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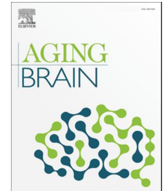

Aging changes the expression of adenosine receptors, insulin-like growth factor 1 (IGF1), and hypoxia-inducible factor 1α (HIF1α) in hypothalamic astrocyte cultures

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ABSTRACT

The aging process induces neurochemical alterations in different brain regions, including hypothalamus. This pivotal area of the central nervous system (CNS) is crucial for detection and integration of nutritional and hormonal signals from the periphery of the body to maintain metabolic homeostasis. Astrocytes support the CNS homeostasis, energy metabolism, and inflammatory response, as well as increasing evidence has highlighted a critical role of astrocytes in orchestrating hypothalamic functions and in gliocrine system. In this study, we aimed to investigate the age-dependent mRNA expression of adenosine receptors, the insulin-like growth factor 1 receptor (IGF1R), and the hypoxia-inducible factor 1α (HIF1α), in addition to the levels of IGF1 and HIF1α in hypothalamic astrocyte cultures derived from newborn, adult, and aged rats. Our results revealed age-dependent changes in adenosine receptors, as well as a decrease in IGF1R/IGF1 and HIF1α. Of note, adenosine receptors, IGF1, and HIF1α are affected by inflammatory, redox, and metabolic processes, which can remodel hypothalamic properties, as observed in aging brain, reinforcing the role of hypothalamic astrocytes as targets for understanding the onset and/or progression of age-related diseases.

1. Introduction

The aging process is characterized by a progressive loss of physiological integrity, leading to increased risk for several diseases [22]. Aging can also markedly affect both the periphery of the body and the hypothalamus, whose neural networks have emerged as key orchestrators of systemic metabolism, food intake, and body weight [13,17]. Hypothalamic astrocytes are versatile cells that can regulate energy metabolism due to its capacity to detect peripheral signals that inform about energy status. They are also part of the gliocrine system, because they are secretory cells and targets of hormone action, such as leptin and insulin [13,24,30,40].

In line with this, the brain, in particular hypothalamus, plays a role in regulating glucose homeostasis. Insulin-like growth factor 1

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(IGF1) and its receptor (IGF1R) are also crucial to maintain neural functions, including development, neurogenesis, and plasticity [1,28]. IGF1 might also induce hypoxia-inducible factor 1 (HIF1), an essential transcription factor for the regulation of oxygen homeostasis, which more recently was associated with inflammation [3,10]. Accumulated evidence indicates that inflammatory process might have a key role in the obesity, as well as in the onset and progression of age-related diseases [7,26]. In this regard, the adenosinergic system can modulate astroglial responses, including those related to inflammation, and changes in the expression of adenosine receptors are associated with aging [4,15].

Our previous publications showed that aging significantly affect the hypothalamic functionality, due to changes in metabolic, inflammatory response, and leptin signaling [18,32,31]; therefore, here, we aimed to expand the knowledge of how aging modulates the expression of adenosine receptors, IGF1, and HIF1α. Using a protocol of hypothalamic astrocyte cultures derived from newborn (2 days old), adult (90 days old), and aged (180 days old) Wistar rats, we concluded that aging changes these parameters, which are related to etiological factors of diabetes, obesity, and neurodegeneration.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium/F12 (DMEM/F12) and other materials for cell cultures were purchased from Gibco/Invitrogen (USA). TRIzol, High Capacity complementary DNA (cDNA) Reverse Transcription Kit and TaqMan real-time RT-PCR system were purchased from Applied Biosystems (USA). ELISA kit for IGF1 was purchased from Invitrogen (USA) and for HIF1ɑ from Elabscience (China). Poly-L-lysine and TRI Reagent were purchased from Sigma-Aldrich (USA). All other chemicals were from common commercial suppliers.

2.2. Animals

Male Wistar rats were obtained from the breeding colony of Department of Biochemistry (UFRGS, Porto Alegre, Brazil) and maintained under a controlled environment (12-h light/12-h dark cycle; 22 ± 1 °C; *ad* libitum access to water and diet – regular laboratory chow). All animal experiments were performed in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals. The Federal University of Rio Grande do Sul Animal Care and Use Committee approved the use of Laboratory animals for this project (process number 29420).

2.3. Hypothalamic primary astrocyte cultures

The protocol was previously described by Santos et al [32]). Wistar rats at 2, 90, and 180 days old were euthanized by decapitation. They subsequently had their hypothalamus dissected, and the meninges removed. A pool of 3 hypothalamus was used to perform a single astrocyte culture (6 independent cultures were used, each culture was derived from 3 hypothalamus). The sample size was 18 Wistar rats per age/group. 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 NaHCO3, 1 % Fungizone®, and 0.04 % gentamicin. Cells were seeded (approximately $2 - 4 \times 10^5$ cells/cm^2 into 6-well plates pre-coated with poly-L-lysine and cultured at 37 °C in a 5 % CO2 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.

2.4. RNA extraction and Quantitative RT-PCR

Total RNA was isolated from newborn, adult, and aged astrocyte cultures using TRIzol reagent. 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 in a 20 μL reaction according to the manufacturer's instructions. The messenger RNA (mRNA) encoding adenosine receptors A_1 (#Rn00567668_m1), A_{2A} (#Rn00583935_m1), A_{2B} (#Rn00567697_m1), and A_3 (#Rn00563680_m1), IGFR1 (#Rn00583837_m1), HIF1α (#Rn01472831_m1), and β-actin (#Rn00667869_m1) were quantified using the TaqMan real-time RT-PCR system using inventory primers and probes purchased from Applied Biosystems. Quantitative RT-PCR was performed using the Applied Biosystems 7500 Fast system. The mRNA levels were expressed relative to the levels of newborn (1 day old) cultures using the $2^{-\Delta\Delta Ct}$ method [20].

2.5. IGF and HIF1α measurement

IGF1 and HIF1α levels were measured in the extracellular medium and intracellular content, respectively, using commercial ELISA kits following manufacturer's instructions with some modifications. IGF1: Invitrogen (#ERIGF1), analytical sensitivity: 30.0 pg/ml,

detection range: 30.72 – 7500 pg/ml; HIF1α: Elabscience (#E-EL-R0513), analytical sensitivity: 4.7 pg/ml, detection range: 7.81 – 500 pg/ml. The results are expressed in pg/mL for IGF1 and in pg/mg of protein for HIF1α.

2.6. Statistical analysis

The results are presented as mean ± standard error of the mean (SEM). To ensure adequate power for statistical analysis at least six independent experiments were performed in triplicate. All attempts at replication were successful. For each experiment, replicates are noted in the figure legends. The normal distribution was confirmed by Shapiro-Wilk test and variance homogeneity was tested using Bartlett's test. Considering the normality and homogeneity of variance, differences among groups were statistically analyzed using one-way analysis of variance (ANOVA), followed by Tukeýs test. All analyses were performed using the GraphPad Prism software version 9 (GraphPad Software). Adjusted P values of post hoc analysis are described in the Results section, only for significant results. P values < 0.05 were considered significant (*a* refers to statistically significant differences from newborn rats – 2 days old; *b* refers to statistically significant differences from 90 days old rats). All graphs were also performed by using GraphPad Prism 9.

3. Results

First, we examined the expression of adenosine receptors. A significant age-dependent decrease in mRNA levels of A_1 was observed ($P = 0.0013$ (newborn vs. 90 days old rats); $P < 0.0001$ (newborn vs. 180 days old rats and 90 days old rats vs. 180 days old rats), Fig. 1A). However, the mRNA levels of A_{2A} presented an age-dependent increase ($P = 0.0003$ (newborn vs. 90 days old rats); $P < 0.0001$ (newborn vs. 180 days old rats and 90 days old rats vs. 180 days old rats), Fig. 1B). The mRNA of A_{2B} did not change in cultured astrocytes and A_3 decreased only in astrocytes from 180 days old ($P < 0.0001$ (newborn vs. 180 days old rats and 90 days old rats vs. 180 days old rats); Fig. 1C and D, respectively).

We also demonstrated an age-dependent decrease in the mRNA expression of IGF1R ($P = 0.0001$ (newborn vs. 90 days old rats); $P < 0.0001$ (newborn vs. 180 days old rats); $P = 0.0009$ (90 days old rats vs. 180 days old rats) and in the extracellular levels of IGF1 ($P < 0.0001$ (newborn vs. 90 days old rats, newborn vs. 180 days old rats and 90 days old rats vs. 180 days old rats), Fig. 2A and B, respectively). The same response was observed for the mRNA expression of HIF1α ($P < 0.0001$ (newborn vs. 90 days old rats and newborn vs. 180 days old rats); $P = 0.0005$ (90 days old rats vs. 180 days old rats), Fig. 2C), as well as for its intracellular levels ($P < 0.0001$ (newborn vs. 90 days old rats, newborn vs. 180 days old rats and 90 days old rats vs. 180 days old rats), Fig. 2D).

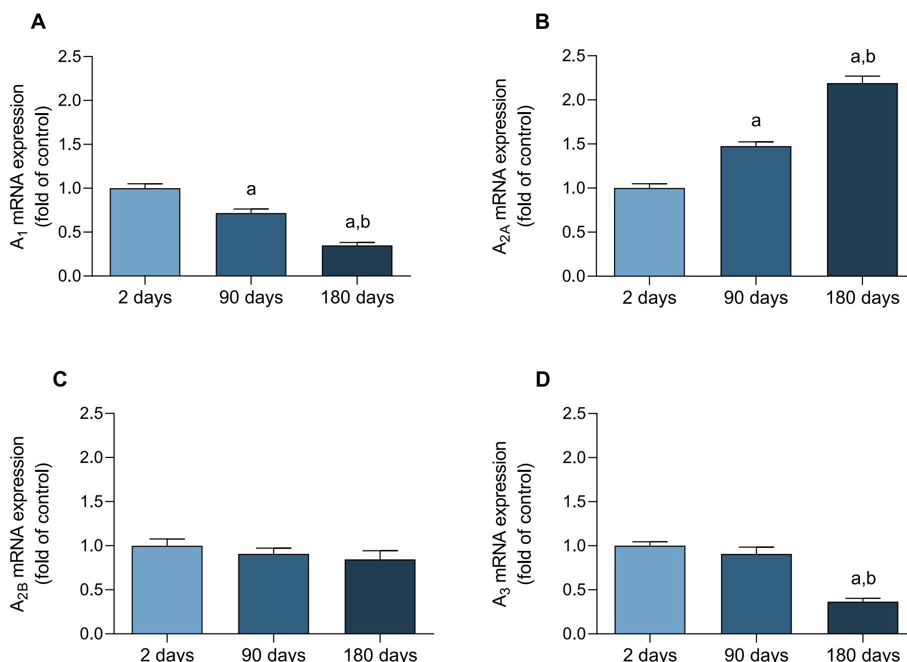

Fig. 1. Age-related changes of adenosine receptors in hypothalamic astrocyte cultures. RT-PCR quantification of mRNA expression of A_1 (A), A_{2A} (B), A_{2B} (C), and A_3 (D). The data represent the mean ± SEM of triplicate samples from 6 independent cultures (each culture derived from 3 hypothalamus) and statically analyzed by one-way ANOVA followed by Tukey's test. *a* refers to statistically significant differences from newborn rats (2 days old); *b* refers to statistically significant differences from 90 days old rats.

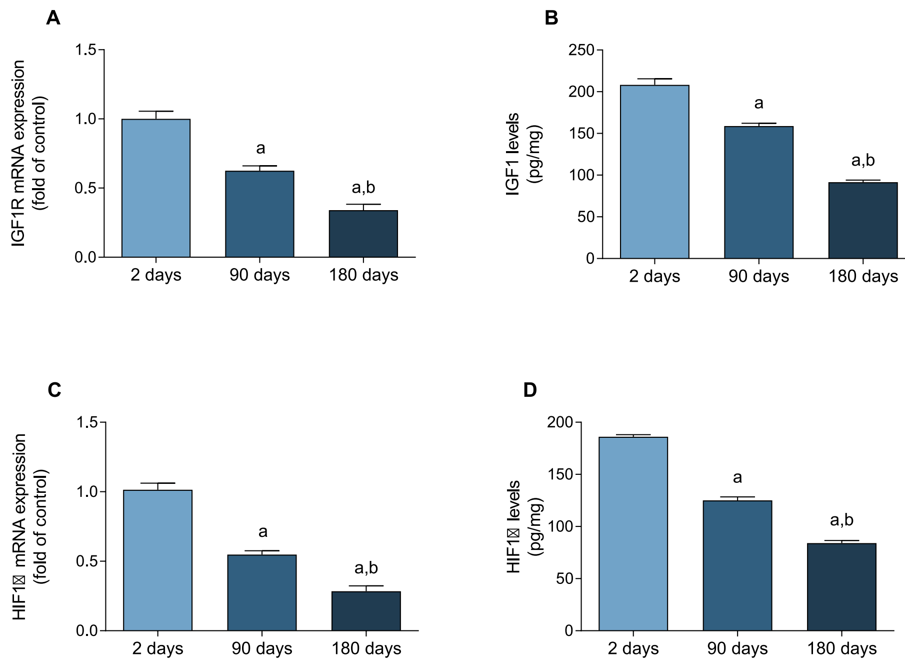

Fig. 2. Aging decreases expression and levels of IGF1 and HIF1α in hypothalamic astrocyte cultures. RT-PCR quantification of mRNA expression of IGF1R (A) and HIF1α (C), extracellular levels of IGF1 (B) and intracellular content of HIF1α (D). For RT-PCR and ELISA measurements, the data represent the mean ± SEM of triplicate samples from 6 independent cultures (each culture derived from 3 hypothalamus). The data were statically analyzed by one-way ANOVA followed by Tukey's test. *a* refers to statistically significant differences from newborn rats (2 days old); *b* refers to statistically significant differences from 90 days old rats.

4. Discussion

Astrocytes are dynamic cells that maintain brain homeostasis and regulate hypothalamic-feeding circuits [2,38]. These cells respond to metabolic signals interacting with neurons, and this communication can alter neural survival and development, reinforcing the role of hypothalamic astrocytes as target for understanding the pathomechanisms of the communication between periphery and brain, including during the aging process [43]. Our previous studies showed that aging promotes changes in neurochemical properties of hypothalamic astrocytes and, here, we described important age-dependent alterations in the expression of adenosine receptors, IGF1, and HIF1α, which could be associated with neurodegeneration/neurodegenerative diseases.

Adenosinergic system can control neuroendocrine signals and participates in the mechanisms of blood pressure variability, sleep disorders, obesity, inflammation, and aging [5,6,8,11,33,35,41]. Hypothalamic astrocytes express adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3), which can orchestrate different cellular responses. Our findings showed that A_1 and A_3 receptors decreased with aging, potentially indicating a dysfunction in inflammatory and metabolic states, since their downregulation promote the production of pro-inflammatory mediators and changes in energy balance [15,16,42]. Moreover, A_1 and A_3 adenosine receptors may be also related to A_{2A} signaling, which presented an age-dependent increase in astrocytes. This receptor is strongly associated with neuroinflammation, obesity, sleep disorder, and cognitive decline [9,19]. Therefore, our results may suggest alterations of the adenosine receptor-mediated signaling and functions in hypothalamic astrocytes during aging. In addition, we recently demonstrated that chronic resveratrol treatment mediates glioprotection in aged astrocytes regulating adenosine receptors [37].

The hormone IGF1 and its receptor, IGFR1, are critical for hypothalamic function, because they are involved in the regulation of synaptic activity and attenuation of neuroinflammation [45]. During aging, the concentration of IGF1 and the expression of IGF1R decrease in different brain areas, and their deficiencies, as well as the defective insulin signaling, have been implicated in the cognitive dysfunction and Alzheimer's disease (Lyra E [23,36]. Although in the Alzheimer's disease the role of IGF1/IGF1R is associated with decline in hippocampus-dependent learning and memory [21], our findings point out hypothalamic astrocytes as key players in the pathophysiology of brain metabolism, with potential impact in neurometabolic disorders. Furthermore, defective IGF1 gliosignaling can induce mitochondrial dysfunction and production of reactive oxygen species [21], which actively participates in the pathomechanisms of neurodegenerative diseases. Our findings are also in accordance with a recent publication that indicated an age-dependent reduction in IGFR1 that may represent a link between the progression of synaptic dysfunction and inflammation in paraventricular hypothalamic nucleus in an experimental model of hypertension, a common condition observed during aging [29]. The IGF signaling can also modulate the activity of HIF1α [3,10]. In this sense, astrocytes from mice with insulin signaling deficiency show higher levels of reactive oxidant species, leading to stimulation of HIF1α, with consequent impact in neurochemical functions [12].

HIF1α is a transcriptional factor that acts as a master regulator of systemic and cellular homeostatic response to hypoxia, consequently impacting on oxygen delivery and metabolic adaptation [34]. In the CNS, HIF 1 and 2 are critically involved in the regulation

of neurogenesis, nerve cell differentiation, and neuronal apoptosis [27]. Particularly in the hypothalamus, HIF1α also controls the expression of pro-opiomelanocortin (POMC) in neurons, with significant impact on the energy homeostasis of the body [39,44]. Here, we demonstrated the age-dependent decrease in the expression and intracellular levels of HIF1α, which can be associated with metabolic, oxidative, and inflammatory functions, which, in turn, are linked to pathomechanisms of neurometabolic diseases [14]. HIF1α Moreover, HIF1α is interconnected with adenosinergic system [13,25] and other signaling pathways, including nuclear factor kappa B (NFκB) and erythroid-derived 2 like 2 (Nrf2), which we recently demonstrated that changed with age in hypothalamic astrocytes [18,32]. It is noteworthy that regulation of the expression and activity of HIF1 may represent a promising neuroprotective tool during ischemic injury [27]. Considering that hypoxia and ischemia are also common diseases in elderly population, the reduction of HIF1α during aging corroborates this hypothesis, particularly due the relevance of astrocytes in the signaling mechanisms related to adaptive functions.

5. Conclusions

In summary, we demonstrated that adenosine receptors, IGF1, and HIF1α changed during aging, impacting the hypothalamic astrocyte functionality, and consequently, the development and progression of age-dependent metabolic brain dysfunctions. Our findings reinforce/complement the knowledge from our previous report, in which we found that cultured hypothalamic astrocytes undergo neurochemical remodeling with age [32]. Therefore, hypothalamic astrocytes may emerge as targets for understanding the brain aging, particularly about neurometabolic conditions.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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