The N-Methyl-(2S, 4R)-trans-4-hydroxy-L-proline-Enriched Methanol Fraction from Sideroxylon obtusifolium Shows an Anticonvulsant Activity Associated with its Anti-inflammatory/Antioxidant Actions

Authors
Pedro Everson Alexandre de Aquino1, Ítalo Rosal Lustosa1, Caren Nádia Soares de Sousa1, Adriano José Maia Chaves-Filho1, Francisco Arnaldo Viana Lima1, Alan Diego da Conceição Santos2, Nilce Viana Gramosa2, Edilberto Rocha Silveira2, Glaucê Socorro de Barros Viana1

Affiliations
1 Laboratory of Neuropharmacology, Faculty of Medicine, Federal University of Ceará, Fortaleza, Brazil
2 Department of Organic and Inorganic Chemistry, Federal University of Ceará, Fortaleza, Brazil

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ABSTRACT
Epilepsy is a neurological disorder characterized by recurrent seizures, resulting from excessive neuronal discharges. Sideroxylon obtusifolium is used in Brazil for its anti-inflammatory/antioxidant properties, known to be involved with epilepsy. The anticonvulsant effects of the methanol fraction from S. obtusifolium leaves, rich in N-methyl-(2S,4R)-trans-4-hydroxy-L-proline, were investigated on pilocarpine- and pentylenetetrazole-induced convulsion models. Mice were pretreated with N-methyl-(2S,4R)-trans-4-hydroxy-L-proline (50, 100, 200 mg/kg, p.o.) and, 1 h later, by pilocarpine (400 mg/kg, i.p.) or pentylenetetrazole (80 mg/kg, i.p.). The animals were observed for latency to the first convulsion and latency to death. Immediately after death, brain areas from the pilocarpine groups were harvested for biochemical measurements. The latency to the first convulsion and latency to death increased after N-methyl-(2S,4R)-trans-4-hydroxy-L-proline treatment compared with the pilocarpine- or pentylenetetrazole-only groups. In both convulsion models, sodium valproate (reference drug) was used as a positive control. Additionally, the decreases in striatal dopamine and 3,4-dihydroxyphenylacetic acid contents observed in the pilocarpine-only group were partially prevented in the N-methyl-(2S,4R)-trans-4-hydroxy-L-proline-treated groups. While brain gamma-aminobutyric acid and glutamate contents decreased and increased, respectively, after pilocarpine only, these changes were also prevented by N-methyl-(2S,4R)-trans-4-hydroxy-L-proline. Similarly, N-methyl-(2S,4R)-trans-4-hydroxy-L-proline reduced the brain oxidative stress by decreasing the levels of nitrite and lipid peroxidation and increasing the glutathione content of the pilocarpine-only group. The increases in hippocampal expressions for interleukin 6, interferon-gamma, and glial fibrillary acidic protein, after pilocarpine only, were decreased to normal levels by N-methyl-(2S,4R)-trans-4-hydroxy-L-proline. In conclusion, the study showed significant anticonvulsant effects for N-methyl-(2S,4R)-trans-4-hydroxy-L-proline, probably related to its anti-inflammatory/antioxidant properties. N-Methyl-(2S,4R)-trans-4-hydroxy-L-proline effects were potentiated by VPA (sodium valproate), thus it may also interact with the GABAergic system, as we had recently shown.
Introduction

According to the National Institute of Neurological Disorders and Stroke (USA), epilepsies are a spectrum of brain disorders, ranging from severe, life-threatening, and disabling to more benign forms. In epilepsy, the normal pattern of neuronal activity becomes disturbed and the individual may show convulsions, muscle spasms, and loss of consciousness. Epilepsy affects 65 000 000 people worldwide and can be a major burden in seizure-related disabilities, mortality, and comorbidities [1]. TLE is the most common and prevalent refractory form of adult focal epilepsy [2]. It involves recurrent seizures, arising in the mesial structures of the hippocampus, amygdala, and entorhinal cortex and, thus, it is better named mesial temporal lobe epilepsy [3, 4].

The main characteristics of TLE are epileptic foci in the limbic system, an initial precipitating injury, the latent period, and the presence of hippocampal sclerosis. All these features are reproduced in rodent models of epilepsy by systemic injection of high doses of Pilo [5]. Therefore, the Pilomodel of epilepsy is a valuable tool for studying the pathogenesis of TLE and evaluating potential antiepileptogenic drugs [6]. Furthermore, the understanding of the disease pathogenesis relies largely on the use of animal models.

Sideroxylon obtusifolium (Humb. ex Roem. & Schult.) T.D. Penn. belongs to the Sapotaceae family and is a medicinal plant used in Brazil due to its analgesic and anti-inflammatory activities, as already demonstrated by us [7, 8] and others [9]. Furthermore, experimental and clinical evidence indicates that there is a close relationship between epileptogenesis and brain inflammation. Epileptic seizures increase inflammatory mediators in the brain and the likelihood of recurrent seizures [10]. Additionally, brain neuronal hyperexcitability and oxidative injury produced by free radicals may play a role in the initiation and progression of epilepsy [11].

Thus, considering the involvement of oxidative stress and neuroinflammation in epileptic seizures, the objectives of the present work were to investigate the possible anticonvulsant properties of the L-proline derivative NMP in models of Pilo- and PTZ-induced convulsions in mice. This compound is present in the decoction and mainly in the methanol fraction from the leaves of S. obtusifolium. In addition, there is also evidence for the involvement of dopaminergic neurotransmission [12, 13] and increased astrocyte activation [14, 15] in these experimental models of convulsions. Thus, we also focused on behavioral, biochemical, and immunohistochemical analyses to detect the possible benefits of NMP, mainly on the Pilo-induced convulsive processes and treated groups.

Results

In Pilo-induced seizures, a model of TLE, two behavioral parameters were analyzed: the latency to the first convolution and the latency to death. While no significant change was noticed with the lowest NMP dose (50 mg/kg, p.o.), we showed 1.5 and 1.7 times increases in the latency to the first convolution after treatment with the doses of 100 and 200 mg/kg, respectively, relative to the untreated control (Pilo-only group). In addition, 2.4 times increases were seen in the VPA group (reference group) compared with the control group [F(4,60) = 17.0, p < 0.0001]. Similarly, while no significant change was observed with the dose of 50 mg/kg, significant increases (1.6 and 1.8 times, respectively) were observed in the latency to death after treatments with the two higher NMP doses (100 and 200 mg/kg, respectively) compared to the control group. Also, increases of 2.5 times were noticed in the VPA group compared with the control [F(4,46) = 17.0, p < 0.0001] (Fig. 1).

Another experiment using the same model but a lower Pilo dose (350 mg/kg, i.p.) was performed after a 7-day daily treatment with NMP, and the results on the latency to the first convulsion and the latency to death were similar to those above (data not shown).

In the PTZ-induced seizure model, increases in the latency to the first convolution ranging from 1.6 to 1.9 times were observed after acute treatments with NMP at doses of 50, 100, and 200 mg/kg, p.o., while the VPA-treated group showed an increase of 1.8 times compared with the control group (PTZ only) [F(4,76) = 15.0, p < 0.0001]. Increases in the latency to death, ranging from 2.3 to 3.4 times, were observed after treatments with NMP (50, 100, 200 mg/kg, p.o.) when compared with PTZ only [F(4,63) = 10.57, p < 0.0001] (Fig. 2). The VPA-treated group showed a 2.8 times increase. In another experiment, we demonstrated that the effect of a lower dose of NMP (25 mg/kg) was potentiated by VPA, suggesting that the GABAergic system might be a pharmacological target for NMP (data not shown).

The dopaminergic system is a neuromodulatory system in the brain and has a significant effect on neuronal excitability. It is known to change in the epileptic brain [16]. The Pilo-only group showed a 63 % decrease in striatal DA contents and this decrease was significantly attenuated (41, 19, 31 %) in the striata after treatments...
with NMP at doses of 50, 100, and 200 mg/kg, respectively [F(4,38) = 4.85, p = 0.0029]. A similar result was demonstrated for DOPAC contents, with a 75% decrease in the Pilo-only group, but only 41, 32, and 23% decreases after NMP treatments with doses of 50, 100, and 200 mg/kg, p.o., respectively [F(4,43) = 8.278, p < 0.0001] (Fig. 3).

GABA and GLU are the main inhibitory and excitatory brain amino acids, respectively, thus, as such, very important for seizure development. We showed high GABA level decreases in the brain areas tested (PFC, hippocampus, and striatum) in the Pilo-only group in relation to normal controls. Thus, while 62 to 65% decreases were observed in the PFC [F(3,15) = 4.773, p = 0.0157] and striatum [F(3,15) = 7.399, p = 0.0029], an even higher decrease (84%) was demonstrated in the hippocampus [F(3,17) = 5.35, p = 0.0087]. These changes were reversed after NMP treatments with doses of 100 and 200 mg/kg. On the contrary, in the Pilo-only group, significant increases were observed in GLU contents (5.7, 4.7, and 6.6 times increases, respectively) in the PFC [F(3,18) = 15.29, p < 0.0001].

![Fig. 1](image1.png)

**Fig. 1** Effects of the methanol fraction, rich in NMP, from *S. obtusifolium* on the latency to the 1st convulsion and latency to death in the model of Pilo-induced convulsions in mice. Groups tested (6 to 20 animals): Pilo only, Pilo + NMP treated, and Pilo + VPA treated.

- **a** Latency to the 1st convulsion (a) vs. Pilo only, p = 0.0003; (b) vs. Pilo only, p < 0.0001; (c) vs. Pilo + NMP 50, p = 0.0016; (d) vs. Pilo only, p < 0.0001; (e) vs. Pilo + NMP 50, p < 0.0001; (f) vs. Pilo + NMP 100, p < 0.0001; (g) vs. Pilo + NMP 200, p = 0.0006. **b** Latency to death (a) vs. Pilo only, p = 0.0045; (b) vs. Pilo only, p < 0.0001; (c) vs. Pilo + NMP 50, p < 0.0001; (d) vs. Pilo only, p < 0.0001; (e) vs. Pilo + NMP 50, p < 0.0001; (f) vs. Pilo + NMP 100, p = 0.0056; (g) vs. Pilo + NMP 200, p = 0.0116 (one-way ANOVA and Tukey multiple comparisons test).

![Fig. 2](image2.png)

**Fig. 2** Effects of the methanol fraction, rich in NMP, from *S. obtusifolium* on the latency to the 1st convulsion and latency to death in the model of PTZ-induced convulsions in mice. Groups tested (6 to 22 animals): PTZ only (control), PTZ + NMP treated, and PTZ + VPA treated.

- **a** Latency to the 1st convulsion (a) vs. PTZ only, p = 0.0023; (b) vs. PTZ only, p < 0.0001; (c) vs. PTZ only, p = 0.0001; (d) vs. PTZ only, p = 0.0002. **b** Latency to death (a) vs. PTZ only, p < 0.0001; (b) vs. PTZ only, p < 0.0001; (c) vs. PTZ only, p = 0.0150 (one-way ANOVA and Tukey multiple comparisons test).
Epilepsy is a neurological disease whose mechanisms involved with seizure generation are not completely understood. Therefore, the search for new antiepileptic drugs for treating around 30% of patients refractory to conventional therapies remains greatly in need. Up to now, the most important action mechanisms for seizure generation focus on the hyperactivity of excitatory amino acid systems, insufficient GABA_A receptor-mediated neurotransmission, and disturbances in the properties of neuronal membranes [18].

In the present work, we aimed at investigating the anticonvulsant activity of the methanol fraction rich in NMP, an L-proline derivative, and major bioactive constituents present in S. obtusifolium leaves. Considering the beneficial properties of S. obtusifolium and the increasing evidence for the inflammatory processes involved with con-
vulsions and epilepsy [19], we showed that the L-proline derivative (NMP) significantly increased latency to the first convulsion and latency to death in a Pilo-induced convulsion model in mice.

Furthermore, the dopaminergic system has been shown to have a seizure-modulating effect that depends upon the brain dopamine receptor subtypes [12]. Evidence indicates that drugs stimulating
Fig. 5 Effects of the methanol fraction, rich in NMP, from *S. obtusifolium* on brain nitrite content in mice subjected to Pilo-induced convulsions. Groups tested (5 to 7 animals): control (normal), Pilo only, and Pilo + NMP treated. a PFC (a) vs. control, p < 0.05; (b) vs. Pilo + NMP 100, p < 0.05; (c) vs. Pilo + NMP 200, p < 0.01. b Hippocampus (a) vs. control, p < 0.05; (b) vs. Pilo + NMP 100, p < 0.01; (c) vs. Pilo + NMP 200, p < 0.01. c Striatum (a) vs. control, p < 0.001; (b) vs. Pilo + NMP 100, p < 0.05; (c) vs. Pilo + NMP 200, p < 0.001; (d) vs. control, p < 0.001; (e) vs. control, p < 0.001 (one-way ANOVA and Tukey multiple comparisons test).

Fig. 6 Effects of the methanol fraction, rich in NMP, from *S. obtusifolium* on brain lipid peroxidation (MDA, nmol/g tissue) in mice subjected to Pilo-induced convulsions. Groups tested (5 to 16 animals): control (normal), Pilo only, and Pilo + NMP treated. a PFC (a) vs. control, p < 0.001; (b) vs. Pilo + NMP 100, p < 0.001; (c) vs. Pilo + NMP 200, p < 0.001. b Hippocampus (a) vs. control, p < 0.01; (b) vs. Pilo + NMP 100, p < 0.01; (c) vs. Pilo + NMP 200, p < 0.01. c Striatum (a) vs. control, p < 0.01; (b) vs. Pilo + NMP 100, p < 0.05; (c) vs. Pilo + NMP 200, p < 0.05 (one-way ANOVA and Tukey multiple comparisons test).
the dopaminergic system, such as apomorphine and amphetamines, as well as antiparkinsonian drugs, such as pergolide and bromocriptine, present antiepileptic and anticonvulsant effects [20, 21]. Dopaminergic neurons also seem to modulate synaptic plasticity, a phenomenon affected by seizure activity [22]. Corroborating with these findings, we showed that the striatum from brains of animals subjected to Pilo-induced convulsions presented decreased DA and DOPAC contents, effects which were partially reversed in the NMP-treated groups.

Besides the neuromodulatory role of the dopaminergic system, an intense change in neuronal activity in excitatory (glutamatergic) and inhibitory (GABAergic) neurotransmissions is also known to occur in epilepsy [16]. Earlier, GLU and GABA were shown to be centrally involved in the kindling process that constitutes a model of complex partial seizures [23]. In the present work, the changes in the contents of brain GABA and GLU, observed in mice after the pilocarpine-induced convulsions, were reversed by NMP treatments.

The oxidative stress and excessive ROS production are involved with the initiation and progression of epileptic seizures [24]. Oxidative stress in the hippocampus was previously observed [25] after Pilo-induced seizures in rats, as evidenced by changes in GSH, nitrite content, and lipid peroxidation. Later, a direct relationship between lipid peroxidation and nitrite content was seen in some brain areas after acute seizure activity [26]. Now, we showed that the Pilo-induced seizures in mice cause higher increases in nitrite and lipid peroxidation and also decreases GSH contents in the mice brain, which are indexes of oxidative stress. The treatment of such Pilo groups with NMP prevented all these changes.

The tripeptide GSH, among other physiological functions, is highly involved in brain protection against ROS [27]. Additionally, a compromised brain GSH system has been connected to the oxidative stress, occurring in neurological diseases, including epilepsy [28]. Furthermore, a widespread impairment of the GHS system in epileptic patients was also demonstrated [29]. We showed that Pilo-induced seizures significantly decreased GSH content and these values were increased and brought towards normality in all brain areas tested after the NMP treatments.

A large body of evidence indicates a role for inflammatory mediators in epileptogenesis [12, 30] and epileptic seizures. In addition, the increase in inflammatory mediators was shown to result in secondary brain damage and the likelihood of recurrent seizures [10]. We observed significant increases in brain IL-6 and IFN-γ levels in the presence of Pilo-induced seizures in the mouse hippocampus. These changes were completely brought back to control levels after NMP treatments. Post-ictal serum increases in cytokine levels, including IL-6 and IFN-γ, were demonstrated in epileptic patients [31], corroborating with our findings.

Previous studies [32] suggested that alterations in gene expression are involved in the genesis of epilepsy and also in the induction of GFAP expression in astrocytes. Earlier evidence [33] reported intense neuronal activity in the hippocampus, leading to a rapid and drastic increase in GFAP expression. Thus, astroglisosis is considered an important feature in epilepsy and may also be involved in the development and persistence of seizures that result in prominent hypertrophy of astrocytes [34]. Although reactive astrocytes are commonly found in epileptic foci, the knowledge of whether...
Astrogliosis is a cause or a consequence of epileptogenesis is still an unsolved question [35].

Even though some studies [15, 36, 48] have shown that astrocyte activation needs some time (hours to days) to occur, we showed that Pilo-treated mice present acute and significant increases in GFAP immunostainings in the hippocampus and TC shortly after seizures. These changes were highly attenuated by treatment with NMP. However, others [37] also detected acute astrocyte activation in the brain by MRI, providing insight into acute and reversible brain injury processes in neurologic patients. It is important to point out that these data corroborate our findings. Furthermore, dysfunctional astrocytes are crucial players in epilepsy and should be considered potential and promising targets in therapies for this neurologic disorder [38].

Altogether, the data of the present work strongly suggest a neuroprotective effect for this L-proline derivative (NMP), highly present in the methanol fraction from S. obtusifolium, in the model of Pilo-induced seizures in mice. Part of this is probably a consequence of the anti-inflammatory property of NMP, as already shown by us [7]. In an earlier study [39], L-proline was shown to suppress clonic tonic and focal clonic seizures in ouabain-induced seizures in rats.

Furthermore, considering the important role of GABA in the mechanism and treatment of epilepsy, we found that NMP may also act through the GABAergic pathway, since it significantly increased both latencies to the first convulsion and death in the PTZ model. In addition, sodium VPA, used as a reference, when combined with a lower NMP dose (25 mg/kg) potentiated the NMP effect on the latency to death. Interestingly, similar findings were also observed with the standard compounds L-proline and trans-4-hydroxy-L-proline and also, as expected, with NMP itself (data are not shown). Most bioactive small molecules, such as NMP, are known to interact with proteins or other macromolecule targets, which results in their final biological effects. Recently, we reported that one of the possible targets for the NMP molecule is the GABA transporter (GAT-1) [40]. These results point again to GABA neurotransmission involvement in the NMP action mechanism.

Furthermore, at least one-third of epileptic patients do not present any benefit from conventional pharmacological treatment [41, 42]. Recently, the amino acid D-leucine was shown to potentially protect mice, when administered before the onset of the kainic acid-induced seizures [43]. The authors found that the D-leucine, present in trace amounts in the brain, worked even better than L-leucine against both kainic acid and electroshock-induced seizures. Earlier, L-proline and derivatives, such as trans-4-hydroxy-L-proline, cis-4-hydroxy-D-proline, and 3,4-dehydro-DL-proline, were shown to be weak anticonvulsants when used alone, but were able to potentiate the anticonvulsant effect of vigabatrin similarly to that reported for glycine [44].

The intracellular L-proline accumulation in mammalian cells has been demonstrated to correlate with decreased ROS levels and increased protection against oxidative stress [45]. L-proline was also shown to improve the survival rate of vitrified mouse oocytes for protecting mitochondrial functions [46]. The coadministration of L-proline and LPS pointed out the ability of L-proline in preventing the harmful effects of LPS [47]. These authors indicated that LPS induces inflammation and oxidative stress in the rat cerebral cortex and cerebellum, and the coadministration with L-proline prevents these LPS effects.

In conclusion, we showed, for the first time, the neuroprotective effects of the proline derivative NMP isolated from S. obtusifolium. These NMP neuroprotective properties are surely associated with its anti-inflammatory and antioxidant effects. However, considering that NMP also presented anticonvulsant activity in the PTZ model, GABAergic neurotransmission is probably an NMP target also involved with the drug anticonvulsant action.
Materials and Methods

Plant material and preparation of the methanol fraction containing the bioactive constituent N-methyl-(2S,4R)-trans-4-hydroxy-L-proline

*S. obtusifolium* leaves were collected and handled as previously described [8] and identified (voucher specimen #10,648) by Maria Arlene Pessoa da Silva, Ph.D., botanist at the Herbarium “Dárdano de Andrade Lima”, Regional University of Cariri (URCA), Crato, Brazil. The methanol fraction from the leaves decoction used in the present work contains the bioactive compound NMP and was obtained according to a procedure previously described [7]. The amount of N-methyl-(2S, 4R)-trans-4-hydroxy-L-proline present in the methanol fraction (NMP) tested was also studied by qHNMR, as shown in Supporting Information. In addition, spectra for both the methanol fraction and the standard compound (NMP) were also carried out (see Supporting Information).

Animals

Male Swiss mice (25–33 g) were maintained at 25 ± 2 °C, under a 12/12-h light/dark cycle. Food and water were provided ad libitum. The study was submitted and approved by the Ethics Committee on Animal Research of the Faculty of Medicine of the Federal University of Ceará (CEUA/UFC), under the number 59/17, on February 26, 2018.

Drugs and reagents

Pilo and PTZ were purchased from Sigma-Aldrich and showed a degree of purity of around 99 %. Depakene (liquid containing 50 mg/mL sodium valproate) was from Abbott Laboratórios do Brasil Ltda. All other drugs and reagents were of analytical grade.

Pilocarpine-induced seizures and behavioral assessment

Mice (6–20 animals per group) were pretreated by gavage with NMP (50, 100, and 200 mg/kg, p.o., dissolved in distilled water, 0.1 mL/100 g) or VPA (10 mg/kg, used as the reference or positive control). After 1 h, seizures were induced in all groups by the systemic administration of Pilo (400 mg/kg, i.p.). The Pilo-only group was the control group. Then, the animals were placed in individual cages and observed for the following behavioral parameters: latency to the first seizure (elapsed time before the first seizure) and latency to death (time elapsed until death). The seizure was characterized primarily by the hind limb extension and/or uncoordinated jump. Immediately after death, the animals were decapitated and
the brain areas (PFC, hippocampus, and striatum) were dissected for biochemical tests.

**Pentylenetetrazole-induced seizures**

Although the present study focused on the Pilo-induced seizure model, we also performed the model of PTZ-induced seizures, since GABA neurotransmission may be a possible target for NMP. PTZ is considered to have an antagonistic action on GABA_\text{A} receptors. For that, mice (6–22 animals per group) were pretreated by gavage with NMP (50, 100, and 200 mg/kg, p.o., dissolved in distilled water) or VPA (10 mg/kg) as a reference or positive control. After 1 h, seizures were induced in all groups by the systemic administration of PTZ (80 mg/kg, i.p., PTZ-only group). The following behavioral parameters observed were: latency to the first seizure (elapsed time before the first seizure) and latency to death (time elapsed until death). The seizure was characterized primarily by the hind limb extension and/or uncoordinated jump. The possible potentiation of the NMP effect by VPA was also determined.

**Neurochemical determinations of dopamine and 3,4-dihydroxyphenylacetic acid by HPLC**

The contents of DA and its metabolite DOPAC were determined by HPLC (electrochemical detector, model L-ECD-6A; Shimadzu) in the striata from 3 to 10 animals per group according to previously described methods [14]. Briefly, homogenates were prepared in 10 % HClO\text{4} and centrifuged at 4 °C (25000 \times g, 15 min). The supernatants were filtered and 20 \mu L were injected into the HPLC column (flow of 0.6 mL/min). Monoamines were quantified by comparison with standards and the results are expressed as ng/g tissue.

**Brain gamma-aminobutyric acid and glutamate determinations by HPLC**

Brain areas (PFC, hippocampus, and striatum) from 4–6 animals were used for the determination of amino acid concentrations. This assay was carried out by reversed-phase HPLC involving pre-column derivatization with orthophthalaldehyde according to a method previously described [48]. The results are expressed as µg/g tissue.

**Determination of brain nitrite contents**

Griess reagent was added to a 96-well plate containing supernatants of brain homogenates (PFC, hippocampus, and striatum areas) from 5–7 animals. The absorbance was measured using a microplate reader at 560 nm. In addition, the same brain areas of the animals treated only with vehicle were dissected in the absence of the seizure inducer drug (basal level group). Previously, a standard curve for nitrite was generated using concentrations of 100, 50, 25, 12.5, 6.25, 3.12, and 1.56 nmol/mL [49]. The results are expressed as nmol/g tissue.

**Determination of brain lipid peroxidation by thiobarbituric acid reactive substances assay**

Lipid peroxidation expresses the oxidative stress induced by ROS reactivity. This method is used for the determination of MDA in biological samples. The brain areas (PFC, hippocampus, and striatum) from 5 to 7 animals were used for the preparation of 10 % homogenates in 1.15 % KCl. Then, 250 \mu L were added to 1 mL 10 % TCA, followed by addition of 1 mL 0.6 % thiobarbituric acid. After agitation, this mixture was maintained in a water bath (95–100 °C, 15 min), cooled on ice, and centrifuged (1500 \times g/5 min). The TBARS content was determined on a plate reader at 540 nm, with results expressed in nmol MDA/g tissue. A standard curve with MDA was also performed [50].

**Determination of the brain concentration of reduced glutathione**

The determination of the GSH concentration was performed in the brains of 12 animals. The assay is based on the reaction of Ellman reagent (DTNB) with the free thiol, giving a disulfide plus 2-nitro-5-thiobenzoic acid mixture. The sample preparation of brain areas (PFC, hippocampus, and striatum) was carried out as follows: 10 % homogenates in phosphate buffer were added to an Eppendorf containing 50 \mu L distilled water and 10 \mu L trichloroacetic acid (50 %). After centrifugation (1000 \times g for 15 min at 4 °C), the supernatants (60 \mu L) were added to the cooled ELISA microplates. Immediately before the readings at 412 nm, 102 \mu L of the mixture of Tris HCl buffer, and 0.65 mL of 0.01 M DTNB in methanol were added to each well. The concentration of reduced GSH is expressed as µg/g tissue based on a standard curve of GSH.

**Immunoassays for hippocampal interferon-gamma and interleukin 6**

The brain area (hippocampus) from 7 animals per group was homogenized in 8 volumes of PBS buffer containing a protease (EMD Biosciences) inhibitor and centrifuged (9000 \times g, 5 min). The concentration of the cytokines in 100 µL samples was determined by an immune-enzymatic assay (ELISA; R&D Systems) according to the manufacturer’s protocol. The results are expressed in pg/g tissue.

**Immunohistochemical assays for glial fibrillary acidic protein**

Hippocampal sections (5 \mu m, 3 animals per group) were fixed in buffered formalin followed by 70 % alcohol. This was followed by deparaffinization, hydration in xylol and ethanol, immersion in 10 % HClO\text{4} for 15 min, rinsing, the sections were stained with 3,3 diaminobenzidine-peroxidase complex. After a final wash with deionized water, the sections were rinsed with PBS (1:200 dilution), washed in PBS, and incubated (30 min) with the conjugated streptavidin-peroxidase complex. After a final washing, the sections were stained with 3, 3 diaminobenzidine-peroxide, dehydrated, and mounted in microscope slides for analyses. The data were semiquantified with the Image J software [51].

**Statistical analyses**

The results are presented as means ± SEM and were analyzed by one-way ANOVA followed by the Tukey test for multiple comparisons. The immunohistochemical data were analyzed by Image J software. All results were considered significant at p < 0.05.
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Conflict of Interest

The authors declare that they have no conflict of interest.

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