CAFFEINE PREVENTS AGE-ASSOCIATED RECOGNITION MEMORY DECLINE AND CHANGES BRAIN-DERIVED NEUROTROPHIC FACTOR AND TIROSINE KINASE RECEPTOR (TrkB) CONTENT IN MICE

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Abstract—The beneficial effects of caffeine on cognition are controversial in humans, whereas its benefit in rodents had been well characterized. However, most studies were performed with acute administration of caffeine and the tasks used to evaluate cognition had aversive components. Here, we evaluated adulthood administration of caffeine up to old age on recognition memory in mice using the object recognition task (ORT) and on brain-derived neurotrophic factor (BDNF) and tirosine kinase receptor (TrkB) immunocontent in the hippocampus. Adult mice (6 months old) received either drinking water or caffeine (1 mg/mL) during 12 months. At 18 months of age both groups were tested for ORT. Our results showed that aged mice exhibited lower performance in the recognition memory compared with adults (6 months old). Furthermore, caffeine-treated mice showed similar performance to adult mice in the ORT and an improvement compared with their age-matched control mice. Caffeine also counteracted the age-related increase in BDNF and TrkB immunocontent. Our results corroborate with other studies and reinforce that caffeine consumed in adulthood may prevent recognition memory decline with aging. This preventive effect may involve a decrease in the hippocampal BDNF and TrkB immunocontent. © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.

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The elderly population is increasing worldwide and there is a concern about strategies that can improve life quality of elderly people, owing to the progressive decline of cognitive and motor functions that happen with aging. There is still no effective clinical treatment for age-associated diseases; thus part of the aging research has been conducted aiming to acquire better knowledge of different factors (molecular, cellular or environmental) that may regulate the process of aging (Dröge and Schipper, 2007; Froy and Miskin, 2007; Rao, 2007). On the other hand, current studies are investigating which interventions could be made to prolong longevity and in a greater extent to minimize the progressive decline of cognitive functions (Hall et al., 2007; Kallus et al., 2005). In this context, the influence of usual diet components on age-related events has been investigated as a strategy to prevent cognitive and motor decline (Morris et al., 2006; Solfrizzi et al., 2006).

Adenosine as a neuromodulator participates in the signaling of many neurotransmitters in the CNS (for review Cunha, 2001). Among the four adenosine metabotropic receptors so far cloned (A₁, A₂A, A₂B and A₃), subtypes A₁ and A₂A are widely expressed in the CNS. Adenosine A₁ receptors are widely distributed in throughout brain while A₂A receptors are highly concentrated in the striatum with a more discrete expression in the limbic system (for review Fredholm et al., 2005).

Caffeine is a psychoactive substance used worldwide, belonging to the class of compounds known as methylxanthines. The main molecular target of the psychostimulant effects of caffeine is the non-selective antagonism of adenosine actions predominantly via A₁ and A₂A receptors (Fredholm, 1980; Snyder et al., 1981). Over the past years, research about habitual consumption of caffeine has deserved much attention, mainly related to its effects in ameliorating cognitive performance (for reviews see Daly, 2007; Ferré, 2008). Studies in humans have shown that caffeine intake can improve the performance of subjects submitted to cognitive tests, but there is a contradiction between its direct effects, tolerance and withdrawal symptoms (Childs and de Wit, 2006; Christopher et al., 2005; Haskell et al., 2005; Heatherley et al., 2005; Warburton et al., 2001). In contrast, some studies did not find any direct effects of caffeine in improving the performance of subjects submitted to cognitive tests (Rogers et al., 2003; Yeomans et al., 2002). Interestingly, two epidemiological studies have suggested that caffeine intake prevents mild cognitive impairment. The first study was performed in a retrospective design in which caffeine intake was associated with a lower risk for developing dementia related to Alzheimer’s disease (Maia and Mendonça, 2002). Recently, the preventive effects of caffeine were confirmed for elderly women in a prospective design where caffeine intake was followed up for 4 years (Ritchie et al., 2007). Our laboratory data and others have also confirmed neuroprotective effects of caffeine in preventing cognitive deficits and neurodegeneration observed in experimental models of Alzheimer’s disease (Arendash et al., 2006; Dall’Igna et al., 2003, 2007).

Other studies performed in animals have shown that administration of caffeine frequently causes an improvement on the cognitive performance, including in aged an-
imals (Higgins et al., 2007; Prediger et al., 2005a,b). However, the beneficial effects of caffeine were investigated in tasks with aversive or reinforcing stimulus (Angelucci et al., 2002; Kopf et al., 1999; Prediger et al., 2005a,b). In fact, tasks in which the natural behavior of the animals could be used for evaluating learning and memory were not fully explored. Recently, caffeine was described to reverse olfactory discrimination and social recognition memory decline in old rats (Prediger et al., 2005c).

The object recognition task is viewed as a working memory that consists of two components: recollection and familiarity. Recollection involves remembering specific contextual details about a prior learning episode; familiarity involves simply knowing that an item was presented, without having available any additional information about the learning episode (Ennaceur and Delacour, 1988). In recent years, the object recognition paradigm has been widely used to test effects of pharmacological and genetic interventions on memory recognition (Bertaina-Anglade et al., 2006; Heldt et al., 2007). This behavioral task consists in quantifying the natural behavior of rodents of readily approaching and exploring a novel object instead of the old object; thus this task deals with the natural motivation of the animals to explore novelty, an innate instinct that drives animals to learn about their environment.

Although the anatomical basis for the exact process that underlies recognition memory is still under investigation, some studies have already characterized the important role of the hippocampus for both recollection and familiarity processes (Buffalo et al., 2006; Mumby, 2001; Rossato et al., 2007; Squire et al., 2007). Since the hippocampus is highly affected by cellular injury and aging, any dysfunction in this brain region usually is strictly implicated in learning and memory processes.

From a wide range of molecules that participate in memory processes, brain-derived neurotrophic factor (BDNF) has highlighted the important role of neurotrophins in the biochemical cascades of consolidation and persistence of the memory (Bekinschtein et al., 2007; Heldt et al., 2007). Indeed, impairment of the BDNF signaling disrupts memory processes in a variety of tasks (Cirulli et al., 2004; Tyler et al., 2002). BDNF signaling participates in physiological functions as well as in pathological events of the CNS (Lindsay, 1994), its levels and expression being widespread in the hippocampus (Hofler et al., 1990; Valenzuela et al., 1993). Recently, it was reported that adenosine seems to participate in the signaling operated by BDNF, since adenosine was able to activate tiro sine kinase receptor (TrkB) receptors in the hippocampal neurons and both A<sub>2A</sub> and TrkB receptors were co-immunoprecipitated (Lee and Chao, 2001). Besides, adenosine A<sub>2A</sub> receptors seem to be crucial for the BDNF-triggered facilitatory effect on the synaptic transmission in young and in aged rats (Diógenes et al., 2004, 2007). Likewise, activation of adenosine A<sub>2A</sub> receptors contributes to the maintenance of normal levels of BDNF and also helps to sustain BDNF-induced potentiation of synaptic transmission in the hippocampus (Tebano et al., 2008).

In spite of the influence of adenosine on the BDNF-mediated effects on synaptic transmission, there are no studies dealing with pharmacological manipulation of adenosine receptors on changes in the BDNF and TrkB receptors in vivo. Considering the important role of BDNF in the memory process, including recognition memory (Heldt et al., 2007) and the reinforcing effects of caffeine on the performance of animals in learning and memory tasks, we sought to investigate whether oral administration of caffeine during adulthood up to old age could prevent the predictable age-associated decline in the recognition memory in mice with relevant changes in the hippocampal BDNF and TrkB content.

**EXPERIMENTAL PROCEDURES**

**Materials**

Caffeine, protease inhibitor cocktail, Tween-20, Ponceau S, mouse anti-BDNF and mouse anti-α-actin were purchased from Sigma (São Paulo, SP/Brazil). Bicinchoninic acid assay (BCA) was from Pierce (São Paulo, SP/Brazil). All reagents and equipment for electrophoresis and immunoblotting were purchased from Bio-Rad Laboratories (São Paulo, SP/Brazil). Nitrocellulose membrane and ECL immunoblotting detection system were from Amersham Biosciences (São Paulo, SP/Brazil). Rabbit anti-TrkB antibody was from Upstate Cell Signaling (Billerica, MA, USA).

**Animals**

Male albino CF1 mice were obtained from State Foundation for Health Research Science (FEEPS, Porto Alegre/RS, Brazil). All experimental procedures were performed according to the NIH Guide for Care and Use of Laboratory Animals and Brazilian Society for Neuroscience and Behavior (SBNeC) Recommendations for Animal Care and approved by the ethical committee from the Federal University of Rio Grande do Sul. Mice were housed in standard cages and kept up to four per cage under a reversed 12-h light/dark cycle with free access to food and water or caffeine solution. All behavioral tests were performed between 8:00 am and 5:00 pm. All the experimental procedures were designed to minimize the number of animals used and their suffering.

**Treatment**

Two groups of adult mice (6 months old) received either caffeine solution or drinking water during 12 months. Another group of adult mice (6 months old) received only drinking water and they were used in all determinations. Caffeine solution (1 mg/mL) was left in the water bottles throughout the weekend being changed daily during the week. Caffeine solution is equivalent to 220 mg/kg/day and the intake for each animal was averaged to be 5 mL/day, which means that each mouse consumed approximately 5 mg of caffeine/day. According to the mice strain used here weighting 40 g, this dose should correspond to 10 cups of coffee/day if normalized to human intake (Finn and Holtzman, 1987; Fredholm et al., 1999). However, the metabolic rate of mice is faster than human and caffeine solution was always replaced between 6:00 and 7:00 pm to avoid disruptions on the circadian cycle of the animals. Caffeine administration was not interrupted during behavioral tests.

**Object recognition task**

The object recognition task was performed by a blinded observer and followed guidelines recently reviewed (Bevins and Besheer, 2006). The apparatus consisted of a painted wood small chamber
with the following dimensions: 25×25 cm; 1×w. A light bulb was switched on during test sessions. The objects were placed near the two corners at either end of one side of the chamber. Mice were placed individually into the chamber facing the center of the opposite wall. The objects presented similar textures, colors and sizes, but different shapes (tower and pyramid built with Lego toys).

In the first week, 6-month- or 18-month-old mice were handled daily and adapted to the procedure in 3 days. Adaptation sessions consisted of placing mice to explore the apparatus during 10 min each day. They were acclimated in the testing room during 2 h before the beginning of the sessions. After 3 days of adaptation, mice were submitted to training session that consisted in leaving the animals in the apparatus containing two identical objects. Each mouse was always placed in the apparatus facing the wall and after 10 min of exploration, the mouse was put back in its home cage. The testing session was performed 90 min after the training session and two dissimilar objects were present, a familiar and a novel one. The total number of trials per animals consisted of three trials of the habituation period (in the absence of objects) and two additional trials that comprised the training and test session (in the presence of objects). Overall, the animals started to explore the objects 1 or 2 min after they had entered in the box. Discrimination ratio for each mouse was expressed by the ratio \( T_F/(T_{F}+T_{N}) \), \( T_F \) time spent exploring familiar object; \( T_{N} \) time spent exploring the novel object. Between trials the objects were cleaned with 10% ethanol solution. Exploration was defined by directing the nose to the object at a distance of more than 2 cm and/or touching the object with the nose or forepaws. Sitting on the object was not considered exploratory behavior.

Immunoblotting

After behavioral tests mice were killed by cervical displacement; the whole hippocampus was dissected out and immediately homogenized in 5% SDS with a protease inhibitor cocktail. The homogenate was frozen at −70 °C and kept at this temperature until the moment of use. After defrosting, the protein content was determined by using bicinchoninic acid assay and bovine serum albumin (BSA) as standard. Hippocampal extracts were diluted to a final protein concentration 2 μg/μl in SDS-PAGE buffer and 70 μg of the samples and dual color prestained molecular weight standards were separated by SDS-PAGE (12% gel). Samples from adult mice, old mice and caffeine-treated old mice were loaded at the same gel. After electro-transfer, the membranes were incubated overnight with Tris-buffered saline (TBS-T) 0.1% containing Tween-20 and 3% BSA. After blocking, membranes were incubated for 24 h at 4 °C with rabbit anti-TrkB antibody (1:1000), mouse anti-BDNF antibody (1:500) or mouse anti-α-actin antibody (1:1000). After primary antibodies incubation, the membranes were washed and incubated with alkaline phosphatase-conjugated secondary antibodies for 2 h at room temperature and developed with ECL kit. The autoradiographic films were scanned and densitometric analyses were performed using public domain NIH Image Program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/). As an additional control of the protein loading, we also stained the membranes with Ponceau S stain. No differences were found in the amount of protein loaded (data not shown).

Statistical analysis

For novel objects statistical analysis was performed using discrimination ratio, while for familiar objects we used the time spent exploring the objects in seconds. Multiple comparisons between groups were analyzed by using parametric analysis (one-way ANOVA) followed by Newman Keuls post hoc test. Statistical differences were considered when \( P<0.05 \).

RESULTS

Effect of caffeine treatment on the total exploration for both objects

In this study, the object recognition memory task was not performed in an open field arena to avoid larger environments, which according to the guidelines can evoke anxiety and stress-related behaviors that compete with object recognition (Beverns and Besheer, 2006). Thus, we tried to discard age-related or caffeine treatment effect on locomotion of the animals by evaluating the total time spent in both objects during the sessions (Fig. 1). In the training session no differences were found in the time spent exploring the objects in all groups of animals (Fig. 1). Accordingly, in the test session performed 90 min after training, mice also did not show statistical differences, albeit caffeine-treated mice showed a slight decrease in the total time of exploration. This slight decrease caused by caffeine treatment could be predicting preliminary effects that were further characterized.

Effect of caffeine treatment on the familiar object recognition memory

Normally behaving mice spent less time exploring the familiar object, unless any impairment had been observed in the locomotion of the animals. The ability of the mice to discriminate familiar objects was analyzed by recording the time spent in the familiar object in the test session performed 90 min after training. In the test session all groups spent less time in the familiar object, but the caffeine-treated mice group recognized markedly the familiar object when compared with their age-matched mice group (Fig. 2). It could be also noticed that adult mice spent less time in the familiar object than age-matched control mice. Notably, caffeine-treated mice showed a similar performance to adult mice in the time spent in the familiar object (Fig. 2) \( F(2,25)=4.178, P=0.0290 \).
Fig. 2. Analysis of the time spent in the familiar object for all groups of mice during 10 min each session. Graphic shows the time spent (in seconds) in exploring the familiar object for each group of mice: adult mice (6 months old); age-matched control mice (18 months old); and aged-mice treated during 12 months with caffeine (1 mg/mL, in the drinking water). Results are presented as means ± S.E.M. of the seconds spent in the familiar object in the training session and in the test session performed 90 min later (n=8–9 animals for each group). *P<0.05 indicates significant difference for the time spent exploring the familiar object in the test session between adult and aged-mice caffeine.

Effect of caffeine treatment on the novel object recognition memory

Regardless of caffeine treatment, discrimination ratio has been described to be minor for aged animals when compared with adult ones (Bevins and Besheer, 2006). In our study, these differences were also observed, even though we had achieved a suitable discrimination ratio for aged-mice by handling and increasing adaptation period before starting the novel object recognition task (Fig. 3). Caffeine-treated mice showed a similar pattern of discrimination ratio compared with adult mice, and there was an increase in the discrimination ratio compared with age-matched control mice (Fig. 3). Hence, caffeine-treated mice showed a better recognition memory for novel objects compared with their age-matched control mice (Fig. 3) [F(2,25)=7.130, P=0.0041].

Effect of caffeine administration on the age-associated effect in BDNF and TrkB immunocontent

Analysis of the BDNF and its receptor TrkB in the whole hippocampus revealed changes in the immunocontent for both proteins. First of all, BDNF and TrkB densities increased with aging (Fig. 4A and B). Extracts from the whole hippocampus of the age-matched control mice presented a twofold increase in the BDNF immunocontent compared with adult mice (Fig. 4A). Hippocampus from caffeine-treated mice showed a 20% decrease in the BDNF immunocontent when compared with their age-matched controls that received only drinking water (Fig. 4A) [F(2,23)=45.03, P<0.0001]. Although with a less pronounced effect, TrkB immunocontent also increased (20%) in the old mice hippocampus when compared with adult mice (Fig. 4B). Similar to that observed for BDNF, caffeine treatment also diminished TrkB immunocontent in old mice hippocampus when compared with their age-matched controls (Fig. 4B) [F(2,23)=12.67, P=0.0002]. Finally, α-actin immunocontent did not differ between samples from all groups (Fig. 4C) [F(2,23)=1.444, P=0.2585].

DISCUSSION

Equivalent age criteria for human and rodents have been difficult to achieve (Coleman, 2004). However it was assumed here that 18-month-old mice would have lower performance than 6-month-old animals, and therefore could potentially better characterize the effects of caffeine on cognition. Even though aged mice spent less time exploring the familiar object, these animals recognized less efficiently the familiar object when compared with adult mice. In addition, aged mice to a certain extend recognized the novel object as seen by the increase in the discrimination ratio between training and testing session, but adult mice presented a higher discrimination ratio. Thus, 18-month-old mice were considered aged-mice because they showed a clear decline in the object recognition task which suits the purpose of studying preventive effects of caffeine.

In our study, caffeine administered during mice adulthood prevented age-associated decline in the recognition memory when evaluated 90 min after training that corresponds to short term memory. Although some reports had evaluated the long term memory in this task, we decided to measure only short term memory since the performance for the novel object recognition deteriorates as the period between training and session increases, with better discrimination values around 60–90 min after training (de Bruin and Pouzet, 2006; Sik et al., 2003). Besides, aged animals usually present a lower discrimination ratio when a testing session is performed 90 min after training.

Aged mice treated with caffeine presented similar performance to adult animals in recognizing the novel object. To our knowledge this is the first report in which caffeine was administered in adulthood and its effects on recognition memory evaluated in aged-animals. Likewise, to the best of our knowledge, it is the first study in which the effects of caffeine on working memory were evaluated in a task with no aversive or reinforcement component (Agenlucci et al., 2002; Castellano, 1976; Prediger et al., 2005a,b). Although our results agree with other studies...
where chronic administration of caffeine prevented cognitive decline in young as well as in old animals, we cannot rule out possible acute effects of this substance, since the animals had access to caffeine solution during the intertrial intervals. Furthermore, it is important to emphasize that caffeine can also trigger anxiogenic-like effects when given at high amounts, but the dose and schedule administered in our study are distinct from other studies where caffeine was able to evoke anxiety (El Yacoubi et al., 2000; Jain et al., 2005). Besides, animals that displayed anxiety-like behavior by caffeine administration also presented a poor performance in the learning and memory tasks (Silva and Frussa-Filho, 2000). Thus, in case caffeine provoked anxiogenic effects, we would expect that mice would have avoided moving toward the objects.

In humans, there are controversial data on whether caffeine intake is beneficial on cognitive functions, because only a few studies found a positive association between caffeine intake and cognitive improvement (Rogers et al., 2003; Yeomans et al., 2002). These discrepancies reflect the difficulties to accurately follow up caffeine intake in humans. Hence, our study performed in rodents found that this substance helped to preserve recognition memory in old mice as compared with age-matched con-
trols. In this context, even though humans and rodents show evident differences in the rate of aging, studies designed to investigate the influence of diet components on cognition have been usually carried out in rodents because they can be better controlled in animals rather than in humans.

Although adenosine A2A receptors are more prevalent in the striatum, their relative scarce density in the hippocampus does not imply a minor role in the information (cognitive) processing in this brain region. In this scenario, a recent study showed that the long-term potentiation of N-methyl-D-aspartate (NMDA)-receptor-mediated synaptic currents (NMDA-EPSCs) between hippocampal mossy fibers and CA3 pyramidal cells depends on postsynaptic adenosine A2A receptors (Rebola et al., 2008). Although the blockade of A1 and A2A adenosine receptors was first attributed to the psychostimulant action of caffeine, recent studies have shown that the effects on arousal as well as on neuroprotection seem to be due to the preferential blockade of A2A receptors (Dall’Igna et al., 2003; Higgins et al., 2007; Huang et al., 2005; Silva et al., 2007). Likewise, prevention of cognitive decline and improvement in this performance by caffeine in animals is often reproduced by selective adenosine A2A antagonists, but not by adenosine A1 antagonists (Dall’Igna et al., 2007; Higgins et al., 2007; Kopf et al., 1999; Prediger et al., 2005a,b).

Western blotting analysis for BDNF in hippocampal extracts from age-matched control mice revealed a robust increase when compared with adult ones. Similarly, TrkB immunocontent also increased in aged-mice albeit to a lesser extend than BDNF. At a first glance, our findings appear to be unmatched to previous reports where BDNF and TrkB levels were compared with 6-month-old animals. However, in most of the cases the mRNA levels were analyzed and hence posterior post-translational modifications cannot be discarded. Even though our results with mice are in line with current reports where BDNF modification with aging (Hattiangady et al., 2005; Kaisho et al., 1994; Lapchak et al., 1993). Nonetheless, these discrepancies may also be responsible for these discrepancies related to BDNF modifications with aging. Additionally, these discrepancies may also be extended to BDNF signal transduction since recently a decrease in TrkB immunocontent in aged-rats was reported, but this neurotrophin was able to enhance field excitatory postsynaptic potentials recorded from the hippocampus of young adults and aged rats, an action triggered by adenosine A2A receptor activation (Diógenes et al., 2007). Thus, the complexity of the signal transduction pathways operated by BDNF makes it difficult to establish a consensus between the functionality of the receptor and its content (Huang and Reichardt, 2003). Besides, there are differences found in the truncated and full-length forms of TrkB in the same sample, and here we just analyzed the full length form.

It has been previously demonstrated that learning activity can modify BDNF and TrkB content in aversive tasks (Croll et al., 1998; Silhol et al., 2007), but we did not seek to investigate whether object recognition task could modify the immunocontent of these proteins in our study, which therefore remains to be elucidated in future studies. Nevertheless, adulthood caffeine administration partially prevented the age-associated increase in the BDNF immunocontent, while it sustained TrkB immunocontent similar to that found in hippocampal extracts from adult mice. The beneficial effects afforded by adulthood caffeine administration observed here were related to prevention of the age-associated increase in the BDNF and TrkB immunocontent.

As mentioned above, some beneficial effects triggered by caffeine were related to the preferential blockade of adenosine A2A receptors, whereby the BDNF signaling seems also to operate, but it remains to be further determined if caffeine could affect some signal transduction pathways operated by BDNF since caffeine seems to modify some proteins involved in the BDNF downstream cascade signaling (Sahin et al., 2007).

Ever since lifespan is increasing worldwide, research on aging is not as unattractive as it was two or three decades ago, with one abiding question being about the interventions that could be made to prevent cognitive decline. Our study showed that a usual diet component that is frequently consumed in the adulthood may prevent the predictable decline in the recognition memory with aging. Considering that our results just evaluated the effects of this long-term treatment with caffeine on some parameters of CNS functioning, it remains important to evaluate if the benefits observed here could be extended to the whole organism. Finally, it is important to know benefits of a usual diet component as a low-cost and simple strategy for the improvement of the life quality of elderly people.

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