



Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.jfda-online.com

JFDA

Original Article

Therapeutic effects of D-aspartate in a mouse model of multiple sclerosis



Sanaz Afraei ^a, Antimo D'Aniello ^{b,*}, Reza Sedaghat ^c, Parvin Ekhtiari ^a, Gholamreza Azizi ^{d,e}, Nakisa Tabrizian ^f, Laura Magliozzi ^g, Zahra Aghazadeh ^a, Abbas Mirshafiey ^{a,*}

^a Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

^b Laboratory of Neurobiology, Zoological Station of Naples “Anton Dohrn”, Villa Comunale, Napoli, Italy

^c Departments of Anatomy and Pathology, Faculty of Medicine, Shahed University, Tehran, Iran

^d Imam Hassan Mojtaba Hospital, Alborz University of Medical Sciences, Karaj, Iran

^e Research Centre for Immunodeficiencies, Pediatrics Centre of Excellence, Children's Medical Centre, Tehran University of Medical Sciences, Tehran, Iran

^f Department of Cellular and Molecular Biology, Kish International Campus, University of Tehran, Tehran, Iran

^g Department of Biology, University of Naples, “Federico II” Via Cinthia, MSA Campus, bldg. 7, Naples, Italy

ARTICLE INFO

Article history:

Received 20 May 2016

Received in revised form

21 October 2016

Accepted 30 October 2016

Available online 27 March 2017

ABSTRACT

Experimental autoimmune encephalomyelitis (EAE) is an animal model of multiple sclerosis. EAE is mainly mediated by adaptive and innate immune responses that leads to an inflammatory demyelination and axonal damage. The aim of the present research was to examine the therapeutic efficacy of D-aspartic acid (D-Asp) on a mouse EAE model. EAE induction was performed in female C57BL/6 mice by myelin 40 oligodendrocyte glycoprotein (35–55) in a complete Freund's adjuvant emulsion, and D-Asp was used to test its efficiency in the reduction of EAE. During the course of study, clinical evaluation was assessed, and on Day 21, post-immunization blood samples were taken from the heart of mice for the evaluation of interleukin 6 and other chemical molecules. The mice were sacrificed, and their brain and cerebellum were removed for histological analysis. Our findings indicated that D-Asp had beneficial effects on EAE by attenuation in the severity and delay in the onset of the disease. Histological analysis showed that treatment with D-Asp can reduce inflammation. Moreover, in D-Asp-treated mice, the serum level of interleukin 6 was significantly lower than that in control animals, whereas the total antioxidant capacity was significantly higher. The data indicates that D-Asp possess neuroprotective property to prevent the onset of the multiple sclerosis.

Copyright © 2017, Food and Drug Administration, Taiwan. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

* Corresponding authors. Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran 14155-6446, Iran (A. Mirshafiey); Laboratory of Neurobiology, Zoological Station of Naples “Anton Dohrn”, Villa Comunale, Napoli, Italy (A. D'Aniello).

E-mail addresses: daniello.antimo@gmail.com (A. D'Aniello), mirshafiey@tums.ac.ir (A. Mirshafiey).

<http://dx.doi.org/10.1016/j.jfda.2016.10.025>

1021-9498/Copyright © 2017, Food and Drug Administration, Taiwan. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Multiple sclerosis (MS) is considered as an autoimmune inflammatory demyelinating disease of the central nervous system (CNS) and of spinal cord that is characterized by relapsing-remitting attacks and worsening neurologic function [1]. MS disease is known by the destruction of the myelin sheath that surrounds neuronal axons in the CNS, a process that results in neurodegeneration and consequently the formation of sclerotic plaques in the brain. Recent investigations showed an association between MS and steroid hormones, namely progesterone, testosterone, and 17 β -estradiol [2–4]. Steroids attenuate neuroinflammation by reducing the pro-inflammatory function of astrocytes. Another research provided the evidence that steroids induce remyelination after demyelination [5–7]. The underlying cellular mechanisms involve interactions with astroglia, insulin-like growth factor-1 responses, and the recruitment of oligodendrocytes [4]. Several studies have shown that a reduction in the testosterone levels in rats induces a decrease in the synthesis of myelin protein [4]. Furthermore, progesterone is capable of protecting the motor neurons in the spinal cord of rats [5,6], and 17 β -estradiol protects oligodendrocytes from the cytotoxicity of cell death [7]. The other series of studies reported that the neurosteroids contribute to the formation of synapses, synaptic plasticity, and cognitive activity in addition to having protective effects on myelin destruction [5,7–16]. These multiplatform studies indicate impaired neurosteroidogenesis in both MS and experimental autoimmune encephalomyelitis (EAE). EAE is an animal model of MS where the disease is mediated by autoantigen-specific T cells, such as myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein [17,18]. EAE is used for the evaluation of mechanism causing MS as well as pharmacological research in MS [19–22].

D-Asp is a natural amino acid present in all animal phyla investigated, including rodents and humans. It was first discovered in the brain of the marine mollusc, *Octopus vulgaris*, in 1977 [23]. Subsequent studies have clarified that this amino acid is mainly present in the nervous tissues and endocrine glands [24–26] where it performs important physiological functions [27,28]. In the nervous tissues, D-Asp is localized in various areas of the brain, including the olfactory bulb, frontal cortex, hippocampus, and cerebellum. In the endocrine glands, D-Asp is localized mainly in the pineal gland, adrenal medulla, posterior pituitary gland, and testis [29]. In the hypothalamus, D-Asp is involved in the synthesis and release of gonadotropin-releasing hormones. In the pituitary gland, it induces the synthesis and release of luteinizing hormone, and in testis, it induces the synthesis and release of testosterone [30]. Furthermore, from the pineal gland, this amino acid is involved in the synthesis and release of melatonin [31], whereas from the pituitary gland, it can increase the synthesis of the melanocortin [32]. In the nervous system instead, D-Asp acts as a novel neurotransmitter of cell-cell signaling [33] and as neuromodulator [34]. Interestingly, D-Asp can also enhance learning and memory in rats [35] as well as improve the long-term potentiation [36] and prevent long-term depression in mice [37]. Lastly, this amino acid also acts as a regulator for

the neurogenesis and is an endogenous factor for the neuronal dendrites growth [38,39].

Previously, studies have established that some hormones, mainly testosterone, progesterone, and 17 β -estradiol, in the CNS (neurosteroids) play a role in the protection of the neurons against neuronal damage caused by dangerous hexogen and endogen molecules, i.e., free radicals, nitric oxide, peroxides, and endogen antibody. Moreover, such neurosteroids are essential elements to keep the myelin sheath healthy that surrounds the neuron and concur in the synthesis of myelin [7–9,13–15]. In the present study, we have obtained the evidence in mice that on oral ingestion of a solution consisting of 20mM sodium D-aspartate instead of tap water, this amino acid crosses the blood brain barrier in male and female mice and increases the synthesis of the neurosteroids (testosterone, progesterone, and 17 β -estradiol) in the brain. Thus, these results suggest that D-Asp may have the property to prevent and reduce the neuronal damage of the myelin sheath, which is the major protein surrounding the neuron. Therefore, the aim of this study was to the therapeutic effects of the oral treatment of sodium D-aspartate in experimental mouse model for MS.

2. Material and methods

2.1. Animal selection and grouping

In this experiment, 24 female C57BL/6 mice, weighing 18–20 g and aged 8 weeks were used. The mice were purchased from the Experimental Animal Center of Pasteur Institute of Iran. These mice were divided into four groups randomly (6 mice per group): I, normal group (healthy control); II, prophylactic group; III, treatment group; and IV, control group. For adaptation, all mice were housed in cages under 12-hour light–dark cycle in the animal house of Tehran University for 2 weeks. During the study period, the same meal plan including pelleted diet soya, carrot, peanuts, and water was used. All the animal-related procedures were conducted in accordance with the protocol approved by the committee on animal experimentation of Tehran University of Medical Sciences.

2.2. EAE induction and treatment protocol

From the first day of adaptation, all animals were weighed, and their weights were recorded. To induce EAE, Hook kit (Hooke Laboratories, Inc, USA) was used. Each kit contained two pre-filled syringes of MOG 35-55 in an emulsion with complete Freund's adjuvant and a vial of lyophilized pertussis toxin (PTX). EAE induction was performed according to instructions: 0.1 mL MOG 35-55 was injected to the left flank area as well as to the right flank area subcutaneously; after 2 hours, the first dose of PTX dissolved in 2.5-cc sterile phosphate-buffered saline (PBS) was injected intraperitoneally into mice. After 24 hours, the second dose of PTX (0.1 mL/mouse) was injected intraperitoneally.

We prepared a stock solution of 1M of sodium D-aspartate as follows: 133.1 g of D-aspartic acid, 99% grade (Sigma, USA), was added to 500 mL distilled water with stirring. Then, it was added under stirring a solution of 2M sodium hydroxide to

5–10 mL to time with stirring until all the D-aspartic acid was solubilized to form the sodium D-aspartate. The solution was then brought to 1 L with distilled water and then filtrated on the filter of 0.42 μ m pores, divided into aliquots, and stored at –20°C. The given dose to animals with the concentration of 20mM was based on our previous study where we established that a solution of 20mM of D-Asp was not dangerous for the animals over a long time period of treatment [36]. Furthermore, 5 days before the induction of EAE, 20mM sodium D-aspartate solution was given daily in drinking water to the prophylactic group. The solution was given until the 20th day. Moreover, from the 5th day after the induction of EAE and until the 20th day, the animal continued to drink the 20mM sodium D-aspartate solution. Mice were checked daily, and their clinical scores were evaluated according to the following criteria: 0, no clinical sign; 1, paralysis of the tail; 2, paralysis of the tail and weakness of the hind limbs, hind limbs get together when lifting the mouse from the tip of the tail; 3, paralysis of the tail and hind limbs and/or paralysis of the tail with one fore limb and one hind limb; 4, complete paralysis of the tail and hind limbs and partially paralyzed fore limbs; 5, complete paralysis.

2.3. Histopathology and *in vitro* determinants evaluation

On 21st day after induction, all mice in normal, control, prophylactic, and treatment groups were anesthetized by chloroform, blood samples were taken from the right ventricle of the heart, and perfusion was performed immediately with the injection of formalin to heart by the scalp vein. Blood samples were centrifuged, and sera were separated from the blood. Sera were used to assay glutathione reductase (GR), total antioxidant capacity (TAC), super oxide dismutase (SOD), and interleukin 6 (IL-6). The brain and cerebellum were removed for histopathological examinations and placed in 10% formalin for fixation. To evaluate the inflammatory criteria and mononuclear cell infiltration, cross sections (5 mm) of brain and cerebellum were prepared, embedded in paraffin, and stained with hematoxylin-eosin. In order to determine demyelination, sections (8 μ m thick) were prepared and stained with luxol fast blue [20]. Lastly, stained slides were examined under a microscope by an expert pathologist.

2.4. Quantification of SOD activity

A ZellBio GmbH SOD kit (Ulm, Deutschland) was prepared to assay SOD in serum. This kit is used for SOD activity assessment in the range of 5–100 U/mL. It can determine the SOD activity in the biological sample with the sensitivity of 1 U/mL (1 KU/L). In this assay, the SOD activity unit was considered as the amount of the sample that will catalyze the decomposition of 1 μ mol of O₂ to H₂O₂ and O₂ in 1 minute. The SOD activity is determined colorimetrically at 420 nm.

2.5. GR assessment

To assay the GR activity, a ZellBio GmbH (Ulm, Deutschland) kit was prepared. Biocore GR assay kit can be used for the activity assessment in the range of 10–15 U/L. This kit can determine

GR in the biological sample with the sensitivity of 1U/L sensitivity. The GR activity is determined photometrically at 340 nm.

2.6. TAC

The TAC measurement in serum using the colorimetric method by the radical cation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) was introduced by Ozgen and co-workers [40]. The experiment is based on eliminating or reviving of cation ABTS + (with a maximum light absorption at wavelengths of 660 nm, 734 nm, and 820 nm) by antioxidant compounds in the serum sample. With reviving radical ABTS, the green-blue solution turns into an achromatic solution. The decrease in optical density is measured by a spectrophotometer and expressed as radical inhibition percentage. The instability of ABTS is a drawback of this method, which is improved by the production of stable ABTS [41]. To produce ABTS cation [2,2 azino-bi(3-ethylbenzolin-6-sulfonicacid)], it is combined with potassium persulfate that is stable at least for 2 days. In this investigation, bovine serum albumin (BSA) was manipulated instead of trolox. For converting the inhibition percentage to g/dL, the standard BSA curve was used [40,42].

2.7. Quantification of IL-6

The Biolegend LEGEND MAX Mouse IL-6 ELISA Kit (BioLegend, Inc. San Diego) is a sandwich enzyme-linked immunosorbent assay (ELISA) with a 96-well strip plate that is precoated with a captured antibody to assay the level of pro-inflammatory cytokine of IL-6 in the sera of control, normal, prevention, and treatment groups. This assay was performed according to the manufacturer's instructions. Absorbance was read at 450 nm in a 96-well microplate ELISA reader.

2.8. Determination of neurosteroids

The brain of each mouse was homogenized 1:5 with PBS using Ultra-Turrax I-125 homogenizer (IKA Werke GmbH & Co, Staufen im Breisgau, Germany) or other homogenizer. Next, 2 mL of the homogenate was mixed vigorously with 10 mL of diethyl ether to extract the hormones and allowed to stand at room temperature for 10 minutes. The upper ether phase was separated from the precipitate. The extraction was repeated one more time with further 10 mL ether. The two ether phases were combined, and ether was allowed to evaporate in petri dishes at room temperature under the hood. The residue was suspended in 500 μ L of PBS containing 5 mg of BSA/mL for the hormones to bind for affinity to the BSA; finally, this sample was used for the determination of the neurosteroids using the specific ELISA for the determination of testosterone, progesterone, and 17 β -estradiol using the reagent kit from the DIAMETRA (Secrate, Milano, Italy) or the reagent kit (Elecsys and Cobas e analyzers) from Roche Diagnostics GmbH, Mannheim (Germany). Using the above ether extraction with each hormone standard added to the sample, the recovery of the sample was 90–95%.

2.9. Determination of D-Asp

The determination of D-Asp in mice brain was performed using a high-performance liquid chromatography (HPLC) enzymatic

method combined with D-aspartate oxidase (D-AspO), according to D'Aniello et al. [33]. In brief, 1 mL of brain homogenate prepared as above was mixed with 1 mL of 0.2M trichloroacetic acid (TCA) and centrifuged for 2 minutes at 13,000g. The supernatant was purified on cation exchange resin: AG 50WX8; (100–200 mesh, Bio-Rad) column 0.5 × 2 cm activated as follows: the column was treated with 10 mL 6M NaOH followed by 3 × 10 mL distilled water, 10 mL 6M HCl, and 3 × 10 mL distilled water. Finally the TCA supernatant was passed on the activated column, followed by washing with 3 × 5 mL 0.01M HCl. The amino acids bound to the resin and then were eluted with 2 × 4 mL 4M NH₄OH. The eluent containing all the amino acids, including D-Asp, was dried on petri dishes on a warm plate (50–60°C). The residue was dissolved in 1 mL distilled water. For the HPLC analysis, 50 μL of this sample was mixed with 100 μL of 0.1M sodium borate buffer (pH 10.0) and 10 μL of o-phthaldialdehyde (OPA)/ N-acetyl-cysteine (NAC) reagent (prepared by mixing 20 mg of OPA with 10 mg of NAC in 1 mL methanol). After 2 minutes (for completing reaction) 20 μL of this mixture was injected into a C18 Supelcosil column (0.45 cm × 25 cm, Supelco Inc., Belafonte, PA, USA) connected to a Beckman-Gold HPLC system. The column was eluted at 1.2 mL/minute with a gradient consisting of: solution A (950 mL 0.05M citrate-phosphate buffer of pH 5.6 and 50 μL acetonitrile) and solution B (90% acetonitrile in H₂O). The program gradient was: 0–10 minutes, 90% A and 10% B; 10–20 minutes; 0% A and 100% B; 20–25 minutes at 100% B and returning to 0% in 1 minute. The fluorescence was read at an excitation wavelength of 330 nm and emission wavelength of 450 nm. D-Asp was eluted with a peak at 5.2 minutes, followed by L-Asp at 6.0 minutes, L-Glu at 7 minutes, and other amino acids. In order to quantify D-Asp in the sample, a standard curve consisting of 50 pmol of D-Asp and 100 pmol of each of the amino acids, namely L-Asp, L-Glu, L-Ser, L-Thr, L-His, L-Gly, L-Arg, L-Ala, L-Val, L-Met, L-Tyr, L-Phe, L-Ile, L-Leu, and L-Lys, was performed in the same assay condition.

2.10. Statistical analysis

Data were expressed as mean ± SD, except for histological scores, which were calculated as mean ± SEM. Statistical

analysis was performed using the Mann–Whitney U test for nonparametric data and Student t test for parametric data. A *p* value < 0.05 was considered statistically significant.

3. Results

3.1. Clinical findings

In this investigation, the induction of EAE was done in C57BL/6 mice by immunizing them with a ready-to-use Hooke kit. Mice in prevention and treatments groups were treated with 20mM D-Asp in their drinking water. The clinical course and intensity of the disease differed consistently between prevention, treatment, and control groups. The mice in prevention and treatment groups showed a significant reduction in the clinical course of EAE compared with those in the control group (Figure 1). EAE onset was delayed in the prevention group ($11.00 \pm .00$ *p* < 0.05) compared with the control group (10.5 ± 0.54 , *p* < 0.05); additionally, EAE onset was delayed significantly in the treatment group (12.00 ± 1.26) compared with the control group (Figure 2). These effects led to a clinical improvement and a delayed disease progression, indicating that D-Asp can inhibit the progression of EAE.

3.2. Histological findings

As shown in Figure 3, the demyelination and inflammation determinants were significantly higher in the control group than in EAE mice treated by sodium D-aspartate. The results demonstrated in Tables 1 and 2 showed that the clinical severity of EAE in control, prevention, and treatment groups is correlated with the severity of inflammation observed in the histopathology of CNS.

3.3. SOD activity

D-Asp therapy showed an increase in the SOD activity in serum; this was in agreement with the clinical findings. As shown in Figure 4, the SOD activity was increased in the prevention (25.43 ± 2.22) and treatment groups (23.39 ± 5.65)

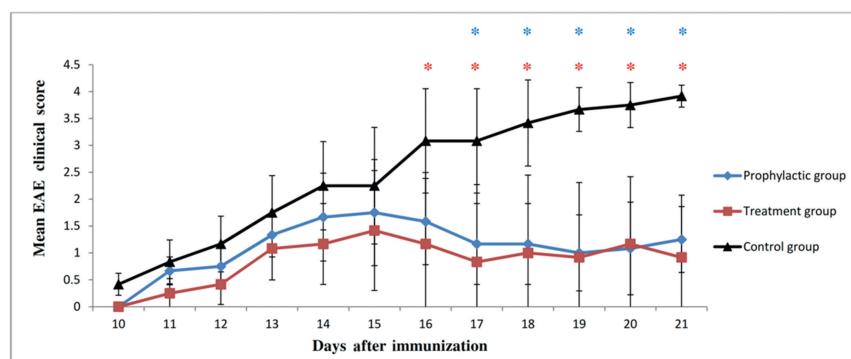


Figure 1 – Effect of D-Asp on the clinical score of experimental autoimmune encephalomyelitis (EAE). Female C57BL/6 mice were administered 20mM D-Asp 5 days before and after EAE induction in the prevention group and treatment group, respectively. Disease severity was assessed by a visual cumulative scoring system. Cumulative scores from Day 10 until Day 21 are given as mean ± SEM and **p* < 0.05. Each data point has been calculated by Mann–Whitney U test with comparing treatment and prevention versus control.

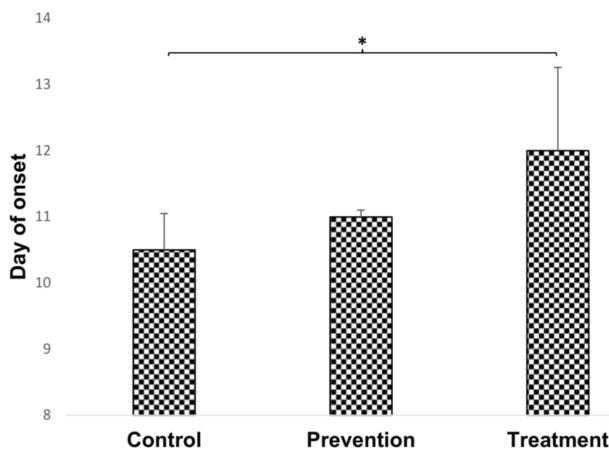


Figure 2 – Effect of D-Asp on experimental autoimmune encephalomyelitis (EAE) onset. In the prevention group, D-Asp therapy showed a delay at onset of disease compared with the control group but was not significant. In the treatment group, D-Asp therapy showed a significant delay at onset of disease compared with the control group.

* $p = 0.033$.

compared with the control group (20.51 ± 4.77); however, these differences were not significant.

3.4. GR

As shown in Figure 5, GR activity was increased in the prevention (33.72 ± 9.37) and treatment groups (25.73 ± 8.78) compared with the control group (20.18 ± 3.36); however, these differences were not statistically significant.

In the normal group, GR activity was significantly higher than that in the control, prevention, and treatment groups ($p < 0.01$).

3.5. TAC evaluation

TAC evaluation was based on ABTS radical cation [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] scavenging on

serum samples of mice. As shown in Figure 6, treatment with D-Asp, increased TAC in the treatment group (2.10 ± 0.21) and prevention group (1.94 ± 0.32) compared with the control group (1.68 ± 0.22); however, these differences were not statistically significant.

3.6. IL-6 evaluation

The effect of D-Asp on IL-6 cytokine concentration in mice sera was evaluated. The analysis was performed using an ELISA kit. As shown in Figure 7, treatment by D-Asp reduced IL-6 production in prevention group (82.98 ± 16.91) and treatment group (70.00 ± 23.6) compared with the control group (138.65 ± 37.35).

3.7. Effects of D-Asp on the neurosteroid production and its accumulation in the brain

Many studies have reported that some neurosteroids, such as progesterone, testosterone, and 17β -estradiol, play an important role in the prevention or reduction of the gravity of MS pathology presumably by acting their specific receptors or by other unknown mechanisms [4,6–10,16]. Previously, our study has demonstrated that D-Asp can increase the concentration of these hormones in the endocrine tissues [30,31,43–45]; therefore, in this study, we wanted to determine whether such a phenomenon also occurred in the CNS. Therefore, we measured the concentration of the neurosteroids in mice brain treated and non-treated with D-Asp, and the results obtained indicated that the concentration of the progesterone and testosterone (1.5-fold; $p < 0.01$) and 17β -estradiol (about 2-fold, $p < 0.01$) in the brain was improved statistically in mice which had orally ingested D-Asp compared with their respective control group mice (Table 3).

In order to know whether D-Asp ingested orally crosses the brain barrier and then triggers neurosteroid synthesis, we also determined the concentration of D-Asp in the brain in mice treated and non-treated orally with D-Asp. The results indicated that the concentration of D-Asp was significantly higher (2-fold) in the brain of mice treated orally with D-Asp than in

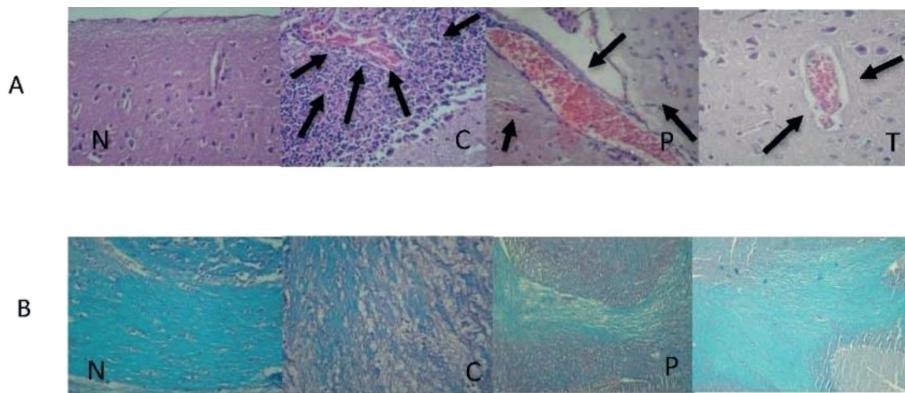


Figure 3 – Representative light microscopic view of histopathological slides of the central nervous system in different groups. (A) Hematoxylin–eosin staining of brains sections showed that D-Asp therapy could suppress the development of inflammation significantly by restricting leukocyte infiltration. (B) Luxol fast blue staining showed that the extent of demyelination could be reduced by D-Asp therapy. C = control group; N = normal group; P = prevention group; T = treatment group.

Table 1 – Comparison of inflammation indexes in histopathology examination in the brain of EAE mice.

Inflammation indexes	Control (Mean ± SD)	Prevention (Mean ± SD)	Treatment	p*
Demyelination	3.85 ± 0.74	1.75 ± 0.86	1.30 ± 0.67	0.007
Degeneration	3.70 ± 0.57	1.75 ± 0.52	1.20 ± 0.73	0.005
Infiltration of inflammatory cells in leptomeninges	3.50 ± 0.64	1.16 ± 0.75	0.50 ± 0.58	0.004
Meningeal vessels hyperemia	3.50 ± 0.61	1.91 ± 0.80	1.60 ± 0.55	0.01
Leukocyte margination	2.65 ± 0.60	1.62 ± 1.02	1.10 ± 0.89	0.006
Perivascular cuffing	3.65 ± 0.85	1.25 ± 0.69	0.50 ± 0.58	0.004
Perivascular edema	3.20 ± 0.57	1.46 ± 0.75	1.60 ± 0.55	0.009
Hypercellularity	4.11 ± 0.70	1.70 ± 1.56	1.55 ± 0.37	0.006
Laminar necrosis	3.90 ± 0.65	1.58 ± 0.37	1.45 ± 1.11	0.004
Endothelial cell hypertrophy	3.60 ± 0.82	1.83 ± 0.49	1.55 ± 0.45	0.006
Spongiosis	3.05 ± 0.67	1.29 ± 0.75	0.85 ± 0.45	0.006

Note. Source: Histopathology examination. 0 = no symptoms; 1 = mild; 2 = moderate; 3 = moderately severe; 4 = severe; 5 = very severe. Demyelination: loss of myelin in the nerve sheaths; Leptomeninges: the two innermost layers of tissue (the arachnoid mater and pia mater) that cover the brain and spinal cord; Hyperemia: too much blood that accumulates in micro circulation as meningeal vessels; Leukocyte margination: accumulation and adhesion of leukocytes to the endothelial cells of blood vessel walls at the site of injury in the early stages of inflammation; Perivascular cuffing: the accumulation of leukocytes around the vessel; Laminar necrosis: necrosis of the selected neuron in particular layer of cerebral cortex; Spongiosis: vacuolation of cerebral gray and white matter; Hypercellularity: increased number of glial and/inflammatory cells in a tissue.

*All differences were statistically significant (*p < 0.05) except for meningeal vessels hyperemia.

Table 2 – Comparison of inflammation indexes in histopathology examination in the cerebellum of experimental autoimmune encephalomyelitis mice.

Inflammation indexes	Control (Mean ± SD)	Prevention (Mean ± SD)	Treatment (Mean ± SD)	p*
Demyelination	3.75 ± 0.66	1.62 ± 0.80	1.35 ± 0.60	0.006
Degeneration	3.80 ± 0.45	1.58 ± 0.49	1.05 ± 0.67	0.005
Infiltration of inflammatory cells in leptomeninges	3.50 ± 0.79	1.37 ± 0.97	0.75 ± 0.71	0.005
Meningeal vessels hyperemia	3.80 ± 0.76	1.75 ± 1.14	1.00 ± 0.50	0.006
Leukocyte margination	3.90 ± 0.89	1.70 ± 1.30	0.95 ± 0.84	0.009
Perivascular cuffing	3.80 ± 0.76	1.54 ± 1.17	0.65 ± 0.60	0.006
Perivascular edema	3.15 ± 0.86	1.62 ± 1.22	1.10 ± 0.74	0.023
Hypercellularity	4.00 ± 0.79	1.58 ± 0.75	0.90 ± 0.67	0.005
Endothelial cell hypertrophy	3.25 ± 0.50	1.58 ± 0.75	1.45 ± 0.51	0.007
Spongiosis	3.30 ± 0.86	1.62 ± 0.90	1.45 ± 0.27	0.007

Note. Source: Histopathology examination. 0 = no symptoms; 1 = mild; 2 = moderate; 3 = moderately severe; 4 = severe; 5 = very severe. Demyelination: loss of myelin in the nerve sheaths; Leptomeninges: the two innermost layers of tissue (the arachnoid mater and pia mater) that cover the brain and spinal cord; Hyperemia: too much blood that accumulates in micro circulation as meningeal vessels; Leukocyte margination: accumulation and adhesion of leukocytes to the endothelial cells of blood vessel walls at the site of injury in the early stages of inflammation; Perivascular cuffing: the accumulation of leukocytes around the vessel; Spongiosis: vacuolation of cerebral gray and white matter; Hypercellularity: increased number of glial and/inflammatory cells in a tissue.

*All differences were statistically significant (*p < 0.05).

the control group (Table 4). Thus, this finding indicated that D-Asp is capable of crossing the brain barrier and accumulate in the brain.

4. Discussion

EAE is an animal model of MS that causes brain inflammation and demyelination mediated by the immune response to brain antigens. Several studies indicated that neurosteroids have protective properties against the neurodegeneration that occurs in some neurodegenerative disorders such as MS [17,43,44]. On the other hand, it was reported that neurosteroids contribute to the formation of neural synapses, synaptic plasticity, and cognitive activity [8]. There is substantial evidence in animal studies suggesting that neurosteroids can affect brain function by neurotransmission and influence on

neuronal survival, glial differentiation, and myelination in the CNS by regulating gene expression of neurotrophic factors and anti-inflammatory molecules [46]. Involvement of neurosteroids in the neurodegenerative and neuroinflammatory processes suggests that they may deserve further investigations as potential therapeutic agents in MS [46]. In our and other previous studies regarding the steroidogenesis of rat, it has been demonstrated that D-Asp can increase some hormones of the hypothalamus–hypophysis–gonadal axis, such as gonadotropin-releasing hormone from the hypothalamus, luteinizing hormone from the hypophysis, and testosterone from the gonads [30] as well as testosterone from Leydig cells [45], α -melanocyte-stimulating hormone, GABA and dopamine release [31], and melanocortin [32]. These results induced us to verify whether D-Asp treatment in mice could also increase the neurosteroids (testosterone, progesterone, and 17 β -estradiol) in mice experimental model of MS.

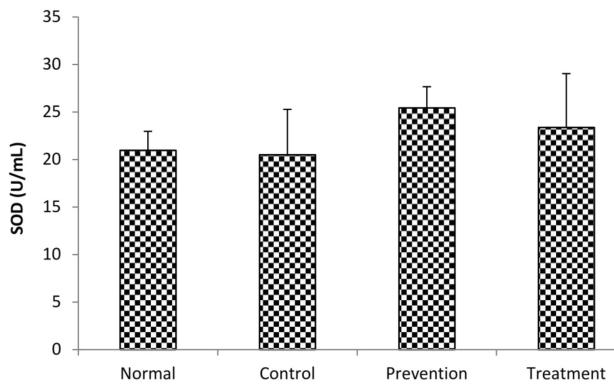


Figure 4 – The effect of D-Asp on serum super oxide dismutase (SOD) activity. It is observed that D-Asp increased SOD activity in prevention (25.43 ± 2.22) and treatment groups (23.39 ± 5.65) compared with control group (20.51 ± 4.77); however, this difference was not statistically significant.

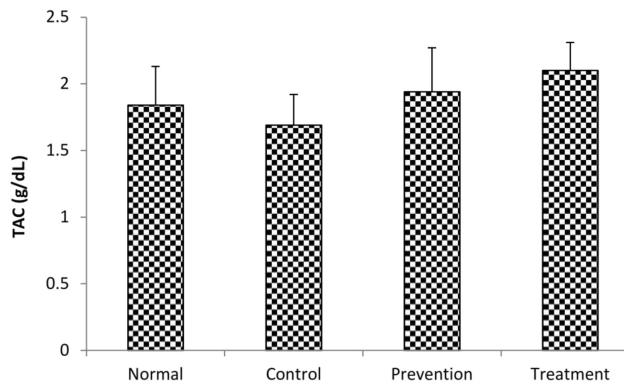


Figure 6 – The effects of D-Asp on serum total antioxidant capacity (TAC). D-Asp increased TAC in prevention (1.94 ± 0.32) and treatment groups (2.10 ± 0.21) compared with the control group (1.68 ± 0.22); however, differences were not significant.

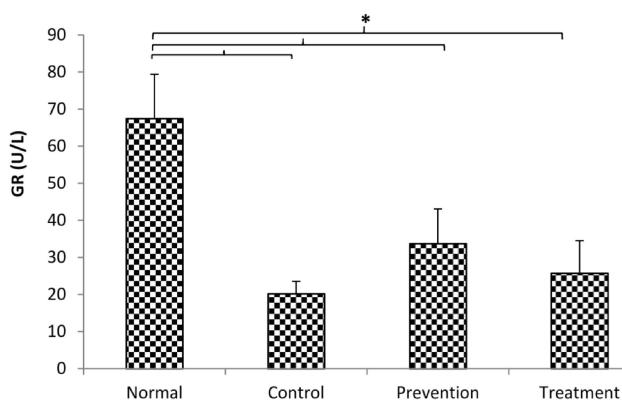


Figure 5 – The effect of D-Asp on serum glutathione reductase (GR) activity. It is observed that D-Asp treatment increased the GR activity in prevention (33.72 ± 9.37) and treatment groups (25.73 ± 8.78) compared with the control group (20.18 ± 3.36) but was not statistically significant. However, the difference between normal group and prevention, treatment and control groups was significant ($p < 0.01$).

We observed that D-Asp can treat EAE by decreasing the intensity and delaying the onset of EAE in C57BL/6 mice. It was observed that D-Asp in drinking water (every day) can significantly decrease the intensity of inflammation determinants in the brain and cerebellum of EAE mice. In another study, the elevated IL-6 levels in EAE were reportedly associated with the disease onset [42]. Other studies showed that neurosteroids can reduce the pro-inflammatory cytokines of astrocytes and decrease neuroinflammation [4].

Tumor necrosis factor- α , a pro-inflammatory cytokine, is involved in the pathology of MS and EAE. Its levels are increased in the serum, cerebrospinal fluid, and also at the site of active lesions of patient with MS disease, and its enhancement is correlated with the severity of the disease

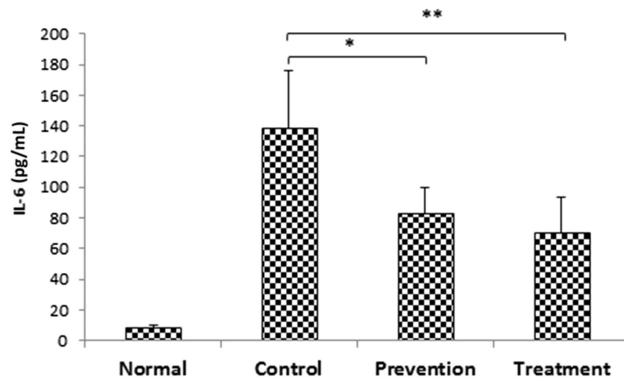


Figure 7 – The effect of D-Asp on serum interleukin 6 (IL-6) concentration. It is observed that D-Asp significantly decreased IL-6 in prevention (82.98 ± 16.91) and treatment groups (70.00 ± 23.6) compared with the control group (138.65 ± 37.35). * $p = 0.01$, ** $p = 0.002$, respectively.

[47]. IL-6 cytokine is an important mediator of many inflammatory processes because it mediates cellular responses during immune activation and inflammation. IL-6 reportedly has a major role in inflammatory reactions, neuroimmunology, and neuroinflammation [48]. It was demonstrated in some studies that in mononuclear cells in the blood and cerebrospinal fluid [48–50] and in the brain tissue of patients with MS [51], the levels of IL-6 were increased. Furthermore, studies involving both human MS patients [49] and mouse models of MS (EAE) suggest that IL-6 levels may correlate with disease severity [52].

In another investigation, the elevated IL-6 levels in EAE were reportedly associated with the disease onset. Our investigation showed that treatment with D-Asp can also decrease the IL-6 level in animal model of MS; this is in agreement with our clinical and histopathological findings. Research showed that in the CNS and peripheral nervous system, neurosteroids are capable of elevating the synthesis of myelin and

Table 3 – Effects of chronic treatment of D-Asp on the synthesis of neurosteroids in the total brain of C57BL/6 female mice and on the accumulation of D-Asp in the brain. EAE = experimental autoimmune encephalomyelitis.

	Progesterone (ng/g)	Testosterone (ng/g)	17 β -Estradiol (pg/g)
Control group	5.5 \pm 2.0	3.4 \pm 1.2	15.0 \pm 4.0
-EAE induction			
Treated group	8.5 \pm 2.8	5.2 \pm 0.8	28.0 \pm 5.2
-EAE induction plus			
D-Asp treatment			
Increased (fold)	1.54	1.52	1.86
<i>p</i>	< 0.01	< 0.01	< 0.01

Values are the mean and standard deviation obtained from C57BL/6 female mice aged 10 weeks. Control group included mice which were induced the EAE and left to drink tap water. After 15 days from the induction of EAE, the mice were sacrificed and neurosteroids were measured in the brain. Treatment group included mice which were left to drink the solution of sodium D-aspartate starting 5 days before induction of EAE and continued for other 15 days from the induction of the EAE.

Table 4 – Effects of chronic treatment of D-Asp on C57BL/6 female mice on its accumulation in the brain. EAE = experimental autoimmune encephalomyelitis.

	D-Aspartic acid (ng/g of brain)
Control group	205 \pm 23
-EAE induction	
Treated group	550 \pm 40
-EAE induction plus	
D-Asp treatment	
Increased (fold)	2.68
<i>p</i>	< 0.001

Values are the mean and standard deviation obtained from the same mice used in the study as reported in the above Table 3. Control group were mice which were induced the EAE and left to drink tap water. After 15 days from the induction of EAE, the mice were sacrificed and concentration of D-Asp was measured using HPLC. Treatment group included mice which were left to drink the solution of sodium D-aspartate starting 5 days before induction of EAE and continued for other 15 days from the induction of EAE.

preventing cellular damage created by free radicals, nitric oxide, and peroxides [7,14]. Research also shows that oxidative stress is created when the balance between SOD and TAC defense is impaired [40]. One of the most important defenses against oxidative stress is to produce antioxidant enzymes that have important role in free radicals, i.e., eradication and detoxification of reactive oxygen species [9].

Our results showed that the treatment with D-Asp could increase TAC, SOD, and GR compared with the control group; however, differences were not statistically significant. Other studies showed that GR is decreased in autoimmunity diseases, such as rheumatoid arthritis and its experimental models collagen-induced arthritis. Finally, our study shows that disease progression in experimental model of MS can be reduced by D-Asp. This finding encourages further investigations on anti-inflammatory effects and remyelinating

capacity of D-Asp as well as its role as a potential candidate for developing an alternative therapeutic strategy against an inflammatory disease, such as MS.

5. Conclusion

The results obtained in this study demonstrate that the treatment by D-Asp prevented the onset of MS in EAE mice. However, the exact molecular mechanism for this phenomenon remains unclear. We hypothesize that D-Asp has an indirect activity in the synthesis and repair of the myelin sheath due to its action in triggering the increase of the cerebral neurosteroids, which in turn are capable of increasing the synthesis of the myelin and simultaneously protecting the neurons against internal and external injury.

Conflicts of Interest

There is no conflict of interest in this work.

Acknowledgments

The authors wish to thank Dr Angelo Seleni, Grimaldi Teresa, and Giovanni Pierro of the clinical laboratory “Igea of Frattamaggiore” (Italy) for the determination of testosterone, progesterone, and 17 β -estradiol by using the “Elecys and cobas e analyzers” Roche.

REFERENCES

- [1] Compston A, Coles A. Multiple sclerosis. *Lancet* 2008;372:1502–7.
- [2] El-Etr M, Vukusic S, Gignoux L, Durand-Dubief F, Achiti I, Baulieu EE, Confavreux C. Steroid hormones in multiple sclerosis. *J Neurol Sci* 2005;233:49–54.
- [3] Simpkins J, Yang SH, Wen Y, Singh M. Estrogens, progestins, menopause and neurodegeneration: basic and clinical studies. *Cell Mol Life Sci* 2005;62:271–80.
- [4] Kipp M, Beyer C. Impact of sex steroids on neuroinflammatory processes and experimental multiple sclerosis. *Front Neuroendocrinol* 2009;30:188–200.
- [5] Gonzalez S, Labombarda F, Deniselle MG, Guennoun R, Schumacher M, De Nicola A. Progesterone up-regulates neuronal brain-derived neurotrophic factor expression in the injured spinal cord. *Neuroscience* 2004;125:605–14.
- [6] Ye JN, Chen XS, Su L, Liu YL, Cai QY, Zhan X, Xu Y, Zhao SF, Yao ZX. Progesterone alleviates neural behavioral deficits and demyelination with reduced degeneration of oligodendroglial cells in cuprizone-induced mice. *PLoS One* 2013;8:e54590.
- [7] Takao T, Flint N, Lee L, Ying X, Merrill J, Chandross KJ. 17 β -estradiol protects oligodendrocytes from cytotoxicity induced cell death. *J Neurochem* 2004;89:660–73.
- [8] Baulieu E. Neurosteroids: a novel function of the brain. *Psychoneuroendocrinology* 1998;23:963–87.
- [9] Svenningsen ÅF, Kanje M. Estrogen and progesterone stimulate Schwann cell proliferation in a sex-and age-dependent manner. *J Neurosci Res* 1999;57:124–30.

[10] Melcangi RC, Magnaghi V, Martini L. Aging in peripheral nerves: regulation of myelin protein genes by steroid hormones. *Prog Neurobiol* 2000;60:291–308.

[11] Melcangi R, Azcoitia I, Ballabio M, Cavarretta I, Gonzalez LC, Leonelli E, Magnaghi V, Veiga S, Garcia-Segura LM. Neuroactive steroids influence peripheral myelination: a promising opportunity for preventing or treating age-dependent dysfunctions of peripheral nerves. *Prog Neurobiol* 2003;71:57–66.

[12] Melcangi R, Ballabio M, Cavarretta I, Gonzalez LC, Leonelli E, Veiga S, Martini L, Magnaghi V. Effects of neuroactive steroids on myelin of peripheral nervous system. *J Steroid Biochem Mol Biol* 2003;85:323–7.

[13] Mellon SH, Griffin LD, Compagnone NA. Biosynthesis and action of neurosteroids. *Brain Res Rev* 2001;37:3–12.

[14] Azcoitia I, Leonelli E, Magnaghi V, Veiga S, Garcia-Segura LM, Melcangi RC. Progesterone and its derivatives dihydroprogesterone and tetrahydroprogesterone reduce myelin fiber morphological abnormalities and myelin fiber loss in the sciatic nerve of aged rats. *Neurobiol Aging* 2003;24:853–60.

[15] Rodriguez-waitkus PM, Lafollette AJ, Ng BK, Zhu TS, Conrad HE, Glaser M. Steroid hormone signaling between Schwann cells and neurons regulates the rate of myelin synthesis. *Ann N Y Acad Sci* 2003;100:340–8.

[16] Giatti S, Caruso D, Boraso M, Abbiati F, Ballarini E, Calabrese D, Pesaresi M, Rigolino R, Santos-Galindo M, Viviani B, Cavaletti G. Neuroprotective effects of progesterone in chronic experimental autoimmune encephalomyelitis. *J Neuroendocrinol* 2012;24:851–61.

[17] Noorbakhsh F, Ellestad KK, Maingat F, Warren KG, Han MH, Steinman L, Baker GB, Power C. Impaired neurosteroid synthesis in multiple sclerosis. *Brain* 2011;134:2703–21.

[18] Vanheel A, Daniels R, Plaisance S, Baeten K, Hendriks JJ, Leprince P, Dumont D, Robben J, Bröne B, Stinissen P, Noben JP. Identification of protein networks involved in the disease course of experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis. *PLoS One* 2012;7:e35544.

[19] Azizi G, Navabi SS, Al-Shukaili A, Seyedzadeh MH, Yazdani R, Mirshafiey A. The role of inflammatory mediators in the pathogenesis of Alzheimer's disease. *Sultan Qaboos Univ Med J* 2015;15:e305.

[20] Afraei S, Azizi G, Zargar SJ, Sedaghat R, Mirshafiey A. New therapeutic approach by G2013 in experimental model of multiple sclerosis. *Acta Neurologica Belgica* 2015;115:259–66.

[21] Azizi G, Goudarzvand M, Afraei S, Sedaghat R, Mirshafiey A. Therapeutic effects of dasatinib in mouse model of multiple sclerosis. *Immunopharmacol Immunotoxicol* 2015;37:287–94.

[22] Mirshafiey A, Aghily B, Namaki S, Razavi A, Ghazavi A, Ekhtiari P, Mosayebi G. Therapeutic approach by Aloe vera in experimental model of multiple sclerosis. *Immunopharmacol Immunotoxicol* 2010;32:410–5.

[23] D'Aniello A, Giuditta A. Identification of D-Aspartic acid in the brain of *Octopus vulgaris* lam. *J Neurochem* 1977;29:1053–7.

[24] Dunlop DS, Neidle A, McHale D, Dunlop DM, Lajtha A. The presence of free D-aspartic acid in rodents and man. *Biochem Biophys Res Commun* 1986;141:27–32.

[25] Hashimoto A, Nishikawa T, Oka T, Hayashi T, Takahashi K. Widespread distribution of free D-aspartate in rat periphery. *FEBS Lett* 1993;331:4–8.

[26] Hashimoto A, Oka T, Nishikawa T. Anatomical distribution and postnatal changes in endogenous free D-aspartate and D-serine in rat brain and periphery. *Eur J Neurosci* 1995;7:1657–63.

[27] Ota N, Shi T, Sweedler JV. D-Aspartate acts as a signaling molecule in nervous and neuroendocrine systems. *Amino Acids* 2012;43:1873–86.

[28] D'Aniello A. D-Aspartic acid: an endogenous amino acid with an important neuroendocrine role. *Brain Res Rev* 2007;53:215–34.

[29] Schell MJ, Cooper OB, Snyder SH. D-aspartate localizations imply neuronal and neuroendocrine roles. *Proc Natl Acad Sci* 1997;94:2013–8.

[30] D'Aniello A, Di Fiore MM, Fisher GH, Milone A, Seleni A, D'Aniello S, Perna AF, Ingrosso D. Occurrence of D-aspartic acid and N-methyl-D-aspartic acid in rat neuroendocrine tissues and their role in the modulation of luteinizing hormone and growth hormone release. *FASEB J* 2000;14:699–714.

[31] Pampillo M, Scimonelli T, Bottino MC, Duvilanski BH, Rettori V, Seilicovich A, Lasaga M. The effect of D-aspartate on luteinizing hormone-releasing hormone, α -melanocyte-stimulating hormone, GABA and dopamine release. *Neuroreport* 2002;13:2341–4.

[32] Huang AS, Beigneux A, Weil ZM, Kim PM, Molliver ME, Blackshaw S, Nelson RJ, Young SG, Snyder SH. D-Aspartate regulates melanocortin formation and function: behavioral alterations in D-aspartate oxidase-deficient mice. *J Neurosci* 2006;26:2814–9.

[33] D'Aniello S, Somorjai I, Garcia-Fernández J, Topo E, D'Aniello A. D-Aspartic acid is a novel endogenous neurotransmitter. *FASEB J* 2011;25:1014–27.

[34] Errico F, Nisticò R, Napolitano F, Mazzola C, Astone D, Pisapia T, Giustizieri M, D'Aniello A, Mercuri NB, Usiello A. Increased D-aspartate brain content rescues hippocampal age-related synaptic plasticity deterioration of mice. *Neurobiol Aging* 2011;32:2229–43.

[35] Topo E, Soricelli A, Di Maio A, D'Aniello E, Di Fiore MM, D'Aniello A. Evidence for the involvement of D-aspartic acid in learning and memory of rat. *Amino Acids* 2010;38:1561–9.

[36] Errico F, Nisticò R, Palma G, Federici M, Affuso A, Brill E, Topo E, Centonze D, Bernardi G, Bozzi Y, D'Aniello A, Di Lauro R, Mercuri NB, Usiello A. Increased levels of D-aspartate in the hippocampus enhance LTP but do not facilitate cognitive flexibility. *Mol Cell Neurosci* 2008;37:236–46.

[37] Errico F, Rossi S, Napolitano F, Catuogno V, Topo E, Fisone G, D'Aniello A, Centonze D, Usiello A. D-aspartate prevents corticostriatal long-term depression and attenuates schizophrenia-like symptoms induced by amphetamine and MK-801. *J Neurosci* 2008;28:10404–14.

[38] Kim PM, Duan X, Huang AS, Liu CY, Ming GL, Song H, Snyder SH. Aspartate racemase, generating neuronal D-aspartate, regulates adult neurogenesis. *Proc Natl Acad Sci* 2010;107:3175–9.

[39] Errico F, Nisticò R, Di Giorgio A, Squillace M, Vitucci D, Galbusera A, Piccinin S, Mango D, Fazio L, Middei S, Trizio S. Free D-aspartate regulates neuronal dendritic morphology, synaptic plasticity, gray matter volume and brain activity in mammals. *Transl Psychiatry* 2014;4:e417.

[40] Ozgen M, Reese RN, Tulio AZ, Scheerens JC, Miller AR. Modified 2, 2-azino-bis-3- ethylbenzothiazoline-6-sulfonic acid (ABTS) method to measure antioxidant capacity of selected small fruits and comparison to ferric reducing antioxidant power (FRAP) and 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) methods. *J Agric Food Chem* 2006;54:1151–7.

[41] Daugherty DJ, Selvaraj V, Chechneva OV, Liu XB, Pleasure DE, Deng W. A TSPO ligand is protective in a mouse model of multiple sclerosis. *EMBO Mol Med* 2013;5:891–903.

[42] Tyrakowska B, Lemańska K, Szymusiak H, Borkowski T, Rietjens IMCM. Modified TEAC test for determination of

the antioxidant properties of dietary polyphenolic compounds over a wide pH range. *Pol J Food Nutr Sci* 2003;12:141–8.

[43] Luchetti S, Huitinga I, Swaab DF. Neurosteroid and GABA-A receptor alterations in Alzheimer's disease, Parkinson's disease and multiple sclerosis. *Neuroscience* 2011;191:6–21.

[44] D'Aniello A, Di Cosmo A, Di Cristo C, Annunziato L, Petrucelli L, Fisher GH. Involvement of D-aspartic acid in the synthesis of testosterone in rat testes. *Life Sci* 1996;59:97–104.

[45] Nagata Y, Homma H, Lee JA, Imai K. D-Aspartate stimulation of testosterone synthesis in rat Leydig cells. *FEBS Lett* 1999;444:160–4.

[46] Noorbakhsh F, Baker CB, Power C. Allopregnanolone and neuroinflammation: a focus on multiple sclerosis. *EMBO Mol Med* 2013;5:891–903.

[47] Beck J, Rondot P, Catinot L, Falcoff E, Kirchner H, Wietzerbin J. Increased production of interferon gamma and tumor necrosis factor precedes clinical manifestation in multiple sclerosis: do cytokines trigger off exacerbations? *Acta Neurologica Scandinavica* 1988;78:318–23.

[48] Erta M, Quintana A, Hidalgo J. Interleukin-6, a major cytokine in the central nervous system. *Int J Biol Sci* 2012;8:1254–66.

[49] Stelmasiak Z, Koziol-Montewka M, Dobosz B, Rejdak K, Bartosik-Psujek H, Mitosek-Szewczyk K, Belniak-Legiec E. Interleukin-6 concentration in serum and cerebrospinal fluid in multiple sclerosis patients. *Med Sci Monit* 2000;6:1104–8.

[50] Ireland SJ, Blazek M, Harp CT, Greenberg B, Frohman EM, Davis LS, Monson NL. Antibody-independent B cell effector functions in relapsing remitting multiple sclerosis: clues to increased inflammatory and reduced regulatory B cell capacity. *Autoimmunity* 2012;45:400–14.

[51] Maimone D, Guazzi GC, Annunziata P. IL-6 detection in multiple sclerosis brain. *J Neurol Sci* 1997;146:59–65.

[52] Yan J, Liu J, Lin CY, Csurhes PA, Pender MP, McCombe PA, Greer JM. Interleukin-6 gene promoter-572 C allele may play a role in rate of disease progression in multiple sclerosis. *Int J Mol Sci* 2012;13:13667–79.