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Ratos púberes de ambos os sexos e ratos envelhecidos apresentam distintas alterações comportamentais e em proteínas sinápticas pelo tratamento crônico com cafeína

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APRESENTAÇÃO

Conforme as normas do Programa de Pós Graduação em Ciências Biológicas: Bioquímica, esta tese de doutorado está organizada em três partes e os resultados estão apresentados na forma de artigo científico.

- ✓ **Parte I**: contém os Resumos, Lista de Abreviaturas, Introdução e Objetivos do trabalho.
- ✓ **Parte II**: contém os capítulos 1, 2 e 3, apresentados na forma artigos científicos publicados e/ou em preparação.
- ✓ **Parte III**: constitui-se da Discussão, Conclusões, Perspectivas e Referências Bibliográficas.

A Introdução apresenta uma revisão bibliográfica sobre os temas discutidos ao longo da tese, e busca dar um embasamento para o entendimento e relevância do trabalho realizado.

Os resultados são apresentados na Parte II, sob a forma de artigos científicos, onde há a descrição da metodologia utilizada, bem como os resultados e interpretações dos mesmos.

A Discussão e Conclusão englobam os capítulos descritos na Parte II, com interpretações e comentários gerais sobre os resultados presentes nos artigos científicos.

As Referências Bibliográficas adicionais ao final desta tese representam as utilizadas apenas na Introdução e Discussão desta tese.

SUMÁRIO

PARTE I

RESUMO

A cafeína é o psicoestimulante mais consumido em todo o mundo, cujos efeitos benéficos nas funções cognitivas têm sido observados em diferentes condições e modelos animais. O consumo de cafeína é difundido entre adultos, idosos, gestantes e mais recentemente, entre crianças e adolescentes. Alguns estudos clínicos e pré-clínicos sugerem que a exposição pré-natal à cafeína apresenta efeitos prejudiciais, como prematuridade, malformações congênitas, baixo peso ao nascer e mesmo teratogenicidade, enquanto outros estudos não demonstraram efeitos deletérios a longo prazo da cafeína. Além disso, poucos estudos têm abordado os efeitos da cafeína nas diferenças de sexo durante a puberdade e/ou adolescência. Devido ao alto consumo de bebidas contendo cafeína por crianças e adolescentes, existe uma grande preocupação a respeito dos seus potenciais efeitos nocivos nessas subpopulações. Assim, considerando que o consumo de cafeína tem crescido nesta população, no primeiro capítulo desta tese investigamos as alterações comportamentais e de proteínas sinápticas em ratos machos e fêmeas púberes expostos à cafeína pelo consumo materno durante a gestação, lactação e na água de beber até o início da puberdade. Ratas Wistar adultas receberam cafeína na água de beber (0.1 e 0.3 g/L) durante o seu ciclo ativo, em dias úteis, duas semanas antes do acasalamento até o desmame, quando então os filhotes passaram a consumir a cafeína até o início da sua puberdade (30-34 dias de idade). A análise comportamental e os níveis de proteínas sinápticas (pró-BDNF, BDNF, GFAP e SNAP-25) foram analisados no hipocampo e córtex cerebral. As fêmeas púberes apresentaram uma atividade locomotora maior e comportamento menos ansioso que os machos. Em ambos os sexos, a cafeína causou hiperlocomoção no campo aberto. Enquanto a cafeína em doses moderadas causou um prejuízo na memória de reconhecimento em fêmeas, foi observado uma melhora na memória de longo prazo em ambas as doses em ratos machos. O comportamento relacionado à ansiedade foi atenuado pela cafeína (0,3 g/L) apenas em fêmeas. Paralelamente com a melhora da memória nos machos, a cafeína aumentou os níveis de pró-BDNF e BDNF no hipocampo e córtex. As fêmeas apresentaram um aumento do pró-BDNF em ambas as regiões avaliadas em comparação aos machos. Embora a proteina GFAP não tenha sido alterada pelas diferenças de sexo e pelo tratamento com cafeína, a cafeína em doses moderadas aumentou o imunoconteúdo de SNAP-25 no córtex das fêmeas. Os resultados demonstram que o consumo de cafeína altera de forma distinta a memória de reconhecimento e o comortamento do tipo ansioso em ratos machos e fêmeas púberes. Além disso, o BDNF e proteínas relacionadas também foram modificados de uma forma dependente do sexo, sugerindo que alterações sinápticas ou de plasticidade podem estar associadas aos efeitos comportamentais. Além dos efeitos da cafeína durante a gravidez e a puberdade, têm sido observados efeitos benéficos da cafeína sobre a memória no envelhecimento normal e no prejuízo observado em em modelos animais de doenças neurodegenerativas. Tendo em vista que os mecanismos subjacentes a estes efeitos da cafeína ainda permanecem desconhecidos, no segundo capítulo investigamos se a administração crônica de cafeína poderia melhorar o desempenho na tarefa de memória avaliada pelo teste da esquiva inibitória em ratos adultos e de meia-idade. Como o BDNF está associado com a formação da memória e as ações do BDNF são moduladas pelos receptores de adenosina, os alvos moleculares para as ações psicoestimulantes da cafeína, neste estudo avaliamos os efeitos da administração crônica de cafeína (1 g/L na água de beber durante 30 dias) na memória de curta e longa duração e nos níveis de pró-BDNF, BDNF maduro,o receptor TrkB e o fator de transcrição CREB no hipocampo de ratos machos adultos (3 meses de idade) e de meia-idade (12 meses) Ambos os grupos

foram submetidos a tarefa de campo aberto e esquiva inibitória. Os ratos de meia-idade apresentaram diminuição da atividade locomotora em relação aos adultos e a cafeína não teve efeitos sobre a locomoção em ambas idades. Na tarefa de esquiva inibitória, avaliou-se a memória de curta e longa duração. Ratos de meia-idade apresentaram um comprometimento total da memória de curta duração, e parcial da memória de longa duração em comparação com ratos adultos. O consumo de cafeína foi capaz de reverter o prejuízo decorrente da idade tanto para a memória de curta quanto de longa duração. O aumento do BDNF hippocampal causado pelo envelhecimento foi prevenido pelo consumo de cafeína, juntamente com um aumento no imunoconteúdo de pró-BDNF e CREB em ambas as idades. Além disso, os níveis de CREB aumentaram com o envelhecimento. Houve uma diminuição no imunoconteúdo de TrkB no hipocampo de ratos de meia-idade quando comparados aos adultos, e a cafeína diminuiu a densidade de TrkB em ambas as idades. Os dados encontrados indicam uma estreita associação entre a modificação do desempenho da memória e imunoconteúdo BDNF. Em conjunto, esses resultados apresentam novos indícios de que o consumo de cafeína promove desfechos comportamentais sexo-específicos em ratos púberes, além de ser capaz de normalizar o desempenho em tarefas de memória e alterações na sinalização do BDNF causadas pelo envelhecimento.

ABSTRACT

Caffeine is the most consumed psychostimulant worldwide, and the beneficial effects of chronic caffeine administration on cognitive function have been observed in different conditions and animal models. Caffeine consumption is widespread among adults, eldery, pregnant women and more recently, children and adolescents. Some clinical and preclinical studies suggest that prenatal exposure to caffeine presents harmful effects, such as prematurity, congenital malformations, low birth weight and even teratogenicity, whereas others studies demonstrated no long-term harmfull effects of caffeine. Besides that, few studies have addressed the effects of caffeine in a sex dependent manner during puberty and/or adolescence. Also, due to the increase in the consumption of caffeine containing drinks by children and adolescents, the potential harmful effects of caffeine in these subpopulations need to be investigated. Considering that caffeine intake has grown in this population, in the first chapter of the this thesis we investigated the behavioral and synaptic proteins changes in pubescent male and female rats after maternal consumption of caffeine. Adult female Wistar rats started to receive caffeine in drinking water (0.1 and 0.3 g/L; low and moderate dose, respectively) during the active cycle in weekdays, two weeks before mating. The treatment lasted up to weaning (21 days) and offspring continued receiving caffeine until the onset of puberty (30-34 days old). Behavioral analysis and synaptic proteins levels (proBDNF, BDNF, GFAP and SNAP-25) were immunodetected in the hippocampus and cerebral cortex. Pubescent females showed hyperlocomotion and less anxiety behavior as compared to males. In both sexes caffeine caused hyperlocomotion in the open field. While moderate caffeine worsened recognition memory in females, an improvement for long-term memory in both doses was observed in male rats. Anxiety-related behavior was attenuated by caffeine (0.3 g/L) only in females. Also, in parallel with memory improvement in males, caffeine increased pro- and BDNF in the hippocampus and cortex. Females presented increased proBDNF in both brain regions as compared to males. While GFAP was not different according to sex or altered by caffeine consumption, moderate caffeine increased SNAP-25 in the cortex of female rats. Our findings revealed that caffeine differently affects recognition memory and anxiety-related behaviors in pubescent male and female rats. In addition, BDNF and related proteins have also changed in a sex dependent manner, suggesting an association with behavioral outcomes. Beyond caffeine effects during pregnancy and puberty, beneficial effects of caffeine on memory processes have been observed in animal models relevant to neurodegenerative diseases and aging, although the underlying mechanisms remain unknown. In the second chapter we investigated whether chronic caffeine consumption could improve the performance in inhibitory avoidance memory task in adult and middle-aged rats. Because brain-derived neurotrophic factor (BDNF) is associated with memory formation and BDNF's actions are modulated by adenosine receptors, the molecular targets for the psychostimulant actions of caffeine, we here compare the effects of chronic caffeine (1 mg/mL drinking solution for 30 days) on short- and long term memory and on levels of hippocampal proBDNF, mature BDNF, TrkB and CREB in young (3 month old) and middle-aged (12 month old) male rats. Both groups were submitted to open field and inhibitory avoidance tasks. Middle-aged rats presented decreased locomotor activity as compared to adults and caffeine was devoid of effect at any age. In the inhibitory avoidance task, short- and long-term memory was evaluated. Middle-aged rats presented impaired performance compared to adult ones for short-term memory. When long-term memory was evaluated, middle-aged rats showed a decreased in their perfomances compared to adult rats, and caffeine treatment was able to improve

it. Western blot analysis showed that BDNF and CREB imunocontent increased in the hippocampus of aged rats and caffeine consumption was able to prevent the changes in BDNF levels. In addition, caffeine treatment increased the pro-BDNF and CREB immunocontent in both ages. Furthermore, CREB densities increased with aging. TrkB immunocontent was decreased in the hippocampus from middle-aged rats when compared to adult ones, and caffeine decreased the density of TrkB in both ages. The present findings indicate a close association between the modification of memory performance and BDNF immunocontent. Therefore, our data suggest caffeine normalyze memory performance upon aging and may be related to the ability of caffeine to normalyze the levels of BDNF. Taken together, these results present new evidence that caffeine consumption promotes sex-specific behavioral outcomes in addition to being able to normalize memory performance during aging and that changes could be related to a modification in BDNF signaling.

LISTA DE ABREVIATURAS

A_1: Receptor metabotrópico de adenosina do subtipo A_1

A_{2A}: Receptor metabotrópico de adenosina do subtipo A_{2A}

A_{2B}: Receptor metabotrópico de adenosina do subtipo A_{2B}

A_3: Receptor metabotrópico de adenosina do subtipo A_3

AMPc: AMP cíclico

BDNF: Fator neurotrófico derivado do encéfalo

CYP450: Citocromo P 450

CYP1A2: Subunidade do citocromo P 450

CREB: proteína ligante ao elemento responsivo ao AMPc

DA: Doença de Alzheimer

DE: Dia embrionário

DPN: Dia pós-natal

EFSA: *European Food Safety Authority*

FDA: *Food and Drug Administration*

FI: Filamentos intermediários

GFAP: proteína ácida fibrilar glial

LTD: Depressão a longa duração

LTM: Memória de longa duração

LTP: Potenciação de longa duração

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

NT-3: Neurotrofina-3

NT-4: Neurotrofina-4

p75NTR : Receptor Pan Neurotrofina

pró-BDNF: Forma precursora o BDNF

RNAm: RNA mensageiro

SCH58261: Antagonista A_{2A}

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*)

SNC: Sistema nervoso central

STM: Memória de curta duração

TrkA: Receptor do tipo tirosina cinase A

TrkB: Receptor do tipo tirosina cinase B

TrkC: Receptor do tipo tirosina cinase C

1. INTRODUÇÃO

1.1. Cafeína

1.1.1. Breve Histórico

Atualmente, a cafeína é a substância psicoestimulante mais consumida no mundo, sendo que o seu consumo pela humanidade data de séculos (Fredholm, 1999). É um alcalóide da família das xantinas encontrado naturalmente em plantas (Fredholm, 2011). Existem mais de 60 espécies de plantas que contém cafeína, sendo as mais conhecidas o café, chá, cacau, erva mate e guaraná. A quantidade de cafeína presente em diversos produtos e bebidas depende da planta utilizada e do modo de preparo (Fredholm, 1999).

Como o uso da cafeína é algo tão profundamente enraizado na nossa cultura, as origens históricas do seu uso são desconhecidas e rodeadas de mito, mas a sua história está fortemente relacionada à história do café (Fredholm, 2011). O mito mais conhecido é sobre um pastor de cabras etíope (talvez árabe) de nome Kaldi, que observou que suas cabras ficavam muito agitadas após ingerirem as bagas de certo arbusto, que seria uma das diversas espécies de planta que teriam cafeína na sua composição. Após essa observação, o próprio pastor resolveu experimentar as bagas, e foi tomado por um estado de euforia, que o fez relatar as sensações experimentadas a um abade de um mosteiro local, e conta-se que, desde então, a cafeína teria ganhado o mundo (Fredholm, 2011; Weinberg e Bealer, 2001).

A palavra "café" deriva de qahva (ou qahwah) que é simplesmente uma palavra que denota uma bebida feita das plantas. Acredita-se que inicialmente os grãos de café foram consumidos na sua forma *in natura*, sendo posteriormente iniciado o processamento em forma de infusões para consumo com água fervente, em torno de

1000 d.C. (Fredholm, 2011). Embora os árabes cultivassem as plantas e as bebidas preparadas a partir de grãos de café, foi apenas por volta do século XIV que o processo de torrefação foi descoberto (Fredholm 2011; Weinberg e Bealer, 2001).

Apesar de ser uma planta originária da Etiópia, foi a Arábia a responsável pela propagação da cultura do café, sendo o seu uso mais difundido nos séculos XV e XVI, e na Europa, nos séculos XVIII e XIX, sendo introduzido principalmente pelos espanhóis e holandeses no período das descobertas (Fredholm, 2011). No Brasil as primeiras sementes de café chegaram em 1727 no Estado do Pará. Devido às condições climáticas favoráveis, o cultivo de café se espalhou rapidamente (Carvalho et al., 1993). Em meados do século XVIII, a produção de café revolucionou a sociedade e a economia do Brasil (Taunay, 1939). Atualmente, o Brasil é o maior exportador de café do mundo, e o segundo maior consumidor do produto (Cecafé, 2016; Ministério da Agricultura, 2016).

1.1.2. Metabolismo

O consumo de cafeína a partir de todas as fontes é bastante variável, sendo que alguns estudos apontam uma média de 70-76 mg/pessoa/dia em todo o mundo ou 5-8 mg/kg/dia (equivalente a 3 xícaras de café) (Chen et al., 2010; Fredholm et al., 1999).

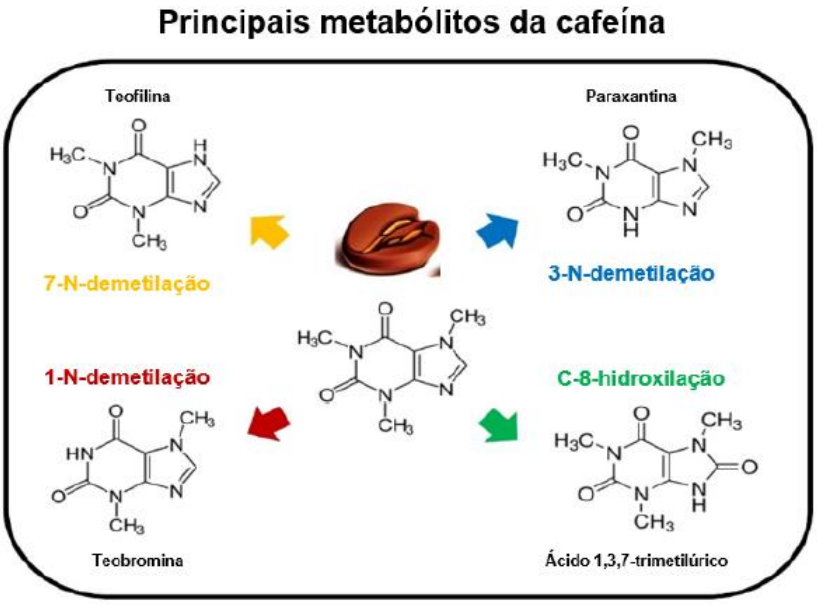
As propriedades hidrofóbicas da cafeína permitem a sua passagem através de todas as membranas biológicas, inclusive da barreira hematoencefálica do adulto ou do feto (Lachance et al., 1983; Tanaka et al., 1984). A cafeína é absorvida de forma rápida e completa pelo trato gastrointestinal, cujo pico de concentração plasmática é atingido entre 15 e 120 minutos após a ingestão oral em seres humanos (Arnaud, 1976; Arnaud e Welsch, 1982; Bonati et al., 1982; Yesair et al., 1984). A absorção, a biodisponibilidade e a excreção urinária e fecal da cafeína não mostraram diferenças entre as espécies (Arnaud et al., 1989). A metabolização da cafeína é completa em humanos adultos,

sendo que menos de 2% do que é ingerido é recuperável na urina de forma inalterada (Arnaud, 1987; Gorodischer et al., 1986; Somani e Gupta, 1988). A cafeína (1,3,7-trimetilxantina) sofre o processo de desmetilação, dando origem aos seus intermediários di-metilados com mais de 80% de cafeína sendo metabolizada a paraxantina (1,7-dimetilxantina) e cerca de 16% sendo convertida em teobromina (3,7 -dimetilxantina) e teofilina (1,3-dimetilxantina) (Benowitz et al., 1995; Lelo et al., 1986). É importante mencionar que em roedores a paraxantina é a principal dimetilxantina, embora os níveis de teofilina também sejam elevados (Bonati et al., 1984; Fredholm et al., 1999). Além disso, os derivados tri-metílicos, produtos da hidroxilação da cafeína, correspondem a 40% dos seus metabólitos em ratos, em comparação a 6% em seres humanos (Arnaud, 1985) (Figura 1).

De uma forma geral, a cafeína é extensamente metabolizada pelos sistemas enzimáticos hepáticos - os citocromos - mais especificamente o CYP1A2. Entretanto, a farmacocinética dessa substância encontra-se alterada em casos específicos, como em mulheres grávidas e nos fetos em desenvolvimento. A CYP1A2, principal enzima do metabolismo da cafeína, tem sua expressão e atividade altamente associada aos estágios do desenvolvimento, fazendo com que o metabolismo da cafeína fique dependente da maturação dessa enzima (Leeder, 2001). Além disso, já foi demonstrado que o metabolismo da cafeína é notavelmente alterado durante a gestação, onde ocorre um aumento na inibição da atividade dessa enzima com o passar do tempo (-32,8% no 1º trimestre, -48,1% no 2º trimestre e -65,2% no 3º trimestre) (Yu et al., 2016). Esses aspectos devem ser levados em consideração, ainda mais que a cafeína e seus metabólitos facilmente atravessam a barreira placentária e são permeáveis sem exceção a todos os tecidos do feto (Nehlig e Debry, 1994; Parsons e Neims, 1981; Soellner et al., 2009; Yu et al 2016).

No tecido fetal, a CYP1A2 apresenta baixa funcionalidade devido à imaturidade desse sistema, consequentemente acarretando numa meia-vida prolongada da cafeína no plasma fetal e um acúmulo no sistema nervoso central (SNC) do feto (Nehlig e Debry 1994; Parsons e Neims 1981; Soellner et al 2009; Yu et al 2016). O metabolismo da cafeína desenvolve-se gradualmente durante o primeiro ano de vida em seres humanos (Pearlman et al., 1989). A meia-vida da cafeína diminui gradualmente no recém-nascido a termo (Aranda et al., 1979; Le Guennec e Billon, 1987), e diminui exponencialmente com a idade pós-natal, reforçando que o metabolismo da cafeína se desenvolve gradualmente durante o primeiro ano de vida (Aldridge et al., 1979; Parsons e Neims, 1981; Pearlman et al., 1989). No entanto, esta substância tem uma meia-vida mais longa em prematuros (Parsons e Neims, 1981). A meia-vida da cafeína em neonatos em aleitamento materno exclusivo é prolongada quando comparada à eliminação da cafeína em neonatos em uso de fórmulas infantis (Blake et al., 2005; Le Guennec e Billon, 1987), e esta diferença foi relacionada a um aumento da expressão de CYP1A *in vitro* pela fórmula infantil (Xu et al., 2005).

Em conjunto, essas modificações no metabolismo da cafeína constituem um risco adicional ao feto, enfatizando a necessidade de maiores investigações (Aldridge et al., 1981; Mioranzza et al., 2014).



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

1.1.3. Mecanismo de Ação

A cafeína produz efeitos farmacológicos complexos, sendo que o seu principal mecanismo de ação é o antagonismo não seletivo dos receptores de adenosina A_1 e A_{2A} (Quarta et al, 2004; Fredholm et al 1999). O bloqueio dos receptores de adenosina pela cafeína pode levar a efeitos secundários importantes sobre muitas classes de

neurotransmissores, incluindo a noradrenalina, dopamina, serotonina, acetilcolina, glutamato e GABA, que interferem em muitas funções fisiológicas (Fredholm et al., 1999). Além disso, em doses elevadas (concentrações 40 vezes maior do que as concentrações atingidas pelo consumo humano regular) a cafeína inibe fosfodiesterases, aumenta a liberação do cálcio intracelular e bloqueia os receptores $GABA_A$ (Fredholm et al, 1999).

A adenosina é considerada um neuromodulador endógeno, controlando a liberação de neurotransmissores, a excitabilidade neuronal e o ritmo circadiano (Cunha, 2001; Fredholm et al., 2005). Até o presente momento, quatro diferentes subtipos de receptores de adenosina foram clonados e identificados em humanos e roedores: A_1,A_{2A}, A_{2B} e A_3 sendo que todos os subtipos são acoplados a proteínas G (Fredholm et al., 2003; Sebastião e Ribeiro, 2009). Os receptores A_1 e A_3 estão acoplados a proteínas G inibitórias enquanto os receptores A_{2A} e A_{2B} estão acoplados a proteínas G estimulatórias (Fredholm et al., 2001). Estudos *in vitro* demonstram que a cafeína possui afinidades semelhantes para os receptores de adenosina do subtipo A_1, A_{2A} e A_{2B} e uma baixa afinidade para o receptor do subtipo A_3 (Fredholm et al., 2001; Solinas et al., 2005). Como concentrações fisiológicas de adenosina podem estimular facilmente os receptores A_1 e A_{2A}, enquanto os receptores A_{2B} somente são ativados com concentrações elevadas de adenosina, parece que os receptores A_1 e A_{2A} são os alvos preferenciais da cafeína no SNC (Fredholm, 1999).

A ativação dos receptores A_1 exerce ações inibitórias sobre a transmissão sináptica enquanto que a dos receptores A_{2A} exerce ações facilitatórias sobre a transmissão sináptica (Cunha et al., 2001). O receptor adenosinérgico do subtipo A_1 é o mais abundante no SNC, principalmente na região do neocórtex, cerebelo, hipocampo e corno dorsal da medula espinhal, enquanto que os receptores do subtipo A_{2A} são

altamente expressos em neurônios pálido-estriatais e no bulbo olfatório, mas também são encontrados em outras regiões do cérebro, como no hipocampo (Fredholm et al., 2005).

Ao longo do desenvolvimento do SNC, assim como durante o envelhecimento ocorrem alterações na densidade dos receptores de adenosina. Os receptores A_1 e A_{2A} estão presentes desde o nascimento em roedores, mas o maior desenvolvimento em termos de densidade e acoplamento aos sistemas de formação de segundos mensageiros ocorre após o nascimento (Adén et al., 2000; Adén, 2011; para revisão, ver Porciúncula et al., 2013). O tratamento com cafeína nesse período pode levar a alterações nesses receptores. Um estudo mostrou que a cafeína causou uma regulação positiva nos receptores A_1 no hipotálamo no dia pós-natal (DPN) 5 e 8 e na região ponto-bulbar no DPN 5. Da mesma forma, a cafeína levou a um aumento do RNAm do receptor A_{2A} no hipotálamo e região ponto-bulbar no DPN 5, juntamente com um aumento moderado no hipotálamo no DPN 8-11 e região ponto-bulbar no DPN 7-11 (Gaytan e Pasaro, 2012). No envelhecimento, é relativamente bem estabelecido que ocorre uma diminuição dos receptores A_1 enquanto ocorre um aumento dos receptores A_{2A} em regiões como o córtex, hipocampo e estriado (para revisão, ver Cunha e Agostinho, 2010). Uma hipótese para esse aumento dos receptores A_{2A}, que exerce efeitos facilitatórios, seria uma estratégia do organismo para compensar a perda geral da eficiência sináptica encontrada no envelhecimento (Cunha e Agostinho, 2010).

1.2. Cafeína e o Sistema Nervoso Central em Desenvolvimento

1.2.1. Adolescência e Puberdade

A adolescência pode ser considerada um período de transição, que ocorre entre o final da infância e início da vida adulta, no qual o indivíduo passa por uma série de mudanças, tanto físicas quanto comportamentais (Drzewiecki et al., 2016; Peper et al., 2011; Piekasrki et al., 2016; Romeo et al., 2016; Semple et al., 2013). Dentre essas mudanças podemos citar as alterações hormonais, alterações na composição corporal, aumento das interações sociais e busca de novidades, assim como instabilidade emocional, destacando-se maior impulsividade, capacidade aparentemente limitada de autocontrole e maior tendência a correr riscos (Lee et al 2014; Semple et al., 2013; Sturman e Moghaddam, 2011).

O período da adolescência é um período muito importante do desenvolvimento pós-natal, onde o cérebro apresenta uma maior plasticidade em relação à vida adulta, o que também lhe confere uma vulnerabilidade única, estando mais suscetível a influências tanto negativas quanto positivas na sua estrutura e função (Andersen, 2003; Lee et al. , 2014; Peper et al., 2011; Piekarski et al., 2016). Por exemplo, alguns estudos mostram que a exposição a ambientes estressantes de forma crônica durante esse período pode contribuir para o surgimento de doenças neurodesenvolvimentais na vida adulta (Giedd et al., 2008; Kessler et al., 2005; Malter Cohen et al., 2013; Turner e Loyd, 2004; revisado em Romeo et al., 2016). Em vista disso, é essencial um maior conhecimento sobre as alterações às quais os adolescentes estão expostos, tanto em relação a suas potencialidades quanto a suas vulnerabilidades, para que possam ser realizadas, quando necessário, intervenções precoces e até mesmo tratamentos, a fim de melhorar a qualidade de vida desses indivíduos (Giedd et al., 2008; Kessler et al 2005; Lee et al., 2014).

Do ponto de vista biológico, não existe uma definição precisa ou mesmo um consenso de quando começa e quando termina a adolescência ou a puberdade, tanto em

humanos quanto em animais. Embora esses períodos estejam intrinsecamente ligados, os termos não são sinônimos, apesar haver uma sobreposição entre eles (Sisk e Zehr, 2005; Spear, 2000). A puberdade refere-se à maturação sexual, quando a capacidade reprodutiva se inicia associado à secreção elevada de hormônios sexuais (Peper et al., 2011; Schulz e Sisk, 2016). Já a adolescência é considerada um período mais amplo, abrangendo não só a maturação reprodutiva, mas também a maturação cognitiva, emocional e social (Sisk e Zehr, 2005; Spear, 2000).

Segundo a Organização Mundial de Saúde (2016), o período da adolescência ocorre entre 10-19 anos, mas esse período tem definições distintas entre os estudos, tendo sido descrita entre 9-18 anos, 10-20 anos e até os 25 anos de idade, havendo diferenças entre homens e mulheres (Semple et al., 2013; Spear, 2000; *World Health Organization*, 2016). Segundo Falkner e Tanner (1986), o período da puberdade ocorre tipicamente entre os 10 e os 17 anos de idade nas mulheres e entre os 12 e os 18 anos nos homens (Falkner e Tanner, 1986).

Em roedores, a definição de adolescência também apresenta variações, sendo que alguns consideram um período mais estreito (dias pós-natal – DPN – 28-42) e outros um período mais longo (DPN 21-60) (Semple et al., 2003; Spear, 2000; Tirelli et al., 2003). A adolescência pode então ser considerada desde o desmame (DPN 21), ainda antes do início da puberdade, até o DPN 60, que ainda não se deve considerar idade adulta. A adolescência ainda pode ser dividida em três estágios diferentes: adolescência precoce (animais pré-púberes, DPN 21 a 34), adolescência intermediária (animais púberes, DPN 34-46) e adolescência tardia (DPN 46-59), conforme ilustrado na Figura 2 (Steinberg, 2005; Tirelli et al., 2003). Assim como em humanos, alguns estudos sugerem que em roeadores, as fêmeas atingiriam a puberdade mais cedo do que os machos. A idade média para o início da puberdade em fêmeas seria no DPN 34,9,

com um intervalo entre 32-38 dias e nos machos seria no DPN 44,9, com um intervalo entre 42-48 dias (Drzewiecki et al 2016; Willing e Juraska 2015). Entretanto, as janelas de tempo mais estreitas são mais comumente empregadas como descrição padrão da adolescência, entre DPN 30-45 ou DPN 35-49 (Evans, 1986; Laws et al., 2003; Reger et al., 2009; Semple et al., 2013; Sengupta, 2011, 2013). Neste trabalho, utilizaremos como parâmetros de adolescência os DPN 31-60, aproximadamente, como está demonstrado na Figura 3.

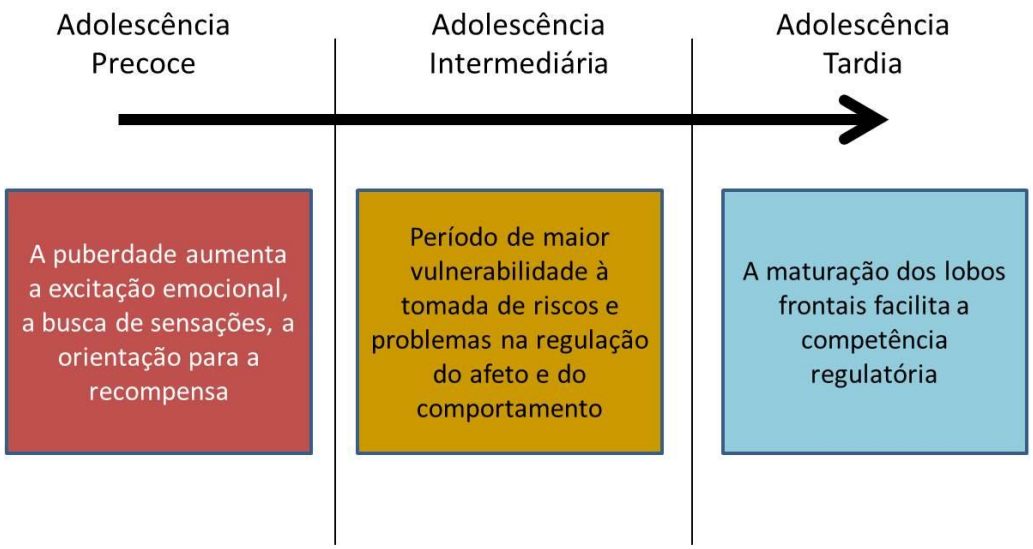


Figura 2. Representação dos estágios da adolescência (Adaptado de Steinberg, 2005).

As diferenças entre roedores e humanos devem ser consideradas quando se compara às idades de maturação do SNC, visto que deve haver uma similaridade e um alinhamento nos marcos do desenvolvimento entre as espécies, bem como o conhecimento das idades em que eles ocorrem (Piekarski et al., 2016; Semple et al., 2013). Felizmente, as fases do desenvolvimento do SNC são altamente conservadas entre espécies de mamíferos, e existem marcos comparáveis que permitem a utilização de modelos animais para os estudos de desenvolvimento. Por exemplo, o estágio de maturação do sistema nervoso em um humano recém-nascido corresponde ao 10º dia

pós-natal em ratos (Tchekalarova et al., 2005; Williams, 2008). Além disso, a primeira e a segunda semana pós-natal em roedores corresponderia ao desenvolvimento do SNC em humanos aos primeiros 3 anos e 6 anos de vida, respectivamente (Marco et al., 2011). De fato, no 21º dia pós-natal (idade típica do desmame) o córtex de ratos atinge aproximadamente 90% do seu peso adulto, enquanto que em seres humanos, o peso cerebral atinge um patamar semelhante aos 2-3 anos de idade (Dekaban et al., 1987; revisado em Semple et al., 2013). Em relação ao desevolvimento do córtex e hipocampo, os dias embrionários 18 e 20 de ratos correspondem ao final do primeiro trimestre e ao início do segundo trimestre gestacional em humanos, respectivamente, (Clancyet al., 2001). Como se pode se observar na Figura 3, apesar de existir diferenças significativas na escala de tempo em que ocorrem os eventos, há uma cronicidade na maturação do cérebro bastante consistente entre humanos e roedores (Semple et al 2013).

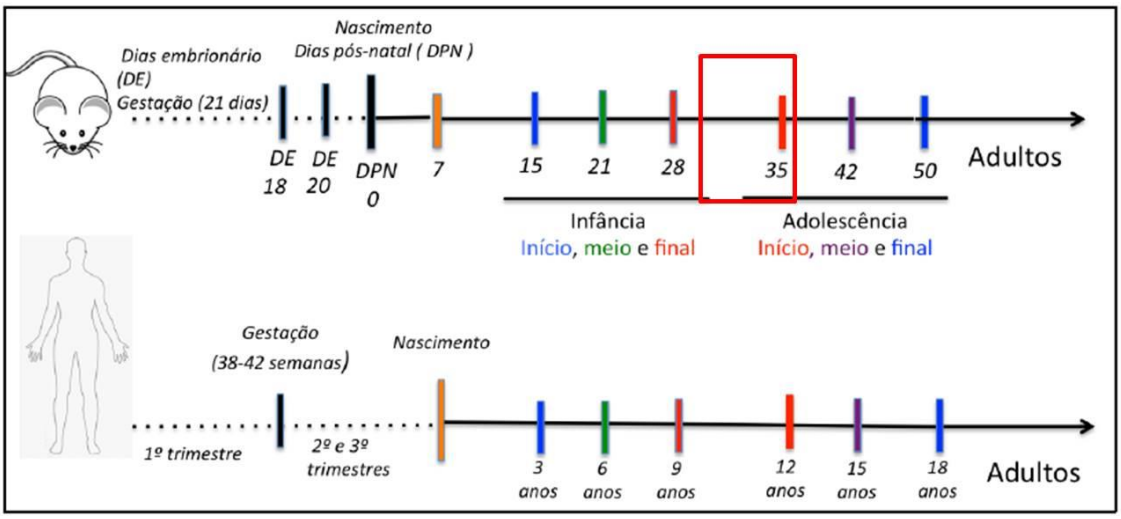


Figura 3: Linha do tempo comparativa entre o desenvolvimento de ratos e humanos (Adaptado de Clancy et al., 2001; Marco et al., 2011; Tchekalarova et al., 2005; Williams, 2008).

1.2.2. Alterações morfológicas e Influência de Gênero

A adolescência é um período de refinamento e maturação contínuos de circuitos neurais, sendo a puberdade um evento central na reorganização do córtex, tanto em

roedores quanto em seres humanos (Blakemore et al., 2010; Juraska e Willing, 2016; Semple et al., 2013; Spear, 2000). Além disso, o aumento da produção dos hormônios sexuais após o início da puberdade exerce efeitos amplos sobre o cérebro em desenvolvimento (Juraska e Willing 2016; Pierkarski et a., 2016; Schulz e Sisk, 2016).

Em humanos, durante a primeira infância, há uma superprodução de axônios e sinapses, atingindo um pico em torno da época da puberdade, seguido por uma rápida poda sináptica na adolescência até a idade adulta (Crews et al 2007; Peper et al., 2009; Huttenlocher e Dabholker, 1997). A poda sináptica refere-se ao processo pelo qual neurônios e conexões sinápticas em excesso são eliminados, a fim de refinar e aumentar a eficiência das transmissões neuronais (Santos e Nogle, 2011).

A maturação e reorganização do cérebro adolescente envolvem diversas mudanças na sua morfologia, que incluem a reorganização da substância cinzenta e substância branca, da arborização dendrítica e sinapses, assim como alterações nos padrões de disparo de células corticais e disponibilidade de neurotransmissores e seus receptores durante esse período (Herting et al., 2015; Huttenlocher e Dabholkar, 1997; Lee et al 2014; Markham et al., 2007; Peper et al., 2009; Sturman e Moghaddam 2011; Willing e Jusraska, 2015). Vários estudos de ressonância magnética indicam que o córtex cerebral humano diminui de tamanho durante a adolescência, incluindo uma redução de volume de substância cinzenta e um aumento de volume de substância branca (Blakemore et al., 2010; Giedd et al., 1999; Jernigan et al., 1991; Peper et al., 2011; Sturman e Moghaddam 2011), sendo que a substância branca continua a aumentar depois da desse período, enquanto que a substância cinzenta do córtex estabiliza quando atinge o início da vida adulta (Juraska e Willing 2016).

Além dos estudos de ressonância magnética, estudos em modelos animais revelaram que o cérebro em desenvolvimento leva a diferenças estruturais significativas

entre os sexos (Drzewiecki et al., 2016; Markham et al., 2007; Willing e Juraska et al., 2016). Por exemplo, tanto os machos como as fêmeas há uma diminuição na densidade dos espinhos dendríticos entre DPN35 e DPN90, mas as fêmeas também perdem ramos dendríticos, indicando uma diminuição ainda maior no número total de espinhos dendríticos (Drzewiecki et al., 2015). Além disso, as perdas de substância cinzenta no córtex pré-frontal, devidas à poda sináptica, são consideravelmente mais proeminentes nas fêmeas do que nos machos (Drzewiecki et al 2016; Wiling e Juraska, 2015).

As mudanças neuroquímicas, estruturais e funcionais que ocorrem ao longo do desenvolvimento geralmente são benéficas, e aprimoram as conexões neurais, apesar de também levar a desequilíbrios transitórios nos circuitos cerebrais durante a adolescência. As exacerbações desses desequilíbrios por fatores biológicos, ambientais e genéticos podem conferir uma maior vulnerabilidade e contribuir para o risco de desenvolvimento de transtornos mentais (Giedd et al., 2008; Lee et al 2014).

1.2.3. Efeitos da cafeína no período do desenvolvimento

Os efeitos do consumo de cafeína sobre as funções cognitivas vêm sendo demonstrados em estudos humanos e animais. Em estudos epidemiológicos, o consumo de cafeína foi associado a um menor risco de desenvolver doença de Alzheimer (Eskelinen et al., 2009; Maia e de Mendonça, 2002; Ritchie et al., 2007), assim como em estudos experimentais com animais envelhecidos, a administração crônica de cafeína foi capaz não só de prevenir e também de reverter o comprometimento de memória relacionado à idade (Costa et al., 2008a, Prediger et al., 2005; Sallaberry et al 2013). Da mesma forma, o tratamento crônico com cafeína também foi eficaz na prevenção da neurodegeneração, formação do peptídeo beta-amilóide e nos déficits mnemônicos em modelos experimentais de doença de Alzheimer (Arendash et al., 2006;

Dall'Igna et al., 2003; Espinosa et al., 2013). Entretanto, chama a atenção que a exposição crônica de cafeína durante o desenvolvimento fetal e até durante o desenvolvimento do SNC pós-natal seja pouco estudada (Mioranzza et al., 2014; revisado em Porciúncula et al. 2013; Temple, 2009).

Mulheres gestantes comumente consomem cafeína, sendo que estudos mostram que cerca de 97% das mulheres afirmam ter consumido cafeína por alguma fonte da dieta durante o ano anterior à gravidez (consumo médio de 129,9 mg/dia no ano), e cerca de 68% continuam o consumo após a concepção (consumo médio de 125 mg/dia durante a gravidez) (Chen et al 2014; Doepker et al 2016; Frary et al 2005; Knight et al 2004). Outra população que passou a consumir regularmente cafeína e em altas quantidades foram as crianças e adolescentes, chegando a representar um aumento de 70% no consumo de cafeína nos últimos 30 anos por esta população (Harnack et al., 1999; Temple, 2009). O surgimento de bebidas energéticas, que contém concentrações elevadas de cafeína, tem contribuído para esse aumento no consumo de cafeína (Heckman et al, 2010; Temple, 2009). Entretanto, os resultados de estudos sobre a segurança do consumo de cafeína durante a gravidez e seus efeitos no desenvolvimento do SNC são controversos ou inconsistentes (Bakker et al., 2010; Chen et al., 2014; Doepker et al., 2016; Fernandes et al., 1998; Lynch and Klebanoff, 2008; Temple, 2009; Yu et al., 2016).

Os estágios de desenvolvimento do SNC dependem de processos que são precisamente orquestrados, por isto este é um período vulnerável onde a exposição a determinadas condições pode inclusive mudar drasticamente sua estrutura e função, reverberando por toda a vida. Essa especulação é reforçada pela crença de que muitas doenças na idade adulta são originadas no desenvolvimento fetal (Godfrey e Barker, 2000; Harding, 2001; Temple, 2009). Isso enfatiza a importância de se investigar e

conhecer em mais detalhes o que realmente acontece nesse período, e como esperar que intervenções que ocorram nesse período venham a ser benéficas ou até mesmo deletérias. Em vista disso, alguns especialistas consideram essas subpopulações – gestantes, crianças e adolescentes - como sendo "de risco", o que exigiria ajustes no consumo de cafeína a fim de moderar e limitar a sua ingestão (Knight et al., 2004; Nawrot et al., 2003).

Um estudo recente avaliou jovens pré-puberes (8-9 anos) e pós-puberes (15-17 anos), de ambos os sexos, nos quais foi feita uma administração aguda de cafeína (1 ou 2 mg/kg) e realizado a aplicação de questionários relacionados aos efeitos subjetivos causados pela cafeína (por exemplo, sensação de bem estar, euforia, vontade de repetir a administração, depressão, etc). Encontrou-se uma diferença entre os sexos, fase da puberdade, fase do ciclo menstrual e de dose, sendo que, de uma forma geral, as meninas relataram maiores alterações nas respostas subjetivas em relação aos meninos, demonstrando que há diferenças comportamentais relacionadas ao sexo antes e após a puberdade (Temple et al., 2015).

Em meados da década de 90, a agência do governo americano responsável pelo controle de indústrias alimentícias e de medicamentos (FDA - *Food and Drug Administration*) com base em resultados de estudos experimentais, desaconselhou o consumo de alimentos e bebidas que contenham grandes quantidades de cafeína, especialmente durante o primeiro trimestre gestacional (Goyan, 1980). Desde então, uma série de estudos epidemiológicos e experimentais têm sido realizados tentando estabelecer se o consumo de cafeína é seguro (Bakker et al., 2010; Doepker et al., 2016). De fato, a cafeína, assim como outras metilxantinas, tem sido usada há mais de 30 anos para tratar a apnéia do recém-nascido (Koppe et al., 1979; Millar e Schmidt, 2004). Entretanto, alguns estudos clínicos demonstram que a exposição pré-natal à

cafeína tem sido associada a efeitos deletérios sobre o feto em desenvolvimento, como aborto espontâneo, prematuridade e baixo peso ao nascer (Fernandes et al., 1998; Hoyt et al., 2014; Weng et al., 2008). Os estudos em animais associando o consumo de cafeína na gestação apresentaram resultados coincidentes com os estudos epidemiológicos, nos quais foram encontradas malformações congênitas, teratogenicidade, perturbações de memória, reabsorção fetal e diminuição do peso fetal (Brent et al., 2011; Fernandes et al 1998; Soellner et al., 2009).Entretanto, devido a limitações metodológicas e erros sistemáticos esses achados parecem ser inconsistentes, e dificultam uma interpretação e conclusão definitiva (Bakker et al., 2010; Chen et al., 2014; Doepker et al., 2016; Einother e Giesbrecht, 2013; Lynch e Klebanoff, 2008). Além disso, outros estudos pré-clinicos e também clínicos não encontraram os efeitos deletérios da exposição pré-natal de cafeína a longo prazo (Linn et al., 1982; Pollock et al., 2010; Savitz et al., 2008). Sendo assim, os efeitos do consumo de cafeína no período gestacional ainda não estão completamente elucidados e permanecem em discussão.

Diversos estudos experimentais demonstram que a cafeína apresenta uma série de efeitos comportamentais durante o desenvolvimento cerebral, incluindo alterações na atividade locomotora, ansiedade, aprendizagem e memória (Laureano-Melo et al., 2016; Porciúncula et al., 2013; Silva et al., 2013; Tchekalarova et al., 2005). Em adultos, a cafeína, quando administrada em doses baixas e moderadas, leva a um aumento da locomoção, enquanto que doses mais altas acarretam em diminuição (Porciúncula et al., 2013). A avaliação da atividade locomotora em animais que receberam cafeína durante a gestação demonstra que ocorrem alterações desse parâmetro, entretanto, essas alterações dependem do período de tratamento bem como da dose de cafeína. Por exemplo, o tratamento com cafeína durante a gestação e a lactação não alterou a atividade locomotora de camundongos na vida adulta no teste de campo aberto

(Laureano-Melo et al., 2016; Silva et al., 2013). Entretando, Tchekalarova et al. (2005) demonstraram que a cafeína também pode causar efeitos distintos quando administrada em diferentes dias pós-natal, sendo que o tratamento com cafeína entre DPN 7 a 11 em ratos provoca um aumento da atividade locomotora, enquanto que o tratamento entre DPN 13 a 17 diminui este parâmetro (Tchekalarova et al., 2005).

Outras alterações comportamentais também foram descritas, como prejuízos em diferentes tipos de memória. Por exemplo, machos e fêmeas que receberam cafeína durante a gestação e lactação (0,075 g/L) apresentaram comprometimento na memória de reconhecimento na vida adulta, avaliado pela tarefa de reconhecimento de objetos (Soellner et al., 2009). Resultados semelhantes também foram encontrados por Silva et al (2013), onde a administração de cafeína (0,3 g/L) administrada apenas durante a gestação e a lactação foi capaz de prejudicar a memória espacial e de reconhecimento em camundongos adultos (Silva et al., 2013).

Além disso, também foram descritas alterações morfológicas de animais tratados com cafeína. Proteínas astrocitárias apresentaram seus níveis reduzidos aos 15 dias pós-natal em animais tratados com cafeína durante 3º até o 10º dia pós-natal, seguido de uma diminuição na proliferação dos astrócitos (Desfrere et al., 2007). Outro estudo mostrou que a administração de 50 mg/kg durante os primeiros doze dias de vida revelaram uma modificação na morfologia dendrítica de neurônios piramidais do córtex pré-frontal dos ratos na adolescência e na vida adulta, com um aumento no comprimento dos dendritos (Juárez-Méndez et al., 2006).

Em conjunto, essas evidências demonstram que deve-se ter moderação no consumo de cafeína por gestantes, assim como por crianças e adolescentes, em vista das possíveis alterações e prejuízos a longo prazo que possam surgir no período do desenvolvimento do SNC e na vida adulta.

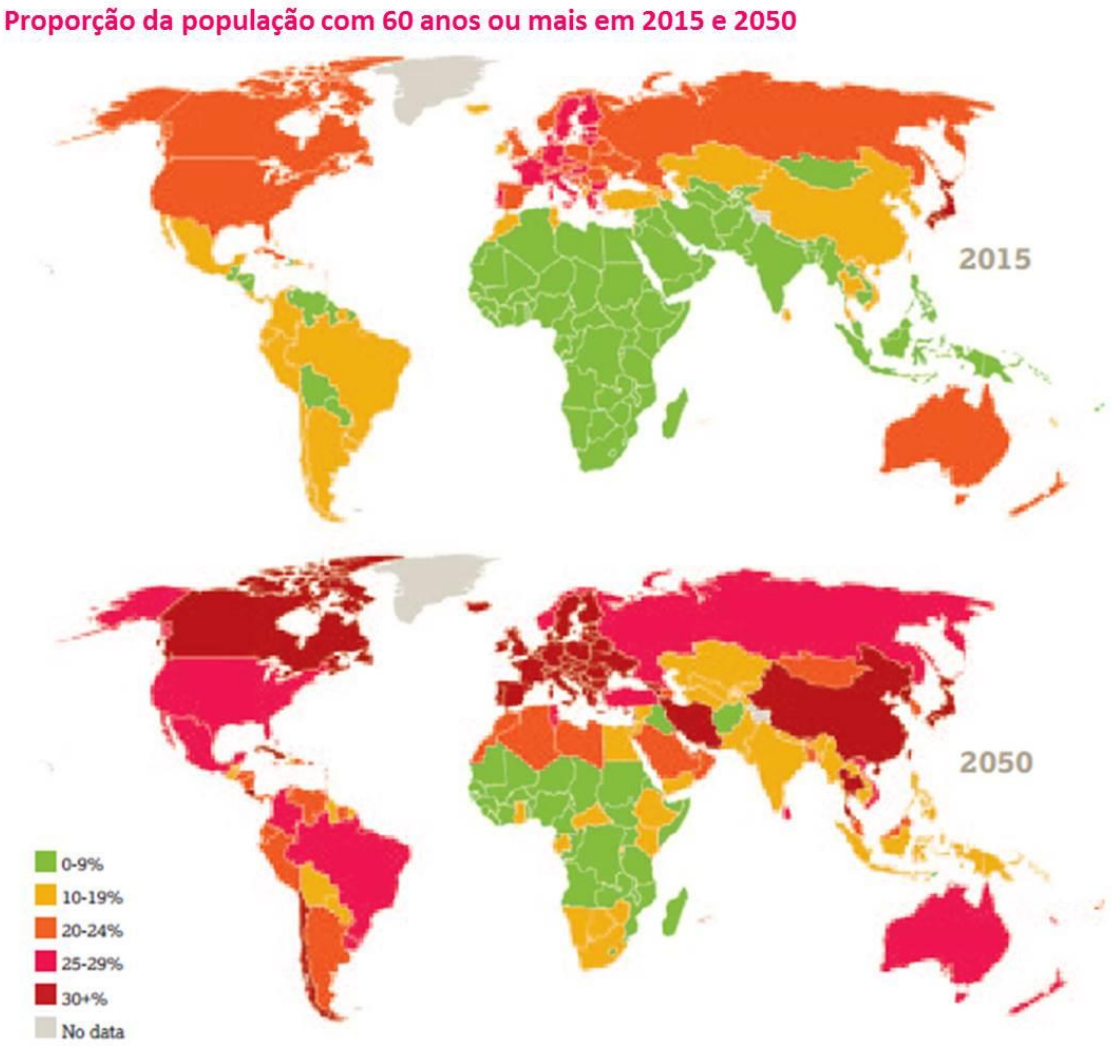
1.3. Cafeína e o Declínio Cognitivo decorrente da idade

1.3.1. Epidemiologia do Envelhecimento

O século XX presenciou um aumento sem precedentes na expectativa de vida, bem como um rápido declínio da fertilidade humana em muitos países do mundo. (Sander et al., 2015). O envelhecimento da população mundial é uma das maiores conquistas da sociedade moderna. Atualmente, as pessoas vivem mais tempo do que viviam a um século, refletindo avanços na Medicina, Nutrição e Tecnologia (Sander et al., 2015; *World Economic and Social Survey* 2007). Mas o envelhecimento representa também grandes desafios e será um tema dominante para o desenvolvimento no século XXI (*Global Agewatch Index* 2015; *World Economic and Social Survey* 2007).

De acordo com o Relatório Global de Saúde e Envelhecimento feito pela Organização Mundial de Saúde de 2015, existem cerca de 901 milhões de pessoas com 60 anos ou mais no mundo, representando 12,3% da população mundial. Até 2030, isso aumentará para 1,4 bilhões ou 16,5%, e até 2050, haverá um aumento para 2,1 bilhões ou 21,5% da população mundial (Figura 4). As pessoas com mais de 60 anos já superam as crianças menores de cinco anos (Figura 5); em 2050, ultrapassarão os de 15 anos. Estas mudanças demográficas serão mais rápidas nos países em desenvolvimento (*Global Agewatch Index* 2015).

A perspectiva de um envelhecimento saudável é contrabalanceada por uma das conseqüências mais assustadoras e potencialmente onerosas de uma expectativa de vida cada vez maior: o aumento das pessoas com demência, especialmente a doença de Alzheimer (*World Health Organization*, 2011). O risco de demência aumenta acentuadamente com o avanço da idade, e, caso não sejam desenvolvidas novas estratégias de prevenção, espera-se que esta condição acarrete em demandas crescentes



e de longo prazo aos serviços de saúde à medida que a população mundial envelhece (*Age international* 2013; *World Health Organization*, 2011).

Figura 4: Representação da população de idosos ao redor do mundo em 2015 e 2050 (Adaptado do *Global Agewatch Index* 2015).

O número de pessoas com demência, em todo o mundo, irá quase dobrar a cada 20 anos, impulsionado por padrões globais de envelhecimento da população. Em 2013 havia cerca de 44,4 milhões de pessoas com demência, e esses índices irão aumentar

para aproximadamente 75,6 milhões em 2030 e 135,5 milhões em 2050 (*Age international* 2013). Além disso, baseado em dados demográficos, a *Alzheimer's Disease International* – Federação das Associações de Alzheimer prevê um aumento no impacto econômico global devido às demências. Em 2010, foi estimado um custo de U$ 604 bilhões de dólares, e para 2030, estima-se um aumento de 85% no custo total, com os países em desenvolvimento tendo uma parte crescente do ônus econômico (Figura 6) (Abbot, 2011).

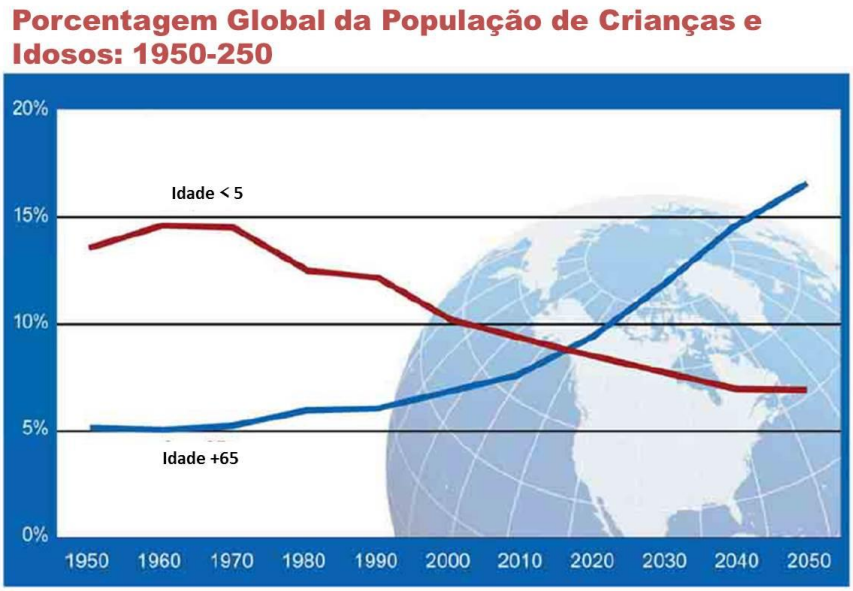


Figura 5: Representação global da população de crianças e idosos de 1950 a 2050 (Adaptado do *World Health Organization* ,2011)

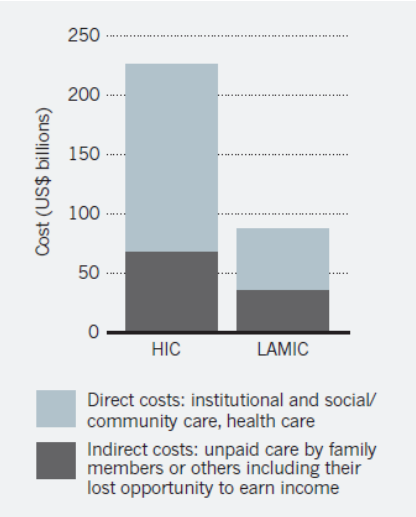


Figura 6: Custos globais com a demência. Há uma grande diferença no custo com cuidados de pessoas entre países de alta renda (HIC) e de baixa e média renda países (LAMIC). HIC - países de alta renda (*high-income countries*); LAMIC – países de baixa e média renda (low- and middle-income countries) (Abbot, 2011).

1.3.2. Envelhecimento e Declínio Cognitivo

No envelhecimento, as habilidades cognitivas tendem a declinar gradualmente, e o desenvolvimento de algum nível de comprometimento cognitivo é esperado com o avanço da idade (Erickson e Barnes, 2003). A partir da quinta década de vida já podem ser observadas alterações na memória, e muitos indivíduos nessa faixa etária se queixam de lapsos de memória à medida que envelhecem. Esses déficits de memória são chamados de "declínio de memória relacionado à idade", uma condição prevalente nas sociedades modernas (Albert, 2002; Crook et al, 1986; Glisky 2007; Rosenzweig e Barnes, 2003).

Os efeitos do envelhecimento no cérebro e na cognição possuem diversas etiologias, e abrangem uma infinidade de processos correlacionados, que em conjunto contribuem para a senescência do cérebro humano, resultando em uma diminuição da diferenciação e integração da função e comportamento cerebral (Lindenberger, 2014; Peters, 2006). O cérebro diminui de tamanho com o avanço da idade e há mudanças

tanto em nível molecular quanto morfológico (Scahill et al. 2003; revisado em Peters, 2006). Há uma perda neuronal substancial em certas regiões subcorticais (por exemplo, o prosencéfalo basal, locus coeruleus,e substancia nigra), o que resulta em diminuição da produção de uma variedade de neurotransmissores e na densidade de seus receptores em numerosas regiões corticais importantes para a função da memória (Albert, 2002; Lindenberger, 2014). Estudos *postmortem* mostram diferenças relacionadas à idade em vários aspectos morfológicos do cérebro, como redução de peso e tamanho, expansão de ventrículos cerebrais e sulcos, deformação e perda de mielina, perda de arborização dendrítica e corpos neuronais regiões-específicas, diminuição da vascularização cerebral e redução da densidade sináptica (Piguet et al., 2009; Raz e Rodrigue, 2006).

As alterações cerebrais não ocorrem na mesma medida em todas as regiões do cérebro (Peters, 2006). Regiões como o hipocampo e a substância branca pré-frontal exibem maiores reduções de volume do que outras áreas neocorticais, enquanto o córtex visual primário e o córtex associativo apresentam relativamente pouca perda de volume, e o corpo caloso não apresenta nenhuma mudança consistente de volume com a idade (Pfefferbaum et al., 2013; Raz et al., 2005).

Embora as mudanças na memória com a idade possam ser variáveis entre os indivíduos, é importante salientar que se deve distinguir os declínios de memória atribuível ao envelhecimento normal daqueles que são indicativos de envelhecimento patológico, como ocorre na doença de Alzheimer (Glisky, 2007). A doença de Alzheimer (DA) é uma doença neurodegenerativa relacionada à idade e é a principal causa de demência (Pike, 2017; Vinters, 2015). É uma doença irreversível e progressiva, que destrói lentamente a memória, que envolve principalmente a formação de placas senis compostas pelo peptídeo beta-amilóide e emaranhados neurofibrilares, gliose crônica, rompimento da barreira hematoencefálica e degeneração da substância branca

(Hashimoto et al., 2009; Mucke e Selkoe, 2012; Pike 2017; Vinters, 2015). Em vista disso, torna-se importante o conhecimento da fronteira entre condições normais e patológicas durante o envelhecimento (Albert, 2002). Até o momento, não há tratamentos eficazes para minimizar o comprometimento de memória associado à idade ou para as doenças demenciais como o Alzheimer, o que enfatiza a importância da concepção e implementação de estratégias de prevenção primária, durante, ou antes, da meia-idade (Kivipelto et al., 2006; Eskelinen et al., 2009; Nooyens et al., 2011).

1.3.3. Efeitos da Cafeína no Envelhecimento

A fim de adiar ou prevenir o declínio cognitivo e, eventualmente, a demência na velhice, as estratégias de prevenção são necessárias mais cedo na vida, durante ou antes da meia idade (Eskelinen et al., 2009; Kivipelto et al., 2006; Nooyens et al., 2011). Neste contexto, a influência de componentes de dieta usuais em eventos relacionados à idade tem sido investigada como uma estratégia para prevenir o declínio cognitivo e motor (Morris et al., 2006). Entre essas estratégias aparece cafeína, um componente da dieta amplamente consumido e que tem sido sugerida como um modulador de funções de aprendizagem e memória (Alhaider, 2011).

A cafeína parece ter um papel na normalização do comprometimento da função da memória durante o envelhecimento e em diferentes condições neurodegenerativas (Cunha e Agostinho, 2010). Em um estudo caso-controle de pequeno porte (Maia e de Mendonça, 2002) foi demonstrado, pela primeira vez, que pacientes diagnosticados com doença de Alzheimer haviam consumido significativamente menos cafeína durante 20 anos anteriores ao diagnóstico do que indivíduos da mesma faixa etária. De acordo com esses achados, estudos longitudinais têm apontado que a ingestão diária de cafeína equivalente a três ou mais xícaras de café reduz o declínio cognitivo em indivíduos idosos (Jarvis, 1993; Johnson-Kozlow et al 2002). Além disso, um estudo prospectivo

que avaliou o consumo de café na meia-idade por um período de 21 anos, até o envelhecimento, demonstrou que a cafeína estaria relacionada a um menor risco de desenvolver demência e doença de Alzheimer em idade avançada em comparação com aqueles indivíduos que não consumiam ou consumiam pouco café (Eskelinen et al., 2009), assim como Ritchie et al. (2007), que demonstraram os efeitos preventivos da cafeína na redução do declínio cognitivo em mulheres idosas quando o seu consumo foi acompanhado durante 4 anos (Eskelinen et al., 2009; Ritchie et al., 2007).

Modelos animais ajudam a identificar mecanismos de declínio cognitivo relacionado à idade no nível celular, visto que os roedores, assim como os humanos, também apresentam um declínio da função cognitiva relacionado à idade (Costa et al 2008a; Erickson e Barnes, 2003). Um estudo avaliou o desempenho da memória de reconhecimento de camundongos envelhecidos submetidos à administração de cafeína durante a vida adulta até o envelhecimento, na tarefa de reconhecimento de objetos. Os resultados desse estudo demonstraram que os camundongos envelhecidos apresentaram um desempenho inferior na memória de reconhecimento em comparação com os adultos e que o tratamento com cafeína durante 12 meses foi capaz de prevenir o comprometimento de memória (Costa et al 2008a). Quando outros tipos de memória foram avaliados, como a memória de reconhecimento social e a discriminação olfatória, também foram encontrados comprometimentos relacionados à idade (ratos de 12 e 18 meses de vida), e o tratamento agudo de cafeína foi capaz de reverter esses prejuízos (Prediger et al., 2005).

Enquanto que os estudos com animais envelhecidos têm sido particularmente bem sucedidos para avaliação dos processos de aprendizagem e memória relacionados à idade, os modelos celulares e animais da doença de Alzheimer permitem uma maior abordagem e investigação da relação causal entre o efeito protetor da cafeína e a

redução do declínio cognitivo em seres humanos (Fredholm, 2011). A primeira evidência *in vitro* de neuroproteção por cafeína em modelos de DA foi realizada em cultura de células cerebelares (Dall'Igna et al., 2003), no qual a cafeína e um antagonista A_{2A} (SCH58261) reduziram a agregação induzida pelo peptídeo β-amilóide, um evento chave associada à patogênese da DA. Além disso, esse efeito protetor foi comprovado em um estudo experimental em que a cafeína e antagonistas A_{2A} preveniram o comprometimento de aprendizagem e de memória induzidas pela infusão intracerebroventricular do peptídeo β-amilóide (Dall'lgna et al., 2007). Considerados em conjunto, esses dados indicam que o consumo de cafeína pode representar uma estratégia de prevenção em potencial para a demência e DA (Cunha e Agostinho, 2010; Fredholm, 2011).

1.4. Proteínas essenciais do Sistema Nervoso Central

1.4.1. Fator neurotrófico derivado do encéfalo (BDNF) e proteínas relacionadas no desenvolvimento e senescência

As neurotrofinas são fundamentais de diversas formas para a função do sistema nervoso central, com papéis críticos na diferenciação celular, na sobrevivência neuronal, na migração, na arborização dendrítica, na sinaptogênese e nas formas dependentes da atividade de plasticidade sináptica (Alonso et al., 2002; Greenberg et al., 2009; Pollock et al., 2001). Estes fatores tróficos apresentam um nível de expressão e de importância elevados durante todo o desenvolvimento do SNC dos mamíferos, e podem representar uma reserva fisiológica do organismo para combater o declínio cognitivo relacionado à idade (Fryer et al., 1996; Knüsel et al., 1994; Silhol et al., 2008; Tang et al., 2010).

Os principais componentes da família de neurotrofinas, entre os mamíferos, são o fator de crescimento neuronal (NGF), fator neurotrófico derivado do encéfalo (BDNF), neurotrofina-3 (NT-3) e NT-4/5. As ações celulares das neurotrofinas são mediadas principalmente pelos seus receptores de alta afinidade, os receptores tirosina cinases (Trk) (Chen e Weber, 2004). Cada Trk é preferencialmente ativado por uma ou mais neurotrofinas - TrkA por NGF, TrkB pelo BDNF e NT-4/5, e TrkC por NT-3 (Greene e Kaplan, 1995). O papel das neurotrofinas tem sido de extremo interesse, visto que, além da importância na manutenção da homeostasia do sistema nervoso central, alterações na sinalização mediada pelas neurotrofinas estão muitas vezes presentes em patologias neurodegenerativas, como na doença de Parkinson e de Alzheimer. Assim, o conhecimento dos mecanismos celulares e moleculares desencadeados pelas neurotrofinas, bem como as alterações das mesmas em situações patológicas, é fundamental para o desenvolvimento de novos alvos terapêuticos (Santos, 2009).

O BDNF é um dos fatores neurotróficos mais investigados devido ao seu papel central nos eventos que envolvem a regulação não só da estrutura, mas também da função e manutenção da sobrevivência de algumas populações de neurônios durante o desenvolvimento e na vida adulta (Lu e Chow, 1999; Poo, 2001; Tyler e Pozzo-Miller, 2003). O BDNF e seu receptor de alta afinidade Trk B são altamente expressos no hipocampo maduro e no córtex cerebral e são essenciais na regulação da sobrevivência e diferenciação neuronal (Greenberg et al., 2009; Pollock et al., 2001). Inicialmente, o BDNF é sintetizado como uma proteína precursora (pré-pró-BDNF) que é clivada no retículo endoplasmático dando origem ao pró-BDNF, que, por sua vez, pode seguir três destinos distintos: a) clivagem intracelular seguida de liberação; b) liberação seguida por clivagem extracelular; ou c) liberação sem clivagem subseqüente (Lu et al, 2005). A clivagem da pró-BDNF origina o BDNF maduro. A secreção de BDNF pode ocorrer de

duas formas: 1) via constitutiva (espontânea), no qual as vesículas contendo as neurotrofinas fundem-se espontaneamente com a membrana plasmática libertando assim o seu conteúdo ou 2) via regulada, onde as neurotrofinas são liberadas em resposta a determinados estímulos, como pelo aumento prolongado dos níveis intracelulares de cálcio ou pela atividade elétrica neuronal (Lessman et al., 2003).

Tanto a forma imatura do BDNF quanto a sua forma madura podem atuar como moléculas sinalizadoras, contudo, com propriedades de sinalização distintas (Lee e Chao, 2001). Evidências sugerem que as proneurotrofinas têm efeitos biológicos opostos aos das neurotrofinas maduras. O pró-BDNF que não sofre clivagem liga-se ao receptor p75NTR e aumenta depressão a longa duração (LTD) e leva a apoptose, enquanto que a ativação de TrkB por BDNF facilita a potenciação de longa duração (LTP) e inibe a depressão a longa duração (LTD) e também leva a sobrevivência neuronal (Lu et al., 2005; Woo et al, 2005). Assim, a proteólise da forma precursora de BDNF torna-se um mecanismo de controle importante para a direção da plasticidade do hipocampo (Woo et al., 2005).

A sinalização do BDNF é fundamental no período de desenvolvimento do SNC. As neurotrofinas e seus receptores apresentam um nível elevado de expressão durante todo esse período, sendo que podem se expressar de forma seletiva conforme o estágio do desenvolvimento e conforme a região cerebral (Fryer et al, 1996; Knüsel et al, 1994; Tang et al., 2010). Os efeitos produzidos pelo BDNF podem variar conforme a fase do desenvolvimento. No início da fase fetal, o BDNF é importante para a formação e maturação dos neurônios em geral, participando do desenvolvimento dos circuitos neurais do encéfalo imaturo e adulto, incluindo sua participação no crescimento e elongação dos dendritos de sinapses inibitórias e excitatórias, no estímulo ao desenvolvimento das espinhas dendríticas e no crescimento dos neuritos (Carvalho et

al., 2008; Ji et al, 2010; Lipsky e Marini, 2007; Singh et al., 2006; Tyler e Pozzo-Miller, 2004). A imunorreatividade ao BDNF já está presente no 13° dia embrionário (DE 13) no SNC de ratos, especialmente na subplaca neocortical e nos neuroblastos da placa cortical em desenvolvimento, e no DE 18 todas as células da placa cortical encontram-se marcadas por esta neurotrofina (Fukumitsu et al., 1998). A superexpressão de BDNF em camundongos transgênicos aumenta o número de sinapses nos gânglios simpáticos e acelera a maturação de vias inibitórias no córtex visual em desenvolvimento (Huang et al., 1999).

Na fase adulta, o BDNF tem papel fundamental no processo de consolidação da memória episódica (Barboza, 2009). Além disso, o BDNF também é essencial para eventos de plasticidade neuronal e funções importantes como o aprendizado e memória (Tyler et al., 2002). De fato, o bloqueio da sua sinalização compromete a persistência da memória (Alonso et al., 2002; Bekinschtein et al., 2007). Além disso, o BDNF é considerado essencial no mecanismo de formação de potenciação de longa duração (LTP), mecanismo essencial na aprendizagem e memória (Barboza, 2009).

As alterações nos níveis de BDNF e/ou na expressão do TrkB têm sido descritas durante o envelhecimento normal e na doença de Alzheimer, e as diminuições na expressão do BDNF foram associadas com atrofia e/ou morte neuronal (Silhol et al., 2008; Tapia-Arancibia et al. 2008). Em ratos envelhecidos, a indução da LTP por estímulos mais fracos (mas não de alta freqüência) liberam BDNF acompanhado por um aumento na magnitude da LTP (Balkowiec e Katz, 2002; Rex et al., 2005). Além disso, tem sido demonstrado que o BDNF endógeno é necessário para a LTP em ratos envelhecidos, juntamente com os receptores A_{2A} de adenosina (Diógenes et al., 2011).

De acordo com a possibilidade de que os efeitos da cafeína sobre a cognição estejam ligados à regulação das neurotrofinas, foi demonstrado que a administração de

cafeína durante o período gestacional 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 no DE 18 e aumento no imunoconteúdo do BDNF cortical no DE 20 (Mioranzza et al., 2014). Além disso, a administração de cafeína em ratos adolescentes (0,3 e 1 g/L) acarretou em um aumento tanto da forma imatura do BDNF quanto da sua forma madura, no hipocampo e córtex cerebral, que foi acompanhado de uma melhoria na memória de reconhecimento destes animais, demonstrando o envolvimento desta via de sinalização com possíveis alterações comportamentais em animais expostos a cafeína durante as fases iniciais do seu desenvolvimento (Ardais et al., 2014).

Em relação aos efeitos da cafeína sobre as neurotrofinas em idades mais avançadas, foi demonstrado que a administração de cafeína em camundongos durante a vida adulta até o envelhecimento preveniu o declínio na memória de reconhecimento relacionado à idade e juntamente com um aumento nos níveis de BDNF e TrkB do hipocampo (Costa et al., 2008a). Além disso, a melhora da memória de reconhecimento após uma administração aguda de cafeína também foi acompanhada por um aumento no imunoconteúdo de BDNF (Costa et al., 2008b).

É amplamente aceito que a formação da memória de longa duração (LTM) exige a participação de uma ampla maquinaria transcricional e translacional nos sistemas neuronais (Izquierdo e Medina, 1997; Viola et al., 2000). Neste contexto, o CREB (proteína de ligação de elemento de resposta cAMP), um fator de transcrição envolvido na formação de memória a longo prazo, é um mediador principal das respostas neuronais de BDNF (Finkbeiner et al., 1997) e é ativado no hipocampo durante a aprendizagem (Alonso et al., 2002; Finkbeiner; 1997; Yamada, 2003). As alterações da atividade do CREB foram documentadas em ratos envelhecidos (Asanuma et al., 1996).

Todas estas moléculas são conhecidas como sinais principais envolvidos na plasticidade neuronal, uma propriedade que é prejudicada com o envelhecimento (Silhol 2008).

1.4.2. Proteína Ácida Fibrilar Glial (GFAP) no desenvolvimento

A proteína ácida fibrilar glial (GFAP) é um dos principais filamentos intermediários (FI) expressos pela glia radial, astrócitos adultos e células-tronco neurais, juntamente com microtúbulos e microfilamentos, que compõem o citoesqueleto da maioria das células eucarióticas (Eng et al., 2000; Hol e Pekny, 2015; Mamber et al., 2012; Middeldorp e Hol., 2011). Os FI adquiriram seu nome de sua forma filamentosa e seu diâmetro intermediário (8-12 nm) entre finos microfilamentos de actina (7 nm) e microtúbulos espessos (25 nm) (Middeldorp e Hol, 2011).

A GFAP é importante na modulação do formato e motilidade dos astrócitos, proporcionando estabilidade estrutural para processos astrocíticos. Além disso, a GFAP pode ser utilizada com um parâmetro de lesão do SNC, visto que é induzido por danos cerebrais, como resultado de trauma, doença, distúrbios genéticos ou insulto químico ou ainda durante a neurodegeneração do SNC (Eng et al., 2000; Middeldorp e Hol, 2011). Nessas situações, os astrócitos tornam-se reativos e respondem de maneira típica, denominada astrogliose. A astrogliose é caracterizada por uma *upregulation* e rearranjo das FIs, acarretando numa síntese rápida de GFAP, vimentina, sinemina e nestina que formam um sistema altamente complexo (Hol e Pekny, 2015). Esta resposta ajuda a manejar o estresse agudo, limitar os danos aos tecidos/órgãos e restaurar a homeostase (Hol e Pekny, 2015).

Os níveis de GFAP são detectados mais cedo em roedores do que em humanos, sendo que em humanos o surgimento se dá em torno de 13 semanas de gestação, enquanto que em animais surgem no período que corresponderia a 4-6 semanas

gestacionais humanas (Clancy et al., 2001) . A expressão de GFAP no cérebro de rato em desenvolvimento segue a progressão básica do desenvolvimento de glia e astrócitos radiais. Apesar de não estar claro a idade exata em que a expressão da proteína GFAP começa, estudos indicam que o RNAm da GFAP pode ser detectados no cérebro de ratos juntamente com o aparecimento da primeira glia radial, entre os dias embrionários 9-11 (Fox et al., 2004; Götz e Barde, 2005). Os níveis de mRNA de GFAP nestes períodos iniciais são muito baixos, mas à medida que o desenvolvimento progride e a gliogenêse começa, tornam-se abundantemente expressos. Foi demonstrado que a expressão de GFAP aumenta abruptamente até DPN 6, após há uma tendência a estabilização mas com um aumento progressivo até DPN 48 em hipocampo de ratos (Kim et al., 2011). A GFAP é primeiro expresso pela glia radial ao redor da zona ventricular telencefálica, no globo pálido medial e fimbria (Mamber et al., 2012). Após a gliogênese, os níveis de GFAP estabilizam no cérebro adulto, mas aumentam progressivamente durante o envelhecimento, provavelmente devido a danos oxidativos decorrentes da idade (Mamber et al., 2012; Middeldorp e Hol, 2011).

1.4.3. Proteína Associada ao Sinaptossoma-25 (SNAP-25) no desenvolvimento

A SNAP-25 (proteína associada ao sinaptossoma-25) é uma proteína do complexo SNARE (do inglês *Soluble N-ethylmaleimidesensitive factor attachment protein receptor*) que participa na regulação da exocitose das vesículas sinápticas (Corradini et al., 2009). As proteínas do complexo SNARE formam a maquinaria base necessária para a fusão dos compartimentos intracelulares de membrana uns com os outros ou com a membrana plasmática (Lin e Scheller, 2000; Söllner et al., 1993). A SNAP-25 é uma proteína ligada à membrana ancorada à superfície citosólica das membranas na região central da molécula, e contribui na formação do complexo

exocitótico de fusão juntamente com a sintaxina-1 e sinaptobrevina, que é necessário para a fusão das vesículas à membrana plasmática, mediando a liberação de neurotransmissores da membrana pré-sináptica para a fenda sináptica, necessário para plasticidade e sinaptogênese neuronal (Braun e Madison, 2000; Corradini et al., 2009; Jahn et al., 2003; Jahn e Scheller, 2006).

Em relação ao desenvolvimento, a expressão da SNAP-25 já pode ser detectada no encéfalo de embriões de ratos de 18-20 dias, com um aumento linear até atingir uma estabilização aos 20 dias pós-natal, sendo que a sua fosforilação e consequente ativação ocorre somente no período pós-natal, a partir dos 4 dias de vida (Kataoka et al., 2006; Oyler et al., 1989). Estudos anteriores demonstraram que a SNAP-25 teria participação no crescimento de neuritos, visto que quando a sua expressão foi inibida em neurônios corticais de ratos impediu a elongação dos mesmos (Hepp e Langley, 2001; Osen-Sand et al., 1993; Tang, 2001).

A SNAP-25, expressa por neurônios no hipocampo, é necessária para a formação de memória a longo prazo e está associado à capacidade cognitiva (Gosso et al., 2006; Zhang et al 2014). Por exemplo, em animais que apresentavam uma super expressão desta proteína foi encontrado um 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). Além disso, é importante destacar que a SNAP-25 também está envolvida na regulação da potenciação de longa duração (LTP) no hipocampo, uma forma de plasticidade sináptica que se acredita estar subjacente à aprendizagem e à memória (Martin et al., 2000). Sendo assim, torna-se interessante a caracterização dessa proteína nos diferentes estágios do desenvolvimento cerebral.

2. OBJETIVO GERAL

Investigar o efeito do consumo de cafeína em diferentes fases da vida de ratos Wistar sobre parâmetros comportamentais e proteínas sinápticas importantes para o funcionamento do Sistema Nervoso Central.

2.1. Objetivos Específicos

1. Avaliar o impacto do consumo de cafeína durante o desenvolvimento do SNC em ratos púberes:

   - nos parâmetros de locomoção, memória e ansiedade;

   - no imunoconteúdo de proteínas sinápticas importantes nos processos de aprendizagem e memória (BDNF, pró-BDNF, GFAP e SNAP-25);

   - associar as alterações neuroquímicas com os achados comportamentais;

   - avaliar a influência do sexo nos parâmetros mencionados acima.

2. Investigar os efeitos da administração crônica de cafeína em ratos adultos e de meia-idade:

   - no declínio cognitivo previsível associado à idade na memória de ratos de meia-idade;

   - nos possíveis efeitos da idade e da administração de cafeína sobre o imunoconteúdo hipocampal do BDNF pró-BDNF, TrkB, e CREB;

   - associar as alterações neuroquímicas com os possíveis prejuízos da memória decorrentes da idade.

PARTE II

CAPÍTULO I

The Janus Face of Caffeine

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Invited review

The Janus face of caffeine

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Learning and memory

ABSTRACT

Caffeine is certainly the psychostimulant substance most consumed worldwide. Over the past years, chronic consumption of caffeine has been associated with prevention of cognitive decline associated to aging and mnemonic deficits of brain disorders. While its preventive effects have been reported extensively, the cognitive enhancer properties of caffeine are relatively under debate. Surprisingly, there are scarce detailed ontogenetic studies focusing on neurochemical parameters related to the effects of caffeine during prenatal and earlier postnatal periods. Furthermore, despite the large number of epidemiological studies, it remains unclear how safe is caffeine consumption during pregnancy and brain development. Thus, the purpose of this article is to review what is currently known about the actions of caffeine intake on neurobehavioral and adenosinergic system during brain development. We also reviewed other neurochemical systems affected by caffeine, but not only during brain development. Besides, some recent epidemiological studies were also outlined with the control of "pregnancy signal" as confounding variable. The idea is to tease out how studies on the impact of caffeine consumption during brain development deserve more attention and further investigation.

1. Introduction

The knowledge of the stimulant effects of caffeine on the central nervous system (CNS) dates back centuries, to the time when Ethiopian shepherds noticed that their sheep stayed awake all night after grazing on wild coffee cherries. Nowadays, it is widely accepted that caffeine is the most regularly consumed psychostimulant in the world being ingested predominantly as coffee. Other caffeine-containing beverages also contain significant amount of caffeine, for example, tea, cocoa beverages, chocolate bars and soft drinks. Coffee and caffeinated beverages take part of the diet in most nations of the world (Fredholm et al., 1999).

Over the past three decades, the pharmacological target responsible for the psychostimulant actions of methylxanthines was proposed (Fredholm, 1980; Fredholm and Persson, 1982). Up to the present moment, the blockade of adenosine receptors is the only known mechanism that is significantly affected by relevant doses of caffeine. Adenosine exerts widespread modulatory effects in the nervous system via four types of guanine nucleotide binding (G) protein-coupled receptors – A_1, A_{2A}, A_{2B}, and A_3 – that trigger several signal transduction pathways (Cunha, 2001; Dunwiddie and Masino, 2001; Fredholm et al., 2005). Caffeine is a non-selective adenosine receptor antagonist, with reported similar affinities for A_1, A_2 receptors and with lower affinity for A_3 receptors. The preferential targets for caffeine are adenosine A_1 (A_1R) and A_{2A} ($A_{2A}R$) being A_1R widely expressed in the brain and $A_{2A}R$ highly concentrated in the striatum (Ferré, 2008; Karcz-Kubicha et al., 2003). Similar to classical psychostimulants, caffeine produces motor-activating, reinforcing, and arousing effects, which depend on the ability of caffeine to counteract multiple effects of adenosine on neurotransmitter systems (Ferré et al., 2010).

Over the last years, the effects of chronic caffeine intake on cognitive functions have been reported in human and animals studies. In epidemiological reports, caffeine intake was associated with a significantly lower risk for developing Alzheimer's disease (Eskelinen et al., 2009; Maia and de Mendonça, 2002; Ritchie et al., 2007). In aged rodents, caffeine chronically administered prevents and also reverses memory impairment (Costa et al., 2008a; Prediger et al., 2005; Sallaberry et al., 2013). Likewise, chronic treatment with caffeine was also effective in preventing neurodegeneration, beta-amyloid production/levels and mnemonic deficits in experimental models of Alzheimer's disease (Arendash et al., 2006, 2009; Dall'Igna et al., 2003, 2007; Espinosa et al., 2013). The preventive effects of caffeine against motor symptoms and loss of dopaminergic neurons were also reported in epidemiological

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studies (Ascherio et al., 2001; Postuma et al., 2012; Ross et al., 2000) and experimental models of Parkinson's disease (Chen et al., 2001; Chen et al., 2008; Joghataie et al., 2004). In most cases the beneficial effects of caffeine were mimicked by the blockade of adenosine A_{2A} receptors, suggesting that this receptor is strictly involved in the beneficial effects of caffeine against memory decline in brain disorders (reviewed in Gomes et al., 2011; Cunha, 2005).

While caffeine seems to restore or prevent memory impairment as a consequence of disturbances in brain homeostasis (Cunha and Agostinho, 2010), its cognitive enhancer properties are still a matter of debate (for more detailed discussion see Einöther and Giesbrecht, 2013; Nehlig, 2010). A wide range of studies in human subjects suggest that the cognitive benefits of caffeine are associated to relief of withdrawal symptoms rather than improvement in the cognitive functions (James and Rogers, 2005; Jarvis, 1993; Rogers et al., 2005, 2013). Besides, moderate to high consumers develop tolerance to caffeine and only low or non-consumers could eventually benefit from an acute administration (Evans and Griffiths, 1992; Griffiths and Mumford, 1996; Robertson et al., 1981; Rogers et al., 2003). In rodents, acute administrations improved the performance in tasks used for evaluating learning and memory (Angelucci et al., 1999, 2002; Botton et al., 2010; Costa et al., 2008b; Kopf et al., 1999).

The stages of development are very sensitive to therapeutic interventions, since the beginning of gestation until the early adulthood. Many diseases in the later years of human life are believed to originate in early fetal life. It is somehow surprising that more detailed studies on the effects of caffeine during brain development remain still scarce (Temple, 2009). In this context, a recent document from The Organization of Teratology Information Specialists (OTIS) reinforces the limited consumption of caffeine for pregnant women. In the 1960's, The US Food and Drug Administration (FDA) focused regulatory attention on caffeine as part of its review of "generally recognized as safe substances" (GRAS). However, caffeine again received close scrutiny in 1987, when the FDA proposed to establish a prior sanction regulation for caffeine.

Of note, the methylxanthines aminophylline, theophylline and caffeine have been used for more than 30 years to treat apnea of prematurity (Koppe et al., 1979; Millar and Schmidt, 2004). Apneas and unstable breathing are the leading causes of hospitalization and morbidity in preterm infants. Methylxanthines are among the most commonly prescribed drugs in neonatal medicine because of their capacity to suppress respiratory depression, reduce periodic breathing and enhance diaphragmatic activity (Darnall et al., 2006; Davis et al., 2010; Leon et al., 2007). Caffeine also increases ventilatory drive (Bairam et al., 1987) and improves sensitivity and/or responsiveness to changes in the level of arterial O_2 (Marchal et al., 1987).

The chronic exposure of the fetal brain to caffeine during this critical time could influence and permanently alter postnatal behavior. If this really occurs, it would not be unpredicted that caffeine exposure in early life is responsible for disturbances in the brain homeostasis which we are currently unaware.

The central mechanisms of action of caffeine in adults have been well documented and reviewed elsewhere (Fredholm et al., 1999; Ribeiro and Sebastião, 2010). Taking into account that adenosine is a neuromodulator and thence the blockade of its receptors influences several neurochemical parameters, many evidences have suggested that the blockade of adenosine receptors is not the single cellular mechanism responsible for the effects of caffeine. In this review, we will outline animal studies on the influence of caffeine in the neurotransmitter systems at different phases of brain development. Although the application to humans in general has to be translated carefully, animal models have long been important for dissecting the underlying mechanisms of many human diseases, as well as in transitioning promising candidate therapies from bench to bedside. In order to better identify dose safety, the manipulations in the dose and schedule of administration for any drug or substance are more feasible in animal studies. In later sections, some recent epidemiological studies on the impact of caffeine consumption during pregnancy will be discussed. Finally, we also sought to tease out how scarce are more detailed studies on the effects of caffeine during brain development.

2. Caffeine metabolism during development

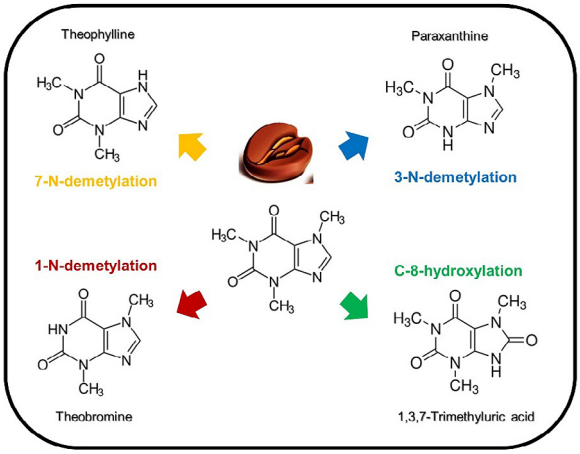
In order to investigate the effects of caffeine intake during pregnancy on fetal weight and development, rats were given 10 or 100 mg/kg/day, either as bolus oral doses, or as four 2.5 or 25 mg/kg doses at 3-h interval. Smaller pups displayed decreased crown-rump length and skeletal ossification from dams that received the highest dose in both schedule of administration (Smith et al., 1987). The frequency of malformations was observed, especially of the limbs and palate, among the offspring of rats or mice treated with caffeine during pregnancy in single daily doses of 80–100 mg/day (equivalent to human consumption of 40 or more cups of coffee daily). Importantly, toxicity was observed in mothers exposed to the highest dose (Nehlig and Debry, 1994). These effects observed in the offspring's exposed to caffeine could be partially explained by age-dependent metabolic differences.

Caffeine is rapidly and completely absorbed by gastrointestinal tract and the excretion is predominantly renal (Arnaud 1976; Yesair et al. 1984). The absorption, bioavailability, and the urinary and fecal excretion of caffeine did not show differences between species (Arnaud, 1985; Arnaud et al., 1989; Walton et al., 2001). The interspecies differences have been reported for pharmacokinetics, which is dose-dependent in animals due to the saturation of metabolic transformation of caffeine (Bortolotti et al., 1985). In adult humans, caffeine is virtually completely metabolized, with less than 2% of the ingested compound being recoverable in urine unchanged (Arnaud, 1987; Gorodischer et al., 1986; Somani and Gupta, 1988). Caffeine (i.e., 1,3,7-trimethylxanthine) is demethylated to its dimethylmetabolic intermediates, with over 80% of orally administered caffeine metabolized to paraxanthine (1,7-dimethylxanthine), and about 16% is converted to theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine) (Benowitz et al., 1995; Lelo et al., 1986). It is also important to mention that paraxanthine is the major metabolite in rodents, but the levels of theophylline are also high (Bonati et al., 1984–1985; Fredholm et al., 1999). Of note, trimethyl derivatives correspond to 40% of the caffeine metabolites in the rat, compared to 6% in humans (Arnaud, 1985) (Fig. 1). Among rodents, paraxanthine glucuronide was identified only in mice strains (Arnaud et al., 1989).

Once caffeine enters the body, it is metabolized by the CYP1A2 from liver being converted into theophylline, theobromine and paraxanthine. Caffeine is commonly used as a pharmacological probe to assess CYP1A2 activity *in vivo* (Nordmark et al., 1999), and the maturation, expression and activity of this enzyme is highly dependent on development (Leeder, 2001). Consequently, the metabolism of caffeine is dependent on the maturation of this enzyme. In general, low functionality of CYP was detected in fetal tissue, with increasing activity apparently triggered by postnatal exposures or events (Blake et al., 2005). In humans and animals, methylxanthines readily passes the placenta barrier and enters all tissues and thus may affect the fetus/newborn at any time during pregnancy or postnatal life (Abdi et al., 1993; Arnaud et al., 1983; Kimmel et al., 1984; Leon et al., 2007).

Caffeine metabolism develops gradually during the first year of life in humans (Pearlman et al., 1989), and can be influenced by

Schematic biochemical pathways of caffeine metabolism



Comparison between human and rodent metabolisms

HUMAN X RODENT	CAFFEINE METABOLITE	MITOCHONDRIAL ENZYMES
	Paraxanthine (aprox. 70%)	CYP1A2
	1,3,7-Trimethyluric acid (aprox. 15%)	CYP1A2, CYP3A4, CYP2C8/9
	Theobromine and Theophylline (7-8%)	CYP1A2, CYP2C8/9, CYP3A4
	1,3,7-Trimethyluric acid (aprox. 70%)	CYP1A2
	Paraxanthine (aprox. 13%)	CYP1A2, CYP2C11
	Theobromine and Theophylline (8-9%)	CYP1A2, CYP2C, CYP3A2

Fig. 1. The molecule of caffeine (at the center), and its conversion into dimethyl metabolites. Summary of the main differences between humans and rodents in the metabolism of caffeine.

various factors (Aldridge et al., 1979). The half-life of caffeine decreases gradually in full-term newborn (Aranda et al., 1977, 1979; Le Guennec and Billon, 1987), and decreases exponentially with postnatal age, reinforcing that caffeine metabolism develops gradually during the first year of life (Aldridge et al., 1979; Parsons and Neims, 1981; Pearlman et al., 1989). However, this substance has a longer half-life in premature infants (Parsons and Neims, 1981). In breast-fed infants caffeine elimination half-life is prolonged compared to formula-fed infants (Blake et al., 2005; Le Guennec and Billon 1987), and this difference was related to an enhanced CYP1A expression *in vitro* by formula-fed but not human milk (Xu et al., 2005).

Although it remains elusive whether caffeine intake during gestation could represent a fetal hazard, there is considerable concern over the widespread use of caffeine due to its prolonged half-life during this period.

3. Neurobehavioral effects during brain development

Phylogenetically, the striatum and motor-associated cortical areas prune earlier than higher-level brain regions associated with cognition (Johnson, 2003; Thompson et al., 2000). Even for cortical regions, there is a clear differential time course of development, suggesting that each brain region has a particular path or trajectory of development. As a result of maturation, the functioning of each brain region and its connectivity to others or skill learning as a specialized function reflected in a developmental shift in regional dominance. Comparatively, this process occurs in all mammalian species, including humans, primates and rats, with relatively the same developmental milestones of puberty onset or the appearance of higher-level cognition. This protracted postnatal period of development renders specific brain areas more or less sensitive to the acute and enduring effects of stimulants depending on the age at which the stimulants are administered. Thus, caffeine as a psychostimulant causes evident changes in the behavior of animals. Considering prenatal or postnatal exposure some factors may interfere with the effects of caffeine on the behavior such as dose–response protocol, exposure period and age when animals are tested. The oral route is considered the only appropriate route for evaluating human risks from exposure to caffeine during pregnancy. Considering developmental toxicology of caffeine in animals, it was identified a No Observed Effect Level (NOEL) of 30 mg/kg/day, and the teratogenic NOEL to be 80–100 mg/kg/day (Christian and Brent, 2001).

3.1. Locomotor activity

Adult animals treated with low to moderate doses of caffeine exhibit a profile of hyperlocomotion, whereas at higher doses prevail hypolocomotion. Over three decades ago, many studies were designed to investigate locomotor activity of the animals from dams treated with caffeine during gestational or lactation periods. These studies reported that young offspring rats treated with caffeine during pregnancy often exhibited alterations in the locomotor and general activity than control subjects. Importantly, these alterations were strictly depended on the age of the animals and the dose of caffeine.

The prenatal exposure to ten daily 20 or 40 mg/kg (i.p.) of caffeine increases locomotor activity at postnatal day 61 (PND 61) along with no effect at PND145 and decreases at PND188 (Hughes and Beveridge, 1990). However, Glavin and Krueger failed to demonstrate any effect of 12.5, 25 or 35 mg/kg/day of caffeine via maternal drinking water on open-field ambulation at PND 48, 68 or 196 (Glavin and Krueger, 1985). In a series of studies, caffeine was ingested by dams in their drinking water during gestation or lactation or in both periods and locomotor activity was assessed 1, 2, 4 and 6 months after birth. All rats exposed to either dose combination of caffeine during both gestation and lactation showed less locomotor and rearing activity (Concannon et al., 1983; Hughes and Beveridge, 1991; Peruzzi et al., 1985; Zimmermberg et al., 1991). In another study, pregnant Sprague–Dawley rats received caffeine-supplemented diet starting from day 9 of gestation until PND93 and had their locomotor activity tested at different times. These results showed that animals presented a profile of hyperlocomotion that lasted until PND 375, i.e. an effect that can last up to aging (Nakamoto et al., 1990).

The profile of locomotor activity is also distinct in animals treated with caffeine after birth, featuring a bell curve (Holloway and Thor, 1982). In a study, caffeine was administered by gavage over postnatal days 2–6 and locomotor activity was increased 12-day-old (Guillet, 1990), but not at 28- or 70–90 days of age (Fischer and Guillet, 1997).

More recently, Tchekalarova and colleagues confirmed that caffeine can also cause distinct effects when administered at different postnatal days. Caffeine (10 and 20 mg/kg) administered between PND7 and 11, which corresponds to the third trimester of human gestation, causes hyperlocomotion, whereas when injected between PND13 and P17 rats were less active than controls at PND25 and P32 (Tchekalarova et al., 2005). Apart from the effects

of caffeine on anxiety-related behavior, the developmental period of caffeine exposure was crucial for the alterations in locomotor activity during ontogenesis. Many of these behavioral changes in specific ages by caffeine treatment might be due to variations in the maturity of the number, binding and density of brain adenosine receptors and possibly also other receptor systems (these topics will be outlined in the next sections).

3.2. Anxiety-related behavior

While the benefits of caffeine on cognitive functions remain under debate, the anxiogenic effects are well documented in animals and human subjects (Bhattacharya et al., 1997; El Yacoubi et al., 2000; Rogers et al., 2006, 2010). Caffeine actions are dose-related and divided into two broad categories: at lower concentrations it stimulates the locomotor activity (Rhoads et al., 2011), whereas it induces an anxiogenic-like profile at higher doses (Kaplan et al., 1997; Sudakov et al., 2001; Rogers et al., 2010). Regarding prenatal exposure to caffeine, in some of the above-cited reports the alterations in the locomotor activity were associated to emotional reactivity. Indeed, rats exposed to caffeine during gestation, lactation or both periods have emotional changes that last up to 6 months of age evidenced by increased number of animals that failed to or took longer than 1 min to emerge into the brightly lit arena in a dark/light task for both males and females, and by increased defecation in the open field task for males (Hughes and Beveridge 1986, 1991). In a recent study, postnatal administration of caffeine (PND 2–6) have induced a decrease in anxiety-related behavior in rats tested at PND 37–42 (adolescence period), which is evidenced by increased time spent in light compartment in the light/dark transition paradigm and by increase time spent in the open arm of elevated plus maze task (Pan and Chen, 2007).

The use of animal models which complement the rodent existent approach have increased recently in order to evaluate anxiety-related behavior. Experiments carried out with zebrafish, an emerging model system in behavioral neuroscience, showed increased thigmotaxis by larvae exposure to caffeine added directly on water (Richendrfer et al., 2012). The knowledge about the behavioral repertoire of zebrafish has been significantly improved and several studies suggest that thigmotaxis may reflect an anxious phenotype for this species (Blaser and Rosemberg, 2012; Cachat et al., 2010; Maximino et al., 2012; Rosemberg et al., 2011).

3.3. Learning and memory

Differently from young adults and elderly population, the consumption of caffeine during brain development on learning and memory deserves more attention. One of the first studies dates from 80's, in which the effects of gestational caffeine intake were investigated on the learning and memory of the offspring. In this study, dams from BALB/c mice were treated during gestation with caffeine (60, 80 and 100 mg/kg/day) in the drinking water. Adult animals from dams that received caffeine (80 mg/kg/day) presented an increased latency to re enter in the dark compartment in the passive avoidance, which suggests learning and memory impairment (Sinton et al., 1981). In adult rats exposed to neonatal caffeine (15–20 mg/kg/day on PND 2–6) females exhibited enhanced memory retention at 24 and 72 h after training, but males exhibited significantly reduced retention at both time periods in the passive avoidance learning (Fisher and Guillet, 1997). However, the same neonatal caffeine treatment did not cause gender specific effects in juvenile rats. Prenatal caffeine exposure (60 mg/kg/day on gestational days 13–19) has also been shown to alter passive avoidance learning in a gender dependent manner. Adult female offspring of caffeine-treated dams showed significantly enhanced retention at 25 days after training when compared to placebo treated controls (Swenson et al., 1990). However, female and male neonates (PND 2–6) treated with caffeine showed worsened performance in the step-through avoidance task at PND 35–37 (Pan and Chen, 2007).

In another study, postnatal administration of caffeine also impaired spatial learning ability in adult Long Evans rats (Zimmermberg et al., 1991). More recently, adult female and male rats that received caffeine from dams (75 mg/mL in the drinking water) showed impairment in the recognition memory as assessed by novel object recognition task. Similarly, they displayed a significant increase of working memory errors and reference errors in a radial arm maze task (Soellner et al., 2009). More recently, caffeine (0.3 g/L) administered only during gestation and lactation was able to impair the performance in adult mice in the following tasks: Y-maze (spatial memory); object displacement, substitution and recognition (spatial and recognition memory) with no evident modifications in the open field (locomotor activity) and elevated plus maze (anxiety-related behavior) (Silva et al., 2013).

4. Effects of caffeine on distinct neurotransmitter systems

While neurotransmitters are released from neurons and mediate neuronal communication, neuromodulators can also be released, but they influence the neuronal signaling. Both neurotransmitters and neuromodulators play a key role in the shaping and wiring of the nervous system during critical windows of the development (Herlenius and Lagercrantz, 2004). The correct and organized set of neurotransmitters and neuromodulators is essential to promote the stimuli needed during neural development. This section will discuss some of the most prominent mechanisms often proposed to account for the neurobehavioral effects of caffeine.

4.1. Adenosinergic system

As mentioned before, the behavioral effects of caffeine occur due to its non-selective antagonism of A_1R and $A_{2A}R$ and also the inhibition of phosphodiesterase and Ca^{2+} mobilization (Francis et al., 2011; Fredholm et al., 1999), but other neurotransmitter systems and transduction signaling pathways are also involved (Fisone et al., 2004; Khaliq et al., 2012; Lorist and Tops, 2003; Simola et al., 2008; Swerdlow et al., 1986). Studies on the effects of caffeine on adenosine receptors in adults have been extensively reviewed elsewhere (Chen et al., 2010; Ribeiro and Sebastião, 2010). Thus, the only ones to be covered are studies on caffeine and adenosine during brain development.

Adenosine is one of the signaling molecules that have the potential to influence the mammals during developing and it is predicted that several of the different adenosine receptor subtypes play important and possibly protective roles during ontogeny (Rivkees and Wendler, 2011). However, most studies have assessed the effects of caffeine on the A_1 receptors, the most abundant adenosine receptor in the brain.

Adenosine A_1 and A_{2A} receptors are present at birth in the rat, but the major development in terms of density and coupling to second messenger-forming systems occurs postnatally (Adén et al., 2000; Adén, 2011). In rats, the expression of A_1 receptors is gradual and regionally specific (Gaytan et al., 2006; Guillet and Kellog, 1991a). The density of receptors in the adult is attained about 24 postnatal days in the cerebellum and by 1 month in the cortex (Guillet and Kellog, 1991a). Although mRNA adenosine A_1 receptor can be detected at embryonic day 14 (E14) and receptors at E18, its levels are very low (Adén et al., 2000; Rivkees, 1995; Weaver, 1996). The gene expression of A_{2A} receptor is much more restricted in fetal rats than A_1R, but there are several sites of overlap

(Weaver, 1996). Transcripts of $A_{2A}R$ achieve adult levels by E18, whereas receptor levels are low or undetectable before birth and increase dramatically until PND14 (Adén et al., 2000).

Even though adenosine receptor interaction with caffeine may not result in teratogenicity, caffeine may affect neuronal growth and neuron interconnections as well as other neurotransmitter signaling pathways during gestation and neonatal periods (Brent et al., 2011). Based on this premise, some studies were performed in order to analyze specific adenosine A_1 binding in fetal rat brain after maternal caffeine intake.

One of the first changes in the adenosine receptors by neonatal exposure to caffeine was reported by using radioligand assays. Aiming to mimic human caffeine intake during brain development, these studies were performed in rat pups that received caffeine (20 mg/kg, i.g) at PND 2 and 15 mg/kg at PND 3–6. Membranes from different brain regions were isolated and adenosine A_1 and A_{2A} receptors binding were assessed at different ages. Caffeine increased specific binding of A_1R in cortex, cerebellum, and hippocampus from 90-day-old rats. Besides, saturation analysis in the cortex demonstrated an increase in maximal A_1 receptor density (Guillet and Kellogg 1991a; Marangos et al., 1984). In another study, adenosine A_1 binding was carried out in membranes isolated from cortex, cerebellum and hippocampus from rats at 14-, 18-, 21- and 28-day-old. Cortical membranes from 18-day-old caffeine-treated rats presented a decrease in the specific binding and increased high-affinity sites (Kd). In the cerebellum and hippocampus, neonatal caffeine exposure did not change A_1 receptors (Guillet and Kellogg, 1991b). In a recent study, caffeine treatment caused an up regulation in the adenosine A_1 receptors in the hypothalamus at PND 5 and 8, and in the ponto-medullary region at PND 5. Likewise, caffeine increased mRNA A_{2A} receptors in the hypothalamus ponto-medulla at PND 5 along with moderate up regulation in the hypothalamus at PND 8–11 and ponto-medulla at PND 7–11 (Gaytan and Pasaro, 2012).

Further studies on the effects of early developmental exposure to caffeine on the ontogeny of the A_1R and $A_{2A}R$ were designed with different doses of treatment. Rat pups were exposed to caffeine (0.3 or 0.8 g/L) during the first 7 days after birth and binding and mRNA levels for both receptors were assessed. While the binding for adenosine A_1 receptor increased only in the cortex, adenosine A_{2A} receptors were not altered in striatal membranes as well as there are no significant changes in the mRNA levels for both receptors (Bona et al., 1995). In newborn rats, chronic daily caffeine administration by oral gavage (15 mg/kg) from postnatal day 2 to 6 increases the number of A_1R-immunopositive neurons in the pons and the NTS (Gaytan et al., 2006). Autoradiography measurements of A_1 receptor expression using [3H] N6-cyclohexyladenosine shows that a similar neonatal caffeine treatment induces a long lasting increase in A_1 receptors in the thalamus of young (1 month-old) and adult rats (Guillet and Kellogg, 1991b). Apart from brainstem (Gaytan et al., 2006), thalamus and cerebellum presented up-regulation of adenosine A_1 receptors by the exposure to caffeine in the early neonatal period (Etzel and Guillet, 1994).

In further studies, caffeine differently affected the number and/or expression of adenosine A_1 receptors in neonatal rats after chronic exposure. Changes in the adenosine receptors were examined by using receptor autoradiography and *in situ* hybridization in the cortex, cerebellum, hippocampus and thalamus from rats at E14, E18, E21, 2 h after birth (P 2 h), P 24 h, PND 3, 7, 14, and 21 (Adén et al., 2000). Caffeine (0.3 g/L) consumption during pregnancy and early postnatal life increased adenosine A_1 binding only in cortical membranes from P 24 h and PND 7 and mRNA levels were decreased in the hippocampus from PND 3–21 (Adén et al., 2000). In subsequent studies, León and coworkers analyzed the effect of caffeine treatment (1 g/L in the drinking water) in pregnant rats (last day of gestation) and fetuses on the density, mRNA levels and functionality of adenosine A_1 receptor in the plasma membranes from the whole brain (León et al., 2002, 2005a). Caffeine decreased the number of adenosine A_1 receptor in maternal and fetal brain, which was related to an increase in the mRNA levels and affinity only in the fetal brain. However, the functionality of A_1 receptors was not altered by the treatment in the fetuses, but a decrease in the adenylyl cyclase activity was detected in the maternal brain (León et al., 2005a). No variation on the levels of mRNA encoding A_{2A} receptor was detected in any case (León et al., 2002). These results agree with data reported by Lorenzo et al. (2010) that analyzed the effects of caffeine beyond gestation period, including lactation period in mothers, male and female neonates. Rat dams that received caffeine only during gestation or lactation, or even throughout gestation and lactation presented a decrease of total adenosine A_1 receptor number, and this finding was accompanied by a significant decrease on A_1 receptor transcripts. In addition, male neonates also presented a decrease of A_1 receptors after chronic caffeine exposure during gestation, lactation and gestation plus lactation. In female neonates, there was a trend toward decrease on adenosine A_1 receptor in response to caffeine exposure accompanied by unaltered mRNA coding for adenosine A_1 receptor in neonates in any case. While radioligand binding assays showed no alterations for adenosine A_{2A} receptor in maternal and neonatal brain in response to caffeine exposure, a significant decrease in mRNA level coding A_{2A} receptor was observed in the dams. In summary, chronic caffeine exposure during gestation and lactation promoted a decrease in adenosine A_1 receptors in whole brain from both dams and neonates (Lorenzo et al., 2010). One possible explanation for the differences between the studies could be the higher caffeine concentration used (1 g/L versus 0.3 g/L). Corroborating with this hypothesis, Kaplan et al. (1993) showed a relationship between caffeine dose and modifications in the adenosine A_1 receptor. Mice receiving caffeine (97 mg/kg/day or 194 mg/kg/day) presented a decrease of adenosine A_1 receptors binding (20% and 69%, respectively) in the cortex. León and coworkers (2002) administered the dose of caffeine close to the minimal effective concentration as Kaplan and coworkers, whereas the plasma concentration of caffeine measured by Adén and coworkers was more than three times lower.

Differently from adenosine A_1 receptor, data from changes in the A_{2A} receptor after caffeine treatment during development are limited due to the restricted distribution of this receptor in rat brain. In fact, A_1 receptors are widespread (Ribeiro, 1999; Svenningsson et al., 1999) and A_{2A} are confined primarily to the striatum, nucleus accumbens and olfactory tubercles (Johansson et al., 1997; Rosin et al., 1998). Besides, $A_{2A}R$ present low or even undetectable levels until birth (Adén et al., 2000). Nevertheless, the fewer studies that analyzed the effects of caffeine in maternal and fetal brain during pregnancy failed to show any changes on A_{2A} receptor (Adén et al., 2000; León et al., 2002, 2005a; Lorenzo et al., 2010). Recently, some alterations were found on gene expression of A_{2A} receptor (Gaytan and Pasaro, 2012; Lorenzo et al., 2010; Picard et al., 2008). Neonatal caffeine exposure (PND 2–6) orally administered was associated to increases on A_{2A} mRNA in brainstem and hypothalamus (Gaytan and Pasaro, 2012). The up-regulation of A_{2A} expression was also reported in the medulla of neonates (PND 24 h) after maternal caffeine treatment in the drinking water (0.2 g/L). These results are distinct from those described by Lorenzo and coworkers (2010), who found that caffeine consumption during gestation and lactation evoked a significant decrease on mRNA level encoding A_{2A} receptor in the dams, while no alterations were detected on A_{2A} expression in the whole brain of neonates (PND 15). The apparent regionally specific discrepancies among studies may be also attributed to the administration, caffeine dosage and duration of exposure (Guillet and Kellog, 1991a,b). Gavage introduce the total amount of caffeine all at once, and the divided

or single dose have different effects on fetuses (Nakamoto, 2004), suggesting that if caffeine were administered through diet or drinking water, the data obtained (Jacombs et al., 1999; Wilkinson and Pollard, 1994) would differ from data obtained with gavage (Nakamoto, 2004). In addition, the sensitivity of a specific region of the brain to caffeine may depend both on the concentration of caffeine in serum (or tissue) and the stage of maturation of each brain region at the time of exposure (Guillet and Kellog, 1991a,b).

Apart from adenosine receptors, studies evaluating the effects of caffeine on the pathways of adenosine metabolism during the development of the CNS are more limited. The ectonucleotidases pathway and bidirectional transporters control the extracellular levels of adenosine, which is a product of ATP catabolism (for reviews see Cunha, 2001; Zimmermann, 2001). The ectonucleotidase family seems to be the major way to control nucleotides and nucleosides availability and includes members of the ectonucleoside triphosphate diphosphohydrolases (E-NTPDase), ectonucleoside pyrophosphatase/phosphodiesterase (E-NPP), alkaline phosphatase and ecto-5-nucleotidase. The expression and activity of E-NTPDases and ecto-5′-nucleotidase have been documented in neural tissues during early neural development (Bruno et al., 2002; Langer et al., 2007; Stanojevic et al., 2011). The effects of caffeine (1 g/L in the drinking water) intake during gestation and lactation were investigated in the hippocampal activity and expression of NTPDases and 5′nucleotidase from rat pups at PND 7, 15 and 21 (da Silva et al., 2012). Although caffeine had increased the activity of 5′nucleotidase in rat pups at PND 7, the expression of this enzyme was not altered by treatment. Interestingly, caffeine decreased ATP and ADP hydrolysis in the hippocampus from rat pups at PND 14, but it increased ATP hydrolysis at PND 21. The expression of NTPDase 1 was decreased by caffeine in both postnatal ages and NTPDase 5 at PND 21 (da Silva et al., 2012). Therefore, caffeine during gestational and lactation period causes important changes in the extracellular catabolism of nucleotides, leading to a transient increase of adenosine in the synaptic cleft via 5′-ectonucleotidase. These findings suggest that more detailed studies must be made focusing on the extracellular catabolism of nucleotides in order to elucidate the effects of caffeine during brain development.

4.2. Cholinergic system

As a neuromodulator adenosine controls the release of several neurotransmitters, including acetylcholine, which is one of the important neurotransmitter during brain development (Lassiter et al., 1998). The cholinergic signaling comprises the early set of neurotransmission systems present at brain development (Herlenius and Lagercrantz, 2004). The cholinergic innervations of the cortex occur about E19 in the mouse and the rat, but mature levels are not reached until 8 weeks after birth (Berger-Sweeney and Hohmann, 1997).

Acetylcholine, the oldest identified neurotransmitter, has been involved in processes of arousal and attention and might be involved in the stimulant properties of caffeine (Acquas et al., 2002; Fibiger, 1991; Hohmann, 2003; Rainnie et al., 1994; Sarter and Bruno, 2000), since central cholinergic neurons are sensitive to adenosine modulation, mainly over ascending cholinergic projections to the thalamus and cortex. Previous studies reported that caffeine promotes acetylcholine release in certain brain areas in adult rodents (Acquas et al., 2002; Carter et al., 1995; Shi and Daly, 1999). The release of acetylcholine is tonically inhibited by adenosine in the hippocampus, and caffeine orally administered enhanced this release via adenosine A_1 receptors (Carter et al., 1995). Thus, the arousal effects of caffeine were associated with increased cholinergic activity in the mammalian cerebral cortex including the hippocampus (Carter et al., 1995). Likewise, chronic ingestion of caffeine (1 g/L in the drinking water) by mice for 7 days caused an increase in the densities of muscarinic and nicotinic receptors in cerebral cortex (Shi and Daly, 1999). It is conceivable that both A_1 and A_{2A} receptors in the brainstem of mouse (Coleman et al., 2006), rat (Marks et al., 2003), and cat (Tanase et al., 2003) contribute to the regulation of arousal (Van Dort et al., 2009).

Maternal caffeine intake during gestation and lactation periods (1 g/L in the drinking water) was investigated on acetylcholinesterase (AChE) activity and expression in the hippocampus from 7-, 14- and 21-day-old neonates. Caffeine promoted an increase on AChE activity in the hippocampus of 21-day-old rats, but mRNA levels were unaltered in all ages (da Silva et al., 2008). These results highlight the ability of maternal caffeine intake to interfere on cholinergic neurotransmission during brain development. Regardless the administration form (gavage, intravenous or intraperitoneal) and dosage of caffeine (0.25–30 mg/kg), two studies reported an increase in the extracellular levels of acetylcholine in hippocampus (Carter et al., 1995) and prefrontal cortex (Acquas et al., 2002) by using microdialysis. Taken together, these findings demonstrate that the tonic inhibitory regulation exerted by the endogenous modulator adenosine on acetylcholine release can be counteracted by caffeine administration.

4.3. Serotonin and catecholamines

Serotonin has been reported to affect neuronal proliferation, differentiation, migration, and synaptogenesis (Bonnin and Levitt, 2011). Serotonergic cells in the raphe are among the earliest to be generated in the brain (about E10–E12 in the mouse). After their generation in the raphe, they start to project diffusely into the spinal cord and the cortex. Excess of serotonin prevents the normal development of the somatosensory cortex, which has been demonstrated in monoamine oxidase knockout mice (Cases et al., 1996).

During critical phases of CNS development, exogenous intervention on neurotransmitter synthesis can lead to permanent changes in proliferation, differentiation and growth of the effector cells (Ruediger et al., 2007). The levels of serotonin must be tightly regulated during the critical period of synaptogenesis and formation of brain connections. Miswiring problems due to excess or inadequate activation of specific 5-hydroxytryptamine (5-HT) receptors during development may be involved in the genesis of psychiatric disorders such as anxiety disorders, drug addiction, and autism (Gaspar et al., 2003).

A number of neurochemical investigations have shown that pronounced increases in brain 5-HT metabolism occur in rats following caffeine administration (Abrams et al., 2005; Haleem et al., 1995; Khaliq et al., 2012; Li et al., 2012; Okada et al., 1999; Shi and Daly, 1999). During critical time of the neurodevelopment, monoamines play an important role in the architecture of the CNS (Herlenius and Lagercrantz, 2004). It was found that the monoamine neurotransmitters levels (5-HT and dopamine) in the brain elevated gradually with the embryo maturity during the development of chicken embryo, especially on the 17th day (Li et al., 2012). Based on these findings, Li and coworkers (2012) evaluated the influence of caffeine on monoamine neurotransmitters development using developmental chicken embryos. Different dosages of caffeine (1.25, 2.5, 5.0, 10.0, 20.0, 40.0 and 80.0 μmol/egg) were injected into the air sac of the incubated 8-day embryos. The embryos were further incubated for 9 days following caffeine treatment, and then the mortality and abnormality rates were detected. Caffeine resulted in defect of neural tube closure and induced disorder of serotonergic system development, increasing the contents of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) under dosage of 10.0 μmol/egg. Moreover, caffeine was not completely metabolized and the magnification may occur by

accumulation in the embryonic brain. This study may provide valuable data for further investigations on toxicology of caffeine during different stages of pregnancy (Li et al., 2012).

The serotonergic system play important roles in modulating behavioral arousal, including behavioral arousal and vigilance associated with anxiety states (Abrams et al., 2005; Nehlig et al., 1992). The behavioral effects of caffeine observed *in vivo*, particularly increased arousal and vigilance, are well known and likely contribute to the widespread use of this substance. In this regard, previous studies have demonstrated that caffeine administration could affect serotonergic system in adult rodents (Abrams et al., 2005; Haleem et al., 1995; Khaliq et al., 2012; Okada et al., 1999; Shi and Daly, 1999). Chronic ingestion of caffeine by mice (1 g/L in drinking water) for 7 days resulted in a significant increase in density of both 5-HT1- and 5-HT2-serotonergic receptors in cerebral cortex (Shi and Daly, 1999). Moreover, Khaliq and coworkers (2012) reported that repeated caffeine administration for 6 days at 30 mg/kg dose significantly increases brain 5-HT and 5-HIAA levels and its withdrawal significantly decreased brain 5-HT levels. Increased levels of 5-HT and its metabolites were also described in a single administration of caffeine (Haleem et al., 1995). Caffeine injected at doses of 20, 40 and 80 mg/kg increased brain levels of 5-HT and 5-HIAA in rat brain (Haleem et al., 1995). A study performed by Reith et al. (1987) demonstrated that caffeine inhibits the carriers involved in the neuronal uptake of 5-HT with an IC_{50} of caffeine of approximately 2×10^{-3} M. Moreover, the administration of caffeine (73, 123, and 162 mg/kg/day) in mice for 3 weeks did not change parameters of monoamine systems, including the 5-HT2-serotonergic receptor in striatum and cortex (Reith et al., 1987). Mice that received acute administration of caffeine (100 and 200 mg/kg caffeine, 30 min, i.p.) presented alterations in the brain regional utilization of monoamines. However, these alterations were not uniform and depended upon the specific neurotransmitter and metabolite studied as well as the brain region. In the olfactory bulb caffeine increased serotonin utilization, whereas in the hypothalamus a decrease was observed (Hadfield and Milio, 1989). The authors suggested that caffeine might also serve as an interesting tool in the bulbectomy model due to the changes on serotonergic parameters detected in olfactory bulb (Hadfield, 1997). A widespread increase of norepinephrine utilizations by caffeine was observed in the olfactory bulb, olfactory tubercles, prefrontal cortex, amygdala, hypothalamus and hippocampus. Likewise, caffeine increased dopamine utilization in the olfactory bulb, olfactory tubercles, prefrontal cortex, septum, hypothalamus and thalamus (Hadfield and Milio, 1989).

Considering that caffeine may facilitate lipolysis and that brain neurotransmitters play pivotal roles in the body weight homeostasis, the use of genetically obese animals is relevant to better understand its role on CNS parameters of these models. Chen et al. (1994) treated obese mice with 12-week of age with caffeine (4 mg/day) in water for 4 weeks. Caffeine decreased the body fat content significantly in obese mice and increased the levels of norepinephrine and epinephrine in brain, even though no significant alterations were detected between obese and lean mice in brain levels of 5-HT, tryptophan, and 5-hydroxyindoleacetic acid (5-HIAA). The effect of caffeine on the expression of tryptophan hydroxylase (TPH), the rate limiting enzyme of serotonin synthesis, was investigated in dorsal and median raphe from rats trained for 6 consecutive days in a treadmill. Subcutaneous injection of 4 mg/kg caffeine inhibited the exercise-induced elevation in TPH expression, which could be a putative ergogenic mechanism of caffeine (Lim et al., 2001).

McNamara and colleagues (2006) observed that the association of 10 mg/kg caffeine with MDMA and MDA (“Love”) markedly reduced 5-HT and 5-HIAA concentrations in different brain structures of rats, as well as increased both acute and long-term toxicity of these amphetamines in terms of hyperthermic response and lethality (McNamara et al., 2006). These data suggest that the interaction of caffeine with other psychostimulants could potentiate toxicity and that caffeine may modulate serotonergic signaling and other neurotransmitter systems concomitantly. Furthermore, these findings provide evidence that caffeine administration could lead developmental and adaptive changes in the serotonergic system.

4.4. Glutamatergic and GABAergic systems

The balance between the levels of excitatory and inhibitory neurotransmitter systems is extremely important in order to ensure the physiological tonus of CNS. In this context, glutamate and γ-aminobutiric acid (GABA) are two amino acids that play a key role in regulating the neuronal signaling. Extracelullar glutamate mediates its effects via ionotropic (AMPA, NMDA, and kainate) and metabotropic (mGluRs) receptors. The actions triggered by glutamate are associated to the increase of Ca^{2+} in the intracellular milieu, which is responsible for mediating several Ca^{2+}-dependent transduction signaling pathways (Ashpole et al., 2012; Sourial-Bassillious et al., 2009; Zonouzi et al., 2011). However, if glutamate concentrations abruptly increase at synaptic cleft, the overstimulation of its receptors may promote excitotoxicity, leading to neuronal damage. Thus, impairment on glutamatergic neurotransmission is involved in acute and chronic neurodegenerative diseases (Danysz, 2001; Loopuijt and Schmidt, 1998; Mattson, 2008; Riederer and Hoyer, 2006). Since adenosine is an endogenous neuromodulator, there is a growing therapeutic interest in the chronic administration of caffeine at moderate doses as an attractive strategy to prevent excitotoxicity mainly due to the blockade of facilitatory A_{2A} receptors in several experimental models (see studies from Dall'Igna et al., 2003; Nobre et al., 2010; Schwarzschild et al., 2003). However, studies aiming to evaluate direct actions promoted by caffeine administration on glutamatergic signaling are still lacking.

Quiroz and colleagues (2006) showed that A_{2A} receptors strongly modulate the efficacy of glutamatergic synapses on striatal enkephalinergic neurons. The authors reported that caffeine (10 mg/kg, i.p.) and MSX-3 (a selective A_{2A} receptor antagonist) administrated 10 min before cortical stimulation, counteracts the cell signaling effects of striatal activation. Considering that the effects of phosphorylation of ERK1/2 and GluR1 induced by stimulation of corticostriatal afferents was dependent of A_{2A} receptor function, caffeine could be deleterious for the normal development of striatal function due to its action as a modulator of synaptic plasticity (Quiroz et al., 2006).

The effects of chronic caffeine intake during gestation on glutamatergic parameters have been also described. Pregnant rats treated with 1 g/L caffeine or theophylline in the drinking water from the gestational day 2 onwards throughout the gestational period presented a down-regulation of $mGluR_{1a}$ and phospholipase C β_1 ($PLC\beta_1$), evidencing a desensitization of mGluR/PLC signaling in the maternal brain (León et al., 2005b). Contrastingly, this same report did not detect significant changes in mGluR/PLC responsiveness, which could be attributed to the immaturity of mGluR/PLC signaling at birth. Interestingly, caffeine also is able to modulate peripheral tissues, such as heart in fetuses since it significantly decrease mGluRs levels and phospholipase C activity in heart (Iglesias et al., 2006).

Studies investigating electrophysiological properties of synaptic transmission have demonstrated that caffeine has been found to induce long-term potentiation (LTP), a physiological phenomenon associated to synaptic plasticity that is thought to underlie learning and memory processes (Lu et al., 1999; Martín and Buño, 2003). Additionally, caffeine may also improve cognitive function

in several models of learning and memory, which is correlated to the modulatory effects of P1 receptors blockade on glutamatergic signaling parameters. Concerning the presynaptic actions of caffeine on A_1 receptors, Wang (2007) showed a facilitation of 4AP-evoked glutamate release possibly through the activation of PKC pathway in rat cerebrocortical synaptosomes. This evidence strongly points for a crosstalk between transduction pathways mediated by adenosine and glutamate, suggesting that different caffeine administrations may exert a fine-tuning regulation of glutamate-mediated neurotransmission, which could, at least in part, be one of the putative mechanisms for mediating its beneficial cognitive processes.

Contrastingly to the actions triggered by glutamate, the GABA-mediated signaling via $GABA_A$ receptor induces the major inhibitory postsynaptic current (IPSC) in vertebrate CNS (Belelli et al., 2009; Bosman et al., 2005). It has been postulated that the GABAergic system plays a role in the regulation of locomotor activity, and that the administration of caffeine (10–40 mg/kg, p.o.) potentiates locomotion due to its antagonism of adenosine receptor and activation of the dopaminergic system, which consecutively, reduces GABAergic activity through the reduction of cholinergic system (Mukhopadhyay and Poddar, 1995). Furthermore, chronic ingestion of caffeine in a dose equivalent to about 100 mg/kg/day in mice promoted several biochemical alterations in the CNS, including an increase of the density of cortical benzodiazepine-binding sites associated with $GABA_A$ receptors (Shi et al., 1993).

Modulatory approaches on GABAergic neurotransmission have been described in different brain regions, and the raise on intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) has been used to mimic these regulations. Caffeine is generally used as a molecule that increases the cellular content of Ca^{2+} by its release from ryanodine-sensitive intracellular sites (Hernández-Cruz et al., 1995). Using preparations from hippocampus of Wistar rats aged 4–12 days, Taketo and colleagues performed whole cell patch clamp recordings to investigate the effects of caffeine and several other $[Ca^{2+}]i$-mobilizing drugs on the IPSCs in acute slices (Taketo et al., 2004). In the respective study, the perfusion with both 1 and 10 mM caffeine inhibited the $GABA_A$ergic IPSCs recorded from CA3 neurons transiently. Interestingly, this inhibitory effect was not observed after treatment with bradykinin, ATP, and acetylcholine, which are molecules able to increase $[Ca^{2+}]i$ by releasing Ca^{2+} from IP3-sensitive store and/or induce Ca^{2+} influx. The inhibition on $GABA_A$ IPSC promoted by caffeine was also observed after Ca^{2+} chelation and concomitant fluorescent analyses suggested that the inhibitory effect occurs independently of intracellular Ca^{2+} mobilization. Although the precise mechanisms of caffeine actions on $GABA_A$ are not fully understood, it has been postulated that cAMP levels and adenosine receptors do not play a crucial role in this phenomenon (Taketo et al., 2004). Although these results are in contrast with previous studies that described the importance of Ca^{2+} on the modulation of $GABA_A$ receptor-channels (De Koninck and Mody, 1996; Vigh and Lasater, 2003), a plausible explanation for these discrepancies could be attributed to the differences in the experimental conditions and also to the analysis performed on distinct brain regions.

Recently, Silva and colleagues showed that caffeine (0.3 g/L) and KW 6002 (istradefylline, 2 mg/kg per day, selective A2AR antagonist) administered during gestation and lactation, delayed migration and insertion of GABA neurons into the hippocampal circuitry in the offsprings (PND 6) (Silva et al., 2013). The authors observed that the number and distribution pattern of GABA neurons (somatostain positive) were similar in the hippocampus of adult control and caffeine-treated mice. The abnormal GABA migration leads to an increase in the frequency of giant depolarizing potential and spontaneous inhibitory and excitatory postsynaptic currents in the hippocampus of caffeine-treated offspring, suggesting evident signs of hyperexcitability. As a consequence, caffeine-treated offsprings were more susceptible to seizures triggered by a convulsant agent (Silva et al., 2013).

Therefore, protocols that aim to evaluate the effects of caffeine on glutamatergic and GABAergic neurotransmitter systems might be relevant to clarify the central actions of this molecule on CNS as well as its potential role in several neurodegenerative diseases models.

4.5. Dopaminergic system

There are evidences showing that several effects mediated by caffeine, such as motor control, level of arousal and vigilance may be attributed to its influence on dopaminegic signaling (Brunyé et al., 2010; Collins et al., 2010; Ferré, 2010; Hsu et al., 2010). Adenosine receptors interact with dopamine D_2 receptors, resulting in multimolecular aggregates, known as receptor heteromers (Fuxe et al., 2012). The A_{2A}-D_2 heteromers are located particularly in the dendritic spines of the striatopallidal GABAergic neurons, exerting a fine-tuning regulation of glutamatergic neurotransmission (Ciruela et al., 2006; Ferré et al., 2009; Fuxe et al., 2007). The molecular integration of both receptors leads to complexes physiological responses, in which the function will be the combination of different chemical–physical signals from distinct cellular microenvironments (Ferré et al., 2004). It is known that the binding of adenosine to A_{2A} receptors alters the phosphorylation state of the dopamine- and cAMP-regulated protein DARPP-32 and upregulates the expression of immediate-early genes (e.g. *c-fos*) in striatal neurons (Lindskog et al., 2002). Activation of either dopamine D_1 or adenosine A_{2A} receptor leads to a protein kinase A (PKA)-dependent phosphorylation of DARPP-32 in separate neurons (Svenningsson et al., 1998).

Considering that A_{2A}R play facilitatory role on CNS (Costenla et al., 2010; Pinto-Duarte et al., 2005) and that caffeine may increase the levels of D2R transcripts (Stonehouse et al., 2003), the use of selective A_{2A} receptors antagonists have emerged as a promising strategy for Parkinson's disease (Schwarzchild et al., 2003).

The pharmacological involvement of the tail of the ventral tegmental area (tVTA) in the caffeine-mediated responses has already been studied (Kaufling et al., 2010). A robust effect on the expression of the transcription factor FosB and its stable truncated splice variant ΔFosB has been acutely detected in this structure after systemic drug administration, such as cocaine (Sato et al., 2011; Sun et al., 2008). Since tVTA output is mainly GABAergic, it may act as a potential control site for dopaminergic activity (Bourdy and Barrot, 2012). As a consequence, tVTA plays a key role for adaptive and goal-directed behaviors, motivation, reward and mood (Grace et al., 2007; Le Moal and Simon, 1991; Nestler and Carlezon, 2006; Schultz, 2007).

Adult male rats treated with caffeine in a range of 2.5–100 mg/kg (i.p.) during 3 h showed a significant increase on FosB/DeltaFosB expression up to the highest dose tested in GABAergic cells (Kaufling et al., 2010). Importantly, this effect was mild when compared to that observed after administration of psychostimulant drugs that directly target amine uptake sites (Kaufling et al., 2010; Sun et al., 2008). The treatment with dopamine transporter inhibitor GBR12909 promoted a strong enhancement on FosB/DeltaFosB expression in the tVTA, showing that this induction is mediated by dopamine (Kaufling et al., 2010). Since caffeine does not promote the inhibition of the monoamine transporter (Nehlig et al., 1992), the neurochemical finding along with behavioral analysis suggest that caffeine may be considered only mildly stimulatory drug and it could not be grouped with other psychostimulant drugs such as amphetamine and cocaine.

Studies involving the combination of caffeine with amphetamines showed that it potentiates the methamphetamine-induced toxicity possibly by increasing oxidative stress and dopamine

release in striatum of rats (Sinchai et al., 2011). Likewise, a synergistic effect of caffeine with 3,4-methylenedioxymethamphetamine (MDMA, "Ecstasy") was reported (Ikeda et al., 2011), which promoted a significant increase of extracellular dopamine and 5-HT, in a way attributed to A_1 receptor blockade (Vanattou-Saïfoudine et al., 2011). Since DA-mediated signaling in the forebrain is a critical component of the brain circuitry regulating behavioral activation, the administration of caffeine reverted the locomotor suppression induced by the D_1 antagonist SCH 39166 (ecopipam) (Collins et al., 2010). As a consequence, the crosstalk between adenosinergic and dopaminergic system in distinct brain structures is considered to be an important factor that elicits the effects mediated by caffeine on several parameters regulated by dopamine signaling, such as locomotion, learning, attention, and arousal (Brunyé et al., 2010; Fisone et al., 2004; Nehlig et al., 1992; Powell et al., 2001; Stonehouse et al., 2003).

More recently, increasing evidence suggests that caffeine might represent important therapeutic tools for the treatment of the Attention deficit and hyperactivity disorder (ADHD) (Lara, 2010; Takahashi et al., 2008), which is the most prevalent psychiatric disorder in children (Polanczyk et al., 2007). ADHD is characterized primarily by a triad of symptoms constituted of hyperactivity, inattention and impulsivity (Biederman and Faraone, 2005). By using spontaneously hypertensive rats (SHR), the most frequently experimental model for ADHD studies, it was observed a reversion of discriminative learning impairment by SHR rats treated chronically with caffeine (Pires et al., 2010). In further study, the ability of caffeine to abrogate behavior deficits in the attention set-shifting task was related to a normalization of density and activity of dopamine transporters (DAT) in the frontal cortex, the brain region more closely associated with sustained attention (Pandolfo et al., 2013).

4.6. Caffeine and signaling pathways

Methylxanthines are molecules that induce pleiotropic effects on CNS. As a consequence, emergent perspectives in clarifying the mechanisms of action triggered by caffeine during the ontogeny exist. The improvement of bioinformatics tools associated with the advance of *omics* and pharmacological strategies allow researchers to design alternative protocols in order to evaluate distinct roles of caffeine on gene and protein expression profiles, as well as on several second messenger systems in different models.

Microarray data from honeybee brain revealed that the biological processes primarily modulated by caffeine treatment for 5 min, 1, 4, and 24 h, include synaptic transmission, Ca^{2+} mobilization, cytoskeletal alterations, and protein/energy metabolism (Kucharski and Maleszka, 2005). Interestingly, although the dose administered of approximately 200 ng/mg body mass is not comparable to the quantities of human consumption, these effects were similar to those previously detected for caffeine-inducible genes in vertebrate models (Lorist and Tops, 2003; Stonehouse et al., 2003). Considering that the current honeybee genome does not present annotated ESTs of adenosine receptors, it is not possible to conclude whether these receptors are affected by caffeine treatment. Moreover, these data lead us to hypothesize that the actions triggered by caffeine in CNS may not be simply explained by its non-selective antagonism of adenosine receptors, suggesting the existence of a pleiotropic effect on other neurotransmitter signaling pathways.

Using neuronal cultures from hippocampus of 3-week-old rodents, Korkotian and Segal (1999) demonstrated that caffeine pulses stimulate a transient rise of $[Ca^{2+}]i$ in dendrites and spines in a 5–10 mM range. Furthermore, this study also verified a significant increase in the size of dendritic spines and the formation of new ones in a mechanism dependent of ryanodine-sensitive Ca^{2+} stores. This mobilization of $[Ca^{2+}]i$ exerted by caffeine can be attributed to the blockade of A_1 receptors and it is known to facilitate somatodendritic dopamine release in the substantia nigra pars compacta (Patel et al., 2009; Vanattou-Saïfoudine, 2011).

It has been reported that caffeine may play its roles via activation of several second messenger systems (Alzoubi et al., 2013; Connolly and Kingsbury, 2010). Studies from our laboratory have focused the attention on the effects promoted by caffeine in memory tasks by modulating neurotrophins. When adult CF1 mice were treated during 4 consecutive days with caffeine (10 mg/kg, i.p., equivalent dose corresponding to 2–3 cups of coffee), the object recognition memory was improved, which was correlated with increased levels of the brain-derived neurotrophic factor (BDNF) and tyrosine kinase receptor (TrkB) in the hippocampus (Costa et al., 2008b). Importantly, these effects occur independently of changes on phospho-CREB (Ca^{2+}/cAMP response element binding protein) immunocontent. The preventive effects of caffeine against memory impairment by aging were related to modifications in BDNF and related proteins in the hippocampus. In both studies, aged- and middle-aged animals presented increase in the BDNF immunocontent and caffeine (1 g/L, drinking water) counteracted at adult animals level (Costa et al., 2008a; Sallaberry et al., 2013). The effect of chronic caffeine treatment (0.3 g/L on drinking water during 4 weeks) on long-term memory deficit associated with 24 h sleep deprivation was reported by Alhaider and colleagues. This study demonstrated that caffeine was able to prevent the impairment of long-term memory as measured by performance in the radial arm water maze task and normalized L-LTP in CA1 region of sleep-deprived anesthetized rats. Importantly, the authors verified that caffeine prevented the effect of sleep-deprivation on the stimulated levels of P-CREB and BDNF (Alhaider et al., 2011).

Pregnant Swiss rats treated with caffeine (25 or 50 mg/kg, i.p.) from 8 to 10 gestational day (GD) presented accelerated neuroepithelium invagination into telencephalic vesicles, which was related to increased expression of protein kinase A (PKA) (Sahir et al., 2000, 2001). However, the peritoneal route of caffeine administration makes it difficult to compare this result with other experimental studies applying the oral route.

Using zebrafish as animal model, Capiotti et al. (2011) showed the expression of adenosine receptors and its indirect targets dopamine and cAMP-regulated phosphoprotein (DARPP-32) and BDNF. The treatment with 100 μM caffeine at 1 hpf did not alter larvae morphology, but significantly increased DARPP-32 and BDNF transcripts at different phases of development. These studies suggest that the modulatory roles of caffeine on neurotrophins are complex and that the different results obtained could be attributed to differences of the experimental protocol (time, method of administration, or dose of caffeine employed) and also to a possible distinct action during ontogenetic development.

CREB is known to mediate the transcription of genes essential for the development and physiology of the CNS, such as BDNF. A study performed by Connolly and Kingsbury (2010) demonstrated that caffeine exhibits a biphasic dose–response curve of CREB activity, in which the maximal stimulation occurred at 10 mM in a mechanisms dependent of Ca^{2+} released from ryanodine sensitive internal stores. Furthermore, quantitative RT-PCR assays revealed that caffeine treatment increase mRNA levels of BDNF in primary cultures of developing mouse cortical neurons (Connolly and Kingsbury, 2010). Based on these findings, we suggest that protocols aiming to investigate the effects of caffeine via Ca^{2+}-dependent proteins can be tempting strategies to elucidate its actions on CNS. Moreover, the identification of new caffeine-sensitive genes, as well as its regulatory networks, might be helpful for discovering putative critical targets for both acute and chronic treatments.

5. Caffeine consumption during pregnancy: epidemiological studies

Over the past decade, many studies were designed to investigate the safety of caffeine consumption during pregnancy and most of them revealed increased risk for miscarriage and spontaneous abortion. In addition, many cases of low body weight at birth were associated to caffeine intake during pregnancy. Since caffeine is found in some daily beverages, a great number of epidemiological studies have been performed to evaluate the reproductive and developmental risks of caffeine intake during pregnancy. Besides controlling confounding factors such as smoking and alcohol intake, according to Brent et al. (2011), it is important to consider the "pregnancy signal". This consists on symptoms (nausea and vomiting) that may occur in the beginning of pregnancy due to high levels of chorionic gonadotropin (HCG) and these symptoms may account to the avoidance of beverages containing caffeine. Here, some recent epidemiological studies will be outlined considering caffeine intake during pregnancy and possible associations with spontaneous abortion (miscarriage or pregnancy loss), congenital malformations, and fetal growth restriction.

5.1. Spontaneous abortion

Giannelli et al. (2003) examined the effect of caffeine and nausea on the risk of miscarriage in a case-control study. In this report, the consumption of cups of coffee, tea and cola drinks were considered caffeine intake and converted into mg of caffeine per day. Daily consumption of more than 300 mg of caffeine increased risk of spontaneous abortion (SA). On the other hand, women who reported mild to moderate nausea had a twofold reduced in risk of miscarriage. The limitation of this study was the gestational age in the interview: cases were interviewed three weeks after spontaneous abortion, mostly in the first trimester period; control participants were interviewed after 13 weeks of pregnancy, corresponding to the second trimester.

In another case-control study of women who had two or more SA, mean caffeine consumption $\geqslant$300 mg/day presented a 2.7-fold increase in odds of repeated miscarriage in nonsmokers, but not in smokers. After adjustment of confounding factors, the significance no longer exists, demonstrating the importance of controlling potential confounding factors (George et al., 2006).

In a population-based case-control study on risk factors for first trimester miscarriage, pregnant women who consumed more than 300 mg of caffeine per day had increased odds ratio (OR) for a miscarriage. The pregnancy signal was considered and after adjusting OR for nausea, the association no longer exists, demonstrating that caffeine exposure was not associated with increased risk of SA (Maconochie et al., 2007). Similar results were found in a cohort study of 1063 women shortly after confirmation of pregnancy (approximately 10 weeks), where risk of SA increased in the exposure group of >200 mg of caffeine per day, but after adjusting for pregnancy signal no statistical difference (Weng et al., 2008). A possible explanation for this result is that when experiencing nausea, especially in the first 12 weeks of pregnancy, women did not tend to drink coffee, the main source of caffeine. In another study, an association between caffeine consumption and the risk of miscarriage was investigated in 2407 women. Caffeine intake was also determined in early gestation. The results were stratified by timing of the pregnancy loss to determine the probability of having a miscarriage at a specific week of pregnancy (Savitz et al., 2008). Three time points of exposure to caffeine were considered: (1) prior to pregnancy, (2) 4 weeks after last menstrual period and (3) at the time of the interview. Changes in caffeine consumption since pregnancy were also determined. The median coffee intake of the entire study population was 350 mg/day prior to pregnancy and 200 mg/day at the time of the interview. Importantly, coffee consumption was similar in women reporting nausea and vomiting in early pregnancy compared to those without nausea. Among all women, caffeine consumption at any of the time points was unrelated to the risk of miscarriage (OR = 0.7 and 1.3). The most elevated odds were for total caffeine intake above the median for the population. The authors did not find strong associations between coffee or caffeine consumption prior to or early in pregnancy and the risk of miscarriage (Savitz et al., 2008).

5.2. Congenital malformations

The National Birth Defect Prevention Study (NBDPS) is an ongoing multisite population-based case-control study that began in 1997 in 10 USA states. Cases were infants with one or more of over 30 different categories of major structural defects, and control infants were liveborn infants without birth defects randomly selected in the same time period and geographic areas as the cases. For caffeine intake information, mothers were asked about their usual intake of coffee, tea, soda, and chocolate during the year before they became pregnant. Caffeine exposure was estimated at 100 mg for a cup of coffee, 37 mg for a cup of tea, 10 mg per ounce of chocolate, and according to the manufacturer or published caffeine contents for soda and other soft drinks by brand and variety. No association was found between maternal caffeine consumption and orofacial clefts (Collier et al., 2009); and various types of cardiac malformations (Browne et al., 2007). In addition, there was no statistical increased risk of bilateral renal agenesis and renal hypoplasia with caffeine exposure (Slickers et al., 2008). Using data from NBDPS, Miller et al. (2009) studied caffeine exposure according to changes in caffeine intake during pregnancy and anorectal atresia. The exposed group (caffeine intake before pregnancy) who consumed the same amount or more caffeine in pregnancy had an association with isolated anorectal atresia. However, this association is due to non-adjusted OR for smoking.

Assessing maternal caffeine intake before pregnancy and the risk of neural tube defects, modest associations with spina bifida were observed for any consumption of caffeine (>10 mg/day; OR = 1.4). When stratified by smoking, alcohol and maternal age, the associations between spina bifida and any caffeine intake were only observed among women without high risk characteristics (i.e., among non-smokers, non-alcohol users and younger aged women). They did not find though dose–response with increased caffeine intake (Schmidt et al., 2009). One recent case-control study on maternal periconceptional factor and risk of spina-bifida showed an association between consumption of more than 3 cups of coffee per day and increased risk for spina-bifida. Nonetheless, they did not specify the amount of caffeine in mg/day, and there was no control for acid folic supplementation (de Marco et al., 2011).

5.3. Fetal growth restriction

The CARE Study Group (2008) was a prospective observational study to examine the association of maternal caffeine intake with fetal growth restriction. The participants were 2635 low risk pregnant women recruited between 8 and 12 weeks of pregnancy. Assessments of caffeine and smoking and tobacco exposure were by self-reporting and by measuring caffeine and cotinine in the saliva. Caffeine intake was collected using the caffeine assessment tool (CAT), previously reported (Boylan et al., 2008). Briefly, it measures caffeine intake from all possible sources of caffeine (coffee, tea, hot chocolate, cola and energy drinks) in a Food Frequency Questionnaire style, considering specific brand, preparation and portion sizes. Three CATs were administered – the first at recruitment (8–12 weeks); the second and third by the participants, that

covered the period 13–28 weeks, and 28 weeks to the end of pregnancy. This time points correspond to first, second and third trimester of pregnancy, respectively. Participants also reported whether they experienced nausea and vomiting during each trimester. This study demonstrated that maternal caffeine intake is associated with an increased risk of fetal growth restriction, after adjustment for smoking and alcohol intake. The risk is increased in pregnant women consuming more than 200 mg/day of caffeine, throughout pregnancy.

Boylan et al. (2012) used the valuable data collected by the CARE study to explore the relationships between nausea and vomiting in pregnancy and caffeine intake. Women who reported nausea and vomiting in the first trimester had lower caffeine intakes compared to those who did not. However, adjusted odds ratio for fetal growth restriction did not show differences in fetal growth restriction and nausea and vomiting.

Bakker et al. (2010) examined the associations of maternal caffeine intake with fetal growth characteristics measured in each trimester of pregnancy and the risks of adverse birth outcomes. This study was embedded in the Generation R Study, a population-based prospective cohort study from the Netherlands with pregnant women enrolled since early pregnancy. Self-reported caffeine intake questionnaires were obtained in early pregnancy (gestational age 18.0 weeks), midpregnancy (gestational age 18.0–24.9 weeks), and late pregnancy (gestational age > 25.0 weeks), which were considered the first-, second-, and third-trimester measurements, respectively. The mothers who reported any coffee or tea consumption were asked to categorize their average number of cups of coffee or tea per day, and what type of coffee or tea they consumed (caffeinated or decaffeinated). In their analyses, they considered 1 cup of coffee containing 90 mg of caffeine, according to their standard values for caffeine contents in beverages. Findings from this large population-based prospective cohort study suggest that caffeine intake of more than 6 units/day during pregnancy is associated with impaired fetal length growth and an increased risk of low birth weight. However, no associations between caffeine intake and the risk of preterm birth were found (Bakker et al., 2010), which is in accordance with another prospective study evaluating self-reporting caffeine intake and urinary caffeine during early and late pregnancy (Bracken et al., 2003).

In a cohort study of Intrauterine Growth Retardation (IUGR), cord blood sample to evaluate caffeine metabolites. No association between IUGR and caffeine intake during the first and the seventh month of pregnancy (Grosso et al., 2001). To determine whether the third-trimester maternal serum concentration of paraxanthine is associated with delivery of a small-for-gestational age infant (SGG, birth weight less than the 10th percentile for gestational age, gender, and ethnicity) and whether this association differs by smoking, the authors studied 2,515 women who participated in the Collaborative Perinatal Project from 1959 to 1966. The women provided a third-trimester serum sample and had been controls for a nested case–control study of spontaneous abortion. The mean serum paraxanthine concentration was greater in women who gave birth to small-for gestational age infants (754 ng/mL) than to appropriately grown infants (653 ng/mL, $p = 0.02$). Nevertheless, the linear trend for increasing serum paraxanthine concentration to be associated with increasing risk of small-for-gestational age birth was confined to women who also smoked ($p = 0.03$), but no caffeine intake information was collected. The authors concluded that maternal third trimester serum paraxanthine concentration, which reflects caffeine consumption, was associated with a higher risk of reduced fetal growth, particularly among women who smoked (Klebanoff et al., 2002).

In a case-control study of association of caffeine intake before pregnancy and during each trimester, no associations between SGA and intake of three or more cups of coffee per day during pregnancy or >4 cups of coffee per day before becoming pregnant were found (Parazzini et al., 2005).

Although most human exposures were measured in cups of coffee per day, it is difficult to define a cup (1 cup in Brazil = 8 fluid ounces); coffee makers measure in 5-oz serving cups. Defining caffeine intake in cups of coffee is not suitable, since coffee is made differently among cultures and countries. It is more suitable presenting the results as mg of caffeine per day. In this regard, each cup of coffee can be converted to mg of caffeine according to the portion size and the preparation of the users from a specific geographic area.

It is also difficult to circumvent all confounding factors in an epidemiological study. Despite some studies are successful in controlling one factor, there are reports that consider other confounding factors. Hence, it is clear that these studies did not reach a unanimous response about what the amount of caffeine can be taken safely during pregnancy.

6. Concluding remarks

In this review, the studies aforementioned clearly pointed for a dualistic action of caffeine. Here, the studies about the benefits of caffeine against age-related cognitive decline and its cognitive enhancer properties were briefly mentioned. The reason is the relatively agreement between animals and human studies pointing to the safety of caffeine consumption during adulthood. In this context, one face of Janus is better established. The other face of Janus comprises the effects of caffeine exposure during the early period of brain development. Basically, the neurochemical and behavioral findings on the effects of pre and postnatal caffeine exposure depended on many variables such as: time of exposure, schedule of administration, dosage and age of the animals. Furthermore, the immaturity of the enzyme machinery leads to accumulation of caffeine in the brain, contributing for the neurochemical and behavioral alterations. While behavioral effects observed in neonatal rats exposed to caffeine were related to ontogenetic modifications in the adenosine A_1 receptors, it remains unclear the participation of adenosine A_{2A} receptors.

Considering that adenosine acts as an inhibitory or facilitatory endogenous neuromodulator, caffeine influences distinct biochemical pathways and, therefore, modulates neurotransmitter signaling, neurotrophins, cell transduction machinery, and gene

Fig. 2. The Janus face of caffeine. Janus is considered the god of the beginnings and transitions. He is usually portrayed as having two faces: one looking forward and one looking backward. The benefits of caffeine consumption in adulthood are relatively well known (the face looking back). However, the safety dose to be consumed during pregnancy and the effects of caffeine consumption by children remains under debate. Thus, the other face is still looking for future more conclusive studies.

expression profile. Recent epidemiological studies corroborates with the idea that the consumption of caffeine during pregnancy deserved more detailed studies, even with the analysis of novel confounding variables. Thus, the other face of Janus is still looking forward advances on the knowledge of the impact of caffeine consumption during early period of brain development (Fig. 2). It is not a simplistic question of whether caffeine is or not safe. But it is important to realize where animal studies are inherently limited and to bridge the gaps between epidemiological and animals investigations. In this regard, the development of experimental protocols aiming to investigate a detailed network of the multiple effects triggered by caffeine during brain development is necessary to complement the rodent existent approach in future translational researches.

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CAPÍTULO II

Sex differences in the effects of pre- and postnatal caffeine on behavior and synaptic proteins in pubescent rats

Artigo em preparação para ser submetido

Sex differences in the effects of pre- and postnatal caffeine on behavior and synaptic proteins in pubescent rats.

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Abstract

Few studies have addressed the effects of caffeine in a sex dependent manner during puberty and/or adolescence. Considering that caffeine intake has grown in this population, we investigated the behavioral and synaptic proteins changes in pubescent male and female rats after maternal consumption of caffeine. Adult female Wistar rats started to receive caffeine in drinking water (0.1 and 0.3 g/L; low and moderate dose, respectively) during the active cycle in weekdays, two weeks before mating. The treatment lasted up to weaning (21 days) and offspring continued receiving caffeine until the onset of puberty (30-34 days old)in drinking water. Behavioral analysis and synaptic proteins levels (proBDNF, BDNF, GFAP and SNAP-25) were immunodetected in the hippocampus and cerebral cortex. In both sexes caffeine caused hyperlocomotion in the open field. Anxiety-related behavior was attenuated by caffeine (0.3 g/L) only in females. While moderate caffeine worsened recognition memory in females, an improvement for long-term memory in both doses was observed in male rats. In parallel with memory improvement in males, caffeine increased the density of pro- and BDNF in the hippocampus and cortex. Females presented increased proBDNF in both brain regions, and caffeine did not change its levels. While GFAP was not altered either by sex differences or caffeine treatment, moderate caffeine increased SNAP-25 in the cortex of female rats. Our findings revealed that caffeine differently affects recognition memory and emotionality in pubescent male and female rats. In addition, BDNF and related proteins have also changed in a sex dependent manner, suggesting an association with behavioral outcomes.

1. Introduction

Caffeine is classified as a psychostimulant drug, which at doses regularly consumed by population exerts its primary effects via non-selective antagonism of adenosine A_1 and A_{2A} receptors (Einother and Giesbrecht, 2013; Fredholm et al., 1999). The prevalence of caffeine consumption is high in the adult population due to its ability to promote arousal, increase vigilance and improve attention and mental performance (Chen et al., 2014; Fredholm et al., 1999; Knight et al., 2004; Temple, 2009).

It has been estimated that 97 % of women consume regularly caffeine before pregnancy and approximately 68 % will persist during this period (Chen et al., 2014; Doepker et al., 2016; Frary et al 2005; Knight et al 2004). Pregnant women and their fetuses are naturally vulnerable to potential harmful effects of caffeine because this methylxantine is metabolized by hepatic enzyme systems namely CYP1A2, whose expression and activity develops at postnatal periods (Leeder 2001). In addition, caffeine and its metabolites can easily cross placenta barrier and cellular membranes, including the fetal brain (Nehlig and Debry 1994; Parsons and Neims 1981; Soellner et al., 2009; Yu et al., 2016). The exposure to caffeine during prenatal period has been associated with spontaneous abortion, prematurity and low birth weight (Fernandes et al., 1998; Hoyt et al., 2014; Weng et al., 2008). In addition, alterations in synaptic proteins essential for brain maturation have been reported by chronic exposure of caffeine during fetal and early postnatal brain development (Mioranzza et al., 2014; Sahir et al., 2000; Silva et al., 2013; for review see Porciúncula et al., 2013; Temple, 2009).

Since other preclinical and even clinical studies have not reported long-term harmful effects of caffeine, the safety of caffeine intake during pregnancy and early periods of brain development is still controversial (Bakker et al 2010; Linn et al., 1982; Pollock et al., 2010; Savitz et al., 2008; Lynch et al, 2008; Yu et al 2016; see recent comment

Rutherford and Mayes, 2016). Of note, the vast majority of studies had been conducted with administrations to pregnant or lactating dams and the offspring undergoes testing at adulthood. This is particularly intriguing considering that children and adolescent population are still increasingly consuming caffeine from coffee, soda and energy drinks (Ahluwalia and Herrick, 2015). In fact, few studies have addressed the effects of caffeine in the pubertal and/or adolescent period (Ardais et al., 2014; O'Neill et al., 2016). Importantly, sex differences in the effects of caffeine are still scarce. Pre and postnatal caffeine exposure increases locomotion in adult male and female rats, decreases anxiety-related behavior and did not affect recognition memory in both sexes at moderate/high dose (Ardais et al., 2016). In the same study, caffeine withdrawal at weaning worsened recognition memory in adult females, and male rats showed increased locomotor activity (Ardais et al., 2016). In another study, caffeine during pregnancy caused memory impairment in both sexes in the adulthood, but only adult rats showed impaired spatial and recognition memory when caffeine was administered from pregnancy up to postnatal day 15 (Soellner et al., 2009). In a recent study, recognition memory was not affected in adolescent rats from both sexes exposed to caffeine, but males were less anxious than females in the light/dark test (Turgeon et al., 2016).

Recently, one study double blind, placebo-controlled dose–response design, investigated the developmental trajectory of sex differences in subjective responses in pre- and post pubertal boys and girls acutely treated with caffeine (Temple et al., 2015). The authors reported that girls showing greater changes in subjective responses after caffeine administration compared with boys, but also they varied as a function of pubertal stage and menstrual cycle phase.

As a part of normal brain development, synaptic density starts to increase during early childhood, peaking around the time of puberty and declines across adolescence into adulthood (Huttenlocher and Dabholkar, 1997; Glantz et al., 2007). Given that every stage of the brain development relies on precisely orchestrated process, each period may suffer alterations from external agents that can dramatically change its structure and function, reverberating for a lifetime. The puberty is a period of intense synaptic remodeling and of high vulnerability to psychoactive substances. In the present study, we investigated the behavioral outcomes and synaptic proteins changes in pubescent rats from both sexes that received caffeine since pregnancy. We hypothesized that caffeine would affect the behavior and synaptic proteins crucial for brain development in a sex selective manner.

2. Materials and Methods

2.1. Animals

Adult Female Wistar rats (70 days old) were mated within our colony at Federal University of Rio Grande do Sul. Animals (adult female rats and their pups) were maintained under 12 hours light-dark-cycle (lights on at 7:00 AM), at constant temperature (22 ± 1 ºC) 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

Caffeine (0.1 or 0.3 g/L) was administered in the drinking water only during their active cycle (lights off at 7:00 PM), with doses regimen corresponding to low and

moderate caffeine intake (0.1 or 0.3 g/L), respectively, acting selectively on adenosine receptors (Fredholm et al., 1999; Ardais et al., 2016). The treatment started 15 days before mating and lasted throughout pregnancy and lactation. 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 lactation. Pups were weaned at postnatal day 21 (PND 21) and they were kept at 3-4 rats 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 two groups: a) pups that received only tap water; b) pups that received caffeine in drinking water up to the onset of puberty (34 days old). The timeline summarizes the schedule of administration and the subsequent behavioral and synaptic proteins levels analysis (Fig. 1).

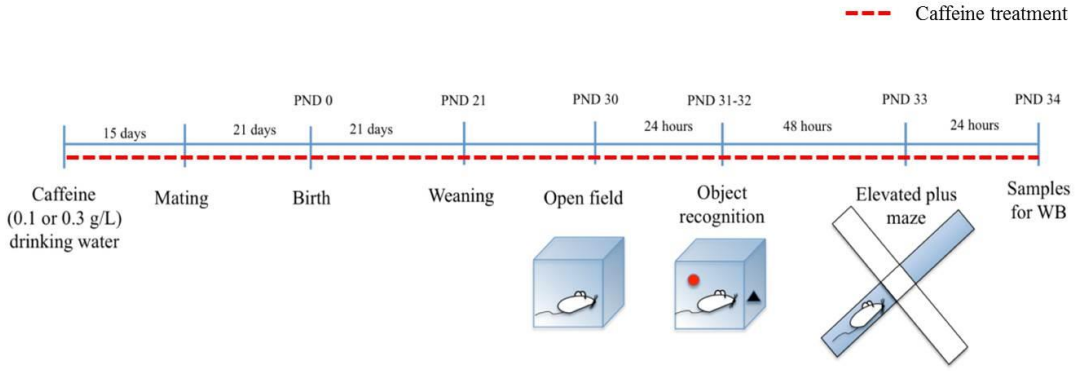

Fig. 1. Schematic overview of the experimental design. Female rats were habituated to caffeine solutions 15 days before mating. Caffeine was available during pregnancy and lactation. At the weaning day (PND 21) litters were separated by sex and divided into two groups: a) pups that received only tap water; b) pups that received caffeine up to the end of puberty (34 days old). 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.

2.3.Behavioral analysis

Behavioral analysis started when rats were 30 days old, which corresponds to the onset of puberty (Quinn, 2005; Spear, 2000). 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). All procedures were carried out during the first period of the light cycle (7:00 to 12:00 AM), in which plasma levels of caffeine are still detected (Ardais et al., 2016).

2.4.Open field

The open field exposure was performed as previously described (Ardais et al., 2014). Briefly, the open field apparatus consisted of a black-painted wooden box (50 x 50 cm) surrounded by a 50 cm wall divided into two areas: center and periphery. Central zone was defined as a rectangular area 20 cm from the wall. Each rat was placed in the center of open field and the traveled distance in the areas was recorded during a single session of 10 minutes.

2.5.Novel object recognition task

The object recognition test was carried out 24 hours after an habituation session to open field apparatus, as previously described (Ardais et al., 2014). 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 minutes. The test session was performed 90 minutes and 24 hours after training and two dissimilar objects were presented, a familiar and a novel one (Ardais et al., 2014; Bevins and Besheer, 2006). The 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 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].

2.6. Elevated plus maze

The elevated plus maze, a pharmacologically validated apparatus for the measurement of anxiety in rodents (Johnston and File, 1991; Lister, 1987) was carried out as previously described (Ardais et al., 2014). The elevated plus-maze apparatus consisted of two open arms (30 cm × 5 cm) and two enclosed arms (30 cm × 5 cm × 10 cm), arranged so that the two arms of each type are positioned oppositely, being separated by a central platform (5 cm × 5 cm). The height of the maze was 70 cm, and the experiments were conducted under dim red light in a quiet room. Each rat was placed in the center of the apparatus facing an open arm. The number of entries and time spent in each apparatus zone (closed and open area) were recorded during one single session of 5 minutes. The entries were recorded when rats have entered with their four paws in each arm.

2.7. Western blot

Twenty-four hours after the end of behavioral tests, rats were sacrificed under anesthesia. The whole hippocampi and cerebral cortex were dissected out and immediately homogenized in a 5 % SDS solution containing a protease and phosphatase inhibitor cocktail (Sigma, São Paulo/SP, Brazil), and frozen at -20 ºC. 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 20 μg (cerebral cortex) or 50 μg (hippocampus) for GFAP and SNAP-25 and 80 μg of protein for 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 hour. 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 (1:5000; Sigma), mouse anti-proBDNF (1:2000; Abcam, São Paulo, Brazil) or mouse anti-BDNF (1:1000; Santa Cruz Biotechnologies, São Paulo, Brazil). 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, São Paulo, Brazil). 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 (1:4000; Santa Cruz Biotechnologies, São Paulo, Brazil), as described above.

2.8. Statistical analysis

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

3. Results

3.1.Open Field Analysis

Sex differences in the water group were found for total traveled distance ($t = 2.183$; $P < 0.05$) and traveled distance in the central zone ($t = 2.511$; $P < 0.05$) (Fig. 2).

In male rats, both total traveled distance [F (2,46) = 7.123; P < 0.01] and distance traveled in the peripheral zone [F (2,46) = 8.949; P < 0.001] were increased by caffeine at 0.3 g/L (Fig. 3). In female rats, both doses of caffeine also caused a similar increase in the total traveled distance [F (2,53) = 5.026; P < 0.01] and in the peripheral zone [F (2,53) = 4.722; P < 0.05] (Fig. 3).

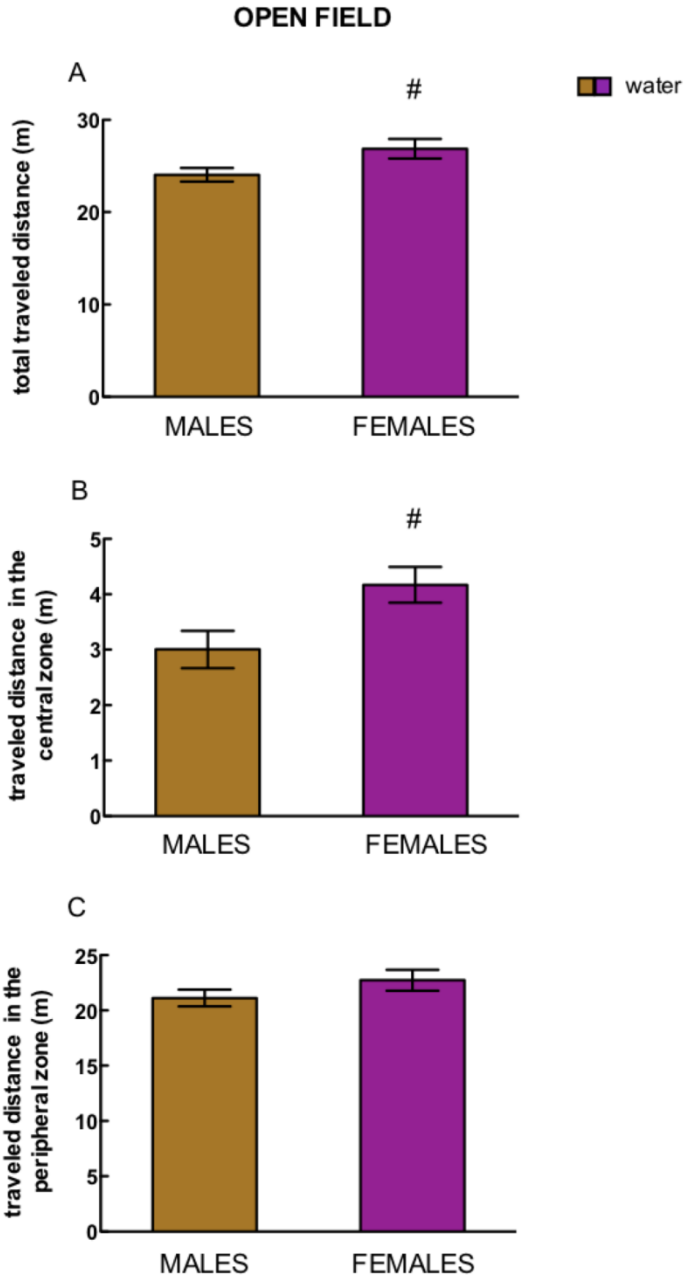

Fig. 2. Sex differences in the locomotor activity displayed by pubescent male and female rats receiving water. Panels show the traveled distance in meters (m) in each area of the open field apparatus during 10 minutes. (A) Total traveled distance); (B) traveled

distance in the center; (C) traveled distance in the periphery. Data are means ± S.E.M (*n* = 20-25 animals per group). # P< 0.05; (Unpaired *t*-test).

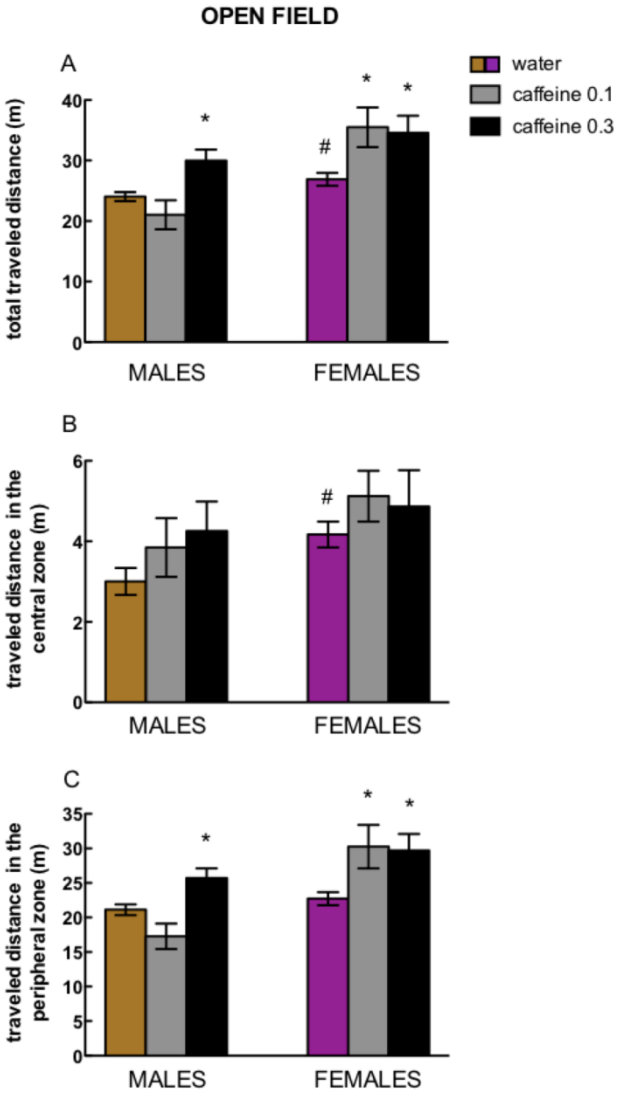

Fig. 3. Sex differences in the locomotor activity displayed by pubescent male and female rats treated with caffeine (0.1 and 0.3 g/L) or water. Panels show the traveled distance in meters (m) in the open field apparatus during 10 minutes. (A) Total traveled distance (m); (B) traveled distance in the center of the open field; (C) traveled distance in the periphery. Data are means ± S.E.M (*n* = 11-25 animals per group). *P< 0.05 – different from the water group with the same sex (One-way ANOVA, Tukey's post hoc test). # P< 0.05 (Unpaired *t*-test).

3.2. Novel object recognition task

Recognition memory was assessed by the novel object recognition task. No differences were found between training and test session in male rats in the water group. However, a paired t-test revealed significant effect of trials when long-term memory (t = 2.125; $P < 0.05$) was assessed in female rats in the water group, presenting a

worsening performance. In male rats, caffeine improved long-term memory at low (0.1 g/L: t = 3.015; $P < 0.05$) and moderate doses (0.3 g/L: t = 3.349; $P < 0.01$) (Fig. 4). No alteration was found in the object recognition task performance of for female rats that consumed caffeine at 0.1 g/L (Fig. 4). However, female rats consuming caffeine at 0.3 g/L presented impairment for long-term memory (Fig. 4).

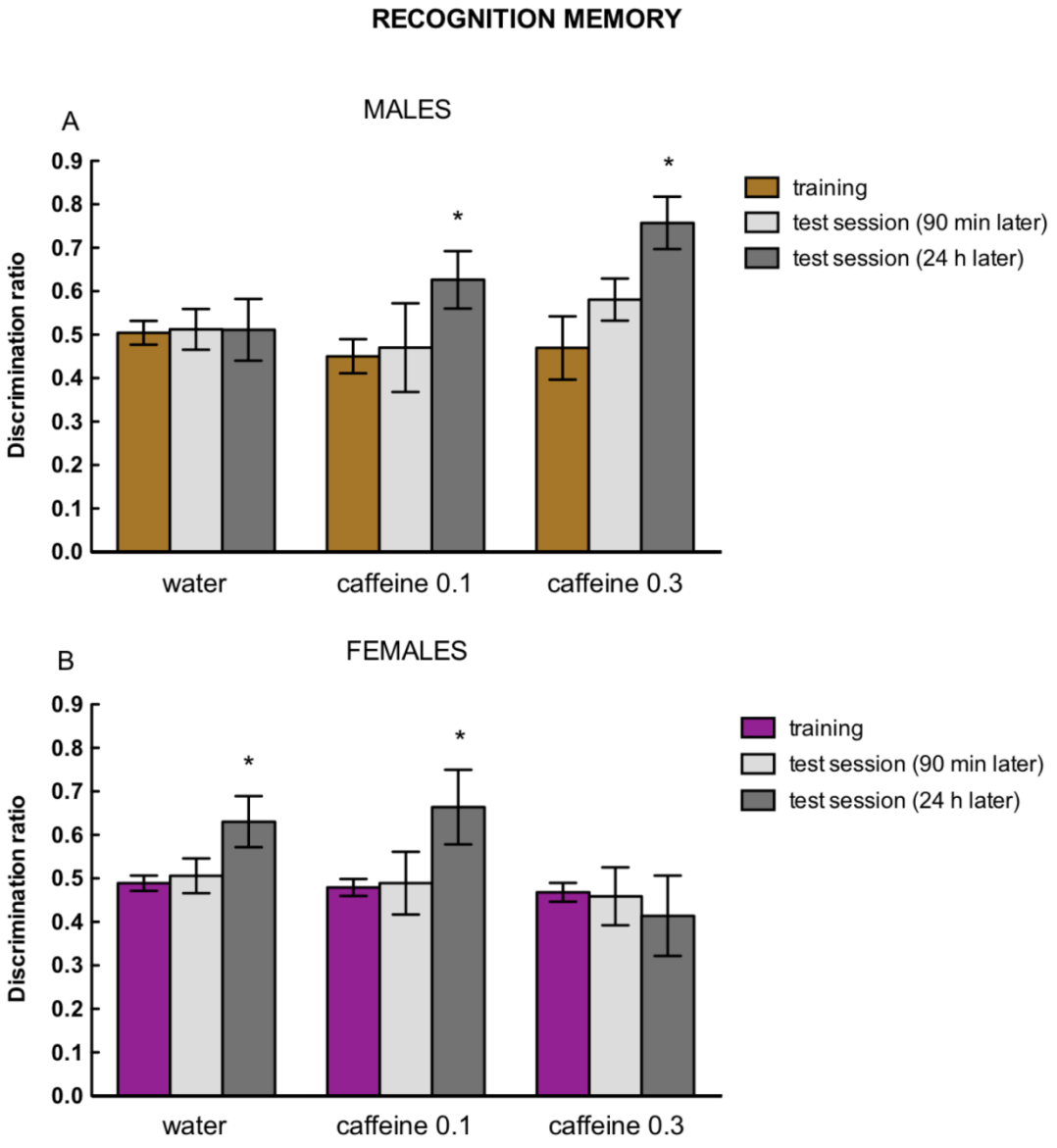

Fig. 4. Performance of the object recognition task for pubescent male and female rats treated with caffeine (0.1 and 0.3 g/L) or water. Panels show the discrimination ratio in the training (brown/purple bars), test session 90 minutes later (light grey bars) or 24 hours later (dark grey bars). Data are means ± S.E.M. of the discrimination ratio (n = 10–24 animals). *P < 0.05 - differences between training and test sessions (Paired t-test).

3.3. Elevated plus maze

The effects of low (0.1 g/L) and moderate (0.3 g/L) doses of caffeine on anxiety-related behavior were evaluated in the elevated plus maze. No differences between sexes were found in all parameters analyzed (Fig 5). Caffeine was devoid of effects in male rats (Fig 5). However, caffeine at 0.3 g/L increased in both the time spent [F (2,45) = 3.341; $P < 0.05$] and the number of entries in the open arms [F (2,45) = 5.343; $P < 0.01$] in female rats. Caffeine at 0.1 g/L decreased the number of entries in the closed arms in female rats [F (2,44) = 4.496; $P < 0.05$] (Fig 5).

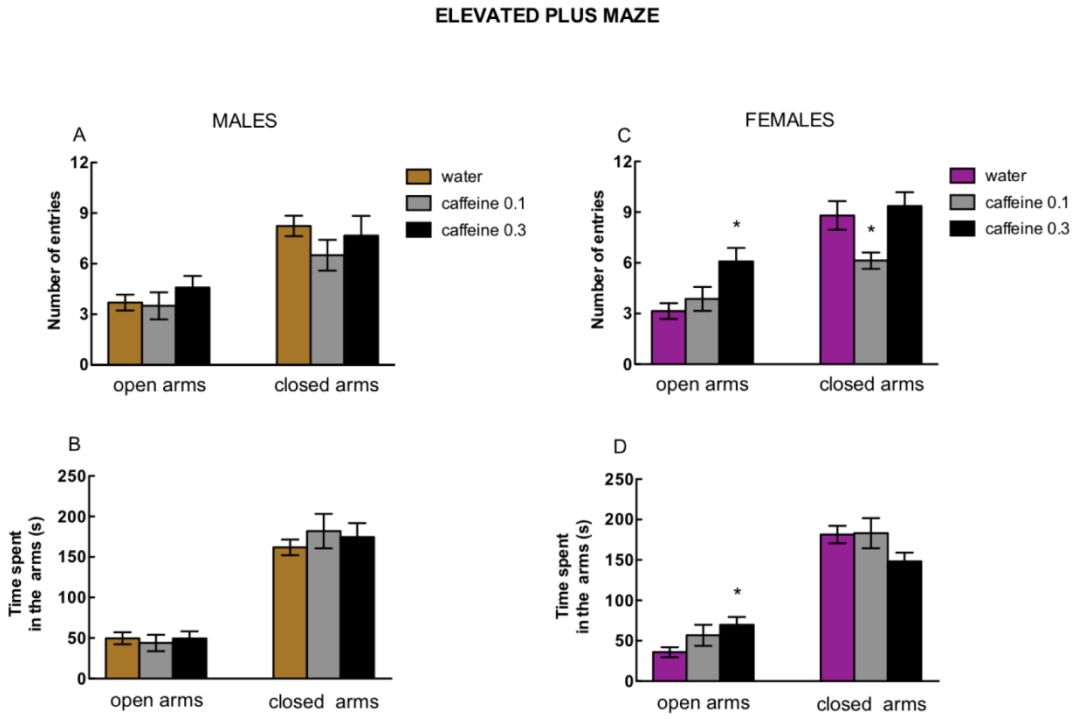

Fig. 5. Anxiety-related behavior in the elevated plus maze displayed by pubescent male and female rats treated with caffeine (0.1 and 0.3 g/L) or water. (A and B) - number of entries and time spent in the open and closed arms for pubescent male rats; (C and D) - number of entries and time spent in the open and closed arms for pubescent female rats. Data are represented as means ± S.E.M. of the time spent in seconds (s) (n = 11–25 animals). *P < 0.05 - differences between water and caffeine groups (One-way ANOVA, Tukey's post hoc test).

3.4. Immunodetection of proteins in the hippocampus and cerebral cortex

3.4.1. BDNF and proBDNF

Sex differences in the water groups were found for proBDNF levels, with female rats presenting an increase in both hippocampus (t = 2.222; $P < 0.05$) and cerebral cortex (t = 2.343; $P < 0.05$)(Fig. 6A and 7A) when compared to males. Males that received caffeine (0.3 g/L) presented increased levels of proBDNF and BDNF in the hippocampus [F (2,14) = 3.882; $P < 0.05$; F (2,14) = 4.621; $P < 0.05$, respectively], while no differences were found for both brain regions in female rats (Fig. 6B and D). In addition, both tested doses of caffeine increased proBDNF and BDNF in the cortex of male rats [F (2,16)= 8.919; $P < 0.01$; F (2,15)= 10.59; $P < 0.01$, respectively] (Fig. 7B and D). In female rats, only the lowest dose of caffeine increased proBDNF in the cerebral cortex [F(2,16) = 6.917; $P < 0.01$], with no effect in the BDNF levels (Fig. 7B and D).

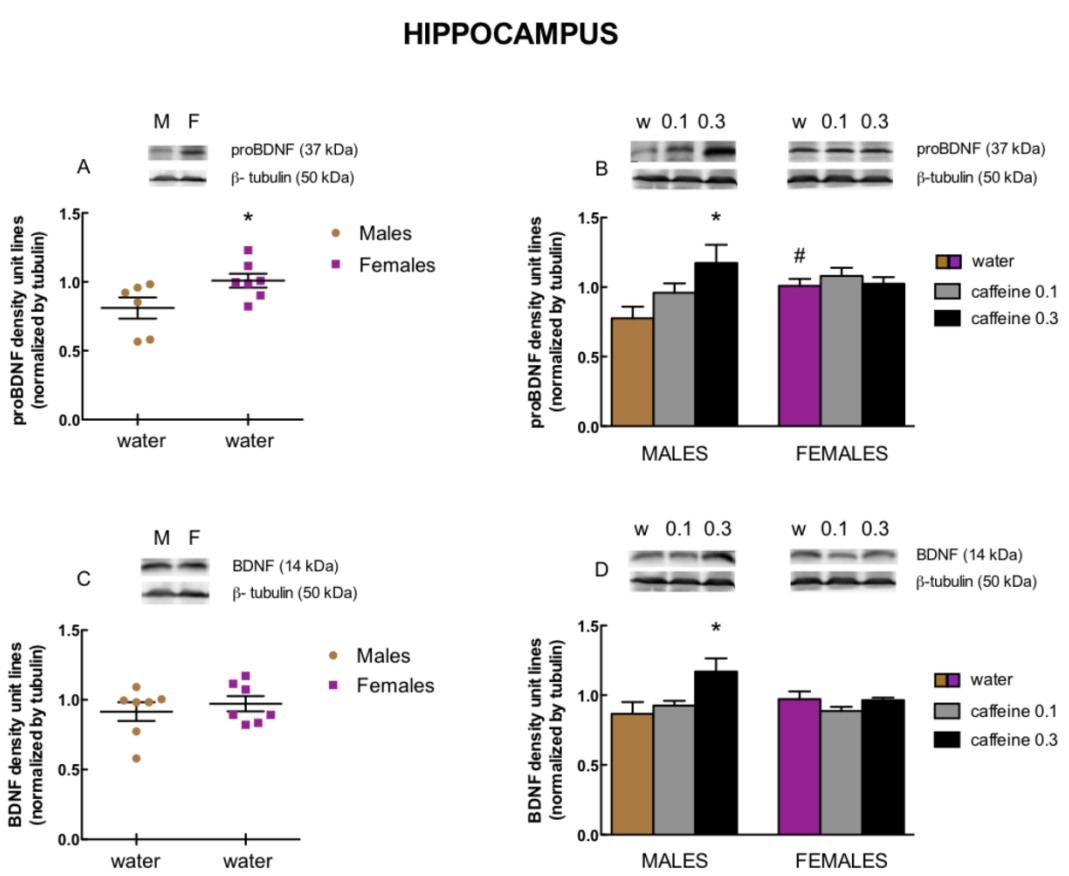

Fig. 6. The immunocontent of proBDNF and BDNF in the hippocampus from pubescent male and female rats treated with caffeine (0.1 and 0.3 g/L) or water.
(A and C) – sex differences in the proBDNF and BDNF levels from hippocampus of water groups; *P < 0.05; (Unpaired- t test). (B and D) - proBDNF and BDNF levels from hippocampus of water and caffeine-treated groups. Data are represented as means ± S.E.M (n = 6–7 animals per group) of density unit lines (normalized by β-tubulin). At the top of each graphic are representative bands for all proteins.
*P < 0.05 - different from the water group (One-way ANOVA, Tukey's post hoc test).
$^{\#}$P < 0.05; (Unpaired- t test).

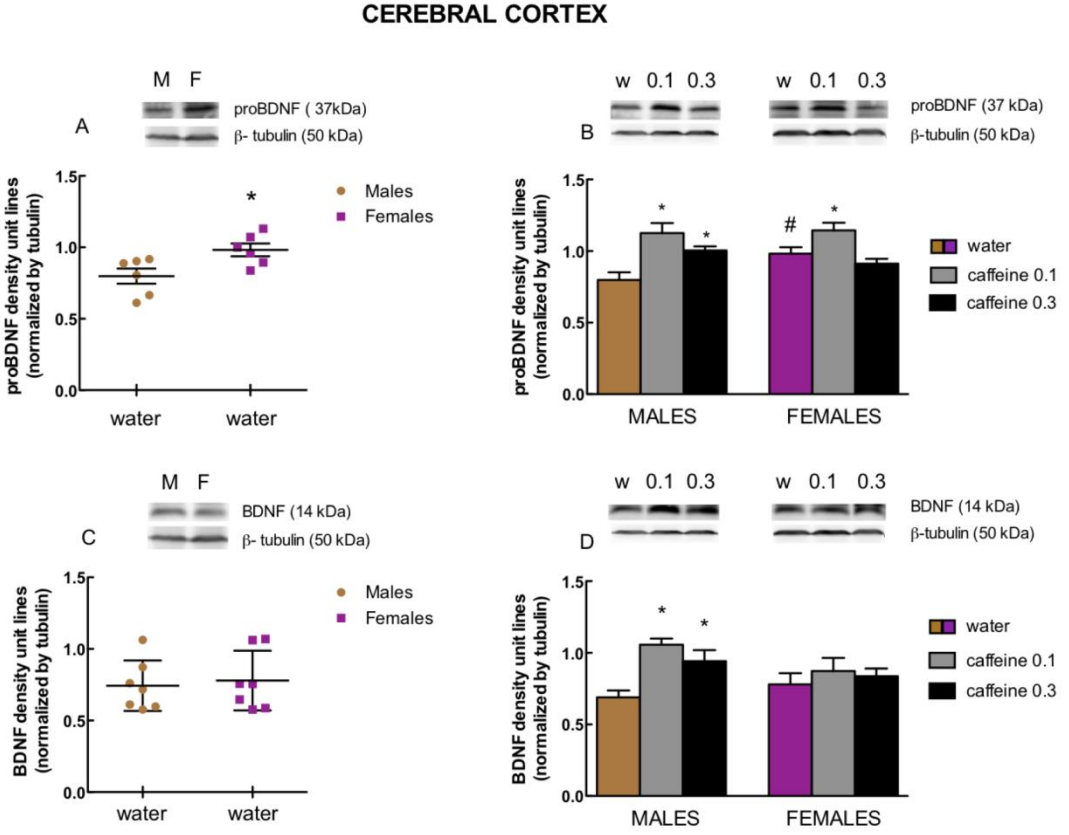

Fig. 7. The immunocontent of proBDNF and BDNF in the cerebral cortex from pubescent male and female rats treated with caffeine (0.1 and 0.3 g/L) or water.
(A and C) - sex differences in the proBDNF and BDNF from hippocampus of water groups; *P < 0.05; (Unpaired- t test). (B and D) - proBDNF and BDNF levels from cortex of water and caffeine-treated groups. Data are represented as means ± S.E.M (n = 6–7 animals per group) of density unit lines (normalized by β-tubulin). At the top of each graphic are representative bands for all proteins. *P < 0.05 - different from the water group (One-way ANOVA, Tukey's post hoc test).

3.4.2. GFAP and SNAP-25

The GFAP levels were not modified either by sex or caffeine treatment (Fig. 8). In addition, SNAP-25 levels were similar in both sexes in the hippocampus and cerebral cortex (Fig. 9 A and C). Caffeine (0.3 g/L) increased SNAP-25 levels in the cerebral cortex from female rats [$F(2,15) = 8.554$; $P < 0.01$](Fig. 9 D).

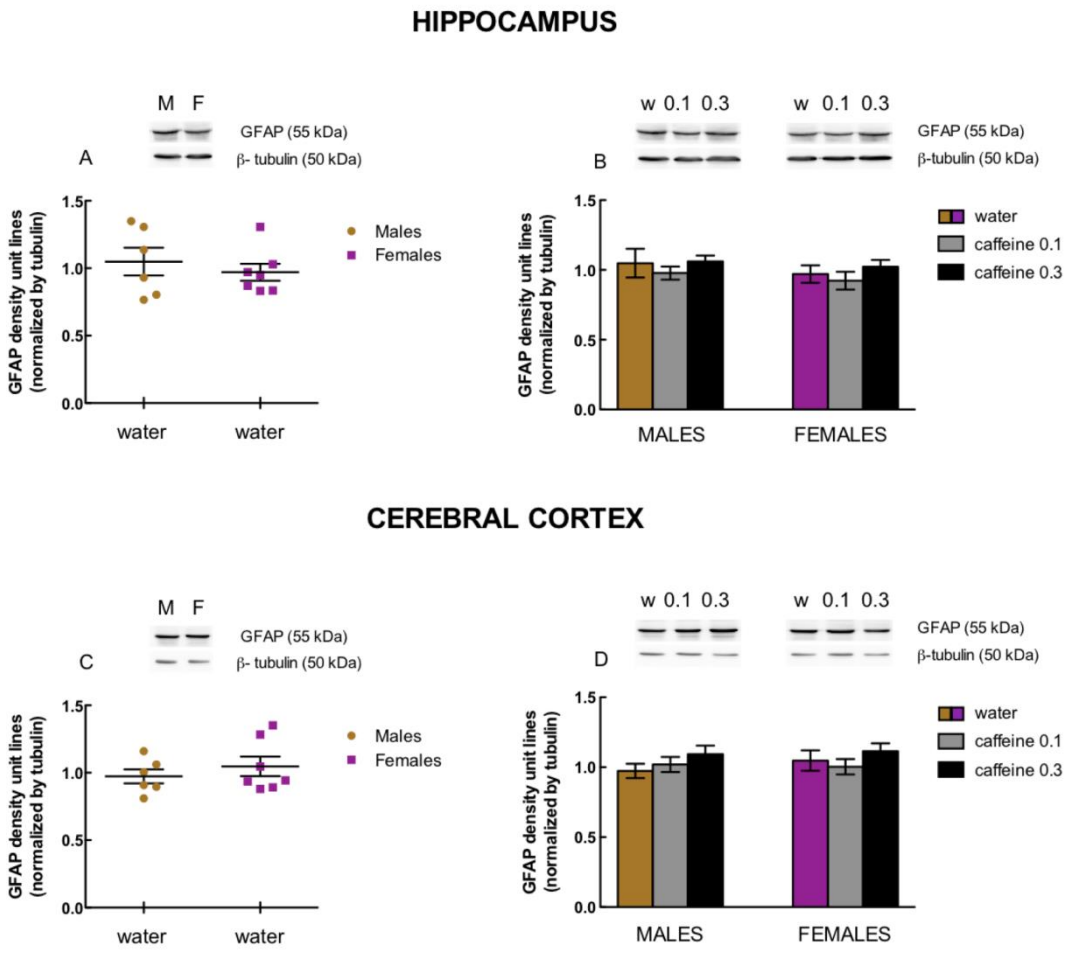

Fig. 8. The immunocontent of GFAP in the hippocampus and cerebral cortex from juvenile male and female rats treated with caffeine (0.1 and 0.3 g/L).
Sex differences in the GFAP levels from hippocampus (A) and cortex (C) of water groups. GFAP levels from hippocampus (B) and cortex (D) of water and caffeine-treated groups. Data are represented as means ± S.E.M (n = 6–7 animals per group) of density unit lines (normalized by β-tubulin). At the top of each graphic are representative bands for all proteins.

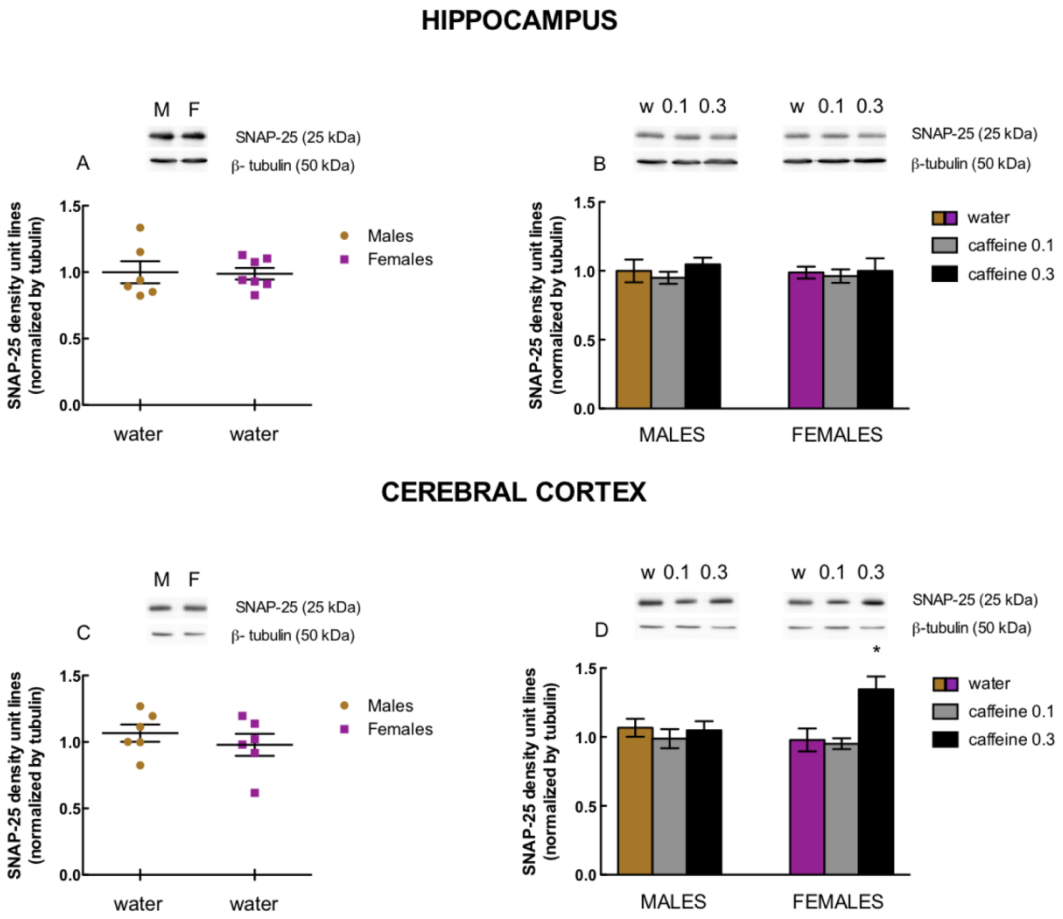

Fig. 9. The immunocontent of SNAP-25 in the hippocampus and cerebral cortex from pubescent male and female rats treated with caffeine (0.1 and 0.3 g/L) or water.
Sex differences in the SNAP-25 levels from hippocampus (A) and cortex (C) of water groups. SNAP-25 levels from hippocampus (B) and cortex (D) of water and caffeine-treated groups. Data are represented as means ± S.E.M (n = 6–7 animals per group) of density unit lines (normalized by β-tubulin). At the top of each graphic are representative bands for all proteins. *P < 0.05 - different from the water group (One-way ANOVA, Tukey's post hoc test).

4. Discussion

In this study, the effects of caffeine in pubescent male and female rats exposed during pre and postnatal period were evaluated on locomotor activity, recognition memory and anxiety-like behavior. In parallel, a set of synaptic proteins was assessed in the hippocampus and cerebral cortex.

Locomotor activity

Rodents at different ages tend to avoid central areas of a new environment and explore preferentially perimeters of a novel environment, a behavior called *thigmotaxis* (Bogdanov et al., 2013; Lamprea et al., 2008; Treit and Fundytus, 1988). Based on this

premise, time spent and traveled distance in the central zone are used as an indicative of anxiety behavior, while the same parameters in the periphery are interpreted as locomotor activity (Prut and Belzung, 2003). Thus, pubescent females showed hyperlocomotion and attenuated anxiety in the open field, which is in line with other reports at different ages (Ardais et al., 2016; Brotto et al., 2000; Brown and Nemes, 2008; Frye and Walf, 2002; Hughes and Beveridge, 1990; Padilha et al., 2009; Simpson and Kelly, 2012).

Caffeine usually shows biphasic effects on locomotion in adult animals, with lower doses promoting increases and higher doses decreasing the locomotor activity (El Yacoubi et al., 2000; Fisone et al., 2004; Marin et al., 2011; Wise, 1988). It can be noted that hyperlocomotion in females was observed at both doses, while in males at moderate dose of caffeine. Caffeine, at doses achieved in normal human consumption, is likely to exert its primary effects through the antagonism of adenosine receptors, specially through A_1 and A_{2A} receptors (Fredholm, 1999). According to the study conducted by El Yacoubi et al. (2000) the stimulant effect of low doses of caffeine could be explained by A_{2A} receptor blockade while the depressant effect seen at higher doses may be associated to A_1 receptor blockade. Psychomotor stimulant properties also occur in response to other psychostimulants, such as amphetamine, nicotine or cocaine (Caldarone et al., 2008; Catlow and Kirstein, 2005; Dietrich et al., 2004; Mori et al., 2004; Schmidt et al., 2010). Notably, females were more responsive to caffeine, since they respond to the lowest dose, while only the highest dose were capable to alter locomotor activity in male rats. These findings may be associated to the well established difference between sex response to drug abuse, being females more vulnerable than males (Anker and Carroll 2011; Becker and Hu 2008). Therefore the present results are in accordance with the body of literature that point to a role of caffeine and other

psychostimulants on locomotor activity.

Anxiety-related behavior

It is relatively established that female rodents are less anxious and fearful than males (Archer, 1975; see recent comments in Shansky and Wooley, 2016), and this pattern of emotionality may already be observed in earlier periods of brain development. Pubescent females were less anxious than males in the open field, but both sexes showed similar behavior in the elevated plus maze, as previously noted (Estanislau and Morato, 2006). Pre and postnatal moderate caffeine attenuated anxiety in the elevated plus maze only in pubescent females, with no effect observed for both sexes in the open field. Interestingly, only adult female rats exposed to low, moderate and high caffeine since prenatal period were less anxious in the open field, and at high dose both sexes showed attenuated anxiety in the elevated plus maze (Ardais et al., 2016). In the same study, caffeine treatment interrupted at weaning did not alter anxiety behavior in the adulthood of both sexes in the elevated plus maze. Apart from differences in the responsiveness to apparatuses, the classical anxiogenic effects of caffeine seem to be more evident in later periods of brain development (Bhattacharya et al., 1997; Noschang et al., 2009; Pechlivanova et al., 2012). For example, male rats receiving caffeine during throughout adolescence period have already showed an exacerbation of anxiety at low and moderate doses (Ardais et al., 2014; O'Neill et al., 2016).

Recognition memory

Another important sex difference was found for recognition memory. We have already reported that adolescent male rats had poor performance in the object recognition task (Ardais et al., 2014). In this study, pubescent females presented a better performance in the object recognition task for long-term memory (LTM), and both

sexes displayed worsened recognition memory when short-term memory (STM) was evaluated. Age differences have been described for the object recognition task, with younger rats presenting a decrement compared to adults (Anderson et al., 2004; Reger et al., 2009), probably due to the relatively slow maturation at this time of brain development of the neural circuits involved in recognition memory (Bachevalier and Beauregard, 1993). Furthermore, it has been demonstrated that sex hormones exert influence in distinct brain areas involved in learning and memory (Duarte-Guterman et al., 2015; Hamson et al., 2016; Sánchez-Andrade and Kendrick, 2011).

Of note, caffeine differently affected memory according to the sex, being pubescent males most benefited by improvements in the recognition memory, similar to previous findings in adolescent male rats (Ardais et al., 2014), whereas pubescent females presented a worsened recognition memory at moderate dose. Likewise, adult females exposed since prenatal period have also showed impairment in the recognition memory when caffeine treatment was interrupted at weaning (Ardais et al., 2016). In the same study, recognition memory was not affected in adult male and female rats exposed continuously to caffeine since prenatal period (Ardais et al., 2016). It becomes increasingly clear that beyond sex differences, there is a time window of brain development in which caffeine affects permanently some types of memory. For example, adult male and female rats exposed to caffeine either gestation and/or lactation showed impaired memory in different tasks (Silva et al., 2013; Soellner et al., 2009). Furthermore, sexual dimorphism in the adult brain are originated from effects of the sexual hormones during specific time windows of development, which include the late embryonic period to the first postnatal weeks, revealing that the influence of sex hormones occurs even before birth (Colciago et al., 2015).

Synaptic proteins levels

Caffeine has altered the levels of BDNF in association with behavioral outcomes at different ages (Costa et al., 2008a,b; Ardais et al., 2014; Sallaberry et al., 2013). Thus, in order to find some association between behavioral outcomes and sex differences by caffeine treatment, BDNF and related proteins were analyzed in the hippocampus and cerebral cortex. BDNF is synthesized as the proneurotrophin proBDNF (Lu et al., 2005) and its signaling is essential for recognition memory (Callaghan and Kelly, 2012; Greenberg et al., 2009).

Regarding the sex differences, pubescent female rats presented increased proBDNF, but not BDNF, suggesting that the proneurotrophin was not necessarily converted into the mature form. These differences between pro- and BDNF have been particularly evident in females during brain development, in which higher levels of proBDNF gradually decrease, while BDNF increases after puberty (Harte-Hargrove et al., 2013; Yang et al., 2009). Importantly, estrogen may regulate BDNF expression via an estrogen-sensitive response element in the BDNF gene (Sohrabji et al., 1995).

Coincident with the increase in both proBDNF and BDNF in the hippocampus, caffeine restored recognition long-term memory in pubescent male rats. It is plausible that both events are associated since BDNF signaling is essential for recognition long-term memory (Callaghan and Kelly, 2012; 2013; Greenberg et al., 2009), an association between both events might have occurred. Importantly, proBDNF is not only an inactive precursor of BDNF, but a signaling protein with specific functions (Lu et al., 2005).

The expression of GFAP abruptly increases until PND 6, then stabilized but increased progressively until PND 48 in the hippocampus (Kim et al., 2011). Caffeine during postnatal period promotes decreases in the GFAP at different ages during development of the hippocampus and neocortex (Desfrere et al., 2007), which also

include decreases in these brain areas from adolescent male rats treated only during this period (Ardais et al., 2014). Differently from adult males, but not females, pre and postnatal caffeine treatment did not modify GFAP in both brain areas (Ardais et al., 2016), suggesting that GFAP may not be involved in these behavioral outcomes in the puberty.

The synaptosomal protein of 25 kDa (SNAP-25) is a crucial component of the ternary soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex, the minimal machinery required for vesicle exocytosis. There is substantial evidence that alterations in the SNAP-25 levels are associated with cognitive impairment, hyperactivity and exacerbation of anxiety-related behaviors (Hess et al., 1996; Kataoka et al., 2011; Braida et al., 2015). Since moderate caffeine increased cortical SNAP-25 only in pubescent female rats, this increase might be associated with worsened recognition memory and less anxiety behavior, two behavioral outcomes found exclusively in females. Although most studies collectively suggest that reduced levels of SNAP-25 may contribute to the cognitive impaiment and anxiety, one study showed memory impairment in young adult rats after overexepression and activity of SNAP-25 induced by infusion of a recombinant adeno-associated virus vector (McKee et al., 2010). While pubescent male rats treated with caffeine showed normal SNAP-25 levels, adolescent male rats showed exacerbated anxiety and decreased cortical SNAP-25 after caffeine treatment (Ardais et al., 2014). Additionally, both sexes that received caffeine since prenatal period showed attenuated anxiety in the adulthood at high dose of caffeine, but only males had increased levels of cortical SNAP-25 (Ardais et al., 2016).

Over the last years, there is a growing body of evidence pointing to the impact of caffeine consumption at different phases of brain development. While pubescent male

rats were more benefited to the cognitive improvements afforded by caffeine than females, the anxiolytic effects of caffeine were observed in females. Regarding to the differences between sexes in drugs responses females are more vulnerable than males (Anker and Carroll, 2011; Becker and Hu, 2008). Our study tried to contribute not only for the knowledge about the effects of caffeine in the puberty, but also to be in line with the rationale for incorporating Sex as a Biological Variable (SABV) in current investigations (Shansky and Wooley, 2016). The knowledge of the impact of this psychostimulant, according to the sex in the immature brain is crucial to establish the safety dose.

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CAPÍTULO III

Chronic caffeine prevents changes in inhibitory avoidance memory and hippocampal BDNF immunocontent in middle-aged rats

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Chronic caffeine prevents changes in inhibitory avoidance memory and hippocampal BDNF immunocontent in middle-aged rats

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Abstract

Beneficial effects of caffeine on memory processes have been observed in animal models relevant to neurodegenerative diseases and aging, although the underlying mechanisms remain unknown. Because brain-derived neurotrophic factor (BDNF) is associated with memory formation and BDNF's actions are modulated by adenosine receptors, the molecular targets for the psychostimulant actions of caffeine, we here compare the effects of chronic caffeine (1 mg/mL drinking solution for 30 days) on short- and long term memory and on levels of hippocampal proBDNF, mature BDNF, TrkB and CREB in young (3 month old) and middle-aged (12 month old) rats. Caffeine treatment substantially reduced i) age-related impairments in the two types of memory in an inhibitory avoidance paradigm, and ii) parallel increases in hippocampal BDNF levels. In addition, chronic caffeine increased proBDNF and CREB concentrations, and decreased TrkB levels, in hippocampus regardless of age. These data provide new evidence in favor of the hypothesis that modifications in BDNF and related proteins in the hippocampus contribute to the pro-cognitive effects of caffeine on age-associated losses in memory encoding.

This article is part of a Special Issue entitled 'Cognitive Enhancers'.

Keywords: Aging, Caffeine, Memory, BDNF, TrkB

1. Introduction

The steady increase in human life expectancy has been paralleled by increasing concern with the prevalence of dementia, likewise rising substantially worldwide. These worries extend beyond the diseases that are prevalent in the elderly, such as Alzheimer's disease (AD), for it is now recognized that the pathological processes that lead to dementia start decades before its clinical manifestations (Eskelinen et al., 2009; Kivipelto et al., 2006; Nooyens et al., 2011). The mild memory deficits commonly experienced by aging people, also referred to as age-associated memory impairment (Crook et al., 1986; Rosenzweig and Barnes, 2003), have important socio-economic costs in contemporary society. To date there is no curative treatment to alleviate age-associated memory impairment or dementia-producing diseases, emphasizing the relevance of preventive strategies that could be adopted before or throughout middle age. In this context, a series of epidemiological studies including small case-control (Maia and de Mendonça, 2002), prospective (Eskelinen et al., 2009; Ritchie et al., 2007) and longitudinal studies (Jarvis, 1993; Johnson-Kozlow et al., 2002) demonstrated that caffeine intake prevents and/or delays the onset of age-related cognitive decline. In agreement with these clinical observations, a series of experiments have produced evidence that chronic caffeine ameliorates both age-related memory decline (Costa et al., 2008a; Prediger et al., 2005) and neurodegeneration (Arendash et al., 2006; Dall'Igna et al., 2003, 2007) in experimental models of aging and AD.

The mode of action of caffeine is complex, including inhibition of phosphodiesterases, antagonism of GABAA and adenosine receptors, and sensitization of induced calcium release through ryanodine-sensitive channels (Daly, 2007; Stone et al., 2009). Nevertheless, it is now generally accepted that the adenosine receptors

are the primary extracellular targets of caffeine, where it acts as a competitive antagonist at A1 and A2A adenosine receptors and thus diminishes endogenous adenosinergic tone (Chen et al., 2010; Fredholm et al., 1999; Fredholm, 2011). The G-protein- coupled A1, A2A, A2B, and A3 adenosine receptors are key in the pathways that make adenosine an important central nervous system (CNS) modulator (Fredholm et al., 2011; Sebastião and Ribeiro, 2009), and A1 and A2A are thought to play a major role in the neuroprotective effects of caffeine (Cunha, 2005; Fredholm et al., 2003).

Brain-derived neurotrophic factor (BDNF), a neurotrophin generated by proteolytic cleavage of proBDNF, along with its high- affinity receptor tyrosine kinase B (TrkB) are thought to be essential in the regulation of neuronal survival and differentiation (Greenberg et al., 2009; Pollock et al., 2001; Teng et al., 2005). The ultimate fates of intracellular proneurotrophins include: intracellular cleavage followed by secretion, secretion followed by extra-cellular cleavage, secretion without subsequent cleavage, or rapid intracellular conversion to mature BDNF (Matsumoto et al., 2008; Lu et al., 2005; Yang et al., 2009). Considerable evidence indicates that released, uncleaved proBDNF enhances long-term depression (LTD) through the receptor p75NTR and leads to apoptosis (Barnes and Thomas, 2008; Lee and Chao, 2001; Rösch et al., 2005; Woo et al., 2005) while released BDNF inhibits long-term depression (LTD), and contributes to cell survival (Lu et al., 2005). However, the extent to which BDNF is released in its pro-form and then cleaved extracellularly to the mature form has been disputed (Matsumoto et al., 2008). Additionally BDNF, via TrkB receptor activation, promotes long-term potentiation (LTP) in the hippocampus (Chen et al., 1999, 2010b; Figurov et al., 1996; Kang et al., 1997; Pang et al., 2004). In particular, BDNF-driven signaling is critical for synaptic plasticity, learning activity

and memory processing in the adult brain (Alonso et al., 2002; Chen et al., 2010b; Tyler et al., 2002), including the persistence of inhibitory avoidance long- term memory in rats (Bekinschtein et al., 2007, 2008).

Changes in BDNF levels and/or the expression of TrkB have been identified during normal aging and AD, and decreases in BDNF expression have been associated with neuronal atrophy and/or death (Silhol et al., 2008; Tapia-Arancibia et al., 2008). In aging rats, LTP induction by weaker (but not high-frequency) stimulus releases BDNF accompanied by an enhancement in the LTP magnitude (Balkowiec and Katz, 2002; Gärtner and Staiger, 2002; Rex et al., 2005). In addition, it has been shown that endogenous BDNF is required for LTP in aged rats, along with adenosine A2A receptors (Diógenes et al., 2011).

In accord with the possibility that caffeine's effects on cognition are linked to the regulation of neurotrophins, it has been reported that caffeine administration to mice from adulthood to old age prevents age-associated declines in object recognition memory, and increases hippocampus levels of BDNF and TrkB (Costa et al., 2008a).

In addition, the enhancement by acute caffeine of novel object recognition memory in adult mice was also accompanied by increases in BDNF immunocontent (Costa et al., 2008b).

While acute caffeine is reported to reverse social recognition and olfactory memory deficits in 12 and 18 month old rats (Prediger et al., 2005), studies evaluating the effects of chronic administration in age-associated impairments of emotional memory are lacking. The purpose of the present study was to investigate whether chronic caffeine alters the predictable age-associated decline in inhibitory avoidance memory in middle-aged rats, mimicking physiological aging. The study was conducted

using young adult (3 month old) and middle-aged (12 month old) rats, and included assessments of changes in hippocampal proBDNF, BDNF, TrkB, and CREB immunocontent.

2. Material and methods

2.1. Animals

Young adult (3 month old) and middle-aged (12 month old) male Wistar rats from our own breeding colony were used in the study. All efforts were made to minimize animal suffering and reduce the number of subjects. The project was approved by the ethical committee of the Universidade Federal do Rio Grande do Sul and experimental procedures were performed according to the NIH Guide for Care and Use of Laboratory Animals as well as the Brazilian Society for Neuroscience and Behavior recommendations for animal care. Animals were maintained in standard animal housing conditions, on a 12-h light/dark cycle (lights on at 7:00 a.m.), five/ cage, with food and water ad libitum. All behavioral tests were performed between 8:00 a.m. and 5:00 p.m.

2.2. Caffeine administration

Young adult and middle-aged rats were given access to drinking bottles containing either 1 mg/mL caffeine solution or tap water during 30 consecutive days; the caffeine solution was prepared daily and bottles refilled between 6:00 and 7:00 p.m.. This treatment regimen is thought to correspond to a moderate caffeine intake in humans, with meaningful effects believed to be restricted to adenosine receptors (Fredholm et al., 1999; Quarta et al., 2004).

2.3. Locomotion

The open field test is widely used for assessing locomotion and exploratory activities in rodents. The apparatus consisted of black-painted Plexiglas measuring 50 x 50 cm, surrounded by 50 cm high walls. The experiments were conducted in a sound-attenuated room under low-intensity light. Each rat was placed in the center of the arena and the distance traveled was recorded during 5 min. The experiment was video recorded from above the arena, and monitored in an adjacent room by an observer blind to treatments. The analysis was performed using a computer-operated tracking system (Any-maze, Stoelting, Woods Dale, IL).

2.4. Inhibitory avoidance

The inhibitory avoidance training apparatus was an acrylic box of 50 x 25 x 25 cm, with a formica platform (7 x 2.5 x 4 cm) fixed at the left end of the grid floor (parallel stainless steel bars, 1 mm diameter, spaced 1 cm apart). Rats were habituated to the room 1 h before starting experiments. In the training session, rats were placed on the platform and the latency to step-down onto the floor with all four paws was measured with an automatic device; immediately after stepping-down rats received a 0.5 mA scrambled footshock for 2 s, and were at once returned to their home cage. Test sessions were performed 90 min (short term memory, STM) or 24 h (long term memory, LTM) later with the same procedure, except that no shock was administered after stepping down; an upper cut-off time was set at 180 s. The drinking bottles (with water or caffeine) were available between test sessions. The same animals were tested for STM and LTM; potential reminder effects that could affect LTM were previously ruled out (Cammarota et al., 2007).

2.5. Immunoblotting

Immediately after the last inhibitory avoidance test session rats were sacrificed by decapitation; the whole hippocampus was dissected out and immediately homogenized in 5% SDS with a protease inhibitor cocktail (Sigma, São Paulo, SP/ Brazil). Homogenates were frozen and kept at -70°C. After defrosting, the protein content was determined by bicinchoninic acid assay (BCA from Pierce, São Paulo/ Brazil), using bovine serum albumin (BSA) as standard. Hippocampus extracts were diluted to a final protein concentration of 2 mg/ml in SDS-PAGE buffer and 80 mg (for pro-BDNF and BDNF) or 40 mg (for CREB and TrkB) of protein and dualcolor pre- stained molecular weight standards were separated by SDS-PAGE. BDNF and proBDNF were separated at 14% while TrkB and CREB at 8% resolving gel; all samples were run at 4% concentrating gel. After electro-transfer, the membranes were incu- bated for 1 h at room temperature with Tris-buffered saline 0.1% Tween-20 (TBS-T) containing 5% BSA. After blocking, the membranes were incubated overnight at 4 C with mouse anti-BDNF antibody (1: 1000, Sigma, São Paulo, Brazil), mouse anti- proBDNF antibody (1: 500, Sigma, São Paulo, Brazil), or rabbit anti-TrkB antibody (1: 1000; Upstate Cell Signaling, São Paulo/Brazil), or rabbit anti-CREB (1: 1000, Cell Signaling, São Paulo/Brazil). As an additional control for protein loading, membranes were incubated with mouse anti-#-actin antibody (1: 2000; Santa Cruz, São Paulo/ Brazil), mouse anti-tubulin (1: 3000; Santa Cruz, São Paulo/Brazil) or mouse anti- GAPDH (1: 2000; Chemicon,

São Paulo/Brazil). Membranes were also stained with Ponceau S. After incubation with primary antibodies, membranes were washed and incubated with horseradish-peroxidase conjugated secondary antibodies for 1 e 2h at room temperature and developed with ECL kit (Amersham, São Paulo/Brazil). The autoradiography films were scanned and densitometry analyses performed by using the public domain NIH Image Program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info. nih.gov/nih-image/).

2.6. Statistics

Open field and immunoblotting data are expressed as means ± SEM and were analyzed by two-way ANOVA, with treatments (caffeine or drinking water) versus age as factors. For inhibitory avoidance, step-down latencies are expressed as medians (interquartile ranges). Differences between training and test latencies were analyzed by Wilcoxon, and KruskaleWallis followed by Dunn's multiple comparison test was used to compare treatments. GraphPad Prism 5 (GraphPad Software, San Diego, CA USA) was used for these analyses and significant differences were considered where $p < 0.05$.

3. Results

Though no significant differences were found in the locomotor activity of rats treated with caffeine or water, two-way ANOVA revealed a significant effect of age [$F(1,57) = 25.33$; $p < 0.0001$] (Fig. 1), with middle-aged rats being less active than young adult animals regardless of treatment.

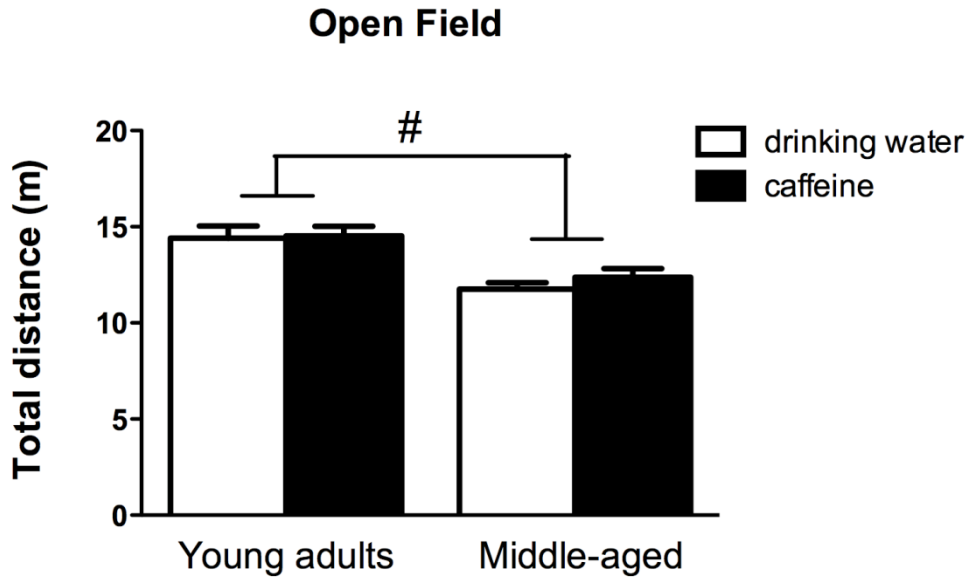

Figure 1. Effects of 30 days caffeine (1 mg/mL) in spontaneous locomotion of adult and middle-aged rats. Data expressed as media + S.E.M. of the traveled distance in meters (n = 13-16). #P < 0.05, denotes a significant difference between middle-aged and adult rats (two-way ANOVA).

Data from the inhibitory avoidance task are presented in Fig. 2. Of note, despite the aforementioned decrease in locomotion, no differences were found in training session latencies regardless of age and/or treatments ($p = 0.84$). Caffeine did not modify either the short- or the long-term step down avoidance memory performance of young rats. The control (water) middle-aged animals had a clear loss of short-term memory ($p = 0.50$; training vs. test scores), and a significant deficit of long-term memory when compared to control young adult rats ($p = 0.008$); no such decline was observed in the caffeine treated middle-aged rats.

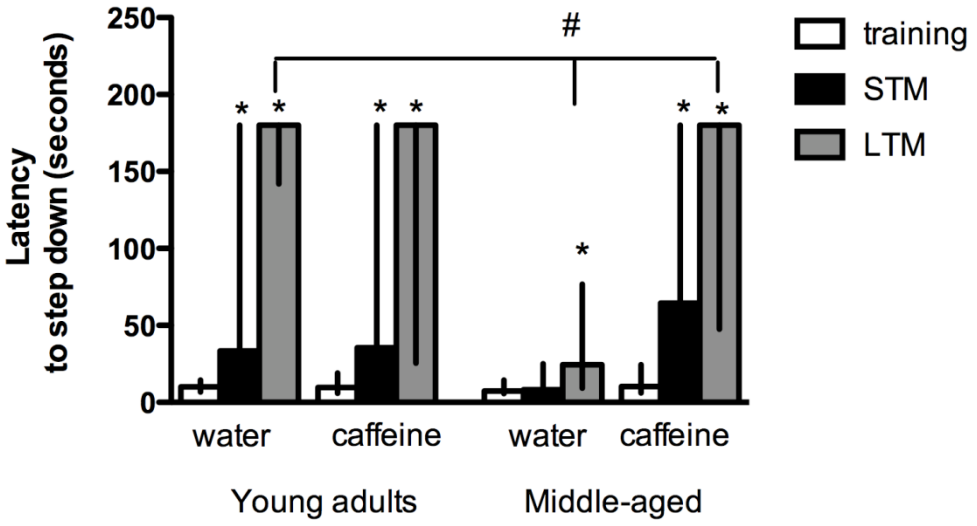

Figure 2. Effects of 30 days (1 mg/mL) caffeine intake on short and long term memories in the step down inhibitory avoidance. Results are expressed as median and interquartile range (n = 13–16). *P < 0.05, differences between latencies of training and test session (Wilcoxon). #P < 0.05, denotes significant differences between middle-aged and adult rats.

Immunoblotting analysis for BDNF revealed a significant effect of age [F(1,31) = 5.98; p = 0.0203], treatment [F(1,31) = 4.61; p = 0.0396] and the interaction [F(1,31) = 4.17; p = 0.0497] between these factors (Fig. 3A). Thus, middle-aged control rats presented a significant increase in BDNF immunocontent (~50%) compared to young adults, while this increase was no longer observed in middle-aged animals that received caffeine (Fig. 3A). As for proBDNF, the analysis revealed only a significant effect of treatment [F(1,34) = 16.30; p = 0.0003] (Fig. 3B), with caffeine increasing the hippocampal proBDNF immunocontent similarly in adult young and middle-aged rats. Regarding TrkB, two-way ANOVA revealed significant effects of age [F (1,22) = 6.35; p = 0.0195] and treatment [F(1,22) = 4.43; p = 0.0470]. TrkB immunocontent was decreased in the hippocampus of middle-aged (~25%) in comparison to young adult rats; there was a significant caffeine-induced decrease in TrkB immunocontent of comparable magnitude in animals of both ages (Fig. 3C). Finally, the data showed an age-associated increase [F(1,24) = 12.84; p =

0.0015] in CREB immunocontent, as well as a caffeine-induced increase in both young adult and middle age rats [F(1,24) = 5.91; p = 0.0229] (Fig. 3D). Representative bands from each immunoblotted protein are provided in Fig. 3E.

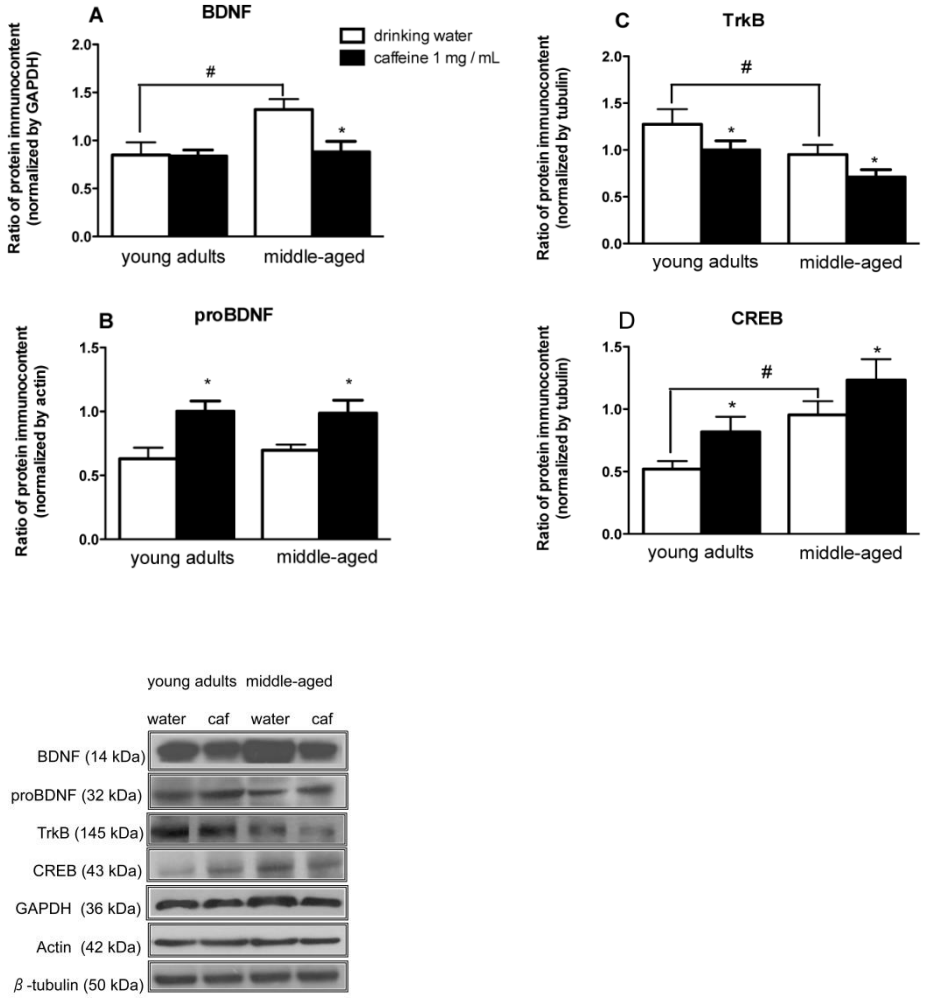

Figure 3. Immunoblotting analysis for BDNF, proBDNF, CREB and TrkB immuncontent in the whole hippocampus from adult and middle-aged rats that received drinking water or caffeine (1 mg/mL) during 30 days. Data are presented as means + S.E.M. of the density unit lines. (A) – immunoblotting for BDNF (n = 9-10). *P < 0.05, differences (two-way ANOVA). (B) – immunobloting for proBDNF (n = 9-11). *P < 0.05, difference within each age group (two-way ANOVA). (C) – immunoblotting for CREB (n = 6-8) - #P < 0.05 difference between adult and middle-aged control group. *P < 0.05, difference within and between each age group (two-way ANOVA). (D) – immunoblotting for TrkB (n = 9-10) - #P < 0.05 difference between adult and middle-aged control group. *P < 0.05, difference within each age group (two-way ANOVA). (E) – Representative bands of the immunoblotting with their respective molecular weights (kDa) for BDNF, proBDNF, CREB, TrkB and the standard proteins used for protein loading control (actin, tubulin and GAPDH). The bands were obtained from extracts of the whole hippocampus from adult and middle-aged rats that receive drinking water (water) or caffeine (1 mg/mL – caf).

4. Discussion

In this study, the effects of chronic caffeine administration on young adult and middle-aged rats were evaluated for short- and long-term memory using a step-down inhibitory avoidance task, along with a set of hippocampal proteins (proBDNF, BDNF, TrkB and CREB) relevant for learning and memory processes. Compared to young adults, middle-aged rats presented a complete short-term memory loss, as well as significant long-term memory impairment.

Differences in the magnitude of age-associated memory deficits have been reported for various experimental learning and memory tasks (Bergado et al., 2011). Though a complete loss of long term memory for inhibitory avoidance was previously observed in 12 month old rats (Moretti et al., 2011), our protocol used a higher footshock intensity, which is likely to explain these differences in memory deficit, since stronger footshocks generate longer lasting memories (Rossato et al., 2009). Middle-aged animals in our studies showed less open field exploratory activity than did younger rats, in agreement with earlier reports (Agafonova et al., 2007; Costa et al., 2012; Stefanova et al., 2010); this effect of aging was left unchanged by chronic caffeine, a result that rules out the possibility that caffeine's actions on memory are secondary to changes in locomotion.

A key and novel finding of this study is that deficits in short- and long-term memory for inhibitory avoidance found in middle-aged rats can be prevented by chronic oral intake of moderate levels of caffeine. The avoidance task is widely used to assess different types of memory associated with aversive stimuli, and is considered to be a form of learning that engages several sensory modalities including spatial and visual perception, sensitivity to pain, and emotional, fear-driven components

(Cammarota et al., 2007). Positive results for caffeine have also been reported for novel object recognition, olfactory discrimination and social preference memory impairments in aged rodents (Costa et al., 2008a; Leite et al., 2011; Prediger et al., 2005). These experimental findings when combined with the outcome of epidemiological studies (Eskelinen et al., 2009; Maia and de Mendonça, 2002; Ritchie et al., 2007) strongly suggest that long term caffeine ingestion has significant therapeutic benefits with regard to age-associated memory losses.

Although cognition enhancing properties of caffeine, typically with acute administration, have been observed in a variety of learning and memory paradigms in young rodents (Angelucci et al., 1999; Botton et al., 2010; Castellano, 1976; Costa et al., 2008b; Kopf et al., 1999; Roussinov and Yonkov, 1976), we found no evidence for this with chronic treatments. Two factors that could potentially contribute to the negative outcome are as follows. First, although experimental results are unavailable, it is possible that tolerance to caffeine (Karcz-Kubicha et al., 2003) develops at different rates, or to degrees, in young and middle-aged animals. Second, our behavioral protocols were adjusted to produce a small but significant degree of long term retention in the control (no caffeine) older rats; it is possible that these arrangements elicited near maximal scores in the young group and thus little room for enhancement. Both ideas are amenable to testing.

The data show an increase in BDNF levels in the middle-aged as compared to young adult hippocampus, while no age-related changes were observed for proBDNF. Age-related changes in hippocampal BDNF mRNA, proBDNF and BDNF immunocontent are controversial (Hattiangady et al., 2005; Kaisho et al., 1994; Katoh-Semba et al., 1998; Lapchak et al., 1993; Obiang et al., 2011; Segovia et al., 2007; Silhol et al., 2005, 2007; Tapia-Arancibia et al., 2008; Zeng et al., 2011). Pertinent to

this, several studies have shown that changes in BDNF mRNA levels are not necessarily correlated with protein content (Alonso et al., 2002; Pollock et al., 2001; Tyler et al., 2002), suggesting that BDNF is regulated at the level of translation as well as transcription. It is not possible to determine if pro and mature BDNF from intra- or extracellular pools are being immunodetected in the whole hippocampus extract samples used in this study. BDNF pools are relevant to interpreta- tion of our aging results because mature BDNF is usually thought to be derived primarily from the cleavage of proBDNF by extracellular proteases (for review see Lu et al., 2005). Nevertheless, recent data suggest that proBDNF in hippocampal neurons is rapidly converted to mature BDNF that accumulates intracellularly, with the latter being the only form secreted upon stimulation (Matsumoto et al., 2008). Currently it is unclear if BDNF processing is modified by aging, and it is not possible to conclude whether the lack of correlation between changes in proBDNF and BDNF levels found in this study reflect an increased BDNF turnover or the lack of translation of the newly synthesized mRNA.

Chronic caffeine prevented the age-related increase in BDNF in middle-aged rats, while increasing proBDNF in both young and middle-aged animals. The preventive effect of caffeine in regard to BDNF increases has also been reported for 18 month old mice (Costa et al., 2008a). The age-induced increase in BDNF in parallel with memory impairment in middle-aged rats observed in this study may suggest age-induced abnormalities in signaling by the neurotrophin, a phenomenon somehow prevented by caffeine. It has been observed that non pathological aging alters the role of BDNF on LTP, so that enhanced LTP in older animals is abolished by diminishing the TrkB receptor signaling (Diógenes et al., 2011). The same authors suggested that the beneficial effects of caffeine and A2A receptor antagonists against aged-induced

memory deficits could be due to a counteraction of dysfunctional higher tonic actions of BDNF upon plasticity (Diógenes et al., 2011). Relevant to this discussion, age-related morphological changes have been documented in hippocampus, including reductions in spine densities, synaptic loss and neuronal death (Erickson and Barnes, 2003; Halbach, 2010; Hattiangady et al., 2005; Jolitha et al., 2009), all of which are likely contributors to age-related memory deficiencies. It has been shown that uncleaved proBDNF serve as an endogenous ligand for p75NTR (Hempstead et al., 1991) which is dynamically regulated upon brain damage, suggesting that a plau- sible role of proneurotrophins is to eliminate damaged cells that express p75NTR (Lu et al., 2005). Given that the balance between cell survival and cell death might depend upon the mature/pro- neurotrophin ratio, the caffeine-induced increase in proBDNF can be understood as part of the underlying mechanism of caffeine neuroprotective effects (Chen and Chern, 2011). Therefore, reducing neurotrophin signaling abnormalities (by minimizing the age-induced increase in BDNF) as well as relevant morphological changes in hippocampus (by increasing hippocampal proBDNF levels) are arguably factors by which caffeine intake prevents age- induced memory deficits in middle-age rats.

In agreement with others (Croll et al., 1998; Silhol et al., 2005, 2008), significant age-related decreases in TrkB receptor immunocontent were detected in the hippocampus; caffeine had a lowering effect in the immunocontent regardless of age. TrkB receptors are usually internalized and rapidly degraded following activation by BDNF (Silhol et al., 2008), and it has been suggested that the age-related loss of TrkB contributes to the age-related memory disruptions presented by middle-aged rats (Croll et al., 1998). It is also conceivable that decreases in the immunocontent of the receptor could be associated with various biochemical modifications that occur

during aging (i.e. glycation, and carbonylation), which prevents its recognition by the antibody and possibly by the endogenous ligand as well (Silhol et al., 2008; Tapia-Arancibia et al., 2008). Curiously, however, neither the effects of age nor those of caffeine on the immunocontent of TrkB receptors are consistent in mice and rats. In fact, TrkB was found to be increased in aging mice (Costa et al., 2008a) while decreased in aging Fischer 344 rats (Zeng et al., 2011), though both species show age-related memory decline.

We also found that aging increases hippocampal CREB immunocontent, as did chronic treatment with caffeine. CREB is a transcriptional factor involved in LTM formation and is activated in the hippocampus during learning (Alonso et al., 2002; Finkbeiner et al., 1997; Viola et al., 2000; Yamada and Nabeshima, 2003). CREB is considered the major mediator of genomic responses to BDNF (Finkbeiner et al., 1997), consistent with the parallel increases in BDNF and CREB observed in middle-aged rats. The fact CREB and proBDNF are increased in caffeine treated rats, while BDNF is decreased, may indicate that the processing of proBDNF into BDNF is somehow affected by the methylxanthine (Connolly and Kingsbury, 2010).

Recent reports indicate that learning releases BDNF (Chen et al., 2010a), that long-term memory for inhibitory avoidance requires BDNF synthesis (Bekinschtein et al., 2007, 2008), and a wide range experiments performed in young adult and aged animals have established a crucial role for BDNF in LTP (Balkowiec and Katz, 2002; Chen et al., 1999; Diógenes et al., 2011; Gärtner and Staiger, 2002; Figurov et al., 1996; Kang et al., 1997; Pang et al., 2004). The present study was not designed to distinguish between the effects of caffeine on experience dependent vs. experience inde- pendent changes in BDNF and related proteins. Thus, the possibility that caffeine

may actually be affecting the changes in BDNF and related proteins produced by learning cannot be discarded.

5. Conclusion

Our study showed that chronic caffeine, a substance frequently consumed worldwide in various forms, prevents the age-related decline in rat emotional memory. Altogether, this study extends previous evidence suggesting a potential benefit of caffeine in relation to age-induced cognitive decline, and corroborates the key participation of BDNF-mediated signaling pathways.

Considering that caffeine consumption is widely available and culturally accepted, it deserves further exploration as a potential strategy to ultimately improve the quality of life of elderly people.

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PARTE III

3. DISCUSSÃO

O consumo de cafeína é amplamente difundido ao redor do mundo e por populações com características bastante distintas entre si, o que torna interessante a avaliação de como os efeitos desse psicoestimulante podem refletir na diversidade de cada população, como, por exemplo, seus efeitos ao longo da gestação, desenvolvimento e envelhecimento. Em vista disso, este trabalho buscou fazer uma abordagem mais abrangente, englobando a avaliação dos efeitos da cafeína nessas três populações.

No primeiro capítulo, foi realizada uma revisão da literatura em que se buscou fazer um levantamento sobre o impacto da administração de cafeína na gestação e no desenvolvimento encefálico, visto que o período de desenvolvimento, que se estende desde o período embrionário até o início da vida adulta, sofre uma série de alterações morfológicas e funcionais, que faz com que esse período seja muito vulnerável a intervenções, ao mesmo tempo em que o impacto que essas intervenções possam acarretar nesse processo minucioso e altamente organizado é muitas vezes imprevisível. Sendo assim, este trabalho buscou contemplar os estudos da cafeína sobre o seu metabolismo na população em geral, gestantes e nos fetos em desenvolvimento, assim como alterações comportamentais em estudos experimentais e os achados mais atuais dos estudos epidemiológicos, bem como a sinalização operada pela cafeína e sua influência sobre os diferentes tipos de sistemas de neurotransmissores.

Embora o metabolismo da cafeína seja realizado por uma família de enzimas do citocromo P450, principalmente pela isoforma CYP1A2, altamente expressas no fígado, existem mudanças consideráveis nesses sistemas enzimáticos durante a gravidez e no feto em desenvolvimento (Blake et al., 2005; Yu et al., 2016; Zanger e Schwab, 2013).

Estudos mostram que conforme a gestação avança, há um aumento da inibição da atividade dessas enzimas, e, associado a isso, a livre passagem que a cafeína tem através dos tecidos, inclusive da barreira placentária, leva a um prolongamento substancial do tempo de meia-vida dessa substância (Arnaud et al., 1983; Nehlig e Debry, 1994). Além disso, o feto também apresenta uma baixa atividade dessa enzima, que tende a aumentar no período pós-natal (Blake et al., 2005). Sendo assim, existem preocupações sobre o tempo de exposição prologado do feto à cafeína.

Recentemente, a EFSA (Autoridade Europeia para a Segurança dos Alimentos - do inglês *European Food Safety Authority*) emitiu um parecer sobre a segurança do consumo de cafeína em cada faixa etária, sugerindo que para a população em geral, a dose de 400 mg/dia seria considerada segura, enquanto que para grávidas essa dose seria de 200 mg/dia (EFSA, 2015). Tanto estudos experimentais quanto epidemiológicos discutem se esses efeitos da cafeína são benéficos ou deletérios a longo prazo, mas ainda permanecem não totalmente elucidados. Os estudos epidemiológicos ainda necessitam um melhor controle dos possíveis fatores de confusão, como o consumo de álcool, cigarro, assim como "os sinais de gravidez" (náuseas, vômitos), que devem ser levados em conta, visto que esses fatores podem contribuir tanto com o aumento quanto com a diminuição do consumo de bebidas que contenham cafeína (Brent et al., 2011). Sendo assim, apesar de existirem muitos estudos apontando uma série de efeitos benéficos da cafeína sobre a cognição (Fredholm, 1999), existem estudos epidemiológicos apontando possíveis efeitos prejudiciais sobre o feto em desenvolvimento (Bakker et al. 2010; Klebanoff et al., 2002; Weng et al., 2008), o único consenso passível de se afirmar é a necessidade de cautela no consumo de cafeína e de maiores estudos nessa área.

No segundo capítulo dessa tese, tendo em vista o impacto que algumas intervenções podem acarretar nos diferentes estágios do desenvolvimento encefálico, o objetivo do trabalho foi avaliar os efeitos da cafeína em ratos púberes, machos e fêmeas, expostos durante o período pré e pós-natal.

O consumo de cafeína por crianças e adolescentes, que há algumas décadas poderia ser considerado irrisório, hoje toma grandes proporções. Houve um aumento substancial no consumo de bebidas que contém cafeína, especialmente refrigerantes, e mais atualmente, as bebidas energéticas, sendo este o segmento da indústria de bebidas que mais cresce (Chandrasekaran 2006; Drewnowski e Rehm, 2016). Essas bebidas contêm grandes quantidades de cafeína, além de outros componentes, e a sua popularidade cresce principalmente entre os adolescentes. Alguns casos de morte relacionados ao uso abusivo de energéticos têm sido relatados, o que está levando a agência responsável pelo controle de indústrias alimentícias e de medicamentos do governo norte americano - FDA (- do inglês *Food and Drug Administration*) a rever o consumo de cafeína por essa população, e buscar estabelecer uma dose que possa ser consumida de forma segura, ou até mesmo sugerir a suspensão do consumo (Temple et al., 2015).

Além das preocupações com a vulnerabilidade associada a esses períodos do desenvolvimento, as questões referentes ao sexo devem ser levadas em conta. Atualmente, no campo das neurociências, há um predomínio de estudos em machos, sendo que o número desses estudos chega a ser cinco vezes maior que em fêmeas ou em ambos os sexos (Beery e Zucker, 2011; Elliot e Richardson, 2016). Entretanto, deve-se ressaltar que existem diferenças consideráveis no que diz respeito ao sexo, desde diferenças comportamentais, a aspectos biológicos e uma predisposição a determinadas doenças, sendo de grande importância avaliar esses aspectos tanto em estudos

experimentais quanto epidemiológicos (Elliot e Richardson, 2016; Shansky e Woolley, 2016). Portanto, este trabalho buscou avaliar, além dos efeitos da cafeína no decorrer do desenvolvimento encefálico, priorizar também a investigação avaliando as diferenças de sexo.

As análises comportamentais avaliadas neste estudo foram: a atividade locomotora, o comportamento relacionado a ansiedade e memória de reconhecimento. Paralelamente um conjunto de proteínas sinápticas foram imunodetectadas no hipocampo e córtex cerebral. Um dos primeiros parâmetros comportamentais que foram avaliados foi a atividade locomotora dos animais na tarefa de campo aberto. De uma forma geral, os roedores tendem a evitar áreas centrais de um novo ambiente e explorar preferencialmente a periferia, um comportamento chamado tigmotaxia, que protege os animais de predadores (Bogdanov et al., 2013; Lamprea et al., 2008; Treit e Fundytus, 1988). Baseado nisso, o tempo gasto e a distância percorrida na zona central do campo aberto são usados como parâmetros de avaliação de ansiedade, enquanto os mesmos parâmetros na periferia são utilizados na avaliação da atividade locomotora (Prut e Belzung, 2003). Assim, as fêmeas púberes apresentaram hiperlocomoção e uma atenuação da ansiedade no campo aberto em relação aos machos, evidenciado pelo aumento significativo da distância total percorrida e distância percorrida na zona central, respectivamente, o que está de acordo com outros estudos anteriores que compararam machos e fêmeas em diferentes idades (Ardais et al., 2016; Brown e Nemes, 2008; Frye e Walf, 2002; Hughes e Beveridge, 1990; Padilha et al., 2009; Simpson e Kelly, 2012). O aumento da locomoção em fêmeas em relação aos machos já foi descrito em ratos de diferentes cepas e diferentes idades, tanto na adolescência quanto na vida adulta e meia-idade (Brotto et al., 2000; Hughes e Beveridge, 1990; Padilha et al., 2009; Simpson e Kelly, 2012). Frye e Walf (2002) observaram um

comportamento menos ansioso em fêmeas adultas. Neste estudo as fases do ciclo estral de ratas fêmeas adultas foram estratificadas na tarefa de campo aberto, e foi encontrado que na fase de pró-estro (quando ocorre um pico de estrogênio) houve um aumento no número de entradas na zona central em relação às fêmeas na fase de diestro (quando ocorrem baixos níveis de estrogênio) e em relação aos machos (Frye e Walf, 2002). Além disso, um estudo com fêmeas ovariectomizadas tratadas com estradiol mostrou que estas permaneceram mais tempo na zona central do que as fêmeas do grupo controle, sugerindo que o estradiol poderia exercer efeitos ansiolíticos (Hiroi e Neumaier, 2005). Apesar de estes trabalhos terem descrito esses resultados para fêmeas em outras idades, o perfil de hiperlocomoção e comportamento menos ansioso parece estar presente já em fases anteriores do desenvolvimento, conforme os resultados do nosso trabalho.

A cafeína geralmente apresenta efeitos bifásicos na locomoção em animais adultos, com doses menores promovendo aumentos e doses mais altas diminuindo a atividade locomotora (El Yacoubi et al., 2000; Fisone et al., 2004; Marin et al., 2011; Wise, 1988). Em doses usualmente utilizadas no consumo humano, a cafeína exerce os seus efeitos primários pelo antagonismo não seletivo dos receptores de adenosina A_1 e A_{2A} (Fredholm 1999). De acordo com o estudo realizado por El Yacoubi (2000), o efeito estimulante da cafeína na locomoção em baixas doses seria pelo bloqueio dos receptores de adenosina do tipo A_{2A}, enquanto que em doses mais elevadas os seus efeitos depressores poderiam estar associados ao bloqueio do receptor A_1 (El Yacoubi, 2000).

A exposição precoce à cafeína pode ter grande impacto na atividade locomotora, A exposição pré e pós-natal à cafeína causou hiperlocomoção na puberdade de forma dependente do sexo e da dose. Nas fêmeas a hiperlocomoção foi observada em ambas as

doses de cafeína, enquanto que em machos apenas com a dose mais alta. Sabe-se que as substâncias psicoestimulantes de uma forma geral (como anfetamina, nicotina ou cocaína) podem apresentar propriedades estimulantes sobre a atividade locomotora, conforme demonstram alguns estudos em animais adultos (Caldarone et al., 2008; Catlow e Kirstein, 2005; Dietrich et al., 2004; Mori et al., 2004; Schmidt et al., 2010). Além disso, apesar da cafeína ser caracterizada como uma substância psicoestimulante, esses resultados poderiam estar associados às diferenças de sexo em resposta a drogas de abuso, onde os estudos mostram que as fêmeas são mais vulneráveis do que os machos (Anker e Carroll, 2011; Becker e Hu, 2008).

É relativamente estabelecido em roedores que as fêmeas apresentam um comportamento de medo e ansiedade atenuados comparados aos machos (Archer, 1975; Brown e Nemes, 2008; ver comentários recentes em Shansky e Wooley, 2016), e esse padrão de emotividade já pode ser observado desde cedo durante o período do desenvolvimento. As fêmeas púberes apresentaram um comportamento menos ansioso do que os machos no campo aberto, mas ambos os sexos mostraram comportamento semelhante no labirinto em cruz elevado, conforme observado anteriormente (Estanislau e Morato, 2006). A exposição à cafeína em doses moderadas no período pré e pós-natal atenuou a ansiedade no labirinto em cruz elevado apenas em fêmeas púberes, sem efeito observado para ambos os sexos no campo aberto. Curiosamente, apenas as ratas adultas expostas à cafeína em doses baixas, moderadas e altas desde o período pré-natal apresentaram menor ansiedade no campo aberto e, em altas doses, ambos os sexos apresentaram ansiedade atenuada no labirinto em cruz elevado (Ardais et al., 2016). No mesmo estudo, o tratamento com cafeína pré e pós-natal interrompido no desmame não alterou o comportamento de ansiedade na idade adulta de ambos os sexos no labirinto em cruz elevado. Independentemente das diferenças de respostas aos aparatos, os efeitos

ansiogênicos clássicos da cafeína parecem ser mais evidentes em períodos posteriores de desenvolvimento do cérebro (Bhattacharya et al., 1997; Noschang et al. 2009; Pechlivanova et al., 2012). Por exemplo, ratos machos que receberam cafeína durante o período da adolescência já mostraram uma exacerbação da ansiedade em doses baixas e moderadas (Ardais et al., 2014; O'Neill et al., 2016).

Outra importante diferença de sexo encontrada foi na memória de reconhecimento. Na tarefa de reconhecimento de objetos foram avaliadas as memórias de curta e de longa duração. Independentemente do sexo e/ou do tratamento, não foram encontradas diferenças na memória de curta duração. Machos púberes apresentaram um baixo desempenho na memória de reconhecimento de longa duração, como já havíamos demonstrado anteriormente para ratos machos adolescentes (Ardais et al., 2014), sugerindo que esse desempenho pode estar relacionado à idade. De fato, alguns estudos demonstraram a existência de uma diferença dependente da idade no desempenho na tarefa de reconhecimento de objetos, com ratos mais jovens apresentando uma diminuição no desempenho em relação aos ratos adultos (Anderson et al., 2004; Reger et al., 2009), provavelmente devido à maturação relativamente lenta dos circuitos neurais envolvidos na memória de reconhecimento neste período do desenvolvimento encefálico (Bachevalier e Beauregard, 1993).

Em contrapartida, as fêmeas apresentaram um melhor desempenho para a memória de longa duração em relação aos machos. As diferenças sexuais na aprendizagem e na memória são amplamente relatadas em estudos clínicos e pré-clínicos, e já foi demonstrado que os hormônios sexuais exercem influência em diferentes áreas cerebrais envolvidas na aprendizagem e na memória (Duarte-Guterman et al., 2015; Hamson et al., 2016; Sánchez-Andrade e Kendrick, 2011). Walf et al. (2006) demonstraram que os esteróides ovarianos melhoram a memória de

reconhecimento, tanto em fêmeas intactas quanto em fêmeas ovariectomizadas que receberam esses hormônios na tarefa de reconhecimento de objetos (Walf et al. 2006).

Embora a idade dos animais utilizados neste trabalho foi considerada como correspondente ao início da puberdade, o início exato da produção de hormônios sexuais não é conhecido e difere entre as espécies (Evans, 1986; Lohmiller e Swing, 2006; Reger et al., 2009; Sengupta 2011, 2013). Independente disso, de acordo com Colciago et al. (2015), o dimorfismo sexual no cérebro adulto é originado a partir dos efeitos dos hormônios sexuais exibidos durante janelas específicas de desenvolvimento, que em ratos correspondem ao período embrionário tardio e às primeiras semanas pós-natal, enfatizando que a influência hormonal está presente mesmo antes do nascimento. Visto que as diferenças na memória de reconhecimento que encontramos são coincidentes com muitos estudos que demonstraram os efeitos dos hormônios esteróides sobre os processos de aprendizagem e memória (Colciago et al., 2015; Duarte-Guterman, 2015 Frick, 2015; Inagaki et al., 2010; Joansson 2005; Sánchez-Andrade e Kendrick 2011; Walf et al., 2006), podemos especular que essas diferenças de sexo poderiam estar associadas a questão hormonal mesmo nessa fase inicial da puberdade.

Neste trabalho a cafeína afetou de forma diferente a memória de acordo com o sexo, sendo os machos púberes mais beneficiados pelos aprimoramentos na memória de reconhecimento em ambas as doses, semelhante aos achados anteriores em ratos adolescentes (Ardais et al., 2014), enquanto as fêmeas apresentaram uma deterioração em dose moderada. Os efeitos da cafeína parecem ser fortemente dependentes do tempo de desenvolvimento do cérebro em que a exposição ocorre. Por exemplo, ratos adultos, machos e fêmeas, expostos à cafeína, tanto na gestação quanto na lactação, apresentaram um prejuízo na memória em diferentes tarefas (Silva et al., 2013; Soellner et al., 2009). No entanto, a memória de reconhecimento não foi afetada em machos e

fêmeas expostos continuamente à cafeína desde o período pré-natal até a vida adulta (Ardais et al., 2016). Além disso, o mesmo estudo mostrou que, quando o tratamento com cafeína foi interrompido no desmame, apenas as fêmeas adultas apresentaram um comprometimento na memória de reconhecimento (Ardais et al., 2016). Em conjunto, esses achados nos levam a afirmar que a cafeína pode ser deletéria para a memória em janelas de tempo específicas do desenvolvimento cerebral, com uma clara influência do sexo.

Com o objetivo de verificar a existência de uma possível associação entre os efeitos comportamentais e alterações do imunoconteúdo de proteínas sinápticas, fizemos a avaliação do fator trófico BDNF, de sua forma imatura, juntamente com a SNAP-25 e GFAP no hipocampo e córtex. Estudos anteriores demonstraram que a cafeína alterou os níveis de BDNF em associação a mudanças comportamentais em diferentes idades (Ardais et al., 2014; Costa et al., 2008a,b; Sallaberry et al., 2013). Assim, a fim de encontrar alguma associação entre os resultados comportamentais e as diferenças de sexo no tratamento com cafeína, o BDNF e suas proteínas relacionadas foram analisadas no hipocampo e córtex cerebral. O BDNF é sintetizado como a proneurotrofina pró-BDNF (Lu et al., 2005) e sua sinalização é essencial para a memória de reconhecimento (Callaghan e Kelly, 2012; Greenberg et al., 2009).

Em relação às diferenças de sexo, as ratas púberes apresentaram um aumento nos níveis de pró-BDNF, mas não de BDNF, sugerindo que a proneurotrofina não foi necessariamente convertida na forma madura. Essas diferenças entre o pró- e o BDNF têm sido particularmente evidentes nas fêmeas durante o desenvolvimento encefálico, visto que os níveis de pró-BDNF estão inversamente relacionados aos níveis de BDNF maduro, com níveis mais elevados de pró-BDNF durante o desenvolvimento precoce e diminuições graduais após a puberdade e a idade adulta (Harte-Hargrove et al., 2009;

Yang et al., 2009). É importante ressaltar que os estrogênios podem regular a expressão de BDNF através de um elemento de resposta sensível ao estrogênio presente no gene BDNF (Sohrabji et al., 1995), mas não é bem compreendido como o estradiol pode influenciar os níveis de pró-BDNF. De fato, especula-se que os aumentos nos níveis circulantes de 17β-estradiol na puberdade seriam os responsáveis pelo aumento dos níveis de pró-BDNF, e que esta proneurotrofina teria então um papel central no desenvolvimento dos circuitos neurais, refinando dendritos e axônios e reduzindo o número de neurônios (Harte-Hargrove et al., 2009; Yang et al., 2009).

Coincidente com o aumento do pró-BDNF e do BDNF no hipocampo, a cafeína restaurou a memória de reconhecimento de longa duração em ratos machos púberes. É plausível que ambos os eventos estejam associados, visto que a sinalização do BDNF é essencial para a memória de reconhecimento de longa duração (Callaghan e Kelly, 2012, 2013; Greenberg et al., 2009), e uma associação entre ambos os eventos pode ter ocorrido. É importante observar que o pró-BDNF não é apenas uma forma precursora do BDNF desprovida de atividade, mas sim uma proteína sinalizadora com funções específicas.

Além dos fatores tróficos, também foi avaliado os níveis de GFAP, um dos principais filamentos intermediários importante na adaptação do cérebro às mudanças neurais que ocorrem no desenvolvimento. A expressão de GFAP aumenta abruptamente até PND 6, depois tende a estabilizar mas segue com aumento progressivo até PND 48 no hipocampo (Kim et al., 2011). A cafeína durante o período pós-natal foi capaz de promover diminuições nos níveis de GFAP em diferentes idades durante o desenvolvimento do hipocampo e neocórtex (Desfrere et al., 2007), e também levar a diminuições nestas áreas cerebrais de ratos machos adolescentes tratados com cafeína apenas durante este período (Ardais et al., 2014). Diferentemente dos machos adultos,

mas não das fêmeas, o tratamento pré e pós-natal da cafeína não modificou o GFAP em ambas as áreas cerebrais (Ardais et al., 2016), sugerindo que as modificações causadas pela cafeína nos níveis GFAP parecem estar ocorrendo em períodos posteriores de desenvolvimento do cérebro, e que os achados de GFAP neste trabalho podem não estar envolvidos nestes resultados comportamentais na puberdade.

A proteína associada ao sinaptossoma de 25 kDa (SNAP-25) é um componente crucial do complexo ternário SNARE (do inglês *Soluble N-ethylmaleimidesensitive factor attachment protein receptor)*, uma maquinaria necessária para a exocitose vesicular. Existem evidências substanciais de que as alterações nos níveis de SNAP-25 estão associadas com comprometimento cognitivo, hiperatividade e exacerbação de comportamentos relacionados à ansiedade (Braida et al., 2015; Hess et al., 1996; Kataoka et al., 2011). Uma vez que a cafeína em doses moderadas aumentou os níveis de SNAP-25 cortical somente nas fêmeas púberes, esse aumento pode estar associado com a piora na memória de reconhecimento e comportamento menos ansioso, dois resultados comportamentais encontrados exclusivamente em fêmeas. Embora a maioria dos estudos sugira coletivamente que níveis reduzidos de SNAP-25 possam contribuir para o prejuízo cognitivo e ansiedade, um estudo mostrou um comprometimento de memória em ratos adultos jovens após a superexpressão e atividade da SNAP-25 induzida pela infusão de um vetor de vírus adeno-associado recombinante (McKee et al., 2010). Enquanto os ratos machos púberes tratados com cafeína apresentaram níveis normais de SNAP-25, ratos machos adolescentes apresentaram ansiedade exacerbada e diminuição dos níveis de SNAP-25 no córtex após o tratamento com cafeína (Ardais et al., 2014). Além disso, ambos os sexos que receberam cafeína desde o período pré-natal apresentaram uma atenuação da ansiedade na idade adulta em altas doses de cafeína,

mas apenas os machos tiveram níveis aumentados de SNAP-25 cortical (Ardais et al., 2016).

Nos últimos anos, há um crescente número de evidências apontando para o impacto do consumo de cafeína em diferentes fases do desenvolvimento encefálico. Enquanto os ratos machos púberes foram mais beneficiados com as melhorias cognitivas proporcionadas pela cafeína do que as fêmeas, os efeitos de atenuação da ansiedade da cafeína foram observados nas fêmeas. Em relação às diferenças entre os sexos nas respostas às drogas, as fêmeas são mais vulneráveis que os machos (Anker e Carroll, 2011; Becker e Hu, 2008). Nosso estudo tentou contribuir não só para o conhecimento dos efeitos da cafeína na puberdade, mas também como um reforço para que ocorra a incorporação da diferença de sexo como uma variável biológica em investigações atuais (Shansky e Wooley, 2016). O conhecimento do impacto desse psicoestimulante, de acordo com o sexo no cérebro imaturo, é crucial para se estabelecer a dose segura.

No terceiro capítulo desta tese foram avaliados os efeitos de uma administração crônica de cafeína em animais adultos e de meia-idade sobre a memória aversiva de curta e longa duração. A proposta de avaliar uma intervenção no decorrer no envelhecimento foi inspirada no aumento constante da expectativa de vida nos dias atuais, que vem sendo acompanhada de um crescente número de casos de demência (Panza et al., 2015). Até os dias de hoje não existe tratamento para o comprometimento da memória ou das doenças que levam à demência, enfatizando a necessidade e relevância de estratégias preventivas que possam ser adotadas antes ou durante a meia-idade. Além disso, sabe-se que o processo de envelhecimento pode ser modificado por vários fatores ambientais, dos quais os componentes presentes na alimentação emergem

como os mais promissores, sendo a cafeína, de fácil consumo e acesso, uma boa alternativa.

O envelhecimento normal é associado a diversas alterações cognitivas, com uma diminuição gradual de algumas habilidades, como a memória e a velocidade de processamento de informações (Carman et al 2014; Silhol et al., 2008). Os déficits de memória comumente experimentados por pessoas idosas, também referidas como declínio cognitivo relacionado à idade (Crook et al., 1986, Rosenzweig e Barnes, 2003), têm importantes custos socio-econômicos na sociedade contemporânea. Esses déficits de memória podem ser associados a modificações estruturais e funcionais que ocorrem durante o envelhecimento, como reduções da substância branca, perda de neurônios e atrofia (Pakkenberg e Gundersen, 1997; revisado em Piguet et al., 2009; Raz et al., 2005). Visto que ainda não existe tratamento clínico eficaz para essas doenças, parte da pesquisa sobre envelhecimento tem sido conduzida com o objetivo de adquirir um melhor conhecimento de diferentes fatores (moleculares, celulares ou ambientais) que possam estar envolvidas no processo de envelhecimento (Dröge e Schipper, 2007; Froy e Miskin, 2007; Rao, 2007). Essas preocupações se estendem além das doenças que são prevalentes nos idosos, como a doença de Alzheimer (DA), pois tem sido reconhecidos que os processos patológicos que levam à demência começam décadas antes de suas manifestações clínicas (Eskelinen et al., 2009; Kivipelto et al., 2006; Nooyens et al., 2011; Panza et al 2015).

Como demonstrado anteriormente, os ratos de meia-idade apresentaram uma menor atividade exploratória em comparação aos ratos mais jovens, estando de acordo com estudos anteriores (Agafonova et al, 2001; Costa et al., 2012; Vila-Luna et al., 2012; Stefanova et al, 2010). Esse resultado já era esperado, visto que é comum em muitas espécies de animais que ocorra uma atenuação da atividade locomotora à medida

que envelhecem, devido às diversas disfunções que podem surgir, como o aumento da rigidez articular e capacidade reduzida de reparação de tecido muscular, entre outras (Horner et al., 2011). O tratamento crônico com cafeína não modificou este efeito do envelhecimento, sendo assim, esse resultado descarta a possibilidade de que as ações da cafeína sobre memória sejam secundárias a mudanças na atividade locomotora.

Nesse trabalho, a retenção da memória aversiva foi avaliada em dois momentos: primeiro aos 90 minutos após o treino para medir a memória de curta duração e novamente em 24 horas para medir a memória de longa duração. O questionamento de o fato de se treinar os animais duas vezes – primeiro 90 minutos após o treino, e novamente 24 horas depois – poderia alterar a memória de longa duração por um efeito de reforço foi descartado baseado em estudos anteriores que demonstraram que intervenções que alteram a memória de longa duração apresentavam os mesmos resultados independentemente de ter sido realizado ou não a avaliação da memória de curta duração (Cammarota et al., 2007). Além disso, os mecanismos envolvidos na formação e manutenção da memória de curta duração são separados dos envolvidos no processo de consolidação da memória de longa duração, e diversos estudos mostraram que a administração de alguns fármacos pode bloquear a formação da memória de curta duração, sem prejuízos à memória de longa duração (Cammarota et al., 2007; Izquierdo et al 2006).

Em comparação com ratos adultos, enquanto os ratos de meia-idade apresentaram um comprometimento total da memória de curta duração, os mesmos apresentaram apenas um comprometimento parcial da memória de longa duração. Diferenças na magnitude dos déficits de memória associados à idade foram relatadas para várias tarefas experimentais de aprendizagem e memória (Bergado et al., 2011). Embora tenha sido demonstrado previamente um comprometimento total da memória

de longa duração em ratos de 12 meses de idade na tarefa de esquiva inibitória (Moretti et al., 2011), nosso protocolo usou uma intensidade de choque maior, o que provavelmente explicaria essas diferenças no déficit de memória, visto que choques de maior intensidade geram memórias mais duradouras (Rossato et al., 2009).

Uma descoberta chave e inovadora deste trabalho é que os déficits de memória de curta e longa duração na tarefa da esquiva inibitória encontrados em ratos de meia-idade foram prevenidos pelo consumo crônico de cafeína em doses moderadas. Esta tarefa é amplamente utilizada para avaliar diferentes tipos de memória após um estímulo aversivo, sendo considerada uma forma de aprendizagem que envolve vários estímulos sensoriais, incluindo a percepção espacial e visual, a sensibilidade à dor e componentes emocionais e de medo (Cammarota et al. , 2007). Resultados positivos da cafeína também foram descritos no comprometimento de memória associado à idade, nos quais foram utilizadas diferentes tarefas para avaliar a memória de reconhecimento, discriminação olfatória e memória social (Costa et al., 2008a;. Leite et al., 2011.; Prediger et al., 2005). Dados do nosso grupo (Costa et al., 2008a) demonstraram que o tratamento com cafeína durante 12 meses foi capaz de prevenir o comprometimento de memória em camundongos envelhecidos quando foi avaliada a memória de reconhecimento. Da mesma forma, Leite et al (2011) demonstraram que a cafeína foi capaz de reverter o comprometimento de memória em ratos envelhecidos após uma administração sub-crônica de 10 dias. Em conjunto, os achados experimentais associados aos resultados de estudos epidemiológicos (Eskelinen et al., 2009; Maia e de Mendonça, 2002; Ritchie et al., 2007) sugerem fortemente que a ingestão prolongada de cafeína apresenta benefícios terapêuticos significativos em relação às perdas de memória associadas à idade.

As propriedades cognitivas da cafeína têm sido observadas em uma variedade de tarefas de aprendizagem e memória em ratos adultos, embora geralmente com a administração aguda (Angelucci et al., 1999; Botton et al., 2010; Castellano, 1976; Costa et al., 2008b; Kopf et al., 1999; Roussinov e Yonkov, 1976). Em contraste com os efeitos da cafeína encontrados em ratos de meia-idade, não foram encontrados efeitos sobre os ratos adultos. Dois fatores poderiam potencialmente contribuir para essa ausência de efeitos. Primeiro, é possível que uma tolerância à cafeína tenha sido desenvolvida após o tratamento crônico (Karcz-Kubicha et al., 2003) em magnitudes diferentes em animais jovens e de meia-idade, embora essa avaliação não tenha sido realizada neste trabalho. Em segundo lugar, os nossos protocolos comportamentais foram ajustados para produzir um pequeno, mas significativo grau de retenção à longo prazo nos animais do grupo controle. Pode-se especular que esses ajustes tenham levado os animais adultos a apresentar um alto desempenho na tarefa, e então, deixando pouca possibilidade para aprimoramentos.

Além da análise comportamental, assim como no segundo capítulo desta tese, foi feita também uma análise do imunoconteúdo de proteínas sinápticas importantes nos processo de aprendizagem e memória - pró-BDNF, BDNF, CREB e TrkB. O BDNF é amplamente expresso no hipocampo maduro e no córtex cerebral (Pollock et al., 2001). Para avaliar esta neurotrofina, nós quantificamos o seu imunoconteúdo no hipocampo, uma das regiões do encéfalo mais suscetível ao envelhecimento (Hattiangady et al., 2005). Neste estudo foi encontrado um aumento do imunoconteúdo de BDNF associado à idade, o que não foi surpreendente, visto que este achado é consistente com dados anteriores de nosso laboratório, em que a análise do imunoconteúdo de BDNF no hipocampo de camundongos envelhecidos com 18 meses de idade revelou um aumento robusto quando comparados com os adultos (Costa et al., 2008). No entanto, existem

controvérsias sobre mudanças relacionadas à idade na expressão e no conteúdo de BDNF no hipocampo de ratos (Hattiangady et al., 2005). Estudos anteriores demonstraram que o conteúdo de BDNF varia em ratos envelhecidos dependendo de sua cepa (Tapia-Arancibia et em., 2008). Por exemplo, Silhol et al (2005) demonstraram que ratos Sprague Dawley apresentaram uma diminuição do conteúdo BDNF no hipocampo e hipotálamo associado à idade, mas ao contrário, Katoh-Semba et al (1998) documentaram um aumento no conteúdo de BDNF no hipocampo de ratos envelhecidos da mesma cepa, ambos os estudos utilizando a mesma técnica de quantificação (ELISA). Hattiangady et al (2005) demonstraram que em ratos Fischer 344 o conteúdo de BDNF diminuiu consideravelmente assim que esses animais alcançaram a meia-idade (12 meses) no giro denteado e nas regiões CA1 e CA3 do hipocampo, sem maiores alterações durante o envelhecimento. Além disso, em ratos Wistar machos, foi relatado um aumento relacionado à idade do conteúdo de BDNF no hipocampo (Silhol et al, 2007; Segovia et al, 2007). Também em ratos Wistar, Perovic et al. (2013) demonstrou não haver um efeito da idade no conteúdo de BDNF até os 24 meses de idade, nem no córtex nem no hipocampo (Perovic et al., 2013). Considerados em conjunto, estes dados indicam que as alterações no conteúdo de BDNF durante o envelhecimento permanecem controversas.

Além disso, muitos estudos analisaram a expressão do BDNF, quantificando o RNAm e não a proteína (Lapchak et al, 1993; Kaisho et al, 1994; Hattiangady et al, 2005). É importante ressaltar que há uma controvérsia a respeito das mudanças relacionadas à idade no RNAm e no imunoconteúdo de BDNF no hipocampo (Hattiangady et al, 2005; Kaisho et al, 1994; Katoh-Semba et al, 1998; Lapchak et al, 1993; Segovia et al, 2007; Silhol et al, 2005; 2007; Tapia-Arancibia et al, 2008). Da mesma forma, vários trabalhos têm mostrado que as mudanças nos níveis de RNAm não

são necessariamente correlacionados com o imunoconteúdo das proteínas (Alonso et al., 2002; Pollock et al., 2001; Tyler et al., 2002), sugerindo que o BDNF é regulado tanto no nível de tradução, quanto no de nível de transcrição.

O tratamento crônico de cafeína preveniu o aumento do BDNF relacionado à idade em ratos de meia-idade. O efeito preventivo da cafeína no que diz respeito ao aumento do BDNF também foi demonstrado em camundongos de 18 meses de idade (Costa et al., 2008a). O aumento induzido pela idade no BDNF juntamente com os prejuízos na memória dos ratos de meia-idade nesse trabalho podem sugerir que o envelhecimento leva a anormalidades na sinalização desta neurotrofina, um fenômeno que de alguma forma foi impedido pela cafeína. Tem sido demonstrado que o envelhecimento altera o papel do BDNF na LTP, sendo que ratos velhos apresentaram um aumento da LTP relacionado ao BDNF, mas não associado a um aprimoramento na memória, ao contrário, foi associado a um prejuízo na memória no labirinto aquático de Morris (Diógenes et al., 2011). Nesse mesmo trabalho foi encontrado que um antagonista do receptor A_{2A} diminuiu de forma significativa a LTP. Baseado nesses resultados, os autores sugeriram que esse aumento da LTP relacionado ao BDNF poderia ser disfuncional, e que os efeitos benéficos da cafeína e dos antagonistas A_{2A} sobre o comprometimento da memória no envelhecimento (Costa et al., 2008a; Prediger et al., 2005) poderiam, pelo menos em parte, estar relacionadas a uma inibição dessas ações tônicas disfuncionais do BDNF sobre a plasticidade (Diógenes et al., 2011). Além disso, foram demonstradas alterações morfológicas relacionadas à idade no hipocampo, incluindo reduções na densidade sináptica, perda sináptica e morte neuronal (Erickson e Barnes, 2003; Halbach, 2010; Hattiangady et al., 2005; Jolitha et al., 2009), todos os quais provavelmente contribuem para os déficits de memória relacionado à idade.

Não foram observadas modificações relacionadas à idade no imunoconteúdo de pró-BDNF, entretanto, esse resultado é coincidente com outros estudos (Obiang et al., 2011; Perovic et al., 2013). Por exemplo, Perovic et al. (2013) avaliaram o imunoconteúdo de pró-BDNF em ratos durante o envelhecimento, aos 6, 12, 18 e 24 meses de idade, e foi encontrado que o pró-BDNF, tanto no hipocampo quanto no córtex, não é modificado com a idade até os 18 meses, só é encontrada uma diminuição aos 24 meses (Perovic al., 2011).

Mesmo sem ter sido observado um efeito da idade, o tratamento da cafeína foi capaz de aumentar o imunoconteúdo do pró-BDNF em ambas as idades. As proneurotrofinas podem desempenhar um papel crítico no direcionamento sináptico e na liberação atividade-dependente nas sinapses (Lu et al., 2005). O pró-BDNF endógeno é rapidamente convertido em BDNF, que promove a potenciação sináptica, sugerindo uma regulação bidirecional da plasticidade sináptica pelo pró-BDNF e pelo BDNF maduro (Woo et al., 2005). Visto que o receptor p75 pode ser dinamicamente regulado em danos no sistema nervoso central, uma função plausível das proneurotrofinas é também eliminar as células danificadas que expressam p75 (Lu et al., 2005). A regulação da expressão ou ativação de proteases específicas em condições fisiológicas ou patológicas específicas (estresse, envelhecimento, neurodegeneração, inflamação ou lesão) pode, portanto, ser extremamente importante na determinação das respostas celulares pró ou anti-apoptótica (Tapia-Arancibia et al., 2008). Dado que o balanço entre a sobrevivência e a morte celular pode depender da proporção de neurotrofinas maduras e proneurotrofinas, o aumento do pró-BDNF induzido pela cafeína pode ser entendido como parte do mecanismo subjacente aos efeitos neuroprotetores da cafeína (Chen e Chern, 2011). Além disso, embora o aumento do pró-BDNF causado pela cafeína em ratos adultos não tenha sido relacionado com alterações comportamentais,

em animais de meia-idade esse aumento poderia ser um aumento precoce para minimizar qualquer dano adicional associado à idade no hipocampo. Da mesma forma, o aumento de BDNF associado à idade também pode ser um sinal precoce para minimizar muitas mudanças morfológicas relacionadas à idade no hipocampo, tais como a perda sináptica seguida de morte neuronal, a redução da densidade dos espinhos dendríticos e diminuição da neurogênese no giro denteado, o que provavelmente contribui o comprometimento de aprendizagem e memória relacionadas à idade (Barnes and McNaughton, 1985; Erickson e Barnes, 2003; Halbach, 2010; Hattiangady et al., 2005; Jolitha et al., 2009). Atualmente não está claro se o processamento BDNF é modificado pelo envelhecimento, e não é possível concluir se a ausência de correlação entre as alterações nos níveis de pró-BDNF e BDNF encontradas neste estudo estariam refletindo um aumento no *turnover* do BDNF ou à falta de tradução do RNAm recém sintetizado. Alternativamente, mudanças na liberação de BDNF podem explicar essa discrepância entre BDNF e pró-BDNF (Alonso et al., 2002; Pollock et al., 2001).

Considerando que o BDNF pode desempenhar um papel no declínio cognitivo relacionado à idade, e, os seus efeitos funcionais dependem criticamente do tempo, quantidade e dos receptores aos quais se liga, é importante entender como o seu receptor de alta afinidade, TrkB, muda com a idade e com o comprometimento de memória associado à idade (Croll et al., 1998; Wei et al., 2017). De acordo com alguns autores (Croll et al., 1998, Silhol et al., 2005, 2008), diminuições significativas do receptor TrkB relacionadas com a idade foram detectados no hipocampo. A cafeína levou a uma diminuição no imunoconteúdo desse receptor independentemente da idade. Os receptores TrkB normalmente são internalizados e rapidamente degradados após a ativação pelo BDNF (Silhol et al., 2008), e foi sugerido que a perda do TrkB relacionada com a idade contribui para o comprometimento de memória apresentadas

por ratos de meia-idade (Croll et al., 1998; Diógenes 2007). Também é concebível que as diminuições no imunoconteúdo do receptor poderiam estar associadas a várias modificações bioquímicas que ocorrem durante o envelhecimento (por exemplo, glicação e carbonilação), o que impede o seu reconhecimento pelo anticorpo e possivelmente pelo ligante endógeno também (Silhol et al., 2008, Tapia-Arancibia et al., 2008). Entretanto, nem os efeitos da idade nem os efeitos da cafeína no imunoconteúdo dos receptores TrkB são coincidentes em camundongos e ratos. De forma contraditória, foi demonstrado um aumento dos níveis de TrkB em camundongos idosos (Costa et al., 2008a) enquanto foi encontrado uma diminuição no envelhecimento em ratos Fischer 344 ratos (Zeng et al., 2011), embora ambas as espécies tenham apresentado comprometimento de memória.

Além disso, deve se levar em consideração a capacidade de *downregulation* do BDNF sobre os receptores de TrkB (Binder et al, 2001;. Chen e Weber, 2004; Knusel et al, 1997). Esta capacidade pode resultar na disfunção do receptor TrkB por si só ou em um dos componentes da sua via de sinalização (Tsai, 2004), e assim, seria razoável especular que o aumento associado à idade dos níveis de BDNF levaria a *downregulation* dos receptores TrkB e redução da sua responsividade, um efeito normalizado pelo tratamento com cafeína que promoveu um impacto positivo na aprendizagem e memória.

Apesar da diminuição do imunoconteúdo de TrkB, a cafeína ainda foi capaz de melhorar o desempenho da memória nos ratos de meia-idade. Este efeito pode estar relacionado ao envolvimento dos receptores TrkB/A_{2A}. Estudos anteriores demonstraram que os efeitos neuroprotetores da cafeína parecem estar relacionados ao bloqueio preferencial dos receptores A_{2A} (Dall'Igna et al, 2003; Higgins et al, 2007; Huang et al, 2005; Silva et al., 2007) e que a função dos receptores TrkB é modulada

pela ativação de receptores de adenosina A_{2A} (Lee e Chao, 2001; Diógenes et al, 2004; Assaife-Lopes et al, 2010). Além disso, foi demonstrado que os receptores A_{2A} são capazes de transativar os receptores TrkB na ausência de BDNF (Lee e Chao, 2001). No entanto, assim como para o BDNF, ainda não foi determinado se a cafeína pode afetar algum sinal nas vias de sinalização operadas pelo TrkB.

Neste trabalho também foi encontrado um aumento do imunoconteúdo de CREB em ratos de meia-idade, assim como um aumento com o tratamento crônico com cafeína. O CREB é um fator de transcrição envolvido na formação da LTM e é ativado no hipocampo durante a aprendizagem (Alonso et al., 2002, Finkbeiner et al.,1997; Viola et al., 2000; Yamada e Nabeshima, 2003). O CREB é considerado o principal mediador da resposta neuronal ao BDNF (Finkbeiner et al., 1997), e seu aumento observado pelo tratamento com a cafeína pode contribuir para os efeitos positivos sobre a memória encontrado neste estudo.

Apesar do tratamento com cafeína aumentar os níveis de CREB e pró-BDNF em ratos adultos, não foram encontradas diferenças nas tarefas comportamentais. Talvez realizando um tempo de tratamento maior, ou mesmo em uma idade mais avançada, estes efeitos poderiam melhorar de alguma forma o desempenho desses animais em tarefas de memória.

Estudos indicam que os processos de aprendizagem estão associados à liberação de BDNF (Chen et al., 2010), que a memória de longa duração na esquiva inibitória requer síntese de BDNF (Bekinschtein et al., 2007, 2008), e uma vasta gama de experimentos realizados em animais adultos e idosos estabeleceu um papel crucial para o BDNF na LTP (Balkowiec e Katz, 2002; Chen et al., 1999; Diógenes et al., 2011; Gärtner e Staiger, 2002; Figurov et al., 1996; Kang et al., 1997; Pang et al., 2004). Em

conjunto, estes achados corroboram estudos anteriores sobre o potencial da cafeína em reverter o declínio cognitivo associado à idade com a participação das vias de sinalização mediadas pelo BDNF.

Estudos recentes têm demonstrado os efeitos do consumo de cafeína e a relação com declínio cognitivo relacionado à idade (Driscoll et al., 2016; Panza et al., 2015; Ritchie et al., 2014). Por exemplo, Ritchie et al. (2014) demonstrou que o consumo de cafeína está relacionado a uma diminuição dos níveis do peptídeo beta-amilóide, e Driscoll et al. (2016) encontrou que a exposição à cafeína, com um consumo médio de 261 mg/dia em mulheres estava associado a um menor risco de desenvolver demência ou qualquer prejuízo cognitivo com a idade, quando comparado as mulheres que tinham um baixo consumo (média de 64 mg/dia) (Driscoll et al., 2016; Panza et al., 2015; Ritchie et al., 2014). Embora ainda não existam dados conclusivos sobre os efeitos preventivos da cafeína no declínio de memória relacionado à idade e em relação às doenças neurodegenerativas, como o Alzhemeir, as pesquisas sugerem, de uma forma geral, que o consumo de cafeína pode ser uma alternativa profilática (Cunha e Agostinho, 2010).

4. CONCLUSÃO

Este trabalho apresentou evidências dos efeitos do consumo de cafeína em diferentes fases da vida de roedores, pela exposição à cafeína desde a prenhez até atingir o período da puberdade dos filhotes, e durante o envelhecimento. Ainda não está totalmente esclarecido se o consumo de cafeína é seguro durante o período gestacional, apesar de muitos órgãos regulatórios delimitarem o seu consumo diário, assim como existem questionamentos sobre a eficácia da cafeína na prevenção dos déficits de memória associado à idade e doenças neurodegenerativas.

No desenvolvimento encefálico, a cafeína apresentou efeitos dicotômicos em relação aos sexos, promovendo efeitos benéficos em machos e deletérios em fêmeas sobre a memória de reconhecimento, apresentando variações conforme a dose. As diferenças de resposta entre os sexos apresentadas neste trabalho demonstram a possibilidade de haver mecanismos distintos envolvidos nos processos de aprendizagem e memória, que poderiam estar relacionados às alterações hormonais que cada sexo apresenta de forma particular, o que enfatiza a importância de se investigar e incluir a variável sexo/gênero em pesquisas futuras.

Os avanços tecnológicos e na área da medicina têm proporcionado um aumento considerável da expectativa da vida da população mundial, que tende a ser cada vez maior. Porém, conforme a população envelhece, os impactos econômicos, sociais e de saúde pública que a sociedade enfrenta devem cada vez mais ser discutidos, a fim de que a população idosa se torne mais preparada para os desafios que irão se apresentar. Essas mudanças demográficas vêm acompanhadas de um crescimento substancial de casos de demências e doenças neurodegenerativas, tornado-se imprescindível a busca por alternativas que tratem essas condições ou mesmo previnam o seu surgimento. No

nosso estudo foi possível observar que a cafeína parece prevenir o início do declínio cognitivo associado à idade. Estas evidências abrem a possibilidade de que de fato as intervenções nos componentes da dieta possam auxiliar na melhora da qualidade de vida das pessoas em idade avançada.

O desenvolvimento e o envelhecimento são períodos distintos da vida do ser humano, que abrangem aspectos muito importantes envolvendo as mais diversas alterações neurobiológicas, estruturais e funcionais. Enquanto há uma expansão no período do desenvolvimento, com uma evolução contínua no que diz respeito a ganhos de capacidades, habilidades, aprendizados e novas descobertas, no período do envelhecimento poderia se fazer uma relação quase inversa, visto que essa fase da vida é associada a muitas defasagens, sejam elas motoras e/ou cognitivas. O aprofundamento do conhecimento de ambos os períodos é de extrema importância e relevância, seja de forma a proporcionar maiores ganhos em cada fase, seja de forma a diminuir as perdas, pois apesar de os processos que governam cada uma dessas fases sejam diferentes, ambas apresentam tanto potencialidades quanto vulnerabilidades a serem exploradas e conhecidas. Assim, o conhecimento dos potenciais efeitos da cafeína nas diversas fases da vida do ser humano deve ser explorado em mais detalhes, a fim de que se possa fazer uso dessa substância de forma a não causar danos e/ou extrair o máximo de benefícios que ela pode proporcionar frente às condições pato-fisiológicas que o ser humano pode experimentar ao longo de sua vida.

5. PERSPECTIVAS

- Fazer um estudo comparativo dos padrões comportamentais e o imunoconteúdo de proteínas sinápticas de ratos púberes expostos ao consumo materno de cafeína somente até o período de lactação e desmame, e avaliar o impacto do sexo;

- Avaliar os padrões comportamentais e o imunoconteúdo de proteínas sinápticas em idades mais avançadas, pela administração crônica de cafeína em ratos velhos;

- Analisar a morfologia hipocampal das proteínas sinápticas ratos púberes e ratos de meia-idade;

- Analisar os níveis de cafeína e metabólitos no encéfalo durante a puberdade e envelhecimento.

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7. ANEXO

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Chronic caffeine prevents changes in inhibitory avoidance memory and hippocampal BDNF immunocontent in middle-aged rats

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ABSTRACT

Beneficial effects of caffeine on memory processes have been observed in animal models relevant to neurodegenerative diseases and aging, although the underlying mechanisms remain unknown. Because brain-derived neurotrophic factor (BDNF) is associated with memory formation and BDNF's actions are modulated by adenosine receptors, the molecular targets for the psychostimulant actions of caffeine, we here compare the effects of chronic caffeine (1 mg/mL drinking solution for 30 days) on short- and long term memory and on levels of hippocampal proBDNF, mature BDNF, TrkB and CREB in young (3 month old) and middle-aged (12 month old) rats. Caffeine treatment substantially reduced i) age-related impairments in the two types of memory in an inhibitory avoidance paradigm, and ii) parallel increases in hippocampal BDNF levels. In addition, chronic caffeine increased proBDNF and CREB concentrations, and decreased TrkB levels, in hippocampus regardless of age. These data provide new evidence in favor of the hypothesis that modifications in BDNF and related proteins in the hippocampus contribute to the pro-cognitive effects of caffeine on age-associated losses in memory encoding.

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1. Introduction

The steady increase in human life expectancy has been paralleled by increasing concern with the prevalence of dementia, likewise rising substantially worldwide. These worries extend beyond the diseases that are prevalent in the elderly, such as Alzheimer's disease (AD), for it is now recognized that the pathological processes that lead to dementia start decades before its clinical manifestations (Eskelinen et al., 2009; Kivipelto et al., 2006; Nooyens et al., 2011). The mild memory deficits commonly experienced by aging people, also referred to as age-associated memory impairment (Crook et al., 1986; Rosenzweig and Barnes, 2003), have important socio-economic costs in contemporary society. To date there is no curative treatment to alleviate age-associated memory impairment or dementia-producing diseases, emphasizing the relevance of preventive strategies that could be adopted before or throughout middle age. In this context, a series of epidemiological studies including small case-control (Maia and de Mendonça, 2002), prospective (Eskelinen et al., 2009; Ritchie et al., 2007) and longitudinal studies (Jarvis, 1993; Johnson-Kozlow et al., 2002) demonstrated that caffeine intake prevents and/or delays the onset of age-related cognitive decline. In agreement with these clinical observations, a series of experiments have produced evidence that chronic caffeine ameliorates both age-related memory decline (Costa et al., 2008a; Prediger et al., 2005) and neurodegeneration (Arendash et al., 2006; Dall'Igna et al., 2003, 2007) in experimental models of aging and AD.

The mode of action of caffeine is complex, including inhibition of phosphodiesterases, antagonism of GABAA and adenosine receptors, and sensitization of induced calcium release through ryanodine-sensitive channels (Daly, 2007; Stone et al., 2009).

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