Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Departamento de Bioquímica

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica

TESE DE DOUTORADO

O IMPACTO DA ADMINISTRAÇÃO DE CAFEÍNA SOBRE O COMPORTAMENTO E PROTEÍNAS SINÁPTICAS EM DIFERENTES FASES DO DESENVOLVIMENTO ENCEFÁLICO DE RATOS

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Doutorando

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"O homem lúcido sabe que a vida é uma carga tamanha de acontecimentos e emoções que nunca se entusiasma com ela, assim como não teme a morte. O homem lúcido sabe que viver e morrer são o mesmo em matéria de valor, posto que a Vida contém tantos sofrimentos que a sua cessação não pode ser considerada um mal.

O homem lúcido sabe que é o equilibrista na corda bamba da existência. Sabe que, por opção ou acidente, é possível cair no abismo, a qualquer momento, interrompendo a sessão do circo.

Pode também o homem lúcido optar pela Vida. Aí então, ele esgotará todas as suas possibilidades. Passeará por seu campo aberto e por suas vielas floridas. Saberá ver a beleza em tudo. Terá amantes, amigos, ideais. Urdirá planos e os realizará. Resistirá aos infortúnios e até às doenças. E, se atingido por algum desses emissários, saberá suportá-los com coragem e mansidão.

Morrerá o homem lúcido de causas naturais e em idade avançada, cercado por filhos e netos que seguirão sua magnífica aventura. Pairará então, sobre sua memória uma aura de bondade. Dir-se-á: aquele amou muito e fez bem às pessoas.

A justa lei máxima da natureza obriga que a quantidade de acontecimentos maus na vida de um homem iguale-se sempre à quantidade de acontecimentos favoráveis. O homem lúcido que optou pela Vida, com o consentimento dos Deuses, tem o poder magno de alterar esta lei. Na sua vida, os acontecimentos favoráveis estarão sempre em maioria.

Esta é uma cortesia que a Natureza faz com os homens lúcidos."

Texto Caldáico século VI a.C.

Dedico esta tese a eles, que incondicionalmente sempre estiveram ao meu lado. Pai e mãe, obrigada pelo apoio, compreensão e tolerância. Obrigada pela vida.

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MUITO OBRIGADA!

APRESENTAÇÃO

Conforme as normas do programa de Pós-Graduação em Ciências Biológicas-Bioquímica, esta tese de doutorado está organizada em 3 partes e os resultados estão apresentando sob a forma de artigos científicos:

- **PARTE I** contém os Resumos, a Lista de Abreviaturas, a Introdução e os Objetivos do trabalho;
- **PARTE II** composta pelos Capítulos 1 e 2 que contém os artigos científicos;
- **PARTE III** contém os tópicos: Discussão, Conclusões, Perspectivas, Referências Bibliográficas e Anexos.

A Introdução apresenta uma revisão bibliográfica geral dos conteúdos científicos necessários para definir a proposta da tese, compreender e esclarecer seus objetivos.

Os resultados são apresentados sob a forma de artigos científicos e encontram-se nos capítulos 1 e 2 da parte II do corpo da tese. Cada artigo científico apresenta suas respectivas seções de Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas. Assim, detalhes técnicos sobre a metodologia empregada podem ser encontrados nos artigos científicos correspondentes.

Sob forma de interpretações, análises e comentários, os tópicos Discussão e Conclusões englobam aos artigos científicos contidos na tese. As referências bibliográficas referem-se unicamente as citações feitas nos tópicos Introdução e Discussão desta tese.

SUMÁRIO

PARTE I

RESUMO

A cafeína é a substância psicoestimulante mais consumida no mundo. Em doses moderadas, ela proporciona efeitos benéficos sobre as funções cognitivas na vida adulta e no decorrer do envelhecimento. No entanto, a ingestão crescente de bebidas contendo cafeína por adolescentes tem causado preocupação, pois os efeitos desta substância sobre as funções cognitivas e a maturação do encéfalo durante a adolescência são pouco conhecidos. A cafeína atravessa a placenta e a barreira hemato-encefálica e o seu consumo tem sido associado ao maior risco de aborto espontâneo e baixo peso ao nascer. Portanto, nos estágios iniciais do desenvolvimento encefálico o consumo de cafeína também carece de maiores eslcarecimentos. Nesta tese, o impacto do consumo de cafeína durante diferentes fases de desenvolvimento do encéfalo foi investigado sobre o comportamento e proteínas sinápticas em ratos. No primeiro capítulo, ratos adolescentes machos consumiram cafeína na água de beber nas doses de 0,1; 0,3 e 1,0 g/L (correspondendo ao consumo baixo, moderado e elevado, respectivamente) somente durante o seu período ativo (das 19 às 7 horas). Nenhuma das doses testadas teve efeito sobre a atividade locomotora, porém todas desencadearam efeitos ansiogênicos. A cafeína (0,3 e 1,0 g/L) melhorou o desempenho na tarefa de reconhecimento ao objeto, enquanto na dose mais elevada (1,0 g/L) os animais não habituaram ao campo aberto, uma forma de avaliar o aprendizado não-associativo. Todas as doses testadas reduziram a densidade de proteína glial fibrilar ácida (GFAP) e proteína associada ao sinaptossoma (SNAP-25) sem causar alterações na imunorreatividade da proteína nuclear específica para neurônios (NeuN) no hipocampo e no córtex cerebral. No hipocampo, a cafeína (em todas as doses testadas) aumentou a densidade de receptor de adenosina A_1 e reduziu a do factor neurotrófico derivado do encéfalo (BDNF) e sua forma precursora (proBDNF) (1,0 g/L). No córtex cerebral, a cafeína (1,0 g/L) reduziu a densidade do receptor A_1 e aumentou a do BDNF e do proBDNF (0,3 e 1,0 g/L). Estes resultados revelam que o consumo de cafeína por ratos adolescentes exacerba a ansiedade, mas provoca diferentes efeitos sobre a memória, melhorando a de reconhecimento e prejudicando o aprendizado não associativo. Parte destes efeitos foi associada às mudanças nos níveis de BDNF, GFAP e SNAP-25, porém sem perda da viabilidade neuronal aparente no hipocampo e no córtex cerebral. No segundo capítulo, o impacto do consumo de cafeína (0,1; 0,3 e 1,0 g/L na água de beber, das 19 às 7 horas) foi investigado sobre o comportamento e proteínas sinápticas na vida adulta dos animais que consumiram cafeína no decorrer do desenvolvimento encefálico. O consumo de três diferentes doses de cafeína iniciou 15 dias antes do acasalamento e permaneceu durante a prenhez e lactação. A partir do desmame os animais foram divididos em dois grupos: os que consumiram cafeína até a vida adulta (ao longo da vida) e os que interromperam o consumo (desenvolvimento). Esses dois grupos também foram subdivididos e analisados de acordo com o sexo. Foram comparados os efeitos destes protocolos sobre o comportamento e a densidade de proteínas sinápticas do hipocampo e córtex de fêmeas e machos adultos. A memória de reconhecimento foi prejudicada nas fêmeas que receberam cafeína (0,3 e 1,0 g/L) durante o desenvolvimento, o que coincidiu com o aumento do proBDNF e níveis inalterados de BDNF no hipocampo. Ambos os protocolos de exposição causaram hiperlocomoção nos machos, enquanto que nas fêmeas somente a exposição ao longo da vida aumentou a atividade locomotora de forma significativa. Já no comportamento relacionado à ansiedade, ambos os sexos apresentaram um perfil ansiolítico ao

consumir cafeína (1,0 g/L) ao longo da vida. Ambos os regimes de administração diminuíram os níveis de GFAP e SNAP-25 no hipocampo dos ratos machos. A densidade do receptor de TrkB foi reduzida no hipocampo em ambos os sexos e protocolos de exposição. No córtex cerebral BDNF e proBDNF aumentaram com o consumo de cafeína ao longo da vida nos machos. Nas fêmeas houve aumento no BDNF, mas não no proBDNF, em ambos regimes de administração. O receptor TrkB diminuiu no córtex dos ratos machos que receberam cafeína somente durante o desenvolvimento. Ambas proteínas – GFAP e SNAP-25 – aumentaram suas densidades nos machos que receberam ambos regimes de administração. Estes resultados revelaram que o consumo de cafeína ao longo da vida pode recuperar o prejuízo na memória de reconhecimento das fêmeas que consumiram a substância durante o desenvolvimento e indicam que a exposição durante um período específico do desenvolvimento do encéfalo promove alterações comportamentais dependentes do sexo, as quais nós relacionamos com modificações na sinalização BDNF. Os resultados desta tese destacam a importância de controlar o consumo de cafeína em períodos críticos para o desenvolvimento encefálico de ratos, e aponta para um efeito dependente do sexo. No entanto, mais estudos são necessários para ampliar nosso conhecimento sobre as possíveis vias de sinalização envolvidas nestes processos.

ABSTRACT

Caffeine is the most consumed psychostimulant substance worldwide, with benefits for cognitive functioning. Caffeine intake at moderate doses also prevents age-related cognitive decline. However, health experts have raised concerns about the growing intake of caffeine-containing drinks by adolescent population. In fact, the effects of caffeine on cognitive functions and neurochemical aspects of late brain maturation during adolescence are poorly understood. In addition, caffeine consumption in the early stages of fetal development has been associated with miscarriage and low birth weight, since it penetrates placenta and blood-brain barrier during pregnancy. Therefore, the impact of caffeine intake was investigated during different stages of brain development. In the first chapter of this thesis, adolescent male rats consumed caffeine in the drinking water (0.1; 0.3 and 1.0 g/L corresponding to low, moderate and high doses, respectively) only during their active period (from 7:00 p.m. to 7:00 a.m.). None of the doses tested had effect on locomotor activity, whereas all triggered anxiogenic effects. Caffeine (0.3 and 1.0 g/L) improved the performance in the object recognition task, but the higher dose of caffeine (1.0 g/L) decreased habituation in open field arena, suggesting a non-associative learning impariment. All tested doses reduced glial fibrillary acidic protein density (GFAP) and synaptosome-associated protein (SNAP-25) without causing any changes in immunoreactivity for neuron-specific nuclear protein (NeuN) in the hippocampus and cerebral cortex. In the hippocampus, caffeine (all doses tested) increased adenosine A_1 receptor density and reduced brain-derived neurotrophic factor (BDNF) and proBDNF (1.0 g/L). In the cerebral cortex, caffeine (1.0 g/L) reduced adenosine A_1 receptor and increased BDNF and proBDNF density (0.3 and 1.0 g/L). These findings document the effects of caffeine consumption in adolescent rats with a dual impact on anxiety and recognition memory, associated with changes in BDNF, GFAP and SNAP-25 levels without apparent neuronal loss in hippocampus and cerebral cortex. In the second chapter, it was tested whether caffeine consumption (0.1; 0.3 and 1.0 g/L in drinking water, from 7:00 p.m. to 7:00 a.m) throughout life may reverse the negative effects caused by the consumption of caffeine in the early stages of development. For this, we used exposure protocols with the end in postnatal days (PND) 21 (development) or 90 (throughout life); both protocols starting 15 days before mating. The effects of these protocols on the behavior and hippocampal synaptic proteins density of adult female and male rats were compared. Recognition memory was impaired in females receiving caffeine (0.3 and 1.0 g/L) during development, which coincided with increased proBDNF levels and unchanged BDNF in the hippocampus. Both exposure protocols caused hyperlocomotion in males, whereas in females only the exposure throughout life significantly increased locomotor activity. Considering the anxiety related behavior, both sexes presented an anxiolytic profile when consuming caffeine (1.0 g/L) throughout life. Both exposure regimens decreased hippocampal GFAP and SNAP-25 of male rats. The hipocampal TrkB receptor was reduced in both sexes and protocols of exposure. In the cortex, both proBDNF and BDNF increased in males receiving caffeine throughout life as well as GFAP and SNAP-25 increased in both treatments regimen. The results revealed that caffeine consumption throughout life can recover the impairment in recognition memory of females that consumed caffeine during development and indicate that exposure for a specific period of brain development promotes sex-dependent behavioral changes, which we relate to alterations in BDNF signaling. The results of this thesis emphasize the importance of controlling caffeine intake during critical periods of brain development of rats and

points to a sex dependent effect. However, more studies are needed to expand our knowledge about the possible signaling pathways involved in these processes.

LISTA DE ABREVIATURAS

A1: Receptor de adenosina do tipo 1

A2A: Receptor de adenosina do tipo 2A

A2B: Receptor de adenosina do tipo 2B

A3: Receptor de adenosina do tipo 3

AMPc: AMP cíclico

APP: Proteína precursora de amilóide (do inglês amyloid precursor protein)

BDNF: Fator neurotrófico derivado do encéfalo (do inglês Brain-Derived Neurotrophic Factor)

BMP: Proteína morfogenética do osso (do inglês bone morphogenetic protein)

CAMKII: Ca^{2+}/calmodulina cinase II (do inglês Ca^{2+}/calmodulin-dependent protein kinase II)

CPI: Célula precursora intermediária

CREB: Proteína de ligação ao AMPc (do inglês cAMP response element- binding protein)

CYP450: Citocromo P 450

CYP1A2: Subunidade do citocromo P 450

D_2 : Receptor de dopamina 2

DA: Doença de Alzheimer

DE: Dia embrionário

DL_{50} : Dose letal que mata 50 % dos animais

DP: Doença de Parkinson

DPN: Dia pós-natal

EM: Esclerose Múltipla

FDA: Food and Drug Administration

FI: Filamentos intermediários.

$GABA_A$: Receptor do ácido γ-aminobutírico do tipo A (do inglês gamma-aminobutiric acid receptor type A)

GFAP: Proteína ácida fibrilar glial (do inglês glial fibrilar acid protein)

hAPP: Proteína precursora de amilóide humano (do inglês human amyloid precursor protein)

K_D: Constante de dissociação

LTD: Depressão de longa duração (do inglês long-term potentiation)

LTP: Potenciação de longa duração (do inglês long-term depression)

mBDNF: BDNF maduro

MCP: Morte celular programada

mGluR1a: Receptor metabotrópico de glutamato 1a

MPTP: 1-metil-4-fenil-tetrahidroxipiridina

NGF: Fator de crescimento do nervo (do inglês Nerve Growth Factor)

NT-3: Neurotrofina-3

NT-4: Neurotrofina-4

proBDNF: precursor BDNF

$p75^{NTR}$: Receptor Pan Neurotrofina

RNAm: RNA mensageiro

Shh: Sonic Hedgehog

SHR: (do inglês Spontaneously Hipertensive Rats)

SNC: Sistema nervoso central

SNAP-25: Proteína associada ao sinaptossoma de 25 kDa (do inglês Synaptossomal-Associated Protein 25)

SNARE: (do inglês Soluble N-ethylmaleimide-sensitive factor attachment protein receptor)

STZ: Estreptozotocina (do inglês streptozotocin)

TDAH: Transtorno de Deficit de Atenção e Hiperatividade

TrkB: Receptor tirosina cinase do tipo B (do inglês Tyrosin kinase B)

1. INTRODUÇÃO

1. 1. Desenvolvimento do Sistema Nervoso Central (SNC): da formação das sinapses aos padrões comportamentais.

O evento que marca o início do desenvolvimento do SNC é a formação da placa neural, na região dorsal média. Suas bordas laterais se elevam para formar as pregas neurais que após fundirem-se, originam o tubo neural. Em humanos, este evento inicia-se na 3ª e 4ª semana de gestação, período equivalente para ratos, aos dias 10,5 e 11,0 do estágio embrionário (DE10,5 e DE11) (revisado por DeSesso et al, 1999; Rice & Barone, 2000). A extremidade cefálica do tubo neural apresenta três vesículas encefálicas primárias que irão constituir o prosencéfalo (encéfalo anterior), mesencéfalo (encéfalo médio) e rombencéfalo (encéfalo posterior) (Lent, 2008; 2010; Moore & Persaud, 2003; Nolte, 2008). A seguir, o prosencéfalo passa a ser constituído pelo telencéfalo e diencéfalo. O primeiro, dará origem aos hemisférios cerebrais, constituídos pelo córtex cerebral e pelos núcleos da base. O segundo, ao pedúnculo cerebral e a cápsula interna, estruturas importantes constituídas por numerosos feixes de fibras e núcleos. O rombencéfalo também passa a ser constituído por duas formações distintas denominadas metencéfalo e mielencéfalo. O metencéfalo dará origem ao cerebelo e a ponte e o mielencéfalo ao bulbo. Por fim, posicionado na parte posterior do mielencéfalo, o tubo neural transforma-se gradativamente na medula espinhal (Lent, 2008; 2010; Moore & Persaud, 2003; Nolte, 2008).

Do ponto de vista celular, o desenvolvimento do SNC pode ser organizado nas seguintes etapas: gênese das células nervosas, migração, maturação, estabelecimento de conexões e eventos regressivos. Do ponto de vista biológico, é de crucial

importância ressaltar que estas etapas não ocorrem dissociadamente, e sim são processos celulares dinâmicos que se sobrepõem uns aos outros.

A diferenciação das células ectodérmicas em células neuroectodérmicas marca o início do desenvolvimento do SNC. Este processo é possível pela existência de um complexo programa que direciona a expressão de genes específicos em determinadas subpopulações celulares. As células do ectoderma produzem e reconhecem proteínas denominadas proteínas morfogenéticas do osso (BMPs, do inglês *bone morphogenetic proteins*), as quais determinam a permanência das células ectodêrmicas e a diferenciação em epiderme. O bloqueio do receptor das BMPs altera o futuro destas células que ao invés de diferenciarem-se em células da epiderme, passarão a constituir o neuroectorma (Lent, 2008; 2010).

Após a fundição completa do tubo neural, as células neuroectodérmicas diferenciam-se em dois tipos de células nervosas: células precursoras neuronais e gliais. As precursoras neuronais proliferam neurônios a partir da divisão celular. A glia radial, como também é conhecida a célula precursora glial, pode sofrer dois tipos de divisão celular: um simétrico, gerando duas novas glias radiais e outro assimétrico diferenciando-se em células precursoras intermediárias (CPIs) ou neurônios pós-mitóticos. As CPIs podem formar novas CPIs ou diferenciarem-se em novos neurônios, processo conhecido como *neurogênese*. Já os neurônios pós-mitóticos, os quais tem o seu ciclo celular interrompido, entrelaçam-se em prolongamentos da glia radial iniciando um processo de deslocamento até alcançarem uma região específica onde finalmente se estabelecerão: *é a migração neuronal*. A *gliogênese*, como é conhecido o processo de geração das células gliais, ocorre de maneira semelhante à neuronal, porém em um período posterior. As células neurogliais originam-se a partir da glia radial após o fim da neurogênese quando esta perde seus prolongamentos

radiais e diferencia-se em astrócitos ou em precursores intermediários que irão originar os oligondrócitos (Lent, 2008; 2010; revisado por Kriegstein & Alvarez-Buylla, 2009).

Durante a migração e potencialmente depois de os neurônios juvenis se estabelecerem em um determinado local, inicia-se o processo de maturação que engloba alterações morfológicas, bioquímicas e funcionais (Lent, 2008; 2010; Moore & Persaud, 2003). A maturação neuronal é caracterizada pela emissão de prolongações dendríticas e axonais, aumento no volume do corpo celular e início da síntese de moléculas que irão garantir maturidade funcional ao neurônio. O processo de maturação dos neurônios consiste em um refinamento na forma e tamanho dos dendritos e axônios e representa uma preparação morfológica para a realização da sinapse. É de crucial importância lembrar que dendritos e axônios de uma mesma célula não só diferem morfológica e funcionalmente, eles também apresentam regulações diferentes em seu crescimento (revisado por Craig & Banker, 1994; Jan & Jan, 2010).

A expansão dendrítica é determinada pelo balanço entre os custos metabólicos a serem gastos para a expansão destas novas ramificações, e a real necessidade de cobrir um determinado campo (Shepherd et al, 2005; Wen & Chklovskii, 2008; revisado por Jan & Jan, 2010). Estudos recentes têm demostrado que esta regulação pode ser feita por fatores neurotróficos, que são proteínas essenciais para o crescimento sobrevivência celular (Luikart et al, 2008; McAllister et al, 1995). Por outro lado, o que determina o crescimento axonal são os cones de crescimento. Ramón e Cajal foi pioneiro em observar estas estruturas em seus preparos histológicos e as descreve como dotadas de sensibilidade química refinada, rápidos movimentos amebóides e relativa força impulsiva, tornando-as capaz de superar

muitos obstáculos até alcançar seu destino final. Esta descrição de Ramón y Cajal serve como base para formular uma das hipóteses mais aceitas sobre como se estabelecem as conexões nervosas durante o desenvolvimento: a hipótese quimiotrópica. Ela sugere que os cones de crescimento axonal contém moléculas capazes de reconhecer sinais químicos atrativos e repulsivos, determinando o trajeto da célula para novas conexões (Ramón y Cajal, 1893; 1995; revisado por Tessier-Lavigne & Goodman, 1996).

Quando se estabelece uma conexão entre duas células neuronais forma-se uma sinapse, processo conhecido como *sinaptogênese*. Em humanos, a sinaptogênese ou proliferação sináptica inicia por volta da 20ª semana de gestação, aumentando consideravelmente após o nascimento. A exemplo da sinaptogênese pós-natal em humanos, destacam-se o córtex visual primário com picos entre os 8º e 12º primeiros meses de vida e o córtex pré-frontal com picos dos 2 aos 4 anos de idade (Huttenlocher, 1979; Huttenlocher et al, 1982). Em ratos, o pico de densidade sináptica ocorre durante as três primeiras semanas pós-natal, quando os níveis chegam a mais de 50 % dos níveis encontrados em adultos (Micheva & Beaulieu, 1996). No entanto, em algumas regiões cerebrais a sinaptogênese se estende até o início da adolescência. Na camada molecular do giro denteado do hipocampo e no córtex somatossensorial de ratos, por exemplo, o número de sinapses atinge a equivalência de um animal adulto nos dias pós-natal 25 e 30 (DPN 25 e 30), respectivamente (Crain et al, 1973; Micheva & Beaulieu, 1996; revisado por Rice & Barone, 2000).

O aumento expressivo de neurônios e sinapses que ocorre durante as primeiras semanas pós-natal, resultaria em um encéfalo infantil com muito mais neurônios e conexões sinápticas do que seria realmente necessário. Assim, a existência de eventos celulares regressivos como a poda sináptica e a morte celular programada ou apoptose

(MCP), os quais se iniciam após o nascimento e permanecem ativos até o fim da adolescência, são essenciais para controlar e lapidar esse significativo aumento (Andersen et al, 2000; Andersen & Teisher, 2004; Cunningham et al, 2002; revisado por Semple et al, 2013). Durante o desenvolvimento, a MCP exerce a função de regular as populações de progenitores durante a neurogênese, corrige erros e promove a otimização das conexões sinápticas (revisado por Kim & Sun, 2011). Já no período da adolescência, podemos observar a atividade destes processos pela maturação estrutural do córtex pré-frontal, redução da densidade sináptica e reorganização dos sistemas de neurotransmissão tais como os glutamatérgico (Kasanetz & Manzoni, 2009), serotoninérgico (revisado por Sodhi & Sanders-Bush, 2004) e GABAérgico (Ben-Ari et al, 2012; Shen et al, 2010). Estudos recentes têm revelado que a MCP é o resultado de uma disputa por fatores tróficos essenciais à sobrevivência celular, os quais são fornecidos de forma limitada pelos neurônios-alvo (revisado por Oppenheim, 1996; Oppenheim et al, 2001). Diferentemente da MCP, a poda sináptica não promove a morte do neurônio, mas uma retração ou degeneração axonal que impede conexões funcionalmente defeituosas (revisado por Hua & Smith, 2004). Assim, a importância dos processos regressivos durante o desenvolvimento está na capacidade de remover sistemas redundantes, conter o desperdício energético com conexões não essenciais (revisado por Buss et al, 2006; Kim & Sun, 2011) e promover o refinamento e maturação de circuitos neuronais e sistemas de neurotransmissão (Huttenlocher, 1979; Petanjek et al, 2011; Uylings & van Eden, 1990).

Os processos celulares, funcionais e comportamentais que acompanham o desenvolvimento cerebral do período embrionário até o fim da adolescência são sensíveis à injurias como trauma isquêmico (Ajao et al, 2012; Pullela et al, 2006; van

der Kooij et al, 2010) a modificações ambientais (Tang, 2001) ou exposição a fatores estressores (revisado por McCormick & Green, 2013). Tais perturbações podem modificar o curso normal do desenvolvimento do encéfalo e culminar em alterações comportamentais permanentes nos animais (Ajao et al, 2012; Pullela et al, 2006; Tang, 2001; van der Kooij et al, 2010).

Durante as fases iniciais da vida ocorrem abundantes adaptações morfológicas e funcionais no encéfalo. Porém, é no período da adolescência que marcadamente inicia-se uma fase de transição, na qual as habilidades e padrões comportamentais do animal adulto, por exemplo, são adquiridas e lapidadas. Este conceito pode ser elaborado a partir da comparação temporal do desenvolvimento morfológico e funcional de diferentes regiões cerebrais com as mudanças comportamentais sofridas pelos animais durante este período.

Do ponto de vista comportamental, a adolescência compreende o período mais importante do desenvolvimento pós-natal. Em roedores, esta fase começa logo após o desmame ainda antes do início da puberdade e deve terminar após o final da maturação sexual, o que implicaria em uma janela de tempo para ratos de DPN 22 a 57 para fêmeas e DPN 28 a 72 para machos (revisado por Schneider, 2013). A figura a seguir mostra um quadro comparativo do desenvolvimento de ratos e humanos (Figura 1).

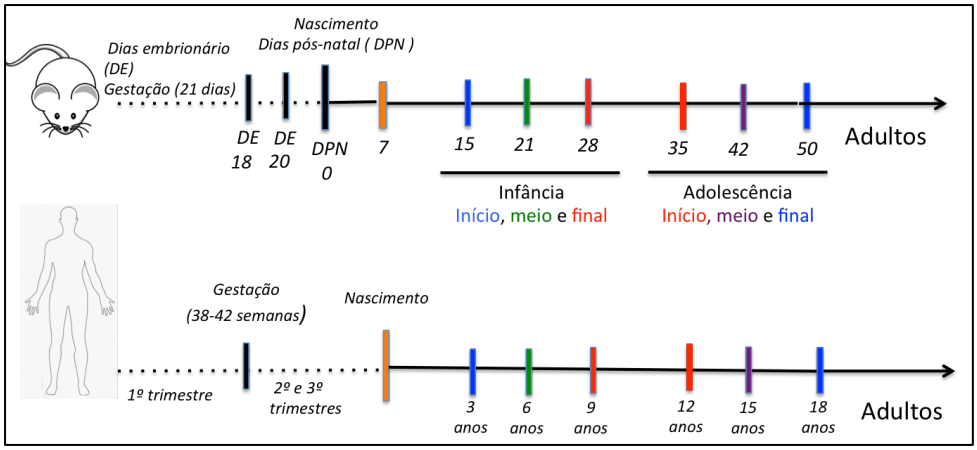


Figura 1. Linha do tempo comparativa entre o desenvolvimento de ratos e humanos (Adaptada de Clancy et al, 2001; de Marco et al, 2011; Tchekalarova et al, 2005; Williams, 2008).

É durante a infância e adolescência que se observam os primeiros sinais do comportamento social e do desenvolvimento cognitivo. Após atingirem a maturidade sexual (DPN 35-56) (Lambert, 2009; revisado por Semple, 2013), por exemplo, os roedores começam a exibir comportamentos tipicamente adolescentes, tornando-se mais impulsivos, com comportamento de risco acentuado e orientados para o acasalamento (revisado por Spear, 2000; Sturman & Muoghaddam 2011; Semple, 2013). É interessante lembrar que estas alterações acontecem paralelamente ao refinamento e maturação do circuito neural em regiões como o córtex cingular anterior, amigdala, insula anterior, córtex pré-frontal medial (revisado por Blakemore, 2008) e dorsolateral (Raznahan et al, 2011; revisado por Laviola et al, 2003).

Um grande número de substâncias disponíveis no ambiente é capaz de ultrapassar a barreira hematoencefálica e a placenta. Dessa forma, estudos em roedores tem demonstrado que a exposição a algumas substâncias tóxicas durante o desenvolvimento, como metais e pesticidas prejudica a locomoção (Betharia & Maher, 2012; Eriksson et al, 1992) e o aprendizado, além de levar os animais a desenvolverem comportamento ansiogênico na vida adulta (Betharia & Maher, 2012) e afetar os sistemas de neurotransmissão serotoninérgico, dopaminérgico,

noradrenérgico (Mohammad & St. Omer, 1985; 1986; St. Omer & Mohammad, 1987) e colinérgico (Ahlbom et al, 1994; 1995; Ricceri et al, 2006; Schneider et al, 2012). Além disso, substâncias aditivas como a nicotina (Adriani et al, 2003; 2004; Eriksson et al, 2000; Mychasiuk et al, 2014), a canabis (Schneider et al, 2008; revisado por Schneider, 2009), o álcool (Coleman et al, 2011; Morris et al, 2010), a cocaína (Salas-Ramirez et al, 2010; García-Cabrerizo et al, 2015) e as anfetaminas (Lloyd et al, 2013) também afetam o desenvolvimento do encéfalo quando consumidas na adolescência e durante a gestação e/ou lactação, podendo provocar alterações comportamentais, neuroquímicas e funcionais permanentes na vida adulta dos animais.

1. 2. Cafeína

1. 2. 1. Breve histórico

A história da cafeína, contada por Weinberg & Bealer no livro "O mundo da Cafeína", descreve especulações antropológicas a respeito do seu consumo e apreciação, datados de 700 mil anos a. C durante a era paleolítica (Weinberg & Bealer, 2001). Atualmente, a cafeína é considerada a substância psicoestimulante mais consumida no mundo (revisado por Fredholm et al, 1999), sendo o consumo de café sua principal fonte na dieta. Porém, ela também pode ser encontrada em chás, frutos de cacau e consequentemente, nos chocolates, bem como em bebidas energéticas ou a base de cola.

Apesar de ser uma planta orginária da Etiópia, as primeiras plantações surgiram no século XIV na Península Arábica, principal responsável pela propagação da cultura cafeeira (Weinberg & Bealer, 2001). Uma lenda popular atribui a descoberta do café a um pastor da Etiópia que teria percebido a agitação do seu rebanho após comer os frutos de um arbusto. Curioso, o pastor provou as frutinhas e

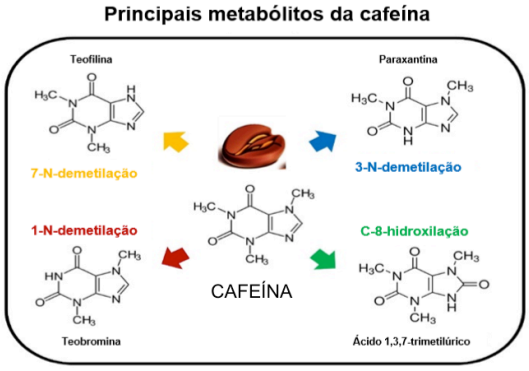
foi acometido por um estado de euforia capaz de perturbar-lhe o sono daquela noite (Weinberg & Bealer, 2001).

A seguir, nos séculos XVI e XVIII, o consumo de café foi amplamente difundido e popularizado nos continentes europeu e latino-americano, respectivamente (Weinberg & Bealer, 2001). Especificamente no Brasil, as primeiras sementes de café chegaram no ano de 1727 e devido as excelentes condições climáticas encontradas aqui, o seu cultivo propagou-se rapidamente (Carvalho et al, 1993). Em meados do século XVIII, a produção de café revolucionou a sociedade e a economia do Brasil que se tornou o maior produtor mundial de café (Taunay, 1939).

1. 2. 2. Metabolismo da cafeína

A cafeína, quimicamente conhecida como 1,3,7-trimetilxantina, é totalmente absorvida pelo trato gastrointestinal (Arnaud 1976, 1985, 1993). Suas propriedades hidrofóbicas permitem passagem através das membranas biológicas, ignorando a existência da barreira placentária e hematoencefálica (Lachance et al, 1983; Tanaka et al, 1984; Ikeda et al, 1982; Kimmel et al, 1984). Em humanos, a cafeína é desmetilada a dimetilxantinas, sendo que mais de 80 % é metabolizada em paraxantina (1,7-dimetilxantina), e cerca de 16 % é convertida em teobromina (3,7-dimetilxantina) e teofilina (1,3-dimetilxantina) (Lelo et al, 1986). Em roedores, o principal metabólito encontrado no plasma é a paraxantina, embora os níveis de teofilina também sejam elevados (Fuhr et al, 1996; revisado por Miners & Birkett, 1996) (Figura 2). Para doses menores do que 10 mg/kg, a meia vida da cafeína em roedores adultos gira em torno de 0,7-1,2 horas e em humanos de 2,5-4,5 horas (Bonati et al, 1984; revisado por Arnaud, 1987), o que implica em uma correção na concentração pela massa corporal, na qual a dose de 3,5 mg/kg para humanos representa 10 mg/kg para ratos (revisado por Fredholm et al, 1999). A cafeína é metabolizada no fígado pelo

complexo enzimático citocromo P450 (CYP450), sendo isoenzima CYP1A2, a principal responsável por este processo (Aranda et al, 1979). Não existe diferença na meia vida intraespécie entre jovens e idosos (Blanchard & Sawers, 1983). Porém, em neonatos a cafeína tem uma meia-vida mais longa, considerando que a CYP1A2 tem sua maturação, expressão e atividade dependente do desenvolvimento (revisado por Leeder, 2001). Em humanos, a metabolização da cafeína diminui gradualmente no recém-nascido (Aranda et al, 1979; Le Guennec & Billon, 1987) e aumenta exponencialmente no primeiro ano de vida (Aldridge et al, 1979; Parsons & Neims, 1981; Pearlman et al, 1989). Dessa forma, embora ainda não exista um consenso se o consumo de cafeína durante a gestação e lactação representa um perigo ao desenvolvimento do feto, o seu uso deve permanecer cauteloso considerando a sua meia-vida prolongada durante esses períodos.



Comparação entre metabolismo humano e de roedores

HUMANOS X ROEDORES	METABÓLITOS DA CAFEÍNA	ENZIMAS MITOCONDRIAIS
	Paraxantina (aprox. 70%) Ácido 1,3,7-trimetilúrico (aprox. 15%) Teobromina e Teofilina (7-8%)	CYP1A2 CYP1A2, CYP3A4, CYP2C8/9 CYP1A2, CYP2C8/9, CYP3A4
	Ácido 1,3,7-trimetilúrico (aprox. 70%) Paraxantina (aprox. 13%) Teobromina e Teofilina (8-9%)	CYP1A2 CYP1A2, CYP2C11 CYP1A2, CYP2C, CYP3A2

Figura 2. Metabólitos da cafeína e principais diferenças entre humanos e roedores no metabolismo da cafeína (Adaptada de Porciúncula et al, 2013).

***1. 2. 3**. Mecanismo de ação: bloqueio dos receptores de adenosina A_1 e A_{2A}*

A ação farmacológica da cafeína acontece pelo bloqueio dos receptores de adenosina, um nucleosídeo que no SNC atua como um neuromodulador controlando a liberação de neurotransmissores, a excitabilidade neuronal e o ritmo circadiano por meio de seus receptores metabotrópicos A_1, A_{2A}, A_{2B} e A_3 (revisado por Cunha, 2001; Fredholm et al, 2005). A ativação dos receptores de adenosina A_1 e A_{2A} (A_1R e $A_{2A}R$) exercem, respectivamente, ações inibitórias e facilitatórias sobre a transmissão sináptica (revisado por Cunha et al, 2001). Estes receptores de adenosina são os mais expressos no SNC e são os principais alvos farmacológicos da cafeína (revisado por Fredholm et al, 1999; Nehlig et al, 1992). Em humanos, doses moderadas de cafeína alcançam níveis séricos na faixa de μM bloqueando os A_1R e $A_{2A}R$ em humanos com K_D próximo a 12 e 2,4 μM, respectivamente (revisado por Fredholm et al, 1999) (Tabela 1). Em doses elevadas, a cafeína inibe fosfodiesterases, aumenta a liberação de cálcio intracelular e bloqueia os receptores $GABA_A$ (revisado por Fredholm et al, 1999).

Subtipo do Receptor	**K_D (Ratos)**	**K_D (Humanos)**
	μM	
Receptor A_1	20	12
Receptor A_{2A}	8.1	2.4
Receptor A_{2B}	17	13
Receptor A_3	190	80

Tabela 1. Tabela comparativa entre o K_D de ratos e humanos para a ação inibitória da cafeína sobre os receptores de adenosina (Adaptada de Fredholm et al, 1999).

Seu efeito farmacológico mais conhecido consiste em aumentar o estado de

alerta, diminuir o sono e a sensação de fadiga (Rogers et al, 2005). Um indivíduo adulto pesando 70 kg que consuma apenas uma xícara de café contendo de 30-175 mg de cafeína, em pouco mais de 30 minutos terá concentrações séricas de cafeína (1-10 μM) suficientes para bloquear os A_1R e $A_{2A}R$ e diminuir o tônus inibitório da adenosina, resultando em uma ação psicoestimulante (revisado por Fredholm et al, 1999).

1. 2. 4. *Alterações promovidas pela cafeína durante desenvolvimento cerebral*

Atualmente, a cafeína é reconhecida como a substância psicoestimulante mais utilizada no mundo e o seu consumo é considerado seguro para a população adulta. Por ser uma substância hidrofóbica que atravessa a barreira placentária e hematoencefálica, a cafeína exerce influência sobre o SNC imaturo (Ikeda et al, 1982; Kimmel et al, 1984; Tanaka et al, 1984; revisado por Fredholm et al, 1999) e adulto (Lachance et al, 1983; revisado por Fredholm et al, 1999). Em neonatos, a cafeína tem uma meia vida mais longa devido a baixa atividade do citocromo P450, um dos principais complexos enzimáticos responsáveis pela metabolização de xenobióticos (revisado por Leeder, 2001). Dessa forma, os indivíduos recém-nascidos são mais vulneráveis à possíveis ações tóxicas desta substância (Aranda et al, 1979; revisado por Fredholm et al, 1999; Porciúncula et al, 2013).

Uma série de estudos epidemiológicos apresentaram evidências de que o consumo de altas doses de cafeína durante a gestação estaria associado a baixos índices de concepção, problemas de fertilidade (Christianson et al, 1989; Jensen et al, 1998; Stanton & Gray, 1995; Williams et al, 1990), aumento no número de abortos espontâneos (Cnattingius et al, 2000; Dlugosz et al, 1996; Fernandes et al, 1998; Srisuphan & Bracken, 1986), malformações congênitas (de Marco et al, 2011;

Schmidt et al, 2009), prejuízos no crescimento fetal e risco aumentado de baixo peso ao nascer (Bakker et al, 2010; Bracken et al, 2003; Vlajinac et al, 1997).

Do ponto de vista neuroquímico, alguns estudos já reportaram efeitos promovidos pela cafeína nas fases pré e pós-natal (revisado por Tchekalarova et al, 2014). As concentrações de cafeína a partir de 105 μM (equivalente a dose de 25 mg/kg, *in vivo*), comprometem o fechamento do tudo neural e aumentam a proliferação de células neuroepiteliais em culturas embrionárias de camundongos (Marret et al, 1997). Uma aceleração na evaginação neuroepitelial foi observada nas vesículas teloencefálicas de embriões cujas mães receberam 12,5, 25 e 50 mg/kg pela via i.p (intraperitoneal), e em culturas embrionárias de camundongos a partir de 105 μM (Marret et al, 1997; Sahir et al, 2000). Além disso, a administração neonatal de 50 mg/kg pela via s.c (subcutânea) por 12 dias modificou a morfologia dendrítica de neurônios piramidais do córtex pré-frontal dos ratos na adolescência e na vida adulta (Juárez-Méndez et al, 2006). A mesma dose administrada pela via i.p, causou morte neuronal em diversas regiões cerebrais de ratos 24 horas após a administração (Kang et al, 2002). Em culturas de astrócitos e neurônios, a cafeína na concentração de 1 mM causou um aumento na expressão do gene para a *Sonic Hedgehog* (*shh*), um hormônio peptídico que é expresso no SNC em desenvolvimento e tem como uma de suas principais funções a diferenciação dos neurônios motores da medula espinhal (Sahir et al, 2004). Além disso, o consumo de 1 g/L de cafeína por ratas gestantes causou um aumento no imunocontúdo da *shh* no hipocampo e no córtex de embriões de dezoito dias. Já em embriões de vinte dias, o aumento foi observado somente no hipocampo para as doses 0,3 e 1 g/L (Mioranzza et al, 2014). León e colaboradores demonstraram ainda, que o consumo de 1 g/L de cafeína por ratas gestantes causou um aumento na expressão dos A_1R dos fetos (León et al, 2002). Resultado este, que

foi contrastante ao encontrado por Lorenzo e colaboradores onde uma redução significativa na expressão deste receptor foi observada nas gestantes e nos filhotes machos em DPN 15 (Lorenzo et al, 2010). Além de exercer influência sobre o sistema de neurotransmissão adenosinérgico (León et al, 2002; Lorenzo et al, 2010), o consumo de cafeína por ratas gestantes promoveu também alterações nos sistemas glutamatérgico e colinérgico, uma vez que, caracterizou-se uma redução no imunoconteúdo do $mGluR_{1a}$ (do inglês *metabotropic glutamate receptor* 1a) nas mães e nos fetos (León et al, 2005) e um aumento a atividade da acetilcolinesterase no hipocampo de filhotes com 21 dias de idade (da Silva et al, 2008). A cafeína administrada durante os primeiros dias pós-natal promoveu um atraso na migração de neurônios GABAérgicos e prejuízos no disparo de circuitos cerebrais, aumentando a susceptibilidade a convulsões (Silva et al, 2013).

Alguns padrões comportamentais foram alterados em ratos que consumiram cafeína durante a gestação e lactação, tais como a redução na atividade locomotora e a exacerbação de comportamentos do tipo ansioso na vida adulta (revisado por Tchekalarova et al, 2014; Hughes & Beveridge, 1991). Os prejuízos na memória espacial e de reconhecimento foram observados na vida adulta de camundongos que foram expostos a 0,3 g/L de cafeína durante os períodos gestacional e lactacional (Silva et al, 2013). Resultados semelhantes também foram encontrados quando a exposição à cafeína ocorreu somente no período pré- ou pós-natal. O consumo de 0,075 g/L de cafeína por ratas gestantes prejudicou as memórias de trabalho, referência e reconhecimento ao objeto dos filhotes na vida adulta (Soellner et al, 2009). Da mesma forma, ratos expostos à 15-20 mg/kg de cafeína pela via oral entre os dias pós-natal 2 e 6 apresentaram hiperalgesia, comportamento ansiolítico e

prejuízo na memória aversiva quando avaliados no período da adolescência (Pan & Chen, 2007).

Ao longo das últimas décadas o uso de cafeína vem sendo popularizado na população juvenil e adolescente por promover a manutenção do estado de alerta e a diminuição do sono. A fase da adolescência é classicamente definida em roedores e humanos como crucial para a maturação cerebral. De fato, é durante este período que o encéfalo passa por intensa remodelação sináptica (revisado por Arain et al, 2013; Christensen et al, 2013), e por isso aumenta a sua vulnerabilidade à ação de substâncias psicoestimulantes. Nos últimos anos houve um aumento considerável no consumo de bebidas energéticas que contém concentrações elevadas de cafeína (Heckman et al, 2010; revisado por Temple, 2009), fato que tem tem despertado o interesse e a preocupação da comunidade científica (Harnack et al, 1999; revisado por Fredholm et al, 1999; Temple, 2009). O consumo exagerado de bebidas energéticas pode provocar a intoxicação do indivíduo causando alucinações, convulsões, arritmia cardíaca e até a morte (Seifert et al, 2011). Os valores plasmáticos exatos necessários para que esses efeitos possam ser desencadeados ainda são desconhecidos, mas há relatos de casos sugerindo que as concentrações plasmáticas maiores ou iguais a 15 mg/L podem ser altamente tóxicas, e as maiores que 80 mg/L podem ser letais (um consumo excedente à 3000 mg de cafeína) (Clausson et al, 2008; Holmgren et al, 2004; Kerrigan & Lindsey, 2005; Riesselmann et al, 1999; revisado por Fredholm et al, 1999). É importante ainda citar que para ratos a DL_{50} (dose letal que mata 50% dos animais testados) da cafeína está em torno de 200 mg/kg (revisado por Fredholm et al, 1999).

Algumas alterações comportamentais já foram observadas após exposição de ratos adolescentes à cafeína. As administrações agudas promovem resposta

locomotora bifásica, aumentando a atividade em doses moderadas (10-30 mg/kg) (Marin et al, 2011; Rhoads et al, 2011) e reduzindo em doses elevadas (60-120 mg/kg (Marin et al, 2011). A exposição crônica causou uma redução da atividade locomotora, o que foi relacionado a um aumento da reatividade emocional, em que machos pareceram ser mais responsivos do que fêmeas (Anderson & Hughes 2008). A cafeína administrada no período da adolescência potencializa os efeitos de outras substâncias, por exemplo, o efeito do álcool sobre a ansiedade (Hughes, 2011). Ainda são escassos os estudos que buscam correlacionar o perfil comportamental dos animais com parâmetros bioquímicos e aspectos funcionais ainda ativos neste período do desenvolvimento. Dessa forma, a utilização de marcadores biológicos que apontem os meios pelos quais a cafeína causa interferências no desenvolvimento do SNC se faz necessário. Todas essas evidências de que o consumo de cafeína pode alterar padrões do desenvolvimento do SNC, reforça que o consumo de cafeína por gestantes e lactantes deve ser feito com parcimônia. Dessa forma, mais investigações são necessárias com o objetivo de determinar se há uma dose segura que não afete o desenvolvimento normal do indivíduo.

1. 2. 5*. Influência do gênero*

Os estudos experimentais recentes têm demonstrado consumo crônico de doses moderadas de cafeína previne o comprometimento cognitivo decorrente da idade (Costa et al, 2008a; Prediger et al, 2005; Sallaberry et al, 2013), em modelos experimentais de doença neurodegenerativa como a Doença de Alzheimer (DA) (Arendash et al, 2006; Dall'Igna et al, 2007; Espinosa et al, 2013), induzidos por estresse (Alzoubi et al, 2013; Kaster et al, 2015) ou em modelos de transtornos neuropsiquiátricos (Pandolfo et al, 2013).

Inicialmente os estudos epidemiológicos correlacionaram o consumo de cafeína (Ascherio et al, 2001; 2004; Benedetti et al, 2000; Ross et al, 2000) ou café (Benedetti et al, 2000) como um fator de redução para o risco de desenvolvimento da Doença de Parkinson (DP), uma doença neurodegenerativa caracterizada pelo comprometimento da transmissão dopaminérgica (revisado por Picconi et al, 2012) e perda neuronal na região nigroestriatal (Bernheimer et al, 1973) que culmina em prejuízos na atividade motora (Rodriguez-Oroz et al, 2009). Do ponto de vista molecular, esse efeito neuroprotetor da cafeína parece estar relacionado ao fato de os $A_{2A}R$ e os receptores de dopamina D_2 (D_2R) estarem colocalizados em neurônios do estriado e formarem heterodímeros, apresentando uma interatividade funcional (Fink et al, 1992; Ferré et al, 1991). A ativação dos $A_{2A}R$ diminui a afinidade da dopamina pelos D_2R e, consequentemente, o bloqueio dos $A_{2A}R$ pela cafeína aumenta a afinidade da dopamina pelos D_2R, potencializando essa neurotransmissão que se encontra deficiente na DP (Ferré et al, 1991; Schwarzschild et al, 2003). A relação entre o consumo de cafeína e o risco reduzido de desenvolver DP tem sido observada em homens. Interessantemente, em mulheres, esta associação está presente somente naquelas que não realizaram reposição de estrógeno após a menopausa, sugerindo uma interação entre as influências do estrógeno e da cafeína sobre o risco de desenvolvevimento da doença (Ascherio et al, 2001; 2003; 2004). Em um importante estudo com modelo animal para DP, observou-se que o tratamento com cafeína atenuou a neurodegeneração dopaminérgica induzida por 1-metil-4-fenil-tetrahidroxipiridina (MPTP). Contudo, doses consideralvelmente mais baixas foram mais eficazes para camundongos machos quando comparado à fêmeas, tanto nos jovens quanto nos animais envelhecidos que se encontravam fora do período reprodutivo (Xu et al, 2006). Ao analizar a interação estrógeno-cafeína

especificamente em fêmeas, o estudo comparou jovens sham e ovariectomizadas (OVX) ou fêmeas envelhecidas OVX com reposição de estrógeno ou placebo. Os resultados apontaram para uma menor eficácia da cafeína em prevenir a depleção de dopamina naquelas fêmeas que mantiveram níveis de estrógeno, ou seja, nas jovens sham e nas ovariecomizadas com reposição (Xu et al, 2006). Nesse mesmo estudo nos machos que receberam o estrógeno a cafeína também foi menos eficaz em prevenir a redução nos níveis de dopamina induzida pelo MPTP (Xu et al, 2006).

Estudos epidemiológicos apresentaram evidências de que o consumo de cafeína ou café está associado à um risco reduzido de desenvolver demência e retardo no progresso dos prejuízos cognitivos leves (Cao et al, 2012; Eskelinen et al, 2009). Além disso, os efeitos da cafeína em retardar a demência parecem ser prevalentes em mulheres acima de 65 anos que consumiram altas doses (Ritchie et al, 2007). Especula-se que esta diferença com relação ao gênero pode indicar que as mulheres são mais suscetíveis aos efeitos da cafeína do que os homens e a análise de uma relação positiva entre o consumo de cafeína e a produção de estrógeno ou tratamento de reposição hormonal tem sido considerada (Ferrini & Barrett-Connor, 1996). Em modelos experimentais com camundongos transgênicos machos para DA, a administração crônica de 0.3 g/L de cafeína reduziu os níveis do peptídeo β-Amilóide no hipocampo (Arendash et al, 2006; 2009), reverteu o prejuízo na memória de trabalho (Arendash et al, 2009) e melhorou o desempenho dos animais em tarefas que requerem a memória espacial (Arendash et al, 2006; Laurent et al, 2014) e o reconhecimento (Arendash et al, 2006). Da mesma forma, a administração de cafeína ou SCH 58261 (antagonista seletivo dos $A_{2A}R$), impediu o déficit cognitivo induzido pela administração intracerebroventricular (i.c.v) do peptídeo β-Amilóide (25-35), o que sugere a participação dos $A_{2A}R$ no mecanismo protetor da cafeína em relação os

prejuízos cognitivos apresentados pelos camundongos machos (Dall'Igna et al, 2007). Em modelo de demência induzido por estreptozotocina (STZ), a administração crônica de 1 g/L de cafeína na água de beber impediu o prejuízo mnemônico de ratos machos na tarefa de reconhecimento ao objeto (Espinosa et al, 2013). Esses estudos permitem destacar a importância da cafeína e de antagonistas dos $A_{2A}R$ como estratégia profilática, mas também como potenciais fármacos para o tratamento da DA. Porém, poucos são os avanços nos estudos experimentais com animais que elucidem os mecanismos pelos quais a cafeína parece retardar o processo de demência de forma dependente do gênero.

Noschang e colaboradores investigaram o impacto do consumo de cafeína sobre parâmetros bioquímicos e comportamentais de ratos machos e fêmeas submetidos à estresse crônico. Os níveis de corticosterona (hormônio de roedores que aumenta em situações de estresse), foram reduzidos pelo consumo de 1 g/L de cafeína tanto nas fêmeas submetidas ao estresse e nos controles, enquanto nos machos nenhuma alteração foi encontrada. Nesse mesmo estudo, o consumo de cafeína (0,3 e 1 g/L) promoveu ansiedade em machos e este efeito foi potencializado pelo estresse, enquanto as fêmeas foram mais resistentes aos efeitos da cafeína e do estresse (Noschang et al, 2009).

Alguns trabalhos em roedores demonstram que o consumo de cafeína durante as fases iniciais da vida provoca alterações comportamentais dependentes do sexo. Por exemplo, a exposição pré-natal à cafeína reduziu a atividade locomotora e exacerbou o comportamento do tipo ansioso somente em ratos machos adultos (Hughes & Beveridge, 1991). Além disso, 15-20 mg/kg de cafeína pela via oral entre DPN 2-6 melhorou o desempenho na retenção da memória 24 e 72 horas após treino de fêmeas testadas na vida adulta (Fisher & Guillet, 1997). Da mesma forma, o

tratamento com cafeína (60 mg/kg entre DE13-19) melhorou a retenção da memória 25 dias após o treino em fêmeas adultas (Swenson et al, 1990). Dessa forma, há indícios de que diferentes resultados podem ser obtidos pela exposição à cafeína considerando-se o sexo. Portanto, é necessário observar atentamente o envolvimento dos gêneros e hormônios sexuais nos efeitos da cafeína sobre o desenvolvimento dos animais, bem como as alterações bioquímicas e comportamentais que podem influenciar na vida adulta.

1. 3. Proteínas essenciais para o desenvolvimento do SNC

1. 3. 1. O fator neurotrófico derivado do encéfalo (BDNF, do inglês *brain-derived neurotrophic fator)*

As neurotrofinas são uma família de proteínas essenciais para o desenvolvimento e manutenção do sistema nervoso. Em mamíferos, foram identificados os seguintes tipos de fatores tróficos neuronais: fator de crescimento neural (NGF, do inglês *nerve growth factor*), fator neurotrófico derivado do encéfalo (BDNF, do inglês *brain-derived neurotrophic factor*), neurotrofina-3 (NT-3) e neurotrofina-4 (NT-4) (revisado por Lu et al, 2005).

O BDNF é um dos fatores neurotróficos mais investigados devido ao seu papel central nos eventos celulares e funcionais do encéfalo imaturo e adulto. Ele é sintetizado como uma proteína precursora (pré-proBDNF) no retículo endoplasmático. Esta proteína precursora é clivada em proBDNF e transportada para o complexo de Golgi onde é armazenada em vesículas secretoras da via constitutiva ou regulada (Mowla et al, 1999). O proBDNF pode ser: i) clivado em mBDNF (BDNF maduro) intracelularmente seguido de secreção, ii) secretado e clivado extracelularmente ou iii) secretado sem subsequente clivagem (revisado por Cunha et al, 2010), embora

ainda haja controvérsias quanto a secreção neuronal do proBDNF (Matsumoto et al, 2008; Yang et al, 2009). Algumas evidências demostram que quando o proBDNF é secretado para o meio extracelular e não sofre clivagem, ele liga-se ao receptor $p75^{NTR}$, aumenta a depressão de longa duração (LTD, do inglês *long-term depression*) (Woo et al, 2005), enfraquece as sinapses (Woo et al, 2005; revisado por Lu et al, 2005) e promove a apoptose (Teng et al, 2005). Porém a liberação do mBDNF e consequente ligação ao receptor tirosina cinase B (TrkB), aumenta a potenciação de longa duração (LTP, do inglês *long-term potentiation*) (Pang et al, 2004), fortalece as sinapses (Tyler & Pozzo-Miller 2001; revisado por McAllister et al, 1999; Lu et al, 2005) e contribui para a sobrevivência celular (Pang, 2004; revisado por Lu et al, 2005). Assim, caracteriza-se uma das principais funções do BDNF: o equilíbrio entre a morte e a sobrevivência neuronal (Figura 3).

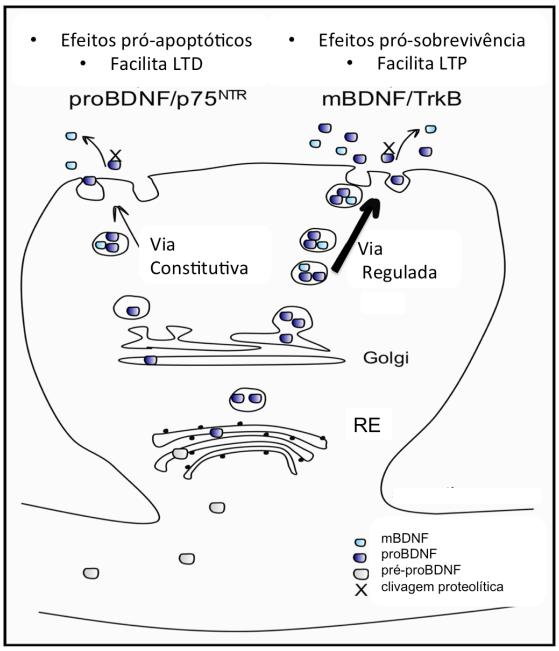


Figura 3. A imagem representa os processos de síntese, armazenamento e secreção do BDNF destacando o seu papel no equilíbrio entre a morte e a sobrevivência neuronal. O BDNF é sintetizado como uma proteína pré-proBDNF que é clivada no retículo endoplasmático (RE). O proBDNF resultante desta clivagem é armazenado no Complexo de Golgi em dois tipos de vesículas secretoras: vesículas da via constitutiva e vesículas da via regulada. O proBDNF armazenado nestas vesículas sofre clivagem proteolítica e é secretado como mBDNF (BDNF maduro) ou é secretado como proBDNF e sofre clivagem extracelular (os processos finais de clivagem estão representados na figura pelo símbolo ×). Uma vez liberado o proBDNF se liga, preferencialmente no receptor p75 e o mBDNF ao receptor TrkB pré e pós-sináptico ativando diferentes cascatas de segundos mensageiros e

desencadeando respostas celulares distintas. LTD (do inglês *long-term depression*); LTP (do inglês *long-term potentiation*) (Adaptada de Cunha et al, 2010).

Durante o desenvolvimento do SNC dos mamíferos, a expressão das neurotrofinas e seus receptores é marcadamente elevada e destaca-se seletivamente de acordo com a região cerebral e o estágio do desenvolvimento em que o indivíduo se encontra (Fryer et al, 1996; Knüsel et al, 1994; Tang et al, 2010). Especificamente em hipocampo de ratos, por exemplo, a imunoreatividade ao BDNF e ao TrkB são inicialmente detectáveis em DE 18 (Ernfors et al, 1992) e DE 13-18 (Ernfors et al, 1992; Fryer et al, 1996), respectivamente.

Como descrito anteriormente, a via de sinalização do BDNF exerce um papel fundamental no equilíbrio entre a morte e a sobrevivência neuronal (Teng et al, 2005; Pang et al 2004; revisado por Lu et al, 2005). Contudo, esta importante neurotrofina participa de muitos outros eventos celulares envolvidos na regulação do desenvolvimento dos circuitos neurais do encéfalo imaturo e adulto incluindo sua participação na diferenciação e sobrevivência de células-tronco neurais (Ahmed et al; 1995; Lachyankar et al, 1997; Shetty & Turner, 1998, revisado por Park & Poo, 2013), na diferenciação, crescimento e direcionamento axo-dendrítico (Shelly et al, 2007; Horch & Katz, 2002; Cohen-Cory & Fraser, 1995; McAllister et al, 1997; Wirth et al, 2003), no estímulo ao desenvolvimento das espinhas dendríticas (Tyler & Pozzo-Miller, 2003; Ji et al, 2010) e no crescimento dos neuritos (Ji et al, 2010). Além disso, o BDNF desempenha um papel crucial na formação (Vicario-Abejon et al, 1998) e maturação (Wang et al, 1995; Sallert et al, 2009) das sinapses por potencializar a neurotransmissão (Kang & Schuman, 1995; Tyler & Pozzo-Miller, 2001) e facilitar a plasticidade sináptica (Tartaglia et al, 2001; Tyler et al, 2002; Ji et al, 2010; revisado por Lu et al, 2008; Park & Poo, 2013). É interessante destacar que esta função exercida pelo BDNF, sugere a sua participação ativa sobre a formação e manutenção

dos processos cognitivos que começam na adolescência e continuam na vida adulta. De fato, estudos recentes têm demonstrado o envolvimento do BDNF na LTP o que o torna essencial para a cognição, uma vez que o bloqueio de sua sinalização compromete a persistência da memória (Alonso et al, 2002a, 2005; Bekinschtein et al, 2007).

Atualmente, há um grande número de evidências sugerindo que a disfunção ou perda sináptica é um dos principais fatores patofisiológicos envolvidos no desencadeamento de doenças neurodegenerativas, como a DA (Terry et al, 1991; revisado por Selkoe, 2002). Modelos para DA utilizando camundongos transgênicos que expressam APP (do inglês *amyloid precursor protein*) tem revelado que a perda sináptica e o declínio cognitivo apresentado pelos animais foram revertidos pela infusão de BDNF no córtex entorhinal (Nagahara et al, 2009) e que o prejuízo na sinalização do receptor TrkB contribuiu para os déficits mnemônicos observados nos animais (Kemppainen et al, 2012). Além disso, esta neurotrofina recuperou o prejuízo na plasticidade sináptica hipocampal induzida pelo peptídeo β-amilóide em camundongos (Zeng et al, 2010) e sua sinalização parece estar envolvida com baixos desempenhos de camundongos na tarefa de reconhecimento ao objeto em decorrência da idade (Costa et al, 2008a). Por estes motivos, a via de sinalização do BDNF tem sido alvo direto de investigações científicas em modelos de doenças neurodegenerativas.

Nos últimos anos, uma série de estudos tem associado prejuízos cognitivos decorrentes da idade (Costa et al, 2012; Sallaberry et al, 2013; Calabrese et al, 2013), induzidos por modelos experimentais da DA (Nagahara et al, 2009; Kemppainen et al, 2012; Rantamäki et al, 2013), dietas hipercalóricas (Tozuka et al, 2010; Moy &

McNay 2013; Noble et al, 2014), privação de sono (Zhang et al, 2013) ou estresse (Andero et al, 2012; Kwon et al, 2013) a disfunções na via de sinalização do BDNF. Curiosamente, Lee & Chao demonstraram que a exposição de cultura de neurônios hipocampais à adenosina ou a um agonista seletivo para os A_{2A}Rs (CGS 21680) promoveu a ativação dos receptores TrkB. Este evento de transativação foi neutralizado na presença de um antagonista seletivo para A_{2A}Rs (ZM 241385) indicando o envolvimento destes receptores no processo (Lee & Chao, 2001). Nos últimos anos, um número crescente de trabalhos tem evidenciado a relação existente entre a via de sinalização do BDNF e os A_{2A}Rs. Em 2004, Diógenes e colaboradores demonstraram que a ativação dos A_{2A}Rs facilitou a modulação da transmissão sináptica exercida pelo BDNF em fatias de hipocampo (Diógenes et al, 2004). Além disso, o envolvimento dos A_{2A}R nas ações positivas do BDNF sobre a plasticidade sinpática pode ser demonstrado diante da descoberta de que aumento na LTP promovido por esta neurotrofina em fatias hipocampais é dependente da ativação dos A_{2A}R pela adenosina endógena (Fontinha et al, 2008) e que o bloqueio destes receptores impede a facilitação da LTP mediada pelo BDNF (Diógenes et al, 2011; Jerónimo-Santos et al, 2014). A integração funcional entre A_{2A}Rs e o BDNF também pode ser observada em culturas corticais primárias de ratos, onde a ativação destes receptores regula a expressão e a liberação do BDNF (Jeon et al, 2011).

Embora a cafeína atue como um antagonista dos receptores de adenosina (revisado por Fredholm et al, 1999), e antagonistas seletivos dos A_{2A}Rs impeçam a ação facilitatória do BDNF sobre a transmissão sináptica e a LTP (Diógenes et al, 2004; 2011; Jerónimo-Santos et al, 2014), uma pesquisa recente demostrou que o BDNF exerce ação neuroprotetora contra a toxicidade induzida pelo peptídeo β-amilóide em cultura de neurônios por um mecanismo independente da ativação ou

inibição dos A_{2A}Rs (Jerónimo-Santos et al, 2015). Dessa forma, os mecanismos de ação que envolvem a participação do BDNF nos efeitos neuroprotetores da cafeína sobre os prejuízos cognitivos decorrentes da idade (Costa et al, 2012; Sallaberry et al, 2013), induzidos por estresse (Alzoubi et al, 2013), ou modelos experimentais para DA (Han et al, 2013) permanecem pouco esclarecidos. Porém, algumas evidências indicam a participação de diversas moléculas de sinalização como a Ca^{2+}/calmodulina cinase II (CaMKII, do inglês *Ca^{2+}/calmodulin-dependent protein*) (Alzoubi et al, 2013) e a proteína de ligação ao AMPc (CREB, do inglês *cAMP-response elemento-binding protein*) (Alhaider et al, 2011; Connolly & Kingsbury, 2010), ambas esseciais aos processos de aprendizado e memória e plasticidade sináptica.

No que diz respeito ao desenvolvimento do SNC, a administração de cafeína durante o período gestacional também provocou alterações na via de sinalização do BDNF. Embriões de ratos cujas mães foram expostas a 1 g/L de cafeína apresentaram redução nos níveis de BDNF corticais e TrkB hipocampais em DE 18 e aumento no imunoconteúdo do BDNF cortical em DE 20 (Mioranzza et al, 2014). Porém, estudos buscando observar o envolvimento desta via de sinalização com possíveis alterações comportamentais de animais adolescentes ou adultos expostos a cafeína durante as fases iniciais do desenvolvimento ainda são incipientes.

1. 3. 2. *Proteína ácida fibrilar glial (GFAP,* do inglês *glial fibrilar acid protein)*

A proteína ácida fibrilar glial (GFAP) é a principal proteína de filamentos intermediários (FI) de 8-9 nm e é encontrada especificamente em astrócitos e glias radiais (Bignami & Dahl, 1974). Foi descrita pela primeira vez em 1971 por Eng e colaboradores e observada mais tarde em casos clínicos de doenças

neurodegenerativas e desmielinizantes, como o DA e a Esclerose Múltipla (EM), respectivamente (Eng et al, 1971; revisado por Eng et al, 2000).

Por muito tempo a principal função da GFAP ficou restrita a estabilização estrutural dos processos astrocíticos, devido a regulação exercida sobre a motilidade e forma dos astrócitos. Porém, estudos mais recentes caracterizaram a sua utilização como um marcador de injuria do SNC e identificaram o seu envolvimento nos processos gliais associados à LTP e a formação sináptica (Emirandetti, 2006; McCall et al, 1996; revisado por Sofroniew, 2009). Quando o tecido cerebral sofre algum dano por trauma, infecção, insulto químico, isquemia ou doença neurodegenerativa, os astrócitos tornam-se reativos e respondem a esses insultos por um processo denominado astrogliose (revisado por Eng et al, 2000). A astrogliose é caracterizada pelo aumento dos filamentos intermediários e proliferação e/ou hipertrofia celular. Classicamente, o grau de severidade da astrogliose pode ser determinado proporcionalmente pelos níveis de GFAP que, quando muito elevados, resultam na formação de cicatrizes gliais (revisado por Pekny & Pekna 2004; Sofroniew, 2009).

As pesquisas que vem sendo realizadas ao longo das últimas décadas tem apresentado um papel dualístico para os astrócitos. Inicialmente a astrogliose foi relatada como um processo prejudicial visto que, a formação das cicatrizes gliais foi descrita por impedir a regeneração axonal (Fidler et al, 1999; McKeon et al, 1991; Smith-Thomas et al, 1994). Porém, novas pesquisas tem indicado que a própria formação das cicatrizes resulta na reorganização da arquitetura tecidual podendo isolar fisicamente a região que sofreu o insulto ou trauma (revisado por Pekny & Nilsson 2005; Silver & Miller, 2004; Sofroniew, 2009). Este papel dualístico também pode ser observado em relação ao estresse oxidativo, podendo os astrócitos reativos desempenharem um papel tanto neuroprotetor pela produção de glutationa (Chen et al,

2001), quanto neurotóxico pelo excesso de produção de espécies reativas de oxigênio em resposta a presença de citocinas (Hamby et al, 2006). Diante dessas considerações, pesquisas científicas mais recentes têm apresentado os astrócitos como futuros alvos potenciais na modulação terapêutica de deenças cerebrais como trauma e doenças neurodegenerativas (Myer et al, 2006; Wharton et al, 2009; revisado por Pekny et al, 2014;). Porém, os avanços têm sido lentos frente a necessidade primária de compreender mais profundamente os mecanismos funcionais associados ao papel dos astrócitos em situações fisiológicas e patológicas.

Durante o desenvolvimento, as células gliais desempenham um papel fundamental na diferenciação das células neurais, participam ativamente do processo de migração neuronal (revisado por Kriegstein & Alvarez-Buylla 2009) e auxiliam no direcionamento dos cones de crescimento (Hung & Colicos, 2008). Além disso, tanto no encéfalo imaturo quanto no adulto exercem um papel essencial na organização, poda (revisado por Burry, 1986) e plasticidade sináptica (Kang et al, 1998; Fellin et al, 2004; Henneberger et al, 2010; Fossat et al, 2012), liberam gliotransmissores (Bonasco et al, 2011; Zorec et al, 2012), fatores de crescimento (Rudge et al, 1992), respondem a liberação pré-sináptica de neurotransmissores (Porter & McCarthy, 1996) e na ausência de excitabilidade elétrica apresentam excitabilidade celular intrínseca baseada em flutuações de Ca^{2+} intracelular (Charles et al, 1991; Parri et al, 2001; Nett et al, 2002). Diante destas considerações importantes acerca do papel astroglial na manutenção e desenvolvimento cerebral, torna-se interessante a caracterização astrocitária identificando alterações morfológico-funcionais como, por exemplo, a astrogliose frente a presença de agentes modificadores sejam eles internos ou externos durante este período.

1. 3. 3. *Proteína associada a sinaptossoma-25 (SNAP-25,* do inglês *synaptosomal-associated protein-25*)

A formação das sinapses no SNC requer a participação de um grande número de proteínas complexas que organizadas em domínios de membranas especializados, promovem a liberação de neurotransmissores inibitórios e excitatórios (Tafoya et al, 2008). As vesículas carregadas com neurotransmissores fundem-se com a membrana plasmática pré-sináptica e liberam seu conteúdo na fenda. Este processo de liberação de neurotransmissores por meio da exocitose vesicular é repetido diversas vezes de forma muito acelerada e é mantido durante todo o tempo de vida da sinapse.

Um conjunto de proteínas denominadas SNARE (do inglês *Soluble N-ethylmaleimide-sensitive factor attachment protein receptor*) são determinantes para a transmissão sináptica por serem responsáveis pelo funcionamento da maquinaria de fusão vesicular que secreta os neurotransmissores (revisado por Sudhof, 2004). De acordo com sua localização, as SNAREs são classificadas como proteínas vesiculares ou de membrana plasmática (V-SNARE e T-SNARE, respectivamente) as primeiras exemplificadas pela sinaptofisina e sinaptobrevina e as segundas pela sintaxina e SNAP-25 (Figura 4).

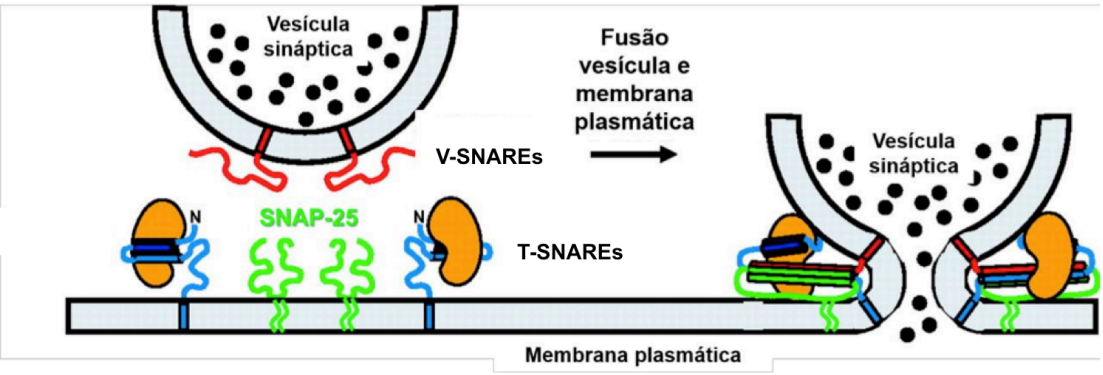


Figura 4. Localização sináptica das proteínas do complexo SNARE (V-SNARE e T-SNARE) envolvidas na fusão vesicular e liberação de neurotransmissores (Adaptada de Dulubova et al, 2007).

Nas últimas décadas, foram identificadas duas isoformas da SNAP-25: SNAP-25*a* e SNAP-25*b*. No encéfalo adulto, a expressão prevalente da SNAP-25*b* caracteriza a participação desta isoforma na transmissão sináptica. Por outro lado, a expressão predominante da SNAP-25*a* durante o período embrionário, sugere o envolvimento desta isoforma no crescimento axonal e na sinaptogênese (Boschert et al, 1996). Além disso, em um estudo com camundongos mutantes heterozigotos com baixos níveis de expressão da SNAP-25, observou-se que esta proteína não é necessária para a liberação do neurotransmissor independente do estímulo, mas é essencial para a transmissão sináptica evocada (Washbourne et al, 2002).

Os prejuízos cognitivos obsevados em modelos experimentais de convulsão (Cognato et al, 2010), diabetes (Duarte et al, 2012), DA (Canas et al, 2009) Transtorno de Déficit de Atenção e Hiperatividade (TDAH) (Barr et al, 2000, Brophy et al, 2002; Kustanovich et al, 2003; Mill et al, 2002), esquizofrenia (Young et al, 1998) e estresse crônico (Kaster et al, 2015) tem sido, frequentemente, acompanhados da perda de terminais nervosos e alterações na densidade da SNAP-25 (Cognato, et al 2010; Duarte et al, 2012; Canas et al, 2009). Além disso, animais mutantes para a SNAP-25 têm apresentado uma série de alterações comportamentais. A super expressão desta proteína em ratos, por exemplo, causou atraso na aquisição da memória espacial e redução no tempo de *freezing* no teste de medo condicionado (McKee et al, 2010). Já a substituição de um único aminoácido na SNAP-25 (utilizando a tecnologia *Knock-in*) em camundongos causou convulsões e promoveu fortes tendências ansiogênicas (Kataoka et al, 2011). A inibição da SNAP-25 através da infusão de um oligonucleotídeo *antisense* na região CA1 e CA3 prejudicou a consolidação e a formação da memória de ratos, respectivamente (Hou et al, 2004; 2006). Camundongos mutantes homozigotos apresentaram fenótipo embriogênico letal. Já os

heterozigotos com baixos níveis de expressão da SNAP-25 apresentaram hiperatividade moderada e prejuízo na memória associativa (Corradini et al, 2014). Assim, a utilização desta proteína como marcador de terminais nervosos, apontando possíveis disfunções sinápticas em diferentes estágios do desenvolvimento cerebral, pode facilitar a compreensão dos mecanismos que levam a danos ao SNC e colaborar para o desenvolvimento de novas estratégias farmacológicas contra a sinaptotoxicidade.

2. OBJETIVOS

2. 1. Objetivo geral

Avaliar o impacto do consumo de cafeína durante fases distintas do desenvolvimento de ratos sobre o comportamento e a densidade de proteínas essenciais para a maturação do SNC.

2. 2. Objetivos específicos

Avaliar por meio de testes comportamentais a atividade locomotora, a memória de reconhecimento e a ansiedade em:

- ratos machos adolescentes de 53 dias de vida expostos à 0,1, 0,3 ou 1,0 g/ L de cafeína durante a adolescência (DPN 28-52);

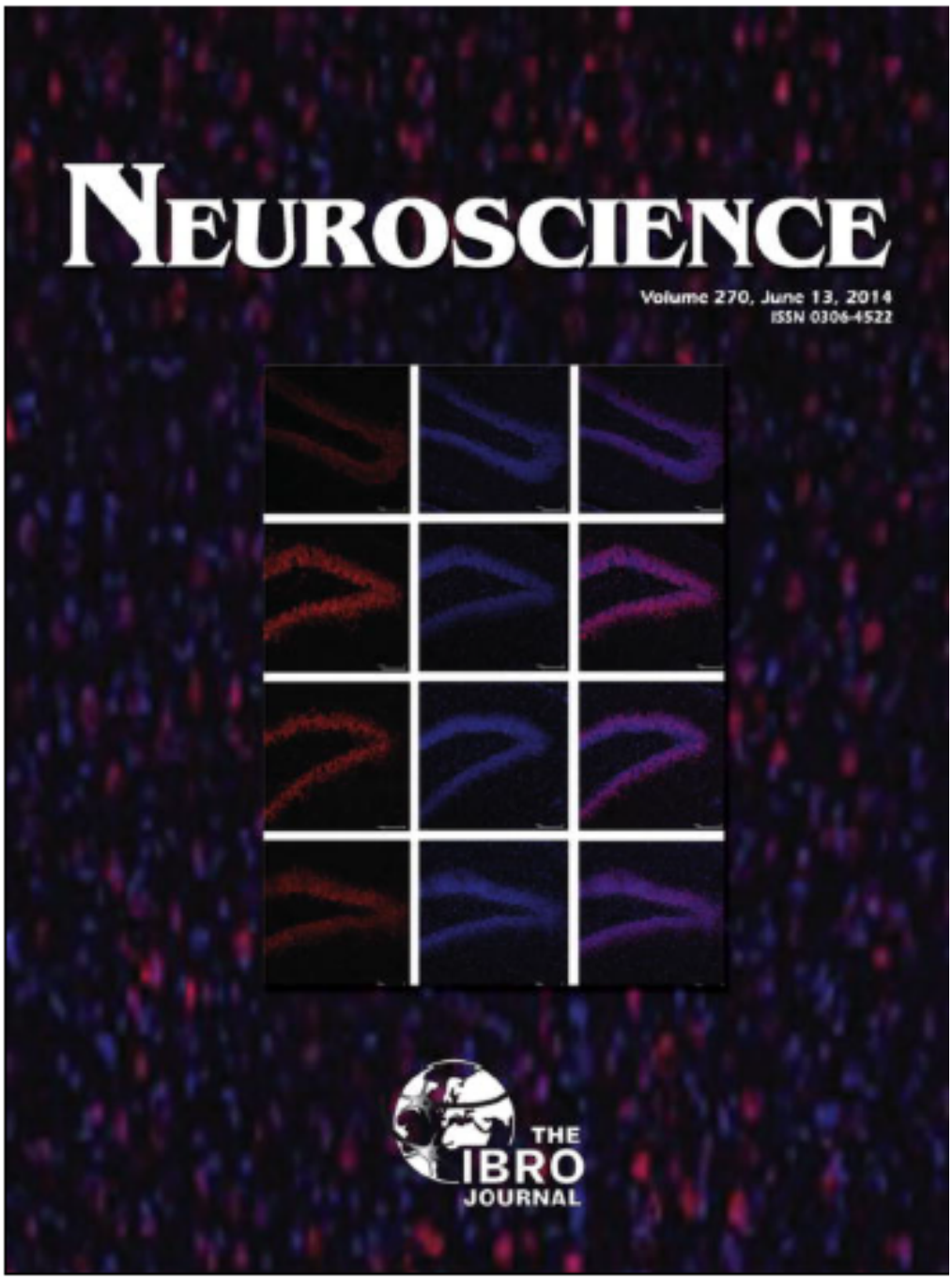
- ratos machos e fêmeas de 90 dias expostos à 0,1, 0,3 ou 1,0 g/ L de cafeína durante o período gestacional e lactacional;

- ratos machos e fêmeas de 90 dias expostos à 0,1, 0,3 ou 1,0 g/ L de cafeína durante toda a vida.

Quantificar e analisar por western blot o imunoconteúdo hipocampal e cortical de proteínas sinápticas (receptores de adenosina A_1, BDNF, proBDNF, TrkB, SNAP-25 e GFAP) e por imunohistoquímica a reatividade para GFAP e NeuN nas idades e administrações de cafeína referidas acima.

PARTE II

CAPÍTULO I



CAFFEINE TRIGGERS BEHAVIORAL AND NEUROCHEMICAL ALTERATIONS IN ADOLESCENT RATS

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CAFFEINE TRIGGERS BEHAVIORAL AND NEUROCHEMICAL ALTERATIONS IN ADOLESCENT RATS

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Abstract—Caffeine is the psychostimulant most consumed worldwide but concerns arise about the growing intake of caffeine-containing drinks by adolescents since the effects of caffeine on cognitive functions and neurochemical aspects of late brain maturation during adolescence are poorly known. We now studied the behavioral impact in adolescent male rats of regular caffeine intake at low (0.1 mg/mL), moderate (0.3 mg/mL) and moderate/high (1.0 mg/mL) doses only during their active period (from 7:00 P.M. to 7:00 A.M.). All tested doses of caffeine were devoid of effects on locomotor activity, but triggered anxiogenic effects. Caffeine (0.3 and 1 mg/mL) improved the performance in the object recognition task, but the higher dose of caffeine (1.0 mg/mL) decreased the habituation to an open-field arena, suggesting impaired non-associative memory. All tested doses of caffeine decreased the density of glial fibrillary acidic protein and synaptosomal-associated protein-25, but failed to modify neuron-specific nuclear protein immunoreactivity in the hippocampus and cerebral cortex. Caffeine (0.3–1 mg/mL) increased the density of brain-derived neurotrophic factor (BDNF) and proBDNF density as well as adenosine A_1 receptor density in the hippocampus, whereas the higher dose of caffeine (1 mg/mL) increased the density of proBDNF and BDNF and decreased A_1 receptor density in the cerebral cortex. These findings document an impact of caffeine consumption in adolescent rats with a dual impact on anxiety and recognition memory, associated with changes in BDNF levels and decreases of astrocytic and nerve terminal markers without overt neuronal damage in hippocampal and cortical regions. © **2014 IBRO. Published by Elsevier Ltd. All rights reserved.**

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Abbreviations: ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; DG, dentate gyrus; PBS, phosphate-buffered saline; PND, postnatal days.

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Key words: caffeine, adenosine, memory, psychostimulants, anxiety, BDNF.

INTRODUCTION

Caffeine is the most popular psychostimulant substance consumed worldwide, being found mainly in coffee and its psychostimulant effect is due to the antagonism of central adenosine A_1 and A_{2A} receptors (A_1R and $A_{2A}R$) (Fredholm et al., 1999). The actions of caffeine include the maintenance of alertness and arousal, decreased reaction times and increased vigilance and attention (Brice and Smith, 2002; Childs and de Wit, 2006). It is a matter of debate if caffeine has net cognitive enhancing properties or if its acute intake simply promotes a relief of withdrawal symptoms (Childs and de Wit, 2006; Rogers et al., 2013). Studies from our group and others found that the acute administration of caffeine improves the performance of adult rodents in various learning and memory tasks (Angelucci et al., 1999; Costa et al., 2008a; Botton et al., 2010), whereas chronic caffeine administration prevents mnemonic deficits in experimental models of Alzheimer's disease as well as age-related cognitive decline (e.g. Prediger et al., 2005; Arendash et al., 2006; Dall'Igna et al., 2007; Costa et al., 2008b; Espinosa et al., 2013; Sallaberry et al., 2013).

Notably, the impact of caffeine consumption during adolescence remains poorly investigated (Porciúncula et al., 2013). Thus is of particular relevance since there has been a substantial increase in caffeine consumption among children and adolescents over the past four decades (Harnack et al., 1999). This phenomenon has been associated with a recent surge of energy drink sales (Heckman et al., 2010), the search for better cognitive performance among college students and shifts in the circadian rhythm (Taylor et al., 2011). Caffeine is the main active ingredient of energy drinks and many of them contain 70–80 mg per 8-oz serving (~3 times the concentration in cola drinks) (Reissig et al., 2009; Seifert et al., 2011) prompting the emergence of caffeine intoxication (Clauson et al., 2008). While moderate caffeine use is "generally recognized as safe" by the FDA, this classification is largely based on studies conducted in adults. In fact, healthy people can tolerate moderate ingestions of caffeine, but heavy caffeine consumption has been associated with serious adverse health effects (Reissig et al., 2009; Seifert et al.,

2011). However, the threshold dose for caffeine intoxication in adolescents is unknown.

This question is particularly pertinent since adolescence is classically defined in rodents as the critical final period of cerebral maturation (Arain et al., 2013) and recent studies have shown that caffeine negatively impacts on neuronal migration and wiring of brain circuits during early development (Silva et al., 2013). Thus, we now evaluated the safety and behavioral impact of caffeine intake during adolescence by testing the impact of three different doses of caffeine in order to mimic low, moderate and moderate/high consumption in humans (Fredholm et al., 1999). Furthermore, we also gauged the impact of caffeine consumption on the density of proteins associated with cognition and synaptic integrity, such as BDNF, SNAP-25, NeuN and GFAP.

EXPERIMENTAL PROCEDURES

Animals

According to previous studies, the time window from postnatal days (PND) 28–60 is considered as the prototypic period during which rats of most breeding stocks exhibit typical adolescent characteristics (Spear, 2000; Schneider, 2013). In this study, male Wistar rats (28-day-old) were maintained at five per cage under a 12-h light/dark cycle (lights on at 7:00 A.M.), constant temperature (22 ± 1 °C) and with free access to food and water beverages (see below). All experimental procedures were designed to minimize the number of animals used and their suffering and were approved by the Committee on the Ethics of Animal Experiments of the Federal University of Rio Grande do Sul (CEUA-UFRGS – Protocol number 20332).

Caffeine treatment

The animals received caffeine (0.1, 0.3 or 1.0 g/L) dissolved in tap water only during the dark cycle, which is their active period, to mimic the pattern of caffeine consumption in humans. During the light cycle, the rats received water *ad libitum*. The night treatment with caffeine was maintained throughout the behavioral tasks (carried out during the day) to avoid both the acute impact of caffeine as well as the effects of caffeine withdrawal, which develops over a period of 24–48 h (Finn and Holtzman, 1986; Johansson et al., 1993). Although we did not quantify the plasma levels of caffeine, the chosen treatment regimens are thought to correspond to a low, moderate and high caffeine intake in humans, with effects believed to be mainly operated through antagonism of adenosine receptors (Fredholm et al., 1999). Fig. 1 summarizes the timeline of treatments and all the subsequent tests and manipulations of the rats in this study.

Behavioral analysis

All behavioral tests were performed between 7:00 A.M. and 12:00 P.M. The behavioral analysis was recorded by using a computer-operated tracking system (Any-maze, Stoelting, Woods Dale, IL) and was ranked by two observers blinded to the treatments. Since our tracking video system can record simultaneously 4 animals, rats from different groups were analyzed in parallel to decrease the impact of shifts of their circadian rhythm.

Open field. Rats (PND 48 and 49) were exposed to an open-field arena during two days in order to evaluate locomotor activity and non-associative learning. The first day corresponded to training and the second day to the test session, with an interval of 24 h. The apparatus was made of black-painted Plexiglas measuring 50 × 50 cm and was surrounded by 50-cm-high walls. Each rat was placed in the center of the arena and the distance traveled in meters was recorded during 10 min. The experiments were conducted in a sound-attenuated room under low-intensity light (12 lux); activity was recorded with a video camera positioned above the arena and monitored in an adjacent room by an observer blinded to the treatment of the animals. The open-field apparatus was cleaned after the end of each session.

Novel object recognition task. The object recognition test was carried out 24 h after the test session (second day) in the open-field apparatus, as previously described by our group (e.g. Costa et al., 2008a,b; Botton et al., 2010). Rats (PND 50) first underwent a training session, in which two identical objects were placed near the two corners of one side of the chamber. Rats were placed individually into the open field facing the center of the opposite wall and allowed to explore the objects during 5 min. The test session was performed 90 min after training and two dissimilar objects were presented, a familiar one and a novel one (Dere et al., 2005; Bevins and Besheer, 2006). Exploration was defined by directing the nose to the object at a distance of at least 2 cm and/or touching the object with the nose or forepaws. Rearing onto the object was not considered as exploratory behavior. The discrimination ratio was defined as: TN/(TN + TF), [TN = time spent exploring the novel object; TF = time spent exploring the familiar object]. After the end of each session, we cleaned both the objects as well as open-field apparatus.

Elevated plus maze task. The plus maze apparatus allows a pharmacologically validated measure of anxiety in rodents (Pellow et al., 1985). It consists of two 50 × 10-cm^2 open arms, and two 50 × 10 × 50-cm^3 enclosed arms, which are elevated to 50 cm (height) with an open roof arranged in such a way that the two arms of each type are opposite to each other. Each rat was placed in the central square facing an open arm. The number of entries in each arm (when all four paws had entered the arm), and time spent in each arm were recorded for 5 min. The experiments were conducted with a low-intensity red light, 24 h after the object recognition task. The maze was thoroughly cleaned before testing another animal.

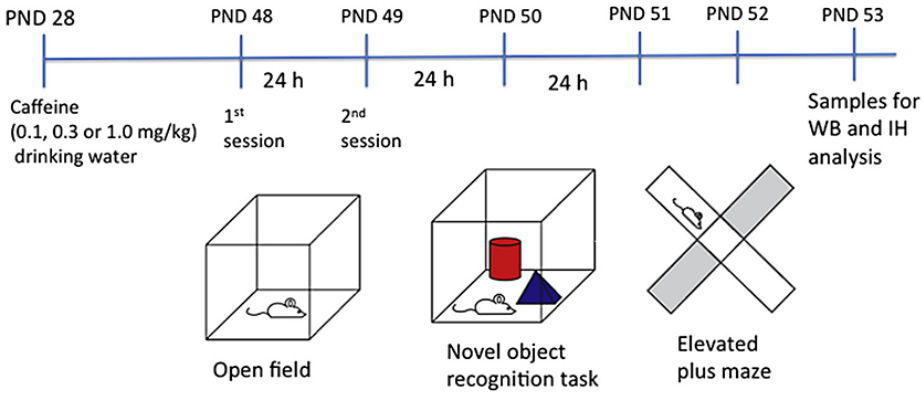

Fig. 1. Schematic overview of the experimental design. Caffeine treatment started from postnatal day 28 (PND 28) to PND 53. Caffeine was available only during the active cycle of the animals. All behavioral tests were carried out between 7:00 A.M. and 12:00 P.M. WB – Western blot; IH – immunohistochemistry.

Immunoblotting

Twenty-four hours after the behavioral tests, rats were sacrificed under anesthesia. The hippocampi and cerebral cortex were dissected out and immediately homogenized in a 5% SDS solution containing a cocktail of protease and phosphatase inhibitors (Sigma, São Paulo/SP, Brazil), and frozen at −20 °C. After defrost, the protein content was determined using the bicinchoninic acid assay (BCA) (Pierce, São Paulo/Brazil). The sample extracts were diluted to a final protein concentration of 2 μg/μL in SDS solution and the amount of protein applied for SDS–PAGE analysis was as follows: 40 μg for A_1R, 50 μg for GFAP and SNAP-25 or 80 μg for TrkB, proBDNF and BDNF. The proteins, together with pre-stained molecular weight standards (Bio-Rad, São Paulo/Brazil), were applied to a 8–12% SDS–PAGE running gel with a 4% concentrating gel. After electro-transfer, membranes were blocked with Tris-buffered saline containing 0.1% Tween-20 and 3% bovine serum albumin (BSA) during 1 h. The nitrocellulose membranes (Amersham, São Paulo/Brazil) were then incubated overnight at 4 °C with rabbit anti-A_1R antibody (1:1000; Affinity Bioreagents, USA), rabbit anti-GFAP antibody (1:2000; Sigma), rabbit anti-SNAP-25 antibody (1:2000; Sigma), rabbit anti-TrkB antibody (1:500; Upstate Cell Signaling, São Paulo/Brazil) mouse anti-proBDNF (1:500; Sigma) or mouse anti-BDNF (1:500; Sigma). The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature and developed with chemiluminescence ECL kit (Amersham). Densitometric analyses were performed using the NIH ImageJ software. After stripping, β-tubulin was quantified as a loading control using a mouse anti-β-tubulin antibody (1:1000; from Santa Cruz Biotechnologies, São Paulo/Brazil), as described above.

Immunohistochemistry

Twenty-four hours after the behavioral tests, rats were anesthetized with sodium pentobarbital and transcardially perfused with 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde diluted in PBS (Sigma). The brains were removed from the skull and post-fixed with 4% paraformaldehyde solution at 4 °C for 24 h. Coronal sections (50 μm) were obtained using a cryostat (CM1850 Leica Microsistemas, São Paulo/Brazil). Six free-floating sections at 400-μm interval were pre-incubated for 40 min in PBS with 1% BSA and 0.3% Triton X-100 and then incubated overnight at 4 °C with rabbit anti-GFAP antibody (1:500; Sigma) or mouse anti-NeuN (1:200; Chemicon, Millipore, São Paulo/Brazil) in PBS. After rinsing five times in PBS, sections were incubated with anti-rabbit secondary antibody conjugated to Alexa Fluor 488 fluorescent dye for GFAP or anti-mouse secondary antibody conjugated to Alexa fluor 594 for NeuN (1:500; Invitrogen, Biogen Porto Alegre/RS, Brazil) in PBS for 2 h at room temperature. After staining, sections were washed in PBS, counterstained with 0.0001% DAPI (Invitrogen) for 5 min, and rinsed again. Coverslips were mounted using a fluorescence mounting medium (Dako, São Paulo/Brazil). For fluorescence image analysis, frames from different hippocampal subfields – CA1, CA3, and dentate gyrus (DG) were created (406 × 334). All images were acquired using a fluorescence Nikon microscope with NIS Elements AR 2.30 software. The fluorescence intensities were quantified using the NIH ImageJ software. The light conditions and magnifications were kept constant and representative images were acquired using an Olympus IX70 confocal microscope with the Fluoview software.

Statistical analysis

Data were analyzed using a two-way analysis of variance (ANOVA) in the open field and novel object recognition to check for possible interactions between treatment and sessions (trials as repeated measures), followed by a Bonferroni post hoc test. A one-way ANOVA was also used and comparisons between groups were performed using Newman–Keuls Multiple Comparison Test. Paired Student's *t*-test was used for the open field when comparisons among days were necessary. Data were

expressed as means ± SEM and differences were considered statistically significant at $P < 0.05$.

RESULTS

Body weight

The average body weight of the rats at the beginning of caffeine administration was 87.36 ± 1.97 g. After 25 days of treatment, the average body weight of the rats consuming tap water was 224 ± 7.31 g, which was similar ($P > 0.05$) to the body weight of rats consuming caffeine at the doses of 0.3 mg/mL (231 ± 14.24 g) or 1.0 mg/mL (200 ± 12.83 g). Although using a different schedule of treatment, our data are in agreement with the previous study, reporting that the consumption of caffeine (1 mg/mL) has no significant impact on weight gain in adolescent rats (Rhoads et al., 2011).

Open-field analysis

In the training session, caffeine did not alter locomotor activity (Fig. 2A, first day, open bars). As a normal behavior, rats decrease the total distance traveled in the second exposure to the open field (test session) due to habituation to the apparatus. This habituation was observed in rats consuming tap water or receiving low (0.1 mg/mL) or moderate doses (0.3 mg/mL) of caffeine (Fig. 2A); a two-way ANOVA revealed an interaction between sessions and treatment [$F(3,54) = 6.228$; $P = 0.0010$]. By contrast, rats treated with caffeine (1.0 mg/mL) did not habituate, as evidenced by similar distance traveled in the training and test sessions (Fig. 2A). An analysis of the distance traveled in the peripheral zone revealed that there was a reduction during the test session (Fig. 2B) in animals consuming tap water and caffeine (0.1 or 0.3 mg/mL) and a two-way ANOVA confirmed an interaction between sessions and treatment [$F(3,54) = 4.80$; $P = 0.0049$]. By contrast, the animals that received caffeine (1.0 mg/mL) displayed a more discrete reduction in the distance traveled in the peripheral zone of the open field compared to other groups [$F(3,54) = 4.857$; $P < 0.005$] (Fig. 2B) together with an increased distance traveled in the central area of the apparatus during the test session (140%; $P < 0.05$ Fig. 2C).

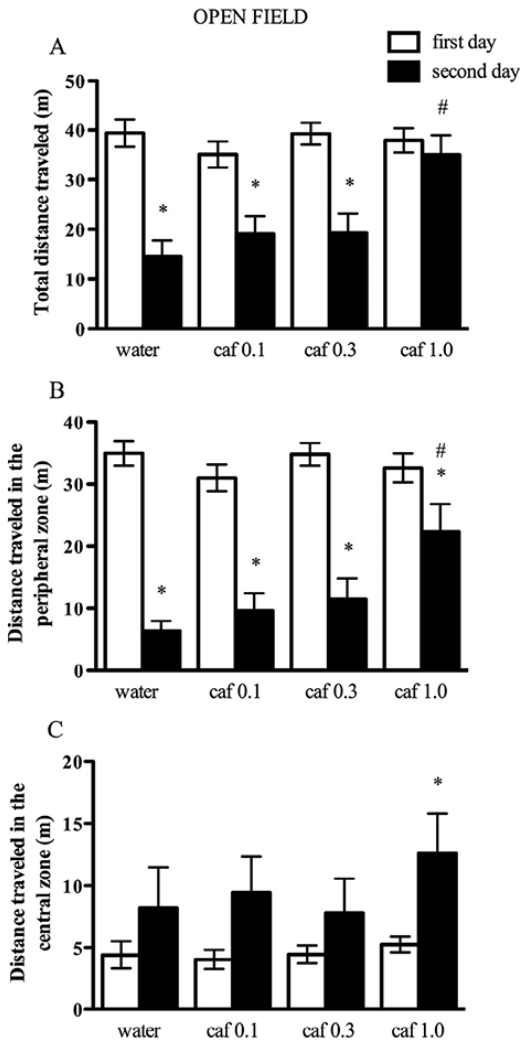

Fig. 2. Evaluation of locomotor activity and non-associative memory in adolescent rats treated with caffeine (0.1–1 mg/mL). The panels show the traveled distance in meters in the open-field apparatus during 10 min of video recording. (A) Total distance traveled. (B) Peripheral distance traveled. (C) Central distance traveled. Data are means + S.E.M. (n = 13–15 animals per group). $^{*}P < 0.05$ – differences between training in the first day (white bars) and test in the second day (white bars). (Paired t-test). $^{\#}P < 0.05$ – indicates differences between water and caffeine within the same day (two and one-way ANOVA).

Novel object recognition task

Recognition memory was assessed by the novel object recognition test (Fig. 3). A two-way ANOVA revealed no significant differences between groups in the time spent in the exploration of both objects during training and test sessions (90 min or 24 h later) (data not shown). However, a two-way ANOVA revealed a significant effect of trials when both short – [$F(1,36) = 6.82$; $P = 0.0130$] and long-term memory [$F(1,36) = 7.17$; $P = 0.0111$] were assessed. For short-term memory, the only difference between the training and test sessions was found in adolescent rats treated with caffeine at moderate (0.3 mg/mL; $t = 2.920$; $P = 0.0193$) or high doses (1.0 mg/mL; $t = 3.866$; $P = 0.0038$) (Fig. 3A). The same results were also found when long-term memory was assessed in rats treated with moderate ($t = 2.589$; $P = 0.0322$) or high dose of caffeine ($t = 5.180$; $P = 0.0006$) (Fig. 3B).

Elevated plus maze

The effects of low (0.1 mg/mL), moderate (0.3 mg/mL) or high doses of caffeine (1.0 mg/mL) on anxiety-related behavior were evaluated in the elevated plus maze. As a natural behavior, adolescent rats spend more time in the closed arms ($P = 0.0064$) (Fig. 4A, open bars). The three different doses of caffeine tested caused a similar decrease of the time spent in the open arms [$F(3,43) = 4.262$; $P = 0.0101$] along with a concomitant

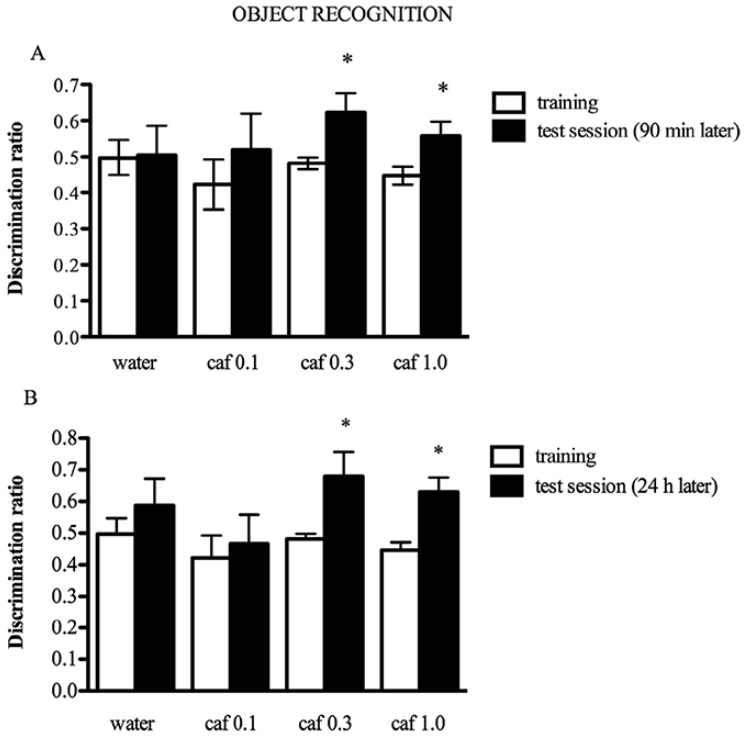

Fig. 3. Performance of the object recognition task in adolescent rats treated with caffeine (0.1–1 mg/mL). The panels show the object discrimination ratio in the training (white bars) and test session (black bars). (A) The test session was performed 90 min after training. (B) The test session was performed 90 min after training. Data are shown as means + S.E.M. of the discrimination ratio (n = 9–11 animals per group). $^{*}P < 0.05$ – differences between training and test sessions (two-way ANOVA and Bonferroni post hoc test).

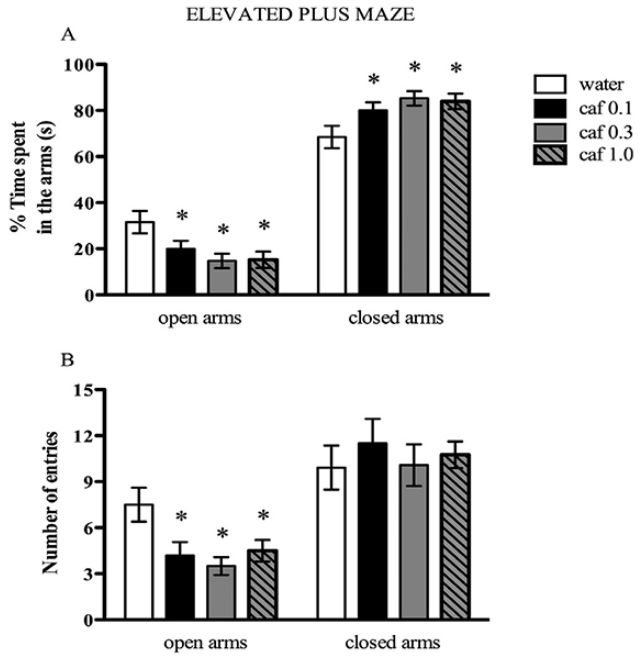

Fig. 4. Analysis of performance in the elevated plus maze by adolescent rats treated with caffeine (0.1–1 mg/mL). Panel (A) represents the percentage of time spent in the open and closed arms. Panel (B) shows the number of entries in the open and closed arms. The session lasted 5 min. Data are represented as means + S.E.M. (n = 11–12 animals per group). $^{*}P < 0.05$ – indicates differences between to water and caffeine groups (one-way ANOVA).

increase of the time spent in the closed arms [$F(3,44) = 4.60$; $P = 0.0112$] (Fig. 4A). When instead of the time spent, we evaluated the number of entries in both arms (Fig. 4B), it was found that adolescent rats drinking tap water did a similar number of entries in both arms ($P = 0.1270$) and the three tested doses of caffeine decreased the number of entries only in the open arms [$F(3,44) = 3.389$; $P = 0.0264$] (Fig. 4B).

Immunodetection of proteins in the hippocampus and cerebral cortex

The density of neuronal and astrocytic proteins, associated with the cognitive and emotional behaviors previously assessed, was analyzed in homogenates from the whole cerebral cortex or hippocampus. All tested doses of caffeine reduced the density of the astrocytic marker GFAP in the hippocampus [$F(3,31) = 3.694$; $P = 0.0221$] and cerebral cortex [$F(3,30) = 5.231$; $P = 0.0050$] (Figs. 5A and 6A, respectively). In addition, all tested doses of caffeine also decreased the density of the presynaptic marker SNAP-25 in the hippocampus [$F(3,30) = 7.576$; $P = 0.0006$] (Fig. 5A), whereas only the highest dose of caffeine decreased SNAP-25 density in the cerebral cortex [$F(3,31) = 3.985$; $P = 0.0164$] (Fig. 6A).

When we investigated the BDNF system, we found the highest dose of caffeine decreased the density of proBDNF [$F(3,29) = 8.029$; $P = 0.0005$] and of BDNF [$F(3,29) = 5.350$; $P = 0.0047$] in the hippocampus (Fig. 5B), whereas both moderate (0.3 mg/mL) and high doses of caffeine (1.0 mg/mL) increased proBDNF [$F(3,28) = 7.094$; $P = 0.0011$] and BDNF [$F(3,28) = 4.325$; $P = 0.0126$] in the cerebral cortex (Fig. 6B). By contrast, the three tested doses of caffeine did not affect the density of TrkB receptors in the hippocampus or cerebral cortex (Figs. 5B and 6B).

Finally we investigated if caffeine consumption affected the density of adenosine A_1 receptors (A_1R), which have been associated with the anxiogenic effects of caffeine (Jain et al., 1995; El Yacoubi et al., 2000; Prediger et al., 2004). All tested doses of caffeine increased the density of A_1R in the hippocampus [$F(3,25) = 3.586$; $P = 0.0278$], whereas only the highest dose of caffeine instead decreased A_1R density in the cerebral cortex [$F(3,32) = 3.629$; $P = 0.0232$] (Fig. 7).

Immunohistochemistry

Immunohistochemical analysis confirmed that all tested doses of caffeine decreased GFAP staining in the hippocampus, a decrease that was observed in all hippocampal subfields: CA1 [$F(3,11) = 7.88$; $P = 0.00044$], CA3 [$F(3,11) = 8{,}433$; $P = 0.00034$] and DG [$F(3,11) = 4.277$; $P = 0.0313$]. Caffeine at moderate (0.3 mg/mL) or high doses (1.0 g/L) also decreased GFAP immunoreactivity [$F(3,11) = 7.979$; $P = 0.0042$] in the motor cortex (Fig. 8A). By contrast to the observed caffeine-induced loss of the presynaptic marker (SNAP-25), none of the tested doses of caffeine caused an overt neuronal loss, as gauged by the lack of alteration of the immunoreactivity of neuron-specific nuclear protein (NeuN) by all tested doses of caffeine (Fig. 8B) ($P > 0.05$).

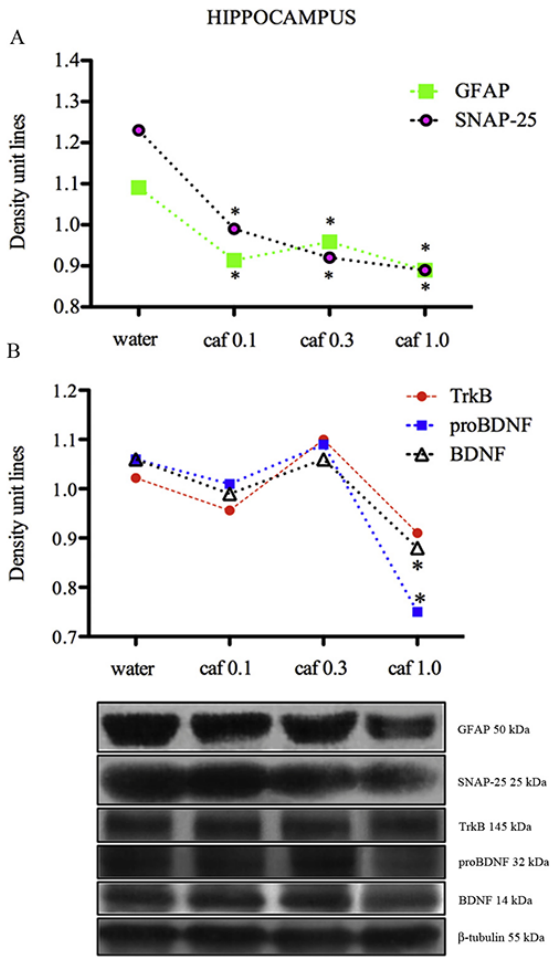

Fig. 5. The immunocontent of synaptic proteins from the hippocampus of adolescent rats treated with caffeine (0.1–1 mg/mL). Panel (A) shows the quantification of GFAP (green) and SNAP-25 (pink) and panel (B) shows the quantification of TrkB (red), proBDNF (blue) and BDNF (black) from the whole cortex of adolescent rats that received drinking water (water) or caffeine at three different doses (0.1, 0.3, 1.0 mg/mL). Data are displayed as means + S.E.M. (n = 8–10 animals per group) of density unit lines (normalized by the β-tubulin immunocontent). At the bottom of the figure are the representative Western blot bands for all proteins, including β-tubulin. $^*P < 0.05$ – indicates significant differences compared to water group (one-way ANOVA). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

DISCUSSION

The present study shows that the regular consumption of caffeine triggers morphological, behavioral and biochemical modifications in adolescent rats. Thus, all tested doses of caffeine decreased the immunoreactivity of an astrocytic marker (GFAP) as well as the density of a synaptic marker (SNAP-25), albeit they did not trigger an overt neuronal loss (lack of modification of the neuronal marker, NeuN). These caffeine-induced morphological modifications translated into modified behavioral profiles, namely an increase in anxiety

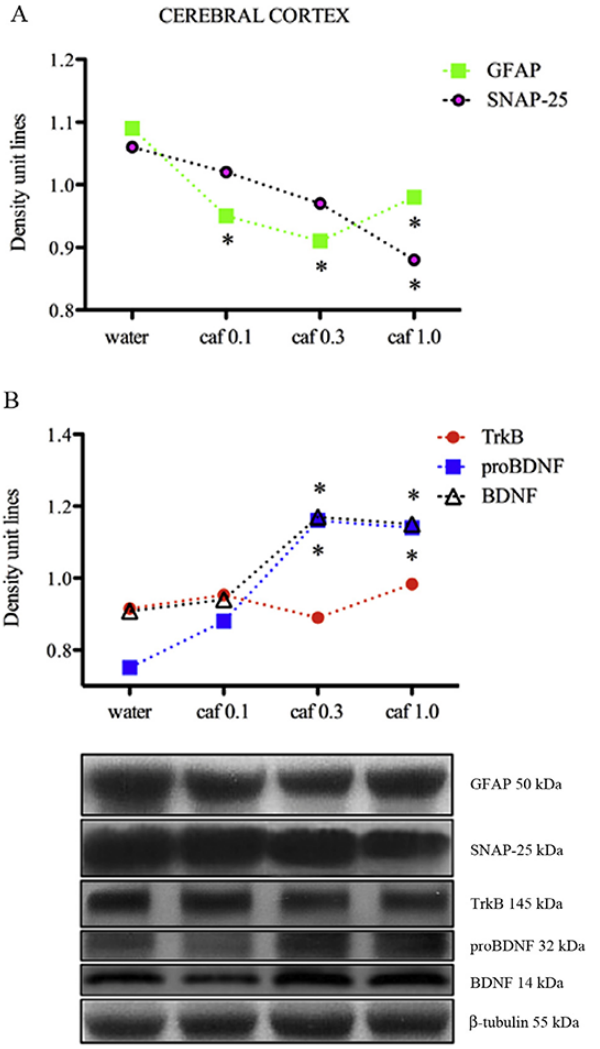

Fig. 6. The immunocontent of synaptic proteins from the cerebral cortex of adolescent rats treated with caffeine (0.1–1 mg/mL). Panel (A) shows the quantification of GFAP (green) and SNAP-25 (pink) and panel (B) shows the quantification of TrkB (red), proBDNF (blue) and BDNF (black) from the whole cortex of adolescent rats that received drinking water (water) or caffeine at three different doses (0.1, 0.3, 1.0 mg/mL). The data are displayed as means + S.E.M. (n = 8–9 animals per group) of density unit lines (normalized by the β-tubulin immunocontent). At the bottom of the figure are the representative Western blot bands for all proteins, including β-tubulin. $^{*}P < 0.05$ – indicates significant differences compared to water group (one-way ANOVA). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

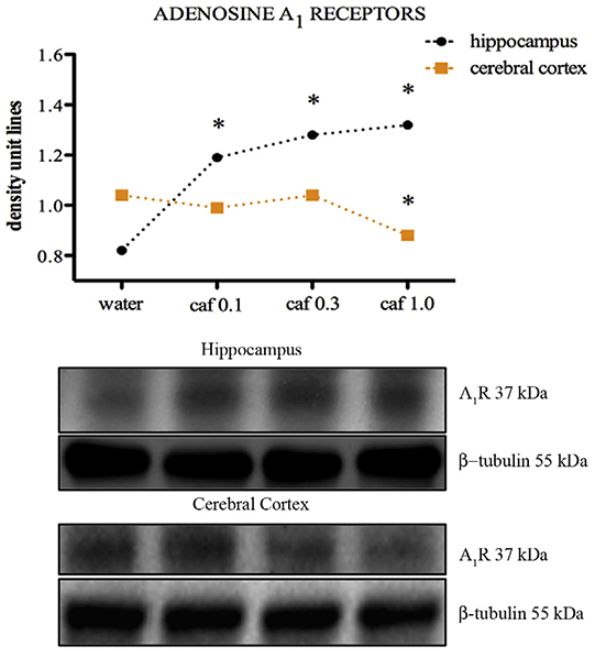

Fig. 7. Immunocontent of adenosine A_1 receptors in adolescent rats treated with caffeine (0.1–1 mg/mL). Quantification of adenosine A_1 receptors in the hippocampus and cerebral cortex from adolescent rats treated with caffeine. The data are displayed as means + S.E.M. (n = 7–10 animals per group) of the density unit lines (normalized by β-tubulin). At the bottom of the figure are the representative Western blot bands for A_1 receptors and β-tubulin. $^{*}P < 0.05$ – indicates significant differences compared to water group (one-way ANOVA).

evaluated in the elevated plus maze together with an increase recognition memory (object recognition) at moderate doses of caffeine and a decrease of habituation in the open field, a form of non-associate learning, at higher doses of caffeine. These behavioral changes were accompanied by biochemical changes in: (i) the density of adenosine A_1 receptors (A_1R), which was increased in the cerebral cortex and decreased in the hippocampus at higher caffeine doses; (ii) the BDNF modulation system, typified by an increase of BDNF and proBDNF levels in the cerebral cortex at moderate doses and a decrease of hippocampal BDNF and proBDNF levels at higher doses of caffeine.

One of the prominent responses to the acute administration of caffeine in rodents is the modification of locomotion (Snyder et al., 1981), but adult rodents display a tachyphylaxis to the locomotor effects of chronic caffeine consumption (Finn and Holtzman, 1986). In a previous report, adolescent male rats submitted to a chronic caffeine regime followed by 24–48 h of caffeine withdrawal, developed a significant decrease of the spontaneous locomotion in the open field, as well as a reduced motor activation when they received an acute caffeine challenge (30 mg/kg), which they interpreted as caffeine tolerance (Rhoads et al., 2011). Considering that the behavioral tasks were always performed during the 5 h after switching the caffeinated solution (dark phase) to water (light phase), it was unlikely that the behavioral outcomes were a manifestation of a withdrawal syndrome, which in adult rats require at least 24 h to develop (Holtzman, 1983). In support of this possibility, we did not find any alterations in the traveled distance during the first exposure to the open field and the number of entries in the closed arms of the elevated plus maze (as an index of locomotor activity) in adolescent rats exposed to a chronic, intermittent caffeine regime, delivered only during their active phase. This observation is also important to rule out changes of locomotion as a basis for other behavioral alterations displayed by adolescent

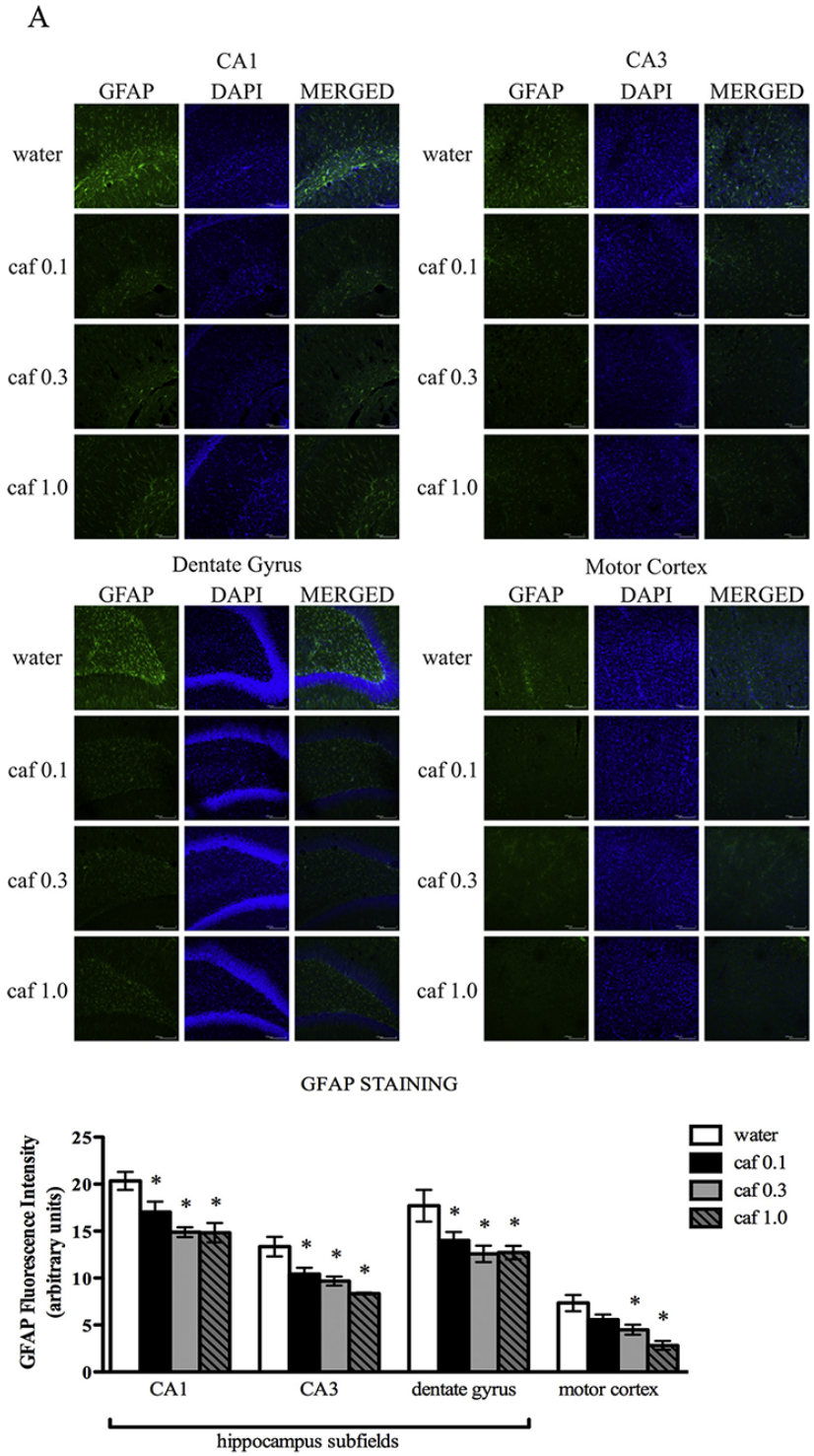

Fig. 8. Immunoreactivity for GFAP and NeuN in slices from rats treated with caffeine (0.1–1 mg/mL). Representative images of the hippocampal subfields (CA1, CA3 and dentate gyrus) and motor cortex for GFAP (green), NeuN (red) and DAPI (blue) staining. Panel (A) shows the quantification of GFAP staining and panel (B) the quantification of NeuN staining. Data are presented as means ± S.E.M (n = 5 animals per group; 6 slices/rat). Scale bar = 50 μm (20 × magnification). $^{*}P < 0.05$ – indicates difference from water group (one-way ANOVA). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

rats exposed to caffeine. One prominent alteration displayed by caffeine-consuming adolescent rats was their increased anxiety profile in the elevated plus maze, which was observed for all tested doses of caffeine. Caffeinism is associated with increased anxiety in humans (Greden, 1974) and the chronic administration of high-caffeine doses (50–100 mg/kg, twice a day) causes an anxiogenic effect in adult animals (El Yacoubi

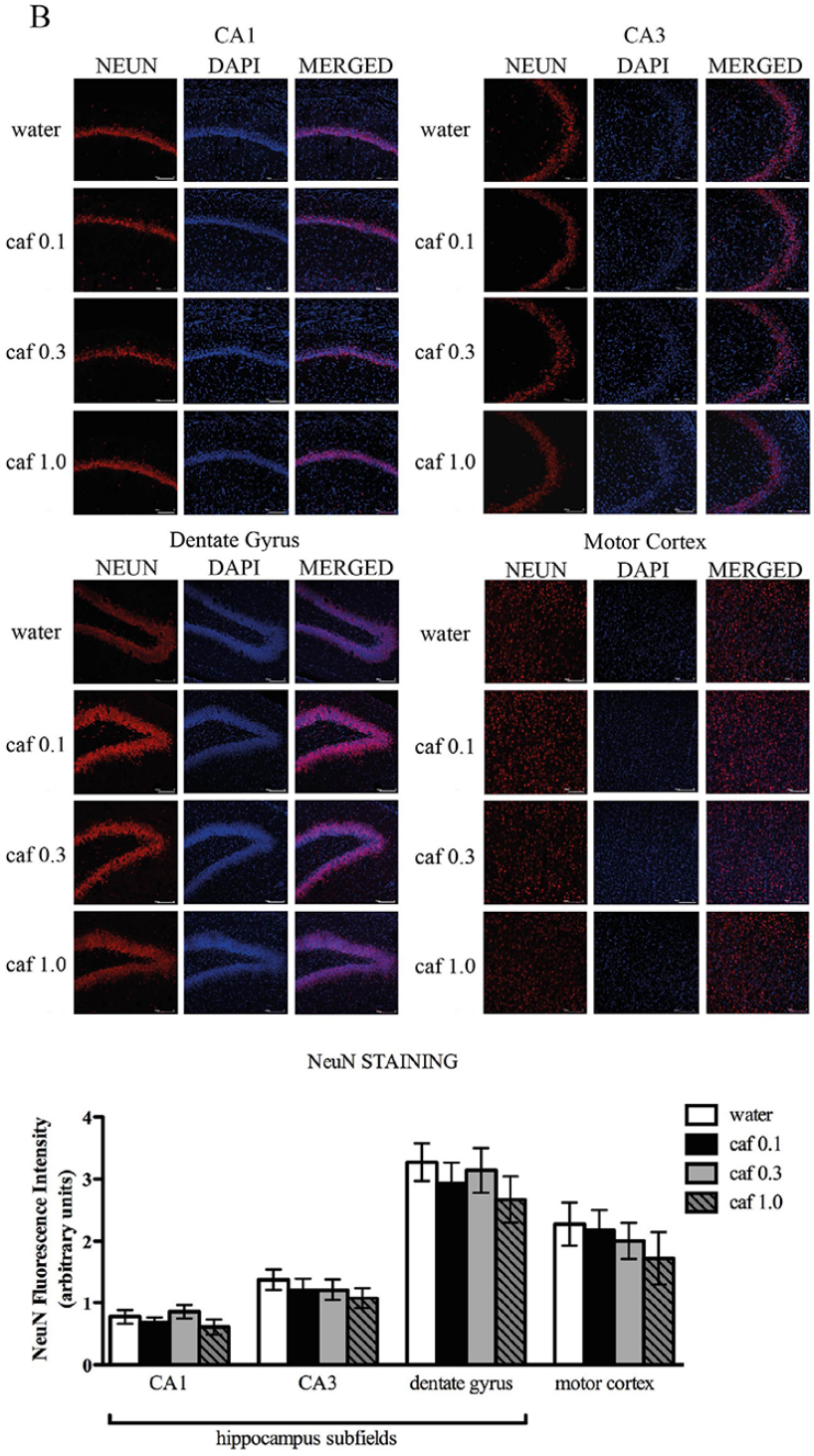

Fig. 8 (*continued*)

et al., 2000); but our data revealed an even greater susceptibility of adolescent rats to the anxiogenic effects of chronic caffeine consumption. This is particularly striking since adolescent rats are relatively resistant to behavioral changes in the elevated plus maze by exogenous variables (Doremus et al., 2004). Differently from adult rats (Bhattacharya et al., 1997), adolescent rats seem to be more refractory to the development of tolerance to the anxiogenic effects triggered by chronic caffeine. We also report that the chronic consumption of moderate doses of caffeine also resulted in beneficial behavioral responses in adolescent rats, since moderate and high

doses of caffeine improved performance in the object recognition test. Notably, adolescent rats drinking tap water performed poorly in the object recognition task, even though they are usually more responsive to novel stimuli and also habituate faster to a novel environment than adults (Douglas et al., 2003; Stansfield and Kirstein, 2006). Since all requirements to provide a good performance were followed as previously recommended for juvenile rats (Ennaceur and Delacour, 1988; Reger et al., 2009), we conclude that this might be a particular characteristic of Wistar rats, which display inferior performance in this task when compared to other strains (Andrews et al., 1995; Ennaceur et al., 2005). This was an actual advantage to highlight the clear and evident improvement of both short-term and long-term recognition memory afforded by chronic caffeine consumption in adolescent rats. Caffeine has been documented to affect learning and memory performance in adults either through a direct control of cognitive processes or by improving attention (reviewed in Takahashi et al., 2008; Cunha and Agostinho, 2010), although other alterations of emotionality might also play a role. In this context, chronic caffeine treatment improved memory (Y-maze test and object recognition task) and attention deficits (set-shifting test) in spontaneously hypertensive rats (SHR), a validated adolescent model of attention-deficit hyperactivity disorder (Pires et al., 2010; Pandolfo et al., 2013). Interestingly, we also observed that only the highest dose of caffeine (but not lower or moderate) decreased habituation in a repeated visit to the same environment, which represents a simple form of non-associative learning (Leussis and Bolivar, 2006). Considering that at the time of behavioral tasks caffeine were still present in the brains of adolescent rats, the lack of locomotor habituation during the second day in the open field of rats that consumed the highest caffeine concentration (1 mg/mL) could also be a reflection of the motor stimulant effects of caffeine due to the sustained blockade of A_1 and A_{2A} adenosine receptors within the CNS. In fact, the direct relationship between plasma caffeine concentrations and the magnitude of motor activation in adult rats has been found (Lau and Falk, 1994). In addition, pharmacokinetic studies revealed that following a single oral dose of caffeine (10 mg/kg) to 40-day-old male Sprague–Dawley rats, the average half-life of caffeine in the plasma is 120 min, and in the brain is 132 min (Latini et al., 1980). This pharmacokinetic parameter remains unchanged in 64-day-old male rats, in which the half-life of a single oral dose of caffeine (4 mg/kg) is 127 min (Bienvenu et al., 1990). In addition, the half-life of caffeine in the plasma and brain lengthens as the dose increases in adult rats (Latini et al., 1978) and this opens the possibility that this might occur in adolescent rats as well, particularly after drinking caffeine solutions at moderate (0.3 mg/mL) and high (1 mg/mL) concentrations.

Caffeine is a non-selective adenosine receptor antagonist with similar *in vitro* affinities for A_1 receptors (A_1R) and $A_{2A}R$ (Fredholm et al., 1999), the main adenosine receptors involved in memory normalization (Cunha and Agostinho, 2010) and in the motor-activating, reinforcing and arousal effects of this drug (Ferré, 2008). We now observed that all doses of caffeine caused an up-regulation of A_1R in the hippocampus of adolescent rats. Chronic caffeine has previously been documented to up-regulate A_1R in different brain areas in adult animals (e.g. Johansson et al., 1993). Notably, the manipulation of A_1R, which is the most abundant and widespread adenosine receptor in the brain (Fredholm et al., 2005), has a prominent impact on anxiety (El Yacoubi et al., 2000; Johansson et al., 2001; Prediger et al., 2004), suggesting that the caffeine-induced up-regulation of hippocampal A_1R might be associated with this caffeine-induced behavioral alteration in adolescent rats. In parallel, the highest tested dose of caffeine caused a down-regulation of A_1R in the cortex; this might be associated with the high caffeine-induced deficits of non-associative memory since chronic A_1R blockade worsens memory performance in adult rodents (von Lubitz et al., 1993; Vollert et al., 2013; but see Hauber and Bareiss, 2001). However, these observations do not rule out the additional involvement of $A_{2A}R$ blockade in alterations of emotional and memory behaviors caused by caffeine in adolescent mice, since $A_{2A}R$ have also been reported to be associated with these behavioral responses in adult mice (reviewed in Cunha et al., 2008; Cunha and Agostinho, 2010).

The neuromodulation system operated by BDNF has been shown to be modified by caffeine consumption (Costa et al., 2008a,b; Han et al., 2013; Sallaberry et al., 2013) and is well recognized to be tightly associated with the control of emotional behavior, namely anxiety, as well as learning and memory (reviewed in Greenberg et al., 2009). This involves a balanced signaling of BDNF through its high-affinity TrkB receptors and often opposite effects on synaptic plasticity operated by its precursor pro-BDNF through p75NTR (Greenberg et al., 2009). We now observed that caffeine consumption by adolescent rats leads to an increase of BDNF levels in the cerebral cortex, in parallel with an enhanced recognition memory and increased anxiety. This parallels the ability of caffeine to normalize the decreased BDNF levels and improve recognition animals in adult and aged-rats (Costa et al., 2008a,b; Sallaberry et al., 2013) and in a rodent model of Alzheimer's disease (Ham et al., 2013), as well as with the parallel normalization of both BDNF levels and of emotional behavior in adult transgenic mice without adenosine A_{2A} receptors (Wei et al., 2013), a putative target of chronic caffeine consumption (Cunha and Agostinho, 2010). We also observed that the highest dose of caffeine caused a decrease of BDNF and pro-BDNF selectively in the hippocampus of adolescent rats, which might be associated with the detrimental effect of this high dose of caffeine on non-associative learning, which involves hippocampal processing (e.g. Vianna et al., 2000). This contrasts with the primary involvement of the perirhinal and entorhinal cortices rather than the hippocampus in the discrimination of familiarity, which is essential for object recognition memory (Balderas et al., 2008; Song et al., 2011). Notably, these cortical regions undergo substantial postnatal development and reorganization in rats, monkeys and humans (Bachevalier and Beauregard, 1993; Brown and Aggleton, 2001), which parallels gradual increases of BDNF from weeks 3–12 in

the cortex, without significant changes in the hippocampus (Hill et al., 2012). Overall, these findings prompt the tentative hypothesis that the impact of caffeine on memory recognition in adolescent rats may be associated with cortical BDNF levels, whereas the detrimental effects of high doses of caffeine on non-associative learning may involve a decrease of hippocampal BDNF levels, a hypothesis that will require direct experimental confirmation.

One of the most evident changes observed in adolescent rats regularly consuming caffeine was a decrease of the cortical and hippocampal density of GFAP, an astrocytic marker, and of SNAP-25, a marker of nerve terminals. The loss of synaptic markers in limbic cortical regions has been identified as an early event associated with memory impairment in aging and in animal models of Alzheimer's disease (reviewed in Cunha and Agostinho, 2010). Younger animals with deficits in SNAP-25 also display a severe memory deficit (Hou et al., 2006), but the over-expression of SNAP-25 in adult rats also impairs memory-associated synaptic plasticity (McKee et al., 2010) and could be one of dysfunctions related with brain disorders such as attention deficit and hyperactivity disorders (Barr et al., 2000). Considering that memory processes are complex, even though moderate- and high-caffeine intake had improved recognition memory, the decrease in the SNAP-25 could be a reflex of alterations of neuronal connectivity in the hippocampus with implications for other aspects of learning and memory (Hou et al., 2004; Delgado-Martínez et al., 2007). Considering that higher caffeine doses impaired non-associative learning, when translating to human adolescents our data suggest that high doses of caffeine may have a negative impact in the learning at school.

Based on the parallel increased anxiety and decreased SNAP-25 density observed in adolescent rats treated with caffeine, the lower SNAP-25 levels could be also related to anxiety-related behavior since one of the behavioral consequences caused by decreased expression of SNAP-25 in mutant mice is the exacerbation of anxiety-related behavior (Kataoka et al., 2011). However, it is important to emphasize that this caffeine-induced loss of a synaptic marker might be indicative of an early sign of synaptotoxicity, suggesting an eventual greater susceptibility to brain insults, but was not accompanied by an overt neuronal loss as indicated by the lack of NeuN immunoreactivity. Whereas global neuronal markers such as NeuN are not affected, we observed a decrease of GFAP density in adolescent rats consuming caffeine, which might reflect adaptive changes in astrocytes (Middeldorp and Hol, 2011). Given that recent studies have highlighted changes in astrocytic function controlling emotional behavior (e.g. Rajkowska and Stockmeier, 2013) and memory performance in adult rodents (e.g. Suzuki et al., 2011) and that we have identified adenosine A_{2A} receptors, a purported target of caffeine (Cunha and Agostinho, 2010), in astrocytes (Matos et al., 2012a) controlling the main astrocytic energizing system (Na^+-K^+-ATPase) (Matos et al., 2013) and memory function in particular (Matos et al., 2012b), it is tempting to consider the possible central importance of astrocytes in the behavioral alterations caused by chronic consumption of caffeine by adolescent rats.

CONCLUSION

Overall this study documents the anxiogenic and recognition memory-enhancing properties of moderate doses of regular caffeine consumption in adolescent mice, together with the negative impact of higher caffeine doses on non-associative learning. The anxiogenic effect found in rats emerges as potential adverse effect of caffeine when translated for adolescent humans that consume energy drinks with a high content of this substance. Our neurochemical analysis suggests that the anxiogenic effects of caffeine might involve an up-regulation of hippocampal A_1R and a down-regulation of SNAP-25 together with a modified astrocytic function typified by a decreased density of GFAP in the hippocampus and cerebral cortex, whereas the enhanced recognition memory might involve an increased density of BDNF in the cortical areas. Furthermore, the decreased non-associative learning caused by higher caffeine doses might be associated with a decreased density of cortical A_1R and hippocampal BDNF levels. Finally, the observed decrease of a synaptic marker together with a decrease of an astrocytic marker by all tested doses of caffeine in adolescent rats, is suggestive of an increased susceptibility to noxious insults; this hypothesis, raising concerns about the consumption of caffeine during adolescence, ought to be experimentally confirmed in future studies, considering that caffeine consumption is growing in this population.

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CAPÍTULO II

CAFFEINE EXPOSURE DURING RAT BRAIN DEVELOPMENT CAUSES MEMORY IMPAIRMENT IN A SEX SELECTIVE MANNER THAT IS OFFSET BY CAFFEINE CONSUMPTION THROUGHOUT LIFE

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Research report

Caffeine exposure during rat brain development causes memory impairment in a sex selective manner that is offset by caffeine consumption throughout life

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HIGHLIGHTS

- Behavior and synaptic proteins were modified by caffeine throughout life and during development.
- Adult female rats receiving caffeine during development displayed impaired recognition memory.
- Caffeine at highest dose throughout life attenuates anxiety-related behavior in both sexes.
- Caffeine treatments modified locomotion in a different manner according to sexes.
- Caffeine treatments change BDNF and related proteins in the hippocampus from both sexes.

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ABSTRACT

Caffeine is the psychostimulant most consumed worldwide. In moderate doses, it affords a beneficial effect in adults and upon aging, but has a deleterious effect during brain development. We now tested if caffeine consumption by rats (0.1, 0.3, 1.0 g/L in the drinking water, only during active cycle and weekdays) during adulthood could revert the potentially negative effects of caffeine during early life. Thus, we compared caffeine intake starting 15 days before mating and lasting either up to weaning (development) or up to adulthood, on behavior and synaptic proteins in male and female rats. Recognition memory was impaired only in female rats receiving caffeine (0.3 and 1.0 g/L) during development, coincident with increased proBDNF and unchanged BDNF levels in the hippocampus. Caffeine in both treatment regimens caused hyperlocomotion only in male rats, whereas anxiety-related behavior was attenuated in both sexes by caffeine (1.0 g/L) throughout life. Both caffeine treatment regimens decreased GFAP (as an astrocyte marker) and SNAP-25 (as a nerve terminals marker) in the hippocampus from male rats. TrkB receptor was decreased in the hippocampus from both sexes and treatment regimens. These findings revealed that caffeine intake during a specific time window of brain development promotes sex-dependent behavioral outcomes related to modification in BDNF signaling. Furthermore, caffeine throughout life can overcome the deleterious effects of caffeine on recognition memory during brain development in female rats.

1. Introduction

Caffeine is the most widely consumed psychoactive drug worldwide, which acts through the antagonism of adenosine receptors to exert arousal effects [1]. A renewed interest in the central effects of caffeine emerged from observations that moderate doses of caffeine attenuate cognitive decline in aging [2–4], Alzheimer's disease [5–8] and neuropsychiatric disorders [9,10]. These beneficial effects

Abbreviations: BDNF, brain-derived neurotrophic factor; TrkB, tropomyosin receptor kinase B; GFAP, glial fibrillar acid protein; SNAP-25, synaptosomal-associated protein 25.

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of caffeine in adults have been associated with a normalization of synaptic activity by antagonism of adenosine A_{2A} receptors [11] and more recently with a control of astrocytic function [12,13]. Caffeine also exerts impact on the BDNF neuromodulation system in adulthood [2,4,14,15] and during brain development [16].

Although caffeine consumption affords general health benefits in the adult population, the safety of caffeine consumption during pregnancy and childhood remains controversial [17,18]. In animal studies, caffeine inhibited adult neurogenesis [19], altered the proliferation of neuronal precursors [20], promoted defective neural tube closures in chick and mouse embryos [21,22] and delayed GABAergic neurons migration [23]. These deleterious effects of caffeine during early brain development seem to trigger long-lasting deficits of brain function; thus, adult rodents that received caffeine during gestation and lactation through their dams, display reduced locomotor activity [24–26], anxiogenic-like profile [27] and impaired performance in learning and memory tasks [23,28,29].

Since caffeine has opposite effects on brain function during brain development (deleterious) and later in life (beneficial), we now tested if caffeine intake throughout life might offset the deleterious effects of caffeine during brain development. Furthermore, since sex differences have previously been reported for caffeine neuroprotection [8,30,31], we also probed for possible sex differences in the effects of caffeine during brain development and later in life.

2. Materials and methods

2.1. Animals

Female Wistar rats, 60–70 days of age, were mated within our colony at Federal University of Rio Grande do Sul. Animals from different ages and both sexes were maintained under 12 h light-dark-cycle (lights on at 7:00 AM), at constant temperature ($22 \pm 1\,^{\circ}C$) and with free access to food, water or caffeinated solution. All experimental procedures were designed to minimize the number of animals used and their suffering and were approved by the Committee on Ethics of Animal Experiments of the Federal University of Rio Grande do Sul (CEUA-UFRGS—Protocol number 20332).

2.2. Caffeine treatment

In order to mimic the pattern of caffeine intake by humans, caffeine (0.1, 0.3 or 1.0 g/L) was administered in the drinking water only during the dark cycle (lights off at 7:00 PM) and only on weekdays. The doses regimen corresponds to low, moderate and high caffeine intake in humans, which low and moderate intake acting selectively on adenosine receptors [1]. The treatment started 15 days before mating and lasted throughout the pregnancy cycle. Tap water was available during the light cycle (lights on at 7:00 AM). After birth, pups received caffeine or tap water from dams throughout the lactation period. Pups were weaned at postnatal day 21 (PND 21) and 3–4 rats were maintained per cage so that rats of the same litter and sex could be housed together. At PND 21, litters were separated by sex and divided into three groups: a pups that received only tap water; b pups that received caffeine up to weaning (development); c pups that received caffeine up to adulthood (throughout life). The timeline summarizes the schedule of administration and the subsequent behavioral and neurochemical analysis (Fig. 1).

2.3. Caffeine measurements

Three to four animals were separated and killed by rapid cervical dislocation and blood samples were collected as follows: a at the morning when replacing water by caffeine (7:00 AM); b at night when shifting to water again (7:00 PM). Samples were centrifuged at $4000 \times g$ for 10 min and the organic layer was lyophilized. Plasma levels of caffeine were analyzed using high-pressure liquid chromatography (HPLC). The chromatographic separation was achieved on a C18 Kinetex® ODS column (4.6 mm × 25 cm i.d., particle size 5 μm), UV detection at 280 nm. The quantification was achieved using a calibration curve and the internal standard was used to estimate the recovery rate of the extraction procedure.

2.4. Behavioral analysis

All behavioral tests were conducted in a sound-attenuated room under low-intensity light (12 lux) and recorded by means of a computer-operated tracking system (Any-maze, Stoelting, Woods Dale, IL) and ranked by two observers blind to the treatments. All procedures were carried out during the light cycle (7:00 to 12:00 AM).

2.5. Open field

The open field exposure was performed as previously described [7,14] in an apparatus made of black-painted Plexiglas, measuring 50 × 50 cm, surrounded by 50-cm-high walls. Each rat was placed in the center of the arena and the distance traveled in meters was recorded during 10 min.

2.6. Novel object recognition task

The object recognition test was carried out 24 h after the test session (second day) in the open field apparatus, as previously described [7,14]. Rats first underwent a training session, in which two identical objects were placed near the two corners at either end of one side of the chamber. Rats were placed individually into the open field facing the center of the opposite wall and allowed to explore the objects for 5 min. The test session was performed 90 min after training and two dissimilar objects were presented, a familiar and a novel one [32,33]. The exploration was defined by directing the nose to the object at a distance of less than 2 cm and/or touching the object with the nose or forepaws. Rearing on to object was not considered exploratory behavior. The discrimination ratio was defined as: TN/(TN + TF), [TN = time spent exploring the novel object; TF = time spent exploring familiar object].

2.7. Elevated plus maze

The elevated plus maze, a pharmacologically validated apparatus for the measurement of anxiety in rodents [34,35], was carried out as previously described [18], 24 h after the object recognition task. The maze consists of two $50 \times 10\,cm^2$ open arms, and two $50 \times 10 \times 50\,cm^3$ enclosed arms, which were elevated at 50 cm (height) with an open roof arranged in such a way that the two arms of each type were opposite to each other. Each rat was placed in the central square facing an open arm. The number of entries in each arm (when all four paws had entered the arm), and the time spent in each arm were recorded for 5 min.

2.8. Western blot

Twenty-four hours after the end of behavioral tests, rats were sacrificed under anesthesia. The hippocampi were dissected out and immediately homogenized in a 5% SDS solution containing a protease and phosphatase inhibitor cocktail (Sigma, São Paulo/SP,

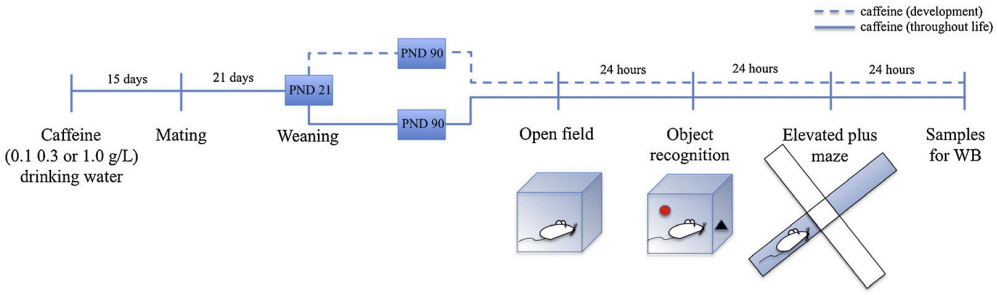

Fig. 1. Schematic overview of the experiment design. Female rats were habituated to caffeine solutions 15 days before mating. Caffeine was available during pregnancy and lactation. At the weaning day (PND 21) animals were divided into two groups: ---- caffeine development (treatment was interrupted at PND 21); —caffeine throughout life (treatment lasted up to adulthood). Caffeine was available only during the active cycle of the animals (lights off 7:00 P.M). All behavioral tests were carried out between 7:00 A.M. and 12:00 P.M. WB—western blot.

Brazil), and frozen at −20 °C. After defrost, the protein content was determined using the bicinchoninic acid assay (BCA, Pierce, São Paulo, Brazil). The extracts were diluted at a final protein concentration of 2 μg/μL in sample buffer and either 50 μg (for GFAP and SNAP-25) or 80 μg of protein (for TrkB, proBDNF and BDNF) were applied along with pre-stained molecular weight standards (Bio-Rad, São Paulo, Brazil) for SDS-PAGE analysis using 8 or 12% running gel at a 4% concentrating gel. After electro-transfer, membranes were blocked with Tris-buffered saline containing 0.1% Tween-20 and 3% bovine serum albumin (BSA) for 1 h. The nitrocellulose membranes (Amersham, São Paulo, Brazil) were then incubated overnight at 4 °C with rabbit anti-GFAP antibody (1:2000; Sigma), rabbit anti-SNAP-25 antibody (1:2000; Sigma), rabbit anti-TrkB antibody (1:500; Upstate Cell Signaling, São Paulo, Brazil) mouse anti-proBDNF (1:500; Sigma) or mouse anti-BDNF (1:500; Sigma). The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature and developed with chemiluminescence ECL kit (Amersham). Densitometric analyses were performed using NIH ImageJ software. β-Tubulin was used as loading control and was quantified using a mouse anti-β-tubulin antibody (1:1000; from Santa Cruz Biotechnologies, São Paulo, Brazil), as described above.

2.9. Statistical analysis

Data were analyzed by using One-way ANOVA followed by Newman–Keuls Multiple Comparison Test. Unpaired *t*-test was used to compare differences between sexes. Paired *t*-test was used object recognition task to compare training and test sessions within groups. Data are expressed as means + SEM and differences were considered statistically significant for $P < 0.05$.

3. Results

3.1. Caffeine measurements

Levels of caffeine were analyzed in the plasma collected in two time points: immediately before replacing caffeine by water (7:00 AM) and immediately before replacing water by caffeine (7:00 PM). Caffeine was not detectable (n/d) in rats treated with water. Caffeine levels were higher at the morning (7:00 AM) than at night (7:00 PM) in all doses administered (Table 1). Rats that consumed caffeine (0.1 and 0.3 g/L) revealed plasma concentrations that selectively act on adenosine receptors, while caffeine (1.0 g/L) intake revealed plasma levels that are acting also on phosphodiesterases [1].

Table 1
Caffeine concentration in the plasma collected at 7:00 PM and 7:00 AM.

Treatment	7:00 PM	7:00 AM
Water	n/d	n/d
Caffeine 0.1 g/L	6.7 μM (±2.34)	0.99 μM (±0.53)
Caffeine 0.3 g/L	27 μM (±9.63)	1.76 μM (±0.94)
Caffeine 1.0 g/L	131 μM (±35.80)	1.51 μM (±0.48)

Data are presented as means ± S.E.M of 3–4 independent plasma samples for each treatment and time point.
*n/d—non detectable.

3.2. Sex-dependent effects of caffeine in promoting hyperlocomotion

Sex differences in the water group were found for total traveled distance ($t = 3.182$; $P = 0.0028$) and traveled distance in the peripheral zone ($t = 4.099$; $P = 0.0002$) (Fig. $S1_{A,B}$). In male rats, the interruption of caffeine (0.3 and 1.0 g/L) at weaning increased both the total traveled distance [$F(3,51) = 8.856$; $P < 0.0001$] and the distance traveled in the peripheral zone [$F(3,51) = 6.277$; $P = 0.001$]; the consumption of all tested doses of caffeine throughout life also caused a similar increase of both total traveled distance [$F(3,51) = 4.165$; $P = 0.0103$] and in the peripheral zone [$F(3,51) = 4.204$; $P = 0.0098$] in male rats (Fig. 2A and C). In female rats, the intake of caffeine only during early life was devoid of effect in locomotor activity, whereas all tested doses of caffeine consumed throughout life increased the total traveled distance [$F(3,59) = 5.536$; $P = 0.002$] and the distance traveled in the central zone [$F(3,59) = 4.628$; $P = 0.0057$] (Fig. 2D and E).

3.3. Highest dose of caffeine throughout life attenuates anxiety-related behavior in both sexes

Female rats spent more time in the open arms than males ($t = 2.141$; $P = 0.0393$) (Fig. S2). No difference between sexes or treatment regimens was found in the total number of entries (Fig. S3A and B). The interruption of caffeine at weaning did not modify the time spent in the open arms in both sexes (Fig. 3A and B). However, caffeine throughout life (1.0 g/L) increased the time spent in the open arms either in both male [$F(3,47) = 5.214$; $P = 0.0034$] and female rats [$F(3,44) = 4.295$; $P = 0.0096$] (Fig. 3C and D).

3.4. Recognition memory is impaired in female rats receiving caffeine only during development

Considering that all groups presented differences between training and test session, no alteration was found in the performance of the object recognition task for male rats that consumed caffeine up to weaning or throughout life (Fig. 4A and C), How-

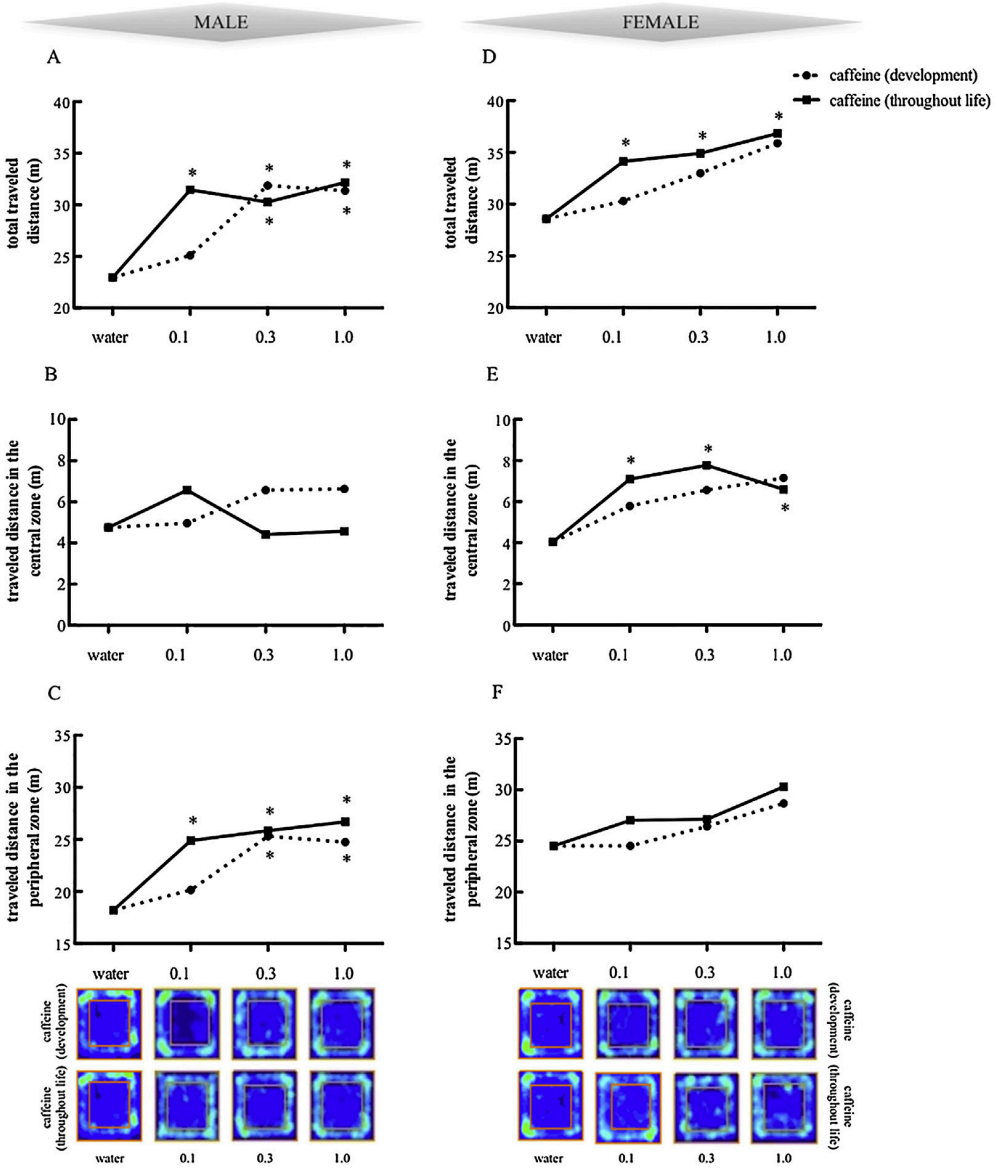

Fig. 2. Locomotor activity displayed by adult male and female rats treated with caffeine (0.1, 0.3 or 1.0 mg/mL) during development (----) or throughout life (—). Panels show the traveled distance in meters in the open field apparatus during ten minutes of video recording. (A and D)—Total traveled distances in meters (m); (B and E)—traveled distance at the center of the open field; (C and F)—traveled distance in the peripheral area. The schematic figure at the bottom represents the occupancy plots of total traveled distance for each group. Data are means + S.E.M. (n = 11–24 animals per group). *P < 0.05–different from the water group (One-way ANOVA, Newman–Keuls post hoc test).

ever, female rats consuming caffeine (0.3 and 1.0 g/L) only during development presented impairment in the object recognition task (Fig. 4B), since no differences were found between training and test session. Remarkably, female rats that consumed caffeine throughout life had a normal performance (Fig. 4D).

3.5. BDNF and related proteins are affected by caffeine in both sexes

The consumption of caffeine decreased the density of TrkB receptors in the hippocampus both male and female rats, both when caffeine was consumed during development (up to weaning) [males: F (3,24) = 4.754, P = 0.0097; females: F (3,24) = 6.841; P = 0.0017] and throughout life [males: F (3,25) = 6.407; P = 0.0023; females: F (3,24) = 10.75; P = 0.0001] (Fig. 5). The levels of proBDNF in the hippocampus were increased in females by caffeine consumption both during development [F (3,24) = 5.322; P = 0.0059] and throughout life [F (3,23) = 4.863; P = 0.0096], whereas the levels of proBDNF in the hippocampus of male rats were unaltered by both schedules of caffeine consumption (Fig. 5). The most striking difference was in the levels of BDNF in the hippocampus: they were unchanged in male rats with both schedules of caffeine consumption as well as in female rats consuming caffeine during development (Fig. 5A–C), but they were increased in female rats consuming caffeine throughout life [F (3,21) = 15.69; P = 0.0001] (Fig. 5D).

In the cortex from male rats receiving caffeine (0.3–1.0 g/L g) during development proBDNF was increased [F (3,22) = 5.729; P = 0.047], and TrkB receptor levels were reduced in all doses [F (3,22) = 3.994; P = 0.0206] (Fig. 6A). Female rats presented an increase in BDNF at 0.3 and 1.0 g/L caffeine doses [F (3,22) = 6.093; P = 0.0035 (Fig. 6C)]. Caffeine throughout life increased BDNF

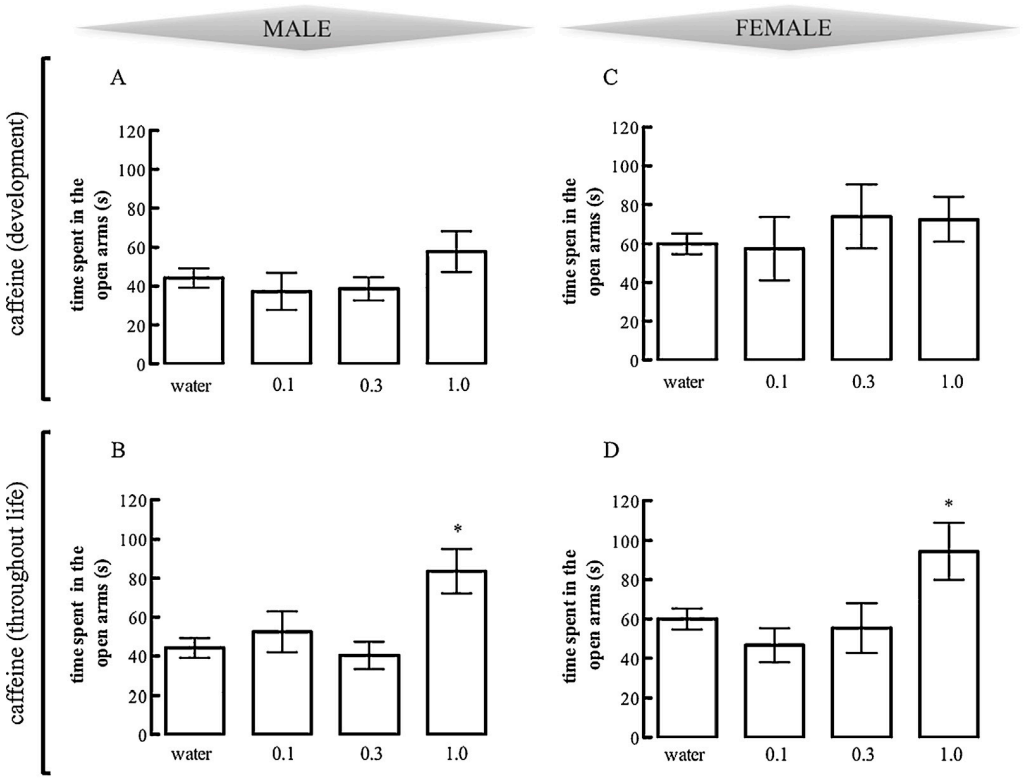

Fig. 3. Performance in the elevated plus maze for adult male and female rats treated with caffeine (0.1, 0.3 or 1.0 mg/mL). (A and B)—time spent in the open arms for male and female receiving caffeine during development; (C and D)—time spent in the open arms for males and females receiving caffeine throughout life. Data are represented as means + S.E.M. of the time spent in seconds (n = 11–21 animals). *P < 0.05—differences between water and caffeine groups (One-way ANOVA, Newman–Keuls post hoc test).

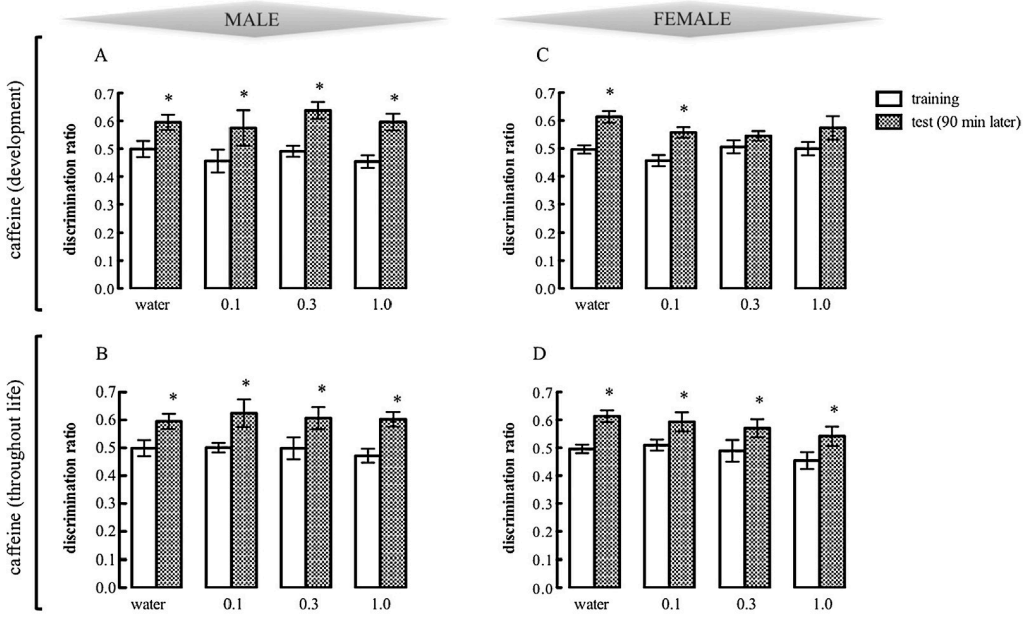

Fig. 4. Performance of the object recognition task for adult male and female adult rats treated with caffeine (0.1, 0.3 or 1.0 mg/mL). Panels show the discrimination ratio in the training (white bars) and test session (black bars). The test session was performed 90 min after training. (A) Male and (C) Female rats—caffeine during development; (B) Male and (D) Female—caffeine throughout life. Data are means + S.E.M. of the discrimination ratio (n = 11–24 animals). *P < 0.05—differences between training and test sessions (Paired t-test).

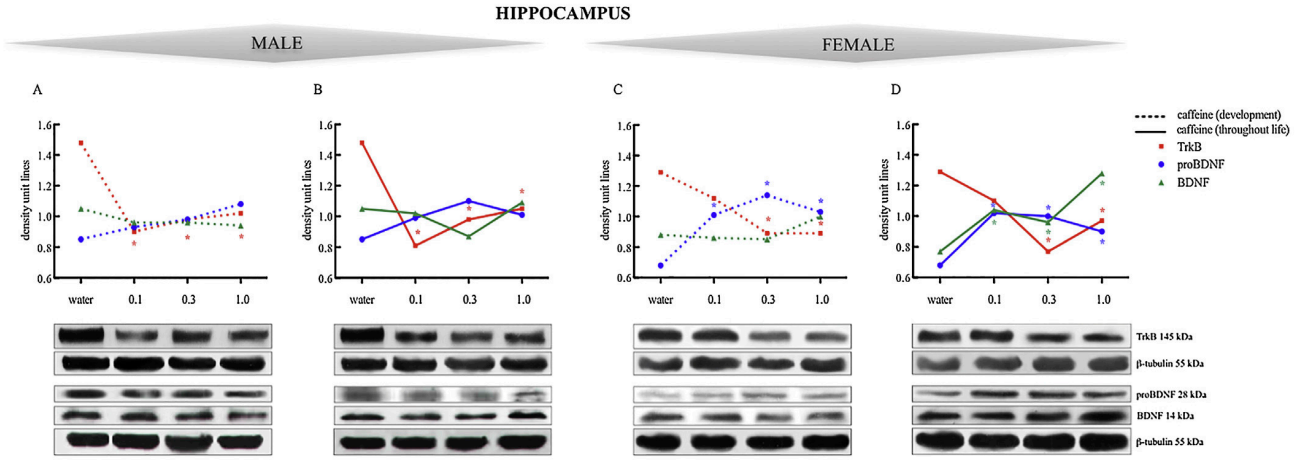

Fig. 5. The immunocontent of TrkB, proBDNF and BDNF in the hippocampus from adult male and female rats. Caffeine (0.1, 0.3 or 1.0 mg/mL) treatment during development (----) or throughout life (—). (A and B)—TrkB, proBDNF and BDNF from male hippocampus; (C and D) TrkB, proBDNF and BDNF from female hippocampus. Data are represented as means (n = 6–9 animals per group) of density unit lines (normalized by the β-tubulin immunocontent). At the bottom of the figure are the representative bands for all proteins. *P < 0.05—different from the water group (One-way ANOVA, Newman–Keuls post hoc test).

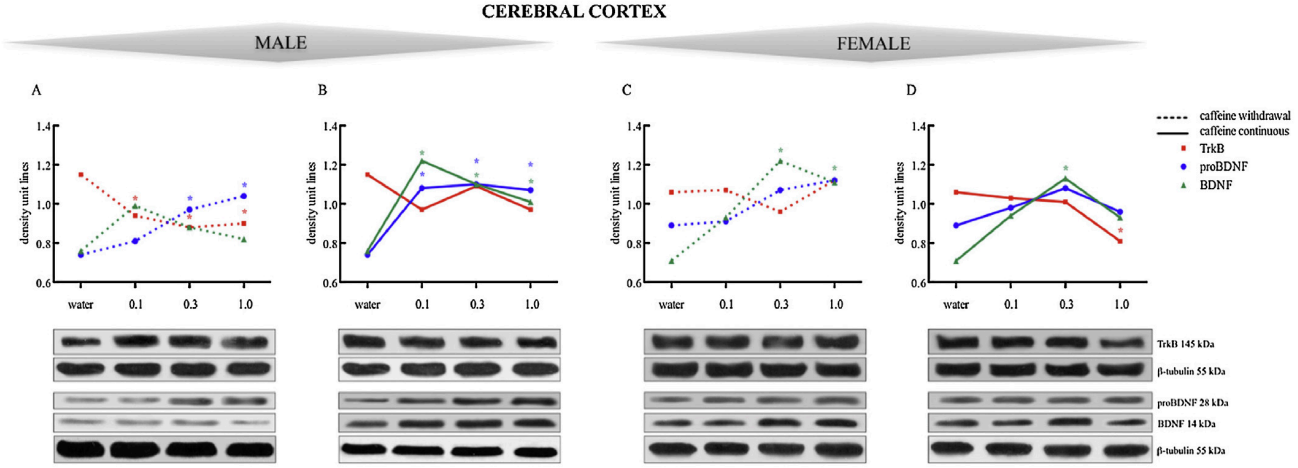

Fig. 6. The immunocontent of TrkB, proBDNF and BDNF in the cortex from adult male and female rats. Caffeine (0.1, 0.3 or 1.0 mg/mL) treatment during development (----) or throughout life (—). (A and B)—TrkB, proBDNF and BDNF from male cortex; (C and D) TrkB, proBDNF and BDNF from female cortex. Data are represented as means (n = 6–9 animals per group) of density unit lines (normalized by the β-tubulin immunocontent). At the bottom of the figure are the representative bands for all proteins. *P < 0.05—different from the water group (One-way ANOVA, Newman–Keuls post hoc test).

[F (3,25) = 5.443; P = 0.0051] and proBDNF [F (3,25) = 4.697; P = 0.0098] in males, with no effect in TrkB receptors (Fig. 6B). In females, BDNF was also increased by caffeine at 0.3 g/L [F (3,23) = 3.505; P = 0.0315], while TrkB levels were reduced at 1.0 g/L [F (3,26) = 3.737; P = 0.0234] (Fig. 6D).

3.6. GFAP and SNAP-25 are affected by caffeine in male rats

In the hippocampus of male rats, the GFAP immunocontent was reduced at all doses by caffeine consumption during development (up to weaning) [F (3,23) = 3.814; P = 0.0236] (Fig. 7A), as well as by caffeine consumption throughout life [F (3,23) = 9.670; P = 0.0003] (Fig. 7B). This contrasted with female rats, where the levels of GFAP were unaltered by both schedules of caffeine consumption (Fig. 6C and D).

The situation was similar for the levels of SNAP-25: in male rats, SNAP-25 levels were unaltered at all doses by caffeine consumption during development (up to weaning) (Fig. 7A). However, SNAP-25 levels were reduced by caffeine consumption throughout life [F (3,25) = 4.125; P = 0.0166] (Fig. 7B); this contrasted with female rats, where SNAP-25 levels were unaltered by both schedules of caffeine consumption (Fig. 7C and D).

In the cortex from male rats, caffeine 0.3 and 1.0 g/L during development increased GFAP [F (3,27) = 10.39; P = 0.0001] and SNAP-25 [F (3,28) = 5.673; P = 0.0036] levels (Fig. 8A). The same increase was also observed in all doses for both GFAP and SNAP-25 immunocontent [F (3,28) = 4.987; P = 0.0068] and [F (3,30) = 5.721; P = 0.0032], respectively (Fig. 8B). No difference was found in female rats (Fig. 8C and D).

4. Discussion

The present study characterized sex differences in the impact of caffeine on memory performance and locomotion, but not of anxiety, and defined the ability of caffeine consumption throughout life to selectively reverse the memory impairment caused by exposure to caffeine during brain development, which was associated with an increase of BDNF levels in the hippocampus.

The exposure to caffeine throughout life caused an hyperlocomotor profile in both sexes, which was proportionally larger in

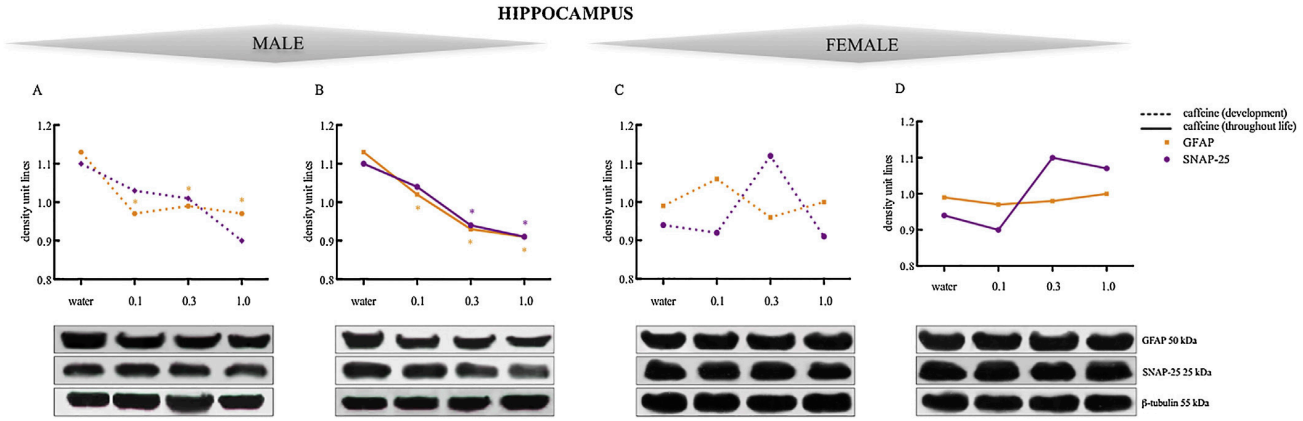

Fig. 7. Quantification of GFAP and SNAP-25 in the hippocampus of adult male and female rats. Caffeine (0.1, 0.3 or 1.0 mg/mL) treatment during development (----) or throughout life (—). (A and B)—GFAP and SNAP-25 from male hippocampus; (C and D)—GFAP and SNAP-25 from female hippocampus. Data are represented as means (n = 6–9 animals per group) of density unit lines (normalized by the β-tubulin immunocontent). At the bottom of the figure are the representative bands for all proteins. *P < 0.05—different from the water group (One-way ANOVA, Newman–Keuls post hoc test).

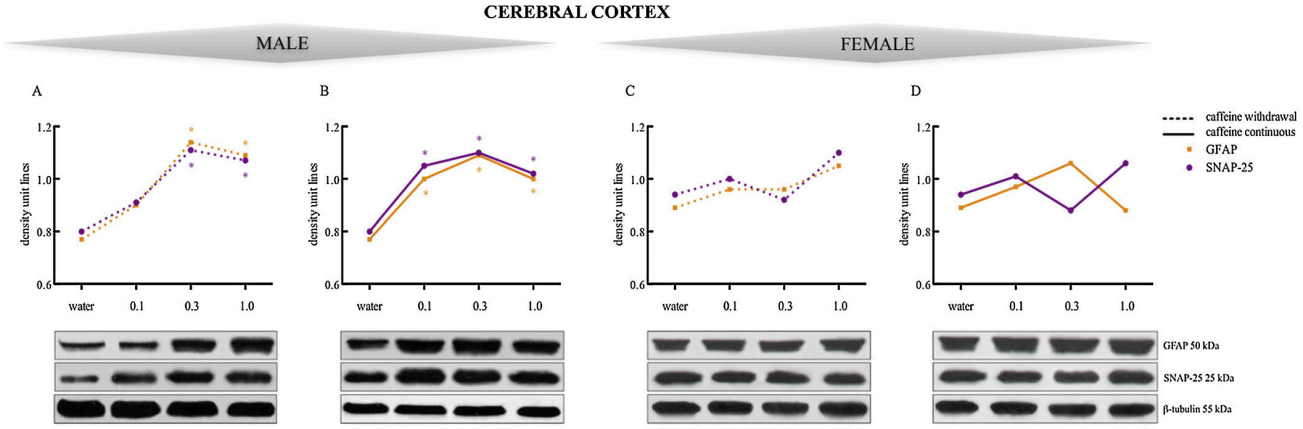

Fig. 8. Quantification of GFAP and SNAP-25 in the cortex of adult male and female rats. Caffeine (0.1, 0.3 or 1.0 mg/mL) treatment during development (----) or throughout life (—). (A and B)—GFAP and SNAP-25 from male cortex; (C and D)—GFAP and SNAP-25 from female cortex. Data are represented as means (n = 6–9 animals per group) of density unit lines (normalized by the β-tubulin immunocontent). At the bottom of the figure are the representative bands for all proteins. *P < 0.05—different from the water group (One-way ANOVA, Newman–Keuls post hoc test).

males in agreement with previous reports [25,36,37], although females traveled greater distances than males in the open field, as previously noted [25,36,38]. When evaluating anxiety-related behavior, we observed that caffeine consumption did not alter the behavior in the elevated plus maze [39], although female rats spent more time in the open arms, as previously reported [34,40,41]. The most striking sex difference associated with caffeine exposure was on memory performance in the object recognition task. In fact, only adult female rats receiving intermitent caffeine during development displayed a worse recognition memory. By contrast, male rats were unaffected by our intermitent caffeine exposure during development. This contrasts with the effect of the continuous exposure to caffeine during pregnancy that impaired recognition and working memory (but not in spatial memory), in both adult male and female rats [29], and also impaired spatial and recognition memory in adult male mice that were continuously exposed to caffeine during pregnancy up to postnatal day 15 [29]. This confirms that caffeine exposure during brain development can cause long-term detrimental effects on memory performance, but the difference susceptibility of males and females might depend on the schedule of caffeine intake. Evidences from animal and clinical studies have pointing to a more susceptibility to the effects of other psychostimulants for females than males [42,43]. In humans, some studies have shown distinct responses to caffeine according to gender [44].

Remarkably, the consumption of caffeine throughout life altered this hyperlocomotion profile in either sexes, but selectively reversed the deterioration of memory performance observed in female rats that were exposed to caffeine during brain development. This was associated with the ability of caffeine intake throughout life to selectively increase the levels in the hippocampus of BDNF, the neurotrophin that is described to control recognition memory [45]. Both schedules of treatment increased proBDNF in the cortex, but only caffeine throughout life increased BDNF, which could be associated to the hyperlocomotion promoted by caffeine in male rats. Similar findings were reported in the cortex from male adolescent rats receiving the same doses and schedule of caffeine treatment [14]. Of note, when compared to other psychostimulants such as cocaine and methylphenidate administered at early ages, the modifications in the BDNF gene and protein expression were also age-dependent and restricted to some brain areas [46]; in some cases hyperlocomotion was the behavioral outcome [47,48].

Although the influence of sex hormones was not directly investigated, previous studies have reported a differencial regulation of BDNF between male and female rats with implications in the

synaptic plasticity in the hippocampus [49,50]. Notably, there was a selective association of BDNF levels with the preservation rather than with the loss of memory performance, in agreement with previous observations that caffeine intake can bolster BDNF levels to recover memory dysfunction either during adolescence [14], upon aging [2,4], in animal models of Alzheimer's disease [51] and after sleep deprivation [52]. We also observed that the beneficial effects of caffeine were selectively associated with the levels of BDNF rather than proBDNF or TrkB receptors, in accordance with the previously observed reduction of hippocampal TrkB receptor density without alteration of memory performance in adults rats receiving chronic caffeine [4].

Although, there are many open questions with respect to how astrocytic changes may impact cognitive functions [53,54], current findings reported increases in GFAP-positive astrocytes when aged-rats were exposed to a spatial memory task [55]. It is important to note that astrocytic A_{2A} receptors (the main target of caffeine) were recently described to regulate memory not only under physiological conditions, but also in memory impairment under pathophysiological conditions [12,13]. Additionaly, adolescent rats exposed to caffeine presented behavioral alterations with decreased GFAP-positive astrocytes [14]. In our findings, we did not find a direct correlation between the levels of synaptic (SNAP-25) and astrocytic markers (GFAP) with the effects of caffeine on memory performance. The ability of caffeine and of selective antagonists of adenosine A_{2A} receptors to preserve memory function in adult animals subject to noxious brain insults, has been tightly related to a recovery of the density of synaptic proteins [7,8,56] and preservation of synaptic function [10]. However, we now failed to observe any correlation between the density of GFAP and of SNAP-25 in the hippocampus and the impact of caffeine on memory performance. Thus, caffeine during brain development affected the density of GFAP and of SNAP-25 only in male rats, whereas memory deterioration occurred in female rather than in male rats; also, caffeine intake throughout life ammeliorated memory function in female rats treated with caffeine during early development, whereas it did not modify the density of either hippocampal or cortical GFAP and SNAP-25.

In summary, the present study shows that the exposure to caffeine during brain development triggers a memory impairment selectively in female rats, which can be recovered by a worktime-like schedule of caffeine consumption throughout life, reinforcing the general neuroprotective potencial of caffeine consumption by adults. Furthermore, our parallel neurochemical analysis suggests that the beneficial effects of caffeine consumption throughout life seem to be selectively associated with an increase of hippocampal levels of BDNF rather than of its precursor (proBDNF) or receptor (TrkB), and also seem unrelated to the control of the density of synaptic or astrocytic markers.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2016.01.026.

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Legends to the supplementary figures.

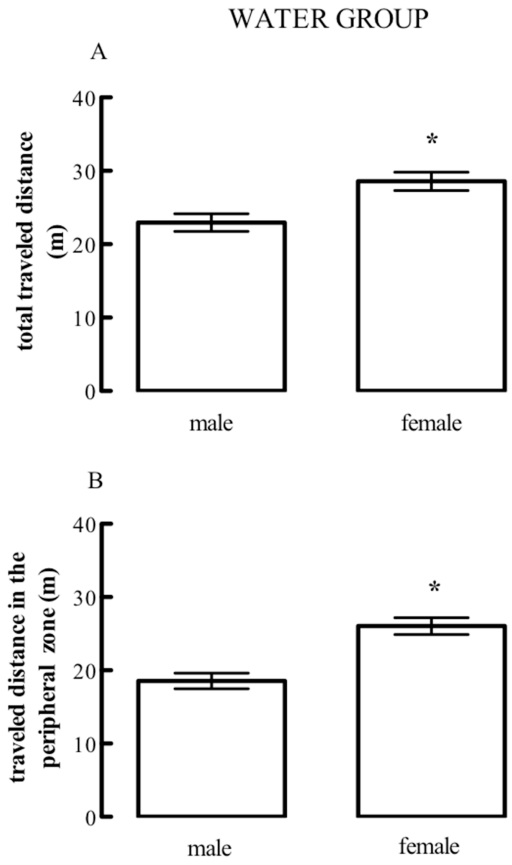

Fig. S1. Analysis of the locomotor activity between adult male and female rats in the open field. (A) total traveled distance in meters (m); (B) traveled distance in the peripheral zone in meters (m). *$P < 0.05$ - differences between sexes (Unpaired-t-test).

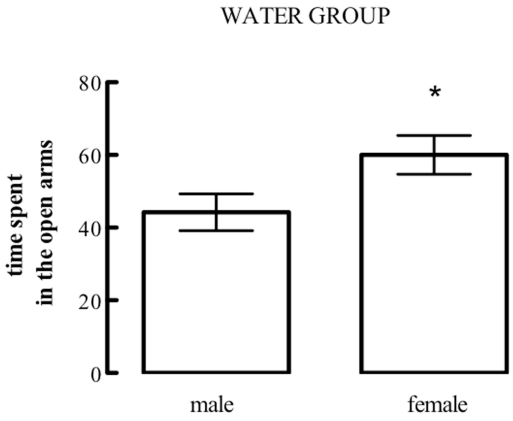

Fig. S2. Analysis of the time spent in the closed arms between adult male and female rats in the elevated plus maze. *$P < 0.05$ - differences between sexes (Unpaired-t-test).

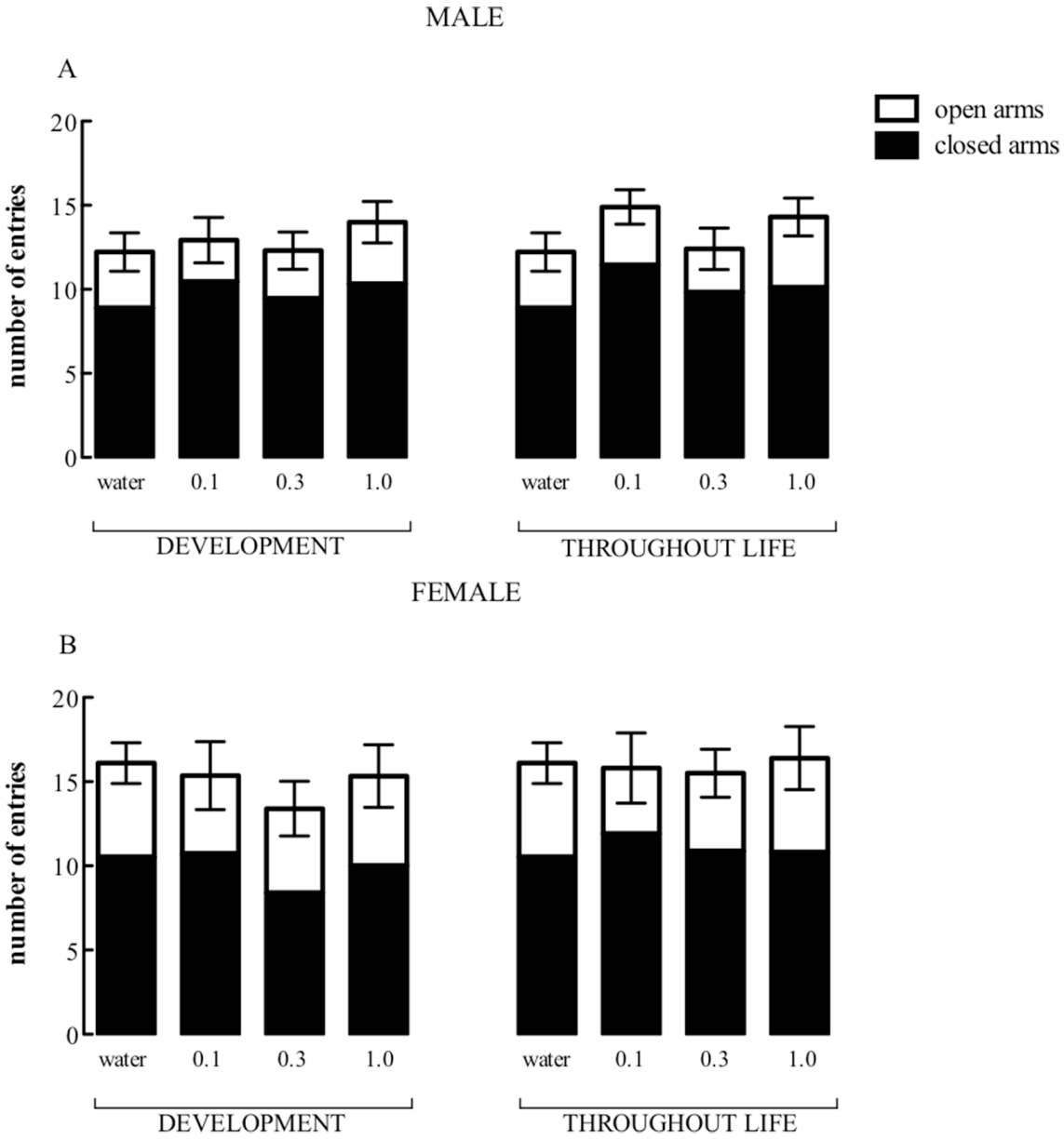

Fig. S3. Analysis of the number of the total entries in both arms of the elevated plus maze between adult male and female rats. Male and female rats received caffeine (0.1-1 mg/mL) during development or throughout life. (A) Male and (B) Female rats. Bars represent the total number of entries in open (white) and closed arms (black). No statistic difference.

PARTE III

3. DISCUSSÃO

Em toda a sua complexidade, o desenvolvimento do sitema nervoso central (SNC) é um processo que envolve a organização espaço-temporal de diversos eventos celulares, tais como: proliferação, migração, diferenciação, sinaptogênese e morte celular programada. É durante os estágios do desenvolvimento encefálico que se observa uma maior vulnerabilidade frente ao consumo de substâncias que causam dependência (Adriani et al, 2003; Coleman et al, 2011; García-Cabrerizo et al, 2015; Schneider et al, 2008), situações ambientais adversas ou alterações na dieta (Georgieff, 2007). A cafeína é a única substância psicoestimulante que faz parte da dieta de todas as populações do mundo (revisado por Fredholm et al, 1999), e, portanto, faz-se necessário investigar o seu impacto quando consumida durante as diferentes fases do desenvolvimento do encéfalo.

O primeiro trabalho desta tese teve como objetivo avaliar o comportamento, o imunoconteúdo e a imunoreatividade de proteínas sinápticas em amostras de córtex e hipocampo de ratos adolescentes após o consumo crônico de três doses diferentes de cafeína mimetizando o consumo baixo, moderado e moderado/alto em humanos (0,1, 0,3 ou 1,0 g/L, respectivamente). Uma das respostas proeminentes da administração aguda de cafeína em roedores é a modificação da locomoção, que é aumentada e diminuída por baixas e elevadas doses de cafeína, respectivamente (Snyder et al, 1981). Porém, após o consumo crônico de cafeína, roedores adultos apresentam taquifilaxia para os efeitos locomotores observados (Finn & Holtzman, 1986). Rhoads e colaboradores observaram que ratos adolescentes machos submetidos à exposição crônica de cafeína, seguida por 24-48 horas de abstinência, reduziram de forma significativa a locomoção espontânea no campo aberto, assim como reduziram a

ativação motora quando receberam uma dose aguda de 30 mg/kg de cafeína, o que foi interpretado como tolerância a esta substância (Rhoads et al, 2011). Dessa forma, considerando que as tarefas comportamentais sempre foram realizadas durante as cinco horas posteriores a troca da solução cafeinada (fase escura) pela água (fase clara), parece improvável que o efeito da cafeína sobre os comportamentos tenha ocorrido devido ao desenvolvimento de uma síndrome de abstinência, visto que em ratos adultos requer pelo menos 24 horas para se manifestar (Holtzman, 1983). Além disso, o fato de a exposição crônica de ratos adolescentes à cafeína não ter resultado em nenhuma alteração na distância percorrida durante a primeira exposição ao campo aberto, e tampouco no número de entradas nos braços fechados no teste do labirinto em cruz elevado, medida que pode ser utilizada como um índice de atividade locomotora, ajudam a suportar esta ideia. Estas observações também são importantes para se desconsiderar alterações locomotoras como uma base para outras mudanças comportamentais apresentadas pelos ratos adolescentes expostos à cafeína.

Na avaliação no labirinto em cruz elevado, os ratos adolescentes que consumiram cafeína em qualquer uma das doses testadas desenvolveram um perfil ansioso, representado pelo maior tempo de permanência dos animais nos braços fechados do aparato. Em humanos, o consumo de cafeína desencadeia ansiedade (Greden, 1974) e, em animais adultos o consumo crônico de doses altas de cafeína (50-100 mg/kg, duas vezes por dia) também promove efeitos ansiogênicos (El Yacoubi et al, 2000). Contudo, a susceptibilidade dos ratos adolescentes aos efeitos ansiogênicos da cafeína foi um resultado surpreendente, pois animais nessa fase são descritos por serem relativamente resistentes às mudanças comportamentais provocadas por fatores exógenos no labirinto em cruz elevado (Doremus et al, 2004). Diferentemente dos ratos adultos (Bhattacharya et al, 1997), os ratos adolescentes

parecem ser mais resistentes ao desenvolvimento de tolerância aos efeitos ansiogênicos da cafeína. Foi também observado que o consumo crônico de cafeína nas doses de 0.3 e 1.0 g/L melhorou o desempenho dos animais na tarefa de reconhecimento ao objeto novo. Notavelmente, os ratos adolescentes que beberam água obtiveram um desempenho inferior no teste do reconhecimento ao objeto, pois ratos adolescentes são descritos por serem mais resposivos a estímulos novos e se habituam mais rápido a ambientes novos do que os ratos adultos (Douglas et al, 2003; Stansfield & Kirstein, 2006). Considerando que o protocolo foi adaptado de acordo com recomendações para proporcionar um bom desempenho em ratos jovens (Ennaceur & Delaceur, 1988; Reger et al, 2009), o desempenho inferior dos animais adolescentes pode ser uma característica particular ratos *Wistar,* visto que estes animais já foram descritos por apresentar desempenho inferior quando comparados aos ratos de outras estirpes (Andrews et al, 1995; Ennaceur et al, 2005). Entretanto, esse comportamento foi considerado vantajoso, pois reforçou os efeitos da cafeína em afetar a memória, seja pelo controle direto dos processos cognitivos, seja por manter o estado de atenção (revisado por Takahashi et al, 2008; Cunha & Agostinho, 2010). Dentro desse contexto, o tratamento crônico com cafeína melhorou a memória (avaliada pelos testes do labirinto em Y e do reconhecimento ao objeto) e o comprometimento da atenção (avaliada pelo teste do *set-shifting*) em ratos adolescentes do tipo SHR (do inglês *spontaneously hipertensive rats*), considerado um modelo experimental de Transtorno de Déficit de Atenção e Hiperatividade (TDAH) (Pires et al, 2010; Pandolfo et al, 2013). Foi também observado que somente os animais que receberam a maior dose de cafeína não reduziram sua atividade locomotora na segunda exposição ao campo aberto. Esse resultado revela uma falta de

habituação e caracteriza um prejuízo em uma forma simples de aprendizado não-associativo (revisado por Leussis & Bolivar, 2006).

Uma vez que no momento em que as tarefas comportamentais foram realizadas a cafeína ainda estava presente no encéfalo dos ratos adolescentes, a ausência da habituação dos animais pela dose mais alta de cafeína poderia ser também um reflexo do seu efeito estimulante motor pelo bloqueio dos receptores de adenosina A_1 e A_{2A} no SNC. De fato, uma relação direta tem sido observada entre as concentrações plasmáticas de cafeína e a magnitude da ativação motora de ratos adultos (Lau & Falk, 1994). Além disso, estudos farmacocinéticos revelaram que após a administração de uma única dose oral de 10 mg/kg de cafeína em ratos *Sprague–Dawley* machos com 40 dias de vida, a cafeína tem uma meia-vida media de 120 e 132 minutos no plasma e no encéfalo, respectivamente (Latini et al, 1980). Este parâmetro farmacocinético permanence inalterado em ratos machos com dois meses, nos quais a meia-vida de uma única dose oral de cafeína (4 mg/kg) é de 127 minutos (Bienvenu et al, 1990). Em ratos adultos, quanto maior a dose de cafeína administrada maior será a sua meia-vida no encéfalo e no plasma (Latini et al, 1978). Esta informação permite considerar que isto possa estar ocorrendo também em ratos mais jovens, especialmente após os animais beberem soluções de doses moderadas e altas de cafeína (0,3 e 1,0 g/L, respectivamente).

A cafeína é um antagonista não seletivo dos receptores de adenosina e tem afinidades similares para os receptores A_1 e A_{2A} *in vitro* (revisado por Fredholm et al, 1999), os quais estão bem documentados pelo envolvimento na normalização da memória (revisado por Cunha & Agostinho, 2010), na ativação motora e na manutenção do estado de alerta (Ferré, 2008). Nesse trabalho todas as doses de

cafeína aumentaram o imunoconteúdo do receptor A_1 no hipocampo dos ratos adolescentes. Johansson e colaboradores (1993) já haviam documentado o mesmo aumento nos níveis do receptor A_1 pela cafeína em diferentes regiões cerebrais de animais adultos (Johansson et al, 1993). Como a manipulação dos receptores A_1 tem um impacto considerável sobre a ansiedade (El Yacoubi et al, 2000; Johansson et al, 2001; Prediger et al, 2004), este aumento aqui observado no hipocampo poderia estar associado aos efeitos ansiogênicos da cafeína observados nos ratos adolescentes. Paralelamente, a maior dose de cafeína testada causou uma redução dos níveis do receptor A_1 no córtex, o que poderia ser associado com os |prejuízos no aprendizado não-associativo provocado pela cafeína. Embora o bloqueio dos receptores A_1 e A_{2A} tenha melhorado o desempenho de ratos em testes que avaliaram a memória espacial (Hauber & Bareiss, 2001), outros estudos estão de acordo com nossos resultados, indicando que o bloqueio contínuo dos receptores A_1 prejudicou o desempenho nas tarefas que avaliam aprendizado e memória em roedores adultos (von Lubitz et al, 1993; Vollert et al, 2013). Contudo, estas observações não excluem a possibilidade de que o bloqueio dos receptores A_{2A} pela cafeína também esteja envolvido nas alterações comportamentais (revisado por Cunha et al, 2008; Cunha & Agostinho, 2010).

O consumo de cafeína e também a manipulação farmacológica seletiva dos receptores de adenosina modifica o sistema de neuromodulação operado pelo fator neurotrófico derivado do encéfalo (BDNF) (Costa et al, 2008a; 2008b; Han et al, 2013; Sallaberry et al, 2013; Diógenes et al, 2004; 2011), o qual está intimamente associado ao controle do comportamento emocional (Siuciak et al, 1997; Chen et al, 2006; Shirayama et al, 2002; Deltheil et al, 2008; 2009; Taliaz et al, 2010), bem como o aprendizado e memória (Kesslak et al, 1998; Mizuno et al, 2000; Alonso et al,

2002a; 2002b; Ma et al, 1998). A sinalização do BDNF participa ativamente desses processos, uma vez que o equilíbrio entre as ligações BDNF-TrkB e proBDNF-$p75^{NTR}$ exercem influência sobre a plasticidade sináptica por estimular a LTP e LTD, respectivamente (Pang et al, 2004; Woo et al, 2005). Os resultados obtidos revelaram também que o consumo de cafeína por ratos adolescentes levou a um aumento dos níveis de BDNF no córtex cerebral, coincidente com a melhora da memória de reconhecimento e exacerbação da ansiedade. Se comparados a outros resultados onde houve normalização nos níveis do BDNF e melhora da memória de reconhecimento em roedores adultos e idosos (Costa et al, 2008a; 2008b; Sallaberry et al, 2013), isto pode ser caracterizado como um efeito normalizador da cafeína. Em outro estudo com o modelo transgênico para Doença de Alzheimer (DA), a cafeína também reverteu o prejuízo na memória espacial e normalizou a expressão do BDNF e do seu receptor TrkB (Han et al, 2013). Em outro modelo, a cafeína normalizou os níveis de BDNF e o comportamento emocional de camundongos transgênicos sem os receptores de adenosina do tipo A_{2A} (Wei et al, 2014), os quais são um importante alvo do consumo crônico de cafeína (revisado por Cunha & Agostinho, 2010). Visto que a maior dose de cafeína reduziu o imunoconteúdo do BDNF e do proBDNF no hipocampo dos ratos adolescentes, estas alterações podem estar associadas aos efeitos prejuidicais da maior dose de cafeína sobre o aprendizado não-associativo cuja participação do hipocampo já foi documentada (Vianna et al, 2000). O envolvimento primário dos córtex perirhinal e entorhinal na discriminação da familiaridade são contrastantes, uma vez que o hipocampo é essencial para a memória de reconhecimento ao objeto (Balderas et al, 2008; Song et al, 2011). No SNC de mamíferos como ratos, macacos e humanos, o desenvolvimento e reorganização dessas regiões corticais inicia-se substancialmente no período pós-natal (Bachevalier and Beauregard, 1993; revisado

por Brown and Aggleton, 2001); isso sobrepõe-se a um aumento gradual de BDNF cortical que ocorre no espaço entre a 3ª e 12ª semanas pós-natal sem alterações significativas no hipocampo (Hill et al, 2012). De maneira geral, nossos resultados incitam a hipótese de que o impacto positivo da cafeína sobre a memória de reconhecimento em ratos adolescentes pode estar associado ao aumento nos níveis de BDNF cortical, ao passo que o prejuízo causado pela dose mais alta de cafeína sobre o aprendizado não-associativo possa envolver a redução nos níveis de BDNF no hipocampo, uma hipótese que requer posteriores confirmações experimentais diretas. Uma das modificações mais evidentes observadas nos ratos adolescentes que consumiram cafeína de forma crônica foi uma redução na densidade hippocampal e cortical da GFAP e da SNAP-25 (marcadores astrocitário e de terminais nervosos, respectivamente). A perda de terminais nervosos em regiões corticais límbicas tem sido indentificada como um evento precoce associado a um comprometimento da memória decorrente da idade ou em modelos experimentais da DA (revisado por Cunha & Agostinho, 2010). Os animais jovens com diminuição na SNAP-25 desenvolvem comprometimento severo na memória (Hou et al, 2006), mas a sua expressão exacerbada em ratos adultos também prejudica a plasticidade sináptica associada a memória (McKee et al, 2010), o que pode ser uma das disfunções relacionadas à distúrbios cerebrais como o TDAH (Barr et al, 2000). Considerando a complexidade e magnitude dos processos mnemônicos, ainda que o consumo de doses altas e moderadas de cafeína tenham melhorado a memória de reconhecimento dos ratos adolescentes, a diminuição encontrada na densidade da SNAP-25 poderia ser um reflexo de alterações na conectividade neuronal no hipocampo com implicações em outros aspectos do aprendizado e memória (Hou et al, 2004; Delgado-Martínez et al, 2007). Dessa forma, levando em conta que a dose mais alta de cafeína comprometeu o

aprendizado não-associativo de ratos adolescentes, podemos sugerir que doses mais altas dessa substância poderiam ter um impacto negativo também no aprendizado de adolescentes humanos.

O comportamento ansioso apresentado pelos ratos adolescentes após o consumo crônico de cafeína também pode estar associado à redução na densidade da SNAP-25, pois esse tipo de comportamento foi observado em camundongos mutantes que apresentam diminuição da expressão da SNAP-25 (Kataoka et al, 2011). Contudo, é importante considerar que a perda desse marcador de terminais nervosos observada nos grupos que ingeriram cafeína pode indicar um sinal precoce de sinaptotoxicidade, sugerindo uma eventual suceptibilidade a insultos cerebrais, mesmo sem perda neuronal evidente. De fato, embora nenhuma diferença na imunoreatividade para NeuN entre os grupos controle e tratados tenha sido encontrada, a redução observada na densidade da GFAP nos ratos adolescentes que consumiram cafeína pode ser reflexo de alterações adaptativas nos astrócitos (revisado por Middeldorp & Hol, 2011). Estudos recentes enfatizam que alterações na função astrocitária controlam o comportamento emocional (Rajkowska & Stockmeier, 2013) e o desempenho mnemônico em roedores adultos (Suzuki et al, 2011), assim como, que os receptores A_{2A} (que são alvos da cafeína) (revisado por Cunha & Agostinho, 2010) participam da modulação da captação de glutamato em cultura de astrócitos (Matos et al, 2012) por exercerem o controle do seu principal sistema energético, a Na^{+}-K^{+}-ATPase (Matos et al, 2013). A partir dessas informações, é plausível considerar a possível importância central dos astrócitos nas alterações comportamentais causadas pelo consumo crônico de cafeína por ratos adolescentes.

No segundo capítulo desta tese, o objetivo foi verificar se o consumo de cafeína ao longo da vida seria capaz de compensar os efeitos prejudiciais causados por esta substância quando ela é consumida durante o desenvolvimento do encéfalo. Adicionalmente, uma vez que as diferenças de sexo foram previamente relatadas como um fator importante no desempenho do papel neuroprotetor exercido pela cafeína (Palacios et al, 2010; Ritchie et al, 2007; Xu et al, 2006), foi também investigado se o sexo tem impacto sobre os efeitos da cafeína quando consumida durante o desenvolvimento do encéfalo ou até a fase adulta. Os ratos machos e fêmeas receberam três doses diferentes de cafeína (0.1, 0.3 ou 1.0 g/L, respectivamente) na água de beber até o desmame ou até atingirem a fase adulta. Na fase adulta foram avaliados os comportamentos e as análises das proteínas sinápticas nas amostras encefálicas.

Os resultados encontrados permitiram identificar uma diferença de sexo nos efeitos exercidos pela cafeína sobre a memória de reconhecimento e a atividade locomotora. Foi possível observar uma diferença entre os protocolos de administração, pois o consumo de cafeína ao longo da vida foi capaz de reverter o prejuízo na memória causado pela exposição a cafeína até o desmame, o que foi associado a um aumento dos níveis de BDNF no hipocampo.

Uma comparação inicial entre os sexos dos grupos controle revelou que as fêmeas percorreram distâncias maiores no campo aberto do que os machos, o que corrobora resultados já descritos anteriormente (Cauli et al, 2013; Hughes & Beveridge, 1990, 1991; Lynn & Brown, 2010). Essa diferença entre os sexos também pode ser explicada pelas observações de que as fêmeas são menos ansiosas e assustadas do que os machos em resposta a ambientes e aparatos novos (Zimmerberg

& Farley, 1993; Aguilar et al, 2003). As fêmeas também apresentam mais motivação para a investigação ou fuga na presença de elementos novos (Brown & Nemes, 2008). Conforme previamente observado (Hughes & Beveridge, 1990, 1991; Glavin & Krueger, 1985) a exposição à cafeína durante o desenvolvimento do encéfalo provocou o aparecimento de um perfil hiperlocomotor em machos. As fêmeas apresentaram apenas uma tendência a hiperlocomoção pela cafeína, provavelmente porque seus os índices locomotores basais já são mais altos do que os machos. Ambos os protocolos de administração de cafeína causaram hiperlocomoção nos machos, enquanto que nas fêmeas a hiperlocomoção foi significativa somente quando administrada durante toda a vida.

Ao analisar o comportamento relacionado a ansiedade, foi observado que o consumo de cafeína durante o desenvolvimento não alterou a atividade dos animais no labirinto em cruz elevado (Pan & Chen, 2007), embora as fêmeas tenham passado mais tempo nos braços abertos, conforme relatado anteriormente (Aguilar et al, 2003; Johnston & File, 1991; Zimmerberg & Farley, 1993). Já os animais que consumiram a maior dose de cafeína durante toda a vida, passaram mais tempo nos braços abertos, independentemente do sexo. A diferença mais marcante da exposição à cafeína associada ao sexo foi no desempenho dos animais na tarefa de reconhecimento de objetos. As fêmeas que receberam cafeína durante o desenvolvimento exibiram um desempenho inferior na realização da tarefa, enquanto os machos apresentaram o desempenho normal. Estes resultados são contrastantes se comparados aos encontrados por Soellner e colaboradores, onde a exposição contínua à cafeína durante a gestação prejudicou as memórias de reconhecimento e de trabalho de fêmeas e machos adultos (Soellner et al, 2009), ou ainda aos encontrados por Silva e colaboradores onde a exposição contínua à cafeína durante a gestação até DPN 15

prejudicou as memórias de reconhecimento e espacial de ratos machos adultos (Silva et al, 2013). Conjuntamente com os nossos resultados, essas observações prévias confirmam que a exposição à cafeína durante o desenvolvimento do encéfalo pode provocar efeitos negativos a longo prazo sobre o desempenho em tarefas que avaliam a memória, mas a susceptibilidade das fêmeas pode depender do protocolo de exposição à cafeína. Evidências de estudos clínicos e com animais tem apontado para uma maior suceptibilidade de fêmeas à ação de outros psicosestimulantes quando a comparado aos machos (Becker, 1999; Bisagno et al, 2003; Carroll et al, 2004; Sell et al, 2010). Além disso, em humanos, alguns estudos tem demonstrado respostas distintas à cafeína de acordo com o sexo (Temple et al, 2010; 2014; Temple & Ziegler, 2011).

O consumo de cafeína durante toda a vida causou hiperlocomoção em ambos os sexos, mas reverteu seletivamente o prejuízo na memória de reconhecimento que foi observado nas fêmeas que receberam cafeína durante o desenvolvimento. Esta diferença observada entre os protocolos de exposição nas fêmeas pode estar relacionada aos níveis de BDNF que foram aumentados no hipocampo dos grupos que consumiram cafeína ao longo da vida, principalmente considerando-se a importância desta neurotrofina para os processos de aprendizado e memória (Callaghan & Kelly, 2012; revisado por Bekinschtein et al, 2014). Nos machos, ambos os protocolos de tratamento aumentaram o imunoconteudo do proBDNF cortical, mas somente a cafeína ao longo da vida aumentou os níveis de BDNF nesta região. Isto poderia estar associado à hiperlocomoção causada pela cafeína nos machos. Resultados similares foram encontrados no córtex de ratos machos adolescentes que receberam as mesmas doses de cafeína entre DPN 28-52 (Ardais et al, 2014). Ao comparar a cafeína a outros psicoestimulantes como a cocaína e o metilfenidato administrados nas fases

iniciais da vida, também observa-se modificações na expressão da proteína e do gene do BDNF de uma forma dependente do gênero e restrita a algumas áreas encefálicas (Banerjee et al, 2009) sendo que em alguns casos a hperlocomoção foi o resultado comportamental encontrado (Simchon-Tenenbaum et al, 2015a; 2015b).

Embora a influência dos hormônios sexuais não tenha sido diretamente investigada, estudos prévios têm relatado que o BDNF pode ser regulado de maneira dependente do sexo e com implicações sobre a plasticidade sináptica hipocampal (Kramár et al, 2012; revisado por Scharfman & MacLusky, 2014). Notavelmente, houve uma associação entre os níveis do BDNF e a preservação da memória, o que está de acordo com resultados anteriores, que mostram que o consumo de cafeína pode reforçar os níveis de BDNF para recuperar disfunções na memória de ratos adolescentes (Ardais et al, 2014) causadas pelo envelhecimento (Costa et al, 2008a; Sallaberry et al, 2013), em modelos experimentais para DA (Han et al, 2013) ou após a privação de sono (Alhaider et al, 2010; Sahu et al, 2013). Estes efeitos benéficos da cafeína parecem estar mais associados aos níveis de BDNF do que de proBDNF ou do receptor TrkB. Isto está de acordo com resultados anteriores do nosso grupo de pesquisa, onde o consumo crônico de cafeína não alterou o desempenho da memória de reconhecimento de ratos adultos ainda que a densidade hipocampal do TrkB tenha sido reduzida (Sallaberry et al, 2013).

Os resultados encontrados neste trabalho não apontam para nenhuma relação direta entre os efeitos da cafeína sobre o desempenho mnemônico dos animais e o imunoconteúdo da SNAP-25 e GFAP. Isto pode ser observado sob dois aspectos: a) nenhum dos protocolos de exposição à cafeína causou qualquer comprometimento de memória nos machos. No entanto, a densidade das proteínas SNAP-25 e GFAP deste

animais foram reduzidas nos ratos expostos à cafeína por toda vida e em ambos os protocolos de exposição, respectivamente; b) nem o prejuízo na memória de reconhecimento apresentado pelas fêmeas que consumiram cafeína durante o desenvolvimento cerebral, tampouco a melhora promovida pelo consumo contínuo até a vida adulta foram acompanhadas de modificações na densidade da GFAP e SNAP-25.

A capacidade da cafeína e de antagonistas seletivos para os receptores de adenosina A_{2A} em preservar a função mnemônica em animais adultos submetidos a insultos cerebrais, tem sido fortemente relacionada com uma recuperação da densidade de uma série de outras proteínas sinápticas (Canas et al, 2009;. Cognato et al, 2010; Duarte et al, 2012; Espinosa et al, 2013; Gonçalves et al, 2013) além do envolvimento na preservação da função sináptica (Batalha et al, 2014; Kaster et al, 2015). Dessa forma, nossos resultados são surpreendentes, uma vez que estudos recentes tem apresentado resultados bastante relevantes, no que diz respeito a forma pela qual alterações sinápticas podem afetar as funções cognitivas. À exemplo, Sampero-Piquero e colaboradores, demonstraram que, sob condições ambientais enriquecidas, o aumento no número de astrócitos reativos para GFAP foi acompanhado de uma melhora na performance mnemônica espacial de ratos idosos (Sampero-Piquero et al, 2014). Além disso, outros estudos observaram que, perturbações na expressão dos transportadores astrocitários para lactato causaram amnésia e comprometimento da LTP (Suzuki et al, 2011), camundongos idosos expressando hAPP (do inglês *human amyloid precursor protein*) apresentaram níveis mais elevados de receptores de adenosina astrocitários do tipo A_{2A} e que a remoção genética destes receptores melhorou a memória de longa duração destes camundongos (Orr et al, 2015). Além disso, outro estudo observou que a deleção dos receptores de

adenosina astrocitários do tipo A_{2A} causou prejuízos cognitivos e psicomotores devido a desequilíbrios na homeostase do glutamato em camundongos (Matos et al, 2015). Em ratos machos adolescentes, o consumo crônico de cafeína também prejudicou o aprendizado não-associativo. Resultado este, que foi acompanhado de uma diminuição da imunoreatividade para GFAP (Ardais et al, 2014).

De maneira geral, os resultados apresentados nos dois trabalhos que compõe esta tese, apontam para a necessidade de um consumo de cafeína parcimonioso durante o desenvolvimento. Apesar de melhorar a memória de reconhecimento, o consumo de cafeína durante a adolescência promove também um aumento no estado ansioso dos animais, prejudica o aprendizado não-associativo e altera diversas proteínas importantes para o desenvolvimento. Além disso, o consumo de cafeína até o desmame prejudica a memória de reconhecimento de fêmeas adultas ainda que o seu consumo estendido durante toda a vida recupere este prejuízo. Este trabalho limita-se a avaliações bioquímicas e testes comportamentais específicos que nos permitem observar os efeitos desta substância sobre o desenvolvimento de maneira parcial. É necessário ampliar o conhecimento sobre o tema, variando parâmetros comportamentais e bioquímicos, assim como, os protocolos de exposição com o objetivo de buscar uma dose segura para o consumo durante os períodos observados.

4. CONCLUSÃO

Acafeína é uma substância psicoestimulante que faz parte da dieta de consumidores em todo o mundo e, portanto, faz-se necessário investigar o seu impacto quando consumida durante diferentes fases do desenvolvimento do encéfalo. Os resultados apresentados nesta tese documentam que o consumo regular de doses moderadas de cafeína promove efeitos ansiogênicos, prejudica o aprendizado não-associativo mas melhora a memória de reconhecimento em ratos adolescentes. Dada a exacerbação da ansiedade observada nos animais como um potencial efeito adverso da cafeína, é importante considerar esses efeitos quando traduzidos para o seu consumo por adolescentes humanos, pois estes atualmente consomem bebidas energéticas que possuem alto teor desta substância. As análises neuroquímicas sugerem que os efeitos ansiogênicos da cafeína possam envolver um aumento da regulação dos receptores de adenosina A_1 do hipocampo e uma sub-regulação de SNAP-25, juntamente com uma função astrocitária modificada caracterizada por uma diminuição da densidade de GFAP no hipocampo e no córtex cerebral. A melhora na memória de reconhecimento parece envolver um aumento da densidade de BDNF nas áreas corticais e o comprometimento do aprendizado não associativo provocado pela dose de cafeína mais elevada parece estar associado com uma diminuição da densidade de receptores de adenosina A_1 do córtex cerebral e de BDNF do hipocampo. A redução da densidade do marcador de terminais e astrocítico no hipocampo por todas as doses de cafeína testadas em ratos adolescentes, sugere uma maior susceptibilidade a insultos cerebrais. Esta hipótese, sustenta as preocupações em relação ao consumo de cafeína durante a adolescência e deveria ser confirmada experimentalmente em estudos futuros, considerando que o consumo de cafeína está crescendo neste grupo populacional.

Nossos resultados mostraram também que a exposição à cafeína durante os períodos gestacional e lactacional desencadeou um prejuízo na memória de reconhecimento de ratas adultas, que pode ser revertido pela exposição continua à cafeína, reforçando o papel neuroprotetor exercido pela cafeína em animais adultos. Além disso, a análise neuroquímica sugere que os efeitos benéficos do consumo de cafeína durante toda a vida parecem estar associados com um aumento dos níveis de BDNF no hipocampo sem envolvimento aparente do seu precursor (proBDNF), ou receptor (TrkB). Os benefícios também parecem não estar relacionados com o controle da densidade de marcadores sinápticos ou astrocíticos.

De maneira geral, os resultados apresentados nesta tese foram referentes ao impacto da administração de cafeína sobre o comportamento e a densidade de proteínas sinápticas em diferentes estágios do desenvolvimento do encéfalo de ratos. Podemos concluir que os efeitos exercidos pela cafeína foram dependentes do protocolo de exposição utilizado. Contudo, a diversidade dos dados mantém a unidade ao alertar sobre o consumo abusivo desta substância psicoestimulante durante os estágios cruciais para o desenvolvimento encefálico dos indivíduos.

5. PERSPECTIVAS

- Fazer um estudo comparativo dos padrões comportamentais apresentados por ratos *wistar* adolescentes e adultos consumidores crônicos de cafeína;
- Avaliar o impacto do gênero sobre os efeitos comportamentais e neuroquímicos exercidos pela cafeína em ratos adolescentes machos e fêmeas;
- Observar o impacto da ovariectomia ou orquiectomia nos efeitos comportamentais e neuroquímicos exercidos pela cafeína consumida durante diferentes estágios do desenvolvimento;

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ANEXOS

Em anexo apresentamos artigos de co-autoria publicados no decorrer da tese, mas que não fazem parte do corpo da tese. O anexo I refere-se ao artigo intitulado *"Caffeine acts through neuronal adenosine A2A receptors to prevent mood and memory dysfunction triggered by chronic stress",* um dos resultados do doutorado sanduíche realizado no Laboratório de Eletrofisiologia da Universidade de Coimbra sob a supervisão do Professor Rodrigo Cunha. No anexo II encontra-se uma lista de todos os artigos científicos de autoria e co-autoria publicados durante o desenvolvimento desta tese.

ANEXO 1

Caffeine acts through neuronal adenosine A2A receptors to prevent mood and memory dysfunction triggered by chronic stress

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Caffeine acts through neuronal adenosine A_{2A} receptors to prevent mood and memory dysfunction triggered by chronic stress

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The consumption of caffeine (an adenosine receptor antagonist) correlates inversely with depression and memory deterioration, and adenosine A_{2A} receptor ($A_{2A}R$) antagonists emerge as candidate therapeutic targets because they control aberrant synaptic plasticity and afford neuroprotection. Therefore we tested the ability of $A_{2A}R$ to control the behavioral, electrophysiological, and neurochemical modifications caused by chronic unpredictable stress (CUS), which alters hippocampal circuits, dampens mood and memory performance, and enhances susceptibility to depression. CUS for 3 wk in adult mice induced anxiogenic and helpless-like behavior and decreased memory performance. These behavioral changes were accompanied by synaptic alterations, typified by a decrease in synaptic plasticity and a reduced density of synaptic proteins (synaptosomal-associated protein 25, syntaxin, and vesicular glutamate transporter type 1), together with an increased density of $A_{2A}R$ in glutamatergic terminals in the hippocampus. Except for anxiety, for which results were mixed, CUS-induced behavioral and synaptic alterations were prevented by (*i*) caffeine (1 g/L in the drinking water, starting 3 wk before and continued throughout CUS); (*ii*) the selective $A_{2A}R$ antagonist KW6002 (3 mg/kg, p.o.); (*iii*) global $A_{2A}R$ deletion; and (*iv*) selective $A_{2A}R$ deletion in forebrain neurons. Notably, $A_{2A}R$ blockade was not only prophylactic but also therapeutically efficacious, because a 3-wk treatment with the $A_{2A}R$ antagonist SCH58261 (0.1 mg/kg, i.p.) reversed the mood and synaptic dysfunction caused by CUS. These results herald a key role for synaptic $A_{2A}R$ in the control of chronic stress-induced modifications and suggest $A_{2A}R$ as candidate targets to alleviate the consequences of chronic stress on brain function.

chronic stress | adenosine A_{2A} receptor | caffeine | synaptic dysfunction | mood dysfunction

Repeated stress elicits neurochemical and morphological changes that negatively affect brain functioning (1, 2). Thus, repeated stress is a trigger or a risk factor for neuropsychiatric disorders, namely depression, in both humans and animal models (2, 3). Given the absence of effective therapeutic tools, novel strategies to manage the impact of chronic stress are needed, and analyzing particular lifestyles can provide important leads. Notably, caffeine consumption increases in stressful conditions (4) and correlates inversely with the incidence of depression (5, 6) and the risk of suicide (7, 8). However, the molecular targets operated by caffeine to afford these beneficial effects have not been defined.

Caffeine is the most widely consumed psychoactive drug. The only molecular targets for caffeine at nontoxic doses are the main adenosine receptors in the brain, namely the inhibitory A_1 receptors (A_1R) and the facilitatory A_{2A} receptors ($A_{2A}R$) (9). $A_{2A}R$ blockade affords robust protection against noxious brain conditions (10), an effect that might result from the ability of neuronal $A_{2A}R$ to control aberrant plasticity (11, 12) and synaptotoxicity (13–15) or from $A_{2A}R$'s impact on astrocytes (16) or microglia (17). The protection provided by $A_{2A}R$ blockade prompts the hypothesis that $A_{2A}R$ antagonism may underlie the beneficial effects of caffeine on chronic stress, in accordance with the role of synaptic (18, 19) or glial dysfunction (20) in mood disorders. Thus, $A_{2A}R$ antagonists prolonged escape behavior in two screening tests for antidepressant activity (21–23) and prevented maternal separation-induced long-term cognitive impact (12). We combined pharmacological and tissue-selective $A_{2A}R$ transgenic mice (24, 25) to test if neuronal $A_{2A}R$ controlled the modifications caused by chronic unpredictable stress (CUS).

Results

Validation of the CUS Model. Chronic stress is expected to cause decreased weight gain, increased corticosterone levels, helpless-like and anxiogenic-like behaviors, and decreased performance in memory tests (2, 3). Accordingly, compared with control (i.e., nonstressed) mice, the mice exposed to the 3-wk CUS protocol (Table S1) displayed (*i*) reduced weight gain (Fig. S1*C*); (*ii*) increased corticosterone plasma levels (Fig. S2*A*); (*iii*) no significant modification in the number of crossings in an open-field task

Significance

Epidemiological studies show that individuals exposed to repeated stress, a major trigger of depression, increase their caffeine intake, which correlates inversely with the incidence of depression. However, the mechanism underlying this protective effect is unknown. We used an animal model of chronic unpredictable stress (CUS) to show that caffeine prevents the maladaptive changes caused by CUS in a manner mimicked by the selective blockade of adenosine A_{2A} receptors ($A_{2A}R$). CUS enhanced $A_{2A}R$ in synapses, and the selective elimination of neuronal $A_{2A}R$ abrogated CUS modifications. Moreover, $A_{2A}R$ blockade also afforded a therapeutic benefit, paving the way to consider $A_{2A}R$ blockers as a strategy to manage the negative impact of chronic stress on mood and memory.

Author contributions: M.P.K., P.A., P.M.C., and R.A.C. designed research; A.L.S.R. provided important advice in planning experiments; M.P.K., N.J.M., H.B.S., A.N., A.P.A., M.S., Â.R.T., P.A., and P.M.C. performed research; Y.B., C.E.M., and J.F.C. contributed new reagents/analytic tools; M.P.K., N.J.M., H.B.S., A.N., A.P.A., A.L.S.R., L.O.P., Â.R.T., P.A., P.M.C., and R.A.C. analyzed data; and N.J.M. and R.A.C. wrote the paper.

The authors declare no conflict of interest.

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(Fig. S3*A*), indicating the absence of altered locomotion that might have confounded analysis in other behavioral tasks; (*iv*) increased immobility time in the forced-swimming test (Fig. 1*A*) and in the tail-suspension test (Fig. 1*B*) indicative of a helpless-like state; (*v*) reduced sucrose preference (Fig. 1*C*), indicative of anhedonia; (*vi*) reduced time spent in the open arms of an elevated-plus maze (Fig. 1*D*), indicative of an anxiogenic state; (*vii*) decreased spatial reference memory, gauged by reduced time spent in the novel arm of a modified Y-maze test (Fig. 1*E*) and by a lower recognition index in an object-displacement test (Fig. 1*F*) (n = 36–39 mice per group).

CUS caused no alteration in neuronal organization (determined by cresyl violet staining), no neuronal damage (shown by the lack of FluoroJadeC staining), and no microgliosis (shown by CD11 immunoreactivity) (Fig. 1*G*) but did cause increased GFAP immunoreactivity (Fig. 1 *H* and *I*), which was confirmed by Western blot analysis (Fig. 1*L*). Notably, CUS decreased the immunoreactivity of synaptophysin, a synaptic marker, mainly in the hippocampus (Fig. 1 *J* and *K*), in accordance with the critical impact of synaptic modifications in stress-induced behavioral alterations (26, 27). This putative hippocampal synaptotoxicity caused by CUS was confirmed further by Western blot analysis (Fig. 1 *M* and *N*). Overall, these behavioral, biochemical, and morphological alterations validate our CUS protocol.

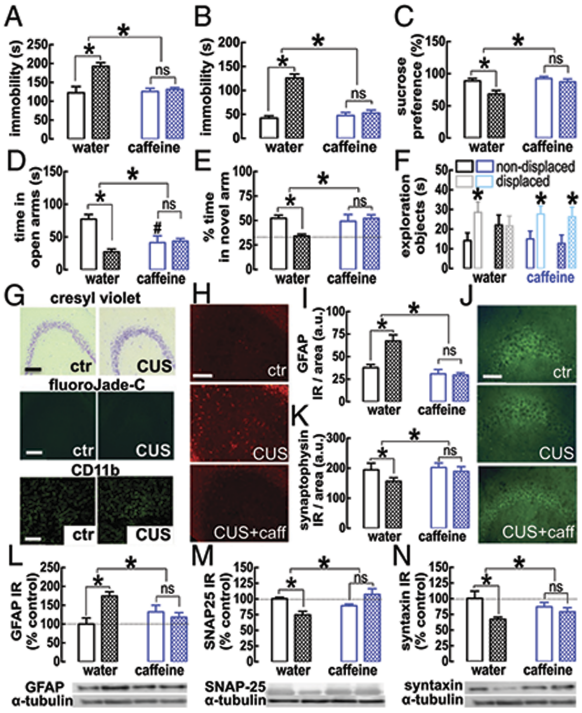

Fig. 1. Mice subjected to CUS display the expected features of depressed mice, which are largely prevented by the regular consumption of caffeine. Male mice (10 wk old) were subjected to a 3-wk period of CUS (Table S1) and were evaluated behaviorally 24 h after the last stressor. Compared with nonstressed control mice (ctr, open bars), CUS-mice (checkered bars) displayed helpless-like behavior as evaluated by the forced-swimming (*A*) and tail-suspension (*B*) tests, anhedonia as evaluated by a sucrose preference test (*C*), anxiety-like behavior as evaluated by the elevated-plus maze test (*D*), and impaired memory performance as evaluated by a modified Y maze test (*E*) and an object-displacement test (Student's *t* test comparing displaced vs. nondisplaced object) (*F*). After mice were killed, the CA3 area of hippocampi from CUS-subjected mice did not display overt neuronal damage, as gauged by the preservation of cresyl violet staining (*G*, *Top Row*) and lack of FluoroJade C staining (*G*, *Middle Row*) or microgliosis as evaluated by CD11b immunoreactivity (*G*, *Bottom Row*) but did display increased GFAP (*H* and *I*) and decreased synaptophysin immunoreactivity (*J* and *K*). Similar findings were obtained in the hippocampal CA1 area. Western blot analysis of whole hippocampal membranes confirmed the increase in GFAP density with CUS (*L*) and the decrease of synaptic markers, namely SNAP25 (*M*) and syntaxin (*N*). The administration of caffeine (1 g/L via the drinking water) to mice beginning 3 wk before CUS and continuing until mice were killed did not modify behavior or histology, except for increased anxiety in the elevated-plus maze (*D*), but did prevent all CUS-induced behavioral and morphological alterations. (Scale bars, 100 μm.) Data are shown as mean ± SEM; n = 9–19 mice per group in the behavioral assays (*A*–*F*); n = 4–7 mice per group in the morphological analysis; n = 5 or 6 mice per group in the neurochemical analysis. $^{*}P < 0.05$ and $^{\#}P < 0.05$ using a two-way ANOVA followed by a Newman–Keuls post hoc test, except when stated otherwise. ns, not significant.

Prophylactic Effect of Caffeine on Chronic Stress. Caffeine consumption in drinking water (1 g/L for 6 wk; n = 18–19 mice per group) did not alter behavioral, morphological, or biochemical parameters in control mice (Fig. 1 and Figs. S1–S3), with the exception of the time spent in the open arms of the elevated-plus maze (Fig. 1*D*), which is suggestive of an anxiogenic effect. In contrast, caffeine prevented the CUS-induced reduction in weight gain (Fig. S1*C*) and the increase in corticosterone plasma levels (Fig. S2*A*) and blunted all other behavioral alterations caused by CUS, namely the increased immobility in the forced-swimming (Fig. 1*A*) and tail-suspension tests (Fig. 1*B*), the anhedonic-like behavior (Fig. 1*C*), the memory impairment in the modified Y maze (Fig. 1*E*) and object-displacement tests (Fig. 1*F*), as well as the loss of synaptic markers gauged by immunohistochemistry (Fig. 1 *J* and *K*) or Western blot (Fig. 1 *M* and *N*).

Alterations of Adenosine Receptors upon Chronic Stress. We tested if caffeine's ability to prevent CUS-induced changes while having little effect in nonstressed mice might be explained by CUS-induced changes in A_1R and $A_{2A}R$ density. CUS decreased the A_1R antagonist [^{3}H]8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX) binding, both in total membranes and in synaptosomal membranes but not in gliosomal [i.e., astrocytic (16)] membranes of the hippocampus (Fig. 2*A*). In contrast, CUS enhanced the $A_{2A}R$ antagonist [^{3}H]5-amino-7-2-phenylethyl.-2-2-furyl-pyrazolo [4,3-ex-1,2,4-triazolo-1,5]pyrimidine ([^{3}H]SCH58261) binding in the synaptosomal membranes (which was 60.1 ± 8.7% larger in CUS-treated mice than in control mice; n = 6, $P < 0.05$) but not in total or in gliosomal membranes of the hippocampus (Fig. 2*B*).

Double-labeling immunocytochemistry of individual nerve terminals further revealed that CUS selectively increased the number of hippocampal glutamatergic [vesicular glutamate transporter 1 (vGluT1)-positive] terminals (Fig. 2*C*) rather than the number of GABAergic [vesicular GABA transporter (vGAT)-positive] terminals (Fig. 2*D*) endowed with $A_{2A}R$.

Neuronal $A_{2A}R$ Control the Burden of Chronic Stress. In accordance with this CUS-induced enhancement of $A_{2A}R$, the selective $A_{2A}R$ antagonist KW6002 (3 mg/kg) mimicked the protection observed with caffeine against CUS-induced alterations (n = 8–10 mice per group) (Fig. 3). Thus, although KW6002 was devoid of effects in nonstressed mice (Fig. 3), it prevented the CUS-induced reduction in weight gain (Fig. S1*D*), the increase in corticosterone plasma levels (Fig. S2*B*), and all other measured behavioral, morphological, and neurochemical alterations caused by CUS (Fig. 3 *A*–*E* and Fig. S4). KW6002 also prevented the CUS-induced decrease in the density of a presynaptic glutamatergic marker, vGluT1 (Fig. 3*F*); this finding is compatible with the synaptic atrophy known to occur in chronic stress (18, 19).

This prophylactic effect on pharmacological $A_{2A}R$ blockade was mimicked by global genetic deletion of $A_{2A}R$ (n = 9–10 mice per group). Thus, CUS did not change the behavior of global $A_{2A}R$-KO

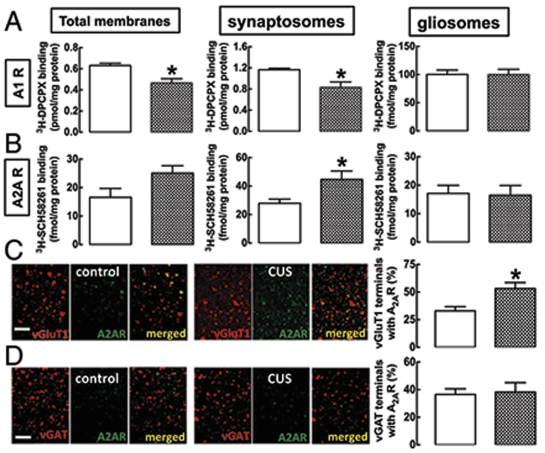

Fig. 2. CUS alters the adenosine neuromodulation system in the hippocampus. Male mice (10 wk old) were subjected to a 3-wk period of CUS (Table S1) and were killed for preparation of total, synaptosomal (i.e., from synapses), and gliosomal (i.e., from astrocytes) membranes from the hippocampus. (*A*) The binding density of A_1R [evaluated with the A_1R antagonist ^{3}H-DPCPX (10 nM)] was decreased in total and synaptosomal membranes and was unaltered in gliosomal membranes of CUS-subjected mice (checkered bars) compared with nonstressed mice (control; open bars). (*B*) In contrast, there was a selective increase in the binding density of the $A_{2A}R$ antagonist ^{3}H-SCH58261 in synaptosomal membranes from CUS-subjected mice, without changes in its binding density in total or gliosomal membranes. (*C* and *D*) Double-labeling immunocytochemical analysis of plated purified nerve terminals confirmed that $A_{2A}R$ are located mostly in glutamatergic (immunopositive for vGluT1) rather than GABAergic (immunopositive for vGAT) nerve terminals and showed that in CUS-exposed mice the number of glutamatergic terminals was enhanced selectively (*C*), rather than GABAergic terminals endowed with $A_{2A}R$ (*D*). Note that this immunocytochemistry approach allows only the relative colocalization of epitopes to be quantified, irrespective of their absolute staining, which varies among groups of plated nerve terminals. We previously validated the selectivity of ^{3}H-SCH58261 and of the anti-$A_{2A}R$ antibodies used, which do not yield any signal in tissue from $A_{2A}R$-KO mice (47). Data are shown as mean ± SEM of 5 or 6 mice per group; $*P < 0.05$ using an unpaired Student's *t* test.

mice in the forced-swimming (Fig. 3*G*), elevated-plus maze (Fig. 3*H*), or modified Y-maze tests (Fig. 3*I*) or the density of synaptic proteins such as syntaxin (Fig. 3*J*) or synaptosomal-associated protein 25 (SNAP-25) (Fig. 3*K*) in the hippocampus, in contrast to the alterations found in wild-type littermates (Fig. 3). In contrast to wild-type mice, $A_{2A}R$-KO mice also failed to display behavioral alterations in the tail suspension, splash, social recognition, and object-displacement tests and in the levels of vGluT1 (Fig. S5). This CUS-induced loss of synaptic markers translated into a synaptic dysfunction, typified by a reduction of long-term potentiation (LTP) amplitude in CUS (16.2 ± 2.8% over baseline, $n = 5$) compared with nonstressed wild-type (control) mice (63.5 ± 6.3% over baseline, $n = 5$) (Fig. 3*L*). As previously reported (11), the acute blockade of $A_{2A}R$ with SCH58261 (50 nM) during LTP induction decreased LTP amplitude in wild-type control mice (by 40.7 ± 4.4% over baseline, $n = 5$; $P < 0.05$ vs. control) but increased LTP amplitude in wild-type mice subjected to CUS (by 36.5 ± 3.1% over baseline, $n = 5$ with SCH58261; $P < 0.05$ compared with 16.2 ± 2.8% without SCH58261) (Fig. 3*L*). Notably, LTP amplitude was unchanged by CUS in global $A_{2A}R$-KO mice (Fig. 3*M*) and in mice drinking KW6002 (Fig. S4).

Given the prominent changes in synaptic proteins and synaptic function paralleling the CUS-induced behavioral changes, together with the changes in the $A_{2A}R$ density mainly in glutamatergic terminals, we hypothesized that neuronal $A_{2A}R$ plays a pivotal role in the emergence of CUS-induced changes. Thus, we tested the impact of CUS on calcium/calmodulin-dependent protein kinase II-α (CaMKII-α) gene promoter-driven forebrain $A_{2A}R$ knockout (hereafter, fb-$A_{2A}R$-KO) mice (n = 7–9 mice per group), in which we previously had shown neuronal $A_{2A}R$ to be eliminated in the forebrain and $A_{2A}R$-mediated control of glutamatergic synapses to be blunted (24, 25). The behavior of fb-$A_{2A}R$-KO mice was similar to that of wild-type mice under control conditions (Fig. 4); however, they did not display behavioral alterations in mood (Fig. 4 *A* and *B*) and memory tests (Fig. 4 *D*–*F* and Fig. S6) after CUS. CUS in these mice also failed to modify the density of synaptic proteins such as syntaxin (Fig. S6), SNAP-25

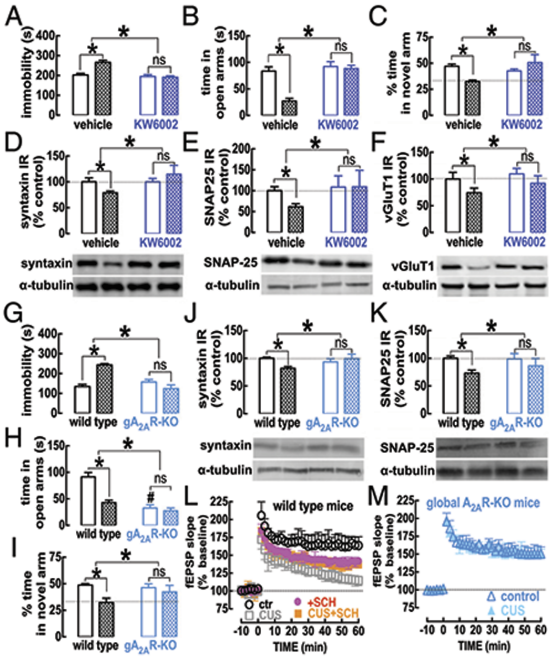

Fig. 3. The pharmacological or genetic blockade of $A_{2A}R$ prevents CUS-induced behavioral, neurochemical, and electrophysiological alterations in the hippocampus. Male mice (10 wk old) were subjected to a 3-wk period of CUS (Table S1) before behavioral evaluation 24 h after the last stressor. In CUS-subjected mice (checkered bars), as compared with vehicle-treated mice (*A*–*F*) (open bars) or wild-type mice (*G*–*M*) (open bars and symbols), the consumption of the $A_{2A}R$ antagonist KW6002 (3 mg/kg, through the drinking water, starting 3 d before CUS until mice were killed) (*A*–*F*), or the genetic elimination of $A_{2A}R$ in global $A_{2A}R$-KO mice (*G*–*M*), prevented the CUS-induced helpless-like behavior evaluated in the forced-swimming test (*A* and *G*), the anxiety-like behavior evaluated in the elevated-plus maze test (*B* and *H*), the impaired memory performance evaluated in a modified Y maze test (*C* and *I*), and the decreases in synaptic markers such as syntaxin (*D* and *J*), SNAP-25 (*E* and *K*), and markers of glutamatergic terminals (vGluT1) (*F*) in hippocampal nerve terminals. Additionally, $A_{2A}R$ blockade with the antagonist SCH58261 (SCH, 50 nM) prevented the CUS-induced depression of LTP [triggered by a high-frequency stimulation train at time 0 in Schaffer fibers (collateral synapses of CA1 pyramidal cells)] of hippocampal slices from wild-type mice (*L*) and CUS failed to modify LTP in $A_{2A}R$-KO mice (*M*) (Student's *t* test). Data are shown as mean ± SEM; n = 8–10 mice per group in the behavioral assays (*A*–*C* and *G*–*I*); n = 5 or 6 mice per group in the neurochemical analyses (*D*–*F*, *J*, and *K*); and n = 5 or 6 mice per group in the electrophysiological analyses (*L* and *M*). $*P < 0.05$ using a two-way ANOVA followed by a Newman–Keuls post hoc test, except when stated otherwise; ns, nonsignificant.

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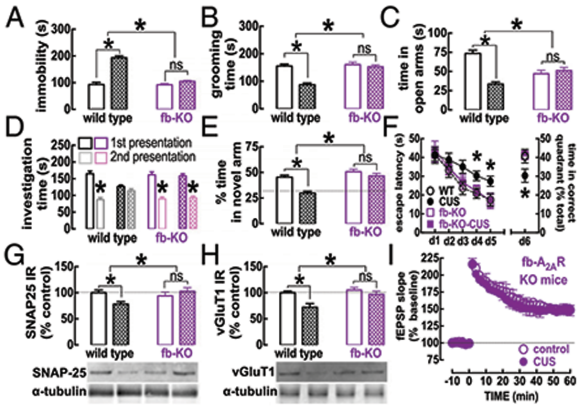

Fig. 4. The selective deletion of neuronal $A_{2A}R$ prevents CUS-induced behavioral, neurochemical, and electrophysiological alterations in the hippocampus. Male mice (10 wk old) were subjected to a 3-wk period of CUS (Table S1) before behavioral evaluation 24 h after the last stressor. In contrast with the impact of CUS in wild-type mice (checkered bars and filled symbols; control: open bars and symbols), in fb-$A_{2A}R$-KO mice (in which neuronal $A_{2A}R$ is eliminated selectively in the forebrain and $A_{2A}R$-mediated control of glutamatergic synapses is blunted) CUS failed to trigger helplessness-like behavior in the forced-swimming test (*A*), anhedonia-like behavior in the splash test (*B*), anxiety-like behavior in the elevated-plus maze test (*C*), impaired social interaction and social interaction memory (Student's *t* test comparing first vs. second presentation of the foreign mouse) (*D*), or impaired memory performance in a modified Y maze (*E*). (*F*) Additionally, in a spatial reference memory version of the Morris water maze test, both the acquisition (days 1–5) and the retention of the location of the hidden platform (day 6) were diminished by CUS in wild-type mice but not in fb-$A_{2A}R$-KO mice. Also in contrast with the deleterious effects of CUS, which decreased the density of synaptic markers such as syntaxin (*G*) and vGluT1 (*H*) in hippocampal nerve terminals in wild-type mice, CUS did not affect the density of either of these synaptic markers (*G* and *H*) or the amplitude of LTP (*I*) in fb$A_{2A}R$-KO mice. Data are shown as mean ± SEM; n = 7–9 mice per group in the behavioral assays (*A*–*F*); n = 5 mice per group in the neurochemical analysis (*G* and *H*) and in the electrophysiological analysis (*I*). $^{*}P <$ 0.05 using a two-way ANOVA followed by a Newman–Keuls post hoc test, with repeated measures for the Morris water maze test. ns, nonsignificant.

(Fig. 4*G*), vGluT1 (Fig. 4*H*), in contrast to the effect of CUS in wild-type mice (Fig. 4 *G* and *H*). Accordingly, hippocampal LTP amplitude in fb-$A_{2A}R$-KO mice (50.9 ± 5.0% over baseline, n = 6) was not affected by either SCH58261 (41.4 ± 4.3% over baseline, n = 5) or CUS (Fig. 4*I*).

$A_{2A}R$ manipulations did not affect ambulation in the open-field test (Fig. S3).

Therapeutic Effect of $A_{2A}R$ Antagonists in Reversing Chronic Stress-Induced Deficits. The impact of CUS on the behavioral, neurochemical, and electrophysiological measures was stable for at least 3 wk after completion of the CUS protocol (Fig. 5), allowing us to test whether treatment with an $A_{2A}R$ antagonist after the CUS protocol reversed the CUS-induced modifications (n = 9–10 mice per group). After the 3-wk CUS protocol, mice showed increased immobility in the forced-swimming test (Fig. 5*A*), decreased time spent in the open arm of an elevated-plus maze (Fig. 5*B*), and decreased time spent in the novel arm of a modified Y maze (Fig. 5*C*). At 6 wk, these alterations were maintained in mice subjected to CUS that were injected daily with vehicle (Fig. 5 *A*–*C*). In contrast, in mice subjected to CUS and then treated with SCH58261 (0.1 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$) for 3 wk, the altered behavior in the forced-swimming (Fig. 5*A*), elevated-plus maze (Fig. 5*B*), and modified Y maze (Fig. 5*C*) tests reverted to values similar to nonstressed mice. At the end of this 6-wk protocol, mice subjected to CUS displayed reduced hippocampal density of syntaxin (Fig. 5*D*), SNAP-25 (−21.15 ± 4.67% compared with nonstressed mice; n = 7–9), and vGluT1 (Fig. 5*E*) and lower amplitude of hippocampal LTP (Fig. 5*F*). Notably, in stressed mice that were treated for 3 wk with SCH58261 (Fig. 5 *D*–*F*), these changes also reverted to values similar to those in nonstressed mice. SCH58261 treatment per se did not cause behavioral or neurochemical alterations in nonstressed mice (Fig. 5).

Discussion

This study shows that caffeine prevents the behavioral, neurochemical, and electrophysiological alterations caused by chronic stress in a manner mimicked by the pharmacological or genetic blockade of adenosine $A_{2A}R$. We also show that neuronal $A_{2A}R$ plays a critical role in controlling the burden of chronic stress in adult mice and also has the ability to reverse the maladaptive changes caused by repeated stress. These findings in a model of CUS that caused a constellation of behavioral changes comparable to these observed in stressed or depressed individuals (28, 29) confirm the previously reported inverse relationship between caffeine consumption and the incidence of depression (5, 6) or suicide (7, 8) and suggest that $A_{2A}R$ has a pivotal role in controlling mood disorders. However, the role of $A_{2A}R$ in anxiety

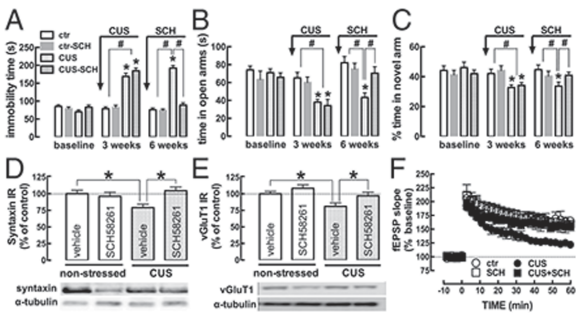

Fig. 5. Blockade of adenosine $A_{2A}R$ reverses CUS-induced alterations. Male mice (10 wk old) were behaviorally evaluated (baseline). Then mice were randomized into two groups. One group (checkered bars) was subjected to a 3-wk period of CUS (Table S1). The mice in the control group (ctr) were handled daily. All mice were behaviorally evaluated after 3 wk. Finally, half of the mice in each group were i.p. injected daily with saline (bars filled with white), and the other half were injected with the $A_{2A}R$ antagonist SCH58261 (SCH, 0.1 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$, gray-outlined bars). All mice were behaviorally evaluated again at 6 wk and then were killed for neurochemical and electrophysiological analysis. Compared with nonstressed (control) mice (noncheckered bars), the mice subjected to CUS (checkered bars) displayed increased immobility in the forced-swimming test [$F_{(2,36)}$ = 182.0, $P < 0.0001$] (*A*), decreased time in the open arms of the elevated-plus maze test [$F_{(1,36)}$ = 77.14, $P < 0.0001$] (*B*), and decreased time spent in the novel arm of a modified Y maze test [$F_{(1,36)}$ = 77.14, $P < 0.0001$] (*C*), both at the end of the CUS protocol (3 wk) and 3 wk later (6 wk). As shown in *A*–*C*, SCH58261 treatment did not affect the behavior of control mice (noncheckered bars) but reversed the CUS-induced alterations in helpless behavior [$F_{(1,36)}$ = 77.14, $P < 0.0001$] (*A*), anxiety [$F_{(2,36)}$ = 21.35, $P < 0.0001$] (*B*), and spatial reference memory [$F_{(2,36)}$ = 14.88, $P = 0.0005$] (*C*) to the level of control (noncheckered bars). SCH58261 treatment also reversed the CUS-induced reduction in the density of the synaptic markers syntaxin [CUS $F_{(1,16)}$ = 4.62; SCH58261 $F_{(1,16)}$ = 10.54; interaction $F_{(1,16)}$ = 18.30] (*D*) and vGluT1 [CUS $F_{(1,16)}$ = 11.60; SCH58261 $F_{(1,16)}$ = 6.25; interaction $F_{(1,16)}$ = 4.54] (*E*) in hippocampal nerve terminal membranes and reversed the CUS-induced decrease in the amplitude of LTP in hippocampal slices [CUS $F_{(1,16)}$ = 49.89; SCH58261 $F_{(1,16)}$ = 16.74; interaction $F_{(1,16)}$ = 29.56] (*F*). Data are shown as mean ± SEM. n = 9 or 10 mice per group in the behavioral assays (*A*–*C*); n = 5 mice per group in the neurochemical analysis (*D* and *E*) and in the electrophysiological analysis (*F*). $^{*}P < 0.05$, $^{\#}P < 0.05$ using a repeated ANOVA followed by a Newman–Keuls post hoc test.

still remains unclear (30), as shown by the inconsistent effects of the different $A_{2A}R$ manipulations.

A major advance provided by this study is the finding that neuronal $A_{2A}R$ controls behavioral dysfunction upon CUS, as demonstrated by the elimination of CUS-induced changes in fb-$A_{2A}R$-KO mice (24, 25). This result excludes a major participation by peripheral adenosine receptors (22) and does not support a prominent role for glial $A_{2A}R$ in controlling CUS-induced alterations, as occurs in animal models of Parkinson's disease (25) or upon treatment with lipopolysaccharide (17). However, the data are compatible with $A_{2A}R$ having a key role in glutamatergic terminals defining the synaptic dysfunction underlying the behavioral alterations associated with repeated stress (26, 27). In accordance with the synaptic atrophy observed in different models of chronic stress (18, 19), CUS led to alterations in synaptic markers and synaptic function in the hippocampus, a brain region known to play a pivotal role in the maladaptive changes upon chronic stress (2, 3). Furthermore, $A_{2A}R$ are located most abundantly in hippocampal nerve endings, and $A_{2A}R$ are selectively engaged to control synaptic plasticity (11). Additionally, noxious brain conditions trigger $A_{2A}R$ up-regulation, which is most evident in synapses (14, 31, 32), particularly in glutamatergic synapses (11, 31), as now shown also under CUS. This $A_{2A}R$ up-regulation is accompanied by an $A_{2A}R$ gain of function (reviewed in ref. 33) that leads to synaptic dysfunction, as demonstrated by the ability of $A_{2A}R$ antagonists to prevent synaptic plasticity dysfunction with aging (11) and maternal separation (12) and by the loss of synaptic markers in different noxious brain conditions (14, 15, 31), namely upon repeated restraint stress (32). How enhanced $A_{2A}R$ function triggers synaptic dysfunction remains to be determined, given our current ignorance of the transducing systems operated by these pleiotropic $A_{2A}R$ (34, 35). This unanswered question has significant importance, because the mechanism seems to be common to the $A_{2A}R$-mediated control of memory and of mood dysfunction.

Overall, this robust ability of $A_{2A}R$ to control CUS-induced alterations provides a rationale explaining the ability of caffeine to attenuate the burden of CUS. The prevention of different alterations caused by repeated stress by regular (not acute) caffeine consumption had been noted previously by others (36–38) and is in tight agreement with the inverse correlation between caffeine intake and the incidence of depression (5, 6). Given that caffeine intake increases in stressed individuals (4), it is tempting to speculate that this increased intake may be a prophylactic antistress measure to normalize mood-related behavioral changes by normalizing synaptic functions via $A_{2A}R$ blockade. The present results also show that the selective antagonism of $A_{2A}R$ offers a therapeutic benefit in stressed rats similar to the reversion of memory impairment in aged rodents (39). Thus we prompt the suggestion that the up-regulated $A_{2A}R$ might be an effective target to correct brain disorders that involve a synaptic dysfunction, as now observed for the maladaptive responses to chronic stress.

Materials and Methods

Animals. Male C57BL/6 mice (10–12 wk old) were obtained from Charles River. Global $A_{2A}R$-KO and fb-$A_{2A}R$-KO mice, both in the C57BL/6 background (24), were raised based on mating of heterozygotes. Mice were handled according to European Union guidelines, as approved by the CNC Ethical Committee for Animal Research (ORBEA-78/2013).

CUS and Administration of Drugs. Prophylactic studies with caffeine or KW6002 were done using previously validated doses (14, 40). Mice were divided into two groups: a drug-free group drinking water or vehicle and a treated group drinking either 1 g/L caffeine (Sigma), starting 3 wk before CUS, or KW6002 (istradefylline; 3 mg/kg, dissolved in 0.5% nitrocellulose), synthesized as described previously (41), starting 4 d before CUS. At the end of treatments, blood samples were collected at 9:00 AM, and the plasma concentrations of caffeine and corticosterone were determined by HPLC (31) and RIA (MP Biomedicals), respectively. Half the animals in each group were subjected to a CUS protocol (42) for 21 d (Table S1). Control (i.e., nonstressed) and stressed mice were housed individually and were submitted to behavioral tests 24 h after the last stressor.

In therapeutic studies, mice first were characterized behaviorally, then were subjected to the 3-wk CUS protocol, and were characterized behaviorally again. They then were randomized into two groups. One group was treated daily with saline, and the other group was treated with a validated selective dose of the $A_{2A}R$ antagonist SCH58261 (0.1 mg/kg, i.p.) for 3 wk (15) before the final behavioral characterization. Then all mice were killed by decapitation after halothane-induced deep anesthesia.

Behavioral Tests. Locomotor and exploratory behavior was monitored using an open-field apparatus (14). Evaluation of anxiety was carried out using the elevated-plus maze, and the helpless-like behavior was evaluated in the tail-suspension and forced-swimming tests (43). Anhedonic-like behavior was evaluated with the sucrose (1.2%) preference test (44) by measuring the intake of sucrose solution versus water intake at the end of a 16-h test period (12-h dark phase plus 4-h light phase) or with the splash test by measuring grooming bouts (head washing and nose/face and body grooming) over 5 min after a 10% sucrose solution was squirted on the dorsal coat (45). Social recognition memory was evaluated as previously described (39), and spatial memory was evaluated using an object-displacement test (40), a reference memory version of the Morris water maze (46), and a two-trials Y-maze test in which mice first explored the maze for 8 min while one arm was blocked and explored it again 2 h later for 8 min with all three arms accessible (14). Behavioral experiments were conducted between 10:00 AM and 5:00 PM in a sound-attenuated room under low-intensity light and were monitored by two researchers who were unaware of phenotypes or drug treatments.

Western Blot Analysis in Hippocampal Membranes. Western blot analyses of total membranes [to evaluate the astrocytic marker GFAP (1:10,000; Sigma)] or Percoll-purified synaptosomal membranes [to probe synaptic markers, using antibodies against syntaxin (1:5,000; Sigma), SNAP-25 (1:5,000; Sigma), and vGluT1 (1:5,000; Chemicon)] were performed as described previously (14, 31). Membranes then were reprobed with α-tubulin (1:10,000; Sigma) as a loading control.

Membrane-Binding Assays. The density of A_1R and $A_{2A}R$ was estimated by radioligand-binding assays using supramaximal concentrations of the A_1R antagonist [^{3}H]DPCPX (10 nM; DuPont NEN) or the $A_{2A}R$ antagonist [^{3}H]SCH58261 (6 nM; provided by E. Ongini, Schering-Plough, Milan, Italy), as described previously (31, 32). Specific binding was determined by the subtraction of nonspecific binding measured in the presence of 3 μM XAC (Tocris), a mixed A_1R/A_2R antagonist.

Immunocytochemistry of Purified Nerve Terminals. Immunocytochemistry of purified nerve terminals was performed as previously described (31) by double labeling with goat anti-$A_{2A}R$ (1:200; Santa Cruz Biotechnology) together with either guinea pig anti-vGAT (1:1,000; Calbiochem) or guinea pig anti-vGluT1 (1:1,000; Chemicon) followed by incubation with Alexa Fluor-labeled secondary antibodies (1:2,000; Molecular Probes). The preparations were examined under a Zeiss Z2 microscope, and each coverslip was analyzed by counting an average of 500 elements (31).

Mouse Brain Histochemistry. Neuronal morphology was assessed using cresyl violet staining of Nissl bodies, and neuronal degeneration was evaluated by FluoroJade-C staining in 20-μm brain sections (14, 32). Immunohistochemical analysis of microglia (CD11-b staining; 1:500; Serotec), astrocytes (GFAP staining; 1:1,000; Sigma), or nerve terminals (synaptophysin staining; 1:200; Sigma) was done as previously described (17, 31).

Electrophysiological Analysis of Synaptic Plasticity. Electrophysiological recordings of synaptic plasticity were performed in 400-μm hippocampal slices, as described previously (11). Briefly, a bipolar electrode was placed onto Schaffer fibers, and the evoked field excitatory postsynaptic potentials (fEPSP) were recorded through an extracellular microelectrode (4 M NaCl; 1–2 MΩ resistance) placed in the CA1 stratum radiatum. LTP was induced with a high-frequency stimulation train (100 pulses at 100 Hz, over a 0.066-Hz basal stimulation) and was quantified as the percentage change between the fEPSP slopes 60 min after and 10 min before the train.

Statistics. Results are given as mean ± SEM of *n* animals, and significance was considered at $P < 0.05$ using Student's *t* test for comparison between two

groups or two-way ANOVA followed by a Newman–Keuls post hoc test for comparison of multiple groups (Table S2).

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Supporting Information

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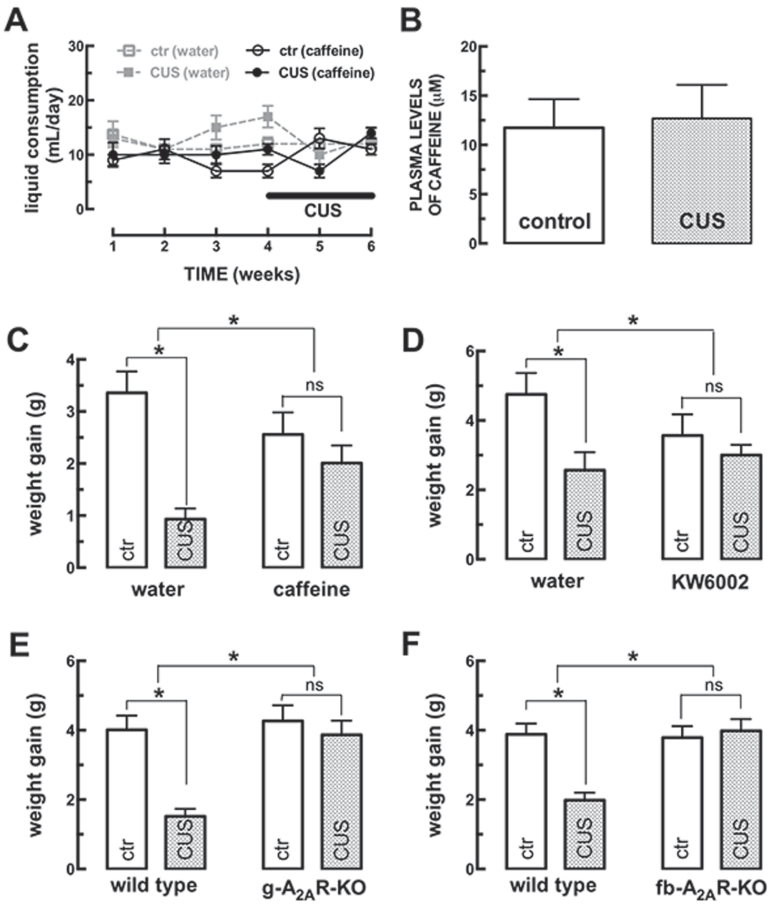

Fig. S1. Mice subjected to CUS do not change their liquid intake and have a decreased weight gain. Male mice (10 wk old) were subjected to a 3-wk period of CUS (Table S1). (*A*) Liquid intake is not significantly altered upon CUS or with caffeine consumption. (*B*) Caffeine reaches similar plasma concentrations in control mice and in mice subjected to CUS, as measured by HPLC. (*C–F*) Compared with control mice (ctr), CUS caused decreased weight gain, which was prevented by the regular consumption of either caffeine (1 g/L) [CUS $F_{(1,70)} = 17.95$; caffeine $F_{(1,70)} = 0.16$; interaction $F_{(1,70)} = 7.15$] (*C*) or the selective $A_{2A}R$ antagonist KW6002 (3 mg/kg) [CUS $F_{(1,33)} = 6.64$; KW6002 $F_{(1,33)} = 0.49$; interaction $F_{(1,33)} = 2.27$] (*D*) and which also was abrogated by the global deletion of $A_{2A}R$ (g-$A_{2A}R$-KO) [CUS $F_{(1,36)} = 14.33$; genotype $F_{(1,36)} = 11.69$; interaction $F_{(1,36)} = 7.50$] (*E*) or by the selective deletion of neuronal $A_{2A}R$ in fb-$A_{2A}R$-KO mice [CUS $F_{(1,32)} = 7.92$; genotype $F_{(1,32)} = 9.89$; interaction $F_{(1,32)} = 11.84$] (*F*). Data are shown as mean ± SEM. $n = 19–27$ mice per group in the liquid consumption experiment (*A*); $n = 6$ or 7 mice per group in the HPLC experiments (*B*); $n = 7–19$ mice per group in the weight-gain determinations (*C–F*). $^*P < 0.05$ using two-way ANOVA followed by a Newman–Keuls post hoc test; ns, nonsignificant.

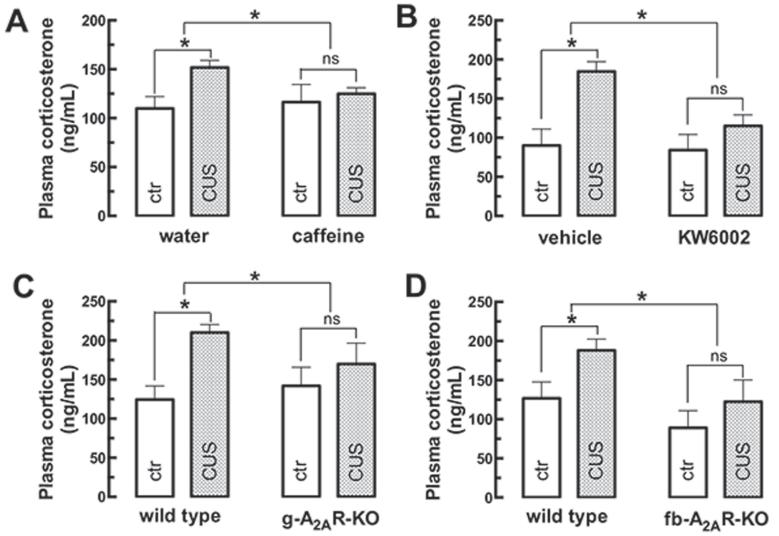

Fig. S2. CUS increased the plasma levels of corticosterone, and caffeine, and $A_{2A}R$ blockade prevented this increase. Male mice (10 wk old) were subjected to a 3-wk period of CUS (Table S1). Three days later, animals were gently immobilized, and blood was collected from the tail vein. Animals were killed at 9:00 AM on the day of blood collection. Compared with control mice (ctr), CUS increased the plasma levels of corticosterone. This increase was prevented by the regular consumption of either caffeine (1 g/L) [CUS $F_{(1,70)} = 7.95$; caffeine $F_{(1,70)} = 2.34$; interaction $F_{(1,70)} = 4.36$] (*A*) or the selective $A_{2A}R$ antagonist KW6002 (3 mg/kg) [CUS $F_{(1,33)} = 15.30$; KW6002 $F_{(1,33)} = 6.78$; interaction $F_{(1,33)} = 4.99$] (*B*) and also was abrogated by the global deletion of $A_{2A}R$ (g-$A_{2A}R$-KO) [CUS $F_{(1,36)} = 14.91$; genotype $F_{(1,36)} = 1.50$; interaction $F_{(1,36)} = 6.41$] (*C*) or by the selective deletion of neuronal $A_{2A}R$ in fb-$A_{2A}R$-KO mice [CUS $F_{(1,32)} = 16.79$; genotype $F_{(1,32)} = 20.55$; interaction $F_{(1,32)} = 4.56$] (*D*). Data are shown as mean ± SEM of 7–19 mice per group. $^*P < 0.05$ using two-way ANOVA followed by a Newman–Keuls post hoc test; ns, nonsignificant.

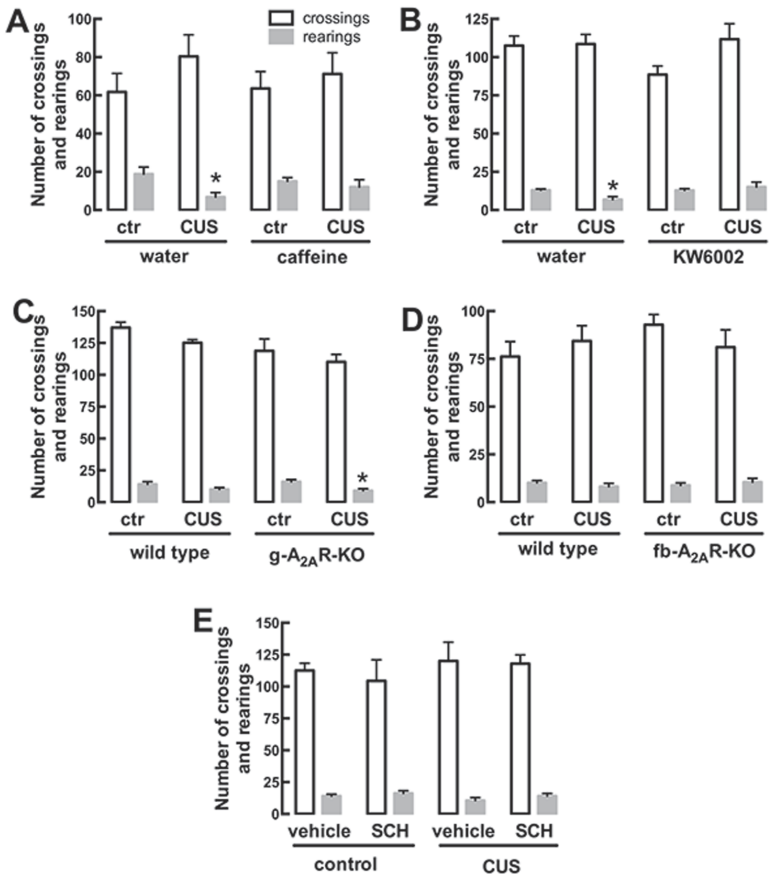

Fig. S3. CUS does not significantly affect spontaneous locomotion. Male mice (10 wk old) were subjected to a 3-wk period of CUS (Table S1) and were evaluated 24 h after the last stressor in an open-field arena. Both the number of crossings and the number of rearing events were globally similar in non-stressed control mice (ctr) and in mice subjected to CUS. Furthermore, neither the regular consumption of caffeine (1 g/L) (*A*) or of the selective $A_{2A}R$ antagonist KW6002 (3 mg/kg) (*B*) nor the global deletion of $A_{2A}R$ (g-$A_{2A}R$-KO) (*C*) or the selective deletion of neuronal $A_{2A}R$ in fb-$A_{2A}R$-KO mice (*D*) affected spontaneous locomotion. (*E*) Likewise, the pattern of spontaneous locomotion was similar in control mice and mice subjected to CUS that subsequently were treated for 3 wk with the $A_{2A}R$ antagonist SCH58261. Data are shown as mean ± SEM of 7–19 mice per group. *$P < 0.05$ using two-way ANOVA followed by a Newman–Keuls post hoc test; ns, nonsignificant.

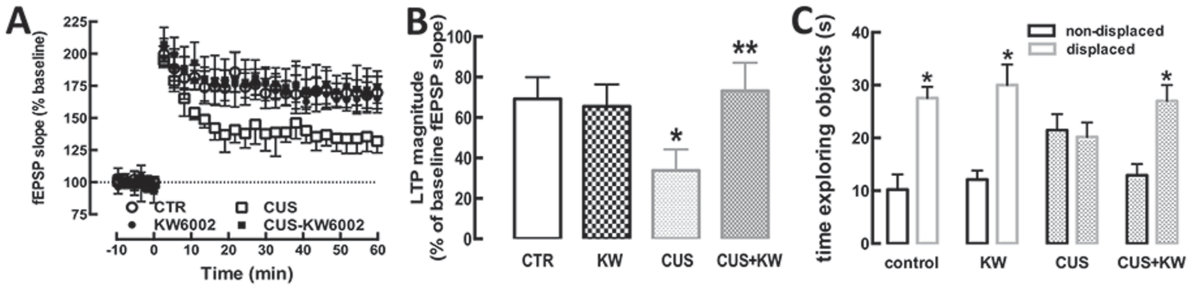

Fig. S4. The pharmacological inhibition of $A_{2A}R$ blunts behavioral and electrophysiological alterations caused by CUS. Male mice (10 wk old) were subjected to a 3-wk period of CUS (Table S1) and were evaluated 24 h after the last stressor in an open-field arena. Mice were killed 8 d after the end of the CUS period. (*A* and *B*) Synaptic plasticity (induced by a high-frequency stimulation train at time 0) recorded in Schaffer fibers (CA1 pyramid synapses) was decreased in hippocampal slices from mice subjected to CUS, and this decrease was prevented in mice consuming the $A_{2A}R$ antagonist KW6002 (KW) (3 $mg \cdot kg^{-1} \cdot d^{-1}$) from 3 d before the beginning of the protocol until the end of the CUS protocol. $n = 5–6$ mice per group. *$P < 0.05$ vs. control; **$P < 0.05$ vs. CUS; two-way ANOVA [CUS $F_{(1,11)} = 5.24$; KW6002 $F_{(1,11)} = 8.71$; interaction $F_{(1,11)} = 12.60$] followed by a Newman–Keuls post hoc test. CTR, control. (*C*) In an object-displacement test, mice were exposed to two identical objects for 3 min and 90 min later were exposed for 3 min to the same objects, but with one of the objects in a different location. Control mice spent more time sniffing/exploring the displaced object than the nondisplaced object. This spatial recognition memory was blunted in mice subjected to CUS but was present in mice consuming KW6002. $n = 8–10$ mice per group. *$P < 0.05$ vs. time exploring the nondisplaced object; Student's *t* test.

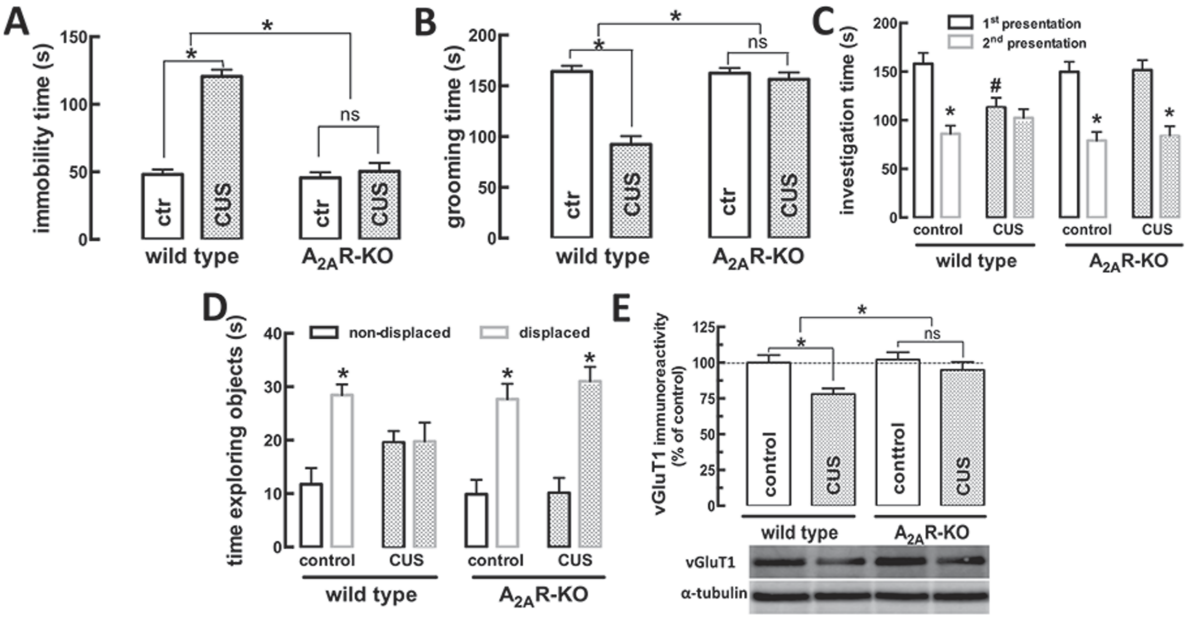

Fig. S5. The genetic deletion of $A_{2A}R$ blunts behavioral and neurochemical alterations caused by CUS. Male mice (10 wk old) were subjected to a 3-wk period of CUS (Table S1) and were evaluated in an open-field arena 24 h after the last stressor. Mice were killed 8 d after the end of the CUS period. In contrast with the effect of CUS (patterned bars) in wild-type mice, in global $A_{2A}R$-KO mice CUS failed to trigger helpless-like behavior in the tail-suspension test (*A*) [CUS $F_{(1,36)}$ = 65.33; genotype $F_{(1,36)}$ = 58.16; interaction $F_{(1,36)}$ = 50.48], anhedonia-like behavior in the splash test (*B*) [CUS $F_{(1,36)}$ = 35.61; genotype $F_{(1,36)}$ = 22.87; interaction $F_{(1,36)}$ = 25.51], or impaired social interaction (*C*) [first presentation, CUS $F_{(1,36)}$ = 4.32; genotype $F_{(1,36)}$ = 2.09; interaction $F_{(1,36)}$ = 5.04; social interaction memory, Student's *t* test comparing first vs. second presentation of the foreign mouse] or impaired short-term spatial memory in the object-displacement test (*D*) (n = 10; $^*P < 0.05$ vs. time exploring the nondisplaced object, Student's *t* test). (*E*) The density of the vGluT1, a marker of glutamatergic terminals, was reduced in the hippocampus of wild-type mice subjected to CUS. This reduction was not observed in $A_{2A}R$-KO mice subjected to CUS. n = 5–6 mice per group. $^*P < 0.05$, two-way ANOVA [CUS $F_{(1,20)}$ = 11.42; genotype $F_{(1,20)}$ = 7.53; interaction $F_{(1,20)}$ = 5.29] followed by a Newman–Keuls post hoc test. n = 5–6 mice per group; nonsignificant (ns) differences at $P > 0.05$, two-way ANOVA [CUS $F_{(1,20)}$ = 0.19; genotype $F_{(1,20)}$ = 0.01; interaction $F_{(1,20)}$ = 0.02] followed by a Newman–Keuls post hoc test.

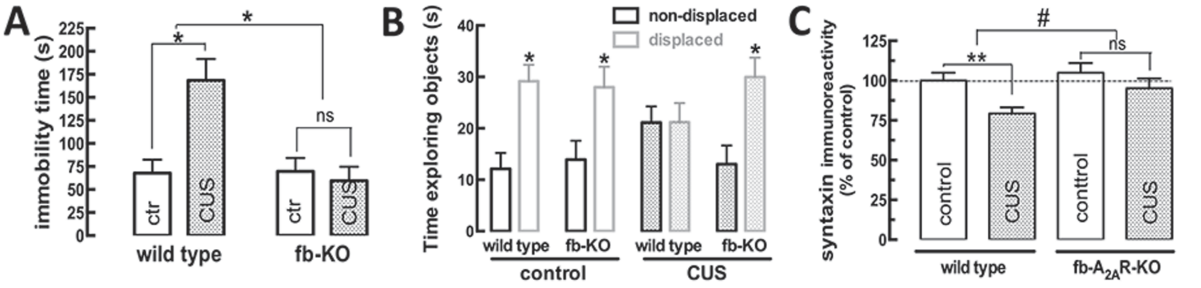

Fig. S6. The selective deletion of neuronal $A_{2A}R$ blunts behavioral and neurochemical alterations caused by CUS. Male mice (10 wk old) were subjected to a 3-wk period of CUS (Table S1) and were evaluated 24 h after the last stressor in an open-field arena. Mice were killed 8 d after the end of the CUS period. In contrast with the impact of CUS in wild-type mice (patterned bars), in fb-$A_{2A}R$-KO mice (in which neuronal $A_{2A}R$ is eliminated selectively in the forebrain and $A_{2A}R$-mediated control of glutamatergic synapses is blunted), CUS failed to trigger helpless-like behavior in the tail-suspension test [CUS $F_{(1,32)}$ = 61.10; genotype $F_{(1,32)}$ = 86.09; interaction $F_{(1,32)}$ = 91.89] (*A*) or impaired short-term spatial memory in the object-displacement test (n = 9; $^*P < 0.05$ vs. time exploring the nondisplaced object; Student's *t* test) (*B*). (*C*) The density of the presynaptic marker syntaxin was reduced in the hippocampus of wild-type subjected to CUS. This reduction was not observed in fb-$A_{2A}R$-KO mice subjected to CUS. n = 5; $^*P < 0.05$, two-way ANOVA [CUS $F_{(1,16)}$ = 19.15; genotype $F_{(1,16)}$ = 10.13; interaction $F_{(1,16)}$ = 4.16] followed by a Newman–Keuls post hoc test.

Table S1. Protocol of CUS showing the schedule of application of the different stressors during the 3-wk CUS period

Week	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Week 1	Damp bedding	Paired housing (1 h)	Restraint stress (2 h)	Cold bath (15 °C, 20 min)	Inescapable shock (0.7 mA)	Apparatus exposure, no footshock	Light/dark cycle inversion
Week 2	Cage tilt (45°)	Food + water deprivation (24 h)	Empty bottle exposure (1 h)	Damp bedding	Paired housing (1 h)	Restraint stress (2 h)	Cold bath (15 °C, 20 min)
Week 3	Inescapable shock (0.7 mA)	Apparatus exposure, no footshock	Food + water deprivation (24 h)	Empty bottle exposure (1 h)	Light/dark cycle inversion	Cage tilt (45°)	Restraint stress (2 h)

Table S2. Statistical values resulting from the two-way ANOVA analysis of the different groups of mice subjected to the 3-wk CUS protocol and treated with different drugs or having different genotypes

Group	Assay	Effect of CUS	Effect of drug or genotype	Interaction
Caffeine	Weight gain	$F_{(1,70)} = 17.95$; $P < 0.0001$	$F_{(1,70)} = 0.1585$; $P = 0.6918$	$F_{(1,70)} = 7.145$; $P = 0.0093$
	Cortisol	$F_{(1,70)} = 7.952$; $P = 0062$	$F_{(1,70)} = 2.336$; $P = 0.1309$	$F_{(1,70)} = 4.361$; $P = 0.0404$
	Forced swim	$F_{(1,43)} = 12.25$; $P = 0.0011$	$F_{(1,43)} = 7.098$; $P = 0.0108$	$F_{(1,43)} = 9.033$; $P = 0.0044$
	Tail suspension	$F_{(1,42)} = 45.40$; $P < 0.0001$	$F_{(1,42)} = 26.25$; $P < 0.0001$	$F_{(1,42)} = 35.18$; $P < 0.0001$
	Sucrose preference	$F_{(1,42)} = 8.368$; $P = 0060$	$F_{(1,42)} = 9.311$; $P = 0.039$	$F_{(1,42)} = 4.552$; $P = 0.0039$
	Elevated-plus maze	$F_{(1,41)} = 13.28$; $P = 0007$	$F_{(1,41)} = 2.183$; $P = 0.1472$	$F_{(1,41)} = 15.77$; $P = 0.0003$
	Modified Y maze	$F_{(1,41)} = 8.268$; $P = 0091$	$F_{(1,41)} = 7.490$; $P = 0.0091$	$F_{(1,41)} = 15.01$; $P = 0.0004$
	GFAP IHC	$F_{(1,21)} = 4.866$; $P = 0.0387$	$F_{(1,21)} = 20.81$; $P = 0.0002$	$F_{(1,21)} = 9.942$; $P = 0.0048$
	Synaptophysin IHC	$F_{(1,21)} = 9.531$; $P = 0.0056$	$F_{(1,21)} = 14.76$; $P = 0.0009$	$F_{(1,21)} = 6.814$; $P = 0.0163$
	GFAP	$F_{(1,19)} = 5.168$; $P = 0.0348$	$F_{(1,19)} = 0.8074$; $P = 0.3801$	$F_{(1,19)} = 11.34$; $P = 0.0032$
	Syntaxin	$F_{(1,19)} = 5.183$; $P = 0.0346$	$F_{(1,19)} = 1.041$; $P = 0.3204$	$F_{(1,19)} = 4.609$; $P = 0.0449$
	SNAP-25	$F_{(1,19)} = 6.754$; $P = 0.0176$	$F_{(1,19)} = 2.212$; $P = 0.1534$	$F_{(1,19)} = 10.79$; $P = 0.0039$
KW6002	Weight gain	$F_{(1,33)} = 6.635$; $P = 0.0147$	$F_{(1,33)} = 0.4935$; $P = 0.4873$	$F_{(1,33)} = 2.274$; $P = 0.1411$
	Cortisol	$F_{(1,33)} = 15.30$; $P = 0.0004$	$F_{(1,33)} = 6.784$; $P = 0.0137$	$F_{(1,33)} = 4.987$; $P = 0.0324$
	Forced swim	$F_{(1,33)} = 11.50$; $P = 0.0018$	$F_{(1,33)} = 22.08$; $P < 0.0001$	$F_{(1,33)} = 14.65$; $P = 0.0005$
	Tail suspension	$F_{(1,33)} = 108.9$; $P < 0.0001$	$F_{(1,33)} = 79.88$; $P < 0.0001$	$F_{(1,33)} = 66.12$; $P < 0.0001$
	Elevated-plus maze	$F_{(1,33)} = 17.65$; $P = 0.0002$	$F_{(1,33)} = 24.00$; $P < 0.0001$	$F_{(1,33)} = 13.50$; $P = 0.0008$
	Modified Y maze	$F_{(1,33)} = 9.987$; $P = 0.0034$	$F_{(1,33)} = 2.501$; $P = 0.1233$	$F_{(1,33)} = 7.112$; $P = 0.0118$
	Syntaxin	$F_{(1,18)} = 12.48$; $P = 0.0024$	$F_{(1,18)} = 0.9993$; $P = 0.3307$	$F_{(1,18)} = 12.76$; $P = 0.0022$
	SNAP-25	$F_{(1,19)} = 13.50$; $P = 0.0016$	$F_{(1,19)} = 6.006$; $P = 0.0241$	$F_{(1,19)} = 6.541$; $P = 0.0192$
	vGluT1	$F_{(1,20)} = 12.15$; $P = 0.0023$	$F_{(1,20)} = 0.4262$; $P = 0.5213$	$F_{(1,20)} = 4.822$; $P = 0.0401$
	LTP amplitude	$F_{(1,11)} = 5.241$; $P = 0.0428$	$F_{(1,11)} = 8.712$; $P = 0.0132$	$F_{(1,11)} = 12.60$; $P = 0.0046$
Global A_{2A}R-KO	Weight gain	$F_{(1,36)} = 14.33$; $P = 0.0006$	$F_{(1,36)} = 11.69$; $P = 0.0016$	$F_{(1,36)} = 7.495$; $P = 0.0096$
	Cortisol	$F_{(1,36)} = 14.91$; $P = 0.0005$	$F_{(1,36)} = 1.497$; $P = 0.2291$	$F_{(1,36)} = 6.413$; $P = 0.0158$
	Forced swim	$F_{(1,36)} = 9.029$; $P = 0.0048$	$F_{(1,36)} = 14.98$; $P = 0.0004$	$F_{(1,36)} = 32.46$; $P < 0.0001$
	Tail suspension	$F_{(1,36)} = 65.33$; $P < 0.0001$	$F_{(1,36)} = 58.16$; $P < 0.0001$	$F_{(1,36)} = 50.48$; $P < 0.0001$
	Splash	$F_{(1,36)} = 35.61$; $P < 0.0001$	$F_{(1,36)} = 22.87$; $P < 0.0001$	$F_{(1,36)} = 25.51$; $P < 0.0001$
	Elevated-plus maze	$F_{(1,36)} = 18.89$; $P = 0.0001$	$F_{(1,36)} = 35.92$; $P < 0.0001$	$F_{(1,36)} = 12.32$; $P = 0.0012$
	Social interaction	$F_{(1,36)} = 4.318$; $P = 0.0449$	$F_{(1,36)} = 2.095$; $P = 0.1564$	$F_{(1,36)} = 5.041$; $P = 0.0310$
	Modified Y maze	$F_{(1,36)} = 12.22$; $P = 0.0013$	$F_{(1,36)} = 2.189$; $P = 0.1477$	$F(1,36) = 4.959$; $P = 0.0323$
	Syntaxin	$F_{(1,20)} = 5.724$; $P = 0.0267$	$F_{(1,20)} = 5.068$; $P = 0.0358$	$F_{(1,20)} = 13.79$; $P = 0.0014$
	SNAP-25	$F_{(1,20)} = 7.820$; $P = 0.0111$	$F_{(1,20)} = 4.318$; $P = 0.0508$	$F_{(1,20)} = 5.318$; $P = 0.0319$
	vGluT1	$F_{(1,20)} = 11.42$; $P = 0.0030$	$F_{(1,20)} = 7.530$; $P = 0.0125$	$F_{(1,20)} = 5.289$; $P = 0.0324$
	Effect of SCH58261 in LTP amplitude, WT	$F_{(1,16)} = 67.12$; $P < 0.0001$	$F_{(1,16)} = 0.1595$; $P = 6949$	$F_{(1,16)} = 46.75$; $P < 0.0001$
Forebrain A_{2A}R-KO	Weight gain	$F_{(1,32)} = 7.924$; $P = 0.0083$	$F_{(1,32)} = 9.886$; $P = 0.0036$	$F_{(1,32)} = 11.84$; $P = 0.0016$
	Cortisol	$F_{(1,32)} = 16.79$; $P = 0.0003$	$F_{(1,32)} = 20.55$; $P < 0.0001$	$F_{(1,32)} = 4.563$; $P = 0.0404$
	Forced swim	$F_{(1,32)} = 84.68$; $P < 0.0001$	$F_{(1,32)} = 53.24$; $P < 0.0001$	$F_{(1,32)} = 51.40$; $P < 0.0001$
	Tail suspension	$F_{(1,32)} = 61.10$; $P < 0.0001$	$F_{(1,32)} = 86.09$; $P < 0.0001$	$F_{(1,32)} = 91.89$; $P < 0.0001$
	Splash	$F_{(1,32)} = 27.69$; $P < 0.0001$	$F_{(1,32)} = 23.91$; $P < 0.0001$	$F_{(1,32)} = 17.14$; $P = 0.0002$
	Elevated-plus maze	$F_{(1,32)} = 21.01$; $P < 0.0001$	$F_{(1,32)} = 1.242$; $P = 0.2735$	$F_{(1,32)} = 31.35$; $P < 0.0001$
	Social interaction	$F_{(1,32)} = 8.525$; $P = 0.0064$	$F_{(1,32)} = 3.907$; $P = 0.0568$	$F_{(1,32)} = 6.147$; $P = 0.0186$
	Modified Y maze	$F_{(1,30)} = 20.43$; $P < 0.0001$	$F_{(1,30)} = 25.82$; $P < 0.0001$	$F_{(1,30)} = 6.918$; $P = 0.0133$
	Syntaxin	$F_{(1,16)} = 19.15$; $P = 0.0005$	$F_{(1,16)} = 3.902$; $P = 0.0658$	$F_{(1,16)} =1$ 0.13; $P = 0.0058$
	SNAP-25	$F(1,16) = 6.482$; $P = 0.0216$	$F_{(1,16)} = 4.215$; $P = 0.0568$	$F_{(1,16)} = 7.443$; $P = 0.0149$
	vGluT1	$F_{(1,16)} = 15.43$; $P = 0.0012$	$F_{(1,16)} = 10.76$; $P = 0.0047$	$F_{(1,16)} = 4.668$; $P = 0.0463$

ANEXO II

Lista de publicações em periódicos durante o doutoramento 2009-2015

LISTA

Stangherlin EC, **Ardais** AP, Rocha JB, Nogueira CW. *Exposure to diphenyl ditelluride, via maternal milk, causes oxidative stress in cerebral cortex, hippocampus and striatum of young rats.* Arch Toxicol 83: 485-491, **2009.**

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Botton PH, Costa MS, **Ardais AP**, Mioranzza S, Souza DO, da Rocha JB, Porciúncula LO. *Caffeine prevents disruption of memory consolidation in the inhibitory avoidance and novel object recognition tasks by scopolamine in adult mice.* Behav Brain Res 214: 254-259, **2010.**

Mioranzza S, Botton PH, Costa MS, Espinosa J, Kazlauckas V, **Ardais AP,** Souza DO, Porciúncula LO. *Adenosine A_1 receptors are modified by acute treatment with methylphenidate in adult mice.* Brain Res 1357: 62-69, **2010.**

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Costa MS, **Ardais AP**, Fioreze GT, Mioranzza S, Botton PH, Portela LV, Souza DO, Porciúncula LO.*Treadmill running frequency on anxiety and hippocampal adenosine receptors density in adult and middle-aged rats.* Prog Neuropsychopharmacol Biol Psychiatry 36: 198-204, **2012.**

Costa MS, **Ardais AP**, Fioreze GT, Mioranzza S, Botton PH, Souza DO, Rocha JB, Porciúncula LO. *The impact of the frequency of moderate exercise on memory*

and brain-derived neurotrophic factor signaling in young adult and middle-aged rats. Neuroscience 222: 100-109, **2012**.

Sallaberry C, Nunes F, Costa MS, Fioreze GT, **Ardais AP**, Botton PH, Klaudat B, Forte T, Souza DO, Elisabetsky E, Porciúncula LO. *Chronic caffeine prevents changes in inhibitory avoidance memory and hippocampal BDNF immunocontent in middle-aged rats*. Neuropharmacology 64: 153-159, **2013.**