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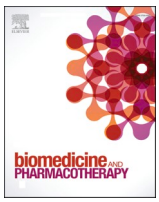

1,3-butanediol administration as an alternative strategy to calorie restriction for neuroprotection – Insights into modulation of stress response in hippocampus of healthy rats

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ABSTRACT

Ketogenic diet has a wide range of beneficial effects but presents practical limitations due to its low compliance, hence dietary supplements have been developed to induce ketosis without nutrient deprivation. The alcohol 1,3-butanediol (BD) is a promising molecule for its ability to induce ketosis, but its effects on brain have been investigated so far only in disease models, but never in physiological conditions. To support BD use to preserve brain health, the analysis of its activity is mandatory. Therefore, we investigated, in healthy rats, the effect of a fourteen-days BD-administration on the hippocampus, an area particularly vulnerable to oxidative and inflammatory damage. Since BD treatment has been reported to reduce energy intake, results were compared with those obtained from rats undergoing a restricted dietary regimen, isoenergetic with BD group (pair fed, PF). Reduced pro-inflammatory signaling pathways and glial activation were revealed in hippocampus of BD treated rats in comparison to control (C) and PF groups. ROS content and the extent of protein oxidative damage were lower in BD and PF groups than in C. Interestingly, higher amounts of nuclear factor erythroid 2-related factor 2 (Nrf2), decreased level of lipid hydroperoxides, lower susceptibility to oxidative insult, higher amounts of superoxide dismutase-2, glutathione reductase and glutathione peroxidase (GPx), and increased GPx activity were observed in BD animals. BD administration, but not dietary restriction, attenuated endoplasmic reticulum stress, reduced autophagic response activation, and was associated with an increase of both the neurotrophin BDNF and pre-synaptic proteins synaptophysin and synaptotagmin. Our results highlight that BD plays a neuroprotective role in healthy conditions, thus emerging as an effective strategy to support brain function without the need of implementing ketogenic nutritional interventions.

1. Introduction

Ketosis, occurring in conditions of low carbohydrate availability, prolonged fasting or undertaking ketogenic diets, is characterized by a state of elevated blood ketones [1]. The ketone bodies (KBs), acetoacetate and β-hydroxybutyrate (βHB), are organic compounds produced endogenously from free fatty acids oxidation in the liver and represent an alternative fuel source to glucose for the brain as well as other peripheral tissues, when glucose is short in supply [2]. Moreover, KBs have been demonstrated to act as hormone-like signalling molecules and exert pleiotropic effects in multiple organs by modulating substrate utilization, inflammation, oxidative stress, catabolic processes, and gene expression [1–3].

Interestingly, ketogenic diet has been proposed as an effective strategy for the treatment of epilepsy, psychological disorders, brain injury, and neurodegenerative diseases [4–7]. Also, nutritional ketosis is

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considered a promising strategy to improve health status, and there has been growing research interest in the last years in its potential application for ameliorating exercise performance and age-related diseases [7,8]. Indeed, it was demonstrated that dietary interventions based on ketosis ameliorate brain network stability [9], and prevent or rescue brain energy deficit [10], thus protecting the aging brain. However, this nutritional regimen presents practical limitations, especially in the long-term, due to its low compliance [5,11], so the effectiveness of oral exogenous ketone supplements in inducing ketosis without dietary restriction has been investigated [12–14]. Exogenous ketone supplements, including ingestible ketone salts and ketone esters, were demonstrated to elevate circulating βHB**,** affecting glucose, free fatty acids and triglyceride concentrations [8,15]. So, their global market has drastically grown in the last decade essentially for their properties such as increased mental clarity, enhanced athletic performance, and appetite control [14]. In this context, it is worth mentioning that the secondary alcohol 1, 3-butanediol (BD), used as component of ketone esters [16,17], represents an alternative method to induce ketosis without nutrient deprivation. Indeed, after ingestion, BD undergoes a series of oxidation steps in the liver leading to the production of βHB and consequent increase of plasma βHB concentration [13,18]. It was previously shown that BD acts as an endothelium-dependent vasodilator [19], enhances the antioxidant capacity of the gonadal white adipose tissue in rat [20], and exerts an anti-inflammatory and neuroprotective effect in rats after spinal cord injury [21]. Furthermore, it plays a leptin-sensitizing effect in the hypothalamus of diet-induced obese mice [22]. However, our understanding of the impact of βHB on the brain in physiological conditions remains limited since recent data showing the beneficial effect of βHB signalling in this organ mainly refer to processes involved in disease conditions, as proceeding from studies in animal models of pathologies [4,6,14]. To fill this gap, a study in which the increase in βHB levels is induced by the administration of its precursors in healthy animals, other than contributing to give further insight into the mechanism by which βHB affects brain physiology, would help to clarify whether this intervention can be considered a valid treatment to prevent or delay the onset of neurological pathologies. The hippocampus is crucial for learning and memory and is particularly vulnerable to dietary influences and/or oxidative and inflammatory damages, thus alterations of its functional or structural integrity can prelude the onset of neurodegenerative events [23]. Considering the central role of hippocampus and the deleterious consequences of perturbations of its homeostasis, it is important to identify molecules or treatments that could play a neuroprotective role on this brain area just using healthy animals to point out the effects detectable in physiological conditions. To this end, we evaluated the effect of exogenous ketosis induced by BD, focusing, in healthy adult rats, on mechanisms involved in the ability of cells to respond to stress, namely inflammation, redox balance, autophagy and endoplasmic reticulum stress. Further, since BD is known to induce a reduction in energy intake [20,24] we compared its effect with those observed in rats undergoing a restricted dietary regimen, isoenergetic with BD group (pair fed, PF), to further unveil the effects of this specific molecule.

2. Materials and methods

2.1. Materials

Bovine serum albumin fraction V (BSA), prestained protein ladder, salts and buffers were purchased from DelTek (Naples, Italy). Rabbit anti-human Haptoglobin and mouse anti-human actin IgGs were from Sigma-Aldrich (St. Louis, MO, USA). The dye reagent for protein titration was from Bio-Rad (Hercules, CA); polyvinylidene difluoride (PVDF) and nitrocellulose membranes were from GE Healthcare (Milan, Italy). Fuji Super RX 100 film, developer and fixer were from Laboratorio Elettronico di Precisione (Naples, Italy). 1,3 BD was purchased by Merck Millipore.

2.2. Experimental design

All experiments were carried out with male Wistar rats (90 days old; about 270 g) purchased from Envigo RMS Srl (Udine Italy). Animals received a standard diet (4RF21 Mucedola) during the experimental phase (14 days). BD treatment was chosen given its ability to significantly increase βHB serum levels [20].

Rats were randomly divided into three groups: 1) control group (C), composed of rats receiving a single i.p. administration of physiological solution at the beginning of the treatment. Rats had free access to water and food; 2) BD treated group (BD), composed of rats that received a single i.p. administration of BD (2.5 mmol/100 g body weight) followed by the oral administration of the compound in drinking water (10 % v/v) for 14 days. Rats had free access to BD drinking solution and food; 3) pair fed group (PF), composed of rats that received a single i.p administration of a physiological solution at the beginning of the treatment and that were pair fed on an isoenergetic basis to the BD group, in order to guarantee equal amounts of energy intake between BD treated and PF group, during the two weeks of treatments. In detail, since BD treatment reduced animal energy intake by 15 % compared to C, the PF group received a daily amount of chow calibrated to match the energy intake assumed daily by BD group. Rats had free access to water and food.

For the evaluation of the energy intake of BD group, the energy of BD assumed trough drinking solution was considered.

During the experimental phase, drinking BD solution (10 % v/v) was prepared and replaced daily. Food and BD drinking solution intake were monitored daily, while animal body weight was monitored three times a week. Rats of BD group assumed on average of 20 mL of BD drinking solution daily.

At the end of the 14 days treatment, the rats were anesthetized, and from a small cut on the tail a drop of blood was collected to detect βHB by using a ketone body meter (Glucomen Areo β-ketone sensor). The animals were then euthanized by decapitation, brains were quickly removed and hippocampus was harvested and dissected as previously described [25]. Aliquots were immediately processed or snap frozen in liquid nitrogen and stored at – 80 °C for further analyses. Blood samples were also collected, serum was isolated by centrifugation (1400 g, 10 min, 4 °C), and then stored at −20 °C until used for determination of inflammatory markers.

This study was carried out under the recommendations in the EU Directive 2010/63 for the Care and Use of Laboratory Animals. All animal protocols were approved by the Committee on the Ethics of Animal Experiments of the University of Naples Federico II and the Italian Minister of Health (Authorization n° 776/2021-PR.). Every effort was made to minimize animal pain and suffering.

2.3. Protein extraction

The homogenization of aliquots of frozen hippocampus (about 40 mg) was carried out in seven volumes (w/v) of cold RIPA buffer (150 mM NaCl, 50 mM Tris- HCl pH 8.0, 0.5 % sodium deoxycholate, 0.5 % NP-40, 0.1 % SDS pH 8.0) containing 1 % Protease Inhibitor Cocktail and 1 % Phosphatase Inhibitor Cocktail (Euroclone, Milan, Italy). Homogenates were incubated (30 min) at 4 °C and then centrifuged (14,000 g, 45 min, 4 °C) [26]. Nuclear extracts used for detecting Nrf2 were isolated as previously reported [27]. In detail, aliquots of frozen hippocampus (about 30 mg) were homogenized in a buffer containing 20 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl2, 0.15 % Igepal CA-630. The homogenates were centrifuged at 700 x g for 5 min. The nuclear pellets were resuspended in half of the volume of nuclear homogenization buffer and were centrifuged as before. This step was repeated twice. The nuclear pellet was extracted in buffer containing 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25 % glycerol (30 min on ice, followed by 10 min at room temperature). The extract was then centrifuged at 20,000 x g (4 °C, 30 min) and the supernatant was collected.

Protein concentration of supernatants was finally evaluated by the colorimetric Bio-Rad Bradford protein assay, using a commercial kit (Bio-Rad, Hercules, CA, USA).

2.4. Western blotting

Electrophoretic fractionation of hippocampal proteins (30 μg) was carried out under denaturing and reducing conditions [28] on 12.5 % polyacrylamide gels (to quantify glial fibrillary acidic protein [GFAP], nuclear factor erythroid 2-related factor 2 [Nrf2], superoxide dismutase-2 [SOD-2], catalase, glutathione peroxidase [GPx], glutathione reductase [GR], synaptophysin, synaptotagmin, brain derived neurotrophic factor [BDNF], beclin, and P62-sequestosome-1 [p62]) or on 10 % polyacrylamide gels (to assay toll-like receptor-4 [TLR4], nuclear factor kappa-light-chain-enhancer of activated B cells [NFkB], eukaryotic initiation factor 2α [eIF2α], post-synaptic density protein 95 [PSD-95], glucose-regulated protein [GRP-78], calnexin). Proteins were then transferred onto PVDF or nitrocellulose membrane, washing and blocking steps were performed according to previously published procedures [29,30].

After blocking the membranes were incubated with primary antibodies (overnight, at 4 °C), washed and then treated (1 h, at 37 °C) with the appropriate peroxidase-conjugated secondary antibodies. The specific dilutions of primary and secondary antibodies are reported in Supplementary Table 1. As the amount of phosphorylated proteins (NFkB, eIF2α) was expressed as relative to the total NFkB or eIF2α, after revelation of the immunocomplexes, the membranes were stripped [28] and then incubated with the specific antibody for the total form of the protein (Supplementary Table 1). For loading control, after detection of each antigen, the membranes were stripped and incubated (overnight, 4 °C) with mouse anti-β-actin IgG (1:1000 in 0.25 % v/v non-fat milk) followed by goat anti-mouse horseradish peroxidase-conjugated IgG (Immunoreagents, Raleigh, NC, USA; 1:30,000 in 0.25 % v/v non-fat milk; 1 h, 37 °C). For loading control of Nrf2 nuclear content, histone H3 levels were used. In detail, membranes were incubated with rabbit anti-Histone H3 (1:1000, in 1 % v/v non-fat milk) followed by goat anti-rabbit horseradish peroxidase-conjugated IgG (Immunoreagents, Raleigh, NC, USA; 1:200,000 in 1 % v/v non-fat milk; 1 h, 37 °C). Signal detection was carried out using the Excellent Chemiluminescent Kit Westar Antares (Cyanagen s.r.l., Bologna, Italy). Densitometric analysis of chemidoc or digital images of X-ray films exposed to immunostained membranes was performed with Un-Scan-It gel software (Silk Scientific, UT, USA).

2.5. Analysis of serum and hippocampal tumor necrosis factor alpha (TNF-alpha) and interleukin 6 (IL-6)

The concentrations of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) in both plasma and hippocampus were evaluated by sandwich enzyme-linked immunosorbent assay (ELISA) using the Duo-Set ELISA kit specific for rats (R&D, DBA Italia; TNF-alpha, catalog number: DY510; IL-6, catalog number: DY506).

In detail, serum samples were diluted 1:15 (in PBS containing 1 % BSA, 0.05 % Tween 20) and used in the assay according to the manufacturer's instructions. TNF-alpha and IL-6 concentrations were reported as pg per mL.

For cytokines quantification in hippocampus, proteins were extracted by homogenizing slices of frozen hippocampus in lysis buffer (100 mM Tris/HCl, pH 7.0, 1 M NaCl, 4 mM EDTA, 2 % Triton X-100, 0.1 % sodium azide) containing 1 % Protease Inhibitor Cocktail and 1 % Phosphatase Inhibitor Cocktail (Euroclone, Milan, Italy). Homogenates were then centrifuged (14,000 g, 30 min, 4 °C), and TNF-alpha and IL-6 concentrations were assessed in the homogenate supernatants diluted 1:20 [31]. Data were reported as pg per mg of total proteins.

A seven point standard curve with recombinant rat TNF-alpha (4000–62.5 pg/mL) or IL-6 (8000–125 pg/mL) was used in the respective assay, according to manufacturer's instructions.

2.6. Evaluation of nitro-tyrosine and haptoglobin (Hpt)

Nitro-tyrosine (N-Tyr) concentration in hippocampus homogenates was measured by ELISA essentially as previously described [32]. In particular, samples were diluted (1:9000, 1:18000, 1:40000) with coating buffer (7 mM Na_2CO_3, 17 mM $NaHCO_3$, 1.5 mM NaN_3, pH 9.6), and aliquots of each dilution (50 μL) were incubated in the wells of a microtitre plate (Immuno MaxiSorp; overnight, 4 °C). Washing and blocking were performed according to Mazzoli et al. [33]. The wells were then incubated (1 h, 37 °C) with 50 μL of rabbit anti-N-Tyr IgG (Covalab, distributed by VinciBiochem, Vinci, Italy; 1: 1000 dilution in 130 mM NaCl, 20 mM Tris-HCl, 0.05 % Tween, pH 7.4, containing 0.25 % w/v BSA) followed by 60 μl of goat anti-rabbit horseradish peroxidase-conjugated IgG (GAR-HRP IgG; Immunoreagents, Raleigh, NC, USA; 1:9000 dilution; 1 h, 37 °C). Peroxidase-catalyzed color development from *o*-phenylenediamine was measured at 492 nm. Data were reported as OD per mg of total proteins.

Haptoglobin (Hpt) was titrated by ELISA in both serum and hippocampus. Samples were diluted (plasma = 1: 20000–1:60000–1:120000; hippocampus = 1: 700, 1:2100, 1:7000) with coating buffer and Hpt was immunorevealed by incubation 50 μL of rabbit anti-human haptoglobin (1:800 in 130 mM NaCl, 20 mM Tris-HCl, 0.05 % Tween, pH 7.4, containing 0.25 % w/v BSA), followed by 60 μL GAR-HRP IgG (1:12000 dilution, 1 h, 37 °C). Peroxidase-catalyzed color development from o-phenylenediamine was measured at 492 nm. The calibration curve was obtained by assaying the immunoreactivity of 0.8, 0.4, 0.2, 0.1, 0.03, 0.01, 0.003 ng of purified haptoglobin.

2.7. Evaluation of lipid oxidative damage and in vitro susceptibility to oxidants

The hydroperoxides (HPs) content in hippocampus homogenates was used to evaluate the extent of lipid oxidative damage [34] by measuring the stoichiometrically correlated reduction in NADPH absorbance, at 340 nm, due to the coupled reactions catalysed by the glutathione peroxidase and glutathione reductase enzymes in the presence of GSH. Briefly, hippocampus homogenate (0.1 mg of protein) was added to 250 μL of 0.2 mM EDTA, 0.124 M Tris-HCl buffer, pH 7.6 supplemented with glutathione peroxidase (0.025 U/mL), GSH (0.425 mM), glutathione reductase (0.025 U/mL) and NADPH (1.5 mM). Variation in the Absorbance was detected at 340 nm by a microplate reader (Synergy™ HTX Multi-Mode Microplate Reader, BioTek). HPs content was expressed as nmol NADPH oxidized•min^{-1}•mg^{-1} proteins.

To estimate the susceptibility of the hippocampus to oxidative stress we evaluated the change in the hydroperoxide levels induced by the treatment of tissue homogenates (0.01 mg) with Fe and ascorbate (Fe/As), at a concentration of 100/1000 μM, for 10 min at room temperature. In such system ascorbate reduced iron, and Fe^{2+} reacted with samples' lipid hydroperoxides producing the hydroxyl radical that, if not scavenged by the antioxidants of the sample, triggered lipid peroxidation. The reaction was stopped by the addition of 0.2 % 2.6-di-t-butyl-p-cresol (BHT) and the hydroperoxide levels were evaluated as previously described [34].

2.8. Determination of reactive oxygen species (ROS) content

ROS content was evaluated by determining the conversion of 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA) to the fluorescent dichlorofluorescein (DCF) induced by ROS [35]. In brief, an amount of hippocampus homogenate (25 μg of proteins) was added to 250 μL of monobasic phosphate buffer (0.1 M, pH 7.4) containing 10 μM DCFH-DA. After the addition of 100 μM FeCl3, the mixture was incubated for 30 min. The ROS- induced conversion of DCFH-DA to the fluorescent product DCF was evaluated by a microplate reader (Tecan

Infinite 200 pro plate reader, λ_{EX}485, λ_{EM}530). The conversion of DCFH to DCF in the absence of homogenate was detected to evaluate the background. The results were expressed as Relative Fluorescent Unit (RFU)/μg protein.

2.9. Evaluation of total antioxidant capacity (TAC)

To estimate TAC, we evaluated the decolorization of the mono-cation radical of 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS•+), induced by hippocampal homogenate antioxidants, at 740 nm. Hippocampus homogenate (0.01 mg) was added to 250 μL of KH_2PO_4 0.1 M, pH 7.4 supplemented with $ABTS^{\bullet+}$(0.437 mM), and variation in absorbance was detected with a microplate reader. $ABTS^{\bullet+}$ was obtained by the reaction between potassium-persulfate (245 mM) and ABTS (7 mM), for 16 hours at room temperature [36]. A calibration curve was obtained using 3,5-di-tert-4-butylhydroxytoluene (BHT, 0.05–2.5 mM). Total antioxidant capacity was expressed as μmol BHT Equivalents/mg protein.

2.10. Antioxidant enzyme activities

Specific activity of the enzyme superoxide dismutase was measured at 550 nm following the decrease in the rate of reduction of cytochrome c (induced by superoxide radicals, produced by the xanthine-xanthine oxidase system) in the presence of hippocampal homogenate. Briefly, hippocampal homogenates (0.1 mg protein) were added to 250 μL of a solution containing 0.1 mM EDTA, 2 mM KCN, 50 mM KH_2PO_4, pH 7.8, 20 mM cytochrome c, 5 mM xanthine, and 0.01 U of xanthine oxidase. Variations in absorbance were followed by a microplate reader A unit of SOD activity corresponds to the enzyme concentration able to inhibit the reduction of cytochrome c by 50 % in the presence of xanthine + xanthine oxidase [37].

Glutathione peroxidase (GPX) specific activity was evaluated using H_2O_2 and reduced glutathione (GSH) as substrates and measuring the rate of NADPH oxidation at 340 nm, catalysed by glutathione reductase (GR) which reduces the oxidized glutathione (GSSG) obtained by the reaction [38], using a microplate reader. Briefly, hippocampus homogenate (0.02 mg of proteins) was added to 250 μL of 0.1 M KH_2PO_4, 1 mM EDTA, pH 7.0 supplemented with NADPH (1.5 mM), H_2O_2 (1.5 mM), GSH (10 mM) and GR (2.4 U/mL).Glutathione reductase specific activity (GR) was evaluated assessing the oxidation rate of NADPH in the reaction of reduction of GSSG [39]. Briefly, hippocampus homogenate (0.02 mg proteins) was added to 250 μL of 0.2 M KH_2PO_4, 2 mM EDTA, pH 7.0 supplemented with NADPH (2 mM) and GSSG (20 mM) [38], and the rate of NADPH oxidation was monitored at 340 nm, by using a microplate reader (Synergy™ HTX Multi-Mode Microplate Reader, BioTek). The results were expressed as μmol NADPH oxidized$\bullet min^{-1}\bullet mg^{-1}$ proteins.

Catalase specific activity was assessed according to Aebi [40] by measuring the decomposition of H_2O_2. In brief hippocampus homogenate (0.1 mg proteins) was added to 1 mL sodium phosphate buffer (50 mM, pH 7) supplemented with H_2O_2 (30 mM). Catalase activity was calculated by determining the changes in the absorbance at 240 nm for 2 min, using extinction coefficient of 39.4 M^{-1} cm^{-1} and expressed as μmol of H_2O_2 reduced per min per mg of proteins at pH 7.0 and 25 °C.

2.11. Statistical analysis

The sample size was defined by a priori power analysis, by using the G*Power software version 3.1.9.4 from the Heinrich Heine University of Dusseldorf (http://www.gpower.hhu.de). The power analysis was based on our previous investigations estimating a large effect size (Cohen's d > 0.8), and using a desired power of 0.80 with a probability of a Type I error of 0.05, implying that 7–8 different samples from each group are sufficient to observe statistically significant differences with the parameters set as such using one-way ANOVA.

Data were expressed as mean values ± SEM. Normal distribution of data was verified by Shapiro-Wilk normality test, using the program GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA). Multiple group comparisons were performed with one-way ANOVA followed by Tukey post-test. $P < 0.05$ was considered significant in the reported analyses.

3. Results

3.1. Titration of beta-hydroxybutyrate (βHB) and inflammatory markers in serum

In order to verify the ketogenic effect of BD, plasma levels of βHB were titrated in the three groups, namely the control group (C), the group of rats that received administration of BD (BD group), and the third group of rats that were pair fed on an isoenergetic basis to the BD group (PF group). βHB levels were found significantly higher in BD group respect to both control and PF ones (Fig. 1A; $p < 0.001$; $F(2, 21) = 17.85$) in line with results showing that BD administration increases plasma βHB concentration [13,18].

Plasma inflammatory status was assessed by measuring the concentrations of cytokines (TNF-alpha and IL-6) and Hpt, an inflammatory marker sensitive to nutritional stress [41]. As reported in Fig. 1 B-D, the levels of these markers were significantly lower in plasma of BD than C rats ($p < 0.01$), with no differences between PF and BD group (Hpt, $F(2, 21) = 169.3$, $P < 0.0001$; TNF-alpha, $F(2,21) = 17.52$, $P < 0.0001$; IL-6, $F(2,21) = 27.62$, $P < 0.0001$).

3.2. Evaluation of hippocampus inflammatory status

Neuroinflammation represents one of the pathological mechanisms underlying aging [42] and neurodegenerative diseases [43,44], and recent studies also demonstrated the involvement of pro-inflammatory conditions in the onset of depressive-like behaviors and anxiety symptoms [45,46]. To verify whether BD treatment influences molecular mechanisms involved in the modulation of hippocampal inflammatory status, we evaluated pro-inflammatory signaling pathways by measuring the amount of TLR4, the degree of NFkB phosphorylation, and the levels of TNF-alpha, IL-6, Hpt and GFAP.

As shown in Fig. 2A-B, TLR4 amount and the ratio between phosphorylated and total NFkB (p-NFkB/NFkB), which is an index of protein pathway activation, were found decreased in BD-treated rats compared to both control and PF groups, while no differences were detected between C and PF (TLR4, $F(2,21) = 13.33$, $P < 0.0001$; -NFkB/NFkB, $F(2,21)$, $F(2,21) = 17.93$, $P < 0.0001$). In line with results obtained in plasma, both TNF-alpha and IL-6 concentrations were lower in hippocampus of BD rats compared to C group (TNF-alpha, $F(2,21) = 14.72$, $P < 0.001$; IL-6, $F(2,21) = 10.18$, $P < 0.01$; Fig. 2C-D). Interestingly, cytokines levels did not differ between PF and BD group, suggesting that their decrease might be due to reduced energy intake, which is common to both BD and PF rats.

Further, lower amounts of GFAP, a marker of astrogliosis, and Hpt were detected in the hippocampus of BD-treated rats respect to both the control and PF rats (GFAP, $F(2,21) = 29.42$, $P < 0.0001$; Hpt, $F(2,21) = 6.944$, $P < 0.01$; Fig. 2E-F).

Altogether, these results demonstrate that BD administration did not induce inflammation, but conversely it exerts a beneficial role modulating the inflammatory response.

3.3. Oxidative status of rat hippocampus

Oxidative stress is a broad inflammation-related mechanism that has been shown to be strongly implicated in aging/neurodegeneration [47,48]. Oxidative stress is a by-product of energy metabolism and occurs when there is an imbalance in the homeostatic processes that balance the cellular production of reactive oxygen species (ROS) and their

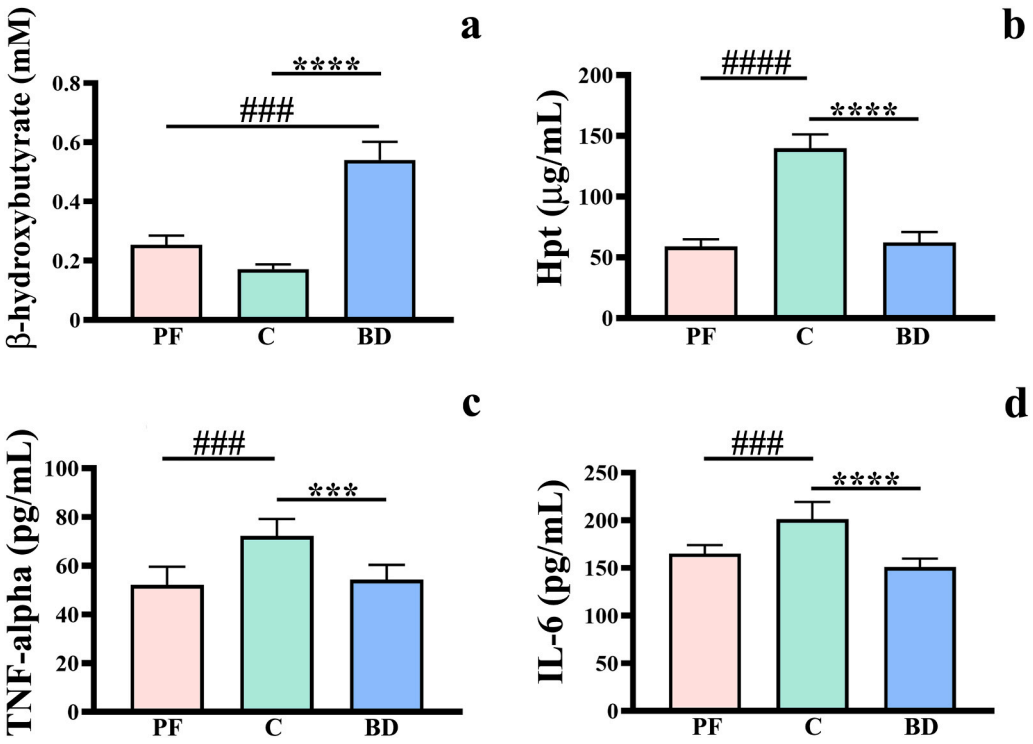

Fig. 1. Titration of beta-hydroxybutyrate (βHB) and inflammatory markers in serum. (A) βHB level in serum was measured by a ketone body meter (Glucomen Areo β-ketone sensor). (B) Haptoglobin concentration was measured by ELISA in serum samples diluted 1: 20000, 1:60000, and 1:120000. Immunodetection was carried out with rabbit anti-Hpt and GAR-HRP IgGs. Data represent means ± SEM of 8 animals from each group. (C) TNF-alpha and (D) IL-6 levels were titrated by sandwich ELISA following the manufacturer's instructions. Data are reported as means ± SEM of 8 different rats from each experimental group. Control rats (C); 1,3-butanediol treated rats (BD); animals pair fed on an isoenergetic basis to the BD group (PF). *** $P < 0.001$, **** $P < 0.0001$ versus BD. ### $P < 0.001$, #### $P < 0.0001$ versus PF (one-way ANOVA followed by Tukey post-test).

buffering by antioxidant activity. To highlight the effect of BD treatment on redox homeostasis in the hippocampus, we evaluated ROS tissue content, N-Tyr amount, as a marker of oxidative damage to proteins induced by peroxynitrite, and lipid hydroperoxides content, as a marker of oxidative damage to lipids. Further, the total tissue antioxidant capacities (TAC), and the *in vitro* susceptibility to an oxidant insult were assessed in order to evaluate the tissue ability to counteract an oxidative attack.

As shown in Fig. 3, total ROS content was reduced to the same extent in both PF and BD groups compared to C ($F(2,21) = 7.731$, $P < 0.01$; Fig. 3A). The observed lower level of ROS was associated with a reduced oxidative damage to lipids and proteins in BD group in respect to the control (lipid hydroperoxides, $F(2,21) = 4.269$, $P < 0.05$; N-Tyr, $F(2,21) = 10.47$, $P < 0.001$; Fig. 3B-C). Interestingly, lipid hydroperoxides levels did not differ between PF and C groups (Fig. 3B). Similarly, susceptibility to oxidative insult, calculated as change in the hydroperoxide levels (ΔHPs) induced by the treatment with Fe/As, was reduced in the hippocampus of BD-treated rats in comparison to both C and PF rats ($F(2,21) = 27{,}58$, $P < 0.0001$; Fig. 3D). Total antioxidant capacity resulted unmodified by both treatments (Fig. 3E).

In order to get further insight into the mechanistic pathway underlying the BD-associated decrease in oxidative condition, we measured the nuclear levels of Nrf2, a fundamental player in the modulation of the antioxidant response. In line with the condition of reduced oxidative stress in the hippocampus of BD-treated rats, we found increased amount of nuclear Nrf2 in hippocampus of rats receiving BD ($F(2,21) = 7.055$) respect to both C and PF rats ($P < 0.01$ and $P < 0.05$ respectively, Fig. 4A). Further, a BD-related increase of the protein content of the enzymes SOD-2 and glutathione reductase (GR) in comparison with both PF and C rats was detected (SOD-2, $F(2,21) = 4792$, $P < 0{,}05$; GR, $F(2,21) = 5378$, $P < 0.05$; Fig. 4B-C). In addition, glutathione peroxidase (GPx) and catalase amounts were higher in BD than in C group (GPx, $F(2,21) = 8.308$, $P < 0.01$; catalase, $F(2,21) = 5.911$, $P < 0.01$), with no differences between PF and BD groups (Fig. 4D-E). Notably, we found an increase in the activity of GPx in hippocampus of BD rats in comparison to C group, while SOD, GR and catalase activities did not differ between the two groups ($F(2,21) = 3.378$, $P < 0.05$; Fig. 5A-D). Further, higher activities of GR and SOD were observed in BD group respect to PF (GR, $F(2,21) = 5.349$, $P < 0.05$; SOD, $F(2,21) = 4.996$, $P < 0.05$).

Taken together these data support the hypothesis that BD plays a neuroprotective role by promoting the increase of nuclear levels of Nrf2, the master regulator of antioxidant systems, thus inducing the expression of enzymes involved in the cellular antioxidant responses. In this way, the shift of oxidative balance towards the increase of antioxidant players contributes to preserving redox homeostasis.

3.4. Endoplasmic reticulum stress and autophagy

Endoplasmic reticulum stress plays a critical role in cell homeostasis [49]. Therefore, we investigated whether BD administration modulates the level of ERS indicators, including the degree of eIF2α phosphorylation and the amount of the two chaperones calnexin and GRP78.

As shown in Fig. 6A-B, lower amounts of both calnexin and GRP78, and reduced p-eIF2α/eIF2α ratio were detected in the hippocampus of BD treated rats, with no differences between control and PF groups (calnexin, $F(2,21) = 6.992$, $P < 0.01$; GRP78, $F(2,21) = 13.17$, $P < 0.001$; p-eIF2α/eIF2α, $F(2,21) = 10.70$, $P < 0.001$). This result suggests that ER stress activation is specifically counteracted/prevented by the compound administration and is not just a consequence of reduced energy intake associated to BD administration.

Since a dysregulation of autophagy can induce ER stress and the rise of inflammatory process, the impact of BD administration on autophagic response has been explored. Autophagy is a fundamental process for

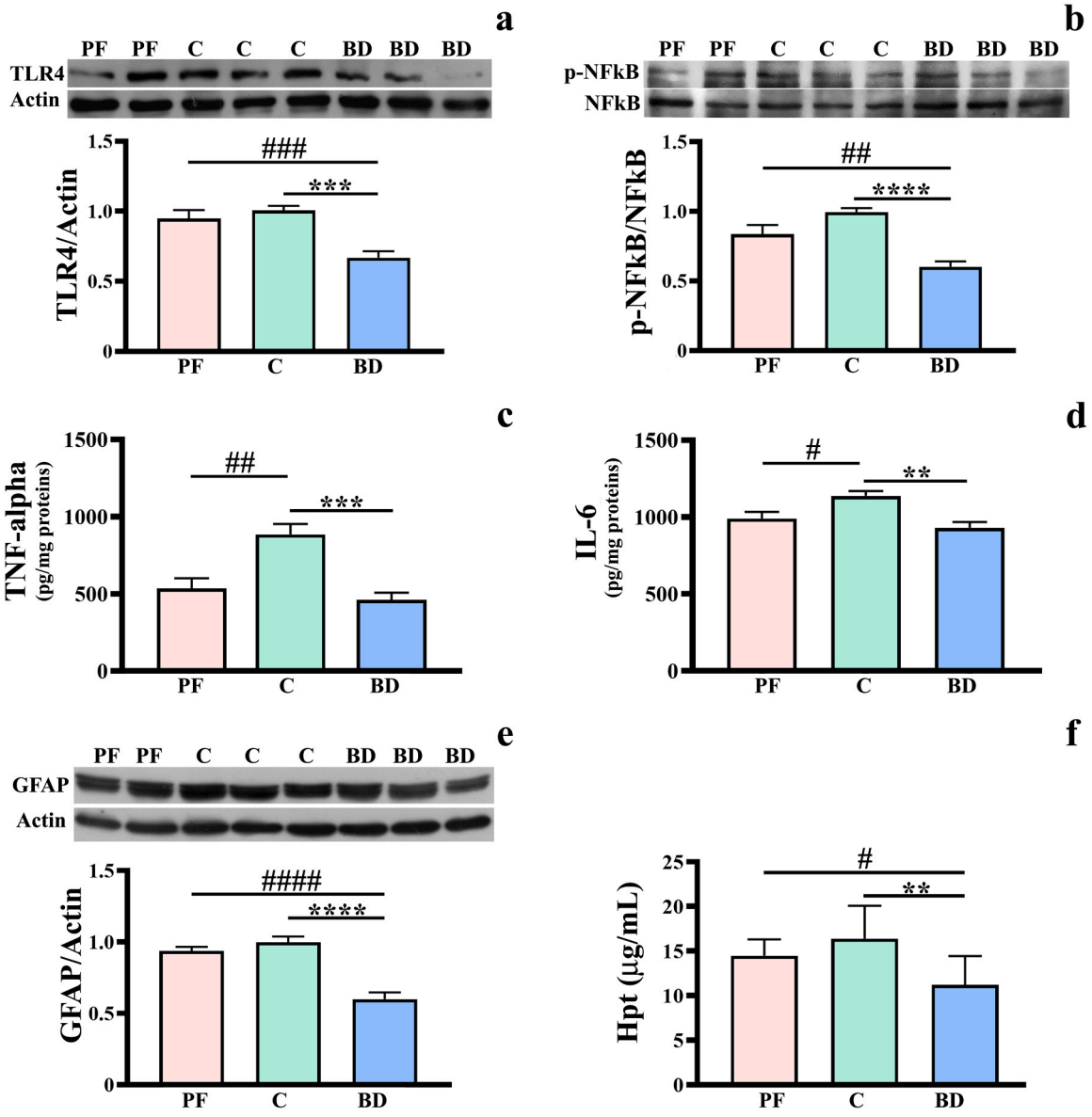

Fig. 2. Evaluation of inflammatory status. (A) TLR-4 amount (representative western blotting and densitometric analysis) in protein extracts from hippocampus of adult rats. Samples were analyzed by 10 % SDS-PAGE and western blotting. After detection of immunocomplexes the membranes were stripped and treated with mouse anti- β-actin as loading control. Values shown are means ± SEM of 8 animals from each group. Data from densitometric analysis were normalized to the value obtained for control animals, set as 1. (B) p-NFkB/NFkB ratio (representative western blotting and densitometric analysis) in protein extracts from hippocampus of adult rats. Samples were analyzed by 10 % SDS-PAGE and western blotting. After revelation of the immunocomplexes (by rabbit anti-phospho NFkB and GAR-HRP IgGs), the membrane was stripped and treated with rabbit anti-NFkB and GAR-HRP IgGs. The amount of phosphorylated NFkB was expressed relative to total NFkB level. Values shown are means ± SEM of 8 animals from each group. Data from densitometric analysis were normalized to the value obtained for control animals, set as 1. (C), TNF-alpha and (D) IL-6 amount was titrated by sandwich ELISA following the manufacturer's instructions. Data are expressed as pg per mg of total proteins and reported as means ± SEM of 8 different rats from each experimental group. (E) GFAP amount (representative western blotting and densitometric analysis). Samples were analyzed by 12.5 % SDS-PAGE and western blotting. After revelation of immunocomplexes (by rabbit anti-GFAP and GAR-HRP IgGs), the membrane was stripped and re-incubated with anti-β-actin. Values shown are means ± SEM of 8 animals from each group. Data from densitometric analysis were normalized to the value obtained for control animals, set as 1. (F) Hpt concentration was measured by ELISA in hippocampus samples diluted 1: 700, 1:2100, and 1:7000. Immunodetection was carried out with rabbit anti-Hpt and GAR-HRP IgGs. Data represent means ± SEM of 8 animals from each group. Control rats (C); 1,3-butanediol treated rats (BD); animals pair fed on an isoenergetic basis to the BD group (PF). $^{**}P < 0.01$, $^{***}P < 0.001$; $^{****}P < 0.0001$ versus BD; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, #### $P < 0.0001$ versus PF (one-way ANOVA followed by Tukey post-test).

maintaining cells homeostasis, mostly balancing energy sources during development and nutritional stress [50]. As shown in Fig. 6C-D, the levels of beclin and p62, two key proteins involved in this mechanism, were significantly lower in BD rats than in both C and PF groups (beclin, $F(2,21) = 33.30$, $P < 0.0001$; p62, $F(2,21) = 9.036$, $P < 0.01$). Interestingly, no differences were evidenced between C and PF group, thus suggesting that the observed effect induced by BD cannot be just ascribed to the reduction in energy intake associated with its administration.

3.5. BDNF and synaptic markers

In order to further clarify whether BD administration influences critical markers of brain functioning, we investigated whether the amount of BDNF, a neurotrophin crucial in the modulation of survival and development of the nervous system [51], is affected by the

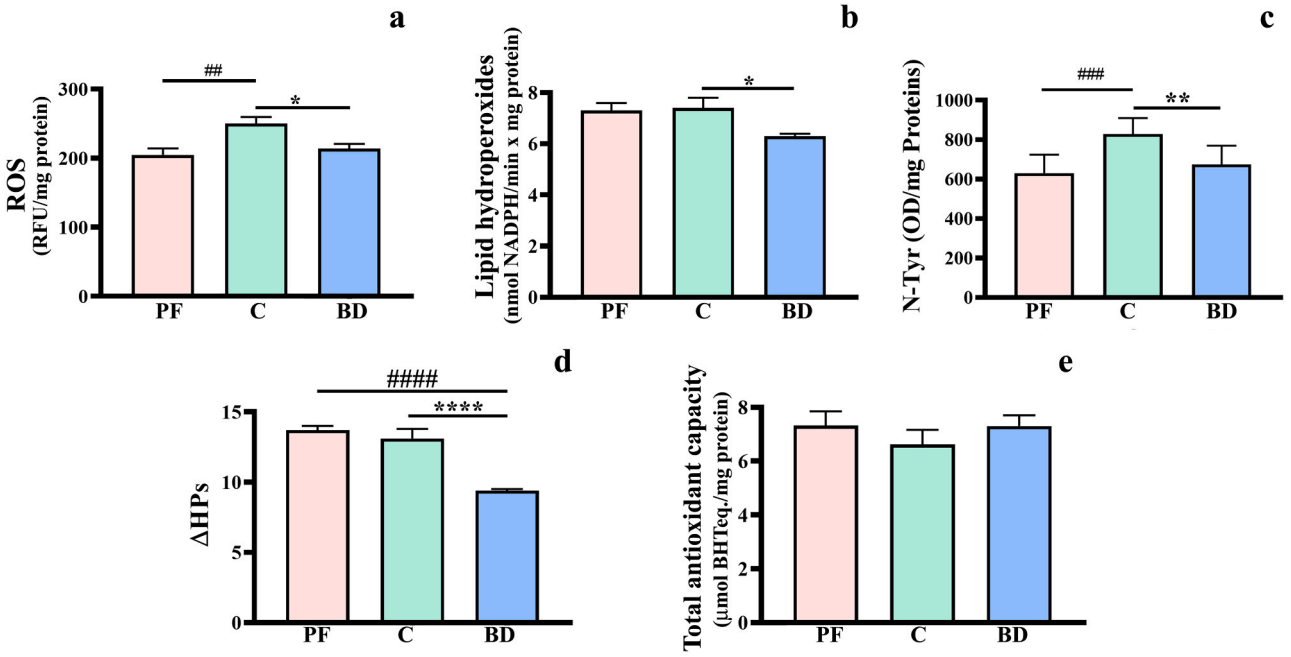

Fig. 3. Evaluation of oxidative status. Quantification of (a) total ROS; (b) lipid hydroperoxides; (c) protein oxidative damage (N-Tyr); (d) oxidative damage susceptibility (ΔHPs); (e) tissue soluble antioxidant capacity (TAC) in hippocampus of adult rats. Control rats (C); 1,3-butanediol treated rats (BD); animals pair fed on an isoenergetic basis to the BD group (PF). Normal distribution of data was verified by Shapiro-Wilk normality test, using the program GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA). Multiple group comparisons were performed with one-way ANOVA followed by Tukey post-test. Values are means ± SEM of 8 animals from each group. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ versus BD; ## $P < 0.01$, ### $P < 0.001$, #### $P < 0.0001$ versus PF.

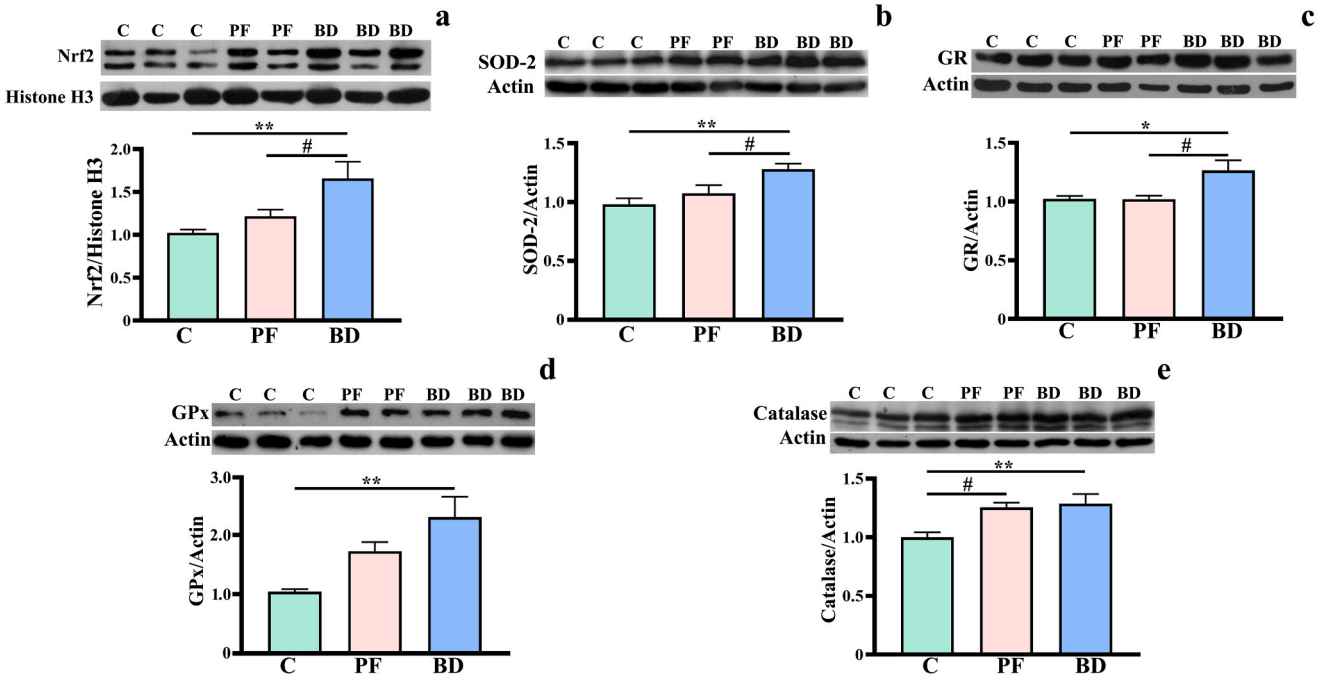

Fig. 4. Quantification of antioxidant enzymes amount. Western blotting quantification of: (a) nuclear factor-E2-related factor 2 (Nrf2), (b) superoxide dismutase (SOD), (c) glutathione reductase (GR), (d) glutathione peroxidase (GPx), (e) catalase in protein extracts from hippocampus of adult rats. Control rats (C); 1,3-butanediol treated rats (BD); animals pair fed on an isoenergetic basis to the BD group (PF). Samples were analyzed by 12.5 % SDS-PAGE and western blotting. After detection of immunocomplexes the membranes were stripped and treated with rabbit anti-Histone H3 (for Nrf2 quantification) or mouse anti-β-actin (for antioxidant enzymes) as loading control. Representative Western blotting and densitometric analysis are shown. Data from densitometric analysis were normalized to the value obtained for control animals, set as 1. Values are the means ± SEM of 8 different rats. * $P < 0.05$, ** $P < 0.01$ versus BD; # $P < 0.05$ versus PF (one-way ANOVA followed by Tukey post-test).

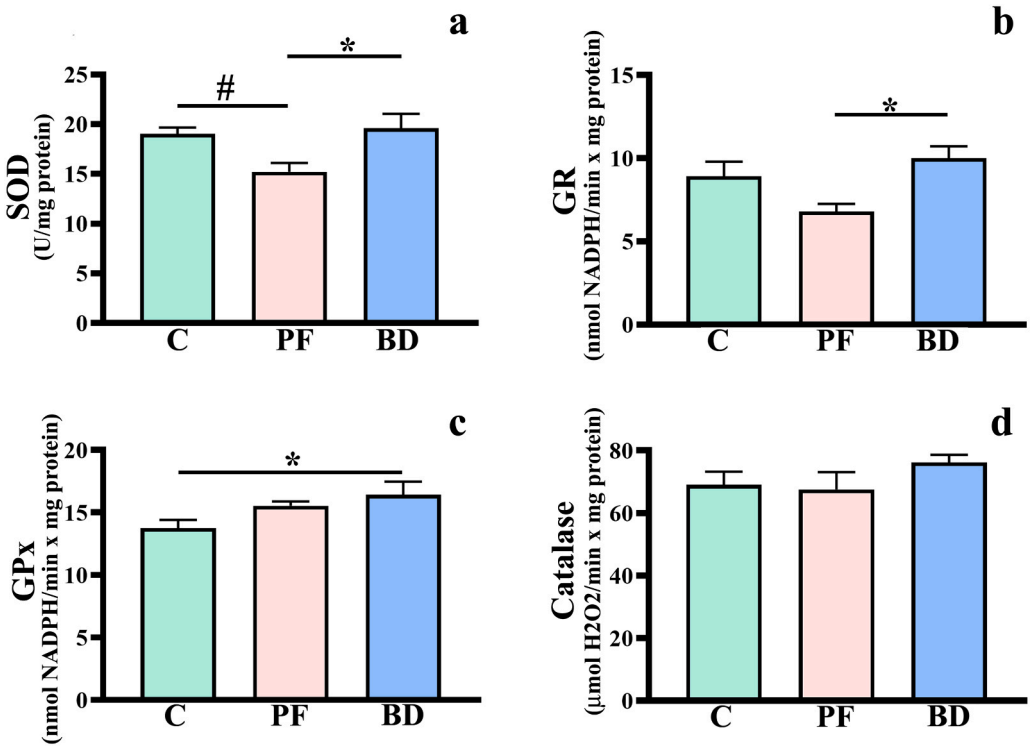

Fig. 5. Quantification of antioxidant enzymes activities The activities of (a) superoxide dismutase (SOD), (b) glutathione reductase (GR), (Cc) glutathione peroxidase (GPx), and (d) catalase were quantified in hippocampus of control rats (C), 1,3-butanediol treated rats (BD), animals pair fed on an isoenergetic basis to the BD group (PF). Normal distribution of data was verified by Shapiro-Wilk normality test, using the program GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA). Multiple group comparisons were performed with one-way ANOVA followed by Tukey post-test. Values are the means ± SEM of 8 different rats. * $P < 0.05$ versus BD; # $P < 0.05$ versus PF.

treatment.

As shown in Fig. 7, BDNF level was found higher in BD than in both C and PF groups ($F(2,21) = 6.641$, $P < 0.01$; Fig. 7A). Since BDNF signalling participates in the modulation of different neurophysiological processes such as synaptic function, dendritic spine maturation and stabilization [52], we evaluated the levels of presynaptic proteins synaptotagmin and synaptophysin, as well as that of the postsynaptic protein PSD-95. BD treatment was associated with a significant increase in the three proteins with respect to C group (synaptotagmin, $F(2,21) = 15.82$, $P < 0.0001$; synaptophysin, $F(2,21) = 8.04$, $P < 0.01$; PSD-95, $F(2,21) = 7.632$, $P < 0.01$; Fig. 7B-D). Notably, the amount of pre-synaptic proteins did not differ between control and PF group, thus suggesting that BD-associated increase cannot be achieved by just a 15 % reduction of energy intake as simulated in PF group.

4. Discussion

The effectiveness of ketogenic regimen in treatment of obesity and refractory epilepsy [6,7], as well as the beneficial and neuroprotective effect in neurological pathologies [4–7] have been ascribed to ability of ketone bodies to replace glucose as brain's main energy source and to act as signaling molecules [2,3]. However, ketogenic diets don't have long-term tolerability, and may present challenges in clinical application [53,54]. As a matter of fact, they may be accompanied by a temporary cluster of symptoms or longer-term effects [5,11,55]. Therefore, alternative dietary supplements have been developed to induce exogenous ketosis [13,56]. In particular, several exogenous ketogenic supplements (EKSs), such as ketone esters (KEs), ketone salts (KSs), and medium chain triglycerides (MCTs) are known to play a therapeutic role thanks to their effectiveness in increasing blood KB level [12,13,57,58,59]. As a matter of fact, KEs, KSs and MCT oils can evoke anti-seizure and anti-epileptic effects [56,60-62], and exert alleviating effects on neurodegenerative diseases [59,60,63,64]. Nevertheless, to date their beneficial effects have been investigated only in disease models, both *in vivo* and *in vitro*.

We believe it is mandatory to clarify the role of these treatments in physiological conditions by using *in vivo* models, given the opportunity to exploit these supplements as future strategies to prevent brain aging and/or delay the development of neurodegenerative diseases. Therefore, we investigated the effect of BD-administration on the hippocampus of adult healthy rats to gain insight into the mechanisms by which BD might affect brain homeostasis in physiological conditions. Since BD administration was associated with a 15 % reduction in animal energy intake, its effect was compared to that of a restricted dietary regimen, isoenergetic with that of BD group (PF group). This allowed us to highlight the specificity of ketone supplements and to discriminate between the BD effects and those induced simply by a regime of dietary restriction.

We report here that BD treatment plays a neuroprotective role by reducing the activation of pro-inflammatory signaling pathways activation. As a matter of fact, treated animals showed lower degree of NFkB phosphorylation and reduced amount of TLR4, as well as reduced glial activation, evidenced by decreased levels of GFAP, and lower concentration of Hpt in comparison to both control and PF animals. So, we evidenced, in hippocampus, a higher protective role of BD in comparison to mild caloric restriction. It is worth mentioning that this is the first study demonstrating a beneficial BD-dependent modulation of inflammatory state in hippocampus, in healthy conditions. Notably, the levels of the inflammatory cytokines TNF-α and IL-6 were found reduced in both BD and PF groups in comparison to the control, in line with previous work showing that caloric restriction correlates with a decrease of pro-inflammatory markers [65,66]. Further, rats receiving BD administration showed lower serum levels of inflammatory markers in comparison to control animals.

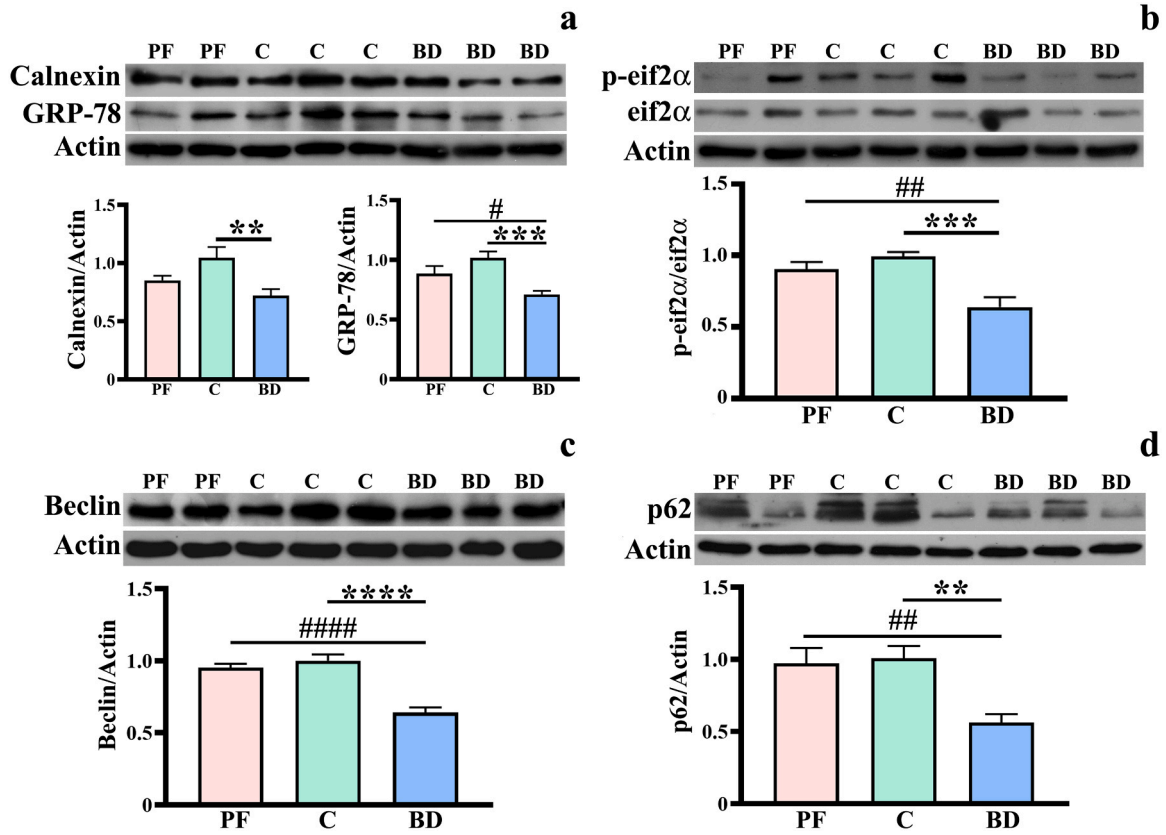

Fig. 6. Evaluation of endoplasmic reticulum stress and autophagy. (A) calnexin and GRP-78, (b) p-eif2α/eif2α, (c) beclin, (d) P62-sequestosome-1 (p62) were measured in protein extracts from hippocampus of control rats (C), 1,3-butanediol treated rats (BD), animals pair fed on an isoenergetic basis to the BD group (PF). Samples were analyzed by 10 % SDS-PAGE and western blotting. After detection of immunocomplexes the membranes were stripped and treated with anti- β-actin as loading control. Representative Western blotting and densitometric analysis are shown. Data from densitometric analysis were normalized to the value obtained for control animals, set as 1. Values are the means ± SEM of 8 different rats. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ versus BD; # $P < 0.05$, ## $P < 0.01$, #### $P < 0.0001$ versus PF (one-way ANOVA followed by Tukey post-test).

Our data suggest that the modulatory effect of BD is ascribable to the βHB rise, which is known to antagonize neuroinflammation [67–69]. In our experimental model, plasma βHB level was found about 3- and 1.5-fold higher in BD and PF groups with respect to C. Therefore, it can be speculated that the observed differences regarding the indices of inflammation between the three groups mainly depend on the differences in plasma levels of βHB, since ketone bodies are able to cross the blood-brain barrier and their influx into the brain has been shown to be proportional to the circulating levels [70–72]. It can be argued that the mechanism underlying the observed BD anti-inflammatory effect in our experimental paradigm might rely on the βHB-mediated inhibition of NFkB pathway, with the consequent suppression of the pro-inflammatory cytokines IL-6 and TNF-alpha. Our results agree with previous works showing that EKSs-generated therapeutic ketosis may evoke beneficial effects on central nervous system diseases [58,59,73,74], thus evidencing that BD may act, through increased βHB level (ketosis), preserving hippocampus physiology.

Redox homeostasis controls multiple cellular signaling pathways and its dysregulation is implicated in the pathogenesis and progression of neurodegenerative disorders [75,76]. The central nervous system is particularly sensible to ROS injury [76] since i) neurons consume large amounts of oxygen, ii) neuronal mitochondria generate large amounts of hydrogen peroxide, and iii) neuronal membranes are rich in poly-unsaturated fatty acids, which are particularly susceptible to oxidative stress. Thus, we investigated whether BD treatment affects hippocampal oxidative status, focusing on both antioxidant defense system and oxidative damage markers. A reduced ROS content together with a lower degree of oxidative damage to proteins was observed in both BD and PF groups. Interestingly, the extent of lipid peroxidation was decreased in BD animals but not in PF rats, maybe because of the increased activity and amount of the enzyme GPx, which might be also responsible for the reduced susceptibility to oxidative insult revealed in this group. Further, BD treatment was found associated with the increase in nuclear levels of nuclear factor-erythroid 2-related factor-2 (Nrf2), the pivotal modulator of antioxidant response, together with higher concentrations of the antioxidant enzymes SOD-2, GR, and catalase. Since BD supplemented rats showed higher plasma levels of βHB, which has been suggested to activate Nrf2 pathway [77], we hypothesize that modulation of the antioxidant response in hippocampus, in our experimental model, occurs through the regulation of Nrf2. Our data agree with the evidence that an increase in ketone levels, obtained by ketogenic diet administration, was able to induce nuclear Nrf2 accumulation in both liver and hippocampus [78], and with the finding that ketogenic diet attenuates oxidative stress and inflammation through the activation of Nrf2 and the consequent suppression of NFkB pathway, in a rat model of spinal cord injury [79]. Notably, we demonstrate, for the first time, that BD administration in physiological conditions, is more effective on Nrf2 with respect to caloric restriction. The unchanged activity of SOD-2, GR, and catalase notwithstanding the increased protein levels could result from a post-translational modification of the proteins and could have an important role in preserving the functionality of the hippocampus in animals not challenged with oxidative stress, as in our experimental conditions. This prevents a huge metabolization of ROS, which would lead to a deleterious reduction in ROS signaling. Indeed, ROS signaling is critical for neuronal development and differentiation [80] and participates in hippocampal synaptic plasticity processes such

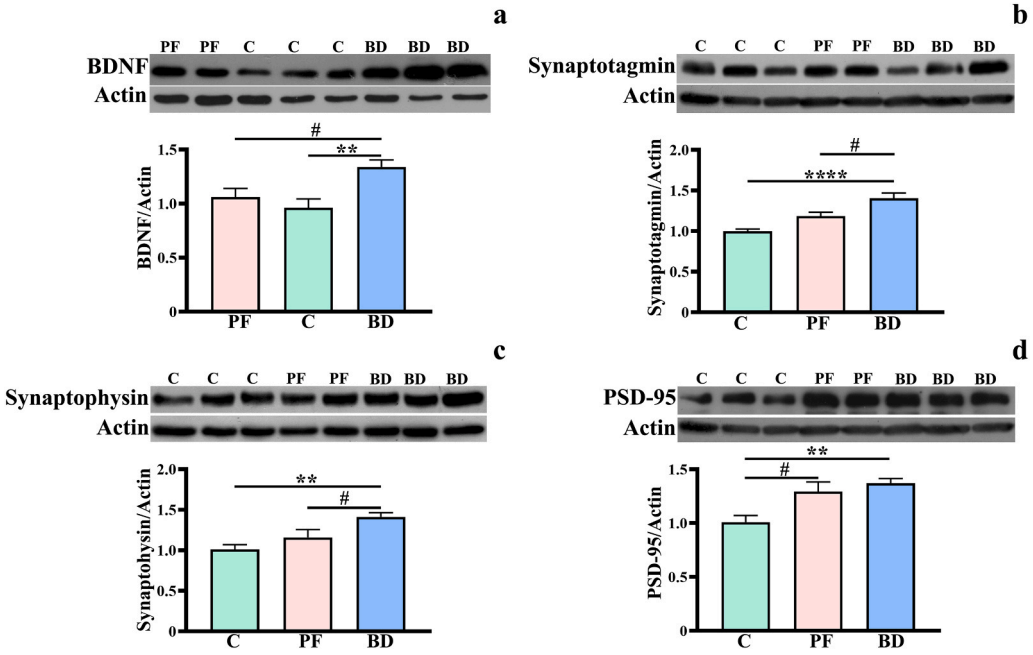

Fig. 7. Brain derived neurotrophic factor (BDNF) and synaptic proteins. (a) BDNF, (b) synaptotagmin, (c) synaptophysin, (d) post-synaptic density protein 95 (PSD-95) were quantified in protein extracts from hippocampus of control rats (C), 1,3-butanediol treated rats (BD), animals pair fed on an isoenergetic basis to the BD group (PF). Samples were analyzed by 12.5 % (BDNF, synaptophysin, synaptotagmin) or 10 % (PSD-95) SDS-PAGE and western blotting. After detection of immunocomplexes the membranes were stripped and treated with anti- β-actin as loading control. Representative Western blotting and densitometric analysis are shown. Data from densitometric analysis were normalized to the value obtained for control animals, set as 1. Values are the means ± SEM of 8 different rats. ** $P < 0.01$, **** $P < 0.0001$ versus BD; # $P < 0.05$ versus PF (one-way ANOVA followed by Tukey post-test).

as long-term potentiation [80,81]. The βHB-dependent neuroprotective modulation of redox balance has been considered implicated also in the ability of ketone bodies to suppress the unfolded protein response associated with endoplasmic reticulum stress and to correct defective autophagy in pathological conditions [82,83]. In our model, BD treatment was associated with a reduction of the degree of eIF2α phosphorylation and the amount of the two chaperones calnexin and GRP78, in agreement with the finding that BD attenuates ER stress in the hypothalamus of diet-induced obese mice [22]. Our data also agree with the evidence that other treatments enhancing βHB, such as the ketogenic diet, protect hippocampus from epilepsy-induced activation of ER stress in rat [84], as well as from hypoglycemia-induced activation of ER stress pathway in mice [85], thus supporting the use of BD as a strategy to preserve hippocampus functionality in healthy conditions.

In addition, a reduction of the autophagy-related proteins beclin and p62 with respect to control rats was observed in BD rats but not in PF rats that underwent a mild caloric restriction. It should be highlighted the novelty of our results, since data available in the literature are limited to the description of a modulatory effect of ketone bodies in pathological conditions characterized by an abnormally prolonged or excessive ER stress response activation or by a dysregulation of autophagic flux. Notably, we report for the first time that BD plays a protective action also in physiological conditions and this could be crucial for guaranteeing the proper functioning of ER and autophagy. In this context, it should be mentioned that ER besides participating in the synthesis, folding, structural maturation, and degradation of proteins folded incorrectly [86], also impacts several cellular processes required for brain function [87], and its homeostasis contributes to neurodevelopmental processes [87]. As representing a major response pathway to cellular stress, autophagy is tightly connected to ER stress activation [49,88]. Both mechanisms operate to maintain homeostasis, but their dysregulation can induce the rise of inflammatory processes, participating in the onset or progression of several diseases [49,88]. Therefore, BD plays a neuroprotective role through the positive modulation of redox homeostasis and the reduction of the integrated activation of the major response pathways related to stress. This beneficial action might also contribute to preserving neuronal plasticity since ER stress and neuroinflammation are associated with synaptic loss as well as the repression of the expression of a synaptic proteins cluster [89,90]. As a matter of fact, we found that BD treatment induces an increase of the neurotrophin BDNF, the presynaptic proteins synaptophysin and synaptotagmin, and the post-synaptic protein PSD-95. Interestingly, in PF group only the amount PSD-95 was found increased, demonstrating the stronger effect of BD on the regulation of neurotrophin and pre-synaptic proteins. Our results are in line with the finding that KBs induce the production of hippocampal BDNF. Indeed, several studies demonstrated the induction of specific BDNF promoters by βHB in hippocampus neuronal cell lines [91–93], and it was shown that systemic administration of βHB in diabetic mice restored retina BDNF levels towards those of non-diabetic animals [94].

Since we observed that BD treatment induced higher βHB plasma levels than caloric restriction, our hypothesis is that differences between PF and BD group can be explained by the different circulating levels of βHB, which acts as molecular mediator of the observed effect. As a matter of the fact, βHB is known to protect the brain from oxidative stress, acting as a free radical scavenger of hydroxyl radicals and superoxide ions [5,77,95]. Further, data obtained in pathological models showed that both the ketogenic diet and βHB treatment are able to prevent or alleviate ER stress activation, both *in vitro* and *in vivo* [96–98], and to modulate autophagy [94,99]. Overall, we postulate that BD modulation of stress response in the healthy animal model occurs through mechanisms based on the regulation of NFkB and Nrf2 pathways. In agreement with our findings, the beneficial effects described for several EKS were proposed to be induced likely through ketosis-evoked neuroprotective effects, for example, by decreased inflammatory processes and decreased oxidative stress [59,74,99,100]. Actually, our data can be also interpreted at the light of the fact that βHB, being an inhibitor of histone deacetylase, also induces an increased expression of specific genes regulating the cellular antioxidant defences, such as catalase, SOD-2, GPx [101,102], and is involved the induction of BDNF

expression [65,103,104], which in turn is a modulator of synaptophysin and synaptotagmin synthesis [105–107]. Most of our results seems to be the consequence of the increased circulating levels of βHB, but it cannot be excluded that other metabolites and/or other signaling pathways in addition to those investigated in this work may occur. This opens the way for further research into additional effects associated with BD administration.

Altogether our results highlight that BD, independently from energy intake reduction associated with its assumption, modulates Nrf-2 and NF-kB pathway, ameliorating redox and inflammatory status, and also promoting neurotrophin expression. Therefore, our findings represent an important advance in the knowledge of BD effects just because point out that this supplement could represent an effective neuroprotective treatment to manage brain function, also in healthy conditions, with possible applications for preventing or delaying aging without the need of implementing ketogenic nutritional interventions.

Ethics approval

The study was conducted according to the guidelines of the Declaration of Helsinki, approved by "Comitato Etico-Scientifico per la Sperimentazione Animale" of the University of Naples Federico II, and authorized by Italian Health Minister (776/2021-PR).

Informed consent statement

Not applicable.

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CRediT authorship contribution statement

Gianluca Fasciolo: Writing – review & editing, Investigation, Formal analysis, Data curation. **Natasha Petecca:** Writing – review & editing, Investigation. **Francesca De Palma:** Writing – review & editing, Investigation. **Luisa Cigliano:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Maria Stefania Spagnuolo:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Assunta Lombardi:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Paola Venditti:** Writing – review & editing, Supervision, Investigation, Formal analysis, Data curation, Conceptualization. **Giuliana Panico:** Writing – review & editing, Investigation, Formal analysis, Data curation.

Declaration of Competing Interest

The authors have no financial or non-financial interests to disclose.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.117774.

Data Availability

Data will be made available on request.

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