Creatine maintains mitochondrial integrity and protects against dysfunction in molecular systems involved in early epileptiform activity and cognitive impairment in young rats submitted to traumatic brain injury

Luiz Fernando Freire Royes (nandoroyes@yahoo.com.br)
Universidade Federal de Santa Maria https://orcid.org/0000-0003-4045-4827
Guilherme Lago Busanello
UFSM: Universidade Federal de Santa Maria
Douglas Godinho
UFSM: Universidade Federal de Santa Maria
Alexandre Seixas Nascimento
UFSM: Universidade Federal de Santa Maria
Gabriel Correa Lima
UFSM: Universidade Federal de Santa Maria
Jane do Socorro dos Navegantes Marçal Cunha
UFPA: Universidade Federal do Para
João Bento Torres Neto
UFPA: Universidade Federal do Para
Marcia Consentino Kronka Sosthenes Sosthenes
UFPA: Universidade Federal do Para
Fernando Gomez-Pinilla
UCLA: University of California Los Angeles
Gokul Krishna
The University of Arizona College of Medicine Phoenix
Ana Flavia Furian
UFSM: Universidade Federal de Santa Maria
Mauro Scneider Oliveira
UFSM: Universidade Federal de Santa Maria
Michele Rechia Fighera
UFSM: Universidade Federal de Santa Maria

Research Article

Keywords: Traumatic Brain Injury, Creatine Supplementation, Mitochondrial Integrity

Posted Date: December 4th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3635710/v1

Abstract

Despite the number of studies supporting the neuroprotective role of creatine in pathophysiology caused by traumatic brain injuries, there is insufficient knowledge of how this ergogenic compound may potentially affect brain-injury complications in adolescence. Thus, we hypothesized that creatine supplementation after a TBI could prevent from deleterious neurological effects such as memory deficits, mitochondrial disfunction and epileptiform activity. Our experimental data revealed, for the first time, that creatine supplementation (300 mg/kg, po) for two weeks after neuronal injury protected against spatial memory dysfunction (Barnes maze test), disruption of hippocampal theta/delta activity, and spontaneous epileptiform activity in 35-day-old rats submitted to severe fluid percussion injury (FPI). Interestingly, the FPI protocol did not cause cell loss (especially parvalbumin-positive neurons) in the CA1 and CA2/CA3 areas but induced mitochondria dysfunction (MTT, Δψ, SDH, complex II, COX, CS, CKm activity inhibition, and CKm immunoreactivity decrease), and mitochondrial oxidative stress (DCFH-DA oxidation, increased 4-hydroxynonenal levels, free -SH groups, and lower GSH levels). Creatine's ability to maintain mitochondrial integrity protected against dysfunctions in molecular systems involved in cellular energy homeostasis (decreased PGC1 and TFAM immunoreactivity), Na+,K+-ATPase activity inhibition, and proteins related to brain plasticity (decreased BDNF, TrkB, and pCREB/CREB immunoreactivity) after FPI. These data suggest that epileptiform activity and cognitive dysfunction in young rats may, at least partly, result from alterations in surviving neurons interfering with creatine-induced secondary injury, which may be an excellent therapeutic strategy against toxicity induced by traumatic brain injury.

1. Introduction

Traumatic brain injury (TBI) is recognized as a global public health problem, affecting 70 million people worldwide yearly [1]. Historically, various lines of research have focused on adult injuries, as it is assumed that greater neuroplasticity potential may partially ameliorate impairments in younger patients [2, 3]. However, TBI is more than a singular event: it is a neurological disease in which those injured at a young age are more prone to long-term behavioral changes, such as attention-deficit/hyperactivity disorder, anxiety, depression, and motor and learning deficits [4]. In fact, epidemiological data have shown that TBI during childhood or adolescence is associated with a 4-fold increased risk of mental disorders in adult males [5]. In addition, clinical studies have demonstrated that disruption of neuronal development in juvenile TBI may increase symptom severity compared to similar injuries in adult patients [6, 7]. Nonetheless, little is known about how TBI in adolescence influences the architecture and brain metabolism of structures related to memory, planning, and social communication. Despite extensive research on cellular and molecular disturbances in the early stages of TBI, the electrophysiological changes after chronic injury remain to be explored, and more specifically, how disturbances in electrophysiological patterns in adolescence may affect memory in adulthood. Given this scenario, animal models are an excellent opportunity to investigate the effects of TBI on neural activity [8].

From a metabolic perspective, experimental juvenile TBI has demonstrated that progressive neuronal loss and impaired axonal transport elicited by cascades related to inflammatory/oxidative stress compromise

neuronal function and induce long-term deficits in cognition in adulthood [9–11]. Even though the causal relationship is not fully known, experimental studies with TBI have suggested that disruption of regional hippocampal excitability is critical for learning, such as the GABAergic pathway in adulthood, which alters inhibitory circuits, reduces information processing [12–14], and induces a cognitive deficit after neurological injury [15, 16].

Given the previous findings from our research group, which demonstrated that excitability increases due to GABAergic system impairment [17], we hypothesize that the reorganization of neuronal synapses in the hippocampus caused by significant cell loss, including GABAergic neurons, may be associated with TBI-induced learning deficits. However, there are caveats when interpreting this pathway's involvement in TBI-induced physiopathology. Understanding the mechanisms through which the brain interacts with systemic physiology during TBI pathophysiology is crucial to establish scientific-based rehabilitative strategies. Some approaches, including creatine supplementation, which decreases neuronal damage in neurological disorders, have therapeutic potential by modulating conditions characterized by brain creatine deficits induced by acute stressors such as TBI [18, 19].

Sakellaris and colleagues (2006) conducted a prospective study on adolescents with TBI and demonstrated that six months of creatine supplementation (0.4 g/kg orally) significantly improved clinical recovery in the categories of cognitive capacity, personality/behavior, self-care, and communication aspects [20]. Creatine supplementation also reduced post-traumatic amnesia and improved recovery, cognitive function, headaches, dizziness and fatigue, and language articulation in children after TBI [21]. In experimental TBI models, creatine supplementation reduced cortical damage (36–50%) by protecting mitochondrial function (i.e., it increased mitochondrial membrane potential, decreased intramitochondrial reactive oxygen species [ROS] and calcium, and maintained adenosine triphosphate [ATP] levels). In another study, higher ATP/PCr levels and mitochondrial membrane maintenance induced by creatine supplementation protected against markers of secondary cellular injury characterized by lactate, free fatty acids, intramitochondrial levels of ROS, and calcium increase induced by TBI [22]. Recently, experimental data from our research group revealed that the anticonvulsant effect of creatine supplementation (300 mg/kg) was associated with its ability to reduce cell loss, including the number of parvalbumin-positive (PARV+) cells in the CA3 region of the hippocampus. The effective protection against reduced glutamic acid decarboxylase (GAD67) levels, GAD activity, and specific [3H] flunitrazepam binding in the hippocampus suggest that this guanidine compound may play a neuroprotective role on brain excitability by controlling the GABAergic function after TBI [17]. However, the potential effect of creatine on oxidative/inflammatory stress-related cascades that impact signal transduction and cognitive behavior after TBI is not fully understood. In order to shed more light on this issue, we decided to investigate whether: a) hippocampal oxidative stress is implicated in signal transduction after TBI; b) these responses are associated with protein levels related to brain plasticity and how these mediators contribute to syndrome-like behaviors and neuronal damage; and c) interference in the secondary injury development elicited by creatine supplementation breaks the progression of neuronal damage and cognitive dysfunction after TBI.

2. Materials and methods

2.1 Animals and reagents

Juvenile male Wistar rats (35 days old; PND35), which are used to model adolescence in humans [23], were kept in a controlled light environment (12:12 h light cycle, 24 ± 1°C, 55% relative humidity) with food and water ad libitum. Experimental procedures were conducted according to national and international legislation (Brazilian College of Animal Experimentation and US Public Health Service Policy on Humane Care and Use of Laboratory Animals guidelines) and approved by the Ethics Committee on Animal Research of the Federal University of Santa Maria (protocol no. 011/2015). The experiments were carried out according to the principles described by Grundy (2015) [24].

2.2 Experimental design

The animals were randomly divided into two groups: sham and TBI animals; the experimental procedures are illustrated in Fig. 1. The animals were trained for four days in the Barnes maze to learn the task and subjected to either sham (n = 8) or TBI (n = 6–8). For this, the animals were submitted to TBI surgery, and 24 h later, the neuronal injury was induced. Twenty-four hours after TBI, the animals were randomly reassigned to receive creatine supplementation (300 mg/kg, po; n = 6–8), suspended in 0.5% CMC or vehicle (CMC; n = 6–8) [25, 26] for two weeks. At the end of the second week after the injury, memory retention was assessed 24 h after the last creatine supplementation. Immediately after the Barnes maze task, the animals were euthanized, and the hippocampus was collected for biochemical and histological assays (Fig. 1).

2.3 Traumatic brain injury

The lateral fluid percussion injury was performed according to the method of D'Ambrosio et al. (2004) [27], with slight modifications. In summary, the animals were anesthetized with a single injection of Equithesin (3 mL/kg, ip; 1% sodium pentobarbital, 4% chloral hydrate, 2% magnesium sulfate, 42.8% propylene glycol, and 11.2% absolute ethanol) and placed in a rodent stereotaxic apparatus. A 3-mm burr hole was drilled on the right convexity, 2 mm posterior to the bregma and 3 mm lateral to the midline, avoiding dura mater injury. A plastic injury cannula was placed over the craniotomy with acrylic cement. The cannula was filled with chloramphenicol when the cement hardened and closed with a proper plastic cap. The animal was then removed from the stereotaxic device and placed in a heated recovery chamber before returning to their cages. After 24 h, the animals were anesthetized with isoflurane; their injury cannula was attached to the fluid percussion injury (FPI) device and placed in a heat pad maintained at 37 ± 0.2°C. The TBI was produced by an FPI device developed in our laboratory. A brief (10–15 ms) transient pressure fluid pulse (3.01 ± 0.29 atm) impact was applied against the exposed dura mater, producing a moderate TBI with an overall mortality of 21.09%. Pressure pulses were measured extracranially by a transducer (Fluid Control, AmScien Instruments, USA) and recorded on a storage oscilloscope (Gould Ltd., Essex, UK). Immediately after restoring righting reflex and responding to a paw pinch after TBI, anesthesia of animals was restored, and the skull was sutured. Neomycin was applied to

the suture, and the animals were placed in a heated recovery chamber before returning to their cages. The sham group underwent the same procedures and was coupled to the injury device, although no fluid pulse was delivered.

2.4 Surgical procedures and electroencephalography

Two weeks after FPI, the animals were anesthetized with an intraperitoneal injection of Equithesin and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, a 3-mm craniectomy was performed on the right convexity parietal cortex, 2 mm posterior to the bregma and 3 mm lateral to the midline, with the utmost care to keep the dura mater intact. A plastic injury cap was placed over the craniotomy with dental acrylic cement and was filled with chloramphenicol. During the surgery to place the plastic injury cap, all animals were implanted with electrodes for electroencephalographic (EEG) recordings. Two screw electrodes were placed over the parietal cortex, one rostral to the craniectomy and the other contralateral to the craniectomy, along with a ground lead positioned over the nasal sinus. Bipolar nichrome wire Teflon-insulated depth electrodes (100 μm) were unilaterally implanted into the ipsilateral hippocampus (coordinates relative to the bregma: AP -4, ML 3, and DV 3 mm) [28]. All electrodes were connected to a multipin socket fixed to the skull with dental acrylic cement. Electroencephalographic recordings were performed three days after surgery. Each rat was transferred to a Plexiglas cage (25 x 25 x 40 cm) and habituated for 30 min before EEG recordings. The EEG recordings were analyzed offline using standard functions of the LabChart 8 software (AD Instruments) [29]. The EEG wave amplitude and EEG spike frequency were automatically calculated using the native LabChart functions Average cyclic height and Event count, respectively. Power spectrum analysis was performed considering the following EEG frequency bands: delta (1–3 Hz) and theta (4–7 Hz), according to [30]. Extracellular recordings were made for 20 min before the Barnes maze assay.

2.5 The Barnes maze test

Barnes maze testing was performed with two daily trials and a 5-min test period, as described elsewhere [31]. The apparatus comprised a circular acrylic plastic table to form a 1.5-cm thick and 115-cm wide disk, with 18 7-cm holes spaced equidistantly around the perimeter; the apparatus was brightly lit by overhead halogen lamps. At the start of each trial period, the rats were individually placed at the center of the maze and covered with an opaque ‘starting’ cylinder for 10 s before it was raised to allow the animal to explore the maze, locate, and enter an escape box hidden beneath the table. The box remained in the same location throughout the testing period. Their movement was simultaneously tracked with a camera connected to an automated video tracking system (ANY-maze, Stoelting Co., USA) installed directly overhead at the center of the maze. The training/acquisition phase ended after the animal entered the escape box or after a maximum test duration (5 min), following which the animal was allowed to stay in the box for 30 s and then returned to the home cage. If the animal failed to enter the escape box within the time limit, it was gently led toward the escape box. The rats were tested twice a day for five consecutive days at approximately the same time every day before the swimming tests and recurrent concussion protocols. Memory retention was then assessed 24 h after the open field task. The arena and boxes were wiped clean using alcohol 30% and distilled water between each training session.

2.6 Tissue processing

Immediately after the behavioral Barnes maze task, a subset of animals under deep anesthesia (thiopental sodium, 100 mg/kg, ip) was transcardially perfused with 600 mL of heparinized saline 0.9%, followed by 600 mL of 4% paraformaldehyde in 0.1 M phosphate-buffered (PB) at pH 7.4. The brains were removed for histological analysis and immunochemistry. For ex vivo assays, the subset of animals was euthanized by decapitation, and the ipsilateral hippocampus was rapidly dissected for neurochemical analysis.

2.7 Na+,K+-ATPase activity

The Na+,K+-ATPase activity was determined according to [32]. The amount of inorganic phosphate released was quantified by the colorimetric method described by Fiske and Subbarow (1925) [33].

2.8 Western blot assay

Western blot analysis was performed according to Funck et al. (2014) [34], with some modifications. Samples were lysed on ice in radio-immunoprecipitation assay and centrifuged for 20 min at 12,700 ×g and 4°C. The protein concentration of each sample was determined by the bicinchoninic acid protein assay (Thermo Fisher Scientific). Samples (30 µg protein) were then subjected to 4–12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane using the Trans-Blot® Turbo™ Transfer System, and equal protein loading was confirmed by Ponceau S solution (Sigma Aldrich - P7170). After specific blocking, the blots were incubated overnight at 4°C with BDNF antibody (1:1000, Cell Signaling), TRKB antibody (1:1000, Cell Signaling), PGC1α (4A8; sc517380; 1:1000 Santa Cruz Biotechnology), CREB antibody (D-4: sc-374227-P 1:1000 Santa Cruz Biotechnology), p-CREB-1 antibody (10E9: sc-81486 1:1000 Santa Cruz Biotechnology), MtCK antibody (sc-373686; 1:1000 Santa Cruz Biotechnology), mitochondrial transcription factor A (mtTFA; sc-166965; 1:1000 Santa Cruz Biotechnology), SIRT1 antibody [19A7AB4] (ab110304), and 4-HNE (SAB 5202472; 1:1000; Sigma Aldrich). Rabbit anti-β-actin (1:10.000, Santa Cruz Biotechnology, USA) was stained as an additional protein loading control. After primary antibody incubation, the membranes were washed with TBS-T (TBS plus 0.1% Tween 20) two times at room temperature for 10 min and incubated with anti-rabbit (Sigma Aldrich - A6154) or anti-mouse (Santa Cruz Biotechnology - sc-2005) secondary antibodies conjugated with horseradish peroxidase (1:5000) for 2 h at room temperature. Bands were visualized by enhanced chemiluminescence using ECL western blotting substrate (Pierce ECL, BioRad), and the signals were captured with an image analysis system (ChemiDoc XRS+, BioRad). The bands were then quantified using the Image Lab software (Bio-Rad).

2.9 Rat brain mitochondria isolation

Rat brain mitochondria were isolated as described by Tonkonogi and Sahlin (1997) [35], with slight modifications. The ipsilateral hippocampus was quickly removed from the skull and homogenized in a buffer containing (in mM): 100 sucrose, 10 EDTA, 100 Tris-HCl, and 46 KCl (pH 7.4). The homogenates were centrifuged at 800 g for 10 min at 4°C, and the precipitate was discarded. The supernatant was

centrifuged at 10,000 g for 10 min at 4°C. The second centrifugation supernatant was used to determine the activity of the Na+,K+-ATPase. The sediment enriched in mitochondria was washed twice with the same buffer and resuspended in Tris-HCl buffer (pH 7.5) and centrifuged for 3 min at 2,000 g (4 °C) to obtain a low-speed supernatant fraction (S1). The S1 was centrifuged for 20 min at 12,000 g (4°C). The pellet was resuspended in a buffer containing (in mM): 100 sucrose, 10 EDTA, 100 Tris–HCl, 46 KCl, and bovine serum albumin (BSA, 0.5%; pH 7.4) and re-centrifuged for 10 min at 12,000 g (4°C). The supernatant was decanted, and the final pellet was resuspended in a buffer containing (in mM): 70 sucrose, 0.02 EDTA, 20 Tris-HCl, 230 mannitol, and 1 K2HPO4 to yield a protein concentration of 30–40 mg/mL. All the isolation procedure was carried out at 4 °C in order to maintain the temperature under control minimize ROS production throughout the process.

2.10 ROS production, nonprotein sulfhydryl (NPSH), and reduced glutathione (GSH) content

The poduction of ROS was estimated in the ipsilateral hippocampus mitochondria with the fluorescence probe 2',7'-dichlorfluorescein diacetate (DCFH-DA), as described by Myhre et al. [36] Free -SH groups were determined according to Ellman and Lysko (1961) [37], with some modifications. The GSH levels were measured according to the method described by Hissin and Hilf was assayed [35].

2.11 Assays for respiratory chain enzyme activities

The activity of succinate dehydrogenase (SDH) and complex II was determined according to the method of Fischer et al. (1985) [38] through the decrease in absorbance of 2,6-dichloroindophenol at 600 nm with 700 nm as the reference wavelength (ε = 19.1 mM-1 cm-1) in the presence of phenazine petassulfato, and the results were expressed as nmol/min/mg protein. The cytochrome c oxidase (COX) activity was determined according to Rustin and collaborators (1994) [39], and enzymatic activity was measured at 25°C for 10 min by the decrease in absorbance due to the oxidation of previously reduced cytochrome c at 550 nm with 580 nm as the reference wavelength (ε = 19.1 mM-1 cm-1). The reaction solution containing 10 mM potassium phosphate, pH 7.0, 0.6 mM n-dodecyl-β-D-maltoside, 2–4 μg of protein homogenized, and the reaction was initiated by adding 7.0 μg of reduced cytochrome c. The results were expressed in nmol/min/mg protein. Citrate synthase (CS) activity was determined spectrophotometrically following the Srere method by measuring the appearance of free CoA.

2.12 Mitochondrial methyl-tetrazolium (MTT) reduction and membrane potential (Δψ)

The MTT assays were carried out with a modified version of the method of Berridge and Tan (1993) [40]; the respiration buffer was used as a stock buffer. Hippocampal mitochondrial ΔΨm determination was estimated by fluorescence changes in safranine-O assayed, as previously described.

2.13 Superoxide dismutase (SOD) and creatine kinase (CK) enzyme activities

The hippocampal SOD activity was measured according to Misra and Fridovich (1972) [41]. The CK activity was estimated according to the colorimetric method described by Hughes (1962) [42]. The incubation medium contained 65 mM Tris-HCl pH 7.5, 7 mM phosphocreatine, 9 mM MgSO4, and ~ 1 µg of protein in a final volume of 0.1 mL.

2.14 Histological procedures

The hemispheres were sectioned on a vibratome and every other parasagittal section (100-µm thick). The sections were collected in histological laminae and prepared for hematoxylin and eosin staining. The histological laminae were dehydrated in ethanol, cleared in xylene, and covered with Canadian balsam. Four images were taken from the CA1 and CA2 regions of each rat using a microscope (200×; CX21, Olympus, Japan) coupled to a micrometric camera (Accu Scope, USA). The cells in the areas of interest (AOI; 31,702 µm2) of each image were counted using the ImageJ 1.46 software (NHI, USA). The data represents the mean of the counted cells in the CA1 and CA2 for each animal; this procedure was adapted from Marcuzzo and colleagues (2010) [43].

2.15 Parvalbumin positive (PARV+) assay

Given that the CA1 and CA2 regions of the dorsal hippocampus constitute the processing network for spatial memory and learning in tests (e.g., the Barnes maze task) [44], we performed immunohistochemical labeling for PARV + neurons. The sections were washed in 0.1 M phosphate buffer and pretreated for 60 min in 0.2 M boric acid solution (pH 9.0 at 70°C). After washing with phosphate-buffered saline plus Triton 5% (PBST 5%), the section was washed thrice (60 min) in 1% casein solution diluted in PBST. The sections were then incubated on the monoclonal primary antibody anti-parvalbumin (Milipore®, MAB1572, 1: 2000 in 0.1% PBST) for 72 h, followed by washing in PBS and incubated with the biotinylated secondary antibody (Vector®, BA- 1400, diluted in PBST 0.05% in 1:200 ratio) for 12 h (overnight).

Afterward, the sections were incubated for 20 min in 1% hydrogen peroxide and washed thrice in PBS for further incubation in avidin-biotin complex (Vector®, ABC kit) for 1 h. After washing to remove the ABC, peroxidase in the antibody-antigen binding sites was developed employing a protocol to enhance the reaction using nickel/glucose oxidase/diaminobenzidine [45]. The reaction was quenched with three washes in 0.1M phosphate buffer at pH 7.2–7.4. The sections were mounted on gelatinized slides, allowed to dry at room temperature, and then dehydrated in alcohol (70, 80, 90, and 100%). The sections were then diaphanized in xylene and covered with Entellan (Merck, Germany) and coverslip for further qualitative and quantitative stereological analyses. Quantitative analysis of the number of PARV + cells was performed using the optical fractionator method. Notably, the CA1 and CA2 regions represent an important hippocampal function involved in neurodegeneration after TBI [46, 47]. The stratum radiatum was delimited using the 4× objective of an optical microscope (Nikon Eclipse 80i, Japan) equipped with motorized platinum and X, Y, and Z (MAC6000, Ludl Electronic Products, USA). This system is coupled to a computer with the Stereoinvestigator software (MBF Bioscience, USA), which records the three-dimensional coordinates and stores the stereological data. By using the 100× objective, the following

counting parameters were used: dissector height (Z; 15 µm), counting frame width (X–Y; 85–60 µm), sampling grid (X–Y; 90–65 µm), guard zone (1 µm).

For the 4-hydroxynonenal (4-HNE) assay, 40-µm serial coronal brain sections were cut on a cryostat and collected in 0.01 M PBS. After washing, free-floating sections were heated to 80°C for 30 min in 10 mM sodium citrate buffer (pH 8.5) to promote antigen binding and returned to room temperature. Nonspecific binding was blocked by incubating the sections with 0.01 M PBS solution containing 0.5% BSA and 0.5% Triton X-100 for 2 h at room temperature. The sections were then incubated with either rabbit polyclonal primary anti-4-HNE (1:250; Abcam, USA) in 0.01 M PBS solution containing 0.2% BSA and 0.6% Triton X-100 at 4°C overnight. The staining was visualized under a microscope (Imager Z1, Zeiss, Germany) using the Axiovision software (version 4.6). The images were collected using a black and white camera (Axiocam MRm, Zeiss, Germany) and pseudocolored purple (4-HNE).

2.16 Protein determination

The protein content was colorimetrically measured using BSA (1 mg/mL) as standard.

2.17 Statistical analyses

Data were expressed as the means ± SEM or median ± interquartile range. Data analyses were conducted using one- or two-way analyses of variance (ANOVA) or non-parametric tests, such as the Kruskal-Wallis test, depending on the experimental design. Post-hoc analyses were performed using Tukey's test when appropriate; $p < 0.05$ was considered significant.

3. Results

3.1 Creatine supplementation prevents TBI-induced cognitive dysfunction

The animals were tested for spatial learning in the Barnes maze test for five days before being subjected to the experimental conditions. All groups showed lower latency time to find the escape hole from the first to last day of training and similar latency time on the last day, indicating that all rats had the same cognitive condition before starting the experiments (data not shown). A Barnes maze test was performed two weeks after injury to evaluate memory retention in the vehicle and creatine-treated animals. Statistical analyses revealed that creatine supplementation had no effect per se on spatial memory but protected against increased latency for escape [$F(1,24) = 16.75$ $p < 0.005$; Fig. 2A] and the mean number of errors [$F(1,24) = 9.05$ $p < 0.005$; Fig. 2B] in animals subjected to TBI. In order to verify the effects of creatine supplementation on FPI-induced EEG pattern alterations, we obtained video-EEG recordings of the ipsilateral cortex of a subgroup of animals two weeks after FPI. Our results revealed that creatine did not alter EEG patterns per se but protected against increased wave amplitude [$F(1,24) = 5.24$; $p < 0.05$; Fig. 3A] and the number of spontaneous epileptiform events [$F(1,24) = 11.55$; $p < 0.05$; Fig. 3B] in animals subjected to TBI. The power spectra analysis revealed that creatine supplementation protected against FPI-induced theta power [$F(1,24) = 9.31$; $p < 0.05$; Fig. 3C] and delta power decreases [$F(1,24) = 8.50$; $p < 0.05$; Fig. 3D]. A representative picture shows a portion of the EEG recording (2 min in the image and a

magnification of 20 s in the box) obtained in the ipsilateral hippocampus of representative CMC/FPI (Figs. 3E and 3G) and the FPI/creatine group (Figs. 3F and 3H).

3.2 Creatine mitochondrial assays (CKm)

The statistical analyses showed that creatine supplementation was effective against FPI-induced mitochondria dysfunction, herein characterized by MTT [$F(1,24) = 4.29$; $p < 0.05$; Fig. 4A] and $\Delta\psi$ [$F(1,21) = 6.68$ $p < 0.05$; Fig. 4B] reduction. Statistical analyses (two-way ANOVA) also revealed that the activities of SDH [$F(1,21) = 6.41$; $p < 0.05$; Fig. 4C], complex I to II [$F(1,21) = 6.05$; $p < 0.05$; Fig. 4D], and COX [$F(1,24) = 23.59$; $p < 0.05$; Fig. 4E] were affected by FPI and that creatine supplementation partially reversed the decrease elicited by this TBI model. Moreover, creatine also increased CKm activity [$F(1,21) = 7.36$; $p < 0.05$; Fig. 5A] per se and protected against FPI-induced citrate synthase [$F(1,22) = 25.40$; $p < 0.05$; Fig. 5B], CKm activity inhibition [$F(1,22) = 6.53$; $p < 0.01$; Fig. 5A], and CKm immunoreactivity decrease [$F(1,21) = 10.01$; $p < 0.006$; Fig. 5C).

3.3 The impact of TBI and creatine treatment on mitochondrial oxidative stress and levels of proteins related to cell energy metabolism

Our findings showed that creatine supplementation prevented DCFH-DA oxidation [$F(1.22) = 13.81$; $p < 0.001$; Fig. 6A], free -SH groups [$F(1,21) = 19.25$; $p < 0.03$; Fig. 6B], GSH levels [$F(1.16) = 25.41$; $p < 0.0001$; Fig. 6C] decrease and protected against 4-HNE [F $F(1.20) = 18.17$; $p < 0.001$; Fig. 7G] immunoreactivity increase induced by FPI. The creatine supplementation protocol also protected against FPI-induced SIRT1 [$F(1,24) = 18.90$; $p < 0.05$; Fig. 7A], PGC1 [$F(1,21) = 21.09$; $p < 0.05$; Fig. 7B], and mit TFAM [$F1.22) = 13.30$; $p < 0.05$; Fig. 7C] immunoreactivity decrease indicating that oxidative stress observed in ipsilateral hippocampus alters the molecular systems engaged in cell energy homeostasis and that creatine treatment protects against these deleterious effects.

3.4 The impact of TBI and creatine treatment on the levels of proteins related to brain plasticity

The statistical analyses showed that creatine supplementation protected against FPI-induced BDNF [$F(1,16) = 5.96$; $p < 0.05$; Fig. 7D], TrkB [$F(1,16) = 16.92$; $p < 0.05$ Fig. 7E], and pCREB/CREB [$F(1,21) = 3.59$; $p < 0.05$; Fig. 7F] ratio decreases. Our experimental data also showed that FPI decreased Na+,K+-ATPase activity [$F(1,22) = 6.44$; $p < 0.05$; Fig. 7H] and that creatine supplementation protected against this deleterious effect.

3.5 The effect of TBI and creatine treatment on the parameters of GABAergic function and damage markers in post-TBI hippocampi

We performed histologic analyses in the hippocampus because secondary injury processes lead to necrotic, apoptotic, and autophagic neuronal cell death and synaptic loss. The statistical analyses demonstrated that neither FPI [$F(1,18) = 0.01$; $p = 0.91$) nor creatine supplementation [($F(1,18) = 0.79$; $p = 0.38$)] altered the total number of PARV + neurons in the CA2/CA1 (Fig. 8); H&E staining did not show any CA1 hippocampal tissue damage by FPI. The experimental data indicate that the stress response in this

TBI model did not cause early hippocampal damage. Nevertheless, it is worth noting that these damages are not perceived in the final stage of the basic trysinaptic circuit of the hippocampus. Despite this, the collapse of ion gradients explains the significant role that the hippocampus may assume in secondary damage after neuronal injury.

4. Discussion

For the first time, an episode of FPI in the parietal cortex of young rats has been shown to alter the molecular systems involved in cellular energy homeostasis, followed by significant oxidative stress in the hippocampus. The progressive alteration of mitochondrial function elicited by cascades related to oxidative stress jeopardizes neural function and induces deficits in the spatial memory of rats, as characterized herein by increased latency time in finding the escape hole in the Barnes maze test. Despite not altering PARV + neurons in the CA2/CA1 areas, the FPI-induced ion gradient collapse suggests that alterations in the redox state of regulatory -SH groups in selected targets, such as Na+, K+-ATPase, contribute to neurological dysfunction characterized by spatial memory impairment and development of epileptiform activity [48].

Additionally, this TBI protocol promoted plasma membrane lipid peroxidation (based on elevated protein and phenotypic expression of 4 HNE), suggesting that the invasive effects of TBI involve a myriad of complex and interconnected reasons that make the developing brain susceptible to toxicity [10, 49]. In line with this view, the observed disruption of hippocampal theta/delta activity after FPI is an interesting explanation for spatial memory impairment since the hippocampal activity is an essential modulator of long-term potentiation [50]. Our experimental data also revealed that the FPI protocol did not cause cell loss (especially PARV + neurons) in the CA2/CA1 areas but increased the susceptibility to the appearance of spontaneous epileptiform activity two weeks after the insult. These data reinforce the idea that the development of epileptiform activity after neuronal injury may, at least in adolescence, result from alterations in surviving neurons and dysfunction of the initial stages.

Considering that adolescence is a period characterized by continuous neuronal development [51], the altered molecular systems engaged in cell energy homeostasis (SIRT1, PGC-1α, and TFAM) followed by dysfunction in neurotrophin metabolism (BDNF, TrkB, and pCREB/CREB ratio decrease) are interconnected events with late secondary manifestations of brain injury, including early epileptiform activity and cognitive impairment. The magnitude of this problem is more significant considering that the altered pattern of development expected in the adolescent's hippocampal organization increases the risk of neurological disorders [52–54]. Understanding post-injury metabolic dysregulation in TBI is crucial to establishing scientific-based rehabilitative strategies. One such strategy may include ergogenic compounds such as creatine, a guanidino compound that alters brain energy, promotes neurogenesis, and improves brain function [55]. Despite the considerable overlap between the role of creatine and the neuropathology of TBI [18, 22], the molecular mechanisms underlying this guanidine compound in the developing brain after TBI remain largely unknown.

Our experimental data revealed that the ability to maintain mitochondrial bioenergetics elicited by creatine supplementation protected against FPI-induced toxicity characterized herein by impaired mitochondrial function, energetic (Na+, K+-ATPase), and plasticity (BDNF, TrkB, and pCREB/CREB) brain-related proteins as well as ROS generation and neurobehavioral impairment (early epileptiform activity and spatial learning). In addition to preclinical data, the ability of creatine to improve brain function in this TBI model corroborate prospective randomized studies with children and adolescents victims of TBI, in which creatine (0.4 g/kg orally) for six months significantly improved clinical recovery in the categories of cognitive, personality/behavior, self-care, and communication [20, 21].

We demonstrated that creatine supplementation was effective against mitochondria dysfunction, herein characterized by MTT and Δψ reduction as well as inhibition of respiratory chain enzyme activities (SDH, complex II, and COX) after neuronal injury. The creatine supplementation protocol increased CKm activity and was effective against Citrate synthase, CKm activity inhibition, and CKm immunoreactivity decrease after neuronal injury. These experimental data suggest that stabilizing enzymes such as CKm, which play a central role in regulating the metabolic feedback of mitochondrial respiration, attenuate TBI-induced toxicity. This idea is based on the ability of this guanidine compound to stabilize the contact sites of the inner and outer mitochondrial membranes and prevent the opening of mitochondrial permeability pores and, consequently, the excess of oxygen free radicals [55–57].

Regarding the involvement of ROS in TBI toxicity, our experimental data showed that animals subjected to FPI had increased oxidation of DCFH-DA (intracellular hydrogen peroxide marker and oxidative stress) and decreased free -SH groups and GSH levels. The present redox state alteration was followed by a significant increase in immunoreactivity of 4-HNE (lipoperoxidation marker) and Na+, K+-ATPase inhibition. In developing brains, the invasive effects induced by severe neurological injury involve a myriad of complex and interconnected reasons that make the brain susceptible to toxicity. Although any cellular constituent can target this toxicity, failure of the selected target, such as Na+, K+-ATPase [28], reinforces the idea that ROS-induced modification of biologically important macromolecules in the hippocampus results in hyperexcitability and neurological damage after TBI. Our experimental data also suggest that the development of compensatory responses to oxidative stress elicited by creatine supplementation reflects on decreased markers of ROS generation, which translates into the maintenance of mitochondrial homeostasis and consequent Na+,K+-ATPase inhibition. This hypothesis is based on experimental and clinical studies that associate reductions in N-acetyl aspartate (NAA)/creatine, choline/creatine, and/or NAA/Cho ratios not only with neuronal cell loss but with energy impairment due to post-traumatic trauma mitochondrial dysfunction [18].

In addition to increasing energy buffering, a non-energetic role has also been proposed for creatine. For instance: researchers have demonstrated that creatine, unlike PCr, has an inherent ability to scavenge free radicals and reduce the increase of 8-hydroxydeoxyguanosine (a biomarker of oxidative DNA damage that accumulates as part of the natural aging process) when creatine is present at high concentrations in the cell [58, 59]. As described previously, the stabilization of mitochondrial membranes via the interaction of Cr/PCr with phospholipid membranes increases the expression of antioxidant enzymes and attenuates

ROS production [60, 61], suggesting that the protective effect of creatine is not only due to improved cellular energy but also more likely related to antioxidant effects. Nonetheless, it is important to consider that events after brain injury are not linear and further research is needed to clarify the involvement of free radical pathways in creatine-induced protection.

One common pathway engaged in synaptic plasticity and susceptible to TBI-induced toxicity is via BDNF expression. Experimental and clinical studies have suggested that significant changes in BDNF levels can accelerate the normal aging process after TBI [62]. In this scenario, our experimental data showed that severe FPI episodes in juvenile rats induced BDNF, TrkB, and pCREB/CREB ratio decreases, and creatine supplementation protects against these deleterious effects. This finding is particularly important, considering that the brain is very metabolically demanding and the BDNF receptor TrkB stimulates mitochondrial bioenergetics by recruiting metabolic activation pathways [63].

Given that memory depends on intact mitochondrial respiratory function, it is plausible that effects from creatine supplementation on memory performance may be related to its ability to influence brain bioenergetics in this neuronal injury model. This assumption is based on animal models that showed the involvement of CREB in spatial memory formation (Barnes maze test) of rats submitted to intrahippocampal injections of creatine in the CA1 subfield [64]. More recently, Snow and colleagues (2018) [65] also revealed that four weeks of creatine supplementation in mice increased coupled respiration in isolated hippocampal mitochondria and improved memory. Considering that dysfunction in this neurotrophin metabolism and mitochondrial function are interconnected events, it is plausible to propose that dietary creatine's ability to maintain mitochondrial integrity protects against BDNF depletion in TBI patients and animal models, events that accelerate the normal process of aging [62]. This effective response reinforces the possibility of creatine monohydrate providing a new natural strategy for treating TBI-induced neurological disorders.

In support of this perspective, our experimental data revealed that two weeks of creatine supplementation (300 mg/kg, po) protected against SEEs. These experimental data corroborate a recent study by our research group that demonstrated that four weeks of creatine supplementation (300 mg/kg, po) after TBI increased the latency to first myoclonic and tonic-clonic seizures, decreased the time spent in tonic-clonic seizure, seizure intensity, and spindle oscillations induced by a sub-convulsant dose of PTZ (35 mg/kg, ip) [17]. In this study, the anticonvulsant effect of creatine was associated with its ability to reduce cell loss, including the number of PARV + cells in the CA3 region of the hippocampus.

Gioardano et al. (2022) [66] demonstrated that axonal neurodegeneration after TBI follows the basic trisynaptic circuit for CA3, CA2, and CA1 afferents. Therefore, it is expected that cellular alterations are initially triggered in CA3 and subsequently in CA2 and CA1, as suggested by Grady et al. (2003) [67], who demonstrated significant neuron loss in the hilus and CA3 regions but not in CA2/CA1 areas. Our experimental data corroborate these findings, indicating that neither FPI nor creatine supplementation altered PARV + neurons in the CA2/CA1 areas. Equally, H&E staining did not show hippocampal tissue CA1 damage induced by FPI, suggesting that epileptiform activity may, at least partly, result from

alterations in surviving neurons and dysfunction of the initial stages after neuronal injury in juvenile rats [48].

Considering that the expected altered pattern of brain development in the adolescent's hippocampal organization increases the risk of neurological disorders in adulthood [53], we suggest that ability of creatine supplementation to suppress long-term epileptiform activity after TBI is due to the bioenergetic maintenance in surviving neurons that reflects on decreased markers of ROS generation and protection against ROS-induced Na+,K+-ATPase inhibition in juvenile rats. This hypothesis has been upheld in animal studies indicating a mechanistic basis for the neuroprotective effects of creatine involving alterations of the insult-induced depletion of cellular ATP, which limits secondary damage and promotes neural repair and behavioral rehabilitation after TBI [18, 55, 68–70].

5. Conclusions

Traumatic brain injury is a devastating disease frequently followed by significant behavioral disabilities characterized by a combination of immediate mechanical dysfunction in brain tissue and secondary damage developed over a longer period of time [71]. Especially in a developing brain, a complex array of responses to injury, especially in a developing brain, may result in an energy crisis that compromises the brain's ability to cope with challenges. In contrast, the development of compensatory responses to oxidative stress elicited by creatine supplementation, which translates into the maintenance of mitochondrial homeostasis and consequent Na+,K+-ATPase inhibition, is critical in maintaining brain function.

Our experimental data also suggest that the ability of creatine to influence molecular systems involved in cellular energy homeostasis (PGC-1α and TFAM) may attenuate the damage caused by neurotrophin metabolism (decreased BDNF, TrkB, and pCREB/CREB ratio), events interconnected with the manifestations secondary to brain injury characterized herein by early epileptiform activity and cognitive impairment. Hence, approaches to counteract or attenuate secondary damage developed for hours to days after a TBI (e.g., creatine) may have preventive and therapeutic potential against TBI-induced toxicity.

Declarations

Authors' contributions

Luiz Fernando Freire Royes and Michele Rechia Fighera: Study concept, study design, molecular data acquisition, data analysis, data interpretation, drafting, revising and final approval of the article Guilherme Lago Busanelloa; Douglas Godinho; Alexandre Seixas Nascimento, Gabriel Correa Lima: Responsible for Creatine treatment and FPI procedure. Jane do Socorro dos Navegantes Marçal Cunha, Marcia Consentino Kronka Sosthenes: data analysis of histologic analysis Gokul Krishna; Ana Flavia Furian,

Mauro Schneider Oliveira: data analysis of Western and mitochondrial functions Fernando Gomez-Pinilla: Data interpretation, drafting, revising and approval of the article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval

Experimental procedures were conducted according to national and international legislation (Brazilian College of Animal Experimentation and US Public Health Service Policy on Humane Care and Use of Laboratory Animals guidelines) and approved by the Ethics Committee on Animal Research of the Federal University of Santa Maria (protocol no. 011/2015). The experiments were carried out according to the principles described by Grundy (2015) [24].

Consent to participate

Not applicated

Consent for publication

Not applicated

Availability of data and materials

The authors declare to make information about the manuscript available upon request, including supplementary information or figure source data files.

Funding

This study was supported by the Ministry of Sports and High-Performance Sports Department (grant: 880509/2018) and CNPq (grants: 311953/2021-2). M.R. Fighera and L.F.F. Royes are recipients of CNPq fellowships.

Acknowledgements

Not applicated

References

1. Dewan MC et al (2018) *Estimating the global incidence of traumatic brain injury*. J Neurosurg, : p. 1–18
2. Giza CC, Prins ML (2006) Is being plastic fantastic? Mechanisms of altered plasticity after developmental traumatic brain injury. Dev Neurosci 28(4–5):364–379

3. Adelson PD et al (2013) Morris water maze function and histologic characterization of two age-at-injury experimental models of controlled cortical impact in the immature rat. Childs Nerv Syst 29(1):43–53
4. Babikian T et al (2015) Chronic Aspects of Pediatric Traumatic Brain Injury: Review of the Literature. J Neurotrauma 32(23):1849–1860
5. Timonen M et al (2002) The association of preceding traumatic brain injury with mental disorders, alcoholism and criminality: the Northern Finland 1966 Birth Cohort Study. Psychiatry Res 113(3):217–226
6. Gerber P, Coffman K (2007) Nonaccidental head trauma in infants. Childs Nerv Syst 23(5):499–507
7. Kramer ME et al (2008) Long-term neural processing of attention following early childhood traumatic brain injury: fMRI and neurobehavioral outcomes. J Int Neuropsychol Soc 14(3):424–435
8. Rowe RK et al (2018) Midline (central) fluid percussion model of traumatic brain injury in pediatric and adolescent rats. J Neurosurg Pediatr 22(1):22–30
9. Hood KN et al (2018) Endoplasmic Reticulum Stress Contributes to the Loss of Newborn Hippocampal Neurons after Traumatic Brain Injury. J Neurosci 38(9):2372–2384
10. Hylin MJ et al (2018) Juvenile Traumatic Brain Injury Results in Cognitive Deficits Associated with Impaired Endoplasmic Reticulum Stress and Early Tauopathy. Dev Neurosci 40(2):175–188
11. Green TRF et al (2021) Age-At-Injury Influences the Glial Response to Traumatic Brain Injury in the Cortex of Male Juvenile Rats. Front Neurol 12:804139
12. Tóth K, Freund TF, Miles R (1997) Disinhibition of rat hippocampal pyramidal cells by GABAergic afferents from the septum. J Physiol 500(Pt 2):463–474
13. Mathias JL et al (2004) Neuropsychological and information processing performance and its relationship to white matter changes following moderate and severe traumatic brain injury: a preliminary study. Appl Neuropsychol 11(3):134–152
14. Witgen BM et al (2005) Regional hippocampal alteration associated with cognitive deficit following experimental brain injury: a systems, network and cellular evaluation. Neuroscience 133(1):1–15
15. Zhang B et al (2011) Impairment of synaptic plasticity in hippocampus is exacerbated by methylprednisolone in a rat model of traumatic brain injury. Brain Res 1382:165–172
16. Hsieh TH et al (2017) Trajectory of Parvalbumin Cell Impairment and Loss of Cortical Inhibition in Traumatic Brain Injury. Cereb Cortex 27(12):5509–5524
17. Gerbatin RR et al (2019) Delayed creatine supplementation counteracts reduction of GABAergic function and protects against seizures susceptibility after traumatic brain injury in rats. Prog Neuropsychopharmacol Biol Psychiatry 92:328–338
18. Freire Royes LF, Cassol G (2016) The Effects of Creatine Supplementation and Physical Exercise on Traumatic Brain Injury. Mini Rev Med Chem 16(1):29–39
19. Dolan E, Gualano B, Rawson ES (2019) Beyond muscle: the effects of creatine supplementation on brain creatine, cognitive processing, and traumatic brain injury. Eur J Sport Sci 19(1):1–14

20. Sakellaris G et al (2006) Prevention of complications related to traumatic brain injury in children and adolescents with creatine administration: an open label randomized pilot study. J Trauma 61(2):322–329
21. Sakellaris G et al (2008) Prevention of traumatic headache, dizziness and fatigue with creatine administration. A pilot study. Acta Paediatr 97(1):31–34
22. Ainsley Dean PJ et al (2017) *Potential for use of creatine supplementation following mild traumatic brain injury*. Concussion, 2(2): p. Cnc34.
23. Doust YV et al (2021) Age-at-Injury Determines the Extent of Long-Term Neuropathology and Microgliosis After a Diffuse Brain Injury in Male Rats. Front Neurol 12:722526
24. Grundy D (2015) Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology. J Physiol 593(12):2547–2549
25. Magni DV et al (2007) Creatine decreases convulsions and neurochemical alterations induced by glutaric acid in rats. Brain Res 1185:336–345
26. Rambo LM et al (2009) Additive anticonvulsant effects of creatine supplementation and physical exercise against pentylenetetrazol-induced seizures. Neurochem Int 55(5):333–340
27. D'Ambrosio R et al (2004) Post-traumatic epilepsy following fluid percussion injury in the rat. Brain 127(Pt 2):304–314
28. de Castro MRT et al (2017) Previous physical exercise alters the hepatic profile of oxidative-inflammatory status and limits the secondary brain damage induced by severe traumatic brain injury in rats. J Physiol 595(17):6023–6044
29. Funck VR et al (2015) Contrasting effects of Na+, K+-ATPase activation on seizure activity in acute versus chronic models. Neuroscience 298:171–179
30. Szu JI et al (2020) Modulation of posttraumatic epileptogenesis in aquaporin-4 knockout mice. Epilepsia 61(7):1503–1514
31. Krishna G et al (2017) 7,8-Dihydroxyflavone facilitates the action exercise to restore plasticity and functionality: Implications for early brain trauma recovery. Biochim Biophys Acta Mol Basis Dis 1863(6):1204–1213
32. Silva LF et al (2013) Treadmill exercise protects against pentylenetetrazol-induced seizures and oxidative stress after traumatic brain injury. J Neurotrauma 30(14):1278–1287
33. King EJ (1932) The colorimetric determination of phosphorus. Biochem J 26(2):292–297
34. Funck VR et al (2014) Long-term decrease in Na+,K+-ATPase activity after pilocarpine-induced status epilepticus is associated with nitration of its alpha subunit. Epilepsy Res 108(10):1705–1710
35. Tonkonogi M, Sahlin K (1997) Rate of oxidative phosphorylation in isolated mitochondria from human skeletal muscle: effect of training status. Acta Physiol Scand 161(3):345–353
36. Myhre O et al (2003) Evaluation of the probes 2',7'-dichlorofluorescin diacetate, luminol, and lucigenin as indicators of reactive species formation. Biochem Pharmacol 65(10):1575–1582

37. Ellman GL et al (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95
38. Fischer JC et al (1985) Differential investigation of the capacity of succinate oxidation in human skeletal muscle. Clin Chim Acta 153(1):23–36
39. Rustin P et al (1994) Biochemical and molecular investigations in respiratory chain deficiencies. Clin Chim Acta 228(1):35–51
40. Berridge MV, Tan AS (1993) Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Arch Biochem Biophys 303(2):474–482
41. Misra HP, Fridovich I (1972) The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 247(10):3170–3175
42. Hughes BP (1962) A method for the estimation of serum creatine kinase and its use in comparing creatine kinase and aldolase activity in normal and pathological sera. Clin Chim Acta 7:597–603
43. Marcuzzo S et al (2010) Different effects of anoxia and hind-limb immobilization on sensorimotor development and cell numbers in the somatosensory cortex in rats. Brain Dev 32(4):323–331
44. Molinaro P et al (2011) Na+ -Ca2 + exchanger (NCX3) knock-out mice display an impairment in hippocampal long-term potentiation and spatial learning and memory. J Neurosci 31(20):7312–7321
45. Shu SY, Ju G, Fan LZ (1988) The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. Neurosci Lett 85(2):169–171
46. Huusko N, Pitkänen A (2014) Parvalbumin immunoreactivity and expression of GABAA receptor subunits in the thalamus after experimental TBI. Neuroscience 267:30–45
47. Huusko N et al (2015) Loss of hippocampal interneurons and epileptogenesis: a comparison of two animal models of acquired epilepsy. Brain Struct Funct 220(1):153–191
48. da Silva Fiorin F et al (2016) The Impact of Previous Physical Training on Redox Signaling after Traumatic Brain Injury in Rats: A Behavioral and Neurochemical Approach. J Neurotrauma 33(14):1317–1330
49. Kamper JE et al (2013) Juvenile traumatic brain injury evolves into a chronic brain disorder: behavioral and histological changes over 6months. Exp Neurol 250:8–19
50. Hyman JM et al (2003) Stimulation in hippocampal region CA1 in behaving rats yields long-term potentiation when delivered to the peak of theta and long-term depression when delivered to the trough. J Neurosci 23(37):11725–11731
51. Eyolfson E et al (2020) Microglia dynamics in adolescent traumatic brain injury. J Neuroinflammation 17(1):326
52. Asdikian R et al (2021) Hippocampal injury and learning deficits following non-convulsive status epilepticus in periadolescent rats. Epilepsy Behav 125:108415

53. van Ierssel J et al (2021) Symptom Burden, School Function, and Physical Activity One Year Following Pediatric Concussion. J Pediatr 228:190–198e3
54. Arski ON et al (2022) Epilepsy disrupts hippocampal phase precision and impairs working memory. Epilepsia 63(10):2583–2596
55. Cassol G et al (2019) Potential therapeutic implications of ergogenic compounds on pathophysiology induced by traumatic brain injury: A narrative review. Life Sci 233:116684
56. Adhihetty PJ, Beal MF (2008) Creatine and its potential therapeutic value for targeting cellular energy impairment in neurodegenerative diseases. Neuromolecular Med 10(4):275–290
57. Marshall RP et al (2022) *Role of Creatine Supplementation in Conditions Involving Mitochondrial Dysfunction: A Narrative Review*. Nutrients, 14(3)
58. Gerber I et al (2005) Stimulatory effects of creatine on metabolic activity, differentiation and mineralization of primary osteoblast-like cells in monolayer and micromass cell cultures. Eur Cell Mater 10:8–22
59. Arazi H, Eghbali E, Suzuki K (2021) *Creatine Supplementation, Physical Exercise and Oxidative Stress Markers: A Review of the Mechanisms and Effectiveness*. Nutrients, 13(3)
60. Guimarães-Ferreira L et al (2012) Short-term creatine supplementation decreases reactive oxygen species content with no changes in expression and activity of antioxidant enzymes in skeletal muscle. Eur J Appl Physiol 112(11):3905–3911
61. Tokarska-Schlattner M et al (2012) Phosphocreatine interacts with phospholipids, affects membrane properties and exerts membrane-protective effects. PLoS ONE 7(8):e43178
62. Wurzelmann M, Romeika J, Sun D (2017) Therapeutic potential of brain-derived neurotrophic factor (BDNF) and a small molecular mimics of BDNF for traumatic brain injury. Neural Regen Res 12(1):7–12
63. Agrawal R et al (2014) Coupling energy homeostasis with a mechanism to support plasticity in brain trauma. Biochim Biophys Acta 1842(4):535–546
64. Souza MA et al (2012) Involvement of hippocampal CAMKII/CREB signaling in the spatial memory retention induced by creatine. Amino Acids 43(6):2491–2503
65. Snow WM et al (2018) Chronic dietary creatine enhances hippocampal-dependent spatial memory, bioenergetics, and levels of plasticity-related proteins associated with NF-κB. Learn Mem 25(2):54–66
66. Giordano KR et al (2022) Time Course of Remote Neuropathology Following Diffuse Traumatic Brain Injury in the Male Rat. Exp Neurobiol 31(2):105–115
67. Grady CL, McIntosh AR, Craik FI (2003) Age-related differences in the functional connectivity of the hippocampus during memory encoding. Hippocampus 13(5):572–586
68. Saraiva AL et al (2012) Creatine reduces oxidative stress markers but does not protect against seizure susceptibility after severe traumatic brain injury. Brain Res Bull 87(2–3):180–186

69. Signoretti S et al (2010) Transient alterations of creatine, creatine phosphate, N-acetylaspartate and high-energy phosphates after mild traumatic brain injury in the rat. Mol Cell Biochem 333(1–2):269–277
70. Tarnopolsky MA, Beal MF (2001) Potential for creatine and other therapies targeting cellular energy dysfunction in neurological disorders. Ann Neurol 49(5):561–574
71. Royes LFF, Gomez-Pinilla F (2019) Making sense of gut feelings in the traumatic brain injury pathogenesis. Neurosci Biobehav Rev 102:345–361

Tables

Table 1: diet ingredients provided *ad libitum*

Crude protein (min)	220 g/kg	Biotin (min)	0,052 mg/kg
Ethereal extract (min)	40 g/kg	Choline (min)	1,950 mg/kg
Crude fiber (max.)	12 g/kg	Vitamin B1 (min)	5 mg/kg
Mineral matter (max)	80 g/kg	Vitamin B12 (min)	23 mcg/kg
Calcium (min)	10 g/kg	Vitamin B2 (min)	8 mg/kg
Calcium (max)	12 g/kg	Vitamin B6 (min)	7 mg/kg
Phosphorus (min)	8.000 mg/kg	Vitamin K3 (min)	3 mg/kg
Humidity (max)	120 g/kg	Cobalt (min)	1,5 mg/kg
Lysine (min)	12,3 g/kg	Copper (min)	10 mg/kg
Methionine (min)	4,1 g/kg	Iron (min)	51 mg/kg
Vitamin A (min)	13.500 UI/kg	Fluorine (max)	80 mg/kg
Vitamin D3 (min)	2.500 UI/kg	Iodine (min)	2 mg/kg
Vitamin E (min)	34 UI/kg	Manganese (min)	62 mg/kg
Vitamin C (min)	50 mg/kg	Selenium (min)	0,05 mg/kg
Folic acid (min)	1 mg/kg	Sodium (min)	2.700 mg/kg
Nicotinic acid (min)	63 mg/kg	Zinc (min)	83 mg/kg
Pantothenic acid (min)	25 mg/kg	BHA and BHT (min)	100 mg/kg

Figures

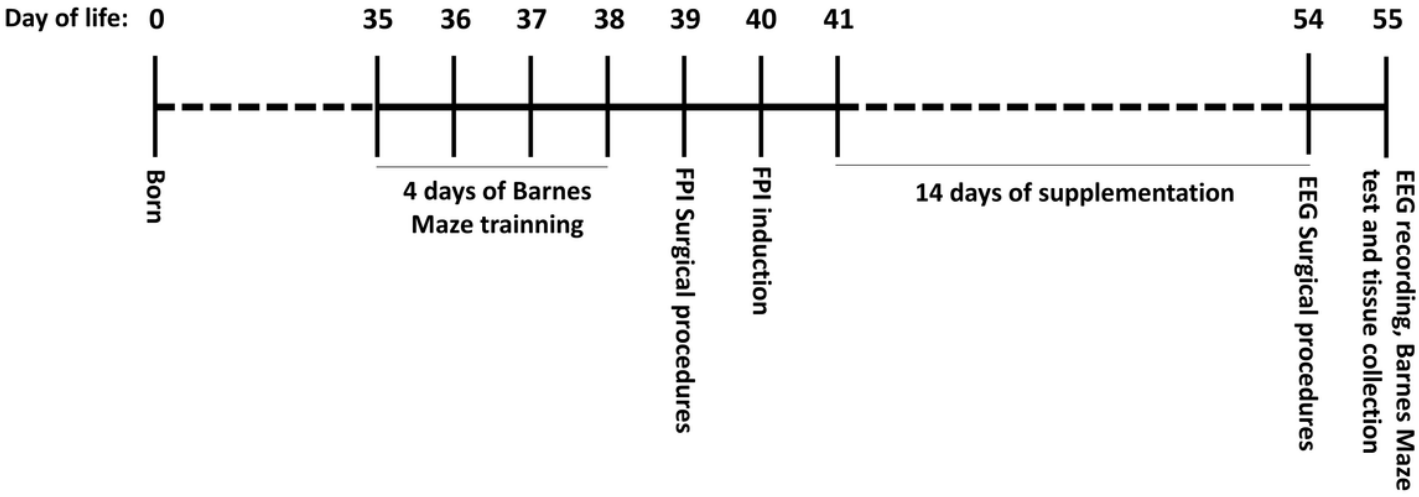

Figure 1

*Experimental Design.*Animals at 35 days of life started the 4 days Barns Maze training, then went through FPI surgical procedure and received creatine supplementation for 14 days after the TBI. At the end of creatine supplementation animals went through surgical procedure to EEG electrode implantation and 24h later EEG recording, behavior test and tissue collection.

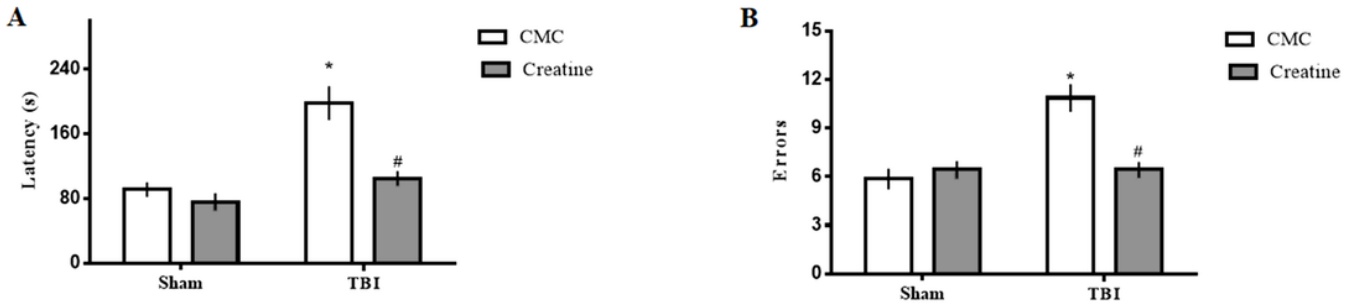

Figure 2

FPI-induced behavior alterations. Barns Maze results of latency to escape (A) and number of errors (B) of animals submitted to TBI and creatine supplementation. Values represent mean ± S.E.M. * = P<0.05 *vs.* Sham/CMC and # = P<0.05 *vs.* TBI/CMC.

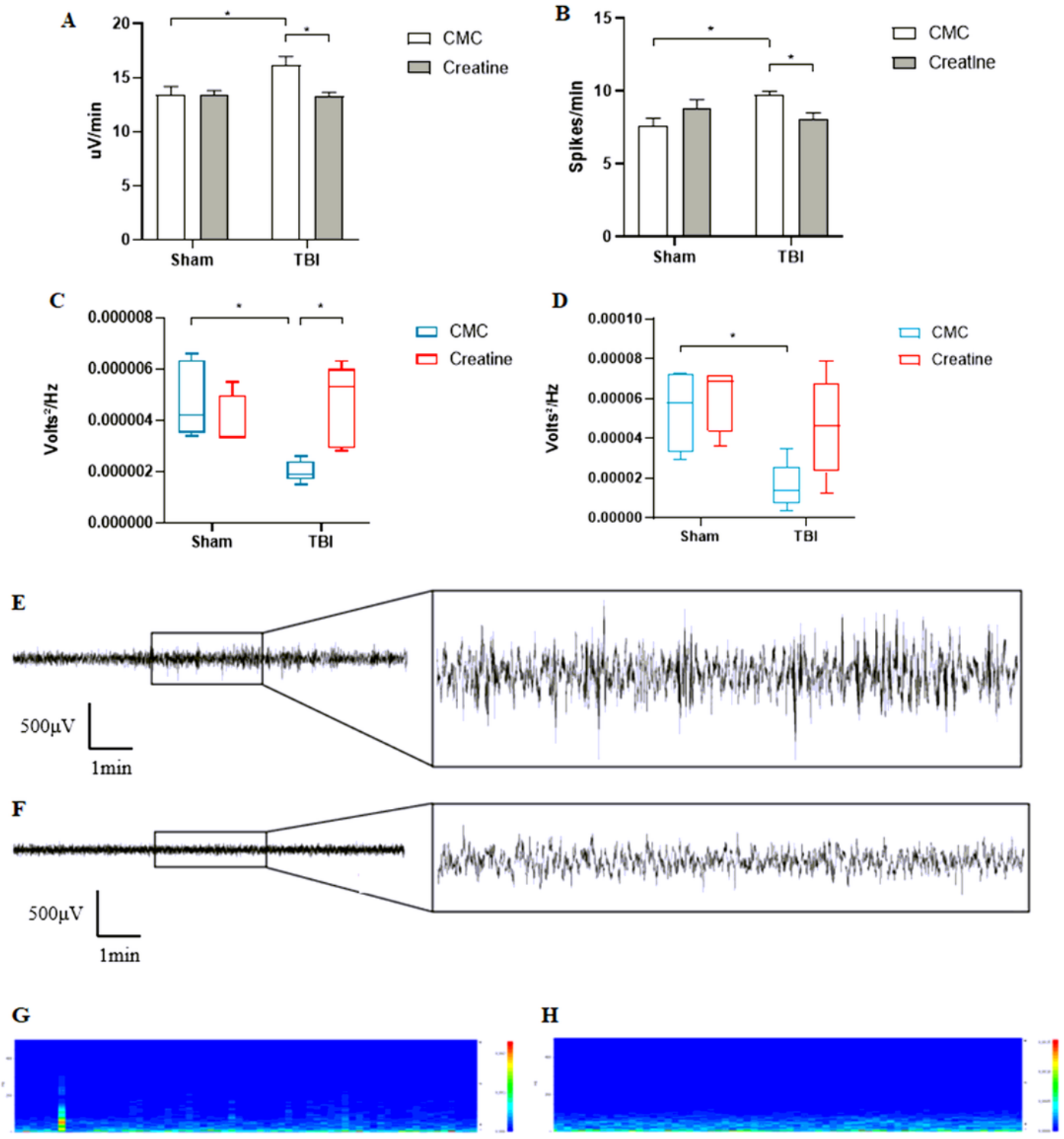

Figure 3

Modulation of epileptiform activity. Effects of creatine supplementation on epileptiform activity (A) and (B) and power spectra delta (C) and theta (D) frequency bands. Representative figure shows a portion of encephalographic recording (one minutes in the left image, and a magnification of 20 seconds in the box on the right) obtained in the cortex of representative animals of vehicle (E) and creatine (F) groups who have received traumatic brain injury and its respectively (G and F) heat representative figures of

amplitude measure. Values represent mean ± S.E.M. * = P<0.05 *vs.* Sham/CMC and # = P<0.05 *vs.* TBI/CMC.

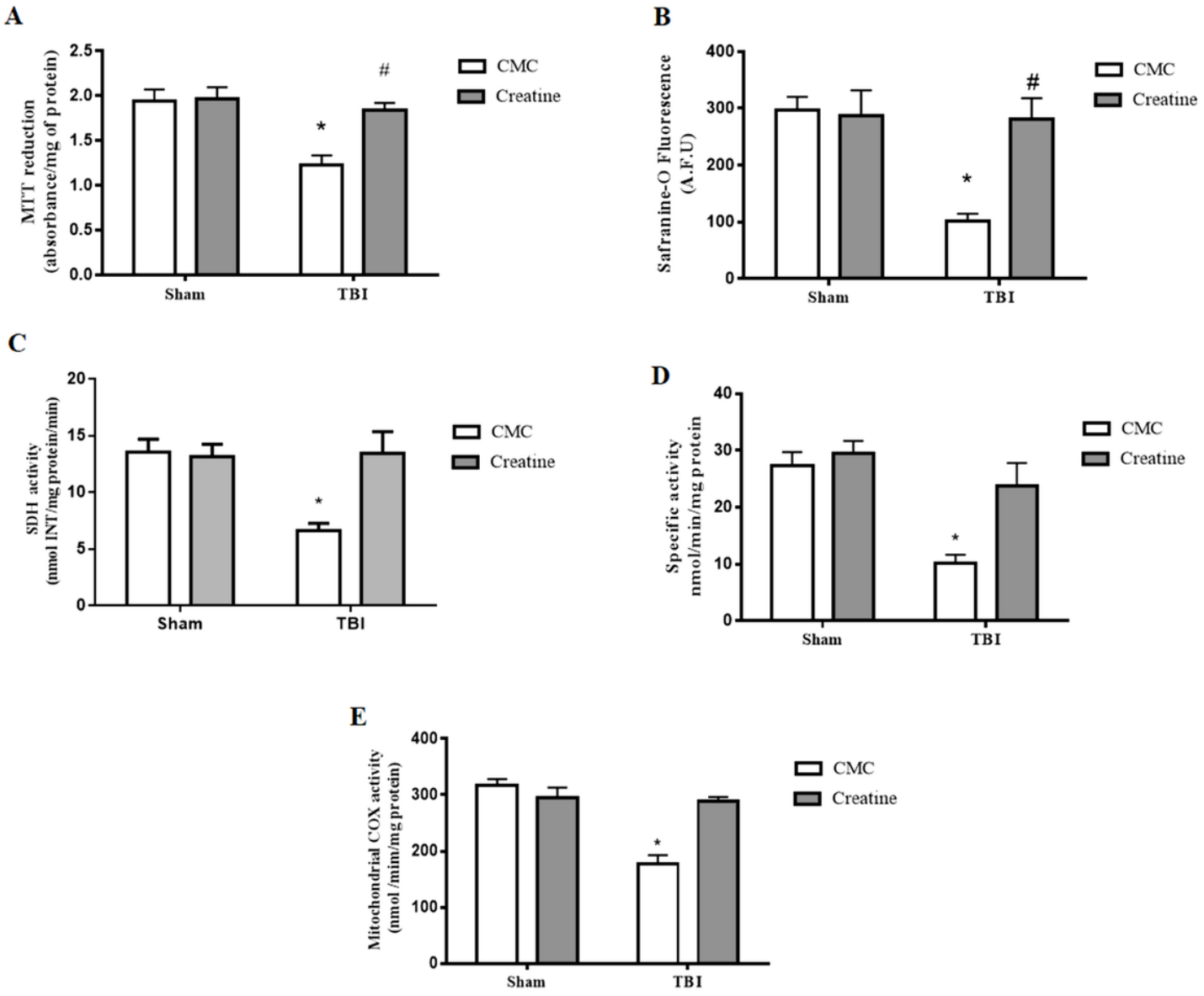

Figure 4

Post-traumatic mitochondrial dysfunction prevented by creatine supplementation. Effects of TBI and creatine supplementation on MTT reduction (A), membrane potential (B), activity of SDH (C), complex I to II (D) and COX (E). Values represent mean ± S.E.M. * = P<0.05 *vs.* Sham/CMC and # = P<0.05 *vs.*TBI/CMC

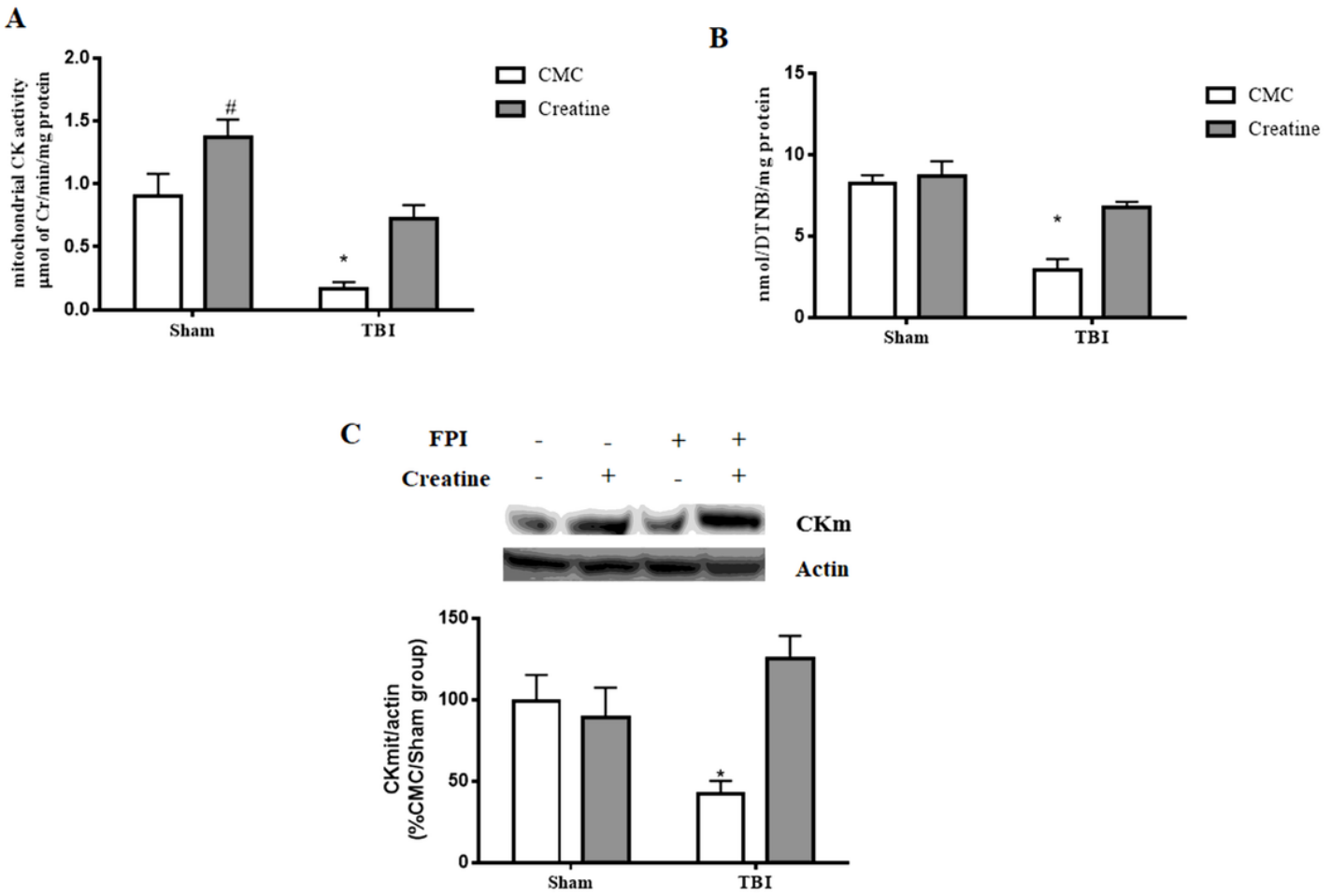

Figure 5

Effects of TBI and creatine supplementation on mitochondrial CK activity (A), citrate synthase activity (B) and immunocontent of mitochondrial CK (C). Values represent mean ± S.E.M. * = P<0.05 *vs.* Sham/CMC and # = P<0.05 *vs.* TBI/CMC.

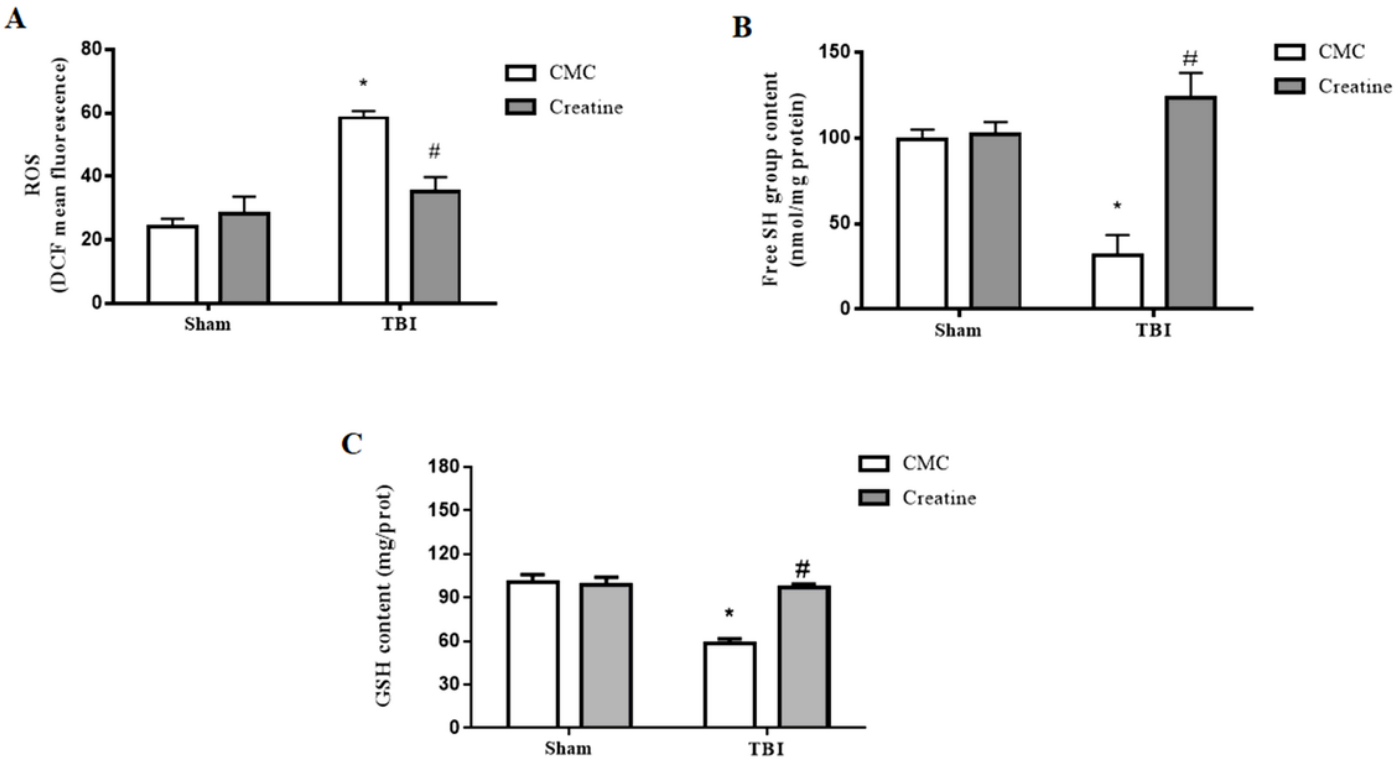

Figure 6

Effects of TBI and creatine supplementation on DCFH-DA oxidation (A), free -SH groups (B) and GSH levels (C). Values represent mean ± S.E.M. * = P<0.05 *vs.* Sham/CMC and # = P<0.05 *vs.* TBI/CMC.

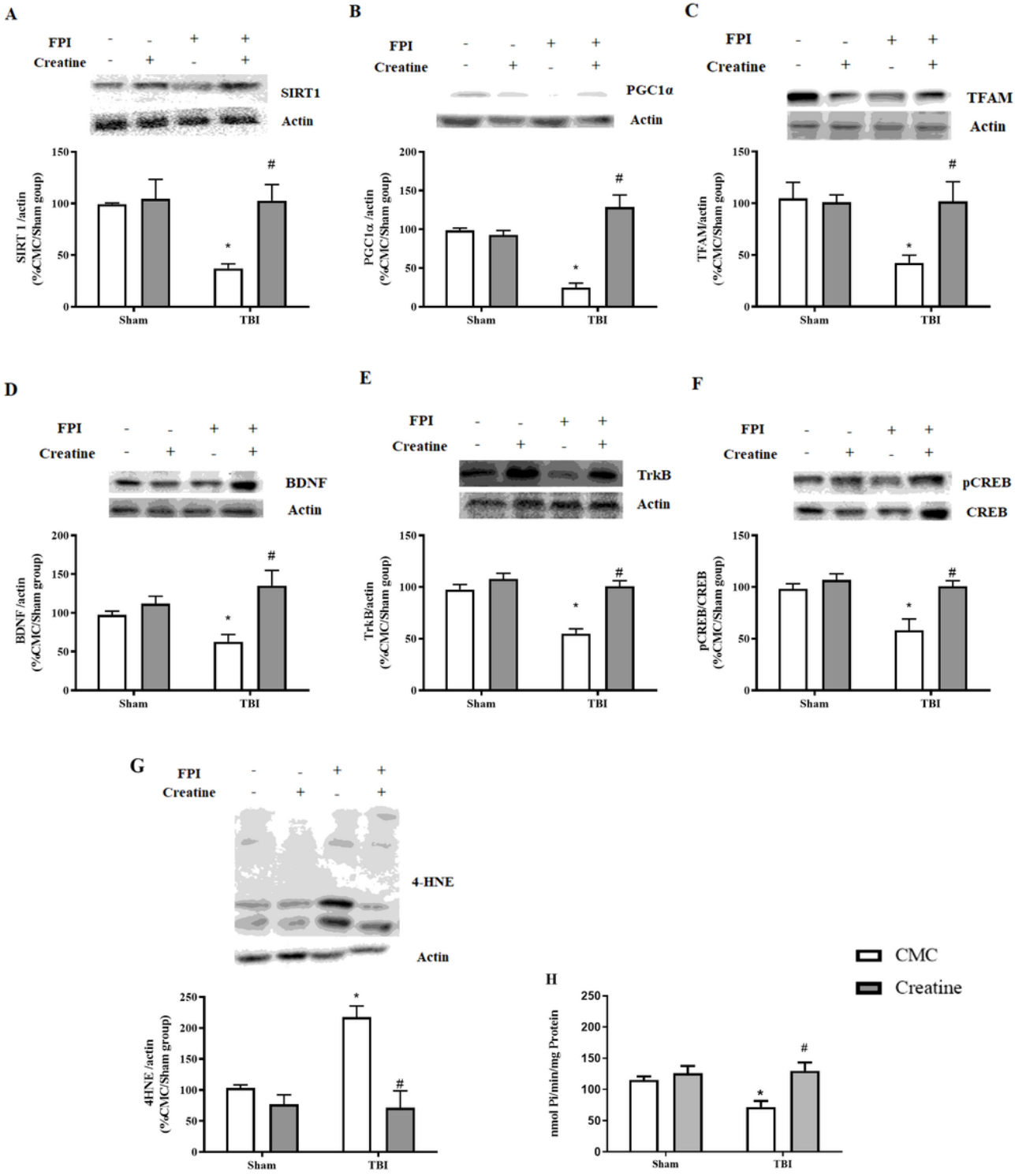

Figure 7

Effects of TBI and creatine supplementation on SIRT1 (A), PGC1α (B), TFAM (C), BDNF (D), TrkB (E), p-CREB1^{Ser133}/CREB ratio (F), 4-HNE (G) immunoreactivity and Na^+, K^+-ATPase activity (H). Values represent mean ± S.E.M. * = P<0.05 *vs.* Sham/CMC and # = P<0.05 *vs.* TBI/CMC.

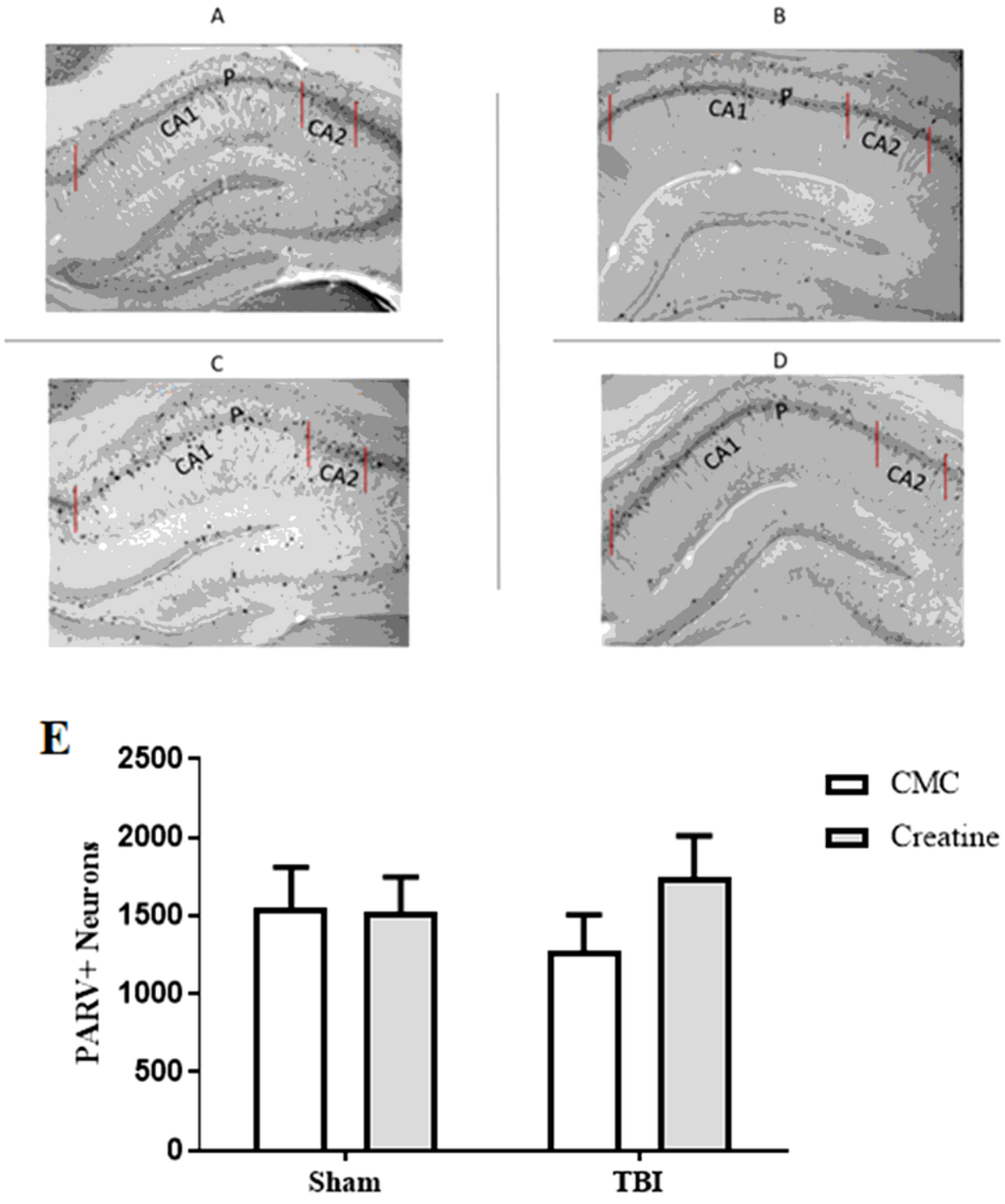

Figure 8

Demonstrative of hippocampal regions of Sham/CMC (A), Sham/Creatine (B), TBI/CMC (C) and TBI/Creatine (D) groups. TBI and creatine supplementation did not affect PARV+ neurons (E). Values represent mean ± S.E.M.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- GraphicalAbstract2.tif
- GraphicalAbstractLegend.docx
- SupplementaryData.docx