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ORIGINAL PAPER

Caffeine Neuroprotection Decreases A2A Adenosine Receptor Content in Aged Mice

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Abstract

Caffeine is a bioactive compound worldwide consumed with effect into the brain. Part of its action in reducing incidence or delaying Alzheimer's and Parkinson's diseases symptoms in human is credited to the adenosine receptors properties. However, the impact of caffeine consumption during aging on survival of brain cells remains debatable. This work, we investigated the effect of low-dose of caffeine on the ectonucleotidase activities, adenosine receptors content, and paying particular attention to its pro-survival effect during aging. Male young adult and aged Swiss mice drank water or caffeine (0.3 g/L) ad libitum for 4 weeks. The results showed that long-term caffeine treatment did not unchanged ATP, ADP or AMP hydrolysis in hippocampus when compared to the mice drank water. Nevertheless, the ATP/ADP hydrolysis ratio was higher in young adult (3:1) compared to the aged (1:1) animals regardless of treatment. The content of A1 receptors did not change in any groups of mice, but the content of A2A receptors was reduced in hippocampus of mice that consumed caffeine. Moreover, the cell viability results indicated that aged mice not only had increased pyknotic neurons in the hippocampus but also had reduced damage after caffeine treatment. Overall, these findings indicate a potential neuroprotective effect of caffeine during aging through the adenosinergic system.

Keywords Aging · Adenosine receptors · Caffeine · Ecto-NTPDase · Ecto-5′-nucleotidase

Introduction

Caffeine (1,3,7-trimethylxanthine) is the most consumed psychoactive substance in the world. Its consumption causes a diverse range of pharmacological effects that are time- and concentration-dependent and reversible. The behavioral effects are similar to those of classical psychostimulants, such as amphetamine, mainly motor activation [1]. There has been a growing interest in studying the neuroprotective effects of caffeine in rodent models of neurodegenerative disorders, such as Parkinson's disease [2], Alzheimer's disease [3] and others including ischemic and hemorrhagic brain injury [4].

There is evidence that the psychomotor stimulant actions of caffeine are mediated by its antagonism at adenosine A1 and mainly A2A receptors [5]. Extracellular adenosine acts through multiple G-protein-coupled receptors (adenosine receptor subtypes A1, A2A, A2B and A3) to exert a variety of physiological effects. The extracellular adenosine may come from two distinct sources: (1) the release via bi-directional nucleoside transporter; and/or (2) degradation of adenine nucleotides released by a chain of ectonucleotidases [6].

Nucleotides as ATP and ADP are extracellular signaling substances present in nearly all tissues including brain that are inactivated by hydrolysis catalyzed by ectonucleotidases [7]. The most relevant ectoenzymes involved in this chain are those of the ectonucleoside triphosphate diphosphohydrolases family (E-NTPDases), which hydrolyze nucleotide tri- and di-phosphates. In mammals, there are eight related and homologous enzymes sharing five apyrase-conserved regions (ACRs), namely NTPDase1–8 that have been cloned and characterized. NTPDase1 (CD39, ATPDase,

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ecto-apyrase or ecto-ATP diphosphohydrolase) NTPDase2 (ecto-ATPase) and NTPDase3 (CD39L3, HB6) are those expressed in the nervous tissue [7]. The AMP produced in the synaptic cleft is hydrolyzed by ecto-5′-nucleotidase (EC 3.1.3.5; or CD73) to generate adenosine [8].

The effects of adenosine in neural cells are operated mainly through its action on either inhibitory A1 receptors or excitatory A2A receptors. A1 receptors are more abundant in the brain and their activation induces inhibition of glutamate release (as well as other neurotransmitters), reduction in calcium influx through voltage-sensitive calcium channels and NMDA receptors and in potassium currents, culminating in membrane hyperpolarization. A2A receptors activation induces enhanced release of different neurotransmitters, as glutamate [9]. Such receptors have received attention because their blockade by caffeine evokes a robust neuroprotection in different neurodegenerative models in rodents by mechanisms that are still unclear [3, 10]. There is evidence pointing to the preferred activation of A2A receptors when adenosine is formed via the ectonucleotidase pathway [11]. Conversely, caffeine induces neuroprotection mainly due to the blockade of A2A receptors rather than to A1 receptors [1]. Thus, long-term caffeine treatment could reduce brain damage effects associated with A2A receptors during aging.

Neurodegenerative diseases are a multifactorial age-related biological process, in which caffeine has shown beneficial effects on human cognition, since low consumption of caffeine in beverages has been associated with a significantly lower risk of Alzheimer's and Parkinson's diseases [12, 13]. In aged rodents, caffeine intake may prevent memory decline with aging, regardless of the occurrence of neurodegenerative diseases [14, 15].

Thus far, the methods of long-term consumption of caffeine in rodents have focused on reverting or preventing cell damage mechanisms after brain injury. Nonetheless, the effect on cell death during senescence has yet to be found.

This study evaluates whether the long-term consumption of low-dose of caffeine (the equivalent to one cup of coffee for human) could modulate adenosine effects. The investigation is focusing on the extracellular production of adenosine, immunocontent of A1 and A2A receptors, and cell viability in hippocampus of aged mice compared with young adult mice.

Materials and Methods

Animals

Sixty-four young adult (32 animals, 2–3 months, 30–40 g) and aged (32 animals, 16–18 months, 35–45 g) male Swiss mice, separated equally in caffeine group or water group of treatment, were caged in groups of 4. The animals were kept on a 12-h light/12-h dark cycle in a room under controlled temperature (23 ± 1 °C), with free access to food and liquid. All experimental procedures were performed on light period between 7:00 a.m. and 5:00 p.m. The project was approved by the ethical committee of the Universidade do Extremo Sul Catarinense (n° 103/2012) and performed according recommendations for animal care on NIH Guide for Care and Use of Laboratory Animals and National Council for the Control of Animal Experimentation (CONCEA, Brazil).

Caffeine Treatment

Young adult and aged mice accessed drinking bottles containing either 0.3 g/L caffeine (Sigma, St. Louis, MO) or ordinary tap water as their only source of fluid for 30 consecutive days [16]. The caffeine solution was prepared every 2 days and the bottles (which are dark to prevent caffeine oxidation) refilled between 7:00 p.m. and 8:00 p.m. The treatment regimens correspond to a moderate caffeine intake in humans, with meaningful effects believed to be restricted to adenosine receptors [17]. The volume of water or caffeine consumed was quantified every 2 days, and the value divided by two (interval between exchange) and the result was divided by four (number of animals in the cage). Mice were killed by decapitation just before the beginning of the biochemical analysis (on the 30th day of caffeine treatment period).

Nucleotides Hydrolysis Assay

After 30 days of caffeine consumption, 6–8 the animals from each group were killed by decapitation and the brain was rapidly removed. The hippocampus was dissected in cold PBS in 0.6% glucose, sliced transversely as 400-µm-thick slices on a Mcllwain tissue chopper and divided into individual slices. Two slices per tube (approx. 0.15 mg protein) were preincubated for 10 min at 37 °C with 400 µL of the pre-warmed HEPES-buffered salt solution with the following composition: 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 10 mM glucose (pH 7.4) and gassed with a 95% O_2/5% CO_2 mixture (incubation medium). To measure ATP, ADP, and AMP hydrolysis, the slices were incubated for 20 min with each nucleotide at a final concentration of 1 mM at 37 °C in freshly pre-warmed incubation medium. An aliquot of the assay medium was removed and mixed with trichloroacetic acid (TCA) to the final concentration of 5% to stop reaction. A sample of the supernatant was taken for the assay of inorganic phosphate (Pi) generated by colorimetric determination [18]. Non-enzymatic Pi released from nucleotide in the assay medium without slices and Pi released from slices incubated without nucleotide were subtracted from the total Pi released during nucleotide hydrolysis to produce specific enzymatic activity values (nmol Pi^{-1} mg

protein^{-1} min). All experimental conditions adapted were previously investigated to ensure the linearity of enzymatic reaction and the preservation of cell viability until the end of the experiment (data not shown). Protein was determined using bovine serum albumin as standard [19].

MTT Reduction Assay

The dehydrogenase activities were measured in another hippocampus from each 6 to 8 young adult and aged mice following 30 days of caffeine or water treatment. The MTT tetrazolium ring [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is cleaved by intracellular dehydrogenases generating formazan product, a blue precipitated into living cells [20]. The hippocampus was sliced as cited above, divided into individual slices and pre-incubated for 10 min in PBS with 0.6% glucose (pH 7.4). Two slices were incubated with MTT (0.5 mg/mL) in fresh pre-warmed PBS for 20 min at 37 °C. The PBS was gently aspirated, the formazan precipitate was solubilized with dimethyl sulphoxide and viable cells were quantified spectrophotometrically at a wavelength of 550 nm. The variability due to differences in the size of the slice was determined by protein quantification and indicated the homogeneity among slices (data not shown).

Immunoblot Analysis

The hippocampi of 4 mice from each group were removed for immunoblot analysis on the 30th day of caffeine consumption. The tissue was homogenized and prepared according to [21] Homogenization was carried out in an ice-cold lysate buffer (10 mM Tris/2 mM EDTA/200 mM NAF, protease inhibitors 0.1 mM PMSF, 2 mM Na_3VO_4, 1% Triton, 10% glicerol) and centrifuged at 12,000×*g* for 10 min at 4 °C. The supernatants were diluted 1/1 (v/v) in 100 mM Tris (pH 6.8), 4 mM EDTA, and 8% SDS and boiled for 5 min. One aliquot was separated from the supernatants for determination of proteins [22], and samples were diluted in storage buffer [40% glycerol, 100 mM Tris, and bromophenol blue (25:100, v/v) and 8% β-mercaptoethanol] and stored at -20 °C for to 30 days. Protein samples (70 µg/track) were separated by SDS-PAGE mini-gel, using polyacrilamide gel (10%), followed by transfer to nitrocellulose membranes. Protein loading and blot transfer efficiency were monitored by staining membranes with Ponceau S (0.5% ponceau: 1% acetic acid) and gel with Stain Solution [50% methanol, 8% acetic acid, 0.1% Comassie Blue R-250 (w/v in water)]. Membranes were blocked for 1 h with TBS-T (Tris-buffered saline and 0.1% Tween-20; pH 7.4) and 0.5% fish gelatin. Membrane blots were incubated with primary antibody anti-A1 receptors (1:500; Millipore California, USA) or anti-A2A receptors (1:500; Millipore California, USA) diluted in 1% albumin/TBS-T and incubated overnight at 4 °C. After washing, the membranes were incubated for 1 h with anti-mouse IgG (1:1000; Santa Cruz Biotechnology, USA), or anti-rabbit IgG (1:1500; Santa Cruz Biotechnology, USA) horseradish peroxidase (HRP)-conjugated secondary antibodies. Immunocomplexes were visualized using the enhancing chemiluminescence detection system (Pierce, USA) as described by the manufacturer. The membranes were then reprobed and tested for β-actin immunoreactivity using a mouse anti-β-actin antibody (1:1000; Santa Cruz Biotechnology, USA), as previously described [23]. Densitometry analysis was performed using Image J software and values for A1 and A2A receptors immunoreactivity were normalized to β-actin immunoreactivity. The total protein concentrations were determined using the method described by [24].

Assessment of Histological Changes

On the 30th day of caffeine consumption, 5 mice per group were anesthetized (50 mg/kg pentobarbital, i.p.) and intracardially perfused with physiological saline (0.9% NaCl) followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). After extraction of the skull, the brain was removed and placed in a vial containing 4% paraformaldehyde solution for 24 h, and subsequently processed and embedded in paraffin wax. Coronal sections (5 µm) were made using a microtome (Leica RM2265, rotary microtome) and stained with toluidine blue (1% toluidine blue-O in distilled water). To examine cell density, hippocampal CA1 region of the brain was visualized under bright field illumination using a Nikon Eclipse Ti-U microscope. The cells were counted on at least three sections, at each level, at 400 magnification by two blind analyzers. Both healthy and pyknotic cells were counted and the estimates of total neuronal number were based on counting nuclei technique, observed in the sections of the CA1. The pyknotic neurons were considered when neuron bodies' pyknosis and cytoplasm were stained darkly [25].

Statistical Analysis

The results were statistically analyzed using a two-way ANOVA with treatment and age as main factors. Tukey HSD post hoc test was used when $p < 0.05$. The data are presented as mean values ± SEM (standard error of the mean). Results were analyzed by STATISTICA® software version 7.0 (StatSoft, Inc., USA).

Results

Mice were observed during all treatment period with respect to health, liquid ingestion (every 2 days), body weight and food intake. Body weight of mice was no significantly

different between mice of the same age for both liquids consumed over the period of 30 days (weight of 2–3 months old mice, water: 32.5 ± 3.2; caffeine: 34 ± 4.1; and 16–18 months old mice, water: 39.8 ± 2.8; caffeine: 41.5 ± 3.2, $p > 0.05$). The means of water or caffeine consumed daily, for 30 days, were estimated for each animal per day through the loss of water from drinking bottles (Table 1). Two-way ANOVA revealed a significant interactive effect of age and treatment on volume of liquid consumed [$F(3,62) = 14.074$; $p < 0.001$]. Post-hoc analysis indicated that young adult mice ingested less caffeine than water; aged mice ingested a similar volume of water and caffeine, but they consumed less water than the young adult mice.

Table 1 Ingestion of water or caffeine (0.3 g/L) in mL per day estimated per mouse measured every 2 days for 30 days' treatment

	Water (mL)	Caffeine (mL)
Young adult	13.39 ± 0.50	11.63 ± 0.27*
Aged	10.90 ± 0.37*	12.06 ± 0.36

Consumption of liquid was measured every 2 days for during 30 days, at the change of caffeine solution, as described in "Materials and Methods" section. Data are expressed as mean of liquid ingested on the day of change ± S.E.M., n = 16

*$p < 0.05$ compared to young adult mice that ingested water

ATP hydrolysis was the same among young adult and aged groups of mice [$F(3,19) = 2,466$; $p = 0.133$] (Fig. 1a). However, two-way ANOVA indicated a significant effect of age on ADP hydrolysis [$F(3,17) = 52.12$; $p < 0.001$]. Post-hoc analysis indicated that ADP hydrolysis increased in hippocampus slices of aged mice (treated with water or caffeine) compared with water-treated young adult animals (Fig. 1b). In the same statistical effect, AMP hydrolysis increased in hippocampus of all aged mice compared with

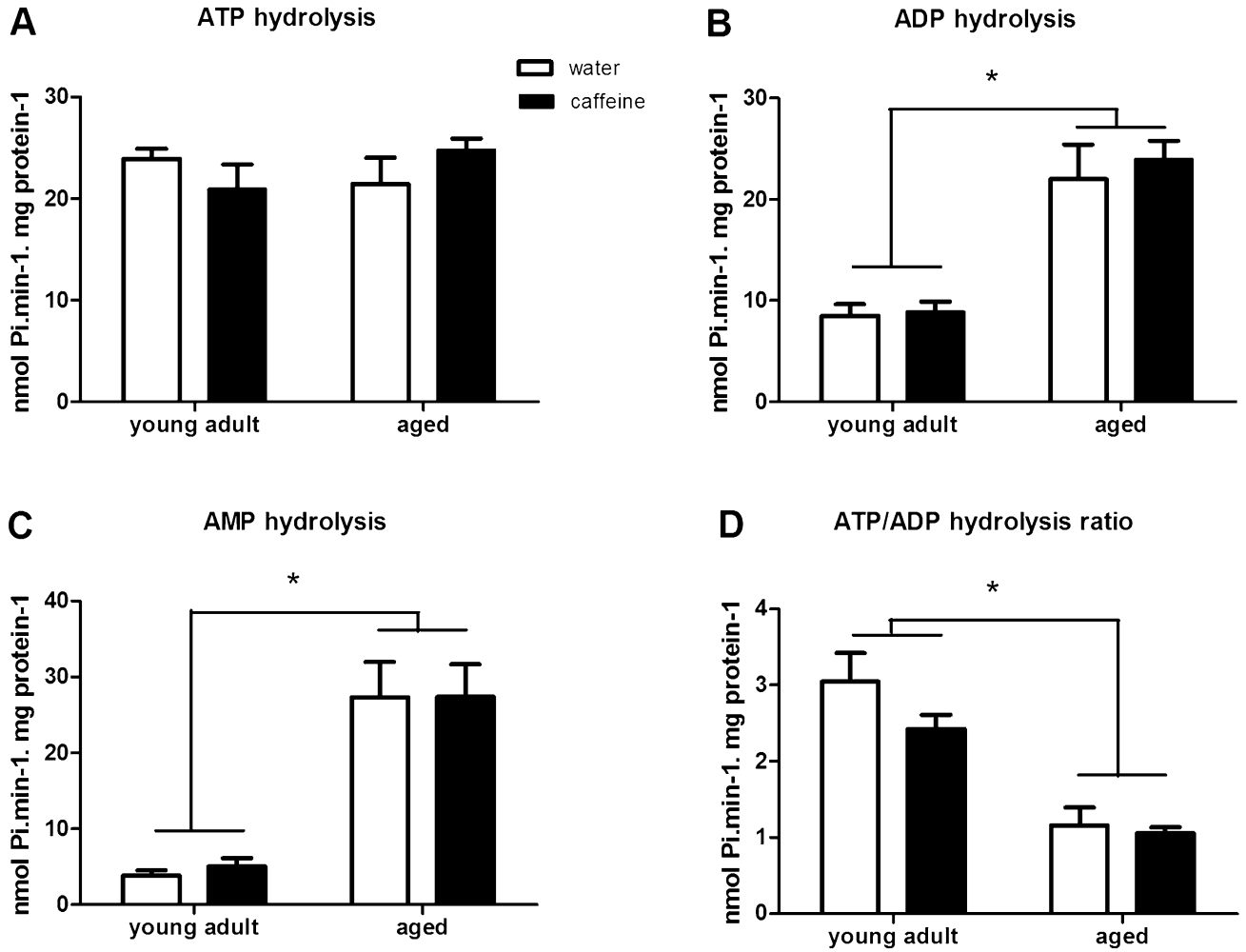

Fig. 1 Effect of the chronic ingestion of caffeine (0.3 g/L) on the nucleotides hydrolysis in slices from hippocampus of young adult and aged mice. Enzymatic activities for **A** ATP, **B** ADP, **C** AMP and **D** ATP/ADP hydrolysis ratio are expressed as mean of nmol Pi^{-1} mg $protein^{-1}$ min values ± S.E.M., and each value reflects the mean of triplicate samples from 6 to 8 animals per group. *$p < 0.05$ compared to young adult mice [consumed water (control) or caffeine] vs. aged mice (consumed water or caffeine)

young adults that ingested water [F(3,17) = 56.00; p < 0.001] (Fig. 1c). ATP/ADP hydrolysis ratio was not the same along aging, because the ratio decreased in hippocampus of aged mice compared with young adults, regardless of treatment [F(3,17) = 34.93; p < 0.001] (Fig. 1d).

Analysis of the adenosine receptors content showed the same effect of age and treatment on A1 receptor levels in hippocampus of mice [F(3,12) = 0.93; p = 0.353] (Fig. 2a). However, two-way ANOVA revealed a significant effect of treatment on the content of A2A receptors in hippocampus of mice [F(3,16) = 18.81; p = 0.0005]. Post-hoc analysis indicated that caffeine induced a reduction in A2A receptor levels in hippocampus regardless of age (Fig. 2b). β-actin content was unaltered among all groups (data not shown).

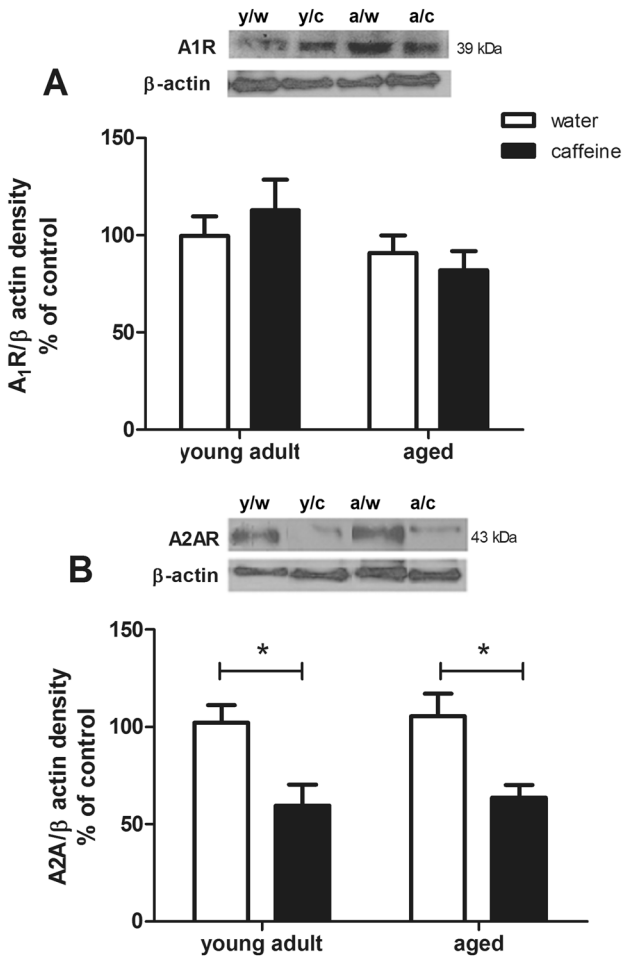

Fig. 2 Effect of the chronic ingestion of caffeine (0.3 g/L) on the adenosine receptors content. **a** A1 receptors and **b** A2A receptors content in hippocampus of young adult and aged mice. Data are presented as mean values ± SEM, and each value reflects the mean from 4 animals per group. *p < 0.05 compared to mice that ingested water (respective young or aged control mice). Insets are bands from representative experiments. *y/w* young adult mice that ingested water, *y/c* young adult mice that ingested caffeine; aged mice that ingested water, *a/c* aged mice that ingested caffeine

Two-way ANOVA showed that neither age nor treatment had effect on MTT reduction of cell living in hippocampus after treatment of mice (p > 0.05) (Fig. 3). However, when tissues were stained with toluidine blue (Fig. 4), two-way ANOVA revealed a significant effect for all factors. Post-hoc analysis indicated augmented pyknotic neurons in CA1 area in aged mice [F(3,16) = 67.38; p < 0.0001], and an important interactive effect of age and treatment [F(3,16) = 4.72; p = 0.045], since the water-treated aged had a greater reduction, than both young adult groups. The reduction of neurons in the hippocampus observed in aged mice is physiological and was prevented by the treatment with low-dose of caffeine for 30 days [F(3,16) = 4.47; p = 0.050] (Fig. 5a, b).

Discussion

In the present study, prolonged low-dose caffeine consumption by aged mice induced modulation of adenosinergic system resulting in less cell damage in the hippocampus. Caffeine has not changed nucleotides hydrolysis but evoked a reduction in adenosine A2A content and in pyknotic neurons in the hippocampus of aged mice. Such findings indicate an important physiological contribution to brain integrity during aging.

Aged mice consumed 12.06 ± 0.36 mL of caffeine daily, corresponding to approximately 3.3 mg/kg/day, to an estimated concentration of 22 µM in hippocampus [26]. Young adult animals ingested less caffeine than water, this effect does not seem to be specifically related to caffeine (two-way ANOVA analysis indicated significance for age versus treatment, for only for age factor, but no significance for the treatment per se), since fluid intake varies between ages, as previously observed [27].

The ectonucleotidases catalyze the adenine nucleotides hydrolysis in stages to produce free extracellular adenosine

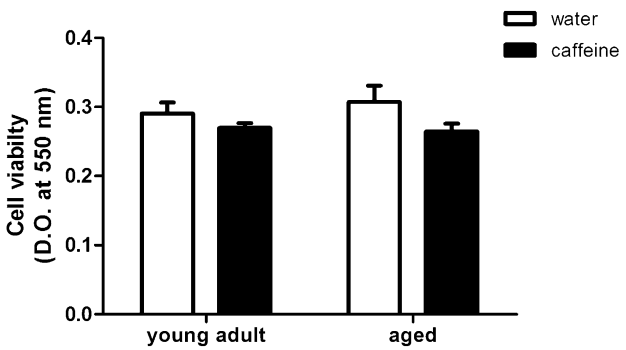

Fig. 3 Effect of the chronic ingestion of caffeine (0.3 g/L) on the MTT reduction in slices from hippocampus of young adult and aged mice. Data are presented as mean values ± SEM, and each value reflects the mean of triplicate samples from 6 to 8 animals per group

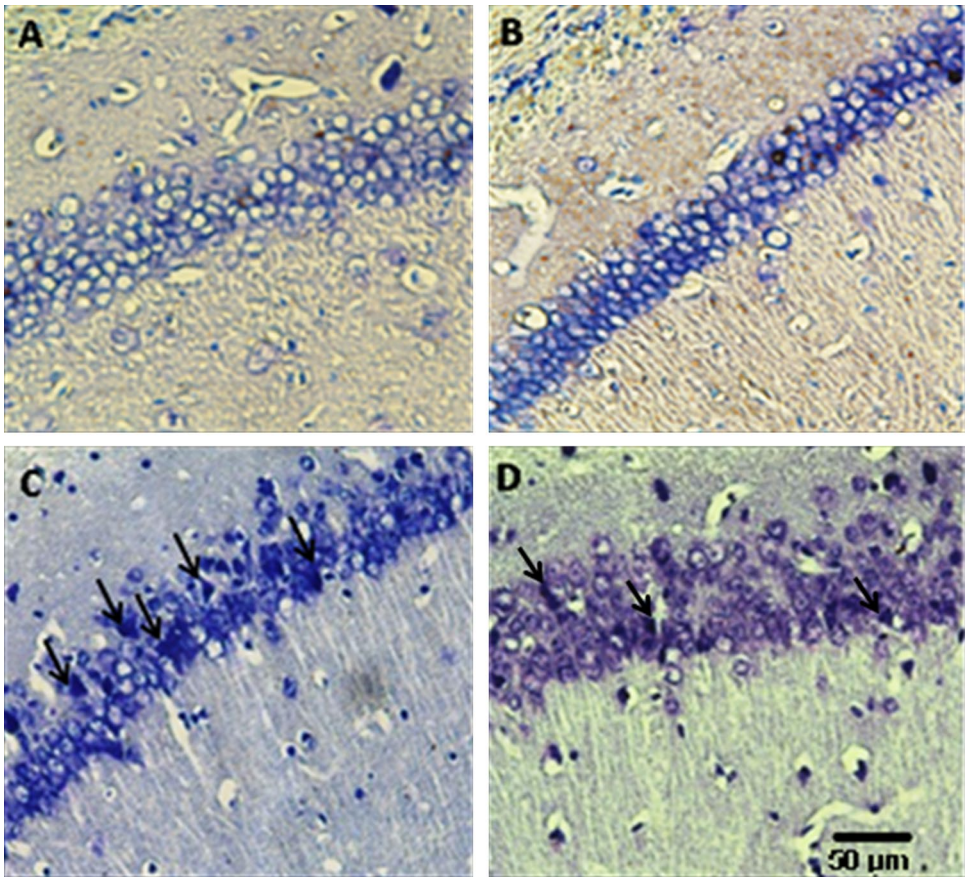

Fig. 4 Effect of the chronic ingestion of caffeine (0.3 g/L) on the toluidine blue staining (Nissl). Photomicrographs (×400) of toluidine blue staining in brain hippocampus CA1 sections (in slices of 5 µm). **a** Young adult mice that ingested water (control group) showing healthy neurons; **b** young adult mice that ingested caffeine (0.3 g/L); **c** aged mice that ingested water shows necrotic neurons body pyknosis and cytoplasm stained darkly (arrows); **d** aged mice that ingested caffeine showing reduction on neurodegeneration induced by aging. Representative photomicrographs are from 5 animals per group

at the synapse cleft [7]. Initially ATP is degraded to ADP by ecto-NTPDases. In the current study, the ATP hydrolysis rates were not changed over aging or after treatment with caffeine. Nevertheless, ADP and AMP hydrolysis rates increased in aged mice compared with young adult animals regardless of the caffeine treatment. Studies have shown a decrease in the levels of ATP in the energy charge of the intact tissue as well as those from hippocampal slices of aged rats [28, 29]. Probably, the decrease in tissue ATP availability observed in aged rats contributes to ATP/ADP hydrolysis ratio reduction observed, however it is important to highlight that there was an increment in the ADP and AMP hydrolysis, and ADP is also an agonist to the P2Y1 receptors expressed in hippocampus [30].

The ATP/ADP hydrolysis ratio was lower in aged compared to young adult animals regardless of treatment, demonstrating a change in the pattern of enzymatic activities over aging. E-NTPDase family (NTPDase1–8) has four cell-surface NTPDases capable of controlling the concentrations of nucleotide agonists near purinergic receptors (NTPDase1–3, 8). NTPDase1 hydrolyzes ATP and ADP about equally well and NTPDase2 prefers triphospho- over diphosphonucleoside by about 30-fold [31, 32]. NTPDase3 catalyzes the hydrolysis of triphosphonucleosides and diphosphonucleosides efficiently with ATP:ADP rate of hydrolysis of 3:1, and NTPDase8 is probably not present in the brain tissue [33]. Thus, E-NTPDase activity can be modulated in different physiological and pathological situations. In the present study, ATP/ADP hydrolysis rate was 1:1 in aged mice, whereas in young adult mice it was 3:1. The dependence of ATP/ADP rate on the brain age can be due to the existence of regulatory mechanisms in the ectoenzymes NTPDase3, preferentially acting in the hippocampus during the young period and ecto-NTPDase1 in the aging period.

Following ATP and ADP hydrolysis, the generated AMP must be hydrolyzed to increase adenosine levels, which is the critical step of its extracellular generation by ecto-5′-nucleotidase [34]. In the present study, the adenosine generation was higher in the hippocampus of aged mice. Some studies have reported the increased adenosine release in old rats [34, 35] and adenosine production via increase in the 5′-nucleotidase activity in brain of aged rat (12–18 months), with small increases in older rats (24–32 months) [36]. Adenosine formed by the ectonucleotidase pathway is considered to bind mainly to adenosine A2A receptor [37]. Thus, is important to associate ectoenzyme activities with adenosine receptors in order to evaluate the effect of any treatment on aging brain. The density of A2A receptors was greater in cholinergic and glutamatergic hippocampal nerve terminals of aged rats (18–24 months) comparing

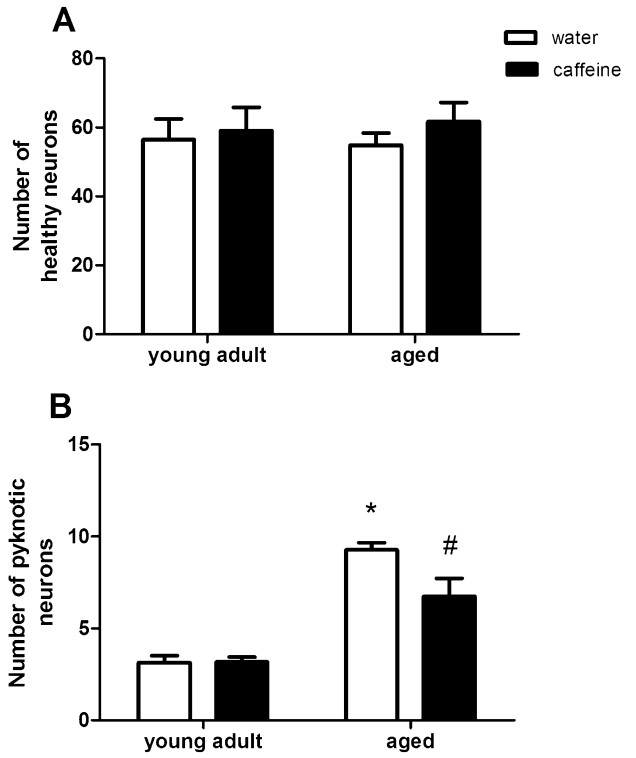

Fig. 5 Effect of the chronic ingestion of caffeine (0.3 g/L) on the toluidine blue staining in hippocampus CA1 sections of young adult and aged mice. **A** Number of healthy neurons; **B** number of pyknotic neurons. Data are presented as mean values ± S.E.M., and each value reflects the mean of triplicate samples from 5 animals per group. *$p < 0.05$ compared to young adult mice [consumed water (control) or caffeine]. #$p < 0.05$ compared to aged mice that consumed water

with young animals (2 months) but not with middle-aged (6–12 months) animals [37, 38]. The current study did not show an effect regarding age, since the content of A1 and A2A receptors remained unchanged between young adult and aged mice. Nonetheless, there are important differences when compared to the latter studies regarding age and species (16–18 months, Swiss mice). Moreover, the content of A2A receptors was reduced in young and aged mice that ingested caffeine comparing to those that drank water. Such A2A downregulation observed herein was also seen after both acute and repeated intraperitoneal injections of caffeine (during 14 days) in Sprague Dawley rats [39]. In the later study, chronic caffeine treatment delivered through the drinking water also led to a decrease in A2A receptors content in hippocampus of pregnant rats [40].

In addition, during aging, the increase in the extracellular levels of adenosine by ecto-5′-nucleotidase activity induces neuroprotection if adenosine preferentially binds to A1 instead of A2A receptors. The observed reduction in the content of A2A receptor caused by the caffeine treatment can be protective in the aged mice. The ability of A2A receptors to modulate neurotransmitter release was also observed in experimental conditions implying in toxicity, as in the model of K^+ stimulated glutamate release using microdialysis in vivo [41]. Excessive glutamate concentrations may result in neuronal dysfunction, excitotoxicity and death, which share common pathogenesis mechanism with neurodegenerative diseases related with aging [42]. Thus, the decrease in the receptor content observed in the current study may suggest an important protective effect of caffeine during aging.

Furthermore, the treatment with A2A receptor antagonists has been emerged as a promising neuroprotective strategy, since it caused neuroprotection against Parkinson's disease [43, 44] and β-amyloid neurotoxicity [3]. The A2A receptor activation leads to multitude of downstream cascades, activating mitogen-activated protein kinase (MAPK p44/42), also known as pERK1/2, through protein kinase A (PKA) pathway. The MAPK family covers three main groups, the extracellular signal-regulated protein kinases (ERK), the stress-activated protein kinases (SAPK; p38) and the c-Jun N-terminal kinases (JNK), which are involved in cell cycle progression, proliferation and differentiation. Thus, adenosine receptor signaling may enhance or inhibit proliferation of a variety of cell types [45]. It has been known that A2A and D2 receptors are functionally antagonistic, as A2A receptor antagonist can exert a similar effect on motor control as D2 agonists for PD treatment [46]. However, the mechanism which A2A antagonism provides neuroprotection in the most of nerodegenerative diseases remains unclear [47].

It has been suggested that reduced neurogenesis in the hippocampus is associated with cognitive deficits in aged rats [48]. CA1 neurons of the hippocampus shown longer and more branched basal dendrites, and greater numbers of spines in rats chronically treated with caffeine [49]. In the present study, toluidine blue staining showed no changes in the number of healthy cells in the CA1 area of the hippocampus, but the number of pyknotic neurons were higher in aged mice. The good condition of the hippocampus is an important factor to either preserve memory. An Italian longitudinal study on aging with old consumers of moderate and regular coffee were related with protective effects against cognitive decline and dementia incidence [50]. The neuroprotective effect of caffeine in aging may also be due to the reversal on DNA damage observed in peripheral blood and hippocampus of aged mice [51].

The current findings, along with the outcome of previous epidemiological studies [12, 52] strongly suggest that prolonged low dose caffeine consumption has significant therapeutic benefits with regard to neuroprotection in the aged. Further studies evaluating other effects should be conducted in order to elucidate the mechanism of adenosine in the neuroprotective role of caffeine in aging.

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Author Contributions MLG: contributions to the conception of the work, acquisition, analysis and interpretation of data for the work; writing and correcting the work; final approval of the version to be published; APD, RP, LR, LLA, MCA: substantial contributions to the acquisition and analysis of data for the work; drafting the work; final approval of the version to be published; VMA: design of study; analysis and interpretation of data; correction of final version of manuscript; supervisor of APD. CRB: conception and design of study; project for funding support; analysis and interpretation of data; writing and correcting of final version of manuscript of manuscript; supervisor of the majority of post-graduation and undergraduation students. The present work was part of Dissertations of MLG. All authors—agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Compliance with Ethical Standards

Disclosure The authors declare that there is no conflict of interests regarding the publication of this paper.

Ethical Approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The project was approved by the ethical committee of the Universidade do Extremo Sul Catarinense (n° 103/2012) and performed according recommendations for animal care on NIH Guide for Care and Use of Laboratory Animals and National Council for the Control of Animal Experimentation (CONCEA, Brazil).

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