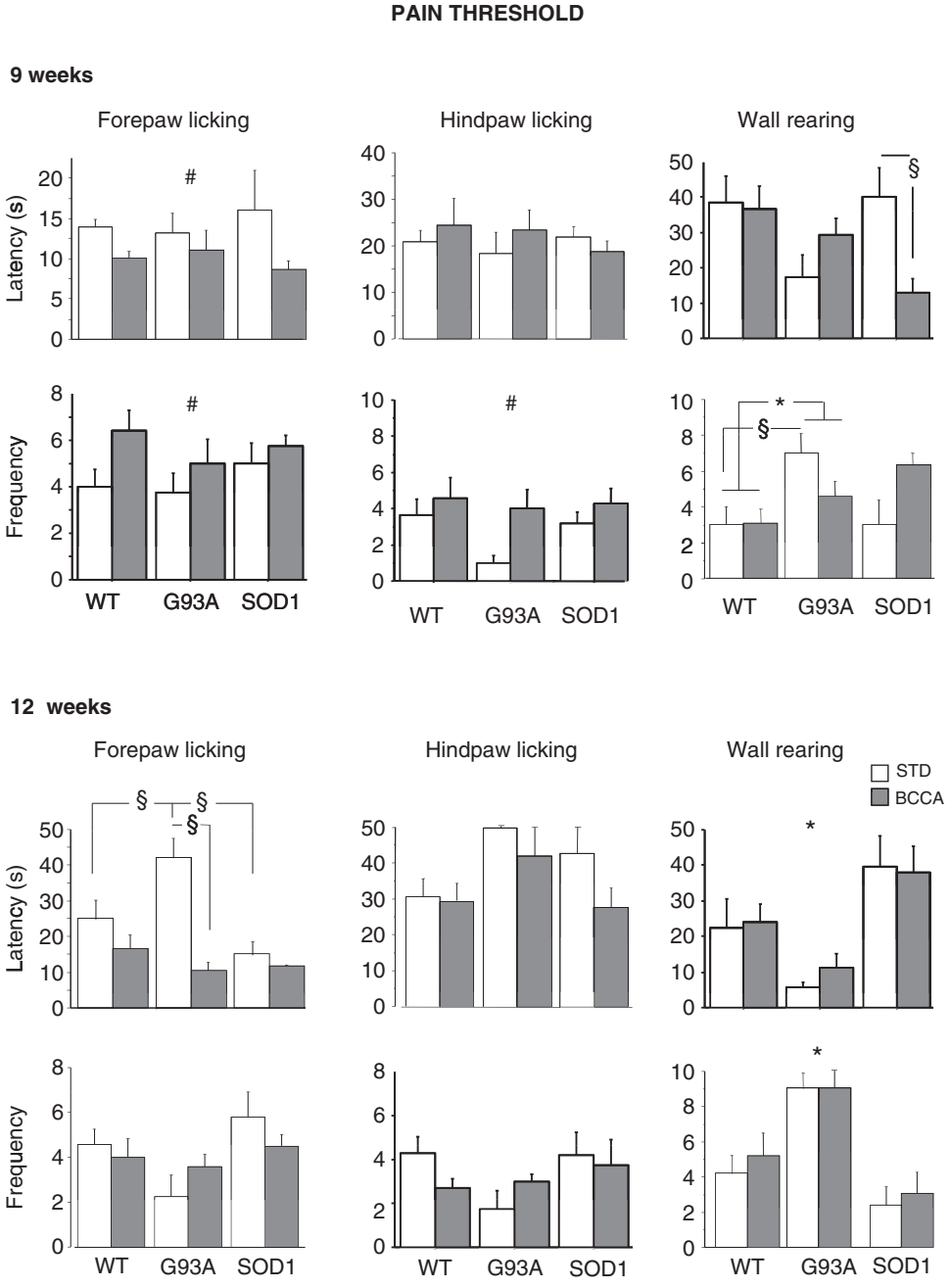

Figure 4. Behavioral responses (mean ± SEM) assessing pain threshold and general activity in a hot-plate apparatus set at 50 ± 1C°. *Significant effect of genotype ($p \leq 0.05$); ♯significant effect of diet ($p \leq 0.05$); §significant effect of genotype × diet interaction ($p \leq 0.05$).

(lane 4). However, G93A-BCAA mice (lane 1) showed up-regulation of GLT-1 expression if compared with lane 3, whereas no difference with their WT littermates was evident (lane 2). GLT-1 was down-regulated also in the striatum and in the spinal cord of G93A-STD (lane 3) with respect to their WT control (lane 4). The protein amount was greater in G93A-BCAA (lane 1) than in G93A-STD (lane 3). In spinal cord, G93A-BCAA (lane 1) neither expressed more GLT-1 protein than WT (lane 2) nor than SOD1 mice treated with STD (lane 3). No significant difference in any of the areas considered was evident between SOD1 mice fed with (lane 5) and without BCAA diet (lane 6). In conclusion, GLT-1 was down-regulated in G93A mice in all brain areas examined, except in the hippocampus, and BCAAs induce GLT-1 overexpression in cortex and striatum in G93A mice only.

As for mGluR5 expression, down-regulation was observed in the cortex and in the striatum of G93A mice (Fig. 6C and D). Spinal cord results are not shown because it

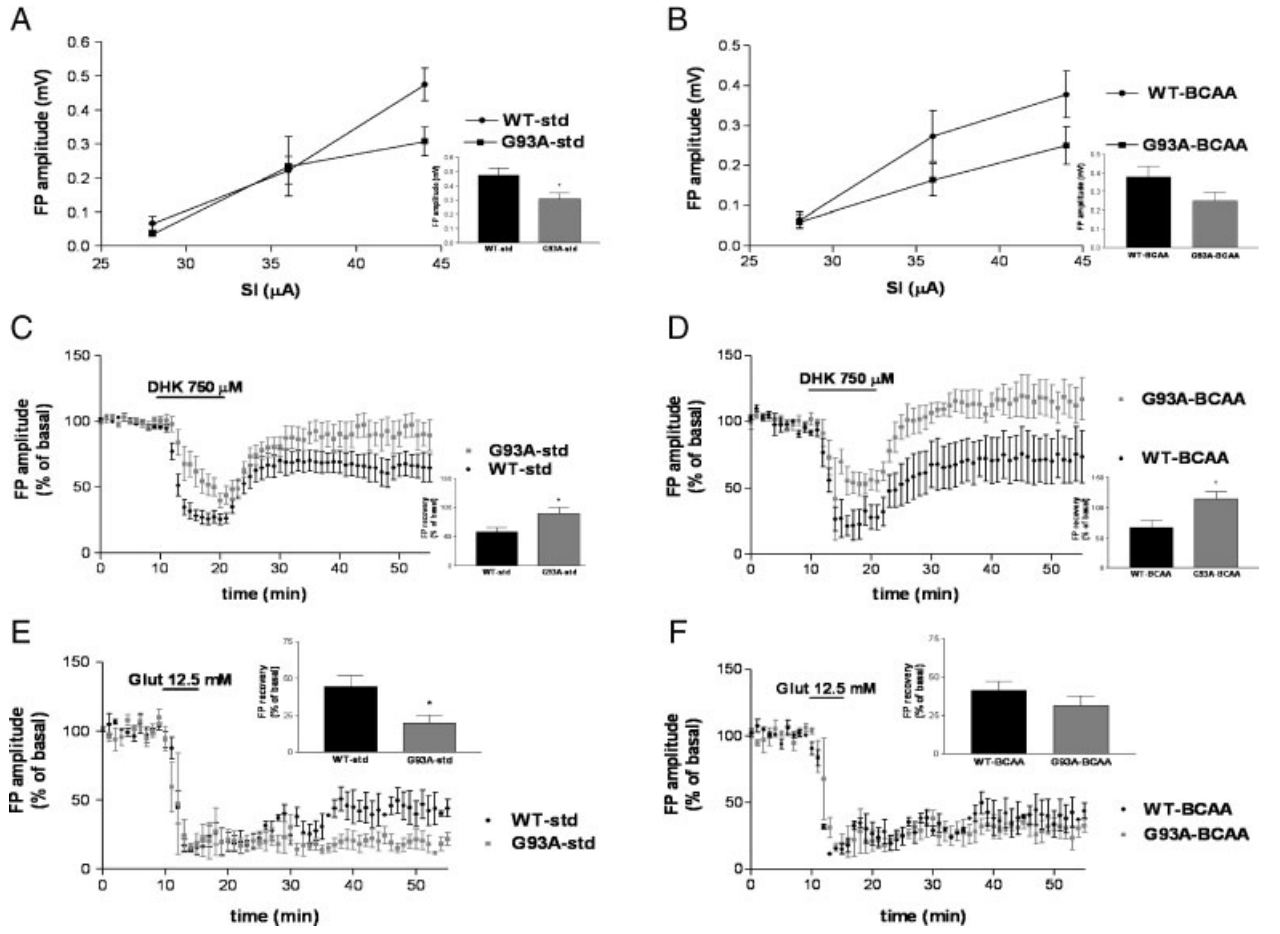

Figure 5. (A) Input/output plots of the FPs' amplitude in slices from 16-wk-old WT-STD (circles) and G93A-STD mice (squares). In the inset, significant impairment of synaptic function in G93A-STD mice compared with age-matched WT-STD. (B) Basal synaptic transmission is not different in WT (circles) and G93A mice (squares) in the BCAA diet condition. (C) Lower vulnerability to DHK in the cortex of G93A-STD *versus* WT-STD mice. (D) FP recovery after DHK was significantly higher also in G93A-BCAA mice with respect to WT-BCAA mice. (E) Increased vulnerability to glutamate in the cortex of G93A *versus* WT mice. (F) FP recovery after glutamate was no longer different in G93A-BCAA mice with respect to WT-BCAA. $^{*}p<0.05$ *versus* WT according to Student's *t*-test.

was not possible to obtain a satisfactory signal. The cortex and the striatum of G93A-STD (lane 3) showed a significantly lower amount of mGluR5 than their control (lane 4); conversely, the G93A-BCAA (lane 1) showed up-regulation of the receptor if compared with G93A–STD (lane 3). No differences were detected in mGluR5 expression in SOD1 mice.

Since mGluR5 overexpression was found in activated astrocytes isolated from spinal cords of ALS patients [23], we attempted to verify if the mGluR5 up-regulation induced by BCAAs in G93A mice could be due to astrogliosis. We evaluated the expression of the GFAP, which is up-regulated in reactive astrocytes. BCAA diet did not influence GFAP expression in hippocampus, cortex and striatum of G93A-BCAA mice with respect to G93A-STD mice (data not shown). Moreover, an overexpression of GFAP was evident only in the spinal cord of G93A mice, irrespective of the diet received.

4 Discussion

It is well accepted that environmental factors could contribute to the occurrence of sporadic ALS. In particular, it has been speculated that the abnormal proportion of football players who are reported to develop this disease could result from the interaction between a specific genetic background and the life-style conditions – *e.g.* physical activity, use of drugs and diet supplementation – which characterize this population of athletes. The focus on the dietary regimen, which includes specifically combination of BCAAs as supplementation, was supported by the role of BCAAs in the synthesis of glutamate, this later reportedly involved in the pathogenesis of ALS [1, 24, 25]. The results of the present study, though not confirming a causative role of BCAA supplementation in inducing/accelerating onset of ALS-like pathology in a transgenic mouse model, clearly indicate that chronic exposure to BCAAs (at doses comparable to human

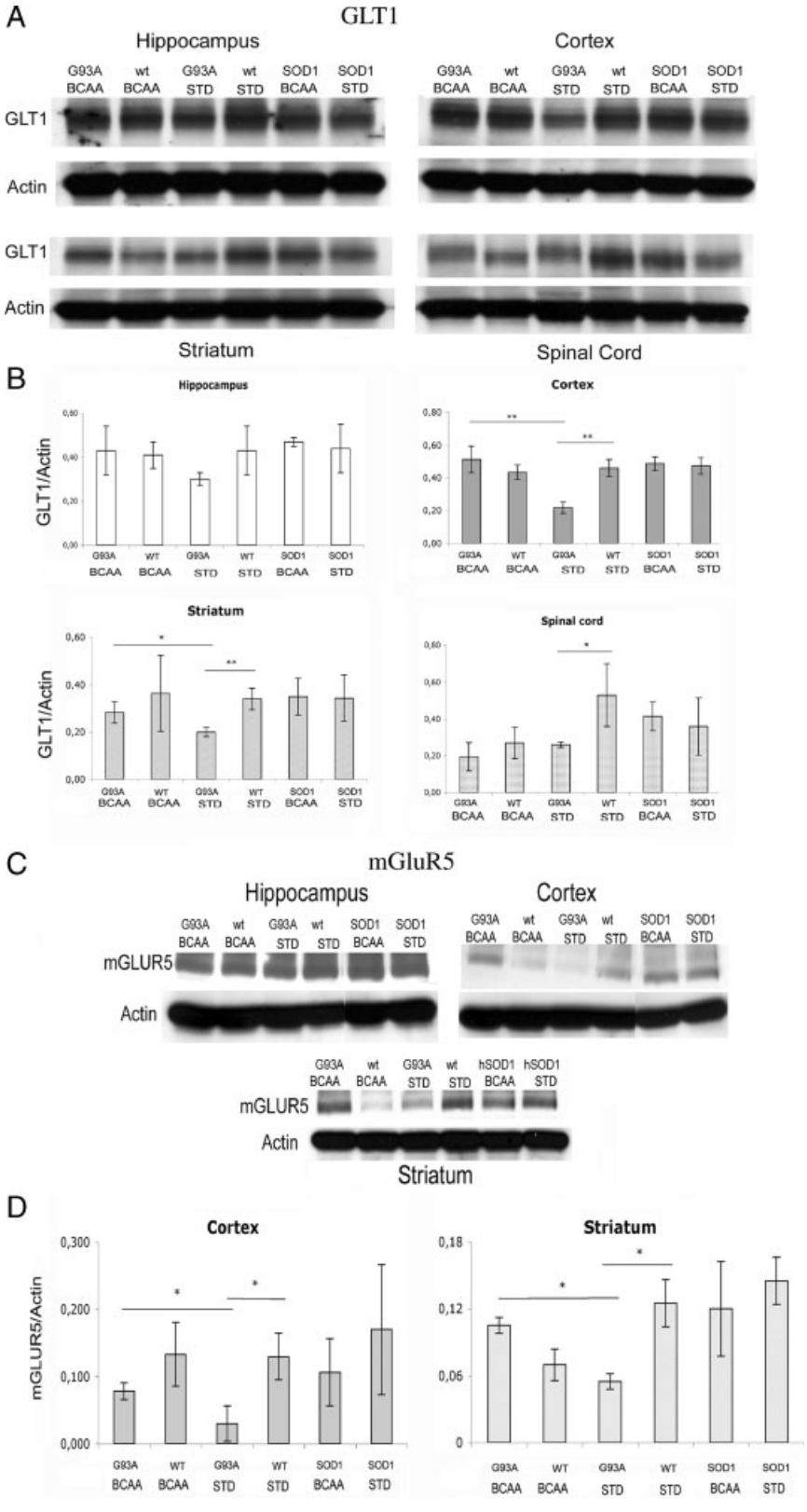

Figure 6. Western blot analysis of GLT-1 (hippocampus, cortex, striatum, spinal cord 70 kDa; (A) and mGLUR5 (hippocampus, cortex, striatum 130 kDa; (C) expression in G93A, *SOD1* and WT mice at 16 wk. An anti-actin antibody was used to identify variations in total protein amounts. One out of three separate experiments conducted on three different mice is represented. (B and D) Data are means ± SEM of three separate experiments ($^{*}p < 0.05$ and $^{**}p < 0.005$).

usage) has significant effects on exposed animals, and that G93A mice show a specific susceptibility to BCAA supplementation.

In agreement with previous reports, G93A mice consuming the STD diet had lower weight than WT and SOD1 mice, an effect counteracted by BCAA supplementation. In addition, G93A consumed more food than their control groups in both diet conditions, thus suggesting higher energetic need, possibly associated with enhanced basal metabolism previously reported in this same mutant strain [26]. The body weight recovery observed in G93A mice supplemented with BCAA was not associated with improved motor coordination. On the contrary, 1 month of BCAA supplementation significantly impaired performance of G93A mice on the first day of rotarod training already at 8 wk of age, though they acquired the task by the third day of training. At 9 wk, an initial sign of motor coordination deficit appeared in the mutant mice at the highest rotation speed, irrespective of the diet received, 2 wk before than usually reported in this strain [1, 27]. A significant decline in motor coordination performance became manifest at 12 wk of age during the second testing session: of notice at this stage G93A performance was significantly worsened by BCAA diet supplementation.

In the open-field test at 9 wk of age, G93A mice were far more active than control mice. The enhanced spontaneous activity in the pre-symptomatic stage is in agreement with recent data indicating higher activity in running wheels by G93A mice than control mice, which preludes to a sharp decline in motor performance 10–20 days prior to the symptomatic stage [28]. BCAA diet *per se* also increased significantly basal activity in the open-field test. At 9 wk of age, BCAA-fed mice had higher crossing frequency than STD-fed mice, irrespective of the gene background. Interestingly, such hyperactivating effect was more marked in the G93A genotype that displayed higher frequency of wall rearing too after BCAA supplementation. Thus, it is also possible that the early motor coordination deficit observed in the rotarod in G93A mice could be ascribed to motor hyperactivity rather than to motor coordination impairment.

At 9 and 12 wk hot-plate data further confirmed the hyperactive profile of G93A mice while BCAA *per se* induced hyperalgesia in all genotypes. At 12 wk of age G93A-STD showed a very high pain threshold and thus BCAA supplementation restored control-like pain sensitivity in G93A mice too.

Electrophysiology and immunoblotting studies performed in the full symptomatic stage highlighted a rather complicated picture, where BCAA supplementation either had no effect or apparently counteracted some of the abnormalities in glutamatergic function already reported for G93A mice. In ALS pathogenesis, an abnormal increase in the glutamatergic tone is believed to occur due to the down-regulation/inactivation of glutamate transporters, and of the GLT-1 subtype in particular [3, 4].

In agreement, we found a significant reduction in GLT-1 expression in the brain and the spinal cord of G93A mice, and increased vulnerability to application of glutamate in cortical slices. The reduction of GLT-1 was completely prevented in the cortex and significantly attenuated in the striatum after BCAA supplementation. Furthermore, the increased glutamate-induced toxicity was no longer observed in cortical slices of G93A-BCAA mice. On their whole, electrophysiology results would suggest a beneficial influence of BCAA diet on the perturbation of glutamate uptake. Again, all the effects elicited by the enriched diet were specific for the G93A genotype, since no differences were observed in WT or SOD1 mice.

mGlu5Rs efficiently modulate glutamate uptake by activating GLT-1, a mechanism that is impaired in cortical astrocytes derived from G93A rats [5]. Based on the above observation, our findings – decreased expression of mGlu5Rs in G93A mice and up-regulation of these receptors after BCAA diet – seem to support a beneficial influence of the diet on the regulation of glutamate uptake. Interestingly, such an up-regulation is not associated with astrogliosis, since identical levels of GFAP were observed in G93A STD-and BCAA-fed. This might indicate that the increased expression of mGlu5Rs induced by BCAAs mostly takes places in neurons. Since neuronal mGlu5Rs also mediates pro-excitotoxic effects [29, 30], the functional nature (*i.e.* beneficial *vs* detrimental) of the presently reported up-regulation remains to be clarified. Furthermore, since because of the limits of the immunoblotting technique used here we could not explore the diet influence on mGlu5R in the spinal cord, an important piece of information is lacking with respect to findings in ALS patients [31, 32].

Electrophysiology also showed lower vulnerability to DHK in the cortex of G93A *versus* WT mice. This finding is in line with the reduced expression of GLT-1 in G93A mice, as its blockade could exert milder effects in this genotype. Based on this interpretation, one would expect that in G93A-BCAA mice, showing increased expression of GLT-1, a 'normal' vulnerability to DHK would be reinstated. This, however, was not the case, and the recovery of the electrical response even resulted increased in the cortex of BCAA-fed mice. This suggests that the expression of GLT-1 *per se* is not a reliable predictor of the functional activity of the uptake system. Alternatively, it is possible that BCAA supplementation not only enhances the expression of GLT-1 but also that of other transporters, thus explaining why the selective blockade of GLT-1 results less effective. Whatever the mechanism, the effects of BCAA supplementation are genotype-specific, since no effects were observed in WT and in SOD1 mice.

To summarize, BCAA supplementation seems to exacerbate the behavioral profile typical of G93A mice, including significant worsening in motor performances and enhancement of hyperactivity, whereas it appears to counteract some of the effects of SOD1 mutation on glutamate neurotransmission. This seems to exclude a

direct correlation between the behavioral changes observed in G93A mice and the altered glutamatergic neurotransmission. On the other hand, a complex brain functional pathway controls motor activity in rodents, involving the interplay among glutamate, GABA, and dopamine neurotransmission. As BCAAs compete with serotonin and catecholamine precursors to enter the brain through the same transporter [12], it is also possible that a rise in BCAAs' plasma levels interferes with the maturation and function of neurochemical systems further to glutamatergic one. Furthermore, behavioral studies have been performed at 9–12 wk of age, while electrophysiology and Western blotting were done in end-stage animals (16 wk). Thus, it is not surprising that the effects of BCCA diet are not the same on behavioral parameters and on glutamate homeostasis, and it is unlikely that a causal link exist between these effects.

The increased pain threshold observed in G93A mice was counteracted by BCAA supplementation. A number of mechanisms are involved in pain response in mammals, and it is hard to advance hypotheses on the mechanisms linking BCAA supplementation, gene background and pain sensitivity. However, as mGlu5 receptors are involved in modulation of nociceptive response [33] and mGlu5R antagonists are analgesic [34], hypoalgesia found in G93A mice at 12 wk might be related to mGluR5 reduction. According to this view, reinstatement of control level pain sensitivity is in full agreement with the ability of chronic BCAA supplementation to increase mGlu5R expression in ALS mice. An additional mechanism that could be considered refers to the fact that BCAAs share the same transporter with neutral amino acids, such as tyrosine, phenylalanine and tryptophan, as mentioned above. An increase in plasma concentration of BCAAs may saturate the transporter and determine a decrease in tryptophan entry and consequently serotonin synthesis [12]. Since experiments with animal models of pain indicate that serotonergic interventions reduce pain-related behavior, a possible decrease in serotonin may further explain reduction of pain threshold in BCAA-fed G93A mice. Pain is a reported symptom in late stage ALS patients; however, to our knowledge there are no evidence of altered pain sensitivity in mutant murine strains. A longitudinal analysis of pain progression in G93A model could validate this marker for therapeutic assessment.

In conclusion, the results of this study do not allow one to draw general conclusions on a possible role of BCAAs in human ALS, as the SOD1 mutation is involved only in a small proportion of sporadic ALS cases. However, our findings clearly indicate that chronic exposure to BCAAs (at doses comparable to human usage) has significant effects on exposed animals, and that G93A mice show a specific, although not univocal, susceptibility to BCAA supplementation. Considering that BCAAs are used in general population as dietary integrators to improve mental and physical performances, the finding that they can induce significant CNS effects raises alert over their spread, chronic use. The peculiar sensitivity of G93A genotype does represent a further matter of concern, suggesting that individuals with defective oxidative stress defense response might be especially vulnerable. Further experimental studies to define thresholds for adverse effects in humans need to be performed.

This research was supported by 9M30 Ministry of Health Grant to PP 'Use of branched chain amino acids during sport and risk of amyotrophic lateral sclerosis'. We thank A. M. Confaloni and N. Vanacore for comments on a first draft of this manuscript.

The authors have declared no conflict of interests.

5 References

[1] Bendotti, C., Carri, M. T., Lessons from models of SOD1-linked familial ALS. *Trends Mol. Med.* 2004, *10*, 393–400.

[2] Gurney, M. E., Pu, H., Chiu, A. Y., Dal Canto, M. C. *et al.*, Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science* 1994, *264*, 1772–1775.

[3] Sala, G., Beretta, S., Ceresa, C., Mattavelli, L. *et al.*, Impairment of glutamate transport and increased vulnerability to oxidative stress in neuroblastoma SH-SY5Y cells expressing a Cu,Zn superoxide dismutase typical of familial amyotrophic lateral sclerosis. *Neurochem. Int.* 2005, *46*, 227–234.

[4] Trotti, D., Rolfs, A., Danbolt, N. C., Brown Jr, R. H., Hediger, M. A., SOD1 mutants linked to amyotrophic lateral sclerosis selectively inactivate a glial glutamate transporter. *Nat. Neurosci.* 1999, *2*, 427–433.

[5] Vermeiren, C., Hemptinne, I., Vanhoutte, N., Tilleux, S. *et al.*, Loss of metabotropic glutamate receptor-mediated regulation of glutamate transport in chemically activated astrocytes in a rat model of amyotrophic lateral sclerosis. *J. Neurochem.* 2006, *96*, 719–731.

[6] Patel, B. P., Hamadeh, M. J., Nutritional and exercise-based interventions in the treatment of amyotrophic lateral sclerosis. *Clin. Nutr. (Ed, Scotland)* 2009, *28*, 604–617.

[7] Tandan, R., Bromberg, M. B., Forshew, D., Fries, T. J. *et al.*, A controlled trial of amino acid therapy in amyotrophic lateral sclerosis: I. Clinical, functional, and maximum isometric torque data. *Neurology* 1996, *47*, 1220–1226.

[8] Sweatt, A. J., Garcia-Espinosa, M. A., Wallin, R., Hutson, S. M., Branched-chain amino acids and neurotransmitter metabolism: expression of cytosolic branched-chain aminotransferase (BCATc) in the cerebellum and hippocampus. *J. Comp. Neurol.* 2004, *477*, 360–370.

[9] Contrusciere, V., Paradisi, S., Matteucci, A., Malchiodi-Albedi, F., Branched-chain amino acids induce neurotoxicity in rat cortical cultures. *Neurotoxicity Res.*, 2010, *17*, 392–398.

[10] Baker, D. H., Tolerance for branched-chain amino acids in experimental animals and humans. *J. Nutr.* 2005, *135*, 1585S–1590S.

[11] Hutson, S. M., Sweatt, A. J., Lanoue, K. F., Branched-chain [corrected] amino acid metabolism: implications for establishing safe intakes. *J. Nutr.* 2005, *135*, 1557S–1564S.

[12] Fernstrom, J. D., Branched-chain amino acids and brain function. *J. Nutr.* 2005, *135*, 1539S–1546S.

[13] Beretta, S., Carri, M. T., Beghi, E., Chio, A., Ferrarese, C., The sinister side of Italian soccer. *Lancet Neurol.* 2003, *2*, 656–657.

[14] Belli, S., Vanacore, N., Proportionate mortality of Italian soccer players: is amyotrophic lateral sclerosis an occupational disease? *Eur. J. Epidemiol.* 2005, *20*, 237–242.

[15] Vanacore, N., Binazzi, A., Bottazzi, M., Belli, S., Amyotrophic lateral sclerosis in an Italian professional soccer player. *Parkinsonism Relat. Disord.* 2006, *12*, 327–329.

[16] Pieri, M., Gaetti, C., Spalloni, A., Cavalcanti, S. *et al.*, Alpha-amino-3-hydroxy-5-methyl-isoxazole-4-propionate receptors in spinal cord motor neurons are altered in transgenic mice overexpressing human Cu,Zn superoxide dismutase (Gly-->93Ala) mutation. *Neuroscience* 2003, *122*, 47–58.

[17] De Lorenzo, A., Petroni, M. L., Masala, S., Melchiorri, G. *et al.*, Effect of acute and chronic branched-chain amino acids on energy metabolism and muscle performance. *Diabetes Nutr. Metab.* 2003, *16*, 291–297.

[18] Tsubuku, S., Hatayama, K., Katsumata, T., Nishimura, N. *et al.*, Thirteen-week oral toxicity study of branched-chain amino acids in rats. *Int. J. Toxicol.* 2004, *23*, 119–126.

[19] Domenici, M. R., Scattoni, M. L., Martire, A., Lastoria, G. *et al.*, Behavioral and electrophysiological effects of the adenosine A2A receptor antagonist SCH 58261 in R6/2 Huntington's disease mice. *Neurobiol. Dis.* 2007, *28*, 197–205.

[20] Tebano, M. T., Domenici, M. R., Popoli, P., SCH 58261 differentially influences quinolinic acid-induced effects in striatal and in hippocampal slices. *Eur. J. Pharmacol.* 2002, *450*, 253–257.

[21] Anderson, W. W., Collingridge, G. L., The LTP program: a data acquisition program for on-line analysis of long-term potentiation and other synaptic events. *J. Neurosci. Methods* 2001, *108*, 71–83.

[22] Wilcox, R. G., *New Statistical Procedures for the Social Sciences*, Erlbaum, Hillsdale, NJ 1987.

[23] Anneser, J. M., Chahli, C., Ince, P. G., Borasio, G. D., Shaw, P. J., Glial proliferation and metabotropic glutamate receptor expression in amyotrophic lateral sclerosis. *J. Neuropathol. Exp. Neurol.* 2004, *63*, 831–840.

[24] Boillee, S., Vande Velde, C., Cleveland, D. W., ALS: a disease of motor neurons and their nonneuronal neighbors. *Neuron* 2006, *52*, 39–59.

[25] Wilson, J. M., Shaw, C. A., Late appearance of glutamate transporter defects in a murine model of ALS-parkinsonism dementia complex. *Neurochem. Int.* 2007, *50*, 1067–1077.

[26] Dupuis, L., Oudart, H., Rene, F., Gonzalez de Aguilar, J. L., Loeffler, J. P., Evidence for defective energy homeostasis in amyotrophic lateral sclerosis: benefit of a high-energy diet in a transgenic mouse model. *Proc. Natl. Acad. Sci. USA* 2004, *101*, 11159–11164.

[27] Stam, N. C., Nithianantharajah, J., Howard, M. L., Atkin, J. D. *et al.*, Sex-specific behavioural effects of environmental enrichment in a transgenic mouse model of amyotrophic lateral sclerosis. *Eur. J. Neurosci.* 2008, *28*, 717–723.

[28] Bruestle, D. A., Cutler, R. G., Telljohann, R. S., Mattson, M. P., Decline in daily running distance presages disease onset in a mouse model of ALS. *Neuromol. Med.* 2009, *11*, 58–62.

[29] Battaglia, G., Busceti, C. L., Molinaro, G., Biagioni, F. *et al.*, Endogenous activation of mGlu5 metabotropic glutamate receptors contributes to the development of nigro-striatal damage induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in mice. *J. Neurosci.* 2004, *24*, 828–835.

[30] Popoli, P., Pintor, A., Tebano, M. T., Frank, C. *et al.*, Neuroprotective effects of the mGlu5R antagonist MPEP towards quinolinic acid-induced striatal toxicity: involvement of pre- and post-synaptic mechanisms and lack of direct NMDA blocking activity. *J. Neurochem.* 2004, *89*, 1479–1489.

[31] Aronica, E., Catania, M. V., Geurts, J., Yankaya, B., Troost, D., Immunohistochemical localization of group I and II metabotropic glutamate receptors in control and amyotrophic lateral sclerosis human spinal cord: upregulation in reactive astrocytes. *Neuroscience* 2001, *105*, 509–520.

[32] Anneser, J. M., Ince, P. G., Shaw, P. J., Borasio, G. D., Differential expression of mGluR5 in human lumbosacral motoneurons. *Neuroreport* 2004, *15*, 271–273.

[33] Hu, H. J., Alter, B. J., Carrasquillo, Y., Qiu, C. S., GereauIV, R. W., Metabotropic glutamate receptor 5 modulates nociceptive plasticity via extracellular signal-regulated kinase-Kv4.2 signaling in spinal cord dorsal horn neurons. *J. Neurosci.* 2007, *27*, 13181–13191.

[34] Montana, M. C., Cavallone, L. F., Stubbert, K. K., Stefanescu, A. D. *et al.*, The metabotropic glutamate receptor subtype 5 antagonist fenobam is analgesic and has improved *in vivo* selectivity compared with the prototypical antagonist 2-methyl-6-(phenylethynyl)-pyridine. *J. Pharmacol. Exp. Ther.* 2009, *330*, 834–843.

