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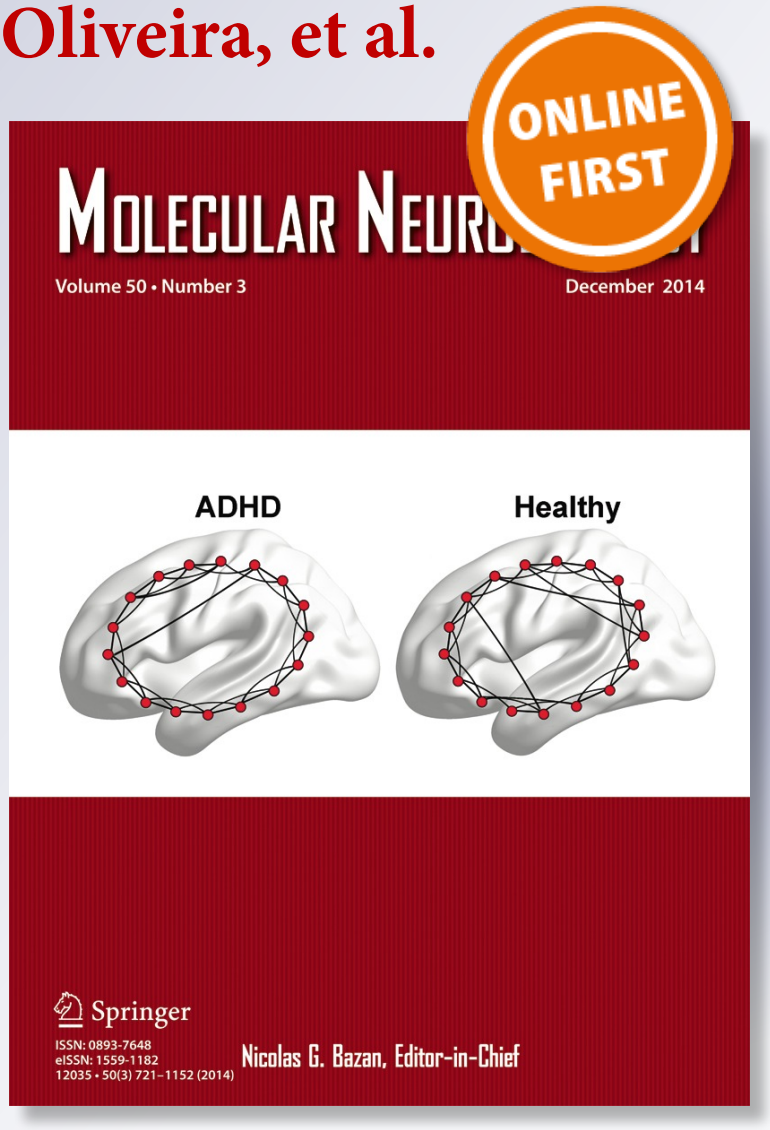

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Crosstalk Among Disrupted Glutamatergic and Cholinergic Homeostasis and Inflammatory Response in Mechanisms Elicited by Proline in Astrocytes

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Abstract Hyperprolinemias are inherited disorder of proline (Pro) metabolism. Patients affected may present neurological manifestations, but the mechanisms of neural excitotoxicity elicited by hyperprolinemia are far from being understood. Considering that the astrocytes are important players in neurological disorders, the aim of the present work was to study the effects 1 mM Pro on glutamatergic and inflammatory parameters in cultured astrocytes from cerebral cortex of rats, exploring some molecular mechanisms underlying the disrupted homeostasis of astrocytes exposed to this toxic Pro concentration. We showed that cortical astrocytes of rats exposed to 1 mM Pro presented significantly elevated extracellular glutamate and glutamine levels, suggesting glutamate excitotoxicity. The excess of glutamate elicited by Pro together with increased glutamate uptake and upregulated glutamine synthetase (GS) activity supported misregulated glutamate homeostasis in astrocytic cells. High Pro levels also induced production/release of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6. We also evidenced misregulation of cholinergic anti-inflammatory system with increased acetylcholinesterase (AChE) activity and decreased acetylcholine (ACh) levels, contributing to the inflammatory status in Pro-treated astrocytes. Our findings highlighted a crosstalk among disrupted glutamate homeostasis, cholinergic mechanisms, and inflammatory cytokines, since ionotropic (DL-AP5 and CNQX) and metabotropic (MCPG and MPEP) glutamate antagonists were able to restore the extracellular glutamate and glutamine levels; downregulate TNFα and IL6 production/release, modulate GS and AChE activities; and restore ACh levels. Otherwise, the non-steroidal anti-inflammatory drugs nimesulide, acetylsalicylic acid, ibuprofen, and diclofenac sodium decreased the extracellular glutamate and glutamine levels, downregulated GS and AChE activities, and restored ACh levels in Pro-treated astrocytes. Altogether, our results evidence that the vulnerability of metabolic homeostasis in cortical astrocytes might have important implications in the neurotoxicity of Pro.

Keywords Proline · Astrocyte · Glutamate · Inflammation · Cholinergic system · Acetylcholine · Acetylcholinesterase · Cytokine mediators · TNF-α · IL-1β · IL-6 · Excitotoxicity · Glutamine synthetase

Introduction

Astrocytes act as multifunctional interactive cells. They are intimately involved in diverse neural functions: nutritive function, allowing the transfer of metabolites from the circulation and supplying energetic substrates to neurons; controlling the extracellular ion balance by buffering neurotransmitter, ion and water concentrations; participating in repair and scarring processes after CNS injury; processing information including release of gliotransmitters and modulating neuronal excitability and synaptic plasticity [1–8].

It is of critical importance that the extracellular concentration of glutamate, the major excitatory neurotransmitters in the mammalian central nervous system, is kept low (nanomolar ranges) [9–12]. Dysregulated excitatory neurotransmission is

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harmful, resulting in increased levels of extrasynaptic glutamate that is thereby toxic [13–15]. Low extracellular level can be maintained by cellular uptake because there is no extracellular metabolism of glutamate [16, 10]. Therefore, the rapid removal of extracellular glutamate must occur on a millisecond time scale to avoid cellular damage. Glutamate is actively removed from the synaptic cleft and transported into the cytosol against its concentration gradient via excitatory amino acid transporters (EAATs), primarily found on synaptically associated astrocytic processes. Five high-affinity transporters have been identified [17–19]. The major glutamate transporters are EAAC1 and EAAC2 that, in rodents, are referred to as GLAST and GLT1, respectively, located predominantly in glial cells [20, 21, 10]. The location of the EAATs relative to the organization of synapse places them in a critical position to prevent glutamate excess and activation of extra-synaptic glutamate transporters [22]. Considering that an individual astrocyte serve large numbers of synapses, the failure of a single astrocyte could impair glutamate removal at thousands of synapses in some brain regions [23].

Glutamate is converted in glutamine, in an ATP-requiring reaction with ammonia, catalyzed by glutamine synthetase (GS), an enzyme located primarily, if not exclusively, in astrocytes [24, 25]. Under appropriate electrophysiological conditions, newly synthesized glutamine is then released from astrocytes, transferred to neurons and hydrolyzed by glutaminase, an enzyme found abundantly in neurons, which reconverts inert glutamine to glutamate for subsequent repackaging into synaptic vesicles. The cycling of glutamate/glutamine in astrocytes and neurons has been termed "the glutamate–glutamine cycle" [26–28].

Astrocytes also release molecules acting not only on neighboring neurons, but also on nearby glial cells and on cellular constituents of the blood brain barrier. These cells work as the sentinels of the brain and react to neuronal stress or injury by providing neuronal metabolic and trophic support [29–31]. In addition to trophic factors, astrocytes release cytokines in response to brain injury and neurodegenerative disease. A number of pro- and anti-inflammatory cytokines are induced in reactive astrogliosis, including tumor necrosis factor-β, IL-6, IL-1β, IL-4, and IL-10 [32–37]. The role of excitotoxicity in neurodegeneration has been well established and evidence supports an increasing role of inflammatory processes in neuronal pathology [38–40]. In addition, it has been demonstrated that IL-6, IL-1β, and TNF-α levels appear to exacerbate cerebral injury with a critical role in coordinating the inflammatory response. Moreover, recent studies have established that acetylcholine (ACh), the classical neurotransmitter in the central and peripheral nervous systems, acts as a suppressor of inflammatory responses, establishing a cholinergic anti-inflammatory pathway that can be a highly robust mechanism for inflammation control [41, 42]. In this context, earlier findings have suggested TNF-α-mediated increase of inflammatory component cascades in cultured astrocytes which was attenuated by addition of ACh to the culture medium [43, 44].

Hyperprolinemia is a phenotype present in two distinct, autosomal recessive metabolic disorders caused by defects in the L-proline (Pro) catabolic pathway. Hyperprolinemia type I (HPI) and type II (HPII) are caused by deficiencies in proline dehydrogenase that catalyze the first step of proline metabolism, and P5C dehydrogenase that catalyzes the oxidation of glutamate semialdehyde to glutamate, respectively. HPI patients can exhibit Pro plasma concentrations in the range of 700 to 2,400 mM, and some individuals are asymptomatic, while others have neurologic, renal, and/or auditory impairments. HPII is causally linked to neurologic manifestations and is associated with an increased incidence of seizures and possibly mental retardation; the Pro plasma concentrations can reach 700 to 3,700 mM [45–49]. Hyperprolinemia is also a risk factor for schizophrenia [50–52].

The underlying mechanisms of brain dysfunction in hyperprolinemias are poorly understood; however, alterations in the glutamatergic homeostasis seem to be involved. In this context, it has been demonstrated that high levels of glutamate are found in the cerebrospinal fluid of patients with HPII [53, 45, 54, 55]. Besides, there are some evidences that Pro serve as a modulator of glutamate neurotransmission by overstimulation of receptors NMDA and AMPA [56, 53, 57, 58, 55]. In addition, in vitro and in vivo models of hyperprolinemia are able to modify glutamate uptake in cerebral cortex and hippocampus of rats [59, 60]. Nevertheless, the exact mechanisms of this glutamatergic excitotoxicity elicited by hyperprolinemia are far from being understood.

Considering that the astrocytes are the main neural cell type responsible for the maintenance of brain homeostasis and are also important players in neurological disorders, the aim of the present work was to study the effects of Pro in concentrations described to be neurotoxic to astrocytes and neurons on the glutamatergic and inflammatory parameters in cultured astrocytes exploring some molecular mechanisms underlying such effects.

Material and Methods

Materials

L-Proline, acetylthiocholine, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), L-glutamate, D-2-amino-5-phosphonopentanoic acid (D,L AP5), acetylsalicylic acid (AAS), ibuprofen, nimesulide, diclofenac sodium, monoclonal mouse anti-β-actin IgG, protease inhibitor cocktail, cell culture chemicals, high-performance liquid chromatography (HPLC), Western blot, and GS assay reagents were purchased from Sigma–

Aldrich (St. Louis, MO, USA). Western blot polyclonal anti-EAAT1 was purchased from Alpha Diagnostic Intl. Inc. (USA). Horseradish peroxidase-conjugated donkey anti-rabbit IgG was obtained from GE Healthcare (USA). The potent competitive non-NMDA iGluR antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), the non-selective group I/group II mGluR antagonist (R,S)-a-methyl-4-carboxyphenylglycine (MCPG) and the metabotropic glutamate receptor 5 antagonist (MPEP) were purchased from Tocris Neuramin (Bristol, UK). L-[3,4-^{3}H]Glutamate (L-[^{3}H]glu) was purchased from PerkinElmer Inc. (USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle´s medium (DMEM), fungizone, and penicillin/streptomycin were purchased from Gibco BRL (Carlsbad, CA, USA). All other chemicals were of analytical grade.

Primary Astrocyte Culture

Astrocyte primary cultures were prepared from cerebral cortex of newborn (0–1-day-old; P0) Wistar rats as described previously [61]. All animals were kept under standard laboratory conditions according to "Principles of Laboratory Animal Care" (NIH publication 85–23, revised 1996), and the experimental protocol was approved by the University's Ethics Committee. Briefly, rats were decapitated; brain structures were removed, and the meninges were carefully stripped off. Mechanically dissociated cells were plated in DMEM/10 % FBS (pH 7.4) supplemented with glucose (33 mM), glutamine (2 mM), and sodium bicarbonate (3 mM) into a 15.6- and 34.8-mm-diameter well (six- and 24-well plates) (Corning Inc., New York, NY), previously coated with polyornithine (1.5 μg/mL, Sigma, St. Louis, MO).

After astrocytes reached confluence, the culture medium was removed by suction, and the cells were incubated 1 h at 37 °C in an atmosphere of 5 % CO_2 in DMEM without FBS in the absence (controls) or presence of different Pro concentrations (30 μM–5 mM) for 1 h. In experiments designed to evaluate glutamatergic and inflammatory involvements on Pro effects, 10 μM DL-AP5, 50 μM MCPG, 50 μM MPEP, 25 μM CNQX, 1.5 μg/ml nimesulide, 10 μM acetylsalicylic acid, 100 μg/ml ibuprofen, 1 μg/ml diclofenac sodium were used. The astrocytes were pre-incubated in the presence of these antagonists or anti-inflammatory drugs for 30 min before Pro treatment.

The anti-inflammatory used in this work has differences in the selectivity to cyclooxygenase enzymes (COX-1 and COX-2) that work suppressing the production of prostaglandins (chemical messengers that mediate inflammation): AAS has pronounced selectivity towards COX-1; diclofenac and ibuprofen have moderate selectivity towards COX-1, and nimesulide has equal inhibition of COX-1 and COX-2. In addition, these non-steroidal anti-inflammatory drugs were chosen because of their ability to inhibit cytokine production [62, 63].

Cytokines (TNF- a, IL-1β, IL-6, and IL-10) Assays

The assays of cytokines were carried out in 100-μl extracellular medium, using a Multiplexed Immunoassay with a commercially available kit, and analyzed on a Luminex 200®TM.

Determination of Acetylcholinesterase (AChE) Activity

The astrocytic cells were gently homogenized on ice in Tris–citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, with citric acid). Acetyl cholinesterase (AChE) activity was measured according to Ellman and colleagues [64] by determining the rate of hydrolysis of acetylthiocholine iodide (0.88 mM) in 300 μL, with 33 μL of 100 mM phosphate buffer, pH 7.5 mixed to 33 μL of 2.0 mM DTNB. Briefly, samples containing 5 μg protein and the reaction medium described above were preincubated for 10 min at 25 °C. The hydrolysis of acetylthiocholine iodide was monitored by the formation of thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s). Controls without the homogenate preparation were performed in order to determine the non-enzymatic hydrolysis of the substrate. Importantly, the linearity of absorbance toward time and protein concentration was previously determined. All reactions were performed in quadruplicate. AChE activity was expressed as micromole of thiocholine (SCh) released per hour per milligram of protein. Protein was measured by the Coomassie Blue method using bovine serum albumin as standard [65].

Determination of Extracellular Acetylcholine (ACh) Levels

Choline/Acetylcholine Assay Kit (Abcam, UK) was used to determine ACh levels in the medium of astrocyte cultures. The assay was carried out in accordance with the manufacturer's instruction. The total and free choline levels were calculated by plotting the fluorescence of each sample in relation to choline standard curve. The measurement of the fluorescence was obtained by SpectraMax M5 plate reader (Molecular Devices) at λ Ex/Em 535/590 nm. The assay was conducted in the absence and presence of AChE in order to identify values of total and free choline to establish the concentration of extracellular ACh levels.

[^{3}H]Glutamate Uptake

The astrocyte monolayer was incubated for 15 min with a Hanks' balanced salt solution (HBSS) solution containing (millimolar)—137 NaCl, 0.63 Na_2HPO_4, 4.17 $NaHCO_3$, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$, and 5.55 glucose, adjusted to pH 7.4; [^{3}H]glutamate

uptake was performed according to Frizzo and colleagues [66] with some modifications. Briefly, uptake was carried out at 35 °C by adding 100 μM unlabeled glutamate and 0.33 μCi/mL [^{3}H] glutamate and proline (30 μM–5 mM) for 5 min. The reaction was stopped by two washes of ice-cold HBSS (4 °C) immediately followed by addition of 0.5 N NaOH. Sodium-independent uptake was determined at 35 °C using HBSS containing *N*-methy-L-D glutamine instead of NaCl and Na_2HPO_4. Sodium-dependent uptake was obtained by subtracting sodium-independent from total uptake.

Incorporated radioactivity was determined with a Packard scintillator (TRI CARB 2100 TR). All experiments were performed in triplicate.

Glutamine Synthetase (GS) Activity

The enzymatic assay was performed as previously described [67]. Briefly, cell homogenate was added to a reaction mixture containing 10 mM $MgCl_2$, 50 mM L-glutamate, 100 mM imidazole-HCl buffer (pH 7.4), 10 mM 2-mercaptoethanol, 50 mM hydroxylamine-HCl, and 10 mM ATP incubated for 15 min at 37 °C. The reaction was stopped by adding a solution containing 370 mM ferric chloride, 670 mM HCl, and 200 mM trichloroacetic acid. After centrifugation, the absorbance of the supernatant was measured at 530 nm and compared with a calibration curve of γ-glutamylhydroxamate treated with ferric chloride reagent. Results are expressed as percentage of the control value.

Glutamate and Glutamine Determination by HPLC

The astrocyte culture medium was removed, and the cell layers were deproteinized with 7 % TFA. The homogenates were centrifuged at 4 °C for 10 min, and the supernatant was collected and neutralized with 1.5 M potassium bicarbonate. Both the medium (extracellular levels) and cellular components (intracellular levels) were derivatized with o-phthalaldehyde, and separation was carried out with a reverse-phase column (Supelcosil LC-18, 250 mm×4.6 mm, Supelco) in a Shimadzu Instruments liquid chromatograph (50 μL loop valve injection). The mobile phase flowed at a rate of 1.4 mL/min, and column temperature was 24 °C. Buffer composition was A—0.04 mol/L $NaH_2PO_4{\cdot}H_2O$ buffer, pH 5.5, containing 20 % of methanol; B·0.01 mol/L $NaH_2PO_4{\cdot}H_2O$ buffer, pH 5.5, containing 80 % of methanol. The gradient profile was modified according to the content of buffer B in the mobile phase—0 % at 0.00 min, 25 % at 13.75 min, 100 % at 15.00–20.00 min, 0 % at 20.01–25.00 min. Absorbance was read at 360 and 455 nm, excitation and emission, respectively, in a Shimadzu fluorescence detector. Samples of 10 or 25 μL were used and concentration was expressed in micromolar per milligram protein [68].

Western Blot Analysis

Astrocytes were homogenized in lysis buffer (5 mM Tris base, 1 mM EDTA, 0.1 % sodium dodecyl sulfate (SDS), and protease inhibitor cocktail; pH 7.0), and protein content was normalized to 2 μg protein/μL. Aliquots were diluted 1:1 in sample buffer (0.01 g % Bromophenol Blue, 60 mM Tris base, 20 % glycerol, 2 % SDS, and 5 % 2-mercaptoethanol, pH 6.8) and were resolved by 10 % SDS-polyacrylamide gel electrophoresis. Proteins were electro-transferred to nitrocellulose membranes (GE Healthcare, USA) using a semi-dry transfer apparatus (Bio-Rad, Trans-Blot SD). After 2 h of incubation in blocking solution containing 5 % powdered milk and 0.1 % Tween-20 in Tris–buffered saline (10 mM Tris base, 30 mM NaCl, pH 7.4), membranes were incubated overnight with anti-EAAT1 IgG (1 μg/mL) (GLAST), or anti-β-actin IgG (1:3,000) at 4 °C overnight. Membranes were exposed to horseradish peroxidase-conjugated anti-rabbit IgG diluted 1:1,000 overnight or horseradish peroxidase-conjugated anti-mouse IgG (1:3,000) for 2 h at 4 °C. The chemiluminescence (ECL, GE Healthcare, USA) was detected using X-ray films (Kodak X-Omat). Films were scanned, and the percentage of band intensity was analyzed using Optiquant software. β-Actin was used as protein loading control.

Statistical Analysis

Data from the experiments were analyzed statistically by *t*test or one-way analysis of variance (ANOVA) followed by the Tukey's test when the *F*-test was significant. Values of $p<0.05$ were considered to be significant. Correlations were assessed with the Pearson correlation coefficient. Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA) statistical packages.

Results

We initially studied the effect of Pro on the glutamate levels in cortical astrocytes of rats. HPLC measurement of intra- and extracellular glutamate levels showed that 1 h of incubation with 1 mM Pro provoked significantly decreased intracellular glutamate and increased extracellular glutamate levels in primary astrocytes (Fig. 1a). Accordingly, Fig. 1b shows that Pro administration promoted similar results concerning the intra- and extracellular glutamine levels compared with controls. However, we observed a decreased extracellular glutamine/glutamate ratio in astrocytes treated with 1 mM Pro (Fig. 1c).

Since it has been previously determined that the great majority (≅80 %) of glutamate taken up by astrocytes is converted to glutamine [69], we measured the GS activity. Results

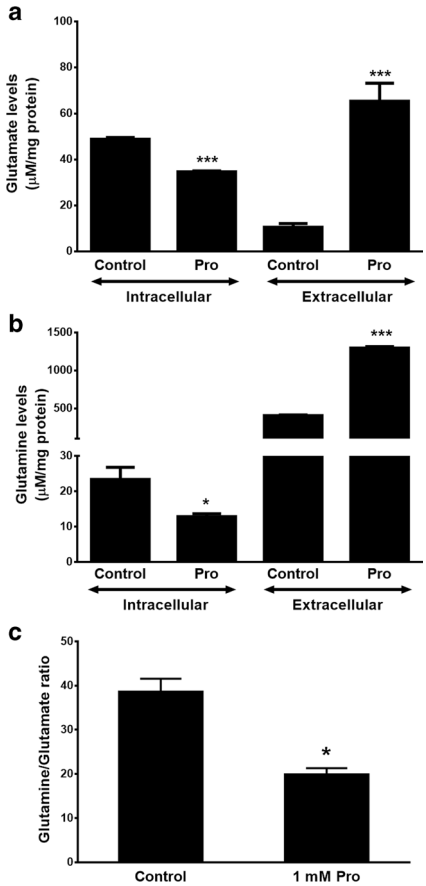

Fig. 1 Effects of Pro on the intra and extracellular glutamate (**a**) and glutamine (**b**) levels in cortical astrocytes of rats. After 1 mM Pro treatment, cellular components (intracellular levels) and medium (extracellular levels) of astrocytes were derivatized with o-phthalaldehyde, and separation was carried out with a reverse-phase column in a Shimadzu Instruments liquid chromatograph. Absorbance was read at 360 and 455 nm, excitation and emission, respectively, in a Shimadzu fluorescence detector. Samples of 10 and 25 μL were used, and concentration was expressed in micromolar per milligram protein. The glutamine/glutamate (Gln/Glu) ratio also was calculated (**c**). Data are reported as means±SEM of four independent different experiments performed in triplicate. Results were statistically analyzed by Student's *t* test. Statistically significant differences from controls, $*P<0.05$; $***P<0.001$

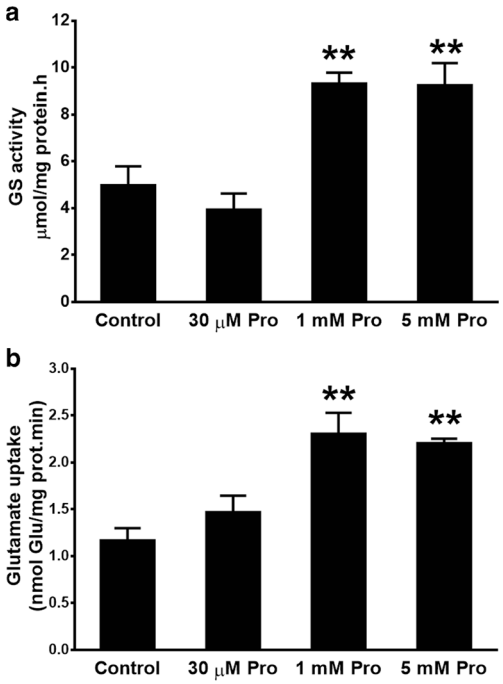

Fig. 2 Effects of Pro on glutamine synthetase (GS) activity (**a**) and glutamate uptake (**b**) in cortical astrocytes of rats. Astrocytes cells were incubated in the presence or absence of 30 μM, 1 and 5 mM Pro for 1 h. Data are reported as means±SEM of five independent experiments performed in triplicate. Results were statistically analyzed by one-way ANOVA followed by Tukey's test. Statistically significant differences from controls, $**P<0.01$

showed increased GS activity in cells treated with 1 and 5 mM Pro while 30 μM Pro failed to alter GS activity (Fig. 2a).

Considering that the astrocytes have a very efficient glutamate uptake system and this is a crucial parameter for the maintenance of extracellular glutamate concentrations below neurotoxic levels, we evaluated the Pro effects on glutamate uptake in cultured astrocytes. Results showed that glutamate uptake was significantly increased in cells treated with 1 and 5 mM Pro (Fig. 2b). Western blot analysis using anti-GLAST antibody was performed to evaluate the effect of high Pro levels on the transporter activity. Surprisingly, results showed that GLAST total immunocontent in 1 mM Pro-treated cells was similar to control values (results not shown).

Since cortical astrocytes treated with Pro present strongly increased extracellular glutamate levels and similar results have been reported in in vivo and in vitro inflammation models [70, 71], we measured inflammatory cytokines (TNF-α, IL-1β, and IL-6) in order to assess the inflammatory response of astrocytes exposed to Pro. Results showed that both 30 μM and 5 mM Pro failed to alter pro-inflammatory cytokines; however, 1 mM Pro significantly increased TNF-α, IL-1β ,and IL-6 levels as compared with control cells (Fig. 3a–c). To confirm the association between glutamate levels and inflammatory response induced by Pro, we analyzed the extracellular glutamate levels in the presence of anti-inflammatory drugs. Results showed that all non-steroidal anti-inflammatory drugs tested [AAS (10 μM), nimesulide (1.5 μg/ml), ibuprofen (100 μg/ml), and diclofenac sodium (1 μg/ml)] dramatically decreased the extracellular glutamate levels per se. Moreover, low extracellular glutamate levels were persistent in astrocytes co-incubated with Pro plus anti-inflammatory drugs (Fig. 4a). In addition, we tested the same anti-inflammatory drugs on the extracellular glutamine levels in Pro-treated astrocytes. Results showed that the anti-inflammatory drugs did not present effect per se; however, they prevented the Pro-induced increase of extracellular glutamine levels, as shown in Fig. 4b.

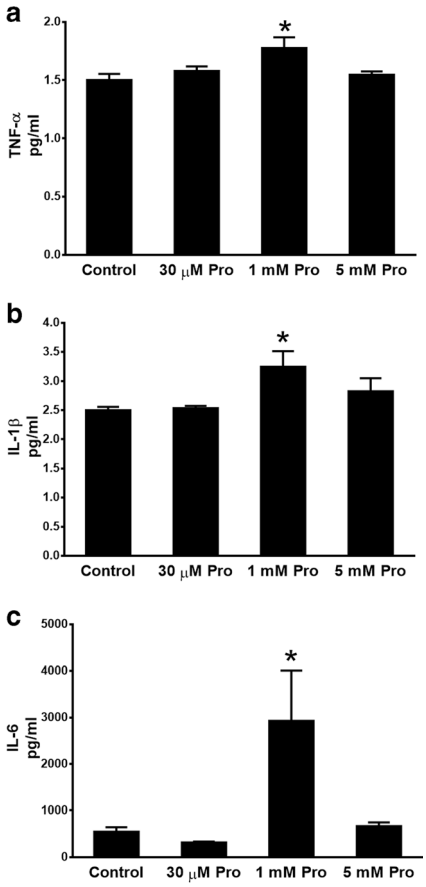

Fig. 3 Effect of Pro administration on cytokines (TNF-α, IL-1β, and IL-6) (**a**–**c**) levels in cortical astrocytes of rats. Astrocytes cells were incubated in the presence or absence of 30 μM, 1 and 5 mM Pro for 1 hour. Results are expressed as mean±SD for five independent different experiments performed in triplicate. Results were statistically analyzed by one-way ANOVA followed by Tukey's test. Different from control, *$pP<0.05$. *TNF-α*: tumor necrosis factor alpha, *IL-1β*: interleukin-1β, *IL-6*: interleukin-6

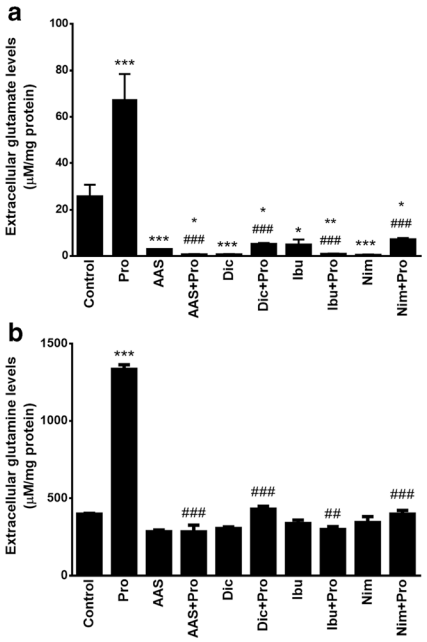

Fig. 4 Prevention of non-steroidal anti-inflammatory drugs on Pro effects in extracellular glutamate (**a**) and glutamine (**b**) levels. The astrocytes were pre-incubated in the presence of 10 μM acetylsalicylic acid (AAS), 1.5 μg/ml nimesulide (Nim), 100 μg/ml ibuprofen (Ibu), and 1 μg/ml diclofenac sodium (Dic) for 30 min before 1 mM Pro treatment. Results are expressed as mean±SD for four independent different experiments performed in triplicate. Results were statistically analyzed by one-way ANOVA followed by Tukey's test. Statistically significant differences from controls are indicated—*$P<0.05$; **$P<0.01$; ***$P<0.001$. Statistically significant differences from Pro:, $^{\#\#}P<0.01$; $^{\#\#\#}P<0.001$

Taking into account that cholinergic signalling seems to be associated with inflammation [41] and that in common neuroinflammation diseases abnormal levels of the AChE, enzyme that degrades ACh neurotransmitter, play a pivotal role in the pathogenesis of neuronal disorders, we determined the effect of Pro administration on AChE activity. Figure 5a depicts that exposure to 1 and 5 mM Pro provoked an increased AChE activity in cortical astrocytes. To test the possibility that increased AChE activity elicited by Pro reduces ACh levels accounts for inflammation, we measured the extracellular ACh levels. Figure 5b shows that 1 mM Pro significantly decreased the ACh levels. To investigate the possible association between AChE activity and inflammation, we performed the correlation between AChE activity and pro-inflammatory cytokines (IL-6 and TNF-α). Pearson correlation analysis shows a significant positive correlation of AChE activity versus IL-6 in astrocytes treated with 1 mM Pro (Pearson $r=0.9838$; $p<0.001$). Line represents linear regression of data ($Y=21{,}369*X-10{,}230$; $r^2=0.9679$) (Fig. 5c). Similarly, we observed a significant positive correlation of AChE activity versus TNF-α levels (Pearson $r=0.9192$; $p=0.0272$). Line represents linear regression of data ($Y=2{,}954*X-0.08150$; $r^2=0.8450$) (Fig. 5d). Comparing extracellular ACh levels and IL-6 and TNF-α levels, we observed a negative correlation respectively (Pearson $r=-0.9839$; $p<0.05$ and Pearson $r=-0.9562$; $p<0.05$) (Fig. 5e and f). Line represents linear regression of data ACh versus IL-6 levels ($Y=-17{,}803*X+11{,}919$; $r^2=0.9680$) and extracellular ACh levels versus TNF-α levels ($Y=-2{,}472*X+2{,}869$; $r^2=0.9144$).

It has been demonstrated that Pro has an excitatory action when applied to different areas of the mammalian brain [72, 55]. This effect may be related to the activation of glutamate receptors since glutamate antagonists are able to block the neurotoxic effects of L-Pro injections into rat hippocampus [73]. In addition, Pro elicited an inward current in dissociated cultured neurons that was partially antagonized by glutamate receptor antagonists D-AP5 and CNQX [74]. Taking into account the glutamate receptor-dependent excitatory action of Pro, we decided to examine the putative involvement of glutamate receptors in Pro-induced alterations on glutamate homeostasis and inflammatory status. We treated cell for 1 h with 1 mM Pro in the presence of glutamate antagonists and evaluated extracellular glutamate and glutamine levels, GS,

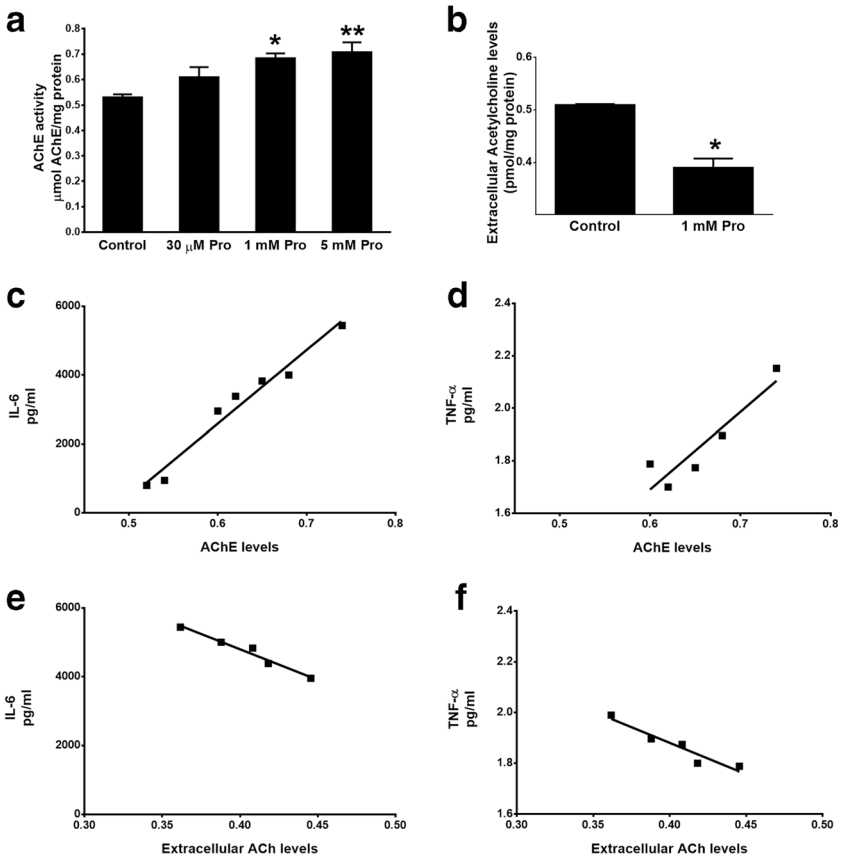

Fig. 5 Effect of Pro administration on AChE (**a**) activity and ACh levels (**b**) and the respective correlations with cytokines levels (**c**–**f**) in cortical astrocytes of rats. Results are expressed as mean±SD for five to seven independent experiments performed in triplicate. Results were statistically analyzed by one-way ANOVA followed by Tukey's test. Pearson correlation analyses were performed. *Line* represents linear regression of data. Different from control, **P*<0.05; ***P*<0.01. *AChE*: acetylcholinesterase. *ACh*: acetylcholine

inflammatory cytokines, AChE activity, and extracellular ACh levels. We observed that the competitive NMDA antagonist DL-AP5 (10 μM), the competitive non-NMDA ionotropic antagonist CNQX (25 μM), and the non-selective group I/group II metabotropic glutamate antagonist MCPG (50 μM) did not present effect per se; however, the antagonists prevented the Pro-induced increase of extracellular glutamate levels. Surprisingly, 50 μM MPEP, the antagonist of metabotropic glutamate receptor 5, induced a dramatically decreased extracellular glutamate levels. In the presence of 1 mM Pro, these low extracellular glutamate levels were still maintained (Fig. 6a). The extracellular glutamine levels were also significantly decreased by each of the glutamatergic antagonists used, and this effect persisted in the presence of Pro (Fig. 6b). We also showed that the glutamatergic antagonists DL-AP5, CNQX, MCPG, or MPEP did not present effect per se on GS activity; however, they prevented the Pro-induced increased GS activity (Fig. 6c).

Considering that anti-inflammatory drugs dramatically decreased the extracellular glutamate levels, we tested whether blocking glutamate receptors could disrupt the inflammatory status induced by Pro. Results showed that high IL-6 and TNF-α levels induced by Pro were prevented by glutamate antagonists (DL-AP5, CNQX, MCPG, or MPEP) (Fig. 7a–b).

Finally, to assay the influence of glutamatergic mechanisms in the altered AChE activity and extracellular ACh levels in Pro-treated astrocytes, we tested the effect of glutamate antagonists on the AChE activity and extracellular ACh levels. Results demonstrated that all of the antagonists tested were able to prevent the increased AChE activity in cortical astrocytes exposure to 1 mM Pro (Fig. 7c). Furthermore, we also observed that DL-AP5 partially prevented and CNQX, MCPG, or MPEP totally prevented the decreased extracellular ACh induced by Pro (Fig. 7d).

As we have shown that 1 mM Pro induced the production of pro-inflammatory cytokines, we investigated the role of the non-steroidal anti-inflammatory drugs on the prevention of Pro effects on GS and AChE activities and extracellular Ach levels. We observed that nimesulide, ibuprofen, and diclofenac sodium totally prevented the 1 mM Pro-induced increase of AChE and GS activities and extracellular ACh levels. In addition, AAS prevented the Pro-induced increased GS activity but only attenuated the Pro effect in AChE activity and extracellular ACh levels (Fig. 8a, b, and c).

Discussion

In the present work, we focused on the effects of Pro on glutamate homeostasis and inflammatory parameters in cortical astrocytes in culture. We exposed primary astrocytes to 1 mM Pro, and we found a crosstalk among disrupted

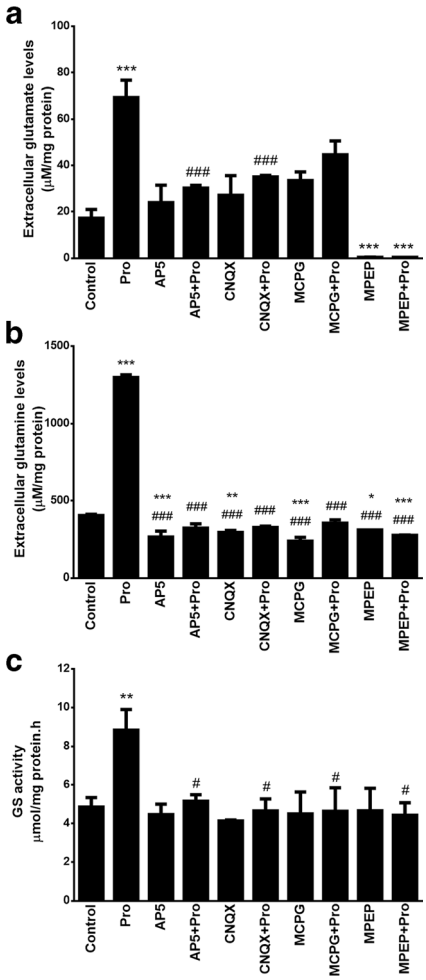

Fig. 6 Prevention of glutamate antagonists on Pro effects in glutamate (**a**) and glutamine levels (**b**), and GS activity (**c**). The astrocytes were pre-incubated in the presence of competitive NMDA ionotropic antagonist DL-AP5 (10 μM), competitive non-NMDA ionotropic antagonist CNQX (25 μM), non-selective group I/group II metabotropic antagonist MCPG (50 μM), or metabotropic glutamate receptor 5 antagonist MPEP (50 μM) for 30 min before 1 mM Pro treatment. Results are expressed as mean± SD for five to seven independent different experiments performed in triplicate. Results were statistically analyzed by one-way ANOVA followed by Tukey's test. Statistically significant differences from controls, $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. Statistically significant differences from Pro, $^{\#}P<0.05$; $^{\#\#}P<0.05$; $^{\#\#\#}P<0.001$. *GS*: glutamine synthetase

glutamate homeostasis, cholinergic anti-inflammatory pathway, and production/release of inflammatory factors, suggesting that the disturbance of Pro homeostasis could affect practically all physiological functions of isolated astrocytes, and this could be associated with the pathological events of hyperprolinemia.

In order to bring about misregulation of glutamate homeostasis, we should consider that Pro metabolism produces glutamate into the cell. Arrieta-Cruz and colleagues [75] have recently described that physiological concentrations of Pro generates glutamate which is subsequently metabolized to alpha-ketoglutarate, which, in turn, enters the tricarboxylic acid cycle for energy production. Alternatively, proline-derived pyruvate is converted to lactate in astrocytes, reinforcing the astrocyte–neuron lactate shuttle [76]. However, our results suggest that, in the presence of 1 mM Pro, glutamate could be preferentially used to form glutamine through activated GS into astrocytes. The metabolic link between glutamine and Pro has been described by Liu and colleagues [77]. These findings are in agreement with previous evidence that hyperprolinemia disrupts physiological metabolic energy system leading to accumulated extracellular glutamate and excitotoxicity. This is consistent with high glutamate levels found in the cerebrospinal fluid of patients with hyperprolinemia [45, 54, 78].

Glutamate/glutamine metabolic cycle between astrocytes and neurons is believed to be vital for preventing neuronal excitotoxicity. We observed that the high levels of extracellular glutamate elicited by Pro were accompanied for high extracellular levels of glutamine, indicating that part of excess glutamate released is converted to glutamine via the GS pathway, since GS activity was also increased by Pro in astrocytes cells. Accordingly, the epileptogenic activity in the hippocampus of patients with epilepsy is associated with the excessive extracellular accumulation of Glu [79], accompanied by decreased extracellular Gln/Glu ratio [80]. In the present study, we also observed decreased extracellular Gln/Glu ratio in response to 1 mM Pro, evidencing a possible exacerbated excitatory activity that could have an important role in the pathomechanisms of hyperprolinemia.

Previous studies from our laboratory showed that in vitro and in vivo administration of Pro reduced glutamate uptake, despite the high levels of glutamate transporters GLAST and GLT-1 in cerebral slices of rats [59, 60]. Otherwise, in the present work, we observed an increase in glutamate uptake without altering GLAST total immunocontent in astrocytes exposed to Pro. This apparent discrepancy of results in different experimental models of exposure to Pro can be ascribed to the different aspects of astrocytic and neuronal metabolism. Astrocytes are able to withstand much higher concentrations of glutamate than neurons. Also, it is important to consider that metabolic and enzymatic compartmentalization exists between neurons and astrocytes. The key outcome of this enzymatic compartmentalization could be the misregulated glutamate/glutamine cycle between neurons and astrocytes [81, 82, 27, 83, 84].

It has been demonstrated that brain injuries found in neurological disorders, such as ischemia [76] and hypoxia [76], cause increased extracellular glutamate levels that can be associated with increased GS activity. In this context, Zou and colleagues [77, 78] also described that excessive glutamate levels induced GS activity and protein expression in cortical astrocyte cultures. Moreover, it was demonstrated that elevated extracellular glutamate levels could rapidly influence astrocyte glutamate uptake capacity mediated by transporter translocation to the cell membrane [79, 80]. Taking into account

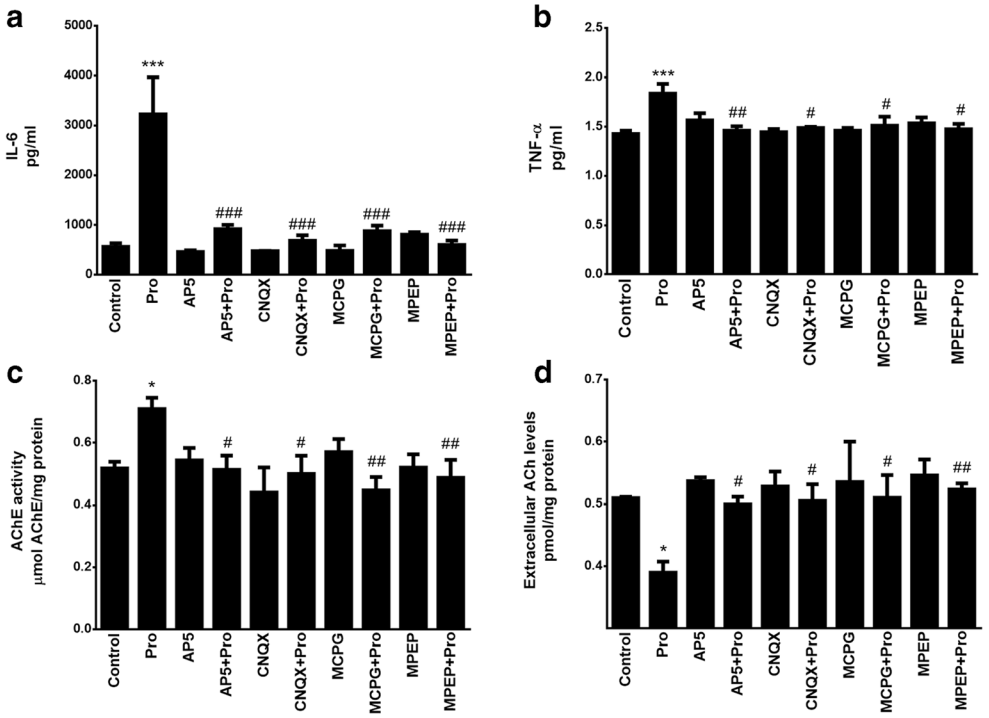

Fig. 7 Prevention of glutamate antagonists on Pro effects in IL-6 (**a**), TNF-α levels (**b**), AChE activity (**c**), and ACh levels (**d**). The astrocytes were pre-incubated in the presence of competitive NMDA ionotropic antagonist DL-AP5 (10 μM), competitive non-NMDA ionotropic antagonist CNQX (25 μM), non-selective group I/group II metabotropic antagonist MCPG (50 μM), or metabotropic glutamate receptor 5 antagonist MPEP (50 μM) for 30 min before 1 mM Pro treatment. Results are expressed as mean±SD for four to six independent different experiments performed in triplicate. Results were statistically analyzed by one-way ANOVA followed by Tukey's test. Statistically significant differences from controls, $^{*}P<0.05$; $^{***}P<0.001$. Statistically significant differences from Pro, $^{\#}P<0.05$; $^{\#\#}P<0.01$; $^{\#\#\#}P<0.001$. *AChE*: acetylcholinesterase. *ACh*: acetylcholine

these evidences, we suggest that the excess of glutamate elicited by Pro could induce increased glutamate uptake and GS activity in astrocytes.

The huge extracellular release of glutamate and glutamine provoked by 1 mM Pro was matched by the fall in intracellular glutamate and glutamine levels. This is consistent with findings by Longuemare and colleagues [85], who described glutamate efflux followed by the decreased intracellular glutamate levels, without involvement of the rapid glutamate catabolism.

Glutamate release in cultured astrocytes can occur from cytosolic pools through plasma membrane channels and transporters, and through Ca^{2+}-dependent exocytosis [86–88].Our findings showed that the high extracellular glutamate and glutamine levels were prevented by ionotropic glutamate antagonists. This is consistent with Ca^{2+}-dependent glutamate exocytosis.

Astrocytic synaptic-like microvesicles were shown to contain glutamate and express proteins for uptake and release of glutamate, including vesicular glutamate transporters (vGluts) [88, 89]. In this context, Santello and colleagues [90] described that glutamatergic gliotransmission and its synaptic effects are controlled not only by astrocyte Ca^{2+} elevations, but also by permissive/homeostatic ambient factors like TNFα. In agreement with this evidence, we described in the present work the significant increase of TNF-α, IL-1β, and IL-6 cytokine levels associated with misregulated glutamate homeostasis induced by Pro. This crosstalk was supported by the role played by anti-inflammatory drugs modulating glutamate and glutamine secretion and restoring their extracellular levels both per se and in the presence of Pro. On the other hand, the affinity of vGluts for glutamate is somewhat higher than of GS [91, 92], so the intracellular glutamate in astrocytes can sufficiently be taken up by vesicles and is thus protected from conversion to glutamine by GS [93]. In agreement with this evidence and considering that the effects of anti-inflammatory drugs in extracellular glutamine levels are similar than extracellular glutamate levels, we can premise that, when anti-inflammatory are incubated with Pro, the Ca^{2+}-dependent glutamate release is disrupted and the excess glutamate goes to the synaptic vesicle through vGluts instead of transforming into glutamine by GS and thus the release of glutamine decreased. This hypothesis can be enhanced due the observation that all the anti-inflammatory drugs tested also prevented the GS increase induced by 1 mM Pro.

Another important finding concerning the evidence that Pro injury was also accomplished by interfering with cholinergic anti-inflammatory pathway through upregulated AChE

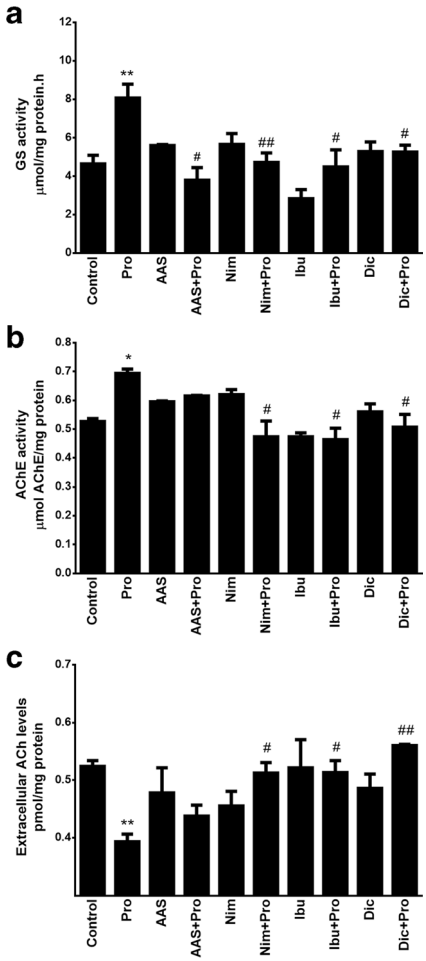

Fig. 8 Prevention of non-steroidal anti-inflammatory drugs on Pro effects on GS (**a**), AChE activities (**b**), and ACh levels (**c**). The astrocytes were pre-incubated in the presence of 10 μM acetylsalicylic acid (AAS), 1.5 μg/ml nimesulide (Nim), 100 μg/ml ibuprofen (Ibu), and 1 μg/ml diclofenac sodium (Dic) for 30 min before 1 mM Pro treatment. Results are expressed as mean±SD for five to seven independent different experiments performed in triplicate. Results were statistically analyzed by one-way ANOVA followed by Tukey's test. Statistically significant differences from controls are indicated: $^{*}P<0.05$; $^{**}P<0.01$. Statistically significant differences from Pro, $^{\#}P<0.05$; $^{\#\#}P<0.01$. *GS*: glutamine synthetase; *AChE*: acetylcholinesterase. *ACh*: acetylcholine

activity and decreased ACh levels. ACh is known to attenuate inflammation by mechanisms dependent on the nicotinic ACh receptor α7 subunit [94]. We therefore propose that both production/release of inflammatory cytokines and downregulated cholinergic transmission account for the inflammatory status in Pro-treated astrocytes.

Altogether, our findings highlight a crosstalk among disrupted glutamate homeostasis, cholinergic mechanisms, and inflammatory cytokines, since glutamate antagonists were able to downregulate TNFα and IL6 production and restore ACh levels. Otherwise, the non-steroidal anti-inflammatory drugs decreased the extracellular glutamate and glutamine levels, downregulated GS and AChE activities, and restored ACh levels in Pro-treated astrocytes.

In an attempt to understand some of the molecular mechanisms underlying this crosstalk, we should first consider several interconnecting actions mediated by increased intracellular Ca^{2+} concentration, mainly from over-stimulated AMPA and NMDA receptors which initiate or potentiate a variety of Ca^{2+}-dependent intracellular cascades associated with cell injury. Therefore, it is plausible that glutamate excitotoxicity and Ca^{2+} overload elicit signaling pathways to produce and release inflammatory mediators [95]. On the other hand, inflammatory molecules can contribute to hyperexcitability leading to increased glutamatergic neurotransmission [96, 97, 39]. Evidences suggest that both IL-1β and IL-6 can functionally interact with glutamate receptors [98–100] and that TNF-α intensifies AMPA-induced toxicity in Purkinje neurons by increasing intracellular glutamate [101].

Pharmacological studies indicate that COX, the major target to the anti-inflammatory drugs used in the present study, is involved in the mechanism leading to glutamate release [102, 103]. On the other hand, Liu and colleagues [104] described that metabotropic glutamate receptor 5 modulates the TNF-α release in microglia in response to inflammatory stimulus. Yoshio and colleagues [105] showed that purified IgG anti-NMDA receptor subunits upregulates the expression of interleukin-6 (IL-6) and IL-8. Werry and colleagues [106] have demonstrated that IL-10 release stimulated by an inflammatory insult is enhanced by glutamate and all subtypes of glutamate receptors. Taking these facts into consideration, together with the evidence that glutamate antagonists are able to prevent the increase of TNF-α and IL-6 production, our results further support the direct involvement of inflammatory mediators and glutamatergic synaptic transmission in astrocytes treated with Pro.

Our present results also showed that 1 mM (not 5 mM) Pro-treated astrocytes induced upregulation of pro-inflammatory cytokines in cortical astrocytes, which could contribute to an exacerbated glutamatergic neurotransmission probably through modulation of glutamate release. Previously, this selective and specific effect of 1 mM Pro has been observed by our group. We observed that 1 mM Pro (not 5 mM), significantly altered antioxidant enzymes, such as catalase (CAT) and superoxide dismutase (SOD) activities [107]. In addition and corroborating our findings, a negative correlation between pro-inflammatory cytokines levels and CAT and SOD activities were found in another study [108]. Taking into account this evidence, we can assign that the specific effect of 1 mM Pro on interleukin levels can be related, at least in part, with specific redox signaling mechanisms elicited by this Pro concentration.

The increased pro-inflammatory cytokine levels might also be associated with upregulated AChE activity in astrocytes treated with 1 mM Pro because the findings showed that there is a positive correlation between AChE activity and IL-6 or TNF-α levels in astrocytes treated with 1 mM Pro. Moreover,

the AChE activity can be related with a consequent reduction in the ACh levels. In agreement with our findings, previous studies demonstrated anti-inflammatory effects for ACh and AChE inhibitors in glial cultures [109, 110]. In this context, AChE inhibitors have also been shown to reduce glial activation and inflammatory cytokine production in experimental models of cerebral injury [111–113]. Overexpression of AChE can impair glutamatergic synaptic structures and transmission, indicating that excessive AChE molecules are a harmful factor in the process of neurodegeneration [114, 115]. Taken together, we can hypothesize that inflammation status predisposes the Pro-treated astrocytes to increased excitability through glutamatergic modulation. On the other hand, the action of glutamatergic antagonists restoring AChE activity reinforces a modulation between glutamatergic and cholinergic systems in Pro-treated astrocytes.

In line with this, we also showed that non-steroid anti-inflammatory drugs were able to prevent Pro effects on GS and AChE activities. In this context, nimesulide reversed the induction of pro-inflammatory cytokine gene expression in a model of Parkinson disease mediating neuroprotective mechanisms in astrocytes [116]. Diclofenac also suppressed LPS-induced elevated TNF-α levels in cultured astrocytes [117]. In addition, Anneken and Gudelsky [118] described that 3,4-methylenedioxy-*N*-methylamphetamine, a popular drug of abuse, produced a sustained increase in the extracellular concentration of glutamate from astrocytes in the hippocampus, and this effect was suppressed by ketoprofen and nimesulide. Taken together, anti-inflammatory drugs reduced extracellular glutamate levels, GS activity, and consequently glutamine extracellular levels.

To further explore the mechanisms underlying the Pro-induced disturbances on glutamate homeostasis, we observed that NMDA, non-NMDA ionotropics, and the non-selective group I/group II metabotropic antagonists prevented the Pro-induced increase of extracellular glutamate levels in astrocytes, giving support to the involvement of glutamate receptors in homeostasis disruption in Pro-treated astrocytes. However, the metabotropic glutamate receptor 5 antagonist MPEP provoked a dramatic decrease in extracellular glutamate levels, and in the presence of Pro, these low levels of glutamate still maintained. In agreement with these results, Cali and colleagues [119] described that metabotropic glutamate receptor 5 is implicated in the glutamate release response to glutamatergic stimulation. This receptor was identified as a mediator of the exocytosis of glutamatergic vesicles in astrocytes, belonging to the G protein-coupled family, which is known to release Ca^{2+} from internal stores [120]. These observations corroborate the role of Ca^{2+}-dependent glutamate release induced by Pro.

Glutamate receptor antagonists also prevented the stimulating effect of Pro on GS activity preventing the high extracellular glutamine levels. We also observed that the glutamatergic antagonists inhibited the extracellular glutamine levels per se, and when co-incubated with Pro, these levels remain low. We do not know yet the exact reason for this phenomenon, but other authors have been demonstrating that glutamate receptors can influence GS status and probably can modulate the extracellular glutamine levels. In accordance with this idea, Muscoli and colleagues [121] demonstrated that activation of NMDA receptor stimulates the expression of GS in astroglial cells. Moreover, Fleischer-Lambropoulos and colleagues [122] described that astrocyte cultures exposed to either glutamate, NMDA, or AMPA/kainate glutamatergic agonists exhibited an increase in GS activity. Another possibility for the prevention in extracellular glutamine levels by glutamate antagonists is that the glutamate levels can stimulate the overflow of glutamine as described previously [25, 123]. Upon overstimulation of glutamate receptors, uncontrolled intracellular signaling cascades involved in neuronal toxicity leads to the production of inflammatory cytokines and reactive oxygen species, causing a further release of intracellular glutamate [124–127], representing a vicious cycle. In this scenario, previous work from our laboratory [107] showed that astrocytes are able to respond to Pro concentrations reorganizing their cytoskeleton and surviving through RhoA- and ERK-mediated mechanisms, and such effects could be mediated, at least in part, by redox signaling mechanisms.

In conclusion, our results demonstrate that cortical astrocytes exposed to 1 mM Pro present misregulated glutamate homeostasis and cholinergic anti-inflammatory pathway associated with production/release of pro-inflammatory cytokines. We explored some molecular mechanisms underlying the disrupted homeostasis of astrocytes exposed to this toxic Pro concentration, and we highlighted an important crosstalk among dysregulated glutamatergic system and pro-inflammatory mechanisms contributing to the cell damage. We propose that the release of inflammatory cytokines might contribute to the potentiation of excitotoxic mechanisms, and otherwise, glutamate excitotoxicity plays a role in eliciting an inflammatory response driven by the cholinergic system and inflammatory cytokines. To our knowledge, this was the first study which examined the interaction of glutamatergic and inflammatory mechanisms in astrocytes exposed to Pro. Taken together, this evidence raises the notion that protecting astrocytes might be key in the treatment against brain damage of hyperprolinemia.

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Conflict of Interest The authors declare that they have no conflict of interest.

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