UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
DEPARTAMENTO DE BIOQUÍMICA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

Avaliação neuromotora e neuroquímica em ratos de diferentes idades submetidos ao tratamento com cafeína durante a gestação e lactação

SABRINA MIORANZZA

Orientadora:

Profa. Dra. Lisiane de Oliveira Porciúncula

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica do Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do título de Doutor em Bioquímica.

Porto Alegre

2014

Dedico esta tese aos meus filhos Elisa e Rafael, que já estão me acompanhando nessa importante fase da minha vida.

Agradecimentos

À Professora Lisiane, pelo apoio e compreensão em todos esses 8 anos de convivência a aprendizado. Muito obrigada por ter me aceitado como tua aluna desde aquele dia em que eu soube que a Bibi iria para Portugal e eu ficaria sem orientadora. Aprendi muita coisa contigo, desde os primórdios do blotting, como apresentar seminários, controlando o nervosismo, e como submeter um artigo com todas figuras em anexo, e o que fazer quando faltasse uma figura para anexar (risos).

Às minhas queridas colegas e amigas desse grupo maravilhoso que formamos nesses anos: Amanda, Ana, Cassia, Dani G., Dani M., Dani P., Déia, Fezinha, Fê Vuaden, Gabi, Jana, Nati, Paula. Obrigada por tantos momentos felizes, de amizade, companheirismo, e também por aturarem meu mau-humor e meus choros, meus gritos (óóó). Com o apoio de cada uma de vocês, consegui chegar até aqui, com o coração já apertado pela menor convivência nesses últimos meses. E aos representantes masculinos do nosso grupo, Marcelo e Paulete, por sempre estarem dispostos a ajudar e a me apoiar também. Foram tantos experimentos em dias de semana, finais de semana, feriados, regados pelo bom e velho cafezinho, com muitas gargalhadas e fofocas, e ainda tantos encontros, comemorações, com a "boca roxa" e contando histórias, que vejo o quanto vocês são importantes e essenciais em toda essa caminhada. Com certeza vocês sempre poderão contar com minha amizade, onde quer que eu esteja.

Às minhas bolsistas queridas que estão do outro lado do mundo estudando e crescendo ainda mais na pesquisa, Gabi e Déia, obrigada por sempre estarem dispostas a me ajudar e a me ensinar também. Aprendi muito com vocês. À minha bolsista Danizinha, que me ajudou muito na fase final desta tese, muito obrigada, de coração!

Aos alunos do Prof. Roska e colegas do Lab. 28 e 24, pelos anos de convivência e amizade que vai além do laboratório.

À minha família, por compreender tantas ausências em finais de semana, festas e eventos. Mesmo à distância, sempre me incentivaram a continuar esse desafio e acreditaram em mim.

À família Torres de Avila, por sempre estarem ao meu lado, me confortarem e me acalmarem, fazendo jantinhas, chazinho, tudo para me agradar e me sentir melhor. Amo vocês!

Ao meu marido Thiago, que tem sido meu maior porto seguro, compreendendo minhas ausências e minhas preocupações, tendo sempre uma palavra de conforto para continuar de cabeça erguida, independente do que acontecesse.

À secretaria do PPG Bioquímica, por sempre estarem à disposição para esclarecimento de dúvidas.

Aos meus amigos e familiares, muito obrigada por de alguma forma me apoiarem e torcerem pelo meu sucesso.

APRESENTAÇÃO

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

A Parte I contém os Resumos, a Lista de Abreviaturas, a Introdução e os Objetivos do trabalho.

A Parte II aborda essencialmente os Materiais e Métodos e os Resultados do trabalho, sendo apresentada na forma de capítulos constituídos por artigos científicos publicados e/ou em preparação. O capítulo 1 refere-se ao artigo intitulado "*The Janus face of caffeine*". *O* capítulo 2 refere-se ao artigo intitulado "*Prenatal caffeine intake differently affects synaptic proteins during fetal brain development*". O capítulo 3 refere-se ao artigo intitulado "*The impact of different doses of caffeine during rat brain development in neurobehavioral development and glutamate receptors and transporter ontogeny.*"

A Parte III constitui-se da interpretação dos resultados (Discussão), Conclusões, Perspectivas e Referências.

As sessões de introdução, discussão e conclusão encontradas nesta tese apresentam interpretações e comentários gerais sobre os resultados presentes nos artigos científicos. As referências bibliográficas adicionais ao final desta tese representam as utilizadas apenas nas partes I e III desta tese.

SUMÁRIO

PARTE I

RESUMO

A cafeína é um psicoestimulante muito consumido na dieta, tendo como principais fontes alimentares o café, o chá verde, refrigerantes de cola e energéticos. Uma vez que a cafeína ultrapassa a placenta e a barreira hematoencefálica, o consumo de cafeína durante a gestação pode afetar o feto em qualquer momento da gravidez. Alguns estudos têm mostrado que o consumo excessivo de cafeína em humanos pode estar associado com o aumento de aborto espontâneo e de baixo peso ao nascer. No entanto, é difícil de controlar fatores de confusão; dessa maneira, estudos experimentais são importantes para avaliar o efeito do consumo de cafeína no desenvolvimento encefálico. No primeiro capítulo desta tese, ratas Wistar foram tratadas com cafeína na água de beber (0,1; 0,3 e 1,0 g/L, o que corresponde à baixa, moderada e elevada ingestão, respectivamente), durante o ciclo escuro em dias de semana, duas semanas antes do acasalamento e durante toda a gestação. As ratas foram sacrificadas no dia embrionário 18 ou 20 (E18 ou E20, respectivamente), e o córtex e o hipocampo dos fetos foram dissecados para análise por Western Blot. Verificamos o imunoconteúdo cortical e hipocampal das seguintes proteínas: Proteína Associada ao Cone de Crescimento (GAP-43), Sonic Hedgehog (*Shh*), Proteína Associada ao Sinaptossoma (SNAP-25), Fator Neurotrófico Derivado do Encéfalo (BDNF) e seu receptor Tirosina Cinase B (TrkB). Além disso, foi avaliado o número de células neuronais e não-neuronais em ambas idades e regiões encefálicas pela marcação de NeuN. Em E18 no hipocampo, a maior dose de cafeína diminuiu TrkB e aumentou *Shh*, e a dose moderada (0,3 g/L) reduziu GAP-43; no córtex, houve uma diminuição de BDNF e um aumento de *Shh* nos embriões expostos à dose mais alta de cafeína nessa idade. No E20, o BDNF cortical foi aumentado pela dose mais elevada, e as doses de 0,3 g/L e 1,0 g/L de cafeína aumentaram *Shh* no hipocampo. Não houve efeito da dose mais baixa (0,1 g/L) no imunoconteúdo das proteínas estudadas em ambas as estruturas encefálicas e idades embrionárias. O número de células neuronais foi aumentado pela dose mais baixa de cafeína no córtex em E20, e a dose moderada aumentou o número de neurônios hipocampais em E18. A exposição de cafeína durante a embriogênese em roedores influenciou em modificações de proteínas cruciais para o desenvolvimento encefálico. Por isso, seria necessário analisar se estas modificações poderiam refletir em alterações pós-natais no desenvolvimento. Para isso, utilizando a mesma escala de tratamento de cafeína durante a gestação e lactação, verificamos o desenvolvimento motor e reflexo, o metabolismo da cafeína e a sinalização glutamatérgica nos dias pós-natal (DPN) 7, 14 e 21. Não houve diferença no desenvolvimento reflexo dos animais que receberam cafeína. A atividade locomotora aumentou com a idade, mas a cafeína não influenciou na locomoção. No entanto, cafeína a 1,0 g/L reduziu o peso corporal dos filhotes nos DPN 14 e 21, enquanto a exposição à 0,3 g/L de cafeína reduziu o peso corporal no DPN 21. Os níveis plasmáticos de cafeína e teobromina não foram afetados pela idade, mas encontramos interação de idade e tratamento nos níveis plasmáticos de paraxantina e teofilina. No DPN 7, a ingestão moderada de cafeína (0,3 g/L) aumentou o GLT-1 e a razão pGluN1/GluN1 no córtex, enquanto que a dose mais elevada (1,0 g/L) diminuiu o GLT-1 e aumentou a razão pGluN1/GluN1 no hipocampo; todas as doses de cafeína aumentaram o GluA1 hipocampal nesta idade. A dose moderada de cafeína aumentou o $mGlu_5$ em ambas as estruturas

encefálicas e aumentou o GluA1 no córtex no DPN 14. Cafeína a 0,3 g/L diminuiu GluA1 e $mGlu_5$ no córtex e aumentou GluN2A hipocampal no DPN 21, e a dose mais elevada também diminuiu o $mGlu_5$ cortical no DPN 21; a dose moderada reduziu o imunoconteúdo hipocampal de GluA1 e $mGlu_5$ nesta idade. A ligação específica ao glutamato (*binding*) foi transitoriamente reduzida no córtex no DPN 14 pela dose moderada (0,3 g/L), retornando a níveis semelhantes ao controle no DPN 21. Com este trabalho enfatizamos a importância de haver um controle do consumo de cafeína durante um período crítico para o desenvolvimento e maturação encefálica, no entanto mais estudos são necessários para investigar o impacto dessas alterações a longo prazo.

ABSTRACT

Caffeine is the most consumed psychostimulant worldwide, present in coffee, green tea, cola soft drinks and energy drinks. Since caffeine freely crosses placenta and blood-brain barrier during pregnancy, epidemiology studies have found association between caffeine intake and miscarriage and low birth weight; however, some confounding factors may affect the results. Therefore, animal studies are an important tool to evaluate caffeine intake during brain development. In the first chapter of this thesis, adult female Wistar rats received caffeine in the drinking water (0.1, 0.3 and 1.0 g/L) during the active cycle in weekdays, two weeks before mating and throughout pregnancy. Cerebral cortex and hippocampus from embryonic stages 18 or 20 (E18 or E20, respectively) were collected for immunodetection of the following synaptic proteins: brain-derived neurotrophic factor (BDNF), TrkB receptor, Sonic Hedgehog (*Shh*), Growth Associated Protein 43 (GAP-43) and Synaptossomal-associated Protein 25 (SNAP-25). We also estimated the number of NeuN-stained nuclei (mature neurons) and non-neuronal nuclei in both brain regions and embryonic periods. At E18 in hippocampus, high caffeine intake (1.0 g/L) decreased TrkB and increased *Shh*, whereas the moderate dose reduced hippocampal GAP-43. In whole cortex, BDNF was decreased and *Shh* was increased in fetuses exposed to the highest dose of caffeine. At E20, BDNF in whole cortex increased at the highest dose, and *Shh* in hippocampus increased at both moderate and high caffeine doses of caffeine. The lowest dose of caffeine has no effect in all proteins assessed in whole cortex and hippocampus for both embryonic stages. NeuN-stained nuclei was increased in the cortex at E20 and in the hippocampus at E18 by lower and moderate doses of caffeine, respectively. Caffeine exposure during rat embryogenesis modified key proteins in brain development. Therefore, it is essential to evaluate whether these changes may reflect in alterations postnatally. For this, using the same protocol of caffeine administration, we studied whether different doses of caffeine intake during pregnancy and lactation may affect reflex and motor development, caffeine metabolism, ontogeny of glutamate receptors and transporter and glutamate *binding* in cortex and hippocampus in pups at post-natal days (PND) 7, 14 and 21. We did not find differences in reflex development. However, the highest dose decreased body weight from PND 14 up to weaning (PND 21), whereas 0.3 g/L caffeine decreased body weight at PND 21. Locomotor activity increased with age, but it was not modified by caffeine at any dose. Caffeine and theobromine plasma levels were unaffected by age, but there was interaction between age and dose in plasma levels of paraxanthine and theophylline. At PND 7, moderate caffeine intake (0.3 g/L) increased GLT-1 and pGluN1/GluN1 ratio in whole cortex, whereas the highest dose decreased GLT-1 and increased pGluN1/GluN1 ratio in hippocampus. All doses of caffeine increased hippocampal GluA1 at this age. Moreover, moderate dose of caffeine increased $mGlu_5$ in both brain structures and increased GluA1 in whole cortex at PND 14. Caffeine 0.3 g/L decreased cortical GluA1 and $mGlu_5$ and increased hippocampal GluN2A at PND 21. The highest dose also decreased cortical $mGlu_5$ at this age. Conversely, the moderate dose decreased GluA1 and $mGlu_5$ in hippocampus at this age. Glutamate binding were transiently reduced in cortex from rat pups exposed to the lowest dose of caffeine up to PND 14, but it was normalized at PND 21. This work emphasizes the importance of controlling caffeine intake during critical periods of rat brain

development and maturation, and more research is needed to evaluate the impact of these changes later in life.

LISTA DE ABREVIATURAS

A_1: Receptor de adenosina do tipo 1

A_{2A}: Receptor de adenosina do tipo 2A

A_{2B}: Receptor de adenosina do tipo 2B

A_3: Receptor de adenosina do tipo 3

Akt: Proteína cinase B

AMPA: Ácido α-amino-3-hidróxi-5-metilisoxazol-4-propiônico

AMPc: AMP cíclico

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

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

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

CYP450: Citocromo P 450

CYP1A2: Subunidade do citocromo P 450

EAAC: Transportador de glutamato (do inglês *Excitatory Amino Acid Carrier*)

EAAT: Transportador de glutamato (do inglês *Excitatory Amino Acid Transporter*)

ERK: Proteína cinase extracelular regulada (do inglês *Ras/extracellular signal-regulated kinase*)

FDA: Food and Drug Administration

GAP-43: Proteína associada ao cone de crescimento de 43 kDa (do inglês *Growth-Associated Protein 43*)

GLAST: Transportador de glutamato (do inglês *Glutamate aspartate transporter*)

GluA1: Subunidade A1 do receptor AMPA

GluA2: Subunidade A2 do receptor AMPA

GluA3: Subunidade A3 do receptor AMPA

GluA4: Subunidade A4 do receptor AMPA

GLT-1: Transportador de glutamato (do inglês *Glutamate transporter subtype 1*)

KA: Cainato (do inglês *kainate*)

MAPK: Proteína cinase ativadora de mitógeno (do inglês *Mitogen-activated protein kinase*)

$mGlu_1$: Receptor metabotrópico de glutamato 1

$mGlu_2$: Receptor metabotrópico de glutamato 2

$mGlu_3$: Receptor metabotrópico de glutamato 3

$mGlu_4$: Receptor metabotrópico de glutamato 4

$mGlu_5$: Receptor metabotrópico de glutamato 5

$mGlu_6$: Receptor metabotrópico de glutamato 6

$mGlu_7$: Receptor metabotrópico de glutamato 7

$mGlu_8$: Receptor metabotrópico de glutamato 8

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

NMDA: N-metil-D-aspartato

GLAST: Transportador de glutamato (do inglês *Glutamate–aspartate Transporter*)

GLT-1: Transportador de glutamato-1 (do inglês *Glutamate Transporter*)

GluN1: Subunidade 1 do receptor de NMDA

GluN2A: Subunidade 2A do receptor de NMDA

GluN2B: Subunidade 2B do receptor de NMDA

GluN3: Subunidade 3 do receptor de NMDA

NT-3: Neurotrofina-3

NT-4/5: Neurotrofina-4/5

RNAm: RNA mensageiro

SNC: Sistema nervoso central

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

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

Shh: Sonic Hedgehog

TrkA: Receptor tirosina cinase do tipo A (do inglês *Tyrosin kinase A*)

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

TrkC: Receptor tirosina cinase do tipo C (do inglês *Tyrosin kinase C*)

1. INTRODUÇÃO

1.1 CAFEÍNA

1.1.1 Classificação e histórico

A cafeína (1,3,7-trimetilxantina) é um alcaloide do grupo das metilxantinas, que são metabólitos secundários de plantas obtido do metabolismo de nucleotídeos de purinas (Ashihara e Cozier, 1999). As metilxantinas são encontradas em quase 100 espécies de plantas diferentes, embora altas concentrações desses compostos estejam restritas a algumas espécies, como *Coffea arabica* (café), *Camellia sinensis* (chá verde) e *Theobroma cacao* (cacau) (Ashihara e Cozier, 1999).

A história do uso da cafeína está fortemente ligada à história do café, principal fonte natural de cafeína. Admite-se que o café, tal como se conhece nos dias de hoje, seja originário da Etiópia tendo-se difundido na península arábica e daí para o resto do mundo. Uma lenda popular atribui a sua descoberta a um pastor de cabras Etíope, chamado Kaldi, que teria percebido que o rebanho ficava agitado após alguns animais terem comido as frutas de um arbusto. Kaldi também quis provar os bagos dessa misteriosa planta e foi imediatamente invadido por uma euforia tendo, nessa noite, dormido menos que o costume. Ele informou ao abade de um mosteiro local o ocorrido e a partir de então começou a conquista pelo território (Weinberg e Bealer, 2001). A palavra "café" deriva da palavra árabe *qahva* (ou *qahwah*), que significa uma bebida feita a partir de plantas. Apesar de ser uma planta originária da Etiópia, foi a Arábia a responsável pela propagação da cultura do café. Somente no século XIV, na

Pérsia, os primeiros grãos de café foram torrados para se transformar em bebida (Weinberg e Bealer, 2001).

Na Europa, o café apareceu no século XVI sendo introduzido, principalmente, pelos espanhóis e holandeses no período das descobertas. A partir da segunda metade do século XVII, principalmente nas grandes cidades o café passou a ser considerado uma bebida de intelectuais, e em países como a França as cafeterias se tornaram locais de reunião de pensadores, entre eles Victor Hugo, Voltaire e Rousseau (Ukers, 1922; Weinberg e Bealer, 2001).

As primeiras sementes de café chegaram ao Brasil em 1727 no Estado do Pará. Devido às condições climáticas favoráveis, o cultivo de cafeeiros se espalhou rapidamente. Em sua trajetória pelo Brasil o café passou pelo Maranhão, Bahia, Rio de Janeiro, expandindo-se pela Serra do Mar até atingir o Vale do Paraíba. A cultura espalhou-se para todo o Sudoeste Brasileiro, passando de uma posição relativamente secundária para a de produto-base da economia brasileira em 1780, destacando-se como o mais importante da história do Brasil (Carvalho, 1993). A exportação brasileira do café começou a crescer a partir de 1816. Na década de 1830-1840, o produto assumiu a liderança das exportações do país, com mais de 40% do total. Em 1840, o Brasil tornou-se o maior produtor mundial de café. Deu-se início ao período áureo do ciclo de café em que as exportações chegaram a representar 70 % das exportações do país (Taunay, 1939).

1.1.2 Principais fontes alimentares e consumo no mundo

A cafeína é uma das substâncias psicoestimulantes mais consumidas mundialmente, presente em bebidas como café, chá, refrigerantes, energéticos

e em alguns alimentos. O teor de cafeína desses alimentos varia de 40-180 mg/150 mL para o café, de 24-50 mg/150 mL para o chá, de 15 a 29 mg/180 mL para bebidas à base de cola, 2-7 mg/150 mL de cacau, e 1 a 36 mg/28 g para o chocolate (Barone e Roberts, 1996; Debry, 1994). O consumo de cafeína a partir de todas as fontes pode ser estimado para cerca de 70-76 mg/pessoa/dia em todo o mundo (Fredholm et al., 1999), mas atinge 210-238 mg/dia nos EUA e Canadá e mais de 400 mg/pessoa/dia na Suécia e Finlândia, onde 80 a 100% do consumo de cafeína é proveniente do café (Barone e Roberts, 1996; Debry, 1994; Viani, 1996, Figura 1). No Reino Unido, o consumo é tão elevado como na Suécia e na Finlândia, porém 55% é advinda do consumo de chá, 43% do café, e 2% de bebidas à base de cola (Barone e Roberts, 1996).

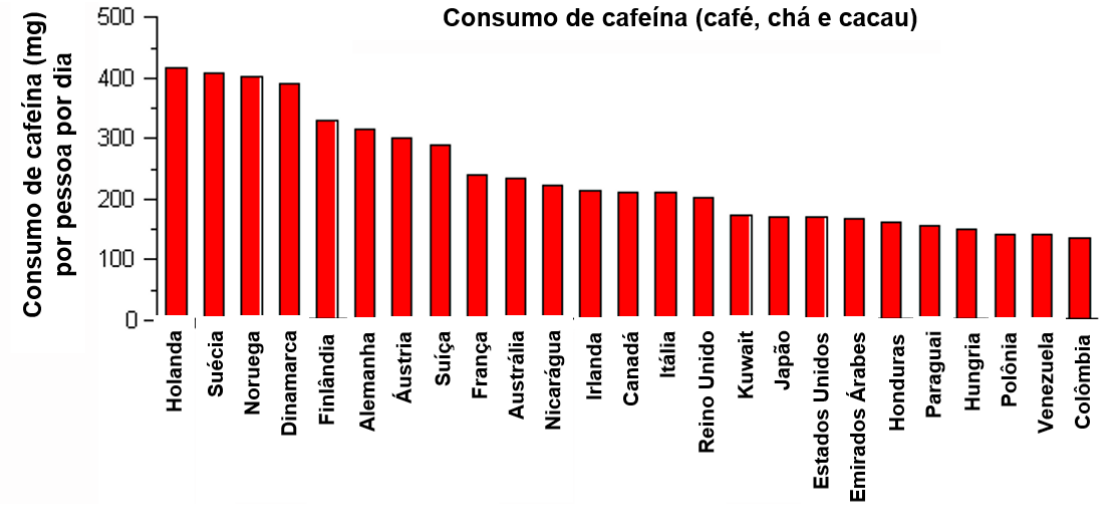


Figura 1. Consumo de cafeína (café, chá, mate e chocolate) por pessoa por dia, em mg. (Adaptado de Fredholm et al., 1999).

No Brasil, dados provenientes da Pesquisa de Orçamentos Familiares de 2008-2009 demonstrou que o consumo médio de cafeína *per capita* (diário) no país foi estimado em 115,57 mg. Moradores da região Sul apresentaram maior ingestão média (128,35 mg) e da Região Norte, menor (99,67 mg) (Sartori e da

Silva, 2013). A tabela 1 representa o conteúdo de cafeína em alimentos consumidos pela população brasileira.

Tabela 1. Conteúdo de cafeína em alimentos populares, refrigerantes e energéticos (Altimari et al., 2001).

Café (xícara de 150 mL)	**Cafeína (mg)**
Expresso	110-150
Coado	64-124
Instantâneo	40-108
Descafeinado instantâneo	2-5
Chá (granel ou saquinhos – xícara de 150 mL)	
Infusão de um minuto	9-33
Infusão de três minutos	20-46
Infusão de cinco minutos	20-50
Chá instantâneo	12-28
Outros produtos	
Refrigerante à base de cola (350 mL)	46
Bebidas energéticas (250 mL)	80
Chocolate ao leite (28 g)	6
Chocolate de confeiteiro (28 g)	35

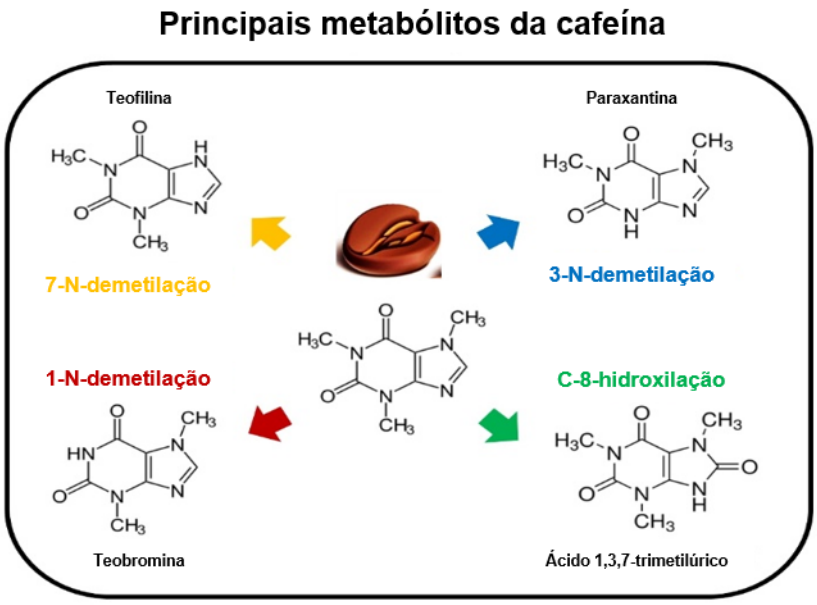
1.1.3 Metabolismo

A cafeína é rápida e completamente absorvida pelo trato gastrintestinal, e a excreção é predominantemente renal (Yesair et al., 1984). A biodisponibilidade da cafeína (5mg/kg) em humanos adultos mostrou uma rápida absorção oral, atingindo o pico plasmático em aproximadamente 30 minutos (Blanchard e Sawers, 1983). Para doses inferiores a 10 mg/kg, a meia-vida da cafeína varia de 0,7-1,2 h em ratos e camundongos, de 3 a 5 h em macaco (Bonati et al., 1984) e de 2,5 a 4,5 h em humanos (Arnaud, 1987). A absorção, a biodisponibilidade e a excreção urinária e fecal de cafeína não mostraram diferenças entre espécies (Arnaud, 1985; Arnaud et al., 1989; Walton et al., 2001). As diferenças entre espécies têm sido relatadas para a farmacocinética, que em animais é dose-dependente devido à saturação da transformação metabólica da cafeína (Bortolotti et al., 1985). A ligação da cafeína à albumina é baixa (10-30%), e seu

volume de distribuição varia de 0,5 a 0,75 L/kg em humanos e 0,9 L/kg em ratos, o que indica que é amplamente distribuída em todo o corpo (Bonati et al., 1984). Devido ao seu caráter lipofílico, a cafeína passa livremente pelas membranas biológicas. Durante a gestação, parece que nenhuma barreira fisiológica limita a passagem de cafeína pelos tecidos, pois um rápido equilíbrio é atingido entre a mãe e o feto, no sangue e em outros tecidos. No entanto, a cafeína atravessa a barreira hemato-encefálica por difusão simples e por transporte mediado por carreadores (McCall et al., 1982). Em humanos adultos, menos de 2% do composto ingerido é excretado na urina como cafeína (Arnaud, 1987; Somani e Gupta, 1988).

A cafeína é metabolizada no fígado pelo sistema da enzima oxidase do citocromo P450 (CYP450), formando as dimetilxantinas teobromina, teofilina e paraxantina. A principal isoenzima do complexo CYP450 responsável pelo metabolismo da cafeína é a CYP1A2; por isso, a cafeína é comumente utilizada como um marcador da atividade dessa enzima *in vivo* (Nordmark et al., 1999). O metabolismo de cafeína é dependente da maturação desta enzima, visto que a maturação, a expressão e a atividade desta enzima é altamente dependente do desenvolvimento (Leeder, 2001). De um modo geral, uma baixa funcionalidade do citocromo foi detectada no feto, com o aumento da atividade aparentemente desencadeada por exposição pós-natal (Blake et al., 2005). Nos seres humanos e em animais, as metilxantinas atravessam facilmente a barreira placentária e todos os tecidos, podendo afetar o feto/recém-nascido em qualquer momento durante a gestação ou logo após o nascimento (Abdi et al., 1993; Arnaud et al., 1983; Kimmel et al., 1984).

Em humanos, a cafeína é demetilada a seus intermediários dimetilxantinas, sendo que mais de 80% da cafeína administrada por via oral é metabolizada a paraxantina (1,7-dimetilxantina), e cerca de 16% é convertida em teobromina (3,7-dimetilxantina) e teofilina (1,3-dimetilxantina) (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 trimetílicos, produtos da hidroxilação da cafeína, correspondem a 40% dos metabólitos da cafeína em ratos, em comparação a 6% em seres humanos (Arnaud, 1985) (Figura 2).



Comparação entre metabolismo humano e de roedores

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

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

Em humanos, o metabolismo da cafeína se desenvolve durante o primeiro ano de vida (Pearlman et al., 1989), e pode ser influenciado por vários fatores (Aldridge et al., 1979). 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, revelando que o metabolismo da

cafeína se desenvolve 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).

Embora permaneça indefinido se a ingestão de cafeína durante a gestação representa um perigo fetal, existe uma preocupação considerável com o uso generalizado de cafeína, devido à sua meia-vida prolongada durante este período: durante o primeiro trimestre, a meia-vida da cafeína varia de 2 a 4,5 horas, mas aumenta para 10 horas na 17ª semana gestacional e acima de 18 horas a partir da 18ª semana de gravidez (Aldridge et al., 1981).

1.1.4 Mecanismo de ação

A ação farmacológica da cafeína consiste no bloqueio dos receptores de adenosina, um nucleosídeo que no sistema nervoso central (SNC) atua como um neuromodulador controlando a liberação de neurotransmissores, a excitabilidade neuronal e o ritmo circadiano por meio de seus receptores metabotrópicos A_1, A_{2A}, A_{2B} e A_3 (Cunha, 2001; Fredholm et al., 2005). Os receptores de adenosina do tipo A_1 e A_{2A} são os mais expressos no SNC, e são os alvos farmacológicos da cafeína (Fredholm et al., 1999; Nehlig et al., 1992). 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). No SNC a cafeína é um potente estimulante aumentando o estado de alerta, diminuindo o sono e a sensação de fadiga (Rogers et al., 2005).

1.1.5 Desenvolvimento do Sistema Nervoso Central – comparação entre humanos e roedores

Uma das principais razões para relacionar os resultados em experimentos em animais com os realizados em humanos é a ideia de que o sistema nervoso de diferentes espécies evolui de ancestrais comuns e que pode apresentar mecanismos moleculares semelhantes. Dentro desse contexto, por exemplo, em experimentos que estudam a influência de drogas psicoativas no sistema nervoso central (SNC), os ratos mostram sinais de dependência química quando submetidos a protocolos de auto-administração de cocaína. Dessa maneira, ratos são excelentes modelos para estudos que visam a compreender como as drogas psicoativas exercem seus efeitos sobre o SNC (Bear et al., 2002).

Considerando o desenvolvimento 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 (Clancy et al., 2001). O nível de maturação do sistema nervoso em um humano recém-nascido corresponde ao dia pós-natal 10 em ratos (Tchekalarova et al., 2005; Williams, 2008). O pico de neurogênese ocorre no final do período embrionário, o pico de crescimento axonal por volta de 7 dias pós-natal e o pico de crescimento dendrítico durante a segunda semana pós-natal (Clancy et al., 2001; Ramsdell, 2010). 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). O esquema abaixo mostra a comparação do período de desenvolvimento do SNC em ratos e humanos (Figura 3).

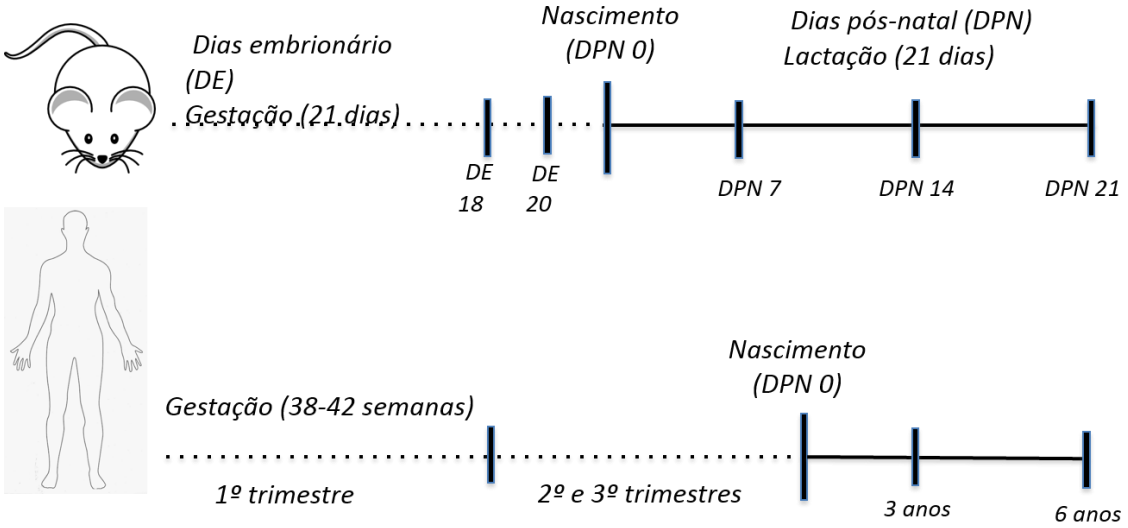


Figura 3. Comparação do período de desenvolvimento do SNC durante a gestação e lactação em ratos e em humanos. DE: dia embrionário; DPN: dia pós-natal (Adaptado de Clancy et al., 2001; Marco et al., 2011; Tchekalarova et al., 2005; Williams, 2008).

Filogeneticamente, o estriado e áreas motoras corticais desenvolvem-se mais cedo do que as regiões do cérebro associadas à cognição (Johnson, 2003; Thompson et al., 2000). Mesmo para regiões corticais, há um tempo diferenciado de desenvolvimento, sugerindo que cada região do cérebro tem um caminho ou trajetória especial de desenvolvimento. Como resultado da maturação, o funcionamento de cada região do cérebro e sua conectividade com outras regiões reflete em uma mudança do desenvolvimento regional. Comparativamente, este processo ocorre em todos os mamíferos, com relativamente os mesmos marcos de desenvolvimento do início da puberdade ou o aparecimento de níveis elevados de cognição. Este período pós-natal prolongado de desenvolvimento torna áreas específicas do cérebro mais ou menos sensíveis aos efeitos agudos e permanentes de estimulantes

dependendo da idade em que os estimulantes são administrados. Assim, a cafeína como um psicoestimulante provoca mudanças evidentes no comportamento dos animais. Considerando a exposição pré-natal ou pós-natal, alguns fatores podem interferir com os efeitos da cafeína sobre o comportamento, como protocolo de dose-resposta, tempo de exposição e idade em que os animais são testados (Porciúncula et al., 2013).

1.1.6 Estudos epidemiológicos – cafeína na gestação

O interesse pelo estudo da cafeína durante o período gestacional começou em meados da década de 70, quando estudos em animais indicaram que a cafeína estaria relacionada a uma diminuição no crescimento intra-uterino, redução do peso ao nascer e anormalidades esqueléticas (Dlugosz e Bracken, 1992; Heller, 1987). Em 1980, com base nos achados de estudos em animais, a agência do governo americano responsável pelo controle de indústrias alimentícias e de medicamentos, o FDA (do inglês *Food and Drug Administration*), desaconselhou o consumo de alimentos e bebidas que contenham quantidades significativas de cafeína especialmente durante o primeiro trimestre gestacional (Goyan, 1980). Mesmo que naquela época ainda fossem incipientes os estudos sobre as implicações do consumo de cafeína para a saúde do feto, a partir dessa determinação da FDA uma série de estudos têm sido feitos tentando estabelecer se o consumo de cafeína pode ser recomendado e a dose diária adequada. De fato, a grande dificuldade de muitos estudos epidemiológicos é monitorar com maior confiabilidade os efeitos do consumo de diferentes doses de cafeína durante o período gestacional sobre o feto e o recém-nascido (Boylan et al., 2008). Sabe-se que a cafeína atravessa facilmente

a barreira da placenta para o feto, mas as gestantes metabolizam mais lentamente essa substância, e o feto ainda não possui totalmente expressas as enzimas necessárias para a metabolização completa (Aldridge et al., 1979; 1981).

Os estudos epidemiológicos sobre os efeitos da cafeína no desenvolvimento do feto parecem indicar associação somente com consumo elevado. O consumo de cafeína acima de 300 mg por dia durante o período gestacional pode estar associado a uma maior incidência de aborto espontâneo (Cnattingius et al., 2000; George et al., 2006; Giannelli et al., 2003; Wen et al., 2001). Entretanto, após ajuste de vieses como os sintomas de gravidez (náusea e vômitos), não há aumento do risco (Maconochie et al., 2007; Weng et al., 2008). A discrepância dos achados epidemiológicos atuais com os anteriores também reside no fato de não se ter avaliado que muitas mulheres que ingerem quantidades maiores de cafeína também são tabagistas, ingerem bebidas alcoólicas e o nível de escolaridade é predominantemente baixo (Fenster et al., 1991). Tabagistas tendem a consumir mais cafeína porque a metabolizam mais rápido do que não-fumantes. Dessa maneira, é importante verificar todos os fatores de confusão que podem influenciar na associação do consumo de cafeína.

Estudos epidemiológicos relacionados à associação entre redução no crescimento fetal e baixo peso ao nascer e consumo de cafeína durante a gestação (acima de 300 mg/dia) mostraram aumento do risco de nascimento de bebês pequenos para a idade gestacional (Care Study Group, 2008; Hoyt et al., 2014; Sengpiel et al., 2013; Xue et al., 2008). No entanto, a redução no peso ao nascer tem baixa relevância clínica; por exemplo, Infante-Rivard (2007) revelou

uma redução de 31 a 38 g de peso corporal do feto para cada 100 mg de cafeína consumida durante o segundo e terceiro trimestres; por outro lado, outros estudos não encontraram associação no baixo peso ao nascer e consumo de cafeína, até mesmo consumo elevado (Bracken et al., 2003; Clausson et al., 2002; Grosso et al., 2001).

Ainda não existe um consenso na comunidade científica sobre os efeitos do consumo de cafeína em doses moderadas durante a gestação, pois muitos estudos não encontraram nenhum efeito significativo quando analisado o crescimento intrauterino, o peso do bebê após o nascimento e a duração do período gestacional (Bracken et al., 2003; Clausson et al., 2002). Entretanto, a maioria dos médicos desaconselha o consumo habitual de bebidas e alimentos que contenham quantidades significativas cafeína especialmente no primeiro trimestre da gestação.

Além do controle dos fatores de confusão, tais como fumo e álcool, de acordo com Brent et al. (2011), é importante considerar “o sinal de gravidez”, que consiste em sintomas (náuseas e vômito) que pode ocorrer no início da gestação devido aos altos níveis séricos de gonadotrofina coriônica (HCG). Esses sintomas podem contribuir para a redução do consumo de bebidas que contenham cafeína, o que pode significar um efeito protetor do “sinal de gravidez” (Brent et al., 2011).

Por ser difícil controlar todos os fatores de confusão em um estudo epidemiológico, alguns estudos são bem sucedidos em controlar um fator, enquanto outros consideraram outros fatores de confusão. Ainda não há um consenso sobre a quantidade segura de ingestão de cafeína durante todo o período gestacional. Desta forma, é necessária a realização de estudos em

animais, nos quais muitos fatores de confusão podem ser facilmente controlados.

1.1.7 Efeitos do consumo perinatal de cafeína em roedores

No que diz respeito ao desenvolvimento do SNC no embrião, a cafeína pode acelerar a formação de vesículas do telencéfalo (Sahir et al., 2000). Estudos *in vitro* utilizando culturas de astrócitos e neurônios tratados com cafeína apresentaram um aumento na expressão do gene para a *Sonic hedgehog*, um morfógeno expresso no SNC em desenvolvimento, cuja função é promover, por exemplo, a diferenciação dos neurônios motores da medula espinhal (Sahir et al., 2004). O tratamento com cafeína durante o período gestacional influencia alguns sistemas de neurotransmissão e neuromodulação cerebrais de ratos, pois fêmeas tratadas durante o período gestacional com cafeína apresentam aumento nos níveis de adenosina endógenos e diminuição na densidade dos receptores de adenosina do tipo A_1 bem como os seus fetos (León et al., 2002; 2005). Além do sistema adenosinérgico, outros sistemas de neurotransmissores podem ser modulados pela administração de cafeína, tais como o sistema glutamatérgico e colinérgico, entre outros. Neste sentido, León et al. (2005), reportou que o imunoconteúdo dos receptores de glutamato metabotrópicos do grupo I foram diminuídos pelo tratamento com cafeína nos encéfalos dos fetos e das fêmeas no final da gestação (León et al., 2005). Adicionalmente, um estudo mostrou que o tratamento com cafeína durante a gestação pode afetar o sistema colinérgico, visto que os animais aos 21 dias de idade apresentaram um aumento na atividade da acetilcolinesterase no hipocampo (da Silva et al., 2008).

Animais neonatos tratados com cafeína também apresentam alterações comportamentais e nos padrões de expressão de proteínas importantes para o funcionamento do SNC. Ratos neonatos que foram tratados com cafeína apresentaram hiperalgesia, menos ansiedade, mas não desempenharam satisfatoriamente uma tarefa que envolve aprendizado e memória (Pan e Chen, 2007). Análises morfológicas de ratos adultos que foram tratados com cafeína durante os primeiros doze dias de vida revelam um aumento no comprimento dos dendritos dos neurônios piramidais do córtex pré-frontal (Juárez-Méndez et al., 2006). Proteínas astrocitárias também 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).

1.2 VIAS DE SINALIZAÇÃO CRUCIAIS PARA O DESENVOLVIMENTO E MATURAÇÃO DO SNC

É essencial o correto estabelecimento de conexões neuronais no sistema nervoso central durante o desenvolvimento, e essas são geradas por meio de uma sequência de eventos que envolve diferenciação e crescimento de axônios e dendritos, inervação alvo, morte celular, sinaptogênese e refinamento sináptico. Muitos fatores são cruciais para este processo de desenvolvimento, dentre eles as neurotrofinas (Zweifel et al., 2005).

1.2.1 BDNF

O primeiro membro da família de neurotrofinas foi o fator de crescimento neurotrófico (NGF, do inglês *Neuronal Growth Factor*), e foi descoberto no início dos anos 1950 como uma proteína que promove a sobrevivência e crescimento de neurônios do sistema simpático e sensorial durante o desenvolvimento (Cohen et al., 1954). O estabelecimento da família de neurotrofinas surgiu com a purificação e caracterização do fator neurotrófico derivado do encéfalo (BDNF, do inglês *Brain-Derived Neurotrophic Factor*) do encéfalo de porco, que tem um efeito neurotrófico semelhante em neurônios sensoriais cultivados, e sua sequência de aminoácidos é altamente homóloga à NGF. As ações celulares das neurotrofinas são mediadas por dois tipos de receptores – um receptor tirosina-cinase de alta afinidade (Trk) e um receptor de pan-neurotrofina de baixa afinidade (p75). Cada Trk é referencialmente ativada por um ou mais neurotrofinas - TrkA por NGF, TrkB pelo BDNF e NT-4/5, e TrkC por NT-3 – e é responsável por mediar a maioria das respostas celulares (Greene e Kaplan, 1995), enquanto o receptor p75 forma um complexo com o receptor do Trk e modula a sua tradução (Barker e Shooter, 1994).

As neurotrofinas são sintetizadas como pré-proproteínas por ambos os tipos de células neuronais e não neuronais (Seidah et al., 1996; Thoenen, 1991). As proteínas produzidas pelos genes que codificam neurotrofinas contêm um péptido sinal para a secreção de proteínas (pré-proteína) e a proteína precursora (pró-proteína). Quando a região hidrofóbica do péptido sinal é removida a partir da pré-proneurotrofina no N-terminal, a pró-neurotrofina é gerada (Bartkowska et al., 2010).

Após a síntese no retículo endoplasmático, o pró-BDNF (precursor do BDNF) liga-se à sortilina intracelular do complexo de Golgi para facilitar o empacotamento correto do domínio maduro. Um motivo no domínio maduro do BDNF se liga a carboxipeptidase E (CPE), uma interação que classifica o BDNF em vesículas densas, que são um componente da via de secreção regulada. Na ausência deste motivo, o BDNF é direcionado para a via constitutiva. A secreção regulada é prevalente em neurônios. Grânulos de secreção contendo neurotrofina são transportados para os dendritos e espinhas dendríticas, e são segregados pós-sinapticamente (Lu et al., 2005).

No retículo endoplasmático, o pró-BDNF é empacotado em vesículas densas para ser transportado e liberado pela via constitutiva (espontânea) ou pela via secretada (induzida por estímulo) (Mowla et al., 1999). Após clivagem para a forma madura, o BDNF liga-se ao seu receptor TrkB, a dimerização deste receptor resulta na ativação da cinase, e o receptor se auto-fosforila em muitos resíduos de tirosina, ativando proteínas alvo envolvidas em cascatas intracelulares de sinalização, tais como proteína cinase extracelular regulada (ERK, do inglês *Ras/extracellular sinal-regulated kinase*) e proteína cinase B (Akt) (Patapoutian e Reichardt, 2001; Reichardt, 2006) (Figura 4).

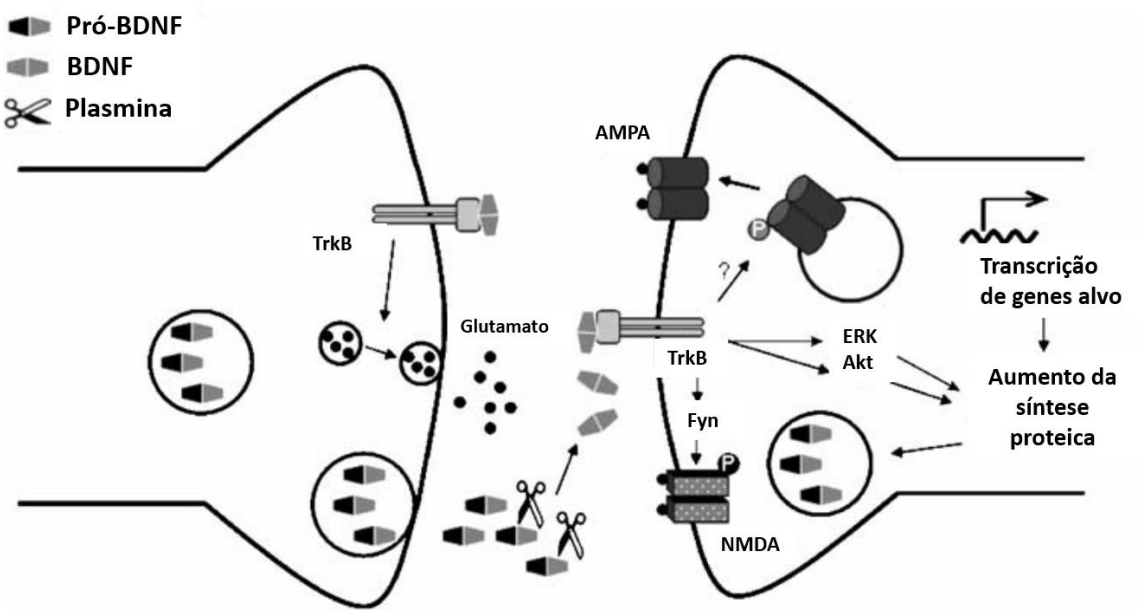


Figura 4. Sinalização do BDNF e de seu receptor TrkB na modulação da sinapse glutamatérgica. Akt: proteína cinase B; AMPA: ácido α-amino-3-hidróxi-5-metilisoxazol-4-propiônico; BDNF: fator neurotrófico derivado do encéfalo; ERK: cinase extracelular regulada; NMDA: N-metil-D-aspartato (Adaptado de Carvalho et al., 2008).

O nível de expressão de neurotrofinas e seus receptores é geralmente elevado durante todo o desenvolvimento do SNC dos mamíferos (Fryer et al., 1996; Knüsel et al., 1994; Numan et al., 2005; Tang et al., 2010). Durante o desenvolvimento, as neurotrofinas são seletivamente expressas em diferentes estágios conforme a estrutura cerebral.

A imunorreatividade ao BDNF já está presente no 13° dia embrionário (E13) no SNC de ratos, especialmente na subplaca neocortical e nos neuroblastos da placa cortical em desenvolvimento, e em E18 todas as células da placa cortical encontram-se marcadas por esta neurotrofina (Fukumitsu et al., 1998). No rato a forma truncada de TrkB não foi detectada antes de E15 e a sua expressão é baixa até o nascimento, aumentando gradualmente durante o desenvolvimento neonatal (Fryer et al., 1996). A proteína de TrkB de comprimento completo (TrkB-FL) é expressa no encéfalo de rato durante o

desenvolvimento embrionário inicial (E13-14) (Fryer et al., 1996; Fukumitsu et al., 1998; Knüsel et al., 1994).

A sinalização pelo BDNF participa como um regulador retrógrado do desenvolvimento das sinapses, sendo essencial para o crescimento e elongação dos dendritos de sinapses inibitórias e excitatórias (Carvalho et al., 2008; Lipsky e Marini, 2007; Singh et al., 2006; Tyler e Pozzo-Miller, 2004). 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). Além disso, o BDNF aumenta a transmissão sináptica excitatória no córtex cerebral e no hipocampo por mecanismos pré- e pós-sinápticos. No terminal pré-sináptico, o BDNF aumenta a liberação de glutamato e a frequência de potenciais excitatórios em neurônios hipocampais e corticais (Lessmann e Heumann, 1998; Takei et al., 1998), e sua ação pós-sináptica é pela fosforilação das subunidades NR1 e NR2B do receptor de glutamato NMDA, levando a um aumento da probabilidade de abertura do canal iônico deste receptor (Levine et al., 1998; Lin et al., 1998). Além disso, o BDNF parece regular a expressão de subunidades do receptor NMDA em neurônios hipocampais (Caldeira et al., 2007).

1.2.2 *Shh*

Shh (Sonic Hedgehog) é um membro da família de glicoproteínas Hedgehog, que está envolvida no desenvolvimento de muitos órgãos e grupos celulares (Echelard et al., 1993; McGowan e McCoy, 2013; Pillai-Kastoori et al., 2014). Nos mamíferos e aves, *Shh* é o único membro da família hedgehog (hh) que é expresso no SNC. Essa proteína é expressa ventralmente em estados

embrionários iniciais e está envolvida no desenvolvimento do cérebro posterior ventral, do mesencéfalo e do prosencéfalo (Ruiz i Altaba, 1998; 1999). A via de sinalização pela *Shh* induz diferentes classes de neurônios no cérebro ventral, regula o tamanho do mesencéfalo ventral, e controla o desenvolvimento dos gânglios basais (Chiang et al., 1996; Hynes et al., 1995; Kotz et al., 1998; Machold et al., 2003). Após este período inicial de expressão de *Shh* no tubo neural ventral, outras regiões do cérebro também começam a expressá-la. Logo após o fechamento do tubo neural, *Shh* é detectado na zona limite intratalâmica, e no final da embriogênese, em neurônios de Purkinje do cerebelo, na amígdala, no giro denteado do hipocampo, placa tectal, bulbo olfatório e neocortex (Ruiz i Altaba et al., 2002). Este morfógeno é altamente expresso no encéfalo de rato a partir do 12° dia embrionário até o 2° dia pós-natal (Petralia et al., 2011).

A proteína *Sonic hedgehog* (*Shh*) está envolvida em vários aspectos da embriogênese. No sistema nervoso central dos mamíferos, Shh tem um papel importante no padrão ventral ao longo da neuraxis, incluindo o telencéfalo (Agarwala et al, 2001; Echelard et al., 1993; Ericson et al., 1995; Roelink et al., 1994; Ruiz i Altaba et al., 1995). Mecanismos moleculares semelhantes podem ser aplicados ao desenvolvimento do neocórtex cerebral. Na verdade, também tem sido relatado que a Shh é um mitógeno para o neocórtex e progenitores tectais, e que ele modula a proliferação celular *in vitro* (Dahmane et al., 2001; Palma e Ruiz i Altaba, 2004).

Em um modelo de camundongos transgênicos com deficiência na sinalização pela *Shh* em células progenitoras em estágios embrionários tardios, a divisão celular foi mais lenta do que em camundongos do tipo selvagem, e alguns neurônios não migraram para a camada superficial do telencéfalo dorsal

(Komada et al., 2008). Por outro lado, a supra-regulação da sinalização de *Shh* promoveu aumento da espessura e tamanho do neocórtex (Shikata et al., 2010). Houve um aumento na proliferação de células-tronco neurais do córtex de ratos no estágio embrionário E18 quando induzidas por *Shh* (Reinchisi et al., 2013). No entanto, quando se avalia o tratamento pré-natal com outro psicoestimulante, a cocaína, a expressão *Shh* no mesencéfalo ventral, estritamente relacionada com a neurotransmissão dopaminérgica, permaneceu inalterada (Koebbe et al., 1999). Culturas de neurônios hipocampais tratadas com *Shh* apresentaram os terminais pré-sinápticos significativamente maiores, com aumentos variáveis no tamanho das vesículas sinápticas (Mitchell et al., 2012). Além disso, a neurogênese durante a idade adulta representa uma única forma de plasticidade no giro dentado hipocampal que requer a presença de células estaminais neurais, e a origem destas células e a sua manutenção no denteado são controlados por fontes distintas de *Shh* durante o período embrionário (Li et al., 2013).

1.2.3 GAP-43

A Proteína Associada ao Crescimento 43 (GAP-43, do inglês *Growth Associated Protein 43*), encontra-se abundante nos cones axonais de crescimento no SNC em desenvolvimento, e desempenha um papel fundamental na diferenciação neuronal, plasticidade e regeneração (Benowitz e Routtenberg, 1997; Dani et al., 1991). A fosforilação de GAP-43 pela proteína cinase C (PKC, do inglês *Phosphoprotein Kinase C*) é importante para várias funções intracelulares, como o direcionamento axonal nas terminações nervosas e a sinaptogênese (Afadlal et al., 2010).

Durante o desenvolvimento do sistema nervoso, os cones de crescimento desempenham um papel central na orientação axonal (Mueller, 1999; Tessier-Lavigne e Goodman, 1996). Eles estão localizados nas pontas dos axônios e dinamicamente mudam a sua morfologia em resposta a estímulos de atração e retração. Tem sido proposto que, durante a formação de neuritos, o alongamento destes depende não só do alongamento do citoesqueleto (Gordon-Weeks e Mansfield, 1992), mas também da inserção de membrana recém-sintetizada no cone de crescimento (Pfenninger e Friedman, 1993).

Durante o período embrionário de ratos, em estágios em que os neurônios estão em intensa divisão e migração celular, os níveis de GAP-43 são baixos; por volta do 17° dia embrionário, uma intensa marcação na camada intermediária do telencéfalo começa a aparecer, assim como no tronco cerebral e nos gânglios basais. No 21° dia gestacional, observa-se um aumento na marcação no colículo superior, mas em algumas áreas do cerebelo, tálamo dorsal e placa cortical, ainda em diferenciação, há pouca marcação desta proteína. Os maiores níveis de GAP-43 são encontrados no período pós-natal, entre o 4° e o 8° dia pós-natal; após este período, os níveis decaem consideravelmente (Dani et al., 1991).

1.2.4 SNAP-25

As proteínas do complexo SNARE (do inglês *Soluble N-ethylmaleimide-sensitive factor attachment protein receptor*) 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 proteína associada a sinaptossoma (SNAP-25, do inglês *Synaptossomal Associated Protein 25 kDa)* pertence à superfamília do complexo SNARE de pequenas proteínas de membrana que participam na regulação da exocitose na vesícula sináptica (Figura 5). 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 (Jahn et al., 2003; Jahn e Scheller, 2006). Em camundongos que não expressam a proteína SNAP-25, observou-se que essa proteína não é necessária para liberação do neurotransmissor independente de estímulo, mas é essencial para a transmissão sináptica evocada (Washbourne et al., 2002).

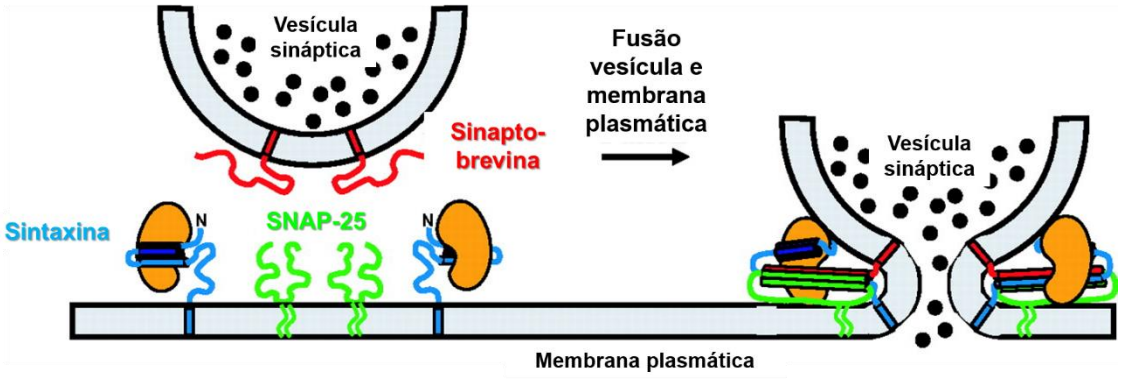


Figura 5. Representação esquemática da fusão da vesícula de neurotransmissor com a membrana plasmática durante a exocitose do neurotransmissor para a fenda sináptica (Adaptado de Dulubova et al., 2007).

A SNAP-25 já é expressa no encéfalo de embriões de ratos de 18-20 dias, com um aumento linear e atingindo um *plateau* aos 20 dias pós-natal (Oyler et al., 1989). Entretanto, sua fosforilação e ativação ocorre somente no período pós-natal, a partir dos 4 dias de vida (Kataoka et al., 2006).

A SNAP-25 parece estar envolvida no crescimento dos neuritos (Hepp e Langley, 2001; Tang, 2001), uma vez que a inibição de sua expressão preveniu a elongação dos neuritos em neurônios corticais de ratos (Osen-Sand et al., 1993), e sua clivagem pela toxina botulínica inibiu o crescimento do axônio (Morihara et al., 1999).

1.3 GLUTAMATO

O desenvolvimento do encéfalo é caracterizado por um nível elevado de plasticidade necessária para a sinaptogênese e está associada a uma maior excitabilidade neuronal. Muitos fatores que governam a transmissão sináptica e excitabilidade neuronal sofrem alterações durante o início do desenvolvimento, como a expressão e composição molecular de receptores de neurotransmissores e transportadores, peptídeos neuromoduladores e canais iônicos dependentes de voltagem e mecanismos de homeostase iônica e de estresse oxidativo (Sanchez e Jensen, 2002).

A sinalização glutamatérgica é importante na regulação do desenvolvimento do sistema nervoso (Komuro e Rakic, 1993; LaMantia, 1995; McDonald e Johnston, 1990), modula migração neuronal (Komuro e Rakic, 1993; Rossi e Slater, 1993), a organização do córtex somatosensorial (Fox et al., 1996), bem como a atividade GABAérgica (Van den Pol et al., 1998). A sensibilidade do cérebro em desenvolvimento à superestimulação glutamatérgica (excitotoxicidade), como no caso de isquemia e hipóxia, é elevada, sendo o hipocampo a estrutura mais sensível aos danos do excesso da ação do

glutamato em torno da segunda semana pós-natal (Ikonomidou et al., 1989; Yager et al., 1996).

O glutamato é o principal neurotransmissor excitatório do SNC, sendo essencial em funções de desenvolvimento como aprendizado e memória (Mattson, 2008; Nakanishi, 1992). O encéfalo contém grandes quantidades de glutamato (cerca de 5-15 mmol por kg de peso, dependendo da região), mas apenas uma pequena fração do glutamato está normalmente presente no meio extracelular (no exterior ou entre as células). As concentrações no fluido extracelular (o que representa 13-22% do volume de tecido cerebral) e no líquido cefalorraquidiano (LCR) são normalmente de cerca de 3-4 µM e cerca de 10 µM, respectivamente (Hamberger et al., 1983; Lehmann et al., 1983). O glutamato exerce suas funções pela ligação aos seus receptores ionotrópicos, que são subdivididos em N-metil-D-aspartato (NMDA), ácido α-amino-3-hidróxi-5-metilisoxazol-4-propiônico (AMPA), e cainato (KA); e receptores metabotrópicos 1-8 (mGlu1-8) (Figura 6). Além de seu papel na transmissão sináptica e plasticidade neuronal, o glutamato é também um importante fator trófico durante a neurogênese, regulando sobrevivência celular, proliferação, migração e diferenciação (Schlett, 2006). Durante o desenvolvimento, o glutamato está presente em altos níveis nas zonas neurogênicas que contenham células progenitoras neurais (Behar et al., 1999; Haydar et al., 2000).

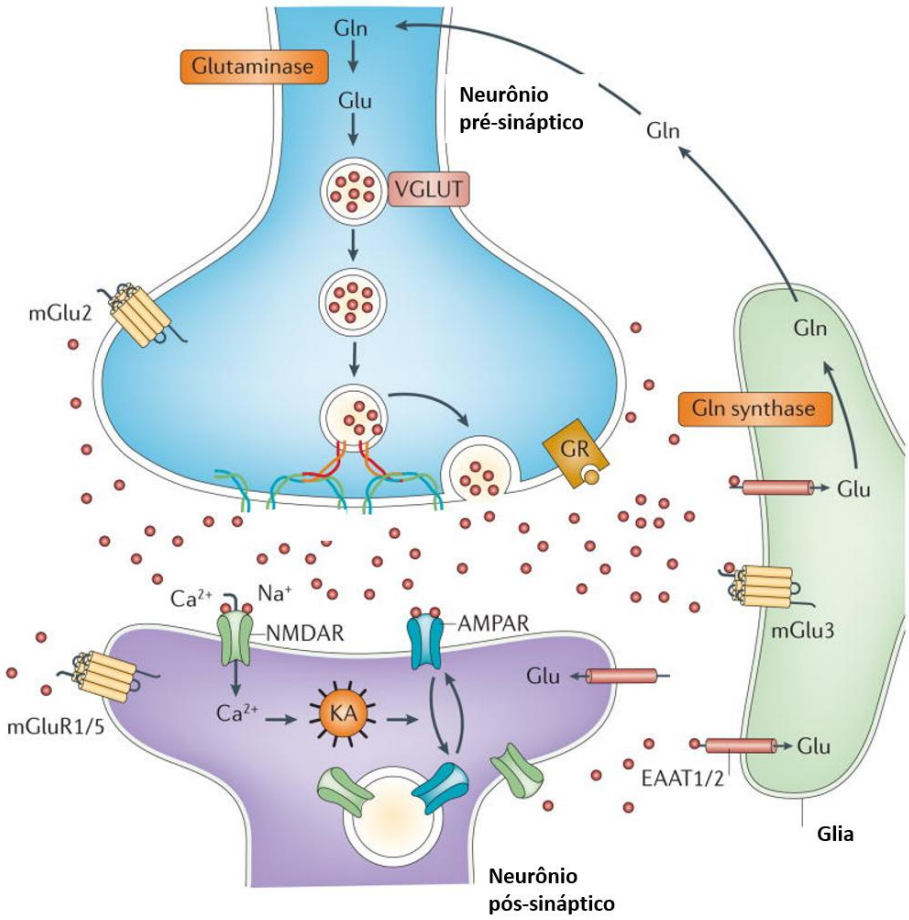


Figura 6. Representação de uma transmissão glutamatérgica com seus principais receptores. AMPAR: ácido α-amino-3-hidróxi-5-metilisoxazol-4-propiônico; EAAT: transportadores de glutamato; Glu: glutamato; mGlu: receptores metabotrópicos de glutamato; KA: proteínas cinases; NMDAR: N-metil-D-aspartato; VGLUT: transportador vesicular de glutamato (Adaptado de Popoli et al., 2011).

Os receptores de glutamato medeiam a maioria da neurotransmissão excitatória no SNC de mamíferos. Eles também participam de mudanças plásticas na eficácia da transmissão sináptica em aprendizado e memória e na formação de redes neurais durante o desenvolvimento (Dingledine et al., 1988; Monaghan et al., 1989). Entretanto, o excesso de glutamato e aminoácidos excitatórios são tóxicos para os neurônios, levando à morte neuronal em situações onde ocorre ativação excessiva de receptores glutamatérgicos, como isquemia cerebral, traumatismo craniano e epilepsia. A neurotoxicidade do glutamato também pode estar envolvida na gênese de várias doenças

neurodegenerativas (Choi e Rothman, 1990; Meldrum e Garthwaite, 1990; Rothman e Olney, 1987). Assim, os receptores de glutamato estão intimamente envolvidos na fisiologia e na patologia das funções cerebrais. Além disso, os receptores de glutamato, em particular, os receptores NMDA, podem atuar sobre a migração de neurônios que não possuem sinapses maduras, e a sua ativação ou bloqueio modula a velocidade de migração (Komuro e Rakic, 1993).

Os receptores de glutamato são classificados em dois grupos distintos de receptores: ionotrópicos e metabotrópicos (Figura 7). Os receptores ionotrópicos (iGluRs) contêm canais de íons cátion-específicos, e são subdivididos em três grupos, de acordo com a especificidade do agonista: ácido α-amino-3-hidróxi-5-metilisoxazol-4-propiônico (AMPA), cainato e N-metil-D-aspartato (NMDA). Os receptores metabotrópicos (mGluRs) são acoplados à proteína G e modulam a produção de mensageiros intracelulares (Hollmann e Heinemann, 1994; Nakanishi, 1992; Seeburg, 1993).

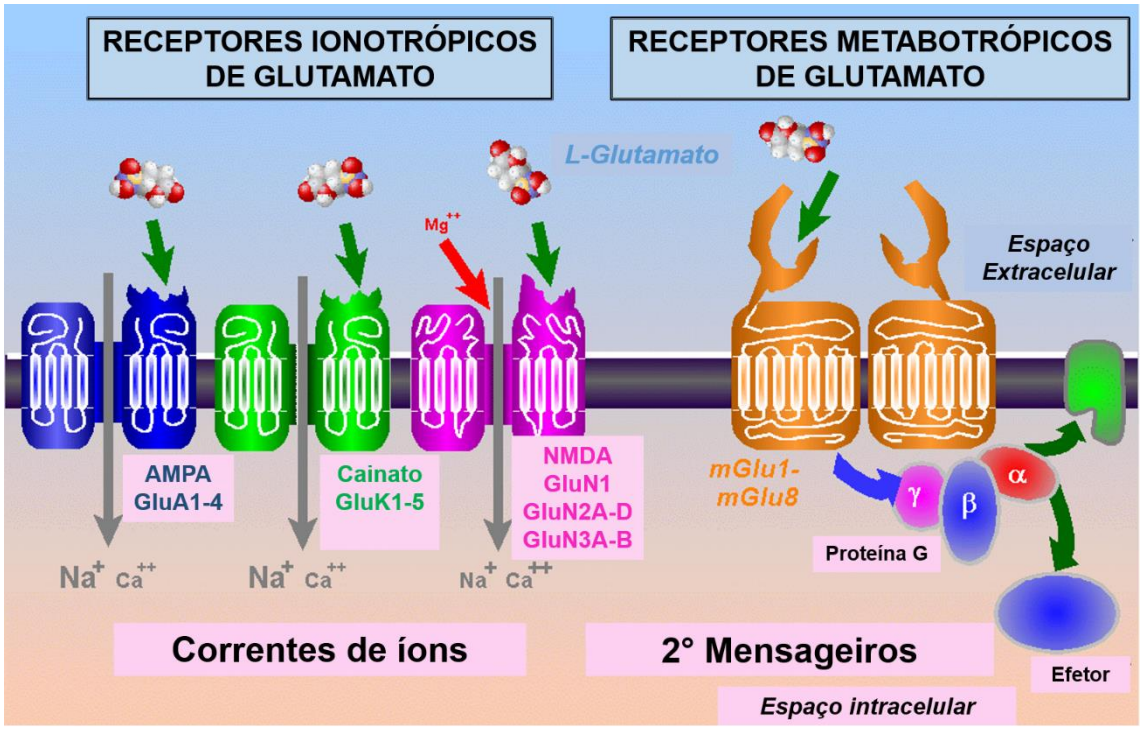


Figura 7. Tipos de receptores de glutamato e seu mecanismo de ação (Adaptado de http://www.ucl.ac.uk/~smgxt01/frameh.htm?page=glutamat.htm).

1.3.1 Receptores ionotrópicos

De acordo com a União Internacional do Comitê de Farmacologia em Nomenclatura de Receptores e Classificação de Drogas (NC-IUPHAR), uma nova classificação das subunidades de receptores ionotrópicos de glutamato foi adotada, conforme a tabela abaixo (Collingridge et al., 2009).

Tabela 2. Nova nomenclatura das subunidades dos receptores ionotrópicos de glutamato (Adaptado de Collingridge et al., 2009).

NMDA		**AMPA**		**CAINATO**	
Antiga	IUPHAR	Antiga	IUPHAR	Antiga	IUPHAR
NR1	GluN1	GluR1	GluA1	GluR5	GluK1
		GluR2	GluA2	GluR6	GluK2
NR2A	GluN2A	GluR3	GluA3	GluR7	GluK3
NR2B	GluN2B	GluR4	GluA4		
NR2C	GluN2C			KA-1	GluK4
NR2D	GluN2D			KA-2	GluK5
NR3A	GluN3A				
NR3B	GluN3B				

Em ratos, os receptores de glutamato ionotrópicos sofrem alterações rápida na sua maturação. O pico de densidade de receptores NMDA ocorre no final da primeira semana pós-natal em várias estruturas do encéfalo anterior, incluindo hipocampo e neocórtex (Insel et al., 1990), enquanto o pico de densidade de receptores AMPA ocorre na segunda semana pós-natal em torno de 10 dias pós-natal (Insel et al., 1990). Ambos os receptores NMDA e AMPA ultrapassam os níveis de expressão quando comparados ao encéfalo de animais adultos, resultando em um aumento na plasticidade mediada pelo glutamato durante o período pós-natal de desenvolvimento (Swann et al., 1999). A regulação da maturação da composição das subunidades dos receptores de glutamato também aumenta a sua capacidade de mediar a plasticidade sináptica dependente de atividade no período pós-natal (Wu et al., 1996). Portanto, no

início da vida pós-natal de roedores, os mecanismos de excitação sináptica predominam sobre os mecanismos inibitórios (Sanchez e Jensen, 2002).

1.3.1.1 AMPA

Os receptores AMPA medeiam a maioria das transmissões sinápticas excitatórias rápidas no SNC dos mamíferos. Sua ativação prolongada é altamente neurotóxica e exerce um papel fundamental na geração e propagação da hiperexcitabilidade (Bernard e Wheal, 1995). AMPA são receptores de glutamato heteroméricos que consistem de quatro subunidades principais: GluA1, 2, 3, e 4, com diferentes estequiometrias (Sommer et al., 1991; Hollmann e Heinemann, 1994). A diversidade de combinações das subunidades deve-se a mecanismos de *splicing* alternativo e edição de RNAm que resultam na formação de receptores com diferentes propriedades biofísicas dos canais iônicos (Kumar et al., 2002). Cada subunidade dos receptores AMPA é codificada pelo seu gene específico e apresentam um padrão dependendo da área cerebral e do estágio de desenvolvimento; portanto, os transcritos das subunidades do receptor AMPA são regulados de forma independente durante o desenvolvimento (Lilliu et al., 2001).

Antes do nascimento e na primeira semana pós-natal, a subunidade GluA4 é expressa em neurônios excitatórios hipocampais e corticais em ratos. Como os níveis de expressão das outras subunidades são muito baixas, os receptores AMPA nesse período é constituído predominantemente por GluA4. Na segunda semana pós-natal, os níveis de expressão das subunidades GluA1, GluA2 e GluA3 começam a aumentar paralelamente e atingem níveis semelhantes ao cérebro adulto na terceira semana pós-natal (Zhu et al., 2000).

Os receptores AMPA são amplamente distribuídos em todo o SNC, sendo o hipocampo a região mais enriquecida nesse receptor de glutamato, principalmente nas regiões de CA1 e CA3, e também a camada superficial do córtex cerebral (Monaghan et al., 1984; Olsen et al., 1987). Durante o desenvolvimento, mudanças na localização subcelular das subunidades GluA1 e GluA2/3 foram examinados em cultura de neurônios hipocampais de embriões de ratos (Craig et al., 1993). GluA1 e GluA2/3 são distribuídos de uma forma relativamente uniforme no corpo celular, nos axônios e em processos menores no início do desenvolvimento; quando os dendritos começam a desenvolver, as subunidades do receptor são polarizadas para os dendritos. Em células piramidais maduras, os “clusters” de GluA1 e GluA2/3 ficam restritos a um subconjunto de locais pós-sinápticos nas espinhas dendríticas. O alvo destas subunidades do receptor parece ocorrer em duas etapas que se desenvolvem sequencialmente: primeiramente, a exclusão do axônio; em seguida, o enriquecimento na densidade pós-sináptica (Craig et al., 1993).

A maioria dos receptores AMPA no SNC contém as subunidades GluA1 e GluA2 ou GluA2 e GluA3 (Lu et al., 2009). Durante a diferenciação neuronal e astrocítica, a expressão de GluA1 é significativamente aumentada (Whitney et al., 2008), e também foi demonstrado que as células progenitoras neurais em diferenciação e migração expressam as subunidades GluA1-3 (Jansson et al., 2011). Além disso, observou-se uma deficiência na dinâmica do cone de crescimento e dos neuritos após o bloqueio do receptor AMPA (Jansson et al., 2013).

Baixos níveis de expressão da subunidade GluA2 do receptor AMPA (portanto, de alta permeabilidade Ca^{2+}) em fases iniciais de desenvolvimento do

SNC (Durand e Zukin, 1993, Monyer et al., 1994) podem participar no aumento da susceptibilidade a episódios que podem levar a crises epilépticas no cérebro imaturo. A maior excitabilidade parece ser provocada pelo aumento da permeabilidade aos íons cálcio pelo receptor AMPA, levando à hiperexcitabilidade e posterior morte de interneurônios gabaérgicos inibitórios (Moshé et al., 1983).

1.3.1.2 NMDA

Os receptores NMDA participam da neurotransmissão excitatória no SNC em diferentes maneiras do que os receptores AMPA. O canal do receptor NMDA tem uma permeabilidade relativamente elevada ao Ca^{2+} e é bloqueado, de forma dependente de voltagem, por Mg^{2+} (MacDermott et al., 1986; Nowak et al., 1984, Figura 8). Em potencial de repouso, o canal de NMDA está bloqueado pelos íons magnésio, e o canal é aberto somente quando a liberação de glutamato e a despolarização pós-sináptica ocorrem, sendo a atividade dos receptores NMDA crucial para a ativação de vias de sinalização dependentes de cálcio, como a potenciação de longa duração (Cummings et al., 1996).

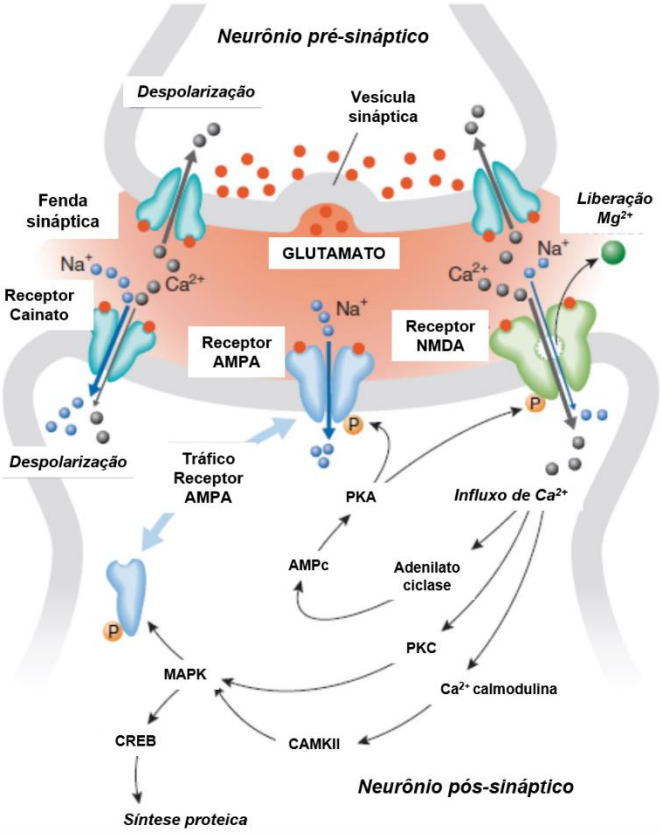


Figura 8. Receptores ionotrópicos de glutamato e seu mecanismo de ação. AMPA: ácido α-amino-3-hidróxi-5-metilisoxazol-4-propiônico; AMPc: AMP cíclico; CAMKII: Ca^{2+}/calmodulina cinase II; CREB: proteína de ligação ao AMPc; MAPK: proteína cinase ativadora de mitógeno NMDA, N-metil-D-aspartato; PKA: proteína cinase A; PKC: proteína cinase C (Adaptado de Voglis e Tavernaraki, 2006).

Os receptores N-metil-D-aspartato (NMDA) são conjuntos heteroméricos das subunidades GluN1, GluN2 e GluN3 que são traduzidos no retículo endoplasmático para formar canais funcionais com diferentes propriedades fisiológicas e farmacológicas e padrões distintos de segmentação sináptica. O requisito mínimo para a expressão funcional eficiente de receptores de NMDA *in vitro* é uma combinação di-heteromérica de GluN1 e, pelo menos, uma variante da subunidade GluN2 (Furukawa et al., 2005; Karakas et al., 2011).

Durante o desenvolvimento, a ativação dos receptores NMDA é necessária para a inserção dos receptores AMPA e cainato (Isaac et al., 1997; Renger et al., 2001; Wu et al., 1996), bem como a maturação dos receptores

GABAérgicos $GABA_A$ (Aamodt et al., 2000). Em roedores, os receptores de NMDA consistem de heterômeros de subunidades GluN1 e GluN2B no nascimento (Monyer et al., 1994; Williams et al., 1993). Durante o período de 3-4 semanas pós-natal, a expressão da subunidade GluN2A aumenta dramaticamente (Stocca e Vicini, 1998).

A expressão de subunidades GluN2 é dependente do tempo de maturação do encéfalo e da estrutura encefálica em desenvolvimento (Monyer et al., 1994; Watanabe et al., 1992). Os níveis de RNAm de GluN2B são elevados em todo o encéfalo durante o período embrionário e no córtex frontal de ratos adultos, ambas regiões de intensa neurogênese, especialmente proliferação celular. Por outro lado, a expressão da subunidade GluN2A começa no período pós-natal e é amplamente distribuída em todo encéfalo de ratos adultos. Por isso, cada tipo de receptor de NMDA contendo a subunidade GluN2A ou GluN2B desempenha um papel diferente durante o desenvolvimento do SNC. De acordo com Fan et al. (2012), GluN2B está envolvido no processo de proliferação celular, enquanto o GluN2A regula o processo de migração celular durante a neurogênese nas primeiras semanas de vida dos roedores.

1.3.2 Receptores metabotrópicos

Os receptores metabotrópicos são receptores transmembrana acoplados à proteína G que modulam a atividade sináptica e plasticidade. A família de receptores metabotrópicos de glutamato (mGlu) consiste de 8 subunidades, que se combinam para formar homodímeros. As subunidades são subdivididas em três grupos baseada na sua sequência homóloga, nos mecanismos de transdução de sinal e nas propriedades farmacológicas (Nakanishi, 1992). O

Grupo I de receptores metabotrópicos consiste das subunidades $mGlu_1$ e $mGlu_5$ e estão acoplados à proteína G_q, que ativa fosfolipase C com produção de inositol 1,4,5-trifosfato (IP_3) e diacilglicerol (DAG) a partir de fosfatidilinositol 4,5-bifosfato (PIP_2). IP_3 libera íons cálcio dos estoques intracelulares, enquanto o DAG ativa proteína cinase C (PKC) (Figura 8). Os receptores do Grupo I estão presentes principalmente na membrana pós-sináptica das sinapses excitatórias. O Grupo II de receptores metabotrópicos é formado pelas subunidades $mGlu_2$ e $mGlu_3$, e o Grupo III inclui as subunidades $mGlu_4$, $mGlu_6$, $mGlu_7$ e $mGlu_8$; todas essas subunidades estão acopladas à proteína G inibitória, que diminuem os níveis de AMPc pela inibição da adenilato ciclase. Os receptores dos grupos II e III estão presentes na membrana pré-sináptica de neurônios glutamatérgicos e GABAérgicos, bem como de células gliais (Hollmann e Heinemann, 1994; Shoepp et al., 1999). Com exceção da subunidade $mGlu_6$, expressa exclusivamente na retina, os receptores metabotrópicos de glutamato são expressos em células neuronais e gliais no encéfalo em desenvolvimento e adulto de maneira distinta conforme a idade e a estrutura encefálica (Di Giorgi-Gerevini et al., 2004; Melchiorri et al., 2007; Yang et al., 2012).

A expressão de $mGlu_5$ é elevada durante o início do desenvolvimento embrionário de roedores. Em cultura de neurônios corticais de embriões de camundongos de 13-18 dias, a migração celular foi promovida por mecanismo dependente de receptores metabotrópicos do Grupo I (Behar et al., 1999). O bloqueio de receptores $mGlu_5$ em células progenitoras neurais de córtex de embriões de camundongos de 20 dias reduziu a proliferação celular e aumentou a morte celular, e a ativação desse receptor aumentou o número de células proliferativas (Di Giorgi-Gerevini et al., 2005). Também foi observado um

aumento da expressão de $mGlu_5$ em células progenitoras neurais de córtex de embriões de ratos de 15 dias em condições de hipóxia, o que promoveu proliferação celular (Zhao et al., 2012). No hipocampo, $mGlu_5$ parece ser essencial para a proliferação de células progenitoras neurais no giro denteado de camundongos durante o desenvolvimento (Xiao et al., 2013). A administração de um ativador alostérico de $mGlu_5$ promoveu o aumento do número de neurônios no giro denteado em camundongos (Nochi et al., 2012).

1.3.3 Transportadores de glutamato

Visto que os receptores de glutamato estão expressos na maior parte dos elementos celulares no SNC (dendritos, terminais nervosos, corpos celulares neuronais, bem como células gliais), é importante que haja um rígido controle nos seus níveis nas fendas sinápticas e no espaço extrassináptico (Danbolt, 2001). No entanto, não existe nenhuma enzima extracelular que metabolize o glutamato após exercer suas ações na fenda sináptica. A única maneira de remover rapidamente o glutamato extracelular é pela captação celular (Johnston, 1981; Logan e Snyder, 1972). A captação de glutamato é o mecanismo responsável pela manutenção a longo prazo de baixas concentrações extracelulares de glutamato. A captação de glutamato é realizada por meio de proteínas transportadoras de glutamato presentes em células gliais e em neurônios (Danbolt, 1994; Schousboe, 1981; Storm-Mathisen, 1977).

Foram clonados cinco tipos de transportadores de glutamato de alta afinidade. Os três transportadores homólogos não-humanos foram denominados GLAST (do inglês *Glutamate aspartate transporter*), GLT-1 (do inglês *Glutamate transporter subtype 1*) e EAAC1 (do inglês *excitatory amino acid carrier 1*), que

correspondem aos transportadores homólogos humanos EAAT1 (do inglês *Excitatory amino acid transporter 1*), EAAT2 e EAAT3, respectivamente. Os transportadores em humanos tem mais dois membros da família, EAAT4 e EAAT5 (Arriza et al., 1997; Fairman et al., 1995; Kanai e Hediger, 1992; Pines et al., 1992; Storck et al., 1992). O GLT-1 é o transportador de glutamato mais expresso no encéfalo (Danbolt et al., 1992; Lehre e Danbolt, 1998; Pines et al., 1992; Tanaka et al., 1997).

A captação de glutamato no encéfalo de ratos é baixa no nascimento e aumenta nas primeiras semanas no período pós-natal (Demarque et al., 2002; Furuta et al., 1997; Juraska e Fifkova, 1979; Owens e Kriegstein, 2001), devido ao período intenso de sinaptogênese (Christensen e de Fonnum, 1992; Kish et al., 1989). Em ratos recém-nascidos, a quantidade de GLT-1 no encéfalo é muito baixa (inferior ao limite de detecção correspondente a cerca de 1% do valor adulto) (Levy et al., 1995), mas as concentrações de ambos transportadores GLT-1 e GLAST aumentam dramaticamente no período mais ativo de sinaptogênese (a partir do final da segunda semana pós-natal até o fim da quarta semana), alcançando os níveis adultos por volta de 35 dias de vida. Os maiores níveis de RNAm de GLT-1 e GLAST são observados no dia pós-natal 14 no córtex de rato (Shibata et al., 1996; Sutherland et al., 1996).

2. JUSTIFICATIVA

Apesar de muitos estudos terem sido focados nos efeitos do consumo de cafeína durante o período gestacional sobre o desenvolvimento do feto, ainda é necessário investigar uma dose de cafeína que não modifique os padrões de desenvolvimento das sinapses durante a vida intrauterina e após o nascimento. De fato, os efeitos da administração de cafeína sobre proteínas, hormônios, vias de sinalização e especialmente fatores neurotróficos que regulam o desenvolvimento e maturação do sistema nervoso central ainda são pouco conhecidos. Dada a importância de proteínas cruciais para a homeostasia do desenvolvimento encefálico, é fundamental conhecer os efeitos da administração de diferentes doses de cafeína sobre os fatores neurotróficos e proteínas envolvidas na formação, maturação e diferenciação das sinapses. Além disso, é importante saber o metabolismo da cafeína durante o período lactacional e as possíveis alterações no desenvolvimento pós-natal pelo consumo de cafeína durante todo o desenvolvimento encefálico.

3. OBJETIVO GERAL

Investigar o efeito do consumo de cafeína durante a gestação e lactação no desenvolvimento do SNC no período embrionário e pós-natal de ratos Wistar.

3.1 OBJETIVOS ESPECÍFICOS

1. Avaliar o impacto do consumo gestacional de cafeína em ratas Wistar:

   - no imunoconteúdo de proteínas envolvidas na formação e manutenção das sinapses;

   - e na maturação neuronal em regiões encefálicas envolvidas na neurogênese nos embriões.

2. Avaliar o impacto do consumo perinatal de diferentes doses de cafeína durante a gestação e lactação:

   - no desenvolvimento reflexo e locomotor;

   - no metabolismo da cafeína em filhotes durante a lactação;

   - no imunoconteúdo de transportadores e receptores de glutamato nas idades pós-natal 7, 14 e 21 nas estruturas cerebrais de córtex e hipocampo de filhotes de ratos Wistar que receberam cafeína durante todo o período de desenvolvimento do SNC;

   - e na sinalização do glutamato nessas regiões encefálicas e nessas idades pelo ensaio de ligação específica ao glutamato (*binding*).

PARTE II

CAPÍTULO I

The Janus face of caffeine

Artigo publicado no periódico *Neurochemistry International 63: 594–609, 2013*

Neurochemistry International 63 (2013) 594–609

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

Invited review

The Janus face of caffeine

Lisiane O. Porciúncula [a,*], Cássia Sallaberry [a], Sabrina Mioranzza [a], Paulo Henrique S. Botton [a], Denis B. Rosemberg [a,b]

[a] Laboratório de Estudos sobre o Sistema Purinérgico, Departamento de Bioquímica/ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-anexo, Porto Alegre/RS, Brazil
[b] Laboratório de Genética e Ecotoxicologia Molecular, Programa de Pós-graduação em Ciências Ambientais, Área de Ciências Exatas e Ambientais, Universidade Comunitária da Região de Chapecó. Avenida Senador Attílio Fontana, 591E, 89809-000 Chapecó/SC, Brazil

ARTICLE INFO

Article history:
Received 8 April 2013
Received in revised form 4 September 2013
Accepted 6 September 2013
Available online 19 September 2013

Keywords:
Caffeine
Adenosine
Brain development
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

* Corresponding author. Tel.: +55 51 3308 5556/5557; fax: +55 51 3308 5540.
E-mail address: loporciuncula@yahoo.com (L.O. Porciúncula).

0197-0186/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neuint.2013.09.009

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

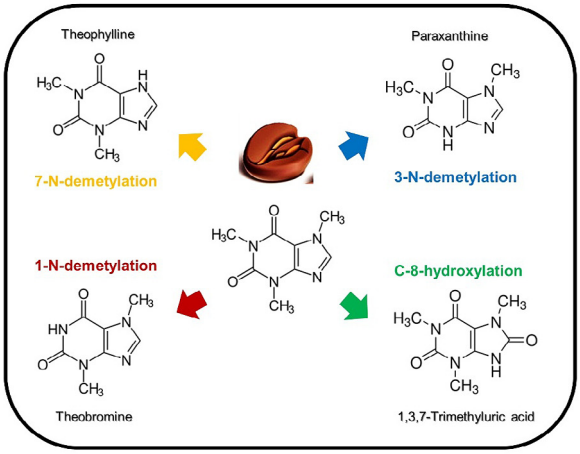
In order to investigate the effects of caffeine intake during pregnancy on fetal weight and development, rats were given 10 or 100 mg/kg/day, either as bolus oral doses, or as four 2.5 or 25 mg/kg doses at 3-h interval. Smaller pups displayed decreased crown-rump length and skeletal ossification from 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.

Acknowledgments

Lisiane O. Porciúncula thanks all her students (beyond those authors of this review) for all efforts and dedication to projects with caffeine: Ana Paula Ardais, Andrea Silva da Rocha, Daniela Pochmann, Fernanda Nunes, Gabriela Tondolo Fioreze, Janaina Espinosa, Marcelo Silveira da Costa, Mauricio Borges, Natália Pagnussat. All authors thank the funding Brazilian agencies: CNPq, FAPERGS, and CAPES.

References

Abdi, F., Pollard, I., Wilkinson, J., 1993. Placental transfer and foetal disposition of caffeine and its immediate metabolites in the 20-day pregnant rat: function of dose. Xenobiotica 23, 449–456.

Abrams, J.K., Johnson, P.L., Hay-Schmidt, A., Mikkelsen, J.D., Shekhar, A., Lowry, C.A., 2005. Serotonergic systems associated with arousal and vigilance behaviors following administration of anxiogenic drugs. Neuroscience 133, 983–997.

Acquas, E., Tanda, G., Di Chiara, G., 2002. Differential effects of caffeine on dopamine and acetylcholine transmission in brain areas of drug-naive and caffeine-preteated rats. Neuropsychopharmacology 27, 182–193.

Adén, U., 2011. Methylxanthines during pregnancy and early postnatal life. In: Fredholm, B.B. (Ed.), Methylxanthines. In: Handbook of Experimental Pharmacology, vol. 200. Springer, London, 200, pp. 384-389

Adén, U., Herlenius, E., Tang, L.-Q., Fredholm, B.B., 2000. Maternal caffeine intake has minor effects on adenosine receptor ontogeny in the rat brain. Pediatr. Res. 48, 177–183.

Aldridge, A., Aranda, J.V., Neims, A.H., 1979. Caffeine metabolism in the newborn. Clin. Pharmacol. Ther. 25, 447–453.

Alhaider, I.A., Aleisa, A.M., Tran, T.T., Alkadhi, K.A., 2011. Sleep deprivation prevents stimulation-induced increases of levels of P-CREB and BDNF: protection by caffeine. Mol. Cell. Neurosci. 46, 742–751.

Alzoubi, K.H., Srivareerat, M., Aleisa, A.M., Alkadhi, K.A., 2013. Chronic caffeine treatment prevents stress-induced LTP impairment: the critical role of phosphorylated CaMKII and BDNF. J. Mol. Neurosci. 49, 11–20.

Angelucci, M.E., Vital, M.A., Cesário, C., Zadusky, C.R., Rosalen, P.L., Cunha, C., 1999. The effect of caffeine in animal models of learning and memory Eur. J. Pharmacol. 373, 135–140.

Angelucci, M.E., Cesario, C., Hiroi, R.H., Rosalen, P.L., Cunha, C., 2002. Effects of caffeine on learning and memory in rats tested in the Morris water maze. Braz. J. Med. Biol. Res. 35, 1201–1208.

Aranda, J.V., Gorman, W., Bergsteinsson, H., Gunn, T., 1977. Efficacy of caffeine in treatment of apnea in the low-birth-weight infant. J. Pediatr. 90, 467–472.

Aranda, J.V., Collinge, J.M., Zinman, R., Watters, G., 1979. Maturation of caffeine elimination in infancy. Arch. Dis. Child. 54, 946–949.

Arendash, G.W., Mori, T., Cao, C., Mamcarz, M., Runfeldt, M., Dickson, A., Rezai-Zadeh, K., Tane, J., Citron, B.A., Lin, X., Echeverria, V., Potter, H., 2009. Caffeine reverses cognitive impairment and decreases brain amyloid-beta levels in aged Alzheimer's disease mice. J. Alzheimers Dis. 17, 661–680.

Arendash, G.W., Schleif, W., Rezai-Zadeh, K., Jackson, E.K., Zacharia, L.C., Cracchiolo, J.R., Shippy, D., Tan, J., 2006. Caffeine protects Alzheimer's mice against cognitive impairment and reduces brain beta-amyloid production. Neuroscience 142, 941–952.

Arnaud, M.J., 1976. Identification, kinetic and quantitative study of [2-14C] and [1-Me-14C] caffeine metabolites in rat's urine by chromatographic separations. Biochem. Med. 16, 67–76.

Arnaud, M.J., Bracco, I., Sauvageat, J.L., Clerc, M.F., 1983. Placental transfer of the major caffeine metabolite in the rat using 6-amino-5[N-formylmethylamino]1,3[Me-14C]-dimethyluracil administered orally or intravenously to the pregnant rat. Toxicol. Lett. 16, 271–279.

Arnaud, M.J., 1985. Comparative metabolic disposition of [1-Me14C]caffeine in rats, mice, and Chinese hamsters. Drug Metab. Dispos. 13, 471–478.

Arnaud, M.J., 1987. The pharmacology of caffeine. Prog. Drug Res. 31, 273–313.

Arnaud, M.J., Bracco, I., Getaz, F., 1989. Synthesis of ring labelled caffeine for the study of metabolic and pharmacokinetics mouse interstrain differences in relation to pharmacologic and toxic effects. In: Baillie, T.A., Jones, J.R. (Eds.), Synthesis and Applications of Isotopically Labeled Compounds. Elsevier, Amsterdam, pp. 645–648.

Ascherio, A., Zhang, S.M., Hernán, M.A., Kawachi, I., Colditz, G.A., Speizer, F.E., Willett, W.C., 2001. Prospective study of caffeine consumption and risk of Parkinson's disease in men and women. Ann. Neurol. 50, 56–63.

Ashpole, N.M., Song, W., Brustovetsky, T., Engleman, E.A., Brustovetsky, N., Cummins, T.R., Hudmon, A., 2012. Calcium/calmodulin-dependent protein kinase II (CaMKII) inhibition induces neurotoxicity via dysregulation of glutamate/calcium signaling and hyperexcitability. J. Biol. Chem. 287, 8495–8506.

Bairam, A., Boutroy, M.J., Badonnel, Y., Vert, P., 1987. Theophylline versus caffeine: comparative effects in treatment of idiopathic apnea in the preterm infant. J. Pediatr. 110, 636–639.

Bakker, R., Steegers, E.A., Obradov, A., Raat, H., Hofman, A., Jaddoe, V.W., 2010. Maternal caffeine intake from coffee and tea, fetal growth, and the risks of adverse birth outcomes: the Generation R Study. Am. J. Clin. Nutr. 91, 1691–1698.

Belelli, D., Harrison, N.L., Maguire, J., Macdonald, R.L., Walker, M.C., Cope, D.W., 2009. Extrasynaptic GABAA receptors: form, pharmacology, and function. J. Neurosci. 29, 12757–12763.

Benowitz, N.L., Jacob, P., Mayan, H., Denaro, C., 1995. Sympathomimetic effects of paraxanthine and caffeine in humans. Clin. Pharmacol. Ther. 58, 684–691.

Berger-Sweeney, J., Hohmann, C.F., 1997. Behavioral consequences of abnormal cortical development: insights into developmental disabilities. Behav. Brain Res. 86, 121–142.

Bhattacharya, S.K., Satyan, K.S., Chakrabarti, A., 1997. Anxiogenic action of caffeine: an experimental study in rats. J. Psychopharmacol. 11, 219–224.

Biederman, J., Faraone, S.V., 2005. Attention-deficit hyperactivity disorder. Lancet 366, 237–248.

Blake, M.J., Castro, L., Leeder, J.S., Kearns, G.L., 2005. Ontogeny of drug metabolizing enzymes in the neonate. Semin. Fetal Neonatal Med. 10, 123–138.

Blaser, R.E., Rosemberg, D.B., 2012. Measures of anxiety in zebrafish (*Danio rerio*): dissociation of black/white preference and novel tank test. PLoS One 7, e36931.

Bona, E., Adén, U., Fredholm, B.B., Hagberg, H., 1995. The effect of long term caffeine treatment on hypoxic–ischemic brain damage in the neonate. Pediatr. Res. 38, 312–318.

Bonati, M., Latini, R., Tognoni, G., Young, J.F., Garattini, S., 1984. Interspecies comparison of in vivo caffeine pharmacokinetics in man, monkey, rabbit, rat, and mouse. Drug Metab. Rev. 15, 1355–1383.

Bonnin, A., Levitt, P., 2011. Fetal, maternal, and placental sources of serotonin and new implications for developmental programming of the brain. Neuroscience 197, 1–7.

Bortolotti, A., Jiritano, L., Bonati, M., 1985. Pharmacokinetics of paraxanthine, one of the primary metabolites of caffeine, in the rat. Drug Metab. Dispos. 13, 227–231.

Bosman, L.W., Heinen, K., Spijker, S., Brussaard, A.B., 2005. Mice lacking the major adult GABAA receptor subtype have normal number of synapses, but retain juvenile IPSC kinetics until adulthood. J. Neurophysiol. 94, 338–346.

Botton, P.H., Costa, M.S., Ardais, A.P., Mioranzza, S., Souza, D.O., da Rocha, J.B., Porciúncula, L.O., 2010. Caffeine prevents disruption of memory consolidation in the inhibitory avoidance and novel object recognition tasks by scopolamine in adult mice. Behav. Brain Res. 214, 254–259.

Bourdy, R., Barrot, M., 2012. A new control center for dopaminergic systems: pulling the VTA by the tail. Trends Neurosci. 35, 681–690.

Boylan, S.M., Greenwood, D.C., Alwan, N., Cooke, M.S., Dolby, V.A., Hay, A.W., Kirk, S.F., Konje, J.C., Potdar, N., Shires, S., Simpson, N.A., Taub, N., Thomas, J.D., Walker, J.J., White, K.L., Wild, C.P., Cade, J.E., 2012. Does nausea and vomiting of pregnancy play a role in the association found between maternal caffeine intake and fetal growth restriction? Matern. Child Health J.. http://dx.doi.org/10.1007/s10995-012-1034-7.

Boylan, S.M., Cade, J.E., Kirk, S.F., Greenwood, D.C., White, K.L., Shires, S., Simpson, N.A., Wild, C.P., Hay, A.W., 2008. Assessing caffeine exposure in pregnant women. Br. J. Nutr. 100, 875–882.

Bracken, M.B., Triche, E.W., Belanger, K., Hellenbrand, K., Leaderer, B.P., 2003. Association of maternal caffeine consumption with decrements in fetal growth. Am. J. Epidemiol. 157, 456–466.

Brent, R.L., Christian, M.S., Diener, R.M., 2011. Evaluation of the reproductive and developmental risks of caffeine. Birth Defects Res. B. Dev. Reprod. Toxicol. 2, 152–187.

Browne, M.L., Bell, E.M., Druschel, C.M., Gensburg, L.J., Mitchell, A.A., Lin, A.E., Romitti, P.A., Correa, A., 2007. National birth defects prevention study. maternal caffeine consumption and risk of cardiovascular malformations. Birth Defects Res. A. Clin. Mol. Teratol. 79, 533–543.

Bruno, A.N., Bonan, C.D., Wofchuk, S.T., Sarkis, J.J., Battastini, A.M.O., 2002. ATP diphosphohydrolase (NTPDase 1) in rat hippocampal slices and effect of glutamate on the enzyme activity in different phases of development. Life Sci. 71, 215–225.

Brunyé, T.T., Mahoney, C.R., Lieberman, H.R., Taylor, H.A., 2010. Caffeine modulates attention network function. Brain Cogn. 72, 181–188.

Cachat, J., Stewart, A., Grossman, L., Gaikwad, S., Kadri, F., Chung, K.M., Wu, N., Wong, K., Roy, S., Suciu, C., Goodspeed, J., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Tan, J., Denmark, A., Gilder, T., Kyzar, E., Dileo, J., Frank, K., Chang, K., Utterback, E., Hart, P., Kalueff, A.V., 2010. Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. Nat. Protoc. 5, 1786–1799.

Study Group, C.A.R.E., 2008. Maternal caffeine intake during pregnancy and risk of fetal growth restriction: a large prospective observational study. BMJ 337, a2332.

Carter, A.J., O'Connor, W.T., Carter, M.J., Ungerstedt, U.J., 1995. Caffeine enhances acetylcholine release in the hippocampus in vivo by a selective interaction with adenosine A1 receptors. Pharmacol. Exp. Ther. 2, 637–642.

Capiotti, K.M., Menezes, F.P., Nazario, L.R., Pohlmann, J.B., de Oliveira, G.M., Fazenda, L., Bogo, M.R., Bonan, C.D., Da Silva, R.S., 2011. Early exposure to caffeine affects gene expression of adenosine receptors, DARPP-32 and BDNF without affecting sensibility and morphology of developing zebrafish (*Danio rerio*). Neurotoxicol. Teratol. 33, 680–685.

Cases, O., Vitalis, T., Seif, I., De Maeyer, E., Sotelo, C., Gaspar, P., 1996. Lack of barrels in the somatosensory cortex of monoamine oxidase A deficient mice: role of a serotonin excess during the critical period. Neuron 16, 297–307.

Chen, J.F., Xu, K., Petzer, J.P., Staal, R., Xu, Y.H., Beilstein, M., Sonsalla, P.K., Castagnoli, K., Castagnoli Jr., N., Schwarzschild, M.A., 2001. Neuroprotection by caffeine and A(2A) adenosine receptor inactivation in a model of Parkinson's disease. J. Neurosci. 21, RC143.

Chen, J.F., Yu, L., Shen, H.Y., He, J.C., Wang, X., Zheng, R., 2010. What knock-out animals tell us about the effects of caffeine. J. Alzheimers Dis. 20 (Suppl. 1), S17–S24.

Chen, M.D., Lin, W.H., Song, Y.M., Lin, P.Y., Ho, L.T., 1994. Effect of caffeine on the levels of brain serotonin and catecholamine in the genetically obese mice. Zhonghua Yi Xue Za Zhi (Taipei) 53, 257–261.

Chen, X., Lan, X., Roche, I., Liu, R., Geiger, J.D., 2008. Caffeine protects against MPTP-induced blood-brain barrier dysfunction in mouse striatum. J. Neurochem. 107, 1147–1157.

Christian, M.S., Brent, R.L., 2001. Teratogen update: evaluation of the reproductive and developmental risks of caffeine. Teratology 64, 51–78.

Ciruela, F., Casadó, V., Rodrigues, R.J., Luján, R., Burgueño, J., Canals, M., Borycz, J., Rebola, N., Goldberg, S.R., Mallol, J., Cortés, A., Canela, E.I., López-Giménez, J.F., Milligan, G., Lluis, C., Cunha, R.A., Ferré, S., Franco, R., 2006. Presynaptic control of striatal glutamatergic neurotransmission by adenosine A1–A2A receptor heteromers. J. Neurosci. 26, 2080–2087.

Coleman, C.G., Baghdoyan, H.A., Lydic, R., 2006. Dialysis delivery of an adenosine A2A agonist into the pontine reticular formation of C57BL/6J mouse increases pontine acetylcholine release and sleep. J. Neurochem. 96, 1750–1759.

Collier, S.A., Browne, M.L., Rasmussen, S.A., Honein, M.A., 2009. National birth defects prevention study. maternal caffeine intake during pregnancy and orofacial clefts. Birth Defects Res. A Clin. Mol. Teratol. 85, 842–849.

Collins, L.E., Galtieri, D.J., Collins, P., Jones, S.K., Port, R.G., Paul, N.E., Hockemeyer, J., Müller, C.E., Salamone, J.D., 2010. Interactions between adenosine and dopamine receptor antagonists with different selectivity profiles: effects on locomotor activity. Behav. Brain Res. 211, 148–155.

Concannon, J.T., Braughler, J.M., Schechter, M.D., 1983. Pre- and postnatal effects of caffeine on brain biogenic amines, cyclic nucleotides and behavior in developing rats. J. Pharmacol. Exp. Ther. 226, 673–679.

Connolly, S., Kingsbury, T.J., 2010. Caffeine modulates CREB-dependent gene expression in developing cortical neurons. Biochem. Biophys. Res. Commun. 397, 152–156.

Costa, M.S., Botton, P.H., Mioranzza, S., Souza, D.O., Porciúncula, L.O., 2008a. Caffeine prevents age-associated recognition memory decline and changes brain-derived neurotrophic factor and tirosine kinase receptor (TrkB) content in mice. Neuroscience 153, 1071–1078.

Costa, M.S., Botton, P.H., Mioranzza, S., Ardais, A.P., Moreira, J.D., Souza, D.O., Porciúncula, L.O., 2008b. Caffeine improves adult mice performance in the object recognition task and increases BDNF and TrkB independent on phospho-CREB immunocontent in the hippocampus. Neurochem. Int. 53, 89–94.

Costenla, A.R., Cunha, R.A., de Mendonça, A., 2010. Caffeine, adenosine receptors, and synaptic plasticity. J. Alzheimers Dis. 20 (Suppl. 1), S25–S34.

Cunha, R.A., 2001. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. Neurochem. Int. 38, 107–125.

Cunha, R.A., 2005. Neuroprotection by adenosine in the brain: from A(1) receptor activation to A (2A) receptor blockade. Purinergic Signal. 1, 111–134.

Cunha, R.A., Agostinho, P.M., 2010. Chronic caffeine consumption prevents memory disturbance in different animal models of memory decline. J. Alzheimers Dis. 20 (Suppl. 1), S95–S116.

da Silva, R.S., Richetti, S.K., Tonial, E.M., Bogo, M.R., Bonan, C.D., 2012. Profile of nucleotide catabolism and ectonucleotidase expression from the hippocampi of neonatal rats after caffeine exposure. Neurochem. Res. 37, 23–30.

da Silva, R.S., Richetti, S.K., da Silveira, V.G., Battastini, A.M., Bogo, M.R., Lara, D.R., Bonan, C.D., 2008. Maternal caffeine intake affects acetylcholinesterase in hippocampus of neonate rats. Int. J. Dev. Neurosci. 26, 339–343.

Dall'Igna, O.P., Porciúncula, L.O., Souza, D.O., Cunha, R.A., Lara, D.R., 2003. Neuroprotection by caffeine and adenosine A2A receptor blockade of beta-amyloid neurotoxicity. Br. J. Pharmacol. 138, 1207–1209.

Dall'Igna, O.P., Fett, P., Gomes, M.W., Souza, D.O., Cunha, R.A., Lara, D.R., 2007. Caffeine and adenosine A(2a) receptor antagonists prevent beta-amyloid (25–35)-induced cognitive deficits in mice. Exp. Neurol. 203, 241–245.

Danysz, W., 2001. Neurotoxicity as a mechanism for neurodegenerative disorders: basic and clinical aspects. Expert Opin. Invest. Drugs 10, 985–989.

Darnall, R.A., Ariagno, R.L., Kinney, H.C., 2006. The late preterm infant and the control of breathing, sleep, and brainstem development: a review. Clin. Perinatol. 33, 883–914.

Davis, P.G., Schmidt, B., Roberts, R.S., Doyle, L.W., Asztalos, E., Haslam, R., Sinha, S., Tin, W.Caffeine for Apnea of Prematurity Trial Group, 2010. Caffeine for apnea of prematurity trial: benefits may vary in subgroups. J. Pediatr. 156, 382–387.

De Koninck, Y., Mody, I., 1996. The effects of raising intracellular calcium on synaptic GABAA receptor-channels. Neuropharmacology 35, 1365–1374.

De Marco, P., Merello, E., Calevo, M.G., Mascelli, S., Pastorino, D., Crocetti, L., De Biasio, P., Piatelli, G., Cama, A., Capra, V., 2011. Maternal periconceptional factors affect the risk of spina bifida-affected pregnancies: an Italian case–control study. Childs Nerv. Syst. 27, 1073–1081.

Dunwiddie, T.V., Masino, S.A., 2001. The role and regulation of adenosine in the central nervous system. Annu. Rev. Neurosci. 24, 31–55.

Einöther, S.J., Giesbrecht, T., 2013. Caffeine as an attention enhancer: reviewing existing assumptions. Psychopharmacology (Berl) 225, 251–274.

El Yacoubi, M., Ledent, C., Parmentier, M., Costentin, J., Vaugeois, J.M., 2000. The anxiogenic-like effect of caffeine in two experimental procedures measuring anxiety in the mouse is not shared by selective A(2A) adenosine receptor antagonists. Psychopharmacology (Berl) 148, 153–163.

Eskelinen, M.H., Ngandu, T., Tuomilehto, J., Soininen, H., Kivipelto, M., 2009. Midlife coffee and tea drinking and the risk of late-life dementia: a population-based CAIDE study. J. Alzheimers Dis. 16, 85–91.

Espinosa, J., Rocha, A., Nunes, F., Costa, M.S., Schein, V., Kazlauckas, V., Kalinine, E., Souza, D.O., Cunha, R.A., Porciúncula, L.O., 2013. Caffeine consumption prevents memory impairment, neuronal damage, and adenosine A2A receptors upregulation in the hippocampus of a rat model of sporadic dementia. J. Alzheimers Dis. 34, 509–518.

Etzel, B.A., Guillet, R., 1994. Effects of neonatal exposure to caffeine on adenosine A1 receptor ontogeny using autoradiography. Brain Res. Dev. Brain Res. 82, 223–230.

Evans, S.M., Griffiths, R.R., 1992. Caffeine tolerance and choice in humans. Psychopharmacology (Berl) 108, 51–59.

Ferré, S., 2010. Role of the central ascending neurotransmitter systems in the psychostimulant effects of caffeine. J. Alzheimers Dis. 20 (Suppl. 1), S35–S49.

Ferré, S., Goldberg, S.R., Lluis, C., Franco, R., 2009. Looking for the role of cannabinoid receptor heteromers in striatal function. Neuropharmacology 56 (Suppl. 1), 226–234.

Ferré, S., 2008. An update on the mechanisms of the psychostimulant effects of caffeine. J. Neurochem. 105, 1067–1079.

Ferré, S., Ciruela, F., Canals, M., Marcellino, D., Burgueno, J., Casadó, V., Hillion, J., Torvinen, M., Fanelli, F., Pd. Benedetti, P., Goldberg, S.R., Bouvier, M., Fuxe, K., Agnati, L.F., Lluis, C., Franco, R., Woods, A., 2004. Adenosine A2A-dopamine D2 receptor–receptor heteromers. Targets for neuro-psychiatric disorders. Parkinsonism Relat. Disord. 10, 265–271.

Fibiger, H.C., 1991. Cholinergic mechanisms in learning, memory and dementia: a review of recent evidence. Trends Neurosci. 14, 220–223.

Fisher, S., Guillet, R., 1997. Neonatal caffeine alters passive avoidance retention in rats in an ageand gender-related manner. Dev. Brain Res. 98, 145–149.

Fisone, G., Borgkvist, A., Usiello, A., 2004. Caffeine as a psychomotor stimulant: mechanism of action. Cell. Mol. Life Sci. 61, 857–872.

Francis, S.H., Sekhar, K.R., Ke, H., Corbin, J.D., 2011. Inhibition of cyclic nucleotide phosphodiesterases by methylxanthines and related compounds. Handb. Exp. Pharmacol. 200, 93–133.

Fredholm, B.B., 1980. Are the effects of methylxanthines due to antagonism of endogenous adenosine? Trends Pharm. Sci. 1, 129.

Fredholm, B.B., Persson, C.G., 1982. Xanthine derivatives as adenosine receptor antagonists. Eur. J. Pharmacol. 81, 673–676.

Fredholm, B.B., Bättig, K., Holmén, J., Nehlig, A., Zvartau, E.E., 1999. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol. Rev. 51, 83–133.

Fredholm, B.B., Chen, J.F., Masino, S.A., Vaugeois, J.M., 2005. Actions of adenosine at its receptors in the CNS: insights from knockouts and drugs. Annu. Rev. Pharmacol. Toxicol. 45, 385–412.

Fuxe, K., Borroto-Escuela, D.O., Marcellino, D., Romero-Fernandez, W., Frankowska, M., Guidolin, D., Filip, M., Ferraro, L., Woods, A.S., Tarakanov, A., Ciruela, F., Agnati, L.F., Tanganelli, S., 2012. GPCR heteromers and their allosteric receptor–receptor interactions. Curr. Med. Chem. 19, 356–363.

Fuxe, K., Ferré, S., Genedani, S., Franco, R., Agnati, L.F., 2007. Adenosine receptor-dopamine receptor interactions in the basal ganglia and their relevance for brain function. Physiol. Behav. 92, 210–217.

Gaspar, P., Cases, O., Maroteaux, L., 2003. The developmental role of serotonin: news from mouse molecular genetics. Nat. Rev. Neurosci. 4, 1002–1012.

Gaytan, S.P., Pasaro, R., 2012. Neonatal caffeine treatment up-regulates adenosine receptors in brainstem and hypothalamic cardio-respiratory related nuclei of rat pups. Exp. Neurol. 237, 247–259.

Gaytan, S.P., Saadani-Makki, F., Bodineau, L., Frugière, A., Larnicol, N., Pásaro, R., 2006. Effect of postnatal exposure to caffeine on the pattern of adenosine A1 receptor distribution in respiration-related nuclei of the rat brainstem. Auton. Neurosci. 126–127, 339–346.

George, L., Granath, F., Johansson, A.L., Olander, B., Cnattingius, S., 2006. Risks of repeated miscarriage. Paediatr. Perinat. Epidemiol. 20, 119–126.

Giannelli, M., Doyle, P., Roman, E., Pelerin, M., Hermon, C., 2003. The effect of caffeine consumption and nausea on the risk of miscarriage. Paediatr. Perinat. Epidemiol. 17, 316–323.
Glavin, G.B., Krueger, H., 1985. Effects of prenatal caffeine administration on offspring mortality, open-field behavior and adult gastric ulcer susceptibility. Neurobehav. Toxicol. Teratol. 7, 29–32.
Gomes, C.V., Kaster, M.P., Tomé, A.R., Agostinho, P.M., Cunha, R.A., 2011. Adenosine receptors and brain diseases: neuroprotection and neurodegeneration. Biochim. Biophys. Acta 1808, 1380–1399.
Gorodischer, R., Zmora, E., Ben-Zvi, Z., Warszwaski, D., Yaari, A., Sofer, S., Arnaud, M.J., 1986. Urinary metabolites of caffeine in the premature infant. Eur. J. Clin. Pharmacol. 31, 497–499.
Grace, A.A., Floresco, S.B., Goto, Y., Lodge, D.J., 2007. Regulation of firing of dopaminergic neurons and control of goal-directed behaviors. Trends Neurosci. 30, 220–227.
Griffiths, R.R., Mumford, G.K., 1996. Caffeine reinforcement, discrimination, tolerance, and physical dependence in laboratory animals and humans. In: Schuster, C.R., Kuhar, M.J. (Eds), Pharmacological Aspects of Drug Dependence: Toward an Integrated Neurobehavioral Approach. In: Handbook of Experimental Pharmacology. Springer, Berlin, Heidelberg, New York, pp. 315–341.
Grosso, L.M., Rosenberg, K.D., Belanger, K., Saftlas, A.F., Leaderer, B., Bracken, M.B., 2001. Maternal caffeine intake and intrauterine growth retardation. Epidemiology 12, 447–455.
Guillet, R., Kellogg, C., 1991a. Neonatal exposure to therapeutic caffeine alters the ontogeny of adenosine A1 receptors in brain of rats. Neuropharmacology 30, 489–496.
Guillet, R., Kellogg, C.K., 1991b. Neonatal caffeine exposure alters developmental sensitivity to adenosine receptor ligands. Pharmacol. Biochem. Behav. 40, 811–817.
Guillet, R., 1990. Neonatal caffeine exposure alters adenosine receptor control of locomotor activity. Dev. Pharmacol. Ther. 15, 94–100.
Hadfield, M.G., 1997. Caffeine and the olfactory bulb. Mol. Neurobiol. 15, 31–39.
Hadfield, M.G., Milio, C., 1989. Caffeine and regional brain monoamine utilization in mice. Life Sci. 45, 2637–2644.
Haleem, D.J., Yasmeen, A., Haleem, M.A., Zafar, A., 1995. 24 h Withdrawal following repeated administration of caffeine attenuates brain serotonin but not tryptophan in rat brain: implications for caffeine-induced depression. Life Sci. 57, PL285–PL292.
Herlenius, E., Lagercrantz, H., 2004. Development of neurotransmitter systems during critical periods. Exp. Neurol. 190, 8–21.
Hernández-Cruz, A., Díaz-Muñoz, M., Gómez-Chavarín, M., Cañedo-Merino, R., Protti, D.A., Escobar, A.L., Sierralta, J., Suárez-Isla, B.A., 1995. Properties of the ryanodine-sensitive release channels that underlie caffeine-induced Ca^{2+} mobilization from intracellular stores in mammalian sympathetic neurons. Eur. J. Neurosci. 7, 1684–1699.
Hohmann, C.F., 2003. A morphogenetic role for acetylcholine in mouse cerebral neocortex. Neurosci. Biobehav. Rev. 27, 351–363.
Holloway Jr., W.R., Thor, D.H., 1982. Caffeine sensitivity in the neonatal rat. Neurobehav. Toxicol. Teratol. 4, 331–333.
Hughes, R.N., Beveridge, I.J., 1986. Behavioral effects of prenatal exposure to caffeine in rats. Life Sci. 38, 861–868.
Hsu, C.W., Wang, C.S., Chiu, T.H., 2010. Caffeine and a selective adenosine A2A receptor antagonist induce sensitization and cross-sensitization behavior associated with increased striatal dopamine in mice. J. Biomed. Sci. 17, 4.
Hughes, R.N., Beveridge, I.J., 1991. Behavioral effects of exposure to caffeine during gestation, lactation or both. Neurotoxicol. Teratol. 13, 641–647.
Hughes, R.N., Beveridge, I.J., 1990. Sex-and age-dependent effects of prenatal exposure to caffeine on open-field behavior, emergence latency and adrenal weights in rats. Life Sci. 47, 2075–2088.
Iglesias, I., León, D., Ruiz, M.A., Albasanz, J.L., Martín, M., 2006. Chronic intake of caffeine during gestation down regulates metabotropic glutamate receptors in maternal and fetal rat heart. Amino Acids 30, 257–266.
Ikeda, R., Igari, Y., Fuchigami, Y., Wada, M., Kuroda, N., Nakashima, K., 2011. Pharmacodynamic interactions between MDMA and concomitants in MDMA tablets on extracellular dopamine and serotonin in the rat brain. Eur. J. Pharmacol. 660, 318–325.
Jacombs, A., Ryan, J., Loupis, A., Pollard, I., 1999. Maternal caffeine consumption during pregnancy does not affect preimplantation development but delays early postimplantation growth in rat embryos. Reprod. Fertil. Dev. 11, 211–218.
James, J.E., Rogers, P.J., 2005. Effects of caffeine on performance and mood: withdrawal reversal is the most plausible explanation. Psychopharmacology (Berl) 182, 1–8.
Jarvis, M.J., 1993. Does caffeine intake enhance absolute levels of cognitive performance. Psychopharmacology (Berl) 110, 45–52.
Joghataie, M.T., Roghani, M., Negahdar, F., Hashemi, L., 2004. Protective effect of caffeine against neurodegeneration in a model of Parkinson's disease in rat: behavioral and histochemical evidence. Parkinsonism Relat. Disord. 10, 465–468.
Johansson, B., Georgiev, V., Lindstrom, K., Fredholm, B.B., 1997. A1 and A2A adenosine receptors and A1 mRNA in mouse brain: effect of long-term caffeine treatment. Brain Res. 762, 153–164.
Johnson, M.H., 2003. Development of human brain functions. Biol. Psychiatry 54, 1312–1316.
Kaplan, G.B., Greenblatt, D.J., Ehrenberg, B.L., Goddard, J.E., Cotreau, M.M., Harmatz, J.S., Shader, R.I., 1997. Dose-dependent pharmacokinetics and psychomotor effects of caffeine in humans. J. Clin. Pharmacol. 37, 693–703.
Kaplan, G.B., Greenblatt, D.J., Kent, M.A., Cotreau-Bibbo, M.M., 1993. Caffeine treatment and withdrawal in mice: relationships between dosage, concentrations, locomotor activity and A1 adenosine receptor binding. J. Pharmacol. Exp. Ther. 3, 1563–1572.
Karcz-Kubicha, M., Antoniou, K., Terasmaa, A., Quarta, D., Solinas, M., Justinova, Z., Pezzola, A., Reggio, R., Müller, C.E., Fuxe, K., Goldberg, S.R., Popoli, P., Ferré, S., 2003. Involvement of adenosine A1 and A2A receptors in the motor effects of caffeine after its acute and chronic administration. Neuropsychopharmacology 28, 1281–1291.
Kaufling, J., Waltisperger, E., Bourdy, R., Valera, A., Veinante, P., Freund-Mercier, M.J., Barrot, M., 2010. Pharmacological recruitment of the GABAergic tail of the ventral tegmental area by acute drug exposure. Br. J. Pharmacol. 161, 1677–1691.
Khaliq, S., Haider, S., Naqvi, F., Perveen, T., Saleem, S., Haleem, D.J., 2012. Altered brain serotonergic neurotransmission following caffeine withdrawal produces behavioral deficits in rats. Pak. J. Pharm. Sci. 25, 21–25.
Kimmel, C.A., Kimmel, G.L., White, C.G., Grafton, T.F., Young, J.F., Nelson, C.J., 1984. Blood flow changes and conceptal development in pregnant rats in response to caffeine. Fundam. Appl. Toxicol. 4, 240–247.
Klebanoff, M.A., Levine, R.J., Clemens, J.D., Wilkins, D.G., 2002. Maternal serum caffeine metabolites and small-for-gestational age birth. Am. J. Epidemiol. 155, 32–37.
Korkotian, E., Segal, M., 1999. Release of calcium from stores alters the morphology of dendritic spines in cultured hippocampal neurons. Proc. Natl. Acad. Sci. U S A 96, 12068–12072.
Kopf, S.R., Melani, A., Pedata, F., Pepeu, G., 1999. Adenosine and memory storage: effect of A(1) and A(2) receptor antagonists. Psychopharmacology 146, 214–219.
Koppe, J.G., de Bruijne, J.I., de Boer, P., 1979. Apneic spells and transcutaneous PO 2: treatment with caffeine, 19-year follow-up. Birth Defects Orig. Artic. Ser. 15, 437–445.
Kucharski, R., Maleszka, R., 2005. Microarray and real-time PCR analyses of gene expression in the honeybee brain following caffeine treatment. J. Mol. Neurosci. 27, 269–276.
Langer, D., Ikehara, Y., Takebayashi, H., Hawkes, R., Zimmermann, H., 2007. The ectonucleotidases alkaline phosphatase and nucleoside triphosphate diphosphohydrolase 2 are associated with subsets of progenitor cell populations in the mouse embryonic, postnatal and adult neurogenic zones. Neuroscience 150, 863–879.
Lara, D.R., 2010. Caffeine, mental health, and psychiatric disorders. J. Alzheimers Dis. 20 (Suppl. 1), S239–S248.
Lassiter, T.L., Barone, Jr., Padilla, S., 1998. Ontogenetic differences in the regional and cellular acetylcholinesterase and butyrylcholinesterase activity in the rat brain. Dev. Brain Res. 105, 109–123.
Leeder, J.S., 2001. Pharmacogenetics and pharmacogenomics. Pediatr. Clin. North Am. 48, 765–781.
Le Guennec, J.-C., Billon, B., 1987. Delay in caffeine elimination in breast-fed infants. Pediatrics 79, 264–268.
Le Moal, M., Simon, H., 1991. Mesocorticolimbic dopaminergic network: functional and regulatory roles. Physiol. Rev. 71, 155–234.
Lelo, A., Birkett, D.J., Robson, R.A., Miners, J.O., 1986. Comparative pharmacokinetics of caffeine and its primary demethylated metabolites paraxanthine, theobromine and theophylline in man. Br. J. Clin. Pharmacol. 22, 177–182.
Leon, A.E., Michienzi, K., Ma, C.X., Hutchison, A.A., 2007. Serum caffeine concentrations in preterm neonates. Am. J. Perinatol. 24, 39–47.
León, D., Albasanz, J.L., Ruiz, M.A., Fernandez, M., Martín, M., 2002. Adenosine A1 receptor down-regulation in mothers and fetal brain after caffeine and theophylline treatments to pregnant rats. J. Neurochem. 82, 625–634.
León, D., Albasanz, J.L., Ruíz, M.A., Martín, M., 2005a. Chronic caffeine or theophylline intake during pregnancy inhibits A1 receptor function in the rat brain. Neuroscience 131, 481–489.
León, D., Albasanz, J.L., Ruíz, M.A., Iglesias, I., Martín, M., 2005b. Effect of chronic gestational treatment with caffeine or theophylline on Group I metabotropic glutamate receptors in maternal and fetal brain. J. Neurochem. 94, 440–451.
Li, X.D., He, R.R., Qin, Y., Tsoi, B., Li, Y.F., Ma, Z.L., Yang, X., Kurihara, H., 2012. Caffeine interferes embryonic development through over-stimulating serotonergic system in chicken embryo. Food Chem. Toxicol. 50, 1848–1853.
Lim, B.V., Jang, M.H., Shin, M.C., Kim, H.B., Kim, Y.J., Kim, Y.P., Chung, J.H., Kim, H., Shin, M.S., Kim, S.S., Kim, E.H., Kim, C.J., 2001. Caffeine inhibits exercise-induced increase in tryptophan hydroxylase expression in dorsal and median raphe of Sprague–Dawley rats. Neurosci. Lett. 308, 25–28.
Lindskog, M., Svenningsson, P., Pozzi, L., Kim, Y., Fienberg, A.A., Bibb, J.A., Fredholm, B.B., Nairn, A.C., Greengard, P., Fisone, G., 2002. Involvement of DARPP-32 phosphorylation in the stimulant action of caffeine. Nature 418, 774–778.
Loopuijt, L.D., Schmidt, W.J., 1998. The role of NMDA receptors in the slow neuronal degeneration of Parkinson's disease. Amino Acids 14, 17–23.
Lorenzo, A.M., León, D., Castillo, C.A., Ruiz, M.A., Albasanz, J.L., Martín, M., 2010. Maternal caffeine intake during gestation and lactation down-regulates adenosine A1 receptor in rat brain from mothers and neonates. J. Neurosci. Res. 88, 1252–1261.
Lorist, M.M., Tops, M., 2003. Caffeine, fatigue, and cognition. Brain Cogn. 53, 82–94.

Lu, K.T., Wu, S.P., Gean, P.W., 1999. Promotion of forskolin-induced long-term potentiation of synaptic transmission by caffeine in area CA1 of the rat hippocampus. Chin. J. Physiol. 42, 249–253.
Maconochie, N., Doyle, P., Prior, S., Simmons, R., 2007. Risk factors for first trimester miscarriage – results from a UK-population-based case-control study. BJOG 114, 170–186.
Maia, L., de Mendonça, A., 2002. Does caffeine intake protect from Alzheimer's disease? Eur. J. Neurol. 9, 377–382.
Marangos, P.J., Boulenger, J.-P., Patel, J., 1984. Effects of chronic caffeine on brain adenosine receptors: regional and ontogenetic studies. Life Sci. 34, 899–907.
Marchal, F., Bairam, A., Vert, P., 1987. Neonatal apnea and apneic syndromes. Clin. Perinatol. 14, 509–529.
Marks, G.A., Shaffery, J.P., Speciale, S.G., Birabil, C.G., 2003. Enhancement of rapid eye movement sleep in the rat by actions at A1 and A2a adenosine receptor subtypes with a differential sensitivity to atropine. Neuroscience 116, 913–920.
Martín, E.D., Buño, W., 2003. Caffeine-mediated presynaptic long-term potentiation in hippocampal CA1 pyramidal neurons. J. Neurophysiol. 89, 3029–3038.
Mattson, M.P., 2008. Glutamate and neurotrophic factors in neuronal plasticity and disease. Ann. N. Y. Acad. Sci. 1144, 97–112.
Maximino, C., Benzecry, R., Matos Oliveira, K.R., Batista, E.J.O., Herculano, A.M., Rosemberg, D.B., de Oliveira, D.L., Blaser, R.E., 2012. A comparison of the light/dark and novel tank tests in zebrafish. Behaviour 149, 1099–1123.
McNamara, R., Kerans, A., O'Neill, B., Harkin, A., 2006. Caffeine promotes hyperthermia and serotonergic loss following co-administration of the substituted amphetamines, MDMA ("Ecstasy") and MDA ("Love"). Neuropharmacology 50, 69–80.
Millar, D., Schmidt, B., 2004. Controversies surrounding xanthine therapy. Semin. Neonatol. 3, 239–244.
Miller, E.A., Manning, S.E., Rasmussen, S.A., Reefhuis, J., Honein, M.A., National Birth Defects Prevention Study, 2009. Maternal exposure to tobacco smoke, alcohol and caffeine, and risk of anorectal atresia: National Birth Defects Prevention Study 1997–2003. Paediatr. Perinat. Epidemiol. 23, 9–17Miller, E.A., Manning, S.E., Rasmussen, S.A., Reefhuis, J., Honein, M.A., National Birth Defects Prevention Study, . Maternal exposure to tobacco smoke, alcohol and caffeine, and risk of anorectal atresia: National Birth Defects Prevention Study 1997–2003. Paediatr. Perinat. Epidemiol. 23, 9–17Miller, E.A., Manning, S.E., Rasmussen, S.A., Reefhuis, J., Honein, M.A., National Birth Defects Prevention Study, . Maternal exposure to tobacco smoke, alcohol and caffeine, and risk of anorectal atresia: National Birth Defects Prevention Study 1997–2003. Paediatr. Perinat. Epidemiol. 23, 9–17Miller, E.A., Manning, S.E., Rasmussen, S.A., Reefhuis, J., Honein, M.A., National Birth Defects Prevention Study, . Maternal exposure to tobacco smoke, alcohol and caffeine, and risk of anorectal atresia: National Birth Defects Prevention Study 1997–2003. Paediatr. Perinat. Epidemiol. 23, 9–17Miller, E.A., Manning, S.E., Rasmussen, S.A., Reefhuis, J., Honein, M.A., National Birth Defects Prevention Study, . Maternal exposure to tobacco smoke, alcohol and caffeine, and risk of anorectal atresia: National Birth Defects Prevention Study 1997–2003. Paediatr. Perinat. Epidemiol. 23, 9–17Miller, E.A., Manning, S.E., Rasmussen, S.A., Reefhuis, J., Honein, M.A., National Birth Defects Prevention Study, . Maternal exposure to tobacco smoke, alcohol and caffeine, and risk of anorectal atresia: National Birth Defects Prevention Study 1997–2003. Paediatr. Perinat. Epidemiol. 23, 9–17Miller, E.A., Manning, S.E., Rasmussen, S.A., Reefhuis, J., Honein, M.A., National Birth Defects Prevention Study, . Maternal exposure to tobacco smoke, alcohol and caffeine, and risk of anorectal atresia: National Birth Defects Prevention Study 1997–2003. Paediatr. Perinat. Epidemiol. 23, 9–17.
Mukhopadhyay, S., Poddar, M.K., 1995. Caffeine-induced locomotor activity: possible involvement of GABAergic-dopaminergic-adenosinergic interaction. Neurochem. Res. 20, 39–44.
Nakamoto, T., 2004. Neurodevelopmental consequences of coffee/caffeine exposure. In: Nehlig, A. (Ed.), Coffee, Tea, Chocolate, and the Brain. CRC Press, London, pp. 97–113.
Nehlig, A., Daval, J.L., Debry, G., 1992. Caffeine and the central nervous system: mechanisms of action, biochemical, metabolic and psychostimulant effects. Brain Res. Brain Res. Rev. 17, 139–170.
Nehlig, A., Debry, G., 1994. Consequences on the newborn of chronic maternal consumption of coffee during gestation and lactation: a review. J. Am. Coll. Nutr. 13, 6–21.
Nehlig, A., 2010. Is caffeine a cognitive enhancer? J. Alzheimers Dis. 20 (Suppl. 1), S85–S94.
Nestler, E.J., Carlezon Jr., W.A., 2006. The mesolimbic dopamine reward circuit in depression. Biol. Psychiatry 59, 1151–1159.
Nobre Jr., H.V., Cunha, G.M., de Vasconcelos, L.M., Magalhães, H.I., Oliveira Neto, R.N., Maia, F.D., de Moraes, M.O., Leal, L.K., Viana, G.S., 2010. Caffeine and CSC, adenosine A2A antagonists, offer neuroprotection against 6-OHDA-induced neurotoxicity in rat mesencephalic cells. Neurochem. Int. 56, 51–58.
Nordmark, A., Lundgren, S., Cnattingius, S., Rane, A., 1999. Dietary caffeine as a probe agent for assessment of cytochrome P4501A2 activity in random urine samples. Br. J. Clin. Pharmacol. 47, 397–402.
Okada, M., Kawata, Y., Murakami, T., Wada, K., Mizuno, K., Kondo, T., Kaneko, S., 1999. Differential effects of adenosine receptor subtypes on release and reuptake of hippocampal serotonin. Eur. J. Neurosci. 11, 1–9.
Pan, H.Z., Chen, H.H., 2007. Hyperalgesia, low-anxiety, and impairment of avoidance learning in neonatal caffeine-treated rats. Psychopharmacology 191, 119–125.
Pandolfo, P., Machado, N.J., Köfalvi, A., Takahashi, R.N., Cunha, R.A., 2013. Caffeine regulates frontocorticostriatal dopamine transporter density and improves attention and cognitive deficits in an animal model of attention deficit hyperactivity disorder. Eur. Neuropsychopharmacol. 23, 317–328.
Parazzini, F., Chiaffarino, F., Chatenoud, L., Tozzi, L., Cipriani, S., Chiantera, V., Fedele, L., 2005. Maternal coffee drinking in pregnancy and risk of small for gestational age birth. Eur. J. Clin. Nutr. 59, 299–301.
Parsons, W.D., Neims, A.H., 1981. Prolonged half-life of caffeine in healthy term newborn infants. J. Pediatr. 98, 640–641.
Patel, J.C., Witkovsky, P., Avshalumov, M.V., Rice, M.E., 2009. Mobilization of calcium from intracellular stores facilitates somatodendritic dopamine release. J. Neurosci. 29, 6568–6579.
Pearlman, S.A., Duran, C., Wood, M.A., Maisels, M.J., Berlin Jr., C.M., 1989. Caffeine pharmacokinetics in preterm infants older than 2 weeks. Dev. Pharmacol. Ther. 12, 65–69.
Peruzzi, G., Lombardelli, G., Abbracchio, M.P., Coen, E., Cattabeni, F., 1985. Perinatal caffeine treatment: behavioral and biochemical effects in rats before weaning. Neurobehav. Toxicol. Teratol. 7, 453–460.
Picard, N., Guenin, S., Larnicol, N., Perrin, Y., 2008. Maternal caffeine ingestion during gestation and lactation influences respiratory adaptation to acute alveolar hypoxia in newborn rats and adenosine A(2A) and GABA(A) receptor mRNA transcription. Neuroscience 156, 630–639.
Pinto-Duarte, A., Coelho, J.E., Cunha, R.A., Ribeiro, J.A., Sebastião, A.M., 2005. Adenosine A2A receptors control the extracellular levels of adenosine through modulation of nucleoside transporters activity in the rat hippocampus. J. Neurochem. 93, 595–604.
Pires, V.A., Pamplona, F.A., Pandolfo, P., Prediger, R.D., Takahashi, R.N., 2010. Chronic caffeine treatment during prepubertal period confers long-term cognitive benefits in adult spontaneously hypertensive rats (SHR), an animal model of attention deficit hyperactivity disorder (ADHD). Behav. Brain Res. 215, 39–44.
Polanczyk, G., de Lima, M.S., Horta, B.L., Biederman, J., Rohde, L.A., 2007. The worldwide prevalence of ADHD: a systematic review and metaregression analysis. Am. J. Psychiatry 164, 942–948.
Postuma, R.B., Lang, A.E., Munhoz, R.P., Charland, K., Pelletier, A., Moscovich, M., Filla, L., Zanatta, D., Romenets, S. Rios, Altman, R., Chuang, R., Shah, B., 2012. Caffeine for treatment of Parkinson disease: a randomized controlled trial. Neurology 79, 651–658.
Powell, K.R., Iuvone, P.M., Holtzman, S.G., 2001. The role of dopamine in the locomotor stimulant effects and tolerance to these effects of caffeine. Pharmacol. Biochem. Behav. 69, 59–70.
Quiroz, C., Gomes, C., Pak, A.C., Ribeiro, J.A., Goldberg, S.R., Hope, B.T., Ferré, S., 2006. Blockade of adenosine A2A receptors prevents protein phosphorylation in the striatum induced by cortical stimulation. J. Neurosci. 26, 10808–10812.
Rainnie, D.G., Grunze, H.C.R., McCarly, R.W., Greene, R.W., 1994. Adenosine inhibition of mesopontine cholinergic neurons: implications for EEG arousal. Science 263, 689–692.
Reith, M.E., Sershen, H., Lajtha, A., 1987. Effects of caffeine on monoaminergic systems in mouse brain. Acta Biochim. Biophys. Hung. 22, 149–163.
Rhoads, D.E., Huggler, A.L., Rhoads, L.J., 2011. Acute and adaptive motor responses to caffeine in adolescent and adult rats. Pharmacol. Biochem. Behav. 99, 81–86.
Ribeiro, J.A., 1999. Adenosine A2A receptor interactions with receptors for other neurotransmitters and neuromodulators. Eur. J. Pharmacol. 375, 101–113.
Ribeiro, J.A., Sebastião, A.M., 2010. Caffeine and adenosine. J. Alzheimers Dis. 20 (Suppl. 1), S3–S15.
Richendrfer, H., Pelkowski, S.D., Colwill, R.M., Creton, R., 2012. On the edge: pharmacological evidence for anxiety-related behavior in zebrafish larvae. Behav. Brain Res. 228, 99–106.
Riederer, P., Hoyer, S., 2006. From benefit to damage. Glutamate and advanced glycation end products in Alzheimer brain. J. Neural Transm. 113, 1671–1677.
Ritchie, K., Carrière, I., de Mendonca, A., Portet, F., Dartigues, J.F., Rouaud, O., Barberger-Gateau, P., Ancelin, M.L., 2007. The neuroprotective effects of caffeine: a prospective population study (the Three City Study). Neurology 69, 536–545.
Rivkees, S.A., Wendler, C.C., 2011. Adverse and protective influences of adenosine on the newborn and embryo: implications for preterm white matter injury and embryo protection. Pediatr. Res. 69, 271–278.
Rivkees, S.A., 1995. The ontogeny of cardiac and neural A1 adenosine receptor expression in rats. Brain Res. Dev. Brain Res. 89, 202–213.
Robertson, D., Wade, D., Workman, R., Woosley, R.L., Oates, J.A., 1981. Tolerance to the humoral and hemodynamic effects of caffeine in man. J. Clin. Invest. 67, 1111–1117.
Rogers, P.J., Heatherley, S.V., Mullings, E.L., Smith, J.E., 2013. Faster but not smarter: effects of caffeine and caffeine withdrawal on alertness and performance. Psychopharmacology (Berl) 226, 229–240.
Rogers, P.J., Hohoff, C., Heatherley, S.V., Mullings, E.L., Maxfield, P.J., Evershed, R.P., Deckert, J., Nutt, D.J., 2010. Association of the anxiogenic and alerting effects of caffeine with ADORA2A and ADORA1 polymorphisms and habitual level of caffeine consumption. Neuropsychopharmacology 35, 1973–1983.
Rogers, P.J., Heatherley, S.V., Mullings, E.L., Wu, Y., Leonards, U., 2006. Caffeine and anxiety. Appetite 47, 274.
Rogers, P.J., Heatherley, S.V., Hayward, R.C., Seers, H.E., Hill, J., Kane, M., 2005. Effects of caffeine and caffeine withdrawal on mood and cognitive performance degraded by sleep restriction. Psychopharmacology (Berl) 179, 742–752.

Rogers, P.J., Martin, J., Smith, C., Heatherley, S.V., Smit, H.J., 2003. Absence of reinforcing, mood and psychomotor performance effects of caffeine in habitual non-consumers of caffeine. Psychopharmacology (Berl) 167, 54–62.
Rosemberg, D.B., Rico, E.P., Mussulini, B.H., Piato, A.L., Calcagnotto, M.E., Bonan, C.D., Dias, R.D., Blaser, R.E., Souza, D.O., de Oliveira, D.L., 2011. Differences in spatio-temporal behavior of zebrafish in the open tank paradigm after a short-period confinement into dark and bright environments. PLoS One 6, e19397.
Rosin, D.L., Robeva, A., Woodard, R.L., Guyenet, P.G., Linden, J., 1998. Immunohistochemical localization of adenosine A2A receptors in the rat central nervous system. J. Comp. Neurol. 401, 163–186.
Ross, G.W., Abbott, R.D., Petrovitch, H., Morens, D.M., Grandinetti, A., Tung, K.H., Tanner, C.M., Masaki, K.H., Blanchette, P.L., Curb, J.D., Popper, J.S., White, L.R., 2000. Association of coffee and caffeine intake with the risk of Parkinson disease. JAMA 283, 2674–2679.
Ruediger, T., Bolz, J., Bagnard, D., 2007. Neurotransmitters and the Development of Neuronal Circuits Axon Growth and Guidance. Springer, New York, pp. 104–114.
Sahir, N., Mas, C., Bourgeois, F., Simonneau, M., Evrard, P., Gressens, P., 2001. Caffeine-induced telencephalic vesicle evagination in early post-implantation mouse embryos involves cAMP-dependent protein kinase (PKA) inhibition. Cereb. Cortex 11, 343–349.
Sahir, N., Bahi, N., Evrard, P., Gressens, P., 2000. Caffeine induces in vivo premature appearance of telencephalic vesicles. Brain Res. Dev. Brain Res. 121, 213–217.
Sallaberry, C., Nunes, F., Costa, M.S., Fioreze, G.T., Ardais, A.P., Botton, P.H., Klaudat, B., Forte, T., Souza, D.O., Elisabetsky, E., Porciúncula, L.O., 2013. Chronic caffeine prevents changes in inhibitory avoidance memory and hippocampal BDNF immunocontent in middle-aged rats. Neuropharmacology 64, 153–159.
Sarter, M., Bruno, J.P., 2000. Cortical cholinergic inputs mediating arousal, attentional processing and dreaming: differential afferent regulation of the basal forebrain by telencephalic and brainstem afferents. Neuroscience 95, 933–952.
Sato, S.M., Wissman, A.M., McCollum, A.F., Woolley, C.S., 2011. Quantitative mapping of cocaine-induced ΔFosB expression in the striatum of male and female rats. PLoS One 6, e21783.
Savitz, D., Chan, R., Herring, A., Howards, P., Hartmann, K., 2008. Caffeine and miscarriage risk. Epidemiology 19, 55–62.
Schmidt, R.J., Romitti, P.A., Burns, T.L., Browne, M.L., Druschel, C.M., Olney, R.S., National Birth Defects Prevention Study, 2009. Maternal caffeine consumption and risk of neural tube defects. Birth Defects Res. A Clin. Mol. Teratol. 85, 879–889.
Schultz, W., 2007. Multiple dopamine functions at different time courses. Annu. Rev. Neurosci. 30, 259–288.
Schwarzschild, M.A., Xu, K., Oztas, E., Petzer, J.P., Castagnoli, K., Castagnoli Jr., N., Chen, J.F., 2003. Neuroprotection by caffeine and more specific A2A receptor antagonists in animal models of Parkinson's disease. Neurology 61, S55–S61.
Shi, D., Daly, J.W., 1999. Chronic effects of xanthines on levels of central receptors in mice. Cell. Mol. Neurobiol. 6, 719–732.
Shi, D., Nikodijević, O., Jacobson, K.A., Daly, J.W., 1993. Chronic caffeine alters the density of adenosine, adrenergic, cholinergic, GABA, and serotonin receptors and calcium channels in mouse brain. Cell. Mol. Neurobiol. 13, 247–261.
Silva, C.G., Métin, C., Fazeli, W., Machado, N.J., Darmopil, S., Launay, P.-S., Ghestem, A., Nesa, M.-P., Bassot, E., Szabó, E., Baqi, Y., Müller, C.E., Tomé, A.R., Ivanov, A., Isbrandt, D., Zilberter, Y., Cunha, R.A., Esclapez, M., Bernard, C., 2013. Adenosine receptor antagonists including caffeine alter fetal brain development in mice. Sci. Transl. Med. 5, 197ra104.
Simola, N., Morelli, M., Seeman, P., 2008. Increase of dopamine D2(High) receptors in the striatum of rats sensitized to caffeine motor effects. Synapse 62, 394–397.
Sinchai, T., Plasen, S., Sanvarinda, Y., Jaisin, Y., Govitrapong, P., Morales, N.P., Ratanachamnong, P., Plasen, D., 2011. Caffeine potentiates methamphetamine-induced toxicity both in vitro and in vivo. Neurosci. Lett. 502, 65–69.
Sinton, C.M., Valatx, J.L., Jouvet, M., 1981. Gestational caffeine modifies offspring behaviour in mice. Psychopharmacology (Berl) 75, 69–74.
Slickers, J.E., Olshan, A.F., Siega-Riz, A.M., Honein, M.A., Aylsworth, A.S., National Birth Defects Prevention Study, 2008. Maternal body mass index and lifestyle exposures and the risk of bilateral renal agenesis or hypoplasia: the National Birth Defects Prevention Study. Am. J. Epidemiol. 168, 1259–1267.
Smith, S.E., McElhatton, P.R., Sullivan, F.M., 1987. Effects of administering caffeine to pregnant rats either as a single daily dose or as divided doses four times a day. Food Chem. Toxicol. 25, 125–133.
Soellner, D.E., Grandys, T., Nuñez, J.L., 2009. Chronic prenatal caffeine exposure impairs novel object recognition and radial arm maze behaviors in adult rats. Behav. Brain Res. 205, 191–199.
Somani, S.M., Gupta, P., 1988. Caffeine: a new look at an age-old drug. Int. J. Clin. Pharmacol. Ther. Toxicol. 26, 521–533.
Sourial-Bassillious, N., Rydelius, P.A., Aperia, A., Aizman, O., 2009. Glutamate-mediated calcium signaling: a potential target for lithium action. Neuroscience 161, 1126–1134.
Stanojevic, I., Bjelobabab, I., Nedeljkovicc, N., Drakulica, D., Petrovica, S., Stojiljkovicb, M., Horvat, A., 2011. Ontogenetic profile of ecto-50-nucleotidase in rat brain synaptic plasma membranes. Int. J. Dev. Neurosci. 29, 397–403.
Stonehouse, A.H., Adachi, M., Walcott, E.C., Jones, F.S., 2003. Caffeine regulates neuronal expression of the dopamine 2 receptor gene. Mol. Pharmacol. 64, 1463–1473.
Sudakov, S.K., Medvedeva, O.F., Rusakova, I.V., Figurina, I.B., 2001. Effect of short-term and chronic caffeine intake on rats with various anxiety level. Bull. Exp. Biol. Med. 132, 1177–1179.
Sun, W.L., Zhou, L., Hazim, R., Quinones-Jenab, V., Jenab, S., 2008. Effects of dopamine and NMDA receptors on cocaine-induced Fos expression in the striatum of Fischer rats. Brain Res. 1243, 1–9.
Svenningsson, P., Nomikos, G.G., Fredholm, B.B., 1999. The stimulatory action and the development of tolerance to caffeine is associated with alterations in gene expression in specific brain regions. J. Neurosci. 19, 4011–4022.
Svenningsson, P., Lindskog, M., Rognoni, F., Fredholm, B.B., Greengard, P., Fisone, G., 1998. Activation of adenosine A2A and dopamine D1 receptors stimulates cyclic AMP-dependent phosphorylation of DARPP-32 in distinct populations of striatal projection neurons. Neuroscience 84, 223–228.
Swenson, R.R., Beckwith, B.E., Lamberty, K.J., Krebs, S.J., Tinius, T.P., 1990. Prenatal exposure to AVP or caffeine but not oxytocin alters learning in female rats. Peptides 11, 927–932.
Swerdlow, N.R., Vaccarino, F.J., Amalric, M., Koob, G.F., 1986. The neural substrates for the motor-activating properties of psychostimulants: a review of recent findings. Pharmacol. Biochem. Behav. 25, 233–248.
Takahashi, R.N., Pamplona, F.A., Prediger, R.D., 2008. Adenosine receptor antagonists for cognitive dysfunction: a review of animal studies. Front. Biosci. 13, 2614–2632.
Taketo, M., Matsuda, H., Yoshioka, T., 2004. Calcium-independent inhibition of GABA(A) current by caffeine in hippocampal slices. Brain Res. 1016, 229–239.
Tanase, D., Baghdoyan, H.A., Lydic, R., 2003. Dialysis delivery of an adenosine A1 receptor agonist to the pontine reticular formation decreases acetylcholine release and increases anesthesia recovery time. Anesthesiology 98, 912–920.
Tchekalarova, J., Kubova, H., Mares, P., 2005. Postnatal caffeine exposure: effects on motor skills and locomotor activity during ontogenesis. Behav. Brain Res. 160, 99–106.
Temple, J.L., 2009. Caffeine use in children: what we know, what we have left to learn, and why we should worry. Neurosci. Biobehav. Rev. 33, 793–806.
Thompson, P.M., Giedd, J.N., Woods, R.P., MacDonald, D., Evans, A.C., Toga, A.W., 2000. Growth patterns in the developing brain detected by using continuum mechanical tensor maps. Nature 404, 190–193.
Van Dort, C.J., Baghdoyan, H.A., Lydic, R., 2009. Adenosine A(1) and A(2A) receptors in mouse prefrontal cortex modulate acetylcholine release and behavioral arousal. J. Neurosci. 29, 871–881.
Vanattou-Saïfoudine, N., Gossen, A., Harkin, A., Neuropsychopharmacology Research Group, 2011. A role for adenosine A(1) receptor blockade in the ability of caffeine to promote MDMA "Ecstasy"-induced striatal dopamine release. Eur. J. Pharmacol. 650, 220–228.
Vigh, J., Lasater, E.M., 2003. Intracellular calcium release resulting from mGluR1 receptor activation modulates GABAA currents in wide-field retinal amacrine cells: a study with caffeine. Eur. J. Neurosci. 17, 2237–2248.
Walton, K., Dorne, J.L., Renwick, A.G., 2001. Uncertainty factors for chemical risk assessment: interspecies differences in the in vivo pharmacokinetics and metabolism of human CYP1A2 substrates. Food Chem. Toxicol. 39, 667–680.
Wang, S.J., 2007. Caffeine facilitation of glutamate release from rat cerebral cortex nerve terminals (synaptosomes) through activation protein kinase C pathway: an interaction with presynaptic adenosine A1 receptors. Synapse 61, 401–411.
Weaver, D.R., 1996. A1 – adenosine receptor gene expression in fetal rat brain. Dev. Brain Res. 94, 205–223.
Weng, X., Odouli, R., Li, D.K., 2008. Maternal caffeine consumption during pregnancy and the risk of miscarriage: a prospective cohort study. Am. J. Obstet. Gynecol. 198 (279), e1–e8.
Wilkinson, J.M., Pollard, I., 1994. In utero exposure to caffeine causes delayed neural tube closure in rat embryos. Teratog. Carcinog. Mutagen. 14, 205–211.
Xu, H., Rajesan, R., Harper, P., Kim, R.B., Lonnerdal, B., Yang, M., Uematsu, S., Hutson, J., Watson-MacDonell, J., Ito, S., 2005. Induction of P 1A by cow milk-based formula: a comparative study between human milk and formula. Br. J. Pharmacol. 146, 296–305.
Yesair, D.W., Branfman, A.R., Callahan, M.M., 1984. Human disposition and some biochemical aspects of methylxanthines. Prog. Clin. Biol. Res. 158, 215–233.
Zimmermann, H., 2001. Ecto-nucleotidases: some recent developments and a note on nomenclature. Drug Dev. Res. 52, 44–56.
Zimmermberg, B., Carr, K.L., Scott, A., Lee, H.H., Weider, J.M., 1991. The effects os postnatal caffeine exposure on growth, activity and learning in rats. Pharmacol. Biochem. Behav. 39, 883–888.
Zonouzi, M., Renzi, M., Farrant, M., Cull-Candy, S.G., 2011. Bidirectional plasticity of calcium-permeable AMPA receptors in oligodendrocyte lineage cells. Nat. Neurosci. 14, 1430–1438.

CAPÍTULO II

Prenatal caffeine intake differently affects synaptic proteins during fetal brain development

Artigo publicado no periódico *Int. J. Devl Neuroscience 36: 45–52, 2014*

Int. J. Devl Neuroscience 36 (2014) 45–52

Contents lists available at ScienceDirect

International Journal of Developmental Neuroscience

journal homepage: www.elsevier.com/locate/ijdevneu

Prenatal caffeine intake differently affects synaptic proteins during fetal brain development

Sabrina Mioranzza, Fernanda Nunes, Daniela M. Marques, Gabriela T. Fioreze, Andréia S. Rocha, Paulo Henrique S. Botton, Marcelo S. Costa, Lisiane O. Porciúncula*

Laboratório de Estudos sobre o Sistema Purinérgico, Programa de Pós-Graduação em Ciências Biológicas/Bioquímica, Porto Alegre, RS 90035-003, Brazil

ARTICLE INFO

Article history:
Received 12 March 2014
Received in revised form 23 April 2014
Accepted 30 April 2014

Keywords:
Caffeine
Brain development
BDNF
Shh
Synaptic proteins

ABSTRACT

Caffeine is the psychostimulant most consumed worldwide. However, little is known about its effects during fetal brain development. In this study, adult female Wistar rats received caffeine in drinking water (0.1, 0.3 and 1.0 g/L) during the active cycle in weekdays, two weeks before mating and throughout pregnancy. Cerebral cortex and hippocampus from embryonic stages 18 or 20 (E18 or E20, respectively) were collected for immunodetection of the following synaptic proteins: brain-derived neurotrophic factor (BDNF), TrkB receptor, Sonic Hedgehog (Shh), Growth Associated Protein 43 (GAP-43) and Synaptosomal-associated Protein 25 (SNAP-25). Besides, the estimation of NeuN-stained nuclei (mature neurons) and non-neuronal nuclei was verified in both brain regions and embryonic periods. Caffeine (1.0 g/L) decreased the body weight of embryos at E20. Cortical BDNF at E18 was decreased by caffeine (1.0 g/L), while it increased at E20, with no major effects on TrkB receptors. In the hippocampus, caffeine decreased TrkB receptor only at E18, with no effects on BDNF. Moderate and high doses of caffeine promoted an increase in *Shh* in both brain regions at E18, and in the hippocampus at E20. Caffeine (0.3 g/L) decreased GAP-43 only in the hippocampus at E18. The NeuN-stained nuclei increased in the cortex at E20 by lower dose and in the hippocampus at E18 by moderate dose. Our data revealed that caffeine transitorily affect synaptic proteins during fetal brain development. The increased number of NeuN-stained nuclei by prenatal caffeine suggests a possible acceleration of the telencephalon maturation. Although some modifications in the synaptic proteins were transient, our data suggest that caffeine even in lower doses may alter the fetal brain development.

1. Introduction

Caffeine is one of the most consumed psychostimulant present in our diet in drinks like coffee, tea and cola soft drinks. The pharmacological actions of caffeine consist in blocking adenosine actions, a nucleoside that acts as a neuromodulator in the central nervous system (CNS). Adenosine controls neurotransmitter release, neuronal excitability and circadian rhythm through its metabotropic receptors A_1, A_{2A}, A_{2B} and A_3 (Cunha, 2001; Fredholm et al., 2005). The major effect of caffeine at the concentrations relevant to daily intake is the antagonism of A_1 and A_{2A} receptors (Fredholm et al., 2005). It is well documented the beneficial effects of caffeine on cognitive functions in different ages in adult animals (Angelucci et al., 1999; Costa et al., 2008a; for review see Cunha and Agostinho, 2010). Chronic caffeine prevents memory disturbances associated with aging (Arendash et al., 2006; Costa et al., 2008b; Prediger et al., 2005; Sallaberry et al., 2013) and experimental models of Alzheimer's disease (Arendash et al., 2009; Dall'Igna et al., 2007; Espinosa et al., 2013).

Regarding effects of caffeine during brain development epidemiological studies have associated caffeine intake and birth outcomes, such as low birth weight, intrauterine growth retardation and miscarriage (Brent et al., 2011; Bakker et al., 2010; Giannelli et al., 2003). However, the effects of caffeine intake during fetal and even postnatal brain development have been poorly investigated (for review see Porciúncula et al., 2013; Temple, 2009). Caffeine enters into all tissues of the fetus (Bracken et al., 2003) since it can easily cross biological membranes including the placental barrier and fetal brain (Arnaud, 1987). Some experimental studies in rodents revealed that caffeine administered during

* Corresponding author at: Universidade Federal do Rio Grande do Sul, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Rua Ramiro Barcelos, 2600 – anexo, Bairro Santana, Porto Alegre, RS 90035-003, Brazil.
Tel.: +55 51 3308 5556; fax: +55 51 3308 5540.
E-mail addresses: loporciuncula@yahoo.com, loporciuncula@gmail.com (L.O. Porciúncula).

http://dx.doi.org/10.1016/j.ijdevneu.2014.04.006
0736-5748/

pregnancy reduced the number of somites and extent of the neural tube in the post-implantation embryo, suggesting intrauterine growth retardation (Jacombs et al., 1999). Besides, caffeine in pregnant females decreased the immunocontent of metabotropic glutamate receptors of group I in the brain in fetuses and pregnant rats (León et al., 2005), and also influenced serotonergic system in chick embryo, increasing the contents of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) (Li et al., 2012). A recent study assessing the effect of moderate caffeine intake during pregnancy on mice pups resulted in delayed migration of cortical γ-aminobutyric acid (GABA) neurons, an increased susceptibility to seizures, and deficits in spatial memory when assessed in adulthood (Silva et al., 2013).

Since caffeine is a non-selective antagonist of A_1 and A_{2A} adenosine receptors and coupling of these receptors to G-proteins occurs postnatally in cortex and hippocampus (Adén et al., 2000; Gaytan et al., 2006), most experimental studies with caffeine during brain development have investigated the impact of caffeine on these receptors at postnatal stages or during adulthood (Adén et al., 2000; Gaytan and Pasaro, 2012; Guillet and Kellogg, 1991). However, it remains to be investigated if the patterns of the development of synapses during intrauterine can be modified by caffeine intake. The effects of caffeine administration on proteins, hormones, signaling pathways and especially neurotrophic factors that regulate the development of the CNS during intrauterine life are still little investigated. Thus, our study aimed to evaluate the effects of administration of different doses of caffeine during pregnancy on proteins involved in the formation and maturation of synapses, namely: (i) the neurotrophin brain-derived neurotrophic factor (BDNF) and its receptor TrkB; (ii) two components of synapse (axons and nerve terminals): Growth Associated Protein 43 (GAP43) and Synaptosomal-associated protein 25 (SNAP-25); (iii) the major morphogen agent Sonic hedgehog (*Shh*). In addition, an estimation of the number of neuronal and non-neuronal cells was assessed in the cortex and hippocampus in fetuses from embryonic state 18 and 20 (E 18 and E 20) exposed to caffeine throughout intrauterine life.

2. Materials and methods

2.1. Animals and caffeine treatment

Female Wistar rats (2 months old) were obtained from the Central Animal House of our Department. They were maintained in standard cages under a standard dark–light cycle (lights on between 7 A.M. and 7 P.M.), at a room temperature of 22 ± 2 °C. All experimental procedures were performed according to the Ethical committee of Universidade Federal do Rio Grande do Sul (Proc. N° 20332) in compliance with Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care.

Adult female Wistar rats were treated with caffeine in drinking water (0.1, 0.3 and 1.0 g/L, corresponding to low, moderate and high intake, respectively) (Fredholm et al., 1999), during the active period (dark cycle) in weekdays, two weeks before mating. Although 1 mg/mL of caffeine is considered high consumption of caffeine, it yielded a plasma concentration of less than 60 μM in adult rats (Costenla et al., 2010; Duarte et al., 2009). At the beginning of light cycle all experimental groups received tap water. The presence of vaginal plug was considered as embryonic day 0 (E0). The same caffeine treatment continued during pregnancy up to embryonic days 18 or 20 (E18 or E20, respectively), when the dams were sacrificed under anesthesia. The fetuses were weighed and whole cortex and hippocampus were immediately dissected out at 4 °C for Western Blot analysis; or the whole brain were immersed in 4% paraformaldehyde in 0.1 M PBS for fixation of the brain tissue to determine total number of neuronal and non-neuronal cells in cortex and hippocampus by the isotropic fractionator. The litter size and number of implantations were also analyzed.

The moderate dose of 0.3 g/L caffeine was chosen considering the plasma concentration of caffeine in rat dams being this dose similar to that found in blood of human mothers drinking three to four cups of coffee per day (Adén et al., 2000). The other two concentrations were chosen to reach approximately 3 times lower and 3 times higher concentrations comparing with the moderate dose (0.1 g/L and 1.0 g/L, respectively), in order to have a wide range of caffeine intake. Female rats received caffeine solutions only during the dark cycle, which is their active period, to mimic the pattern of caffeine consumption in humans. During the light cycle, caffeine solutions were replaced by water *ad libitum*. All experimental groups consumed similar amounts of caffeinated-water and drinking water. Although we did not quantify the plasma levels of caffeine, the chosen treatment regimens are thought to correspond to a low, moderate and high caffeine intake in humans, with effects believed to be mainly operated through antagonism of adenosine receptors (Fredholm et al., 1999).

2.2. SDS-PAGE (sodium dodecyl sulfate-polyacrilamide) immunoblotting

Hippocampus and whole cortex were dissected out from E18 or E20 (*pool* of two fetuses per litter, considering one litter as one subject), were immediately homogenized in 5% SDS with a protease and phosphatase inhibitor cocktail (Sigma, São Paulo/Brazil) and frozen at −70 °C. After defrost, the protein content was determined by Bicinchoninic acid assay using bovine serum albumin (BSA) as standard (Pierce, São Paulo/Brazil). Sample extracts were diluted to a final protein concentration of 2 μg/μL in SDS-PAGE buffer. Aliquots corresponding to 20–40 μg protein for hippocampus and whole cortex samples were separated by SDS-PAGE (12% running gel with a 4% concentrating gel) and electro-transferred to nitrocellulose membranes. After blocking with Tris-buffered saline 0.1% Tween-20 (TBS-T) containing 3% bovine serum albumin for 1 h, the membranes were incubated for 24 hours at 4 °C with the following primary antibodies: mouse anti-BDNF (1:500, Sigma, São Paulo/Brazil); rabbit anti-TrkB (1:1000, Abcam, São Paulo/Brasil); rabbit anti-GAP-43 (1:2000, Sigma, São Paulo/Brazil); mouse anti-Shh (1:500, Abcam, São Paulo/Brazil); or rabbit anti-SNAP-25 (1:1000, Sigma, São Paulo/Brazil). The membranes were then washed with TBS-T, incubated with horseradish peroxidase conjugated secondary antibody for 1 h at room temperature and developed with chemiluminescence ECL kit (Amersham, São Paulo/Brazil). Membranes were re-probed for β-tubulin immunoreactivity. The autoradiographic films were scanned and densitometric analyses were performed using public domain NIH Image Program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/). Band intensity was normalized to β-tubulin as a loading control to assess protein levels. The data used in statistical analysis were obtained from the ratio of the protein studied and β-tubulin density unit lines.

2.3. Quantification of neuronal and non-neuronal nuclei

We used the isotropic fractionator method to estimate total number of cells and neurons, as previously described (Herculano-Houzel and Lent, 2005). Whole cortex and hippocampus from the fixed brain were weighed and mechanically dissociated in a saline solution with 0.1% Triton X-100 and turned into an isotropic suspension of isolated nuclei. The total number of nuclei in suspension – and therefore the total number of cells in the original tissue – was estimated by determining the density of nuclei in small aliquots stained with the fluorescent DNA marker DAPI (4,6-diamidino-2-phenylindole dihydrochloride), under the microscope with a 40× objective, using a hemocytometer for quantification. Neuronal nuclei from an aliquot of the suspension were selectively immunolabeled overnight, at room temperature, with mouse monoclonal anti-NeuN antibody (Millipore, MAB377) at a dilution of 1:200 in PBS. After washing the nuclei in PBS, they were incubated for 2 h at room temperature with AlexaFluor 488 anti-mouse IgG secondary antibody (Invitrogen, Biogen São Paulo/Brazil), at a dilution of 1:500 in PBS in the presence of 10% normal goat serum. The neuronal fraction in each sample was estimated by counting NeuN-labeled nuclei in at least 500 DAPI-stained nuclei. Image analysis and quantification were manually performed and also confirmed by using software from Nikon Eclipse E600. The results were presented as number of cells per mg tissue.

2.4. Statistical analysis

Statistical analysis was performed by using One-way ANOVA and Newman–Keuls *post hoc* test. The statistical significance was considered for $P < 0.05$.

3. Results

3.1. Reproductive data

The litter size and the number of implantations were not affected by chronic caffeine, as shown in Table 1. The fetal weight was decreased by 16% in embryos (E20) from dams that received caffeine at the highest dose (1.0 g/L) (Table 1).

3.2. The impact of different caffeine doses during pregnancy on BDNF signaling and on important proteins for synaptic development

Prenatal caffeine intake differently affected proteins crucial to fetal brain development in two distinct embryonic stages in whole cortex and hippocampus. At E18 in hippocampus, high caffeine

Table 1
Reproductive data[a]

	Water	Caffeine 0.1 g/L	Caffeine 0.3 g/L	Caffeine 1.0 g/L
No. of dams				
Embryonic day 18	7	7	7	7
Embryonic day 20	7	7	7	7
No. of implantations				
Embryonic day 18	10.44 ± 0.48	10.36 ± 0.61	10.73 ± 0.78	10.67 ± 0.44
Embryonic day 20	10.00 ± 0.52	10.25 ± 0.48	10.50 ± 0.72	10.09 ± 0.86
Mean fetal weight (g)				
Embryonic day 18	1.47 ± 0.09	1.47 ± 0.01	1.41 ± 0.05	1.41 ± 0.04
Embryonic day 20	3.88 ± 0.13	3.84 ± 0.16	3.71 ± 0.07	3.26 ± 0.13[b]

[a] Data are presented as mean ± S.E.M.
[b] Different from control group ($P < 0.05$).

intake (1.0 g/L) decreased TrkB (Fig. 1A, black square) and increased *Shh* (Fig. 2A), whereas the moderate dose reduced hippocampal GAP-43 (Fig. 3A); in whole cortex BDNF was decreased (Fig. 1C, black circle) and Shh was increased (Fig. 2B) in fetuses exposed to the highest dose of caffeine. When assessing embryonic stage 20 (E20), BDNF in whole cortex was increased (Fig. 1D) by the highest dose, and both moderate and high caffeine dose (0.3 g/L and 1.0 g/L, respectively) increased *Shh* (Fig. 2A) in hippocampus at E20. The immunocontent of SNAP-25 was not affected by caffeine intake (Fig. 4A and B). The lowest dose of caffeine was devoid of effect in all proteins assessed in whole cortex and hippocampus for both embryonic stages.

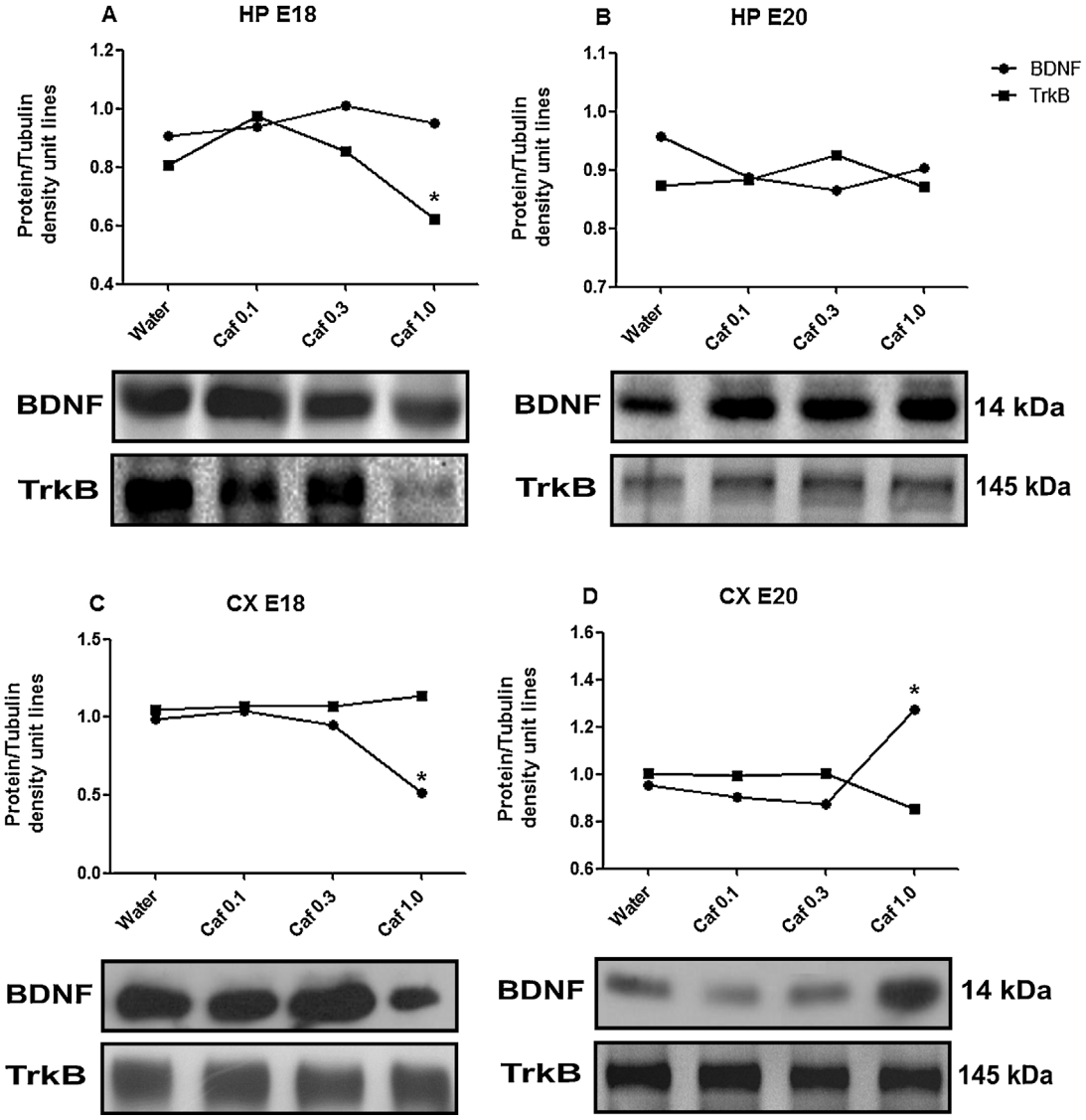


Fig. 1. Immunoblotting analysis of BDNF (black circle) and its receptor, TrkB (black square) in the hippocampus at E18 (A) and E20 (B), and in the whole cortex at E18 (C) and E20 (D) from fetuses exposed to water or caffeine. Data are means ± S.E.M ($n = 5$–6 samples/group) of density unit lines obtained from protein/tubulin ratio. *$P < 0.05$, significantly different from control group (water). One-way ANOVA followed by Newman–Keuls multiple comparison test.

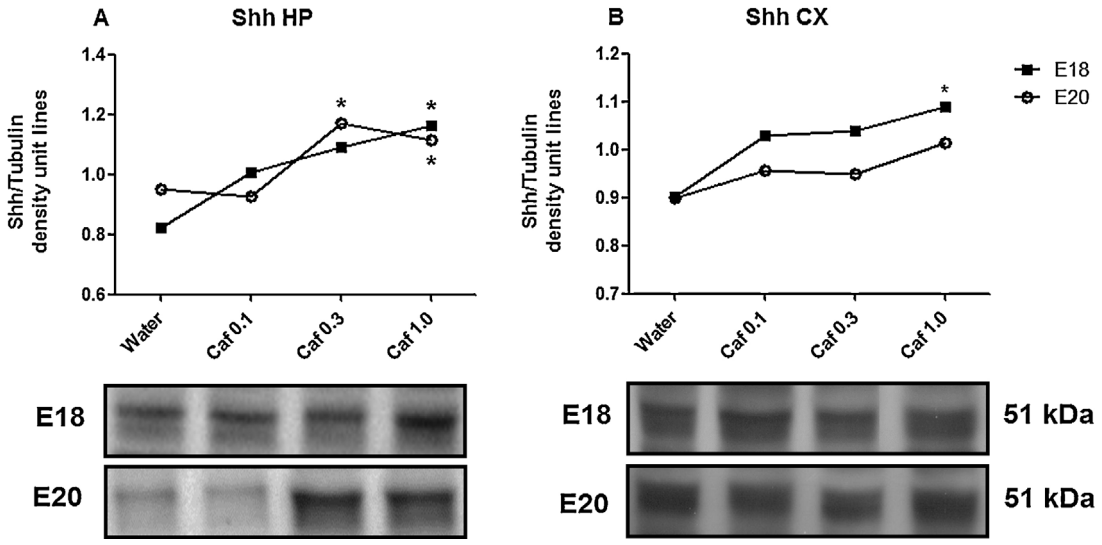


Fig. 2. Immunoblotting analysis of *Shh* in the hippocampus (A) at E18 (black square) and E20 (white circle), and in the whole cortex (B) at E18 (black square) and E20 (white circle) from fetuses exposed to water or caffeine. Data are means ± S.E.M (n = 5–6 samples/group) of density unit lines obtained from Shh/tubulin ratio. *P < 0.05, significantly different from control group (water). One-way ANOVA followed by Newman–Keuls multiple comparison test.

3.3. Number of neuronal and non-neuronal cells is affected by caffeine intake in cortex and hippocampus at different embryonic stages

The impact of prenatal caffeine intake at different doses was also evaluated in number of neuronal and non-neuronal cells. Caffeine 0.3 g/L increased total number of cells and neuronal cells in hippocampus from fetuses exposed to caffeine up to E18 (Fig. 5A). The lowest dose of caffeine increased neuronal cells and total number of cells in cortex at E20 (Fig. 5D). We did not found any effect of caffeine treatment in hippocampus at E20 or in cortex at E18 (Fig. 5B and C, respectively).

4. Discussion

In this study, the immunocontent of key proteins in synaptic formation and the total number of neuronal and non-neuronal cells were assessed in whole cortex and hippocampus from rat embryos exposed to different doses of caffeine. Translating to human brain development, the limbic system and cortex from rat embryos at E18 correspond to the first trimester of pregnancy in humans, while these brain areas at E20 correspond to the end of first and the beginning of second trimester (Clancy et al., 2001). These embryonic stages comprise the period of high neuronal differentiation rate at E18 for whole cortex, while the neuronal migration and laminar organization occur at E20 (Naveau et al., 2014). In the hippocampus during the prenatal period, neurons for the Ammon's horn are generated, while the majority of those destined for the dentate gyrus are formed postnatally (Bayer et al., 1993).

In agreement with previous studies, albeit with different schedules of administration, the absence of effect on the number of implantations and litter size was also observed in animals treated with caffeine (Evereklioglu et al., 2003; Lorenzo et al., 2010). One of the expected effects of high dose of caffeine is the decrease in the body weight, which was confirmed at E20. This phenomenon was previously reported for pregnant Wistar rats that received caffeine

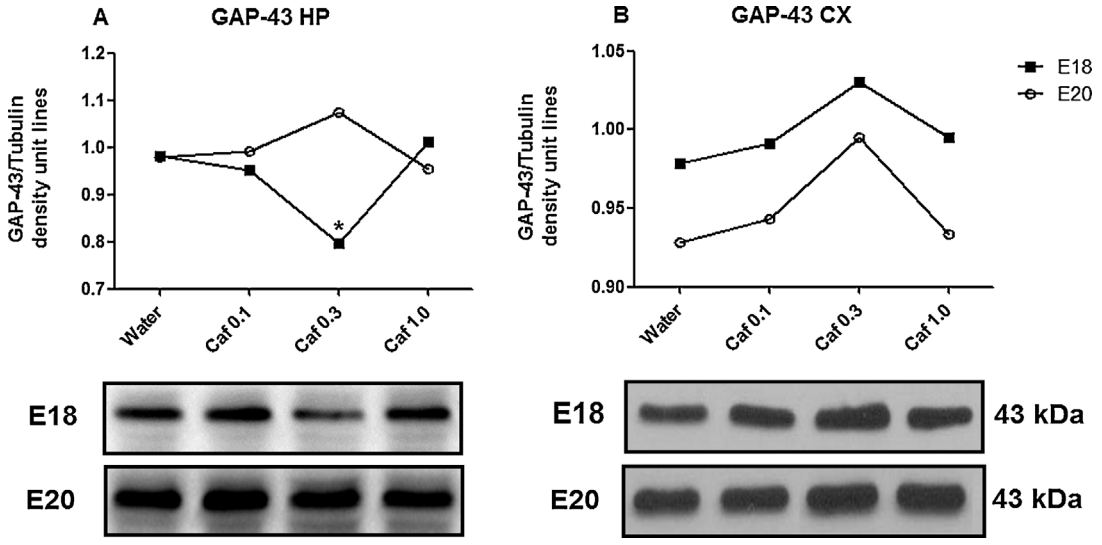


Fig. 3. Immunoblotting analysis of GAP-43 in the hippocampus (A) at E18 (black square) and E20 (white circle), and in the whole cortex (B) at E18 (black square) and E20 (white circle) from fetuses exposed to water or caffeine. Data are means ± S.E.M (n = 5–6 samples/group) of density unit lines obtained from GAP-43/tubulin ratio. *P < 0.05, significantly different from control group (water).

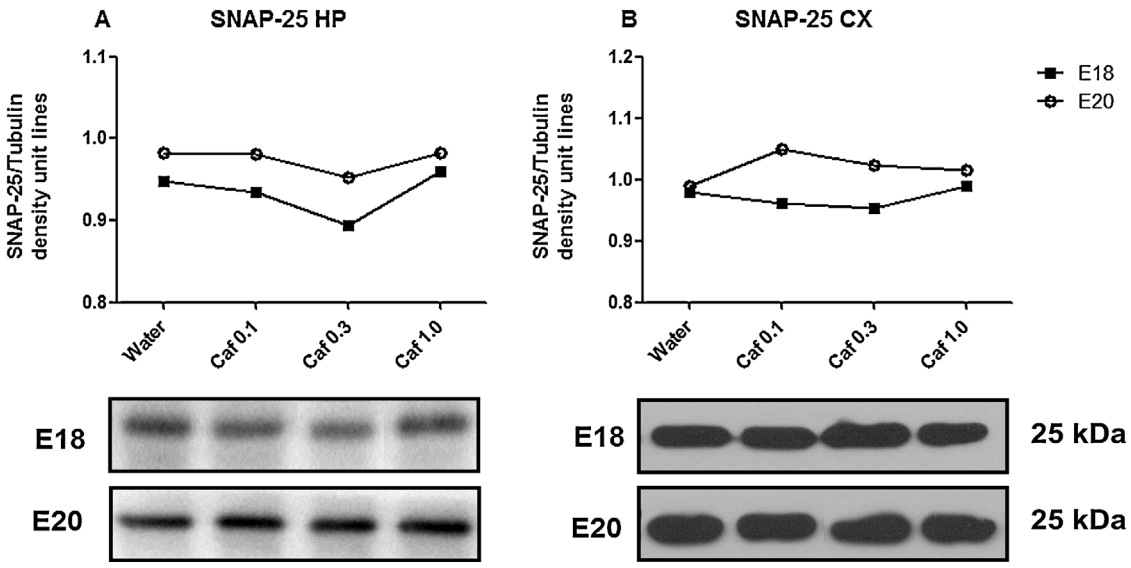


Fig. 4. Immunoblotting analysis of SNAP-25 in the hippocampus (A) at E18 (black square) and E20 (white circle), and in the whole cortex (B) at E18 (black square) and E20 (white circle) from fetuses exposed to water or caffeine. Data are means ± S.E.M (n = 5–6 samples/group) of density unit lines obtained from SNAP-25/tubulin ratio. No statistical differences were found.

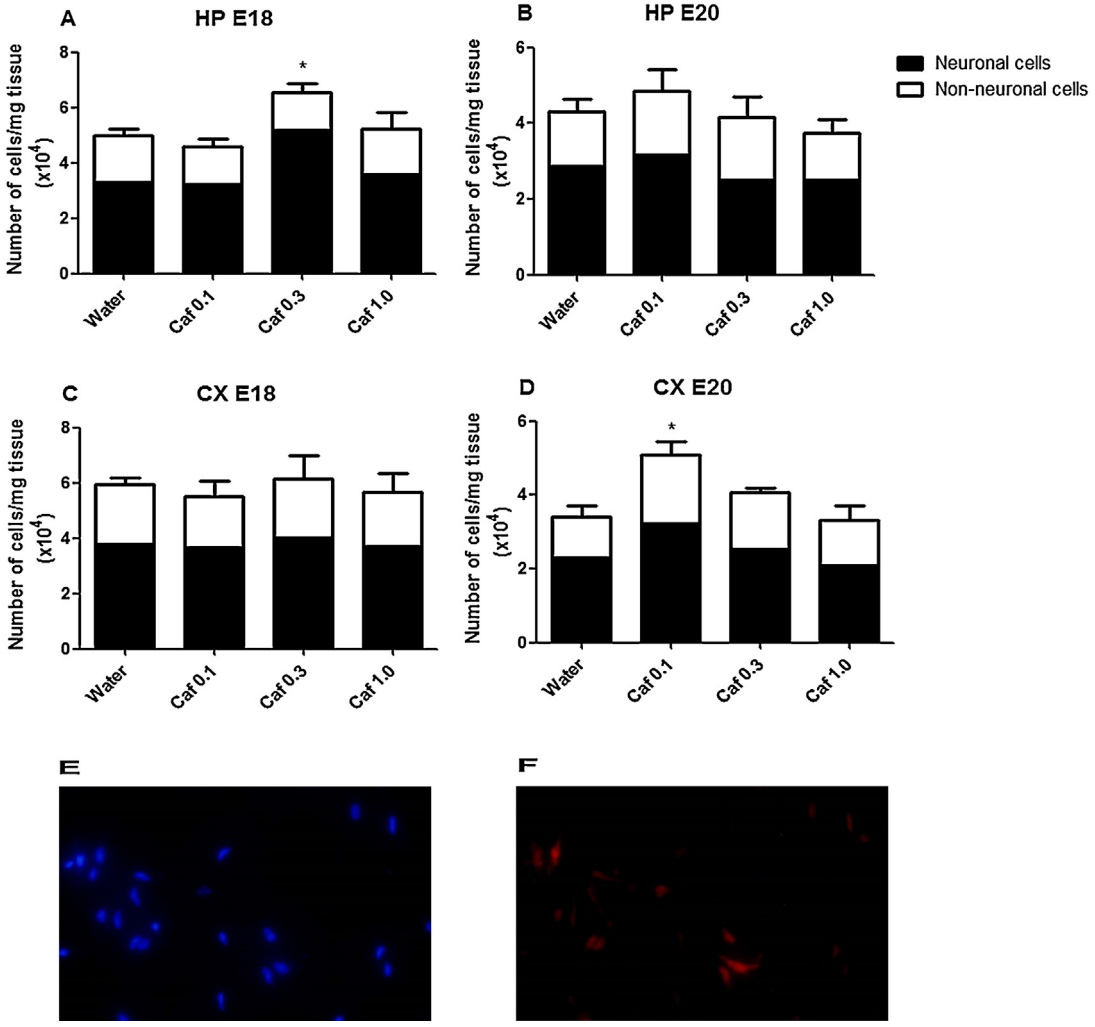


Fig. 5. Number of NeuN-stained nuclei (estimated as neuronal cells) and Dapi-stained nuclei (estimated as non-neuronal cells) (per mg tissue) in the hippocampus at E18 (A) or E20 (B), and in the whole cortex at E18 (C) and E20 (D) from fetuses exposed to water or caffeine. *P < 0.05, significantly different from control group (water). One-way ANOVA followed by Newman–Keuls multiple comparison test.

intragastrically at the concentrations 20, 60, and 180 mg/kg/day from gestational day 11 (GD11) to GD20. In that study, all treatments reduced fetal body weight at embryonic day 20 (E20) and there was an increase in glucocorticoid receptor in the fetal hippocampus (Xu et al., 2012). Although it is difficult to discriminate a more direct effect of caffeine from the loss of body weight, the modifications found in synaptic proteins were not restricted to embryos at E20.

The analysis of some proteins involved in the assembly of the synapses was investigated in two important brain areas from embryos that received caffeine. The brain-derived neurotrophic factor (BDNF) is one of the main proteins involved in the regulation, structure and maintenance of synapses during brain development and also in adulthood (Lu and Chow, 1999; Poo, 2001; Tyler and Pozzo-Miller, 2003). Signaling by BDNF also participates as a regulator of synaptic retrograde development and is essential for growth and elongation of dendrites of excitatory and inhibitory synapses (Carvalho et al., 2008; Lipsky and Marini, 2007; Singh et al., 2006). It is well known that BDNF signaling through its receptor TrkB can influence the morphology and synaptic connectivity of developing neurons (Huang and Reichardt, 2001; Poo, 2001).

Shh is a major morphogen agent, and is thought to be a key regulator of neural and oligodendroglial progenitors across development (Desouza et al., 2011). Possible alterations in nerve terminals were also assessed by the immunocontent of synaptosomal-associated protein 25 (SNAP-25), which is a protein component of the soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptor (SNARE) complex; the increased levels of SNAP-25 are related to synaptogenesis and maturation of functional synaptic connectivity during brain development (Catsicas et al., 1991). We also assessed the impact of caffeine intake during pregnancy in Growth Associated Protein 43 (GAP-43), another protein component of axons and nerve terminals (reviewed in Benowitz and Routtenberg, 1997; Oestreicher et al., 1997), which is associated with neurite outgrowth and synapse formation in brain (Baetge et al., 1992; McGuire et al., 1988).

We investigated the impact of different doses of caffeine in proteins related to fetal hippocampal development. At E18, moderate and high caffeine intake differently affected TrkB, GAP-43 and *Shh*. One of the consequences of lacking expression of GAP-43 is the inhibition of proliferation of certain neural precursors and disturbances in the maturation of differentiated neurons (Maier et al., 1999). This protein is crucial for axonal guidance, since GAP-43 knock out mice show disrupted cortical maps and absence of cortical barrels (Maier et al., 1999). Besides, an increase in GAP-43 expression potentiated neurite outgrowth in cultured hippocampal neurons (Korshunova et al., 2007). We supposed that possible deleterious effects by caffeine-induced decrease of GAP-43 might have been offset by the gradual increase in the hippocampal *Shh*. Of note, the changes observed at E18 in the TrkB and GAP-43 immunocontent were no longer observed at E20.

Since adding *Shh* in cultured hippocampal neurons become the presynaptic terminals significantly larger, with variable increases in the size of synaptic vesicles (Mitchell et al., 2012), we assessed SNAP-25 that can be easily immunodetected at early stages of brain development (Prescott and Chamberlain, 2011). Notably, caffeine in all doses administered did not modify SNAP-25 in both ages and brain regions, suggesting that at this stage of brain development there was no impact of this psychostimulant on nerve terminals.

Differently from the hippocampus, higher caffeine increased cortical BDNF at E18 and decreased this protein at E20, with no effect on TrkB receptors. Coincidently, *Shh* was increased in the cortex from fetuses at E18, but returned to normal levels at E20. One of the consequences of increased BDNF and *Shh* caused by caffeine may be an acceleration of telencephalon development, an event previously reported in *in vitro* studies (Marret et al., 1997; Sahir et al., 2000, 2001). *Shh* modulates cell proliferation and the migration of progenitor cells in adult brain (Charytoniuk et al., 2002; Dave et al., 2011; Palma et al., 2005; Traiffort et al., 2001). This morphogen is highly expressed in rat brains from E12 to postnatal day 2 (P2) (Petralia et al., 2011), and recently its expression has been identified in cortical pyramidal neurons (Garcia et al., 2010). Our results are also in line with *in vitro* studies in which caffeine at relatively high concentrations promoted an overexpression of *Shh* cultured neurons and astrocytes (Sahir et al., 2004). Importantly, *Shh* is related to induction of GABAergic interneuron differentiation in telencephalon at early embryonic stages (E11.5) (Mady and Kohtz, 2007). One of the consequences of increasing this morphogen in late pregnancy might be an abnormal distribution of neurons in cortical layers before birth, which may impact GABA neuron migration postnatally. Interestingly, moderate caffeine intake (0.3 g/L) during pregnancy and lactation delayed GABA neuron migration in the hippocampus and superficial cortical layers at postnatal day 6 (PND6) (Silva et al., 2013).

The participation of other proteins and signaling cascades in the increase of NeuN-stained nuclei in the cortex by lower caffeine deserves more investigation. Given the complexity of cerebral cortex compared to hippocampus, it is not possible to infer if this increase could reflect changes postnatally especially because other developmental milestones were not assessed. However, it is important to note that no effect was found for lower caffeine in the neurotrophins and the other proteins involved in the synaptic development such as *Shh* pathway, which coordinates differentiation of neural stem cells in the neocortex. In fact, the upregulation of *Shh* signaling was described to promote the increase of thickness and size of the neocortex (Shikata et al., 2010).

From our knowledge, this is the first study reporting effects of prenatal caffeine in the number of NeuN-stained nuclei (as estimation of the number of mature neurons). The impact of chronic caffeine has already been investigated in the adult neurogenesis, with different findings. For example, chronic administration of caffeine suppressed hippocampal subgranular zone (SGZ) cell proliferation and neurogenesis in rats (Han et al., 2007; Kochman et al., 2009). On the other hand, in a more detailed study, the effects of caffeine on proliferation of neuronal precursors were dose dependent; moderate doses depressing while higher doses increasing proliferation (Wentz and Magavi, 2009). In the same study, the survival and differentiation of neurons following caffeine administration was not affected, which contrasts with data from Han et al. (2007). Here, the increased number of NeuN-stained nuclei by caffeine was also dependent of the dose and specific according to brain region and embryonic period. It is important to take into account that the fetal metabolism of caffeine is not fully mature, and certainly its effects are more pronounced given the prolong half-life in the fetus (Arnaud, 1987, 2011).

Overall, our data show that caffeine exposure throughout the period of prenatal brain development can produce significant, but transitory effects on fetal brain development. It is important to note that snapshots of events during brain development may yield partial and sometimes conflicting information. The data presented also highlight the relevance of caffeine intake on important synaptic proteins. Until now, the vast majority of most studies focused on the effects of caffeine in the mature brain. Certainly, the impact of caffeine intake on synapse remodeling or modulation of synaptic efficacy and plasticity in the immature brain deserves more attention.

Acknowledgments

This research was supported by CAPES, FAPERGS (PRONEX and PRONEM), and CNPq (INCT).

References

Adén, U., Herlenius, E., Tang, L.Q., Fredholm, B.B., 2000. Maternal caffeine intake has minor effects on adenosine receptor ontogeny in the rat brain. Pediatr. Res. 48, 177–183.

Angelucci, M.E., Vital, M.A., Cesário, C., Zadusky, C.R., Rosalen, P.L., Da Cunha, C., 1999. The effect of caffeine in animal models of learning and memory. Eur. J. Pharmacol. 373, 135–140.

Arendash, G.W., Schleif, W., Rezai-Zadeh, K., Jackson, E.K., Zacharia, L.C., Cracchiolo, J.R., Shippy, D., Tan, J., 2006. Caffeine protects Alzheimer's mice against cognitive impairment and reduces brain beta-amyloid production. Neuroscience 142, 941–952.

Arendash, G.W., Mori, T., Cao, C., Mamcarz, M., Runfeldt, M., Dickson, A., Rezai-Zadeh, K., Tane, J., Citron, B.A., Lin, X., Echeverria, V., Potter, H., 2009. Caffeine reverses cognitive impairment and decreases brain amyloid-beta levels in aged Alzheimer's disease mice. J. Alzheimers Dis. 17, 661–680.

Arnaud, M.J., 1987. The pharmacology of caffeine. Prog. Drug Res. 31, 273–313.

Arnaud, M.J., 2011. Pharmacokinetics and metabolism of natural methylxanthines in animal and man. Handb. Exp. Pharmacol. 200, 33–91.

Baetge, E.E., Hammang, J.P., Gribkoff, V.K., Meiri, K.F., 1992. The role of GAP-43 in the molecular regulation of axon outgrowth and electrical excitability. Perspect. Dev. Neurobiol. 1, 21–28.

Bakker, R., Steegers, E.A., Obradov, A., Raat, H., Hofman, A., Jaddoe, V.W., 2010. Maternal caffeine intake from coffee and tea, fetal growth, and the risks of adverse birth outcomes: the Generation R Study. Am. J. Clin. Nutr. 91, 1691–1698.

Bayer, S.A., Altman, J., Russo, R.J., Zhang, X., 1993. Timetables of neurogenesis in the human brain based on experimentally determined patterns in the rat. Neurotoxicology 14, 83–144.

Benowitz, L.I., Routtenberg, A., 1997. GAP-43: an intrinsic determinant of neuronal development and plasticity. Trends Neurosci. 20, 84–91.

Bracken, M.B., Triche, E.W., Belanger, K., Hellenbrand, K., Leaderer, B.P., 2003. Association of maternal caffeine consumption with decrements in fetal growth. Am. J. Epidemiol. 157, 456–466.

Brent, R.L., Christian, M.S., Diener, R.M., 2011. Evaluation of the reproductive and developmental risks of caffeine. Birth Defects Res. B: Dev. Reprod. Toxicol. 2, 152–187.

Carvalho, A.L., Caldeira, M.V., Santos, S.D., Duarte, C.B., 2008. Role of the brain-derived neurotrophic factor at glutamatergic synapses. Br. J. Pharmacol. 153, S310–S324.

Catsicas, S., Larhammar, D., Blomqvist, A., Sanna, P.P., Milner, R.J., Wilson, M.C., 1991. Expression of a conserved cell-type-specific protein in nerve terminals coincides with synaptogenesis. Proc. Natl. Acad. Sci. U. S. A. 88, 785–789.

Charytoniuk, D., Porcel, B., Rodríguez Gomez, J., Faure, H., Ruat, M., Traiffort, E., 2002. Sonic Hedgehog signalling in the developing and adult brain. J. Physiol. Paris 96, 9–16.

Clancy, B., Darlington, R.B., Finlay, B.L., 2001. Translating developmental time across mammalian species. Neuroscience 105, 7–17.

Costa, M.S., Botton, P.H., Mioranzza, S., Ardais, A.P., Moreira, J.D., Souza, D.O., Porciúncula, L.O., 2008a. Caffeine improves adult mice performance in the object recognition task and increases BDNF and TrkB independent on phospho-CREB immunocontent in the hippocampus. Neurochem. Int. 53, 89–94.

Costa, M.S., Botton, P.H., Mioranzza, S., Souza, D.O., Porciúncula, L.O., 2008b. Caffeine prevents age-associated recognition memory decline and changes brain-derived neurotrophic factor and tirosine kinase receptor (TrkB) content in mice. Neuroscience 153, 1071–1078.

Costenla, A.R., Cunha, R.A., de Mendonça, A., 2010. Caffeine, adenosine receptors, and synaptic plasticity. J. Alzheimers Dis. S1, S25–S34.

Cunha, R.A., 2001. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. Neurochem. Int. 38, 107–125.

Cunha, R.A., Agostinho, P.M., 2010. Chronic caffeine consumption prevents memory disturbance in different animal models of memory decline. J. Alzheimer's Dis. 20 (S1), S95–S116.

Dall'Igna, O.P., Fett, P., Gomes, M.W., Souza, D.O., Cunha, R.A., Lara, D.R., 2007. Caffeine and adenosine A(2a) receptor antagonists prevent beta-amyloid (25–35)-induced cognitive deficits in mice. Exp. Neurol. 203, 241–245.

Dave, R.K., Ellis, T., Toumpas, M.C., Robson, J.P., Julian, E., Adolphe, C., Bartlett, P.F., Cooper, H.M., Reynolds, B.A., Wainwright, B.J., 2011. Sonic hedgehog and notch signaling can cooperate to regulate neurogenic divisions of neocortical progenitors. PLoS ONE 6, e14680.

Desouza, L.A., Sathanoori, M., Kapoor, R., Rajadhyaksha, N., Gonzalez, L.E., Kottmann, A.H., Tole, S., Vaidya, V.A., 2011. Thyroid hormone regulates the expression of the sonic hedgehog signaling pathway in the embryonic and adult Mammalian brain. Endocrinology 152, 1989–2000.

Duarte, J.M., Carvalho, R.A., Cunha, R.A., Gruetter, R., 2009. Caffeine consumption attenuates neurochemical modifications in the hippocampus of streptozotocin-induced diabetic rats. J. Neurochem. 111, 368–379.

Espinosa, J., Rocha, A., Nunes, F., Costa, M.S., Schein, V., Kazlauckas, V., Kalinine, E., Souza, D.O., Cunha, R.A., Porciúncula, L.O., 2013. Caffeine consumption prevents memory impairment, neuronal damage, and adenosine A2A receptors upregulation in the hippocampus of a rat model of sporadic dementia. J. Alzheimers Dis. 34, 509–518.

Evereklioglu, C., Sari, I., Alasehirli, B., Güldür, E., Cengiz, B., Balat, Z., Bagci, C., 2003. High dose of caffeine administered to pregnant rats causes histopathological changes in the cornea of newborn pups. Med. Sci. Monit. 9, BR168–BR173.

Fredholm, B.B., Chen, J.F., Cunha, R.A., 2005. Adenosine and brain function. Int. Rev. Neurobiol. 63, 191–270.

Fredholm, B.B., Battig, K., Holmen, J., Nehlig, A., Zvartau, E.E., 1999. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol. Rev. 51, 83–133.

Garcia, A.D., Petrova, R., Eng, L., Joyner, A.L., 2010. Sonic hedgehog regulates discrete populations of astrocytes in the adult mouse forebrain. J. Neurosci. 30, 13597–13608.

Gaytan, S.P., Pasaro, R., 2012. Neonatal caffeine treatment up-regulates adenosine receptors in brainstem and hypothalamic cardio-respiratory related nuclei of rat pups. Exp. Neurol. 237, 247–259.

Gaytan, S.P., Saadani-Makki, F., Bodineau, L., Frugière, A., Larnicol, N., Pásaro, R., 2006. Effect of postnatal exposure to caffeine on the pattern of adenosine A1 receptor distribution in respiration-related nuclei of the rat brainstem. Auton. Neurosci. 126-127, 339–346.

Giannelli, M., Doyle, P., Roman, E., Pelerin, M., Hermon, C., 2003. The effect of caffeine consumption and nausea on the risk of miscarriage. Pediatr. Perinat. Epidemiol. 17, 316–323.

Guillet, R., Kellogg, C., 1991. Neonatal exposure to therapeutic caffeine alters the ontogeny of adenosine A1 receptors in brain of rats. Neuropharmacology 30, 489–496.

Han, M.E., Park, K.H., Baek, S.Y., Kim, B.S., Kim, J.B., Kim, H.J., Oh, S.O., 2007. Inhibitory effects of caffeine on hippocampal neurogenesis and function. Biochem. Biophys. Res. Commun. 356, 976–980.

Herculano-Houzel, S., Lent, R., 2005. Isotropic fractionator: a simple, rapid method for the quantification of total cell and neuron numbers in the brain. J. Neurosci. 25, 2518–2521.

Huang, E.J., Reichardt, L.F., 2001. Neurotrophins: roles in neuronal development and function. Annu. Rev. Neurosci. 24, 677–736.

Jacombs, A., Ryan, J., Loupis, A., Pollard, I., 1999. Maternal caffeine consumption during pregnancy does not affect preimplantation development but delays early postimplantation growth in rat embryos. Reprod. Fertil. Dev. 11, 211–218.

Kochman, L.J., Fornal, C.A., Jacobs, B.L., 2009. Suppression of hippocampal cell proliferation by short-term stimulant drug administration in adult rats. Eur. J. Neurosci. 29, 2157–2165.

Korshunova, I., Novitskaya, V., Kiryushko, D., Pedersen, N., Kolkova, K., Kropotova, E., Mosevitsky, M., Rayko, M., Morrow, J.S., Ginzburg, I., Berezin, V., Bock, E., 2007. GAP-43 regulates NCAM-180-mediated neurite outgrowth. J. Neurochem. 100, 1599–1612.

León, D., Albasanz, J.L., Ruíz, M.A., Iglesias, I., Martín, M., 2005. Effect of chronic gestational treatment with caffeine or theophylline on Group I metabotropic glutamate receptors in maternal and fetal brain. J. Neurochem. 94, 440–451.

Li, X.D., He, R.R., Qin, Y., Tsoi, B., Li, Y.F., Ma, Z.L., Yang, X., Kurihara, H., 2012. Caffeine interferes embryonic development through over-stimulating serotonergic system in chicken embryo. Food Chem. Toxicol. 50, 1848–1853.

Lipsky, R.H., Marini, A.M., 2007. Brain-derived neurotrophic factor in neuronal survival and behavior-related plasticity. Ann. N. Y. Acad. Sci. 1122, 130–143.

Lorenzo, A.M., León, D., Castillo, C.A., Ruiz, M.A., Albasanz, J.L., Martín, M., 2010. Maternal caffeine intake during gestation and lactation down-regulates adenosine A1 receptor in rat brain from mothers and neonates. J. Neurosci. Res. 88, 1252–1261.

Lu, B., Chow, A., 1999. Neurotrophins and hippocampal synaptic transmission and plasticity. J. Neurosci. Res. 58, 76–87.

Mady, R., Kohtz, J.D., 2007. Isolation of rat telencephalic neural explants to assay Shh GABAergic interneuron differentiation-inducing activity. Methods Mol. Biol. 397, 47–53.

Maier, D.L., Mani, S., Donovan, S.L., Soppet, D., Tessarollo, L., McCasland, J.S., Meiri, K.F., 1999. Disrupted cortical map and absence of cortical barrels in growth-associated protein (GAP)-43 knockout mice. Proc. Natl. Acad. Sci. U. S. A. 96, 9397–9402.

Marret, S., Gressens, P., Van-Maele-Fabry, G., Picard, J., Evrard, P., 1997. Caffeine-induced disturbances of early neurogenesis in whole mouse embryo cultures. Brain Res. 773, 213–216.

McGuire, C.B., Snipes, G.J., Norden, J.J., 1988. Light-microscopic immunolocalization of the growth- and plasticity-associated protein GAP-43 in the developing rat brain. Brain Res. 469, 277–291.

Mitchell, N., Petralia, R.S., Currier, D.G., Wang, Y., Kim, A., Mattson, M.P., Yao, P.J., 2012. Sonic hedgehog regulates presynaptic terminal size, ultrastructure and function in hippocampal neurons. J. Cell Sci. 125, 4207–4213.

Naveau, E., Pinson, A., Gérard, A., Nguyen, L., Charlier, C., Thomé, J.P., Zoeller, R.T., Bourguignon, J.P., Parent, A.S., 2014. Alteration of rat fetal cerebral cortex development after prenatal exposure to polychlorinated biphenyls. PLOS ONE (March), e91903.

Oestreicher, A.B., De Graan, P.N., Gispen, W.H., Verhaagen, J., Schrama, L.H., 1997. B-50, the growth associated protein-43: modulation of cell morphology and communication in the nervous system. Prog. Neurobiol. 53, 627–686.

Palma, V., Lim, D.A., Dahmane, N., Sánchez, P., Brionne, T.C., Herzberg, C.D., Gitton, Y., Carleton, A., Alvarez-Buylla, A., Ruiz i Altaba, A., 2005. Sonic hedgehog controls stem cell behavior in the postnatal and adult brain. Development 132, 335–344.

Petralia, R.S., Schwartz, C.M., Wang, Y., Mattson, M.P., Yao, P.J., 2011. Subcellular localization of Patched and Smoothened, the receptors for Sonic Hedgehog Signaling, in the hippocampal neuron. J. Comp. Neurol. 519, 3684–3699.

Poo, M.M., 2001. Neurotrophins as synaptic modulators. Nat. Rev. Neurosci. 2, 24–32.

Porciúncula, L.O., Sallaberry, C., Mioranzza, S., Botton, P.H.S., Rosemberg, D., 2013. The Janus face of caffeine. Neurochem. Int. 63, 594–609.

Prediger, R.D., Batista, L.C., Takahashi, R.N., 2005. Caffeine reverses age-related deficits in olfactory discrimination and social recognition memory in rats. Involvement of adenosine A1 and A2A receptors. Neurobiol. Aging 26, 957–964.
Prescott, G.R., Chamberlain, L.H., 2011. Regional and developmental brain expression patterns of SNAP25 splice variants. BMC Neurosci. 12, 35.
Sahir, N., Evrard, P., Gressens, P., 2004. Caffeine induces sonic hedgehog gene expression in cultured astrocytes and neurons. J. Mol. Neurosci. 24, 201–205.
Sahir, N., Mas, C., Bourgeois, F., Simonneau, M., Evrard, P., Gressens, P., 2001. Caffeine-induced telencephalic vesicle evagination in early post-implantation mouse embryos involves cAMP-dependent protein kinase (PKA) inhibition. Cereb. Cortex 11, 343–349.
Sahir, N., Bahi, N., Evrard, P., Gressens, P., 2000. Caffeine induces in vivo premature appearance of telencephalic vesicles. Dev. Brain Res. 30, 213–217.
Sallaberry, C., Nunes, F., Costa, M.S., Fioreze, G.T., Ardais, A.P., Botton, P.H., Klaudat, B., Forte, T., Souza, D.O., Elisabetsky, E., Porciúncula, L.O., 2013. Chronic caffeine prevents changes in inhibitory avoidance memory and hippocampal BDNF immunocontent in middle-aged rats. Neuropharmacology 64, 153–159.
Shikata, Y., Okada, T., Hashimoto, M., Ellis, T., Matsumaru, D., Shiroishi, T., Ogawa, M., Wainwright, B., Motoyama, J., 2010. Ptch1-mediated dosage-dependent action of Shh signaling regulates neural progenitor development at late gestational stages. Dev. Biol. 349, 147–159.
Silva, C.G., Métin, C., Fazeli, W., Machado, N.J., Darmopil, S., Launay, P.S., Ghestem, A., Nesa, M.P., Bassot, E., Szabó, E., Baqi, Y., Müller, C.E., Tomé, A.R., Ivanov, A., Isbrandt, D., Zilberter, Y., Cunha, R.A., Esclapez, M., Bernard, C., 2013. Adenosine receptor antagonists including caffeine alter fetal brain development in mice. Sci. Transl. Med. 5, 197ra104.
Singh, B., Henneberger, C., Betances, D., Arevalo, M.A., Rodríguez-Tébar, A., Meier, J.C., Grantyn, R., 2006. Altered balance of glutamatergic/GABAergic synaptic input and associated changes in dendrite morphology after BDNF expression in BDNF-deficient hippocampal neurons. J. Neurosci. 26, 7189–7200.
Temple, J.L., 2009. Caffeine use in children: what we know, what we have left to learn, and why we should worry. Neurosci. Biobehav. Rev. 33, 793–806.
Traiffort, E., Moya, K.L., Faure, H., Hässig, R., Ruat, M., 2001. High expression and anterograde axonal transport of aminoterminal sonic hedgehog in the adult hamster brain. Eur. J. Neurosci. 14, 839–850.
Tyler, W.J., Pozzo-Miller, L., 2003. Miniature synaptic transmission and BDNF modulate dendritic spine growth and form in rat CA1 neurons. J. Physiol. 553, 497–509.
Wentz, C.T., Magavi, S.S., 2009. Caffeine alters proliferation of neuronal precursors in the adult hippocampus. Neuropharmacology 56, 994–1000.
Xu, D., Zhang, B., Liang, G., Ping, J., Kou, H., Li, X., Xiong, J., Dongcai, H., Liaobin, C., Magdalou, J., Wang, H., 2012. Caffeine-induced activated glucocorticoid metabolism in the hippocampus causes hypothalamic-pituitary-adrenal axis inhibition in fetal rats. PLOS ONE 7, 1–10.

CAPÍTULO III

Caffeine during pregnancy and lactation: influence in the neurobehavioral development and glutamate signaling in the offspring

Artigo em preparação para ser submetido

Caffeine during pregnancy and lactation: influence in the neurobehavioral development and glutamate signaling in the offspring.

Sabrina Mioranzza, Andréia S. Rocha, Daniela M. Marques, Fernanda Nunes, Cassio M. Loss, Diogo L. Oliveira, Daniela Pochmann, Lisiane O. Porciúncula*

Universidade Federal do Rio Grande do Sul, Laboratório de Estudos sobre o Sistema Purinérgico, Instituto de Ciências Básicas da Saúde Departamento de Bioquímica, Programa de Pós-Graduação em Ciências Biológicas – Bioquímica, Porto Alegre/RS, Brazil 90035-003.

*Correspondence should be addressed to Lisiane O. Porciúncula
Federal University of Rio Grande do Sul, Health and Basic Sciences Institute, Department of Biochemistry, Laboratory of Studies on the Purinergic System, Rua Ramiro Barcelos, 2600 – anexo, Bairro Santana, Porto Alegre/RS
Brazil 90035-003.
Phone.: + 55 51 3308 5556
Fax.: + 55 51 3308 5540
e-mail.: loporciuncula@yahoo.com

Abstract

Although caffeine is the most consumed psychostimulant worldwide, its effect on brain development remains elusive. In this study, adult female Wistar rats received caffeine in drinking water (0.1, 0.3 and 1.0 g/L) during the active cycle in weekdays, throughout pregnancy and lactation. Maternal and progeny body weight, developmental milestones, locomotor activity was recorded and plasma levels of caffeine and metabolites were determined at postnatal days (PND) 7, 14 and 21. Glutamate binding and immunodetection of glutamate transporter GLT-1 and receptors (NMDA subunits, AMPA subunit and mGlu5) were performed in cortex and hippocampus from rat pups (PND 7, 14 or 21). Caffeine (1.0 g/L) decreased maternal body weight at late pregnancy and pups body weight in PND 14, 18 and 21. Developmental milestones and locomotor activity were not modified by caffeine. At PND 7, caffeine (0.3 g/L) increased GLT-1 and GluN1 in cortex, while the highest dose decreased GLT-1 and increased GluN1 in hippocampus. All doses of caffeine increased hippocampal GluA1 at this age. At PND 14, caffeine (0.3 g/L) increased mGlu5 in both brain structures and increased GluA1 in the cortex. At PND 21 caffeine (0.3 g/L) decreased cortical GluA1 and mGlu5 and increased hippocampal GluN2A, and caffeine (1.0 g/L) decreased cortical mGlu5. In the hippocampus caffeine (0.3 g/L) decreased GluA1 and mGlu5. Membranes from rat pups at PND 14 treated with caffeine (0.1 g/L) showed reduced glutamate binding. Although caffeine intake during pregnancy and lactation did not alter the course of developmental milestones and locomotor activity in the progeny, significant changes were observed in the glutamatergic signaling. Given that glutamate is the main excitatory

neurotransmitter more research is needed to evaluate the impact of these alterations in adulthood.

Keywords: caffeine, glutamate, hippocampus, cortex, brain development.

Introduction

Caffeine is the most widely consumed psychostimulant, and its effects are mediated by the blockade of adenosine A_1 and A_{2A} receptors. As a neuromodulator, adenosine controls neurotransmitter release, neuronal excitability and circadian rhythm through its metabotropic receptors A_1, A_{2A}, A_{2B} and A_3 (Cunha, 2001; Fredholm et al., 2005).

Caffeine at low doses is commonly used as a respiratory stimulant to treat neonatal apneas of premature infants (Francart et al., 2013; Schoen et al., 2014). However, there are concerns about the caffeine intake during pregnancy. Various epidemiological studies have been conducted in order to better establish the safety of caffeine intake during pregnancy. Some studies have found negative birth outcomes correlated with caffeine intake, such as low birth weight, prematurity and miscarriage (Bakker et al., 2010; Bracken et al., 2003; Brent et al., 2011; Hoyt et al., 2014; Sengpiel et al., 2013). Some limitations from epidemiological studies have produced different conclusions (Savitz et al., 2008; Weng et al., 2008), which include the difficulty of performing a randomized clinical trial with pregnant women, measurement of caffeine intake (self-reported consumption), weeks of pregnancy in the beginning of the study and control for confounding variables (smoking, educational level, arterial pressure).

Metabolism of caffeine is similar between rats and humans, therefore rat is a suitable model to study the impact of caffeine during brain development. In both humans and rats, caffeine is demethylated into three dimethylxanthines: theophylline, theobromine and paraxanthine (Bonati et al., 1984). Caffeine and its metabolites cross freely through the placenta to the fetus and it is secreted in the breast milk (Abdi et al., 1993; McNamara et al., 1992). Due to the immaturity

of hepatic enzyme systems, namely cytochrome P-450, caffeine half-life is increased in the neonatal period in both animals and humans (Nehlig and Debry, 1994). All these alterations in caffeine metabolism constitute an additional risk to the fetus especially to organs under development in late gestation, such as the brain (Aldridge et al., 1981).

Behavioral alterations have been found in neonates exposed to high dose of caffeine during various periods of brain development including pregnancy and/or lactation and also long-lasting effects in adulthood (Bjorklund et al., 2008; Concannon et al., 1983; Hughes and Beveridge, 1991; Peruzzi et al., 1985; Zimmemberg et al., 1991). The neurochemical alterations that may underlie behavioral features caused by caffeine, such as changes in neurotransmission systems crucial for brain homeostasis during development, are dependent of doses and schedule of administration (for review see Porciúncula et al., 2013). Glutamate is the major excitatory neurotransmitter in the mammalian brain, and the effects of caffeine on glutamatergic system development are still poorly investigated. Glutamate exerts its actions through ionotropic and metabotropic glutamate receptors. Ionotropic glutamate receptors (iGlu) are integral membrane proteins composed of four large subunits that form a central ion channel pore, being subdivided into *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainate (KA) and δ-receptors (for review see Traynelis et al., 2010). Metabotropic glutamate receptors (mGlu) are members of G-protein-coupled receptor (GPCR) superfamily and consist of eight receptor sub-types, which can be subdivided into three groups based on sequence homology, pharmacology and G-protein coupling specificity (reviewed by Nicoletti et al., 2011). The group I includes the mGlu1 and mGlu5 receptor

subtypes, which are coupled to G_{q11} proteins, while Group II and -III include mGlu2 and mGlu3, and mGlu4, mGlu6, mGlu7 and mGlu8 receptor subtypes, which are all coupled to $G_{i/o}$ proteins (Niswender and Conn, 2010).

In order to maintain the homeostasis of excitatory neurotransmission, synaptic levels of glutamate are kept low via a family of Na^+-dependent glutamate transporters (for review, see Danbolt, 2001; Sheldon and Robinson, 2007). In fact, high extracellular levels of glutamate can cause neuronal death by excessive stimulation of glutamate receptors and the perfect functioning of glutamate transporters and thus the clearance of glutamate at the synaptic cleft, thereby preventing and avoiding excitotoxicity (Attwell et al., 1993; for review, see Choi, 1992; Conti and Weinberg, 1999). One of the four sodium-dependent glutamate transporters is GLT-1, which is localized primarily in astrocytes (Lehre et al., 1995). During development, glutamate is involved in promoting and/or inhibiting the proliferation, survival, migration and differentiation of neural progenitor cells (NPC) in neurogenic zones (for review, see Jansson and Akerman, 2014).

Considering that caffeine is a non-selective antagonist of adenosine A_1 and A_{2A} receptors and that adenosine is a neuromodulator that controls neurotransmitter release in some neurotransmission systems such as glutamate, the effects of caffeine intake on glutamatergic signaling during brain development have been studied (Di Giorgi-Gerevini et al., 2004; León et al., 2005).

Therefore, our study aimed to evaluate the effects of caffeine at different doses during pregnancy and lactation on developmental milestones and locomotor activity. We also analyzed ontogeny of glutamate receptors and transporter and caffeine metabolism in pups during lactation.

Experimental Procedures

Animals and caffeine treatment

Female Wistar rats (2-3 months old) were obtained from the Central Animal House of our Department. They were maintained in standard cages under a standard dark–light cycle (lights on between 7:00 a.m. and 7:00 p.m.), at a room temperature of 22 ± 2° C. All experimental procedures were performed according to the Ethical committee of Universidade Federal do Rio Grande do Sul (Proc. Nº 20332) in compliance with Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care.

Adult female Wistar rats (n=9) were treated with caffeine in drinking water (0.1; 0.3; and 1.0 g/L, corresponding to low, moderate and high intake, respectively), during dark cycle in week days, two weeks before mating. All experimental groups received tap water during the light cycle. Caffeine solutions were freshly prepared in glass bottles every day. This caffeine administration schedule was chosen in order to mimic human caffeine consumption, which is usually taken during the active period of the day. The presence of vaginal plug was considered as gestational day (GD) 0. The same caffeine treatment continued during pregnancy and lactation. From GD 21 until delivery, each pregnant female was checked twice daily (at 09:00 and 17:00 h) for completion of or difficulties in parturition. The day of parturition was defined as postnatal day (PND) 0. When parturition was complete, the numbers of stillborn and live pups in each litter were taken. The dams continued receiving the same doses of caffeine during lactation. We measured maternal body weight during pregnancy and pups body weight during lactation. The dose of 0.3 g/L of caffeine was chosen based on the plasma concentration of caffeine measured in the mothers.

This dose is similar to the blood levels found in pregnant women taking three to four cups of coffee a day (Adén et al., 2000). The other two concentrations were chosen to reach approximately 3 times lower and 3 times higher concentrations comparing with the moderate dose (0.1 g/L and 1.0 g/L, respectively), in order to have a wide range of caffeine intake.

Reflex development

Reflex development was tested according to Bahaarnoori et al. (2012), with some modifications. Four rats per litter (n=9) were evaluated daily starting from PND 6. The following parameters were tested every day in the same pup: a) righting reflex (PND 6–8): pups were placed in a supine position, and positive response was obtained when the animal returned to prone position, with all paws on the ground; b) forelimb grasp reflex (PND 6–8): the reflex was considered fully developed when the pups grasped the barrel of the 16-gauge needle as the barrel was rubbed against the palm of the forepaw; c) negative geotaxis (PND 7–12): the pups were placed head down on an inclined surface (45 degrees) covered with wired mesh. Each pup was observed for 180 seconds to turn and move toward the upper end of the surface; and d) cliff avoidance (PND 7–12): pups were positioned with forepaws and snout over the edge of a shelf (70 cm height), and a positive response consisted of turning and crawling away from the edge.

Locomotor activity

Locomotor activity was performed between PND 6 and PND 10. Each pup was placed in the center of an open field (50 x 50 cm plywood surface subdivided into 25 squares for scoring purposes). Twenty-eight pups in each group (n = 7

litter per treatment) were observed for 3 minutes by an examiner blind to treatment conditions. Number of crossing and number of squares visited was evaluated, and the locomotion index was obtained using the following formula: Locomotion index = (number of crossing)/(number of squares visited – 1). When the value of locomotion index was higher than 1, it implies that the animal presented linear locomotion.

Plasma concentration of caffeine and metabolites

The extraction and chromatographic separation were performed according to the experimental protocol described by Perera et al. (2010) with modifications. Blood from the pups was collected in heparinized tubes and centrifuged. The Internal standard (Benzotriazole) was added to the samples and the compounds were extracted from plasma using ethyl acetate. Samples were collected 4 hours after the end of the dark cycle from pups at PND 7, 14 and 21. They were centrifuged (4000 x *g*, 10 min) and the organic layer was lyophilized. The residue was reconstituted in distilled water. Plasma concentrations of caffeine and the metabolites theophylline, theobromine and paraxanthine were analyzed using high pressure liquid chromatography, with the Shimadzu Class-VP chromatography system, consisting of a quaternary gradient pump with vacuum degassing and piston desalting modules, Shimadzu SIL-10AF autoinjector valve with 50 mL loop and a UV detector (Shimadzu, Kyoto, Japan). Separation was achieved on a C_{18} Kinetex® ODS column (4.6mm X 25cm i.d., particle size 5 µm, Supelco, St Louis, MO, USA). In the procedure, two mobile phases were used, the first one, Mobile phase A, consisted of acetonitrile:acetic acid:distilled water (50:1:949), and the Mobile phase B, consisted of the same compounds but in a

different concentration (75:1:924). Operation conditions were: detector UV, 280nm; mobile phase flow rate, 1.5 mL/min; injection volume, 50 µL; mobile phase A was used until the minute 20, after that mobile phase B was used. The quantification was achieved using a calibration curve and the internal standard was used to estimate the recovery rate of the extraction procedure.

SDS-PAGE (sodium dodecyl sulfate-polyacrilamide) immunoblotting

Whole cortex and hippocampus were dissected out from pups at PND 7, 14 and 21 and immediately homogenized in 5 % SDS with a protease and phosphatase inhibitor cocktail (Sigma, São Paulo/Brazil) and frozen at – 70º C. After defrost, the protein content was determined by Bicinchoninic acid assay (BCA) using bovine serum albumin (BSA) as standard (Pierce, São Paulo/Brazil). Sample extracts were diluted to a final protein concentration of 2 µg/µL in SDS-PAGE buffer. Aliquots corresponding to 40 µg or 80 µg protein for hippocampus and whole cortex samples were separated by SDS-PAGE (10 % running gel with a 4 % concentrating gel) and electro-transferred to nitrocellulose membranes. After blocking with Tris-buffered saline 0.1 % Tween-20 (TBS-T) containing 3 % bovine serum albumin for 1 h, the membranes were incubated for 24 hours at 4º C with the following primary antibodies: mouse anti-glutamate receptor 1 (GluR1; 1:1000, Abcam, São Paulo/Brazil); rabbit anti-metabotropic glutamate receptor 5 (mGluR5; 1:1000, Abcam, São Paulo/Brazil); rabbit anti-GLT-1 (1:60,000, Sigma, São Paulo/Brazil); rabbit anti-phosphoNR1 (1:1,000, Millipore); mouse anti-NR1 (1:1,000, Millipore); rabbit phosphoNR2B (1:1,000, Millipore); mouse anti-NR2B (1:1,000, Millipore); or rabbit anti-NR2A (1:1,000, Millipore). The membranes were then washed with TBS-T, incubated with horseradish peroxidase

conjugated secondary antibody for 1 h at room temperature and developed with chemiluminescence ECL kit (Amersham, São Paulo/Brazil). Membranes were re-probed for β-tubulin immunoreactivity. The autoradiographic films were scanned and densitometric analyses were performed using public domain NIH Image Program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/). Band intensity was normalized to β-tubulin as a loading control to assess protein levels. The data used in statistical analysis were obtained from the ratio of the protein immunodetected and β-tubulin density unit lines. For phosphoproteins, the ratio of phosphoprotein and total protein was analyzed.

[^{3}H] Glutamate binding

The binding assay of [^{3}H] glutamate was performed at 35° C in small polycarbonate tubes (total incubation volume 500 µL) containing 10 mM Tris/HCl pH 7.4 and 40 nM [^{3}H]glutamate (Amersham, São Paulo, Brazil), according to Ganzella et al. (2012). Briefly, plasma membrane preparation was obtained from whole cortex and hippocampus homogenate in an appropriate buffer (10mM Tris/HCl pH 7.4; 0.32 mM saccharose; 1mM $MgCl_2$). The homogenate was centrifuged at 2,900 rpm for 15 min, and both supernatants were centrifuged at 15,000 rpm for 15 min three times. The resultant pellet was resuspended in 10 mM Tris/HCl pH 7.4 and frozen at – 70° C until the day of *binding* assay. Incubation was started by the addition of plasma membrane preparation (50 µL) of cortex and hippocampus from rat pups at PND 7, 14 and 21. After 30 min, tubes were centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant was discarded and the walls of the tubes and the surface of the pellets were quickly

and carefully rinsed with cold distilled water. The pellets were solubilized with 0.2 mL of 0.5 M NaOH overnight. Bound radioactivity was measured by using a Wallac scintillation counter. Unspecific binding (10–20% of the total binding) was determined by adding 200 μM nonradioactive glutamate to the medium in a parallel assay. Specific binding was considered as the difference between total and unspecific binding. Experiments were performed in triplicate. Protein concentration of the plasma membrane preparation was determined by Bicinchoninic acid assay (Pierce, São Paulo/Brazil). The results were presented as pmol glutamate per mg protein.

Statistical analysis

For the tests of reflex development, Fisher´s exact test was used to analyze the reflex of the last day within the time window. Two-way ANOVA with repeated measures were performed on maternal weight during pregnancy, progeny body weight gain, and locomotor activity. For HPLC analysis two-way ANOVA was used and other data were analyzed by using One-way ANOVA and Newman-Keuls *post hoc* test. The statistical significance was considered for $P < 0.05$.

Results

Reproductive data and developmental milestones

The length of gestation, number of live and stillborn pups and implantation sites were not affected by chronic caffeine intake, as shown in Table 1. Besides, none of the developmental milestones analyzed was influenced by caffeine (data not shown).

Maternal and progeny body weight

Overall, maternal body weight during pregnancy increased with gestational age [$F(6, 336) = 150.4$; $P < 0.0001$; Figure 1A], but it was observed a treatment effect [$F(3, 48) = 2.799$; $P < 0.05$]. Pregnant rats receiving the highest dose of caffeine (1.0 g/L) presented a reduction in body weight gain at GD18 and GD20 comparing with control pregnant rats. We found age [$F(6, 262) = 690.6$, $P < 0.0001$] and treatment effect [$F(3, 262) = 19.01$, $P < 0.0001$] in the mean body weight of the progeny (Fig. 1B). Bonferroni´s post hoc test showed a significant decrease in body weight in pups exposed to caffeine 1.0 g/L at PNDs 14, 18 and 21, whereas pups subjected to caffeine at the moderate dose (0.3 g/L) presented decreased body weight only at PND 21.

Reflex development

Caffeine treatment did not significantly affect the reflexes studied (righting reflfex, forelimb grasp reflex, negative geotaxis and cliff avoidance, Fig. 2A, 2B, 2C and 2D, respectively) during early postnatal life.

Locomotor activity

As a normal behavior, the number of crossing [$F(4,92) = 50.98$; $P < 0.0001$], squares visited [$F(4,92) = 56.52$; $P < 0.0001$], and locomotion index [$F(4,92) = 11.14$; $P < 0.0001$] increased with age. Caffeine in all doses administered did not promote any effect (Figure 3).

HPLC analysis

As shown in Figure 4, plasma levels of caffeine and its metabolites were determined for all doses in three different ages. Plasma levels of caffeine were not affected by age and the only difference found was an increase in the levels according to the dosage [$F(2, 27) = 32.67$; $P < 0.0001$, Fig. 4A]. The same profile was observed for theophylline plasma levels [$F(2,27) = 22.01$; $P < 0.0001$, Fig. 4D]. The age effect was observed for theobromine, since lower levels were detected at PND 14 [$F(2,27) = 7.126$; $P=0.0033$]. As caffeine increases, the levels of theobromine had also increased [$F(2,27) = 35.68$; $P < 0.0001$, Fig. 4C] with an interaction between dosage and age [$F(4,27) = 3.218$; $P = 0.0278$]. The same profile was also found for paraxanthine metabolite, in which an interaction was observed between age and dosage [$F(4,27) = 3.789$; $P = 0.0143$, Fig. 4B].

Caffeine intake during pregnancy and lactation differently affected glutamate receptors and GLT-1 transporter depending on the dose, postnatal age and brain structure

At PND 7, moderate caffeine intake (0.3 g/L) increased GLT-1 (Fig. 5A, blue circle) and pGluN1/GluN1 ratio (Fig. 5B, blue circle, dashed lines) in whole cortex, whereas the highest dose decreased GLT-1 (Fig. 6A, blue circle) and increased pGluN1/GluN1 ratio (Fig. 6D, blue circle, dashed lines) in hippocampus; all doses of caffeine increased hippocampal GluA1 at this age (Fig. 6B, red square). Moreover, moderate dose of caffeine has major effect at PND 14, increasing mGlu5 in both brain structures (Fig. 7A and 8A, green triangle, dashed lines) and increasing GluA1 in whole cortex (Fig. 7A, red square). Caffeine 0.3 g/L decreased cortical GluA1 and mGlu5 in whole cortex (Fig. 9A, red square, and

9A, green triangle, dashed lines, respectively), and the highest dose also decreased cortical mGlu5 at PND 21 (Fig. 9A, green triangle, dashed lines); conversely, the moderate dose decreased GLT-1, GluA1 and mGlu5 (Fig. 10A, blue circle, red square and green triangle, respectively) and increased GluN2A (Fig. 10B, red square, dashed line) in hippocampus at this age. GluN2B was not affected by any caffeine treatment in none of the postnatal ages and brain structures studied.

Glutamate binding assay was transiently affected by the lowest dose of caffeine at PND14 in whole cortex

The lowest dose of caffeine given to pregnant rats have led to a transient decrease of glutamate binding in whole cortex from pups at PND14 (Table 2), with no other alterations in whole cortex and hippocampus (Tables 2 and 3, respectively).

Discussion

The effect of perinatal caffeine intake (pregnant and offspring) was studied upon some developmental milestones, metabolic profile and glutamate signaling during ontogenesis in offspring rats. Female rats treated with different doses of caffeine from preconception to lactation presented reductions in the body weight gain in late pregnancy and also in the body weight of the progeny. Previous studies have also found these alterations along with intrauterine growth retardation and increased levels of serum corticosterone in the dams (Kou et al., 2014; Tan et al., 2012; West et al., 1986). In fact, intrauterine programming of glucose and lipid metabolism may be attributed to the high level of circulatory

glucocorticoid in utero (Reynolds, 2010). Moreover, the effect of caffeine on adipogenesis was evaluated using primary rat adipose-derived stem cells (ADSCs) and a mouse bone marrow stromal cell line (M2-10B4) *in vitro*. ADSCs and M2-10B4 continuously exposed to caffeine during adipogenic differentiation presented a dose-dependent decrease in various adipogenesis-related genes (Su et al., 2013). Caffeine is also known to affect thermogenesis by inhibiting the enzyme phosphodiesterase, leading to increase in cAMP concentration and inactivate hormone-lipase sensitive lipase (HLS), which promotes lipolysis (Acheson et al., 2004).

Differently from other studies, caffeine did not promote any alterations in developmental milestones (Peruzzi et al., 1985; West et al., 1986). Recently, Silva et al. (2013) showed that motor and neurological reflexes development were not affected by caffeine exposure during pregnancy and lactation when assessing righting reflex and cliff avoidance in mice offspring. Probably, our schedule of administration with caffeine being available only during active cycle at weekdays may explain the different effects of caffeine treatment. For the same reason, any effect of caffeine in the locomotor activity was not observed. Alterations in the locomotor activity are usually observed in adults and juvenile rats treated with caffeine during brain development (Björklund et al., 2008; Holloway and Thor, 1983; Hughes and Beveridge, 1991; Pan and Chen, 2007; Peruzzi et al., 1985). According to Tchekalarova and colleagues (2005), the effects of caffeine on locomotor activity are dependent on the stage of brain development.

According to previous reports, our findings confirm that plasma levels of caffeine and its metabolites were elevated at PND 7, while lower levels were found at PND 14, when activity and expression of hepatic enzymes are increased

(Bienvenu et al., 1993; Wilkinson and Pollard, 1993). High metabolite levels of caffeine could be found at PND 21 because of two sources of caffeine available (drinking water and breast milk). The CYP450 complex is not completely mature in the first postnatal week, which leads to accumulation of caffeine and its metabolites in the plasma of infants and also pups (Bienvenu et al., 1993; Wilkinson and Pollard, 1993). As previously observed, all metabolites increased with the dose, having the highest levels in pups receiving 1.0 g/L caffeine since intrauterine life (Bienvenu et al., 1993). Caffeine and theophylline levels did not change with age, but age and dosage modified theobromine and paraxanthine levels. Our results are in line with Svenningsson et al. (1999) that treated adult Sprague-Dawley rats with caffeine 0.3 g/L in drinking water for 14 days. They found that after a caffeine challenge of 7.5 mg/kg i.p., rats presented lower levels of plasma of caffeine comparing with rats receiving only the caffeine challenge dose. The other caffeine metabolites (theobromine, paraxanthine and theophylline) were not different between caffeine-treated animals and rats drinking only water. They suggest that caffeine is metabolized more rapidly in tolerant rats (the ones that received caffeine in drinking water for 14 days) and penetrates the blood-brain barrier better than its metabolites (Svenningsson et al., 1999). Therefore, our results for plasma caffeine, which did not change with age, may be explained by tolerance of caffeine in rat pups.

Among the changes that occur as a normal process of brain development, it is known that during the first postnatal week in rodents GABA acts as an excitatory neurotransmitter (Ben-Ari et al., 2007), leading to membrane depolarization and removal of magnesium ion (Mg^{2+}) from NMDA receptors, which allows their activation (Leinekugel et al., 1997). Here, caffeine increased

phosphorylated form of GluN1 subunit of NMDA receptor (pNR1) in both brain regions at PND 7, with no evident changes at PND 14 and 21. The activation of this receptor subunit seems to be restricted to this period of brain development. In addition, caffeine induced alterations in the AMPA receptor with increase in GluA1 subunit at PND 7 and decrease at PND 21 in the hippocampus. In cortical samples, GluA1 increased at the moderate dose at PND 14 followed by a decrease at PND 21.

It is possible that this increase of NMDA and AMPA subunits by caffeine could be a compensation for the reduction of GABA signaling also caused by similar administration of this substance. In a previous study, a delayed migration of the hippocampal GABAergic neurons was observed in mice at PND 6 previously exposed to caffeine (Silva et al., 2013). As mentioned before, GABA in the early development behaves as an excitatory neurotransmitter and glutamate is an important trophic factor (Behar et al., 1999; Ben-Ari et al., 2007; Haydar et al., 2000). The GluN1 subunit of the NMDA receptor in the hippocampus increases considerably in the first postnatal weeks, reaching levels similar to the adult animal about 20 days of age (Ibaraki et al., 1999). Of note, the changes caused by caffeine were observed in this period of life in which this subunit is more expressed, returning to normal levels at subsequent ages.

Furthermore, there was an increase in NMDA receptor subunit GluN2A in the hippocampus of pups that received moderate dose of caffeine (0.3 g/L). According to Fan et al (2012), the GluN2A subunit peaks of expression in neurogenic areas in PND 14, which is related to the formation of mature neurons. Another common alteration of glutamatergic synapses during the first postnatal week is the increase of GluA1 subunit in rat hippocampus between PND 7 and

20 (Ibaraki et al., 1999), but AMPA glutamate receptors are "silenced" at this stage (Ben-Ari et al., 1997). In cultured neurons, one of the consequences of overexpression of GluA1 AMPA receptor subunit is an increase of dendritic segments and the number of proteins in the postsynaptic density of glutamatergic synapses, suggesting that glutamate receptor may facilitate synaptogenesis (Chen et al., 2009). Thus, caffeine effect on this receptor may be accelerating the formation of the synapses via modifications in AMPA receptors.

Changes in the glutamate transporter GLT-1 were only observed at PND 7. Moderate dose of caffeine induced the increase in GLT-1 in the cortex, while higher dose decreased GLT-1 in the hippocampus. Considering that the brain is more sensitive to excitability during the first postnatal week, these region-specific changes in GLT-1 immunocontent may have a role in seizure susceptibility. In fact, acute caffeine treatment at PND 2-6 have led to a decreased susceptibility to seizure depending on the convulsant agent tested in young and adult rats (Guillet, 1995). In recent study, seizure susceptibility was found in mouse pups at P6 after perinatal caffeine treatment (0.3 g/L in drinking water) (Silva et al., 2013). However, when caffeine is given at PND 7-11, they found a pro- or anti-convulsant effect of caffeine depending on the postnatal period when the seizure was induced (Tchekalarova et al., 2006; 2007; 2010; 2011). Furthermore, GLT-1 expression is reduced when synapses are lost following removal of glutamatergic afferents (Ginsberg et al., 1996; Yang et al., 2009), while enhancing synaptic activity increases GLT-1 expression (Genoud et al., 2006). These results may suggest that GLT-1 expression is regulated by neurons to ensure sufficient local uptake capacity.

The changes in mGlu5 metabotropic receptors occurred only after the second postnatal week, during which synaptic plasticity is maximum with the increased number of dendritic spines and expression of proteins that participate in the mechanism of the long-term potentiation (LTP), such as PKC and PKA (Harris and Teyler, 1984; Yasuda et al., 2003). Excessive activation of metabotropic glutamate receptors can result in loss of response, known as desensitization (De Blasi et al., 2001). It was previously reported that chronic treatment of caffeine in the drinking water (1.0 g/L) reduced the immunocontent mGlu1a in fetal brain and there was a compensatory increase in receptor affinity (León et al., 2005). In our study, $mGlu_5$ was changed by the moderate and high doses of caffeine depending on postnatal age and brain structure. The formation of heteromeric complexes between A_{2A} and $mGlu_5$ receptor have been found in the striatum (Ferré et al., 2002) and the activation of mGlu5 potentiates signaling of adenosine A_{2A} receptor-dependent MAPK (Nishi et al., 2003). We observed that at PND 21 the moderate dose of caffeine caused a reduction of mGlu5 receptor in the cortex and hippocampus, and the high dose (1.0 g/L) increased the mGlu5 in the cortex. As previously mentioned, during this period the animals had two sources of caffeine: breast milk and drinking water, increasing the plasma levels of caffeine and its metabolites, which may lead to a compensatory mechanism to reduce these receptors in order to prevent glutamatergic hyperexcitability. The specific binding to glutamate was assessed in membrane preparations from cortex and hippocampus of pups at PND 7, 14 and 21. The only observed change was a transient increase in glutamate binding from the cortex of pups at PND 14 who received the lowest dose of caffeine (0.1 g/L). Since we found no changes in the immunocontent of glutamate receptors and

transporter studied in this brain structure and at this postnatal age, other subunits of glutamate receptors may be involved, as well as kainite receptor, which should be further investigated.

Overall, our results showed that moderate and high caffeine intake throughout rat brain development differently affected maternal body weight and also body weight of the progeny. Although we did not found any effect on growth and reflex development during lactating period by the lowest dose, more research is needed to evaluate whether the changes observed by moderate and high caffeine intake may reflect in behavioral alterations later in life. Concerning glutamatergic system, our data showed that caffeine affected the ontogeny of ionotropic and metabotropic glutamate receptors, as well as its transporter, depending on the postnatal age. Since caffeine is one of the most consumed psychostimulant worldwide present in our diet, its effects on other neurotransmitters systems during critical periods of brain development deserves more attention, once caffeine induces changes at this period that could affect brain homeostasis later in life.

Acknowledgments

This research was supported by CAPES, FAPERGS (PRONEX andPRONEM), and CNPq (INCT).

References

Abdi F, Pollard I, Wilkinson J. Placental transfer and foetal disposition of caffeine and its immediate metabolites in the 20-day pregnant rat: function of dose. Xenobiotica. 23 (4): 449-456, 1993.

Acheson KJ, Gremaud G, Meirim I, Montigon F, Krebs Y, Fay LB, Gay LJ, Schneiter P, Schindler C, Tappy L. Metabolic effects of caffeine in humans: lipid oxidation or futile cycling? Am J Clin Nutr 79: 40–46, 2004.

Adén U, Herlenius E, Tang LQ, Fredholm BB. Maternal caffeine intake has minor effects on adenosine receptor ontogeny in the rat brain. Pediatr. Res. 48: 177–183, 2000.

Aldridge A, Bailey J, Neims AH. The disposition of caffeine during and after pregnancy. Semin Perinatol 5: 310-314, 1981.

Attwell D, Barbour B, Szatkowski M. Nonvesicular release of reported in several conditions including focal cerebral neurotransmitter. Neuron 11: 401–407, 1993.

Baharnoori M, Bhardwaj SK, Srivastava LK. Neonatal behavioral changes in rats with gestational exposure to lipopolysaccharide: a prenatal infection model for developmental neuropsychiatric disorders. Schizophrenia Bulletin 38 (3): 444–456, 2012.

Bakker R, Steegers EA, Obradov A, Raat H, Hofman A, Jaddoe VW. Maternal caffeine intake from coffee and tea, fetal growth, and the risks of adverse birth outcomes: the Generation R Study. Am. J. Clin. Nutr. 91: 1691–1698, 2010.

Behar TN, Scott CA, Greene CL, Wen X, Smith SV, Maric D, Liu QY, Colton CA, Barker JL. Glutamate acting at NMDA receptors stimulates embryonic cortical neuronal migration. J Neurosci 19: 4449-4461, 1999.

Ben-Ari Y, Khazipov R, Leinekugel X, Caillard O, Gaiarsa JL. $GABA_A$, NMDA and AMPA receptors: a developmentally regulated 'ménage à trois'. Trends Neurosci. 20: 523–529, 1997.

Bienvenu T, Pons G, Rey E, Thiroux G, Olive G. Caffeine metabolism in liver slices during postnatal development in rats. Drug Metab Dispos. 21 (1): 178-180, 1993.

Bjorklund O, Kahlstro¨m J, Salmi P, Fredholm BB. Perinatal caffeine, acting on maternal adenosine A_1 receptors, causes long-lasting behavioral changes in mouse offspring. PLoS ONE 3 (12): e3977, 2008.

Bonati M, Latini R, Tognoni G, Young JF, Garattini S. Interspecies comparison of in vivo caffeine pharmacokinetics in man, monkey, rabbit, rat, and mouse. Drug Metab. Rev. 15: 1355–1383, 1984.

Bracken MB, Triche EW, Belanger K, Hellenbrand K, Leaderer BP. Association of maternal caffeine consumption with decrements in fetal growth. Am. J. Epidemiol. 157: 456–466, 2003.

Brent RL, Christian MS, Diener RM. Evaluation of the reproductive and developmental risks of caffeine. Birth Defects Res. B Dev. Reprod. Toxicol. 2: 152–187, 2011.

Chen W, Prithviraj R, Mahnke AH, McGloin KE, Tan JW, Gooch AK, Inglis FM. AMPA glutamate receptor subunits 1 and 2 regulate dendrite complexity and spine motility in neurons of the developing neocortex. Neuroscience 159 (1): 172-182, 2009.

Choi DW. Excitotoxic cell death. J Neurobiol. 23 (9): 1261-1276, 1992.

Concannon JT, Braughler JM, Schechter MD. Pre- and postnatal effects of caffeine on brain biogenic amines, cyclic nucleotides and behavior in developing rats. The Journal of Pharmacolgy and Experimental Therapeutics. 226: 673-679, 1983.

Conti F, Weinberg RJ. Shaping excitation at glutamatergic synapses. Trends Neurosci. 22 (10): 451-458, 1999.

Cunha RA. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. Neurochem. Int. 38: 107-125, 2001.

Danbolt NC. Glutamate uptake. Prog Neurobiol 65 (1): 1-105, 2001.

De Blasi A, Conn PJ, Pin J, Nicoletti F. Molecular determinants of metabotropic glutamate receptor signaling. Trends Pharmacol Sci. 22 (3): 114-20, 2001.

Di Giorgi-Gerevini VD, Caruso A, Cappuccio I, Ricci Vitiani L, Romeo S, Della Rocca C, Gradini R, Melchiorri D, Nicoletti F. The mGluR5 metabotropic glutamate receptor is expressed in zones of active neurogenesis of the embryonic and postnatal brain. Developmental Brain Research 150: 17-22, 2004.

Fan H, Gao J, Wang W, Li X, Xu T, Yin X. Expression of NMDA receptor and its effect on cell proliferation in the subventricular zone of neonatal rat brain. Cell Biochem Biophys. 62 (2): 305-316, 2012.

Ferré S, Karcz-Kubicha M, Hope BT, Popoli P, Burgueño J, Gutiérrez MA, Casadó V, Fuxe K, Goldberg SR, Lluis C, Franco R, Ciruela F. Synergistic interaction between adenosine A_{2A} and glutamate mGlu5 receptors: implications for striatal neuronal function. Proc Natl Acad Sci U S A. 99 (18): 11940-11945, 2002.

Francart SJ, Allen MK, Stegall-Zanation J. Apnea of prematurity: caffeine dose optimization. J Pediatr Pharmacol Ther. 18 (1): 45-52, 2013.

Fredholm BB, Chen JF, Cunha RA. Adenosine and brain function. Int. Rev. Neurobiol. 63: 191-270, 2005.

Ganzella M, Moreira JD, Almeida RF, Böhmer AE, Saute JAM, Holmseth S, Souza DO. Effects of 3 weeks GMP oral administration on glutamatergic parameters in mice neocortex. Purinergic Signalling 8: 49-58, 2012.

Genoud C, Quairiaux C, Steiner P, Hirling H, Welker E, Knott GW. Plasticity of astrocytic coverage and glutamate transporter expression in adult mouse cortex. PLoS Biol. 4 (11): e343, 2006.

Ginsberg SD, Rothstein JD, Price DL, Martin LJ. Fimbria-fornix transections selectively down-regulate subtypes of glutamate transporter and glutamate receptor proteins in septum and hippocampus. J Neurochem. 67 (3): 1208–1216, 1996.

Guillet R. Neonatal caffeine exposure alters seizure susceptibility in rats in an age-related manner. Brain Res Dev Brain Res. 89 (1): 124-128, 1995.

Harris KM, Teyler TJ. Developmental onset of long-term potentiation in area CA1 of the rat hippocampus. J Physiol. 346: 27-48, 1984.

Haydar TF, Wang F, Schwartz ML, Rakic P. Differential modulation of proliferation in the neocortical ventricular and subventricular zones. J Neurosci 20: 5764–5774, 2000.

Holloway WR Jr, Thor DH. Caffeine sensitivity in the neonatal rat. Neurobehav Toxicol Teratol. 4 (3): 331-333, 1983.

Hoyt AT, Browne M, Richardson S, Romitti P, Druschel C; The National Birth Defects Prevention Study. Maternal Caffeine Consumption and Small for Gestational Age Births: Results from a Population-Based Case–Control Study. Matern Child Health J. 2014 18 (6): 1540-1551, 2014.

Hughes RN, Beveridge IJ. Behavioral effects of exposure to caffeine during gestation, lactation or both. Neurotoxicol. Teratol. 13: 641-647, 1991.

Ibaraki K, Otsu Y, Nawa H. A novel two-site enzyme immunoassay reveals the regional distributions of and developmental changes in GluR1 and NMDAR1 protein contents in the rat brain. J Neurochem 73 (1): 408-417, 1999.

Jansson LC, Åkerman KE. The role of glutamate and its receptors in the proliferation, migration, differentiation and survival of neural progenitor cells. J Neural Transm. 121 (8): 819-836, 2014.

Kou H, Liu Y, Liang G, Huang J, Hu J, Yan YE, Li X, Yu H, He X, Zhang B, Zhang Y, Feng J, Wang H. Maternal glucocorticoid elevation and associated blood metabonome changes might be involved in metabolic programming of intrauterine growth retardation in rats exposed to caffeine prenatally. Toxicol Appl Pharmacol. 275 (2): 79-87, 2014.

Lehre KP, Levy LM, Ottersen OP, Storm-Mathisen J, Danbolt NC. Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. J Neurosci. 15 (3 Pt 1): 1835-1853, 1995.

Leinekugel X, Medina I, Khalilov I, Ben-Ari Y, Khazipov R. Ca2+ oscillations mediated by the synergistic excitatory actions of GABA(A) and NMDA receptors in the neonatal hippocampus. Neuron 18 (2): 243-255, 1997.

León D, Albasanz JL, Ruíz MA, Martín M. Chronic caffeine or theophylline intake during pregnancy inhibits A_1 receptor function in the rat brain. Neuroscience. 131 (2): 481-489, 2005.

McNamara PJ, Burgio D, Yoo SD. Pharmacokinetics of caffeine and its demethylated metabolites in lactating adult rabbits and neonatal offspring. Predictions of breast milk to serum concentration ratios. Drug Metab Dispos 20 (2): 302-328, 1992.

Nehlig A, Debry G. Potential teratogenic and neurodevelopmental consequences of coffee and caffeine exposure: a review on human and animal data. Neurotoxicol Teratol. 16 (6): 531-543, 1994.

Nicoletti F, Bockaert J, Collingridge GL, Conn PJ, Ferraguti F, Schoepp DD, Wroblewski JT, Pin JP. Metabotropic glutamate receptors: from the workbench to the bedside. Neuropharmacology 60 (7-8): 1017–1041, 2011.

Nishi A, Liu F, Matsuyama S, Hamada M, Higashi H, Nairn AC, Greengard P. Metabotropic mGlu5 receptors regulate adenosine A_{2A} receptor signaling. Proc Natl Acad Sci U S A. 100 (3): 1322-1327, 2003.

Niswender CM, Conn PJ. Metabotropic glutamate receptors: physiology, pharmacology, and disease. Ann. Rev. Pharmacol. Toxicol. 50: 295–322, 2010.

Pan HZ, Chen HH. Hyperalgesia, low-anxiety, and impairment of avoidance learning in neonatal caffeine-treated rats. Psychopharmacology (Berl). 191 (1): 119-125, 2007.

Perera V, Gross AS, McLachlan AJ. Caffeine and paraxanthine HPLC assay for CYP1A2 phenotype assessment using saliva and plasma. Biomed Chromatogr. 24 (10): 1136-1144, 2010.

Peruzzi G, Lombardelli G, Abbracchio MP, Coen E, Cattabeni F. Perinatal caffeine treatment: behavioral and biochemical effects in rats before weaning. Neurobehav. Toxicol. Teratol. 7: 453-460, 1985.

Porciúncula LO, Sallaberry C, Mioranzza S, Botton PHS, Rosemberg D. The Janus face of caffeine. Neurochem. Int. 63: 594-609, 2013.

Reynolds, RM. Corticosteroid-mediated programming and the pathogenesis of obesity and diabetes. J. Steroid Biochem. Mol. Biol. 122: 3–9, 2010.

Savitz D, Chan R, Herring A, Howards P, Hartmann K. Caffeine and miscarriage risk. Epidemiology 19: 55–62, 2008.

Schoen K, Yu T, Stockmann C, Spigarelli MG, Sherwin CM. Use of methylxanthine therapies for the treatment and prevention of apnea of prematurity. Paediatr Drugs. 16 (2): 169-177, 2014.

Sengpiel V, Elind E, Bacelis J, Nilsson S, Grove J, Myhre R, Haugen M, Meltzer HM, Alexander J, Jacobsson B, Brantsaeter AL. Maternal caffeine intake during pregnancy is associated with birth weight but not with gestational length: results from a large prospective observational cohort study. BMC Med. 11: 42, 2013.

Sheldon AL, Robinson MB. The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention. Neurochem Int. 51 (6-7): 333-355, 2007.

Silva CG, Métin C, Fazeli W, Machado NJ, Darmopil S, Launay PS, Ghestem A, Nesa MP, Bassot E, Szabó E, Baqi Y, Müller CE, Tomé AR, Ivanov A, Isbrandt D, Zilberter Y, Cunha RA, Esclapez M, Bernard C. Adenosine receptor antagonists including caffeine alter fetal brain development in mice. Sci Transl Med. 5 (197): 197ra104, 2013.

Su SH, Shyu HW, Yeh YT, Chen KM, Yeh H, Su SJ. Caffeine inhibits adipogenic differentiation of primary adipose-derived stem cells and bone marrow stromal cells. Toxicol In Vitro. 27 (6): 1830-1837, 2013.

Svenningsson P, Nomikos GG, Fredholm BB. The stimulatory action and the development of tolerance to caffeine is associated with alterations in gene expression in specific brain regions. J Neurosci. 19 (10): 4011-4022, 1999.

Tan Y, Liu J, Deng Y, Cao H, Xu D, Cu F, Lei Y, Magdalou J, Wu M, Chen L, Wang H. Caffeine-induced fetal rat over-exposure to maternal glucocorticoid and histone methylation of liver IGF-1 might cause skeletal growth retardation. Toxicol Lett. 214 (3): 279-287, 2012.

Tchekalarova J, Kubová H, Mares P. Postnatal caffeine exposure: effects on motor skills and locomotor activity during ontogenesis. Behav Brain Res. 160 (1): 99-106, 2005.

Tchekalarova J, Kubová H, Mares P. Biphasic effect of chronic postnatal caffeine treatment on cortical epileptic afterdischarges during ontogeny in rats. Brain Res. 1082 (1): 43-49, 2006.

Tchekalarova J, Kubová H, Mares P. Effects of postnatal caffeine exposure on seizure susceptibility in developing rats. Brain Res. 1150: 32-39, 2007.

Tchekalarova J, Kubová H, Mares P. Effects of early postnatal caffeine exposure on seizure susceptibility of rats are age- and model-dependent. Epilepsy Res. 88 (2-3): 231-238, 2010.

Tchekalarova J, Kubová H, Mares P. Transient changes of cortical interhemispheric responses after repeated caffeine administration in immature rats. Physiol Res. 60 (6): 961-969, 2011.

Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, Dingledine R. Glutamate receptor ion channels: structure, regulation, and function. Pharmacol. Rev. 62: 405-496, 2010.

Weng, X., Odouli, R., De-Kun, L. Maternal caffeine consumption during pregnancy and the risk of miscarriage: A prospective cohort study. American Journal of Obstetrics & Gynecology, 198 (3): e1–e8, 2008.

West GL, Sobotka TJ, Brodie RE, Beier JM, O'Donnell MW Jr. Postnatal neurobehavioral development in rats exposed in utero to caffeine. Neurobehav Toxicol Teratol. 8 (1): 29-43, 1986.

Wilkinson JM, Pollard I. Accumulation of theophylline, theobromine and paraxanthine in the fetal rat brain following a single oral dose of caffeine. Brain Res Dev Brain Res. 75 (2): 193-199, 1993.

Yang Y, Gozen O, Watkins A, Lorenzini I, Lepore A, Gao Y, Vidensky S, Brennan J, Poulsen D, Won Park J, Li Jeon N, Robinson MB, Rothstein JD. Presynaptic regulation of astroglial excitatory neurotransmitter transporter GLT1. Neuron 61 (6): 880–894, 2009.

Yasuda H, Barth AL, Stellwagen D, Malenka RC. A developmental switch in the signaling cascades for LTP induction. Nat Neurosci. 6 (1): 15-16, 2003.

Zimmermberg B, Carr KL, Scott A, Lee HH, Weider JM. The effects of postnatal caffeine exposure on growth, activity and learning in rats. Pharmacol. Biochem. Behav. 39: 883-888, 1991.

Figures

Fig. 1. Effect of perinatal caffeine intake in drinking water (0.1, 0.3 or 1.0 g/L, during dark cycle) on maternal body weight gain (A) and body weight of the progeny (B). Data are presented as mean ± S.E.M. (n=10-12 litters/group). *different from control group ($P < 0.05$), One-way ANOVA and Newman-Keuls post hoc.

Fig. 2. Effect of perinatal caffeine in drinking water (0.1, 0.3 and 1.0 g/L, during dark cycle) on reflex development by the following tests: (A) Righting reflex; (B) Forelimb grasp reflex; (C) Negative geotaxis; (D) Cliif avoidance. Data are presented as mean ± S.E.M. (n=10-12 litters/group) of the mean age from each litter to achieve the task (n=10-12 litters/group). Control group received only water. Fisher´s exact test was used to analyze the reflex of the last day within the time window.

Fig. 3. Effect of perinatal caffeine in drinking water (0.1, 0.3 and 1.0 g/L, during dark cycle) on locomotor activity, represented as number of crossing (A), number

of squares visited (B), and locomotor index (C). Data are presented as mean ± S.E.M. (n = 10-12 litters/group), and were analyzed by two-way ANOVA followed by Bonferroni´s test.

Fig. 4. Plasma levels of caffeine (A) and its metabolites paraxanthine (B), theobromine (C), and theophylline (D) in rat pups submitted to caffeine since intrauterine growth in the dam´s drinking water (0.1, 0.3 and or 1.0 g/L, during dark cycle). Data are presented as mean ± S.E.M. (n=4 litters/group), and were analyzed by two-way ANOVA followed by Bonferroni´s test.

Fig. 5. Immunoblotting analysis of glutamate transporter GLT-1 (A, blue circle), GluR1 subunit of AMPA ionotropic glutamate receptor (A, red square), metabotropic receptor mGluR5 (A, green triangle) and NMDA glutamate receptor subunits (B): pNR1/NR1 ratio (blue circle, dashed line), NR2A (red square, continuous line) and pNR2B/NR2B ratio (green triangle, dashed line) in the whole cortex at PND 7 from pups exposed to water or caffeine. Data are means ± S.E.M (n=6-8 samples/group) of density unit lines obtained from protein/β-tubulin ratio. *P < 0.05, significantly different from control group (water). One-way ANOVA followed by Newman-Keuls multiple comparison test.

Fig. 6. Immunoblotting analysis of glutamate transporter GLT-1 (A, blue circle), GluR1 subunit of AMPA ionotropic glutamate receptor (A, red square), metabotropic receptor mGluR5 (A, green triangle) and NMDA glutamate receptor subunits (B): pNR1/NR1 ratio (blue circle, dashed line), NR2A (red square, continuous line) and pNR2B/NR2B ratio (green triangle, dashed line) in

hippocampus at PND 7 from pups exposed to water or caffeine. Data are means ± S.E.M (n = 6-8 samples/group) of density unit lines obtained from protein/β-tubulin ratio. Representative Western blot bands were presented below. *P < 0.05, significantly different from control group (water). One-way ANOVA followed by Newman-Keuls multiple comparison test.

Fig. 7. Immunoblotting analysis of glutamate transporter GLT-1 (A, blue circle), GluR1 subunit of AMPA ionotropic glutamate receptor (A, red square), metabotropic receptor mGluR5 (A, green triangle) and NMDA glutamate receptor subunits (B): pNR1/NR1 ratio (blue circle, dashed line), NR2A (red square, continuous line) and pNR2B/NR2B ratio (green triangle, dashed line) in the whole cortex at PND 14 from pups exposed to water or caffeine. Data are means ± S.E.M (n=6-8 samples/group) of density unit lines obtained from protein/β-tubulin ratio. Representative Western blot bands were presented below. *P < 0.05, significantly different from control group (water). One-way ANOVA followed by Newman-Keuls multiple comparison test.

Fig. 8. Immunoblotting analysis of glutamate transporter GLT-1 (A, blue circle), GluR1 subunit of AMPA ionotropic glutamate receptor (A, red square), metabotropic receptor mGluR5 (A, green triangle) and NMDA glutamate receptor subunits (B): pNR1/NR1 ratio (blue circle, dashed line), NR2A (red square, continuous line) and pNR2B/NR2B ratio (green triangle, dashed line) in hippocampus at PND 14 from pups exposed to water or caffeine. Data are means ± S.E.M (n=6-8 samples/group) of density unit lines obtained from protein/β-tubulin ratio. Representative Western blot bands were presented below. *P <

0.05, significantly different from control group (water). One-way ANOVA followed by Newman-Keuls multiple comparison test.

Fig. 9. Immunoblotting analysis of glutamate transporter GLT-1 (A, blue circle), GluR1 subunit of AMPA ionotropic glutamate receptor (A, red square), metabotropic receptor mGluR5 (A, green triangle) and NMDA glutamate receptor subunits (B): pNR1/NR1 ratio (blue circle, dashed line), NR2A (red square, continuous line) and pNR2B/NR2B ratio (green triangle, dashed line) in the whole cortex at PND 21 from pups exposed to water or caffeine. Data are means ± S.E.M (n=6-8 samples/group) of density unit lines obtained from protein/β-tubulin ratio. Representative Western blot bands were presented below. $*P < 0.05$, significantly different from control group (water). One-way ANOVA followed by Newman-Keuls multiple comparison test.

Fig. 10. Immunoblotting analysis of glutamate transporter GLT-1 (A, blue circle), GluR1 subunit of AMPA ionotropic glutamate receptor (A, red square), metabotropic receptor mGluR5 (A, green triangle) and NMDA glutamate receptor subunits (B): pNR1/NR1 ratio (blue circle, dashed line), NR2A (red square, continuous line) and pNR2B/NR2B ratio (green triangle, dashed line) in hippocampus at PND 21 from pups exposed to water or caffeine. Data are means ± S.E.M (n=6-8 samples/group) of density unit lines obtained from protein/β-tubulin ratio. Representative Western blot bands were presented below. $*P < 0.05$, significantly different from control group (water). One-way ANOVA followed by Newman-Keuls multiple comparison test.

Fig. 1

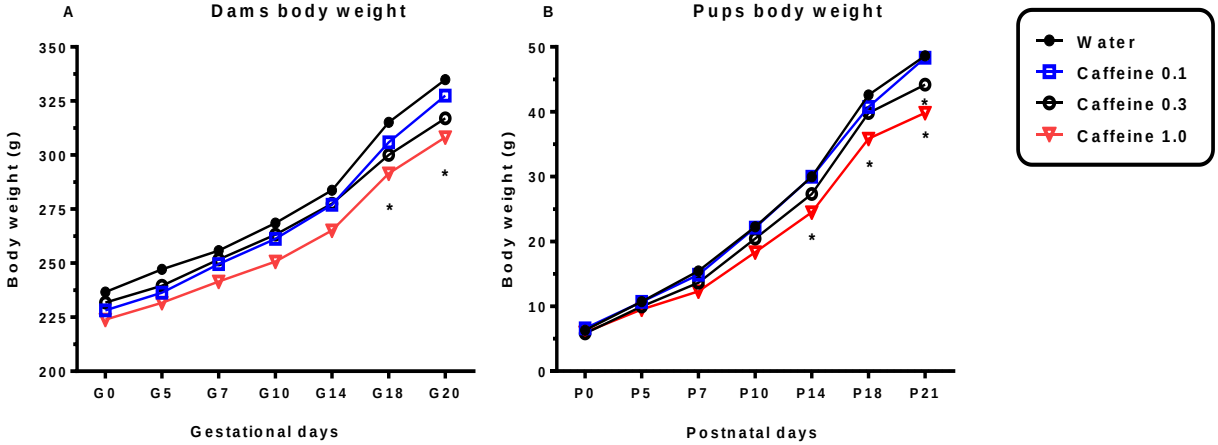

A
Dams body weight
Body weight (g)
350
325
300
275
250
225
200
G0
G5
G7
G10
G14
G18
G20
Gestational days
B
Pups body weight
Body weight (g)
50
40
30
20
10
0
P0
P5
P7
P10
P14
P18
P21
Postnatal days
Water
Caffeine 0.1
Caffeine 0.3
Caffeine 1.0

Fig. 2

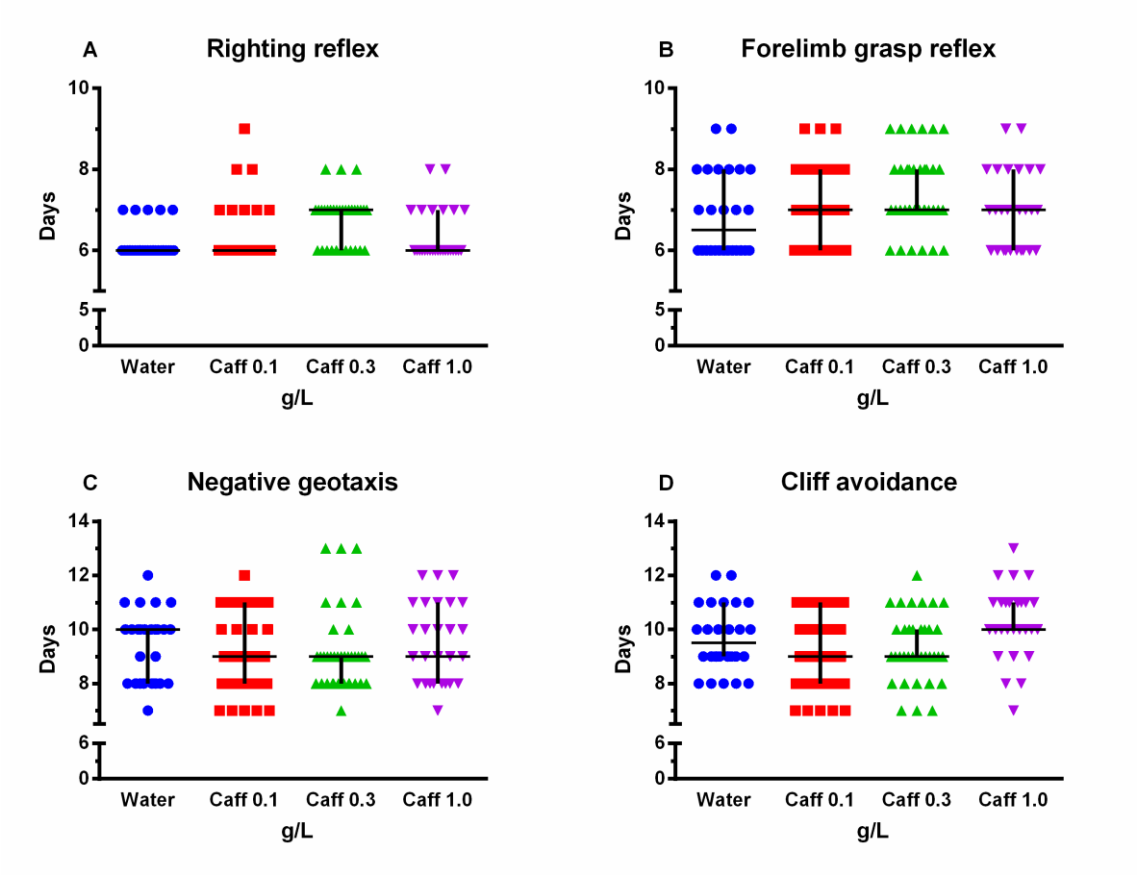

A
Righting reflex
B
Forelimb grasp reflex
C
Negative geotaxis
D
Cliff avoidance
Days
Water
Caff 0.1
Caff 0.3
Caff 1.0
g/L

Fig.3

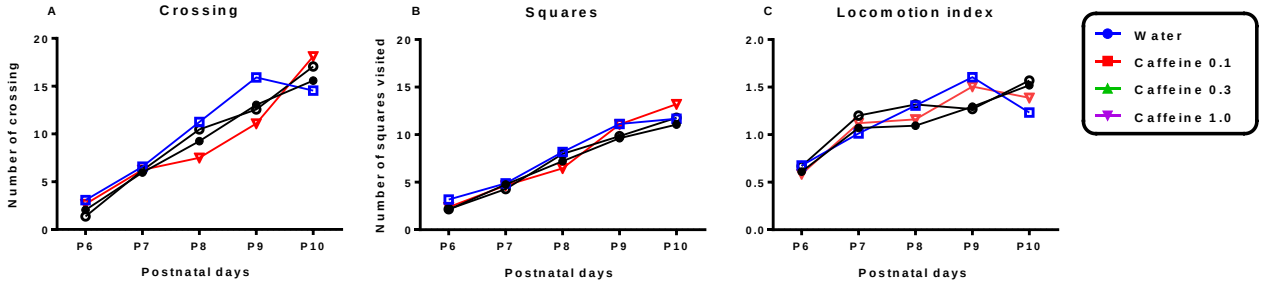

A
Crossing
Number of crossing
20
15
10
5
0
P6
P7
P8
P9
P10
Postnatal days
B
Squares
Number of squares visited
20
15
10
5
0
P6
P7
P8
P9
P10
Postnatal days
C
Locomotion index
2.0
1.5
1.0
0.5
0.0
P6
P7
P8
P9
P10
Postnatal days
Water
Caffeine 0.1
Caffeine 0.3
Caffeine 1.0

Fig. 4

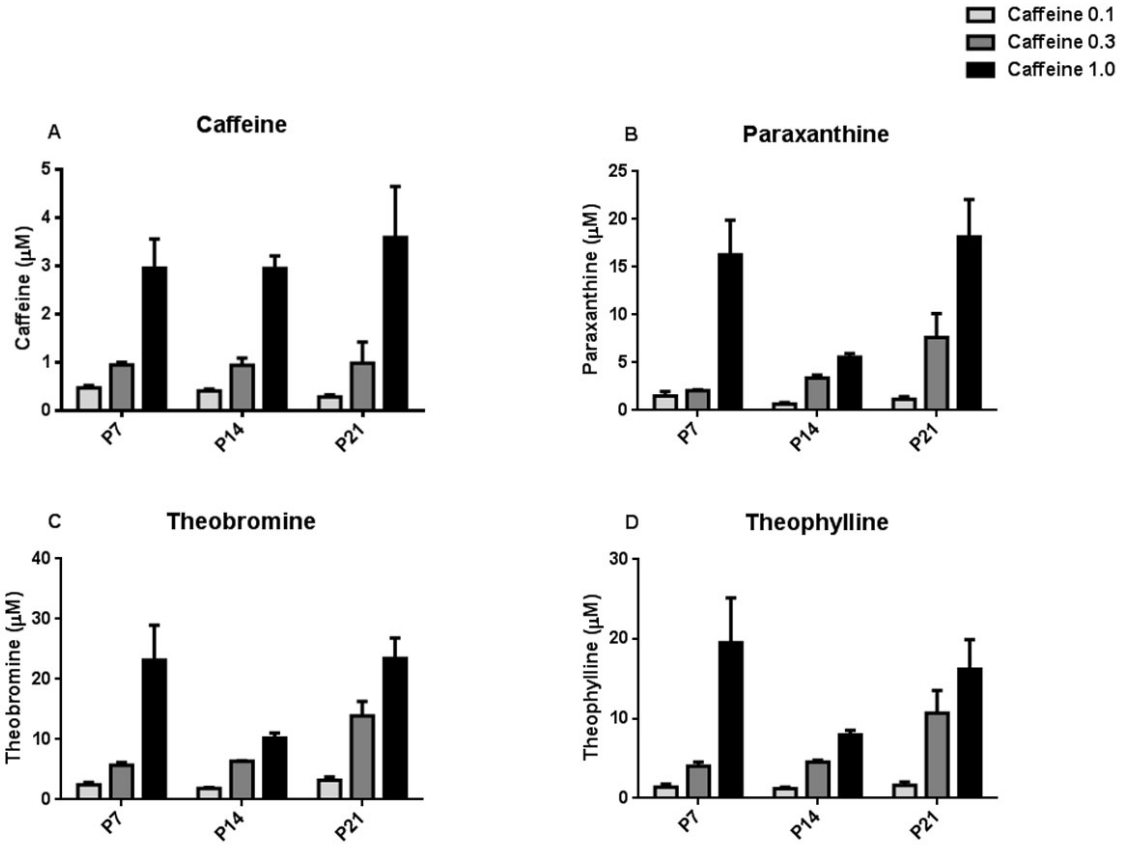

Caffeine 0.1
Caffeine 0.3
Caffeine 1.0
A
Caffeine
Caffeine (µM)
0
1
2
3
4
5
P7
P14
P21
B
Paraxanthine
Paraxanthine (µM)
0
5
10
15
20
25
P7
P14
P21
C
Theobromine
Theobromine (µM)
0
10
20
30
40
P7
P14
P21
D
Theophylline
Theophylline (µM)
0
10
20
30
P7
P14
P21

Fig. 5

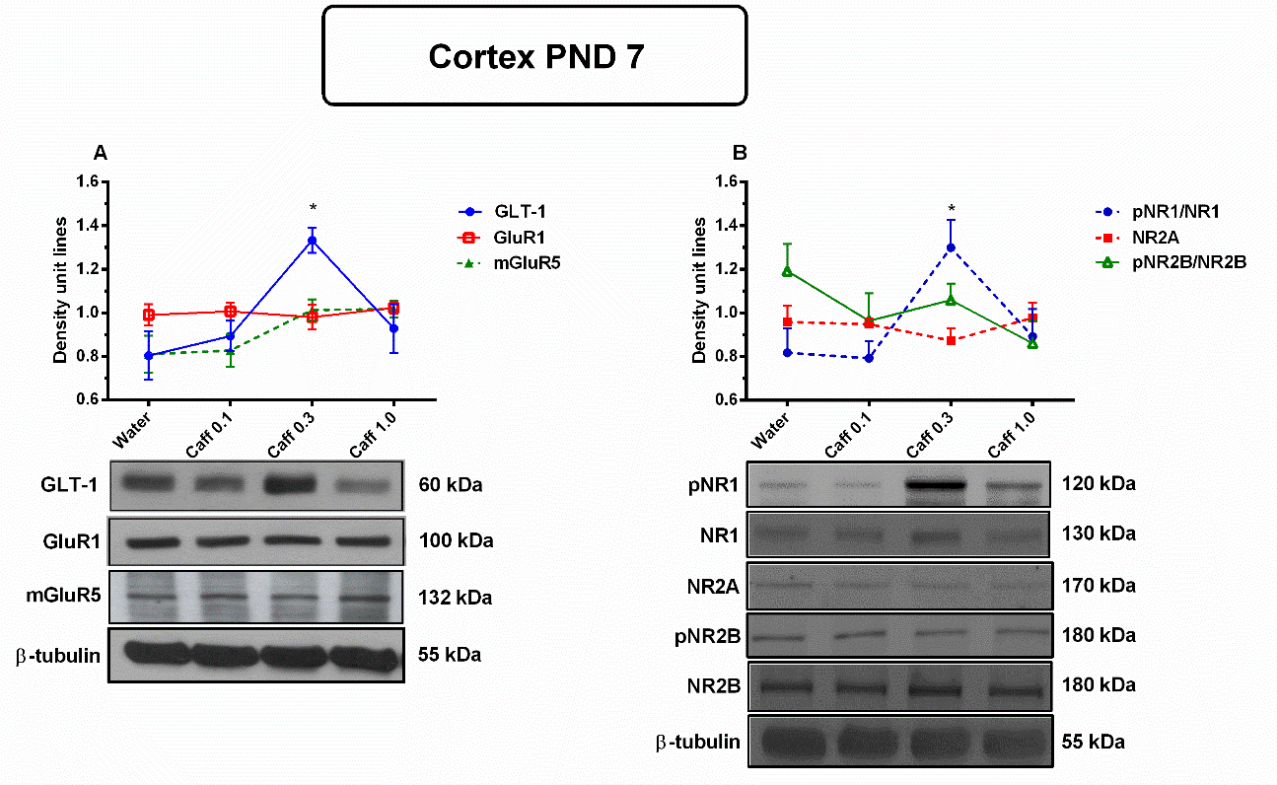

Cortex PND 7
A
Density unit lines
1.6
1.4
1.2
1.0
0.8
0.6
*
GLT-1
GluR1
mGluR5
Water
Caff 0.1
Caff 0.3
Caff 1.0
GLT-1
60 kDa
GluR1
100 kDa
mGluR5
132 kDa
β-tubulin
55 kDa
B
Density unit lines
1.6
1.4
1.2
1.0
0.8
0.6
*
pNR1/NR1
NR2A
pNR2B/NR2B
Water
Caff 0.1
Caff 0.3
Caff 1.0
pNR1
120 kDa
NR1
130 kDa
NR2A
170 kDa
pNR2B
180 kDa
NR2B
180 kDa
β-tubulin
55 kDa

Fig. 6

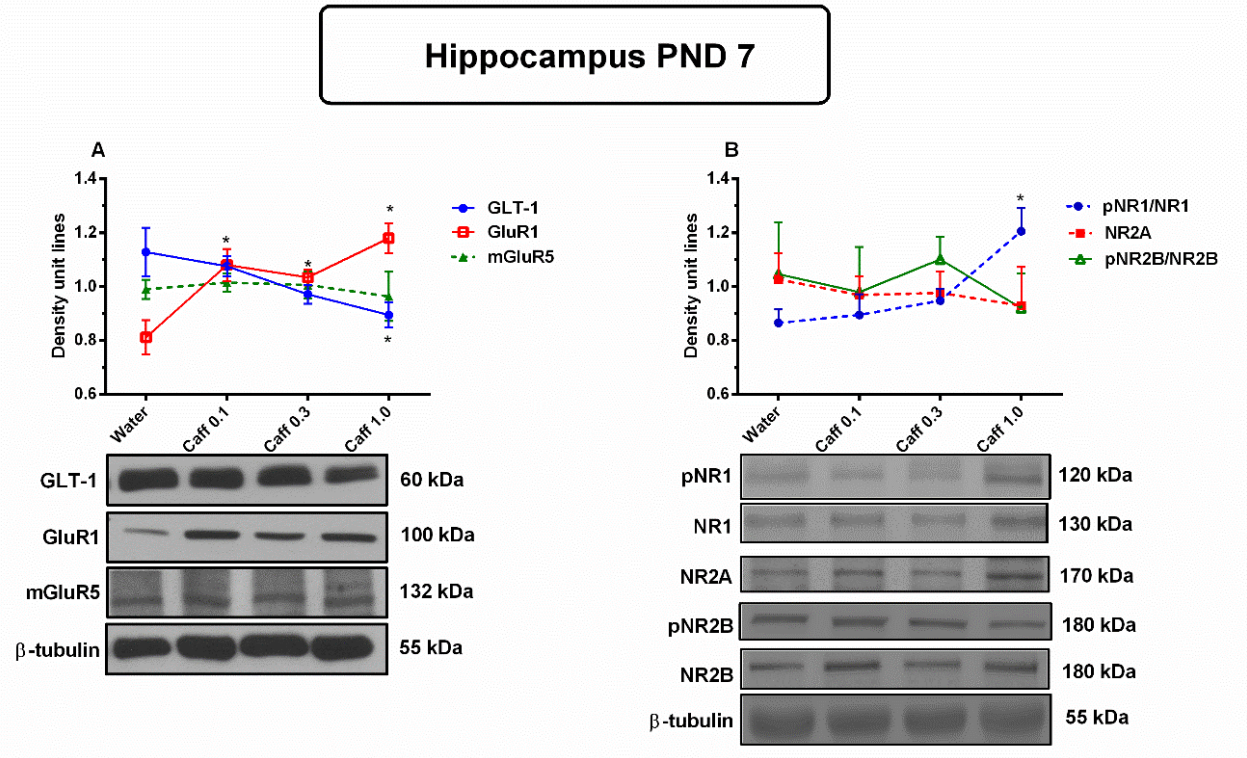

Hippocampus PND 7
A
Density unit lines
1.4
1.2
1.0
0.8
0.6
GLT-1
GluR1
mGluR5
Water
Caff 0.1
Caff 0.3
Caff 1.0
GLT-1
GluR1
mGluR5
β-tubulin
60 kDa
100 kDa
132 kDa
55 kDa
B
Density unit lines
pNR1/NR1
NR2A
pNR2B/NR2B
pNR1
NR1
NR2A
pNR2B
NR2B
β-tubulin
120 kDa
130 kDa
170 kDa
180 kDa
180 kDa
55 kDa

Fig. 7

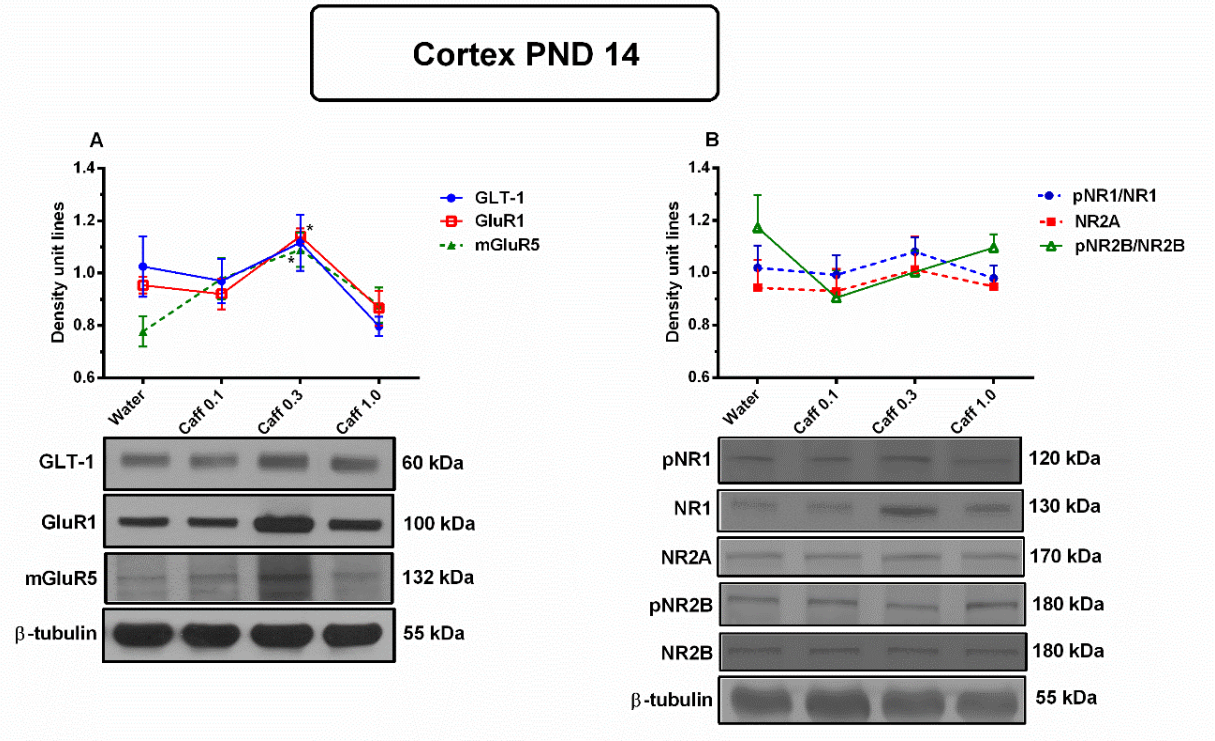

Cortex PND 14
A
Density unit lines
1.4
1.2
1.0
0.8
0.6
Water
Caff 0.1
Caff 0.3
Caff 1.0
GLT-1
GluR1
mGluR5
*
*
GLT-1
60 kDa
GluR1
100 kDa
mGluR5
132 kDa
β-tubulin
55 kDa
B
Density unit lines
1.4
1.2
1.0
0.8
0.6
Water
Caff 0.1
Caff 0.3
Caff 1.0
pNR1/NR1
NR2A
pNR2B/NR2B
pNR1
120 kDa
NR1
130 kDa
NR2A
170 kDa
pNR2B
180 kDa
NR2B
180 kDa
β-tubulin
55 kDa

Fig. 8

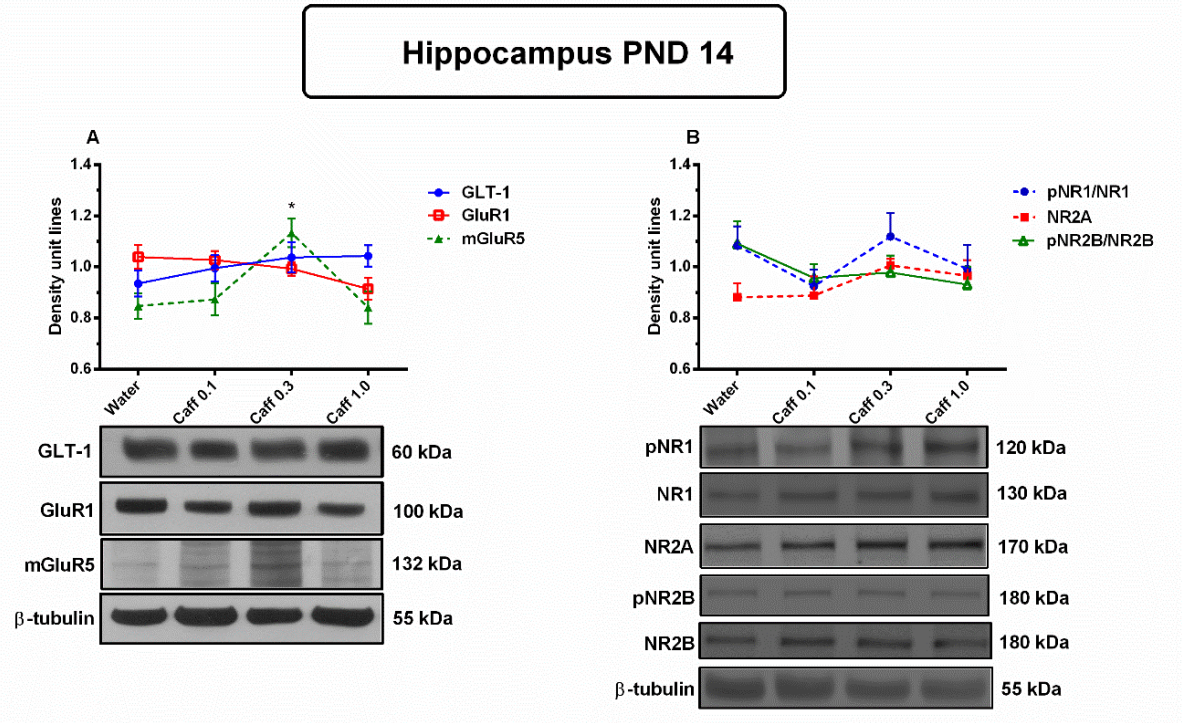

Hippocampus PND 14
A
Density unit lines
1.4
1.2
1.0
0.8
0.6
*
GLT-1
GluR1
mGluR5
Water
Caff 0.1
Caff 0.3
Caff 1.0
GLT-1
60 kDa
GluR1
100 kDa
mGluR5
132 kDa
β-tubulin
55 kDa
B
Density unit lines
1.4
1.2
1.0
0.8
0.6
pNR1/NR1
NR2A
pNR2B/NR2B
Water
Caff 0.1
Caff 0.3
Caff 1.0
pNR1
120 kDa
NR1
130 kDa
NR2A
170 kDa
pNR2B
180 kDa
NR2B
180 kDa
β-tubulin
55 kDa

Fig. 9

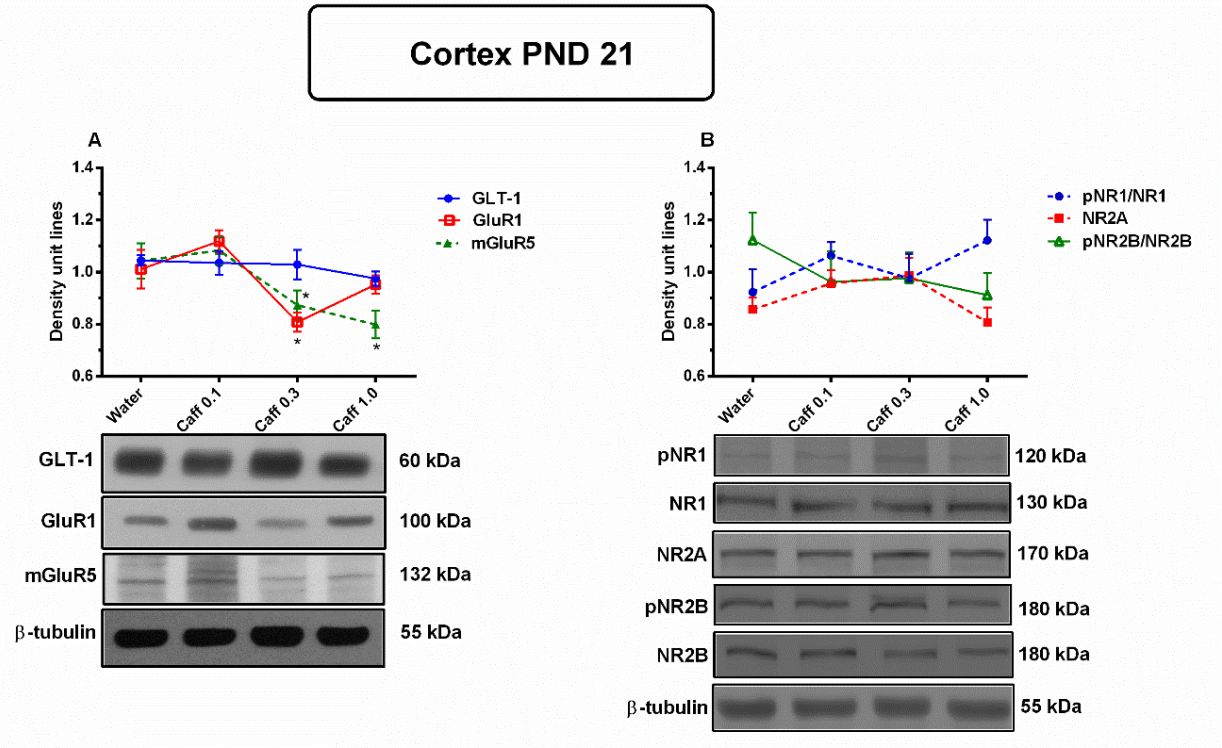

Cortex PND 21
A
Density unit lines
1.4
1.2
1.0
0.8
0.6
Water
Caff 0.1
Caff 0.3
Caff 1.0
GLT-1
GluR1
mGluR5
GLT-1
GluR1
mGluR5
β-tubulin
60 kDa
100 kDa
132 kDa
55 kDa
B
Density unit lines
pNR1/NR1
NR2A
pNR2B/NR2B
pNR1
NR1
NR2A
pNR2B
NR2B
β-tubulin
120 kDa
130 kDa
170 kDa
180 kDa
180 kDa
55 kDa

Fig. 10

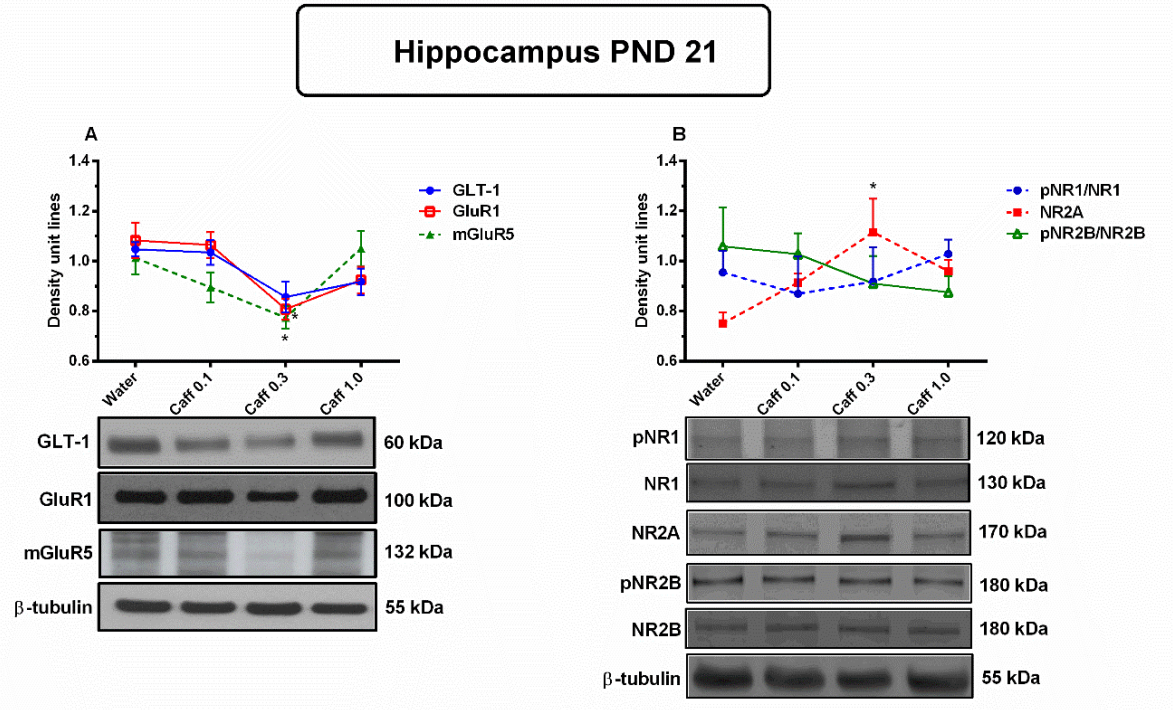

Hippocampus PND 21
A
Density unit lines
1.4
1.2
1.0
0.8
0.6
GLT-1
GluR1
mGluR5
Water
Caff 0.1
Caff 0.3
Caff 1.0
GLT-1
60 kDa
GluR1
100 kDa
mGluR5
132 kDa
β-tubulin
55 kDa
B
Density unit lines
pNR1/NR1
NR2A
pNR2B/NR2B
pNR1
120 kDa
NR1
130 kDa
NR2A
170 kDa
pNR2B
180 kDa
NR2B
180 kDa
β-tubulin
55 kDa

Table 1 – Reproductive data[a]

	Water	Caffeine 0.1 g/L	Caffeine 0.3 g/L	Caffeine 1.0 g/L
No. of dams	12	12	12	12
Gestational duration (days)	21.92 ± 0.08	21.86 ± 0.10	21.71 ± 0.12	21.77 ± 0.12
No. of live pups	8.31 ± 0.96	8.79 ± 0.52	8.00 ± 0.83	9.00 ± 1.24
No. of stillborn pups	0.46 ± 0.31	0.07 ± 0.07	0.93 ± 0.37	1.00 ± 0.34
Implantation sites	10.08 ± 0.79	10.43 ± 0.67	10.64 ± 0.75	10.69 ± 0.83
Index birth/implantation	0.81 ± 0.05	0.86 ± 0.04	0.71 ± 0.06	0.85 ± 0.07

[a]Data are presented as mean ± S.E.M.

Table 2 – Changes in levels of the specific ^{3}H-glutamate binding (pmol glutamate/mg protein) in whole cortex from rat pups receiving water (control group) or caffeine in drinking water (0.1, 0.3 or 1.0 g/L) during lactation.

	Water	Caffeine 0.1 g/L	Caffeine 0.3 g/L	Caffeine 1.0 g/L
Postnatal day (PND)				
PND 7	2.52 ± 0.28	2.33 ± 0.33	2.23 ± 0.41	2.21 ± 0.33
PND 14	5.02 ± 0.32	3.85 ± 0.29*	4.40 ± 0.18	4.92 ± 0.35
PND 21	4.40 ± 0.34	4.67 ± 0.31	4.15 ± 0.44	3.74 ± 0.31

Data are presented as mean ± S.E.M of pmol glutamate/mg protein (n = 6 animals/group).

*Different from control group ($P < 0.05$).

Table 3 - Changes in levels of the specific ^{3}H-glutamate binding (pmol glutamate/mg protein) in hippocampus from rat pups receiving water (control group) or caffeine in drinking water (0.1, 0.3 or 1.0 g/L) during lactation.

	Water	**Caffeine 0.1 g/L**	**Caffeine 0.3 g/L**	**Caffeine 1.0 g/L**
Postnatal day (PND)				
PND 7	5.19 ± 0.76	5.01 ± 0.91	4.73 ± 0.53	3.96 ± 0.49
PND 14	6.59 ± 0.83	5.79 ± 0.76	5.48 ± 0.63	4.57 ± 0.72
PND 21	3.92 ± 0.53	4.00 ± 0.56	3.60 ± 0.78	3.75 ± 0.78

Data are presented as mean ± S.E.M of pmol glutamate/mg protein (n=6 animals/group).

PARTE III

4. DISCUSSÃO

O desenvolvimento do sistema nervoso é um processo altamente complexo que envolve a coordenação temporal e espacial da proliferação, migração, diferenciação, sinaptogênese e a morte celular programada. Períodos de maior vulnerabilidade durante o desenvolvimento do sistema nervoso central são sensíveis às agressões ambientais, condições adversas e modificações na dieta (Georgieff, 2007). Tendo em vista que a cafeína é um psicoestimulante presente na dieta da população, é importante estudar o impacto do consumo de cafeína durante as diferentes fases do desenvolvimento encefálico.

No primeiro capítulo desta tese fizemos uma revisão da literatura sobre o impacto da administração de cafeína durante o período gestacional e lactação de roedores. O artigo manteve seu enfoque em alterações comportamentais e nos sistemas de neurotransmissores mais abundantes no SNC de mamíferos durante o desenvolvimento do encéfalo. Nesta revisão vários aspectos foram relatados. A administração de cafeína por diferentes vias (intraperitoneal, gavagem, na água de beber) é capaz de provocar alterações no comportamento dos animais, com predomínio da atividade locomotora visto que essa pode ser melhor observada em animais pequenos, mas sempre foi dependente da dose. O sistema adenosinérgico, alvo farmacológico da cafeína, apresenta modificações quando administrada durante o desenvolvimento do encéfalo, e essas modificações são dependentes do período de desenvolvimento de cada estrutura encefálica. Houve um aumento nos receptores A_1 na ponte e no núcleo do trato solitário de ratos neonatos de 6 dias que receberam cafeína por gavagem (15 mg/kg) entre os DPN 2-6 (Gaytan et al., 2006). O consumo de

cafeína na água de beber (0,3 g/L) durante a gestação e lactação aumentou a ligação ao receptor A_1 em membranas corticais de filhotes no DPN 1 e DPN 7, mas no hipocampo de filhotes com 21 dias houve uma diminuição no RNAm de A_1 (Adén et al., 2000). Em outro estudo, com uma dose mais elevada de cafeína na água de beber (1,0 g/L) durante a gestação, houve um aumento do número de receptores A_1 no encéfalo materno e fetal, este último foi relacionado a um aumento da afinidade pela cafeína (Lorenzo et al., 2010). O efeito do tratamento com cafeína no período embrionário e pós-natal nos receptores A_{2A} também levou a alterações de acordo com a idade pós-natal e a estrutura encefálica. A cafeína na água de beber (0,2 g/L) levou ao aumento da expressão de A_{2A} na medula de recém-nascidos (Picard et al., 2008). A exposição neonatal de cafeína entre os DPN 2-6 aumentou o RNAm de A_{2A} no tronco cerebral e no hipotálamo (Gaytan e Pasaro, 2012). Outros sistemas de neurotransmissores são afetados pela administração de cafeína, particularmente o sistema glutamatérgico reúne uma quantidade significativa de estudos. Em ratas grávidas tratadas com cafeína na água de beber (1,0 g/L) durante toda a gestação, observou-se uma redução no conteúdo do receptor metabotrópico de glutamato no encéfalo de fetos de 20 dias embrionário, mas essa redução não alterou a responsividade do receptor (León et al., 2005). No que diz respeito aos estudos epidemiológicos estes ainda permanecem tentando controlar uma infinidade de variáveis com o objetivo de estabelecer um consenso sobre a quantidade segura de cafeína que pode ser consumida. Diferentemente da década de 70, os estudos atuais consideram o tabagismo como um fator de confusão entre outros como o consumo de álcool, nível de escolaridade e atividade fisica. Os estudos atuais também levam em consideração os “sintomas da gravidez” (náusea e vômito), além de fazer um

acompanhamento mais frequente ao longo da gestação, e a coleta de dados de consumo de cafeína também ser mais controlada. A partir desses estudos ainda permanece controverso se a cafeína pode ser consumida durante a gravidez e se o seu consumo está diretamente relacionado ao baixo peso ao nascer e incidência de aborto espontâneo.

No segundo capítulo a proposta principal foi avaliar o imunoconteúdo de proteínas importantes para a formação das sinapses no córtex e hipocampo de embriões de ratos no 18° e 20° dia gestacional. Considerando que entre as alterações mais evidentes que ocorrem pelo tratamento com cafeína são as flutuações no peso corporal, primeiramente foi feito o registro do peso dos embriões de ratos de 18 e 20 dias. Foi possível observar uma redução no peso fetal somente nos embriões de 20 dias e que receberam a dose mais elevada de cafeína (1,0 g/L). Alguns estudos anteriores já haviam observado que ratas que receberam o tratamento com cafeína entre o 11° e o 20° dia gestacional tiveram embriões com peso reduzido nas três doses administradas (20, 60 e 180 mg/kg por via intragástrica), além da diminuição no ganho de peso das ratas nas duas doses mais altas (Kou et al., 2014; Liu et al., 2012). Em outro estudo, utilizando o mesmo protocolo de tratamento de cafeína e as mesmas doses, foi encontrada uma inibição do eixo hipotálamo-pituitário-adrenal, pois os fetos das ratas grávidas que receberam as doses de 60 mg/kg e 180 mg/kg apresentaram uma redução na expressão do hormônio liberador de corticotrofinas (Xu et al., 2012). Nesse mesmo estudo foi observado um aumento nos níveis séricos de corticosterona nas fêmeas que se refletiram nos níveis de corticosterona no plasma fetal e aumento no receptor de glicocorticoide no hipocampo. É possível que o excesso de corticosterona sérica no feto, vindo da placenta da mãe pelo

consumo de cafeína, levou a alterações na regulação do eixo hipotálamo-pituitária-adrenal, e esse evento pode ter provocado o retardo no crescimento intrauterino observado nos embriões de 20 dias. Ainda que a dose mais baixa tenha apresentado menor impacto nos resultados, os autores desaconselham o consumo de cafeína durante a gestação. Em outro estudo, utilizando a mesma escala de administração de cafeína, porém em uma única dose (entre o dia gestacional 11 e 20, 120 mg/kg de cafeína por via intragástrica), houve uma redução no comprimento do fêmur de embriões de 20 dias e na expressão de proteínas envolvidas na sinalização do fator de crescimento semelhante à insulina tipo 1 (IGF-1, do inglês *Insulin Growth Factor 1*) (Tan et al., 2012). De acordo com Reynolds (2010), altos níveis de glicocorticoides séricos durante a gestação podem levar a uma programação intrauterina alterada para o metabolismo de glicose e lipídios, o que pode explicar a redução no peso corporal dos embriões. Além disso, os efeitos *in vitro* da cafeína na adipogênese foram avaliados em células tronco derivadas de tecido adiposo de ratos, as quais foram continuamente expostas à cafeína em diferentes concentrações (0,1–1 mM) durante a diferenciação adipogênica. O tratamento com cafeína diminuiu a expressão de genes relacionados à adipogênese, como PPAR-γ, lipoproteína lipase, leptina e TNF-α de maneira dose-dependente (Su et al., 2013). A cafeína também é conhecida por afetar a termogênese através inibição da enzima fosfodiesterase, o que leva a um aumento no AMPc intracelular e à inativação da lipase-hormônio sensível, que promove a lipólise (Acheson et al., 2004). Embora nosso trabalho não teve como objetivo investigar as alterações de peso, nossos achados são similares aos outros trabalhos e uma possível hipótese para essas alterações de peso corporal são os níveis séricos elevados de

glicocorticoides nas fêmeas pelo consumo de cafeína. De acordo com alguns autores essas alterações podem estar diretamente envolvidas na programação metabólica intra-uterina e na maior susceptibilidade a doenças metabólicas na vida adulta, como a esteatohepatite não-alcoólica (Wang et al., 2014) e diabetes tipo 2 (Sun et al., 2014).

Dando continuidade ao nosso trabalho fizemos a imunodetecção de algumas proteínas cruciais para o desenvolvimento, maturação e diferenciação das sinapses: BDNF (e seu receptor TrkB), *Shh*, GAP-43 e SNAP-25. As neurotrofinas são fatores de crescimento com papel crucial no desenvolvimento e na maturação do encéfalo (Cohen-Cory et al., 2010). O fator neurotrófico derivado do encéfalo (BDNF) e seu receptor do tipo tirosina cinase (TrkB) participa como um regulador retrógrado do desenvolvimento das sinapses, sendo essencial para o crescimento e elongação dos dendritos de sinapses inibitórias e excitatórias (Carvalho et al., 2008; Tyler e Pozzo-Miller, 2003). Os neurônios em desenvolvimento no início do crescimento dos neuritos expressam níveis altos da Proteína Associada ao Cone de Crescimento (GAP-43) (Dani et al., 1991; Perrone-Bizzozzero et al., 1986). Além disso, a sinalização da proteína *Shh* (Sonic Hedgehog) é importante para a formação das camadas corticais nos estágios embrionários tardios (Komada et al., 2008) e para a origem de neurônios hipocampais responsáveis pela neurogênese na idade adulta (Li et al., 2013). A proteína associada ao sinaptossoma de 25 kDa (SNAP-25) faz parte do complexo SNARE para a liberação de neurotransmissores na fenda sináptica, mas também está envolvida na extensão e crescimento de neuritos (Osen-Sand et al., 1993).

Através de nosso trabalho foi possível demonstrar que o consumo de cafeína durante a gestação reduziu o imunoconteúdo de GAP-43, TrkB e *Shh* no hipocampo dos embriões com 18 dias de idade. Em um modelo *in vitro* para investigar o crescimento de neuritos, observou-se que o tratamento com o fator de crescimento neuronal (NGF) induziu o crescimento de neuritos e o aumento da expressão de GAP-43, e esse processo foi mediado pela ativação de receptores de adenosina A_{2A} (O'Driscoll e Gorman, 2005). Partindo dessas observações sugerimos que embora a cafeína seja um antagonista não-seletivo dos receptores de adenosina (A_1 e A_{2A}), o bloqueio via receptores A_{2A} pode ter levado à redução de GAP-43. Uma vez que a diminuição de GAP-43 e TrkB não foi mais observada no hipocampo dos embriões de 20 dias, sugerimos que essas alterações transitórias em ambas proteínas podem ter sido contrabalançadas, por exemplo, pelo aumento gradativo de *Shh* observado nas duas idades embrionárias. O impacto desse aumento da *Shh* durante o período embrionário a longo prazo, após o desenvolvimento do sistema nervoso, ainda deve ser estudado.

Tendo em vista que a adição de *Shh* em culturas de neurônios hipocampais leva ao aumento da espessura dos terminais pré-sinápticos, com aumento do tamanho das vesículas sinápticas (Mitchell et al., 2012), investigamos o imunoconteúdo da proteína SNAP-25 que pode ser facilmente detectada nos estágios iniciais do desenvolvimento encefálico (Prescott e Chamberlain, 2011). Nenhuma das doses de cafeína estudadas modificaram o imunoconteúdo de SNAP-25 nas estruturas cerebrais de ambas idades embrionárias, sugerindo que nesse estágio do desenvolvimento não houve influência desse psicoestimulante na integridade dos terminais nervosos.

Comparado ao hipocampo, o tratamento gestacional com a maior dose de cafeína (1,0 g/L) aumentou o imunoconteúdo de BDNF no córtex dos embriões de 18 dias e o reduziu aos 20 dias, sem alterar o seu receptor TrkB. Coincidentemente, *Shh* foi aumentada no córtex dos embriões de 18 dias, e sugerimos que uma das consequências possíveis do aumento dessas proteínas pela cafeína poderia ser a aceleração do desenvolvimento do telencéfalo. De fato, já foi observado em outros estudos *in vitro* que a superexpressão de *Shh* leva a uma aceleração do desenvolvimento do telencéfalo (Marret et al., 1997; Sahir et al., 2000; 2001; 2004). Além disso, a *Shh* participa da indução de diferenciação de interneurônios GABAérgicos no telencéfalo (Mady e Kohtz, 2007). Uma das consequências do aumento deste morfógeno no final da gestação pode ser uma distribuição anormal de neurônios nas camadas corticais antes do nascimento, o que pode afetar a migração dos neurônios GABAérgicos após o nascimento. Isso pode ser observado em estudo recente, em que o consumo moderado de cafeína (0,3 g/L) durante a gravidez e lactação levou a um atraso na migração dos neurônios GABAérgicos no hipocampo e camadas corticais superficiais em camundongos no 6° dia pós-natal (Silva et al., 2013).

Com o objetivo de estimar o impacto das alterações dessas proteínas no número de neurônios, foi utilizada uma técnica em que os núcleos celulares foram isolados por subfracionamento celular e a partir disso identifica-se como núcleos neuronais pela marcação com anticorpo para a Proteína Nuclear Neuronal. (em inglês NeuN). O antígeno nuclear neuronal (NeuN) reconhece uma proteína nuclear neurônio-específica em vertebrados, em períodos do desenvolvimento que correspondem à iniciação de diferenciação de neurônios pós-mitóticos (Mullen et al., 1992). Em nosso trabalho, observamos que os

embriões de 20 dias que receberam a dose moderada de cafeína (0,3 g/L) apresentaram um aumento no número de núcleos totais e neuronais no hipocampo, enquanto os embriões de 20 dias tratados com a menor dose de cafeína (0,1 g/L) apresentaram aumento nos núcleos no córtex. A exposição prenatal a outros psicoestimulantes, como etanol e nicotina, também levou a alterações no número de neurônios no hipotálamo e no sistema límbico no período pós-natal de filhotes (Chang et al., 2012; 2013). Esses autores sugerem que o aumento da neurogênese no período de desenvolvimento do SNC pode aumentar o risco dos filhotes desenvolverem abuso ao consumo dessas substâncias na vida adulta**.** Porém a cafeína não apresenta um efeito tão deletério como esses outros psicoestimulantes, mas aumenta a susceptibilidade a convulsões por agentes convulsivantes na vida adulta de camundongos (Silva et al., 2013). A participação de outras proteínas e cascatas de sinalização no aumento de núcleos NeuN no córtex pela menor dose de cafeína merece maior investigação, já que não foi encontrada nenhuma alteração no imunoconteúdo de proteínas envolvidas no desenvolvimento das sinapses com essa dose.

Dando continuidade ao estudo dos efeitos da cafeína no decorrer do densenvolvimento encefálico, no terceiro capítulo as investigações foram direcionadas para avaliar os efeitos da administração de cafeína durante o período perigestacional sobre o desenvolvimento do reflexo e desenvolvimento motor de roedores.

Os ensaios comportamentais são importantes em toxicologia, teratologia, rastreio de fenótipo, e em outras aplicações que requerem o estado funcional dos animais. No entanto, quando a avaliação envolve animais no período de desenvolvimento, ela pode ser especialmente difícil porque um animal imaturo é

mais frágil, propenso a fadiga e por apresentar limites sensoriais e na capacidade motora, eles respondem diferentemente aos desafios padrão tais como privação de alimento. Além disso, os testes que envolvem treinamento são frequentemente inaplicáveis na fase de desenvolvimento porque o próprio treinamento pode exigir dias ou semanas, o que inviabiliza a avaliação do desenvolvimento em tempo hábil. Assim, a avaliação de animais neonatos deve considerar as características inatas, expressas como respostas reflexas (Moser, 1990; Rogers et al., 1997; Vohers, 1985).

O reflexo é uma resposta motora do SNC a estímulos internos ou externos (Zehr e Stein, 1999). As ações motoras reflexas mais simples são mediadas pela medula espinhal, e podem ser respostas inconscientes que servem como mecanismos de proteção e sobrevivência (Nicholls et al., 1992; Swedlow e Mark, 1998). A avaliação dos reflexos motores durante as primeiras semanas de vida é importante para verificar alterações induzidas por agentes farmacológicos no desenvolvimento de roedores (Ten et al., 2003).

Para a avaliação de desenvolvimento do reflexo, é mais importante saber se os animais completaram o teste dentro da faixa de idade previamente estipulada. De acordo com Allam e Abo-Eleneen (2012), os ratos albinos completam o teste entre 6-8 dias de idade. Dessa maneira, o tratamento com cafeína não alterou o desenvolvimento reflexo em todos os testes analisados. Em um outro tratamento com diferentes doses de cafeína na água de beber (0,2; 0,4 e 0,8 g/L) durante o período gestacional e lactacional em ratos, os efeitos no desenvolvimento do reflexo foram dependentes da dose, com a máxima produzindo o efeito máximo, e a atividade locomotora avaliada na arena de campo aberto foi reduzida no desmame (Peruzzi et al., 1985). No entanto, o

desenvolvimento motor e do reflexo não foi afetado pela exposição perinatal de cafeína (0,3 g/L na água de beber) quando avaliado pelos testes de reflexo da virada e aversão à queda (Silva et al., 2013). Diferentes vias de administração, tempo e dose de tratamento de cafeína e diferentes linhagens de animais podem ser fatores que explicam a diferença entre os resultados. Além disso, nosso protocolo de administração de cafeína somente no ciclo escuro, a fim de mimetizar o consumo humano, pode ter atenuado o efeito da cafeína no desenvolvimento do reflexo observado em outros estudos.

O consumo materno de cafeína durante o desenvolvimento do encéfalo aumenta a atividade locomotora em ratos jovens (Holloway e Thor, 1983; Pan e Chen, 2007; Peruzzi et al, 1985; Tchekalarova a el, 2005). As doses moderadas de cafeína (0,3 g/L) administradas na água de beber em ratas durante a gestação e lactação levaram a um aumento na atividade locomotora dos filhotes na idade adulta, e esses animais também apresentaram uma maior resposta após ação estimulante da cocaína (Björklund et al., 2008). Por outro lado, a atividade locomotora de ratos adultos (1 a 6 meses de idade) expostos à cafeína durante o período embrionário e/ou durante a lactação (0,2-0,4 g/L na água de beber) foi reduzida na exposição a arena de campo aberto somente nos filhotes machos aos 25 dias de idade (Hughes e Beveridge, 1991). De acordo com Tchekalarova e colaboradores (2005), os efeitos na atividade locomotora da cafeína são dependentes do estágio de desenvolvimento encefálico. Entre os DPN 7 e 11 o tratamento agudo com cafeína (20 mg/kg i.p.) aumentou a locomoção dos animais com 25 dias, enquanto que houve uma hipolocomoção nessa mesma idade após tratamento com cafeína entre os DPN 13 e 17. No nosso trabalho, não foi encontrada nenhuma alteração na atividade locomotora nos ratos com 6

a 10 dias de vida, submetidos ao tratamento com cafeína desde o período embrionário. Essa ausência de efeito sobre a atividade locomotora por quaisquer doses de cafeína utilizadas nesse trabalho podem ser explicada pelo diferencial do nosso protocolo de administração, no qual as ratas foram submetidas à cafeína somente no ciclo escuro, a fim de mimetizar o consumo humano.

No que diz respeito ao metabolismo da cafeína durante a lactação sabe-se que na primeira semana pós-natal o complexo CYP450 não está completamente maduro, o que leva ao acúmulo de cafeína e seus metabólitos no plasma de neonatos (Bienvenu et al., 1993). Em consonância com a literatura, os níveis plasmáticos de cafeína e seus metabólitos estavam elevados encontrados nos filhotes de 7 dias de idade. Os níveis mais baixos dos metabólitos da cafeína foram encontrados no dia pós-natal (DPN) 14, quando a atividade e expressão das enzimas hepáticas responsáveis pelo metabolismo da cafeína estão aumentadas. Os níveis elevados dos metabólitos de cafeína encontrados no DPN 21 podem ser explicados pelo início da alimentação mista, pois nessa idade os filhotes são capazes de alcançar a garrafa de água, e dessa forma há duas fontes de cafeína: da água de beber e do leite materno. O tratamento de ratos adultos Sprague-Dawley com cafeína de 0,3 g/L na água de beber durante 14 dias, e após uma dose de ataque 7,5 mg/kg ip, apresentaram menores níveis de cafeína no plasma em comparação com os ratos que receberam uma única dose de cafeína (Svenningsson et al., 1999). A quantidade dos outros metabólitos da cafeína (teobromina, teofilina e paraxantina) foram similares aos animais que beberam apenas água tratada com cafeína. A explicação sugerida é que a cafeína é metabolizada mais rapidamente em ratos

tolerantes, ou seja, que já estão recebendo o tratamento crônico e que essa substância atravessa mais facilmente a barreira hemato-encefálica do que os seus metabólitos (Svenningsson et al., 1999).

Dando segmento aos parâmetros do desenvolvimento do encéfalo, decidiu-se investigar o impacto do consumo de cafeína sobre o sistema glutamatérgico, visto que o glutamato é um aminoácido que é o principal neurotransmissor excitatório no SNC de mamíferos.

Dentre as alterações que ocorrem como um processo normal do desenvolvimento encefálico, sabe-se que durante a primeira semana pós-natal de roedores o GABA age como um neurotransmissor excitatório (Ben-Ari et al., 2007), levando à despolarização da membrana e à remoção do íon magnésio (Mg^{2+}) do receptor NMDA, o que possibilita a sua ativação (Leinekegel et al., 1997). Em nosso trabalho, o consumo de cafeína afetou diferentemente os receptores de glutamato e o transportador GLT-1 no córtex e no hipocampo dos ratos de 7 dias de idade. No córtex, houve aumento tanto no imunoconteúdo da forma fosforilada da subunidade GluN1 do receptor NMDA como no transportador de glutamato GLT-1 nos filhotes tratados com cafeína na água de beber na dose de 0,3 g/L. Também foi encontrado um aumento na fosforilação da subunidade GluN1 do receptor NMDA no hipocampo, mas o GLT-1 foi diminuído pela dose mais alta, e a subunidade GluA1 do receptor AMPA foi aumentada em todas as doses estudadas.

É possível que o aumento no imunoconteúdo das subunidades dos receptores NMDA e AMPA no DPN 7 causado pela cafeína pode ter sido um mecanismo compensatório por causa da redução na sinalização GABAérgica observada em um estudo recente com tratamento com cafeína no período

gestacional e lactacional (Silva et al., 2013). Nesse estudo, foi observada um retardo na migração de neurônios GABAérgicos hipocampais em camundongos de 6 dias de idade submetidos à cafeína desde o período (Silva et al., 2013).

A subunidade GluN1 do receptor NMDA no hipocampo aumenta consideravelmente na primeira semana pós-natal, atingindo níveis semelhantes ao animal adulto por volta de 20 dias de idade (Ibaraki et al., 1999). Dessa maneira, observamos alterações nessa subunidade na fase em que ela está mais aumentada no desenvolvimento, retornando aos níveis semelhantes ao controle nas idades subsequentes. O impacto dessas alterações na vida adulta dos animais ainda precisa ser mais estudado.

Uma característica das sinapses glutamatérgicas durante a primeira semana pós-natal é que os receptores de glutamato AMPA estão “silenciados” (Ben-Ari et al., 1997). Uma vez que o aumento da subunidade GluA1 do receptor AMPA no hipocampo de ratos ocorre entre os dias pós-natal 7 e 20 (Ibaraki et al., 1999), nossos resultados mostraram que os filhotes de 7 dias de idade submetidos às três doses de cafeína apresentaram um aumento nos níveis da subunidade GluA1 do receptor AMPA no hipocampo comparado aos filhotes de mesma idade que receberam apenas água. A superexpressão da subunidade GluA1 do receptor AMPA em cultura de neurônios do neocortex de embriões de ratos de 17 dias aumentou o número de segmentos dendríticos e o número de proteínas na densidade pós-sináptica das sinapses glutamatérgicas, sugerindo que esse receptor glutamatérgico pode facilitar a sinaptogênese (Chen et al., 2009).

Considerando o transportador de glutamato GLT-1, observamos alterações somente no DPN 7. No córtex, a dose de 0,3 g/L induziu um aumento

de GLT-1, enquanto que a dose mais alta reduziu o GLT-1 no hipocampo. Visto que o encéfalo é mais sensível à excitabilidade durante a primeira semana pós-natal, essas alterações específicas de cada estrutura pode ter um importante papel na susceptibilidade a episódios convulsivos. De fato, o tratamento agudo com cafeína entre os DPN 2-6 levou a uma menor susceptibilidade a convulsões dependendo do agente convulsivante utilizado em ratos jovens e adultos (Guillet, 1995). Em outro estudo, foi observado um aumento da susceptibilidade a convulsões em camundongos de 6 dias que receberam cafeína no período gestacional e lactacional (Silva et al., 2013). Entretanto, quando o tratamento com cafeína ocorreu entre os DPN 7-11, foi encontrado efeito pró- ou anti-convulsivante da cafeína dependendo do período pós-natal em que houve a indução da convulsão (Tchekalarova et al., 2006; 2007; 2010; 2011). Além disso, a expressão de GLT-1 é reduzida quando há remoção de aferentes glutamatérgicos, levando à perda da conectividade sináptica (Ginsberg et al., 1996; Yang et al., 2009); e o aumento da atividade sináptica aumenta a expressão de GLT-1 (Genoud et al., 2006). Esses resultados sugerem que a expressão de GLT-1 é regulada pelos neurônios para garantir a capacidade de captação de glutamato para evitar a hiperexcitabilidade.

No que diz respeito aos receptores metabotrópicos $mGlu_5$ as alterações que ocorreram foram observadas somente após a segunda semana pós-natal, período em que a plasticidade sináptica é máxima, pois há o aumento do número de espinhas dendríticas e da expressão de proteínas que participam do mecanismo do potencial de longa duração, como as cinases PKA e PKC (Harris e Teyler, 1984; Yasuda et al., 2003). Em nosso trabalho, o tratamento com a dose moderada de cafeína (0,3 g/L) aumentou o imunoconteúdo de $mGlu_5$ no

córtex e hipocampo dos filhotes de 14 dias. A ativação excessiva de receptores metabotrópicos de glutamato pode resultar em perda da resposta, conhecida como dessensibilização (De Blasi et al., 2001). O tratamento crônico de cafeína na água de beber (1,0 g/L) reduziu o imunoconteúdo de mGlu1a no encéfalo fetal, sem alterações na sua expressão. Além disso, houve uma redução na sinalização mediada por esse receptor. No entanto, foi observado um aumento compensatório na afinidade do receptor (León et al., 2005). Em nosso trabalho, $mGlu_5$, que também é um receptor metabotrópico de glutamato da classe I, foi alterado diferentemente pela dose moderada e alta de cafeína de acordo com a idade pós-natal e com a estrutura encefálica em estudo. Considerando que a cafeína é um antagonista não-seletivo dos receptores A_1 e A_{2A} de adenosina e que esses receptores estão acoplados à proteína G após a segunda semana pós-natal (Adén et al., 2001), encontramos alterações no receptor metabotrópico de glutamato $mGlu_5$ somente após esse período. Além disso, a formação de complexos heteroméricos entre o receptor A_{2A} e $mGlu_5$ encontradas no estriado (Ferré et al., 2002) pode sugerir uma colaboração desses neurotransmissores na sinalização glutamatérgica, uma vez que a ativação de $mGlu_5$ potencia a sinalização do receptor A_{2A} dependente de MAPK (Nishi et al., 2003).

No período de desmame, aos 21 dias de idade, observamos que a dose moderada de cafeína causou uma redução no imunoconteúdo da subunidade GluA1 do receptora AMPA e do receptor $mGlu_5$ no córtex e no hipocampo, e a dose elevada (1,0 g/L) também aumentou o $mGlu_5$ no córtex. Conforme mencionado anteriormente, nesse período os animais recebem duas fontes de cafeína: o leite materno e a água de beber, aumentando os níveis de cafeína e seus metabólitos no plasma, o que pode levar a um mecanismo compensatório

de reduzir o imunoconteúdo desses receptores para evitar a hiperexcitabilidade glutamatérgica. Além disso, houve um aumento na subunidade GluN2A do receptor NMDA no hipocampo de filhotes que receberam a dose moderada de cafeína (0,3 g/L). De acordo com Fan e colaboradores (2012), a subunidade GluN2A atinge o pico de expressão em zonas neurogênicas no DPN 14, que está relacionado à formação dos neurônios maduros. O impacto desse aumento na vida adulta deve ser investigado, uma vez que o tratamento crônico com cafeína na água de beber durante gestação e lactação levou a uma diminuição da hiperlocomoção induzida por um antagonista do receptor NMDA nos filhotes adultos (da Silva et al., 2005).

A ligação específica ao glutamato foi avaliada pelo método de *binding* em preparações de membranas de córtex e hipocampo dos filhotes de 7, 14 e 21 dias. A única alteração observada foi um aumento transitório da ligação do glutamato no córtex dos filhotes de 14 dias que receberam a menor dose de cafeína (0,1 g/L). Visto que nessa estrutura e nessa idade não houve nenhuma alteração nas subunidades dos receptores e transportador de glutamato estudados, outras subunidades podem estar envolvidas, bem como o receptor de glutamato do tipo cainato, o que deve ser melhor investigado.

5. CONCLUSÃO

Considerando que a cafeína é um psicoestimulante presente na dieta de praticamente todas as populações do mundo, é importante investigar os efeitos de seu consumo durante o desenvolvimento encefálico. Em estudos epidemiológicos existe a dificuldade em controlar adequadamente a quantidade de cafeína consumida durante todo o período de desenvolvimento encefálico, pois a coleta de dados para a pesquisa. Nesse ponto, os estudos experimentais são de extrema importância para estabelecer alguns critérios necessários antes de serem aplicados em ensaios clinicos. Além disso, é possível ter um maior controle das variáveis presentes nesses estudos epidemiológicos. Portanto, o conjunto dos nossos resultados contribuem para o melhor conhecimento dos efeitos da cafeína no período de desenvolvimento do encéfalo com ênfase em proteínas cruciais para o desenvolvimento e maturação de sinapses em córtex e hipocampo. Nossos resultados demonstraram que a exposição materna de diferentes doses de cafeína durante o período embrionário leva a alterações mesmo que transitórias em proteínas que participam do desenvolvimento e maturação das sinapses. Os efeitos do consumo perigestacional, ou seja, durante a gestação e lactação, no desenvolvimento do reflexo e na sinalização glutamatérgica em córtex e hipocampo foram dependentes da idade em que os animais foram avaliados. Também foi observado que o metabolismo da cafeína na primeira semana de vida é incompleto levando ao acúmulo de seus metabólitos e estes também podem ser responsáveis pelos efeitos observados. Com este trabalho enfatizamos a importância da haver um controle do consumo de cafeína durante um período crítico para o desenvolvimento e maturação

encefálica, no entanto mais estudos são necessários para investigar o impacto dessas alterações a longo prazo.

6. PERSPECTIVAS

- Analisar os níveis de cafeína e metabólitos no encéfalo de embriões e na placenta, nos dias embrionários 18 e 20;

- Investigar a ontogenia dos receptores GABAérgicos, pois é o principal neurotransmissor inibitório, nas mesmas idades pós-natal estudadas para os receptores de glutamato (principal neurotransmissor excitatório) no córtex e no hipocampo;

- Comparar por meio de tarefas comportamentais o desempenho cognitivo dos animais em diferentes idades (DPN 21, DPN 30 e DPN 60), que receberam cafeína desde o período embrionário até o desmame (DPN 21).

Referências

Aamodt, SM, Shi J, Colonnese MT, Veras W, Constantine-Paton M. Chronic NMDA exposure accelerates development of GABAergic inhibition in the superior colliculus. J. Neurophysiol. 83: 1580–1591, 2000.

Abdi F, Pollard I, Wilkinson J. Placental transfer and foetal disposition of caffeine and its immediate metabolites in the 20-day pregnant rat: function of dose. Xenobiotica 23: 449–456, 1993.

Acheson KJ, Gremaud G, Meirim I, Montigon F, Krebs Y, Fay LB, Gay LJ, Schneiter P, Schindler C, Tappy L. Metabolic effects of caffeine in humans: lipid oxidation or futile cycling? Am J Clin Nutr 79: 40–46, 2004.

Adén U, Herlenius E, Tang LQ, Fredholm BB.Maternal caffeine intake has minor effects on adenosine receptor ontogeny in the rat brain. Pediatr. Res. 48: 177–183, 2000.

Adén U, Leverin AL, Hagberg H, Fredholm BB. Adenosine A_1 receptor agonism in the immature rat brain and heart. Eur J Pharmacol 426:185-192, 2001.

Afadlal Z, Polaboon N, Surakul P, Govitrapong P, Jutapakdeegul N. Prenatal stress alters presynaptic marker proteins in the hippocampus of rat pups. Neuroscience Letters 470: 24-27, 2010.

Agarwala S, Sanders TA, Ragsdale CW. Sonic hedgehog control of size and shape in midbrain pattern formation. Science 291: 2147-2150, 2001.

Aldridge A, Aranda JV, Neims AH. Caffeine metabolism in the newborn. Clin Pharmacol Ther. 25: 447-453, 1979.

Aldridge A, Bailey J, Neims AH. The disposition of caffeine during and after pregnancy. Semin Perinatol. 5: 310-314, 1981.

Allam AA, Abo-Eleneen RE. The development of sensorimotor reflexes in albino mice; albino rats and black-hooded rats. Int J Dev Neurosci 30 (7): 545-553, 2012.

Altimari LR, Cyrino ES, Zucas SM, Okano AH, Burini RC. Cafeína: ergogênico nutricional no esporte. Revista Brasileira Ciência e Movimento 9 (3): 57-64, 2001.

Aranda JV, Collinge JM, Zinman R, Watters G. Maturation of caffeine elimination in infancy. Arch. Dis. Child. 54: 946–949, 1979.

Arnaud MJ, Bracco I, Sauvageat JL, Clerc MF. Placental transfer of the major caffeine metabolite in the rat using 6-amino-5[N-formylmethylamino]1,3[Me-14C]-dimethyluracil administered orally or intravenously to the pregnant rat. Toxicol. Lett. 16: 271–279, 1983.

Arnaud MJ. Comparative metabolic disposition of [1-Me14C]caffeine in rats, mice, and Chinese hamsters. Drug Metab. Dispos. 13: 471–478, 1985.

Arnaud MJ. The pharmacology of caffeine. Prog Drug Res 31: 273-313, 1987.

Arnaud MJ, Bracco I, Getaz F. Synthesis of ring labelled caffeine for the study of metabolic and pharmacokinetics mouse interstrain differences in relation to pharmacologic and toxic effects. In: Baillie, T.A., Jones, J.R. (Eds.), Synthesis and Applications of Isotopically Labeled Compounds. Elsevier, Amsterdam, pp. 645–648, 1989.

Arriza JL, Eliasof S, Kavanaugh MP, Amara SG. Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. Proc. Natl. Acad. Sci. USA 94: 4155–4160, 1997.

Ashihara H, Crozier A. Biosynthesis and metabolism of caffeine and related purine alkaloids in plants. Adv Bot Res 30: 117-205, 1999.

Barker PA, Shooter EM. Disruption of NGF binding to the low affinity neurotrophin receptor p75LNTR reduces NGF binding to TrkA on PC12 cells. Neuron 13: 203–215, 1994.

Barone JJ, Roberts HR. Caffeine consumption. Food Chem Toxicol 34: 119-129, 1996.

Bartkowska K, Turlejski K, Djavadian RL. Neurotrophins and their receptors in early development of the mammalian nervous system. Acta Neurobiol Exp (Wars) 70 (4): 454-467, 2010.

Bear MF, Connors BW, Paradiso MA. Introdução às neurociências. In: Neurociências: desvendando o sistema nervoso. 2. Ed. Porto Alegre: Artmed, cap. 1: 3-22, 2002.

Behar TN, Scott CA, Greene CL, Wen X, Smith SV, Maric D, Liu QY, Colton CA, Barker JL. Glutamate acting at NMDA receptors stimulates embryonic cortical neuronal migration. J Neurosci 19: 4449–4461, 1999.

Ben-Ari Y, Khazipov R, Leinekugel X, Caillard O, Gaiarsa JL. $GABA_A$, NMDA and AMPA receptors: a developmentally regulated 'ménage à trois'. Trends Neurosci. 20: 523–529, 1997.

Benowitz LI, Routtenberg A. GAP-43: An intrinsic determinant of neuronal development and plasticity. Trends Neurosci. 20: 84–91, 1997.

Bernard C, Wheal HV. Plasticity of AMPA and NMDA receptor-mediated epileptiform activity in a chronic model of temporal lobe epilepsy. Epilepsy Res. 21 (2): 95-107, 1995.

Bienvenu T, Pons G, Rey E, Thiroux G, Olive G. Caffeine metabolism in liver slices during postnatal development in rats. Drug Metab Dispos. 21 (1): 178-180, 1993.

Blake MJ, Castro L, Leeder JS, Kearns GL. Ontogeny of drug metabolizing enzymes in the neonate. Semin. Fetal Neonatal Med. 10, 123–138, 2005.

Blanchard J, Sawers SJA. The absolute bioavailability of caffeine in man. Eur J Clin Pharmacol 24 (1): 93-98, 1983.

Bonati M, Latini R, Tognoni G, Young JF, Garattini S. Interspecies comparison of in vivo caffeine pharmacokinetics in man, monkey, rabbit, rat, and mouse. Drug Metab. Rev. 15: 1355–1383, 1984.

Bortolotti A, Jiritano L, Bonati M. Pharmacokinetics of paraxanthine, one of the primary metabolites of caffeine, in the rat. Drug Metab. Dispos. 13: 227–231, 1985.

Boylan SM, Cade JE, Kirk SF, Greenwood DC, White KL, Shires S, Simpson NA, Wild CP, Hay AW. Assessing caffeine exposure in pregnant women. Br J Nutr. 11: 1-8, 2008.

Bracken MB, Triche EW, Belanger K, Hellenbrand K, Leaderer BP. Association of maternal caffeine consumption with decrements in fetal growth. Am J Epidemiol 157: 456–466, 2003.

Brent RL, Christian MS, Diener RM. Evaluation of the reproductive and developmental risks of caffeine. Birth Defects Res B Dev Reprod Toxicol. 92 (2): 152-187, 2011.

Caldeira MV, Melo CV, Pereira DB, Carvalho RF, Carvalho AL, Duarte CB. BDNF regulates the expression and traffic of NMDA receptors in cultured hippocampal neurons. Mol Cell Neurosci 35:208-219, 2007.

Carvalho A, Fazuoli LC. Café. In: Furlani AMC, Viégas GP (Eds). O melhoramento de plantas no Instituto Agronômico. Campinas: Instituto Agronômico. v. 1, p. 29-76, 1993.

Carvalho AL, Caldeira MV, Santos SD, Duarte CB. Role of the brain-derived neurotrophic factor at glutamatergic synapses. Br J Pharmacol. 153 (S1): S310-324, 2008.

CARE Study Group. Maternal caffeine intake during pregnancy and risk of fetal growth restriction: a large prospective observational study. BMJ 337:a2332, 2008.

Chang GQ, Karatayev O, Liang SC, Barson JR, Leibowitz SF. Prenatal ethanol exposure stimulates neurogenesis in hypothalamic and limbic peptide systems: possible mechanism for offspring ethanol overconsumption. Neuroscience 222: 417-428, 2012.

Chang GQ, Karatayev O, Leibowitz SF. Prenatal exposure o nicotine stimulates neurogenesis of orexigenic peptide-expressing neurons in hypothalamus and amygdala. J Neurosci 33 (34): 13600-13611, 2013.

Chen W, Prithviraj R, Mahnke AH, McGloin KE, Tan JW, Gooch AK, Inglis FM. AMPA glutamate receptor subunits 1 and 2 regulate dendrite complexity and spine motility in neurons of the developing neocortex. Neuroscience 159 (1): 172-182, 2009.

Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, Beachy PA. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature 383: 407–413, 1996.

Choi DW, Rothman SM. The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. Ann. Rev. Neurosci. 13: 171-182, 1990.

Christensen H, de Fonnum F. The ontogeny of the uptake systems for glutamate, GABA, and glycine in synaptic vesicles isolated from rat brain. Neurochem. Res. 17, 457–462, 1992.

Clancy B, Darlington RB, Finlay BL. Translating developmental time across mammalian species. Neuroscience 105: 7-17, 2001.

Clausson B, Granath F, Ekbom A, Lundgren S, Nordmark A, Signorello LB, Cnattingius S. Effect of caffeine exposure during pregnancy on birth weight and gestational age. Am J Epidemiol 155: 429–436, 2002.

Cnattingius S, Signorello LB, Annerén G, Clausson B, Ekbom A, Ljunger E, Blot WJ, McLaughlin JK, Petersson G, Rane A, Granath F. Caffeine intake and the risk of first-trimester spontaneous abortion. N Engl J Med. 343: 1839-1845, 2000.

Cohen S, Levimontalcini R, Hamburger VA. Nerve growth stimulating factor isolated from sarcomas 37 and 180. Proc. Natl Acad. Sci. USA 40: 1014–1018, 1954.

Cohen-Cory S, Kidane AH, Shirkey NJ, Marshak S. Brain-derived neurotrophic factor and the development of structural neuronal connectivity. Dev Neurobiol. 70 (5): 271-288, 2010.

Collingridge GL, Olsen RW, Peters J, Spedding M. A nomenclature for ligand-gated ion channels. Neuropharmacology 56: 2-5, 2009.

Craig AM, Blackstone CD, Huganir RL, Banker G. The distribution of glutamate receptors in cultured rat hippocampal neurons: postsynaptic clustering of AMPA-selective subunits. Neuron 10: 1055-1068, 1993.

Cummings JA, Mulkey RM, Nicoll RA. Malenka RC. Ca^{2+} signaling requirements for long-term depression in the hippocampus. Neuron 16: 825–833, 1996.

Cunha RA. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. Neurochem Int. 38: 107-125, 2001.

Dahmane N, Sanchez P, Gitton Y, Palma V, Sun T, Beyna M, Weiner H, Ruiz i Altaba A. The Sonic Hedgehog-Gli pathway regulates dorsal brain growth and tumorigenesis. Development 128: 5201-5212, 2001.

Danbolt NC. The high affinity uptake system for excitatory amino acids in the brain. Prog. Neurobiol. 44: 377–396, 1994.

Danbolt NC. Glutamate uptake. Prog Neurobiol. 65 (1): 1-105, 2001.

Danbolt NC, Storm-Mathisen J, Kanner BI. An [Na+-K+]coupled L-glutamate transporter purified from rat brain is located in glial cell processes. Neuroscience 51: 295–310, 1992.

Dani JW, Armstrong DM, Benowitz LI. Mapping the development of the rat brain by GAP-43 imunocytochemistry. Neuroscience 40: 277-287, 1991.

De Blasi A, Conn PJ, Pin J, Nicoletti F. Molecular determinants of metabotropic glutamate receptor signaling. Trends Pharmacol Sci. 22 (3): 114-20, 2001.

Debry G. In *Coffee and Health* (Debry G ed) pp 1–538, John Libbey, Paris, 1994.

Demarque M, Represa A, Becq H, Khalilov I, Ben-Ari Y, Aniksztejn L. Paracrine intercellular communication by a Ca^{2+} and SNARE independente release of GABA and glutamate prior to synapse formation. Neuron 36: 1051–1061, 2002.

Desfrere L, Olivier P, Schwendimann L, Verney C, Gressens P. Transient inhibition of astrocytogenesis in developing mouse brain following postnatal caffeine exposure. Pediatr Res 62 (5): 604-649, 2007.

Di Giorgi-Gerevini VD, Caruso A, Cappuccio I, Ricci-Vitiani L, Romeo S, Della Rocca C, Gradini R, Melchiorri D, Nicoletti F. The mGlu5 metabotropic

glutamate receptor is expressed in zones of active neurogenesis of the embryonic and postnatal brain. Brain Res Dev Brain Res 150: 17–22, 2004.

Di Giorgi-Gerevini V, Melchiorri D, Battaglia G, Ricci-Vitiani L, Ciceroni C, Busceti CL, Biagioni F, Iacovelli L, Canudas AM, Parati E, De Maria R, Nicoletti F. Endogenous activation of metabotropic glutamate receptors supports the proliferation and survival of neural progenitor cells. Cell Death Differ 12: 1124–1133, 2005.

Dingledine R, Boland LM, Chamberlin NL, Kawasaki K, Kleckner NW, Traynelis SF, Verdoorn TA. Amino acid receptors and uptake systems in the mammalian central nervous system. CRC Crit. Rev. Neurobiol. 4: 1-96, 1988.

Dlugosz L, Bracken MB. Reproductive effects of caffeine: a review and theoretical analysis. Epidemiol Rev 14: 83-100, 1992.

Dulubova I, Khvotchev M, Liu S, Huryeva I, Südhof TC, Rizo J. Munc18-1 binds directly to the neuronal SNARE complex. PNAS 104 (8): 2697-2702, 2007.

Durand GM, Zukin RS. Developmental regulation of mRNAs encoding rat brain kainate/AMPA receptors: a northern analysis study. J Neurochem. 61 (6): 2239-2246, 1993.

Echelard Y, Epstein DJ, St-Jacques B, Shen L, Mohler J, McMahon JA, McMahon AP. Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. Cell 75: 1417-1430, 1993.

Ericson J, Muhr J, Placzek M, Lints T, Jessell TM, Edlund T. Sonic hedgehog induces the differentiation of ventral forebrain neurons: a common signal for ventral patterning within the neural tube. Cell 81: 747-756, 1995.

Fairman WA, Vandenberg RJ, Arriza JL, Kavanaugh MP, Amara SG. An excitatory amino-acid transporter with properties of a ligand-gated chloride channel. Nature 375: 599–603, 1995.

Fan H, Gao J, Wang W, Li X, Xu T, Yin X. Expression of NMDA receptor and its effect on cell proliferation in the subventricular zone of neonatal rat brain. Cell Biochem Biophys. 62 (2): 305-316, 2012.

Fenster L, Eskenazi B, Windham GC, Swan SH. Caffeine consumption during pregnancy and fetal growth. Am J Public Health. 81: 458-461, 1991.

Ferré S, Karcz-Kubicha M, Hope BT, Popoli P, Burgueño J, Gutiérrez MA, Casadó V, Fuxe K, Goldberg SR, Lluis C, Franco R, Ciruela F. Synergistic interaction between adenosine A_{2A} and glutamate mGlu5 receptors: implications for striatal neuronal function. Proc Natl Acad Sci U S A. 99 (18): 11940-11945, 2002.

Fox K, Schlaggar BL, Glazewski S, O'Leary DDM. Glutamate receptor blockade at cortical synapses disrupts development of thalamocortical and columnar organization in somatosensory cortex. Proc. Natl. Acad. Sci. USA 93: 5584–5589, 1996.

Fredholm BB, Battig K, Holmen J, Nehlig A, Zvartau EE. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol Rev. 51: 83-133, 1999.

Fredholm BB, Chen JF, Cunha RA, Svenningsson P, Vaugeois JM. Adenosine and brain function. Int Rev Neurobiol. 63: 191-270, 2005.

Fryer RH, Kaplan DR, Feinstein SC, Radeke MJ, Grayson DR, Kromer LF. Developmental and mature expression of full-length and truncated TrkB receptors in the rat forebrain. J Comp Neurol 374: 21–40, 1996.

Fukumitsu H, Furukawa Y, Tsusaka M, Kinukawa H, Nitta A, Nomoto H, Mima T, Furukawa S. Simultaneous expression of brain-derived neurotrophic factor and neurotrophin-3 in Cajal-Retzius, subplate and ventricular progenitor cells during early development stages of the rat cerebral cortex. Neuroscience 84 :115–127, 1998.

Furukawa H, Singh SK, Mancusso R, Gouaux E. Subunit arrangement and function in NMDA receptors. *Nature*, 438 (7065): 185-92, 2005.

Furuta A, Rothstein JD, Martin LJ. Glutamate transporter protein subtypes are expressed differentially during rat CNS development. J Neurosci 17: 8363–8375, 1997.

Gaytan SP, Pasaro R. Neonatal caffeine treatment up-regulates adenosine receptors in brainstem and hypothalamic cardio-respiratory related nuclei of rat pups. Exp. Neurol. 237: 247–259, 2012.

Gaytan SP, Saadani-Makki F, Bodineau L, Frugière A, Larnicol N, Pásaro R. Effect of postnatal exposure to caffeine on the pattern of adenosine A_1 receptor distribution in respiration-related nuclei of the rat brainstem. Auton. Neurosci. 126–127: 339–346, 2006.

Genoud C, Quairiaux C, Steiner P, Hirling H, Welker E, Knott GW. Plasticity of astrocytic coverage and glutamate transporter expression in adult mouse cortex. PLoS Biol. 4 (11): e343, 2006.

George L, Granath F, Johansson ALV, Olander B, Cnattingius S. Risks of repeated miscarriage. Paediat Perinat Epidemiol 20: 119–126, 2006.

Georgieff MK. Nutrition and the developing brain: nutrient priorities and measurement. Neonatology 92 (2): 73-82, 2007.

Giannelli M, Doyle P, Roman E, Pelerin M, Hermon C. The effect of caffeine consumption and nausea on the risk of miscarriage. Paediatr Perinat Epidemiol. 17 (4): 316-323, 2003.

Ginsberg SD, Rothstein JD, Price DL, Martin LJ. Fimbria-fornix transections selectively down-regulate subtypes of glutamate transporter and glutamate receptor proteins in septum and hippocampus. J Neurochem. 67 (3): 1208–1216, 1996.

Gordon–Weeks PR, Mansfield SG. Assembly of microtubules in growth cones: the role of microtubule-associated proteins. In Macagno ER, ed. The Nerve Growth Cone. New York, Raven Press, 55–64, 1992.

Goyan JE. Food and Drug Administration News release number P80-36. Washington DC: Food and Drug Administration; 1980.

Greene LA, Kaplan DR. Early events in neurotrophin signaling via Trk and p75 receptors. Curr. Opin. Neurobiol. 5: 579–587, 1995.

Grosso LM, Rosenberg KD, Belanger K, Saftlas AF, Leaderer B, Bracken MB. Maternal caffeine intake and intrauterine growth retardation. Epidemiology 12: 447–455, 2001.

Guillet R. Neonatal caffeine exposure alters seizure susceptibility in rats in an age-related manner. Brain Res Dev Brain Res. 89 (1): 124-128, 1995.

Hamberger A, Berthold CH, Karlsson B, Lehmann A, Nystrom B. Extracellular GABA, glutamate and glutamine in vivo - perfusion-dialysis of rabbit hippocampus. Neurol. Neurobiol. 7: 473–492, 1983.

Harris KM, Teyler TJ. Developmental onset of long-term potentiation in area CA1 of the rat hippocampus. J Physiol. 346: 27-48, 1984.

Haydar TF, Wang F, Schwartz ML, Rakic P. Differential modulation of proliferation in the neocortical ventricular and subventricular zones. J Neurosci 20: 5764–5774, 2000.

Heller J. What do we know about the risks of caffeine consumption in pregnancy? Br J Addict 82: 885-889, 1987.

Hepp R, Langley K. SNAREs during development. Cell Tissue Res 305: 247–253, 2001.

Hollmann M, Heinemann S. Cloned glutamate receptors. Annu Rev Neurosci 17: 31–108, 1994.

Holloway WR Jr, Thor DH. Caffeine sensitivity in the neonatal rat. Neurobehav Toxicol Teratol. 4 (3): 331-333, 1983.

Hoyt AT, Browne M, Richardson S, Romitti P, Druschel C; National Birth Defects Prevention Study. Maternal caffeine consumption and small for gestational age births: results from a population-based case-control study. Matern Child Health J. 18 (6): 1540-1551, 2014.

Huang ZJ, Kirkwood A, Pizzorusso T, Porciatti V, Morales B, Bear MF, Maffei L, Tonegawa S. BDNF regulates the maturation of inhibition and the critical period of plasticity in mouse visual cortex. Cell 98: 739–755, 1999.

Hughes RN, Beveridge IJ. Behavioral effects of exposure to caffeine during gestation, lactation or both. Neurotoxicol. Teratol. 13: 641-647, 1991.

Hynes M, Porter JA, Chiang C, Chang D, Tessier-Lavigne M, Beachy PA, Rosenthal A. Induction of midbrain dopaminergic neurons by Sonic hedgehog. Neuron 15: 35-44, 1995.

Ibaraki K, Otsu Y, Nawa H. A novel two-site enzyme immunoassay reveals the regional distributions of and developmental changes in GluR1 and NMDAR1 protein contents in the rat brain. J Neurochem 73 (1): 408-417, 1999.

Ikonomidou C, Mosinger JL, Salles KS, Labruyere J, Olney JW. Sensitivity of the developing rat brain to hypobaric ischemic damage parallels sensitivity to N-methyl-aspartate neurotoxicity. J. Neurosci. 9: 2809–2818, 1989.

Infante-Rivard C. Caffeine intake and small-for-gestational-age birth: modifying effects of xenobiotic-metabolising genes and smoking. Paediatr Perinat Epidemiol 21: 300–309, 2007.

Insel TR, Miller LP, Gelhard RE. The ontogeny of excitatory amino acid receptors in the rat forebrain I: *N*-methyl-D-aspartate and quisqualate receptors. Neuroscience 35: 31–43, 1990.

Isaac JT, Crair MC, Nicoll RA, Malenka RC. Silent synapses during development of thalamocortical inputs. Neuron 18: 269–280, 1997.

Jahn R, Lang T, Sudhof TC. Membrane fusion. Cell 112: 519–533, 2003.

Jahn R, Scheller RH. SNAREs-engines for membrane fusion Nat Rev Mol Cell Biol 7:631–643, 2006.

Jansson LC, Wigren HK, Nordström T, Akerman KE. Functional a-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors in differentiating embryonic neural progenitor cells. Neuroreport 22: 282–287, 2011.

Jansson LC, Louhivuori L, Wigren HK, Nordstro¨m T, Louhivuori V, Castrén ML, Akerman KE. Effect of glutamate receptor antagonists on migrating neural progenitor cells. Eur J Neurosci 37: 1369–1382, 2013.

Johansson B, Georgiev V, Lindström K, Fredholm BB. A_1 and A_{2A} adenosine receptors and A_1 mRNA in mouse brain: effect of long-term caffeine treatment. Brain Res 762: 153-164, 1997.

Johnson MH. Development of human brain functions. Biol. Psychiatry 54: 1312–1316, 2003.

Johnston GAR. Glutamate uptake and its possible role in neurotransmitter inactivation. In: Roberts, P.J., Storm-Mathisen, J., Johnston, G.A.R. (Eds.), Glutamate: Transmitter in the Central Nervous System. Wiley, Chichester, UK, pp. 77–87, 1981.

Juárez-Méndez S, Carretero R, Martínez-Tellez R, Silva-Gómez AB, Flores G. Neonatal caffeine administration causes a permanent increase in the dendritic length of prefrontal cortical neurons of rats. Synapse 60 (6): 450-455, 2006.

Juraska JM, Fifkova E. An electron microscope study of the early postnatal development of the visual cortex of the hooded rat. J Comp Neurol 183: 257–267, 1979.

Kanai Y, Hediger MA. Primary structure and functional characterization of a high-affinity glutamate transporter. Nature 360: 467–471, 1992.

Karakas E, Simorowski N, Furukawa H. Subunit arrangement and phenylethanolamine binding in GluN1/GluN2B NMDA receptors. *Nature*, 475 (7355): 249-53, 2011.

Kataoka M, Kuwahara R, Matsuo R, Sekiguchi M, Inokuchi K, Takahashi M. Development- and activity-dependent regulation of SNAP-25 phosphorylation in rat brain. Neurosci Lett. 407 (3): 258-262, 2006.

Kimmel CA, Kimmel GL, White CG, Grafton TF, Young JF, Nelson CJ. Blood flow changes and conceptal development in pregnant rats in response to caffeine. Fundam. Appl. Toxicol. 4: 240–247, 1984.

Kish PE, Kim SY, Ueda T. Ontogeny of glutamate accumulating activity in rat brain synaptic vesicles. Neurosci. Lett. 97, 185–190, 1989.

Knüsel B, Rabin SJ, Hefti F, Kaplan DR. Regulated neurotrophin receptor responsiveness during neuronal migration and early differentiation. J Neurosci 14: 1542–1554, 1994.

Koebbe MJ, Golden JA, Bennett G, Finnell RH, Mackler SA. Effects of prenatal cocaine exposure on embryonic expression of sonic hedgehog. Teratology 59 (1): 12-19, 1999.

Kohtz JD, Baker DP, Corte G., Fishell G. Regionalization within the mammalian telencephalon is mediated by changes in responsiveness to Sonic Hedgehog. Development 125: 5079–5089, 1998.

Komada M, Saitsu H, Kinboshi M, Miura T, Shiota K, Ishibashi M. Hedgehog signaling is involved in development of the neocortex. Development 135 (16): 2717-27, 2008.

Komuro H, Rakic P. Modulation of neuronal migration by NMDA receptors. Science 260: 95–97, 1993.

Kou H, Liu Y, Liang G, Huang J, Hu J, Yan YE, Li X, Yu H, He X, Zhang B, Zhang Y, Feng J, Wang H. Maternal glucocorticoid elevation and associated blood metabonome changes might be involved in metabolic programming of intrauterine growth retardation in rats exposed to caffeine prenatally. Toxicol Appl Pharmacol 275 (2): 79-87, 2014.

Kumar SS, Bacci A, Kharazia V, Huguenard JR. A developmental switch of AMPA receptor subunits in neocortical pyramidal neurons. J Neurosci. 22 (8): 3005-3015, 2002.

LaMantia AS. The usual suspects: GABA and glutamate may regulate proliferation in the neocortex. Neuron 15: 1223–1225, 1995.

Leeder JS. Pharmacogenetics and pharmacogenomics. Pediatr. Clin. North Am. 48: 765–781, 2001.

Le Guennec JC, Billon B. Delay in caffeine elimination in breast-fed infants. Pediatrics 79: 264–268, 1987.

Lehmann A, Isacsson H, Hamberger A. Effects of in vivo administration of kainic acid on the extracellular amino acid pool in the rabbit hippocampus. J. Neurochem. 40, 1314–1320, 1983.

Lehre KP, Danbolt NC. The number of glutamate transporter subtype molecules at glutamatergic synapses: chemical and stereological quantification in young adult rat brain. J. Neurosci. 18: 8751–8757, 1998.

Leinekugel X, Medina I, Khalilov I, Ben-Ari Y, Khazipov R. Ca2+ oscillations mediated by the synergistic excitatory actions of GABA(A) and NMDA receptors in the neonatal hippocampus. Neuron 18 (2): 243-255, 1997.

Lelo A, Birkett DJ, Robson RA, Miners JO. Comparative pharmacokinetics of caffeine and its primary demethylated metabolites paraxanthine, theobromine and theophylline in man. Br. J. Clin. Pharmacol. 22: 177–182, 1986.

León D, Albasanz JL, Ruíz MA, Fernández M, Martín M. Adenosine A_1 receptor down-regulation in mothers and fetal brain after caffeine and theophylline treatments to pregnant rats. J Neurochem. 82: 625-634, 2002.

León D, Albasanz JL, Ruíz MA, Iglesias I, Martín M. Effect of chronic gestational treatment with caffeine or theophylline on Group I metabotropic glutamate receptors in maternal and fetal brain. J Neurochem. 94: 440-451, 2005.

Lessmann V, Heumann R. Modulation of unitary glutamatergic synapses by neurotrophin-4/5 or brainderived neurotrophic factor in hippocampal microcultures: presynaptic enhancement depends on pre-established paired-pulse facilitation. Neuroscience 86: 399-413, 1998.

Levine ES, Crozier RA, Black IB, Plummer MR. Brain-derived neurotrophic factor modulates hippocampal synaptic transmission by increasing N-methyl-D-aspartic acid receptor activity. Proc Natl Acad Sci USA 95: 10235-10239, 1998.

Levy LM, Lehre KP, Walaas SI, Storm-Mathisen J, Danbolt NC. Down-regulation of glial glutamate transporters after glutamatergic denervation in the rat brain. Eur. J. Neurosci. 7: 2036–2041, 1995.

Li G, Fang L, Fernández G, Pleasure SJ. The ventral hippocampus is the embryonic origin for adult neural stem cells in the dentate gyrus. Neuron 78 (4): 658-672, 2013.

Lilliu V, Pernas-Alonso R, Trelles RD, di Porzio U, Zuddas A, Perrone-Capano C. Ontogeny of AMPA receptor gene expression in the developing rat midbrain and striatum. Brain Res Mol Brain Res. 96 (1-2):133-141, 2001.

Lin RC, Scheller RH. Mechanisms of synaptic vesicle exocytosis. Annu Rev Cell Dev Biol. 16: 19-49, 2000.

Lin SY, Wu K, Levine ES, Mount HT, Suen PC, Black IB. BDNF acutely increases tyrosine phosphorylation of the NMDA receptor subunit 2B in cortical and hippocampal postsynaptic densities. Brain Res Mol Brain Res 55: 20-27, 1998.

Lipsky RH, Marini AM. Brain-derived neurotrophic factor in neuronal survival and behavior-related plasticity. Ann N Y Acad Sci. 1122: 130-143, 2007.

Liu Y, Xu D, Feng J, Kou H, Liang G, Yu H, He X, Zhang B, Chen L, Magdalou J, Wang H. Fetal rat metabonome alteration by prenatal caffeine ingestion probably due to the increased circulatory glucocorticoid level and altered peripheral glucose and lipid metabolic pathways. Toxicol Appl Pharmacol 262 (2): 205-216, 2012.

Logan WJ, Snyder SH. High affinity uptake systems for glycine, glutamic and aspartic acids in synaptosomes of rat central nervous tissues. Brain Res. 42: 413–431, 1972.

Lorenzo AM, León D, Castillo CA, Ruiz MA, Albasanz JL, Martín M. Maternal caffeine intake during gestation and lactation down-regulates adenosine A_1 receptor in rat brain from mothers and neonates. J. Neurosci. Res. 88: 1252–1261, 2010.

Lu B, Pang PT, Woo NH. The yin and yang of neurotrophin action. Nature Ver Neurosci 6: 603–614, 2005.

Lu W, Shi Y, Jackson AC, Bjorgan K, During MJ, Sprengel R, Seeburg PH, Nicoll RA. Subunit composition of synaptic AMPA receptors revealed by a single-cell genetic approach. Neuron 62:254–268, 2009.

MacDermott AB, Mayer ML, Westbrook GL, Smith SJ, Barker JL. NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurones. Nature (Lond.) 321: 519-522, 1986.

Maconochie N, Doyle P, Prior S, Simmons R. Risk factors for first trimester miscarriage-results from a UK-population-based-case-control study. Br J Obstet Gynaecol 114: 170–186, 2007.

Machold R, Hayashi S, Rutlin M, Muzumdar MD, Nery S, Corbin JG, Gritli-Linde A, Dellovade T, Porter JA, Rubin LL, Dudek H, McMahon AP, Fishell G. Sonic hedgehog is required for progenitor cell maintenance in telencephalic stem cell niches. Neuron 39 (6): 937-950, 2003.

Mady R, Kohtz JD. Isolation of rat telencephalic neural explants to assay Shh GABAergic interneuron differentiation-inducing activity. Methods Mol. Biol. 397: 47-53, 2007.

Marco EM, Adriani W, Ruocco LA, Canese R, Sadile AG, Laviola G. Neurobehavioral adaptations to methylphenidate: the issue of early adolescent exposure. Neurosci Biobehav Rev. 35 (8): 1722-1739, 2011.

Marret S, Gressens P, Van-Maele-Fabry G, Picard J, Evrard P.Caffeine-induced disturbances of early neurogenesis in whole mouse embryo cultures. Brain Res. 773 : 213-216, 1997.

Martin TR, Bracken MB. The association between low birth weight and caffeine consumption during pregnancy. Am J Epidemiol 126: 813-821, 1987.

Mattson MP. Glutamate and neurotrophic factors in neuronal plasticity and disease. Ann N Y Acad Sci 1144: 97–112, 2008.

McCall AL, Millington WR, Wurtman RJ. Blood-brain barrier transport of caffeine: dose-related restriction of adenine transport. Life Sci. 31 (24): 2709-2715, 1982.

McDonald JW, Johnston MV. Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. Brain Res. Rev. 15: 41–70, 1990.

McGowan SE, McCoy DM. Platelet-derived growth factor-A and sonic hedgehog signaling direct lung fibroblast precursors during alveolar septal formation. Am J Physiol Lung Cell Mol Physiol 305 (3): L229-239, 2013.

Melchiorri D, Cappuccio I, Ciceroni C, Spinsanti P, Mosillo P, Sarichelou I, Sale P, Nicoletti P. Metabotropic glutamate receptors in stem/progenitor cells.Neuropharmacology 53: 473–480, 2007.

Meldrum B, Garthwaite J. Excitatory amino acid neurotoxicity and neurodegenerative disease. Trends Pharmacol. Sci. 11: 379-387, 1990.

Mitchell N, Petralia RS, Currier DG, Wang Y, Kim A, Mattson MP, Yao PJ. Sonic hedgehog regulates presynaptic terminal size, ultrastructure and function in hippocampal neurons. J. Cell Sci. 125: 4207–4213, 2012.

Monaghan DT, Bridges RJ, Cotman CW. The excitatory amino acid receptors: their classes, pharmacology and distinct properties in the function of the central nervous system. Ann. Rev. Pharmacol. Toxicol. 29: 365-402, 1989.

Monaghan DT, Yao D, Cotman CW. Distribution of [3H]AMPA binding sites in rat brain as determined by quantitative autoradiography. Brain Res. 324: 160-164, 1984.

Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron 12: 529–540, 1994.

Morihara T, Mizoguchi A, Takahashi M, Kozaki S, Tsujihara T, Kawano S, Shirasu M, Ohmukai T, Kitada M, Kimura K, Okajima S, Tamai K, Hirasawa Y, Ide C. Distribution of synaptosomal-associated protein 25 in nerve growth cones and reduction of neurite outgrowth by botulinum neurotoxin A without altering growth cone morphology in dorsal root ganglion neurons and PC-12 cells. Neuroscience 91 (2): 695-706, 1999.

Moser VC. Approaches for assessing the validity of a functional observational battery. Neurotoxicol Teratol 12 (5): 483-488, 1990.

Moshé SL, Albala BJ, Ackermann RF, Engel J Jr. Increased seizure susceptibility of the immature brain. Brain Res. 283 (1): 81-85, 1983.

Mowla SJ, Pareek S, Farhadi HF, Petrecca K, Fawcett JP, Seidah NG, Morris SJ, Sossin WS, Murphy RA. Differential sorting of nerve growth factor and brain-derived neurotrophic factor in hippocampal neurons. J. Neurosci. 19: 2069-2080, 1999.

Mueller BK. Growth cone guidance: first steps towards a deeper understanding. Annu Rev Neurosci 22:351–388, 1999.

Mullen RJ, Buck CR, Smith AM. NeuN, a neuronal specific nuclear protein in vertebrates. Development 116 (1): 201-211, 1992.

Nakanishi S. Molecular diversity of glutamate receptors and implications for brain function. Science 258: 597–603, 1992.

Nehlig A, Daval JL, Debry G. Caffeine and the central nervous system: mechanisms of action, biochemical, metabolic and psychostimulant effects. Brain Res. Brain Res. Rev. 17, 139-170, 1992.

Nicholls J, Martin A, Wallace B. From Neuron to Brain, 3rd ed. Sinauer Associates Inc., Massachusettes, 1992.

Nishi A, Liu F, Matsuyama S, Hamada M, Higashi H, Nairn AC, Greengard P. Metabotropic mGlu5 receptors regulate adenosine A_{2A} receptor signaling. Proc Natl Acad Sci U S A. 100 (3): 1322-1327, 2003.

Nochi R, Kato T, Kaneko J, Itou Y, Kuribayashi H, Fukuda S, Terazono Y, Matani A, Kanatani S, Nakajima K, Hisatsune T. Involvement of metabotropic glutamate receptor 5 signaling in activity-related proliferation of adult hippocampal neural stem cells. Eur J Neurosci 36: 2273–2283, 2012.

Nordmark A, Lundgren S, Cnattingius S, Rane A. Dietary caffeine as a probe agent for assessment of cytochrome P4501A2 activity in random urine samples. Br. J. Clin. Pharmacol. 47: 397–402, 1999.

Nowak L, Bregestovski P, Ascher P, Herbet A, Prochiantz A. Magnesium gates glutamate-activated channels in mouse central neurons. Nature (Lond.) 307: 462-465, 1984.

Numan S, Gall CM, Seroogy KB. Developmental expression of neurotrophins and their receptors in postnatal rat ventral midbrain. J Mol Neurosci 27: 245–260, 2005.

O'Driscoll CM, Gorman AM. Hypoxia induces neurite outgrowth in PC12 cells that is mediated through adenosine A2A receptors. Neuroscience 131 (2): 321-329, 2005.

Olsen RW, Szamraj O, Houser CR. [3H]AMPA binding to glutamate receptor subpopulations in rat brain. Brain Res. 402: 243-254, 1987.

Osen-Sand A, Catsicas M, Staple JK, Jones KA, Ayala G, Knowles J, Grenningloh G, Catsicas S. Inhibition of axonal growth by SNAP-25 antisense oligonucleotides in vitro and in vivo. Nature 364 (6436): 445-448, 1993.

Owens DF, Kriegstein AR. Maturation of channels and receptors: consequences for excitability. Int Rev Neurobiol 45:43–87, 2001.

Oyler GA, Higgins GA, Hart RA, Battenberg E, Billingsley M, Bloom FE, Wilson MC. The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. J Cell Biol. 109 (6 Pt 1): 3039-3052, 1989.

Palma V, Ruiz i Altaba A. Hedgehog-GLI signaling regulates the behavior of cells with stem cell properties in the developing neocortex. Development 131, 337-345, 2004.

Pan HZ, Chen HH. Hyperalgesia, low-anxiety, and impairment of avoidance learning in neonatal caffeine-treated rats. Psychopharmacology 191: 119–125, 2007.

Parsons WD, Neims AH. Prolonged half-life of caffeine in healthy term newborn infants. J. Pediatr. 98: 640–641, 1981.

Patapoutian A, Reichardt LF. Trk receptors: Mediators of neurotrophin action. Curr. Opin. Neurobiol. 11: 272-280, 2001.

Pearlman SA, Duran C, Wood MA, Maisels MJ, Berlin CMJ. Caffeine pharmacokinetics in preterm infants older than 2 weeks. Dev. Pharmacol. Ther. 12: 65–69, 1989.

Perrone-Bizzozero NI, Finklestein SP, Benowitz LI. Synthesis of a growth-associated protein by embryonic rat cerebrocortical neurons in vitro. J Neurosci 6: 3721–3730, 1986.

Peruzzi G, Lombardelli G, Abbracchio MP, Coen E, Cattabeni F. Perinatal caffeine treatment: behavioral and biochemical effects in rats before weaning. Neurobehav Toxicol Teratol. 7 (5): 453-460, 1985.

Petralia RS, Schwartz CM, Wang Y, Mattson MP, Yao PJ. Subcellular localization of Patched and Smoothened, the receptors for Sonic Hedgehog Signaling, in the hippocampal neuron. J. Comp. Neurol. 519: 3684–3699, 2011.

Pfenninger KH, Friedman LB. Sites of plasmalemmal expansion in growth cones. Brain Res Dev Brain Res 71: 181–192, 1993.

Picard N, Guenin S, Larnicol N, Perrin Y. Maternal caffeine ingestion during gestation and lactation influences respiratory adaptation to acute alveolar hypoxia in newborn rats and adenosine A(2A) and GABA(A) receptor mRNA transcription. Neuroscience 156: 630–639, 2008.

Pillai-Kastoori L, Wen W, Wilson SG, Strachan E, Lo-Castro A, Fichera M, Musumeci SA, Lehmann OJ, Morris AC. Sox11 is required to maintain

proper levels of Hedgehog signaling during vertebrate ocular morphogenesis. PLoS Genet 10 (7): e1004491, 2014.

Pines G, Danbolt NC, Bjøra°s M, Zhang Y, Bendahan A, Eide L, Koepsell H, Storm-Mathisen J, Seeberg E, Kanner BI.Cloning and expression of a rat brain L-glutamate transporter. Nature 360: 464–467, 1992.

Popoli M, Yan Z, McEwen BS, Sanacora G. The stressed synapse: the impacto f stress and glucocorticoids on glutamate transmission. Nat Ver Neurosci. 13 (1): 22-37, 2011.

Porciúncula LO, Sallaberry C, Mioranzza S, Botton PH, Rosemberg DB. The Janus face of caffeine. Neurochem Int. 63 (6): 594-609, 2013.

Prescott GR, Chamberlain LH. Regional and developmental brain expression. Patterns of SNAP-25 splice variants. BMC Neurosci. 12: 35, 2011.

Ramsdell JS. Neurological disease rises from ocean to bring model for human epilepsy to life. Toxins 2: 1646-1675, 2010.

Reichardt LF. Neurotrophin-regulated signalling pathways. Philos Trans R Soc Lond B Biol Sci 361: 1545–1564, 2006.

Reinchisi G, Parada M, Lois P, Oyanadel C, Shaughnessy R, Gonzalez A, Palma V. Sonic Hedgehog modulates EGFR dependent proliferation of neural stem cells during late mouse embryogenesis through EGFR transactivation. Front Cell Neurosci. 7: 166, 2013.

Renger JJ, Egles C, Liu G. A developmental switch in neurotransmitter flux enhances synaptic efficacy by affecting AMPA receptor activation. Neuron 29: 469–484, 2001.

Reynolds RM. Corticosteroid-mediated programming and the pathogenesis of obesity and diabetes. J. Steroid Biochem. Mol. Biol. 122: 3–9, 2010.

Roelink H, Augsburger A, Heemskerk J, Korzh V, Norlin S, Ruiz i Altaba A, Tanabe Y, Placzek M, Edlund T, Jessell TM, Dodd J. Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord. Cell 76: 761-775, 1994.

Rogers DC, Fisher EM, Brown SD, Peters J, Hunter AJ, Martin JE. Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment, Mamm. Genome 8: 711 –713, 1997.

Rogers PJ, Heatherley SV, Hayward RC, Seers HE, Hill J, Kane M. Effects of caffeine and caffeine withdrawal on mood and cognitive performance degraded by sleep restriction. Psychopharmacology (Berl). 179: 742-752, 2005.

Rossi DJ, Slater NT. The developmental onset of NMDA receptor-channel activity during neuronal migration. Neuropharmacology 32: 1239–1248, 1993.

Rothman SM, Olney JW. Excitotoxicity and the NMDA receptor. Trends Neurosci. 10: 299-302, 1987.

Ruiz i Altaba A. Combinatorial Gli gene function in floor plate and neuronal inductions by Sonic hedgehog. Development 125 (12): 2203-2212, 1998.

Ruiz i Altaba A. Gli proteins encode context-dependent positive and negative functions: implications for development and disease. Development 126: 3205-3216, 1999.

Ruiz i Altaba A, Jessell TM, Roelink H. Restrictions to floor plate induction by hedgehog and winged-helix genes in the neural tube of frog embryos. Mol. Cell. Neurosci. 6: 106-121, 1995.

Ruiz i Altaba A, Palma V, Dahmane N. Hedgehog-Gli signalling and the growth of the brain. Nat Rev Neurosci 3 (1): 24-33, 2002.

Sahir N, Bahi N, Evrard P, Gressens P. Caffeine induces in vivo premature appearance of telencephalic vesicles. Brain Res Dev Brain Res. 121: 213-217, 2000.

Sahir N, Evrard P, Gressens P. Caffeine induces sonic hedgehog gene expression in cultured astrocytes and neurons. J Mol Neurosci. 24: 201-205, 2004.

Sahir N, Mas C, Bourgeois F, Simonneau M, Evrard P, Gressens P. Caffeine-induced telencephalic vesicle evagination in early post-implantation mouse embryos involves cAMP-dependent protein kinase (PKA) inhibition. Cereb Cortex 11 (4): 343-349, 2001.

Sanchez RM, Jensen FE. Maturational aspects of epilepsy mechanisms and consequences for the immature brain. Epilepsia 42: 577–585, 2001.

Sanchez RM, Jensen FE. Maturational regulation of glutamate receptors and their role in neuroplasticity. In: Sheridan, P. (Ed.), Glutamate Receptors. Academic Press, New Jersey, in press, 2002.

Sartori AGO, da Silva MV. Consumo de cafeína no Brasil. Nutrire 38 (Suplemento): 71-71, 2013.

Schlett K. Glutamate as a modulator of embryonic and adult neurogenesis. Curr Top Med Chem 60:949–960, 2006.

Schoepp DD, Jane DE, Monn JA. Pharmacological agentes acting at subtypes of metabotropic glutamate receptors. Neuropharmacology 38: 1431–1476, 1999.

Schousboe A. Transport and metabolism of glutamate and GABA in neurons and glial cells. Int. Rev. Neurobiol. 22: 1–45, 1981.

Seeburg PH. The molecular biology of mammalian glutamate receptor channels. Trends Neurosci. 16: 359-365, 1993.

Seidah NG, Benjannet S, Pareek S, Savaria D, Hamelin J, Goulet B, Laliberte J, Lazure C, Chrétien M, Murphy RA. Cellular processing of the nerve growth factor precursor by the mammalian pro-protein convertases. Biochem J 314 (3): 951-960, 1996.

Sengpiel V, Elind E, Bacelis J, Nilsson S, Grove J, Myhre R, Haugen M, Meltzer HM, Alexander J, Jacobsson B, Brantsaeter AL. Maternal caffeine intake during pregnancy is associated with birth weight but not with gestational length: results from a large prospective observational cohort study. BMC Med. 11: 42, 2013.

Shibata T, Watanabe M, Tanaka K, Wada K, Inoue Y. Dynamic changes in expression of glutamate transporter mRNAs in developing brain. Neuroreport 7: 705–709, 1996.

Shikata Y, Okada T, Hashimoto M, Ellis T, Matsumaru D, Shiroishi T, Ogawa M, Wainwright B, Motoyama J. Ptch1-mediated dosage-dependent action of Shh signaling regulates neural progenitor development at late gestational stages. Dev. Biol. 349: 147-159, 2010.

da Silva RS, Hoffman A, de Souza DO, Lara DR, Bonan CD. Maternal caffeine intake impairs MK-801-induced hyperlocomotion in young rats. Eur J Pharmacol 509 (2-3): 155-159, 2005.

da Silva RS, Richetti SK, da Silveira VG, Battastini AM, Bogo MR, Lara DR, Bonan CD. Maternal caffeine intake affects acetylcholinesterase in hippocampus of neonate rats. Int J Dev Neurosci. 26 (3-4): 339-43, 2008.

Silva CG, Métin C, Fazeli W, Machado NJ, Darmopil S, Launay PS, Ghestem A, Nesa MP, Bassot E, Szabó E, Baqi Y, Müller CE, Tomé AR, Ivanov A, Isbrandt D, Zilberter Y, Cunha RA, Esclapez M, Bernard C. Adenosine receptor antagonists including caffeine alter fetal brain development in mice. Sci. Transl. Med. 5: 197ra104, 2013.

Singh B, Henneberger C, Betances D, Arevalo MA, Rodríguez-Tébar A, Meier JC, Grantyn R. Altered balance of glutamatergic/GABAergic synaptic input and associated changes in dendrite morphology after BDNF expression in BDNF-deficient hippocampal neurons. J Neurosci. 26: 7189-7200, 2006.

Söllner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE. SNAP receptors implicated in vesicle targeting and fusion. Nature 362 (6418): 318-324, 1993.

Somani SM, Gupta P. Caffeine: a new look at an age-old drug. Int. J. Clin. Pharmacol. Ther. Toxicol. 26: 521–533, 1988.

Sommer B, Köhler M, Sprengel R, Seeburg PH. RNA editing in brain controls a determinant of ion flow in glutamate-gated channels. Cell 67: 11–19, 1991.

Stocca G, Vicini S. Increased contribution of NR2A subunit to synaptic NMDA receptors in developing rat cortical neurons. J. Physiol. 507: 13–24, 1998.

Storck T, Schulte S, Hofmann K, Stoffel W. Structure, expression, and functional analysis of a Na+-dependent glutamate/aspartate transporter from rat brain. Proc. Natl. Acad. Sci. USA 89: 10955–10959, 1992.

Storm-Mathisen, J. Localization of transmitter candidates in the brain: the hippocampal formation as a model. Prog. Neurobiol. 8: 119–181, 1977.

Su SH, Shyu HW, Yeh YT, Chen KM, Yeh H, Su SJ. Caffeine inhibits adipogenic differentiation of primary adipose-derived stem cells and bone marrow stromal cells. Toxicol In Vitro. 27 (6): 1830-1837, 2013.

Sun T, Guo J, Chen H, Zhang J, Zhang X, Jiang X, Wang F, Xu Z, Huang X, Sha J, Chan HC. Maternal caffeine exposure impairs insulin secretion by pancreatic β-cells and increases the risk of type II diabetes mellitus in offspring. Cell Biol Int. 2014. doi: 10.1002/cbin.10321. [Epub ahead of print]

Sutherland ML, Delaney TA, Noebels JL. Glutamate transporter mRNA expression in proliferative zones of the developing and adult murine CNS. J. Neurosci. 16: 2191–2207, 1996.

Svenningson P, Nomikos GG, Fredholm BB. The stimulatory action and the development of tolerance to caffeine is associated with alterations in gene expression in specific brain regions. J Neurosci 19 (10): 4011-4022, 1999.

Swann JW, Pierson MG, Smith KL, Lee CL. Developmental neuroplasticity: roles in early life seizures and chronic epilepsy. Adv. Neurol. 79: 203–216, 1999.

Swerdlow N, Mark A. Using an animal model of deficient sensorimotor gating to study the pathophysiology and new treatments of schizophrenia. Schizophrenia Bulletin 24 (2): 285–301, 1998.

Takei N, Numakawa T, Kozaki S, Sakai N, Endo Y, Takahashi M, Hatanaka H. Brain-derived neurotrophic fator induces rapid and transient release of glutamate through the non-exocytotic pathway from cortical neurons. J Biol Chem 273: 27620-27624, 1998.

Tan Y, Liu J, Deng Y, Cao H, Xu D, Cu F, Lei Y, Magdalou J, Wu M, Chen L, Wang H. Caffeine-induced fetal rat over-exposure to maternal glucocorticoid and histone methylation of liver IGF-1 might cause skeletal growth retardation. Toxicol Lett 214 (3): 279-287, 2012.

Tanaka K, Watase K, Manabe T, Yamada K, Watanabe M, Takahashi K, Iwama H, Nishikawa T, Ichihara N, Hori S, Takimoto M, Wada K. Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. Science 276: 1699–1702, 1997.

Tang BL. Protein trafficking mechanisms associated with neurite outgrowth and polarized sorting in neurons. J Neurochem 79: 923–930, 2001.

Tang S, Machaalani R, Waters KA. Immunolocalization of pro- and mature-brain derived neurotrophic fator (BDNF) and receptor TrkB in the human brainstem and hippocampus. Brain Res 1354: 1–14, 2010.

Taunay AE. História do café no Brasil Imperial 1822-1972. Departamento Nacional do Café, v.3, 1939.

Tchekalarova J, Kubova H, Mares P. Postnatal caffeine exposure: effects on motor skills and locomotor activity during ontogenesis. Behav Brain Res.160 (1): 99-106, 2005.

Tchekalarova J, Kubová H, Mares P. Effects of early postnatal caffeine exposure on seizure susceptibility of rats are age- and model-dependent. Epilepsy Res. 88 (2-3): 231-238, 2010.

Ten V, Bradley-Moore M, Gingrich J, Stark R, Pinsky D. Brain injury and neurofunctional deficit in neonatal mice with hypoxic-ischemic encephalopathy. Behavioural Brain Research 145 (1–2): 209–219, 2003.

Tessier–Lavigne M, Goodman CS. The molecular biology of axon guidance. Science 274:1123–1133, 1996.

Thoenen H. The changing scene of neurotrophic factors. Trends Neurosci. 14: 165–170, 1991.

Thompson PM, Giedd JN, Woods RP, MacDonald D, Evans AC, Toga AW. Growth patterns in the developing brain detected by using continuum mechanical tensor maps. Nature 404: 190–193, 2000.

Tyler WJ, Pozzo-Miller L. Miniature synaptic transmission and BDNF modulate dendritic spine growth and form in rat CA1 neurones. J Physiol. 553: 497-509, 2004.

Ukers WH. All about coffee. The Tea and Coffee Trade Journal Company, New York, 1922.

Van den Pol AN, Gao XB, Patrylo PR, Ghosh PK. Obrietan K. Glutamate inhibits GABA excitatory activity in developing neurons. J. Neurosci. 18: 10749–10761, 1998.

Viani R. Caffeine consumption, in Proceedings of the Caffeine Workshop Thai FDA and ILSI, Bangkok, 1996.

Voglis G, Tavernarakis N. The role of synaptic ion channels in synaptic plasticity. EMBO Rep 7 (11): 1104-1110, 2006.

Vorhees CV. Comparison of the collaborative behavioral teratology study and Cincinnati behavioral teratology test batteries, Neurobehav. Toxicol. Teratol. 7: 625– 633, 1985.

Walton K, Dorne JL, Renwick AG. Uncertainty factors for chemical risk assessment: interspecies differences in the in vivo pharmacokinetics and metabolism of human CYP1A2 substrates. Food Chem. Toxicol. 39: 667–680, 2001.

Wang L, Shen L, Ping J, Zhang L, Liu Z, Wu Y, Liu Y, Huang H, Chen L, Wang H. Intrauterine metabolic programming alteration increased susceptibility to

non-alcoholic adult fatty liver disease in prenatal caffeine-exposed rat offspring. Toxicol Lett 224 (3): 311-318, 2014.

Washbourne P, Thompson PM, Carta M, Costa ET, Mathews JR, Lopez-Bendito G, Molnar Z, Becher MW, Valenzuela CF, Partridge LD, Wilson MC Genetic ablation of the t-SNARE SNAP-25 distinguishes mechanisms of neuroexocytosis. Nat Neurosci 5: 19–26, 2002.

Watanabe M, Inoue Y, Sakimura K, Mishina M. Developmental changes in distribution of NMDA receptor channel subunit mRNAs. Neuroreport 3: 1138-1140, 1992.

Weinberg BA, Bealer BK.The world of caffeine. The science and culture of the world's most popular drug. Routledge, New York, 2001.

Wen W, Shu XO, Jacobs DR Jr, Brown JE. The association of maternal caffeine consumption and nausea with spontaneous abortion. Epidemioloogy 12: 38–42, 2001.

Weng X, Odouli R, Li DK. Maternal caffeine consumption during pregnancy and the risk of miscarriage: a prospective cohort study. Am J Obstet Gynecol 198: 279–286, 2008.

Whitney NP, Peng H, Erdmann NB, Tian C, Monaghan DT, Zheng JC. Calcium-permeable AMPA receptors containing Q/Runedited GluR2 direct human neural progenitor cell differentiation to neurons. FASEB J 22: 2888–2900, 2008.

Williams GR. Neurodevelopmental and neurophysiological actions of thyroid hormone. J Neuroendocrinol. 20 (6): 784-794, 2008.

Williams K, Russell SL, Shen YM, Molinoff PB. Developmental switch in the expression of NMDA receptors occurs in vivo and in vitro. Neuron, 10: 267–278, 1993.

Wu G, Malinow R, Cline HT. Maturation of a central glutamatergic synapse. Science 274, 972–976, 1996.

Xiao XL, Ma DL, Wu J, Tang FR. Metabotropic glutamate receptor 5 (mGluR5) regulates proliferation and differentiation of neuronal progenitors in the developmental hippocampus. Brain Res 1493: 1–12, 2013.

Xu D, Zhang B, Liang G, Ping J, Kou H, Li X, Xiong J, Dongcai H, Liaobin C, Magdalou J, Wang H. Caffeine-induced activated glucocorticoid metabolism in the hippocampus causes hypothalamic-pituitary-adrenal axis inhibition in fetal rats. Plos One 7 (9): e44497, 2012.

Xu H, Rajesan R, Harper P, Kim RB, Lonnerdal B, Yang M, Uematsu S, Hutson J, Watson-MacDonell J, Ito S. Induction of P 1A by cow milk-based formula: a comparative study between human milk and formula. Br. J. Pharmacol. 146: 296–305, 2005.

Xue F, Willett WC, Rosner BA, Forman MR, Michels KB. Parental characteristics as predictors of birthweight. Hum Reprod 23: 168–177, 2008.

Yager JY, Shuaib A, Thornhill J. The effect of age on susceptibility to brain damage in a model of global hemispheric hypoxia-ischemia. Dev. Brain Res. 93: 143–154, 1996.

Yang P, Zhang J, Zhao L, Jiao Q, Jin H, Xiao X, Zhang H, Hu M, Lu H, Liu Y. Developmental distribution pattern of glutamate receptor 5 in prenatal human hippocampus. Neurosci Bull 28: 704–714, 2012.

Yang Y, Gozen O, Watkins A, Lorenzini I, Lepore A, Gao Y, Vidensky S, Brennan J, Poulsen D, Won Park J, Li Jeon N, Robinson MB, Rothstein JD. Presynaptic regulation of astroglial excitatory neurotransmitter transporter GLT1. Neuron 61 (6): 880–894, 2009.

Yasuda H, Barth AL, Stellwagen D, Malenka RC. A developmental switch in the signaling cascades for LTP induction. Nat Neurosci 6 (1): 15-16, 2003.

Yesair DW, Branfman AR, Callahan MM. Human disposition and some biochemical aspects of methylxanthines. Prog. Clin. Biol. Res. 158: 215–233, 1984.

Zehr E, Stein R. What functions do reflexes serve during human locomotion. Progress in Neurobiology 58: 185–205, 1999.

Zhao L, Jiao Q, Chen X, Yang P, Zhao B, Zheng P, Liu Y. mGluR5 is involved in proliferation of rat neural progenitor cells exposed to hypoxia with activation of mitogen-activated protein kinases signaling pathway. J Neurosci Res 90: 447–460, 2012.

Zhu JJ, Esteban JA, Hayashi Y, Malinow R. Postnatal synaptic potentiation: delivery of GluR4-containing AMPA receptors by spontaneous activity. Nat Neurosci 3: 1098–1106, 2000.

Zweifel LS, Kuruvilla R, Ginty DD. Functions and mechanisms of retrograde neurotrophin signalling. Nat Rev Neurosci 6 (8): 615-625, 2005.

ANEXOS

Apresentamos em anexo dois artigos publicados no decorrer da tese, mas que não fazem parte do corpo da tese. Foi avaliada a participação do sistema adenosinérgico nos efeitos comportamentais (ansiedade e memória) do metilfenidato, que é um psicoestimulante que vem sendo amplamente utilizado como uma droga recreativa. O anexo I refere-se ao artigo intitulado "*Adenosine A_1 receptors are modified by acute treatment with methylphenidate in adult mice*". O anexo II refere-se ao artigo intitulado "*Blockade of adenosine A_1 receptors prevents methylphenidate-induced impairment of object recognition task in adult mice*".

ANEXO I

Adenosine A_1 receptors are modified by acute treatment with methylphenidate in adult mice

Artigo publicado no periódico *Brain Research 1357: 62-69, 2010*

BRAIN RESEARCH 1357 (2010) 62–69

available at www.sciencedirect.com

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Adenosine A_1 receptors are modified by acute treatment with methylphenidate in adult mice

Sabrina Mioranzza[a], *Paulo Henrique S. Botton*[a], *Marcelo S. Costa*[a], *Janaina Espinosa*[a,b], *Vanessa Kazlauckas*[a], *Ana Paula Ardais*[a], *Diogo O. Souza*[a], *Lisiane O. Porciúncula*[a,*]

[a]*Federal University of Rio Grande do Sul, Health and Basic Sciences Institute, Department of Biochemistry, Graduation Program in Biological Sciences-Biochemistry, Laboratory of Studies on the Purinergic System, Bairro Santana, Brazil*
[b]*Graduation Program in Biological Sciences-Neurosciences, Porto Alegre/RS 90035-003, Brazil*

ARTICLE INFO

Article history:
Accepted 2 August 2010
Available online 7 August 2010

Keywords:
Adenosine
Cognition
Psychostimulants
Learning
Memory
Anxiety
Methylphenidate

ABSTRACT

In recent years misuse of methylphenidate (MPH) has been reported. The main pharmacological target of methylphenidate is the dopaminergic system. Adenosine is a neuromodulator that influences the dopaminergic neurotransmission, but studies on MPH and adenosine are still lacking. In this study, adult mice were acutely treated with MPH (5 mg/kg, i.p.) and to model misuse, they received an acute overdosage (50 mg/kg, i.p). The involvement of adenosine A_1 receptors in anxiety-related behavior and locomotor and exploratory activity was examined. The administration of methylphenidate (5 and 50 mg/kg) 30 min before the exposure to open field arena did not modify locomotor activity. The anxiolytic-like behavior was observed with both doses of MPH as revealed by the increase on the number of entries and the time spent in the open arms in the elevated plus-maze. Pre treatment with selective adenosine A_1 receptor antagonist (DPCPX 1 mg/kg, i.p.) did not prevent anxiolytic effect caused by MPH 50 mg/kg. Immunoblotting of frontal cortex and hippocampal extracts revealed that MPH 50 mg/kg increased 88% adenosine A_1 receptor density in the frontal cortex. Extracts from hippocampus did not reveal any differences in the adenosine A_1 receptor density. Our findings ruled out the participation of adenosine A_1 receptors on the MPH-triggered anxiolytic effects. However, the density of adenosine A_1 receptors increased in a brain area strictly involved in the MPH-mediated effects. Thus, the adenosinergic system may play a role in the methylphenidate actions in the central nervous system.

1. Introduction

Methylphenidate (MPH; Ritalin®) is the most commonly prescribed drug for the treatment of Attention deficit/hyperactivity disorder (ADHD) (Accardo and Blondis, 2001). Similar to other psychostimulants, one of the main targets of pharmacological action for MPH is the dopaminergic system (Gatley et al., 1999), albeit the participation of other neurotransmitters may also take place (Pascoli et al., 2005; Prieto-Gómez et al., 2005; Yano and Steiner, 2005). Some reports have suggested its potential in

* *Corresponding author*. Federal University of Rio Grande do Sul, Health and Basic Sciences Institute, Department of Biochemistry, Laboratory of Studies on the Purinergic System, Rua Ramiro Barcelos, 2600-anexo, Bairro Santana, Porto Alegre/RS 90035-003, Brazil. Fax: +55 51 3308 5540.
E-mail address: loporciuncula@yahoo.com (L.O. Porciúncula).

0006-8993/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2010.08.004

eliciting drug dependence (Kollins et al., 2001; Kollins, 2003). Currently, the motivation for the misuse and abuse of MPH by college students includes recreational reasons or improvement of cognitive functions (Babcock and Byrne, 2000; Barrett et al., 2005; Dupont et al., 2008; Klein-Schwartz and McGrath, 2003; Wu et al., 2007). In rodents, the administration of MPH has promoted behavioral alterations that include memory impairment and anxiety-like behavior (Britton and Bethancourt, 2009; Chuhan and Taukulis, 2006; Gray et al., 2007; LeBlanc-Duchin and Taukulis, 2007, 2009; McFadyen-Leussis et al., 2004).

Adenosine is a neuromodulator in the central nervous system (CNS), which via mainly adenosine A_1 and A_{2A} metabotropic receptors control synaptic transmission of neurotransmitters such as dopamine and glutamate (Cunha, 2001). Adenosine A_1 receptors are expressed throughout brain while adenosine A_{2A} is more restricted to the basal ganglia. The existence of selective heteromerization of A_{2A} and D_2 receptors and A_1 and D_1 receptors was firstly demonstrated in transfected cells (Canals et al., 2003; Ginés et al., 2000). Subsequently, biochemical characteristics could also be identified of these receptor heteromers, called "intramembrane receptor-receptor interactions" in the striatum (Agnati et al., 2003), therefore demonstrating the existence of A_{2A}–D_2 and A_1–D_1 receptor heteromers in the brain (Ferré et al., 1997; Franco et al., 2007). The A_1–D_1 heteromeric receptor complex may therefore give the molecular basis for the well-documented antagonistic A_1–D_1 receptor/receptor interactions found in the neuronal networks of the brain (Ferré et al., 1997; Fuxe et al., 1998; Franco et al., 2000). The understanding of these receptor/receptor interactions have been useful for the development of novel treatments for some neuropathologies that include ADHD, Parkinson's disease, dyskinesias, schizophrenia and drug addiction (for reviews see Ferré et al., 2008; Fuxe et al., 2007; Maggio et al., 2009).

The stimulation of adenosine receptors counteracts the behavioral effects of dopamine receptor stimulation (Cao et al., 2007; Ferré et al., 1997). Likewise, adenosine receptor agonists counteract whereas adenosine receptor antagonists potentiate pharmacological effects of psychostimulants like cocaine and amphetamines (Poleszak and Malec, 2003; Popoli et al., 1994; Rimondini et al., 1998). Particularly, behavioral alterations caused by other psychostimulants acting on dopaminergic system were related to adenosine A_1 receptors (Kuzmin et al., 1999; Poleszak and Malec, 2003).

The participation of adenosinergic system in the ADHD has been suggested in studies with spontaneously hypertensive rats (SHR) as an experimental model of ADHD. Caffeine (a non selective adenosine receptors antagonist) and selective adenosine receptors antagonists reversed the memory impairment in this strain (Pires et al., 2009; Prediger et al., 2005).

The involvement of adenosine receptors on MPH-mediated behavioral alterations has been reported in studies where administration of caffeine induces cross tolerance and cross desensitization to MPH (Boeck et al., 2009; Jain and Holtzman, 2005). Apart from these studies, possible modifications in the adenosine receptors density or even a pharmacological characterization of adenosine receptors subtype in the MPH-mediated effects are still incipient.

Considering the misuse of MPH, the present study was designed to assess if acute treatment and an acute overdosage of MPH in adult mice could modify the performance in the elevated plus-maze. Besides, the immunocontent of adenosine A_1 receptors was investigated in two brain areas involved in the MPH-mediated effects (frontal cortex and hippocampus) (Hewitt et al., 2005) as well as the involvement of adenosine A_1 receptors in the MPH-mediated effects in the elevated plus-maze.

2. Results

2.1. *Elevated plus-maze task*

Effects of MPH were also evaluated in the elevated plus-maze. The number of entries in the arms was not affected by treatment with MPH 5 mg/kg but mice treated with MPH 50 mg/kg showed an increase on the number of entries in the open arms [F (2,38)=4.745, P=0.0148], coupled with a decrease on the number of entries in the closed arms (Fig. 1A) [F (2,38)=9.147, P=0.0006]. MPH 5 mg/kg increased the percentage of the time spent in the open arms [F (2,38)=32.24, P<0.0001] with a concomitant decrease in the time spent in the closed arms [F (2,38)=37.42, P<0.0001] (Fig. 1B). MPH 50 mg/kg caused a robust increase on the percentage of the time spent in the open arms with a concomitant decrease on the time spent in the closed arms (Fig. 1B). MPH 50 mg/kg also decreased the number of rearing in the elevated plus-maze [F (2,38)=6.223, P=0.0048] (Fig. 1C). The total number of entries in the arms was not influenced by treatments (Fig. 1D) [F (2,38)=1.116, P=0.3385].

The role of adenosine A_1 receptors was also investigated on the effects caused by MPH 50 mg/kg in the elevated plus-maze. Pre administration of DPCPX (1 mg/kg, i.p) was not able to suppress the increase on the number of entries in the open arms and percentage of the time spent in open arms caused by MPH 50 mg/kg (Fig. 2A and B). Pre administration of DPCPX did not modify the decrease on the number of rearing caused by acute administration of MPH 50 mg/kg (Fig. 2C). DPCPX administered alone was devoid of effect in all parameters analyzed in the elevated plus-maze.

2.2. *Open field*

In order to assess the locomotor activity of mice, analysis of the traveled distance was performed 90 min after mice had received MPH 5 and 50 mg/kg. Each mouse was monitored during 10 min in the open field arena. No differences were found in the traveled distance between vehicle (control) and MPH-treated mice in both doses (Fig. 3) [F (2,23)=2.55; P=0.1007].

2.3. *Immunoblotting*

Immunoblotting analysis was carried out in the frontal cortex and hippocampus homogenates from mice treated acutely with vehicle or different doses of MPH. Differently from hippocampus, frontal cortex homogenates from mice treated with MPH 50 mg/kg presented an increase in the density of adenosine A_1 receptors (88% compared to vehicle) [F (2,20)=5.861, P<0.0110]. Acute treatment with 5 mg/kg of MPH did not cause any modifications in the adenosine A1 receptors in the hippocampus as well as in the frontal cortex (Fig. 4).

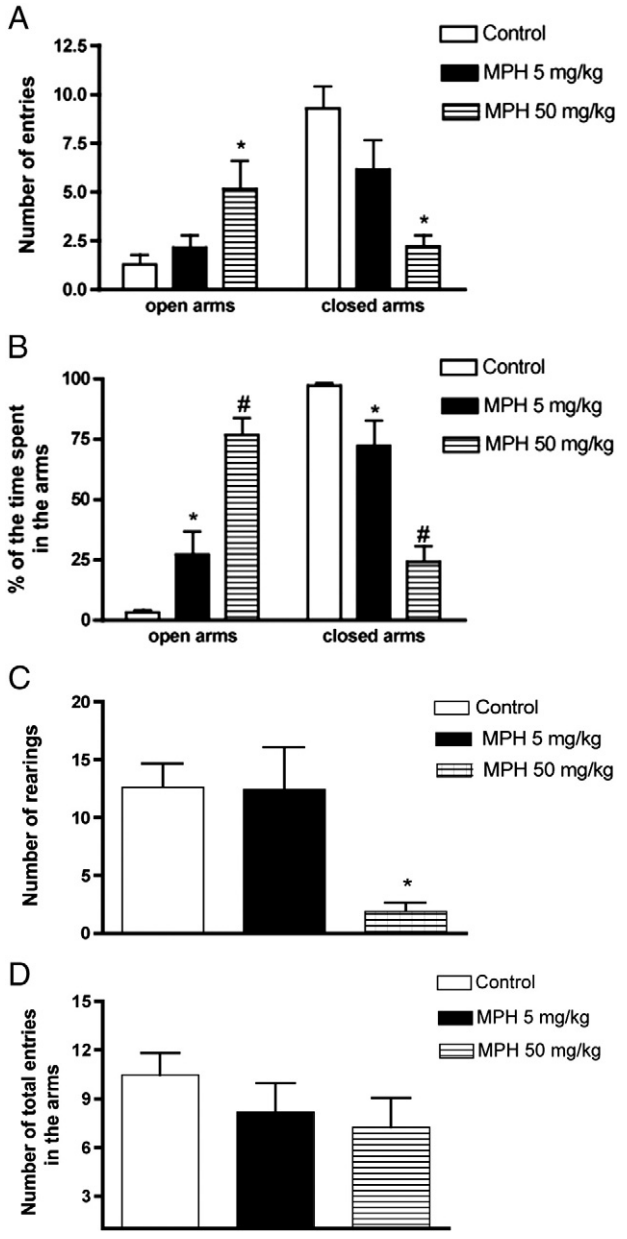


Fig. 1 – Performance of adult mice treated acutely either with vehicle (control) or methylphenidate (MPH, 5 or 50 mg/kg, i.p) in the elevated plus-maze during 5 min. (A) Data represent means ± S.E.M. of the number of entries in the arms. (B) Data represent means ± S.E.M. of the percentage of time spent in the arms. (C) Data represent means ± S.E.M. of the number of rearings. (D) Data represent means ± S.E.M. of the total number of entries in the arms. Statistical comparison was performed with one-way ANOVA followed by Newman–Keuls multiple comparison test ($n = 12–15$ animals per group). $^{*}P < 0.05$, significantly different from control. $^{\#}P < 0.05$, significantly different from control and MPH 5 mg/kg.

3. Discussion

In the present study, acute of MPH and an acute overdosage to model misuse were investigated on locomotor activity and anxiety-like behavior in adult mice. Besides, the density and pharmacological manipulation of adenosine A_1 receptors were evaluated in order to ascertain whether adenosine could play a role in the effects of MPH.

Our findings showed that both doses of MPH acutely administered triggered an anxiolytic-like effect as evidenced by the increase on the number of entries and the time spent in the open arms in the elevated plus-maze. Besides, animals treated with MPH 50 mg/kg exhibited reduced exploratory activity as evidenced by decrease on the number of rearings. It is unlikely that the anxiolytic effect arises from hyperlocomotion in MPH-treated mice since the number of total entries in the arms was not affected by the treatment. Accordingly, MPH did not modify locomotor activity in the open field arena. Anxiolytic properties of MPH 3 mg/kg were reported in mice that presented anxiety-like behavior triggered by social isolation (Koike et al., 2009). In this study, MPH was administered 30 min before placing mouse in the elevated plus-maze (exactly the same treatment regimen from our study) and reversed anxiety-like behavior by social isolation with no effect *per se* on the locomotor activity and the time spent in the arms. In our study, MPH administered alone caused behavioral alterations in the elevated plus maze most certainly because the dose used was higher than in the study aforementioned. Considering that the total time exploration was affected by an overdosage, a decrease on novelty seeking or anhedonia caused by an overdosage of MPH could not be discarded as well as the possibility that methylphenidate has affected motivation, attention, and sensorimotor function. Indeed, previous study showed that adult rats exposed to repeated doses of MPH during their juvenile were less responsive with respect to motor activation exhibited by animals when first exposed to a novel environment (Bolaños et al., 2003).

Despite a different treatment regimen, MPH triggered anxiety-like behavior and enhanced the exploratory activity of the animals (Britton et al., 2007; Britton and Bethancourt, 2009). Interestingly, MPH administered during development up to 35 days old caused a decrease in the anxiety-like behavior when rats were tested at 135 days old (Gray et al., 2007). On the other hand, prenatal exposure to MPH increased exploratory activity but also evoked anxiety-related behavior with no benefits on learning and memory tasks in mice (McFadyen-Leussis et al., 2004). Thus, behavioral findings of the administration of methylphenidate are clearly distinct and dependent on the schedule of administration and age of the animals. It is important to emphasize that the experimental design used in other studies differs from ours and they were designed to evaluate effects of the MPH administered during development on behavior of these animals in the adulthood. Our study aimed to investigate effects of acute administration as well as an overdosage of MPH in adult animals in order to find behavioral alterations in adult animals and to model misuse.

The participation of adenosine A_1 receptors in anxiety behavior is clearly demonstrated in mice lacking adenosine A_1 receptors (Giménez-Llort et al., 2002; Johansson et al., 2001; Lang et al., 2003) followed by the anxiogenic action of adenosine receptor antagonists (File et al., 1988; Florio et al., 1998; Jain et al., 1995). Thus, the role of adenosine A_1 receptors was also investigated on behavioral alterations of MPH in the elevated plus-maze. The blockade of adenosine A_1 receptors was

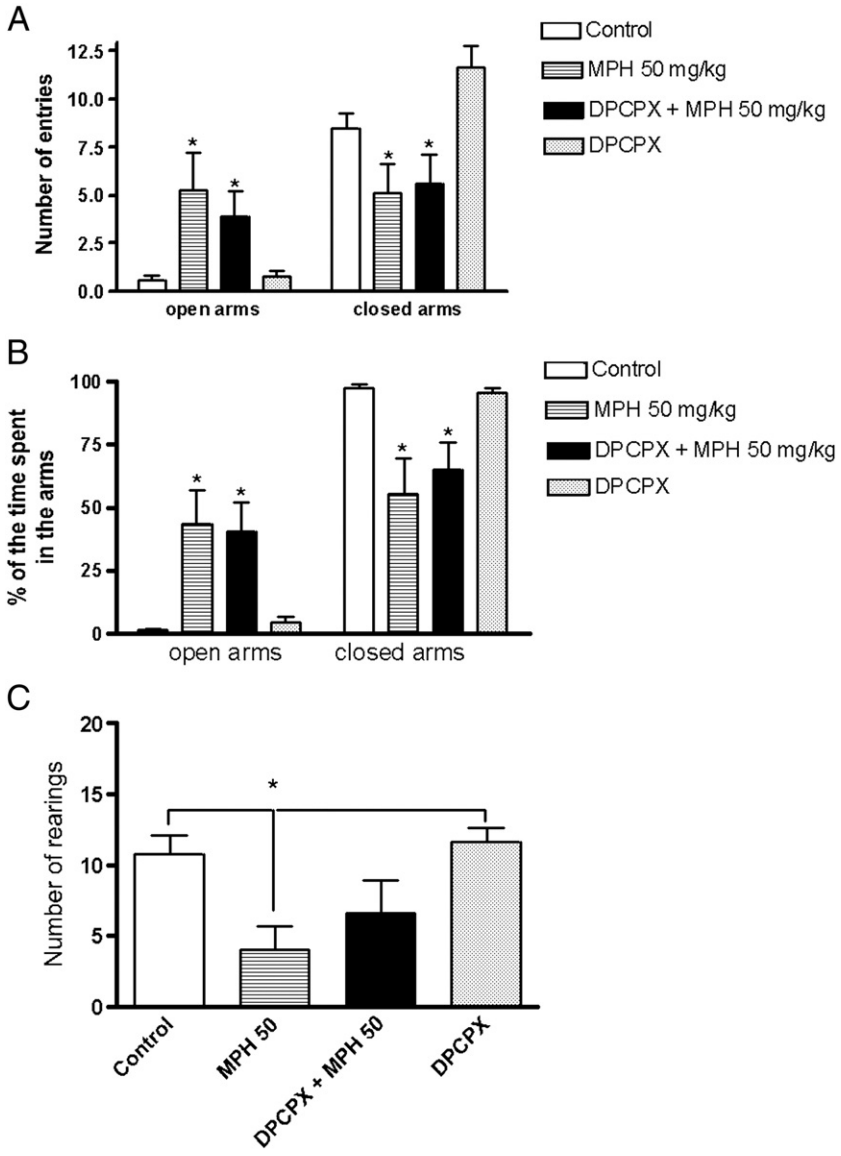


Fig. 2 – The lack of involvement of adenosine A_1 receptors on the MPH-induced behavioral alterations in the elevated plus-maze. DPCPX (1 mg/kg, i.p.) was administered to mice 30 min prior to MPH (50 mg/kg, i.p.), which was administered 30 min before exposing mice to the elevated Plus-maze during 5 min. Data represent means ± S.E.M. of the number of entries in the arms. (B) Data represent means ± S.E.M. of the percentage of the time spent in the arms. (C) Data represent means ± S.E.M. of the number of rearings. Statistical comparison was performed with one-way ANOVA followed by Newman–Keuls multiple comparison test (n = 9–14 animals per group). *$P < 0.05$, significantly different from control and DPCPX.

ineffective in suppressing behavioral alterations caused by acute administration of MPH 50 mg/kg in the elevated plus-maze. Thus, the pharmacological manipulation of adenosine A_1 receptors did not evidence its role on anxiolytic effects caused by MPH.

The immunocontent of adenosine A_1 receptors was differently affected by the treatment with MPH according to the brain region analyzed. In the frontal cortex, acute treatment with an overdosage of MPH increased the immunocontent of adenosine A_1 receptors whereas no modification was found in the hippocampus. Prefrontal cortex is one of the brain regions targets of MPH effects as evidenced by a recent study using pharmacological magnetic resonance imaging (phMRI). They found an increase in the blood-oxygenation level dependent (BOLD) signal in the frontal cortex and nucleus accumbens from adult rats treated with MPH, but not in the hippocampus (Canese et al., 2009). In addition, prefrontal cortex and hippocampus also play an important role in the memory recognition as well as in the anxiety-related behavior (Barker and Warburton, 2008; Bergami et al., 2008; Winters et al., 2008). Since activation of adenosine A_1 receptors trigger anxiolytic effects in rodents (Jain et al., 1995), this transient up-regulation of adenosine A_1 receptors could be involved in the anxiolytic effects of MPH. It remains to be determined if acute treatment with MPH could increase brain adenosine levels and if a continuous administration could trigger the desensitization of adenosine A_1 receptors. It is interesting to note that caffeine, another well-known psychostimulant and a

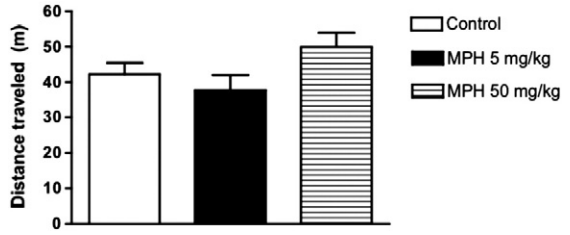


Fig. 3 – Performance of adult mice treated with vehicle (control) or methylphenidate (MPH 5 or 50 mg/kg, i.p) in the open field arena. Data represent means ± S.E.M. of the traveled distance in meters during 10 min of video recording. (n = 8–9 animals per group). Statistical comparison was performed with one-way ANOVA followed by Newman–Keuls multiple comparison test. No significant differences were found between groups.

non-selective adenosine antagonist, also up-regulates adenosine A_1 receptors (Svenningsson et al., 1999). Likewise, caffeine is also able to evoke anxiety-related behavior (El Yacoubi et al., 2000), suggesting that MPH and caffeine may be sharing some molecular targets. However, differently from MPH, previous results from our group and others have reported the cognitive enhancer properties of caffeine in humans and rodents (Costa et al., 2008; Haskell et al., 2005). Other abuse drugs such as cocaine and amphetamine have changed the expression and density of adenosine receptors (Manzoni et al., 1998), and pharmacological interactions between cocaine and methamphetamine with adenosine agonists and antagonists have been reported (Justinova et al., 2003; Kuzmin et al., 1999). Although methylphenidate shares similar pharmacological action with cocaine and amphetamine, it is the first report where administration of MPH focusing on adenosine receptors was investigated.

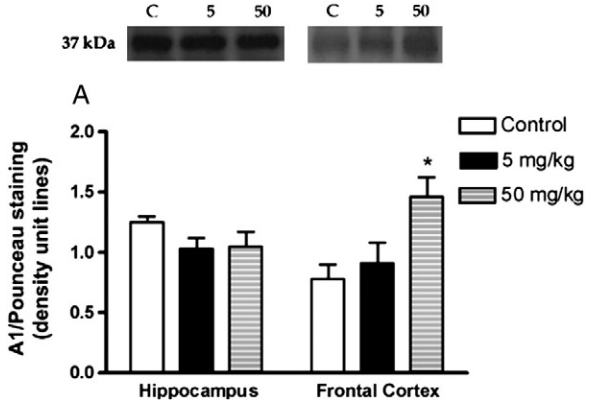


Fig. 4 – Immunoblotting analysis of adenosine A_1 receptor density in frontal cortex and in hippocampus from adult mice. Mice acutely treated either with vehicle or methylphenidate (MPH, 5 or 50 mg/kg, i.p). Data represent means + S.E.M of density unit lines obtained from adenosine A_1 receptor/ Ponceau staining (n = 6 mice/group for hippocampus), (n = 7 mice/group for frontal cortex); At the top of figure are representative bands for adenosine A_1 receptor at 37 kDa (C—vehicle-treated mice; 5 and 50—methylphenidate-treated mice). Statistical comparison was performed with one-way ANOVA followed by Newman–Keuls multiple comparison test *P < 0.05, means significant difference between MPH-treated mice and control.

Our data demonstrated that adult mice treated with MPH presented modifications on adenosine A_1 receptor immunocontent, reporting for the first time that administration of this drug widely prescribed to treat ADHD and used as a cognitive enhancer can affect the adenosinergic system. It needs to be further determined whether this up-regulation of adenosine A_1 receptor in the frontal cortex by acute administration could influence the signaling operated by adenosine.

Thus, it becomes important to investigate the participation of adenosinergic system in the ADHD and MPH treatment, since adenosine is a neuromodulator in the CNS acting in the regulation of dopaminergic neurotransmission, a system that is specially affected in ADHD and one of the main pharmacological targets of MPH.

4. Experimental procedures

4.1. *Animals*

Male albino CF1 mice (3–4 months old) were obtained from Stated Foundation for Health Science Research (FEPPS, Porto Alegre/RS, Brazil). All experimental procedures were performed according to the NIH Guide for Care and Use of Laboratory Animals and to Brazilian Society for Neuroscience and Behaviour (SBNeC) recommendations for animal care. Ethical committee of Federal University of Rio Grande do Sul approved experimental procedures and all adequate measures were taken to minimize pain or discomfort. Mice were housed in standard cages (4 animals per cage) under a reversed 12/12-h light/dark cycle with free access to food and water. All behavioral tests were performed between 8:00 am and 5:00 pm. Separate groups of mice were used for each behavioral task.

4.2. *Drugs*

Methylphenidate hydrochloride (MPH, 5 or 50 mg/kg, i.p), diluted in saline with a drop of Tween 20 or saline (0.9 g%, i.p). Given the fast metabolism of mice (Faraj et al., 1974), a dose of 5 mg/kg should be approximately equivalent to a clinically relevant dose in humans, which ranges from 0.3 up to 1 mg/kg (Solanto, 2000). Besides, MPH 5 mg/kg has been widely used in behavioral studies in mice at different ages (Guerriero et al., 2006; McFadyen-Leussis et al., 2004). The selective antagonist of adenosine A_1 receptors 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 1 mg/kg, i.p) (Tocris, São Paulo, Brazil). DPCPX was daily prepared from a stock solution diluted in DMSO (10% v/v) plus saline. At this dose DPCPX did not promote any locomotor alterations in mice (El Yacoubi et al., 2000). Control mice received saline (0.9 g%) with a drop of Tween 20 or saline (0.9 g%) and for DPCPX experiments control mice received DMSO 1% diluted in saline (0.9 g%) or saline (0.9 g%) with a drop of Tween 20.

4.3. *Elevated plus-maze*

DPCPX (1 mg/kg, i.p) was administered 30 min before MPH 50 mg/kg for Elevated plus-maze. MPH (5 and 50 mg/kg, i.p) was administered 30 min before placing mouse in the elevated plus maze.

The plus maze apparatus which is a pharmacologically validated anxiety measure in rodents (File, 2001; Lister, 1987) consisted of a black painted Plexiglas, with two open arms 65 cm long and 14 cm wide and two closed arms enclosed by 45 cm walls, with an open roof arranged in such a way that the two arms of each type were opposite each other. Each mouse was placed in the central square facing an open arm. Number of entries in each arm (when all four paws had entered the arm), and time spent in each arm were recorded for 5 min. Two observers blind to the drug treatment performed the behavioral analysis.

4.4. *Open field*

MPH (5 or 50 mg/kg, i.p) or vehicle was administered in mice and after 90 min animals were placed in the open field arena. The open field test represents a widely used model for the evaluation of locomotor activity. The apparatus was made of black-painted Plexiglas measuring 50×50 cm and was surrounded by 50 cm high walls. The experiments were conducted in a sound-attenuated room under low-intensity light (12 lx). Each mouse was placed in the center of the arena and the distance traveled was recorded during 10 min. The experiment was recorded with a video camera positioned above the arena and monitored in an adjacent room by an observer blind to the drug treatment of the animals. The analysis was performed using a computer-operated tracking system (Any-maze, Stoelting, Woods Dale, IL).

4.5. *SDS-PAGE (sodium dodecyl sulfate-polyacrylamide) immunoblotting*

Twenty-four hours after behavioral tests, mice were sacrificed by cervical displacement; the hippocampus and prefrontal cortex were dissected out and immediately homogenized in 5% SDS with a protease inhibitor cocktail (Sigma, São Paulo/Brazil) and frozen at –70 °C. After defrost, the protein content was determined by Bicinchoninic acid assay using bovine serum albumin (BSA) as standard (Pierce, São Paulo/Brazil). Samples extracts were diluted to a final protein concentration of 2 μg/μL in SDS-PAGE buffer. Forty micrograms of the samples and prestained molecular weight standards (Bio-Rad, São Paulo/Brazil) were separated by SDS-PAGE (12% with 4% concentrating gel). After electro-transfer, the membranes were blocked with Tris-buffered saline 0.1% Tween-20 (TBS-T) containing 5% milk for 1 h. The membranes were then incubated for 24 h at 4 °C with rabbit anti-adenosine A_1 receptor antibody (1:1000; Affinity Bioreagents, USA). After primary antibodies incubation, membranes were washed and incubated with horseradish-peroxidase conjugated secondary antibodies for 2 h at room temperature and developed with chemiluminescence ECL kit (Amersham, São Paulo/Brazil). The autoradiographic films were scanned and densitometric analyses were performed using public domain NIH Image Program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/). The control of protein loading was carried out with Ponceau S. stain. Membranes with Ponceau S. were scanned at 37 kDa and the values used obtain adenosine A_1 receptor density/Ponceau S. density ratio. No differences were found in the amount of protein loaded (data not shown).

4.6. *Statistical analysis*

Statistical analysis was performed by paired Student's *t*-test when differences within groups were analyzed. One-way ANOVA followed by Newman–Keuls post hoc test was used when differences between groups were analyzed. Statistically significant differences were considered for $P<0.05$.

Acknowledgments

This work was supported by CNPq (Lisiane Porciúncula, Proc. no. 472216/2009-0), IBNnet (Neuroscience Brazilian Network), CNPq/INCTEN, PROPESQ/UFRGS and FAPERGS/PRONEX.

REFERENCES

Accardo, P., Blondis, T.A., 2001. What's all the fuss about Ritalin? J. Pediatr. 138, 6–9.

Agnati, L.F., Ferré, S., Lluis, C., Franco, R., Fuxe, K., 2003. Molecular mechanisms and therapeutical implications of intramembrane receptor/receptor interactions among heptahelical receptors with examples from the striatopallidal GABA neurons. Pharmacol. Rev. 55, 509–550.

Babcock, Q., Byrne, T., 2000. Student perceptions of methylphenidate abuse at a public liberal arts college. J. Am. Coll. Health 49, 143–145.

Barker, G.R., Warburton, E.C., 2008. NMDA receptor plasticity in the perirhinal and prefrontal cortices is crucial for the acquisition of long-term object-in-place associative memory. J. Neurosci. 28, 2837–2844.

Barrett, S.P., Darredeau, C., Bordy, L.E., Pihl, R.O., 2005. Characteristics of methylphenidate misuse in a university student sample. Can. J. Psychiatry 50, 457–461.

Bergami, M., Rimondini, R., Santi, S., Blum, R., Götz, M., Canossa, M., 2008. Deletion of TrkB in adult progenitors alters newborn neuron integration into hippocampal circuits and increases anxiety-like behavior. Proc. Natl Acad. Sci. USA 105, 15570–15575.

Boeck, C.R., Marques, V.B., Valvassori, S.S., Constantino, L.C., Rosa, D.V., Lima, F.F., Romano-Silva, M.A., Quevedo, J., 2009. Early long-term exposure with caffeine induces cross-sensitization to methylphenidate with involvement of DARPP-32 in adulthood of rats. Neurochem. Int. 55, 318–322.

Bolaños, C.A., Barrot, M., Berton, O., Wallace-Black, D., Nestler, E.J., 2003. Methylphenidate treatment during pre- and periadolescence alters behavioral responses to emotional stimuli at adulthood. Biol. Psychiatry 54, 1317–1329.

Britton, G.B., Segan, A.T., Sejour, J., Mancebo, S.E., 2007. Early exposure to methylphenidate increases fear responses in an aversive context in adult rats. Dev. Psychobiol. 49, 265–275.

Britton, G.B., Bethancourt, J.A., 2009. Characterization of anxiety-related responses in male rats following prolonged exposure to therapeutic doses of oral methylphenidate. Pharmacol. Biochem. Behav. 93, 451–459.

Canals, M., Marcellino, D., Fanelli, F., Ciruela, F., de Benedetti, P., Goldberg, S.R., Neve, K., Fuxe, K., Agnati, L.F., Woods, A.S., Ferré, S., Lluis, C., Bouvier, M., Franco, R., 2003. Adenosine A_{2A}-dopamine D_2 receptor-receptor heteromerization: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. J. Biol. Chem. 278, 46741–46749.

Canese, R., Adriani, W., Marco, E.M., De Pasquale, F., Lorenzini, P., De Luca, N., Fabi, F., Podo, F., Laviola, G., 2009. Peculiar response to methylphenidate in adolescent compared to adult rats: a phMRI study. Psychopharmacology (Berl). 203, 143–153.

Cao, Y., Xie, K.Q., Zhu, X.Z., 2007. The enhancement of dopamine D_1 receptor desensitization by adenosine A1 receptor activation. Eur. J. Pharmacol. 562, 34–38.

Chuhan, Y.S., Taukulis, H.K., 2006. Impairment of single-trial memory formation by oral methylphenidate in the rat. Neurobiol. Learn. Mem. 85, 125–131.

Costa, M.S., Botton, P.H., Mioranzza, S., Ardais, A.P., Moreira, J.D., Souza, D.O., Porciúncula, L.O., 2008. Caffeine improves adult mice performance in the object recognition task and increases BDNF and TrkB independent on phospho-CREB immunocontent in the hippocampus. Neurochem. Int. 53, 89–94.

Cunha, R.A., 2001. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. Neurochem. Int. 38, 107–125.

Dupont, R.L., Coleman, J.J., Bucher, R.H., Wilford, B.B., 2008. Characteristics and motives of college students who engage in nonmedical use of methylphenidate. Am. J. Addict. 17, 167–171.

El Yacoubi, M., Ledent, C., Parmentier, M., Costentin, J., Vaugeois, J. M., 2000. The anxiogenic-like effect of caffeine in two experimental procedures measuring anxiety in the mouse is not shared by selective A(2A) adenosine receptor antagonists. Psychopharmacology (Berl). 148, 153–163.

Faraj, B.A., Israili, Z.H., Perel, J.M., Jenkins, M.L., Holtzman, S.G., Cucinell, S.A., Dayton, P.G., 1974. Metabolism and disposition of methylphenidate-14C: studies in man and animals. J. Pharmacol. Exp. Ther. 191, 535–547.

Ferré, S., Fredholm, B.B., Morelli, M., Popoli, P., Fuxe, K., 1997. Adenosine-dopamine receptor–receptor interactions as an integrative mechanism in the basal ganglia. Trends Neurosci. 20, 482–487.

Ferré, S., Quiroz, C., Woods, A.S., Cunha, R., Popoli, P., Ciruela, F., Lluis, C., Franco, R., Azdad, K., Schiffmann, S.N., 2008. An update on adenosine A2A–dopamine D2 receptor interactions: implications for the function of G protein-coupled receptors. Curr. Pharm. Des. 14, 1468–1474.

File, S.E., 2001. Factors controlling measures of anxiety and responses to novelty in the mouse. Behav. Brain Res. 125, 151–157.

File, S.E., Baldwin, H.A., Johnston, A.L., Wilks, L.J., 1988. Behavioral effects of acute and chronic administration of caffeine in the rat. Pharmacol. Biochem. Behav. 30, 809–815.

Florio, C., Prezioso, A., Papaioannou, A., Vertua, R., 1998. Adenosine A_1 receptors modulate anxiety in CD1 mice. Psychopharmacology (Berl). 136, 311–319.

Franco, R., Casado, V., Cortes, A., Ferrada, C., Mallol, J., Woods, A., Lluis, C., Canela, E.I., Ferré, S., 2007. Basic concepts in G-protein-coupled receptor homo- and heteromerization. Sci. World J. 7, 48–57.

Franco, R., Ferré, S., Agnati, L., Torvinen, M., Ginés, S., Hillion, J., Casadó, V., Lledó, P., Zoli, M., Lluis, C., Fuxe, K., 2000. Evidence for adenosine/dopamine receptor interactions: indications for heteromerization. Neuropsychopharmacology 23 (4 Suppl), S50–S59.

Fuxe, K., Ferré, S., Zoli, M., Agnati, L.F., 1998. Integrated events in central dopamine transmission as analyzed at multiple levels. Evidence for intramembrane adenosine A2A/dopamine D2 and adenosine A1/dopamine D1 receptor interactions in the basal ganglia. Brain Res. Rev. 26, 258–273.

Fuxe, K., Ferré, S., Genedani, S., Franco, R., Agnati, L.F., 2007. Adenosine receptor–dopamine receptor interactions in the basal ganglia and their relevance for brain function. Physiol. Behav. 92, 210–217.

Gatley, S.J., Volkow, N.D., Gifford, A.N., Fowler, J.S., Dewey, S.L., Ding, Y.S., Logan, J., 1999. Dopamine-transporter occupancy after intravenous doses of cocaine and methylphenidate in mice and humans. Psychopharmacology (Berl) 146, 93–100.

Giménez-Llort, L., Fernández-Teruel, A., Escorihuela, R.M., Fredholm, B.B., Tobeña, A., Pekny, M., Johansson, B., 2002. Mice lacking the adenosine A_1 receptor are anxious and aggressive, but are normal learners with reduced muscle strength and survival rate. Eur. J. Neurosci. 16, 547–550.

Ginés, S., Hillion, J., Torvinen, M., Le Crom, S., Casadó, V., Canela, E.I., Rondin, S., Lew, J.Y., Watson, S., Zoli, M., Agnati, L.F., Verniera, P., Lluis, C., Ferré, S., Fuxe, K., Franco, R., 2000. Dopamine D_1 and adenosine A_1 receptors form functionally interacting heteromeric complexes. Proc. Natl Acad. Sci. USA 97, 8606–8611.

Gray, J.D., Punsoni, M., Tabori, N.E., Melton, J.T., Fanslow, V., Ward, M.J., Zupan, B., Menzer, D., Rice, J., Drake, C.T., Romeo, R.D., Brake, W.G., Torres-Reveron, A., Milner, T.A., 2007. Methylphenidate administration to juvenile rats alters brain areas involved in cognition, motivated behaviors, appetite, and stress. J. Neurosci. 27, 7196–7207.

Guerriero, R., Hayes, M., Dhaliwal, S., Ren, J., Kosofsky, B., 2006. Preadolescent methylphenidate versus cocaine treatment differ in the expression of cocaine-induced locomotor sensitization during adolescence and adulthood. Biol. Psychiatry 60, 1171–1180.

Haskell, C.F., Kennedy, D.O., Wesnes, K.A., Scholey, A.B., 2005. Cognitive and mood improvements of caffeine in habitual consumers and habitual non-consumers of caffeine Psychopharmacology (Berl). 179, 813–825.

Hewitt, K.N., Shah, Y.B., Prior, M.J., Morris, P.G., Hollis, C.P., Fone, K.C., Marsden, C.A., 2005. Behavioural and pharmacological magnetic resonance imaging assessment of the effects of methylphenidate in a potential new rat model of attention deficit hyperactivity disorder. Psychopharmacology (Berl). 180, 716–723.

Jain, N., Kemp, N., Adeyemo, O., Buchanan, P., Stone, T.W., 1995. Anxiolytic activity of adenosine receptor activation in mice. Br. J. Pharmacol. 116, 2127–2133.

Jain, R., Holtzman, S.G., 2005. Caffeine induces differential cross tolerance to the amphetamine-like discriminative stimulus effects of dopaminergic agonists. Brain Res. Bull. 65, 415–421.

Johansson, B., Halldner, L., Dunwiddie, T.V., Masino, S.A., Poelchen, W., Giménez-Llort, L., Escorihuela, R.M., Fernández-Teruel, A., Wiesenfeld-Hallin, Z., Xu, X.J., Hårdemark, A., Betsholtz, C., Herlenius, E., Fredholm, B.B., 2001. Hyperalgesia, anxiety, and decreased hypoxic neuroprotection in mice lacking the adenosine A_1 receptor. Proc. Natl. Acad. Sci. U. S. A. 98, 9407–9412.

Justinova, Z., Ferre, S., Segal, P.N., Antoniou, K., Solinas, M., Pappas, L.A., Highkin, J.L., Hockemeyer, J., Munzar, P., Goldberg, S.R., 2003. Involvement of adenosine A1 and A2A receptors in the adenosinergic modulation of the discriminative-stimulus effects of cocaine and methamphetamine in rats. J. Pharmacol. Exp. Ther. 307, 977–986.

Klein-Schwartz, W., McGrath, J., 2003. Poison centers' experience with methylphenidate abuse in pre-teens and adolescents. J. Am. Acad. Child Adolesc. Psychiatry 42, 288–294.

Koike, H., Ibi, D., Mizoguchi, H., Nagai, T., Nitta, A., Takuma, K., Nabeshima, T., Yoneda, Y., Yamada, K., 2009. Behavioral abnormality and pharmacologic response in social isolation-reared mice. Behav. Brain Res. 202, 114–121.

Kollins, S.H., MacDonald, E.K., Rush, C.R., 2001. Assessing the abuse potential of methylphenidate in nonhuman and human subjects: a review. Pharmacol. Biochem. Behav. 68, 611–627.

Kollins, S.H., 2003. Comparing the abuse potential of methylphenidate versus other stimulants: a review of available evidence and relevance to the ADHD patient. J. Clin. Psychiatry 64 (Suppl 1), 114–118.

Kuzmin, A., Johansson, B., Zvartau, E.E., Fredholm, B.B., 1999. Caffeine, acting on adenosine A_1 receptors, prevents the extinction of cocaine-seeking behavior in mice. J. Pharmacol. Exp. Ther. 290, 535–542.

Lang, U.E., Lang, F., Richter, K., Vallon, V., Lipp, H.P., Schnermann, J., Wolfer, D.P., 2003. Emotional instability but intact spatial

cognition in adenosine receptor 1 knock out mice. Behav. Brain Res. 145, 179–188.
LeBlanc-Duchin, D., Taukulis, H.K., 2007. Chronic oral methylphenidate administration to periadolescent rats yields prolonged impairment of memory for objects. Neurobiol. Learn. Mem. 88, 312–320.
LeBlanc-Duchin, D., Taukulis, H.K., 2009. Chronic oral methylphenidate induces post-treatment impairment in recognition and spatial memory in adult rats. Neurobiol. Learn. Mem. 91, 218–225.
Lister, R.G., 1987. The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl) 92, 180–185.
Maggio, R., Aloisi, G., Silvano, E., Rossi, M., Millan, M.J., 2009. Heterodimerization of dopamine receptors: new insights into functional and therapeutic significance. Parkinsonism Relat. Disord. Suppl 4, S2–S7.
Manzoni, O., Pujalte, D., Williams, J., Bockaert, J., 1998. Decreased presynaptic sensitivity to adenosine after cocaine withdrawal. J. Neurosci. 18, 7996–8002.
McFadyen-Leussis, M.P., Lewis, S.P., Bond, T.L., Carrey, N., Brown, R.E., 2004. Prenatal exposure to methylphenidate hydrochloride decreases anxiety and increases exploration in mice. Pharmacol. Biochem. Behav. 77, 491–500.
Pascoli, V., Valjent, E., Corbillé, A.G., Corvol, J.C., Tassin, J.P., Girault, J.A., Hervé, D., 2005. cAMP and extracellular signal-regulated kinase signaling in response to d-amphetamine and methylphenidate in the prefrontal cortex in vivo: role of beta 1-adrenoceptors. Mol. Pharmacol. 68, 421–429.
Pires, V.A., Pamplona, F.A., Pandolfo, P., Fernandes, D., Prediger, R.D., Takahashi, R.N., 2009. Adenosine receptor antagonists improve short-term object-recognition ability of spontaneously hypertensive rats: a rodent model of attention-deficit hyperactivity disorder. Behav. Pharmacol. 20, 134–145.
Poleszak, E., Malec, D., 2003. Effects of adenosine receptor agonists and antagonists in amphetamine-induced conditioned place preference test in rats. Pol. J. Pharmacol. 55, 319–326.
Popoli, P., Pèzzola, A., de Carolis, A.S., 1994. Modulation of striatal adenosine A_1 and A_2 receptors induces rotational behaviour in response to dopaminergic stimulation in intact rats. Eur. J. Pharmacol. 257, 21–25.
Prediger, R.D., Pamplona, F.A., Fernandes, D., Takahashi, R.N., 2005. Caffeine improves spatial learning deficits in an animal model of attention deficit hyperactivity disorder (ADHD)—the spontaneously hypertensive rat (SHR). Int. J. Neuropsychopharmacol. 8, 583–594.
Prieto-Gómez, B., Vázquez-Alvarez, A.M., Martínez-Peña, J.L., Reyes-Vázquez, C., Yang, P.B., Dafny, N., 2005. Methylphenidate and amphetamine modulate differently the NMDA and AMPA glutamatergic transmission of dopaminergic neurons in the ventral tegmental area. Life Sci. 77, 635–649.
Rimondini, R., Ferré, S., Giménez-Llort, L., Ogren, S.O., Fuxe, K., 1998. Differential effects of selective adenosine A1 and A2A receptor agonists on dopamine receptor agonist-induced behavioural responses in rats. Eur. J. Pharmacol. 347, 153–158.
Solanto, M.V., 2000. Clinical psychopharmacology of AD/HD: implications for animal models. Neurosci. Biobehav. Rev. 24, 27–30.
Svenningsson, P., Nomikos, G.G., Fredholm, B.B., 1999. The stimulatory action and the development of tolerance to caffeine is associated with alterations in gene expression in specific brain regions. J. Neurosci. 19, 4011–4022.
Winters, B.D., Saksida, L.M., Bussey, T.J., 2008. Object recognition memory: neurobiological mechanisms of encoding, consolidation and retrieval. Neurosci. Biobehav. Rev. 32, 1055–1070.
Wu, L.T., Pilowsky, D.J., Schlenger, W.E., Galvin, D.M., 2007. Misuse of methamphetamine and prescription stimulants among youths and young adults in the community. Drug Alcohol Depend. 89, 195–205.
Yano, M., Steiner, H., 2005. Methylphenidate (Ritalin) induces Homer 1a and zif 268 expression in specific corticostriatal circuits. Neuroscience 132, 855–865.

ANEXO II

Blockade of adenosine A_1 receptors prevents methylphenidate-induced impairment of object recognition task in adult mice

Artigo publicado no periódico *Progress in Neuro-Psychopharmacology & Biological Psychiatry 35: 169–176, 2011*

Progress in Neuro-Psychopharmacology & Biological Psychiatry 35 (2011) 169–176

Contents lists available at ScienceDirect

Progress in Neuro-Psychopharmacology & Biological Psychiatry

journal homepage: www.elsevier.com/locate/pnp

Blockade of adenosine A_1 receptors prevents methylphenidate-induced impairment of object recognition task in adult mice

Sabrina Mioranzza [a], Marcelo S. Costa [a], Paulo Henrique S. Botton [a], Ana Paula Ardais [a], Vanessa Lague Matte [a], Janaina Espinosa [a,b], Diogo O. Souza [a], Lisiane O. Porciúncula [a,b,*]

[a] Federal University of Rio Grande do Sul, Health and Basic Sciences Institute, Department of Biochemistry, Graduation Program in Biological Sciences — Biochemistry, Laboratory of Studies on the Purinergic System, Porto Alegre/RS, Brazil 90035-003
[b] Graduation Program in Biological Sciences — Neurosciences, Porto Alegre/RS, Brazil 90035-003

ARTICLE INFO

Article history:
Received 13 August 2010
Received in revised form 25 October 2010
Accepted 25 October 2010
Available online 31 October 2010

Keywords:
Adenosine receptors
Attention-deficit/hyperactivity disorder
Memory
Methylphenidate

ABSTRACT

Methylphenidate (MPH) is the preferred treatment used for attention-deficit/hyperactivity disorder (ADHD). Recently, misuse for MPH due to its apparent cognitive enhancer properties has been reported. Adenosine is a neuromodulator known to exert influence on the dopaminergic neurotransmission, which is the main pharmacological target of MPH. We have reported that an overdosage of MPH up-regulates adenosine A_1 receptors in the frontal cortex, but this receptor was not involved in its anxiolytic effects. In this study, the role of adenosine A_1 receptor was investigated on MPH-induced effects on aversive and recognition memory in adult mice. Adult mice received acute and chronic (15 days) administration of methylphenidate (5 mg/kg, i.p.), or an acute overdosage (50 mg/kg, i.p) in order to model misuse. Memory was assessed in the inhibitory avoidance and object recognition task. Acute administration 5 mg/kg improved whereas 50 mg/kg disrupted recognition memory and decreased performance in the inhibitory avoidance task. Chronic administration did not cause any effect on memory, but decreased adenosine A_1 receptors immunocontent in the frontal cortex. The selective adenosine A_1 receptor antagonist, (DPCPX 1 mg/kg, i.p.), prevented methylphenidate-triggered recognition memory impairment. Our findings showed that recognition memory rather than aversive memory was differently affected by acute administration at both doses. Memory recognition was fully impaired by the overdosage, suggesting that misuse can be harmful for cognitive functions. The adenosinergic system via A_1 receptors may play a role in the methylphenidate actions probably by interfering with dopamine-enhancing properties of this drug.

1. Introduction

Methylphenidate (MPH) has a long history of being an effective medication for attention-deficit/hyperactivity disorder (ADHD). The increase in stimulant prescriptions has resulted in a corresponding intensification of illicit use, particularly among college students (Advokat et al., 2008; Rabiner et al., 2009; Teter et al., 2006).

The nonmedical use of MPH has increased in the American college setting because most students report using stimulant medications in an attempt to enhance academic performance, specifically to increase concentration, organization, and the ability to stay up longer and study (Dupont et al., 2008; Godfrey, 2009). Besides, stimulants are also consumed for recreational reasons and they are often in combination with alcohol. Although stimulant therapy in childhood does not increase the risk for subsequent drug abuse in youth with ADHD (Barkley et al., 2003; Wilens et al., 2003), the recent escalation in use among adolescents and young adults has raised concern about the prevalence of stimulant diversion and misuse, and initiated debate about the ethical implications of using drugs to improve academic performance.

Recently, a systematic review was focused on MPH administration on cognitive functions in healthy humans, and the available data and the analysis performed do not allow for a conclusion to be drawn about its cognitive enhancer property (Repantis et al., 2010). In rodents, recent developmental studies demonstrating MPH effects on object recognition memory (Britton et al., 2007; Heyser et al., 2004; LeBlanc-Duchin and Taukulis, 2007) and memory for learned contextual fear associations (Britton et al., 2007) suggest that hippocampal-sensitive tasks are affected by MPH exposure during adolescence.

Similar to other psychostimulants, the dopaminergic system is one of the main targets of pharmacological action for MPH (Gatley et al.,

Abbreviations: ADHD, Attention-deficit/hyperactivity disorder; DMSO, dimethyl sulfoxide; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; MPH, Methylphenidate; SHR, Spontaneously hypertensive rats; VMAT2, Vesicular monoamine transporter 2.

* Corresponding author. Federal University of Rio Grande do Sul, Health and Basic Sciences Institute, Department of Biochemistry, Laboratory of Studies on the Purinergic System, Rua Ramiro Barcelos, 2600 anexo, Bairro Santana, Porto Alegre/RS, Brazil 90035-003. Tel.: +55 51 3308 5556; fax: +55 51 3308 5540.
E-mail address: loporciuncula@yahoo.com (L.O. Porciúncula).

0278-5846/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.pnpbp.2010.10.022

1999), albeit the participation of other neurotransmitters may also take place (Pascoli et al., 2005; Prieto-Gómez et al., 2005). Adenosine is a neuromodulator in the central nervous system (CNS), which via mainly adenosine A_1 and A_{2A} metabotropic receptors control synaptic transmission of neurotransmitters such as dopamine and glutamate (Cunha, 2001).

Adenosine A_1 receptors are expressed throughout the brain while adenosine A_{2A} are more restricted to the basal ganglia. The existence of selective heteromerization of A_{2A} and D_2 receptors and A_1 and D_1 receptors was firstly demonstrated in transfected cells followed by biochemical analysis (Agnati et al., 2003; Canals et al., 2003; Ginés et al., 2000), therefore demonstrating the existence of A_{2A}–D_2 and A_1–D_1 receptor heteromers in the brain (Ferré et al., 1997; Franco et al., 2007). The A_1–D_1 heteromeric receptor complex may therefore give the molecular basis for the well-documented antagonistic A_1–D_1 receptor/receptor interactions found in the neuronal networks of the brain (Ferré et al., 1997; Fuxe et al., 1998). The understanding of these receptor/receptor interactions has been useful for the development of novel treatments for some neuropathologies that include ADHD, Parkinson's disease, dyskinesias, schizophrenia, and drug addiction (for reviews see Ferré et al., 2008; Fuxe et al., 2007; Maggio et al., 2009).

The participation of adenosinergic system in the ADHD has been suggested in studies with spontaneously hypertensive rats (SHR) as an experimental model of ADHD. Caffeine (a non-selective adenosine receptors antagonist) and selective adenosine receptors antagonists reversed the memory impairment in this strain (Pires et al., 2009; Prediger et al., 2005).

The involvement of adenosine receptors on MPH-mediated behavioral alterations has been reported in studies where administration of caffeine induces cross tolerance and cross desensitization to MPH (Boeck et al., 2009; Jain and Holtzman, 2005). In addition, our group has reported that acute administration of MPH increases adenosine A_1 receptors density in the frontal cortex but its blockade did not blunt MPH-induced anxiolytic effect (Mioranzza et al., 2010). Apart from these studies, possible modifications in the adenosinergic system in the MPH-mediated effects are still incipient.

Considering the misuse of MPH as a cognitive enhancer, the present study was designed to assess if acute and chronic treatment MPH and an acute overdosage of MPH in adult mice could promote beneficial effects on aversive and recognition memory. Besides, the involvement of adenosine A_1 receptors was investigated in the MPH-mediated behavioral alterations.

2. Methods

2.1. Animals

Male albino CF1 mice (3–4 months old) were obtained from Stated Foundation for Health Science Research (FEPPS, Porto Alegre/RS, Brazil). All experimental procedures were performed according to the NIH Guide for Care and Use of Laboratory Animals and to the Brazilian Society for Neuroscience and Behaviour (SBNeC) recommendations for animal care. Experimental procedures were approved by the ethical committee of Federal University of Rio Grande do Sul and all adequate measures were taken to minimize pain or discomfort. Mice were housed in standard cages (4 animals per cage) under a reversed 12/12 h–light/dark cycle with free access to food and water. The lights are turned on at 7:00 p.m. All behavioral tests were performed between 8:00 am and 5:00 p.m. Separate groups of mice were used for each behavioral task.

2.2. Drugs

A single injection of methylphenidate hydrochloride [MPH, 5 or 50 mg/kg, i.p., diluted in saline (0.9 g %, i.p) with a drop of Tween 20] or saline was administered to mice immediately after training sessions for behavioral analysis. For chronic treatment, MPH (5 mg/kg, i.p) was administered during 15 consecutive days. MPH 5 mg/kg has been widely used in behavioral studies in mice at different ages (Guerriero et al., 2006; McFadyen-Leussis et al., 2004). In order to avoid possible acute effects of the drug, the last injection was performed 12 h before mice had been submitted to behavioral tests. The adenosine A_1 receptor selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 1 mg/kg, i.p) (Tocris, São Paulo, Brazil) was used and prepared at the day of experiments from a stock solution diluted in DMSO (10% v/v) plus saline. This dose was chosed based on previous studies in which no behavioral alterations were observed (El Yacoubi et al., 2000; Mioranzza et al., 2010; Pires et al., 2009). Control mice received saline (0.9 g %) with a drop of Tween 20 or saline (0.9 g %). The final solution of DPCPX administered to mice contained DMSO 0.1% diluted in saline (0.9 g %).

2.3. Inhibitory avoidance task

Methylphenidate hydrochloride (MPH, 5 or 50 mg/kg, i.p) was administered to mice immediately after training session. For chronic treatment, MPH (5 mg/kg, i.p) was administered during 15 consecutive days and the last injection was performed 12 h before mice had been submitted to training session.

The inhibitory avoidance task was assessed in an apparatus that consisted of an acrylic box (50×25×25 cm) whose floor contains parallel caliber stainless-steel bars (1 mm diameter) spaced 1 cm apart. A platform (2 cm high and 4 cm×6 cm wide) was placed in the center of the box. In the training session, mice were placed on the platform and the latency to step-down on the floor with the four paws was measured with an automatic device immediately after stepping-down mice received a 0.5 mA, 2 s foot shock. After they had received the foot shock, mice were immediately placed back in their home cage. The test session was carried out 90 min after training (short-term memory) or 24 h after training (long-term memory). No foot shock was given in the test session, and step-down latencies (180 s ceiling) were taken as a measure of retention.

2.4. Object recognition task

DPCPX was administered thirty minutes prior to the training session. A single injection of methylphenidate hydrochloride (MPH 5 or 50 mg/kg, i.p) was administered to mice immediately after training sessions. For chronic treatment, MPH (5 mg/kg, i.p) was administered during 15 consecutive days. In order to avoid possible acute effects of MPH, the last injection was performed 12 h before mice had been submitted to training session. The object recognition task was performed according to previously reported (Costa et al., 2008) and following the guidelines previously recommended (Bevins and Besheer, 2006). The apparatus consisted of a painted wood small chamber with the following dimensions: 25×25 cm; (lenght×width). Mice had been acclimated in the apparatus during ten minutes twenty-four hours before training session. The training session consisted of placing a mouse in the apparatus containing two identical objects, and allowed it to explore for 10 min. The objects were positioned in two adjacent corners, 9 cm from the wall. Each mouse was always placed in the apparatus facing the wall. The test session was performed 90 min after training, and two dissimilar objects were present, the familiar (one of the objects used in the training session) and a novel one. Both objects presented similar textures, colors and sizes, but different shapes in the test session (Duplo Lego toys). The objects and the apparatus were cleaned with 10% ethanol solution between trials. Exploration was defined as directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose or forepaws. Sitting or leaning on the object without focused was not considered as exploratory behavior. For training session the index was calculated by

the ratio between the time spent on the object that will be the familiar in the test session and the total time of exploration. Considering that during training session both objects are novel, the time spent on both objects should be similar and the recognition index should be around 0.5. For calculating training index, the time spent on the object that will be the familiar in the test session was used. Recognition index for the novel object each mouse was expressed by TN / (TN + TF) ratio [TF = time spent exploring familiar object; TN = time spent exploring the novel object]. Two experienced observers blind to the drug treatment performed the behavioral analysis.

2.5. SDS-PAGE (sodium dodecyl sulfate-polyacrilamide) immunoblotting

Twenty-four hours after behavioral tests, mice were sacrificed by cervical displacement; the hippocampus and pre-frontal cortex were dissected out and immediately homogenized in 5% SDS with a protease inhibitor cocktail (Sigma, São Paulo/Brazil) and frozen at −70 °C. After defrost, the protein content was determined by Bicinchoninic acid assay using bovine serum albumin (BSA) as standard (Pierce, São Paulo/Brazil). Samples extracts were diluted to a final protein concentration of 2 μg/μL in SDS-PAGE buffer. Forty micrograms of the samples and prestained molecular weight standards (Bio-Rad, São Paulo/Brazil) were separated by SDS-PAGE (12% with 4% concentrating gel). After electro-transfer, the membranes were blocked with Tris-buffered saline 0.1% Tween-20 (TBS-T) containing 3% BSA for 1 h. The membranes were then incubated for 24 h at 4 °C with rabbit anti-adenosine A_1 receptor antibody (1:1000; Affinity Bioreagents, U.S.A). After primary antibodies incubation, membranes were washed and incubated with horseradish-peroxidase conjugated secondary antibodies for 2 h at room temperature and developed with chemiluminescence ECL kit (Amersham, São Paulo/Brazil). The autoradiographic films were scanned and densitometric analyses were performed using public domain NIH Image Program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/). The control of protein loading was carried out with Ponceau S. stain. Membranes with Ponceau S. were scanned at 37 kDa and the values used to obtain adenosine A_1 receptor density/Ponceau S. density ratio. No differences were found in the amount of protein loaded (data not shown).

2.6. Statistical analysis

For the inhibitory avoidance task some animals reached the ceiling of 180 s and the data distribution did not follow Gaussian curve. Thus, the step-down latencies were expressed as medians (interquartile ranges) and non-parametric analysis was performed by using Wilcoxon test for differences between training and test latencies of the same group. Kruskal–Wallis followed by Dunn's Multiple comparison test was used to compare treatments. For object recognition test, two-way ANOVA (treatment × trials) as repeated measures (as independent variables) was performed. For immunoblotting, data were analyzed by using Student's t-test between groups. Graphpad Prism 5 and SPSS were the softwares used and significant differences were considered when $P<0.05$.

3. Results

3.1. Acute administration of methylphenidate in the inhibitory avoidance task performance

The administration of MPH 5 mg/kg immediately after training did not modify the latencies between training and test compared to saline-treated mice when test session was performed 90 min (Fig. 1A) as well as 24 h after training (Fig. 1B). However, latencies were statistically different between mice treated with an overdosage of

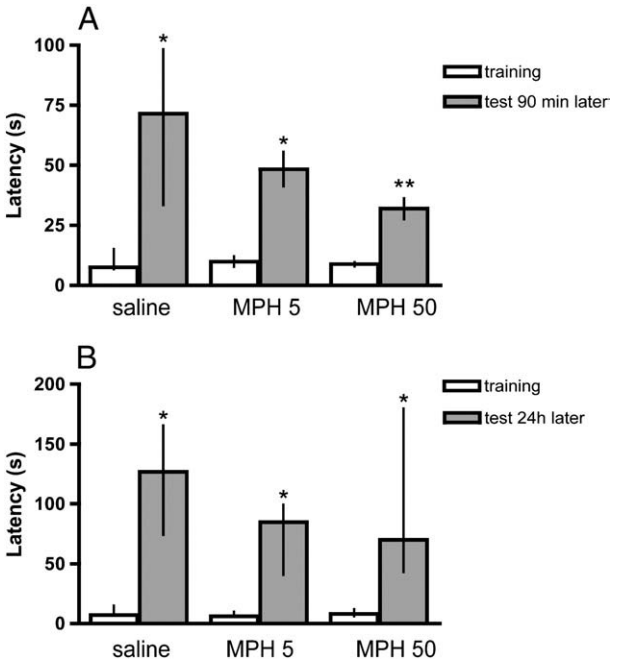


Fig. 1. Acute treatment with methylphenidate (MPH) on the performance in the inhibitory avoidance task. Adult mice receive a single injection of MPH (5 or 50 mg/kg, i.p) or vehicle immediately after training session. A — Test session performed 90 min after training (short-term memory). Results are median and interquartile ranges of step-down latency in seconds (s) from 11 to 12 mice per group. B — Test session performed 24 h after training (long-term memory). Results are median and interquartile ranges of step-down latency in seconds (s) from 11 to 12 mice per group. $^{**}P<0.05$; different from latencies obtained in the test session for saline-treated mice (Kruskal–Wallis followed by Dunn's post hoc test). $^{*}P<0.05$; differences between latencies from training and test sessions within group (Wilcoxon test).

MPH 50 mg/kg and saline only for test session performed 90 min (Fig. 1A) but not for 24 h after training (Fig. 1B).

3.2. Chronic administration of methylphenidate in the inhibitory avoidance task performance

Mice submitted to the chronic treatment with MPH 5 mg/kg were also evaluated in the inhibitory avoidance task. The latencies of training and test session between saline and MPH-treated mice were not statistically significant for test session performed 90 min after training as well as for 24 h (data not shown).

3.3. Acute treatment of methylphenidate in the object recognition task

The influence of chronic and acute treatment with MPH 5 mg/kg and an acute overdosage of 50 mg/kg was investigated in the novel object recognition task that consists on a non-aversive task. Two-way ANOVA analysis for the total exploration time in both objects for acute administration of MPH 5 mg/kg revealed a significant effect of trials (as repeated measures) [$F(1,41)=46.88$; $P<0.001$] but no significant interaction between trials and treatment (Fig. 2A). As a normal behavior, mice spent less time on the familiar object in the test session when comparing to training. Two-way ANOVA treatment × trials (as repeated measures) revealed only a significant main effect of trials [$F(1,41)=77.59$; $P<0.001$] (Fig. 2B). For the object recognition index, two-way ANOVA revealed a significant main effect of trials and significant interaction [$F(1,41)=4.24$; $P=0.0405$] (Fig. 2C). As observed, acute administration of MPH 5 mg/kg caused an increase in the object recognition index.

The acute administration of MPH 50 mg/kg did not affect the total exploration time in both objects since two-way ANOVA analysis revealed only significant effect of trials [$F(1,20)=88.87$; $= P<0.001$]

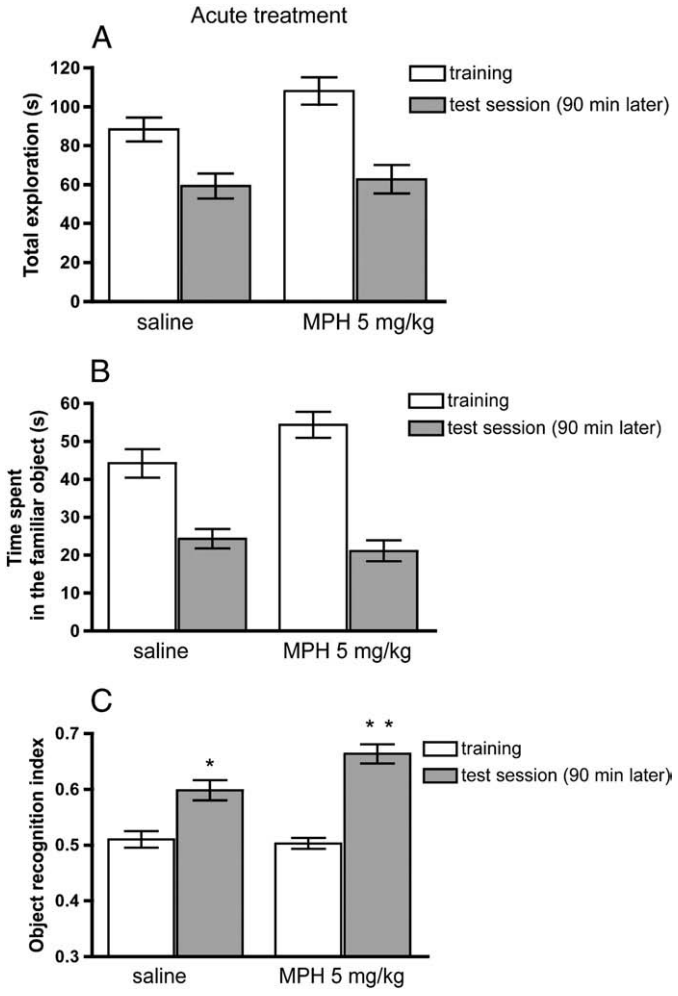


Fig. 2. Acute treatment with methylphenidate (MPH) on the performance in the object recognition task. Adult mice received a single injection of MPH (5 mg/kg, i.p) or vehicle immediately after training session. A – Total time spent in both objects during training and test session performed 90 min after training. Results are mean ± S.E.M from 21 mice per group. B — Time spent in the familiar object in both sessions (training and test session) Results are mean ± S.E.M from 21 mice per group. C — Recognition index obtained from training and test session. Results are mean ± S.E.M from 21 per group mice. *$P<0.05$; differences within group (training and test session) (two-way ANOVA). **$P<0.05$; differences within groups and from saline-treated mice recognition index (two-way ANOVA).

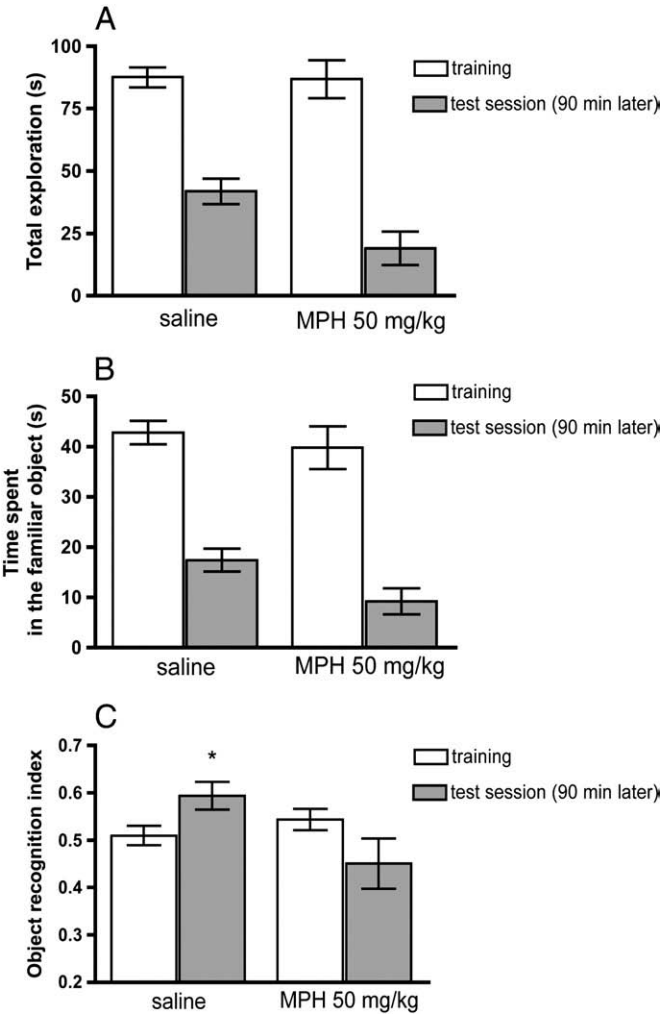


Fig. 3. Acute treatment with methylphenidate (MPH) on the performance in the object recognition task. Adult mice received a single injection of MPH (50 mg/kg, i.p) or vehicle immediately after training session. A — Total time spent in both objects during training and test session performed 90 min after training. Results are mean ± S.E.M from 10 to 12 mice per group. B — Time spent in the familiar object in both sessions (training and test session) Results are mean ± S.E.M from 10 to 12 mice per group. C — Recognition index obtained from training and test session. Results are mean ± S.E. M from 10 to 12 mice per group. *$P<0.05$; different from MPH-treated mice and training session within group (two-way ANOVA).

(Fig. 3A). However, the interaction was almost significant [$F(1,20) = 3.35$; $P<0.09$]. The same result was observed for the time spent in the familiar object, since two-way ANOVA revealed only significant effect of trials [$F(1,20) = 97.88$; $= P<0.001$] (Fig. 3B). For the object recognition index, two-way ANOVA revealed a significant interaction [$F(1,20) = 7.07$; $P = 0.0151$] but no significant effect of trials (Fig. 3C). Thus, the overdosage of MPH 50 mg/kg decreased object recognition index in the test session.

3.4. Chronic treatment with methylphenidate in the object recognition task

The chronic treatment with MPH 5 mg/kg was also evaluated on recognition memory. Analysis of time spent on the familiar object revealed a significant effect of trials [$F(1,16) = 69.95$; $= P<0.001$] (Fig. 4A). Likewise, two-way ANOVA analysis for recognition index also revealed only a significant effect of trials [$F(1,16) = 5.84$; $= P = 0.0279$] (Fig. 4B).

3.5. The effect of blockade of adenosine A_1 receptors on MPH-induced recognition memory impairment

The involvement of adenosine A_1 receptors in the recognition memory impairment by MPH 50 mg/kg was investigated with the selective adenosine A_1 receptor antagonist DPCPX (1 mg/kg, i.p) administered 30 min before training session. MPH (50 mg/kg, i.p) was administered immediately after training. According to previous data, MPH 50 mg/kg did not modify the time spent in the familiar object and administration of DPCPX alone did not cause any effect (data not shown). Two-way ANOVA analysis of the recognition index revealed a significant effect of trials [$F(1,48) = 33.42$; $P<0.001$] and interaction [$F(1,48) = 3.47$; $P = 0.0206$]. Consequently, pre administration of DPCPX prevented the decrease on the recognition index caused by post training administration of MPH 50 mg/kg (Fig. 5).

3.6. Chronic administration of methylphenidate on adenosine A_1 receptors immunocontent

Immunoblotting analysis was carried out in the frontal cortex and hippocampus homogenates from mice treated chronically with saline

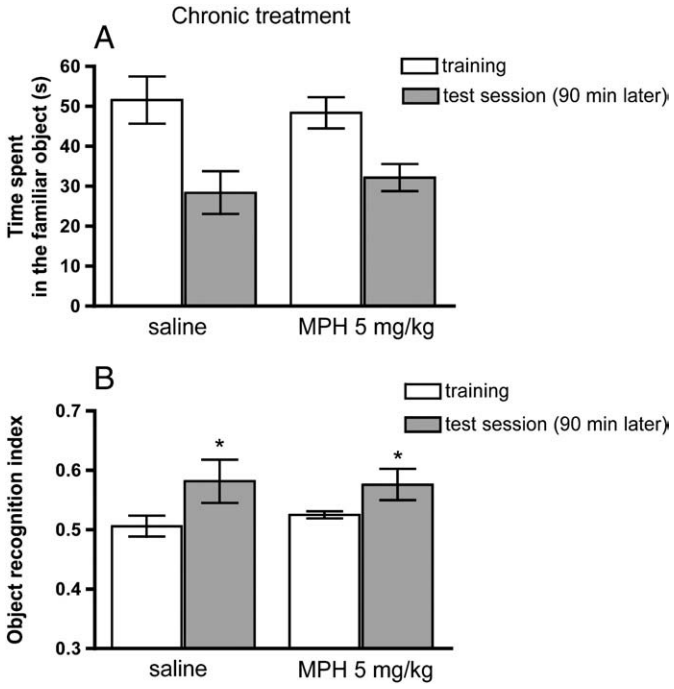


Fig. 4. Chronic treatment with methylphenidate (MPH) on the performance in the object recognition task. Adult mice received a single injection of MPH 5 mg/kg or vehicle during 15 consecutive days. The last dose of MPH was administered 12 h prior to training session. A — Time spent in the familiar object in both sessions (training and test session). Results are mean ± S.E.M from 9 mice per group. B — Recognition index obtained for training and test session. Results are mean ± S.E.M from 9 mice per group. *$P<0.05$; different from training session within group (two-way ANOVA).

or MPH (5 mg/kg) ($t=0.53$; $P>0.05$). Chronic treatment with MPH 5 mg/kg decreased the density of adenosine A_1 receptor only in the hippocampus (15%) compared to saline-treated mice (Fig. 6) ($t=2.855$; $P=0.03$).

4. Discussion

In this study, the acute and chronic administration as well as an overdosage of methylphenidate caused behavioral alterations in adult animals. Here, acute and chronic administration of MPH 5 mg/kg and an acute overdosage to model misuse presented distinct effects according to the task used for evaluating memory. Besides, the role of adenosine A_1 receptors was involved in the effects of MPH on recognition memory.

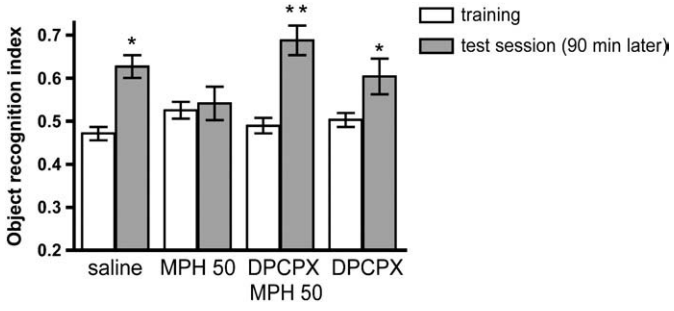


Fig. 5. Blockade of adenosine A_1 receptors prevents MPH-induced impairment on memory recognition. DPCPX (1 mg/kg, i.p) was administered 30 min before training session. MPH (50 mg/kg, i.p) was administered immediately after training. Recognition index obtained from training and test session performed 90 min later. Results are mean ± S.E.M from 12 to 15 mice per group. *$P<0.05$; different from training session within group (two-way ANOVA). **$P<0.05$; different from recognition index in the test session obtained for MPH-treated mice (two-way ANOVA).

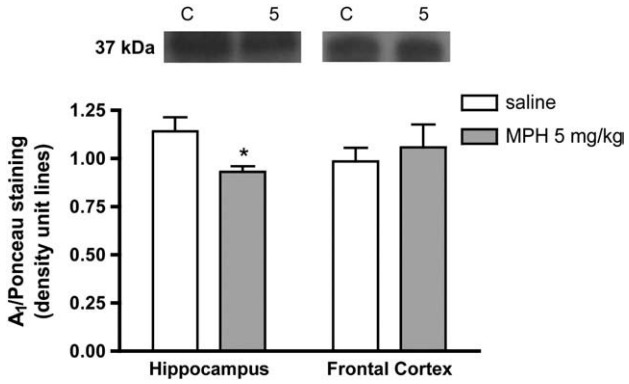


Fig. 6. Immunoblotting analysis of the adenosine A_1 receptor density in the frontal cortex and hippocampus from adult mice. Mice chronically treated either with vehicle or methylphenidate (MPH, 5 mg/kg, i.p. 15 days). Data are mean ± S.E.M of density unit lines obtained for adenosine A_1 receptor/Ponceau staining ($n=5$ mice/group for hippocampus), ($n=6$ mice/group for frontal cortex). At the top of figure are representative bands for adenosine A_1 receptor at 37 kDa (saline-treated mice; 5-methylphenidate-treated mice). *$P<0.05$, means significant difference between MPH- and saline-treated mice (Student's t-test).

Recognition memory was assessed in the novel object recognition task, which deals with the natural ability of animals to explore novelties. In this study, chronic administration of methylphenidate in a dose used in behavioral studies with juvenile mice did not cause any effect on both types of memory in adult mice whereas acute administration improved recognition memory. One explanation for this improvement could be attributed to both dopamine and norepinephrine-enhancing properties of the drug. Stimulant drugs, such as methylphenidate, raise extracellular dopamine levels supposedly by blockade of the dopamine transporter. This mechanism prevents reuptake of dopamine into the neuron, which results in higher extracellular dopamine levels (Madras et al., 2005; Volz et al., 2005). Additionally, acute administration of methylphenidate redistributes vesicular monoamine transporter 2 (VMAT2) protein from membrane associated vesicles fraction to cytoplasmic vesicles, which results in an increase in dopamine content in both fractions (Fleckenstein et al., 2009; Volz et al., 2008). Recent experimental evidence have demonstrated that dopamine is essential for memory consolidation in a variety of behavioral tasks, such as the hidden version of the water maze, the object–place association, and the one-trial inhibitory avoidance tasks (Dalley et al., 2005; Ferretti et al., 2005; Setlow and McGaugh, 2000). Besides, dopamine signaling is crucial for memory persistence as evidenced in the one-trial inhibitory avoidance task (Rossato et al., 2009).

Interestingly, the acute overdosage impaired both types of memory but the magnitude of memory impairment was dependent on the type of memory assessed, since recognition memory was fully impaired, while in the inhibitory avoidance task memory was partially impaired. In fact, in the inhibitory avoidance task mice treated with the overdosage of methylphenidate presented differences between training and test latencies indicating that they recalled the aversive stimuli (foot shock), but not at the same level of saline-treated mice. Therefore, MPH disrupts recognition memory and worsens the performance in the inhibitory avoidance task when short-term memory was assessed. Aversive and recognition memories share the functioning of some brain areas such as the hippocampus, ventral tegmental area, *striatum* and pre-frontal cortex. Differently from object recognition task, the amygdala is highly involved in memories with emotional component such as those assessed in the inhibitory avoidance task. The differences found for the effects of methylphenidate according to the type of memory assessed could be due to the fact that this drug administered intraperitoneally increases the extracellular dopamine levels in the striatum, nucleus accumbens and pre-frontal cortex, with no clear evidences of increase in the

amygdala (Bymaster et al., 2002; Koda et al., 2010). Recently, local administration of methylphenidate in the lateral amygdala enhanced cue-reward learning through dopamine D1 receptor-dependent mechanisms and suppressed task-irrelevant behavior through D2 receptor-dependent mechanisms. These findings suggest distinct roles for dopamine receptor subtypes in mediating methylphenidate-induced enhancements of neural transmission and learning performance (Tye et al., 2010). It is likely that overactivation of dopamine and norepinephrine may contribute to the disruption of learning and memory as well (for review see Arnsten, 2001).

The long-term memory was preserved by the overdosage of MPH probably due to the fast clearance of this drug, since pharmacokinetic analysis studies have shown that the half-life of MPH in rodents after intraperitoneal administration is estimated to be around 1 h (Thai et al., 1999). In fact, when long-term memory was assessed mice had received the injection at least 36 h prior to test session.

Chronic administration of MPH 5 mg/kg in adult mice did not cause any effect on both types of memory. Conflicting results with chronic administration of MPH in different dosing regimen and age have been reported. For example, the administration of similar regular dose in previous studies has shown to impair recognition memory, but the age (periadolescent rats) at the beginning of treatment differs from our study (Heyser et al., 2004; LeBlanc-Duchin and Taukulis, 2007). However, adult rats treated with MPH 5 and 10 mg/kg showed impairment on recognition memory but this effect was evident only 14 days later (LeBlanc-Duchin and Taukulis, 2009). In a recent study, rats presented lower performance in the Water maze test after they had received MPH (2 mg/kg) from the 15th to the 45th day of age (Scherer et al., 2010). However, male and female rats treated with 3 mg/kg of MPH from the 22nd to the 39th day of age presented an improvement on the radial arm maze performance after seven days of treatment (Zhu et al., 2007). The schedule of administration, differences between animal species (rats versus mice), age at the beginning of treatments and strains may take part of the discrepancies found between behavioral findings. Prominently, animals treated with MPH presented distinct behavior depending on the circadian cycle. Similar to our findings, in the one-trial inhibitory avoidance task chronic treatment of adult rats during 28 days with MPH (2 mg/kg) did not cause any effect, but in a multiple trial protocol long-term memory was impaired when animals were tested at night (Gomes et al., 2010). Interestingly, in the same study the long-term memory in young rats was impaired in the one-trial as well as in the multiple trial of the inhibitory avoidance task. As ontogenetic differences are considered one of the main factors responsible for distinct psychopharmacological sensitivity in a variety of species, adolescent rodents seem to be more sensitive than their adult counterparts to effects caused by psychostimulants (for review see Spear, 2000).

Alterations in the locomotor activity of mice could interfere in the performance of the object recognition task, but previous results from our group revealed that the same dose and schedule of administration did not alter locomotor activity (Mioranzza et al., 2010). However, a decrease on novelty seeking or anhedonia caused by an overdosage of methylphenidate could not be discarded as well as the possibility that methylphenidate affected motivation, attention, sensorimotor function, or memory retrieval. Indeed, previous study showed that adult rats exposed to repeated doses of methylphenidate during their juvenile were less responsive with respect to motor activation exhibited by animals when first exposed to a novel environment (Bolaños et al., 2003).

In our previous study, anxiolytic-like effect caused by MPH was not blunted by the blockade of adenosine A_1 receptors and there was an up-regulation in the frontal cortex of this receptor by an overdosage of methylphenidate (Mioranzza et al., 2010). Thus, the up-regulation of this receptor could be involved in the impairment of recognition memory observed in this study. Although chronic administration of MPH was devoid of effect on memory, the immunocontent of adenosine A_1 receptors was decreased in the hippocampus. Overall, it remains to be determined if acute treatment with MPH could increase brain adenosine levels and a continuous administration could trigger the desensitization of adenosine A_1 receptors. It is interesting to note that caffeine, another well-known psychostimulant and a non-selective adenosine antagonist, also up-regulates adenosine A_1 receptors (Svenningsson et al., 1999).

The stimulation of adenosine receptors counteracts the behavioral effects of dopamine receptor stimulation (Cao et al., 2007; Ferré et al., 1997). Likewise, adenosine receptor agonists counteract whereas adenosine receptor antagonists potentiate pharmacological effects of psychostimulants like cocaine and amphetamines (Poleszak and Malec, 2003; Popoli et al., 1994; Rimondini et al., 1998). Particularly, behavioral alterations caused by other psychostimulants acting on dopaminergic system were related to adenosine A_1 receptors (Kuzmin et al., 1999; Poleszak and Malec, 2003). Our results are in agreement with recent report where DPCPX did not promote any effect on recognition memory, but its administration was effective in ameliorating the impairment of novel object recognition in spontaneously hypertensive rats (SHR), used as a model of ADHD (Pires et al., 2009).

In human adults with and without ADHD, there are only few studies that have examined the effects of methylphenidate on cognitive functions. In adult ADHD some studies found methylphenidate to improve working memory (Kurscheidt et al., 2008; Mehta et al., 2000; Turner et al., 2005). In healthy subjects, methylphenidate enhanced performance in a test of spatial working memory and planning whereas impaired attention and fluency tests (Elliott et al., 1997). In our study, improvement on recognition memory was observed with a single dose of methylphenidate acutely administered while chronic administration did not show any effect. Therefore, our findings suggest that chronic use of MPH as a cognitive enhancer did not offer evident advantages for healthy animals.

5. Conclusion

As a cognitive enhancer, our data showed that methylphenidate acutely administered in a single dose promoted improvement on recognition memory. However, the overdosage caused disturbances in aversive as well as non-aversive memories at least when mice were under influence of this drug, suggesting that misuse of MPH may impair important cognitive functions. The chronic administration did not promote neither detrimental nor beneficial effects on memory. Importantly, methylphenidate-induced impairment on recognition memory involved adenosine A_1 receptors suggesting that this receptor plays a role in the mnemonic deficits caused by MPH. Since adenosine is a neuromodulator that controls the dopaminergic neurotransmission, which is one of the main pharmacological targets of MPH, it is important to detail the participation of adenosinergic system in the ADHD and methylphenidate-mediated actions in the CNS.

Disclosure of conflicts of interest

The authors have no disclosures to declare.

Acknowledgements

This work was supported by CNPq (Lisiane O. Porciúncula, Proc. no. 472216/2009-0), IBN.net (Neuroscience Brazilian Network), CNPq/INCTEN, and FAPERGS/PRONEX. Vanessa Lague Matte was supported by FAPERGS fellowship.

References

Advokat C, Martino L, Guidry D. Licit and illicit use of attention-deficit hyperactivity (ADHD) medication by college students. J Am Coll Health 2008;56:601–6.

Agnati LF, Ferré S, Lluis C, Franco R, Fuxe K. Molecular mechanisms and therapeutical implications of intramembrane receptor/receptor interactions among heptahelical receptors with examples from the striatopallidal GABA neurons. Pharmacol Rev 2003;55:509–50.

Arnsten AFT. Dopaminergic and noradrenergic influences on cognitive functions mediated by prefrontal cortex. In: Solanto MV, Arnsten AFT, Castellanos FX, editors. Stimulant drugs and ADHD: basic and clinical neuroscience. New York: Oxford University Press; 2001, pp. 185–208.

Barkley RA, Fletcher K, Fischer M, Smallish L. Does the treatment of attention-deficit/hyperactivity disorder with stimulants contribute to drug use/abuse? A 13-year prospective study. Pediatrics 2003;111:97-109.

Bevins RA, Besheer J. Object recognition in rats and mice: a one-trial non-matching-to-sample learning task to study 'recognition memory'. Nat Protoc 2006;1:1306–11.

Boeck CR, Marques VB, Valvassori SS, Constantino LC, Rosa DV, Lima FF. Early long-term exposure with caffeine induces cross-sensitization to methylphenidate with involvement of DARPP-32 in adulthood of rats. Neurochem Int 2009;55:318–22.

Bolaños CA, Barrot M, Berton O, Wallace-Black D, Nestler EJ. Methylphenidate treatment during pre- and periadolescence alters behavioral responses to emotional stimuli at adulthood. Biol Psychiatry 2003;54:1317–29.

Britton GB, Segan AT, Sejour J, Mancebo S. Early exposure to methylphenidate increases fear responses in an aversive context in adult rats. Dev Psychobiol 2007;49:265–75.

Bymaster FP, Katner JS, Nelson DL, Hemrick-Luecke SK, Threlkeld PG, Heiligenstein JH, et al. Atomoxetine increases extracellular levels of norepinephrine and dopamine in prefrontal cortex of rat: a potential mechanism for efficacy in attention deficit/hyperactivity disorder. Neuropsychopharmacology 2002;27:699–711.

Canals M, Marcellino D, Fanelli F, Ciruela F, de Benedetti P, Goldberg SR, et al. Adenosine A2A-dopamine D2 receptor–receptor heteromerization: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. J Biol Chem 2003;278:46741–9.

Cao Y, Xie KQ, Zhu XZ. The enhancement of dopamine D1 receptor desensitization by adenosine A1 receptor activation. Eur J Pharmacol 2007;562:34–8.

Costa MS, Botton PH, Mioranzza S, Ardais AP, Moreira JD, Souza DO, et al. Caffeine improves adult mice performance in the object recognition task and increases BDNF and TrkB independent on phospho-CREB immunocontent in the hippocampus. Neurochem Int 2008;53:89–94.

Cunha RA. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. Neurochem Int 2001;38:107–25.

Dalley JW, Lääne K, Theobald DE, Armstrong HC, Corlett PR, Chudasama Y, et al. Time-limited modulation of appetitive Pavlovian memory by D1 and NMDA receptors in the nucleus accumbens. Proc Natl Acad Sci 2005;102:6189–94.

Dupont RL, Coleman JJ, Bucher RH, Wilford BB. Characteristics and motives of college students who engage in nonmedical use of methylphenidate. Am J Addict 2008;17:167–71.

El Yacoubi M, Ledent C, Parmentier M, Costentin J, Vaugeois JM. The anxiogenic-like effect of caffeine in two experimental procedures measuring anxiety in the mouse is not shared by selective A(2A) adenosine receptor antagonists. Psychopharmacology (Berl) 2000;148:153–63.

Elliott R, Sahakian BJ, Matthews K, Bannerjea A, Rimmer J, Robbins TW. Effects of methylphenidate on spatial working memory and planning in healthy young adults. Psychopharmacology (Berl) 1997;131:196–206.

Ferré S, Fredholm BB, Morelli M, Popoli P, Fuxe K. Adenosine-dopamine receptor–receptor interactions as an integrative mechanism in the basal ganglia. Trends Neurosci 1997;20:482–7.

Ferré S, Quiroz C, Woods AS, Cunha R, Popoli P, Ciruela F, et al. An update on adenosine A2A-dopamine D2 receptor interactions: implications for the function of G protein-coupled receptors. Curr Pharm Des 2008;14:1468–74.

Ferretti V, Florian C, Costantini VJ, Roullet P, Rinaldi A, De Leonibus E, et al. Co-activation of glutamate and dopamine receptors within the nucleus accumbens is required for spatial memory consolidation in mice. Psychopharmacology 2005;179:108–16.

Fleckenstein AE, Volz TJ, Hanson GR. Psychostimulant-induced alterations in vesicular monoamine transporter-2 function: neurotoxic and therapeutic implications. Neuropharmacology 2009;56:133–8.

Franco R, Casado V, Cortes A, Ferrada C, Mallol J, Woods A, et al. Basic concepts in G-protein-coupled receptor homo- and heteromerization. Sci World J 2007;7:48–57.

Fuxe K, Ferré S, Zoli M, Agnati LF. Integrated events in central dopamine transmission as analyzed at multiple levels. Evidence for intramembrane adenosine A2A/dopamine D2 and adenosine A1/dopamine D1 receptor interactions in the basal ganglia. Brain Res Rev 1998;26:258–73.

Fuxe K, Ferré S, Genedani S, Franco R, Agnati LF. Adenosine receptor–dopamine receptor interactions in the basal ganglia and their relevance for brain function. Physiol Behav 2007;92:210–7.

Gatley SJ, Volkow ND, Gifford AN, Fowler JS, Dewey SL, Ding YS, et al. Dopamine-transporter occupancy after intravenous doses of cocaine and methylphenidate in mice and humans. Psychopharmacology (Berl) 1999;146:93-100.

Ginés S, Hillion J, Torvinen M, Le Crom S, Casadó V, Canela EI, et al. Dopamine D1 and adenosine A1 receptors form functionally interacting heteromeric complexes. Proc Natl Acad Sci USA 2000;97:8606–11.

Godfrey J. Safety of therapeutic methylphenidate in adults: a systematic review of the evidence. J Psychopharmacol 2009;23:194–205.

Gomes KM, Comim CM, Valvassori SS, Réus GZ, Inácio CG, Martins MR, et al. Diurnal differences in memory and learning in young and adult rats treated with methylphenidate. J Neural Transm 2010;117:457–62.

Guerriero R, Hayes M, Dhaliwal S, Ren J, Kosofsky B. Preadolescent methylphenidate versus cocaine treatment differ in the expression of cocaine-induced locomotor sensitization during adolescence and adulthood. Biol Psychiatry 2006;60:1171–80.

Heyser CJ, Pelletier M, Ferris JS. The effects of methylphenidate on novel object exploration in weanling and periadolescent rats. Ann NY Acad Sci 2004;1021:465–9.

Jain R, Holtzman SG. Caffeine induces differential cross tolerance to the amphetamine-like discriminative stimulus effects of dopaminergic agonists. Brain Res Bull 2005;65:415–21.

Koda K, Ago Y, Cong Y, Kita Y, Takuma K, Matsuda T. Effects of acute and chronic administration of atomoxetine and methylphenidate on extracellular levels of noradrenaline, dopamine and serotonin in the prefrontal cortex and striatum of mice. J Neurochem 2010;114:259–70.

Kurscheidt JC, Peiler P, Behnken A, Abel S, Pedersen AT, Suslow T, et al. Acute effects of methylphenidate on neuropsychological parameters in adults with ADHD: possible relevance for therapy. J Neural Transm 2008;115:357–62.

Kuzmin A, Johansson B, Zvartau EE, Fredholm BB. Caffeine, acting on adenosine A(1) receptors, prevents the extinction of cocaine-seeking behavior in mice. J Pharmacol Exp Ther 1999;290:535–42.

LeBlanc-Duchin D, Taukulis HK. Chronic oral methylphenidate administration to periadolescent rats yields prolonged impairment of memory for objects. Neurobiol Learn Mem 2007;88:312–20.

LeBlanc-Duchin D, Taukulis HK. Chronic oral methylphenidate induces post-treatment impairment in recognition and spatial memory in adult rats. Neurobiol Learn Mem 2009;91:218–25.

Madras BK, Miller GM, Fischman AJ. The dopamine transporter and attention-deficit/hyperactivity disorder. Biol Psychiatry 2005;57:1397–409.

Maggio R, Aloisi G, Silvano E, Rossi M, Millan MJ. Heterodimerization of dopamine receptors: new insights into functional and therapeutic significance. Parkinsonism Relat Disord 2009(Suppl 4):S2–7.

McFadyen-Leussis MP, Lewis SP, Bond TL, Carrey N, Brown RE. Prenatal exposure to methylphenidate hydrochloride decreases anxiety and increases exploration in mice. Pharmacol Biochem Behav 2004;77:491–500.

Mehta MA, Calloway P, Sahakian BJ. Amelioration of specific working memory deficits by methylphenidate in a case of adult attention deficit/hyperactivity disorder. J Psychopharmacol 2000;14:299–302.

Mioranzza S, Botton PH, Costa MS, Espinosa J, Kazlauckas V, Ardais AP, et al. Adenosine A_1 receptors are modified by acute treatment with methylphenidate in adult mice. Brain Res 2010;1357:62–9.

Pascoli V, Valjent E, Corbillé AG, Corvol JC, Tassin JP, Girault JA, et al. cAMP and extracellular signal-regulated kinase signaling in response to d-amphetamine and methylphenidate in the prefrontal cortex in vivo: role of beta 1-adrenoceptors. Mol Pharmacol 2005;68:421–9.

Pires VA, Pamplona FA, Pandolfo P, Fernandes D, Prediger RD, Takahashi RN. Adenosine receptor antagonists improve short-term object-recognition ability of spontaneously hypertensive rats: a rodent model of attention-deficit hyperactivity disorder. Behav Pharmacol 2009;20:134–45.

Poleszak E, Malec D. Effects of adenosine receptor agonists and antagonists in amphetamine-induced conditioned place preference test in rats. Pol J Pharmacol 2003;55:319–26.

Popoli P, Pèzzola A, de Carolis AS. Modulation of striatal adenosine A1 and A2 receptors induces rotational behaviour in response to dopaminergic stimulation in intact rats. Eur J Pharmacol 1994;257:21–5.

Prediger RD, Pamplona FA, Fernandes D, Takahashi RN. Caffeine improves spatial learning deficits in an animal model of attention deficit hyperactivity disorder (ADHD) — the spontaneously hypertensive rat (SHR). Int J Neuropsychopharmacol 2005;8:583–94.

Prieto-Gómez B, Vázquez-Alvarez AM, Martínez-Peña JL, Reyes-Vázquez C, Yang PB, Dafny N. Methylphenidate and amphetamine modulate differently the NMDA and AMPA glutamatergic transmission of dopaminergic neurons in the ventral tegmental area. Life Sci 2005;77:635–49.

Rabiner DL, Anastopoulos AD, Costello EJ, Hoyle RH, McCabe SE, Swartzwelder HS. The misuse and diversion of prescribed ADHD medications by college students. J Atten Disord 2009;13:144–53.

Repantis D, Schlattmann P, Laisney O, Heuser I. Modafinil and methylphenidate for neuroenhancement in healthy individuals: a systematic review. Pharmacol Res 2010;62:187–206.

Rimondini R, Ferré S, Giménez-Llort L, Ogren SO, Fuxe K. Differential effects of selective adenosine A1 and A2A receptor agonists on dopamine receptor agonist-induced behavioural responses in rats. Eur J Pharmacol 1998;347:153–8.

Rossato JI, Bevilaqua LR, Izquierdo I, Medina JH, Cammarota M. Dopamine controls persistence of long-term memory storage. Science 2009;325:1017–20.

Scherer EB, da Cunha MJ, Matté C, Schmitz F, Netto CA, Wyse AT. Methylphenidate affects memory, brain-derived neurotrophic factor immunocontent and brain acetylcholinesterase activity in the rat. Neurobiol Learn Mem 2010;94:247–53.

Setlow B, McGaugh JL. D2 dopamine receptor blockade immediately post-training enhances retention in hidden and visible platform versions of the water maze. Learn Mem 2000;7:187–91.

Spear LP. The adolescent brain and age-related behavioral manifestations. Neurosci Biobehav Rev 2000;24:417–63.

Svenningsson P, Nomikos GG, Fredholm BB. The stimulatory action and the development of tolerance to caffeine is associated with alterations in gene expression in specific brain regions. J Neurosci 1999;19:4011–22.

Teter CJ, McCabe SE, LaGrange K, Cranford JA, Boyd CJ. Illicit use of specific prescription stimulants among college students: prevalence, motives, and routes of administration. Pharmacotherapy 2006;26:1501–10.

Thai DL, Yurasits LN, Rudolph GR, Perel JM. Comparative pharmacokinetics and tissue distribution of the D-enantiomers of para-substituted methylphenidate analogs. Drug Metab Dispos 1999;27:645–50.

Turner DC, Blackwell AD, Dowson JH, McLean A, Sahakian BJ. Neurocognitive effects of methylphenidate in adult attention-deficit/hyperactivity disorder. Psychopharmacology (Berl) 2005;178:286–95.
Tye KM, Tye LD, Cone JJ, Hekkelman EF, Janak PH, Bonci A. Methylphenidate facilitates learning-induced amygdala plasticity. Nat Neurosci 2010;13:475–81.
Volz TJ, Bjorklund NL, Schenk JO. Methylphenidate analogs with behavioral differences interact differently with arginine residues on the dopamine transporter in rat striatum. Synapse 2005;57:175–8.
Volz TJ, Farnsworth SJ, Hanson GR, Fleckenstein AE. Methylphenidate-induced alterations in synaptic vesicle trafficking and activity. Ann NY Acad Sci 2008;1139:285–90.
Wilens TE, Faraone SV, Biederman J, Gunawardene S. Does stimulant therapy of attention-deficit/hyperactivity disorder beget later substance abuse? A meta-analytic review of the literature. Pediatrics 2003;111:179–87.
Zhu N, Weedon J, Dow-Edwards DL. Oral methylphenidate improves spatial learning and memory in pre- and periadolescent rats. Behav Neurosci 2007;121:1272–9.