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EFEITO DA ADMINISTRAÇÃO DE CAFEÍNA AGUDA E CRÔNICA SOBRE A MEMÓRIA DE RECONHECIMENTO E O IMUNOCONTEÚDO HIPOCAMPAL DE BDNF E TrkB EM CAMUNDONGOS ADULTOS E IDOSOS

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SUMARIO

RESUMO

A cafeína é o psicoestimulante mais consumido no mundo, cujo alvo molecular é o bloqueio não-seletivo dos receptores de adenosina A_1 e A_{2A}. A administração de cafeína parece melhorar o desempenho cognitivo em humanos e animais, embora esse efeito tenha sido mais bem caracterizado em animais do que em humanos e, sugere-se que parte desses efeitos seja pelo bloqueio preferencial dos receptores A_{2A} e não dos receptores A_1. Recentemente, com o auxílio de técnicas de eletrofisiologia relatou-se a participação dos receptores de adenosina na transmissão sináptica pelo fator neurotrófico derivado do cérebro (BDNF). O BDNF faz parte da família das neurotrofinas e sua sinalização em seus receptores do tipo tirosina cinase B (TrkB) é fundamental para os processos de aprendizado e memória. Neste estudo procurou-se primeiramente verificar se a administração aguda de cafeína ou ao longo da vida adulta até o envelhecimento poderia melhorar o desempenho de camundongos expostos à tarefa de reconhecimento de objetos. A grande maioria dos estudos feitos com animais para avaliar os efeitos da cafeína até o presente momento utilizou tarefas com componentes aversivos ou estímulos de reforço. A tarefa de reconhecimento de objetos consiste em analisar a habilidade natural dos animais em reconhecer uma novidade em um ambiente que foi previamente apresentado. Além disso, a análise do imunoconteúdo do BDNF e do seu receptor TrkB foi realizada com o intuito de relacionar os efeitos comportamentais com possíveis alterações nessas proteínas que estão envolvidas em processos de aprendizado e memória. Na tarefa de reconhecimento de objetos, camundongos adultos (3-4 meses de idade) tratados agudamente com cafeína apresentaram melhores índices de reconhecimento e maiores níveis de BDNF e TrkB do que seus respectivos controles. Em camundongos, que foram tratados com cafeína durante doze meses (dos seis aos dezoito meses de idade), foi verificado um efeito preventivo ao declínio cognitivo decorrente da idade, pois os animais aos 18 meses de idade tratados com cafeína durante a vida adulta apresentaram melhor desempenho na tarefa de reconhecimento de objetos e ainda semelhante ao grupo de camundongos adultos. Este dado mostra que a cafeína melhora a memória de reconhecimento, sendo este efeito relacionado a um aumento no imunoconteúdo hipocampal de BDNF e TrkB. A cafeína preveniu o aumento do imunoconteúdo de BDNF e TrkB relacionados com a idade. Estes resultados indicam que o consumo de cafeína na idade adulta pode prevenir o declínio da memória de reconhecimento que ocorre com o envelhecimento e este efeito preventivo pode envolver uma diminuição no imunoconteúdo hipocampal de BDNF e TrkB.

ABSTRACT

Caffeine is one of the most psychostimulants consumed in the world, and its molecular target is the non-selective A_1 and A_{2A} adenosine receptors blockade. Caffeine administration seems to improve the cognitive performance in humans and animals, although this effect had been better characterized in animals than in humans, and it was suggested that some of these effects were preferential A_{2A} and not A_1 blockade. Recently, electrophysiology studies reported the participation of adenosine receptors in the synaptic transmission by brain-derived neurotrophic factor (BDNF). BDNF is one of neurotrophins family members and its signaling in their tyrosine kinase B (TrkB) receptor type is essential to the learning and memory processes. At the moment, animal studies evaluated the effects of caffeine in tasks with aversive or reinforcement components. This study verified if acute administration of caffeine or throughout adult life to the aging could improve performance in the object recognition task. The object recognition task examines the natural ability of the animals to recognize a novelty in an environment that was previously presented. Furthermore, the analysis of BDNF and its receptor TrkB immunocontent was held with the aim of linking the behavioral effects with possible changes in these proteins that are involved in learning and memory processes. In the object recognition task, adult mice (3-4 months-old) treated acutely with caffeine had better rates of recognition and increased BDNF and TrkB immunocontent than their respective controls. Chronic caffeine treatment (from six to eighteen months-old) was found a preventive effect to age-related cognitive decline. 18 months-old mice treated with caffeine during adulthood showed better performance in object recognition task and yet similar to the adult group. This finding shows that caffeine improves recognition memory, and this effect was related to an increase in the hippocampal BDNF and TrkB immunocontent. Caffeine prevented age-related increase in BDNF and TrkB immunocontent. These results indicate that consumption of caffeine in adulthood may prevent the decline of recognition memory that occurs with aging and this preventive effect may involve a decrease in hippocampal BDNF and TrkB immunocontent.

LISTA DE ABREVIATURAS

5’NT	5’nucleotidase
A_1	Receptor metabotrópico de adenosina do subtipo A_1
A_{2A}	Receptor metabotrópico de adenosina do subtipo A_{2A}
A_{2B}	Receptor metabotrópico de adenosina do subtipo A_{2B}
A3	Receptor metabotrópico de adenosina do subtipo A_3
AC	Adenilato ciclase
ADK	Adenosina cinase
AMPc	Adenosina monofosfato cíclica
ATP	Adenosina trifosfato
BDNF	Fator neurotrófico derivado do cérebro
D1	Receptor metabotrópico de dopamina do subtipo D1
D2	Receptor metabotrópico de dopamina do subtipo D2
EctoN	Ectonucleotidases
ENT	Transportador de nucleosídeos
GABA	Ácido gama-aminobutírico
LTP	Potenciação de longa duração
NMDA	N-metil-D-aspartato
PKA	Proteína cinase A
SNC	Sistema nervoso central
TrkB	Receptor do tipo tirosina cinase B

INTRODUÇÃO

História da cafeína

Conforme descrito no livro de Weinberg e Bealer (2001), achados antropológicos sugerem que a cafeína já era consumida e apreciada na Idade Paleolítica, cerca de 700 mil anos a.C., pelo homem da pedra que se alimentava de plantas ricas desta substância. Posteriormente, com o advento da técnica de infusão com água quente, bebidas a base de cafeína se tornaram populares, tais como o café, chá, coca-cola, chocolate, guaraná e mate.

O café, a principal fonte mundial de cafeína, provém de uma árvore do gênero *Coffea* e dentre as várias espécies conhecidas, as mais comercializadas são *Coffea arabica* e *Coffea canephora* sendo popularmente conhecidas como arábica e robusta, respectivamente. Apesar da planta ser originária da Etiópia, onde ainda hoje faz parte da vegetação natural, as primeiras plantações de café denominadas de “Kaweh”, apareceram na península Arábica no século XIV, e eram usadas como alimento na fabricação de vinho, remédio e para fazer uma bebida árabe denominada “*qahwa”*, conhecida por prevenir o sono. Posteriormente, difundiu-se através do Iêmen e dos países árabes para o resto do mundo.

O hábito de tomar café foi condenado pela ortodoxia islâmica, mas posteriormente, chegou a ser considerado como algo providencial para rezar sem cair em sonolência e como um excelente substituto das bebidas alcoólicas.

Na Europa, o café apareceu no século XVI sendo introduzido, principalmente, pelos espanhóis e holandeses no período das descobertas. Antes disso, o café

era consumido de maneira restrita e a bebida nobre era o chá. Inicialmente, o consumo de café encontrou uma forte oposição em alguns países protestantes, como a Alemanha, Áustria e Suíça, nações essas que chegaram mesmo a castigar o seu comércio e consumo.

Com o passar do tempo, todas as proibições acabaram por desaparecer na Europa e muitos estabelecimentos passaram a oferecer espaços públicos para o consumo de café. Assim, a partir da segunda metade do século XVII, principalmente nas grandes cidades o café passou a ser considerado como uma bebida intelectual, com países como a França onde os cafés se tornaram locais de reunião dos intelectuais, entre eles Victor Hugo, Voltaire, Rousseau.

Em 1736 surgiram as primeiras plantações na América Latina, mais precisamente em Porto Rico, onde o café se tornou o principal produto de exportação do país. O café chegou ao norte do Brasil pela cidade de Belém, em 1727, trazido da Guiana Francesa para o Brasil a pedido do governador do Maranhão. Já naquela época o café possuía grande valor comercial. Devido às nossas excelentes condições climáticas para o cultivo o plantio de café se espalhou rapidamente, com produção voltada para o mercado doméstico. Em sua trajetória pelo Brasil, o café percorreu os estados do Maranhão, Bahia, Rio de Janeiro, São Paulo, Minas Gerais e Paraná.

O primeiro protótipo de uma máquina de café expresso foi criado na França, em 1822, mas só em 1905 surge um modelo comercial na Itália. O café descafeinado foi descoberto na Alemanha em 1903 após investigações que visavam obter um processo que permitisse remover a cafeína sem destruir o verdadeiro sabor do café.

Em 1938, o café instantâneo (Nescafé) foi inventado pela companhia Nestlé que pretendia ajudar o governo brasileiro a escoar o seu excedente de café.

Outra fonte importante de cafeína, encontrada e apreciada muito pela população da América do Sul, especialmente da região sul do Brasil, é o chimarrão, uma bebida preparada a partir de uma planta (*Ilex paraguariensis*) com água quente, de modo semelhante ao chá e café (Martín et al., 2007).

Atualmente, a cafeína é consumida e apreciada em diferentes países estando este hábito inserido nas mais variadas práticas culturais. Além disso, a sua produção é vital para a economia de alguns países, que sobrevivem a partir da exportação do produto para muitos países.

Principais fontes de cafeína

Existem mais de 60 espécies de plantas que fornecem cafeína, sendo as mais conhecidas o café, o chá, o cacau, a erva mate e o guaraná. O conteúdo de cafeína presente em diversos produtos e bebidas depende da planta utilizada e do modo de preparo.

O café é a principal fonte de cafeína e pode fornecer de 40 a 180 mg de cafeína por 150 ml de bebida. Esta diferença deve-se ao tipo de grão utilizado e ao modo de preparo. O chá, outra bebida muito apreciada mundialmente, contém de 24 a 50 mg/150 ml. Bebidas à base de cola, tais como coca-cola e pepsi cola, contêm aproximadamente 40 mg por 350 ml. Já nas conhecidas bebidas energéticas como o Red Bull, encontramos 80mg de cafeína por 250 ml. Um chocolate de 28 gramas pode ter até 36 mg (Barone e Roberts, 1996). O chimarrão contém cerca de 0,93 mg/ml (Martín et al., 2007).

O consumo mundial de cafeína é estimado em 70 a 76 mg/pessoa/dia, sendo que nos Estados Unidos e Canadá é de 210 a 238 mg/dia e pode chegar a mais de 400 mg/pessoa/dia em países como Suécia e Finlândia (Barone e Roberts, 1996). A dose letal de cafeína é em torno de 200 mg/kg, o que equivale a 80-100 copos médios de café (Fredholm et al., 1999).

Metabolismo da cafeína

A cafeína é completamente absorvida pelo trato gastrointestinal após 45 minutos de sua ingestão. A meia vida varia entre as faixas etárias, na gravidez, em combinação com alguns medicamentos e com a integridade hepática. Nos adultos saudáveis a meia vida é de aproximadamente 3-4 horas, enquanto que em ratos é mais curta (cerca de 1 hora). Em mulheres que tomam anticoncepcionais é de 5-10 horas e naquelas em gestação de 9-11 horas. Em recém-nascidos a meia-vida é de 30 horas. Nos indivíduos com doença hepática, a meia-vida da cafeína pode chegar até 96 horas. O tabagismo encurta a meia-vida da cafeína (Fredholm et al., 1999).

Por ser uma molécula hidrofóbica a cafeína tem sua passagem facilitada em todas as membranas biológicas, até mesmo entre o sangue e o cérebro de adulto ou feto (Lachance et al., 1983; Tanaka et al., 1984). Em ratos adultos, por exemplo, a concentração de cafeína no plasma é semelhante à encontrada no líquido cérebro-espinhal (Liu et al., 2006).

A cafeína é metabolizada principalmente no fígado pelas enzimas do sistema citocromo P450 em dimetilxantinas, como a paraxantina, teobromina e teofilina. Cada um destes metabólitos tem suas funções no organismo, sendo excretados

na urina após metabolizados. Nos humanos, a paraxantina é o metabólito predominante (72 a 80%), enquanto em roedores, apesar da paraxantina ser o metabólito plasmático predominante, os níveis de teofilina também estão elevados.

Geralmente uma dosagem de 10 mg/kg em ratos representa uma dosagem de 3,5 mg/kg nos humanos, que corresponde de 2 a 3 copos de café (Fredholm et al., 1999).

Mecanismo de ação da cafeína

As metilxantinas são estruturalmente similares aos nucleotídeos cíclicos e têm sido extensivamente estudadas pela sua capacidade de interagir com as fosfodiesterases. Cafeína e teofilina atuam como inibidores competitivos das isoenzimas da fosfodiesterase em vários tecidos, incluindo o cérebro (Vernikos-Danellis e Harris, 1968). Porém, a sua afinidade pelas fosfodiesterases é baixa, necessitando concentrações na faixa dos milimolar para serem vistos efeitos significativos (Cardinali, 1980). Da mesma forma, concentrações na faixa de 350–500 mM são necessárias para mobilizar o cálcio de seus estoques intracelulares, efeito que é mediado pela ativação dos canais sensíveis a rianodina (McPherson et al., 1991; Sitsapesan et al., 1995; Marangos et al., 1979). Para ser considerada letal, a concentração sanguínea de cafeína deve atingir 500 mM (Dews, 1982). Porém, a ingestão habitual média de café que correspondente a três copos ou 300 mg de cafeína, provoca um pico plasmático de cafeína que não excede 30 mM (Bonati et al.,1982).

Sob condições fisiológicas normais, o efeito exercido pela cafeína no sistema nervoso central (SNC) depende da sua capacidade de atuar como um antagonista dos receptores metabotrópicos de adenosina, principalmente os do subtipo A_1 e A_{2A}. O bloqueio dos receptores de adenosina pela cafeína pode levar a efeitos secundários importantes sobre muitas classes de neurotransmissores, incluindo a noradrenalina, dopamina, serotonina, acetilcolina, glutamato e GABA, que interferem em muitas funções fisiológicas (Fredholm et al., 1999).

Efeitos do consumo crônico de cafeína

Por ser uma substância muito consumida mundialmente a cafeína tem sido alvo de muitos estudos epidemiológicos e experimentais. Estes estudos têm concluído que a ingestão crônica de cafeína está associada com um menor risco de desenvolvimento da doença de Alzheimer (Maia e de Mendonça, 2002) e Parkinson (Xu et al., 2005). Porém, parece que o consumo de cafeína não reduz o risco de desenvolvimento da doença de Parkinson em mulheres que fazem reposição hormonal durante a menopausa (Xu et al., 2005).

Também tem sido verificada uma melhora no desempenho da memória de idosos (Johnson-Kozlow et al., 2002) e uma redução do declínio cognitivo em mulheres idosas sem demência (Ritchie et al., 2007). Além disso, a ingestão de cafeína está associada com o aumento no estado de alerta, melhora da atenção e no desempenho psicomotor e cognitivo (Daly, 2007; Ferré, 2008; Smith, 2002; Smith et al., 2005; Takahashi et al., 2008).

Sistema adenosinérgico

A adenosina é uma purina ribonucleosídeo ubíqua e essencial para as células vivas. Como conseqüência desta distribuição ubíqua e por causa de sua ligação ao pool energético, a adenosina também atua como um importante mensageiro para a sinalização extracelular. A adenosina é considerada um neuromodulador endógeno, pois embora não possa ser armazenada em vesículas sinápticas como os neurotransmissores clássicos, ela exerce bastante influência em muitas funções no SNC tais como o controle da liberação de neurotransmissores e da excitabilidade neuronal (Fredholm et al., 2005). Porém, a adenosina por não ser um neurotransmissor clássico, não transfere informação unidirecionalmente do terminal pré-sináptico ao terminal pós-sináptico e não atua somente ou predominantemente nas sinapses (Cunha, 2001).

Três mecanismos são responsáveis pela formação de adenosina no meio extracelular: a sua liberação por meio de transportadores de nucleosídeos presentes nas membranas celulares após um aumento de seus níveis intracelulares ou gradiente de sódio reverso; pela via das ectonucleotidases quando ocorre a liberação de nucleotídeos da adenina, especialmente adenosina trifosfato (ATP); e, finalmente, a formação de adenosina a partir da adenosina monofosfato cíclica (AMPc) após a sua liberação no meio extracelular (Dunwiddie e Masino, 2001; Latini e Pedata, 2001).

Os receptores de adenosina foram reconhecidos nos anos 70 pela capacidade das metilxantinas, teofilina e cafeína, atuarem como antagonistas não seletivos (Fredholm et al., 1980). Até o presente momento, quatro diferentes subtipos de receptores de adenosina (A_1, A_{2A}, A_{2B} e A_3) foram clonados e identificados em

humanos e roedores, sendo que todos os subtipos são acoplados a proteínas G. Os receptores A_1 e A_3 estão acoplados a proteínas G inibitórias enquanto os receptores A_{2A} e A_{2B} estão acoplados a proteínas G estimulatórias (Fredholm et al., 2001).

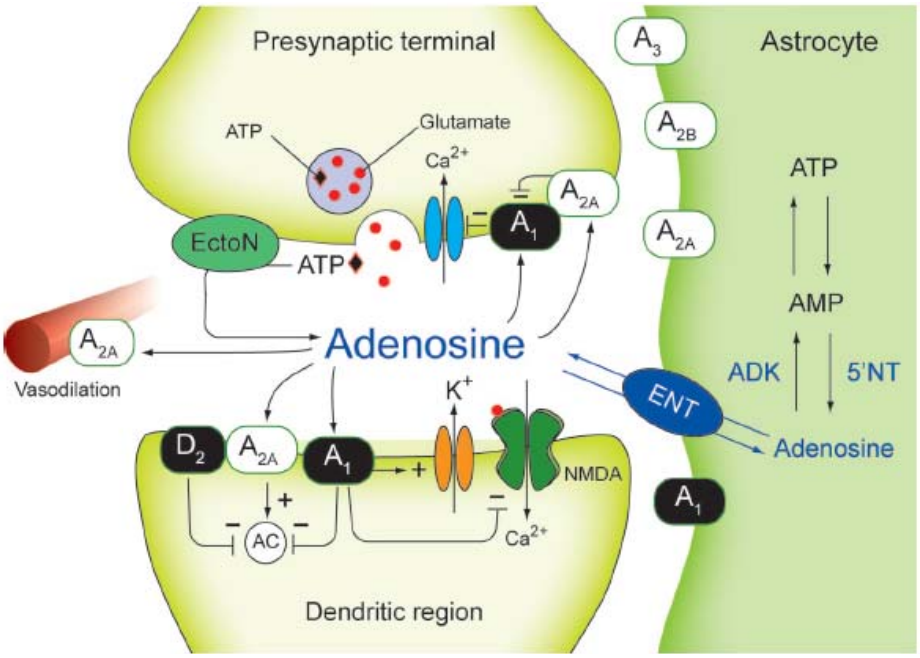

Figura 1. Representação esquemática da síntese de adenosina e seu mecanismo de ação no sistema nervoso central. 5'NT – 5'nucleotidase; AC - adenilato ciclase; ADK - adenosina cinase; AMP – adenosina monofosfato; ATP - adenosina trifosfato; EctoN - ectonucleotidases; ENT – transportador de nucleosídeos; NMDA - N-metil-D-aspartato (Benarroch, 2008).

As ações da adenosina no SNC parecem ser mediadas principalmente pelos receptores A_1 e A_{2A}, que possuem elevada afinidade pela adenosina e são altamente expressos em diversas regiões do cérebro.

O receptor adenosinérgico do subtipo A_1 é o mais abundante no SNC, principalmente na região do neocórtex, cerebelo, hipocampo e corno dorsal da coluna vertebral, enquanto que os receptores do subtipo A_{2A} são altamente expressos em neurônios pálido-estriatais e no bulbo olfatório, mas também são

encontrados em outras regiões do cérebro, como no hipocampo (Fredholm et al., 2005).

Estudos *in vitro* demonstram que a cafeína possui afinidades semelhantes para os receptores de adenosina do subtipo A_1, A_{2A} e A_{2B} e uma baixa afinidade para o receptor do subtipo A_3 (Fredholm et al., 2001; Solinas et al., 2005). Como concentrações fisiológicas de adenosina podem estimular facilmente os receptores A_1 e A_{2A}, enquanto os receptores A_{2B} somente são ativados com concentrações elevadas de adenosina, parece que os receptores A_1 e A_{2A} são os alvos preferenciais da cafeína no SNC.

Em estudos que avaliam diferentes padrões de comportamentos e função cognitiva mostram que a adenosina e seus receptores no SNC parecem ter um papel modulatório fundamental (Cunha et al., 2008; de Mendonça et al., 1997; de Mendonça e Ribeiro, 1994; Kuzmin et al., 2006).

Apesar da cafeína ser considerado um antagonista não seletivo dos receptores de adenosina, o receptor preferencial para a cafeína desencadear seu efeito psicoestimulante ainda é controverso, pois o antagonismo seletivo de um ou de ambos receptores demonstram efeitos psicoestimulantes semelhantes aos da cafeína, enquanto que a utilização de agonistas dos receptores A_1 e A_{2A} apresenta um efeito depressor (Ferré, 2008).

Spealman (1988) demonstrou que o bloqueio do receptor A_{2A} foi o principal responsável pela estimulação motora desencadeada pela administração de cafeína e estudos recentes demonstraram que antagonistas do receptor A_{2A} (e não do receptor A_1) reproduzem efeitos comportamentais e bioquímicas semelhantes aos da cafeína (Svenningsson et al., 1977; El Yacoubi et al., 2000).

Com o avanço da tecnologia da deleção genética tem-se confirmado que o receptor A_{2A} é o alvo molecular predominante dos efeitos da cafeína e, de fato, camundongos deletados para o gene do receptor A_{2A} não apresentam aumento na atividade locomotora com a administração de cafeína (Ledent et al., 1997). Além disso, alguns resultados sugerem que antagonistas do receptor A_1 induzem à depressão motora, podendo ser o responsável pela indução da depressão motora observada com altas doses de cafeína (El Yacoubi et al., 2000).

Porém, a exposição crônica de cafeína resulta em tolerância parcial de seus efeitos sobre o aumento da locomoção que é semelhante à administração de antagonista do receptor A_1, mas não de A_{2A} (Karcz-Kubicha et al., 2003), sugerindo que a tolerância à ativação motora mediada pela cafeína pode ser via bloqueio do receptor A_1 e que a ativação motora residual induzida pela cafeína em indivíduos tolerantes poderia ser principalmente via bloqueio do receptor A_{2A}.

Contudo, antagonistas A_1 também podem induzir ativação motora e potencializar a ativação motora desencadeada por antagonistas A_{2A} (Jacobson et al., 1993; Popoli et al., 1998), sugerindo que ambos receptores A_1 e A_{2A} estão envolvidos no aumento da atividade locomotora com a administração de cafeína e a exposição crônica à cafeína modifica o seu efeito sobre a atividade locomotora que é dependente do bloqueio dos receptores A_1 e A_{2A}.

A adenosina também parece modular os efeitos sobre a memória e aprendizagem, pois agonistas dos receptores de adenosina, principalmente A_1, prejudicam a memória e aprendizagem em roedores (Homayoun et al., 2001; Normile e Barraco, 1991; Ohno e Watanabe, 1996; Zarrindast e Shafaghi, 1994), enquanto que o bloqueio não seletivo dos receptores de adenosina pela cafeína ou teofilina, assim como o bloqueio seletivo dos receptores A_1 e A_{2A} facilitam a

memória e aprendizagem nas tarefas de esquiva passiva (Kopf, 1999; Nehlig et al., 1992; Suzuki et al., 1993), esquiva inibitória (Pereira et al, 2002) e no labirinto aquático de Morris (Angelucci et al., 2002; Dudley et al., 1994; Hauber e Bareiss, 2001). Também tem sido demonstrado que antagonistas do receptor A_{2A} podem prevenir a deterioração da memória que é verificada em animais idosos (Prediger et al., 2005a) e em modelo experimental da doença de Alzheimer (Dall'Igna et al., 2007; Arendash et al., 2006).

Estudos farmacológicos têm indicado que o bloqueio combinado dos receptores A_1 e A_{2A} exerce efeitos facilitatórios sobre o desempenho de ratos em testes de memória espacial, e ambos os subtipos poderiam estar envolvidos na potenciação de longa duração (long-term potentiation – LTP) hipocampal (Arai et al., 1990; Kessey et al., 1997; Rebola et al., 2003). Além disso, há evidências da participação destes receptores na LTP estriatal (d'Alcantara et al., 2001).

A ativação de receptores adenosinérgicos A_1 (Zarrindast e Shafaghi, 1994) ou de ambos receptores A_1 e A_{2A} (Prediger e Takahashi, 2005) está diretamente relacionada a prejuízos cognitivos, enquanto o antagonismo dos receptores A_{2A} (Prediger et al, 2005a) ou de ambos receptores A_1 e A_{2A} (Prediger e Takahashi, 2005) é capaz de melhorar o desempenho de animais submetidos a tarefas de aprendizado e memória.

Porém, a administração de antagonista seletivo do receptor A_1, mas não do A_{2A}, produziu efeitos discriminativos semelhantes ao da administração de cafeína (30 mg/kg, i.p.) 30 minutos antes da tarefa discriminativa em ratos. Ainda foi encontrada uma redução sobre o efeito discriminativo mediado pela cafeína com a administração de um agonista seletivo A_1, mas não A_{2A}, propondo que o

receptor A_1 pode ser o subtipo mais envolvido em estímulos discriminativos (Solinas et al., 2005).

A integridade do sistema dopaminergético parece ser essencial para os efeitos psicoestimulantes da cafeína, pois antagonistas adenosinérgicos modulam a atividade dos receptores dopaminérgicos (Ferré et al., 1992; Cunha 2005), devido à co-localização entre ambos receptores, que podem formar heterodímeros entre si (A_1/D_1 e A_{2A}/D_2), ocasionando alterações alostéricas que afetam a afinidade e o acoplamento à proteína G. Desta forma essa interação parece modular a eficácia de ativação de ambos os receptores (Fuxe et al, 1998), implicando em processos de aprendizado e de formação de memória (Franco et al., 2000; Hillion et al., 2002; Franco et al., 2007). Assim, os agonistas dos receptores de adenosina produzem efeitos comportamentais semelhantes aos dos antagonistas dopaminérgicos, enquanto que os efeitos dos antagonistas da adenosina assemelham-se àqueles induzidos por agonistas dopaminérgicos (Fuxe et al., 1998).

A cafeína também parece atenuar o declínio cognitivo que é verificado com o envelhecimento (Riedel e Jolles, 1996) e pela administração do antagonista de receptores muscarínicos escopolamina (Riedel et al., 1995). A escopolamina é um agente amnésico clássico, pois compromete a função colinérgica induzindo uma deterioração aguda da memória (Sitaram et al., 1978). Interessantemente, o bloqueio seletivo do receptor A_{2A} preveniu a disfunção da memória causada por peptídeo β-amilóide, mas não foi capaz de prevenir a disfunção da memória induzida por escopolamina ou MK-801 (antagonista de receptores NMDA) no teste de labirinto em Y (Cunha et al., 2008). O mecanismo proposto para a contribuição do sistema adenosinérgico na deterioração cognitiva e maior

ocorrência de dano cerebral com o envelhecimento parece estar relacionado ao aumento do número de receptores A_{2A} concomitantemente com a redução do receptor A_1 em ratos velhos (Cunha et al., 2001; Takahashi et al., 2008).

Apesar de muitos estudos demonstrarem que a cafeína melhora a memória (Gevaerd et al., 2001; Prediger et al., 2005b; Riedel et al., 1995; Riedel e Jolles, 1996), o seu consumo crônico poderia prejudicar a aprendizagem e a memória que é dependente do hipocampo e está associado à redução da neurogênese com a administração de cafeína durante quatro semanas (Han et al., 2007).

Fator neurotrófico derivado do cérebro (BDNF)

O fator neurotrófico derivado do cérebro (*brain-derived neurotrophic factor* - BDNF) é um membro de uma família de neurotrofinas que participa da regulação não só da estrutura, mas também da função e manutenção da sobrevivência de algumas populações de neurônios durante o desenvolvimento e na vida adulta (Lu e Chow, 1999; Poo, 2001; Tyler e Pozzo-Miller, 2003). Esse fator neurotrófico, também, é essencial para eventos de plasticidade neuronal e funções importantes como o aprendizado e memória (Tyler et al., 2002). De fato, o bloqueio da sua sinalização compromete a persistência da memória (Alonso et al., 2002, 2005; Bekinschtein et al., 2007). Muitas vias de sinalização parecem ser operadas pela ligação do BDNF em seus receptores do tipo tirosina cinase B (TrkB) (Blanquet, 2000; Pizzorusso et al., 2000) e, dada a importância do funcionamento dessa sinalização para a manutenção das sinapses, o seu comprometimento tem sido observado em uma série de patologias do SNC, tais como, a Doença de Alzheimer e Parkinson, estresse agudo e crônico; bem como

alterações normais que ocorrem durante o envelhecimento parecem coincidir com alterações nos níveis de BDNF (Enna et al., 2006; Laske et al., 2006; Lee et al., 2005; Baquet et al., 2005; Hattiangady et al., 2005; Peng et al., 2005).

Dessa forma, este fator neurotrófico tem sido proposto como uma estratégia de neuroproteção (Hennigan et al., 2007; Fumagalli et al., 2006a,b), mas a sua administração farmacológica ainda é difícil, devido a sua impermeabilidade à barreira hemato-encefálica (Wu e Pardridge, 1999). Por este motivo, outras vias de sinalização possíveis de serem moduladas pelo BDNF estão sendo investigadas e os receptores de adenosina sugerem participação, pois a ativação dos receptores A_{2A} facilita a transmissão sináptica pelo BDNF (Diógenes et al., 2004) e ambos receptores A_{2A} e TrkB são co-imunoprecipitados (Lee e Chao, 2001). Neste mesmo estudo, foi observado que a adenosina e agonistas de seus receptores podem induzir a fosforilação do receptor TrkB com a participação do receptor A_{2A} (Lee e Chao, 2001). Embora os receptores de adenosina do subtipo A_{2A} são encontrados predominantemente no estriado, o hipocampo também parece estar sob o controle neuromodulatório da adenosina (Sebastião e Ribeiro, 2000). Além disso, a ação excitatória do BDNF sobre a transmissão sináptica no hipocampo pode ser induzida pela despolarização pré-sináptica e é dependente da ativação do receptor A_{2A} através de um mecanismo que requer a formação de AMPc e ativação da proteína cinase A (PKA) (Diógenes et al., 2004).

A tarefa de reconhecimento de objetos novos

A tarefa de reconhecimento de objetos novos, originalmente propostos por Ennaceur e Delacour (1988) e recentemente revisado por Besheer e Bevins (2006), é uma ferramenta experimental de bastante utilidade. O protocolo fundamental deste modelo é bastante simples e utiliza basicamente um aparato semelhante a uma caixa, cujo tamanho depende do animal a ser analisado e os objetos a serem explorados. A tarefa consiste em analisar a tendência natural do animal em explorar o ambiente e discriminar novidades. Nessa tarefa o animal é apresentado aos objetos idênticos e, posteriormente, na segunda fase o animal é apresentado a um dos objetos apresentado previamente juntamente com um objeto novo. Os objetos são do mesmo tamanho, mas podem ter a forma e/ou cor diferente na segunda fase.

Primeiramente, os animais são habituados ao aparato para a familiarização com o ambiente proporcionando, desta forma, uma diminuição da ansiedade e do estresse que normalmente são observados quando o animal é exposto a um ambiente novo. Se for percebida, uma menor interação com os objetos durante os testes; este tempo de familiarização deve ser maior. Normalmente, camundongos necessitam de um tempo maior de familiarização e um aparato menor do que o utilizado para ratos, pois eles são mais ansiosos e estressados.

Posteriormente à familiarização ao aparato, submetem-se os animais a dois testes diferentes: no primeiro, chamado de teste 1 ou sessão de treinamento (treino), os animais são postos no aparato com dois objetos similares que serão explorados durante um dado período de tempo que permita uma exploração suficiente e igual de ambos objetos (fase de apresentação dos objetos); no

segundo, conhecido como teste 2 ou sessão de teste (teste), um dos objetos é substituído por um outro, diferente o suficiente para que os animais o percebam e explorem mais este (fase de reconhecimento dos objetos). O intervalo entre o treino e o teste depende do tipo de memória que desejamos investigar: memória de curta ou longa duração. O tempo que o animal explora cada um dos objetos é registrado para as análises pertinentes ao design do estudo.

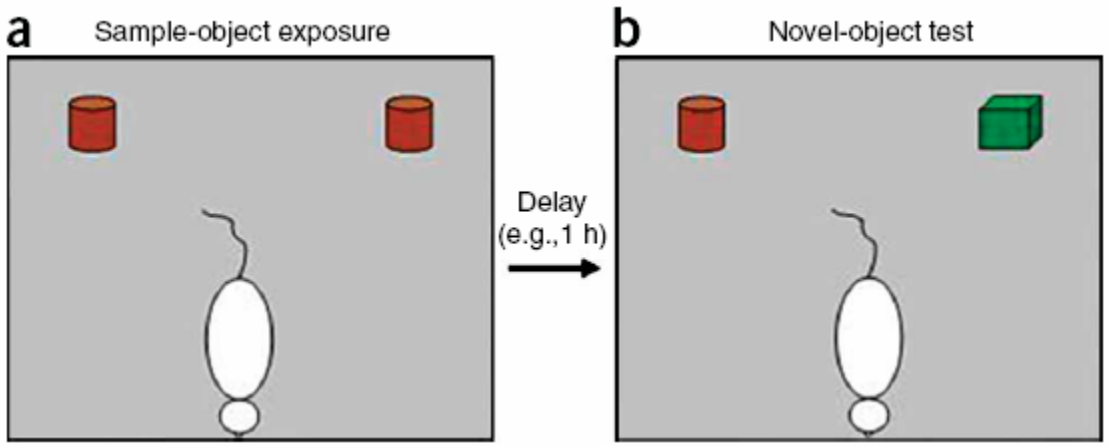

Figura 2. Representação esquemática da tarefa de reconhecimento de objeto; **a** = treino e **b** = teste (Bevins e Besheer, 2006).

A popularidade desta tarefa tem aumentado, pois o animal não precisa ser exposto a estímulos aversivos, restrição de água ou de alimentos, não necessita de várias sessões de treino e ainda pode ser facilmente reproduzido em diversos laboratórios, utilizando ratos ou camundongos (Bevins e Besheer, 2006).

A memória de reconhecimento é conhecida como uma memória de trabalho que consiste de dois componentes distintos, o ato de recordar e a familiaridade (Ennaceur e Delacour, 1988; Eichenbaum et al., 2007). Recordar envolve uma recuperação consciente de associações e contexto, enquanto familiaridade é um senso não contextual de uma exposição anterior. As bases anatômicas que

envolvem estes processos ainda vêm sendo estudadas, mas a participação do hipocampo parece fundamental (Squire et al., 2007), pois um comprometimento na atividade ou lesão desta região cerebral prejudica o desempenho da memória na tarefa de reconhecimento de objetos (Clark et al., 2000; Hammond et al., 2004).

Desta forma, esta tarefa experimental de simples execução e que envolve aspectos de locomoção, atenção, aprendizado e memória é bastante útil para avaliação de diversas drogas que atuam sobre o sistema nervoso central e afetam, principalmente, o desempenho cognitivo.

OBJETIVOS

Os objetivos deste trabalho são:

1. Verificar se a cafeína pode melhorar o desempenho em uma tarefa de aprendizado e memória que corresponde à memória de trabalho, onde se avalia, basicamente, o instinto natural dos animais em discriminar novidades em um ambiente previamente conhecido;

2. Verificar se a administração oral de cafeína durante a fase adulta até o envelhecimento pode prevenir o declínio na memória de reconhecimento relacionada à idade;

3. Verificar se a administração de cafeína pode modificar o imunoconteúdo hipocampal do BDNF e seu receptor, TrkB;

4. Comparar o efeito da cafeína sobre o conteúdo de BDNF e TrkB com as alterações do envelhecimento.

ARTIGO I

Caffeine improves adult mice performance in the object recognition task and increases BDNF and TrkB immunocontent in the hippocampus

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Abstract

Caffeine is one of the most psychostimulants consumed in all world that usually presents positive effects on cognition. In this study, effects of caffeine on mice performance in the object recognition task were tested in different intertrial intervals. Besides, it was analyzed the effects of caffeine on brain derived neurotrophic factor (BDNF) and its TrkB receptor immunocontent to try establish a connection between the behavioral finding with one of the neurotrophins strictly involved in the memory and learning process. CF1 mice were treated during four days with saline (0.9 g %, i.p) or caffeine (10 mg/kg, i.p, equivalent dose corresponding to 2-3 cups of coffee). Caffeine treatment was interrupted 24 hours before the object recognition task analysis. In the test session performed 15 minutes after training caffeine-treated mice recognized more efficiently as the familiar as the novel object. In the test session performed 90 minutes and 24 hours after training caffeine did not changes the time spent in the familiar object but increased the object recognition index. Western blotting analysis of the hippocampus from caffeine-treated mice revealed an increase in the BDNF and TrkB immunocontent compared to their saline-matched controls. Our results suggest that acute treatment with caffeine improves recognition memory, and this effect may be related to an increase of the BDNF and TrkB immucontent in the hippocampus.

Key words: caffeine; neurotrophins; growth factors; psychostimulants; discrimination memory; recognition memory.

Caffeine is one of the most widely consumed psychoactive substances in the world with stimulant effects on the central nervous system (CNS) such as increase of vigilance and arousal (Daly and Fredholm, 1998). Its actions seem to be primarily due to antagonism at adenosine receptors (Fredholm et al., 1980). Among the four different adenosine receptors that have been cloned and pharmacologically identified as A_1, A_{2A}, A_{2B} and A_3, the first two are likely to be the primary targets of psychostimulant actions of caffeine (Fredholm, 1995). Physiological actions of adenosine are exerted by activation of adenosine A_1 and A_{2A} receptors, being considered a neuromodulator in the CNS controlling neuronal excitability and release of several neurotransmitters (for review see Fredholm et al., 2005).

Evidences from the past years have supported the cognitive enhancer properties of caffeine in a variety of behavioral tasks utilized for evaluation of the learning and memory in rodents (Angelucci et al., 1999; 2002; Castellano, 1976; Kopf et al., 1999, Paré, 1961; Roussinov and Yonkov, 1976). More recently, administration of caffeine prevented cognitive deficits and neurodegeneration in a variety of models of neurodegenerative disorders such as Alzheimer´s and Parkinson's disease (Arendash et al., 2006, Dall´Igna et al., 2003; 2007; Gevaerd et al., 2001). Epidemiological studies also confirmed that caffeine intake is inversely correlated with dementia, with more pronouncing results in elderly woman and in decreasing the risk for development of Parkinson's disease (Ascherio et al., 2001; Maia and Mendonça, 2002; Ritchie et al., 2007).

Studies in healthy subjects usually present controversial data on the positive effects of caffeine in improving cognitive functions (Jarvis, 1993; van Boxtel., 2003). Thus, a wide range of studies have investigated the variables that

contribute to the conflicting results about the cognitive enhancer properties of caffeine. Overall, caffeine presents positive effects on cognition depending on the schedule of administration, the amount of caffeine taken in by regular and non-regular users and withdrawal (Attwood et al., 2007; Childs and de Wit., 2006; Christopher et al., 2005; Haskell et al., 2005).

Since it was firstly described and further better characterized, the object recognition task has been widely used for studies on interferences in the recognition memory (Ennaceur and Delacour, 1988). The task is based on the spontaneous behavior of the animals to explore a novel object more than a familiar one. As such, this task has been widely used to assess normal physiological events as well as genetic and pharmacological interventions in the recognition memory including (Chuhan and Taukulis, 2006; de Bruin & Pouzet, 2006; Palchykova et al., 2006).

Brain neurotrophic derived factor (BDNF) is one of the neurotrophins family members essential for the neuronal growth, survival and differentiation. Neurotrophins regulate neuronal cell survival and synaptic plasticity through activation of Trk, receptor tyrosine kinase (Bramham and Messaoudi, 2005). Besides its role in the development of the CNS, BDNF signaling also participates in the activity-dependent plasticity being involved in the memory and learning process, including recognition memory (Bekinschtein et al., 2007; Heldt et al., 2007; Tyler et al., 2002).

The pioneering study showing that adenosine may influence the signaling operated by neurotrophins was performed in cultured hippocampal neurons, in which incubation with adenosine and A_{2A} receptors agonist activated Trk receptors (Lee and Chao, 2001). From this, other studies have confirmed that adenosine

modulates synaptic transmission by BDNF in rat hippocampal slices (Diógenes et al., 2004). Likewise, adenosine A_{2A} receptor knockout mice did not show BDNF-induced increase in the slope of excitatory post-synaptic field potentials (fEPSPs) compared to wild-type mice (Tebano et al., 2008). Data from our group showed that caffeine administered in the drinking water during 12 months prevented aged-cognitive decline with modification in the hippocampal BDNF and TrkB immunocontent (Costa et al., 2008).

This study was designed to investigate whether a short administration of caffeine could enhance mice performance in the object recognition task in different intertrial intervals. For it, adult mice were treated with caffeine in a dose that corresponds to 2-3 cups of coffee taken in by humans. In addition, we sought to evaluate if caffeine could modify the immunocontent of BDNF and TrkB in the hippocampus of adult mice.

2. Experimental procedures

2.1 – Animals and treatment

CF1 albino mice from our colony were used (3-4 months-old). Mice receive a single dose of caffeine (10 mg/ kg, i.p) or vehicle (saline 0.9 g%, i.p) during four consecutive days. This dose corresponds to a regular human intake of 2-3 cups of coffee (Fredholm et al., 1999; Finn and Holtzman, 1987). The last administration of caffeine was performed 45-60 minutes before the habituation session. The treatment was interrupted after the habituation period to avoid effects of acute administration of caffeine on the locomotor activity in the training session. All procedures were carried out according to NIH Guide for Care and Use of Laboratory Animals and Brazilian Society for Neuroscience and Behavior

(SBNeC) recommendations for animal care and also were approved by the ethical committee of Federal University of Rio Grande do Sul.

2.2 – Object recognition task

The object recognition task was performed according to the protocol recently reviewed (Bevins and Besheer, 2006). Briefly, adult mice were submitted to a habituation period for ten minutes 24 hours before training session. The task was performed in an apparatus consisted of a painted wood box (25 x 25 cm; 1 x w). The training session was performed 24 hours after habituation period and testing session was performed 15, 90 minutes or 24 hours after training (intertrial intervals). The duration of each session was always 10 minutes. During training session the apparatus contained two identical objects, while in the test session two dissimilar objects were present: a familiar and a novel one. The session starts when a mouse is placed in the apparatus facing the wall at the middle of the front segment. At the end of session, mouse was immediately put back in its home cage. Recognition object index was calculated by the following ratios: the time spent exploring novel object (T_N) by the time spent exploring the familiar (T_F) and the novel one in the test session (index= T_N/ T_N + T_F). Recognition was defined as directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose. Sitting on the object was not considered recognition. Different groups of mice were used for each intertrial interval.

2.3 – Western blotting analysis

After behavioral tests mice were sacrificed by cervical displacement; the whole hippocampus from both groups was dissected out immediately after the end of the test sessions performed either 90 minutes or 24 hours after training. Hippocampus were homogenized in 5 % SDS with a protease inhibitor cocktail

(Sigma, São Paulo/Brazil) and kept at – 70º C. Further analysis revealed that there was no significant difference between the immunocontent of the BDNF and TrkB from the hippocampus collected as in the test session performed 90 minutes as well 24 hours after training. Protein content was further determined by using Bicinchoninic acid assay using bovine serum albumin (BSA) as standard (Pierce, São Paulo/Brazil). Hippocampal extracts were diluted to a final protein concentration 2 µg /µl in SDS-PAGE buffer and 85 µg of the samples and dual-color prestained molecular weight standards (Bio-Rad, Porto Alegre/Brazil) were separated by SDS-PAGE (12 % with 4 % concentrating gel). After electro-transfer, the membranes were incubated overnight with Tris-buffered saline 0.1 % Tween-20 (TBS-T) containing 3 % BSA. After blocking, the membranes were incubated for 24 hours at 4º C with rabbit anti-TrkB antibody (1:1000; Upstate Cell Signalling, NY, U.S.A), mouse anti-BDNF antibody (1:500, Sigma, São Paulo/Brazil) or mouse anti-α-actin antibody (1:1000; Sigma, São Paulo/Brazil). After primary antibodies incubation, membranes were washed and incubated with alkaline phosphatase-conjugated secondary antibodies for 2 h at room temperature and developed with ECL (Amersham, São Paulo/Brazil). 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, membranes were stained with Ponceau S or mouse anti-α-actin antibody (1:1000; Sigma, São Paulo/Brazil). The results were presented by calculating the ratio of the α-actin by BDNF or TrkB density unit lines.

2.4 – Statistical analysis

Statistical analysis was performed by Student t-test (unpaired), One-Way ANOVA followed by Newman-Keuls Multiple Comparisons test. Statistical significance was considered for $P < 0.05$.

3. Results

3.1 – Analysis of the total time of exploration in both sessions

The total exploration in the training and test session was analyzed. Caffeine treatment (10 mg/kg, i.p) during four days did not affect the total time of exploration for both objects in the training as well in the test sessions performed 15 and 90 minutes after training (Fig. 1A) [$F(3,18)= 1.723$, $P = 0.1808$] and (Fig 1B) [$F(3,19)= 0.5802$, $P = 0.6311$]). However, the group of mice treated with caffeine which performed test session 24 hours after training presented an increase in the total exploration time in the training session (Fig. 1C) [$F(3,19)= 9.815$, $P < 0.0001$].

3.2 – Time spent in the familiar object

The time spent in the familiar object was evaluated in all intervals after training session. As a normal behavior, rodents usually spent less time exploring the familiar object in the test session, which implies that they recognized the object previously presented. Saline-treated mice did not discriminate the familiar object since there was no significant difference between training and test session in the time spent in the familiar object (Fig 2A). Caffeine-treated mice discriminated the familiar object since they spent less time in the familiar object in the test session performed 15 minutes after training, compared to both groups in the training session (Fig. 2A) [$F(3,18)= 4.580$, $P = 0.0085$]. For the group of mice which performed test session 90 minutes after training, both groups of mice

recognized similarly the familiar object, since both groups of mice spent less time in the familiar object in the test session (Fig. 2B) [$F(3,19)= 9.388$, $P = 0.0001$]. For the third group of mice, caffeine treatment did not cause any difference in the time spent in the familiar object compared to their saline-matched mice in the test session performed 24 hours after training. Although both groups of mice recognized the familiar object, the effect of caffeine in increasing the total time of exploration for both objects previously described was reflected here in the time spent in the familiar object in the training session (Fig. 2C) [$F(3,19)= 12.36$, $P < 0.0001$].

3.3 – Object recognition index

In general, there were no differences between the object recognition indexes for saline- and caffeine-treated mice in the training session. Caffeine treatment increased the object recognition index compared to their saline-matched mice in the test session performed 15 minutes after training (Fig. 3A) [$F(3,18)= 4.154$, $P = 0.0131$]. Saline-treated mice showed significant differences in the indexes obtained between training and test session performed 90 minutes after training (Fig. 3B). Besides, caffeine treatment increased the object recognition index in the test session when compared to their saline-matched mice (Fig. 3B) [$F(3,19)= 9.151$, $P = 0.0001$]. In addition, a similar pattern could be observed when the test session was performed 24 after training, since saline-treated mice presented differences in the indexes between training and test session (Fig. 3C). Likewise, caffeine-treated mice obtained an increase in the object recognition index when compared to their saline-matched mice in the test session (Fig. 3C) [$F(3,19)= 8.846$, $P = 0.0002$].

3.4 – Western blotting analysis

Protein immunocontent was presented as the ratio from the density unit lines obtained for BDNF or TrkB to the density unit lines obtained for α-actin. Statistical analysis of these ratio revealed that caffeine treatment caused an increase in the BDNF immunocontent around 45 % compared to control samples. Likewise, the immunocontent of TrkB was increased (65 %) in the hippocampus from caffeine-treated mice compared to their saline-matched controls (Fig. 4).

4. Discussion

In this study, effects of caffeine were evaluated in the object recognition task and in the immunocontent of BDNF and its receptor TrkB. As a general rule, the performance for the novel object recognition deteriorates as the delay between training and session increases, with better indexes obtained around 90 minutes after training (de Bruin & Pouzet, 2006; Sik et al., 2003). These observations could be noticed here since saline-treated mice which performed test session 15 minutes after training revealed did not show significant differences in the time spent in the familiar object between training and test session, suggesting that they did not recognize the familiar object. This observation has been confirmed in other studies in which animals usually present a poor performance when test session is performed at short after training (de Bruin & Pouzet, 2006; Sik et al., 2003). Interestingly, caffeine-treated mice spent significantly less time in the familiar object and the object recognition index was increased in the test session performed 15 minutes after training, whereas their saline-matched controls did not reach a sufficient index in the test session to be different from training. Therefore, caffeine treatment enhanced the performance of the animals in an intertrial interval where they naturally did not present good results,

suggesting that this methylxanthine improved the natural behavior of the animals in exploring novelties.

In all groups of mice treated with caffeine, the treatment was interrupted 24 hours before training session to avoid any effects of this methylxanthine on the locomotor activity, but one group of mice presented an increase in the total time of exploration in the training session. Caffeine is widely described to cause biphasic effects on locomotion, with low doses increasing and high doses decreasing locomotor activity (Nikodijevic et al., 1993; El Yacoubi et al., 2002), but in our study the schedule and dose of caffeine administered usually did not cause any effect on the locomotor activity of the animals. Therefore, this particular group of mice could have presented naturally a high exploratory activity. Nevertheless, this phenomenon did not mismatch further analysis of the behavior of the animals in the task. Even though this group of mice treated with caffeine had spent more time exploring the objects, analyzing the time of exploration in the training session and time spent in the familiar object in the test session, it could be noticed that half of time was equally spent in each object. In the test session performed 24 hours after training both groups spent less time in the familiar object, indicating that both groups recognized similarly the familiar object. It is unlikely that caffeine had triggered an anxiety-like behaviour because the treatment differs from other studies where caffeine clearly provokes anxiety (El-Yacoubi et al., 2000). In addition, anxious animals treated with anxiogenic doses of caffeine presented an unsatisfactory performance in learning and memory task (Silva and Frussa-Filho, 2000).

In the test session performed 90 minutes and 24 hours after training saline-treated mice spent less time in the familiar object and showed an increase

in the object recognition index. Our results are in agreement with other studies in which the best performance in this task is achieved when animals performed test session 90 minutes after training, but with a slight decrease when it is performed 24 hours after training (de Bruin & Pouzet, 2006; Sik et al., 2003). Caffeine-treated mice did not show differences in the time spent in the familiar object when compared to their saline-matched control, which means that both groups of mice efficiently recognized the familiar object. However, caffeine treatment increased the object recognition index compared to their saline-matched controls, reinforcing its positive effects on short- and long- term memory in discriminating novelties.

Some studies have inquired whether the positive effects of caffeine on cognition could be due to reversal of withdrawal rather than its direct effects. However, other reports have pointed to its direct positive effects in increasing cognitive performance in habitual and non-habitual consumers (Haskel et al., 2005; Childs and de Wit 2006). Recent findings from our group showed that caffeine administered in adult mice up to 18 months-old prevented cognitive decline in the object recognition memory (Costa et al., 2008). In this study, caffeine administered during four days with an interruption of the treatment for 24 or 48 hours in adult mice before test session improved their performance in the same task. In our study, caffeine confirmed its cognitive enhancer properties even though its administration had been interrupted before behavioral tests.

Although our results can not infer on the effects of caffeine in the functionality of the signaling operated by BDNF, caffeine-treated mice achieved better recognition memory performance with a concomitant increase in the hippocampal BDNF and TrkB immunocontent than their saline-matched controls. Learning activity was able to modify BDNF levels (Chen et al., 2007), but it

remains still unknown if learning activity in the object recognition task *per se* could modify the immunocontent of these proteins. Up to the present moment, BDNF seems to be essential in this task, because mice lacking BDNF gene presented impairment in the object recognition task (Heldt et al., 2007).

The main target for the psychostimulant effects of caffeine is the antagonism of adenosine A_1 and A_{2A} receptors. Some evidences have pointed to the role of adenosine A_{2A} receptors in the effects of caffeine, inkling its classical effects in maintaining arousal (Huang et al., 2005). Up to the present moment, antagonists of adenosine A_{2A} receptors often present the same positive effects on learning and memory (Dall´Igna et al., 2007; Prediger et al., 2005). As a neuromodulator adenosine controls the neurotransmission operated by a wide range of neurotransmitters including the signaling operated by BDNF. In fact, adenosine via A_{2A} receptors modulates the BDNF-mediated facilitatory effect on the synaptic transmission (Diógenes et al., 2004) and both adenosine A_{2A} and TrkB receptors are co-immunoprecipitated (Lee and Chao, 2001). Since caffeine is a non-selective antagonist of adenosine receptors, in this study we did not seek to characterize pharmacologically what subtype of adenosine receptors could be involved in the behavioral and BDNF and TrkB immunocontent findings. Our work was focused mainly on the positive effects of caffeine as a usual diet component in the recognition memory using a task that basically explores a natural motivation of the animals to discriminate novelties with alterations in one of the neurotrophins specially involved in learning and memory processes. The increase in the BDNF levels and an up-regulation of its receptors was reported in animals that achieve better performances in learning and memory tasks by some stimulus such as exercise (Berchtold et al., 2005; Ogonovszky et al., 2005; Radak et al., 2006). On

the other hand, rats exposed to stressful stimulus presented a decrease in BDNF levels and expression with impairment in learning and memory (Marmigère et al., 2003).

Our study reports a connection between cognitive enhancer properties of a short administration of caffeine and withdrawal with a concomitant increase in the in the immunocontent of BDNF and its receptor TrkB. Considering that caffeine is a usual diet component and one of the most consumed cognitive enhancers, it is important to identify if its administration could promote changes in proteins involved in the maintenance of normal cognitive functions such as learning and memory.

Acknowledgments

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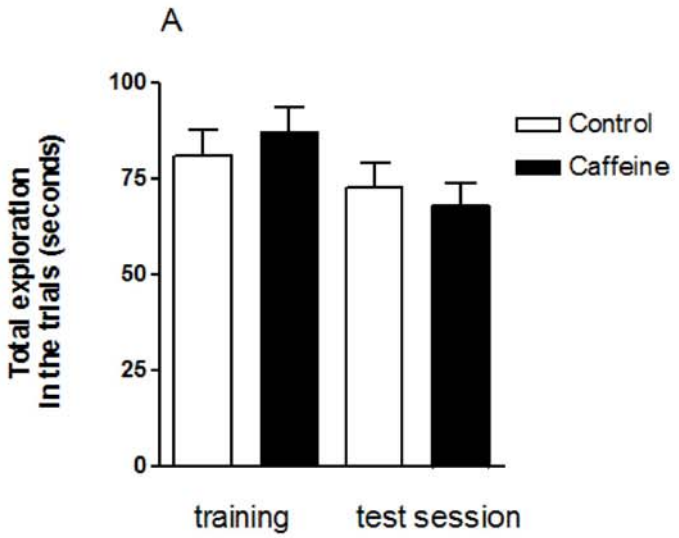
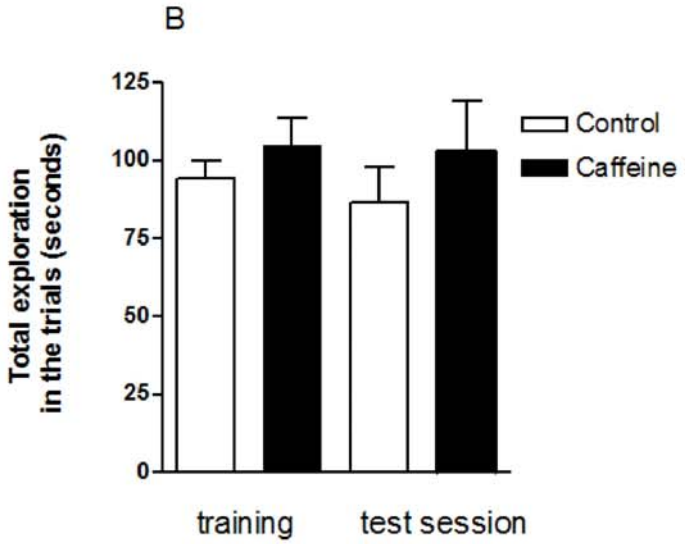

A
Total exploration in the trials (seconds)
100
75
50
25
0
Control
Caffeine
training
test session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125
100
75
50
25
0
Control
Caffeine
training
test session

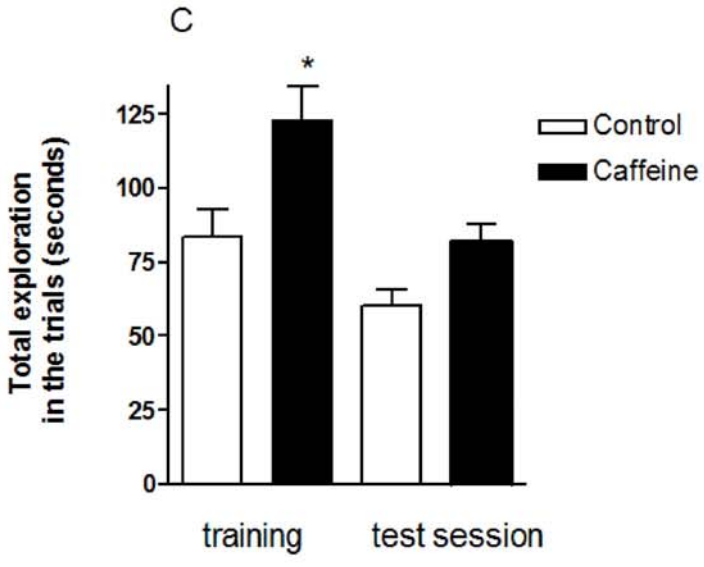
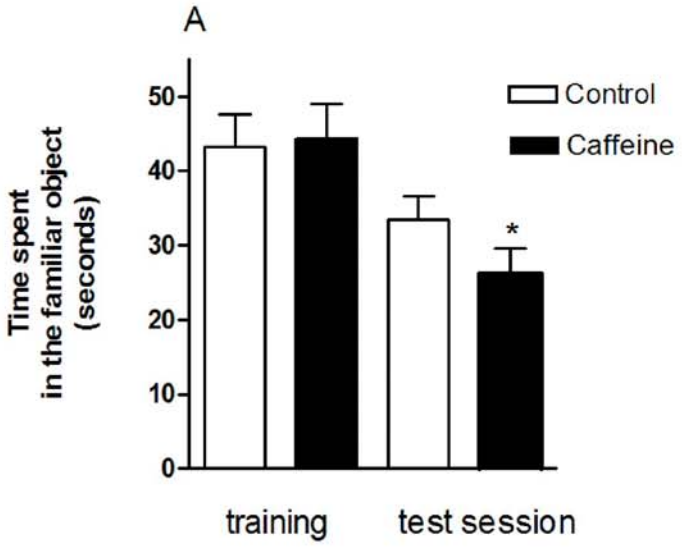
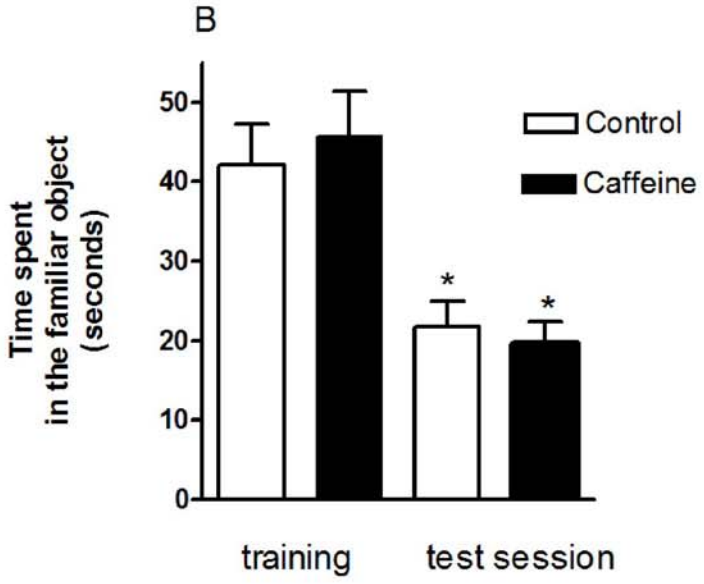

C
*
Total exploration in the trials (seconds)
125
100
75
50
25
0
Control
Caffeine
training
test session

Fig. 1 – Total exploration in both objects in the training and test session in different intertrial intervals.

Test session performed 15 minutes (A), 90 minutes (B) or 24 hours after training session (C). Habituation period was performed 24 hours before training session. Results are means ± S.E.M of the seconds spent in both objects in the training and test session performed in different intertrial intervals. Different number of animals was used for each intertrial intervals: 9-10 (A); 12 (B) and 10 animals for each group (C).

* $P < 0.01$, indicates significant different from all groups in the graphic C.


A
Time spent in the familiar object (seconds)
50
40
30
20
10
0
Control
Caffeine
*
training
test session


B
Time spent in the familiar object (seconds)
50
40
30
20
10
0
Control
Caffeine
*
*
training
test session

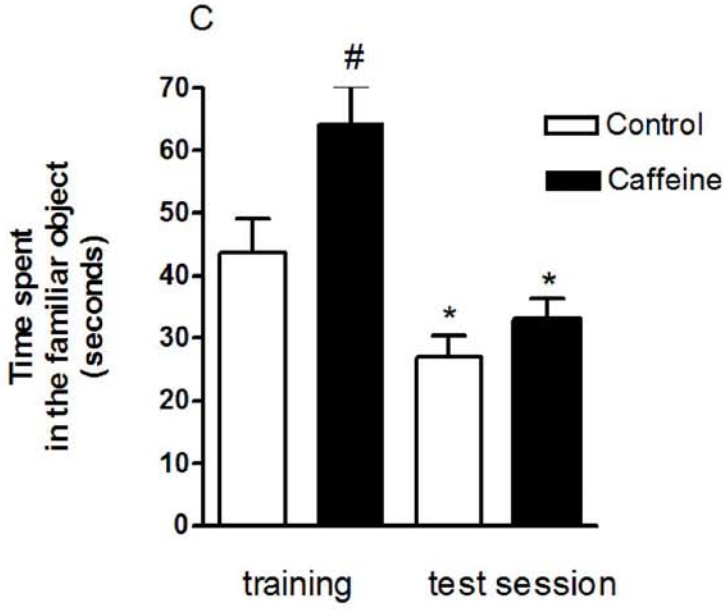
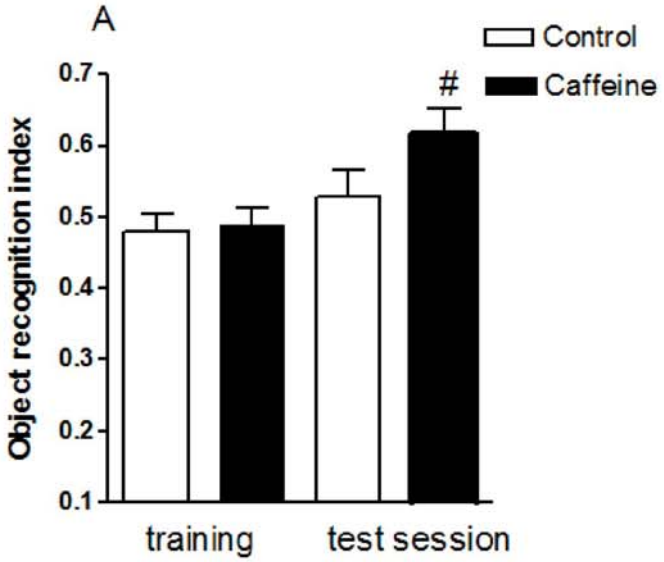
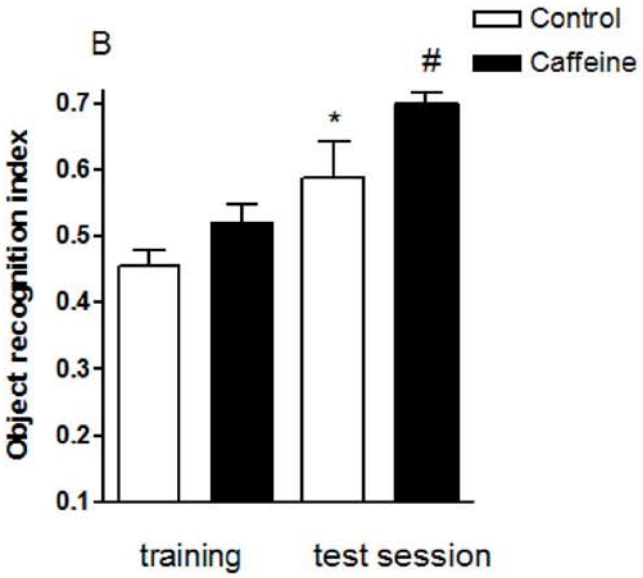

C
Time spent in the familiar object (seconds)
#
70
60
50
40
30
20
10
0
Control
Caffeine
*
*
training
test session

Fig. 2 – The time spent in the familiar object in the test session performed in different intertrial intervals. Test session performed 15 minutes (A), 90 minutes (B) or 24 hours after training session (C). Results are means ± S.E.M of the seconds spent in the object in the training and test session. Different number of animals was used for each intertrial intervals: 9-10 (A); 12 (B) and 10 animals for each group (C).

* P< 0.01, indicates difference between groups when compared to training session (A,B and C).

# P < 0.01, indicates difference between all groups as in the training as in the test session. (One-way ANOVA followed by Newman Keuls test).


A
Control
Caffeine
#
Object recognition index
0.7
0.6
0.5
0.4
0.3
0.2
0.1
training
test session


B
Control
Caffeine
#
*
Object recognition index
0.7
0.6
0.5
0.4
0.3
0.2
0.1
training
test session

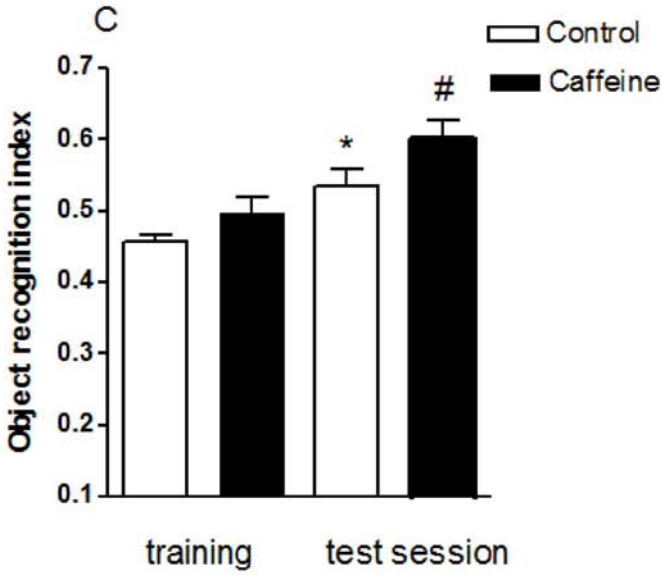

C
Control
Caffeine
#
*
Object recognition index
0.7
0.6
0.5
0.4
0.3
0.2
0.1
training
test session

Fig. 3 – The novel object recognition index obtained by control and caffeine-treated mice in different intertrial intervals. Test session performed 15 minutes (A), 90 minutes (B) or 24 hours after training (C). Results are means ± S.E.M of recognition index obtained by the following ratio: index= (T_N/ T_N + T_F), T_N = time spent exploring novel object at each intertrial interval; T_F = time spent exploring the familiar object at each intertrial interval. Different number of animals was used for each intertrial intervals: 9-10 (A); 12 (B) and 10 animals for each group (C).

* P <0.05, difference between both groups in the training session;

# $P < 0.05$, difference between all groups in the training and test session;

(One-way ANOVA followed by Newman Keulls test).

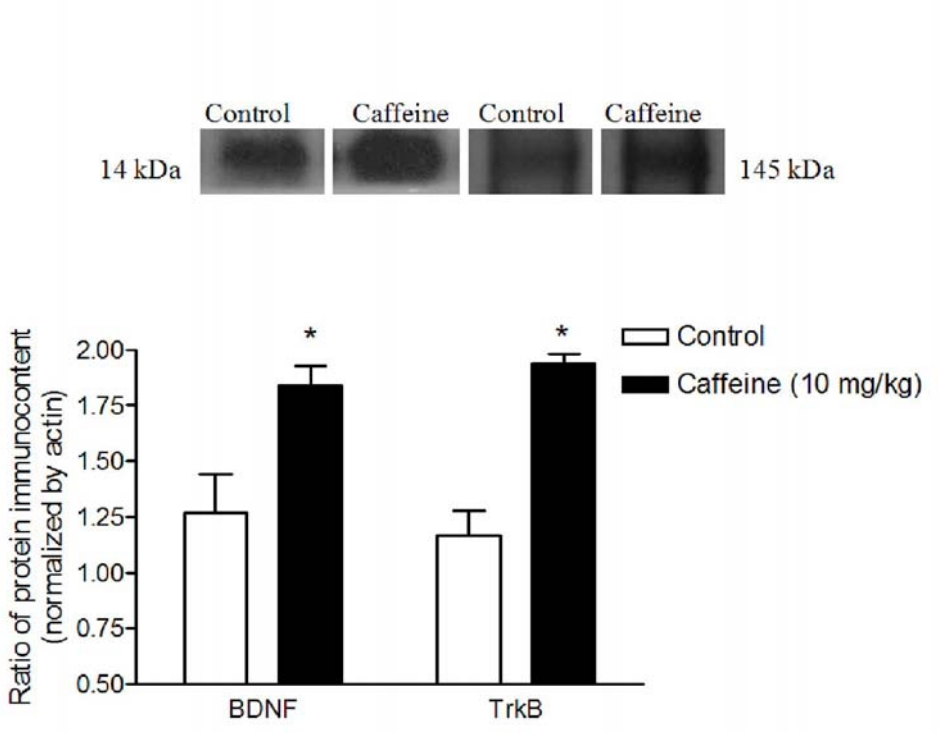


Fig. 4 – Representative and quantitative western blotting analysis of the hippocampal BDNF and TrkB immunocontent from saline- and caffeine-treated mice. At the top of the figure representative bands: BDNF at 14 kDa; TrkB at 145 kDa. Graphic represents means ± SEM of the ratio of optical densities of each band by α-actin quantified in the autoradiographic film of 10 different hippocampal extracts from both groups of mice that performed test session 90 minutes and 24 hours after training. Hippocampal extracts were obtained immediately after test sessions performed 90 minutes or 24 hours.

* $P < 0.05$, difference between white and black bars (Student t- test unpaired).

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ARTIGO II

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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 (BNDF) 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.

Key words: caffeine, aging, cognition, memory, neurotrophins, adenosine.

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 (A_1, A_{2A}, A_{2B} and A_3), subtypes A_1 and A_{2A} are widely expressed in the CNS. Adenosine A_1 receptors are widely distributed in throughout brain while A_{2A} 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 A_1 and A_{2A} 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-

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Abbreviations: BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; NMDA, N-methyl-D-aspartate; TrkB, tirosine kinase receptor.

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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 widely expressed in the hippocampus (Hofer 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 tirosine kinase receptor (TrkB) receptors in the hippocampal neurons and both A_{2A} and TrkB receptors were co-immunoprecipitated (Lee and Chao, 2001). Besides, adenosine A_{2A} 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_{2A} 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, Pounceau 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 Science Research (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

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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 $T_F/(T_N+T_F)$, [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 no 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 $P<0.05$.

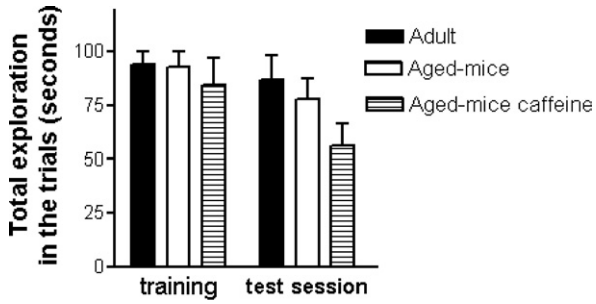

Fig. 1. Total time recorded for the exploration of the objects in the training and test session. Graphic shows the time spent (in seconds) for adult, age-matched control mice and mice treated during 12 months with caffeine (1 mg/mL, in drinking water). Results are presented as means±S.E.M. of the seconds spent in both objects during 10 min of observation as in the training as in the test session (n=8–9 animals in each group). No statistical differences were found for all groups of animals.

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 (Bevins 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) [$F(2,25)=4.178$, $P=0.0290$].

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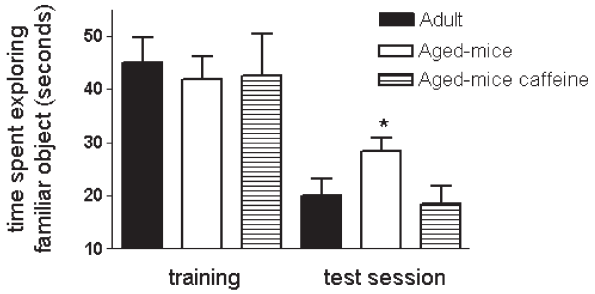

Fig. 2. Analysis of the time spent in the familiar object for all groups of mice during 10 min each session. Graphic shows the time spent (in seconds) in exploring the familiar object for each group of mice: adult mice (6 months old); age-matched control mice (18 months old); and aged-mice treated during 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 (Angelucci et al., 2002; Castellano, 1976; Prediger et al., 2005a,b). Although our results agree with other studies

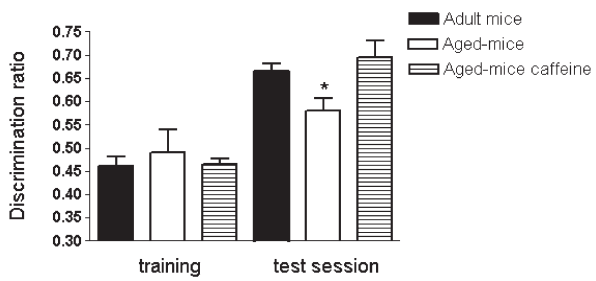

Fig. 3. Performance of all groups of mice for the novel object recognition memory. Graphic shows the discrimination ratio for the objects in the training and test session for the following groups of mice: adult mice (6 months old); age-matched control mice (18 months old); and aged-mice treated during 12 months with caffeine (1 mg/mL, in the drinking water). Results are presented as means±S.E.M. of the discrimination ratio in the training session and in the test session performed 90 min later (n=8–9 animals for each group). * P<0.05, different from the discrimination ratio for adult mice and aged-mice treated with caffeine in the test session.

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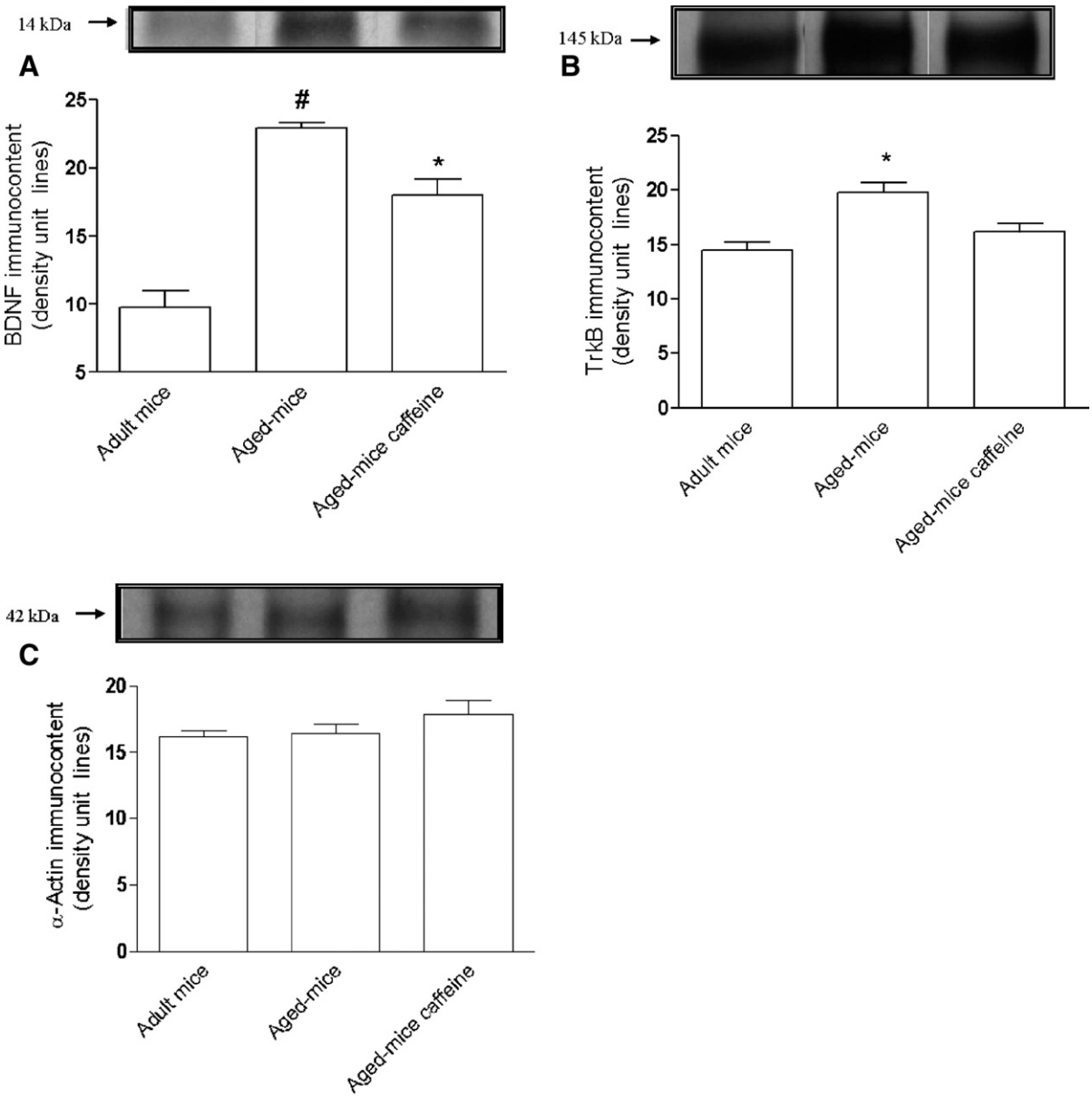

Fig. 4. Western blotting analysis for BDNF, TrkB and α-actin immunocontent in extracts from the whole hippocampus of all groups of mice. On the top of the each graphic are illustrated the bands of the equivalent molecular weights: (A) 14 kDa for BDNF; (B) 145 kDa for TrkB; (C) 42 kDa for α-actin, with their respective quantifications for the density of the bands from the scanned autoradiographic films. Results are means±S.E.M. of eight extracts of the whole hippocampus for each group of mice. * $P<0.05$, denotes a significant difference from all groups. # $P<0.05$, denotes a significant difference from adult mice.

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-

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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 A_{2A} 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 A_{2A} receptors (Rebola et al., 2008). Although the blockade of A_1 and A_{2A} adenosine receptors was first attributed to the psychostimulant actions 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 A_{2A} 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 A_{2A} antagonists, but not by adenosine A_1 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 expression either decreased or unchanged with aging (Hattiangady et al., 2005; Kaisho et al., 1994; Lapchak et al., 1993). 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 15 to 18-month-old rats presented an increase on BDNF and TrkB levels compared with 6-month-old animals (Segovia et al., 2007; Silhol et al., 2007), it is important to take into account that different rat and mice strains 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 A_{2A} 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 A_{2A} 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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DISCUSSÃO

Neste trabalho, a cafeína administrada por via oral, dissolvida na água de beber dos camundongos, desde a fase adulta até a idade de dezoito meses preveniu o declínio da memória de reconhecimento associado à idade. O efeito só foi observado na memória de curta-duração, quando o teste foi realizado noventa minutos após a sessão de treino. Apesar de muitos estudos avaliarem a memória de longa duração nesta tarefa, a escolha pela memória de curta duração deve-se ao fato de que um maior tempo entre o treino e o teste deteriora o desempenho de reconhecimento do objeto novo, sendo os melhores resultados encontrados entre sessenta a noventa minutos após o treino (de Bruin e Pouzet, 2006; Sik et al., 2003).

Dadas às limitações de se estabelecer uma correlação entre a idade dos roedores com a idade dos seres humanos (Coleman, 2004), foi importante para o nosso trabalho verificar que os camundongos idosos usados como controle mostraram um declínio na memória de reconhecimento. Já os camundongos idosos tratados com cafeína apresentaram um desempenho na tarefa de reconhecimento do objeto novo semelhante ao dos camundongos adultos. Este é o primeiro trabalho a descrever os efeitos da administração de cafeína desde a fase adulta até o envelhecimento na memória de reconhecimento e, diferentemente de outros estudos (Angelucci et al., 2002; Castellano, 1076; Prediger et al., 2005a.; 2005b), esta tarefa avalia a memória de trabalho sem a necessidade de um componente de reforço ou aversivo.

A abordagem experimental para estudar o aprendizado e memória e, especialmente a administração de cafeína diferem de outros estudos, pois se

sabe que conforme a dose e a administração (aguda ou crônica) a cafeína provoca ansiedade nos animais (El Yacoubi et al., 2000a; Jain et at., 2005), sendo relatado que animais que ficam ansiosos com a administração de cafeína apresentam um menor desempenho em tarefas de memória e aprendizagem (Silva e Frussa-Filho, 2000). Se os efeitos observados neste estudo sobre a tarefa de reconhecimento de objetos fossem relacionados a efeitos desencadeadores de ansiedade pela cafeína, esperava-se que os animais tivessem um aumento na locomoção e um menor desempenho no reconhecimento do objeto novo. Porém, este efeito não foi observado porque não foi encontrada uma diferença entre os grupos estudados no tempo de exploração total dos objetos, que pode ser utilizado como um índice de locomoção dos animais descartando, desta forma, uma possível interferência da locomoção ou ansiedade mediada pela cafeína sobre o reconhecimento do objeto novo.

No estudo agudo realizado em camundongos machos adultos, os quais receberam durante quatro dias consecutivos cafeína (10 mg/ kg i.p.), foi verificada uma melhora na memória de curta e longa duração na tarefa de reconhecimento de objetos. De fato, os camundongos adultos tratados com cafeína apresentaram um melhor desempenho na memória de reconhecimento inclusive na sessão de teste onde os animais normalmente apresentam um desempenho menos satisfatório (quinze minutos após o treino).

A administração de cafeína foi interrompida 24 horas antes do início do teste para evitar uma interferência da maior ansiedade e atividade locomotora que podem ser desencadeados com o tratamento com cafeína. A interrupção do tratamento antes da exposição à tarefa de reconhecimento de objeto evita algum efeito sobre a atividade locomotora dos animais que poderia ser incompatível

com a interpretação dos resultados na tarefa de reconhecimento de objetos (Bevins e Besheer, 2006). Além disso, o aumento na atividade locomotora causado pela cafeína também não poderia explicar os melhores índices de reconhecimento neste estudo, pois os animais tratados com cafeína não apresentaram diferenças no tempo total de exploração dos objetos. O aumento da atividade motora tem sido verificado quando metilxantinas são administradas agudamente, mas em uma dose maior do que a utilizada neste trabalho (El Yacoubi et al., 2000b; Svenningsson et al., 1995).

O imunoconteúdo de BDNF hipocampal nos camundongos idosos controle apresentou um valor maior do que o encontrado nos camundongos adultos. O receptor TrkB apresentou o mesmo aumento, porém em uma extensão menor. Estes resultados diferem de alguns estudos anteriores que mostram uma diminuição ou nenhuma alteração na expressão de BDNF e TrkB com o envelhecimento (Hattiangady et al., 2005; Kaisho et al., 1994; Lapchak et al., 1993). Contudo, na maioria dos casos, foram analisados os níveis de RNA mensageiro e, desta forma, não pode ser descartada uma modificação pós-transdução. Entretanto, esses dados estão de acordo com estudos recentes onde foi observado um aumento dos níveis de BDNF e TrkB em ratos de 15 a 18 meses de idade quando comparados com ratos de 6 meses de idade (Segovia et al., 2007; Silhol et al., 2007). É importante considerar que diferenças entre espécies (ratos e camundongos) podem ser responsáveis por estas discrepâncias relatadas pelas modificações no BDNF e seu receptor TrkB com o envelhecimento. Adicionalmente, estas discrepâncias podem ser estendidas a sinalização operada pelo BDNF, pois recentemente encontrou-se uma diminuição no imunoconteúdo de TrkB apesar do BDNF ter aumentado a transmissão

sináptica em fatias de hipocampo de ratos idosos (Diógenes et al., 2007). Assim, a complexidade da via de transdução de sinal operada pelo BDNF dificulta estabelecer um conceito entre a funcionalidade e o conteúdo do receptor TrkB (Huang e Reichardt, 2003).

Tem sido demonstrado que a atividade de aprendizagem podem modificar o conteúdo de BDNF e TrkB em tarefas que requerem exposição a estímulos aversivos (Croll et al., 1998; Silhol et al., 2007), mas não foi verificado neste estudo se a tarefa de reconhecimento de objeto por si só poderia modificar o imunoconteúdo destas proteínas. Entretanto, a administração de cafeína desde a fase adulta preveniu parcialmente o aumento no imunoconteúdo de BDNF que está relacionado com a idade, enquanto o imunoconteúdo de TrkB permaneceu similar ao de camundongos adultos controle. O efeito benéfico da administração de cafeína desde a fase adulta descrita neste trabalho pode estar relacionado à prevenção do aumento do imunoconteúdo de BDNF e TrkB associado à idade.

Diferentemente do tratamento crônico com cafeína nos animais que foram analisados aos dezoito meses, o imunoconteúdo hipocampal do BDNF e TrkB estão aumentados nos animais de seis meses tratados agudamente com a maior dose de cafeína. Apesar deste trabalho também não verificar o efeito da cafeína sobre a sinalização do BDNF, a melhora observada pela administração aguda de cafeína no desempenho da tarefa de reconhecimento de objetos foi relacionada a um aumento no imunoconteúdo do BDNF e de seu receptor TrkB. Portanto, nossos dados demonstram que o imunoconteúdo do BDNF e TrkB é modificado conforme a dose, o tempo de administração e a idade dos animais.

Ainda é desconhecido se a tarefa de reconhecimento de objeto poderia, por si só modificar o imunoconteúdo destas proteínas, apesar de outras tarefas que

avaliam aprendizado e memória dos animais serem capazes de modificar os níveis de BDNF (Chen et al., 2007). É importante ressaltar que a sinalização operada pelo BDNF é essencial para os processos de aprendizado e memória (Bekinschtein et al., 2007; Rossato et al., 2007), inclusive a memória de reconhecimento, pois camundongos que não expressam o gene desta proteína apresentaram prejuízo na tarefa de reconhecimento de objeto (Heldt et al., 2007).

Embora nosso trabalho não teve como objetivo fazer uma caracterização farmacológica de qual dos subtipos de receptor de adenosina (A_1 ou A_{2A}) estarem envolvidos na prevenção do declínio cognitivo decorrente da idade, e, a melhora no desempenho da tarefa de reconhecimento de objetos em camundongos adultos pela cafeína, muitos estudos sugerem que o efeito preventivo dessa substância sobre memória e aprendizagem é coincidente com a administração de antagonista seletivo do receptor A_{2A}, mas não para antagonista do receptor A_1 (Cunha et al., 2008; Dall'Igna et al., 2007; Kopf et al., 1999; Prediger et al., 2005a). Portanto, apesar da cafeína ser considerada um antagonista não seletivo dos receptores de adenosina, e seus efeitos psicoestimulantes serem firmemente atribuídos ao antagonismo dos receptores A_1 e A_{2A}, parece que o seu efeito excitatório e neuroprotetor pode ser atribuído ao bloqueio preferencial do receptor A_{2A} (Dall'Igna et al., 2003; Higgins et al., 2007; Huang et al., 2005; Silva et al., 2007).

Várias evidências sugerem que a adenosina participa da transmissão sináptica pelo BDNF por meio de sua sinalização em receptores do subtipo A_{2A} tanto em ratos adultos como idosos (Diógenes et al., 2004; 2007). Além disso, os receptores TrkB e A_{2A} são co-imunoprecipitados e a ativação do receptor A_{2A} promove a sinalização para a fosforilação do receptor TrkB (Liu e Chao, 2001).

Considerando que alguns dos efeitos benéficos observados pela cafeína estão relacionados ao bloqueio preferencial do receptor de adenosina A_{2A}, a qual a sinalização pelo BDNF também parece participar, ainda é necessário determinar se a cafeína poderia afetar alguma via de transdução de sinal operada pelo BDNF, sendo que a cafeína parece modificar algumas proteínas envolvidas na cascata sinalizada pelo BDNF (Sahin et al., 2007).

Apesar disso, ainda não tinha sido realizado nenhum estudo *in vivo* com a abordagem de explorar os efeitos da manipulação farmacológica dos receptores de adenosina sobre possíveis alterações moleculares no BDNF e no seu receptor TrkB.

Assim sendo, este trabalho é o primeiro que mostra uma relação entre os efeitos positivos da cafeína sobre a memória de reconhecimento com as alterações no imunoconteúdo do BDNF e seu receptor, TrkB.

CONCLUSÕES

O trabalho realizado demonstra que o consumo diário de cafeína durante a vida adulta até o envelhecimento previne o declínio cognitivo na memória de reconhecimento associada à idade em camundongos. Este efeito benéfico sobre a função cognitiva pode estar associado à prevenção do aumento do BDNF e do seu receptor (TrkB) que ocorre com o avanço da idade.

Este é o primeiro trabalho a mostrar uma relação entre os efeitos positivos de uma longa administração de cafeína sobre a memória de reconhecimento, uma tarefa que explora o comportamento natural do animal em discriminar novidades em um ambiente previamente conhecido, sem a necessidade de utilizar componentes de reforço ou aversivos, com as mudanças no imunoconteúdo do BDNF e seu receptor, TrkB. Além disso, é o primeiro estudo onde a manipulação farmacológica dos receptores de adenosina *in vivo* esteve implicada em modificações dessas proteínas. Os estudos realizados até o presente momento utilizaram técnicas de eletrofisiologia para estabelecer o papel da adenosina na transmissão sináptica pelo BDNF.

Os resultados da administração aguda de cafeína em camundongos adultos sugerem que a melhora da memória de reconhecimento pode ser observada por uma administração aguda dessa substância, mesmo quando o tempo entre a sessão de teste e treino não favorece um melhor desempenho. Interessantemente, os efeitos agudos da cafeína sobre o imunoconteúdo do BDNF e TrkB hipocampais são distintos da administração ao longo da vida, pois a cafeína aumentou o imunoconteúdo de ambas proteínas. Portanto, os efeitos agudos e crônicos da administração de cafeína podem não afetar distintamente a

memória de reconhecimento, mas parecem modular de forma diferente a densidade de um dos fatores tróficos mais importantes para persistência da memória. É importante ressaltar que nossos resultados focaram apenas os efeitos da cafeína sobre uma função importante do sistema nervoso central bem como sobre proteínas altamente expressas em muitas regiões cerebrais. Estudos de uma administração crônica da cafeína sobre parâmetros do sistema periférico também se fazem necessários, e não podemos descartar algum possível efeito negativo de uma administração tão longa dessa substância nesse sistema.

Por ser um componente usual da dieta de muitas populações em todo o mundo, se torna importante estudar os efeitos da cafeína sobre a função cognitiva e as modificações de proteínas envolvidas no processo da aprendizagem e memória. Para países em desenvolvimento, tais como o Brasil, que é um dos grandes produtores e consumidores de cafeína é importante conhecer o benefício do consumo usual deste componente dietético de baixo custo para a melhora da qualidade de vida de pessoas idosas.

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