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Involvement of the Cholinergic Parameters and Glial Cells in Learning Delay Induced by Glutaric Acid: Protection by *N*-Acetylcysteine

Fernanda Silva Rodrigues[1,2,3,4] · Viviane Nogueira de Zorzi[1,2] · Marla Parizzi Funghetto[1,2] · Fernanda Haupental[1,2] · Alexandra Seide Cardoso[1,2] · Sara Marchesan[5] · Andréia M. Cardoso[5] · Maria Rosa C. Schinger[5] · Alencar Kolinski Machado[6] · Ivana Beatrice Mânica da Cruz[6] · Marta Maria Medeiros Frescura Duarte[6] · Léder L. Xavier[7] · Ana Flavia Furian[6] · Mauro Schneider Oliveira[6] · Adair Roberto Soares Santos[3,4] · Luiz Fernando Freire Royes[2,4,5,6] · Michele Rechia Fighera[1,2,4,5,6]

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Abstract

Dysfunction of basal ganglia neurons is a characteristic of glutaric acidemia type I (GA-I), an autosomal recessive inherited neurometabolic disease characterized by deficiency of glutaryl-CoA dehydrogenase (GCDH) and accumulation of glutaric acid (GA). The affected patients present clinical manifestations such as motor dysfunction and memory impairment followed by extensive striatal neurodegeneration. Knowing that there is relevant striatal dysfunction in GA-I, the purpose of the present study was to verify the performance of young rats chronically injected with GA in working and procedural memory test, and whether *N*-acetylcysteine (NAC) would protect against impairment induced by GA. Rat pups were injected with GA (5 μmol g body weight^{-1}, subcutaneously; twice per day; from the 5th to the 28th day of life) and were supplemented with NAC (150 mg/kg/day; intragastric gavage; for the same period). We found that GA injection caused delay procedural learning; increase of cytokine concentration, oxidative markers, and caspase levels; decrease of antioxidant defenses; and alteration of acetylcholinesterase (AChE) activity. Interestingly, we found an increase in glial cell immunoreactivity and decrease in the immunoreactivity of nuclear factor-erythroid 2-related factor 2 (Nrf2), nicotinic acetylcholine receptor subunit alpha 7 (α7nAChR), and neuronal nuclei (NeuN) in the striatum. Indeed, NAC administration improved the cognitive performance, ROS production, neuroinflammation, and caspase activation induced by GA. NAC did not prevent neuronal death, however protected against alterations induced by GA on Iba-1 and GFAP immunoreactivities and AChE activity. Then, this study suggests possible therapeutic strategies that could help in GA-I treatment and the importance of the striatum in the learning tasks.

Keywords Glutaric acid · Striatum · *N*-acetylcysteine · Procedural learning · Memory · Acetylcholinesterase activity · α7nAChR · Inflammation

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✉ Michele Rechia Fighera
mrfighera@yahoo.com.br

[1] Centro de Ciências da Saúde, Departamento de Neuropsiquiatria, Laboratório de Neuropsiquiatria Experimental e Clínico, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil

[2] Centro de Educação Física e Desportos, Departamento de Métodos e Técnicas Desportivas, Laboratório de Bioquímica do Exercício (BIOEX), Universidade Federal de Santa Maria, Santa Maria, RS, Brazil

[3] Centro de Ciências Biológicas, Laboratório de Neurobiologia da Dor e Inflamação, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil

[4] Centro de Ciências Biológicas, Programa de Pós-Graduação em Neurociências, Universidade Federal de Santa Catarina, Florianópolis, SC 88040-900, Brazil

[5] Centro de Ciências Naturais e Exatas, Programa de Pós-graduação em Ciências Biológicas: Bioquímica Toxicológica, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil

[6] Centro de Ciências da Saúde Programa de Pós-Graduação em Farmacologia, Departamento de Fisiologia e Farmacologia, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil

[7] Faculdade de Biociências, Laboratório Central de Microscopia e Microanálise, Departamento de Ciências Fisiológica, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS 90610-000, Brazil

Introduction

Basal ganglia (BG) dysfunctions are often caused by imbalance between dopaminergic and cholinergic neuromodulation. While the role of the basal ganglia regions has been restricted to motor function [1], recent research has suggested that BG alterations are also involved in a variety of cognitive impairment [2–5]. Furthermore, studies show that memory impairment presented by patients with neurological diseases may be caused, at least in part, by functional changes in the cholinergic system [6–8].

Studies have also revealed, which in some inborn errors of metabolism (IEM), patients present cognitive dysfunctions and changes in cholinergic transmission [9–11], as well as damage in neurons and glia cells in BG [2, 3, 5].

In this context, glutaric acidemia type I (GA-I), an IEM due to deficiency of glutaryl-CoA dehydrogenase (GCDH) activity [12–14], causes an accumulation of glutaric acid (GA) and related metabolites in the brain of GA-I patients [14], accompanied frequently by neurological symptoms. The affected patients present clinical manifestations such as motor dysfunction and memory impairment followed by progressive cortical and extensive striatal neurodegeneration [14–17].

Studies have showed that procedural memory (PM) deficit is one of the symptoms observed in inherited metabolic diseases and other conditions involving the basal ganglia dysfunction [4, 18, 19]. The main input component of the basal ganglia is the striatum, and this cerebral structure exhibits a critical role in neuronal signal orchestration from cortical and subcortical regions that underlie procedural learning, working memory, and attentional set-shifting [1, 20, 21]. Functional and structural striatal lesions, as low release on acetylcholine (ACh), are related with cognitive process impairment, such as procedural learning. It is believed that ionotropic nicotinic receptors are responsible for fast cholinergic transmission in the striatum [22], and thus are intimately involved in the cognitive processes dependent of the striatum. In fact, previous studies have demonstrated that degeneration of cholinergic neurons [23] in Parkinson's disease brains is associated with the development of psychosis and cognitive disturbance [24, 25]. These findings indicate that the cholinergic system is essential to cognition and pathological conditions in which striatal ACh signaling is disrupted which causes impairment in memory and learning. In this sense, it is known that GA accumulation acts as a potent neurotoxic agent leading damage in cellular function, especially in the striatum.

Furthermore, the cholinergic pathway also plays an important role in the regulation of cognitive process and inflammation. Cholinergic pathway is one of the pathways involved in the regulation of neuroinflammation [26]. The nicotinic acetylcholine receptor subunit alpha 7 (α7nAChR) is expressed on both neurons and non-neuronal cells, including (but not limited to) glia and microglia [27]. In this way, the α7nAChR activation is thought to decrease the abnormal activation of microglia and astrocytes and consequently to induce decrease in pro-inflammatory molecules release, as cytokines and reactive oxygen or nitrogen species (ROS or RNS) [26, 28]. Although the cholinergic system is a key point in neurological diseases that affect the basal ganglia and compromise the cognitive process of patients [5, 7, 10], there are still no studies in the literature on the relationship of the situations mentioned above and GA accumulation in GA-I.

Besides participation in neuroinflammation, the role of glial cells in cognition process as learning and memory also has been discussed [29, 30]. Glial cells play key roles in brain development, actively participating in the generation of synapses, blood-brain barrier formation, and axon development [31]. Glial damage has been implicated in several developmental and perinatal central nervous system (CNS) disorders [32, 33], and neurons are expressively impaired by a toxicity of glial cell dysfunction [34] condition that has been shown in animal models of GA-I [3, 33, 35].

Therefore, considering all data mentioned above, we could consider that disturbances in the cholinergic system contribute, along with other mechanisms, to the neurological dysfunction characteristic of the EIMs. However, there is yet no data in the literature regarding the cholinergic system in GA-I. Thus, the purpose of the present study was to verify the performance of young rats injected with GA in working and procedural memory tests, and involvement of the cholinergic parameters and glial cells in striatal dysfunction, as well as if the NAC treatment could improve biochemical and behavioral parameters induced by GA.

Experimental Procedures

Ethics Statement

Laboratory experiments were performed in accordance with national and international legislations (Brazilian College of Animal Experimentation (COBEA) and the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals-PHS Policy) and approved by the Ethics Committee for Animal Research of Universidade Federal de Santa Maria (UFSM; Permit Number: 116/2010) and Universidade Federal de Santa Catarina (UFSC; Permit Number: 5386180317). Indeed, animal handling and laboratory assays were carried out in such a way that all efforts were made to minimize suffering.

Reagents

Unless otherwise stated, reagents were purchased from Sigma (St. Louis, MO, USA).

Animals

We utilized Wistar rats with 5 days of life. Pregnant Wistar rats were housed in individual cages and left undisturbed during gestation. Forty-eight hours after delivery, litters were culled to eight male pups. Mother fed pups since birth until 21 days of life when they were weaned. Animals were divided in order to have the same number of rats for each treatment in each cage. Animals had free access to water and to a standard commercial chow and were maintained on a 12:12-h light/dark cycle in an air-conditioned constant temperature (24 ± 1 °C, 55% relative humidity) colony room.

In Vivo Treatment

GA Injection and NAC Administration

Buffered GA, pH 7.4 (5 μmol g body weight^{-1}), was administered subcutaneously, twice a day, from the 5th to the 28th day of life to produce brain concentrations of GA around 0.6 μmol g^{-1}, ~ 0.72 mM [36], similar to concentrations found in patients with GA-I. Control animals received saline subcutaneously in the same volumes and frequency. All solutions were prepared so that each animal received 10 μL solution g body weight^{-1}. Besides, the rat pups were supplemented with NAC (150 mg/kg) or vehicle (saline 0.9%), by intragastric gavage, one per day, from the 5th to the 28th day of life [37].

Cognitive Task

Behavioral experiments were carried out between 9:00 AM and 6:00 PM at least 1 h after animal habituation to the room. Animals were assessed on the radial maze on postnatal days 21–32 and another set of animals was assessed on the water maze task on postnatal days 28–32.

Radial Arm Maze Task

We used this task to evaluate working and procedural memory of rat pups. The maze consists of a wooden eight-arm radial maze that was secured to a wooden base and elevated 100 cm from the floor. Cornflakes chips were used as reinforces which were placed in circular plastic Petri dishes that were attached to the ends of the maze arms. Four open Petri dishes, without lids, were used to house the obtainable cornflakes chips in the reinforced arms. In the non-reinforced arms, four other Petri dishes with lids that had several small holes drilled had cornflakes chips inside of them. This meant that the rats could not obtain them but the odor of the cornflakes chips was allowed to permeate from the dishes preventing the rats from solving the task using the smell of the cornflakes. The center hub of the maze and the arms were wiped clean using alcohol 30% both between each training session for a given rat and between each rat. A digital stopwatch was used to record time taken to consume the first cornflakes chips and the amount of time it took a rat to complete a trial. The protocol test was realized according to Kay et al. [38] with some modifications. The protocol was composed of 12 days: 1 day of habituation, 7 days of training, and 4 days of testing. During the acquisition period, the animals were trained to find the cornflakes pellets in the labyrinth arms. On the test days, four arms were randomly selected to contain a readily available pellet, while in the other four arms the cornflakes was not available. The animals were submitted to three screenings of 5 min/day or even to consume all four cornflakes. During the test, working memory is required to prevent entry into arms that never contained reinforcement or re-entry into the pellet arms. Thus, a working memory error was defined as the entry and re-entry in the arms that never contained the pellet and also the re-entry in the arms with the cornflakes pellet. The time the animal took to consume the first pellet and the total time to complete the test were also analyzed as a way to evaluate the procedural learning pattern of the animals.

Water Maze Task

Another group of rats was submitted to a cued version of the water maze as previously described by Prediger et al. [39] which evaluate the procedural memory. The water maze task was performed in a black circular swimming pool similar to that described by Morris et al. [40]. The apparatus (1.7 m inside diameter/0.8 m high) was filled to a 0.6-m water depth maintained at 24 °C (± 2). North (N), south (S), east (E), and west (W) were established as four starting points and divided the maze surface into four quadrants: northeast (NE), northwest (NM), southeast (SE), and southwest (SW). The platform used in the test was transparent acrylic (10 × 10 cm) and remained submerged 1–1.5 cm from the surface of the water. As a visual clue, a white ball (7 cm in diameter) was attached to the upper surface of the submerged platform, allowing the ball to remain visible on the surface of the water. The apparatus was located in a room with indirect incandescent illumination.

The animals were submitted to four consecutive training sessions per day for 4 days in the water maze. In each training, the animals were released from the four starting points (N, S, E, W) and a visible platform's position was to be changed in each training between the four different quadrants NE, NM, SE, and SW. The animals were left in the tank facing the wall and allowed to swim freely until they find a platform or up to a maximum time of 60 s. When an animal did not find a platform at that time, it was gently guided to it, where it remained for 10 s. The latency times until the animal found the platform were recorded. After 10 s, the animal was removed from the platform and placed in a box outside the water maze for 20 s.

In this interval, the platform position was changed in the next training. The starting position and position of the platform were arranged so that distances (proximal and distal) were counterbalanced between training. The data are shown as mean of the latencies (s) of the four training sessions of the day.

Western Blot Analyses

Western blot analysis was performed according to Leite et al. [41] with some modifications. Samples of the striatum were lysed on ice in RIPA (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). The samples (20 μg of protein) and pre-stained molecular weight standards (Bio-Rad) were then subjected to a 14% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane using Trans-Blot® Turbo™ Transfer System (25 V; 1.0 A; 35 min for proteins above 25 kDa or 5 min for proteins below 25 kDa) and equal protein loading was confirmed by Ponceau S staining. After specific blocking (5% bovine serum albumin solution or 5% non-fat dry milk), the blots were incubated overnight at 4 °C with rabbit anti-Iba-1 (ionized calcium-binding adapter molecule 1) (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-GFAP (glial fibrillary acidic protein) (1:1.000; Dako), rabbit anti-α7nAChR (nicotinic acetylcholine receptor alpha 7) (1:5.000; Abcam), and mouse anti-NeuN (neuronal nuclear protein) (1:2.000; Millipore). Mouse anti-actin (1:5000, Cell-Signaling) was stained as additional control of the protein loading. After primary antibody incubation, membranes were washed with TBS-T (TBS plus 0.1% Tween 20) three times at room temperature for 15 min and incubated with anti-rabbit (Sigma-Aldrich—A6154) or anti-mouse (Santa Cruz Biotechnology—sc2005) secondary antibodies conjugated with horseradish peroxidase (1:5.000 for anti-Iba-1 and α7nAChR, 1:10.000 for anti-GFAP and 1:20.000 for anti-NeuN) for 2 h at room temperature. Bands were visualized by enhanced chemiluminescence using ECL Western Blotting Substrate (Pierce ECL, Bio-Rad) and the signals were captured with fotodocumentador ChemiDoc XRS+ (Bio-Rad). Then the bands were quantified by using Image Lab software (Bio-Rad). Values are expressed as a percentage of the control.

IL-1β and TNF-α Immunoassay

The striatum was weighted and homogenized (1:10) in a solution containing bovine serum albumin (BSA 10 mg/mL), EGTA (2 mM), EDTA (2 mM), and PMSF (0.2 mM) in phosphate-buffered saline (PBS, pH 7.4) using a Potter homogenizer. The striatum homogenate was centrifuged (3.000*g* for 10 min) and cytokines were determined in supernatant. Cytokine levels were measured using a commercially available ELISA Kit from Rand D Systems (Minneapolis, MN) using an antibody selective against rat IL-1β and TNF-α, according to the manufacturer's protocol. Results are expressed in picograms/milligram of protein for striatum homogenate assays. Absorbance was read at 450 nm. The detection limit was 4 ng/mL.

Acetylcholinesterase Activity

The acetylcholinesterase (AChE) enzymatic assay was determined by a modification of the spectrophotometric method of Ellman et al. [42]. The reaction mixture (2 mL final volume) contained 100 mM K^+-phosphate buffer, pH 7.5, and 1 mM 5,5′-dithio-bis-nitrobenzoic, measured by absorbance at 412 nm during 2-min incubation at 25 °C. The enzyme (40–50 μg of protein) was pre-incubated for 2 min. The reaction was initiated by adding 0.8 nM acetylthiocholine iodide. All samples were run in duplicate or triplicate and the enzyme activity were expressed in micromole AcSCh/h/mg of protein.

Measurement of Oxidative Stress

We estimated the content of thiobarbituric acid reactive substances (TBARS), protein carbonyl, and NOx (NO_2 plus NO_3 levels) as a marker of NO on the striatum of rat pups. We also evaluated the superoxide dismutase (SOD), catalase (CAT) activity, and NPSH content. The description of experimental procedures can be found in the supplementary materials.

Isolation and Cell Culture

For the isolation and cell culture, the striatum of rat pups was treated previously with GA and/or NAC. The animals were euthanized and the structure was removed using sterile materials. After removal, the specimens were placed in Petri dishes containing 2 mL of Hanks solution glycosylated, sectioned, and slightly macerated.

Shortly thereafter, the materials were filtered and the content was prepared in 15-mL falcon tubes. In order, 4 mL of cell culture medium Dulbecco's modified Eagle's medium (DMEM) was added, with 10% fetal bovine serum (FBS) and supplemented with 1% penicillin/streptomycin and 1% antifungal amphotericin B. The content was homogenized and centrifuged for 10 min at 2000 rpm, and then the supernatant was removed and the cell pellet was resuspended in complete culture medium.

Cells isolated from each frame of interest were prepared on sterile 96-well plates, with 200 μL per well, and kept at optimal cell culture conditions in CO_2 incubator at 37 °C and saturation of 5% CO_2 for 24 h to stabilize the cells. After the incubation period, the cultures were tested for apoptosis-related proteins, caspase-1, caspase-3, and caspase-8,

including starting and effector caspases, using a specific fluorimetric method founded on activation of ICE family caspases in mammalian cells (BioVision Incorporated, Milpitas, CA, USA). This method uses specific known molecules conjugated to AFC (7-amino-4-trifluoromethylcoumarin) substrate and is based on the capacity of caspase-1, caspase-3, or caspase-8 to recognize and cleave this structure. The result of substrate cleavage is a yellow light emission, which is quantified through a fluorescence plate reader at λmax = 505 nm when excited at λmax = 400 nm. The quantitative procedure was performed through the comparison of treated groups to untreated samples after protein amount normalization [43].

Protein Determination

Protein content was measured colorimetrically by the method of Bradford [44] using bovine serum albumin (1 mg/mL) as a standard.

Statistical Analysis

The statistical analysis was carried out by two-way analysis of variance (ANOVA) and only F values of $p < 0.05$ are presented. Post hoc analysis was carried out by Duncan's multiple comparisons test, when appropriate. The data were expressed as mean ± S.E.M or as a percentage of the control. Statistical analyses were performed utilizing the SPSS 20 software in a PC-compatible computer and the figures were performed using Graph Pad Prism software (version 6.0).

Results

Effect of Chronic GA Injection on Radial Arm Maze Task

To investigate whether GA treatment affects working memory and procedural memory formation, the rat pups were evaluated in the radial arm-maze task. Statistical analysis (two-way analysis of variance—ANOVA) revealed that no one treatment altered working memory errors ($F(1,49) = 0.23$; $p > 0.05$; Table 1). However, when we examine the procedural memory, statistical analysis showed that GA increased the time of first latency to consume the cornflakes chip in the bait ($F(1,49) = 9.41$; $p < 0.05$; Fig. 1) as compared with control group. Post hoc analysis also revealed the effect of NAC treatment on the first latency to consume the cornflakes chip in the bait to the first day of the test ($F(3,49) = 5.92$; $p < 0.05$; Fig. 1b) and the second day ($F(3,49) = 2.99$; $p < 0.05$; Fig. 1c). Furthermore, statistical analysis revealed that GA increased the total time taken to consume the cornflakes chip of all four baits (completion time in seconds) ($F(1,49) = 5.38$; $p < 0.05$; Fig. 2) as compared with the control group in radial arm-maze task. Post hoc analysis revealed the effect of NAC on the first day of the test ($F(3,49) = 6.72$; $p = 0.001$) and second day ($F(3,49) = 3{,}41$; $p < 0.05$) on the delay to consume the cornflakes chip induced by GA (Fig. 2b, c).

Effect of Chronic GA Injection on Water Maze Task

To confirm whether GA treatment affects procedural memory formation, the rat pups were evaluated in water maze task**.** Statistical analysis (two-way analysis of variance—ANOVA) revealed that GA increased the latency to escape ($F(1,49) = 9.80$; $p < 0.01$; Fig. 3) as compared to the control group. However, post hoc analysis showed that the NAC treatment prevented the increase of latency to escape on the second day ($(3,49) = 4.94$; $p < 0.01$) and third day of the test ($(3,49) = 5.11$; $p < 0.01$).

Effect of Chronic GA Injection on GFAP and Iba-1 Immunoreactivities

Statistical analysis showed higher GFAP ($(1,26) = 5.36$; $p < 0.05$; Fig. 4(a)) and Iba-1($(1,16) = 5.77$; $p < 0.05$; Fig. 4(b)) immunoreactivity induced by GA as compared to the control group. Statistical analysis showed that NAC treatment was able to decrease GFAP ($(1,26) = 4.20$; $p = 0.05$) and Iba-1 ($(1,16) = 4.31$; $p < 0.05$) immunoreactivities in the striatum of animals treated with GA.

Effect of Chronic GA Injection on Inflammatory Parameters

The effect of GA on TNF-α and IL-1β concentration in the striatum of rat pups is shown in Fig. 5.

In addition, the statistical analysis revealed that NAC administration prevented the increase induced by GA on TNF-α ($F(1,24) = 2.87$; $p = 0.05$; Fig. 5a) and IL-1β ($F(1,28) = 48.86$; $p < 0.001$; Fig. 5b) when compared with control group.

Effect of NAC on Markers of Oxidative Damage

The supplementary Fig. 1 showed protective effect of NAC on increased TBARS content ($F(1,28) = 6.37$; $p < 0.05$; Suppl. Fig. 1a), on the content of protein carbonyl GA ($F(1,27) = 7.01$; $p < 0.05$; Suppl. Fig. 1b), and on NOx levels ($F(1,22) = 4.13$; $p < 0.05$; Suppl. Fig. 1a) induced by GA on the striatum of rat pups. Please to see the Supplementary Material for more details.

Effect of NAC on Nrf2 and Antioxidant Defenses

The supplementary Fig. 2 showed that NAC prevented the decreased Nrf2 immunoreactivity in the striatum of animals

Table 1 Effects of early postnatal chronic GA and NAC administration on number of working memory errors of rat pups in the radial arm maze task

Number of working memory errors					
Group	Day 1	Day 2	Day 3	Day 4	*N*
SAL	1.28 ± 0.9	0.76 ± 0.6	0.78 ± 0.7	2.06 ± 2.4	13
AG	1.76 ± 1.2	1.14 ± 0.8	1.27 ± 1.0	2.05 ± 1.0	14
NAC	1.29 ± 1.0	0.71 ± 0.7	0.59 ± 0.4	2.5 ± 2.5	12
GA + NAC	0.78 ± 0.5	1.05 ± 0.9	1.17 ± 1.2	2.6 ± 3.4	14

Data represent means ± S.E.M. for $n = 12–14$ in each group. No significant differences between groups were detected

((1,29) = 5.11; $p > 0.05$). The NAC treatment prevented the decrease of SOD ($F(1,24) = 44.94$; $p < 0.001$; Suppl. Fig. 3a), and CAT activity ($F(1,24) = 6.15$; $p < 0.05$; Suppl. Fig. 3b), as well as decrease of NPSH content ($F(1,24) = 19.26$, $p < 0.001$; Suppl. Fig. 3c) induced by GA. For more details, see the Supplementary Material.

Effect of Chronic GA Injection on AChE Activity and α7nAChR

Since studies have shown the involvement of the cholinergic pathways in brain immune responses [45, 46] and in the process of learning [47], we decide to determine the AChE activity and α7nAChR immunoreactivity in the striatum of rat pups (Fig. 6). The statistical analysis showed that GA ($F(1,28) = 7.27$; $p < 0.05$) increased the AChE activity and treatment with NAC prevented this effect ($F(1,28) = 5.81$; $p < 0.01$; Fig. 6(a)). Western blot analysis revealed a decrease in α7nAChR immunoreactivity induced by GA ($F(1,33) = 4.68$; $p < 0.05$; Fig. 6(b)) and NAC treatment was able to prevent this effect ($F(1,33) = 5.47$; $p < 0.05$).

Effect of Chronic GA Injection on the Apoptotic and Neuronal Biomarkers

Effect of NAC on the apoptotic markers in the striatum of rat pups is shows in Fig. 7. The statistical analysis revealed that NAC protected against an increase on caspase-1 (Fig. 7(b)), caspase-3 (Fig. 7(c)), and caspase-8 (Fig. 7(d)) induced by GA ($F(1,24) = 13.08$; $p < 0.001$; $F(1,24) = 6.19$; $p < 0.05$; $F(1,24) = 16.19$; $p < 0.0001$; respectively). Statistical analysis also revealed lower NeuN immunoreactivity induced by GA ($F(1,26) = 5.82$; $p < 0.05$; Fig. 7(a)). Unfortunately, NAC treatment did not prevent this effect ($F(1,26) = 1.04$; $p > 0.05$).

Discussion

We provide evidence for the first time that GA chronic injection induced cognitive impairment, neuroinflammation, higher AchE activity, and lower α7nAChR immunoreactivity associated to glial and neuronal dysfunction in the striatum of rat pups. Interestingly, NAC treatment was able to attenuate glial cell proliferation and cytokine levels, as well as to

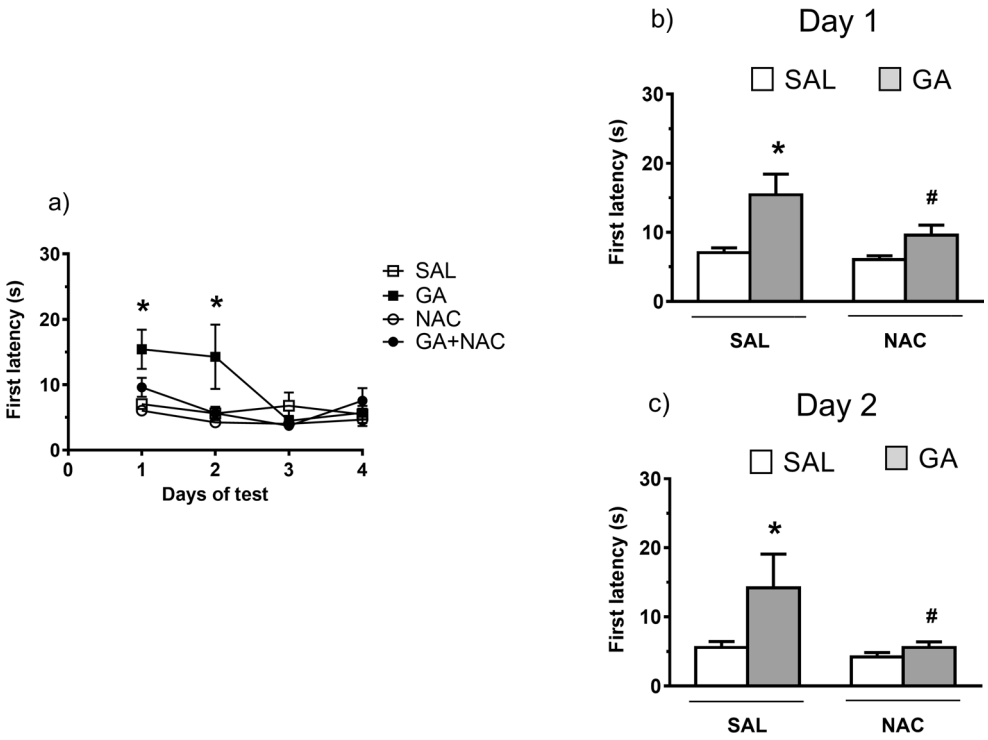

Fig. 1 Effects of early postnatal chronic GA and NAC administration on time taken to consume the first cornflakes chip in the bait in radial arm maze task (first latency). $^{*}p < 0.05$ compared with saline-treated group, $^{\#}p < 0.05$ compared with GA-treated group (Duncan's multiple comparisons test). Data are presented as means ± S.E.M. for $n = 12–14$ in each group. **b**, **c** shows an amplification of day 1 (**b**) and day 2 (**c**) of test, respectively

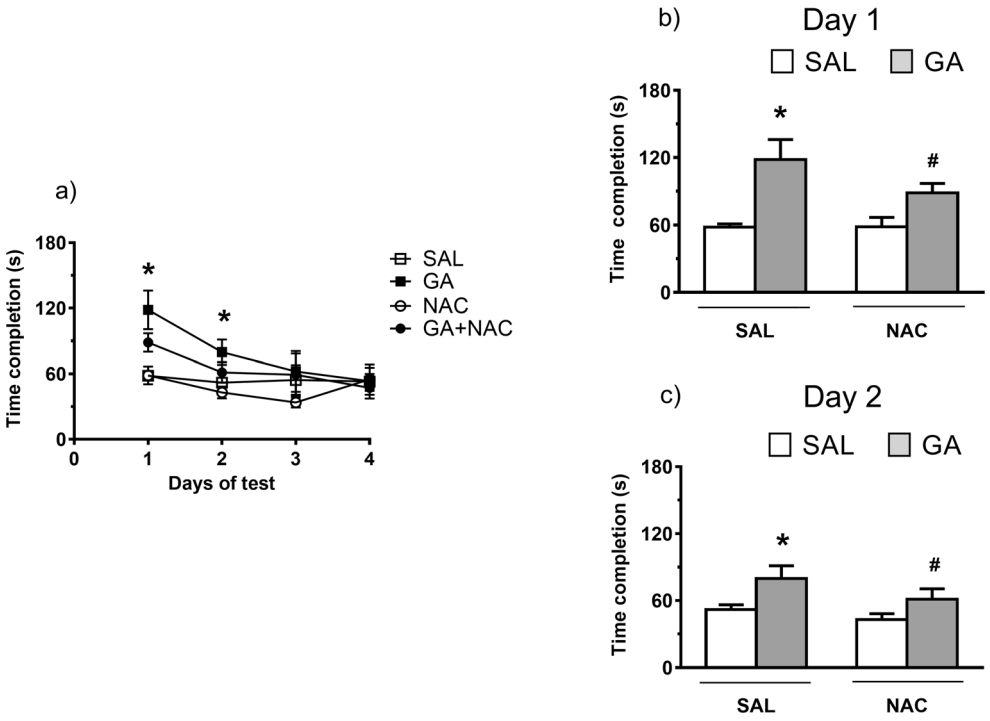

Fig. 2 Effects of early postnatal chronic GA and NAC administration on time taken to consume all four baits in radial arm maze task (time completion). $^{*}p < 0.05$ compared with the saline-treated group, $^{\#}p < 0.05$ compared with the GA-treated group (Duncan's multiple comparisons test). Data are presented as means ± S.E.M. for $n = 12–14$ in each group. **b**, **c** shows an amplification of day 1 (**b**) and day 2 (**c**) of test, respectively

normalize cholinergic parameters and caspase activation and improve the cognitive performance.

It is important to note that our group already showed that chronic GA and NAC administration did not cause effect on body weight of rat pups, providing an indication that the chronic injection did not cause malnutrition in animals. Similarly, we also showed that the same treatment had no effect in locomotor activity and was not apparently anxiogenic in animals [48]. These observations are important since malnourished and anxious animals may behave differently in neurobehavioral tests [49, 50], as well as altered locomotor activity may mask results in behavioral tests, since the resolution of tasks depends on the locomotion of animals.

In this work, we submitted the rat pups to radial arm maze (RAM) task to evaluate working and procedural memory (PM). The PM is the capacity to acquire cognitive and behavioral skills that subsequently operate automatically [51]. The procedural memory system recruits cortico-basal ganglia loops. The encoding of PM is initiated in cortical regions that send information to the basal ganglia where encoding largely occurs within the striatum [52]. Pathologies that damage the striatum exhibit PM deficit [4, 18]. Interestingly, in RAM task, we found that latency to consumption of the first cornflakes chip, designated as first latency (Fig. 1), and total time taken to consume cornflakes in the maze (Fig. 2) were higher after GA injection. Accordingly, it has been demonstrated that the PM

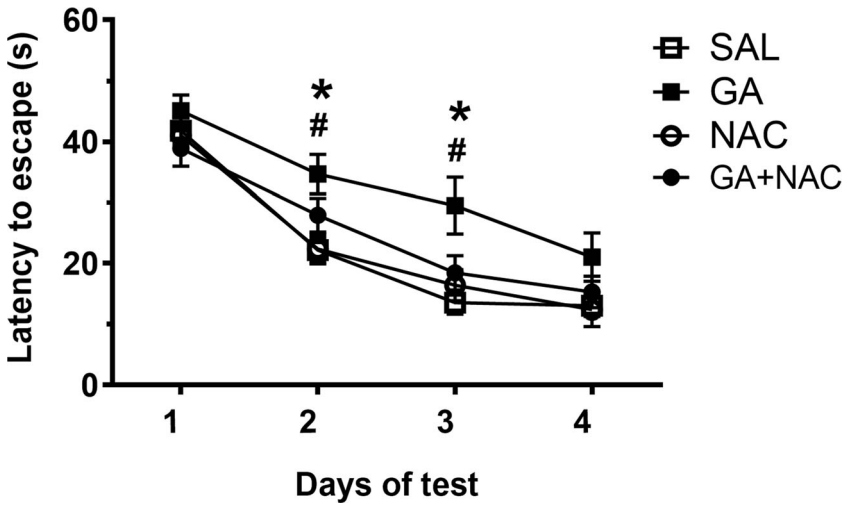

Fig. 3 Effects of early postnatal chronic GA and NAC administration on latency to escape (s) in procedural memory version of water maze task. $^{*}p < 0.01$ compared with the saline-treated group, $^{\#}p < 0.01$ compared with the GA-treated group (Duncan's multiple comparisons test). Data are presented as means ± S.E.M. for $n = 12–15$ in each group

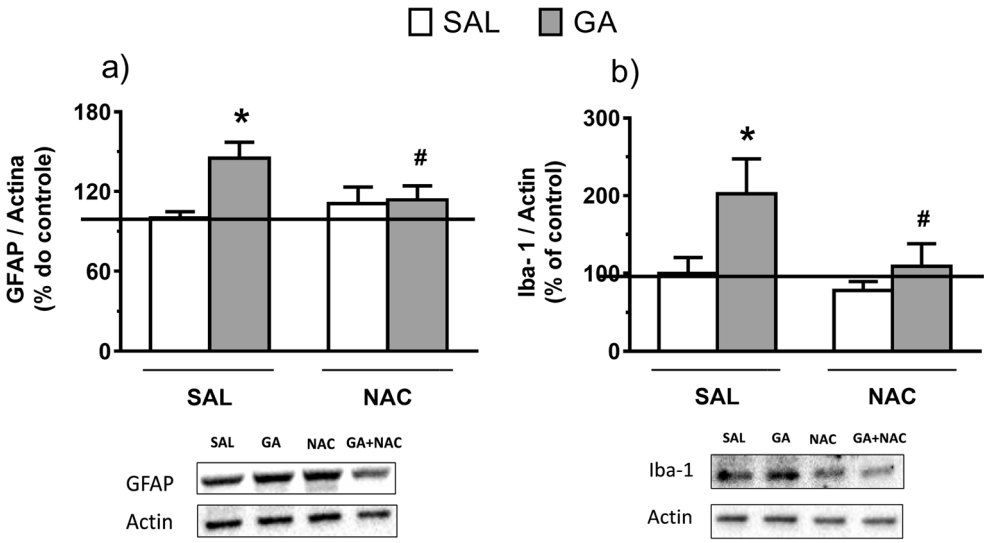

Fig. 4 Effect of early postnatal chronic GA and NAC administration on GFAP (a) and Iba-1 (b) immunoreactivity in rat pups' striatum. NAC prevented the increase of the GFAP and Iba-1 immunoreactivity induced by GA. $^{*}p < 0.05$ compared with saline-treated group, $^{\#}p = 0.05$ compared with GA-treated group (Duncan's multiple comparisons test). Data are expressed as percentage of the control for $n = 7–8$ (GFAP) and n = 5 (Iba-1) in each group

deficit is expressed as a disturbance of executive functions as result of striatal dysfunction [53], as well as being interpreted as a delay to initiate a response [54, 55]. Thus, delay to start and to complete the task exhibited by GA-treated animals can be understood as a delay in learning procedural. To confirm this hypothesis, the animals were also submitted to water maze task. In fact, we found that GA induced delay in procedural learning, as evidenced by increase in latency to find escape platform (Fig. 3).

Interestingly, GA administration did not modify the number of working memory errors in RAM task (Table 1). In agreement with this result, a recent developmental study of neuropsychological functions, specifically in relation to WM, revealed that patients with GA-I did not exhibit impaired performance in visual working memory task [56], suggesting that WM may be preserved in GA-I, even in patients with striatal degeneration. Then, our result suggests that animals injected with GA are able to have memory acquisition; however, learning process is slower due to PM impairment.

The control of PM and WM is performed by the striatum, the key input station in the basal ganglia [1, 20, 21], since it integrates cortical information and transmits it to basal ganglia output nuclei that generate more efficient behavioral responses [57]. One neurotransmitter that is highly enriched in the striatum and strongly modulates striatal integration is ACh [58]. The main source of striatal ACh is cholinergic interneurons (ChIs), which, although represent a low percentage of the striatal neuronal population, provide to this area one of the highest Ach levels in the brain [59, 60]. Thus, it is plausible to propose that striatal cell damage may change neurotransmitter levels, such as ACh, and cause procedural learning deficits [53]. In fact, studies have shown that a selective disruption of the cholinergic system may contribute to cognitive deficit in neurodegenerative diseases, including procedural learning [8, 61, 62].

One way to control the extracellular levels of acetylcholine is through the action of AChE, which catalyzes ACh hydrolysis in the synaptic cleft [21]. In our study, we found a higher

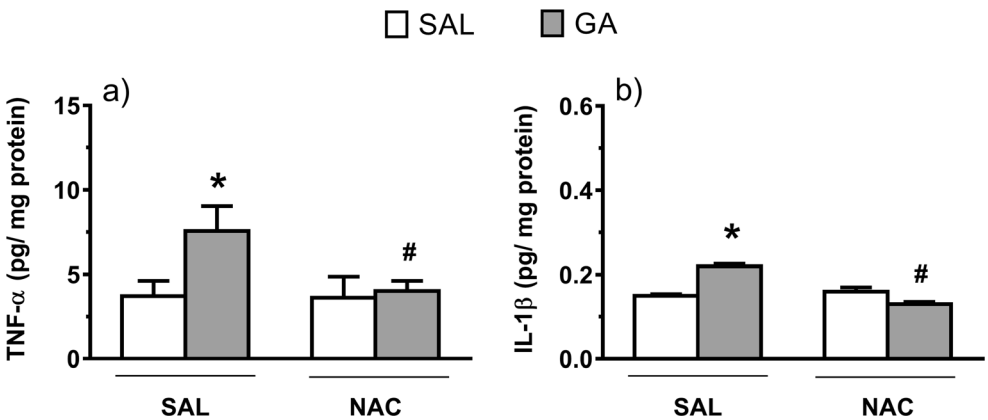

Fig. 5 Effect of early postnatal chronic GA and NAC administration on cytokine concentration in rat pups' striatum. NAC prevented the increase in TNF-α (**a**) and IL-1β (**b**) concentration induced by GA. $^{*}p = 0.05$ compared with the saline-treated group, $^{\#}p < 0.05$ compared with GA-treated group (Duncan's multiple comparisons test). Data are presented as means ± S.E.M. for $n = 7–8$ in each group

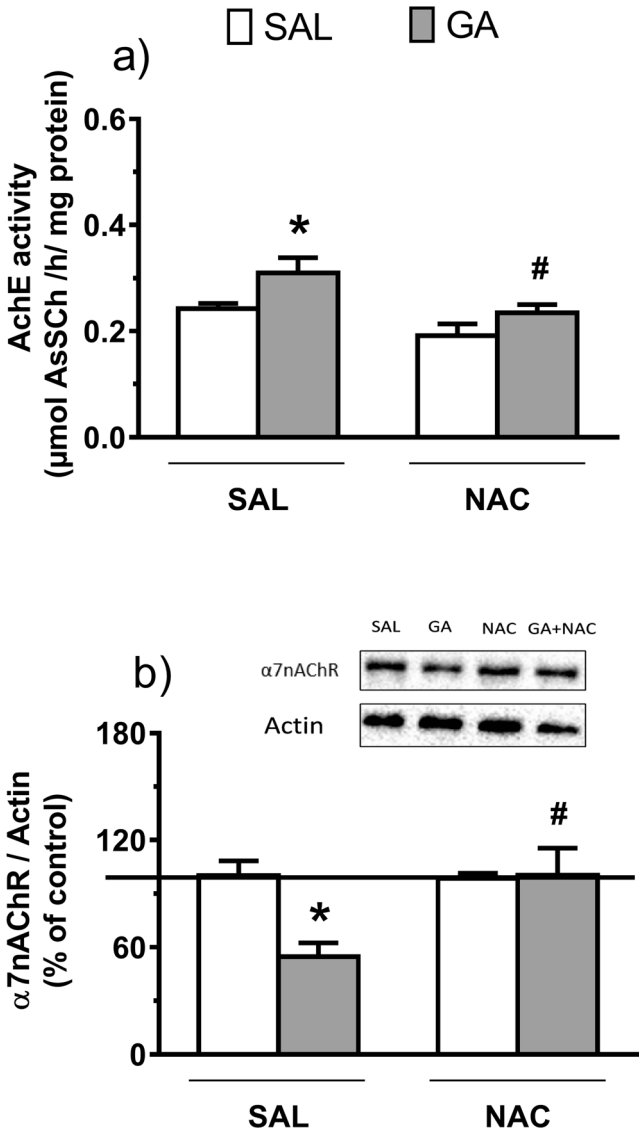

Fig. 6 Effect of early postnatal chronic GA and NAC administration on cholinergic markers. NAC prevented the changes in AChE activity (**a**) and α7nAChR immunoreactivity (**b**) induced by GA in the striatum of rat pups. $^{*}p < 0.05$ compared with the saline-treated group, $^{\#}p < 0.05$ compared with the GA-treated group (Duncan's multiple comparisons test). Data are expressed as means ± S.E.M. for $n = 8$ in each group (**a**) and as percentage of the control for $n = 7$–8 in each group (b)

AChE activity after GA treatment (Fig. 6(a)), suggesting that this biochemical alteration can lead to a lower content of ACh in the synaptic cleft and contribute to procedural learning deficit. Current evidence indicates that ACh is involved in the process of learning, controlling a repertoire of responses to novel information [47, 63]. In the striatum, ACh signaling induces to synaptic plasticity [64] and contributes to the cognitive functions of the striatum [57].

According to this line of view, the ACh can regulate neuronal excitability throughout the nervous system by acting on both the cys-loop ligand-gated nicotinic ACh receptor channels (nAChRs) and the G protein-coupled muscarinic ACh receptors (mAChRs) [65]. Researches have already shown that there is a decrease in cholinergic receptors in CNS pathologies such as Alzheimer's disease (AD) and Parkinson's disease (PD) and this dysfunction can be associated to clinical manifestations [8, 66]. In this sense, pharmacological strategies that aim to modulate cholinergic receptors, in particular the α7AChRs, have been studied in an attempt to minimize the neurological damages [66, 67]. In the striatum, α7AChRs is found in cortical excitatory afferents [68] and participates in induction of long-term potentiation (LTP), contributing to striatum-dependent learning [69]. In fact, Young et al. [63] showed that α7nAChR knockout (KO) mice exhibited delayed procedural learning, suggesting that this cholinergic receptor in the striatum is important for procedural learning. In this sense, we shall suggest that one of the hypotheses to explain the delayed learning induced by GA may be due to higher AChE activity associated to reduced α7nAChR immunoreactivity in the striatum of animals (Fig. 6(b)).

Beyond the role in memory process, signaling through nAChRs also plays an important role in the regulation of inflammation [70]. Cholinergic system has been suggested as mediator of neuro-immune interactions or internal regulator of immune responses [71]. It has been proposed that cholinergic anti-inflammatory pathway occurs through the activation of α7nAChR, which inhibits nuclear translocation of NF-κB and suppresses cytokine pro-inflammatory release, both peripherally in monocytes and macrophages, as in CNS in microglia and astrocyte [26, 27, 45, 46]. In this line, our results showed that GA induced a higher AChE activity and lower α7nAChR immunoreactivity (Fig. 6), as well as higher TNF-α and IL-1β levels in the striatum (Fig. 5(a, b)). Indeed, loss of cholinergic neurons is associated with occurrence of seizures and mental retardation in patients with inherited metabolic disorders [72]. For these reasons, it is plausible to propose that the increased concentration of these inflammatory cytokines can be associated to lower ACh levels and consequent disability of learning [26, 63].

In addition, extensive evidences demonstrated that besides neuroinflammatory process, oxidative damage has a potential role for dysfunction or death of neuronal cells causing cognitive impairment in various neurodegenerative diseases [3, 73–75]. In this report, we also revealed that GA treatment induced higher lipid peroxidation, protein carbonylation, and NOx levels (Fig. 1 Supplementary Material). In fact, some studies show GA-induced excitotoxicity results in oxidative stress and neuroinflammation [73, 76–78]. Moreover, impairment in spatial learning also was associated to increase of TNF-α and IL-1β and oxidative stress in the hippocampus of animals chronically treated with GA [48].

Furthermore, it has also been shown that activation of astrocytes and microglia in the striatum causes cognitive impairment [10]. In this sense, it is well established that astrocytes play an important role in CNS; they regulate the homeostasis

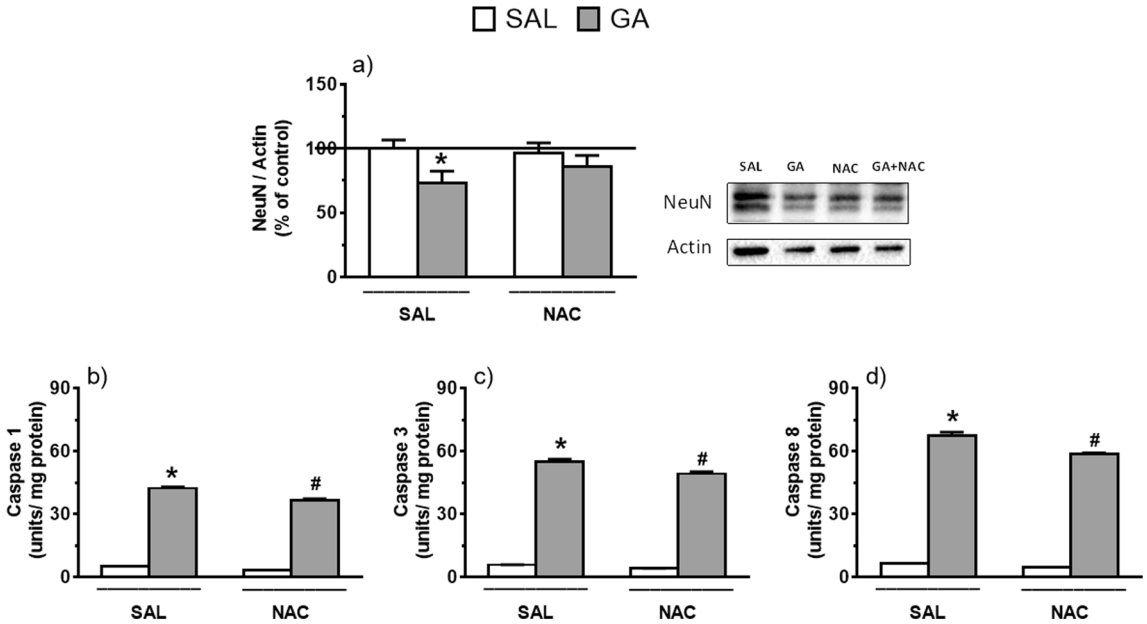

Fig. 7 Effect of early postnatal chronic GA and NAC administration on apoptotic markers and NeuN immunoreactivity of rat pups' striatum. NAC did not prevent the decrease of the NeuN immunoreactivity (a) induced by GA and partially protected against the increase of staining for caspase-1 (b), caspase-3 (c), and caspase-8 (d). *$p < 0.05$ compared with the saline-treated group, $^{\#}p < 0.05$ compared with the GA-treated group (Duncan's multiple comparisons test). Data are expressed as percentage of the control for $n = 5$ in each group (NeuN) and as means ± S.E.M. for $n = 7$ in each group (caspases)

of neurotransmitters and provide metabolic support for the neurons and fundamental factors for the appropriate development of neurons as well as the network between them [31]. In this line of view, we decided to investigate if microglia, astrocytes, and neurons could be dysfunctional in the striatum after GA treatment.

We observed that GA treatment induced higher GFAP and Iba-1 immunoreactivities in the striatum of animals (Fig. 4(a, b)). Since the activation of microglia and astrocyte produces inflammatory mediators [79] and the exposure to GA activates glial cells [33], we also can suggest that higher levels of cytokines induced by GA may be due to microglial and astrocytic activation. In fact, under neurotoxic conditions as neuroinflammation, astrocytes and microglia become overactivated and release a variety of oxidants and activate genes as well as proteins, including iNOS and pro-inflammatory cytokines like IL-1β and TNF-α [80–82]. Accordingly, Gabbi et al. [10] have also showed a higher glial activation and cytokine levels in the striatum associated to working memory impairment in an experimental model of methylmalonic acidemia.

Moreover, higher levels of pro-inflammatory and oxidative biomarkers induced by GA may also have been the trigger for a higher caspase-1, caspase-3, and caspase-8 activation in the striatum of rat pups (Fig. 7(b–d)). In fact, activation of caspase-1 may occur by its substrates, such as TNF-α and IL-1β, and reflect an increase of cellular inflammatory response. Furthermore, the caspase-8, combined with its ability to induce apoptosis through the extrinsic pathway, also triggers the intrinsic apoptosis pathway by cleaving the pro-apoptotic Bcl-2 family members to initiate mitochondria-induced apoptosis [83, 84]. These events induce a subsequent reactive species increase and release of mitochondrial apoptogenic factors to the cytosol and, consequently, lead to caspase-3 activation and cellular dysfunction [85]. Accordingly, Tian et al. [86] showed an upregulation in expression of caspase-3 and an apoptosis dependent of caspase-3 induced by GA in the striatum.

Indeed, the increase of caspases may be related to reduction of NeuN immunoreactivity in the striatum of the animals treated with GA (Fig. 7(a)). The damage caused by GA in the striatum was sufficient for triggering neuronal death, at least by method used in this work. Our data are according to Olivera-Bravo et al. [3] that have shown that acute GA administration induced neuronal death in the striatum of young animals. Thus, we suggest that inflammation and oxidative stress generated by astrocyte and microglia activation, as well as by imbalance in cholinergic parameters GA-induced, trigger activation of the apoptotic pathway that culminated in the death of striatal neurons, mechanisms that possibly are underlying to delay procedural learning.

Since using the same experimental model of GA-I, we found beneficial effects of NAC treatment in relation to oxidative and inflammatory hippocampal changes as well as spatial memory improvement [48]; we evaluated if NAC would maintain its protective effect on the striatum, since this structure is particularly sensitive to GA effects. Interestingly, we observed that NAC supplementation was effective in preventing the delay learning, increase of neuroinflammation,

oxidative mediators, and caspase levels. In addition, NAC also protected against the alterations induced by GA on Iba-1 and GFAP immunoreactivities, as well as on the AChE activity alteration and α7nAChR immunoreactivity induced by GA in the striatum of rat pups.

Accordingly, studies have shown positive effects of NAC as adjuvant treatment in the pathophysiology of neurodegenerative diseases such as AD and PD [87, 88], probably by modulating antioxidant [89, 90], inflammatory [91], and cholinergic system [92]. In this sense, NAC has prevented the cognitive disturbance by restoring the cholinergic system in the cortex, hippocampus, and the striatum of mouse [88], and it inhibited non-competitive AChE activity [93].

In addition, we showed that NAC prevented the reduction of α7nAChR immunoreactivity in the striatum, an important modulator of neuroinflammation [94]. Despite the studies about the involvement of the cholinergic system in GA-I which are only beginning, the alterations in AChE activity and α7nAChR immunoreactivity may suggest that these parameters possibly contribute to both neuroinflammation and cognitive impairment in this disorder.

Then, considering all data found in this work, we should suggest that the increase in inflammatory markers induced by GA might have resulted from activated microglia and astrocyte, increased AChE activity, and decreased α7nAChR immunoreactivity, leading to striatal neuronal death and delay of procedural learning that was responsive to NAC supplementation (Fig. 8). Although many questions remain unknown regarding cholinergic parameters in the pathogenesis of GA-I, this work reports the crucial role of the striatum in the learning tasks and reveals interesting neurochemical alterations as well as therapeutic strategies that could help in the treatment of this inherited metabolic disease. However, further studies are needed to better elicit how the response of the cholinergic

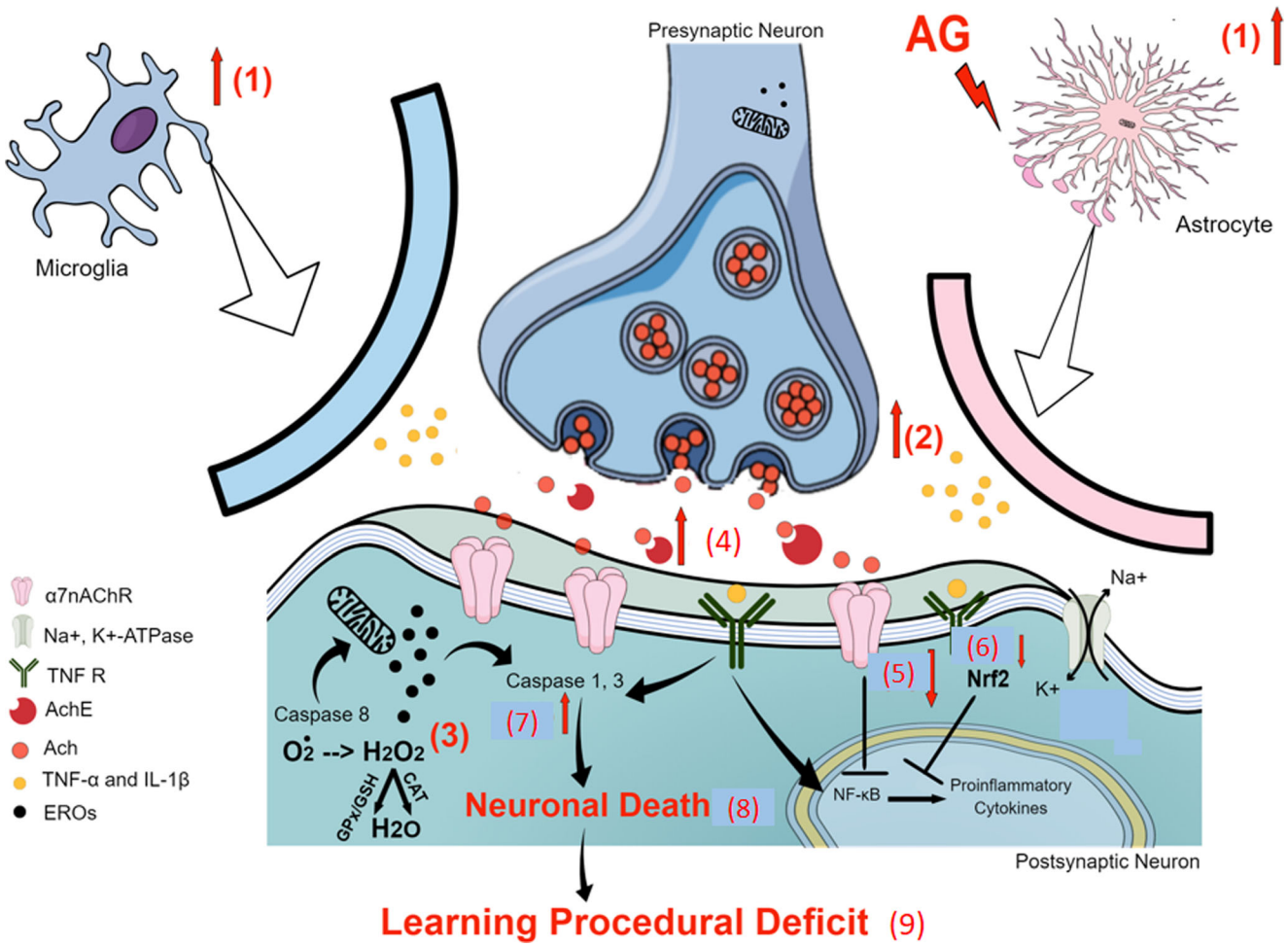

Fig. 8 Schematic illustration of the protective effect of NAC against neurotoxicity and learning deficit induced by GA in the striatum of rat pups. GA-induced increase in GFAP and Iba-1 immunoreactivity (1), increase cytokine TNF-α and IL-1β (2), oxidative stress (3), increase in AchE activity (4), reduced α7nAChR (5) and Nrf2 (6) immunoreactivity, activation of caspase-1, caspase-3, and caspase-8 (7) and neuronal death (8), and subsequent procedural learning deficit (9). NAC treatment prevented delay learning by decreased neuroinflammation, oxidative mediators, and caspase levels. In addition, NAC also protected against glial cell activation and AchE activity alteration induced by GA. (red up arrow = increased, red down arrow = decreased)

system to GA accumulation occurs and better understand the molecular mechanisms whereby the glutaric acid leads to neuronal death and disability of learning.

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Compliance with Ethical Standards

Laboratory experiments were performed in accordance with national and international legislations (Brazilian College of Animal Experimentation (COBEA) and the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals-PHS Policy) and approved by the Ethics Committee for Animal Research of Universidade Federal de Santa Maria (UFSM; Permit Number: 116/2010) and Universidade Federal de Santa Catarina (UFSC; Permit Number: 5386180317).

Conflict of interest All authors confirm that there is no competing financial conflict of interest.

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