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Differences between cultured astrocytes from neonatal and adult Wistar rats: focus on in vitro aging experimental models

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Abstract

Astrocytes play key roles regulating brain homeostasis and accumulating evidence has suggested that glia are the first cells that undergo functional changes with aging, which can lead to a decline in brain function. In this context, in vitro models are relevant tools for studying aged astrocytes and, here, we investigated functional and molecular changes in cultured astrocytes obtained from neonatal or adult animals submitted to an in vitro model of aging by an additional period of cultivation of cells after confluence. In vitro aging induced different metabolic effects regarding glucose and glutamate uptake, as well as glutamine synthetase activity, in astrocytes obtained from adult animals compared to those obtained from neonatal animals. In vitro aging also modulated glutathione-related antioxidant defenses and increased reactive oxygen species and cytokine release especially in astrocytes from adult animals. Interestingly, in vitro aged astrocytes from adult animals exposed to pro-oxidant, inflammatory, and antioxidant stimuli showed enhanced oxidative and inflammatory responses. Moreover, these functional changes were correlated with the expression of the senescence marker p21, cytoskeleton markers, glutamate transporters, inflammatory mediators, and signaling pathways such as nuclear factor κB (NFκB)/nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase 1 (HO-1). Alterations in these genes are remarkably associated with a potential neurotoxic astrocyte phenotype. Therefore, considering the experimental limitations due to the need for long-term maintenance of the animals for studying aging, astrocyte cultures obtained from adult animals further aged in vitro can provide an improved experimental model for understanding the mechanisms associated with aging-related astrocyte dysfunction.

Keywords Aging · Glutamate homeostasis · Inflammatory response · In vitro astrocytes · Oxidative stress

Introduction

Astrocytes comprise the most abundant glial subtype and are involved in a variety of physiological functions, thus playing a critical role for the central nervous system (CNS) homeostasis (Verkhratsky and Nedergaard 2018). They regulate synaptic function and plasticity, maintain glutamate neurotransmitter homeostasis, and provide metabolic, antioxidant, and neurotrophic support (Verkhratsky and Nedergaard 2018; Liu *et al*. 2023). In addition, astrocytes can respond to protective and/or injury stimuli by triggering several signaling pathways, including nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase 1 (HO-1), nuclear factor κB (NFκB), mitogen activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3K)/Akt, which may result in both defense and pathological processes (Quincozes-Santos *et al*. 2021).

In this regard, dysfunctional astrocytes and the loss of their protective capacity have been closely related to neuropathologies, including age-related and neurodegenerative diseases (Palmer and Ousman 2018; Lau *et al*. 2023). In fact, aging has been associated with substantial changes of astrocyte functionality and with changes in

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the expression of senescence markers, including p21. A common event underlying aging is a low-grade inflammatory response, which has been referred as inflammaging (Franceschi *et al*. 2018). In this context, astrocytes are an important source of pro-inflammatory mediators in the CNS and their chronic production, along with increased reactive oxygen/nitrogen species (ROS/RNS), may have detrimental effects on neighboring cells (Guerrero *et al*. 2021).

Previous studies from our group have shown that primary astrocyte cultures obtained from animals of different ages are a useful model to study the age-related functional changes of astrocytes and their underlying molecular mechanisms (Souza *et al*. 2013; Bellaver *et al*. 2017; Santos *et al*. 2018; Bobermin *et al*. 2022; Leite Santos *et al*. 2023; Sovrani *et al*. 2023). However, it is important to consider experimental limitations due to the need for long-term maintenance of the animals. Therefore, considering the relevance of understanding the mechanisms involved in cellular alterations of astrocytes during the aging process, the in vitro model of aging astrocytes (continuous cultivation of cells after confluence) may be an additional and important tool (Gottfried *et al*. 2002; Pertusa *et al*. 2007; Souza *et al*. 2015; Matias *et al*. 2022, 2023). In this regard, since aging phenotypes may already appear in middle aged rodents (Souza *et al*. 2013; Bellaver *et al*. 2017; Yanai and Endo 2021), the utilization of astrocyte cultures obtained from mature animals for further in vitro aging can provide an improved experimental model.

In this study, we investigated functional and neurochemical changes in astrocytes from neonatal and adult Wistar rats aged in vitro. We functionally characterized astrocytes by evaluating glucose and glutamate uptake, glutamine synthetase (GS) activity, ROS/RNS production, antioxidant defenses, inflammatory response, and the expression of genes associated with these processes. In addition, we evaluated the response of the cells to oxidative, inflammatory, and protective stimuli by using hydrogen peroxide (H_2O_2), lipopolysaccharide (LPS), and resveratrol, respectively, as well as the participation of PI3K, p38 MAPK, HO-1 and Toll-like receptor 4 (TLR4) in the mechanisms of these effects.

Materials and methods

Animals Male Wistar rats (1–2 and 90 old) were obtained from the breeding colony of Department of Biochemistry (UFRGS, Porto Alegre, Brazil), maintained under controlled environment (12 h light/12 h dark cycle; 22 ± 1 °C; ad libitum access to food and water). All animal experiments were performed in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Federal University of Rio Grande do Sul Animal Care and Use Committee (process number: 37665).

Primary astrocyte cultures Neonate (1–2 old) and adult (90 old) Wistar rats had their cortices aseptically dissected from cerebral hemispheres, followed by meninges removal. The tissues were digested in Hank's balanced salt solution (HBSS) containing 0.003% DNase using trypsin (0.05%) and papain (40 U/mL), as previously described (Souza *et al*. 2013; Bobermin *et al*. 2020). After mechanical dissociation and centrifugation, the cells were resuspended in DMEM/F12 [10% fetal bovine serum (FBS), 15 mM HEPES, 14.3 mM $NaHCO_3$, 2.5 µg/mL Fungizone and 0.05 mg/mL gentamicin; Gibco, Grand Islands, NY], plated on 6- or 24-well plates pre-coated with poly-L-lysine at a density of $3–5 \times 10^5$ cells/cm^2. Astrocytes were cultured at 37 °C in a 5% CO_2 incubator. During the first week, the culture medium was replaced once every two, and from the second week on, it was replaced once every four when astrocytes received medium supplemented with 20% FBS until they reached confluence (at approximately 21 in vitro – DIV).

In vitro aging For the experimental in vitro model of aging, astrocyte cultures from both neonate and adult animals were maintained for additional 21 in vitro (DIV) after reaching confluence, when the experiments were performed (Fig. 1). Therefore, control astrocytes were maintained in culture for 21 days (21 DIV) while in vitro aged astrocytes maintained for 42 (42 DIV). Primary astrocyte cultures were alternatively subjected to oxidative or inflammatory challenges with H_2O_2 (100 µM for 3 h) and LPS (10 µg/ml for 3 h) or incubated with the antioxidant resveratrol (100 µM for 3 h). Pharmacological

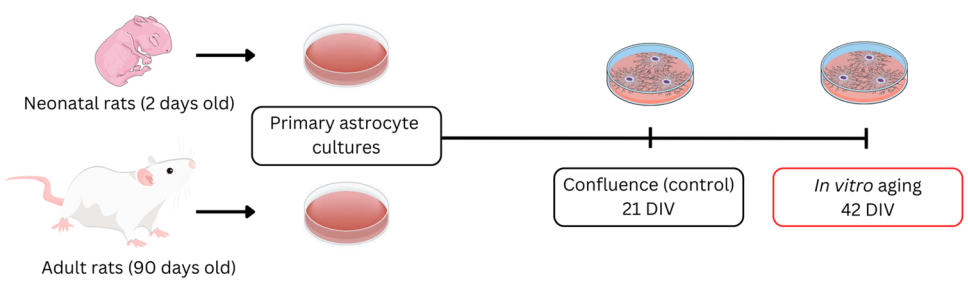

Figure 1. In vitro aging experimental design. Astrocytes obtained from neonate and adult animals were cultured until reach the confluence (at approximately 21 DIV – control condition) or they were maintained for additional 21 in vitro (totalizing 42 DIV – in vitro aging model).

inhibitors for PI3K (LY294002, 10 μM for 3 h), p38 MAPK (SB203580, 5 μM for 3 h), HO-1 (ZnPP IX, 10 μM for 3 h) and TLR4 (CLI-095, 5 μM for 3 h) were also used as indicated, according with our previous studies (Quincozes-Santos *et al*. 2013, 2014; Souza *et al*. 2013; Bellaver *et al*. 2015; Rosa *et al*. 2018).

MTT reduction assay MTT (methylthiazolyldiphenyl-tetrazolium bromide; Sigma-Aldrich, St. Louis, MO) was added to the medium at a concentration of 50 μg/mL and cells were incubated for 3 h at 37 °C in an atmosphere of 5% CO_2. Subsequently, the medium was removed and the MTT crystals were dissolved in dimethyl-sulfoxide. Absorbance values were measured at 560 nm and 650 nm (Bobermin *et al*. 2020). The results are expressed as percentages relative to the control conditions.

Glucose uptake Glucose uptake was assessed as previously described using 2-Deoxy-D-[1,2-^{3}H]-glucose ([^{3}H]-2DG; Amersham, Buckinghamshire, UK) (Souza *et al*. 2013). Briefly, the cell medium was replaced with fresh DMEM/F12 1% FBS for 2 h at 37 °C. Astrocytes were incubated with DMEM/F12 1% FBS containing 1 mCi/ml [^{3}H]-2DG for 20 min at 37 °C. After incubation, the cells were rinsed with HBSS and lysed overnight with NaOH 0.3 M. The incorporated radioactivity was measured in a scintillation counter. Cytochalasin B (10 mM) was used as a specific glucose transporter inhibitor. Glucose uptake was determined by subtracting the uptake in the presence of cytochalasin B from the total uptake.

Glutamate uptake The glutamate uptake was performed as previously described (Souza *et al*. 2013). Briefly, the cells were incubated at 37 °C in Hank's balanced salt solution (HBSS) containing the following components (in mM): 137 NaCl, 5.36 KCl, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$, 0.63 Na_2HPO_4, 0.44 KH_2PO_4, 4.17 $NaHCO_3$, and 5.6 glucose, adjusted to pH 7.4. The assay was started by the addition of 0.1 mM L-glutamate and 0.33 μCi/ml L-[2,3-^{3}H] glutamate (Amersham, Buckinghamshire, UK). The incubation was stopped after 7 min by removal of the medium and rinsing twice the cells with ice-cold HBSS. The cells were then lysed in a solution containing 0.5 M NaOH. Incorporated radioactivity was measured in a scintillation counter. Sodium-independent uptake was determined using ice-cold N-methyl-D-glucamine instead of sodium chloride. Sodium-dependent glutamate uptake was obtained by subtracting the sodium-independent uptake from the total uptake.

Glutamine synthetase activity The activity of GS was determined as previously described (dos Santos *et al*. 2006; Kleinkauf-Rocha *et al*. 2013). Briefly, the 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. The addition of 10 mM ATP started the reaction, which was continued for 15 min at 37 °C. A solution containing 370 mM ferric chloride, 670 mM HCl and 200 mM trichloroacetic acid was then added to stop the reaction. After centrifugation, the absorbance of the supernatant was measured at 530 nm. A calibration curve was prepared using γ-glutamyl hydroxamate (Sigma-Aldrich) and treated with ferric chloride reagent. The results are expressed in μmol/mg protein/h.

Glutathione levels Intracellular levels of glutathione (GSH) were assessed in cell lysates suspended in a sodium phosphate (100 mM)/KCl (140 mM) buffer, pH 8.0, containing 5 mM EDTA. Protein was precipitated with 1.7% meta-phosphoric acid, followed by centrifugation. The supernatant was then incubated with o-phthaldialdehyde (Sigma-Aldrich, at a concentration of 1 mg/ml methanol) at 22 °C for 15 min (Browne and Armstrong 1998). A calibration curve (GSH solutions from 0 to 500 μM; Sigma-Aldrich) was performed and fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. The results are expressed in nmol/mg protein.

Glutamate cysteine ligase activity Glutamate cysteine ligase (GCL) was assayed according to Seelig *et al*., with slight modifications (Seelig and Meister 1985). Cell lysate, suspended in a sodium phosphate buffer containing 140 mM KCl, was diluted with 100 mM sodium phosphate buffer (pH 8.0) containing 5 mM EDTA. The enzyme activity was determined after monitoring the NADH oxidation at 340 nm in sodium phosphate/KCl (pH 8.0) containing 5 mM ATP-Na_2, 2 mM phosphoenolpyruvate, 10 mM L-glutamate, 10 mM L-α-amino-butyrate, 20 mM $MgCl_2$, 2 mM EDTA-Na_2, 0.2 mM NADH, and 17 μg of pyruvate kinase/lactate dehydrogenase. The results are expressed in nmol/mg protein/min.

Glutathione peroxidase activity Glutathione peroxidase (GPx) activity was measured using the RANSEL kit from Randox (Autrim, UK). The concentration of GPx in lysed cells is assessed by measuring the absorption of NADPH at 340 nm. The results are expressed as U/mg protein.

DCFH oxidation Intracellular ROS levels were detected using 2′-7′-dichorofluorescein diacetate (DCFH-DA; Sigma-Aldrich), which was added to the culture medium at a concentration of 10 μM and incubated for 30 min at 37 °C. The intensity of fluorescence was measured in a plate reader with excitation at 485 nm and emission at 520 nm (Bobermin *et al*. 2022). The results are expressed as percentages relative to the control conditions.

ELISA assays The extracellular levels of the cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-10 (IL-10), as well as of the trophic factor GDNF, were measured in the culture medium of astrocytes using ELISA commercial kits. The assay ranges for the kits are the following: 16 to 2000 pg/ml for TNF-α (Invitrogen, Waltham MA catalog #88–7340–22); 31.3 to 2000 pg/ml for IL-1β (Invitrogen, catalog #BMS630); 31.3 to 2000 pg/ml for IL-6 (Invitrogen, catalog #BMS625); 15.6 to 1000 pg/ml for IL-10 (Invitrogen, catalog #BMS629); 31.2 to 2000 pg/ml for GDNF (Abcam; Cambridge, UK catalog #ab213901). The results are expressed in pg/ml.

RNA extraction and quantitative RT-PCR Total RNA was isolated from astrocyte cultures using TRIzol Reagent (Invitrogen). The concentration and purity of the RNA were determined spectrophotometrically at a ratio of 260:280. Then, 1 µg of total RNA was reverse transcribed using Applied Biosystems High Capacity complementary DNA (cDNA) Reverse Transcription Kit (Applied Biosystems, Waltham, MA) in a 20 µL reaction according to manufacturer's instructions. The messenger RNA (mRNA) encoding each target genes was quantified using the TaqMan real-time RT-PCR system with inventory primers and probes purchased from Applied Biosystems as summarized in Table 1. Quantitative RT-PCR was performed using the StepOne System from Applied Biosystems during 40 cycles of amplification. Target mRNA levels were normalized to β-actin levels. Results were analyzed employing the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Table 1. Genes analyzed by quantitative RT-PCR (qRT-PCR)

mRNA target	Assay ID
Aquaporin 4 (AQP4)	Rn00563196_m1
β-actin	Rn00667869_m1
Cyclooxygenase-2 (COX-2)	Rn01483828_m1
Glial fibrillary acidic protein (GFAP)	Rn00566603_m1
Glutamate aspartate transporter (GLAST)	Rn00570130_m1
Glutamate transporter 1 (GLT-1)	Rn00691548_m1
Glutamine synthetase (GS)	Rn01483107_m1
Heme oxygenase-1 (HO-1)	Rn01536933_ m1
Inducible nitric oxide synthase (iNOS)	Rn00561646_m1
Interleukin-1β (IL-1β)	Rn00580432_m1
Nestin	Rn00564394_m1
Nuclear factor erythroid 2-related factor 2 (Nrf2)	Rn00582415_m1
Nuclear factor kappa B p65 subunit (NFκB p65)	Rn01502266_m1
p21	Rn00589996_m1
Tumor necrosis factor-α (TNF-α)	Rn99999017_m1
Vimentin	Rn00667825_m1

Protein assay Protein content was measured using Lowry's method with bovine serum albumin as a standard (Lowry *et al*. 1951).

Statistical analyses Differences among groups were statistically analyzed using two-way analysis of variance (ANOVA) followed by Tukey's test or Student's t test. All analyses were performed using the GraphPad Prism 9 (GraphPad Software, Inc., La Jolla, CA). Values of $P < 0.05$ were considered significant; * refers to statistically significant differences between control and aged in vitro astrocytes (same age) and # refers to statistically significant differences between ages (astrocytes from neonatal *versus* adult animals).

Results

Astrocytes from neonatal and adult animals aged in vitro showed glial functional changes In vitro aging (astrocytes cultured for 42 days) did not affect the cell viability of primary astrocyte cultures obtained from both neonatal and adult animals compared to control conditions (21 days in vitro; data not shown). However, in vitro aging reduced glucose uptake, but only in astrocytes from neonatal animals (Fig. 2*A*; $P = 0.0003$). At control conditions, glucose uptake is decreased in astrocytes from adult animals compared to astrocytes from neonates ($P = 0.0017$). Regarding glutamate uptake, at control conditions, glutamate uptake was also decreased in astrocytes from adult animals compared to those obtained from neonates (Fig. 2*B*; $P < 0.0001$). In vitro aging produced opposite effects; in astrocytes derived from neonatal rats, glutamate uptake was increased ($P < 0.0001$), while in astrocytes obtained from adults it was decreased ($P = 0.0065$). To investigate the involvement of PI3K and p38 MAPK signaling in the changes in glutamate uptake induced by in vitro aging, we incubated astrocytes with LY294002 and SB203580 inhibitors. Inhibition of PI3K pathway prevented the effect of in vitro aging on glutamate uptake in neonatal astrocytes (table insert, Fig. 2*B*). In contrast, p38 MAPK inhibition prevented the impairment of glutamate uptake in adult astrocytes aged in vitro (table insert, Fig. 2*B*). In neonatal astrocytes, in vitro aging did not change the activity of GS, but it was decreased in adult astrocytes (Fig. 2*C*; $P < 0.0001$). Comparing control neonatal and adult astrocytes, the activity of GS was decreased in the cultures obtained from adult animals ($P = 0.0001$).

In vitro aging affected redox homeostasis in astrocytes GSH content was markedly decreased by in vitro aging in astrocytes obtained from adult animals ($P = 0.0017$), but not from neonates (Fig. 3*A*). There was also an age-dependent decrease of GSH levels at control conditions ($P < 0.0001$). In agreement with that, the activity of GCL (Fig. 3*B*)

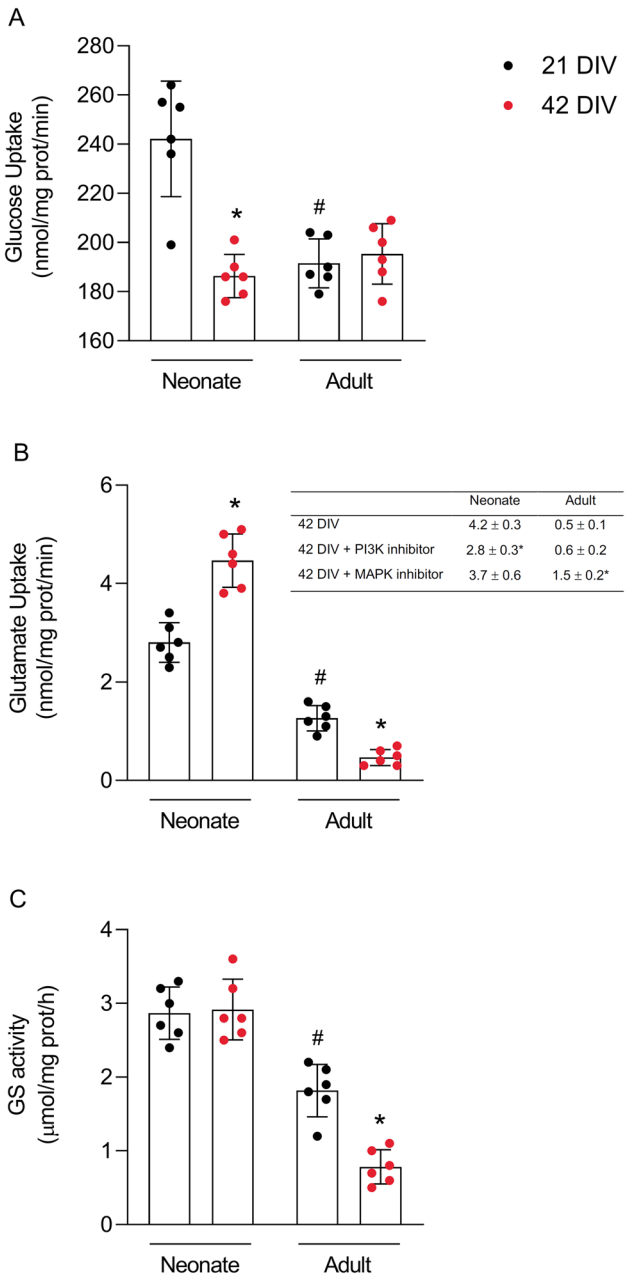

Figure 2. Effects of in vitro aging on glucose and glutamate uptake and GS activity in astrocytes obtained from neonatal and adult animals. Astrocyte cultures obtained from neonatal and adult animals were cultured for 21 (control) or 42 (in vitro aging). Glucose uptake (*A*), glutamate uptake (*B*), and GS activity (*C*) were evaluated. Additionally, astrocytes were incubated with LY294002 (10 µM) or SB203580 (5 µM) for 3 h to examine the modulatory roles of PI3K and p38 MAPK, respectively, on glutamate uptake (insert table). Data are presented as mean ± S.D. and differences among groups were statistically analyzed using two-way analysis of variance (ANOVA), followed by Tukey's test (n = 6 independent cultures and, at least, duplicate of treatments). Values of $P < 0.05$ were considered significant. * refers to statistically significant differences between control and aged in vitro astrocytes (same age) and # refers to statistically significant differences between ages (astrocytes from neonatal versus adult animals).

was decreased in astrocytes derived from adults, both upon in vitro aging ($P = 0.0034$) and at control conditions ($P < 0.0001$). However, the activity of GPx was increased in astrocytes from adults aged in vitro ($P = 0.0008$), as well as it was increased at basal conditions relative to astrocytes obtained from neonatal animals (Fig. 3*C*; $P = 0.0001$).

In vitro aging also increased ROS production in both astrocytes obtained from neonatal and adult rats (Fig. 3*D*; $P = 0.0033$ and $P < 0.0001$, respectively). An age-dependent increase of ROS production was also observed at control conditions ($P < 0.0001$). Astrocytes aged in vitro were then oxidatively challenged with H_2O_2, which further increased ROS production in astrocytes from adult rats ($P < 0.0001$), but not in astrocytes from neonatal rats (Fig. 3*E*).

Astrocytes aged in vitro were also incubated with the antioxidant resveratrol, including in the presence of the inhibitor of HO-1 (ZnPP IX), which has been described as an important mediator of its protective effects. Resveratrol decreased ROS production in astrocytes derived from neonatal and adult animals and aged in vitro ($P < 0.0001$), but not in the presence of HO-1 inhibitor (Fig. 3*F*).

In vitro aging induced changes in inflammatory response and GDNF release in astrocytes Oxidative stress and inflammatory responses are tightly linked processes. Astrocytes aged in vitro released increased amounts of TNF-α compared to their respective controls, with a greater effect observed in astrocytes from adult rats (Fig. 4*A*; $P = 0.0053$ and $P < 0.0001$). The extracellular levels of this cytokine were also increased between ages at control conditions ($P = 0.0044$). LPS challenge potentiated the release of TNF-α only in astrocytes from adult rats aged in vitro (Fig. 4*B*; $P < 0.0001$). Moreover, incubation with TLR4 inhibitor prevented TNF-α release induced by LPS (Fig. 4*B*). When aged astrocytes were incubated with resveratrol, an HO-1-dependent decrease of TNF-α ($P < 0.0001$) was observed only in astrocytes obtained from adult animals (Fig. 4*C*).

In vitro aging also increased the release of IL-1β (Fig. 4*D* $P < 0.0001$), which was further increased by LPS, in both astrocytes obtained from neonatal ($P = 0.0001$) and adult ($P < 0.0001$) animals. Of note, LPS challenge induce an increase in IL-1β release three-fold higher than in astrocytes from neonatal rats. At control conditions, extracellular levels of IL-1β were also augmented comparing astrocyte cultures obtained from both ages ($P < 0.0001$).

After characterizing the effects of in vitro aging in neonatal and adult astrocytes relative to control conditions, the subsequent analyses were performed comparing only astrocytes aged in vitro from both ages. The extracellular levels of IL-6 were higher in in vitro aged astrocytes from adult animals compared to those obtained from neonatal animals (Fig. 4*E*; $P < 0.001$). However, the extracellular levels of

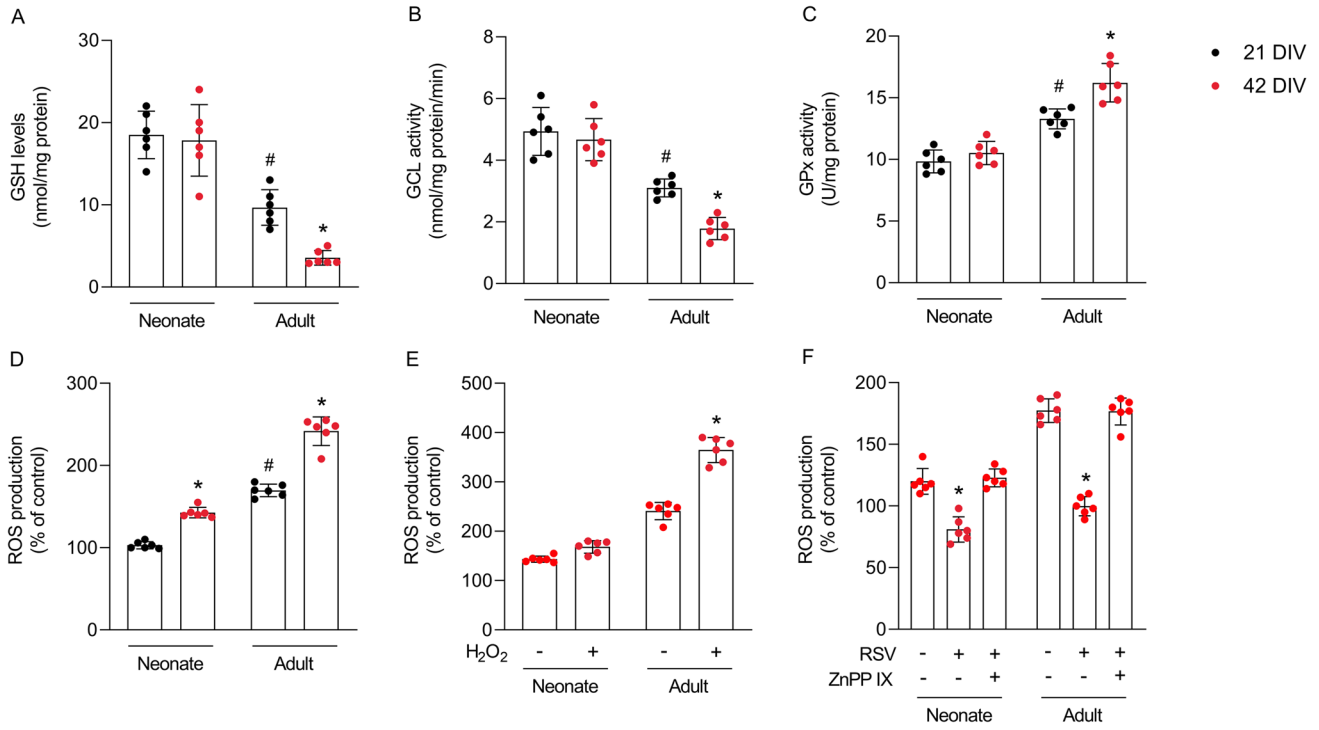

Figure 3. Effects of in vitro aging on astrocyte redox homeostasis. Astrocyte cultures obtained from neonatal and adult animals were cultured for 21 days (control) or 42 days (in vitro aging). GSH content (*A*), GCL activity (*B*), GPx activity (*C*), and ROS production (*D*-*F*) were evaluated. Additionally, astrocytes were incubated with H_2O_2 (100 µM), resveratrol (100 µM) or HO-1 inhibitor (10 µM) for 3 h to assess ROS production. Data are presented as mean ± S.D. and differences among groups were statistically analyzed using two-way analysis of variance (ANOVA), followed by Tukey's test (n = 6 independent cultures and, at least, duplicate of treatments). Values of $P < 0.05$ were considered significant. * refers to statistically significant differences between control and aged in vitro astrocytes (same age) and # refers to statistically significant differences between ages (astrocytes from neonatal versus adult animals).

the anti-inflammatory cytokine IL-10 and the trophic factor GDNF were decreased in astrocytes aged in vitro derived from adult rats compared to neonatal rats (Fig. 4*F* and *G*, respectively, $P < 0.001$).

Astrocytes from adult animals aged in vitro showed differences in the expression of key genes compared to astrocytes from neonatal animals aged in vitro We then compared the expression of genes involved in key astrocyte functions between astrocytes isolated from neonatal and adult rats aged in vitro. The expression of senescence marker p21 was increased in astrocytes from adults aged in vitro (Fig. 5*A*; $P < 0.001$). However, the mRNA levels of cytoskeleton proteins GFAP (Fig. 5*B*; $P < 0.001$), vimentin (Fig. 5*C*; $P = 0.005$), and nestin (Fig. 5*D*; $P = 0.005$) were decreased. Glutamate transporters GLAST and GLT-1 were also markedly downregulated (Fig. 5*E* and *F*, respectively, $P < 0.001$), while the expression of GS did not change (Fig. 5*G*) and the mRNA levels of AQP4 increased (Fig. 5*H*; $P < 0.001$). The expressions of inflammatory genes TNF-α (Fig. 5*I*), IL-1β (Fig. 5*J*), COX-2 (Fig. 5*K*), and iNOS (Fig. 5*L*), as well as of the p65 NFκB (Fig. 5*M*), were increased in astrocytes obtained from adult animals aged in vitro compared to astrocytes from neonatal animals aged in vitro ($P < 0.001$). In contrast, mRNA levels of cytoprotective molecules Nrf2 (Fig. 5*N*) and HO-1 (Fig. 5*O*) were decreased ($P < 0.001$).

Discussion

Recent evidence suggests that glia are the first cells that undergo a functional and neurochemical remodeling with aging, which may be tightly linked to an impaired protective capacity (Soreq *et al*. 2017; Clarke *et al*. 2018). Cultured astrocytes have been a widely used experimental approach to study aging-related changes in astrocyte functions, but since they are commonly isolated from newborn rodents, they are not exposed to challenging experiences throughout life and during aging process. In this regard, our group has demonstrated that primary astrocyte cultures obtained from adult and aged animals (90 to 2 old) can more reliably reproduce the alterations of glial functionality observed in aging (Bellaver *et al*. 2017; Santos *et al*. 2018; Bobermin *et al*. 2022; Sovrani *et al*. 2023), but it is important to consider the need for long-term maintenance of animals. Thus, the

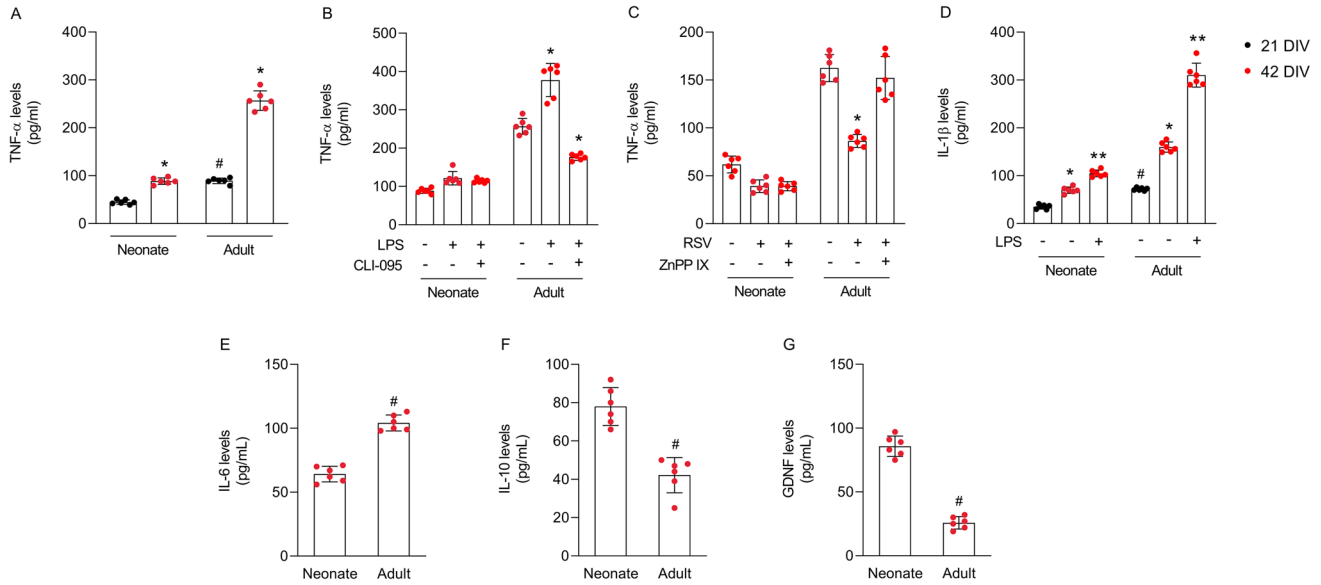

Figure 4. In vitro aging changed the release of cytokines and GDNF. Astrocyte cultures obtained from neonatal and adult animals were cultured for 21 (control) or 42 (in vitro aging). Extracellular levels of TNF-α (*A-C*), IL-1β (*D*), IL-6 (*E*), IL-10 (*F*) and GDNF (*G*) were measured. Additionally, astrocytes were incubated with LPS (100 µM), TLR4 inhibitor, resveratrol (100 µM) or HO-1 inhibitor (10 µM) for 3 h to assess TNF-α or IL-1β release. Data are presented as mean ± S.D. and differences among groups were statistically analyzed using two-way analysis of variance (ANOVA), followed by Tukey's test (n = 6 independent cultures and, at least, duplicate of treatments). Values of $P < 0.05$ were considered significant. * refers to statistically significant differences between control and aged in vitro astrocytes (same age); ** refers to statistically significant differences between aged in vitro astrocytes without LPS and with LPS (same age); and # refers to statistically significant differences between ages (astrocytes from neonatal versus adult animals).

establishment of other in vitro models of aging is relevant to study the underlying mechanisms related to the transition from functional to potentially dysfunctional astrocytes. Astrocytes maintained in vitro after confluence (long-term astrocyte culture models) can show features of cellular senescence (Pertusa *et al*. 2007; Matias *et al*. 2022). Of note, the expression of p21 is a well-established senescence marker and its upregulation has been observed in astrocytes obtained from aged animals (Bellaver *et al*. 2017; Bobermin *et al*. 2022; Sovrani *et al*. 2023). Here, we found that in vitro aged astrocytes obtained from adult animals showed a markedly upregulation of p21 compared to in vitro aged astrocytes obtained from neonatal animals, thus they represent an advantage in culture methods for studying aged astrocytes. Moreover, other different cellular and molecular effects in astrocytes derived from neonatal and adult animals were observed, mainly regarding to glutamatergic, oxidative, and inflammatory parameters, which are commonly associated age-related brain diseases (Mattson and Arumugam 2018; Salas *et al*. 2020).

Hypometabolism has been observed during brain aging (Cunnane *et al*. 2020; Zhang *et al*. 2021) with implications in glial-to-neurons metabolic support. Of note, in addition to energy supply, glucose metabolism is important for maintaining brain redox homeostasis through NADPH generation by the pentose-phosphate pathway (Gonçalves *et al*. 2018). Aging can drive metabolic dysfunctions and affect the metabolic machinery of astrocytes (Chen *et al*. 2023). In this regard, glucose uptake activity affects the capacity of glycolysis, and it was observed a decrease in glucose uptake only in astrocytes aged in vitro obtained from neonatal animals. Metabolic alterations have also been observed in other in vitro models of aging in astrocytes, including a decrease in glucose transporter (GLUT-1) expression, which is essential for the entry of glucose into the CNS and is present in astrocytes (Cao *et al*. 2019). However, it is important to note that astrocytes of adult animals showed a decreased glucose uptake compared to cells from neonatal rats at control conditions, indicating that changes in astrocyte metabolism may precede aging.

Accumulated evidence has also demonstrated the relationship between astrocyte aging and glutamatergic system (Potier *et al*. 2010; Limbad *et al*. 2020). Astrocytes are responsible for the uptake of extracellular glutamate due to their high-affinity glutamate transporters, GLAST and GLT-1 (Danbolt 2001). Reinforcing the age-related effects on glutamate homeostasis, we observed that astrocytes from adult rats have a decreased glutamate uptake activity compared with astrocytes from neonatal rats at basal conditions. The in vitro model of aging caused opposite effects on glutamate uptake in astrocytes obtained from neonatal and adult rats. In agreement with previous findings (Gottfried *et al*.

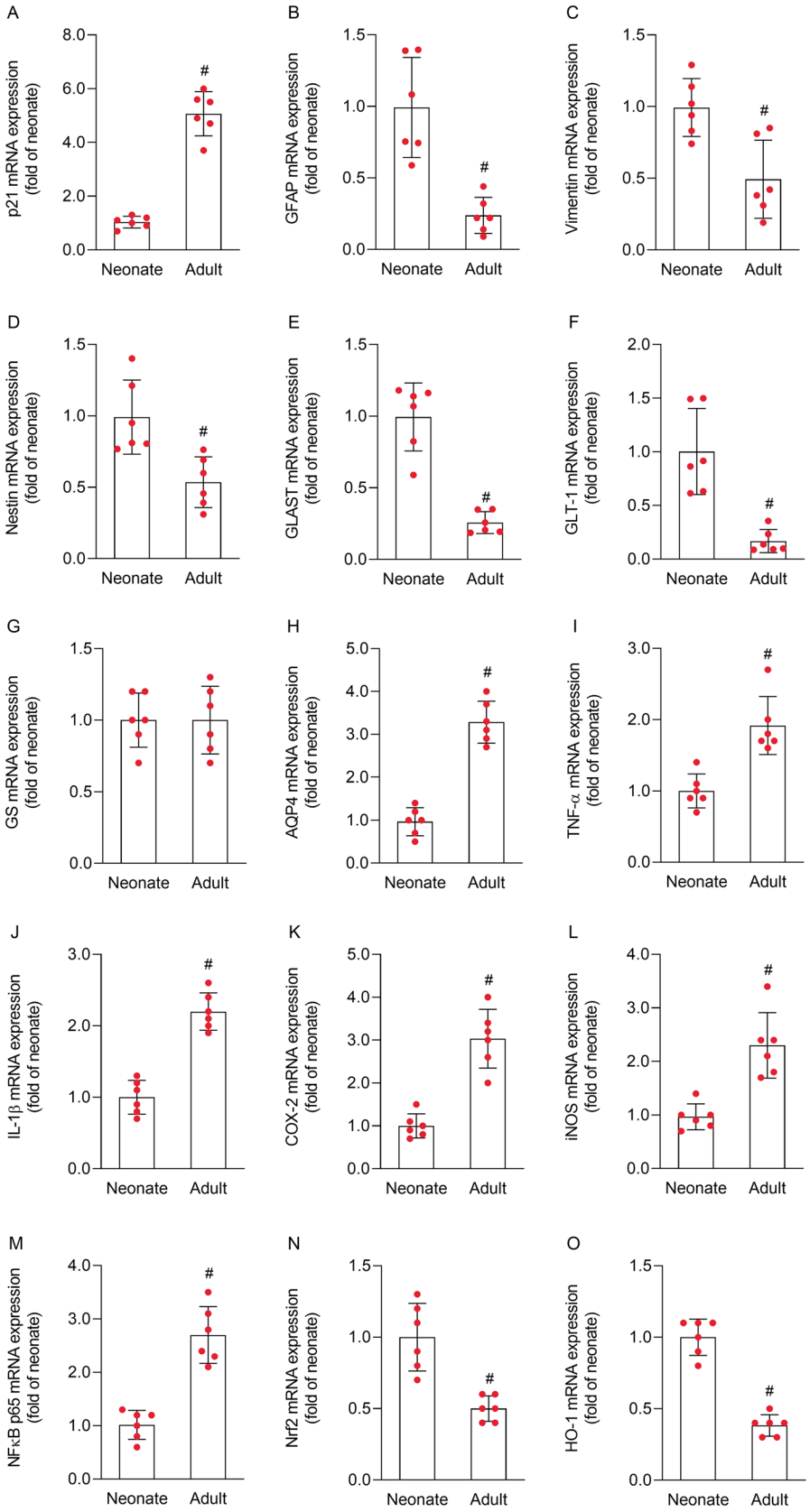

Figure 5. In vitro aging changes the expression of genes involved in key astrocyte functions. Astrocyte cultures obtained from neonatal and adult animals were cultured for 42 (in vitro aging). The mRNA expression of p21 (*A*), GFAP (*B*), vimentin (*C*), nestin (*D*), GLAST (*E*), GLT-1 (*F*), GS (*G*), AQP4 (*H*), TNF-α (*I*), IL-1β (*J*), COX-2 (*K*), iNOS (*L*), p65 NFκB (*M*), Nrf2 (*N*), and HO-1 (*O*). Data are presented as mean ± S.D. and differences among groups were statistically analyzed using Student's t test (n = 6 independent cultures and, at least, duplicate of treatments). Values of $P < 0.05$ were considered significant. # refers to statistically significant differences between ages (astrocytes from neonatal versus adult animals).

2002; Matias *et al*. 2023), astrocytes from neonatal animals aged in vitro showed an increased glutamate uptake, which could represent a neuroprotective mechanism of astrocytes against excitotoxicity. However, in astrocytes obtained from adult animals, in vitro aging decreased glutamate uptake. Interestingly, mice transcriptome datasets show that GLT-1 expression increased from 1 to 9 old of age but drastically decreased in the astrocytes of 2-old animals (Matias *et al*. 2023). Therefore, glutamate uptake function in astrocytes seems to vary dependent on the stage of aging and considering the marked downregulation of GLAST and GLT-1 found in astrocytes obtained from adult animals aged in vitro, these cells may reproduce the glutamatergic alterations that occur in advanced ages and that increased the susceptibility to excitotoxicity.

Glutamate uptake can be also modulated by redistribution of glutamate transporters to or from the plasma membrane, which is under regulation of many signaling pathways (Robinson 2006). We found that the changes in glutamate uptake activity induced by in vitro aging model in astrocytes isolated from neonatal and adult animals were dependent on different signaling pathways. While the inhibition of PI3K prevented the increase of glutamate uptake in astrocytes from neonates, the inhibition of p38 MAPK prevented the impairment of glutamate uptake in astrocytes from adults. PI3K signaling may lead to changes in glial cell functions, and it was already demonstrated that PI3K modulates GLT-1 mRNA levels (Peng *et al*. 2019) and protein content in cell surface (Guillet *et al*. 2005). In contrast, p38 MAPK plays a key role in pathological processes and has been associated with downregulation of GLT-1 (Zhang *et al*. 2019) and GLAST (Piao *et al*. 2015). Noteworthy, both glutamate transporters and p38 MAPK are redox-sensitive (Trotti *et al*. 1998; Vaziri and Rodríguez-Iturbe 2006), thus an increased ROS production may be linked to the glutamate uptake impairment. In line with this, astrocytes exposed to H_2O_2 show a decreased glutamate uptake, which was potentiated under an in vitro aging condition (Pertusa *et al*. 2007). Thus, it seems likely that in vitro aging activates signaling pathways depending on cellular context, which may result in differential effects on glutamate transporter regulation.

GS is a key enzyme that catalyzes the conversion of glutamate into glutamine in astrocytes. The glutamate-glutamine cycle is essential for the replenishment of presynaptic glutamate and physiological synaptic activity, while its impairment is also associated with glutamate excitotoxicity (Jayakumar and Norenberg 2016; Cheung *et al*. 2022). In vitro senescence models have demonstrated both upregulation and downregulation of GS in astrocytes (Shen *et al*. 2014; Cao *et al*. 2019; Matias *et al*. 2023). In the current study, we observed that in vitro aging did not change GS activity in astrocytes from neonatal rats, although there was an increase in glutamate uptake. In astrocytes from adult animals, accompanying the impairment in glutamate uptake, in vitro aging caused a decrease in GS activity. However, there was no difference in GS mRNA expression comparing astrocytes aged in vitro derived from neonatal and adult rats. This result suggests that the decreased activity may be a consequence of oxidative stress (Frieg *et al*. 2021). Moreover, in accordance with our previous data (Bellaver *et al*. 2017; Santos *et al*. 2018; Bobermin *et al*. 2020), astrocytes from adult animals showed a reduction of GS at basal conditions, reinforcing age-related changes in its functioning.

Redox imbalance and oxidative stress are hallmarks of brain aging (Mattson and Arumugam 2018). Although astrocytes notably participate in antioxidant defenses, they may also be an important source of ROS in injury conditions (Rizor *et al*. 2019). In vitro aging increased ROS production in astrocytes from both neonatal and adult rats, as well as an age-related difference was observed at basal conditions. The challenge with H_2O_2 further increased ROS production only in astrocytes from adult rats aged in vitro, suggesting a higher sensitivity of these cells to oxidative stimuli. In contrast, the well-known antioxidant resveratrol was able to decrease ROS production in astrocytes aged in vitro derived from both neonatal and adult animals in a manner dependent on HO-1, thus supporting its already demonstrated gliioprotective effects during aging (Bobermin *et al*. 2022; Sovrani *et al*. 2023). Nrf2/HO-1 signaling has been proposed as an important mechanism underlying the protective roles of resveratrol and to prevent age-related oxidative stress (Bobermin *et al*. 2019, 2022; Liu *et al*. 2021; Zhao *et al*. 2021). Of note, the gene expression of Nrf2 and HO-1 was decreased in astrocytes aged in vitro from adult rats compared to astrocytes aged in vitro from neonatal rats, which may be explain at least in part the increased susceptibility to the oxidative stimulus with H_2O_2. In addition, intracellular levels of GSH and the activity of GCL, the limiting enzyme of GSH biosynthesis, were decreased only in astrocytes aged in vitro from adult animals. It has been reported that the levels of GSH in astrocytes and in brain tissue decline with age, being potentially associated with neurodegenerative disorders (Lee *et al*. 2010; Currais and Maher 2013; Bellaver *et al*. 2017). Moreover, although we found that in vitro aging increased GPx activity in astrocytes from adult rats, possibly as a compensatory effect, it seems to be insufficient to counteract H_2O_2-induced ROS production.

Inflammatory response is another hallmark of aging, as well as a key element in the pathogenesis of neurodegenerative diseases (López-Otín *et al*. 2013; Ransohoff 2016). Astrocytes have been pointed as a significant source of the pro-inflammatory mediators, including TNF-α, IL-1β, and IL-6, involved in neuro-inflammaging (López-Teros *et al*. 2022). Here, we confirm that *in vitro* aging can induce the characteristic pro-inflammatory profile in astrocytes, particularly regarding to TNF-α and IL-1β release, obtained

from both neonatal and adult animals. These data are in line with our previous study in astrocytes obtained from 1 old animals (Bobermin *et al*. 2022). On the other hand, resveratrol mediated an anti-inflammatory effect via HO-1 only in astrocytes from adult rats, which is also consistent with our previous findings (Bobermin *et al*. 2022; Sovrani *et al*. 2023). Of note, inflammatory response upon LPS stimulation seems to be exacerbated in aged astrocytes (Clarke *et al*. 2018; Bobermin *et al*. 2022). Interestingly, we observed that incubation with LPS potentiated cytokine release particularly in in vitro aged astrocytes obtained from adult animals via TLR4 receptor, whose expression has been also correlated with age (Letiembre *et al*. 2007). Thus, considering that aging affects the ability of astrocytes to respond to acute inflammatory stimuli and can sensitize the brain to infections or stress (Sparkman and Johnson 2008; Bobermin *et al*. 2022; Quincozes-Santos *et al*. 2023), in vitro cellular models are essential for studying alterations in inflammatory responses of astrocytes with aging.

Therefore, there are obvious differential effects of in vitro aging in astrocytes from neonatal and adult rats. Based on these results, another set of experiments regarding to gene expression was performed to investigate potential molecular mechanisms associated with such differences, i.e., the specific features of in vitro aged astrocytes from adult rats that distinguish them from in vitro aged astrocytes from neonatal rats. Compared to astrocytes from neonates, astrocytes from adult animals aged in vitro show upregulation of inflammatory-related genes (TNF-α, IL-1β, p65 NFκB, iNOS, COX-2), in addition to the senescence marker p21. The expression of AQP4 was also upregulated in astrocytes from adult rats, and this water channel has been implicated in neuroinflammation and neurodegenerative diseases (Valenza *et al*. 2020). Additionally, astrocytes derived from adult animals aged in vitro had a decreased release of the anti-inflammatory cytokine IL-10 and the trophic factor GDNF, which may compromise their supportive functions (Lawrence *et al*. 2023). We also observed a marked decrease of GFAP mRNA levels in astrocytes from adult rats aged in vitro, in agreement with our previous studies that showed an age-dependent decrease of GFAP content in astrocyte cultures (Souza *et al*. 2015; Bellaver *et al*. 2017). The expression of vimentin and nestin, which decreases with age as they are progressively replaced by GFAP, was also downregulated in astrocytes aged in vitro. Although an increase of GFAP has been found in aging (Salas *et al*. 2020), a decrease of GFAP expression has been detected at the early stages of age-related neurodegenerative diseases (Rodríguez *et al*. 2014; Rodríguez-Arellano *et al*. 2016). Thus, it is important to consider that other cellular alterations may precede the GFAP upregulation. Furthermore, the profile of GFAP expression in astrocyte culture is not a consensus (Orre *et al*. 2014; Rodríguez-Arellano *et al*. 2016; Bellaver *et al*. 2017) and it is not an absolute marker of reactivity, since basal expression of GFAP is variable depending on the brain region and there are discrepancies between mRNA and protein levels (Escartin *et al*. 2021).

Emerging evidence has supported that astrocytes can be functionally heterogeneous (Matias *et al*. 2019). Although the nomenclature A1 and A2 for reactive astrocytes is dichotomic and may not represent the diversity of phenotypes that these cells can assume depending on age and or in response to pathologic conditions, A1 phenotype has been related to neurotoxic roles while A2 has neuroprotective properties. In this regard, A1-like astrocytes show upregulation of pro-inflammatory mediators (IL-1β, TNF-α, and IL-6) and NFκB, inhibition of PI3K-Akt pathway, downregulation of GDNF and other trophic factors, and changes in the expression of AQP4, among other hallmarks (Lawrence *et al*. 2023). Therefore, our data from in vitro aged astrocytes, particularly those obtained from mature animals, are consistent with an induction of A1-like astrocyte reactivity, which in turn is associated with a loss of supportive roles.

Changes in astrocyte functions associated with aging process and their impacts on vulnerability of brain to specific diseases and cognitive decline are only beginning to be understood. Despite of the complexity of aging process, cell culture remains a valuable tool for elucidating its underlying mechanisms. Here, we confirm age-related changes in astrocyte functions, comparing cells obtained from neonatal and adult rats, which can impact the experimental limitations in long-term maintenance of the animals to perform cellular and molecular studies about aging. In addition, we showed that in vitro aging can produce different effects in astrocyte cultures obtained from neonatal and adult Wistar rats, as well as differential responses to oxidative, inflammatory, and protective stimuli. In summary, primary astrocyte cultures obtained from adult animals and further aged in vitro may better reproduce glial changes and represent an innovative tool to study astrocyte aging, as well as potential glioprotective and/or anti-aging strategies.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no conflict of interest.

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