Neurochemical Research (2024) 49:2505–2518
https://doi.org/10.1007/s11064-024-04196-8

ORIGINAL PAPER

Glioprotective Effects of Sulforaphane in Hypothalamus: Focus on Aging Brain

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Received: 7 November 2023 / Revised: 4 June 2024 / Accepted: 10 June 2024 / Published online: 17 June 2024

Abstract

Sulforaphane is a natural compound with neuroprotective activity, but its effects on hypothalamus remain unknown. In line with this, astrocytes are critical cells to maintain brain homeostasis, and hypothalamic astrocytes are fundamental for sensing and responding to environmental changes involved in a variety of homeostatic functions. Changes in brain functionality, particularly associated with hypothalamic astrocytes, can contribute to age-related neurochemical alterations and, consequently, neurodegenerative diseases. Thus, here, we investigated the glioprotective effects of sulforaphane on hypothalamic astrocyte cultures and hypothalamic cell suspension obtained from aged Wistar rats (24 months old). Sulforaphane showed anti-inflammatory and antioxidant properties, as well as modulated the mRNA expression of astroglial markers, such as aldehyde dehydrogenase 1 family member L1, aquaporin 4, and vascular endothelial growth factor. In addition, it increased the expression and extracellular levels of trophic factors, such as glia-derived neurotrophic factor and nerve growth factor, as well as the release of brain-derived neurotrophic factor and the mRNA of TrkA, which is a receptor associated with trophic factors. Sulforaphane also modulated the expression of classical pathways associated with glioprotection, including nuclear factor erythroid-derived 2-like 2, heme oxygenase-1, nuclear factor kappa B p65 subunit, and AMP-activated protein kinase. Finally, a cell suspension with neurons and glial cells was used to confirm the predominant effect of sulforaphane in glial cells. In summary, this study indicated the anti-aging and glioprotective activities of sulforaphane in aged astrocytes.

Keywords Aging · Astrocytes · Glioprotection · Hypothalamus · Sulforaphane

Introduction

The hypothalamus is a crucial brain region that regulates energy balance, body temperature, circadian rhythm, reproduction, as well as vision and emotions [1–8]. It acts as a bridge between the central nervous system (CNS) and the periphery of the body by receiving information and generating an appropriate response. Evidence suggests that the hypothalamus actively participates in the aging process and can be a target for development of anti-aging and therapeutical strategies [9, 10]. In this sense, aging is a biological process that leads to a progressive decline

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in physical and cognitive abilities, resulting in impaired adaptive responses to environmental stressors. In the CNS, aging changes several neural functions, and hypothalamic astrocytes can contribute to age-related neurochemical alterations [11–13].

Astrocytes are a type of glial cells that play multiple roles in maintaining brain homeostasis. They regulate synaptic plasticity, neurotransmitter metabolism, and ionic balance; provide metabolic support to neurons; produce and release growth factors, inflammatory mediators, and antioxidants; and maintain the blood–brain barrier [11, 14–18]. In particular, hypothalamic astrocytes are fundamental for sensing and responding to environmental changes since they have receptors and transporters for hormones and molecules involved in a variety of homeostatic functions [19–23]. To promote an overall improvement of the CNS functions, glioprotection relies on specific responses of glial cells to protect themselves and neighbouring cells from damage, and it can be achieved by using specific molecules [17].

Sulforaphane is a naturally occurring compound found in cruciferous vegetables, such as broccoli, kale, and Brussels sprouts as a system of defense against pathogen attack [24, 25]. It has antioxidant and anti-inflammatory properties and has been shown to have several health benefits, including the improvement of cardiovascular health [17, 25–30]. The concentration of sulforaphane in these dietary sources varies depending on the type of cruciferous vegetable and even on the degree of maturation [31]. In rats, after oral administration of 50 µmol sulforaphane, the peak of plasma concentration reaches 20 µM after 4 h, a concentration that is relevant in in vitro cell studies [32]. Additionally, sulforaphane can cross the blood–brain barrier and reach the CNS to exert neuroprotective effects in pathological conditions such as stroke, traumatic brain injury, and neurodegenerative diseases [33–35]. However, the effects of sulforaphane on aged astrocytes remain unclear.

In this context, the purpose of the present study was to characterize the glioprotective effects of sulforaphane in the hypothalamus of aged rats, focused on astrocytes. For this, we evaluated neurochemical parameters related to inflammation and trophic factors (extracellular content and mRNA expression), as well as mRNA expression of genes associated with antioxidant activity/redox homeostasis, cytoprotective responses, astroglial and senescence markers in hypothalamic astrocyte cultures obtained from aged Wistar rats. In addition, to confirm the predominant effect of sulforaphane on glial cells, we evaluated the expression of the same parameters measured in cultured astrocytes in a cellular suspension of hypothalamic tissue containing both neurons and glial cells obtained from aged Wistar rats. To our knowledge, this is the first study that explores the anti-aging and glioprotective activities of sulforaphane in aged astrocytes.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium/F12 (DMEM/F12), TRIzol Reagent, other materials for cell culture, ELISA kits for interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), BDNF, nerve growth factor brain-derived neurotrophic factor (BDNF) and tumor necrosis factor-α (TNF-α) were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). Poly-L-lysine, sulforaphane and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). ELISA kit for glia-derived neurotrophic factor (GDNF) was purchased from R&D Systems (Minneapolis, MN, USA). High Capacity cDNA Reverse Transcription Kit, TaqMan real-time RT-PCR system, primers and probes were purchased from Applied Biosystems (Thermo Fisher Scientific).

All other chemicals were from common commercial suppliers.

Animals

Male Wistar rats (24 months old) were obtained from the breeding colony of Department of Biochemistry (Federal University of Rio Grande do Sul, Porto Alegre, Brazil) and maintained under a controlled environment (12 h-light/12 h-dark cycle, 22 ± 1 °C; ad libitum access to food and water). The animals received regular laboratory chow (Nuvilab-CR1, from Nuvital, Brazil). 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 35387).

Primary Hypothalamic Astrocyte Cultures from Aged Rats

The protocol described previously by Santos et al. [11] was used. Wistar rats at 24 months old had their hypothalamus dissected, and the meninges removed. The tissue was enzymatically digested in Hank's balanced salt solution (HBSS) containing 0.05% trypsin at 37 °C for 7 min. The tissue was then mechanically dissociated for 7 min and centrifuged at 100×*g* for 5 min. The pellet was resuspended in HBSS and again mechanically dissociated until complete homogenization, and then centrifuged at 100×*g* for 5 min. Then, cells were resuspended in DMEM/F12, supplemented with 10% fetal bovine serum (FBS), 15 mM HEPES, 14.3 mM $NaHCO_3$, 2.5 µg/mL amphotericin B, and 0.05 mg/mL

gentamicin. Cells were seeded (approximately 2–4 × 10^5 cells/cm^2) into 6-well plates pre-coated with poly-L-lysine and cultured at 37 °C in a 5% CO_2 incubator. After 24 h, the culture medium was exchanged; during the first week, the medium was replaced once every 2 days, and from the second week on, once every 4 days. From the second week on, the astrocytes received medium supplemented with 20% FBS until they reached confluence (at approximately the fourth week). No dibutyryl-cAMP was added to the culture medium. To determine whether the culture contained microglia or neurons after reaching confluence, we used anti-ß-tubulin III, anti-NeuN, and anti-CD11, and less than 5% of cells were labeled [11].

Hypothalamic Cell Suspension from Aged Rats

In addition to the primary culture, a cell suspension from the hypothalamic tissue was prepared. The hypothalamus from 24 months old Wistar rats were enzymatically digested in HBSS containing 0.05% trypsin, followed by a mechanical dissociation and centrifugation 100×*g* for 5 min. Cells were resuspended in serum-free DMEM/F12 and immediately incubated with sulforaphane.

Sulforaphane Treatments

To evaluate the effect of sulforaphane on hypothalamic astrocyte cultures, the cells were incubated in the absence (control conditions) or presence of 5 µM sulforaphane dissolved in dimethyl sulfoxide (DMSO) during 24 h at 37 °C in an atmosphere with 5% CO_2 in serum-free DMEM/F12 medium [36]. The hypothalamic cell suspension was incubated in the presence or absence of 5 µM sulforaphane dissolved in DMSO during 1 h at 37 °C in an atmosphere with 5% CO_2 in serum-free DMEM/F12 medium. It is noteworthy that a shorter time of incubation with sulforaphane was used in cell suspension from hypothalamus due to the viability of this tissue preparation. It should be also noted that the final concentration of DMSO (used as vehicle) did not present any effect on astroglial cells neither hypothalamic tissue [36, 37]. The concentration of sulforaphane utilized in this study is readily achievable in rat plasma after a single dose of sulforaphane [32, 38].

Cell Viability and Membrane Integrity Assays

The viability of astrocyte cultures following sulforaphane treatment was determined by performing the MTT reduction assay. MTT was added at a final concentration of 50 µg/mL and incubated for 3 h at 37 °C in a 5% CO_2 atmosphere. After removing the medium, the MTT crystals were dissolved in DMSO, and the absorbance was measured at 560 and 650 nm [39].

Furthermore, the integrity of the cell membrane was evaluated using the propidium iodide (PI) incorporation assay. Astrocyte cultures were incubated with 7.5 µM PI for 30 min before the end of the sulforaphane treatment at 37 °C in an atmosphere with 5% CO_2. Loss of membrane integrity results in fluorescent nuclei labeling with PI, which was quantified using OptiQuant software (Packard Instrument Company).

Trophic Factors and Inflammatory Response Measurements

BDNF, GDNF, and NGF levels were measured in the extracellular medium of astrocyte cultures, using commercial ELISA kits. The results are expressed in pg/mL. Cytokine levels were measured in the extracellular medium of astrocyte cultures using ELISA kits for TNF-α, IL-1β, IL-6, and IL-10 and the results are expressed in pg/mL.

RNA Extraction and Quantitative RT-PCR

Total RNA was isolated from astrocyte cultures or hypothalamic cell suspension using TRIzol Reagent. Extracted RNA (1 µg) was submitted to cDNA synthesis by High-Capacity cDNA Reverse Transcription Kit. Quantitative PCR determination of the mRNAs encoding adenosine receptors A_1 (#Rn00567668_m1), A_{2A} (#Rn00583935_m1), and A_{2B} (#Rn00567697_m1), aldehyde dehydrogenase 1 family member L1 (ALDH1L1) (#Rn00674034_m1), AMP-activated protein kinase (AMPK) (#Rn00576935_m1), aquaporin 4 (AQP4) (#Rn00563196_m1), β-actin (#Rn00667869_m1), BDNF (#Rn02531967_s1), cyclooxygenase 2 (COX-2; #Rn01483828_m1), glutamate-cysteine ligase (GCL; #Rn00689046_ m1), GDNF (#Rn07311775_m1), glial fibrillary acidic protein (GFAP; #Rn00566603_m1), glutamine synthetase (GS; #Rn01483107_m1), heme oxygenase-1 (HO-1) (#Rn01536933_ m1), IL-1β (#Rn00580432_m1), IL-6 (#Rn01410330_m1), IL-10 (#Rn00563409_m1), inducible nitric oxide synthase (iNOS; #Rn00561646_m1), NGF (Rn01533872_m1), nuclear factor kappa B p65 subunit (NFκB p65) (#Rn01502266_m1), nuclear factor erythroid-derived 2-like 2 (Nrf2) (#Rn00582415_m1), peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α; #Rn00580241_m1), p21 (#Rn 00589996_m1), sirtuin 1 (SIRT1; #Rn01428096_ m1), superoxide dismutase 1 (SOD1; #Rn00566938_m1), superoxide dismutase 2 (SOD2; #Rn00690588_g1), SRY-box transcription factor 10 (SOX10; #Rn00569909_m1), TNF-α (#Rn99999017_m1), tyrosine protein kinase receptor A (TrkA; #Rn00572130_m1), tyrosine protein kinase receptors B (TrkB; #Rn01441749_m1), and vascular endothelial growth factor (VEGF) (#Rn01511602_m1) were performed using the TaqMan real-time RT-PCR system with inventory

primers and probes, as referred for each gene. Target mRNA levels were normalized to β-actin levels. Results were analyzed employing the $2^{-\Delta\Delta Ct}$ method [40] and expressed relative to the control levels.

Statistical Analyses

Results are presented as mean ± standard deviation (S.D). The normal distribution was confirmed by Shapiro–Wilk test and then data were statistically analyzed using Student's t-test. P values < 0.05 were considered significant. *Indicates differences between control and sulforaphane groups. All analyses were performed using GraphPad Prism 9.

Results

Anti-inflammatory Effects of Sulforaphane in Hypothalamic Astrocyte Cultures from Aged Rats

First, we tested the cellular viability (measured by MTT reduction) and membrane integrity (by PI incorporation) of three concentrations of sulforaphane (1, 5, and 10 µM) for 24 h in astrocyte cultures. Only 10 µM of sulforaphane decreased MTT reduction and increased PI incorporation (data not shown). Therefore, based on these data and a previous study of our group with sulforaphane in C6 astroglial cells [36], we choose the safely applicable concentration of 5 µM of sulforaphane to treat aged hypothalamic astrocyte cultures. In addition, 5 µM is a commonly used dose since higher doses were proven to be cytotoxic in certain experimental models [28, 36, 37, 41].

In line with this, as shown in the Fig. 1a–d, sulforaphane was able to decrease both the release and mRNA expression of the pro-inflammatory mediators TNF-α ($P < 0.001$ and $P = 0.01$) and IL-1β (both $P < 0.001$), and the release of IL-6 ($P < 0.001$; Fig. 1e). In contrast, sulforaphane increased the release and mRNA expression of the anti-inflammatory cytokine IL-10 ($P < 0.001$ and $P = 0.004$, respectively; Fig. 1g and h).

The mRNA expression of COX-2 was also measured and sulforaphane was able to decrease it ($P = 0.002$; Fig. 1i). In addition, we observed that sulforaphane increased the mRNA expression of both adenosine receptors A_1 and A_{2A} ($P = 0.009$ and $P = 0.002$; Fig. 1j and k), while A_{2B} was not affected (Fig. 1l).

Antioxidant-Related Effects of Sulforaphane in Astrocyte Cultures from Aged Rats

Our results showed that sulforaphane did not induce changes in the mRNA expression of SOD 1 and 2 (Fig. 2a and b). However, we found that sulforaphane was able to increase the mRNA expression of GCL and PGC-1α (both $P < 0.001$; Fig. 2c and e), and to decrease the mRNA expression of iNOS ($P < 0.001$; Fig. 2d).

Effects of Sulforaphane on mRNA of Proteins Related to Astrocyte Markers

Our results showed that sulforaphane had not effect on GFAP and GS mRNA expressions (Fig. 3a and b). On the other hand, sulforaphane increased ALDHL1 ($P < 0.001$; Fig. 3c), SOX10 ($P = 0.002$; Fig. 3d) and VEGF ($P = 0.002$; Fig. 3f) mRNA expressions and dowregulated AQP4 ($P = 0.03$; Fig. 3e).

Effects of Sulforaphane on Trophic Factors

In this study, we observed that sulforaphane was able to increase the mRNA expression of GDNF and NGF ($P < 0.001$; Fig. 4d and f), in addition to increase the release of these trophic factors and BDNF ($P < 0.001$ for all; Fig. 4a, c and e). Moreover, sulforaphane increased TrkA mRNA expression ($P < 0.001$; Fig. 4g), but did not change TrkB mRNA levels (Fig. 4h).

Potential Mechanisms Associated with Sulforaphane-Induced Glioprotection

Sulforaphane has a broad range of protective effects, which have been linked to several signaling pathways, including Nrf2, which activate cytoprotective genes [26, 42]. Furthermore, Nrf2 is known to act as an upstream signal for NFκB and HO-1 [43]. Here, we observed that sulforaphane increased the mRNA expressions of Nrf2 and HO-1 (both $P < 0.001$; Fig. 5a and b), while in the p65 NFκB expression ($P < 0.001$; Fig. 5c) it showed an opposite effect.

In addition, sulforaphane did not alter SIRT1 mRNA expression (Fig. 5d), but decreased AMPK and p21 mRNA expressions ($P = 0.004$ and $P < 0.001$, respectively; Fig. 5e and f).

Effects of Sulforaphane on Hypothalamic Tissue

We further evaluated the effects of sulforaphane in hypothalamic tissue, by using a cell suspension containing both neurons and glial cells. The cell suspension was incubated with 5 µM sulforaphane for 1 h to maintain complete viability of this hypothalamic preparation. Specific parameters were evaluated, and some differences were observed. Figure 6 displays that sulforaphane increased the mRNA expression of TNF-α ($P < 0.001$; Fig. 6a), IL-6 ($P < 0.001$; Fig. 6c), IL-10 ($P < 0.001$; Fig. 6d), Nrf2 ($P < 0.001$; Fig. 6f), HO-1 ($P < 0.001$; Fig. 6g), SIRT1 ($P = 0.02$; Fig. 6j), GDNF ($P < 0.001$; Fig. 6o), NGF ($P = 0.002$; Fig. 6p) and TrkA

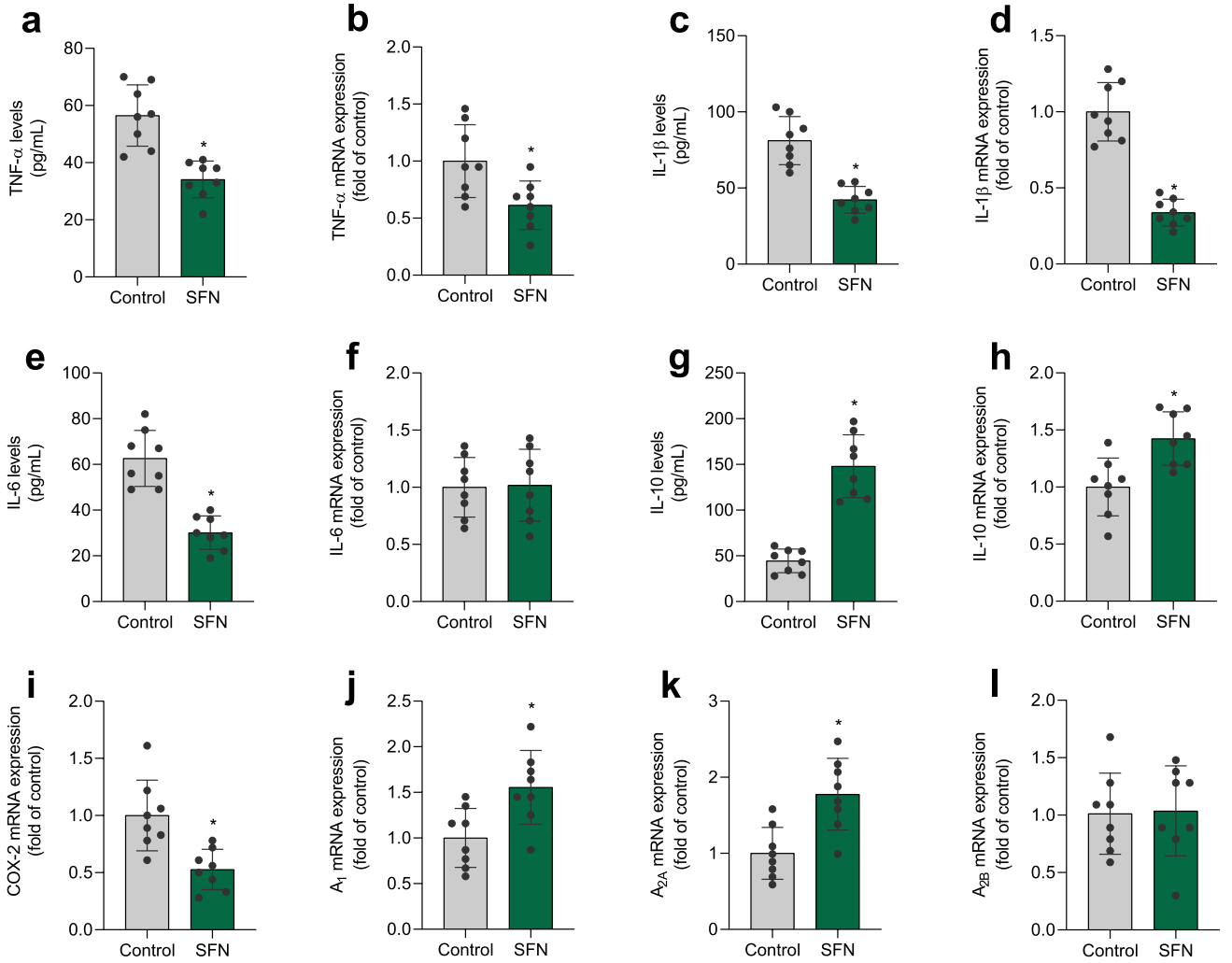

Fig. 1 Anti-inflammatory effects of sulforaphane in hypothalamic astrocyte cultures from aged rats. The release and mRNA expression of TNF-α (**a** and **b**), IL-1β (**c** and **d**), IL-6 (**e** and **f**), IL-10 (**g** and **h**), the mRNA of COX-2 (**i**), A_1 (**j**), A_{2A} (**k**) and A_{2B} (**l**) in the absence or presence of 5 µM of sulforaphane for 24 h in cultured astrocytes were evaluated. The data represent the mean ± SD of 8 independent experiments, performed in triplicate and statistically analyzed by Student's t-test. *Indicates differences between control and sulforaphane groups. *SFN* sulforaphane

(P = 0.002; Fig. 6q). In contrast, sulforaphane decreased TrkB mRNA expression (P < 0.001; Fig. 6r) and did not alter IL-1β (Fig. 6b), COX-2 (Fig. 6e), p65 NFκB (Fig. 6h), GFAP (Fig. 6k), ALDHL1 (Fig. 6l), SOX10 (Fig. 6m), BDNF (Fig. 6n) and iNOS (Fig. 6i).

Discussion

Aging is closely associated with progressive changes in the biology of cells, including astrocytes. These cells are critical to maintain brain homeostasis, and accumulating evidence suggests the role of hypothalamic astrocytes in the progression of aging due to its specific activities on metabolic and inflammatory functions [11, 44–47]. Therefore, protective strategies have been increasingly explored, and the identification of glioprotective molecules, such as sulforaphane, can prevent and/or avoid early events associated with aging [17]. In this regard, this study investigated the effects of sulforaphane on hypothalamic astrocyte cultures and on hypothalamic tissue from aged Wistar rats (24 months). Our findings indicate that sulforaphane has significant anti-inflammatory, antioxidant, and trophic functions, and can mediate glioprotection in the hypothalamus of aged rats.

Sulforaphane exhibits hormetic properties, with opposite effects depending on its concentration. At low doses, such as 5 µM, sulforaphane has shown cytoprotective effects and did not affect cellular viability and integrity at concentrations at least up to 10 µM [36, 41, 48, 49], in agreement with our data. Higher concentrations of sulforaphane (20 µM

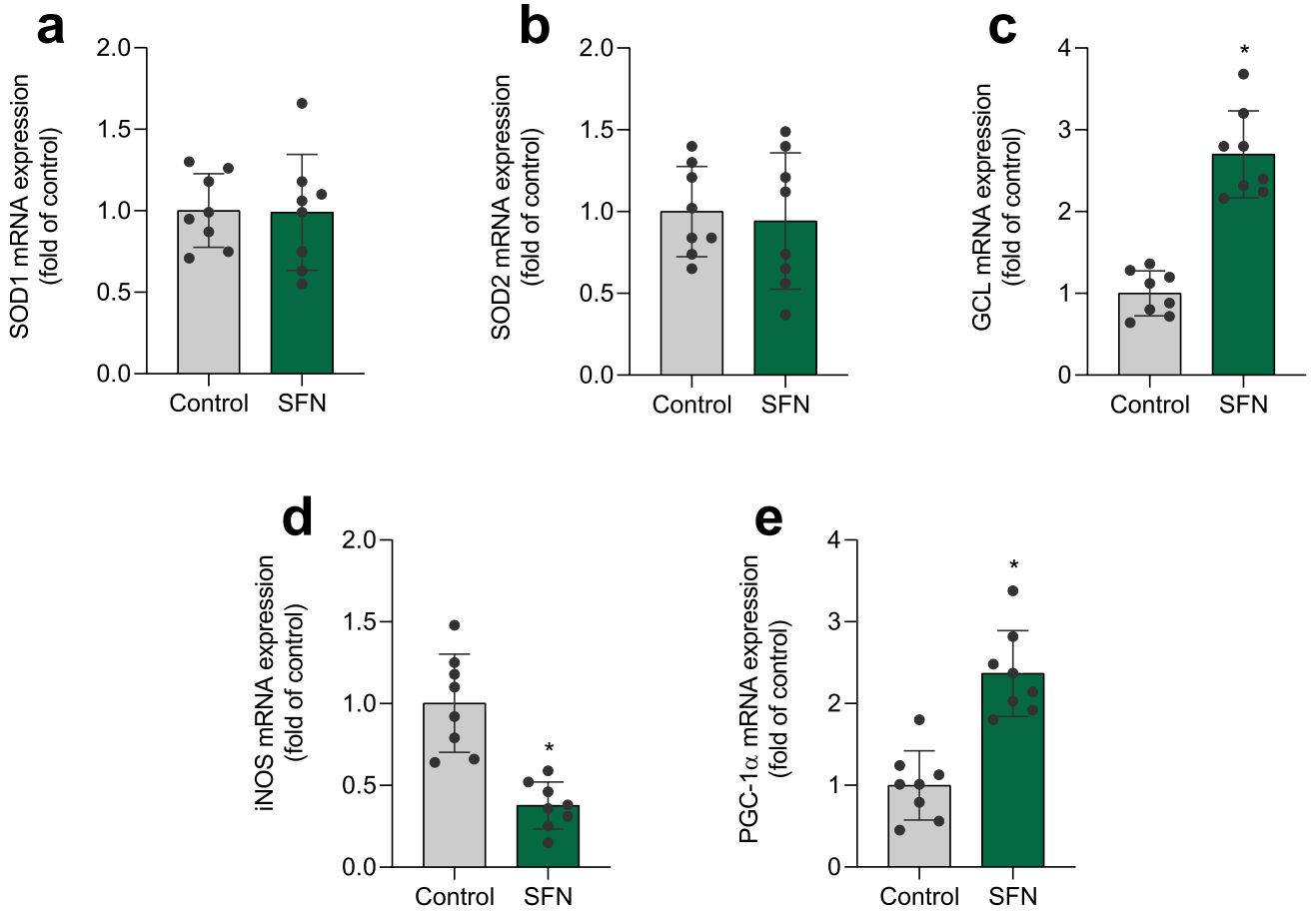

Fig. 2 Effects of sulforaphane on the expression of antioxidant systems. mRNA expression of SOD1 (**a**), SOD2 (**b**), GCL (**c**), iNOS (**d**) and PGC1-α (**e**) in hypothalamic astrocyte cultures from aged Wistar rats in the absence or presence of 5 μM of sulforaphane for 24 h were measured. The data represent the mean ± SD of 8 independent experiments, performed in triplicate and statistically analyzed by Student's t-test. *Indicates differences between control and sulforaphane groups. *SFN* sulforaphane

or more) can induce cytotoxicity through several mechanisms, including excessive ROS production, autophagy, and cell cycle arrest [31, 48, 50]. Concerning the time of incubation, the use of primary astrocyte cultures derived from 24-months old Wistar rats involves some limitations to perform evaluations in different time points, thus we choose 24 h. Although we could not rule out earlier and/or later evaluations in our study, this time of incubation is reasonable to assume changes in gene expression and release of cytokines and trophic factors and is particularly interesting for action of glioprotective molecules, as our group have demonstrated [51–57].

Particularly regarding aging, sulforaphane can modulate the expression of various biomarkers linked to inflammation, cellular senescence, and oxidative stress. Here, we observed that sulforaphane was able to decrease p21 expression, an important senescence marker that is associated with interruption of cell proliferation and acceleration of inflammatory process [58], suggesting a potential approach for treating age-related conditions [58–60]. The aging process is also characterized by an increase in the inflammatory responses, and in line with this, our previous research has demonstrated that hypothalamic astrocytes from mature animals exhibit a pro-inflammatory profile of cytokine and chemokine production [11]. Many pro-inflammatory cytokines are induced through the NFκB pathway, and this transcription factor was enhanced in an age-dependent way in our previous study [11]. On the contrary, the downregulation of NFκB has been associated with an improvement of inflammatory conditions, and sulforaphane was able to decrease not only NFκB expression but also TNF-α, IL-1β and IL-6. We also verified that sulforaphane decreased the mRNA expression of COX-2, an enzyme involved in the synthesis of inflammatory mediators, which may be a transcriptional target of NFκB [61]. On the contrary, in another study, sulforaphane increased the secretion of several chemokines in astrocytes subjected to a stress-induced premature senescence [62]. Therefore, the effects of this compound specifically on

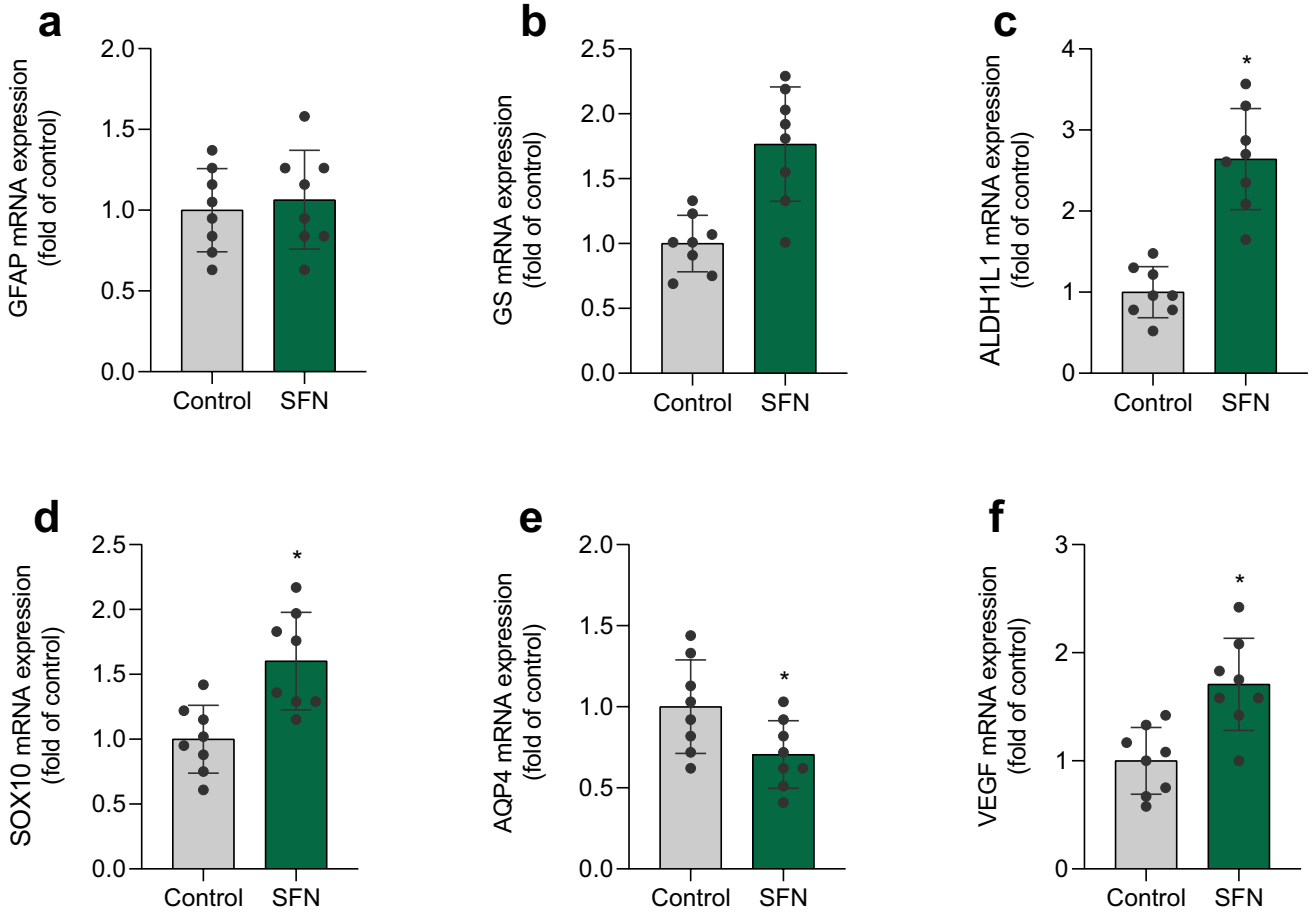

Fig. 3 Effects of sulforaphane on astroglial markers. mRNA expression of GFAP (**a**), GS (**b**), ALDHL1 (**c**), SOX10 (**d**), AQP4 (**e**) and VEGF (**f**) in hypothalamic astrocyte cultures from aged Wistar rats in the absence or presence of 5 μM of sulforaphane for 24 h were evaluated. The data represent the mean ± SD of 8 independent experiments, performed in triplicate and statistically analyzed by Student's t-test. *Indicates differences between control and sulforaphane groups. *SFN* sulforaphane

chemokines production and release might be better characterized in future studies using astrocytes derived from aged rats. Our results also showed an increase in the mRNA expression of both A_1 and A_{2A} adenosine receptors, which are known to have anti-inflammatory effects in glial cells [63, 64]. These results reinforce the glioprotective role of sulforaphane on astroglial cells.

Sulforaphane is a well-recognized activator of Nrf2, which is able to increase the expression of cytoprotective enzymes and factors that may control the redox balance and inflammatory process, such as GCL, NFκB, HO-1, among others [43, 65]. An upregulation of SOD1 and SOD2 by sulforaphane could be also expected [66]. However, it is important to note that astrocytes present highly adaptive antioxidant mechanisms thus changes in gene expression may be fast and transient, returning to basal levels after 24 h. Particularly regarding SOD1 and SOD2, based on previous in vitro data from C6 astroglial cells and cardiomyocytes, it is reasonable to assume that their gene expression, protein content and activity are not direct targets of sulforaphane modulation, since no differences in this antioxidant enzyme were observed in the two cellular models after sulforaphane incubation for 1, 4 or 24 h [27, 36].

HO-1 is responsible for producing cellular responses against stressful conditions and has been related to the protective effects of another glioprotective molecule, resveratrol [44, 67]. We observed that sulforaphane increased the mRNA expression of Nrf2 and HO-1, while p65 NFκB showed an opposite effect. It is noteworthy that Nrf2 and HO-1 exert an upstream control of NFκB, negatively modulating its activation. Together, Nrf2 and NFκB are considered the major transcriptional factors to induce cellular responses, including metabolic responses. In line with this, AMPK signaling is also closely related to energy balance, in addition to regulate mitochondrial function, detoxification, and antioxidant defenses, promoting cellular homeostasis and, consequently, healthy aging [68]. Although AMPK activation has been related to sulforaphane effects in adipose

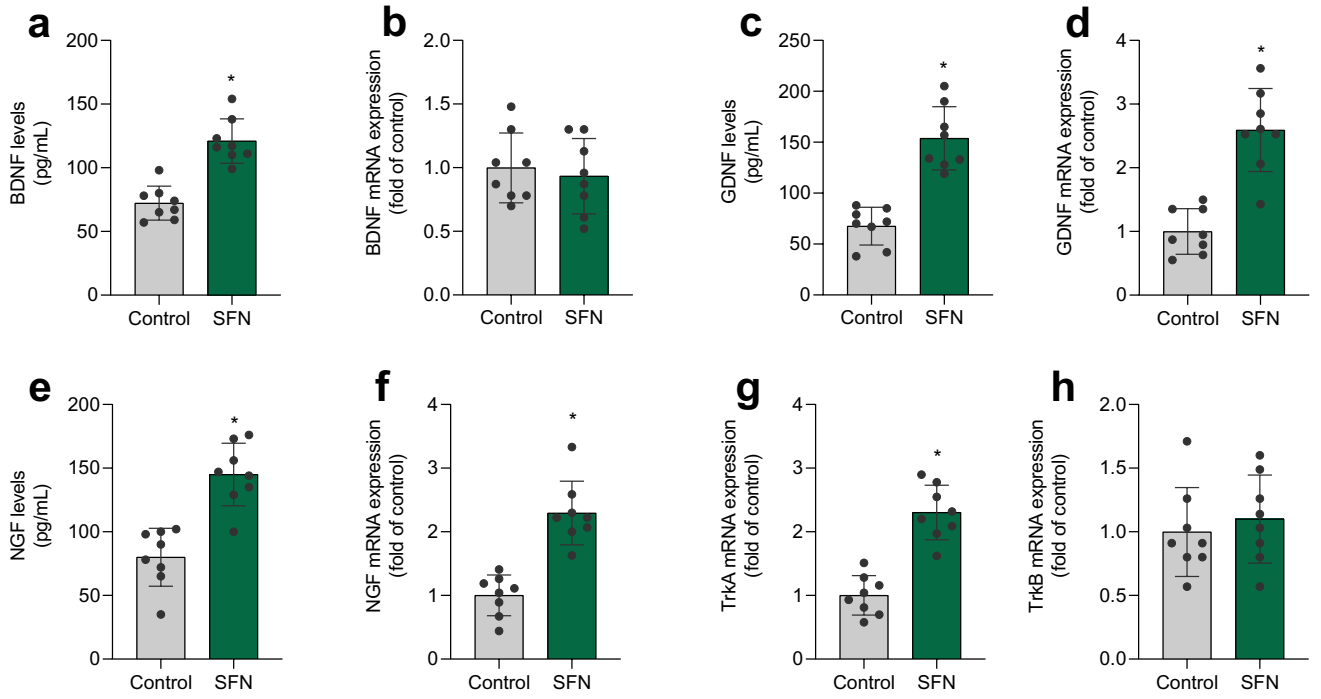

Fig. 4 Effect of sulforaphane on trophic factors. The release and mRNA expression of BDNF (**a** and **b**), GDNF (**c** and **d**), and NGF (**e** and **f**), as well as the mRNA expression of TrkA (**g**) and TrkB (**h**) in hypothalamic astrocyte cultures from aged Wistar rats in the absence or presence of 5 μM of sulforaphane for 24 h were evaluated. The data represent the mean ± SD of 8 independent experiments, performed in triplicate and statistically analyzed by Student's t-test. *Indicates differences between control and sulforaphane groups. *SFN* sulforaphane

and hepatic tissues [69, 70], we observed a downregulation of AMPK expression in hypothalamic astrocytes. Of note, there is a crosstalk between Nrf2 and AMPK signaling, in which the activation of Nrf2 may supress AMPK mRNA levels [69], which may explain the downregulation induced by sulforaphane. Nrf2 also regulates the transcription of the enzyme GCL, which participates in the synthesis of glutathione, a crucial antioxidant molecule that protects cells from oxidative stress and is primarily produced by astrocytes in the CNS [71]. PGC-1α can also control the expression of various enzymes involved in eliminating reactive oxygen species, as well as the induction of mitochondrial biogenesis through interaction with Nrf2 and SIRT1 pathways [72, 73]. Here, sulforaphane was able to upregulate GCL and PGC-1α.

The expression of iNOS can be triggered by different stimuli, including inflammation, cytokines, and oxidative stress [74, 75]. Our observations indicated that sulforaphane reduces the mRNA expression of this enzyme, which is downstream of Nrf2/HO-1, being a relevant target in pathological processes associated with inflammation and redox imbalance [76]. Therefore, sulforaphane was effective in our experimental model in the modulation of different signaling pathways that may prevent age-dependent functional alterations in astrocytes. In addition, modulation of endogenous cellular defense mechanisms represents an innovative approach to therapeutic intervention in diseases causing chronic tissue damage, such as in neurodegeneration due to accumulation of toxic products (including β-amyloid and α-synuclein) and in the aging process. Considering that sulforaphane can also attenuate microglial activation [77–79], it can help in the development of neuroprotective/glioprotective therapeutic strategies through Nrf2 stimulation to restore redox equilibrium or to control neuroinflammation in CNS [80–83].

Besides regulating important functions associated with inflammatory response and redox homeostasis in cultured astrocytes, sulforaphane was able to modulate the expression of glial markers. Although sulforaphane did not change the expression of classical astrocytic markers, such as GFAP and GS, it modulated the mRNA levels of ALDH1L1, SOX10, AQP4, and VEGF. ALDH1L1 is an enzyme highly expressed in astrocytes and sulforaphane was able to increase its expression. This enzyme participates in the folate metabolim, influencing nucleotide biosynthesis and, consequently, the responses of neuron and glial cells, with potential impacts in regenerative process of the brain [71, 84]. In our study, we also observed that sulforaphane increased mRNA expression of SOX10. Although this transcription factor has been

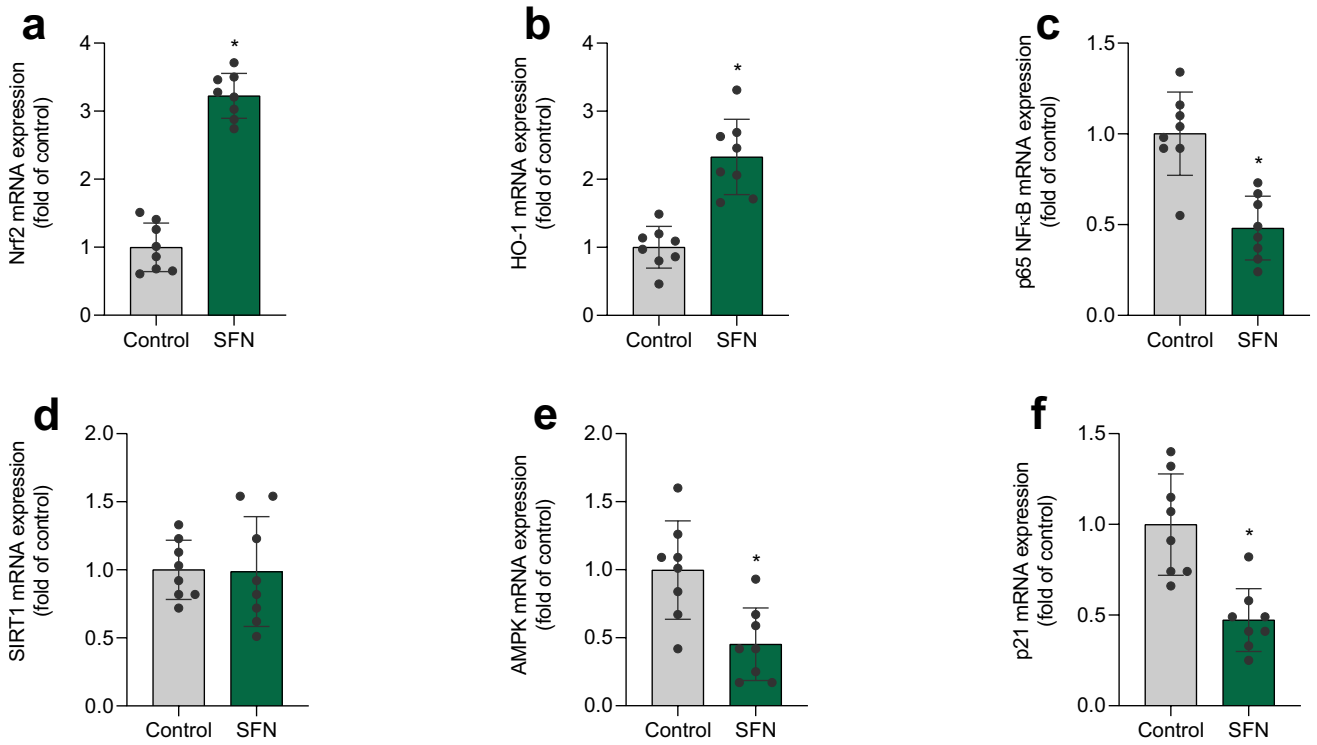

Fig. 5 Potential mechanisms associated with sulforaphane-induced glioprotection. mRNA expressions of Nrf2 (**a**), HO-1 (**b**), p65 NFκB (**c**), SIRT1 (**d**), AMPK (**e**) and p21 (**f**) in hypothalamic astrocyte cultures from aged Wistar rats in the absence or presence of 5 μM of sulforaphane for 24 h were evaluated. The data represent the mean ± SD of 8 independent experiments, performed in triplicate and statistically analyzed by Student's t-test. *Indicates differences between control and sulforaphane groups. *SFN* sulforaphane

used as a marker of oligodendrocyte precursor cells, it is also involved in the development and maintenance of various cell types in the nervous system, including astrocytes [85, 86]. It is important to note that SOX10 perfoms many essential functions, including remodeling neural plasticity [87, 88], and our data reinforce the effects of sulforaphane against the deficits observed in brain aging.

The water channel AQP4, a protein expressed by astrocytes, is an important astrocytic functional parameter [89]. In addition to its role in maintaining water homeostasis, this protein has also been associated with neuroinflammation and neurodegeneration processes [90, 91]. Previous work from our group has shown that astrocytes derived from adult animal cultures express more AQP4 than those from neonatal animals, and furthermore, we also demonstrated that compounds such as resveratrol is capable of decreasing the expression of this protein under such condition [44, 92]. This data supports the result found in the present study, since sulforaphane downregulated AQP4 expression, possibly to maintain astrocytic homeostasis. In addition, VEGF plays a crucial role in regulating the formation and maintenance of the neurovascular unit, promoting the formation of new blood vessels in response to injury or disease, which can restore blood flow to damaged areas of the brain [93]. Moreover, astrocyte-derived VEGF may regulate inflammation and there is an interplay between VEGF and Nrf2 [94], both upregulated by sulforaphane.

The production and release of trophic factors by astrocytes is involved in their support functions, since these factors play critical roles during development and seem to mediate protective and/or reparative responses in mature cells. Trophic factors can act through high-affinity cell surface receptors TrkA and TrkB, supporting survival and plasticity of neural cells, and preventing cell death after injuries. In this study, we observed that sulforaphane increased the mRNA expression and the extracellular levels of GDNF and NGF, in addition to increase BDNF release. Besides that, sulforaphane increased TrkA mRNA expression [95]. It has been reported that the decrease in BDNF, GDNF, and NGF may be associated with the pathophysiology of neurodegeneration, while their increased levels may protect neural cells against toxic insults. Additionally, the expression of the trophic factors may be modulated by Nrf2/HO-1, which were also increased by sulforaphane [17].

Finally, we demonstrated that sulforaphane modulated gene expression in hypothalamic cells ex vivo in a similar way compared to cultured astrocytes, although a different profile of expression was observed for some genes, such as TNF-α, IL-6 and TrkB. Of note, these differences may be related to the presence of other cell types, such

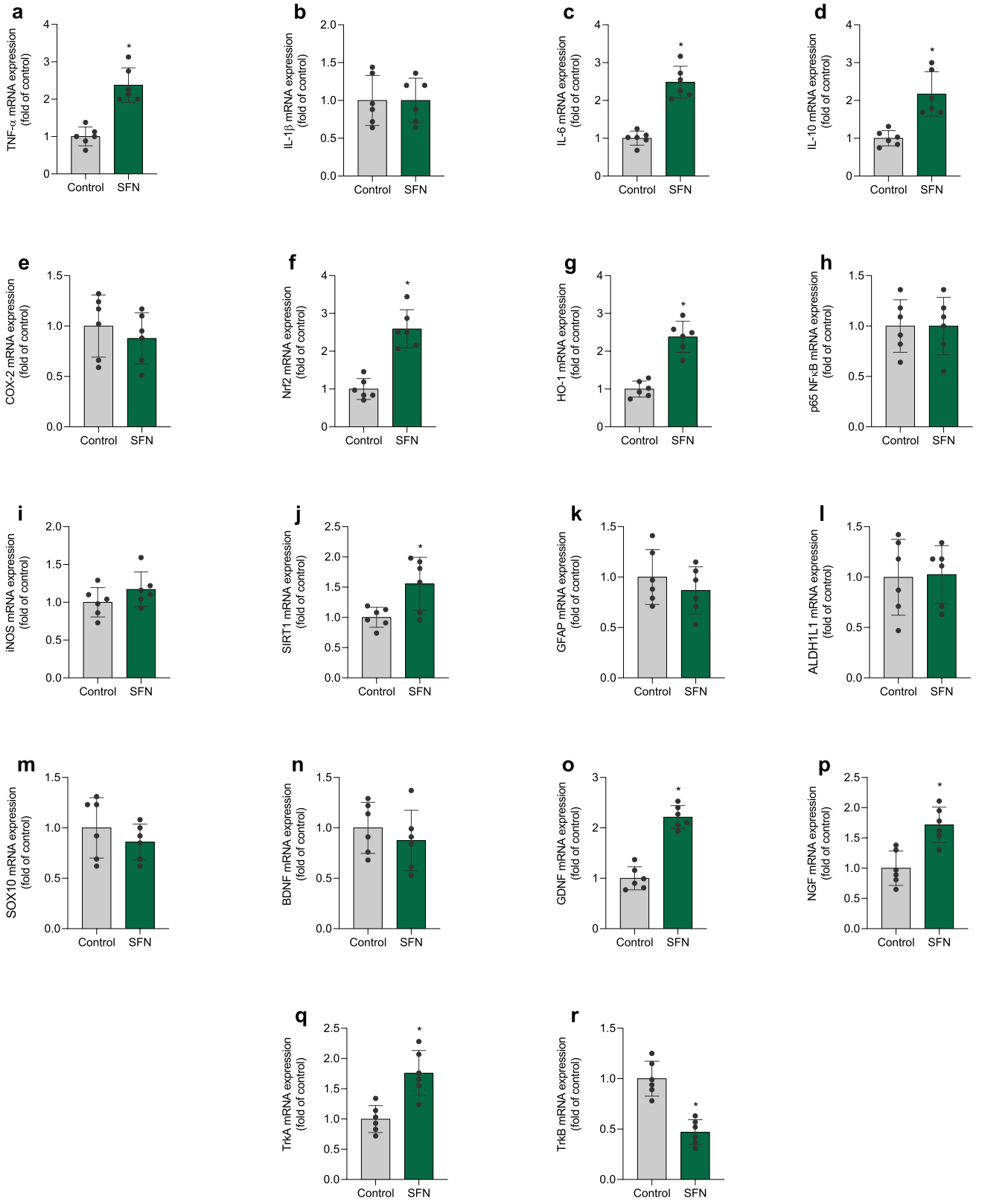

Fig. 6 Effects of sulforaphane on hypothalamic tissue. The mRNA expression of TNF-α (**a**), IL-1β (**b**), IL-6 (**c**), IL-10 (**d**), COX-2 (**e**), Nrf2 (**f**), HO-1 (**g**), p65 NFκB (**h**), iNOS (**i**) SIRT1 (**j**), GFAP (**k**), ALDHL1 (**l**), SOX10 (**m**), BDNF (**n**), GDNF (**o**), NGF (**p**), TrkA (**q**), and TrkB (**r**) in the cell suspension from hypothalamic tissue obtained from aged Wistar rats in the absence or presence of 5 μM of sulforaphane for 1 h were evaluated. The data represent the mean ± SD of 6 independent experiments, performed in triplicate and statistically analyzed by Student's t-test. *Indicates differences between control and sulforaphane groups. *SFN* sulforaphane

as microglia and neurons, and to the shorter incubation time with sulforaphane. However, in general, this set of experiments demonstrated the ability of sulforaphane in modulating genes involved in important astroglial functions and suggests astrocyte responses with a focus on neuron-glia communication. These findings lead to the conclusion that sulforaphane presents glioprotective effects on hypothalamic tissue by modulating the expression of crucial genes related to inflammation, antioxidant defense, and neurotrophic factors in astrocytes, as well as signaling pathways that regulate these effects. Although further studies are needed to fully understand the mechanisms of sulforaphane in the CNS, our work indicate that this compound can contribute to the functional maintenance of aged hypothalamic astrocyte. In summary, our study highlights the potential benefits of sulforaphane in promoting glioprotection, reinforcing that sulforaphane may represent a promising experimental therapeutic intervention against aging-related brain dysfunctions and contributing to a healthier brain aging.

Acknowledgements The authors of this study are supported by Universidade Federal do Rio Grande do Sul (UFRGS), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), and Instituto Nacional de Ciência e Tecnologia para Excitotoxicidade e Neuroproteção (INCTEN/CNPq).

Author Contributions CLS, ABK, LDB, and AQS conceptualized the study. CLS, FBW, and LDB performed the experiments. CLS, LDB, and AQS performed statistical analysis and written the original draft of the manuscript. ABK and AQS provided resources and materials/chemicals. All authors revised, edited, and approved the manuscript.

Funding This research was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) and Instituto Nacional de Ciência e Tecnologia para Excitotoxicidade e Neuroproteção.

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

Declarations

Competing Interests The authors have no relevant financial or non-financial interests to disclose.

Ethical Approval This study protocol was reviewed and approved by the Federal University of Rio Grande do Sul Animal Care and Use Committee (process number 35387).

Consent to Participate Not applicable.

Consent to Publish Not applicable.

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