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Cross-talk between guanidinoacetate neurotoxicity, memory and possible neuroprotective role of creatine

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ABSTRACT

Guanidinoacetate Methyltransferase deficiency is an inborn error of metabolism that results in decreased creatine and increased guanidinoacetate (GAA) levels. Patients present neurological symptoms whose mechanisms are unclear. We investigated the effects of an intrastriatal administration of 10 μM of GAA (0.02 nmol/striatum) on energy metabolism, redox state, inflammation, glutamate homeostasis, and activities/immunocontents of acetylcholinesterase and Na^+,K^+-ATPase, as well as on memory acquisition. The neuroprotective role of creatine was also investigated. Male Wistar rats were pretreated with creatine (50 mg/kg) or saline for 7 days underwenting stereotactic surgery. Forty-eight hours after surgery, the animals (then sixty-days-old) were divided into groups: Control, GAA, GAA + Creatine, and Creatine. Experiments were performed 30 min after intrastriatal infusion. GAA decreased SDH, complexes II and IV activities, and ATP levels, but had no effect on mitochondrial mass/membrane potential. Creatine totally prevented SDH and complex II, and partially prevented COX and ATP alterations. GAA increased dichlorofluorescein levels and decreased superoxide dismutase and catalase activities. Creatine only prevented catalase and dichlorofluorescein alterations. GAA increased cytokines, nitrites levels and acetylcholinesterase activity, but not its immunocontent. Creatine prevented such effects, except nitrite levels. GAA decreased glutamate uptake, but had no effect on the immunocontent of its transporters. GAA decreased Na^+,K^+-ATPase activity and increased the immunocontent of its α3 subunit. The performance on the novel object recognition task was also impaired. Creatine partially prevented the changes in glutamate uptake and Na^+,K^+-ATPase activity, and completely prevented the memory impairment. This study helps to elucidate the protective effects of creatine against the damage caused by GAA.

1. Introduction

Guanidinoacetate (GAA) is highly involved in the metabolism of creatine, being its direct precursor [1]. *N*-guanidinoacetate methyltransferase (GAMT) deficiency is a rare metabolic disease of the metabolism of creatine that leads to a decrease in creatine levels associated with tissue accumulation of GAA [2,3]. In cerebral spinal fluid (CSF) and plasma, GAA concentrations are up to 200 times and 10 to 20 times the upper limit of the reference range, that are 0.020–0.090 μmol/l and 0.9–3.5 μmol/l, respectively [4,5]. Symptoms include intractable epilepsy, intellectual and memory impairment, autism, extra pyramidal syndrome, slurred speech and hypotonia [6–8].

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; ANOVA, analysis of variance; ATP, adenosine triphosphate; CAT, catalase; CK, creatine kinase; CNS, central nervous system; COX, cytochrome *c* oxidase; CSF, cerebral spinal fluid; DCF, dichlorofluorescein; GAA, guanidinoacetate; GAMT, *N*-guanidinoacetate methyltransferase; H_2DCF, 2′,7′-dichlorofluorescein; H_2DCF-DA, 2′,7′-dichlorofluorescein diacetate; HBSS, Hank's balanced salt solution; IEM, inborn error of metabolism; iNOS, inducible nitric oxide synthase; PCr, phosphocreatine; Pi, inorganic phosphate; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDH, succinate dehydrogenase; SOD, superoxide dismutase assay; TBARS, thiobarbituric acid reactive substances; TBS, tris-buffered saline; T-TBS, tween-20 tris-buffered saline

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As the brain is a high energy demanding organ, creatine in low levels may lead to mitochondrial dysfunctions and other serious consequences [9,10]. In this context, it has been shown that energy metabolism alterations are often correlated with several neurodegenerative diseases [11,12]. Despite the fact that decreased levels of creatine play a key role in the physiopathology of GAMT deficiency, the evidence shows that an elevation in the level of GAA in the brain is the major responsible for triggering the neurological symptoms of patients affected by this disease. Although GAA may possess direct and indirect antioxidant properties [13], studies from our group demonstrated that the intrastriatal injection of GAA inhibited the activity of crucial enzymes for the generation of energy in the central nervous system (CNS), such as complex II and II-III of the mitochondrial respiratory chain [14], suggesting that oxidative stress is involved in the mechanisms underlying these changes [15]. Neuroinflammation, or more specifically, activation of the neuroimmune cells microglia and astrocytes into proinflammatory states, has been implicated not as an initiator, but as a pathological contributor for progression of several neurodegenerative diseases, and it is often caused by accumulation of a toxic molecule [16]. Uncontrolled inflammation may result in the production of neurotoxic factors that amplify underlying disease states, like the pro-inflammatory cytokines TNF-α, IL1β and IL6, as well as nitrites [17,18]. Furthermore, the cholinergic system play a key role in learning and memory mechanisms, and more recently has been associated with the modulation of inflammatory pathways [19,20]. Acetylcholinesterase (AChE) is the enzyme responsible for the degradation of acetylcholine (ACh) released in the synaptic cleft [21]; and an increase in its activity is related to neurodegenerative diseases and inborn errors of metabolism, including GAMT deficiency [22–24].

Cytokines and nitric oxide appear to affect astrocytes, oligodendrocytes, and microglia, exerting considerable effects on extracellular glutamate concentrations [25,26]. Glutamate transport through its most frequent carriers (GLAST and GLT-1) is dependent on the Na^+ gradient provided by the enzyme Na^+,K^+-ATPase, thus it can be affected by fluctuations in the activity of this enzyme. However, GAA decreases membrane fluidity and reports from our laboratory have shown that intrastriatal administration of GAA inhibits Na^+,K^+-ATPase activity embedded in the plasma membrane in the rat striatum [27]. Supplementation with creatine has been used as pharmacological treatment in GAMT deficiency for a long time [28,29]. The system creatine/PCr provides an energy reservoir, protecting against adenosine triphosphate (ATP) depletion and delayed membrane depolarization [30,31].

In the present study, we extended our previous reports investigating the effects of an intrastriatal administration of GAA on some parameters of energy metabolism, mitochondrial function, redox state and inflammation, glutamate homeostasis, and activities and immunocontents of Na^+,K^+-ATPase and AChE, as well as the process of memory acquisition (via novel object recognition task). The neuroprotective role of creatine on the biochemical changes observed in this model was also investigated. Our hypothesis is that the alterations caused by GAA in the striatum energy metabolism are closely associated with oxidative insults and inflammatory processes, leading to alterations in the glutamatergic and synaptic homeostasis, and consequently relating with memory impairment. Since the creatine/PCr system is of great value in disease states or situations where there is disruption of the cellular energy metabolism [32,33], we speculate that creatine might prevent such alterations. Striatum was used because patients with GAMT deficiency present basal ganglia abnormalities [34].

2. Materials and methods

2.1. Animals and reagents

Male Wistar rats were obtained from the Central Animal House of the Department of Biochemistry, Institute of Basic Health Sciences, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1 °C) colony room. Rats had free access to a 20% (w/w) protein commercial chow and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology following the Guide for Care and Use of Laboratory Animals and Arouca Law (11794/2008). The project was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil (number 29887). All efforts were made to minimize the number of animals used and their suffering. The chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Pretreatment with creatine

The animals (fifty-one-day-old Male Wistar rats) used for experiments in this study were subjected to a pretreatment for 7 days, receiving a daily intraperitoneal injection of creatine (50 mg/kg) or saline [35]. During this pretreatment, stereotaxic surgery was performed in the animals in order to facilitate the administration of GAA as described below.

2.3. Surgery and intrastriatal administration

Surgery and intrastriatal infusion were performed, according to Folbergrova and colleagues [36] and Zugno and colleagues [27]. During the pretreatment with creatine the animals were anesthetized with an intraperitoneal injection of equithesin solution contained thiopental (2,5 mg/kg i.p). The heads of the animals were fixed in a stereotaxic apparatus, the skin of the skull was removed and a 27-gauge 9-mm guide cannula was then placed above the striatum (AP: −0.5 mm; L: ± 2.5 mm; DV: −2.5 mm). The cannula was fixed with acrylic cement. The correct position of the needle was tested by 0.5 ml of methylene blue injection (4% in saline solution) and carrying out histological analysis. GAA was administered 48 h after surgery when the animals then completed sixty days of age. A 30-gauge cannula was fitted into the guide cannula and connected by a polyethylene tube to a 5 μl Hamilton micro syringe. The tip of the infusion cannula protruded 1.0 mm beyond the guide cannula towards the striatum. The animals were divided into four groups: Control (pretreatment with saline and intrastriatal infusion of saline), GAA (pretreatment with saline and intrastriatal infusion of 10 μM of GAA [0.02 nmol/striatum]), GAA + Creatine (pretreatment with creatine and intrastriatal infusion of 10 μM of GAA [0.02 nmol/striatum]), and Creatine (pretreatment with creatine and intrastriatal infusion of saline). The volume administered intrastriatally (saline or GAA solution) was 2 μl. Thirty minutes after intrastriatal infusion, the rats were decapitated without anesthesia (for biochemical studies) or subjected to the behavioral assessment.

2.4. Assay of the activities of the mitochondrial respiratory chain complexes

Striata were homogenized (1:20, w/v) in SETH (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 IU ml^{-1} heparin) buffer, pH 7.4. The homogenates were centrifuged at 800 × *g* for 10 min, and the supernatants were kept frozen until determinations. The activities of the respiratory chain complexes were calculated as nanomoles per minute per milligram of protein.

2.4.1. Complex II (succinate: 2,6-dichloroindophenol oxidoreductase) activity

Homogenates are following the decrement in absorbance due to the reduction of 2,6-dichloroindophenol at 600 nm with 700 nm as reference wavelength ($\varepsilon = 19.1\ mM^{-1}\ cm^{-1}$), in accordance to Fischer and colleagues [37]. The reaction mixture containing 40 mM potassium phosphate, pH 7.4, 16 mM succinate, and 8 μM 2,6-dichloroindophenol

was pre-incubated with 40–80 μg homogenate protein for 20 min at 30 °C. After, 4 mM sodium azide and 7 μM rotenone were added. After adding 40 μM 2,6-dichloroindophenol, the reaction initiated and was monitored for 5 min. All samples were run in triplicate.

2.4.2. Succinate dehydrogenase (SDH) activity

SDH activity was measured as described by Fischer and colleagues [37]. Samples were frozen and thawed three times to break mitochondrial membranes. The enzymatic activity was determined following the decrease in absorbance due to the reduction of 2,6-dichloroindophenol at 600 nm with 700 nm as reference wavelength ($\varepsilon = 19.1\ mM^{-1}\ cm^{-1}$) in the presence of phenazine methasulfate. The reaction mixture containing 40 mM potassium phosphate, pH 7.4, 16 mM succinate and 8 μM 2,6-dichloroindophenol was preincubated with 40–80 μg homogenate protein for 20 min at 30 °C. Then, 4 mM sodium azide, 7 μM rotenone, and 40 μM 2,6-dichloroindophenol were added. After adding 1 mM phenazine methasulfate, the reaction initiated and was monitored for 5 min. All samples were run in triplicate.

2.4.3. Cytochrome c oxidase (COX) activity

COX activity was measured according to Rustin and colleagues [38]. The activity of this enzyme was determined at 25 °C for 10 min by following the decrease in absorbance due to oxidation of previously reduced cytochrome *c* at 550 nm with 580 nm as reference wavelength ($\varepsilon = 19.1\ mM^{-1} \times cm^{-1}$). The reaction buffer consisted of 10 mM potassium phosphate, pH 7.0, 0.6 mM *n*-dodecyl-β-D-maltoside, 2–4 μg homogenate protein. Reaction initiated after addition of 0.7 μg reduced cytochrome *c*. All samples were run in triplicate.

2.5. ATP levels assay

Striata were immediately dissected and frozen in liquid nitrogen. Each striatum was weighed and homogenized in 1 ml of 0.1 M NaOH (to inactivate cellular ATPases activity). Samples were assayed using the ATPlite Luminescence ATP detection assay system (Perkin-Elmer, Waltham, MA, USA) according to Witt and colleagues [39]. The measurement of chemiluminescence was performed using a Perkin-Elmer Microbeta Microplate Scintillation Analyzer. ATP concentrations were calculated from a standard curve, normalized against wet tissue weights in grams and expressed as micromoles per gram of tissue. All samples were run in triplicate.

2.6. Mitochondrial mass and mitochondrial membrane potential measurements

Striata were mechanically dissociated in PBS containing collagenase to yield digestion to a density of about 200,000 cells/ml. The dissociated contents were then filtered into sterile 50-ml Falcon tubes through a 40-μm nylon cell strainer and kept on ice until mitochondrial staining. Dissociated cells were stained with 100 nM MitoTracker Green and 100 nM MitoTracker Red (diluted from 1 mM stock solutions in dimethylsulfoxide) for 45 min at 37 °C (and in the dark), according to the method described by Keij and colleagues [40] and Pendergrass and colleagues [41] with some modifications, to determine mitochondrial mass and mitochondrial membrane potential, respectively. Immediately after staining, cell suspensions were analyzed on a FACSCalibur flow cytometer, using red (670 nm long pass) and green (530 nm/30) filters. Controls stained with a single dye were used to set compensation. For each sample, 10,000 events corresponding to intact cells (as gated in FSC versus SSC plots) were analyzed. All flow cytometric acquisition and analyses were performed using CELLQuest Pro data acquisition and FlowJo software.

2.7. Oxidative stress parameters

2.7.1. Tissue preparation

The striatum was homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl, to determine the oxidative stress parameters. The homogenate was centrifuged at 750 × *g* for 10 min at 4 °C; the pellet was discarded and the supernatant was immediately separated and used for the measurements.

2.7.2. Superoxide dismutase (SOD) assay

SOD activity assay is based on the auto-oxidation ability of pyrogallol, a process highly dependent on superoxide, which is the substrate for SOD [42]. The inhibition of this compound autoxidation occurs in the presence of SOD, whose activity is then indirectly assayed at 420 nm using SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, CA, USA). A calibration curve was performed with purified SOD as standard in order to calculate the activity of SOD present in the samples. The results were reported as units per mg of protein. All samples were run in triplicate.

2.7.3. Catalase (CAT) assay

CAT activity was assayed using SpectraMax M5/M5 Microplate Reader. The method is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer pH 7.0, and 0.1–0.3 mg protein/ml [43]. One CAT unit is defined as one μmol of hydrogen peroxide consumed per minute and the specific activity is calculated as CAT units/mg protein. All samples were run in triplicate.

2.7.4. 2′,7′-Dichlorofluorescein (H_2DCF) oxidation assay

Reactive species production were measured second to LeBel and colleagues [44] method, based on the oxidation of H_2DCF. Samples (60 μl) were incubated for 30 min at 37 °C in the dark with 240 μl of 100 μM 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA) solution in a 96 wells plate. H_2DCF-DA is cleaved by cellular esterases and the resultant H_2DCF is eventually oxidized by reactive species presenting in samples. The last reaction produces the fluorescent compound dichlorofluorescein (DCF) which was measured at 488 nm excitation and 525 nm emission and the results were represented by nmol DCF/mg protein. All samples were run in triplicate.

2.8. Cytokines levels measurements

The samples were homogenized (1:5, w/v) in phosphate buffered saline (PBS, pH 7.4). The homogenates were centrifuged at 800 × *g* for 10 min at 4 °C, and the supernatants were used for analysis. TNF-α, IL-1β and IL-6 levels in the striata were measured by kit rat high-sensitivity enzyme-linked immunoabsorbent assays (ELISA) with commercially available kits (Sigma-Aldrich®). All samples were run in triplicate.

2.9. Nitrite assay

The striatum was homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. The homogenate was centrifuged at 750 × *g* for 10 min at 4 °C. 100 μl of supernatant was mixed with 100 μl Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) and incubated in 96-well plates for 10 min at room temperature [45,46]. The absorbance was measured on a microplate reader at a wavelength of 543 nm. Nitrite concentration was calculated using sodium nitrite standards. All samples were run in triplicate.

Table 1
Effect of the GAA intrastriatal injection on cellular energy metabolism.

	Control	GAA	GAA + creatine	Creatine
Complex II activity (nmol/min/mg protein)	150.89 ± 39.02	66.51 ± 18.41*	104.68 ± 29.18#	129.44 ± 42.19
SDH activity (nmol/min/mg protein)	7.35 ± 1.65	4.12 ± 0.97*	6.36 ± 2.07#	7.47 ± 1.33
COX activity (nmol/min/mg protein)	150.89 ± 39.02	66.51 ± 18.41*	104.68 ± 29.18	129.44 ± 42.19
ATP levels (mmol/g tissue)	3.07 ± 0.60	1.92 ± 0.19*	2.37 ± 0.53	2.64 ± 0.32

Results are expressed as means ± standard deviation for 6–9 animals in each group.
* $p < 0.05$ when compared with control group.
# $p < 0.05$ compared to GAA group (two-way ANOVA followed by Tukey's post hoc test).

Table 2
Effect of the GAA intrastriatal injection on redox state.

	Control	GAA	GAA + creatine	Creatine
SOD activity (units/mg protein)	16.19 ± 0.88	12.76 ± 1.78*	12.82 ± 1.71*	16.05 ± 1.91
CAT activity (units/mg protein)	2.70 ± 0.80	1.22 ± 0.43*	2.35 ± 0.62#	2.4 ± 0.95
DCF levels (nmol/mg protein)	1525.52 ± 385.41	2215.07 ± 199.03*	1440.57 ± 240.33	1589.60 ± 278.84

Results are expressed as means ± standard deviation for 6–9 animals in each group.
* $p < 0.05$ when compared with control group.
# $p < 0.05$ compared to GAA group (two-way ANOVA followed by Tukey's post hoc test).

Table 3
Effect of the GAA intrastriatal injection on inflammatory markers.

	Control	GAA	GAA + creatine	Creatine
TNF-α levels (pg/mg protein)	10.06 ± 1.11	17.67 ± 5.59*	11.52 ± 0.28#	10.94 ± 2.52
IL-1β levels (pg/mg protein)	182.64 ± 70.93	293.43 ± 60.08*	157.05 ± 23.14#	169.46 ± 63.24
IL6 levels (pg/mg protein)	36.55 ± 5.74	59.08 ± 8.97*	42.74 ± 9.88#	37.59 ± 8.95
Nitrite levels (μmol/mg protein)	15.19 ± 2.08	23.07 ± 3.20*	21.22 ± 1.60	14.95 ± 0.59

Results are expressed as means ± standard deviation for 6–9 animals in each group.
* $p < 0.05$ when compared with control group.
# $p < 0.05$ compared to GAA group (two-way ANOVA followed by Tukey's post hoc test).

2.10. AChE activity assay

AChE activity was determined by the method of Ellman and colleagues [47]. For AChE assay, striatum was homogenized in 10 volumes (1:10, w/v) of 0.1 mM potassium phosphate buffer, pH 7.5, and centrifuged for 10 min at 1000 ×*g*, being the supernatants used for the enzymatic AChE analyses. Hydrolysis rate was measured at ACh (S) concentration of 0.8 mM in 1 ml assay solutions with 100 mM phosphate buffer (pH 7.5) and 1.0 mM DTNB. Fifty microliters of striatum homogenate was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s) at 25 °C. All samples were run in triplicate.

2.11. Glutamate uptake

Glutamate uptake was performed according to previous reports [48,49]. The uptake assay was carried out by adding 0.66 μCi ml^{-1} l-[^{3}H] glutamate and 100 μM unlabeled glutamate (final concentration) in 20 μl Hank's balanced salt solution (HBSS) at 37 °C. Incubation proceeded at 35 °C and was stopped after 3 min by two ice-cold washes with 1 ml HBSS, immediately followed by addition of 0.5 N NaOH, which was kept overnight. Incorporated radioactivity was measured using a scintillation counter (Wallac 1400). Sodium-independent uptake was determined in parallel assays using *N*-methyl-D-glucamine instead of sodium chloride. This uptake was subtracted from the total uptake to obtain the sodium-dependent uptake. All experiments were performed in triplicate.

2.12. Na^+,K^+-ATPase activity assay

The samples were homogenized (1:10, w/v) in 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.5. Homogenates were centrifuged at 1000 ×*g* for 10 min at 4 °C. Supernatants were taken for Na^+,K^+-ATPase activity assay. Reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM MgCl2, 80.0 mM NaCl, 20.0 mM KCl, and 40.0 mM Tris–HCl, pH 7.4, in a final volume of 200 μl. After 10 min of pre-incubation at 37 °C, the reaction was started by the addition of ATP to a final concentration of 3.0 mM and was incubated for 20 min. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays [50]. Inorganic phosphate (Pi) released was measured by the method of Chan and colleagues [51] and enzyme-specific activity was expressed as nanomoles Pi per minute per milligram of protein. All samples were run on triplicate.

2.13. Behavioral procedures

Behavioral procedures were performed between 10 a.m. to 3 p.m. in a controlled light and sound room, by a researcher blind to the animal's experimental condition. GAA was injected 30 min before the training session, in order to evaluate the process of memory acquisition. The test session was performed 1 h after the training to assess short-term memory. One day before the training session, all animals were habituated to walk freely in the empty arena for 5 min. The arena used was a black wooden box (50 × 50 × 50 cm). In the training session, two identical objects (objects A1 and A2) were placed equidistant from the sidewalls. In this chamber, each animal performed a trial of 5 min. After each trial, the apparatus was cleaned to alleviate olfactory cues. In the

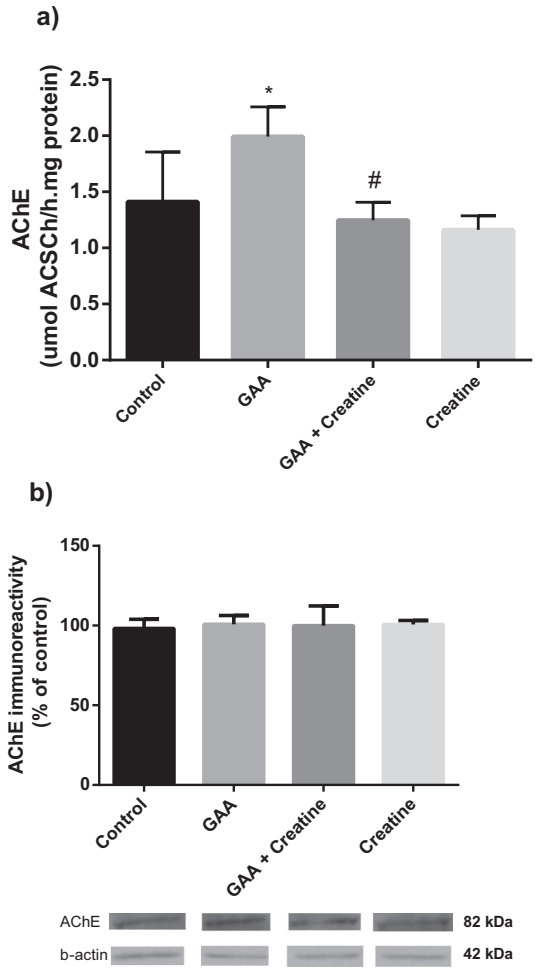

Fig. 1. Effect of GAA intrastriatal infusion and/or creatine administration on AChE activity (a) and on immunocontent (b) in homogenates of the striatum of 60-day-old rats. Results are expressed as means ± standard deviation for 6–9 animals in each group. *p < 0.05 when compared with control group; #p < 0.05 compared to GAA group (two-way ANOVA followed by Tukey's post hoc test).

second trial, the test session, one of the objects (object A2) was substituted by a new and different one (object B). This object had a different form, but was similar in complexity and had the same color and texture as the object A1. An experimenter registered the time of object exploration, i.e., touching it with paws or exploring it by olfaction with direct contact of the snout [52]. The object discrimination index was calculated in the test session, as follows: the difference in exploration time divided by the total time spent exploring the two objects {[(B − A1)/(A1 + B)] where B is the new object and A1 is the familiar object}. Rats without memory impairment explore the new object for more time when compared with the old one [53].

2.14. Western blot analysis

Western blotting was performed as described by Biasibetti-Brendler and colleagues [54]. The striatum was homogenized in 200 µl of a lysis solution containing 2 mM EDTA, 50 mM Tris–HCl, pH 6.8, and 4% SDS. For electrophoresis analysis, samples were dissolved in 25% (v/v) of a solution containing 40% glycerol, 5% mercaptoethanol, and 20 mM Tris–HCl, pH 6.8 and boiled for 3 min. Equal protein concentrations were loaded onto 10% polyacrylamide gels and analyzed by SDS-PAGE (30 µg/lane of total protein) and transferred (Trans-Blot SD Semi-Dry Transfer Cell, Bio-Rad) to nitrocellulose membranes for 1 h at 15 V in transfer buffer (48-mM Trizma, 39-mM glycine, 20% methanol, and 0.25% SDS). The blot was then washed twice for 5 min in 0.05% Tween-20 Tris-buffered saline (T-TBS) and twice for 5 min with Tris-buffered saline (TBS) (0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by a 2-h incubation in blocking solution (TBS plus 5% bovine serum albumin, fraction V). Then, membranes were washed for 10 min with TBS and incubated with primary antibody overnight at 4 °C: 1:1000 rabbit anti-AChE, 1:1000 mouse anti-Na^+,K^+-ATPase (α3 subunit), 1:1000 rabbit anti-GLAST and rabbit anti-GLT-1 glutamate transporters, and 1:2000 mouse anti-β-actin. The blot was then washed twice for 5 min with T-TBS and twice for 5 min with TBS, followed by an incubation for 2 h in antibody solution containing peroxidase-conjugated anti-mouse IgG or peroxidase-conjugated anti-rabbit IgG diluted 1:2000. The blot was then washed twice for 5 min with T-TBS and twice for 5 min with TBS, and then it was developed using a chemiluminescence kit (Immobilon Western Chemiluminescent HRP Substrate, Millipore) and detected by ImageQuant LAS 4000 (GE Healthcare Life Sciences). Band intensities were analyzed using Image J software (developed at the US National Institutes of Health and available on the website http://rsb.info.nih.gov/nih-image/). Band intensity was normalized to β-actin as a loading control to assess protein levels.

2.15. Protein determination

Protein concentration was measured by the method of Lowry and colleagues [55] using bovine serum albumin as standard for most methods. For AChE and Na^+,K^+-ATPase activities, proteins were measured by the Coomassie Blue method according to Bradford and colleagues [56].

2.16. Statistical analysis

The parametric data for four groups were analyzed by two-way analysis of variance (ANOVA) followed by post hoc Tukey test when F-test was significant. Values of $p < 0.05$ were considered statistically significant. All analyses and graphics were performed using GraphPad Prism 6.0 software program in a compatible computer. All samples were run in triplicate.

3. Results

In the present study we addressed the ability of creatine in preventing the deleterious action of GAA in striatum of rats. Firstly, we evaluated the effect of GAA intrastriatal injection on cellular energy metabolism as shown in Table 1. The results showed that GAA decreased the activities of complex II ($F(3,29) = 17.08$ $p < 0.05$), SDH ($F(3,29) = 18.14$ $p < 0.05$) and COX ($F(3,21) = 16.69$ $p < 0.05$). Creatine totally prevented the impairment in complex II ($F(3,29) = 4.181$ $p < 0.05$) and SDH ($F(3,29) = 4.305$ $p < 0.05$), but had only a partial prevention of COX ($F(3,21) = 4.977$ $p < 0.05$). ATP levels were decreased in rats submitted to an intrastriatal injection of GAA ($F(3,18) = 12.7$ $p < 0.05$), and creatine was able to partially prevent such effect ($F(3,18) = 4.918$ $p < 0.05$). To study if the enzymatic effect were due physiological changes in the mitochondria, we assess mitochondrial mass and mitochondrial membrane potential, but these parameters remained unaltered when compared to the control group ($p > 0.05$).

Since mitochondria is the main site for generation of reactive species, and that redox imbalance may originate from the energetic disturbances like the ones that we observed [57], our next step was to evaluate if GAA and this imbalance in the enzymes of the mitochondrial respiratory chain could lead to alterations in oxidative stress parameters and if creatine could prevent them. GAA led to a decrease in SOD ($F(3,28) = 29.36$ $p < 0.05$) and CAT ($F(3,28) = 7.650$ $p < 0.05$) activity, but creatine was only able to prevent the decrease in CAT activity ($F(3,28) = 6.745$ $p < 0.05$). Furthermore, the reactive species

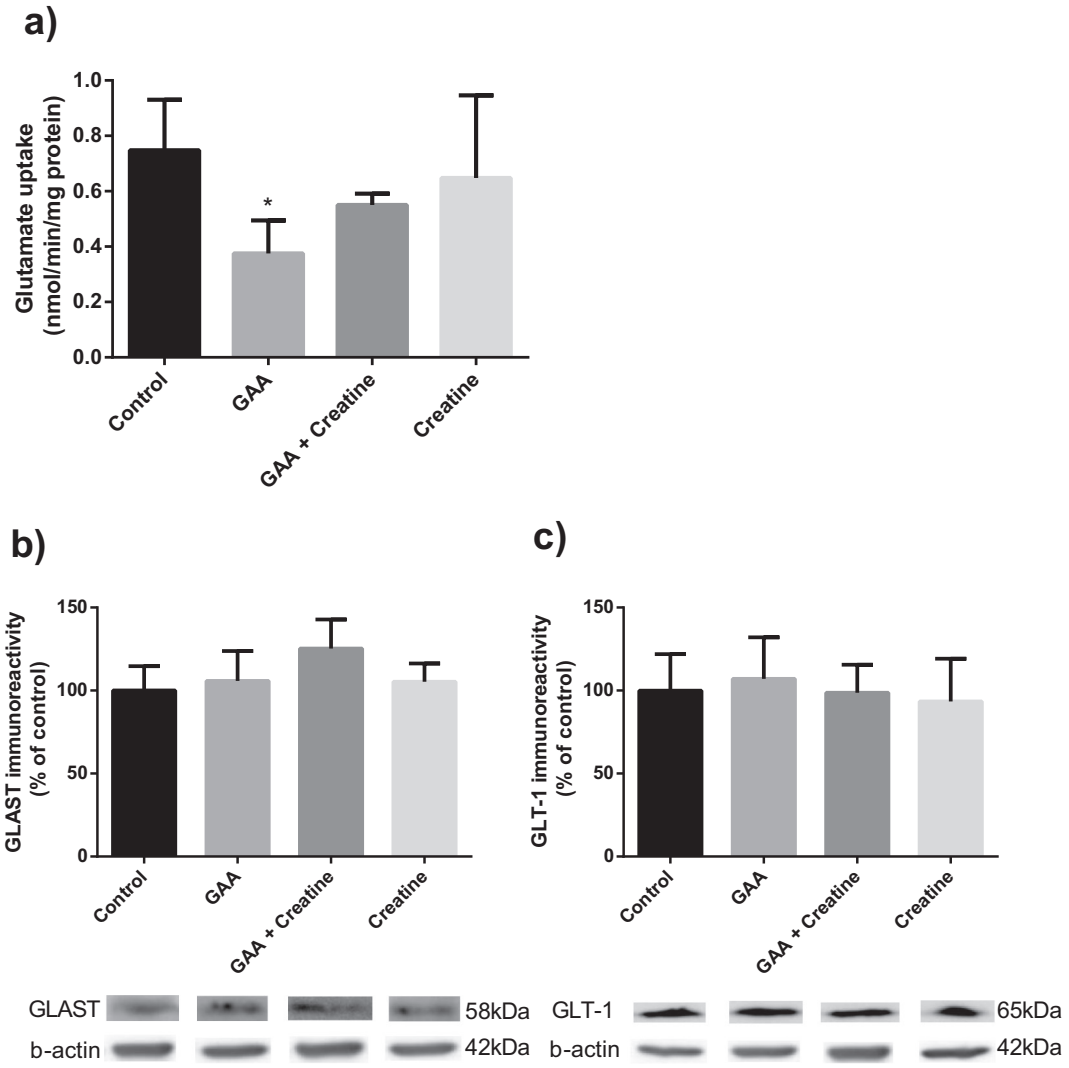

Fig. 2. Effect of GAA intrastriatal infusion and/or creatine administration on glutamate uptake (a) and on glutamate transporters' immunocontent of GLAST (b) and GLT-1 (c) in homogenates of the striatum of 60-day-old rats. Results are expressed as means ± standard deviation for 6–9 animals in each group. *$p < 0.05$ when compared with control group (two-way ANOVA followed by Tukey's post hoc test).

production was increased in rats that suffered GAA intrastriatal infusion when compared with control group, as indicated by the increase of DCF levels ($F(3,21) = 5.071$ $p < 0.05$). Creatine was able to prevent such alteration ($F(3,21) = 12.21$ $p < 0.05$). These results are summarized in Table 2.

Changes in redox homeostasis are highly interconnected with inflammatory processes, so we measured several inflammatory markers in order to investigate if this was the case in our acute model, as can be seen in Table 3. GAA increased the levels of cytokines TNF-α ($F(3,18) = 8.680$ $p < 0.05$), IL1β ($F(3,20) = 5.351$ $p < 0.05$) and IL6 ($F(3,20) = 15.05$ $p < 0.05$), and creatine completely prevented these alterations ($F(3,18) = 6.264$ $p < 0.05$; $F(3,20) = 8.390$ $p < 0.05$; $F(3,20) = 5.932$ $p < 0.05$, respectively). GAA also increased nitrite content ($F(3,18) = 54.56$ $p < 0.05$), which was not prevented by creatine ($p > 0.05$).

In addition, we evaluated the effect of GAA intrastriatal injection on AChE activity and immunocontent. GAA infusion increased AChE activity ($F(3,23) = 10.264$ $p < 0.05$) and creatine prevented this effect ($F(3,23) = 5.743$ $p < 0.05$) (Fig. 1a). However, GAA had no effect on AChE immunocontent ($p > 0.05$) (Fig. 1b).

Having established the overall energetic, redox and inflammatory state, we went on to see possible alterations in the glutamatergic system or in the Na^+,K^+-ATPase activity. Fig. 2a shows that GAA intrastriatal injection decreased glutamate uptake in the striatum ($F(3,23) = 12.78$ $p < 0.05$) when compared to the control group, and that creatine was able to partially prevent this alteration ($F(3,23) = 4.359$ $p < 0.05$). Immunocontents of GLAST and GLT-1 transporters were also evaluated, and the results showed that GAA did not alter the immunocontents of these transporters ($p > 0.05$), as shown in Fig. 2b and c.

Regarding Na^+,K^+-ATPase activity, Fig. 3a shows that GAA significantly inhibited this enzyme ($F(3,20) = 8.645$ $p < 0.05$), and creatine was able to provide only a partial prevention ($F(3,20) = 7.007$ $p < 0.05$). Examination of Na^+,K^+-ATPase α3 subunit by immunoblot also revealed that this protein content was increased in the striatum of rats submitted to the intrastriatal injection of GAA ($F(3,24) = 31.40$ $p < 0.05$) and that creatine did not prevent this increase, as can be seen in Fig. 3b.

Finally, we addressed whether the decreased glutamate uptake and the alterations in crucial enzymes of the CNS could lead to memory deficits as the ones observed in patients, and if creatine would be able to successfully prevent them. In the object recognition test, control rats spent less time on the familiar object in the test session (Fig. 4b) when compared to the training (Fig. 4a), as highlighted by the discrimination index (Fig. 4c). Therefore, the time exploring the novel object increased in the control group while the same did not happened in animals that received intrastriatal infusion of GAA ($F(3,34) = 9.328$ $p < 0.05$).

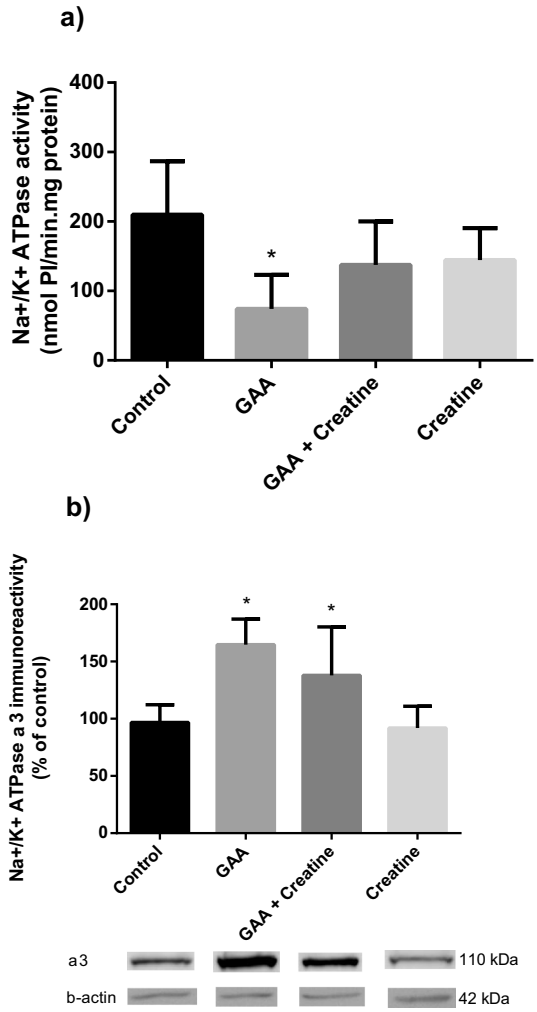

Fig. 3. Effect of GAA intrastriatal infusion and/or creatine administration on Na^+,K^+-ATPase activity (a) and on immunocontent of its α3 subunit (b) in homogenates of the striatum of 60-day-old rats. Results are expressed as means ± standard deviation for 6–9 animals in each group. *$p < 0.05$ when compared with control group (two-way ANOVA followed by Tukey's post hoc test).

Creatine per se exerts no effect on this parameter, but when associated with GAA it was able to prevent the alterations ($F(3,34) = 4.345$ $p < 0.05$).

4. Discussion

Neurological symptoms are common and variable among individuals with GAMT deficiency, but the pathophysiology of this disease is still unclear. Previous work from our laboratory had already found alterations in redox state and in energy metabolism in rats submitted to an intrastriatal injection of GAA [14,15,58]. In the present study, we expanded previous reports by verifying creatine's preventive potential on these effects and on inflammation, as well as the effect on a non-aversive memory test.

Intrastriatal injection of GAA led to decreased activity in the enzymes of mitochondrial respiratory chain (Complex II, SDH and COX). The incapacity of this chain to support the cell with a sufficient amount of energy can promote accumulation of intracellular Ca^{2+}, thus generating reactive species that have the power to cause oxidative stress and cytochrome C dissociation from the inner mitochondrial membrane [59,60]. The increase in reactive species generation, indicated by the increase in DCF levels may lead to alterations of components of the plasma membrane in which enzymes are anchored and decrease of sulphydryl groups, important for enzymatic activity [61]. This becomes even more of a problem when the antioxidant enzymes SOD and CAT present diminished activity, like in our model. These impairments are probably due to reactive species causing a site-specific amino acid modification [62]. These results suggest that GAA provokes an antioxidant imbalance in striatum. This can cause further impairment in the electron transport chain complexes since they are included in the group of enzymes vulnerable to free radicals attack [63]. COX catalyzes a rate-limiting step, being responsible for transferring electrons from cytochrome *c* to molecular oxygen [64]. In this context, the inhibition of this enzyme may lead to an incomplete reduction of oxygen, and a consequent increase in the formation of free radicals, which makes COX a special target for oxidative damage [65]. The role that COX has as a key player in the formation of ROS may be the cause why creatine was not able to fully restore its functionality. To verify if the enzymatic changes were due to changes in the mitochondrial morphology or biology we tested mitochondrial mass and mitochondrial membrane potential, but these parameters remained unaltered when compared to the control group. This indicates that the interval of time between the injection and the death of the animals was insufficient to induce profound changes in the mitochondrial network structure. Nonetheless, the observed activity decline of the enzymes of the mitochondrial respiratory chain was able to generate a drop in ATP levels in the striatum. Taking all this into account, we suggest that high levels of GAA affect brain bioenergetics and ROS generation.

Creatine completely restored complex II and SDH activities, and partially restored the decrease in COX activity and ATP levels. The main destination of supplemented creatine is the system creatine/PCr which strengthens cellular energetics via a temporal and spatial energy buffer that can restore cellular ATP and protects against energy depletion and delayed membrane depolarization [30,31]. Additionally, it has been shown that creatine prevents or delays mitochondrial permeability transition pore opening, an early event in apoptosis [66]. Therefore, the boost of creatine provided by our pre-treatment have potential therapeutic value as it appears to replenish cellular ATP without a reliance on oxygen, helping to prevent the overflow of the mitochondrial respiratory chain [67,68]. In addition to the effects of creatine as an enhancer of cellular energetics, one of the major roles of creatine is also as both a direct and an indirect antioxidant [69]. In this study, we have observed that creatine was able to prevent the decrease in CAT, but not in SOD. Nonetheless, DCF levels were normalized by creatine, and this may be due to the fact that creatine not necessarily acts as an antioxidant by increasing or preventing drops on the activities of antioxidant enzymes [70]. Still in the context of the energy metabolism alterations, studies showed that creatine significantly protects mitochondrial DNA from oxidative damage in a dose dependent manner [59,71].

Since neuroinflammation is closely related to oxidative stress and that both have been implicated as a pathological contributor in several neurodegenerative diseases that present common features with GAMT deficiency [72–74], we have measured several inflammatory markers. All pro-inflammatory cytokines observed (TNF-α IL1β and IL6) had their levels increased in rats that suffered the GAA intrastriatal injection. TNF-α is the key initiator of immune-mediated inflammation in several organs, including the brain [75]. It acts synergistically with IL-1β to induce IL-6 expression [76]. It is worth to mention that recent studies have shown that TNFα leads to the activation of an unknown downstream tyrosine kinase that phosphorylates COX on Tyr304 leading to strong enzyme inhibition, like the one observed in this study [77]. Therefore, this excessive production of proinflammatory cytokines and free radicals can be interpreted as an indicator of uncontrolled activation of microglia, and it may be suggested that GAA is a toxic metabolite with potential to induce neuroinflammation. This is reinforced by the fact that GAA increased nitrite content, implying activation of iNOS. Of note, it is known that activation of toll-like

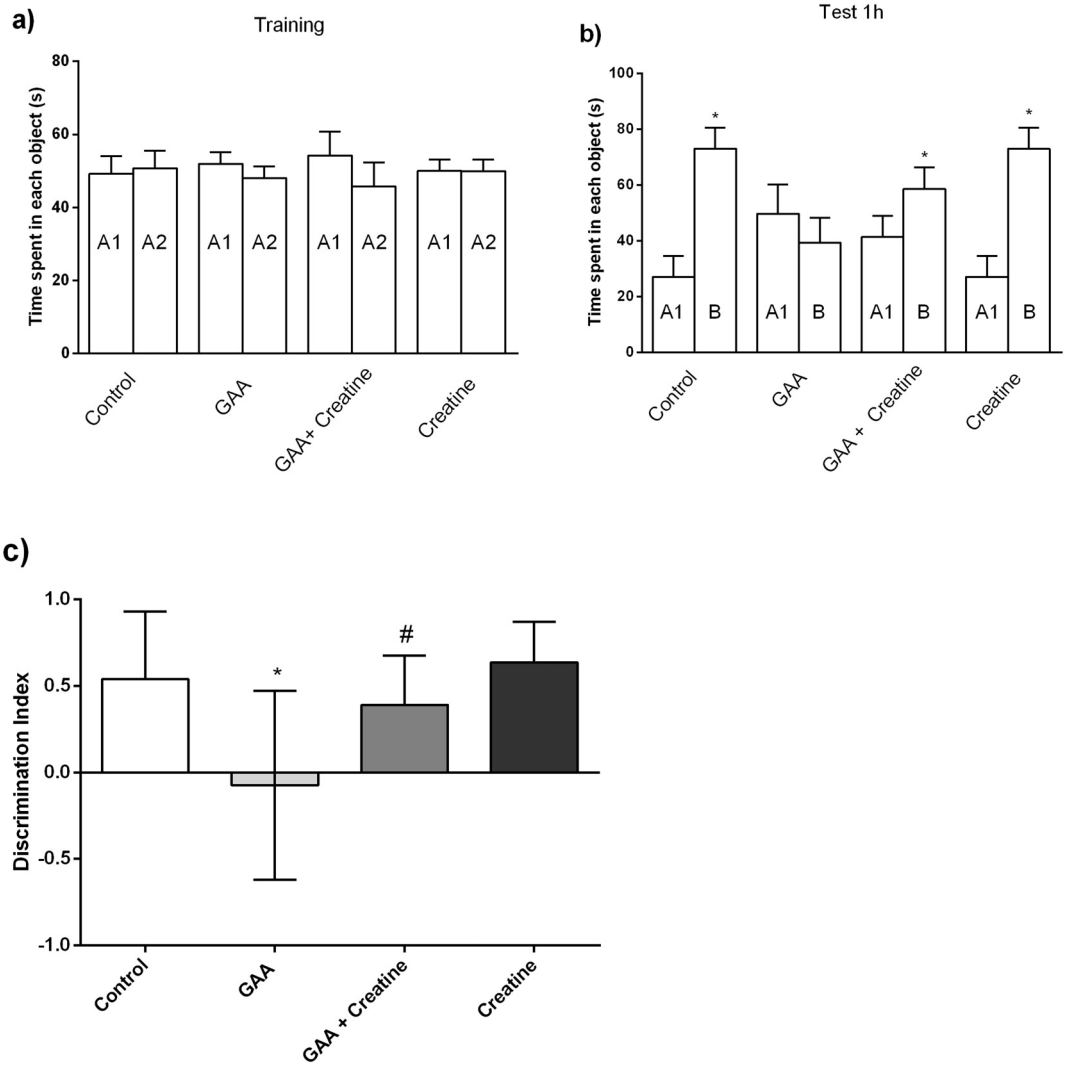

Fig. 4. Effect of GAA intrastriatal infusion and/or creatine administration on the novel object recognition test. (a) Results in the training session. (b) Results in the test session. (c) Discrimination index. Data are expressed as mean ± standard deviation for 8–12 animals in each group.*$p < 0.05$ between objects A1 and B for graph b; *$p < 0.05$ when compared with control group; #$p < 0.05$ compared to GAA group for graph c (two-way ANOVA followed by Tukey's post hoc test).

receptors in microglia and also in astrocytes leads to excessive production of nitric oxide via iNOS [78]. In addition, ACh is a well-known neurotransmitter for signal transduction and also plays a crucial role in regulating immune and inflammatory reactions [19,79,80]. Our results confirmed that GAA increases AChE activity in the striatum of adult rats, suggesting a reduction in the ACh levels, which would contribute to a proinflammatory state. This is supported by studies that have shown that AChE inhibition reduces microglial production of TNF-α in a hypoxia model [81] and that AChE inhibitors suppress systemic cytokine levels during endotoxemia [82]. Furthermore, the cholinergic system plays a crucial role in cognitive function, including memory [83]. Previous studies have shown that cholinergic neurotransmission modulates aversive conditioning [84]. Our results showed that GAA significantly increased AChE activity, implying that GAA accumulation affects memory processing, and that AChE probably plays a role in this alteration.

Although the mechanisms involved are not yet fully understood, in recent years the action of creatine as not only an antioxidant, but also as an immunomodulatory agent has gained increasing attention. Creatine can inhibit endothelial permeability, neutrophil adhesion to endothelial cells, and adhesion molecule expression, as well as diminish the increase in the levels of some inflammatory markers like C-reactive protein, TNFα, INFα, IL-1β and PGE_2 [85–88].

Our studies are in agreement with previous reports that showed that Na^+,K^+-ATPase, an essential enzyme for the maintenance of brain functions [89], is diminished by an intrastriatal injection of GAA, which also may play a role in the memory impairment observed in this study [90]. The inhibition of Na^+,K^+-ATPase activity was inversely correlated to the immunocontent of its α3 subunit, a subunit highly expressed in the striatum [91]. This result suggests that the GAA-induced decrease in Na^+,K^+-ATPase activity did not occur by altering the overall number of molecules of this enzyme, but is a post-translational inhibition. It is possible that the inhibition of this enzyme is associated with alteration of components of the plasma membrane where Na^+,K^+-ATPase is anchored [61]. Furthermore, the active site of Na^+,K^+-ATPase presents several cysteine residues, which makes it an easy target for irreversible oxidation of SH-groups, causing protein degradation [15,92]. The capacity of creatine to partially prevent such alterations may be due its antioxidant capacity. However, since Na^+,K^+-ATPase relies on high amounts of ATP in order to maintain resting potential in neurons, the inability of creatine to fully restore ATP levels may be one of the reasons why it was not able to fully restore this enzyme activity. Besides, the up-regulation in translation with consequent increase in the amount of the enzyme probably indicates the development of an adaptive compensatory mechanism. We also evaluated glutamate uptake and immunocontent of the most important glutamate transporters

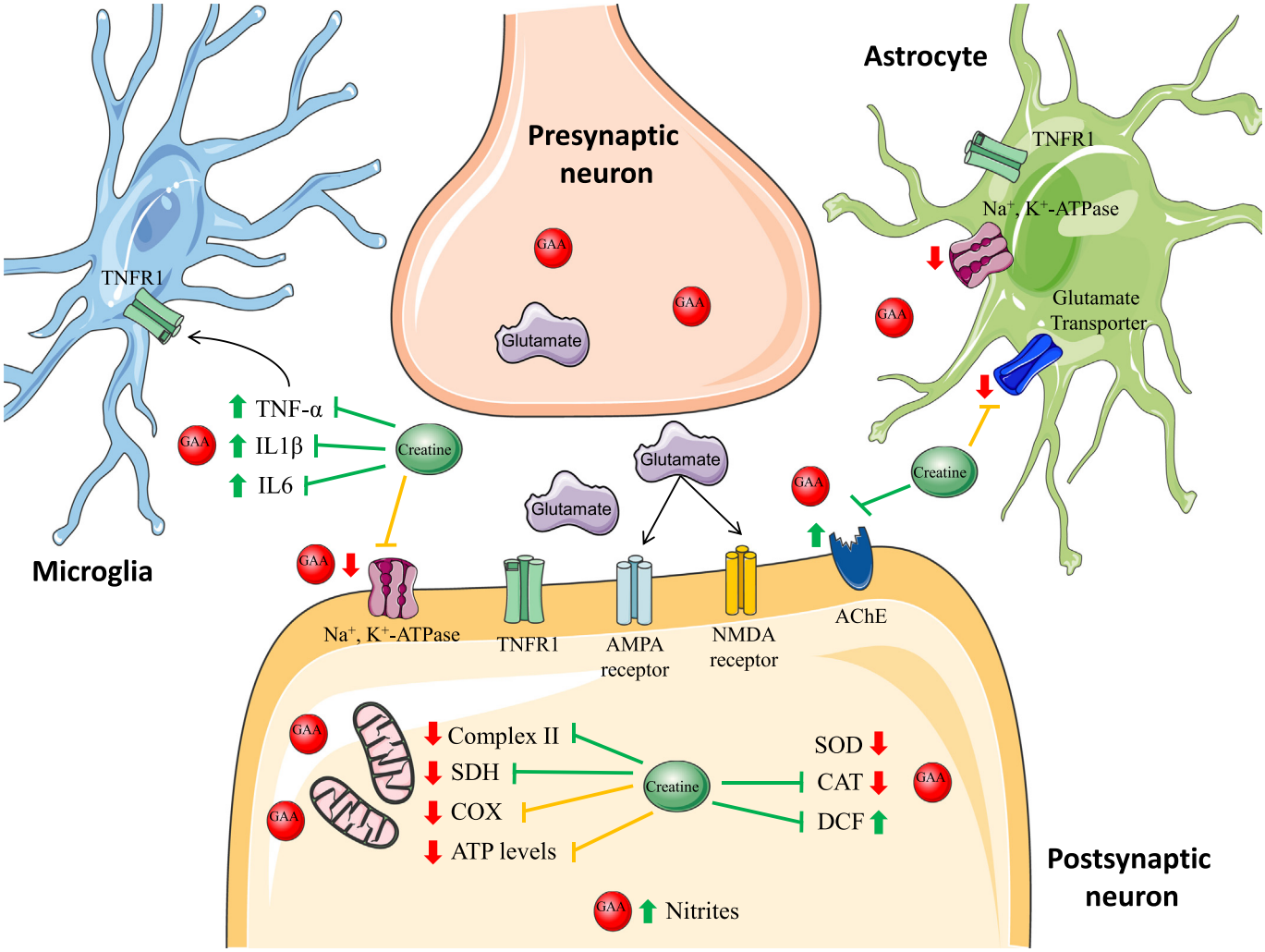

Fig. 5. Summary of the effects of GAA and creatine. Accumulation of GAA may exert its actions mainly by four possible pathomechanisms, namely energy deficit, oxidative stress, inflammation and excitotoxicity. GAA inhibit key enzymes of the mitochondrial respiratory chain, leading to diminished ATP levels while increasing the generation of reactive oxygen species and reducing tissue antioxidant defenses. These processes appear to be highly interconnected inflammation, with increase in nitrite levels, release of pro-inflammatory cytokines and the inhibition of AChE. TNF-α can interact with its receptor TNFR1 in order to amplify the inflammatory response with the release of more cytokines by the microglia, more glutamate by the astrocytes, or to indirectly stimulate AMPA and NMDA receptors in neurons. Since GAA also inhibits glutamate reuptake by its astrocytes transporters, it is possible that GAA cause excitotoxicity via overstimulation of NMDA receptors. Furthermore, Na^+,K^+-ATPase, a key enzyme for neuronal function, is impaired by GAA probably via oxidative stress and ATP depletion, while the immunocontent of its α3 subunit is increased, indicating an adaptive compensatory mechanism. Creatine had no effect on nitrite levels or SOD activity. Even so, creatine appears to be a strong neuroprotector, completing preventing almost every alteration caused by GAA, with the exception of partial preventions on Na^+,K^+-ATPase activity, COX activity, drop in ATP levels and on the inhibition of glutamate uptake.

for extracellular glutamate clearance, GLAST and GLT-1, in the striatum of rats submitted to the injection of GAA. Since no alterations in the immunocontent of these transporters were observed, we suggest that the drop in glutamate clearance was due to alterations in the function of the transporters induced by GAA, and not due to a decrease in the number of transporters available at the synaptic cleft. It is possible that glutamate transport through its carriers is being affected by fluctuations of Na^+,K^+-ATPase activity since glutamate clearance is dependent on the Na^+ gradient provided by this enzyme [93]. Consequently, the decline in Na^+,K^+-ATPase activity in rat striatum could be linked to the reduction of glutamate uptake observed in our work. In addition, glutamate transport can also be impaired by oxidative stress [94]. Prolonged impairment of astrocytic functions could increase the vulnerability of dopaminergic neurons in the substantia nigra, accelerating their degeneration, which may play a role in some of the motor symptoms presented by GAMT deficiency patients [95]. In the context of inflammation, astrocytes present receptors for several immune-derived molecules including cytokines, chemokines, complement, and acute-phase proteins. Once activated by cytokines, astrocytes are able to secrete a considerable number of innate immune inflammatory mediators including several complement cascade proteins and further cytokines like the ones that we found increased in this study [96–98]. This activation is frequently related with impaired glutamate clearance and oxidative stress, both of which contribute to excitotoxicity. Lower clearance and excessive release of glutamate by glial cells during immune activation may lead to glutamate boost and promote abnormal extrasynaptic signaling through ionotropic and metabotropic glutamate receptors. These alterations can ultimately cause severe synaptic dysfunction [99,100]. This synaptic dysfunction mediated by glutamate may play a part in the memory impairment observed in our study, since cortico inputs to and from the basal ganglia play a role in cognitive function, such as working memory and attention [101].

In the CNS, creatine develops a fundamental role in the regeneration of ATP for glutamate clearance over excitatory synaptic transmission [102]. In this context, knock-out mice for CK isoforms showed behavioral abnormalities, including spatial learning impairment and

alterations in the arrangement and preservation of hippocampal mossy fiber connections [103,104]. This may explain the partial prevention that creatine had on glutamate uptake in the striatum. In addition, studies suggest that creatine may play a part as a neurotransmitter or neuromodulator, regulating GABAergic and glutamatergic receptors [105,106]. Therefore, creatine may act not only preventing some of the biochemical alterations found in this study, but could also be a modulator of synapses, which would help to prevent the memory impairment in the object recognition test. Nonetheless, it is important to highlight that creatine supplementation has been shown to decrease the rate of GAA endogenous synthesis [107,108]. Therefore, future studies would benefit from determination of GAA and creatine intrastriatal concentration in order to clarify the degree by which creatine directly protects against GAA exposition.

5. Conclusion

In summary, in the present study we demonstrated that GAA intrastriatal injection impairs the mitochondrial respiratory chain, leading to depleted ATP levels, redox imbalance and inflammatory processes. In addition, we have confirmed that AChE activity, glutamate uptake and Na^+,K^+-ATPase activity in the striatum of young adult rats are inhibited after GAA exposure, probably due to the depletion of ATP levels and attack from radical species, contributing for the memory impairment observed. Nonetheless, immunocontent of AChE, and glutamate transporters GLAST and GLT-1 were not affected by GAA, while immunocontent of α3 subunit of Na^+,K^+-ATPase was increased, indicating that this enzyme underwent an adaptive compensatory mechanism. Creatine appeared to act as an energy reservoir, antioxidant and anti-inflammatory agent, as well as a neuromodulator, since it was able to prevent almost every biochemical alteration detected in this study, as shown by Fig. 5. Creatine is a safe approach to improve the quality of life of GAMT deficiency patients, and our work may have put some light upon how creatine acts in the presence of high concentrations of GAA in the CNS, specifically in the striatum.

Compliance with ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The ethical standards followed the official governmental guidelines issued by the Brazilian Federal of Societies for Experimental Biology, following the Guide for Care and Use of Laboratory Animals and Arouca Law (Law no. 11.794/2008) and the experimental protocol was approved by the University's Ethics Committee (CEUA) under the project #29887.

Transparency document

The Transparency document associated with this article can be found, in online version.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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