



Oxidative Stress and Lymphocyte Alterations in Chronic Relapsing Experimental Allergic Encephalomyelitis in the Rat Hippocampus and Protective Effects of an Ethanolamine Phosphate Salt

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Received: 28 May 2019 / Accepted: 1 September 2019 / Published online: 10 September 2019
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Abstract

Chronic relapsing experimental allergic encephalomyelitis (CR-EAE) exhibits neuropathological and immunological dysfunctions similar to those found in multiple sclerosis (MS) and has been used as an animal model of MS. Inflammatory infiltrates and oxidative stress have been linked to the development of both diseases. Ethanolamine plasmalogen derivatives have been shown to be powerful antioxidants and immunomodulators. Therefore, the objective of this study was to analyse inflammatory infiltrates, the state of the oxidative defences and the possible protective effects of calcium, magnesium and phosphate ethanolamine (EAP) in the CR-EAE rat hippocampus. To this aim, we evaluated, by immunohistochemistry, T cell infiltrates, Iba-1⁺ (a marker of activated microglia) immunoreactivity and TUNEL (+) cells. We also measured the protein levels and activity of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GP) and glutathione reductase (GR). In addition, reduced (GSH) and oxidized (GSSG) glutathione levels, lipid peroxidation and cholesterol as well as desmosterol content were determined. We found an increase in T cell infiltrates and Iba-1⁺ immunoreactivity, lipid peroxidation, SOD, GP and GR activities as well as enhanced cholesterol levels and a decrease in CAT activity, GSH and desmosterol levels in the first and second attack in the CR-EAE rat hippocampus. Pretreatment of CR-EAE rats with EAP led to a delay in the onset of the clinical signs of the disease as well as a decrease in inflammatory infiltrates and alterations of the antioxidant defences in the hippocampus. Altogether, the present results suggest a protective role of EAP in the CR-EAE rat hippocampus.

Keywords CR-EAE · Hippocampus · Multiple sclerosis · Oxidative stress · Antioxidant defences · Ethanolamine phosphate

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12035-019-01774-8>) contains supplementary material, which is available to authorized users.

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Introduction

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating disease of the central nervous system (CNS) that represents the most frequent cause of chronic disability in young adults [1]. The chronic relapsing form of experimental allergic encephalomyelitis (CR-EAE) is an autoimmune disease widely used as an animal model of MS due to the autoimmune, histopathological, genetic and clinical similarities [2–4]. MS is considered a two-stage disease that consists of an inflammatory phase and a neurodegenerative phase [5]. At the histological level, inflammatory infiltrates accumulate within the CNS. These consist mainly of T cells [6], macrophages [7], B cells and granulocytes [8]. Activated macrophages and microglial cells have both been shown to produce a number of factors that can contribute to tissue damage, such as cytokines (TNF- α , IFN- γ), chemokines, nitroxides and reactive oxygen species (ROS) [9–14]. Although ROS play a

physiological role in numerous cellular regulatory processes [15], oxidative stress occurs when the free radical generation exceeds the capacity of the antioxidant defences, leading to subsequent damage of different macromolecules, like proteins, lipids and nucleic acids [16].

MS has been classically regarded as a white matter disease. Recent studies, however, have convincingly shown that grey matter regions are also heavily affected [17]. In fact, some abnormalities in the grey matter have been consistently found with *in vivo* magnetic resonance [17]. Moreover, approximately 50–65% of MS patients experience cognitive deficits; among these, memory dysfunction is especially common [18, 19]. The cause of memory dysfunction is currently unknown, but recent studies have revealed hippocampal atrophy and neurodegeneration in MS [20] as well as hippocampal CA1 atrophy and synaptic loss during EAE [21]. The hippocampus, a brain area that acts as a memory device, seems to be strongly affected by the deleterious effects of oxidative insult [22]. In rats with the acute form of EAE, our group has previously reported alterations of the hippocampal somatostatinergic system, which is involved in behaviour and memory [23, 24].

Despite progress in recent years, an effective therapy that heals MS is not yet available. Most treatments against MS used today are based on immunomodulation. There are treatments, for example, that reduce the transfer of immune cells into the CNS parenchyma by interfering with the $\alpha 4\beta 1$ -integrin receptor on the surface of T cells [25–27] whereas other treatments sequester lymphocytes in lymph nodes, preventing them from contributing to an autoimmune reaction [28, 29]. Unfortunately, some of these treatments pose serious problems. Treatment with natalizumab, for instance, increases the risk of a potentially serious viral infection of the brain called progressive multifocal leukoencephalopathy in people who are positive for antibodies to the causative agent, the JC virus [30]. In EAE, a series of studies have revealed a protective effect of exogenous antioxidants [16, 31, 32]. However, despite promising results in the treatment of EAE, evidence for successful antioxidant therapies in MS patients is very limited.

Ethanolamine is a precursor in the biosynthesis of anandamide, an endocannabinoid whose levels are decreased in MS patients [33]. A study of the sclerotic plaques found in white matter of MS patients has revealed alterations in the composition of ethanolamine phosphoglycerides as compared to normal tissue [34–37]. In rat brain, certain evidence indicates that the plasmalogen phosphatidylethanolamine protects against oxidative stress due to its antioxidant effects against lipid peroxidation [38, 39]. In addition, ethanolamine plasmalogens have been shown to reduce membrane oxidizability and to have anti-inflammatory effects [39, 40] whereas certain phosphoethanolamine derivatives are immunomodulators that regulate macrophage activation [41, 42].

In view of the above, the aim of the present study was to analyse the inflammatory infiltrates, the state of the antioxidant defence mechanisms and the possible preventive effects of a calcium, magnesium and phosphate ethanolamine salt (EAP) in the hippocampus of rats with CR-EAE at three different phases along the progression of the disease: first attack, remission and second attack.

Materials and Methods

Materials

Complete Freund's adjuvant (CFA) and *Mycobacterium tuberculosis* H37RA were obtained from Difco (Detroit, MI, USA). Thiobarbituric acid, nitro blue tetrazolium (NBT), cacodylate, glutathione reductase (GR), trichloroacetic acid (TCA), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Madrid, Spain). Specific antibodies against catalase (C-0979) and GAPDH (SAB2500451) were purchased from Sigma-Aldrich (Madrid, Spain). Specific antibodies against superoxide dismutase (sc-8637), glutathione reductase (sc-133245) and glutathione peroxidase (sc-74498) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-goat (401515), anti-rabbit (401315) and anti-mouse (401215) secondary antibodies were obtained from Calbiochem (Madrid, Spain). Amersham Hybond-p PVDF membranes were purchased from GE-Healthcare Life Science (Barcelona, Spain). Specific antibodies against CD3⁺ (Sigma-Aldrich (C7930)) and CD8⁺ (Millipore.), Iba1⁺ (Wako, Japan) and immunoglobulin G (Becton, Dickinson, USA) were used for immunohistochemistry. To determine cell death, the DeadEnd™ Fluorometric TUNEL System (Promega, Wisconsin USA) was used. To amplify the signal, we used MaxVision, a rabbit polymer-peroxidase system (Master Diagnostica, Granada, Spain).

Experimental Animals

The animal experiments performed in the present study conform to the guidelines set by the Animal Care Committee of Alcalá University as well as to the International guidelines on the ethical use of animals set by the Council of Europe (Protection of Animals Used for Experimentation, 1986 ETS No. 123), and all experimental protocols were previously approved. Extreme efforts were made to minimize the number of animals used and their suffering. Sixty 6-week-old female Lewis rats were purchased from Harlan Interfauna Ibérica, S.L. (Barcelona, Spain). Rats were maintained on a 12-h light/dark cycle (07:00–19:00 h) regime and were provided food and water *ad libitum*. Rats were divided into the following experimental groups: control rats; rats with chronic

relapsing experimental allergic encephalomyelitis (CR-EAE); rats with CR-EAE pretreated with a calcium, magnesium and phosphate ethanolamine salt (EAP) and sacrificed at the same time as the CR-EAE rats (CR-EAE + EAP_{grade 1}); rats with CR-EAE pretreated with EAP and sacrificed in grade 3 (CR-EAE + EAP_{grade 3}) and control rats pretreated with EAP. Rats slightly narcotized with halothane were immunized with an emulsion of guinea-pig spinal cord tissue (GPSC) at a final concentration of 290 mg/ml buffered in PBS with an equal volume of complete Freund's adjuvant (CFA) containing 11 mg/ml of *Mycobacterium tuberculosis* (strain H37RA) in a final volume of 100 μ l [43, 44]. Control rats were injected only with PBS/CFA. Animals were weighed and monitored daily for clinical signs of CR-EAE, assessed according to the following criteria: 0, no clinical signs; 1, limp tail; 2, hind limb weakness; 3, complete hind limb paralysis [45].

EAP was freshly prepared in distilled water and buffered to approximate neutrality using HCl. EAP was injected i.p. at a dose of 600 mg/kg/day to CR-EAE and control rats for 2 days prior to induction. Rats were decapitated at the first or third stage of the disease. Rats from each group were sacrificed on days 15–17 following induction (at the first attack) or on day 24 after induction (at the remission phase). The group of CR-EAE + EAP_{grade 3} rats was sacrificed in grade 3 on days 18–20. On day 28–30, rats from each group were sacrificed (at the second attack). The brains of the sacrificed rats were rapidly removed, and the hippocampus was dissected on ice according to the method of Glowinski and Iversen [46].

Immunohistochemistry

The brains were quickly removed, fixed with 10% (v/v) formaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin wax. In this study, we evaluated similar areas of the medial section of the hippocampus rostro-caudal areas (Supplementary Fig. 1). Sections (5 μ m) were deparaffined with xylene and rehydrated using graded ethanol concentrations. Antigen retrieval was achieved by incubation with 10 mM citrate buffer for 2 min using an express cooker. Endogenous peroxidase was inhibited with 3% H₂O₂ in H₂O for 10 min and 0.3% H₂O₂ in methanol for 10 min at room temperature. After rinsing with Tris-buffered saline (TBS), the sections were incubated with blocking solution (3% normal donkey serum (NDS) plus 0.05% Triton X-100 in TBS) for 60 min to prevent non-specific background. Afterwards, the sections were incubated overnight at room temperature with the primary antibodies diluted in 0.3% NDS–0.05% triton X-100 in TBS: monoclonal rabbit anti-Iba-1 (Wako Chemicals USA) diluted 1:1500, polyclonal rabbit anti-CD3 (Sigma) diluted 1:1000, monoclonal mouse anti-CD8 (Millipore) diluted 1:1000 and polyclonal anti-IgG (Millipore) diluted 1/200. After washing with TBS, samples were incubated under dark conditions for 60 min at room temperature with the

corresponding secondary antibody. For the immunoenzymatic detection method, the MaxVision rabbit polymer-peroxidase system (Master Diagnostica, Granada, Spain) was used. Peroxidase activity was detected with diaminobenzidine (DAB kit, Master Diagnostica). For determination of apoptosis in situ, brains embedded in paraffin wax were cut into 5- μ m slices. Colorimetric apoptosis detection was performed with the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) reaction according to the manufacturer's instructions (Promega). After the reaction, slides were counterstained with Harris' haematoxylin, dehydrated with increasing concentrations of ethanol and xylene, mounted with DePeX and observed under an optical microscope.

Preparation of Membrane and Cytoplasmic Fractions

The hippocampus was homogenized in a solution of 10 mM HEPES–KOH buffer (10% w/v) (pH 7.6) with a Brinkmann Polytron homogenizer (setting 5, 15 s). The homogenate was spun at 600 \times g for 5 min at 4 °C, and the resultant supernatant was subsequently centrifuged at 48,000 \times g for 30 min at 4 °C. The cytoplasmic fraction was aliquoted and immediately stored at –80 °C. For each experimental animal, one aliquot was used to determine protein concentration following the Lowry method [47]. The resulting pellet was resuspended in 10 mM HEPES–KOH buffer (pH 7.6) (10% w/v), centrifuged as before, and the pellet was resuspended in 50 mM Tris–HCl buffer (pH 7.5). Aliquoted samples were immediately stored at –80 °C until the day of assay. For each experimental animal, one aliquot was used to determine protein concentration following the Lowry method [47].

Measurement of Lipid Peroxidation

Lipid peroxidation was measured by assessing the levels of reactive species to thiobarbituric acid according to a slightly modified method of Ottolenghi [48]. Briefly, 100 μ l of hippocampal membranes was suspended in 200 μ l of trichloroacetic acid (TCA), sonicated during 15 s, incubated on ice for 15 min and then centrifuged in a tabletop centrifuge at 2200 \times g at 4 °C for 15 min. A small volume of each sample (200 μ l) was mixed with an equal volume of thiobarbituric acid (TBA). The mixture was then placed in a boiling water bath for 10 min. The absorbance of the solution was read at 535 nm. The results were expressed in mmol of malondialdehyde per total milligrams of protein.

Superoxide Dismutase Activity

To measure hippocampal superoxide dismutase (SOD) activity, the cytoplasmic fraction of the enzyme was first extracted by eluting samples of the homogenized hippocampus in a

solvent mix of 500 μ l cold ethanol and 250 μ l chloroform during 2 min. The mixture was then centrifuged at 18,000 \times g during 1 h at 4 °C, and the supernatants were stored at –20 °C. SOD activity was measured according to the method described by Minami and Yoshikawa [49]. Briefly, a small volume of the samples (150 μ l) was mixed with Triton X-100 (16%), 0.98 mM nitro blue tetrazolium (NBT) and 1.8 mM pirogallol and buffered with Tris-cacodylate buffer (pH 8.2). The absorbance was then measured at 540 nm. The solution was incubated for another 5 min at 37 °C. A stop solution made of 80% 2 M formic acid and 16% Triton X-100 was added and the absorbance of the solution was measured again at 540 nm. SOD activity was then calculated following the method of McCord and Fridovich [50].

Catalase Activity

Catalase (CAT) activity was measured by a method based on the reduction of hydrogen peroxide (Aebi, 1984). In a quartz cuvette, 0.1 ml of each sample was added to 0.9 ml of 50 mM potassium phosphate buffer (pH 7.0) and 0.1 ml of 19 mM hydrogen peroxide, and the absorbance was measured at 240 nm for 5 min. The absorbance decay observed is called “k” and reflects CAT activity. CAT activity was expressed as “k” per milligram of protein, following the method described by Aebi [51].

Determination of GP and GR Activity

Glutathione peroxidase (GP) and glutathione reductase (GR) activities were measured using commercial kits from BioVision Company (Catalogue number: no. 762-100 for GP activity and no. k761-100-200 for GR activity). To measure GP activity, 100 μ l of sample was mixed with GPX assay Buffer, NADPH solution, GR solution and GSSG solution, according to the manufacturer’s instructions. The absorbance of the mixture was measured during 5 min at 340 nm at 25 °C. The results are expressed as μ mol NADPH oxidized/min/mg protein.

To determine GR activity, 100 μ l of sample was mixed with 5 μ l of 3% H₂O₂ and incubated 5 min at 25 °C. Subsequently, a GR assay buffer, DTNB solution, NADPH-GNERAT™ solution and GSSG were added to 100 μ l of pretreated samples and the absorbance was measured during 10 min at 450 nm at room temperature. GR activity is expressed as μ mol NADPH oxidized/min/mg protein.

Glutathione Levels

Glutathione levels were measured using the cyclic method of Griffith [52]. For this purpose, it was necessary to deproteinize the samples by mixing them with a volume of 20% TCA during 20 min at 4 °C and subsequently centrifuging at

11,000 \times g for 10 min at 4 °C. The supernatants were then transferred to Eppendorf tubes and stored at –80 °C.

Determination of T-GSH, GSSG, and GSH Levels

To assess t-GSH levels, 50 μ l of each deproteinized sample obtained above was mixed with a reaction mixture made up of 0.30 mM NADPH, 0.225 mM DTNB and 1.6 U/ml glutathione reductase (GR) and incubated for 5 min at 37 °C. Afterwards, the absorbance was measured at 405 nm during 5 min. To assess GSSG levels, the deproteinized samples were mixed with 4-vinylpyridine (4-VP) (at a concentration 10 times the GSX levels) and 0.2 N NaOH and incubated during 1 h at room temperature. Afterwards, 50 μ l of the solution was mixed with a reaction mixture containing 0.30 mM NADPH, 0.225 mM DTNB and 1.6 U/ml GR and incubated for 5 min at 37 °C. The absorbance was then measured as described previously. GSH levels were calculated, on the one hand, by subtracting the oxidized glutathione from total glutathione and, on the other hand, by measuring GSH levels using a fluorometric method as described by Hissin and Hilf [53].

Protein Immunodetection

Membranes and cytosolic or tissue extracts (100 μ g) were solubilized in sodium dodecyl sulphate (SDS)-sample buffer, and the proteins were then run on an 8–12% SDS-polyacrylamide gel. The transfer of proteins to PVDF membranes and the immunodetection of proteins using specific antibodies were carried out as described elsewhere [54, 55]. Briefly, after protein transfer, the PVDF membranes were blocked with blotting buffer (TTBS) [50 mM Tris–HCl, pH 7.5; 150 mM NaCl; 0.05% (v/v) Tween-20] and 5% (w/v) non-fat dry milk. PVDF membranes were incubated overnight at 4 °C with primary antibodies diluted 1:1000 in blotting buffer [50 mM Tris–HCl, pH 7.5; 150 mM NaCl; 0.05% (v/v) Tween-20]. Subsequently, excess antibody was removed and membranes rinsed with blotting buffer. After washing, secondary horseradish peroxidase-conjugated antibodies diluted 1:2000 in blotting buffer were added for 60 min at 4 °C for the immunodetection of the target protein. After washing unbound antibody, the proteins were detected by chemiluminescence using an ECL western blotting detection kit, according to the manufacturer’s instructions. Quantification of the bands was carried out by densitometric analysis using the Scion Image computer program (Scion Inc., Frederick, MD, USA).

HPLC Analysis of Sterol Content

Hippocampal membranes from each experimental animal were washed twice with ice-cold PBS and resuspended in 0.5 ml of 10% (w/v) KOH. Ergosterol was added as an

internal standard. Samples were treated sequentially, as described by Sánchez-Wandelmer [56], with a solvent mixture of chloroform-methanol in a volumetric proportion of 2:1 and distilled water to obtain the lipid and hydrosoluble fractions. The lipid extract was further separated into a saponifiable and non-saponifiable fraction. Non-saponifiable lipids were resuspended in hexane and sterol separation was performed by HPLC. Sterol separation was accomplished by reverse-phase HPLC with a Luna-Pack 5 μm pore size C18 column (250 mm X 4.60 mm; Phenomenex). Lipids were eluted with acetonitrile-water (95:5, v/v) for the first 37 min and then with methanol at a flow rate of 1.2 ml/min. Eluents were monitored by ultraviolet-absorption spectroscopy (Beckman 168 variable-wavelength detector; Beckman Instruments). Eluted sterols were identified by comparing retention times and ultraviolet spectra with those of pure standards.

Statistical Analysis

Statistical comparisons of enzyme activity and protein levels were carried out by a nonparametric test, the *U* Mann Whitney test. Differences among groups were considered significantly different when the *P* value was less than 0.05. Each individual experiment was performed in duplicate.

Evaluation of Iba-1⁺ cells and the IgG signal was performed using a system of subjective grading, ranging from no labelling intensity (–) to the maximum intensity (+ + +). This evaluation was performed by two independent observers. Subsequently, the intensity levels obtained were grouped into different levels of expression for further analysis and interpretation, following the grading scale: – = 0; –/+ , + = 1; +/++ , ++ = 2; ++/+++ , +++ = 3. For evaluating microglia activation, Iba-1⁺ cells were classified, according to its morphology, into 4 scores ranging from 1 (highly ramified microglia –“surveillance” state-) to 4 (amoeboid phenotype) and, then, grouped and analyzed. Differences among groups were evaluated by ANOVA test followed by Bonferroni’s test.

Results

Clinical Evolution

In the present study, motor impairments were used as indicators to assess disease progression, using the criteria described in materials and methods. In this animal model of MS, CR-EAE, 100% of the Lewis rats immunized with GPSC showed the first signs of the disease between days 10 and 12 post-immunization, while more severe signs were detected on day 17, as shown in Fig. 1, panel c. The appearance of the clinical signs of CR-EAE correlated with a decrease in both body weight (Fig. 1, panel a) and food intake (Fig. 1, panel b). Five percent of rats with CR-EAE pretreated with the EAP salt

(CR-EAE + EAP) did not show any clinical signs of the disease whereas the remaining 95% showed a delayed onset of the clinical signs when compared to CR-EAE. CR-EAE + EAP rats manifested the most severe signs of the disease between days 19 to 20 following immunization (Fig. 1, panel d). Once again, the appearance of the first clinical signs correlated with a decrease in body weight and food intake (Fig. 1, panels a, b). Both rats with CR-EAE and CR-EAE + EAP rats recovered around day 24 post-immunization. The results show a shortening in the duration of the first attack in rats with CR-EAE + EAP as well as a delay in the onset of the clinical signs. The clinical signs of the second attack were observed around days 26–27 in CR-EAE rats, and around days 28–30 in rats with CR-EAE + EAP (Fig. 1, panel c). The analysis of the rats at different stages of the disease revealed that 3.5% of CR-EAE rats remained in grade 1, no rats remained in grade 2, and 96.5% of the rats reached grade 3. However, only 19.0% of the rats with CR-EAE + EAP reached grade 3, as shown in Fig. 1, panel d. Both control and control + EAP rats showed a progressive increase in body weight in correlation with increased food intake.

Autoimmune Response

The study of sections immunolabeled for CD3⁺T cells (including CD4⁺ and CD8⁺) revealed no presence of T-lymphocytes in the hippocampus of control animals (Fig. 2, panels a, b). In CR-EAE rats, scarce and scattered CD3⁺ cells were observed in the parenchyma (Fig. 2, panels a, b; Supplementary Fig. 2) in the first attack. In the second attack, however, there was a significant increase in CD3⁺ T and CD8⁺ cell infiltrates in the hippocampus, especially in the hippocampal commissure and molecular layer, whereas the rats pretreated with EAP showed a marked decrease of CD3⁺ T cells as compared with CR-EAE rats (Fig. 2, panels a, b).

Microglial activation can occur as a result of immune activation, as has been reported for EAE and MS [57–61], but there is little data available regarding the early stages of the disease in the hippocampus [62]. Since microglia are the resident, immune cells in the brain and their morphology changes when activated, we first investigated if their morphology was altered in the hippocampus early on in the disease course, (first attack, remission and second attack). To this end, hippocampal sections were analyzed for Iba-1⁺ immunoreactivity (a marker of activated microglia) (Fig. 2, panel c). Activated microglia can be morphologically identified by their thick, retracted processes and hypertrophied soma (Supplementary Fig. 3). We did not find any evidence for microglial activation in the control group, whereas a high level of microglial activation was observed in both the first and second attack (Fig. 2, panel d). Interestingly, EAP pretreatment decreased microglial activation when compared with the CR-EAE group.

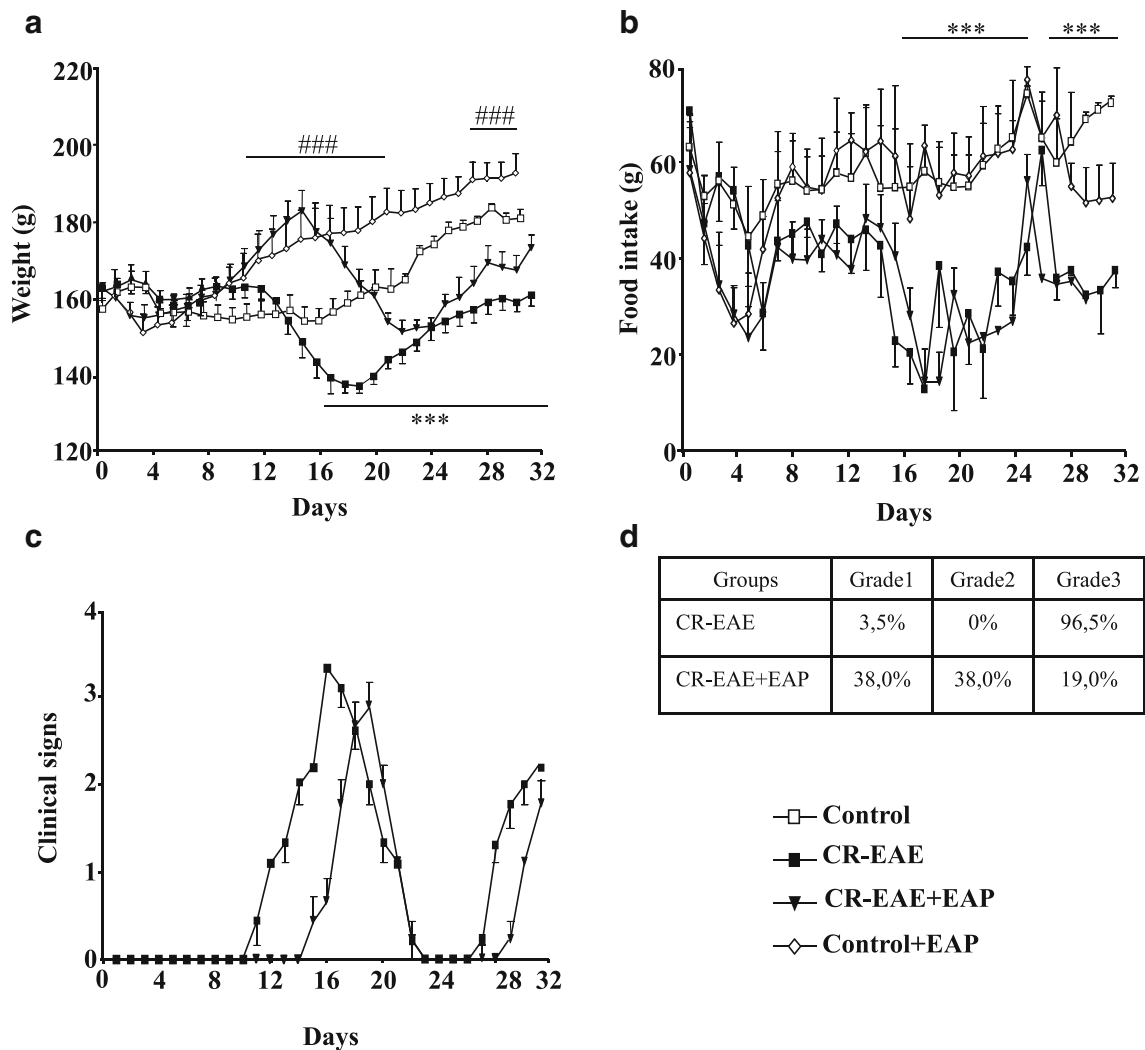


Fig. 1 Temporal evolution of body weight (panel **a**), food intake (panel **b**), clinical signs (panel **c**) and percentage of rats in different stages of the disease (panel **d**) in chronic relapsing experimental allergic encephalomyelitis and effects of an EAP salt. Control rats (white square, $n = 10$), CR-EAE rats (black square, $n = 10$), CR-EAE rats

pretreated with EAP (black inverted triangle, $n = 10$), control rats pretreated with EAP (white diamond, $n = 10$). Statistical significance: $***p < 0.001$ for comparison between control rats and rats with CR-EAE; $###p < 0.001$ for comparison between CR-EAE rats and CR-EAE rats pretreated with EAP

As shown in Fig. 2, panel e and Supplementary Fig. 4, there was no presence of or very scarce amount of immunoglobulin G (IgG) immunoreactivity in the hippocampus of the control group. However, IgG immunoreactivity was significantly increased in the hippocampus of CR-EAE rats and markedly decreased in those rats pretreated with EAP.

Superoxide Dismutase and Catalase Activity and Protein Levels

In view of the above results, we next evaluated total SOD activity, which represents the first line of antioxidant defence. A moderate increase in total SOD activity was

detected during the first attack in the hippocampus of CR-EAE rats when compared to control animals (Fig. 3). This increase disappeared during the remission phase but was once again observed during the second attack (Fig. 3). CR-EAE + EAP rats did not exhibit any increase in SOD activity in any of the phases studied. CR-EAE rats pretreated with EAP (CR-EAE + EAP_{grade 3}) showed the same degree of SOD activity as CR-EAE rats in the three stages of the disease (Fig. 3). Next, SOD protein levels were measured. The results reveal an increase in SOD protein levels in the CR-EAE rat hippocampus in the first attack, which was not found in either the remission stage or in the second attack (Fig. 3).

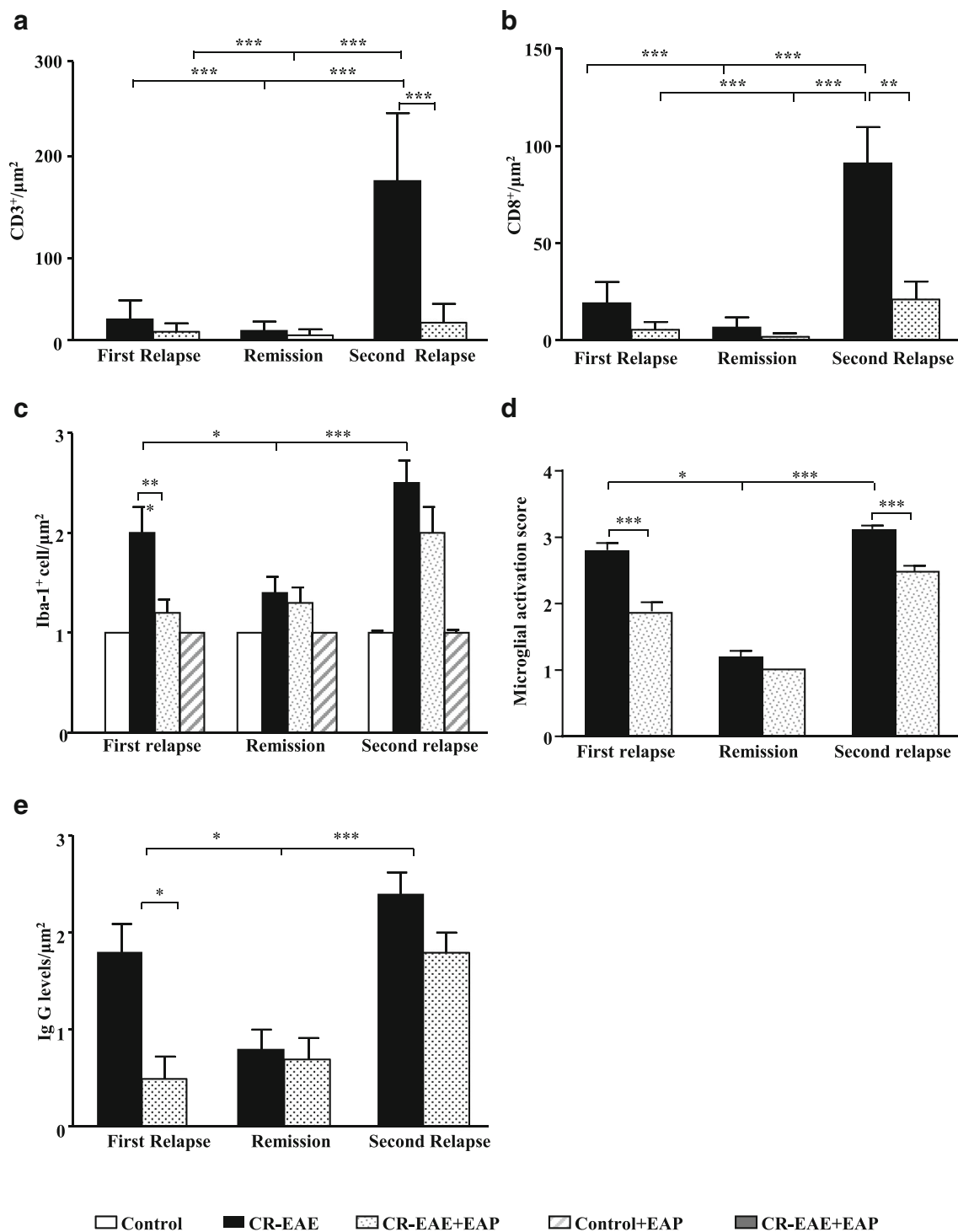


Fig. 2 CD3⁺ infiltrates (panel a), CD8⁺ infiltrates (panel b) and Iba1⁺ cells (panel c) in the hippocampus of rats with chronic relapsing experimental allergic encephalomyelitis (CR-EAE) and effects of an EAP salt. Panel d: Activated Iba1⁺ cells in the hippocampus of rats with chronic relapsing experimental allergic encephalomyelitis (CR-EAE) and effects of an EAP salt. The activity score was determined as described in Materials and Methods.

Panel e: Ig G levels in the hippocampus of rats with chronic relapsing experimental allergic encephalomyelitis (CR-EAE) and effects of an EAP salt. For each treatment, values are presented as the mean ± S.E.M of five to ten fields per rat of five rats per group. Statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Bonferroni's test)

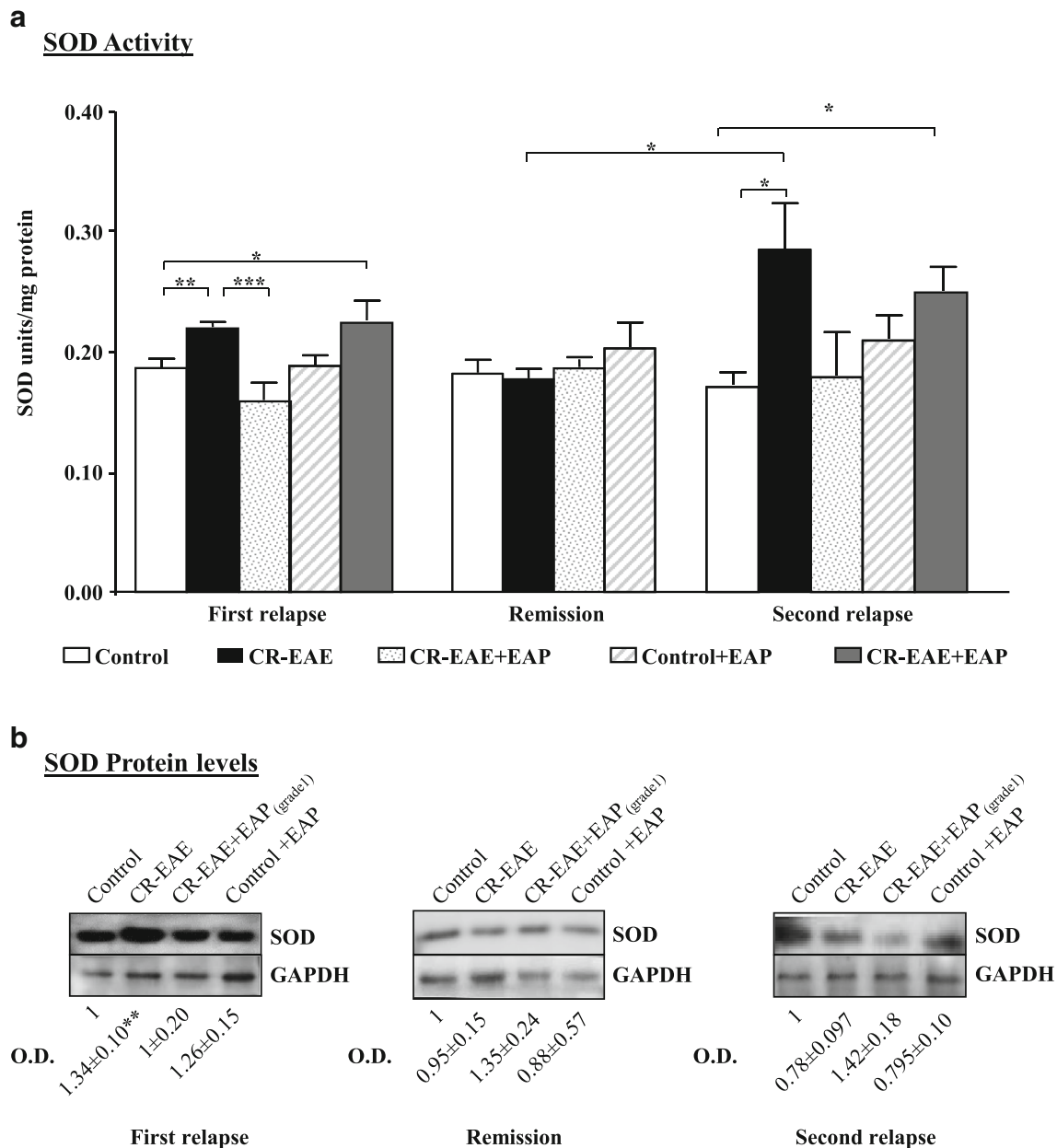


Fig. 3 Superoxide dismutase (SOD) activity and protein levels in the three stages of chronic relapsing experimental allergic encephalomyelitis (CR-EAE) and effects of an EAP salt. Total SOD activity and SOD protein levels in the hippocampus of control rats (open bars), rats with CR-EAE (black bars), rats with CR-EAE pretreated with ethanolamine phosphate (EAP) and sacrificed at the same time as the CR-EAE rats

(CR-EAE + EAP_{grade 1}, speckled bars), rats with CR-EAE pretreated with EAP and sacrificed at grade 3 (CR-EAE + EAP_{grade 3}, solid grey bars) and control rats pretreated with EAP (hatched bars). For each treatment, values are presented as the means ± S.E.M of five rats. Statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Mann-Whitney's U-test)

H₂O₂ is a by-product of SOD activity and is involved in oxidative stress. Consequently, we measured the activity of catalase, an enzyme which catalyses the decomposition of H₂O₂ into oxygen and water. CAT activity was diminished during the first attack, remission stage and second attack in the hippocampus of rats with CR-EAE (Fig. 4). This decrease was less pronounced in the CR-EAE rats pretreated with EAP than in CR-

EAE rats. CR-EAE + EAP_{grade 3} rats, like CR-EAE rats and CR-EAE + EAP_{grade 1} rats, showed a decrease in CAT activity in the first and second attack (Fig. 4). CAT protein levels were next measured by western blot. The results showed a decrease in CAT protein levels in CR-EAE rats as compared with control levels in the three stages of the disease (Fig. 4). This decrease was also found in CR-EAE + EAP_{grade 1} rats (Fig. 4).

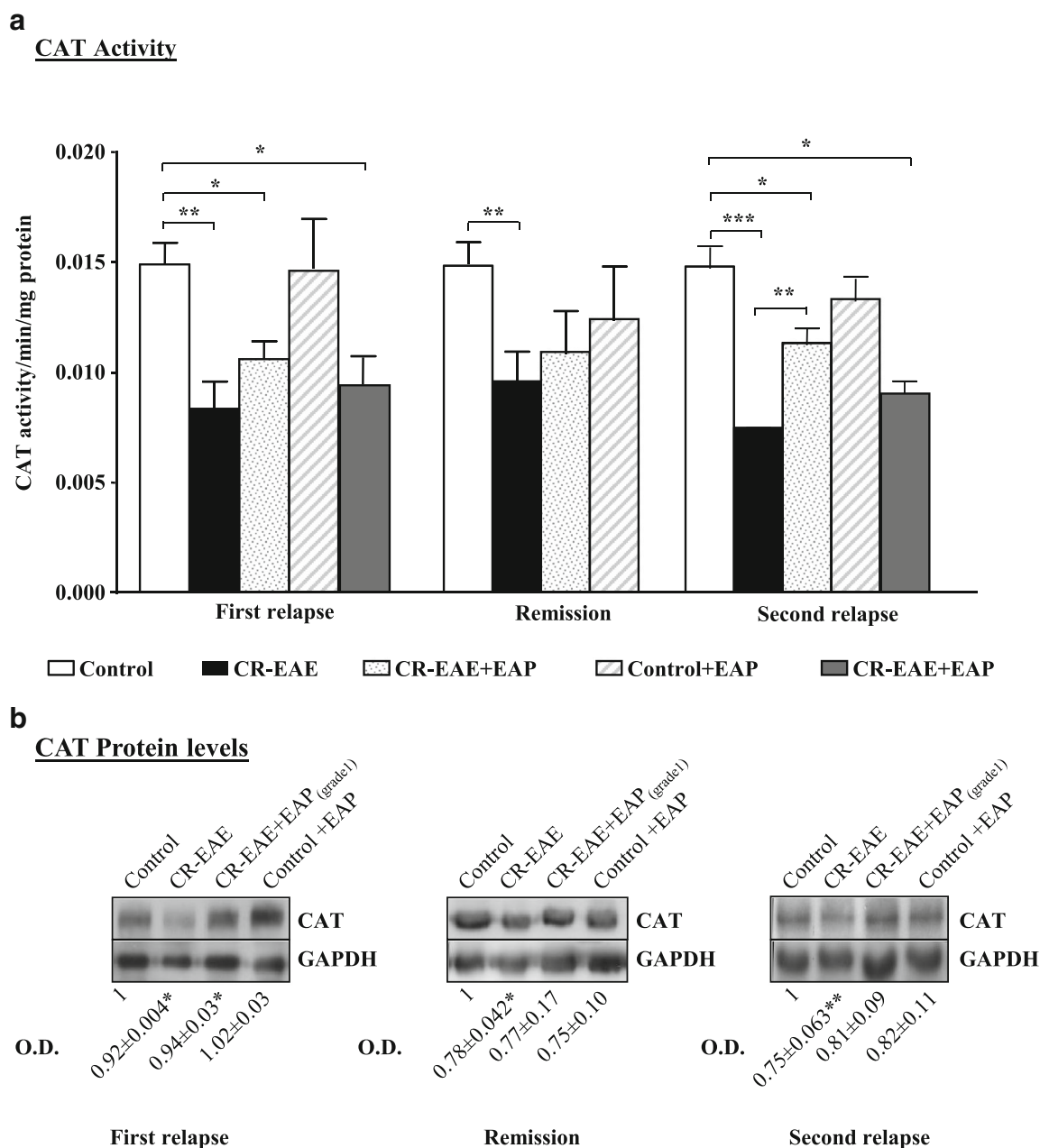


Fig. 4 Catalase (CAT) activity and protein levels in the three stages of chronic relapsing experimental allergic encephalomyelitis (CR-EAE) and effects of an EAP salt. CAT activity and CAT protein levels in the hippocampus of control rats (open bars), rats with CR-EAE (black bars), rats with CR-EAE pretreated with ethanalamine phosphate and sacrificed at the same time as the CR-EAE rats (CR-EAE + EAP_{grade 1}, speckled bars),

rats with CR-EAE pretreated with EAP and sacrificed at grade 3 (CR-EAE + EAP_{grade 3}, solid grey bars) and control rats pretreated with EAP (hatched bars). For each treatment, values are presented as the mean ± S.E.M of five rats. Statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Mann-Whitney's U-test)

Glutathione System

Another system involved in the decomposition of H_2O_2 is the glutathione system. Hence, we also measured glutathione peroxidase (GP) activity (Fig. 5) and glutathione reductase (GR) activity (Fig. 6). Both enzyme activities were found to be significantly increased in CR-EAE rats in the first attack as compared to the controls. In the second attack, GP was

reduced in CR-EAE rats as compared to controls, although GR was increased. No significant changes in GP activity were detected in the remission stage whereas GR activity was reduced. CR-EAE + EAP_{grade 1} rats showed values similar to controls in the three stages of the disease. CR-EAE + EAP_{grade 3} rats showed an increase of GP and GR in the first attack, like CR-EAE rats, whereas a decrease in GP activity, concomitant with an increase in GR activity, was found in the

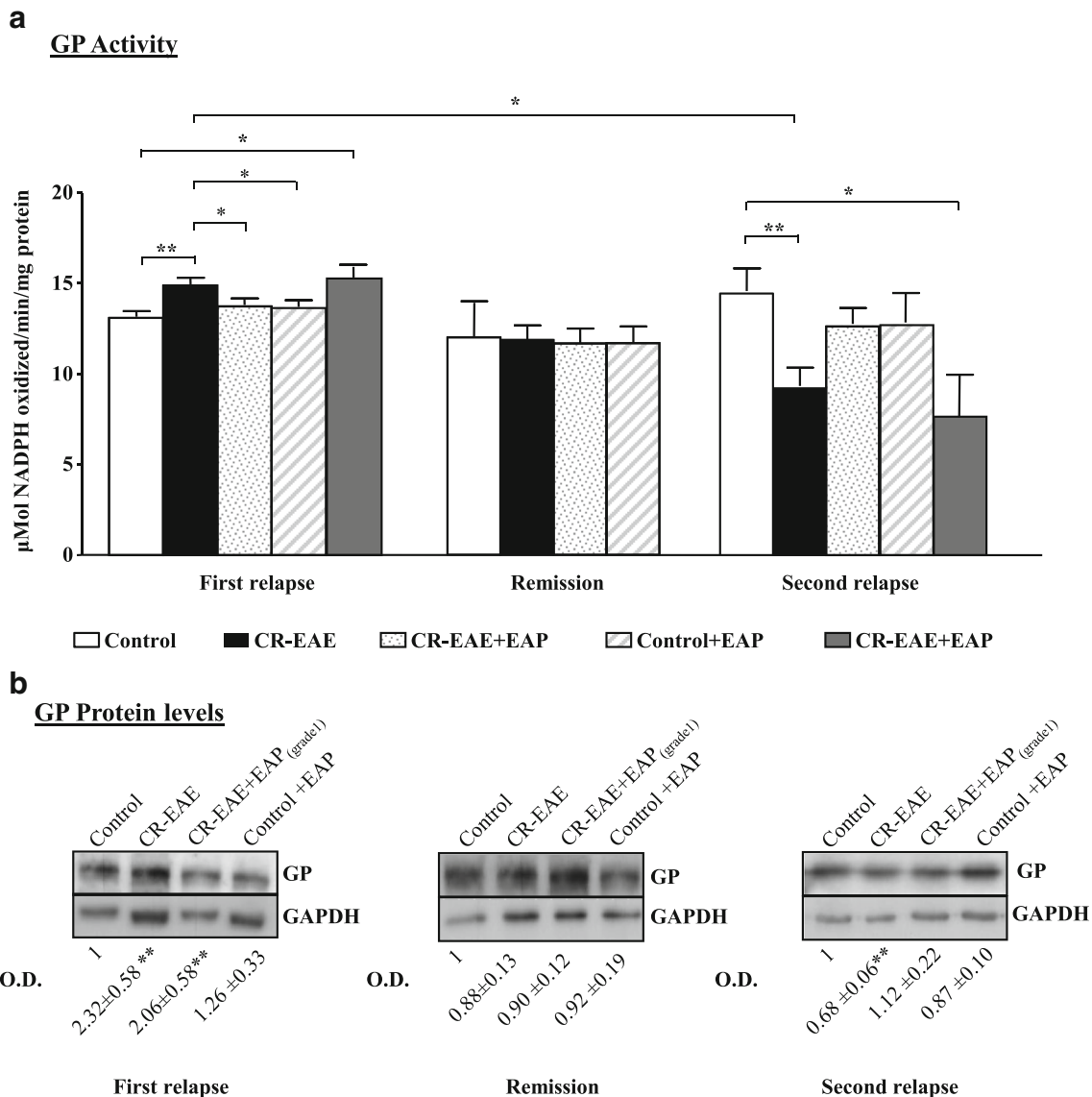


Fig. 5 Glutathione peroxidase (GP) activity and protein levels in the three stages of chronic relapsing experimental allergic encephalomyelitis (CR-EAE) and effects of an EAP salt. Total GP activity and GP protein levels in the hippocampus of control rats (open bars), rats with CR-EAE (black bars), rats with CR-EAE pretreated with ethanolamine phosphate and sacrificed at the same time as the CR-EAE rats (CR-EAE + EAP_{grade 1},

speckled bars), rats with CR-EAE pretreated with EAP and sacrificed at grade 3 (CR-EAE + EAP_{grade 3}, solid grey bars) and control rats pretreated with EAP (hatched bars). For each treatment, values are presented as the mean ± S.E.M of five rats. Statistical significance: * $p < 0.05$; ** $p < 0.01$ (Mann-Whitney's U-test)

second attack. GP and GR protein levels were also measured. The results showed an increase in GP and GR levels in CR-EAE and CR-EAE + EAP_{grade 1} rats in the first attack when compared to controls (Figs. 5 and 6). In the second attack, however, CR-EAE rats showed a decrease in GP levels (Fig. 5, bottom panel).

In view of the above results, we next measured the levels of total glutathione (t-GSH), reduced glutathione (GSH) and oxidized glutathione (GSSG). No changes in t-GSH levels were detected in the hippocampus of rats with CR-EAE throughout the progression of the disease

when compared to control rats (Fig. 7, top panel). In the first attack, however, a decrease in GSH levels was observed in the hippocampus of CR-EAE rats when compared with control rats, whereas in the second attack, a high increase was detected (Fig. 7, middle panel). GSSG levels augmented in the hippocampus of rats with CR-EAE in the first attack when compared to control rats (Fig. 7, bottom panel), whereas CR-EAE + EAP_{grade 3} rats showed a decrease in GSH levels and an increase of GSSG in this same stage of the disease. No changes in GSSG were found in any of the experimental groups in

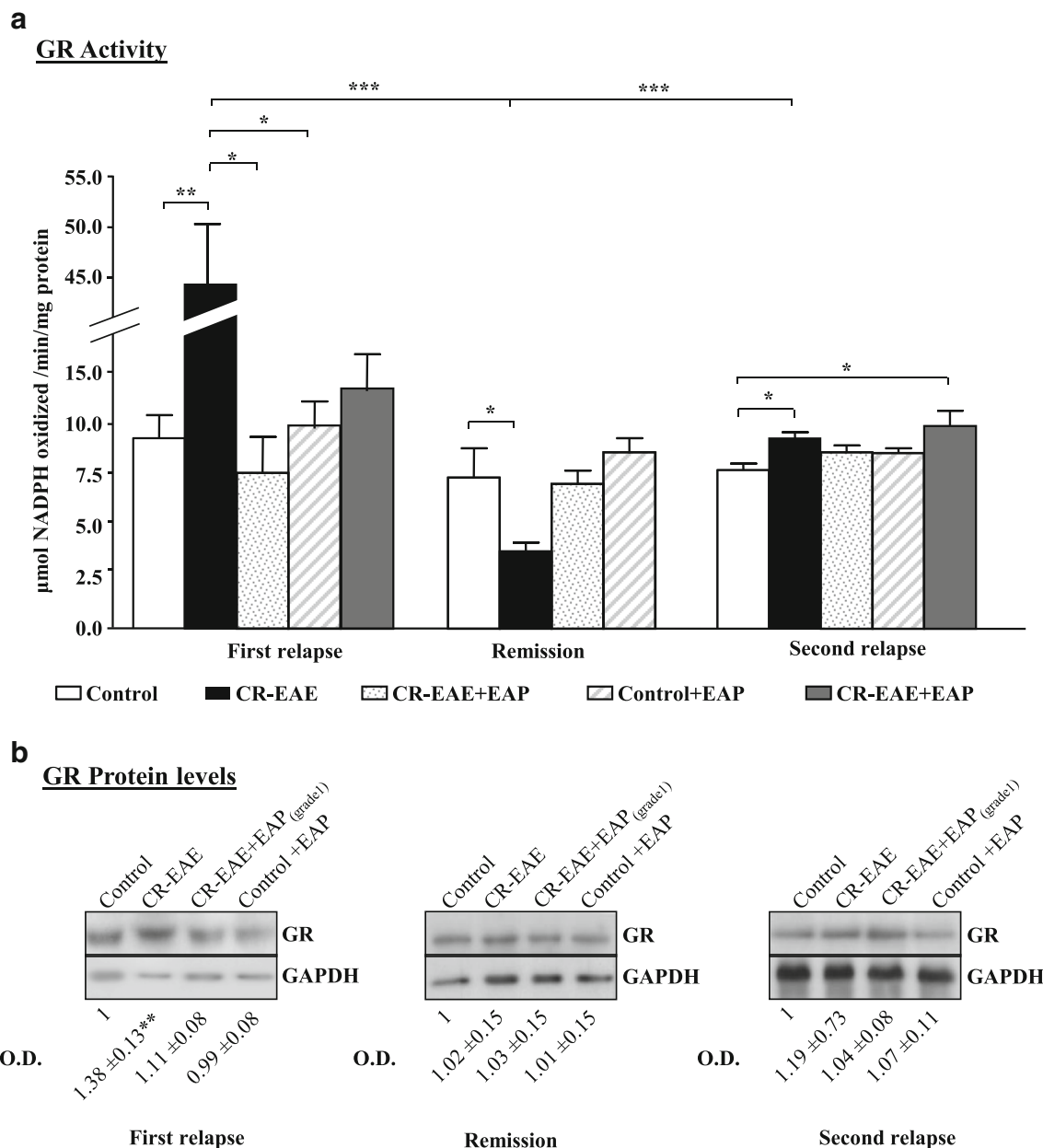


Fig. 6 Glutathione reductase (GR) activity and protein levels of in the three stages of chronic relapsing experimental allergic encephalomyelitis (CR-EAE) and effects of an EAP salt. Total GR activity and GR protein levels in the hippocampus of control rats (open bars), rats with CR-EAE (black bars), rats with CR-EAE pretreated with ethanolamine phosphate and sacrificed at the same time as the CR-EAE rats (CR-EAE + EAP_{grade}

1, speckled bars), rats with CR-EAE pretreated with EAP and sacrificed at grade 3 (CR-EAE + EAP_{grade 3}, solid grey bars) and control rats pretreated with EAP (hatched bars). For each treatment, values are presented as the mean ± S.E.M of five rats. Statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Mann-Whitney's U-test)

the second attack when compared to control values (Fig. 7, bottom panel).

Lipid Peroxidation

Our results showed significant alterations in the antioxidant defences, especially in the second attack. The next parameter assessed was lipid peroxidation, an oxidative

stress indicator, which was determined by measuring the amount of hippocampal malondialdehyde produced. The results of the present study show a significant increase in lipid peroxidation in the hippocampus of rats with CR-EAE during the first attack, when compared to control rats (Fig. 8), which was not found during the remission phase. Lipid peroxidation increased significantly once again in the second attack of CR-EAE rats. These values

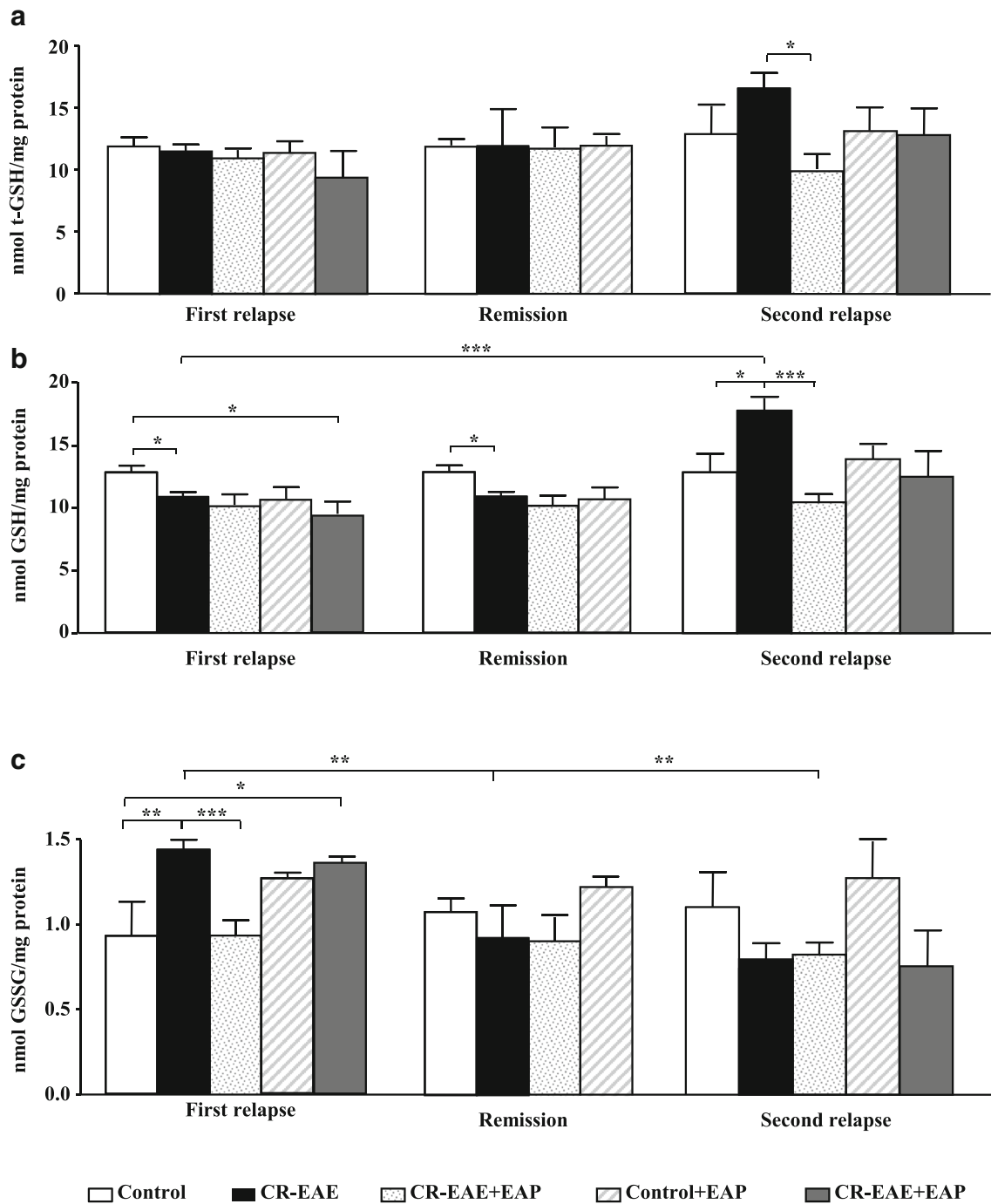


Fig. 7 Total glutathione (t-GSH), reduced glutathione (GSH) and oxidized glutathione (GSSG) levels in the three stages of chronic relapsing experimental allergic encephalomyelitis (CR-EAE) and effects of an EAP salt. Hippocampal t-GSH levels (Top panel), GSH levels (middle panel) and GSSG levels (bottom panel), of control rats (open bars), rats with CR-EAE (black bars), rats with CR-EAE pretreated with ethanalamine phosphate and sacrificed at the same time as the CR-EAE rats (CR-EAE +

EAP_{grade 1}, speckled bars), rats with CR-EAE pretreated with EAP and sacrificed at grade 3 (CR-EAE + EAP_{grade 3}, solid grey bars) and control rats pretreated with EAP (hatched bars) at different phases of the disease (first attack, remission and second attack). For each treatment, values are presented as the mean \pm S.E.M of five rats. Statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Mann-Whitney's U-test)

were significantly greater than those obtained during either the first attack or the remission phase. In CR-EAE + EAP rats, no increase was registered at any of these

stages. CR-EAE + EAP_{grade 3} rats showed the same degree of lipid peroxidation as CR-EAE rats in the three stages of the disease (Fig. 8).

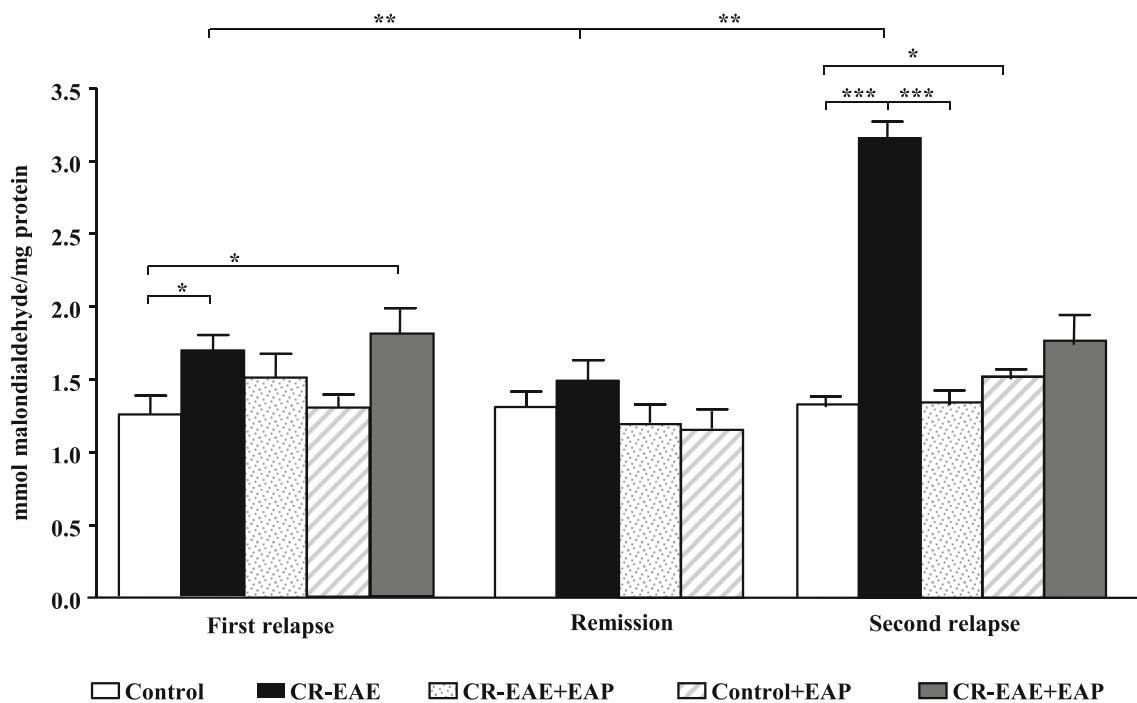


Fig. 8 Lipid peroxidation levels in the rat hippocampus and effects of an EAP salt. Levels of reactive species to thiobarbituric acid in the hippocampus of control rats (open bars), rats with CR-EAE (black bars), rats with CR-EAE pretreated with ethanolamine phosphate and sacrificed at the same time as the CR-EAE rats (CR-EAE + EAP_{grade 1}, speckled bars), rats with CR-EAE pretreated with EAP and sacrificed at grade 3

(CR-EAE + EAP_{grade 3}, solid grey bars) and control rats pretreated with EAP (hatched bars) at different phases of the disease (first attack, remission and second attack). For each treatment, values are presented as the mean \pm S.E.M of five rats. Statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Mann-Whitney's U-test)

Sterol Levels

An increase of oxidative stress is known to induce cholesterol accumulation in membranes. Hence, we measured, by HPLC, sterol levels in hippocampal membranes of control rats, CR-EAE rats, CR-EAE rats pretreated with EAP and control rats pretreated with EAP. Cholesterol levels were increased in CR-EAE rats in the first and second attack, returning to the initial values in the remission phase (Fig. 9, top panel). These changes were accompanied by a decrease in desmosterol levels in CR-EAE rats in the first and second attack (Fig. 9, bottom panel).

Cell Death

In view of the increase in T cell infiltrates and Iba1+ immunoreactivity, lipid peroxidation, SOD, GP and GR activities as well as enhanced cholesterol levels and the decrease in CAT activity, GSH and desmosterol levels in the first and second attack in the CR-EAE rat hippocampus, when compared to controls, we explored the possibility that this impairment might be affecting hippocampal cell survival. To evaluate this hypothesis, we analysed cell death by TUNEL staining. In situ cell detection showed an increase in the number of apoptotic nuclei in the

hippocampus of rats with CR-EAE, especially in the molecular stratum and radiatum stratum in the second attack. The number of apoptotic cells in CR-EAE rats pretreated with EAP was lower than in CR-EAE rats in the three stages of the disease (Fig. 10, supplementary Fig. 5).

Discussion

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating disease of the CNS that represents the most frequent cause of chronic disability in young adults. The present study shows changes in the hippocampus of an animal model of MS, chronic relapsing experimental allergic encephalomyelitis (CR-EAE). These changes include T lymphocyte infiltrates and increased Iba1⁺ cells, high IgG levels and alterations in the antioxidant defences, at three different phases along the progression of the disease: first attack, remission and second attack. Our results also show that EAP attenuates these changes.

At present, the antigen which induces activation of the immune system against the CNS is unknown. Several studies on T cell infiltrates in MS plaques have shown that these cells are activated against myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP) [63, 64]. For this

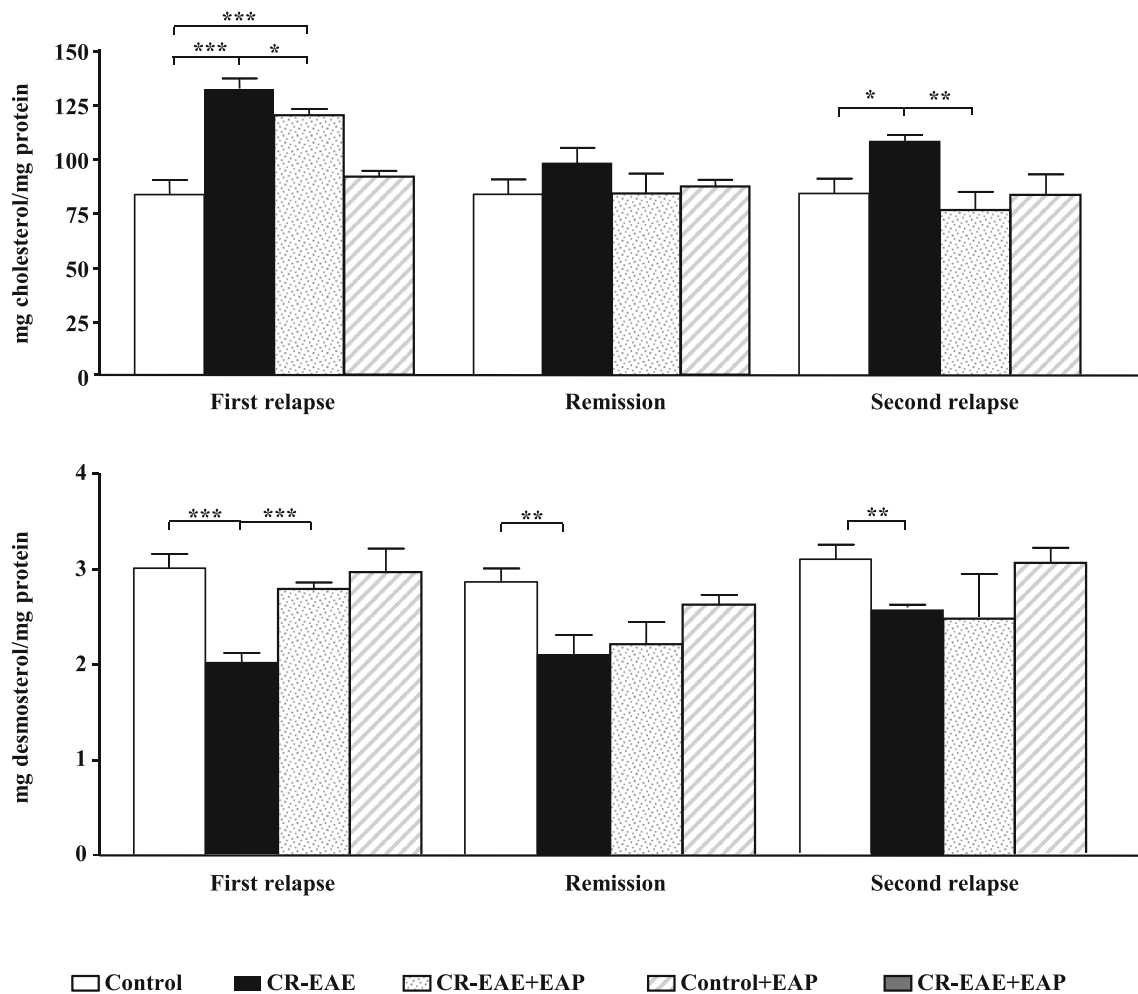


Fig. 9 Hippocampal sterols levels in chronic relapsing experimental allergic encephalomyelitis (CR-EAE) and effects of an EAP salt. Cholesterol levels (top panel), and desmosterol levels (bottom panel) in the hippocampus of control rats (open bars), rats with CR-EAE (black bars), rats with CR-EAE pretreated with ethanolamine phosphate and sacrificed at the same time as the CR-EAE rats (CR-EAE + EAP_{grade 1},

speckled bars) and control rats pretreated with EAP (hatched bars) at different phases of the disease (first attack, remission and second attack). Values are presented as the mean \pm S.E.M of five rats per each group. Statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Mann-Whitney's U-test)

reason, we used an animal model in which EAE was induced with an emulsion of guinea-pig spinal cord tissue (GPSC) [43] since it has all the potential encephalitogenic peptides.

Our results showed that all the rats immunized with the GPSC emulsion developed the chronic form of the disease; in particular, 96.5% of them reached grade 3 of the clinical score. In these CR-EAE rats, the greatest weight loss and lowest food intake coincided with the days in which the rats presented the most severe clinical signs. In the case of CR-EAE rats pretreated with EAP, our results showed a delay in the onset of the clinical signs, a reduction in the duration of the attack and a decrease in the severity of the clinical signs: only 19.0% of the rats reached grade 3 of the clinical score. In these rats, the greatest weight loss and lowest food intake coincided with the days in which the rats presented the most severe clinical signs.

The immunohistochemical analysis of the hippocampus revealed the presence of T cell infiltrates and activated microglial cells in the three stages of the disease in the CR-EAE group, especially in the second attack. The increase in Iba1+ cells found in our study may be due to either migration of microglial cells to that area, to proliferation of pre-existing microglia in the hippocampus or to infiltration of peripheral macrophages. Microglial cells activated by T cells can secrete pro-inflammatory cytokines and free radicals, which could produce direct damage to oligodendrocytes, astrocytes and neurons [9–14]. Our results showed an increase in IgG levels in the hippocampus, which could increase the damage exerted on these cells. In the second attack, the infiltrates, Iba1+ cells and IgG levels are higher and are especially located in the molecular layer and *girus dentatus* of the hippocampus; interestingly, those hippocampal areas involved in memory

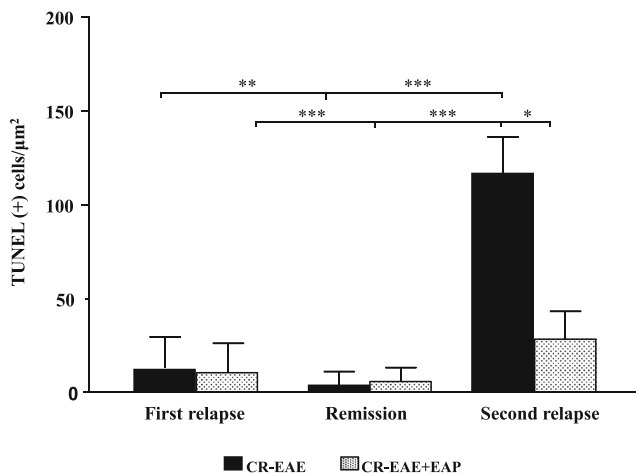


Fig. 10 TUNEL (+) cells in the hippocampus of rats with chronic relapsing experimental allergic encephalomyelitis (CR-EAE) and effects of an EAP salt. For each treatment, values are presented as the mean \pm S.E.M of five to ten fields per rat of five rats per group. Statistical significance: * p <0.05; ** p <0.01; *** p <0.001 (Mann-Whitney's U-test)

processes. These results might partly explain the increase of lipid peroxidation seen in the first and second attack of CR-EAE, which is more abrupt during the second attack. This increase also correlates with a dysfunction in the antioxidant defences. In line with this, superoxide anion ($O_2^{\cdot-}$) production has been previously shown to increase during EAE [65–67] and $O_2^{\cdot-}$ is known to inhibit CAT [68, 69].

In addition, we found a significant increase in total SOD activity in the hippocampus of rats with CR-EAE as compared with control animals (Supplementary Figs. 6 and 7). An increased $O_2^{\cdot-}$ production may favour SOD activity, since it is the major SOD allosteric activator [70]. Here, we have shown that CR-EAE induces a decrease in CAT activity in both the first and second attack. The concomitant increase in SOD activity and decrease in CAT activity might result in an increased H_2O_2 concentration, since the production of the latter from $O_2^{\cdot-}$ by SOD is increased and its elimination by CAT is decreased. In MS, a significantly enhanced expression of SOD1 has been observed in active demyelinating lesions [71–73]. In brain homogenates of rats suffering from EAE, a decreased peroxisomal function was demonstrated, which was accompanied by both a reduction in CAT gene expression and activity [74]. Recently, a decrease in CAT activity has been found in the hippocampus of rats with acute EAE induced with MOG [21] (Sajad et al., 2009). Several studies have shown beneficial effects of CAT treatment on the development of EAE [75–77].

The reduced CAT activity found in the present study might be associated with a compensatory increased activity of GP, which acts as a defence against H_2O_2

formation. GP plays a central role in the removal of toxic peroxides, and the increase of its activity in the EAE hippocampus affected by oxidation could possibly reflect the cells' attempt to counteract increased lipid peroxide products (Supplementary Figs. 6 and 7).

On the other hand, GR has an important role in cellular antioxidant protection because it catalyses the regeneration of GSH from GSSG. The elevation of GR activity observed in the second attack of rats with CR-EAE can partly explain the increase in GSH in this episode, although the recovery of GSH seems to be insufficient for alleviating oxidative stress. In our study, GSH depletion was limited to the first attack. This finding is in agreement with that reported in mice [78] and in patients suffering from MS [79]. Actually, the GSH decrease is considered an early event of the disease [21]. Since GSH inactivates a number of oxidizing species, this data is in accordance with observations showing that the levels of ROS indicators are elevated in EAE [80, 81].

This study also demonstrates that the cholesterol content in hippocampal membranes increases in the first attack of CR-EAE, returning nearly to initial values in the remission phase and increasing, once again, in the second attack, although to a lesser extent (Supplementary Figs. 6 and 7). These results are in accordance with the fact that brain membrane-associated oxidative stress induces cholesterol accumulation [82, 83]. In fact, studies of non-neuronal cells have demonstrated that oxidative stress enhances cholesterol accumulation [84–87]. In addition, previous studies in brain tissue from patients suffering MS indicate that the cholesterol content increases when compared to control patients [88].

Coincident with the increase in brain cholesterol, we found a decrease in the desmosterol content. Conversion of desmosterol into cholesterol is catalysed by 3β -hydroxysterol- Δ 24 reductase (DHCR24; alias hDiminuto/Seladin-1) [89–91]. Endogenous DHCR24/seladin-1 levels are highly upregulated upon acute oxidative stress [92, 93], while its expression declines to very low levels upon chronic exposure [92], suggesting that DHCR24/seladin-1 plays a role in integrating cellular responses to oxidative stress. Several studies seem to indicate that increased cholesterol synthesis potentially counteracts lipid peroxidation associated with oxidative stress, thus maintaining plasma membrane integrity [83].

In the first attack of CR-EAE rats, we did not observe a higher cell death, probably due to increased cholesterol content and a good functioning of the glutathione system. On the other hand, we observed an increase in TUNEL (+) cells in the second attack, which could be caused by the lymphocyte infiltrates and microglial activation as well as by the alterations in the antioxidant defences.

Interestingly, the corpus callosum of CR-EAE mice, which is in close proximity to the hippocampus, was reported by Mangiardi et al. [94] to contain many

inflammatory demyelinating lesions, 30–40% of the infiltrating cells being CD3+ T cells.

Our results demonstrate a delay in the appearance of the clinical signs of CR-EAE in rats that were pretreated with EAP and less severity of the symptoms. The histological results of these rats show less inflammatory infiltrates and a decrease in the levels and activation of microglial cells and IgG. The antioxidant defence mechanisms in the CR-EAE + EAP_{grade 1} rats were similar to control levels whereas the CR-EAE + EA_{grade 3} rats showed alterations in both the activity and protein levels of the antioxidant enzymes, albeit to a lesser extent than CR-EAE rats. This might be attributed to modulation of the immune system by ethanolamine, which is part of anandamide, exerting immunomodulatory effects on T and B cells. It is known that activation of antigen-specific T cells requires a reorganization of the “lipid rafts” in the plasma membrane, the CD4 receptor [95], CD8 [96] and other proteins involved in the early stages of activation [97, 98]. These lipid rafts, where these receptors are located, are microdomains rich in cholesterol and different sphingolipids. Dipalmitoyl phosphatidylethanolamine and *N*-acylethanolamine are known to be involved in the rearrangement and compartmentalization of lipid rafts [98–100]. Variations in the ratios of these components could trigger the disintegration of the lipid rafts and, thereby, modify associated signalling pathways [101].

On the other hand, several studies have shown that ethanolamine derivatives such as palmitoylethanolamine reduce the inflammation induced by PPAR- α [102]. Previous studies from our research group have shown that in rats with acute EAE, EAP treatment blunts the increase in TNF- α and IFN- γ [103]. Taken together, this might suggest that the delay in the onset of the disease is due, at least partly, to decreased T and B cell infiltration and a reduction of microglial cell activity. Alternatively, it might be due to a scavenger role of the EAP salt that scavenges the ROS, although this role is not sufficiently efficient to prevent the alterations in the antioxidant defences.

Ethanolamine plasmalogens have been demonstrated to possess antioxidant properties [38]. Our results reveal that EAP acts as an antioxidant in the hippocampus of rats with CR-EAE. It has been proposed that the plasmalogens serve as endogenous antioxidants to protect cells from oxidative stress, especially cholesterol-rich areas of the lipid membrane, by scavenging radicals at the vinyl ether linkage [39, 104, 105]. Ethanolamine plasmalogens also tend to form a hexagonal phase that contributes to stabilizing the structure of the lipid rafts [39]. These data may help explain the decrease in the cholesterol content in membranes from the CR-EAE + EAP rats.

In the hippocampus of CR-EAE rats pretreated with EAP, we observed less TUNEL(+) cells than in CR-EAE rats in the three stages of the disease. This result could be

explained by a decrease in lymphocyte infiltrates and less impairment of the antioxidant defences.

In conclusion, the present study demonstrates a significant impairment in the antioxidant defence mechanisms in CR-EAE and suggests a protective role of EAP in the rat hippocampus.

Conclusion

In conclusion, the hippocampus, a brain area related with memory processes, is affected by lymphocyte infiltrates and alterations in the antioxidant defences in CR-EAE rats, especially in the second attack. Moreover, hippocampal membrane cholesterol levels are increased, concomitant with lipid peroxidation, at the three stages of the disease. Our results suggest that the EAP salt delays the onset and severity of the clinical signs of CR-EAE. This salt also decreased the lymphocyte infiltrates, possibly due to a decreased activation of lymphocyte subsets in peripheral blood, and maintained the activity of the antioxidant defences. Hence, the present results show that CR-EAE is an ideal model for the study of the chronic course of MS as well as for the evaluation of potential therapeutic treatments such as EAP. Moreover, this salt could be a potential candidate for the treatment of MS.

Acknowledgments We wish to thank the staff of the Animal Center of the Universidad de Alcalá for their support with the animal care and handling. CIBEROBN is an initiative of the Instituto de Salud Carlos III (ISCIII), Spain. MOIR Mechanisms of Insulin Resistance.

Funding information This work was supported by the Ministerio de Ciencia e Innovación (SAF2010-22277), (SAF2011-29951) and (S2010/BMD-2423).

Compliance with ethical standards The animal experiments performed in the present study conform to the guidelines set by the Animal Care Committee of Alcalá University as well as to the International guidelines on the ethical use of animals set by the Council of Europe (Protection of Animals Used for Experimentation, 1986 ETS No. 123), and all experimental protocols were previously approved.

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