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Uma abordagem neuroquímica, neurofuncional e comportamental sobre a influência do sexo e do tratamento com cafeína no modelo murino do Transtorno de Déficit de Atenção e Hiperatividade (TDAH)

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“If you can dream it, you can do it.”

Walt Disney

APRESENTAÇÃO

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

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

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

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

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

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

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PARTE I

RESUMO

O Transtorno de Déficit de Atenção e Hiperatividade (TDAH) é um transtorno neuropsiquiátrico clinicamente caracterizado por sintomas de desatenção, hiperatividade e impulsividade. É frequentemente mais diagnosticado em meninos devido à presença mais pronunciada dos sintomas de hiperatividade/impulsividade. Embora o fármaco metilfenidato seja a primeira escolha de tratamento, cerca de 30% dos pacientes diagnosticados com TDAH não respondem ou toleram o tratamento. Portanto, outras estratégias farmacológicas ainda se fazem necessárias para o tratamento dos sintomas do TDAH. A cafeína é um dos psicoestimulantes mais consumidos mundialmente e curiosamente pacientes diagnosticados com algum transtorno neuropsiquiátrico tendem a consumir mais cafeína do que a população normal. No caso do TDAH, os pacientes diagnosticados consomem cafeína de forma habitual com o objetivo de melhorar o estado de alerta e atenção e sua administração em modelos animais do TDAH se mostrou eficaz contra prejuízos cognitivos. No entanto, pouco foi investigado sobre os efeitos da cafeína considerando as diferenças de sexo no modelo animal do TDAH. Sendo assim, nesta tese buscou-se avaliar o efeito do tratamento com cafeína durante a infância e/ou adolescência em um modelo animal do TDAH em ambos os sexos. Foram analisados parâmetros comportamentais (atividades locomotoras e exploratórias, memória de reconhecimento, memória espacial e comportamento de tomada de decisão) bem como o imunoconteúdo de transportadores, receptores, proteínas sinápticas e fatores tróficos envolvidos no transtorno. Por fim, ainda realizamos registros da atividade elétrica do córtex frontal por meio de eletroencefalograma (EEG). Ratos SHR, de ambos os sexos, receberam cafeína (0,3 g / L) na água de beber a partir do dia 15 pós-natal (DPN) até 28 (infância) ou 55-59 (final da adolescência). No primeiro trabalho, os testes comportamentais foram realizados em duas fases, nos DPN 28-30 e nos 50-52. No segundo trabalhos, os testes foram realizados a partir do desmame. Nossos resultados mostraram uma hiperlocomoção, comprometimento da memória de reconhecimento e espacial em ratos SHR adolescentes de ambos os sexos, porém as fêmeas apresentaram um maior prejuízo na memória espacial de curta duração. A cafeína reverteu o comprometimento da memória de reconhecimento em ambos os sexos, e o prejuízo da memória espacial nas fêmeas SHR. O hipocampo dos animais SHR de ambos os sexos apresentou um aumento no imunoconteúdo do BDNF, na forma truncada e fosforilada do receptor TrkB e nos níveis de fosfo-CREB. A cafeína diminuiu o BDNF no hipocampo dos machos SHR e na forma truncada do receptor TrkB em ambos os sexos. No segundo trabalho, os animais SHR apresentaram hiperlocomoção, mas por meio de análise de componentes principais observamos que em alguns parâmetros os machos SHR foram mais hiperativos que as fêmeas. A cafeína foi capaz de reverter os parâmetros de hiperatividade tanto nos machos quanto nas fêmeas SHR. Na tarefa de cavar, utilizada para avaliar comportamentos de tomada de decisão, as fêmeas SHR apresentaram prejuízo tanto na fase de aprendizado quanto na fase de mudança de estratégia e flexibilidade de escolha entre duas opções. O tratamento com cafeína melhorou o desempenho nas fêmeas na fase de aprendizagem na tarefa de cavar, mas não na fase de mudança de estratégia e flexibilidade. Os machos SHR que tomaram cafeína tiveram melhor desempenho na fase de mudança de estratégia e flexibilidade. Em relação aos achados de imunoquímica, observamos um aumento no receptor de dopamina D4 e no transportador de dopamina no córtex frontal, pois os animais SHR de ambos os sexos apresentaram

diminuição nestas proteínas em preparações de membranas totais. O tratamento com cafeína previne a diminuição dos níveis de DAT em ambos os sexos, e nos níveis dos receptores D4 nos machos SHR. Dentre as principais alterações encontradas nas oscilações no córtex frontal foram as diminuições no poder de ondas delta nas fêmeas SHR em contraste com um aumento nos machos SHR durante a atividade locomotora. O poder de ondas gamma também foi diminuído nas fêmeas SHR durante a atividade locomotora. A cafeína diminui o poder de delta e de gamma nos machos SHR, tendo pouco efeito sobre as oscilações nas fêmeas SHR. Portanto, a cafeína foi capaz de alterar o receptor de dopamina D4, a atividade locomotora, a tarefa de tomada de decisão e as oscilações no córtex frontal de maneira dependente de sexo. Paralelamente observamos que os machos SHR apresentam mais hiperatividade e as fêmeas um pior desempenho em tarefas que requerem tomada de decisão e memória espacial. Com este trabalho evidenciamos a importância de estudar ambos os sexos separadamente não somente para o modelo do TDAH. Finalmente, nossos dados reforçam o potencial da cafeína como uma estratégia adjuvante ou para o tratamento do TDAH observando as particularidades de acordo com o sexo.

ABSTRACT

Attention Deficit Hyperactivity Disorder (ADHD) is a neuropsychiatric disorder clinically characterized by symptoms of inattention, hyperactivity and impulsivity. It is often more diagnosed in boys since they are more prone to show hyperactivity/impulsivity symptoms. Although methylphenidate is the first choice of treatment, about 30% of patients will not respond or tolerate the treatment. Thus, it is still under investigation other pharmacological alternatives for the treatment of ADHD symptoms. Caffeine is one of the most consumed psychostimulants worldwide. Curiously, neuropsychiatric patients tend to consume more caffeine than normal population. In the case of ADHD, diagnosed patients consume caffeine aiming to increase alertness state. In animal models of ADHD, caffeine was described to reverse cognitive and attentional impairments. However, sex differences about the beneficial effects of caffeine in ADHD model were less investigated. In this thesis, we sought to evaluate the effect of caffeine treatment during childhood and/or adolescence in both sexes of the ADHD model. Behavioral parameters (locomotor and exploratory activities, recognition memory, spatial memory and decision making behavior) were analyzed as well as the immunocontent of transporters, receptors, synaptic proteins and trophic factors involved in the disorder. Finally, we still record electrical activity of the frontal cortex by electroencephalogram (EEG). SHR rats of both sexes received caffeine (0.3 g / L, drinking water) from postnatal day 15 (PND) to 28 (childhood) or up to 55-59 (late adolescence). In the first study, behavioral tests were carried out at PND 28-30 and 50-52. In the second work, behavioral analysis started from PND 26 up to 55-59. Our results revealed hyperlocomotion, recognition and spatial memory impairment in adolescent SHR rats from both sexes, but female SHR presented a worsened spatial memory. Caffeine reversed recognition memory impairment in both sexes, and spatial memory impairment in SHR females, but exacerbated hyperlocomotion in SHR females. In the hippocampus from SHR of both sexes increases in the immunocontent of the BDNF, truncated and phosphorylated forms of the TrkB receptor also in the phospho-CREB levels were found. Caffeine normalized BDNF in males SHR males and the truncated form of TrkB receptor in both sexes. In the second study, although SHR animals have displayed hyperlocomotion, male SHR were slightly more hyperactive than females according to principal component analysis (PCA). Caffeine was able to reverse hyperactivity in both males and females SHR. Dig task was carried out in order evaluate decision-making behavior. Females SHR showed impairment in both discriminating and reversal phase of the task, which implicates in difficulties in the learning and of strategy change and flexibility phases. Caffeine improved performance in females SHR only in the learning phase while male SHR had improvements in the reversal phase. We also detected decreased in the dopamine D4 receptor and dopamine transporter in the frontal cortex from SHR animals of both sexes. Caffeine reversed the decreases in DAT levels in both sexes, and D4 receptor levels only in males SHR. Regarding frontal cortex oscillations decreases in delta wave power were found in females SHR while increases were detected in male SHR during locomotor activity. Gamma-wave power was also decreased in females SHR during locomotor activity. Caffeine decreased delta and gamma power in males SHR, and no evident effect was observed for females SHR. Therefore, caffeine changed dopamine D4 receptor levels, locomotor activity, decision-making task and frontal cortex oscillations in a sex-dependent manner. In parallel, we observed that males

SHR presented more hyperactivity while females SHR presented a worsened performance in decision making and spatial memory tasks.
In this thesis, we highlight the importance of studying both sexes in ADHD model as well as our data reinforce the potential of caffeine as an adjuvant strategy or treatment for ADHD taking into account sex differences.

LISTA DE ABREVIATURAS

A_1 - Receptor metabotrópico de adenosina do subtipo A_1

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

AMPc - AMP cíclico

BDNF - Fator neurotrófico derivado do encéfalo

CREB - proteína ligante ao elemento responsivo ao AMPc

D1 - Receptor de dopamina 1

D2 - Receptor de dopamina 2

D4 - Receptor de dopamina 4

D5 - Receptor de dopamina 5

DA - Dopamina

DAT - Transportador de dopamina

DPN - Dia pós-natal

DRD4 - Receptor de dopamina 4

EEG - Eletroencefalograma

p75NTR - Receptor Pan Neurotrofina

pró-BDNF - Forma precursora do BDNF

RNAm - RNA mensageiro

SCH58261 - Antagonista A_{2A}

SHR - do inglês *spontaneoulsy hypertensive rats* ou ratos espontaneamente hipertensos

SNAP-25 - Proteína associada ao sinaptossoma de 25 kDa (do inglês Synaptossomal-

Associated Protein 25)

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

SNC - Sistema nervoso central

TrkB - Receptor do tipo tirosina cinase B

TDAH - Transtorno de Déficit de Atenção e Hiperatividade

1. INTRODUÇÃO

1.1 Histórico do Transtorno de Déficit de Atenção e Hiperatividade (TDAH)

O primeiro relato sobre o Transtorno de Déficit de Atenção e Hiperatividade (TDAH) foi feito pelo pediatra George Frederick Still e publicado no periódico Lancet em 1902. Naquela ocasião, Still descreveu 43 crianças que apresentavam um defeito anormal de controle da moral, o qual estava associado a um “prejuízo do intelecto e atraso mental”. De acordo com relatos de pediatra, algumas crianças apresentavam uma inteligência “quase normal”, e ele ainda enfatizou a possibilidade desse “defeito” ser herdado ao nascer (revisado em Still et al., 2006). Essas crianças descritas por Still em 1906, provavelmente seriam diagnosticadas clinicamente com esse transtorno e, portanto, seriam consideradas as primeiras descrições médicas sobre o TDAH.

A partir desses relatos, inúmeros pesquisadores se voltaram para investigar as crianças que desenvolviam comportamentos semelhantes aos descritos por Still, mas com uma causa já estabelecida como uma lesão cerebral ou epilepsia. Entretanto, somente com a publicação do Manual Diagnóstico e Estatístico de Transtornos Mentais (do inglês, Diagnostic and Statistical Manual of Mental Disorders, Second Edition- DSM-II) que reação hipercinética ficou estabelecido como um termo oficial para descrever uma desordem "caracterizada por hiperatividade, inquietação, distração e uma atenção por pequenos períodos de tempo, especialmente durante a infância” (DSM II, Revisado em Doyle, 2004). Contudo, foi somente em 1970 que surgiu o termo Transtorno de Déficit de Atenção e Hiperatividade (TDAH, do inglês, Attention Deficit and Hyperactivity Disorder -ADHD), primeiramente descrito por Wender, que subdividiu esse “dano cerebral” nas seguintes categorias: 1) motor; 2)

cognitivo atenção-perceptual; 3) problemas de aprendizagem; 4) controle de impulso; 5) relações interpessoais e 6) emoção (Wender et al., 1971; revisado em Doyle, 2004). Entretanto, somente na terceira edição revisada do Manual Diagnóstico e Estatístico de Transtornos Mentais (DSM-III-R), que o TDAH foi realmente se distinguindo de outras desordens psiquiátricas e inserido na categoria das desordens de comportamentos disruptivos onde era apenas classificado quanto a sua severidade. Posteriormente, na quarta edição foi estabelecido que mais de dois sintomas deveriam estar presentes além de causar um prejuízo em mais de duas áreas cerebrais (Doyle et al., 2004).

O tratamento para este transtorno começou a ser estudado em 1937, quando o psiquiatra Charles Bradley administrou sulfato de benzidrina em pacientes classificados com problemas comportamentais. Porém, o intuito era aliviar as fortes dores de cabeça, mas foi observada uma melhora emocional tanto na escola como no convívio social desses pacientes. Anos mais tarde, esse medicamento foi reconhecido como o precursor do uso de anfetaminas para o tratamento do TDAH (revisado em Strohl et al., 2011). Décadas depois surgiu o metilfenidato com resultados promissores no tratamento de crianças com distúrbios comportamentais severos. Este medicamento, além de melhorar o comportamento, a motricidade e a atenção, não demonstrou um efeito tóxico significativo após seis meses de tratamento (Zimmerman et al., 1958).

Atualmente, os fármacos psicoestimulantes são a intervenção de primeira escolha para o alívio dos sintomas do TDAH (Pliszka, 2007; Sagvolden et al., 2008), sendo o próprio metilfenidato o mais utilizado há várias décadas em adultos e o mais recomendado para crianças e adolescentes (Seixas et al., 2012). O mecanismo de ação do metilfenidato é baseado no bloqueio dos transportadores de dopamina (DAT)

e de norepinefrina (NET) (Solanto, 1998). Este bloqueio pode chegar a uma redução de 75% da atividade dos transportadores (Volkow et al., 1998), aumentando então a disponibilidade de dopamina extracelular (Volkow et al., 2001), reforçando a sua eficácia terapêutica. Embora o seu uso permanece relativamente seguro a curto e médio prazos, alguns efeitos adversos são descritos, tais como: distúrbios do sono e apetite, problemas de crescimento, cardiovasculares e o desenvolvimento de distúrbios neuropsiquiátricos, sendo a maioria destes transitórios (revisados em Groenman et al., 2017; Huss et al., 2017). Cerca de 70% das crianças respondem positivamente ao metilfenidato, apresentando uma melhora dos sintomas (Genro et al., 2010), sendo considerada uma das drogas mais eficazes dentro da Psiquiatria e da Medicina (Leucht et al., 2012). Contudo, alguns pacientes não respondem ou não toleram este medicamento em função dos efeitos colaterais descritos (revisado em Wender et al., 1998, Wilens et al., 2002).

1.2 Prevalência do TDAH em âmbito mundial e no Brasil

Nas últimas décadas, crianças e adolescentes afetados por transtornos mentais têm sido o foco de inúmeros estudos. Uma meta-análise recente estimou que cerca de 13,4 % de crianças e adolescentes são afetados por transtornos mentais em âmbito mundial (Polanzyc et al., 2014), e em torno de 3,4% apresentam TDAH. Antigamente, acreditava-se que esse transtorno afetava igualmente todas as classes sociais independente da etnia, perfil socioeconômico e raça (Polanzyc e Jensen, 2008). Atualmente, sabe-se que o perfil socioeconômico e o histórico parental de TDAH são considerados fatores de risco importantes para o desenvolvimento deste transtorno (Rowland et al., 2017).

Evidências sugerem a persistência dos sintomas do TDAH durante a vida adulta, mesmo naqueles que tiveram remissão completa durante a adolescência. Em estudos de meta-análise longitudinal, pesquisadores encontraram uma prevalência em torno 15% quando era confirmado o diagnóstico completo (aqueles que preenchiam todos os critérios para serem classificados com TDAH), porém quando avaliaram juntamente com a remissão completa, essa prevalência aumenta para 40 a 60% (Faraone et al., 2006; Caye et al., 2016). No entanto, a prevalência geral entre a população adulta baseada somente no critério faixa etária é cerca de 2,5 a 3% (Simon et al., 2010; Caye et al., 2016; Katzman, et al. 2017), sendo que a maioria destes apresentam outra comorbidade associada (Katzman et al., 2017).

No Brasil, a ausência de estudos sobre a prevalência do transtorno, além da falta de padronização dos critérios, levou a pesquisadora Ana Guardiola et al. (2000), a fazer uma outra proposta que permitiu o estabelecimento de novos critérios de diagnóstico. Este estudo verificou uma prevalência de 18% deste transtorno em crianças do primeiro ano de diferentes escolas utilizando o DMS-III e essa prevalência diminuiu para 3.5% a 3.9% ao se incluir outros critérios. Diversos estudos foram realizados em escolas do Brasil demonstrando diferentes intervalos de prevalência. Rohde et al. (1999), avaliaram 94 escolas utilizando como critério diagnóstico o DSM-IV, e estimaram uma prevalência do TDAH em adolescentes (12-14 anos) de 5.8%. No entanto, alguns estudos observaram uma faixa etária mais ampla, 5-6 até 12-14 anos de idade e obtiveram diferentes prevalências como: 26.8% (Vasconcelos et al., 2003), 1.8% (Fleitlich-Bilyk et al., 2004), 0.9% (Goodman et al., 2005), 13% (Fontana et al., 2007) (revisados em Polanczyk et al., 2008).

As diferentes metodologias empregadas na avaliação dos dados favorecem essas variabilidades nas prevalências encontradas, bem como as diferentes

populações de estudo e os diferentes critérios utilizados para inclusão e exclusão. Particularmente, um importante critério que influencia na prevalência é o gênero. No estudo de Biederman et al. (1994) foi primeiramente salientada a viabilidade e a importância da identificação de indivíduos do sexo feminino com TDAH. De fato, a diferença de gênero não é surpreendente, pois vários estudos relatam que meninos são mais afetados durante a infância numa proporção de 4:1, e durante a adolescência essa proporção se equivale entre meninos e meninas (Gaub et al, 1997; Andersen et al., 2000). Acredita-se que essa mudança na proporção se deva ao diagnostico realizado mais precocemente em meninos por apresentarem o sintoma hiperativo/impulsivo mais evidente, enquanto que as meninas apresentam mais a falta de atenção (Biederman et al., 2004).

1.3 Sintomatologia e critérios diagnóstico do TDAH

O TDAH é caracterizado por um padrão de falta de atenção e hiperatividade acima do esperado quando comparado com indivíduos da mesma idade. As crianças acometidas pelo TDAH são geralmente mais distraídas, têm dificuldades em manter a atenção durante longos períodos de tempo, eles se levantam frequentemente quando devem permanecer sentados, lutam para permanecerem imóveis, perturbam as atividades dos outros ou respondem sem pensar e de forma desorganizada (Elosúa et al., 2017). Em virtude disso, o TDAH se tornou um dos transtornos neuropsiquiátricos mais comuns durante o desenvolvimento e mais investigados dentro da medicina, e sua tríade sintomática clássica abrange os sintomas de desatenção, hiperatividade e impulsividade (Goldman et al., 1998). A junção destes sintomas acaba resultando em prejuízos sociais, acadêmicos e ocupacionais (APA, 1994). De acordo com o DSM-V são caraterizados três subtipos do TDAH: predominantemente desatento,

predominantemente hiperativo/impulsivo, e o subtipo combinado que reúne os sintomas desatento e hiperativo/impulsivo.

Os sintomas do TDAH são quase sempre evidentes durante os anos pré-escolares (Barkley, 2006), e a sua gravidade principalmente o hiperativo/impulsivo, geralmente diminui com a idade (Hinshaw et al., 2006). Em adultos, os sintomas do TDAH se apresentam mais heterogêneos e sutis do que na população infantil, pois a hiperatividade que é observada em crianças, nos adultos apresenta-se como estar em constante atividade, excesso de programações e busca por cargos atarefados, enquanto que a impulsividade se manifesta por meio de término prematuro de relacionamentos e impaciência para execução de tarefas que requeiram tempo. A desatenção é bem evidente nas tarefas que exigem organização e atenção sustentada ao longo do tempo, bem como dificuldades com a gestão do tempo e da memória e frequente procrastinação (Weiss & Weiss, 2004).

Embora os avanços das metodologias nas áreas da genética, biologia molecular, neurofarmacologia e neuroimagem venham contribuindo significativamente para uma melhor compreensão das bases biológicas do TDAH, o seu diagnóstico ainda é essencialmente clínico (Kieling et al., 2010; Kieling & Rohde, 2012). Os critérios diagnósticos já sofreram inúmeras modificações, e, portanto, é fundamental serem considerados a intensidade dos sintomas e as suas consequências no processo diagnóstico (Kieling et al., 2010; Levy et al., 1997), pois estes podem ser encontrados em indivíduos normais (Barkley et al., 2008; Wender, 1995). No Brasil são utilizados os seguintes sistemas: a CID, da Organização Mundial da Saúde (Who, 1992) e o DSM, da Associação de Psiquiatria Americana (APA, 2013).

Além disso, já foram relatadas diferenças significativas em relação ao gênero na expressão do TDAH (Biederman et al., 2004), porém, quando a diferença foi

encontrada, o declínio dos sintomas ocorreu de forma mais intensa em meninos (Riddle et al., 2013). Outro ponto interessante quanto às diferenças devido ao gênero é que os domínios do TDAH tiveram curso similar nas meninas, contrastando achados prévios dos mesmos pesquisadores, que em uma amostra de meninos os sintomas de hiperatividade e impulsividade tinham um declínio mais acentuado que os de desatenção (Biederman et al., 2000; Biederman et al., 2012).

1.4 Modelos animais para o TDAH

Os modelos animais têm contribuído significativamente para o avanço dos estudos de diversas desordens psiquiátricas. Embora os animais não possam demonstrar essas desordens, eles podem fornecer informações relevantes a partir da aplicação de algumas metodologias inviáveis de serem aplicadas em seres humanos. Apesar de possuírem um sistema nervoso menos complexo, os animais possuem um circuito neural responsável por controlar funções comportamentais básicas, similar ao dos humanos (Russel, 2011). Um modelo animal ideal deve mimetizar, embora de forma mais simples, os fundamentos das características comportamentais (Validade de face), deve estar em conformidade com a literatura (validade de constructo), e ainda, deve ser capaz de prever aspectos do comportamento, neurobiologia e genética previamente desconhecidos pela clínica (validade preditiva) (revisado em Sagvolden et al, 2000).

Diversos modelos animais têm sido desenvolvidos com o intuito de aprofundar o conhecimento sobre o TDAH. Na tabela abaixo, adaptada de Sagvolden et al. 2005, encontra-se uma comparação entre as características predominantes em crianças com TDAH e aquelas descritas em alguns dos modelos animais já estabelecidos para este transtorno.

Tabela 1 - Características comportamentais dos modelos animais para TDAH

	Impulsividade	Falta de atenção (prejuízo na aquisição)	Hiperatividade		
			Ambiente novo	Ao longo do tempo	Diminui com psicoestimulantes
Crianças com TDAH	✓	✓	✗	✓	✓
SHR	✓	✓	✗	✓	✓
WKHA	✗	✗	✗	✓	✗
NHE	✗	✗	✓	✓	
DAT-KO		✓	✓	✗	✓
Camundongo *Coloboma*	✗	✓	✓		✓
Camundongo *Alcallosal*	✓	✓	✗	✓	
Rato com comportamento Hiposexual	✗	✓	✓		✗
Performance fraca no 5-CSRT	✓	✓	✓	✗	✗
Ratos *Wig*		✓	✓	✗	
Lesão com 6-OHDA		✓	✗	✓	✓
Expostos ao PCB	✓	✗	✗	✓	✓
Expostos ao chumbo			✓		
Anóxia		✓	✓	✗	
Raio-X			✓		✓
Isolamento	✗	✓	✓	✓	
Lesão noradrenérgica		✓			
Lesão Colinérgica		✓			
Lesão do Núcleo Accumbens	✓	✗	✓		
Lesão Subtalâmica	✓	✓			

SHR, ratos espontaneamente hipertensivos; WKHA, ratos Wistar-Kyoto hiperativos; NHE, ratos Niples de alta excitabilidade; DAT-KO, ratos knockout para os transportadores de dopamina; 5-CSRT, tarefa de tempo de reação em série de cinco opções; 6-OHDA, 6-hidroxidopamina; PCB, bifenil policlorado.
*Adaptada de Sagvolden et al., 2005.

Dentre os diversos modelos animais existentes, o modelo animal mais validado para os estudos do TDAH é a linhagem de ratos espontaneamente hipertensa (SHR, do inglês *spontaneoulsy hypertensive rats*). Esses animais desenvolvem hipertensão somente na idade adulta mais tardia, sendo que antes dessa fase eles podem ser utilizados como modelo experimental para o TDAH durante a infância e até o início da

vida adulta. Essa linhagem de ratos preenche o critério de validade de face, pois apresentam os principais sintomas do TDAH: desatenção, hiperatividade e impulsividade, mimetizando as características comportamentais do transtorno (Johansen et al 2005; Li et al., 2007; Sagvolden 1998; 2000; 2005; Van den Bergh et al., 2006). Também apresentam alterações na neurotransmissão dopaminérgica que incluem diminuições da dopamina no estriado, núcleo caudato e accumbens (Fujita et al., 2003; Linthorst et al., 1991; Pandolfo et al., 2013), em conformidade com a fundamentação teórica (validade de construto). Os ratos SHR também preenchem o critério de validade preditiva, pois o modelo é capaz de prever correlatos do TDAH em seres humanos no que se refere a comportamento, genética e funções neuronais não mostrados anteriormente na prática clínica (Sagvolden 2000; 2009).

1.5 Bases neurobiológicas do TDAH

Uma das hipóteses mais válidas para a sintomatologia apresentada pelos indivíduos acometidos pelo TDAH seria um prejuízo inicial nas funções executivas (Seidman et al., 2005; Willcutt, 2005). As funções executivas compreendem um conjunto de mecanismos de controle que regulam a cognição e a ação, e geralmente estão vinculados a atividade do córtex pré-frontal (Sergeant et al., 2002; Miyake, 2012). Em uma meta-analise foi constatada que uma disfunção nas funções executivas está significantemente associada ao TDAH, mas que somente essa disfunção não seria capaz de causar o TDAH em todos os indivíduos que apresentam esse transtorno (Willcutt, 2005). No entanto, já foi relatado que lesões no lobo frontal podem causar hiperatividade, distração ou impulsividade, podendo estes sintomas serem apresentados isolados ou combinados. A melhor hipótese para as diferenças no TDAH consiste nas medidas da função cerebral, principalmente das catecolaminas

(norepinefrina e dopamina) (Pennington e Ozonoff, 1996). A região frontocortical, responsável pelo controle da atenção e do sistema motor, é rica em catecolaminas (Faraone e Birmann, 1998), que também são conhecidas por regularem as funções dependentes do córtex, tais como, atenção, memória de trabalho, a tomada de decisão e o controle inibitório (Xing et al., 2016). Sua liberação depende do estado de excitação do cérebro (Arnsten et al., 2011) e evidências sugerem que, tanto em animais quanto em humanos, alterações nas vias das catecolaminas podem estar associadas ao transtorno, uma vez que o bloqueio dessas vias parece ser benéfico em reverter as disfunções observadas nos pacientes com TDAH (Arnsten, 2005; Seidman et a., 2005; Fernando et al., 2012; Bari et al., 2013).

1.5.1 Via noradrenégica

Historicamente, a via noradrenérgica origina-se no *Locus coeruleus,* por sua vez localizado na massa cinzenta da ponte (Aston-Jones et al., 2005). A noradrenalina (NE) pode exercer sua ação em três tipos de receptores: α1, α2, e β, sendo que os receptores α2 ainda podem ser subdivididos em α2A, α2B e α2C. É importante salientar que esses receptores podem estar localizados na região pré-sináptica em neurônios noradrenérgicos, dendritos e terminais dos axônios ou na região pós-sináptica em neurônios que recebem *input* noradrenégico (Arsnten et al., 2011). Dentre esses receptores, foi descrito que a NE apresenta uma maior afinidade por receptores α2 e uma menor afinidade pelo α1 e β (Arnsten, 2000). No entanto, o tipo de receptor ao qual a NE irá atuar dependerá da quantidade de NE liberada (Arnsten, 2011).

Em diversos estudos com humanos, as catecolaminas têm sido relacionadas com alguns dos sintomas presentes em pacientes com TDAH. Recentemente, foi

verificado que um maior número de cópias de uma variação do gene para dopamina beta-hidroxilase, que converte DA em NE estava relacionado com um maior número de erros em uma tarefa de atenção (Greene et al., 2009) e uma piora nas funções executivas (Kieling et al.,2008). Ademais, em um modelo animal para o TDAH, a administração de bloqueadores da recaptação da noradrenalina foi capaz de melhorar a performance dos animais em testes de atenção (Bari et al., 2013). Portanto, esses achados evidenciam a participação da NE na patofisiologia do transtorno.

1.5.2 Via dopaminérgica

A dopamina (DA) é uma catecolamina que era considerada um simples precursor da NE (Benes, 2001). Atualmente, sabe-se que a DA tem um papel importante no controle de ações motoras finas e funções cognitivas, como aprendizado, memória de trabalho, atenção, tomada de decisão e aspectos relacionados ao sistema de recompensa (Tritsch e Sabatini, 2012). A DA atua em duas famílias de receptores acoplados a proteína G que são os receptores D1 (D1 e D5) e os receptores D2 (D2, D3 e D4) (Arnsten et al., 2011). Originalmente, esses receptores foram classificados de acordo com seu efeito sobre a produção de AMP cíclico (cAMP). De fato, os receptores da família D1 estão acoplados à uma proteína G estimulatória (G_s) levando a um aumento dos níveis de cAMP, enquanto que a família dos receptores D2, por sua vez, estão acoplados à uma proteína G inibitória (Gi) e levam a uma diminuição de cAMP (Callier et al., 2003). O transportador de dopamina (DAT ou *SLC6A3*) tem como função regular a disponibilidade da DA na fenda sináptica por remover a DA e “devolver” para o neurônio pré-sináptico ou liberar a DA no espaço extracelular (Amara et al., 1993; Giros et al., 1993; Jaber et al., 1997). O sistema dopaminérgico é bem estabelecido e pode ser descrito em três

passos em relação a sua neurotransmissão: 1) liberação da DA na fenda sináptica, a qual ativa receptores específicos; 2) o transportador recapta a DA liberada regulando sua distribuição no espaço extracelular; 3) enzimas agem para neutralizar a ação da DA, inativando-a. Qualquer anormalidade em alguma dessas etapas pode resultar numa disfunção na transmissão dopaminérgica (Swanson et al., 2007).

Muitos estudos apontam a disfunção dopaminérgica como uma das prováveis causas do TDAH. De fato, estudos em animais indicam um papel proeminente da transmissão dopaminérgica nos processos de controle motor, atenção e aprendizado (Chudasama & Robbins 2006; Takamatsu et al., 2015; Mereu, 2017). Por meio de estudos de neuroimagem, foi possível verificar anormalidades nos pacientes com TDAH, entre elas, um menor volume encefálico, hipofunção, diminuição o fluxo sanguíneo, em áreas do cérebro com predominante inervação dopaminérgica como o córtex pré-frontal, giro do cíngulo anterior e gânglios da base (Arnsten, 2006; Cherkasova, 2017). Ademais, estudos caso-controle e de frequência de alelos de base familiar identificaram diferentes genes relacionados com a transmissão dopaminérgica (por exemplo, os receptores de dopamina e transportador) entre os genes associados com maior risco de desenvolver o TDAH (Burgueño et al., 2007; Faraone et al., 2005). Além disso, o uso de estimulantes para o tratamento dos sintomas do TDAH interfere, principalmente, na transmissão dopaminérgica (Burgueño et al., 2007; Wilens, 2006). Considerando as associações acima citadas, se torna evidente que a alteração na sinalização da dopamina pode influenciar o desenvolvimento do TDAH.

1.5.3 Evidências genéticas

A etiologia deste transtorno ainda não está elucidada, mas há evidências de que influências ambientais como baixo peso ao nascer, complicações na hora do parto, exposição a toxinas, entre outros, podem ter participação (Faraone et al., 1998). Todavia, é considerado um transtorno de alta herdabilidade, com estimativas de 71 a 90% (Bierdman et al., 2005; Faraone et a., 2005). Os dados obtidos em estudos com gêmeos revelaram uma alta herdabilidade durante a infância até o início da vida adulta de problemas relacionados com a atenção (Chang et al., 2013; Larsson et al., 2013), e também com hiperatividade e impulsividade (Nikolas e Burt, 2010; Larsson et al., 2013). Aliás, o TDAH é considerado um dos transtornos do neurodesenvolvimento com maior predisposição genética e estudos vêm demonstrando uma forte contribuição de mutações e polimorfismos na etiologia do transtorno. Dentre as alterações genéticas mais estudadas, estão mutações de genes do sistema dopaminérgico (D4, D2, D5, DAT) além de genes do complexo SNARE, como a proteína associada ao sinaptossoma de 25 KDa (SNAP-25) e outros genes relacionados ao neurodesenvolvimento como o do fator neurotrófico derivado do encéfalo (BDNF) (Gizer et al., 2009; Faraone et al., 2010; Tsai, 2016).

Em relação ao sistema dopaminérgico, os polimorfismos no genótipo do transportador de dopamina (DAT ou *SLC6A3*) e no receptor D4 (DRD4) (Shaw et al., 2007; Hasler et al., 2014; Van der meer et al., 2017) são bastante estudados. Foram encontrados polimorfismos em genes de DAT e DRD4 que também são responsáveis por regular a expressão de sintomas de impulsividade e raiva (Hasler et al., 2014). As variantes mais estudadas do transportador de dopamina são duas regiões de repetição em tandem de número variável (do inglês, variable tandem repeat polymorphism ou VTRP) de 40 e 30 pares de bases e os alelos 10R, 9R, 6R e 5R são os mais comuns (Bonvicini et al., 2016). O alelo 10R e 6R tem sido associado a

susceptibilidade ao desenvolvimento do TDAH em crianças (Gizer et al., 2009). Particularmente em relação ao DRD4, uma região altamente polimórfica no terceiro exón do receptor tem sido relacionada ao TDAH (Bonvicini et al., 2016). Além disso, tem sido reproduzida uma associação entre os pacientes com TDAH e a presença de uma isoforma do receptor, o alelo 7R (Thapar et al., 2007; Brookes et al., 2006; Grady et al., 2003). O fato é que este alelo do receptor D4-7 apresenta uma incidência mais elevada em probandos do TDAH sugerindo risco genético para o transtorno, o que corrobora com uma meta-análise que confirma o receptor D4-7 como um gene de susceptibilidade para TDAH (Faraone et al., 2001; Li et al., 2006; Gizer et al.,2009). Em uma outra meta-análise foi demonstrada uma forte associação entre o alelo 7 e o subtipo combinado do TDAH (Smith, 2010).

Parte do complexo SNARE junto com a sinaptobrevina, SNAP-25, sintaxina e sinaptogamina, são responsáveis pela exocitose de neurotransmissores e a ausência desta proteína pode resultar num bloqueio da transmissão sináptica (revisado em Antonucci et al., 2016). Evidências da influência da SNAP-25 surgiram principalmente devido ao desenvolvimento de um modelo animal mutante (coloboma) que demonstrava hiperatividade similar a reportada no TDAH. Esse modelo animal, apresentava uma deleção no cromossomo 2 em uma região que estava localizado o gene para a SNAP-25 e, por isso, ele apresenta uma redução de 50% na atividade dessa proteína (Hess et al., 1992). Recentemente, estudos têm avaliado diversos polimorfismos para o gene da SNAP-25 e mostraram uma associação de alguns deles com o TDAH (Kim et al., 2007; Herken et al., 2014; Ye et al., 2016; Liu et al., 2017).

O BDNF faz parte de uma família de neurotrofinas e tem como uma de suas funções o equilíbrio entre a morte e a sobrevivência celular (Figura 1).

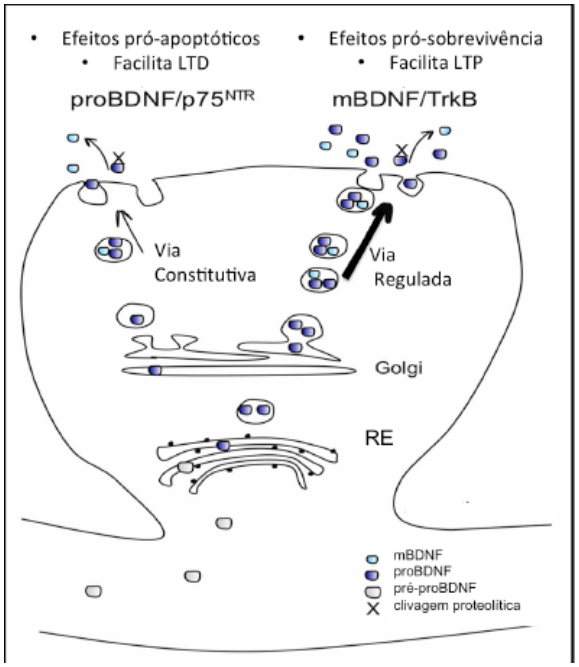

Figura 1- A imagem representa os processos de síntese, armazenamento e secreção do BDNF destacando o seu papel no equilíbrio entre a morte e a sobrevivência neuronal. O BDNF é sintetizado como uma proteína pré-proBDNF que é clivada no retículo endoplasmático (RE). O proBDNF resultante desta clivagem é armazenado no Complexo de Golgi em dois tipos de vesículas secretoras: vesículas da via constitutiva e vesículas da via regulada. O proBDNF armazenado nestas vesículas sofre clivagem proteolítica e é secretado como mBDNF (BDNF maduro) ou é secretado como proBDNF e sofre clivagem extracelular (os processos finais de clivagem estão representados na figura pelo símbolo). Uma vez liberado o proBDNF se liga, preferencialmente no receptor p75 e o mBDNF ao receptor TrkB pré e pós-sináptico ativando diferentes cascatas de segundos mensageiros e desencadeando respostas celulares distintas. LTD (do inglês *long-term depression*); LTP (do inglês *long-term potentiation*) (Adaptada de Cunha et al., 2010).

Devido ao seu papel preponderante no desenvolvimento e formação das sinapses e sua participação nos fenômenos de plasticidade sináptica, o BDNF tem sido investigado na patofisiologia do TDAH (Skaper, 2008; Numakawa et al., 2010; Balaratnasingam et al., 2012; Tsai, 2016). Os polimorfismos no gene BDNF que poderiam contribuir para a suscetibilidade do TDAH têm sido investigados, mas os resultados ainda são controversos (Tsai, 2016). Os estudos em polimorfismos do gene para o BDNF verificaram uma associação entre os polimorfismos de nucleotídeos únicos (SNPs) rs11030101 e rs10835210 e o TDAH (Cho et al., 2010; Know et al., 2015). Além disso, foi visto que o polimorfismo rs11030101 está relacionado com um maior número de erros, no teste de desempenho contínuo, somente em meninas (Cho et al., 2010).

1.5.4 Achados de neuroimagem

Estudos empregando técnicas de neuroimagem quantitativa ao longo do desenvolvimento cerebral têm auxiliado na compreensão das alterações neuro-anatômicas observadas no TDAH, e também, têm complementado avalições clínicas e comportamentais do transtorno. Além disso, a utilização de técnicas de imagem vem sendo aprimorada ao longo dos anos, e as primeiras evidências apontaram para diferenças no desenvolvimento da região frontal do encéfalo de pacientes com TDAH (Castellanos et al., 1996; Castellanos et al., 2001; Sowell et al., 2003; Mostofsky et al., 2002). Recentemente já vem sendo descrito o envolvimento de outras regiões do encéfalo, particularmente as subcorticais (Seidman et al., 2005; Halperin e Schulz 2006; Valera et al., 2007; Hoogman et al, 2017). Na figura abaixo, adaptada de Shaw et al. (2007) é possível visualizar um atraso para atingir a espessura cortical máxima no grupo TDAH (um marco no desenvolvimento), principalmente nas regiões corticais.

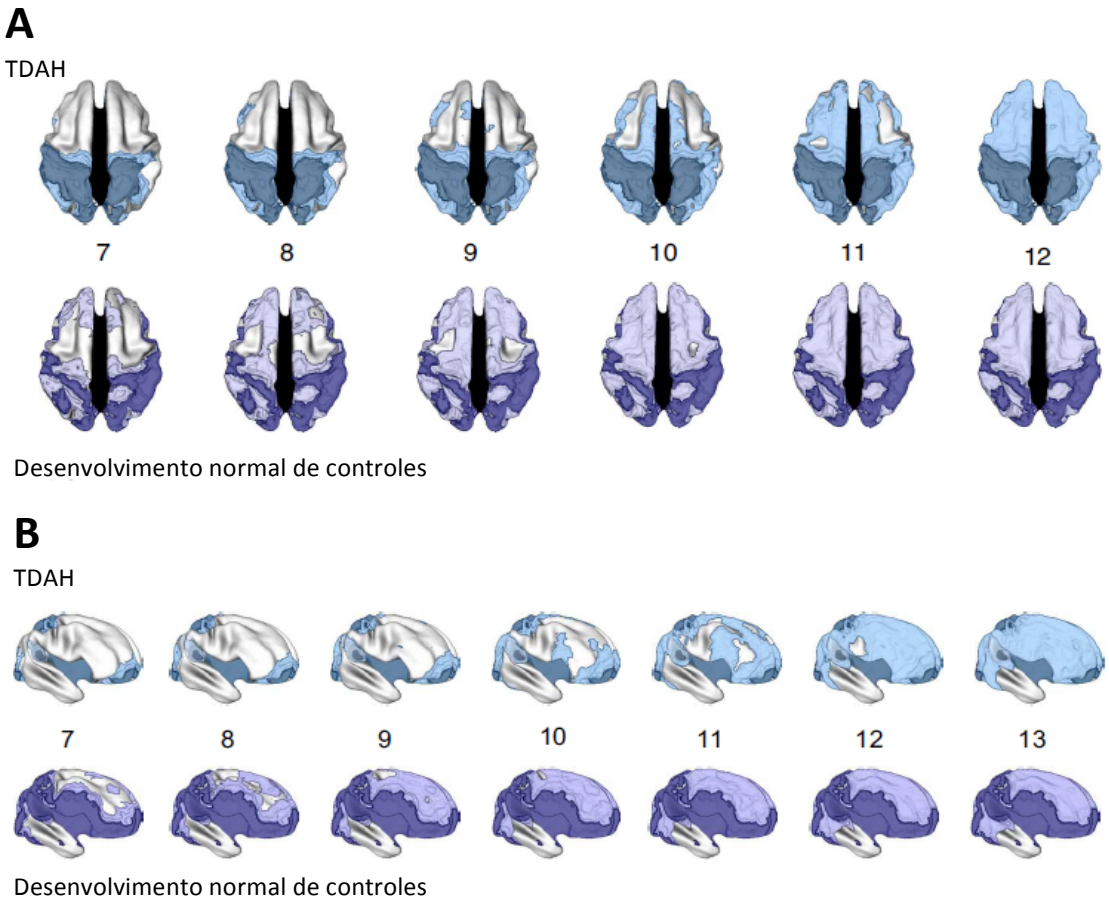

Figura 2 – A imagem representa a idade ao atingir a espessura cortical máxima em crianças com TDAH em comparação com crianças com um desenvolvimento normal. (A) vista dorsal das regiões corticais onde a espessura do pico foi atingida em cada idade (mostrada, idades 7-12) no TDAH (acima) e nos controles com desenvolvimento normal (abaixo). As cores mais escuras indicam regiões onde um modelo quadrático não era apropriado (e, portanto, uma idade de pico não pôde ser calculada), ou a idade de pico foi estimada por estar fora da faixa etária escolhida. Ambos os grupos apresentaram uma sequência similar das regiões que alcançaram a espessura do pico, mas o grupo de TDAH mostrou um atraso considerável ao atingir esse marco do desenvolvimento. (B) Vista lateral direita das regiões corticais onde a espessura do pico foi atingida em cada idade (mostrada, idades 7-13) no TDAH (acima) e nos controles com desenvolvimento normal (abaixo). Novamente, é evidente o atraso no grupo TDAH na obtenção da espessura cortical máxima. (Adaptada de Shaw et al., 2007).

Outros estudos reportaram que pacientes acometidos pelo transtorno apresentam uma diminuição do volume total quando comparados com pessoas normais da mesma idade (Castellanos et al., 1996; Castellanos et al., 2001; Sowell et al., 2003; Mostofsky et al., 2002). Atualmente, estudos de ressonância magnética revelaram um desenvolvimento anormal do lobo frontal com uma diminuição tanto da camada cinzenta quanto da camada branca em meninos e meninas com TDAH (Mahone et al., 2011; Dirlikov et al., 2014; Villemonteix et al., 2015). Anomalias no eletroencefalograma (EEG) também foram relatadas nos pacientes com TDAH quando comparados com controles (Barry et al., 2009; Hermens et al., 2004). Corroborando com os achados citados acima, estudos funcionais demonstraram uma menor atividade na região frontal de adultos com TDAH (Wolf et al., 2009; Valera et al., 2010).

Em relação ao gênero, sabe-se que meninos possuem um maior volume cerebral do que meninas (Gieed et al.,1997). Apesar de os pacientes com TDAH apresentarem diferenças em relação as pessoas sem o transtorno, essas alterações podem ser influenciadas pelo gênero. Recentemente, foi demonstrado que as meninas apresentavam um menor volume da camada cinzenta numa região motora que pode resultar em um maior número de erros em testes específicos, enquanto que meninos, demonstraram um menor volume da camada branca, por sua vez, pode estar relacionada a uma disfunção do controle das funções executivas (Mahone et al.,

2011). Em um outro estudo, foi observado que as meninas apresentavam uma redução global do córtex pré-frontal enquanto os meninos uma redução mais global do córtex pré-motor (Dirlikov et al., 2014). As diferenças entre os gêneros também foram encontradas em estudos utilizando EEG tanto em crianças quanto em adultos com TDAH (Barry et al., 2009; Hermens et al., 2004). Achados funcionais com adultos acometidos pelo TDAH também revelaram uma influência do gênero, com os homens apresentando uma menor atividade cerebral quando comparado aos controles (Valera et al., 2010).

1.6 TDAH e as diferenças de sexo

A diferença na prevalência entre os sexos têm sido uma grande incógnita dentro dos transtornos psiquiátricos (Martel et al., 2009). Segundo a APA (2000), os meninos são mais propensos a desenvolver transtornos de déficit de atenção e hiperatividade (TDAH), transtorno desafiador opositivo (TOD), transtornos de conduta, Autismo e geralmente apresentando prejuízos de aprendizagem. Por outro lado, as meninas estão mais vulneráveis a desenvolver distúrbios emocionais, como transtorno depressivo maior (DDM), transtornos de ansiedade e alimentares. Sendo assim, os hormônios gonadais podem desempenhar um papel na prevalência dos transtornos psiquiátricos.

Várias teorias sobre a diferenciação sexual foram desenvolvidas, dentre elas: (i) a teoria clássica que descreve que a presença hormônios andrógenos favorece o desenvolvimento masculino enquanto que a sua ausência, está relacionada com o desenvolvimento feminino; (ii) a teoria da feminização que sugere um papel fundamental dos hormônios ovarianos promovendo feminização dos circuitos neurais; e a (iii) teoria de gradiente, que postula diferenças de comportamento

conforme a quantidade de cada um dos hormônios de meninos e meninas, por exemplo, as meninas com maiores níveis de hormônios andrógenos podem apresentar características mais masculinas (Collaer e Hines, 1995; revisado em Martel et al., 2009). No entanto, a ideia primordial é que os hormônios esteroides organizavam a estrutura do cérebro durante um período mais vulnerável do desenvolvimento e ativavam o comportamento durante a puberdade e a vida adulta (Schulz et al., 2009).

Durante o desenvolvimento encefálico, a adolescência é considerada uma das fases extremamente lábeis, onde ocorre o refinamento e maturação dos neurônios, com o intuito de aprimorar a função cognitiva (Casey et al., 2005; Amso e Casey, 2006; Khundrakpam et al., 2013; Lee et al., 2014). Assim como a maioria dos receptores sinápticos, existem evidências de que os receptores dopaminérgicos (D1 e D2) também aumentam sua expressão durante o final da infância e início adolescência para depois serem eliminados, durante a maturação cerebral (Teicher et al., 1995). Coincidentemente, é nessa fase de intensa plasticidade que a maioria das doenças psiquiátricas começam a se desenvolver (Giedd et al., 2008). Além disso, é coincidente com essas mudanças na estrutura e funcionalidade do cérebro que ocorre o aumento dos hormônios gonadais (Casey et al., 2005), os quais também contribuem para diferenças na transmissão e circuitos dopaminérgicos (Waddel, 2012). Os hormônios gonadais, estrógeno e testosterona, podem influenciar a sinalização dopaminérgica diminuindo a expressão de receptores e transportadores (Sinclair et al., 2014). De fato, já foram observadas diferenças na distribuição de receptores de dopamina entre os sexos, em estudos experimentais com animais, onde os machos demonstraram um aumento mais expressivo desses

receptores durante a puberdade quando comparados com fêmeas (Andersen e Tiecher, 2000).

No entanto, ainda permanece desconhecido o vínculo exato entre os mecanismos hormonais e o desenvolvimento do déficit cognitivo presente no TDAH. Isso se deve a carência de estudos focados nesta abordagem abrangendo ambos os sexos (Martel et al., 2009), visto que a maioria dos trabalhos inclui somente o sexo masculino. Atualmente, tem-se discutido a importância da inclusão de ambos os sexos nos estudos experimentais, uma vez que a maioria dos avanços com modelos animais têm se limitado ao sexo masculino (Shanky e Wooley, 2016). Sendo assim, comparar o desenvolvimento dos encéfalos de meninos e meninas é uma oportunidade para entender essas diferenças nas prevalências destes transtornos psiquiátricos, uma vez que hormônios sexuais podem contribuir para o desenvolvimento de diversas patologias (Waddel, 2012). Recentemente, foi relatado que essas diferenças observadas na prevalência podem ser causadas por um fator protetor desenvolvido por indivíduos do sexo feminino, tornando-as mais resistentes, em comparação com sexo masculino. Sendo assim, os indivíduos do sexo feminino precisariam de uma maior carga genética para desenvolver o transtorno. Apesar das variantes genéticas autossômicas estarem claramente envolvidas em ambos os sexos, foi visto que irmãos de indivíduos do sexo feminino que tem TDAH apresentam maiores chances de desenvolver o transtorno do que irmãos de indivíduos do sexo masculino acometidos pelo TDAH (Martin et al., 2018).

1.7. Cafeína

A cafeína é o psicoestimulante mais consumido no mundo sendo encontrada em vários componentes da dieta, como chocolate, chá verde, bebidas à base de

cola além do café (Fredholm et al., 1999). O seu consumo a partir de todas as fontes é bastante variável, sendo que alguns estudos apontam uma média de 70-76 mg/pessoa/dia em todo o mundo ou 5-8 mg/kg/dia (equivalente a 3 xícaras de café) (Chen et al., 2010; Fredholm et al., 1999). 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, com a ativação de A_1 exercendo ações inibitórias enquanto os A_{2A} exercem ações facilitatórias sobre a transmissão sináptica (Cunha, 2001; Fredholm et al., 1999; Nehlig et al., 1992).

Nos últimos anos, os efeitos da cafeína em prevenir a neurodegeneração e os prejuízo cognitivos e motores têm sido observados em modelos experimentais da doença de Parkinson e da Doença de Alzheimer, duas das mais prevalentes doenças crônicas neurodegenerativas (Aguiar et al.,2006; Arendash et al., 2006; Chen et al., 2001; Dall´Igna et al., 2007; Espinosa et al., 2013; Joghataie et al., 2004), e prejuízos cognitivos decorrentes da idade (Costa et al., 2008a; Prediger et al., 2005a; Sallaberry et al., 2013).

Os efeitos da cafeína sobre parâmetros comportamentais e neuroquímicos no decorrer do desenvolvimento encefálico também vem sendo investigados nos últimos anos, com contribuições do nosso grupo de pesquisa (Porciúncula et al., 2013). Enquanto a administração aguda de cafeína em camundongos adultos melhora o desempenho da memória de reconhecimento (Botton et al., 2010; Costa et al., 2008b), houve prejuízo em tarefas que envolvem a memória de reconhecimento com

administrações de cafeína feitas durante a gestação e lactação de roedores (Soellner et al., 2009; Silva et al., 2013). Logo, a melhora no desempenho em tarefas de aprendizado e memória pela administração de cafeína parece ser mais restrita a administrações agudas, onde foi observada melhora no desempenho na tarefa de esquiva inibitória e no labirinto aquático de Morris (Angelucci et al., 1999; 2002). A cafeína pode causar efeitos distintos quando administrada em diferentes dias do período pós-natal, entre DPN 7 a 11 em ratos provoca um aumento da atividade locomotora, enquanto que o tratamento entre DPN 13 a 17 diminui (Tchekalarova et al., 2005).

Em relação as diversas doenças neuropsiquiátricas, a manipulação farmacológica dos receptores A_{2A} de adenosina vêm sendo discutida uma vez que existe uma interação física e funcional com receptores de dopamina D2, receptores afetados no TDAH (Ferré et al., 2004; 2011). Geralmente, se observa que os pacientes neuropsiquiátricos têm um aumento do consumo de cafeína em relação a população em geral e, pacientes com TDAH acabam por consumir cafeína em doses adequadas para alterar seu estado de alerta e cognição (Rihs et al., 1996; Broderick & Benjamin, 2004; Magon & Müller, 2012). Os adolescentes com diagnóstico de TDAH são duas vezes propensos a consumir cafeína e nicotina do que os adolescentes sem o transtorno (Walker et al., 2010).

Para o TDAH, foi em 1973 que surgiu o primeiro estudo descrevendo os efeitos do café em 11 crianças hiperativas previamente tratadas com metilfenidato e os efeitos colaterais (Schnackenberg, 1973). Todos os participantes que beberam café ou utilizaram metilfenidato apresentaram melhora dos sintomas (menor pontuação em uma escala hipercinesia), sem diferenças na resposta entre ambos, mas somente as crianças que beberam café não apresentaram efeitos colaterais. Nos anos

subsequentes, esses dados incentivaram uma série de estudos com o objetivo de explorar o potencial da cafeína no contexto clínico. Estes estudos foram realizados, em sua grande maioria, avaliando os efeitos da cafeína ou dos estimulantes (principalmente metilfenidato e anfetaminas), em pequenas amostras de crianças com TDAH. Os resultados obtidos no estudo de Schnackenberg (1973) foram subsequentemente contestados pela falha em reproduzir o resultado positivo da cafeína (Arnold et al, 1978; Baer, 1987; Conners, 1975, 1979; Firestone et al, 1978; Fras, 1974; Garfinkel et ai, 1975a,b; Huestis et al, 1975). Os estudos de Firestone et al. (1978) e Arnold et al. (1978) relataram informalmente que um sujeito respondeu positivamente somente ao tratamento com cafeína e não os outros estimulantes. A cafeína também foi ineficaz em um pequeno estudo com crianças que apresentavam deficiência de leitura e déficits de atenção, mas curiosamente num subgrupo hiperativo a cafeína se mostrou eficaz (Kupietz e Winsberg, 1977). Em outro estudo também com pequeno grupo, houve diferença numérica para a cafeína quando comparado seu efeito sobre o estímulo ao reconhecimento entre as crianças normais e “hipercinéticas”, mas os autores discutem que o resultado foi estatisticamente insignificante possivelmente devido a um poder estatístico limitado (Reichard e Elder, 1977). Outros estudos obtiveram resultados encorajadores indicando superioridade da cafeína sozinha versus placebo (Harvey e Marsh, 1978), ou em tratamento combinado com outros estimulantes contra os estimulantes sozinhos (Garfinkel et al, 1981; Schechter e Timmons, 1985). Geralmente muitos pacientes com TDAH são bastante propensos a consumir cafeína em doses adequadas para alterar seu estado de alerta e cognição, e, essencialmente, os seus sintomas de TDAH pelo consumo habitual (Broderick e Benjamin, 2004).

Recentemente foi investigado o efeito da cafeína e a manipulação de seus receptores em modelos animais do TDAH. A administração de cafeína e antagonistas de receptores de adenosina A_1 e A_{2A} reverteu os prejuízos cognitivos observados nos ratos SHR em tarefas de atenção, memória e aprendizado (Pandolfo et al., 2013; Pires et al., 2009; 2010; Prediger et al., 2005b). Além disso, as alterações frontocorticais observadas na sinalização dopaminérgica nos animais SHR foram normalizadas pelo tratamento com cafeína (Pandolfo et al., 2013). Nesse contexto, a hipótese de que a manipulação dos receptores de adenosina A_{2A} pode ser uma nova estratégia terapêutica e/ou adjuvante para o tratamento do TDAH é promissora tendo em vista o uso de administração de cafeína para tratar esta condição (Garfinkel et al., 1981; Gross, 1975), uma vez que nem todos os pacientes toleram o tratamento com o metilfenidato.

2. OBJETIVOS

2.1. Objetivo Geral

Investigar o impacto do tratamento com cafeína no modelo experimental do Transtorno do Déficit de Atenção e Hiperatividade (TDAH) enfocando as diferenças de sexo, analisando parâmetros neuroquímicos, comportamentais e neurofuncionais.

2.2. Objetivos específicos

a) Investigar o efeito da administração de cafeína no início da infância até o final da adolescência/início da vida adulta na hiperatividade locomotora e exploratória de ratos machos e fêmeas SHR e Kyoto e o desempenho nas tarefas de reconhecimento de objeto novo (memória de reconhecimento) e na tarefa de cavar (Dig task), em ratos SHR e Kyoto machos e fêmeas no início da idade adulta.

b) Avaliar o padrão de expressão de proteínas sinápticas, fatores tróficos e proteínas importantes para a diferenciação e maturação das sinapses nas estruturas cerebrais mais relacionadas a sintomatologia do TDAH, de ratos machos e fêmeas SHR e Kyoto tratados com cafeína durante a infância e adolescência.

d) Analisar a atividade elétrica do córtex frontal cerebral por meio de registros eletroencefalográficos em ratos machos e fêmeas SHR e Kyoto adultos que receberam tratamento com cafeína durante a infância e adolescência.

PARTE II

Artigo I

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Differential Behavioral and Biochemical Responses to Caffeine in Male and Female Rats from a Validated Model of Attention Deficit and Hyperactivity Disorder

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Abstract
Epidemiological studies suggest sex differences in attention deficit and hyperactivity disorder (ADHD) symptomatology. The potential benefits of caffeine have been reported in the management of ADHD, but its effects were not properly addressed with respect to sex differences. The present study examined the effects of caffeine (0.3 g/L) administered since childhood in the behavior and brain-derived neurotrophic factor (BDNF) and its related proteins in both sexes of a rat model of ADHD (spontaneously hypertensive rats—SHR). Hyperlocomotion, recognition, and spatial memory disturbances were observed in adolescent SHR rats from both sexes. However, females showed lack of habituation and worsened spatial memory. Although caffeine was effective against recognition memory impairment in both sexes, spatial memory was recovered only in female SHR rats. Besides, female SHR rats showed exacerbated hyperlocomotion after caffeine treatment. SHR rats from both sexes presented increases in the BDNF, truncated and phospho-TrkB receptors and also phospho-CREB levels in the hippocampus. Caffeine normalized BDNF in males and truncated TrkB receptor at both sexes. These findings provide insight into the potential of caffeine against fully cognitive impairment displayed by females in the ADHD model. Besides, our data revealed that caffeine intake since childhood attenuated behavioral alterations in the ADHD model associated with changes in BDNF and TrkB receptors in the hippocampus.

Keywords ADHD · Caffeine · BDNF · Sex differences · Adolescence

Introduction

Attention deficit and hyperactivity disorder (ADHD) is a neurodevelopmental disorder reaching 7.2% of children and adolescents worldwide [1]. The core symptoms of ADHD are persistent inattention and/or hyperactivity/impulsivity [2]. The higher prevalence is partly explained by the hyperactivity/impulsivity and the externalization of problems that are more exacerbated in boys, since girls generally internalize problems and are more inattentive [3, 4]. Anatomical differences during brain development between sexes have been found in the gray matter, in which girls showed an earlier peak in the frontal lobe than males, and a higher rate of change throughout childhood and adolescence in male brains [5]. These findings could affect the prevalence of ADHD for males and females at different ages, once the brain development is different for each sex [6, 7].

The etiology of ADHD comprises complex interactions of neurotransmitter systems and neuroanatomical changes. Dopamine transporters and dopamine D4 and D5 receptors are one of the hallmarks involved in the pathogenesis of ADHD, with many polymorphisms linked to clinical features [8]. Other proteins have also been implicated in ADHD etiopathology, including those involved in the maturation of synapses during brain development such as brain-derived neurotrophic factor (BDNF). BDNF belongs to the neurotrophins family playing a crucial role in the neurogenesis, neuronal differentiation, and synaptic plasticity, and this neurotrophin has been considered an emergent candidate in the etiology of some psychiatric and neurodevelopmental disorders [9, 10]. For instance, some studies have already reported polymorphisms in the BDNF gene in ADHD patients [11–13] and that they may be contributing to the susceptibility of ADHD disorder [14], while others have not found any association between them [15]. Besides, variations in the circulating levels of BDNF have been reported in ADHD patients [16–18].

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Caffeine is the most consumed psychostimulant worldwide [19]. Caffeine has been described to improve the performance in tasks requiring attention and thus, its potential for the treatment of ADHD has promptly been investigated in the past years [20]. Over the past years, the potential of caffeine as a treatment and/or a second line of treatment for ADHD was investigated, and epidemiological studies showed some efficacy in relieving symptoms related to ADHD [21, 22], while others have failed to find superior effects when compared to the first line of treatment [23, 24]. In a recent study, there was no association between maternal caffeine consumption during pregnancy and increased prevalence of ADHD in children [25].

In ADHD experimental studies, the effects of caffeine have been investigated in spontaneously hypertensive rat (SHR) strain [26], which is the most used animal model to mimic the symptoms of ADHD [27, 28]. A series of studies showed that caffeine administered in different schedules and doses was able to reduce memory deficits and improve attention and memory in SHR rats [29–32]. Similar to epidemiological data, most of the studies have been conducted on male animals resulting in a limited knowledge regarding sex differences connected with ADHD. Particularly for ADHD in which gender differences are remarkable in the symptomatology, it becomes important to distinguish behavioral and synaptic protein differences in ADHD models. Furthermore, responsiveness to caffeine deserves more attention since the evaluation of the potential of caffeine in alleviating cognitive deficits in ADHD model was performed mainly in male rats. Therefore, in this study, we investigated the influence of sex in the effects of caffeine on memory, locomotion, and BDNF and its related proteins, during childhood and adolescence of the ADHD rat model.

Material and Methods

Animals

Male and female SHR (NCrl) and Wistar-Kyoto rats (WKY/NIcoCrl) (60–70 days old) were mated within our colony at Federal University of Rio Grande do Sul. Animals were maintained under 12/12-h light-dark cycle (lights on at 7:00 AM), at constant temperature (22 ± 1 °C) and with free access to food and water. The authors state that all animal experiments and protocols were approved and they were carried out in accordance with the Institutional Animal Care and Use Committee of Federal University of Rio Grande do Sul (CEUA-UFRGS Ethical Protocol number 29196), based on the guide for the care and use of laboratory animals from the National Institutes of Health (NIH Publication No. 85-23, revised 1996). The experimental procedures were designed to minimize the number of animals used and their suffering.

Caffeine Treatment

The schedule of caffeine administration, experimental groups, and subsequent behavioral and protein immunodetection were summarized in the Fig. 1. After birth, male and female SHR rat pups at postnatal day 15 (PND 15) started to receive caffeine (0.3 g/L) in the drinking water from their SHR and Kyoto dams until weaning (PND 25). At PND 28–29, animals were divided into the following groups: (i) animals that received only water, (ii) animals that received caffeine only from PND 15 up to PND 28–29 (caff/water), and (iii) animals that received caffeine from PND 15 up to PND 54 (caff/caff); Kyoto rats were used as a control strain and they did not receive any treatment with caffeine.

Behavioral Analysis

Male and female rats from both strains were evaluated at PND 28–29 in the open field test, novel object recognition and Y-maze task. The apparatuses were always cleaned out with 70% alcohol after each animal tested. All behavioral tests were conducted by a trained observer, in a sound-attenuated room under low-intensity light and recorded by means of a computer-operated tracking system (Any-maze, Stoelting, Woods Dale, IL).

Locomotor Activity and Habituation

Locomotor activity and habituation were evaluated in rats at PND 28–29 and 50–51. Animals were exposed to open field arena during two consecutive days, in order to evaluate locomotor activity and habituation. The apparatus was made of black-painted wooden arena measuring 60-cm diameter and surrounded by 50-cm high walls. Each rat was placed in the periphery of the open field and the total travelled distance (periphery and center) was recorded during 5 min. Locomotor activity was evaluated by analyzing the travelled distance in the periphery and habituation was analyzed by total travelled distance in the open field. The experiments were conducted in a sound-attenuated room under low-intensity light; activity was recorded with a video camera positioned above the apparatus and monitored in an adjacent room by an observer blinded to the treatment and strains. The open field apparatus was always cleaned after the end of each session performed by each animal.

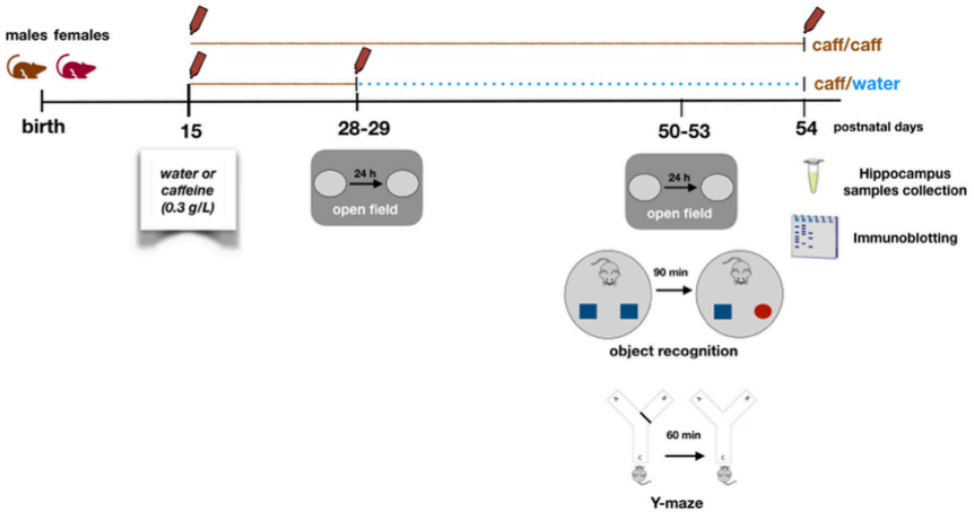


Fig. 1 Schematic timeline of caffeine treatment and experimental groups. Male and female SHR rat pups received water or caffeine (0.3 g/L) from their dams 15 days after birth. At postnatal days (PND), 28–29 animals were divided into three groups: (i) rats receiving only water; (ii) rats treated with caffeine from PND 15 up to PND 28–29 (caff/water); (iii) rats that received caffeine from PND 15 up to 54 (caff/caff). Male and female Kyoto rats were used as a control strain and they received only water. Open field test was carried out at PND 28–29 and 50–51. Novel object recognition and Y-maze task were performed at PND 52 and 53. Samples from hippocampus were collected at PND 54 for protein immunodetection

Novel Object Recognition Task

The novel object recognition test was carried out 24 h after the second session of open field test. Rats at PND 52 first underwent a training session, in which two identical objects were placed in the center of the open field. They were placed individually into the open field facing the center and allowed to explore both objects during 5 min. The test session was performed 90 min after training and two dissimilar objects were presented, a familiar one and a novel one. Exploration was defined by directing the nose to the object at a distance of at least 2 cm and/or touching the object with the nose or forepaws. Rearing onto the object was not considered as exploratory behavior. The discrimination ratio was defined as TN/(TN + TF), [TN = time spent exploring the novel object; TF = time spent exploring the familiar object]. After the end of each session, both objects and apparatus were cleaned.

Modified Y-maze Test

The test was carried out 24 h after novel object recognition task. Rats at PND 53 were exposed to an enclosed Y-maze, which consisted of three arms (50 cm long, 10 cm wide, and 20 cm high) made of wood covered with impermeable Formica elevated to a height of 50 cm above the floor. The task consisted of two trials (training and test) of 5 min each separated by an interval of 60 min. Arm entry was monitored by placing a rat into the end of one arm and by allowing the access to that arm and another arm. During the training trial, a removable door was blocking the third arm (the novel), while during the test the third arm was opened; then the animals were once again placed at the first arm and allowed to explore freely the three arms during 5 min. The following parameters were recorded: (i) total number of entries in the three arms, (ii) the number of entries in the novel arm, and (iii) the time spent in the novel arm. Total entries in the arms, percentage of the number of entries, and time spent in the novel arm were calculated during test session.

Immunoblotting

Twenty-four hours after the end of behavioral tests, rats at PND 54 were sacrificed under anesthesia. The hippocampi were dissected out and immediately homogenized in a 5% SDS solution containing a cocktail of protease and phosphatase inhibitors (Sigma, São Paulo/SP, Brazil) and frozen at -20ºC. After defrost, the protein content was determined using the bicinchoninic acid assay (BCA) (Pierce, São Paulo/Brazil). The sample extracts were diluted to a final protein concentration of 2 μg/uL in SDS solution and the amount of protein applied for SDS–PAGE analysis was as follows: 40–60 μg for BDNF, proBDNF, truncated, full length and phospho-TrkB, and CREB and phospho-CREB. The proteins, together with pre-stained molecular weight standards (Bio-Rad, São Paulo/Brazil), were applied to a 10–14% SDS–PAGE running gel with a 4%

concentrating gel. After electro-transfer, membranes were blocked with Tris-buffered saline containing 0.1% Tween-20 and 3% bovine serum albumin (BSA) during 1 h. The nitrocellulose membranes (Amersham, São Paulo/Brazil) were then incubated overnight at 4 °C with mouse anti-BDNF (1:1000; Abcam, São Paulo/Brazil), mouse anti-proBDNF (1:1000; Abcam), rabbit anti-TrkB (1: 5000; Abcam, São Paulo/Brazil), anti-rabbit phosphoTrkA/TrkB (Cell Signaling, São Paulo/Brazil), and rabbit anti-CREB and anti-phospho-CREB (1:1000; Cell Signaling, São Paulo/Brazil). The membranes were washed and incubated with horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature and developed with chemiluminescence ECL kit (Amersham). Densitometric analyses were performed using the NIH ImageJ software after images acquisition in the ImageQuant LAS 4000 (Amersham, São Paulo/Brazil). After stripping, ß-tubulin was quantified as a loading control using a mouse anti-ß-tubulin antibody (1:1000; from Santa Cruz Biotechnologies, São Paulo/Brazil), as described above.

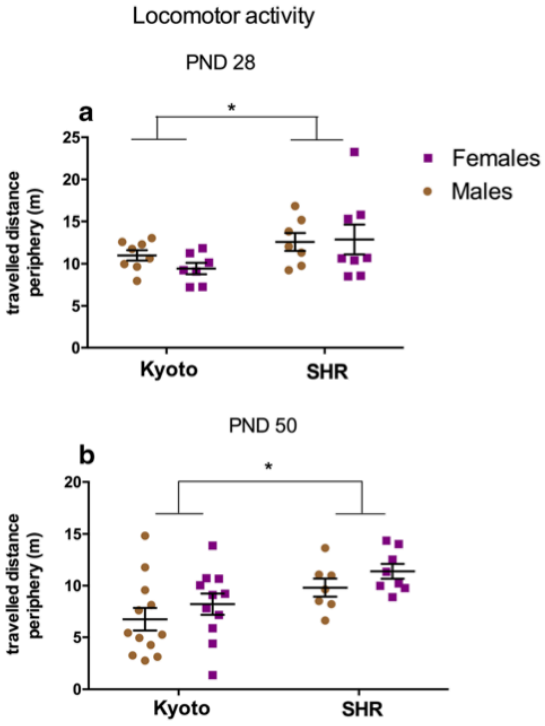


Fig. 2 Strain differences in the locomotor activity. Panels show the total travelled distance in meters in the open field during 5 min. **a** Male and female Kyoto and SHR rats at PND 28. Data are means ± S.E.M ($n = 7–8$ SHR; 11–12 Kyoto rats). **b** Male and female Kyoto and SHR rats at PND 50. Data are means ± S.E.M ($n = 7–8$ SHR; 12–13 Kyoto rats). $^{*}P < 0.05$; two-way ANOVA revealed significant effect of strain at both ages

Statistical Analyses

Data were analyzed using two-way ANOVA to compare sex and strain. One-way ANOVA was used to analyze protein immunoblotting. Paired *t* test was used to compare training and test sessions within groups in the object recognition task and habituation in the open field task. Percentage of the number of entries and time spent in the novel arm in the Y-maze task in the test session were compared to a chance (33%). Data are expressed as means ± SEM and differences were

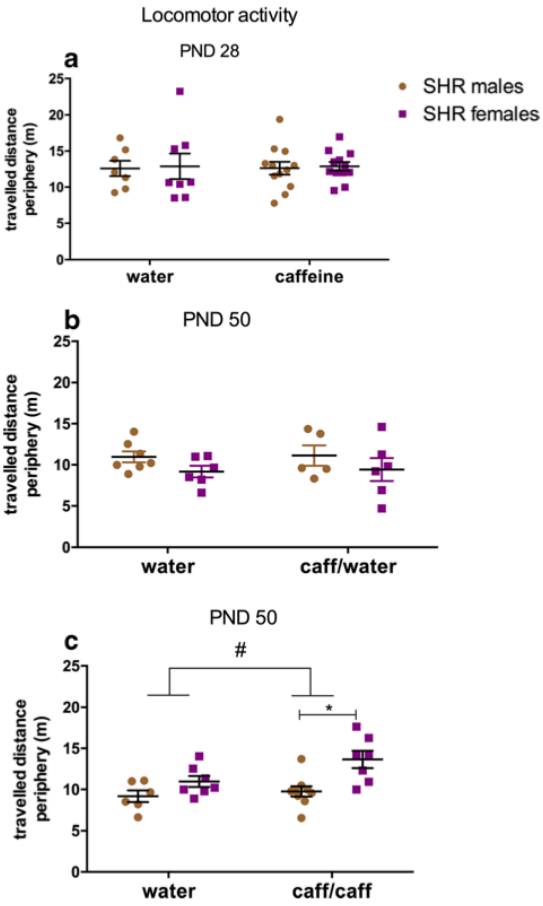


Fig. 3 Effects of caffeine (0.3 g/L) intake started at PND 15 in the locomotor activity of male and female SHR rats. Panels show the total distance travelled in meters (m) in the open field during 5 min. **a** Locomotor activity evaluated at PND 28 after receiving water or caffeine. Data are means ± S.E.M ($n = 7–12$ animals). No significant differences. **b** Locomotor activity evaluated at PND 49–50 after receiving water or caffeine up to PND 28 (caff/water). Data are means ± S.E.M ($n = 5–7$ animals). No significant differences. **c** Locomotor activity evaluated at PND 50 after receiving water or caffeine up to PND 50 (caff/caff). Data are means ± S.E.M ($n = 6–9$ animals). $^{\#}P < 0.05$; two-way ANOVA; treatment differences; $^{*}P < 0.05$; differences between sexes within group

considered for $P < 0.05$. Graph pad prism 6.0 was the software used for statistical analysis and figures.

Results

Locomotor Activity Is Altered by Caffeine in a Sex-Dependent Manner

Locomotor activity was evaluated in both late childhood (PND 28) and at the end of adolescence (PND 50). Two-way ANOVA revealed only significant strain effect for the travelled distance in the periphery at PND 28 [$F(1,26) = 4.724$; $P < 0.05$] and PND 50 [$F(1,36) = 9.340$; $P < 0.01$] (Fig. 2a, b).

SHR rats that received caffeine since PND 15 did not show differences in the travelled distance in the periphery either when they evaluated at PND 28 (Fig. 3a) or when caffeine treatment was interrupted at PND 28 and locomotor activity was evaluated at PND 50 (Fig. 3b, caff/water group). Two-way ANOVA revealed a significant effect of treatment [$F(1,25) = 4.373$; $P < 0.05$] and sex [$F(1,25) = 13.19$; $P < 0.01$] in the travelled distance in the periphery by SHR rats that received caffeine from PND 15 to PND 55 and locomotor activity was evaluated at PND 50 (caff/caff group) (Fig. 3c).

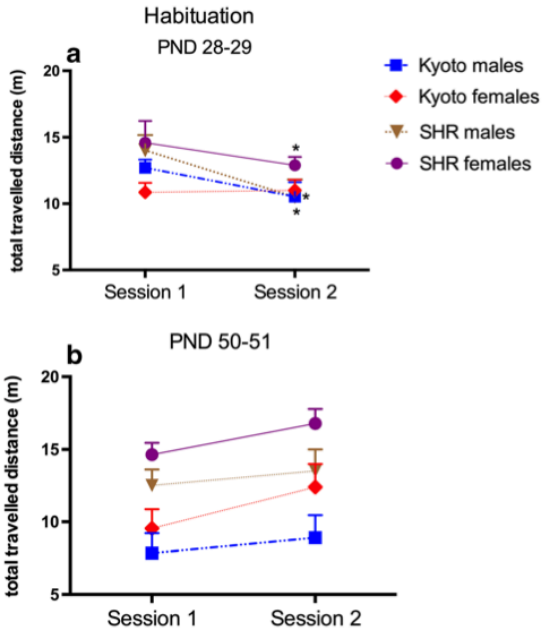


Fig. 4 Habituation of male and female Kyoto and SHR rats. Panels show the total distance travelled in meters (m) in the open field during 5 min in two consecutive days. **a** Habituation evaluated at PND 28–29. Data are means ± S.E.M ($n = 7$–8 SHR; 11–12 Kyoto rats animals). **b** Habituation evaluated at PND 50–51. Data are means ± S.E.M ($n = 7$–8 SHR; 12–13 Kyoto rats). *$P < 0.05$; paired t test, differences between sessions

Caffeine During Childhood Restores Habituation in SHR Female Rats

Habituation was analyzed by recording the total travelled distance during two consecutive days of exposure to the open field. Except for female Kyoto rats, male Kyoto ($t = 2.985$; $P < 0.01$) and male/female SHR rats ($t = 2.788$; $P < 0.05$; $t = 3.823$; $P < 0.01$, respectively) decreased total travelled distance

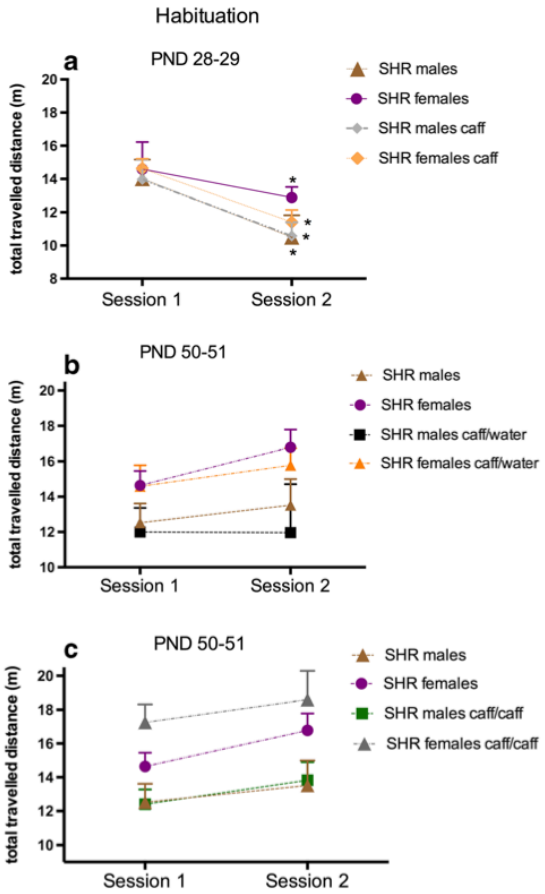


Fig. 5 Habituation of male and female SHR rats during different schedules of caffeine treatment. **a** Habituation evaluated at PND 28 after receiving water or caffeine. Data are means ± S.E.M ($n = 7$–12 animals). *$P < 0.05$; differences between the first and second day of open field exposure. Paired t test. **b** Habituation evaluated at PND 50–51 in rats receiving water or caffeine up to PND 28 (caff/water). **c** Habituation evaluated at PND 50–51 in rats receiving water or caffeine up to PND 50–51 (caff/caff). Data are means ± S.E.M ($n = 5$–7 animals caff/water group), ($n = 6$–9 animals caff/caff group). Paired t test. No significant differences

from session 1 to session 2 at PND 28–29 (Fig. 4a), but not at PND 50–51 (Fig. 4b).

Female SHR rats decreased the travelled distance between sessions after treatment with caffeine since PND 15 to 28 ($t = 3.823$; $P < 0.05$) (Fig. 5a). Caffeine either administered up to PND 28 (caff/water) or PND 55 (caff/caff) did not modify the travelled distance between sessions evaluated at PND 50–51 (Fig. 5b, c).

Both schedules of caffeine treatment restore recognition memory in SHR rats from both sexes.

Recognition memory was evaluated at PND 52. Different from Kyoto rats from both sexes, SHR rats did not show differences in the discrimination ratio between training and test sessions (Fig. 6a). Differences between sessions were observed in SHR rats from both sexes that received caffeine since PND 15 up to PND 28–29 (caff/water group) ($t = 6.883$; $P < 0.001$, females), ($t = 5.943$; $P < 0.001$, males) and also for continuous caffeine (caff/caff group ($t = 7.302$; $P < 0.001$, females) and ($t = 4.711$; $P < 0.001$, males) (Fig. 6b).

Sex Differences in the Effects of Caffeine in the Spatial Working Memory

Spatial memory was evaluated in the Y-maze task at PND 53. Two-way ANOVA revealed a significant effect of sex [$F(1,35) = 6.848$; $P < 0.05$] in the total number of entries in the three arms during test session (Fig. 7a). While Kyoto rats from both sexes and SHR male rats did not increased the percentage of number of entries in the novel arm, female SHR rats showed a significant decrease in this percentage ($t = 2.542$; $P < 0.05$) (Fig. 7b). There was no sex and strain difference in the time spent in the novel arm (Fig. 7c).

Caffeine was devoid of effect in SHR rats from both sexes with respect to the total number of entries in the three arms (Fig. 8a). Female SHR rats, but not males, increased the percentage of entries in the novel arm after caffeine intake from PND 15 up to PND 55 (caff/caff) ($t = 3.843$; $P < 0.05$) (Fig. 8b). They also increased the percentage of time spent in the novel arm, but when caffeine treatment lasted up to PND 28–29 (caff/water) ($t = 2.795$, $P < 0.05$) (Fig. 8c).

BDNF and Its Related Proteins Were Altered in SHR Rats from Both Sexes

BDNF and its related proteins were evaluated in the hippocampus of Kyoto and SHR rats from both sexes at PND 55. Two-way ANOVA revealed a significant effect of strain on BDNF levels [$F(1,31) = 8.357$; $P < 0.01$] (Fig. 9a), while the precursor form (proBDNF) remained unaltered (Fig. 9b). The TrkB receptor full length (TrkB-FL), phospho-TrkB, and truncated form of TrkB receptors were immunodetected in the

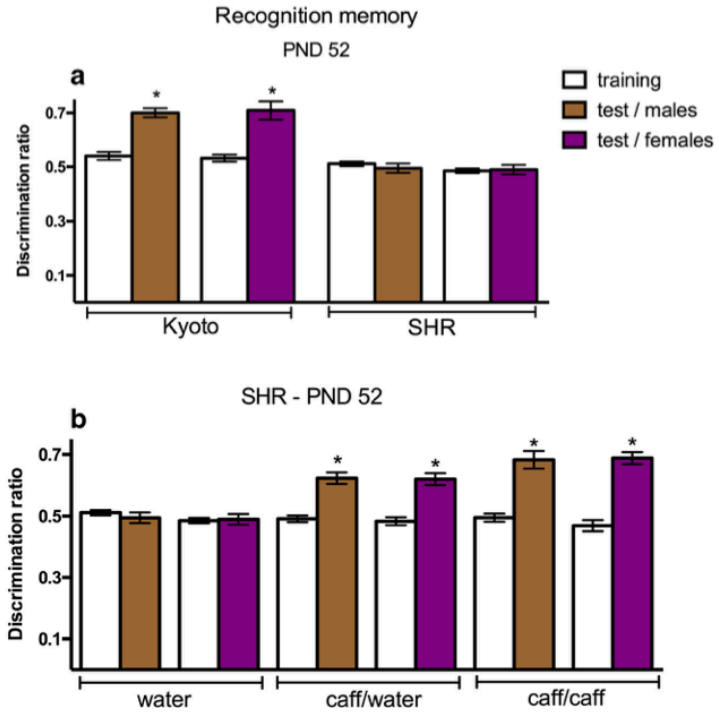


Fig. 6 Recognition memory in male and female Kyoto and SHR rats evaluated at PND 52. Panels show the discrimination ratio in the training (white bars), and test session (brown/purple bars). **a** Discrimination ratio of male and female SHR and Kyoto rats. Data are means ± S.E.M ($n = 13$–15 Kyoto rats), ($n = 7$–8 SHR rats). **b** Discrimination ratio of male and female SHR rats that received water, caffeine up to PND 28 (caff/water) or caffeine up to PND 51 (caff/caff). Data are means ± S.E.M ($n = 7$–8 animals, caff/water group), ($n = 9$–12 animals, caff/caff group). *$P < 0.05$; paired t test, differences between sessions

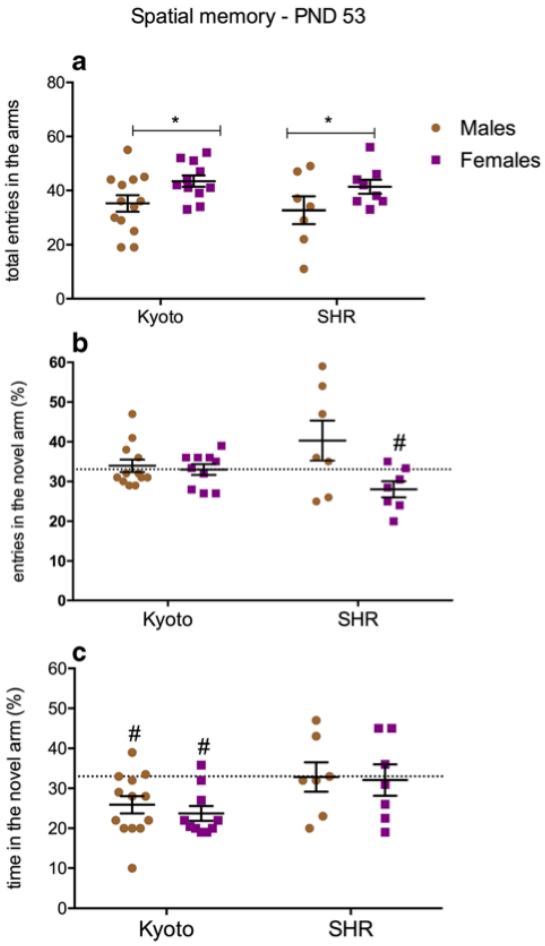


Fig. 7 Spatial memory in male and female Kyoto and SHR rats evaluated at PND 53. **a** Total number of entries in the three arms. **b** Percentage of number of entries in the novel arm. **c** Time spent in the novel arm during the test session. Data are means ± S.E.M ($n = 10$–13 Kyoto rats; $n = 7$ SHR rats). $^{*}P < 0.05$, two-way ANOVA, sex differences within a strain. $^{\#}P < 0.05$; one-way ANOVA, compared to 33% chance

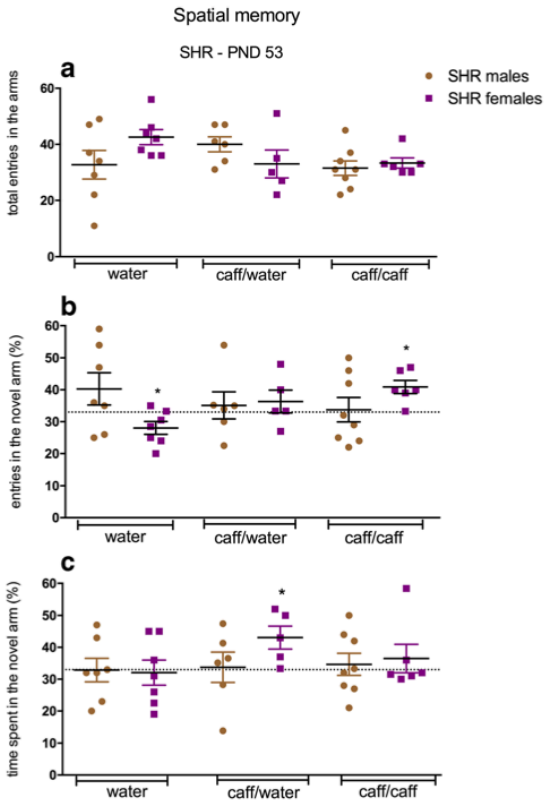


Fig. 8 Spatial memory in male and female SHR rats evaluated at PND 53 after caffeine (0.3 g/L) treatment since PND 15. **a** Total number of entries in the three arms. **b** Percentage of the number of entries in the novel arm. **c** Time spent in the novel arm during the test session. Male and female SHR rats received water, caffeine up to PND 28 (caff/water group) or caffeine up to PND 52 (caff/caff group). $^{*}P < 0.05$, two-way ANOVA, sex differences within a strain. $^{\#}P < 0.05$; one-way ANOVA, compared to 33% chance

hippocampus of Kyoto and SHR rats from both sexes. Two-way ANOVA revealed a significant effect of strain for the truncated form $F(1,44) = 10.57$; $P < 0.01$)] and also for phospho-TrkB [$F(1,22) = 4.499$; $P < 0.05$)] (Fig. 9d, e). In addition, the transcription factor CREB was not altered either by strain or sex (Fig. 9f), but its phosphorylated form (phospho-CREB) was increased in the hippocampus of SHR rats from both sexes [$F(1,22) = 9.190$; $P < 0.01$)] (Fig. 9g).

Sex-Related Differences in the Effects of Caffeine on BDNF and TrkB Receptors

Based on previous findings, we evaluated the impact of caffeine only in the BDNF levels and TrkB receptors (TrkB-FL, phospho-TrkB, and TrkB-T). Caffeine administered since PND 15 up to PND 55 (caff/caff) decreased BDNF levels in the hippocampus from SHR male rats [$F(2,19) = 4.451$; $P < 0.05$] (Fig. 10a), while BDNF levels were unaltered in SHR female rats in both schedules of treatment (Fig. 10a). In male rats, caffeine in both schedules of treatment did not change either TrkB-FL or TrkB-T levels (Fig. 10b, c), while females SHR rats showed decreased TrkB-FL and TrkB-T forms by caffeine treatments (Fig. 10b, c). Both increased phospho-TrkB and CREB were not modified in the hippocampus from SHR rats after caffeine treatments (data not shown).

Discussion

Considering that the symptomatology in ADHD patients appears to be sexually dimorphic, we attempted to find some sex

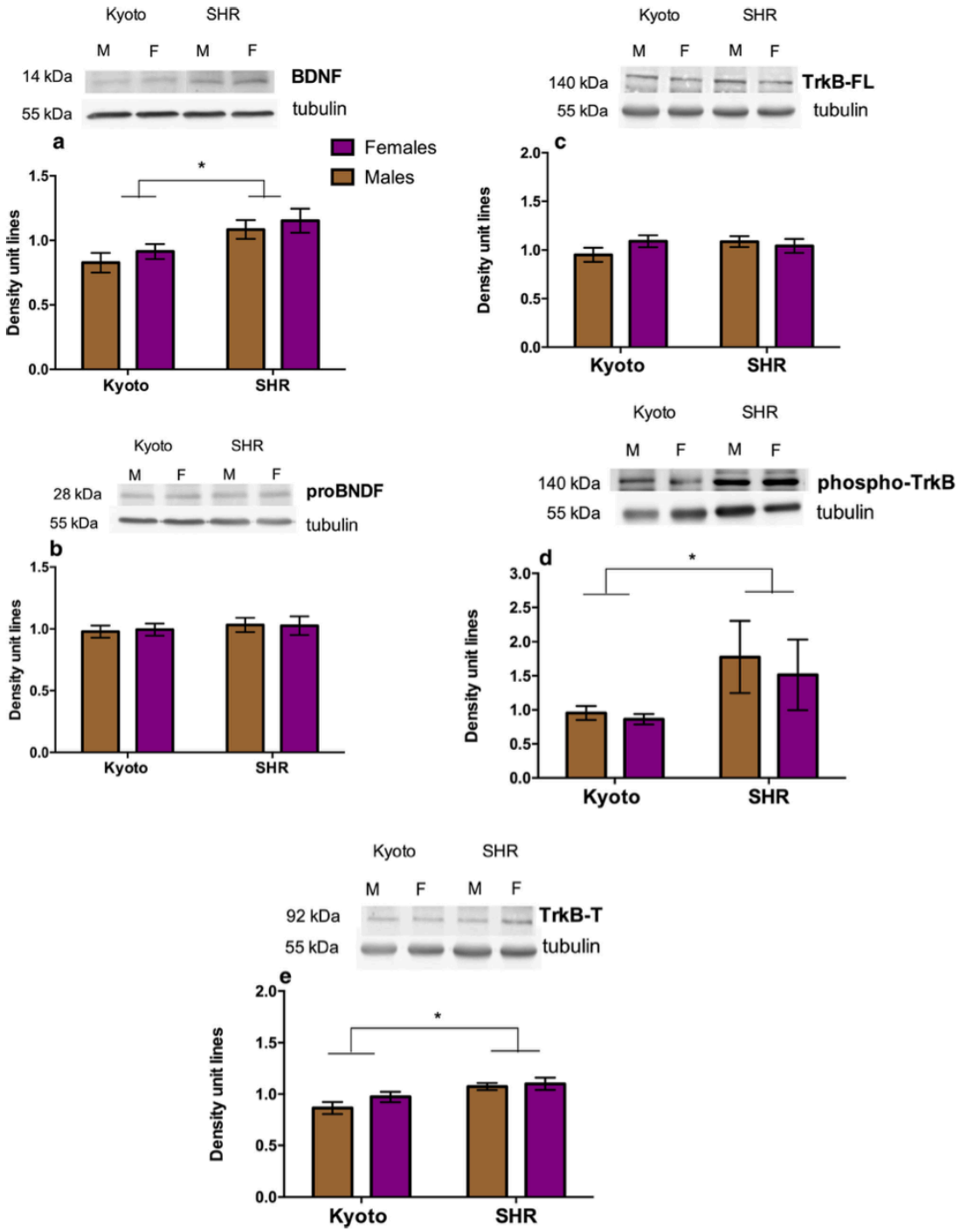


Fig. 9 BDNF, proBDNF, TrkB receptors, and CREB in the hippocampus of male and female Kyoto and SHR rats. Samples from the hippocampus were collected at PND 55. **a** BDNF. **b** proBDNF. **c** TrkB receptors full length (TrkB-FL). **d** Phospho-TrkB (p-TrkB). **e** Truncated form (TrkB-T). **f** CREB. **g** phospho-CREB. Data are represented as means ± S.E.M (n = 5–10 animals per group) of the density unit lines (normalized by β-tubulin). At the top of the figures are the representative bands for all proteins. *$P < 0.05$; two-way ANOVA, strain differences

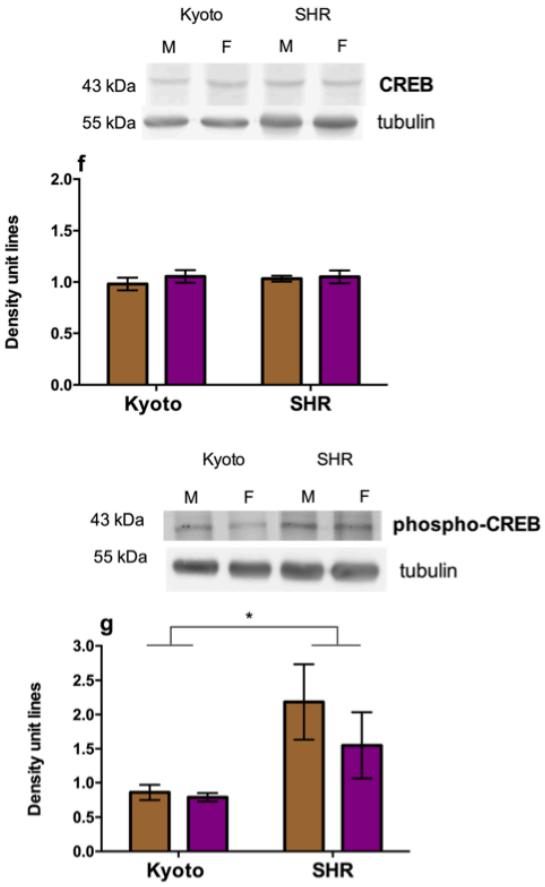


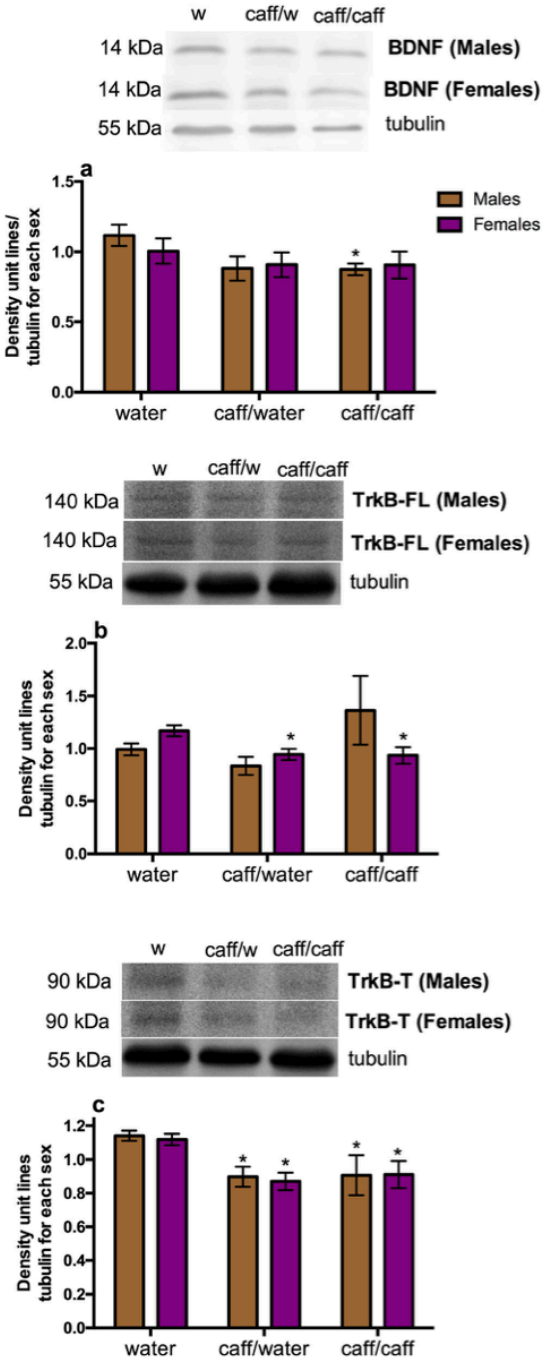
Fig. 9 (continued)

differences in the behavior and neurochemical correlates, namely BDNF and its related proteins, in the most validated experimental model to study ADHD. Besides, we also evaluated the impact of caffeine in these parameters.

The hyperactivity is one of the cardinal symptoms of ADHD patients, and both SHR male and female rats displayed hyperlocomotion from childhood up to adolescence, which is in accordance with previous studies [33–36]. The habituation of the animals can be provided by a second exposure to the open field test, which is a form of non-associative learning. It could be observed a sex and age difference in the non-associative learning, with SHR female rats showing lack of habituation since childhood, while SHR male rats showed lack of habituation only in the adolescence. Probably, two consecutive days of open field exposure would not be sufficient to provide habituation for SHR female rats. Some studies have supported that SHR females need more days to improve their performance, suggesting that female rats are slower in the learning process [37]. In one study, the ability of rats to learn a simple stimulus-reward conditioned task has showed that male SHR rats exhibited more conditioned performance than female SHR rats [38]. Other studies have reported better performance for male SHR rats than females [30, 39, 40]. Importantly, it cannot be discarded that the hyperactivity may be contributing for the lack of habituation.

Caffeine promoted distinct effects according to sex and age in SHR rats. The interruption of the caffeine treatment did not cause long lasting effects in the locomotor activity to both sexes. Caffeine did not affect hyperlocomotion in both sexes during childhood, but continuous treatment exacerbated the hyperactivity in adolescent female SHR rats. Previous studies have also shown that caffeine did not modify locomotor activity in adolescent males [29] and pubertal females [30]. These findings suggest that caffeine intake since childhood may exacerbate the hyperactivity in females but only if the administration persists up to adolescence. The lack of habituation observed in females was reversed by caffeine treatment during childhood. Thus, caffeine may be useful in the management of non-associative learning impairment in earlier periods of brain development, but it is more limited for the management of motor disturbances in females.

SHR rats from both sexes showed impairment in the novel object recognition, while both strains showed a poor performance in the Y-maze task. Of note, female SHR rats showed the worst performance in the Y-maze. Recognition memory has been repeatedly found impaired in SHR rats from both sexes [30, 31], but a series of studies have challenged spatial memory deficits in SHR rats in different paradigms, having some authors attributed distinct performances to hyperactivity as a confound factor [41–45]. In our case, total entries in the arms were similar between strains, and therefore, hyperactivity may not be involved in the performance presented by both rat strains. Nevertheless, our data confirmed previous findings, which show that continuous caffeine intake improves recognition memory in SHR rats [29, 30, 32], and they also add new information to this study. Here, caffeine intake restricted to childhood restored recognition memory in adolescent SHR rats from both sexes. Another novel finding in this study is that caffeine effects on spatial memory was observed only in female SHR rats, but this methylxanthine do not affect all aspects of Y-maze behavior in the same way. That is, caffeine intake during childhood has selective effect on one component of Y-maze behavior, namely on the inspective behavior, as evidenced by the increased number of entries in the novel arm. With regard to the inquisitive behavior, as gauged by increased time spent in the novel arm, the continuous caffeine intake had effect. Both measurements represent spatial recognition but with distinct behavioral responses, and therefore, female SHR rats were the only benefited from caffeine intake.



◀ **Fig. 10** BDNF and TrkB receptors in the hippocampus of male and female SHR rats after caffeine (0.3 g/L) treatment. Samples from the hippocampus were collected at PND 55. **a** BDNF levels. **b** TrkB receptors full-length (TrkB-FL). **c** Truncated form (TrkB-T). Male and female rats that received water, caffeine up to PND 28–29 (caff/water group) or caffeine up to PND 55 (caff/caff group). Data are represented as means ± S.E.M ($n = 6$–9 animals per group) of density unit lines (normalized by β-tubulin). $^*P < 0.05$, one-way ANOVA, difference from water group

Few studies have investigated modifications in the BDNF and its related proteins in animal models of ADHD. In one study, prefrontal cortex of male adolescent SHR rats presented normal levels of BDNF [42], while BDNF and TrkB receptors were decreased in the hippocampus of adult SHR male rats [46]. Our data showed that BDNF and the truncated form of TrkB receptors increased in the hippocampus of adolescent SHR rats from both sexes. Plasma levels of BDNF were increased in untreated children diagnosed with ADHD [47], while adult patients presented decreased BDNF levels [48].

The hyperactivity and memory deficits presented by SHR rats could be linked to the increase of BDNF, truncated and phosphorylated forms of TrkB and phospho-CREB, suggesting an overactivation of the BDNF pathway in the ADHD model. For instance, BDNF levels can vary among ADHD subgroups [49, 50] and higher plasma levels of BDNF have been hypothesized to a lower treatment response, particularly the hyperactivity symptom [16]. It is noteworthy that BDNF is crucial for the normal brain development and also memory processes [51–53], but prolonged or excessive activation of BDNF pathways causes aberrant signaling, which leads to a decreased dendritic and axonal branching [54]. If the increase in the BNDF and TrkB could be related to the transcription factor CREB, and consequently to behavioral alterations, caffeine should rescue these phenotypes. In previous study, caffeine was able to stimulate CREB activity in mouse cultured cortical neurons [55]. Interestingly, sex differences related to the effects of caffeine were observed for locomotor activity and spatial memory, in which adolescent female SHR rats were most benefited ones. The truncated TrkB receptor lacks the catalytic tyrosine kinase domain and there are evidences showing that TrkB-FL/TrkB-T imbalance and aberrant TrkB signaling may be causally linked to neurodegeneration and neuropsychiatric disorders [56–58].

Recently, an overactivation of noradrenergic activity was reported by the increase of phospho-CREB/CREB in the locus coeruleus of PI3Kγ knockout mice, which resembling most of the ADHD behavioral phenotypes [59]. In the same study, ADHD behavioral phenotypes were rescued in PI3Kγ knockout mice after blocking CREB activity with an adeno-associated viral vector. Although caffeine did not change both phospho-TrkB and CREB (data not shown), it decreased the truncated TrkB receptor in SHR rats from both sexes, coincident with restoring recognition memory. Likewise, full-length TrkB receptors levels were decreased by caffeine only in female SHR rats as well as improvements in their spatial memory. Concurrently, continuous caffeine treatment prevented increases in the BDNF levels only in male SHR rats, and a

trend towards decrease was observed in adolescent male rats that received caffeine only during childhood. Thus, it cannot be discarded that caffeine was devoid of effect in both behavioral outcomes by a decrease on BDNF levels in adolescent male SHR rats. Similarly, decreased BDNF levels were observed in juvenile rats treated with methylphenidate [60]. In another study, even though basal levels of BDNF had not been compared to Kyoto control strain, methylphenidate decreased BDNF expression in the prefrontal cortex of SHR male rats, which was similar to our findings with caffeine [61]. Although psychostimulants have been reported to increase BDNF levels in adults [13, 60, 62], childhood comprises a phase of intense synaptic plasticity and exposure to psychostimulants, which may induce opposite effects when compared to adulthood [6, 63, 64].

Over the past years, many epidemiological and experimental studies have highlighted the potential of caffeine in preventing memory impairment in Alzheimer's disease and also age-related cognitive decline [65–69]. Most recently, studies from our group and others on the effects of caffeine have been conducted during brain development in order to establish a safety dose, which produces minimal effects on behavior and brain homeostasis in puberty, adolescence and/or adulthood [70–74]. On the other hand, few studies have been designed to investigate the effects of caffeine in neurodevelopmental disorders.

In this study, female SHR rats showed more cognitive impairments than males, which is similar to epidemiological studies that girls show more predominantly the inattentive subtype [75, 76]. ADHD is usually treated with psychostimulants, but almost 30% of children have been reported to not tolerate or respond to the first line of treatment [77, 78]. Our data strongly highlight the potential of caffeine as an adjuvant or an alternative treatment for ADHD, especially for those patients who are non-responsive to classical treatment. Considering different results for male and female SHR rats and distinct responses to caffeine treatment, our data reinforces the importance of including both sexes in further studies with ADHD models. Finally, pharmacological manipulation of adenosine receptors could be a potential target for the development of new drugs for the treatment of ADHD.

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Author's Contributions F.N., D.P., A.S.A, and D.M.M. performed the experiments and revised the manuscript. F.N and L.O.P designed the study, analyzed and interpreted data, and wrote the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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Artigo 2 – Manuscrito em preparação.

Caffeine recovers frontal cortex-mediated cognitive and functional impairment in a sex dependent manner in the murine model of Attention Deficit and Hyperactivity Disorder

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Abstract

Attention deficit hyperactivity disorder (ADHD) comprises a triad of symptoms inattention, hyperactivity and impulsivity that show differences between both sexes. Caffeine is the most psychostimulant consumed worldwide with beneficial effects in ADHD models. Females are usually underrepresented in experimental studies focusing on new strategies for the treatment of neurological disorders. In this study, we examine the effects of caffeine since childhood in the behavioral, neurochemical and neurofunctional correlates associated with frontal cortical functioning in adolescent male and female spontaneously hypertensive rats (SHR, a validated ADHD animal model). While male SHR were slightly more hyperactive than their female counterparts, caffeine was able to prevent the hyperactivity in both sexes. In a decision-making task, females SHR presented a worsened performance than males SHR, but caffeine improved learning in females and strategy change and flexibility traits in males SHR. Presynaptic protein SNAP-25, dopamine transporter (DAT) and D4 receptors were decreased in the frontal cortex of SHR from both sexes. While caffeine restored DAT levels in both sexes, D4 receptors were recovered only in males SHR. Electroencephalogram revealed reductions in delta and gamma power in females SHR and increases in delta and gamma power in males SHR. Caffeine decreases delta and gamma power in males SHR. Our findings revealed important sex-dependent differences in the ADHD model. Caffeine was able to recover some of these correlates related to frontal cortex functioning in a sex dependent manner. These findings shed light on the importance of sex as biological variable when making treatment decisions in neuropsychiatric disorders.

Keywords: ADHD, caffeine, dopamine, adenosine, sex differences

Introduction

Attention deficit and hyperactivity disorder (ADHD) is one of the most commonly diagnosed neurodevelopmental disorder with childhood onset, which can persist in some cases up to early adulthood (Biederman et al., 2010). The symptomatic triad of ADHD is characterized by inattention, hyperactivity and impulsivity, but patients can present inattention or the combined subtype with hyperactivity and impulsivity (American Psychiatric Association, 2013).

The worldwide prevalence is estimated around 3.4 % in children and adolescents (Polanczyk et al., 2015), with a male-to-female ratio of 3:1 in population based studies and between 5:1 to 9:1 in clinical samples, which seems not be restricted to prevalence (Gaub and Carlson, 1997; Sandberg, 2002). While girls often receive a diagnosis later than boys since they are more prone to have difficulties with inattentive symptoms (Bierderman et al., 2002; Gaub and Carlson, 1997; Nussbaum, 2012), boys are more hyperactive and impulsive, evidencing these disparate clinical courses between the sexes (Gershon, 2002; Skogli et al., 2013). Importantly, these sex differences are relevant in the attentional performance because inattention is assessed for diagnostic purposes and also determines the course of treatment.

Although ADHD is considered a complex and multifactorial disorder with an unknown etiology, the heritability is estimated being around 70 – 75 % in twin studies (Bierderman and Faraone, 2005; Faraone et al., 2005; Larsson et al., 2014; Nikolas and Burt, 2010), suggesting genetic influence in the pathophysiology (Faraone and Mick, 2010). Once confirmed high heritability, some gene polymorphisms have been associated with the severity of symptoms, which include the synaptosomal-associated protein of 25 kDa (SNAP-25), dopamine transporter (DAT) and dopamine receptor D4 (DRD4). In fact, polymorphisms in DAT and DRD4 were considered as risk factors for the development of this disorder (Albrecht et al., 2014; Gizer et al., 2009; Shaw et al., 2007). DRD4 has revealed to be closely related to the inattentive subtype of ADHD (Gizer et al., 2012) and DAT gene expression changes with the risk of ADHD development (Chen et al., 2003; Li et al., 2006).

Functional neuroimaging and quantitative electroencephalogram (EEG) have been performed in order to support clinical diagnosis and to better understand neurobiological substrates of ADHD. From these studies, some brain abnormalities in ADHD patients have been found such as smaller sized basal ganglia, corpus callosum, prefrontal cortex (PFC) and cerebellum (Castellanos et al., 1996; Filipek et al., 1997). In a recent cross-sectional mega-analysis, reductions were also found in limbic areas when compared to normal individuals (Hoogman et al., 2017). The DSM-5 highlights that individuals with ADHD typically show increased slow-wave electroencephalogram (EEG) (American Psychiatric Association, 2013), but significant EEG heterogeneity exists across ADHD-diagnosed individuals and the extent to which these EEG indicators are useful in clinical settings remains under investigation (Banaschewski and Brandeis, 2007).

Considering that dopaminergic projections from midbrain ventral tegmental area (VTA) to the striatal and prefrontal cortical areas play a major role in motor control, attention and impulsivity, alterations in the dopaminergic system are strictly involved in the ADHD symptomatology (Arnsten and Pliszka, 2011). The treatment for ADHD is usually with stimulant drugs being methylphenidate the first-choice drug, which the mechanism of action involves primarily inhibition of dopamine reuptake. However, it is estimated that approximately 30 % of patients show little or no symptomatic improvement after receiving stimulant medication, and also they do not tolerate the side effects caused by methylphenidate (*for review see* Wender, 1998; Wilens et al., 2002).

Caffeine is considered the psychostimulant most consumed worldwide, a non selective adenosine A_1 and A_{2A} receptors antagonist (A_1R and $A_{2A}R$) with psychostimulant effects (Fredholm et al., 1999). Caffeine has improved cognitive functions and performance in tasks requiring attention in several paradigms and protocols. Over the past years, the relief of ADHD symptoms by caffeine have shown conflicting results, which varied from studies showing some efficacy when compared to placebo treatment to others failing to find superior

effects when compared to the first line of treatment (*for review see* Ioannidis et al., 2014; Leon et al., 2000).

The use of caffeine as an alternative treatment for ADHD could arise from the premise that the habitual consumption of caffeine by ADHD patients may be a strategy to increase the ability to sustain attention and improve cognition, and also from its promising results with animal models (Nunes et al., 2018; Pandolfo et al., 2013; Pires et al., 2010). In our previous study, the efficacy of caffeine in preventing cognitive impairment associated with hippocampal functioning was observed predominantly in adolescent female SHR (Nunes et al., 2018). Here, our study aimed to assess sex differences in the most validated ADHD animal model regarding cognitive and motor alterations involving frontal functioning and neurochemical changes (Sagvolden et al., 2008). In parallel, we sought to investigate the impact of caffeine administration in these predictable sex differences in neurochemical and behavioral correlates involving frontal cortex functioning.

2. Material and Methods

2.1 Animals

Male and female SHR (NCrl) and Wistar-Kyoto rats (WKY/NIcoCrl) (90 days old) were mated within our colony at Federal University of Rio Grande do Sul. Animals were maintained under 12/12 h light-dark cycle (lights on at 7:00 AM), at constant temperature (22 ± 1 ºC) and with free access to food and water. The authors state that all animal experiments and protocols were approved and they were carried out in accordance with the Institutional Animal Care and Use Committee of Federal University of Rio Grande do Sul (CEUA-UFRGS Ethical Protocol number 29196), based on the guide for the care and use of laboratory animals from the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Caffeine treatment

After birth, pups at postnatal day 15 (PND 15) started to receive caffeine (0.3 g/L) or water from their SHR and WYK dams until weaning (PND 26). At PND 26, animals started to receive caffeine in the tap water and the treatment was maintained up to the end of behavioral tasks (around PND 55-59) (Fig. 1). Importantly, caffeine was available during behavioral tasks.

2.3 Behavioral analysis

Male and female rats started to be tested in the behavioral tasks after weaning (PND 26). The apparatus was cleaned out with 70 % alcohol after each animal tested. All behavioral tests were conducted by a trained observer, in a sound-attenuated room under low-intensity light. For the dig task, the scores of each animal were manually registered. For locomotor activity, all parameters were video recorded and analyzed by means of a computer-operated tracking system (Any-maze, Stoelting, Woods Dale, IL).

2.3.1 Testing decision-making behavior: dig task

The dig task relies on the basic operant: principles of learning and decision-making. All guidelines were detailed followed in agreement with previously reported (Martens et al., 2012; 2013). This task is based on scent discrimination paradigm in order to evaluate frontal cognition deficits (Martens et al., 2012; 2013). Training and testing phases were conducted in a modified operant chamber (350 x 280 x 500 cm), with two PCV pipes end-caps in one extremity to put reinforcers, in our case fruit-loops cereal. The food restriction should be initiated three days before training, and the animals need to be maintained at 90 % of free feeding during all testing phase. At PND 26-27 animals were firstly exposed to the reinforcer three to five days before initiating to learn digging. After training sessions, animals should be

able to dig faster and eat just 1/3 of fruit-loops as a reinforcer. In the testing phase, cacao and cumin were paired into sand (1 g of each scent for 110 g of sand), each one in each PVC end-caps. Testing phase comprises discrimination and reversal phases. For the discrimination phase, cacao was the reinforcer, whereas cumin was the reinforcer in the reversal phase. The rat was put in the chamber faced to the wall, in the opposite side and equidistant of the cups. Once the rat found out the reinforcer in the correct scent within 30 seconds, it was allowed to consume it; then rat was placed back into holding apparatus. If the animal discriminates incorrectly, it was immediately removed from the holding apparatus for 30 seconds in order to avoid that it "corrects" itself. Once a rat achieves a pre-determined criterion (i.e. > 80% accuracy per day for three consecutive days), the next discrimination will proceed and the above process will repeat for 8 trails/day. To move on to reversal phase, rats must have 90 % of accuracy in three consecutive days, i. e., they need to hit 7 in 8 attempts. Likewise, the testing phase will finish when the animals reached the same criterion described above. Results were represented as the number of trials required to reach the criterion in each phase, and also percentage of the right choices.

2.3.2. Lomocotor activity and non-associative learning: open field-testing

Locomotor activity and non-associative learning was monitored in an open field arena during two consecutive days (24 h intertrial interval), namely day 1 and day 2. The apparatus was made of black-painted wooden arena measuring 60 cm diameter and surrounded by 50 cm high walls. Each rat was placed in the periphery of the open field and the total travelled distance (periphery and center) was recorded during 5 min. Locomotor activity was evaluated by analyzing the travelled distance only in the periphery and habituation was analyzed by total travelled distance in both areas (center and periphery). Habituation was calculated by the ratio of day 1/day 2 measurements. The experiments were conducted in a sound-attenuated room under low-intensity light; activity was recorded with a video camera positioned above the

apparatus and monitored in an adjacent room by an observer blinded to the treatment and strains. The open field apparatus was always cleaned after the end of each session performed by each animal. The following parameters were recorded in each day: travelled distance in the periphery and center, distance and number of inter-stops, number and time spent rearing.

2.4 Neurochemical analysis

2.4.1 Immunoblotting

DAT and D4DR were evaluated in the frontal cortex (FC) and striatum (ST) of Kyoto and SHR rats from both sexes at the end of behavioral dig task (PND 56-59). Twenty-four hours after the end of dig task, rats were sacrificed under anesthesia. The frontal cortex (FC) was dissected out and immediately homogenized in a 5 % SDS solution containing a cocktail of protease and phosphatase inhibitors (Sigma, São Paulo/SP, Brazil), and frozen at -20º C. After defrosting, the protein content was determined using the bicinchoninic acid assay (BCA) (Pierce, São Paulo/ Brazil). The sample extracts were diluted to a final protein concentration of 2 µg/uL in SDS solution and 60 µg of protein were applied for SDS–PAGE analysis together with pre-stained molecular weight standards (Bio-Rad, São Paulo/Brazil), to a 10–12 % SDS–PAGE running gel with a 4 % concentrating gel. After electrotransferring, membranes were blocked with Tris-buffered saline containing 0.1 % Tween-20 and 3 % bovine serum albumin (BSA) during 1 h. The nitrocellulose membranes (Amersham, São Paulo/Brazil) were then incubated overnight at 4° C with rabbit anti-dopamine receptor D4 (D4DR) (1:1000; Abcam, São Paulo/Brazil), mouse anti-dopamine transporter (DAT) (1:1000; Abcam, São Paulo/Brazil). The membranes were washed and incubated with horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature and developed with chemiluminescence ECL kit (Amersham, São Paulo/Brazil). Densitometric analyses were performed using the NIH ImageJ software. After stripping, ß-tubulin was quantified as a loading control using a mouse anti - ß-tubulin antibody (1:1000; from Santa Cruz Biotechnologies, São Paulo/Brazil), as described above.

2.4.2 Immunohistochemistry

Rats were transcardially perfused with phosphate-buffered saline (PBS) pH 7.4 and the brains were removed from the skull and post-fixed with 4% paraformaldehyde (Sigma Aldrich, São Paulo/Brazil) solution at 4° C for 24 h. After this period, the brain was cryopreserved in 30 % sucrose solution containing 0.02 % sodium azide at 4°C. Coronal sections (30 µm thick) were obtained in a vibratome (Leica, São Paulo/Brazil) and mounted on slides coated with 5 % gelatin with chromium and potassium sulfate. Four mounted-slices at 180-µm interval from frontal córtex were incubated with antigen retrieval solution (10 mM citric acid, pH 6.0) for 15 min, rinsed in PBS (pH 7.4) and permeabilized with 1 % Triton X-100. Blockade was carried out in 5 % normal goat serum (Sigma Aldrich, São Paulo/Brazil) with 0.3 % Triton X-100 during 1 h at room temperature and then incubated with rabbit anti-synaptosomal-associated protein of 25 kDa (SNAP-25) (1: 1000, Sigma Aldrich, São Paulo/Brazil) or rabbit anti-synaptophysin (1: 400; Sigma Aldrich, São Paulo/SP, Brazil) at 4 °C for 48 h, in a humid chamber. After rinsing five times in PBS (pH 7.4), sections were incubated with anti-rabbit secondary antibody conjugated to Alexa Fluor 488 or 594 (1:500; Invitrogen, São Paulo/ Brazil), in PBS for 2 h at room temperature. After staining, sections were washed in PBS (for 3 × 5 min), counterstained with 0.001 % DAPI for 15 min. Coverslips were mounted using fluorescence mounting medium (Dako, São Paulo/Brazil). All images were acquired using a fluorescence Nikon microscope with NIS Elements AR 2.30 software. The Tiff images (1372 × 1024 pixels) were captured at an objective lens magnification of 20×. The fluorescence intensities were quantified using the NIH ImageJ software, using the setup calibration information of the NIS Elements AR 2.30 software of 0.322 µm/pixel for the objective 20x.

2.5 Functional analysis

2.5.1 Electroencephalography

2.5.1.1. Electrodes implantation

Animals were anesthetized with ketamine (90 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and carefully placed on the stereotaxic apparatus for electrodes implantation. Four recording stainless steel subdural electrodes were implanted in the frontal cortex (AP + 3.4) and hippocampal area (AP -3.6, LL ± 2.2). The reference electrode was placed in the occipital bone and one screw was placed in frontal bone for fixation. Electrodes were fixed in place with dental cement. After surgery, each animal was place in an individual Plexiglass cage for recovery. Baseline video-EEG recordings were performed 7 days after surgery.

2.5.1.2. Video-EEG recordings

Each animal was transferred to the open field arena, the electrodes connected to an amplifier (MAP-32, Plexon, Inc.). Baseline video-EEG recordings were performed in all animals for 10 minutes (10 minutes of basal plus 10 minutes of test in the open field for each session). EEG signals were filtered at 0.01-100Hz followed by digitalization at 1 kHz for posterior analysis. The power of each individual band frequency was expressed as power ratio of baseline and the respective band frequency in each session of the open field testing (non moving and moving).

2.5.1.3. Data analysis

The data were analyzed in MATLAB (MathWorks Inc.) using built-in and custom-written routines. Power Spectrum Density was calculated by means of the Welch periodogram method using the pwelch.m function from the Signal Processing Toolbox (50 % overlapping,

4-s Hamming windows). The analysis was performed in two different time-periods (non-moving and freely moving, 2 seconds as minimal interval time record and 10 minutes as maximal interval time record) (Zhong et al., 2017). The decomposed signal was quantified in six frequencies bands: delta (1-4 Hz), theta (4-10 Hz), three different gamma sub bands: slow (30-50 Hz), middle (50-90 Hz), high gamma (90-150 Hz) and ripples (160-250 Hz). Alpha bands were not represented because different from humans, alpha band is replaced by a wider range of the theta band (usually 4–12 Hz) in rats (Buzsáki, 2006; Corsi-Cabrera et al., 2001).

3. Statistical Analysis

Data from Dig task and differences in power spectra analyses were evaluated by using two-way ANOVA followed by Bonferroni´s post hoc test to firstly compare strain and sex; then SHR were compared with treatment and sex as a factors. Data from differences between strain and sex proteins for protein immunocontent were evaluated by using two-way ANOVA, but comparisons between treatments on SHR samples were analyzed by one-way ANOVA. Data from all groups tested in open field testing (OFT) were first analyzed by Principal Component Analysis (PCA) with Varimax Rotation and Kaizer Normalization (Loss et al., 2015). If data were not fitted in the D'Agostino-Pearson omnibus normality test, they were transformed to assume Gaussian distribution and then submitted to Pearson correlation analysis. After setting the correlation among variables, PCA was performed in a set of 20 behavioral variables (8 from OFT day 1, 9 from OFT day 2 and 3 from OFT habituation OF1/OF2). Principal Components (PC) scores were extracted by using regression method. Data from PC scores were separately analyzed for strain and sex comparison (Two-way ANOVA [between-subjects factor 1: Strain; between-subjects factor 2: sex]) and for treatment and sex comparisons (Two-way ANOVA [between-

subjects factor 1: Treatment; between-subjects factor 2: sex]). A significance level of 0.05 was set for all analyses.

4. Results

4.1 Strain and sex differences between SHR and Kyoto at both phases of scent discrimination.

Scent discrimination was evaluated from late childhood up to early adulthood. Based on the premise that SHR rats would perform the task, but in a more delayed manner, a cut-off day was not set for any one-scent discrimination. Two-way ANOVA revealed a significant strain and sex differences, with only females from ADHD model requiring more trials to conclude discrimination phase [$F(1,28) = 7.838$; $P = 0.0092$; strain differences] and [$F(1,28) = 9.549$; $P < 0.0045$, sex differences] (Fig. 2A). However, two-way ANOVA showed that both sexes from ADHD model needed more trials to complete the task in the reversal phase [$F(1,28) = 30.77$; $P < 0.0001$, strain differences] (Fig. 2B). The number of right choices was also evaluated in both phases. While no differences were found in the discriminating phase (Fig. 2C), the number of right choices was significantly smaller in females from ADHD model in the reversal phase [$F(1,28) = 7.334$; $P < 0.0114$; strain differences] and [$F(1,27) = 5.338$; $P < 0.0287$; interaction] (Fig. 2D).

4.2 Caffeine improved learning and decision-making in SHR in a sex dependent manner.

SHR from both sexes received caffeine from PND 15 to PND 55-59. It can be observed that the number of trials required to achieve the criterion in the discrimination phase was higher only for females from ADHD receiving water [$F(1,24) = 14.12$; $P = 0.0010$; sex effect] and [$F(1,24) = 5.52$; $P = 0.0273$; treatment effect] (Fig. 3A). Otherwise, the number of trials in

order to achieve the criterion was smaller for males from ADHD model treated with caffeine in the reversal phase [$F(1,24) = 5.201$; $P = 0.0317$, interaction effect] and [$F(1,24) = 5.436$; $P = 0.0285$, treatment effect] (Fig. 3B). Caffeine treatment had no effect on the percentage of right choices performed by ADHD model from both sexes (Fig. 3C and D).

4.3. Caffeine counteracted hyperactivity in both sexes from ADHD model

Five principal components (PC) were obtained from principal component analysis (PCA), which corresponded to 87.6 % of total data variability (Supplementary Figures 1 A-I). After two-way ANOVA analysis, from five PC only two showed statistically significant findings for strain, sex and/or treatment. According to PC1 that gathered exploratory activities and locomotor parameters (Fig. 4A), the hyperactivity was observed for ADHD model (SHR) from both sexes [$F(1,35) = 33.56$; $P < 0.0001$; strain difference] (Fig. 4B) and fully prevented by caffeine treatment [$F(1,33) = 4.144$; $P < 0.05$; treatment effect] (Fig. 4C). According to PC4 that incorporates habituation to the number of stops (Fig. 4 D), male SHR presented the same number of stops between first and second open field sessions, which means that male SHR did not habituate [$F(1,35) = 5.098$; $P = 0.0303$; interaction effect] (Fig. 4E). However, male SHR after receiving caffeine treatment were able to habituate regarding the number of stops [$F(1,33) = 5.614$; $P = 0.0238$; interaction effect] (Fig. 4F).

4.4 – EEG of the frontal cortex from ADHD model (SHR) was modified by caffeine in a sex dependent manner

During the ECoG data collection, rats were placed in a black-painted wooden arena measuring 60 cm diameter and surrounded by 50 cm high walls, within which they could freely move. The ECoG quantification in the frontal region from SHR during the open field testing showed differences in specific brain oscillations. The theta/delta ratio was higher when female SHR were at resting state [$F(1,9) = 11.50$, $P = 0.0088$; non-moving 1, interaction] and [$F(1,9) = 9.592$, $P = 0.0128$; non-moving 2, interaction] (Fig. 5A and B). Similarly, theta/delta ratio was persistently higher in female SHR during locomotor activity in

both open field testing sessions [F(1,9) = 32.15, P = 0.0003; freely moving 1, interaction] and [F(1,9) = 39.32, P = 0.0001; freely moving 2, interaction] (Fig. 5C and D). Besides, male SHR presented lower theta/delta ratio during locomotor activity [F(1,9) = 7.486, P = 0.0230; freely moving 1, strain], [F(1,9) = 10.60, P = 0.0099; freely moving 1, sex] and [F(1,9) = 35.09, P = 0.0002; freely moving 2, sex] (Fig. 5C and D).

While female SHR exhibited decrease in delta power, male SHR showed higher delta power in the habituation (second day of open field testing) [F(1,9) = 7.841, P = 0.0207; non-moving 2, interaction] (Supplementary Fig. 3c). During locomotor activity, female SHR exhibited overall lower delta power, while male SHR exhibited higher delta power [F(1,9) = 8.022, P = 0.0196; freely moving 1, interaction], [F(1,9) = 10.39, P = 0.0091; freely moving 2, interaction] (Supplementary Fig. 3 c, e and g).

Caffeine increased theta/delta ratio in SHR from both sexes in the first open field session at rest [F(1,8) = 6.578, P = 0.0334; non moving 1, treatment] (Fig. 6A). In the second open field session, caffeine was devoid of effect in SHR from both sexes [F(1,8) = 10.79, P = 0.0111; non moving 2, sex effect] (Fig. 6B). Since caffeine decreased delta power in male SHR [F(1,8) = 5.380, P = 0.0490; freely moving 1, interaction effect] and a trend toward increase in the theta power was observed for female SHR [F(1,8) = 4.577, P = 0.0648; freely moving 1, interaction effect] (Supplementary Fig. 4e and f), caffeine did not change theta/delta ratio overall. On the other hand, male SHR treated with caffeine showed decreased delta power during locomotor activity in the second open field session [F(1,8) = 13.66, P = 0.0061; freely moving 2, interaction effect] and [F(1,8) = 6.768, P = 0.0315; freely moving 2, sex effect] (Supplementary Fig. 4g). Consequently, an increase in the theta/delta ratio was observed for male SHR treated with caffeine [F(1,8) = 7.883, P = 0.0229; freely moving 2, interaction effect] (Fig. 6D).

The higher gamma power was observed in male SHR at rest, during habituation (second day of open field testing) [F(1,9) = 5.895, P = 0.0381; freely moving 2, strain] and [F(1,9) = 16.54, P = 0.0028; freely moving 2, sex] (Fig. 7B). The lower gamma power in the frontal

region was observed only in females from ADHD model during locomotor activity [F(1,9) = 7.907, P = 0.0203; freely moving 1, interaction], [F(1,9) = 8.939, P = 0.0152; freely moving 1, strain] and [F(1,9) = 6.649, P = 0.0298; freely moving 1, sex], which persists during the second day of open field testing [F(1,9) = 6.829, P = 0.0281; freely moving 2, interaction] and [F(1,9) = 10.04, P = 0.0114; freely moving 2, sex] (Fig. 7 C and D).

Regarding to gamma power, caffeine was devoid of effect in both open field sessions at resting state (Fig. 8 A-C), but it promoted a decrease only in males from ADHD model (SHR) during habituation of locomotor activity (second open field session) [F(1,8) = 18.96, P = 0.0024; freely moving 2, interaction], [F(1,8) = 25.80, P = 0.0010; freely moving 2, treatment] and [F(1,8) = 25.80, P = 0.0010; freely moving 2, sex] (Fig. 8D).

4.5. Caffeine differently changes dopamine transporter (DAT) and dopamine D4 receptors (D4DR) levels according to sex dependent in ADHD model

Dopamine transporters (DAT) and D4 receptors (D4DR) were analyzed in the frontal cortex. DAT was decreased in the frontal cortex from ADHD model in both sexes when compared to Kyoto rats [F(1,33) = 17.34; P = 0.0002; strain differences] as well as D4DR [F(1,37) = 7.525; P = 0.0093; strain differences] (Fig. 9A and B). Based on these findings, we evaluated the impact of caffeine on DAT and D4DR in the frontal cortex. Caffeine treatment was able to restore DAT levels in males (t = 2.107; P < 0.05) and in females from ADHD model (t = 2.520; P < 0.05) (Fig. 10A and B). While caffeine restored D4DR males from ADHD model (t = 2.573; P < 0.05) (Fig.10 C), D4DR receptors were unaltered in females receiving caffeine (Fig. 10D).

4.6. Caffeine affected SNAP-25 in frontal cortex of SHR in a sex dependent manner

It can be observed that the synaptic vesicles marker synaptophysin was robustly increased in the frontal cortex of female SHR [F(1,10) = 17.73; P = 0.0018; strain differences] and [F(1,10) = 17.00; P = 0.0021; sex effect] (Fig. 11B, insert graphic). Caffeine had no impact on synaptophysin immunoreactivity in SHR from both sexes (Fig. 11C). Presynaptic protein

SNAP-25 was decreased in the frontal cortex from males and females from ADHD model [F(1,17) = 5.242; P = 0.0341; strain differences] (Fig. 11E, insert graphic). While SNAP-25 in females was not affected by caffeine treatment, a decrease was observed in the frontal cortex from males from ADHD model [F(1,17) = 6.376; P = 0.0218; treatment effect] and [F(1,17) = 5.188; P = 0.0360; interaction] (Fig. 11F).

Discussion

In the present study, we evaluated the impact of caffeine administrated from early childhood up to the end of adolescence on cognitive and motor functioning, neurochemical correlates and brain oscillations in both sexes from the most validated animal model for ADHD studies.

Sex differences in the murine ADHD model

One of the cardinal features of ADHD symptomatology is the impairment of executive functions dependent on the prefrontal cortex, such as decision making and learning (Barkley, 2006, Chess et al., 2011; Hauser et al., 2014). Recent study using computational modeling and neuroimaging revealed that adolescents with ADHD have a less-fine-grained decision processed in the prefrontal cortex causing deficits in decision-making and learning (Hauser et al., 2014). Similarly, rats with frontal lesion showed retardation in set shifting and they performed more errors (Birrel and Brown, 2000). This support the evidence that decision-making process involves a cognitive control network (Patros et al., 2017). In an attempt to find some sex differences, a two-choice decision paradigm was used to evaluate basic principles of learning and decision-making associated with frontal deficits in the rat ADHD model (Martens et al., 2012; 2013). In this study, females from ADHD model presented a more pronounced impairment in learned discrimination, which is in agreement with previous

findings showing that females needed more days to improve their performance due to slower learning processes (Bucci et al., 2008a). Likewise, the ability of rats to learn a simple stimulus-reward conditioned task has showed that males from ADHD model exhibited a more conditioned performance than females (Bucci et al., 2008b). Interestingly, both sexes from ADHD model showed impaired performance along with lower percentage of right choices at the reversal phase, revealing that difficulties in the behavioral flexibility revealed by the disability to learn a new strategy (while inhibiting the execution of a previous one) were not sex dependent. Based on the fact that females from ADHD model required more trials to discriminate, which enables to explore sex differences in ADHD, these findings may corroborate with clinical reports in which girls usually presented the inattentive subtype (Rucklidge et al., 2010; Weiss et al., 2003). In a recent study, girls with ADHD exhibited impaired spatial working memory in a delayed in real-time rewards task when compared to boys, which can contribute for slower learning (Patros et al., 2017).

In our previous study, sex differences in the locomotor activity were not noticeable simply by recording travelled distance (Nunes et al., 2018). Here, the lack of habituation to the number of stops revealed that males from ADHD model were slightly more hyperactive than their female counterparts. When considering sex-based differences in neuropsychological functioning in children with ADHD, girls generally show more deficits in planning and strategy mediated by prefrontal circuits, while boys present greater impairments in response control mediated by motor/premotor circuits (Hasson and Fine, 2012; O'Brien et al., 2010; Seymour et al., 2016). Regarding to motor alterations, these findings revealed slight, but discernible sexual dimorphism in this rodent model.

In order to correlate behavioral outcomes with underlying deficits in frontal cortical networks, EEG activity was recorded in the frontal cortex from both sexes of the ADHD model. Analysis from delta and theta waves in ADHD have presented some inconsistences, which varies from no alteration to reduction and increase in the fronto-central and parietal regions of adolescents and adults with ADHD (Bresnahan et al., 1999; Buyck and Wiersema

2014; Clarke et al., 2005; 2006; Kitsune et al., 2015; Koehler et al., 2009; Skirrow et al., 2015; Shephard et al., 2018).

In our males from ADHD model increases in delta power were observed, which is in agreement with previous findings (Vorobyov et al., 2011). Besides, male from ADHD model showed elevated gamma power during locomotor activity. Overall, females from ADHD model presented an increased theta/delta ratio and decreased delta power at resting state and during locomotor activity and a decrease in gamma waves. Since increased slow-wave activity is frequently associated with a lack of inhibitory control over behavior and gamma power has been shown to be higher in fast-moving rats (Molina et al., 2014), probably both alterations in delta and gamma may be contributing for slightly but distinguishable hyperactivity observed in males from ADHD model. The increase in the delta band power could be explained as a progressive cortical disconnection due to the slowing of the conduction along subcortical connecting pathways. It can also be argued that these alterations are reflecting a delayed brain maturation in both sexes. Even though slow waves activity tends to decrease across the lifespan as the brain maturation takes place (Clarke et al., 2001), females would maintain higher levels of EEG slow-wave activity across most mammal species (Ehlers et al., 1993; Knyazev, 2007). In healthy subjects, delta oscillations presented a negative association with connectivity within default mode network (DMN), the idling brain network with prominent activity during rest, which becomes deactivated during cognitive tasks (Hlinka et al., 2010). Translating into our rodent model, those reductions in delta observed for females from ADHD model might be associated with atypical functional connectivity within the DMN, which results in attentional problems as previously reported for children with ADHD (Barber et al., 2015; Castellanos and Proal, 2012; Sonuga-Barke and Castellanos, 2007; Uddin et al., 2008).

Both sexes showed reductions in DAT and D4DR, which was previously found in other brain areas from male SHR rats (Li et al., 2007; Simchon et al., 2010). Dopamine needs to "spillover" the synaptic release site to activate its receptors predominantly located at

extrasynaptic sites. This process is mediated by a competitive kinetics between the dopamine transporters and diffusion, the latter having more influence in determining the net extracellular dopamine levels and the amount of dopamine that reaches its receptors (Cragg and Rice, 2004). Therefore, these lower DAT levels have been suggested as a compensatory mechanism to overcome decreased dopamine release (Li et al., 2007; Simchon et al., 2010).

Alterations in the dopaminergic system promote, as hypoactivity affects delta activities (Knyazev, 2007), and direct application of light-uncaged dopamine in the medial prefrontal cortex strengthens phase-amplitude comodulation between delta and gamma oscillations (Andino-Pavlovsky et al., 2017). Thus, these changes in DAT and D4DR levels may also be reflecting in EEG alterations found in both sexes from ADHD model. It is noteworthy in females from ADHD model because cognitive impairments were ameliorated during adulthood of males adolescent DAT+/- mice, but not females, which suggest a clearly sex-dependent manner susceptibility to dopaminergic hypofunctioning (Mereu et al., 2017).

Gamma oscillations have also been investigated in ADHD, and significant associations of the genetic polymorphisms for DRD4 and DAT were found with alterations in gamma waves responses (Demiralp et al., 2007; Yordanova et al., 2001). In most cortical regions gamma frequency oscillations predominate since childhood; then they decrease during adolescence and increase in early adulthood. In this period, dopamine exerts more control over excitation/inhibition balance and thus contributes for the selection of adequate behavioral responses (Furth et al., 2013; O'Donnell, 2010). Regarding males from ADHD model, the slightly but distinguishable hyperactivity may also be associated with gamma waves, given that increased gamma power in the rodent prefrontal cortex increases with running speed, and gamma power has been shown to be higher in fast-moving rats (Molina et al., 2014). Although EEG was not recorded during a task requiring more cognitive input, it cannot be discarded that decreased gamma waves might be involved with a worsened performance in the decision-making task displayed by females from ADHD model, since they presented

discrimination, novel learning and flexibility impairments and also lack of habituation (Furth et al., 2013).

Aiming to associate behavioral abnormalities with synaptic proteins, synaptophysin and SNAP-25 were analyzed in the frontal cortex. Polymorphisms in both proteins have a significant association with ADHD symptomatology (Choi et al., 2007; Faraone et al., 2005; Guan et al., 2009; Liu et al., 2017).

Both sexes from ADHD model showed reductions in SNAP-25, but females showed a slightly more reduction in SNAP-25 and a robust increase in synaptophysin. Thus, reductions in SNAP-25 observed in the frontal cortex from both sexes may be involved in the hyperactivity, which matches with ADHD patients (Hawi et al., 2013) and with mouse carrying a deletion of SNAP-25 gene that develops hyperactivity being used as an ADHD animal model (Hess et al., 1992; Wilson, 2000).

Normal brain development involves a coordinated maturation of many cellular events, in a temporally and regionally dependent manner; thus, this imbalance between increased synaptophysin (synaptic vesicles marker) and decreased SNAP-25 (nerve terminals marker) observed only in females from ADHD model could contribute for aberrant synaptic transmission and/or delayed frontal cortical maturation.

Synaptophysin is considered a marker for the ontogenetic development of synapses (Knaus et al., 1986), and sex differences were found over the course of rat frontal cortical development, with females showing a peak in synaptic numbers at P35 (their average age of pubertal onset), followed by a drop at P45 that persisted into adulthood (Drzewiecki et al., 2016). Likewise, SNAP-25 also progressively increases during cortical postnatal development (Corradini et al., 2014), playing an important role not only in the presynaptic regulation of synaptogenesis, but it is also essential for neurotransmitter storage and release (Söllner et al., 1993).

Caffeine prevents functional and behavioral alterations in the murine ADHD model in a sex dependent manner

Although the ability of caffeine in preventing memory and attentional impairments have been demonstrated in different ADHD models (Callabero et al., 2011; Pandolfo et al., 2013; Pires et al., 2010; Prediger et al., 2005), the differences between sexes have been poorly investigated. In our previous study, caffeine was able to recover recognition and spatial working memory in females from ADHD model, since they presented a worsened performance when compared to their male counterparts (Nunes et al., 2018). Here, caffeine reversed the worsened discrimination in the decision-making task in females from ADHD model, but it was ineffective in preventing novel learning and flexibility impairments. Different from our previous work, caffeine was now able to counteract hyperactivity in both sexes from ADHD model (Nunes et al., 2018). Since psychostimulant effects of caffeine include increased alertness and wakefulness, this methylxanthine classically decrease slow waves activity, with a more pronounced effect on delta waves (Landolt et al., 1995). Considering that females from ADHD model seems to be reached a threshold in delta waves, it is difficult to observe any impact of caffeine. However, caffeine decreased both delta and gamma power along with attenuation in decreased D4DR receptors only in males from ADHD model. Considering that lower dopamine D4DR receptor protein expression in the frontal cortex may translate into a relatively lower inhibitory dopaminergic influence, leading to hyperactivity in the ADHD model, this effect of caffeine caffeine in counteracting hyperactivity may involve D4DR receptors levels. As a nonselective A_1R and $A_{2A}R$ antagonist, caffeine had no evident impact on dopamine levels (Acquas et al., 2002; De Luca et al., 2007). In fact, dopaminergic and adenosinergic systems are closely interplayed through a well documented functional and molecular interaction between striatal adenosine A1R/A2AR and dopamine D1/D2 receptors (Ferré et al., 1997; Fuxe et al., 2003). Of note, from this molecular interaction caffeine and adenosine $A_{2A}R$ antagonists have been considered as promising agents for motor alterations and dopaminergic degeneration in Parkinson´s disease (*for reviews see* Chen, 2014;

Prediger et al., 2010). Although there are no direct evidences about a molecular and functional interaction between adenosine receptors and D4DR in frontal cortical regions, most recently, a synergistic interaction (rather than antagonistic as for striatum) was found at least for $A_{2A}R–D_2R$ on the glutamatergic neurotransmission in the prefrontal cortex (Real et al., 2018). Besides, blockade of adenosine $A_{2A}R$ also reversed behavioral alterations by dopamine antagonists, including those involving decision-making processes (Mott et al., 2009; Worden et al., 2009). Thus, future studies recording from more than one brain region involved in ADHD and a strict interaction between adenosine and D4DR receptors will hopefully shed light on the altered frontal cortical communication after caffeine treatment.

Regarding to synaptic proteins, while caffeine exacerbated the decreased SNAP-25 observed in frontal cortex from males from ADHD model, SNAP-25 and synaptophysin levels were not altered by treatment in females from ADHD model. Caffeine at similar dose with different treatment schedules have decreased SNAP-25 levels during brain development of male Wistar rats and hyperlocomotion (Ardais et al., 2014; 2016). Therefore, the attenuation on hyperactivity by caffeine seems not to be strictly related to changes in SNAP-25.

Convergent clinical literature indicates sexual dimorphism in the symptomatology of ADHD, with boys presenting the combined subtype and girls presenting predominantly inattention. Given how closely our data on behavioral and neurochemical/functional correlates changes suggest some similarity with those from studies involving ADHD patients, it is possible to investigate novel therapeutic strategies taking into account sex differences. In this sense, the ability of caffeine to restore some aspects of frontal cortical functioning may offer a promising translational tool in preclinical research that could improve attention and motor disturbances. Novel therapeutic strategies that could deal with these sex differences may bring a better functional outcome for ADHD patients.

Fig. 1

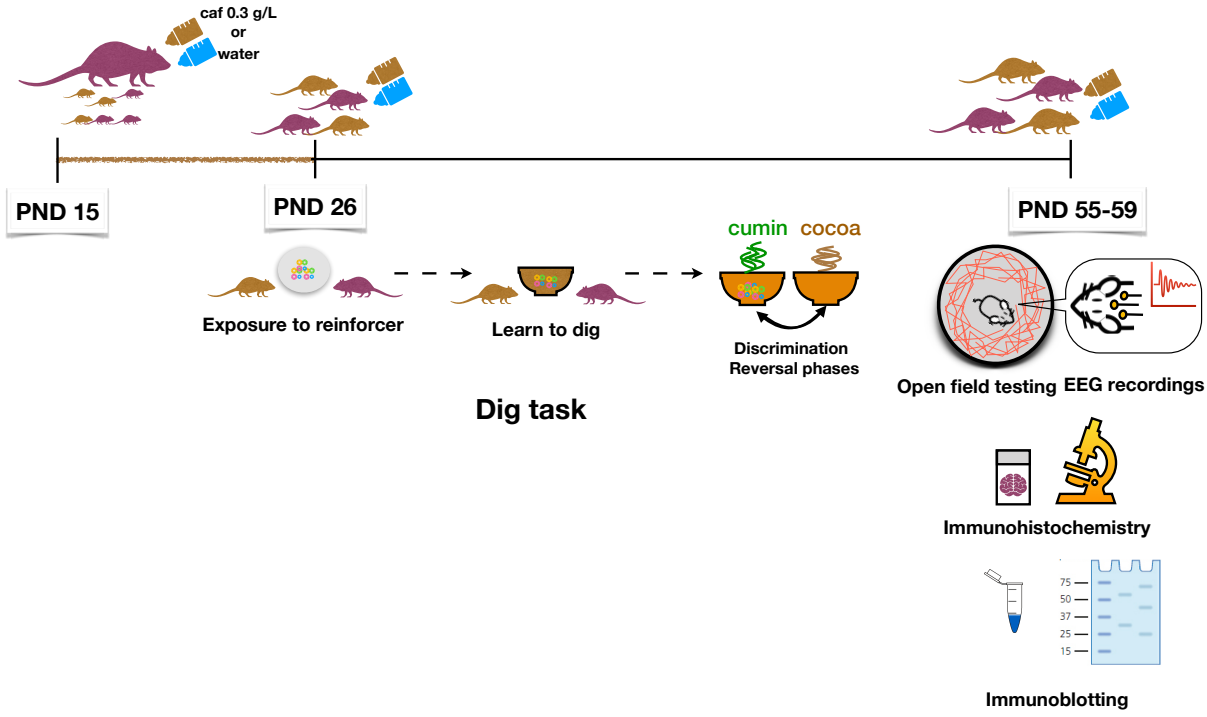

Figure 1 – Caffeine administration in ADHD model (SHR) from both sexes.

At postnatal day 15 (PND 15) SHR pups received caffeine (0.3 g/L) or drinking water from their dams. At weaning (PND 26) SHR from both sexes received water or caffeine in the drinking water up to PND 59. Kyoto rats from both sexes received only drinking water. Dig task started at PND 26 with rats being trained to firstly recognize the reinforcer (froot loops) and later learning to dig in order to find the reinforcer. The discrimination phase started at PND , with the reinforcer placed in the cumin scent. The reversal phase started for each group of rats after the end of discrimination phase, and the reinforcer was placed in the cocoa scent. Locomotor activity started at PND 56 -57 and some animals were simultaneously evaluated regarding locomotor activity and electroencephalographic recordings (EEG). Open field testing comprised two sessions (24 h of interval) and 10 minutes of duration for each session. Samples from the frontal cortex were processed for immunoblotting and immunohistochemistry.

Fig. 2

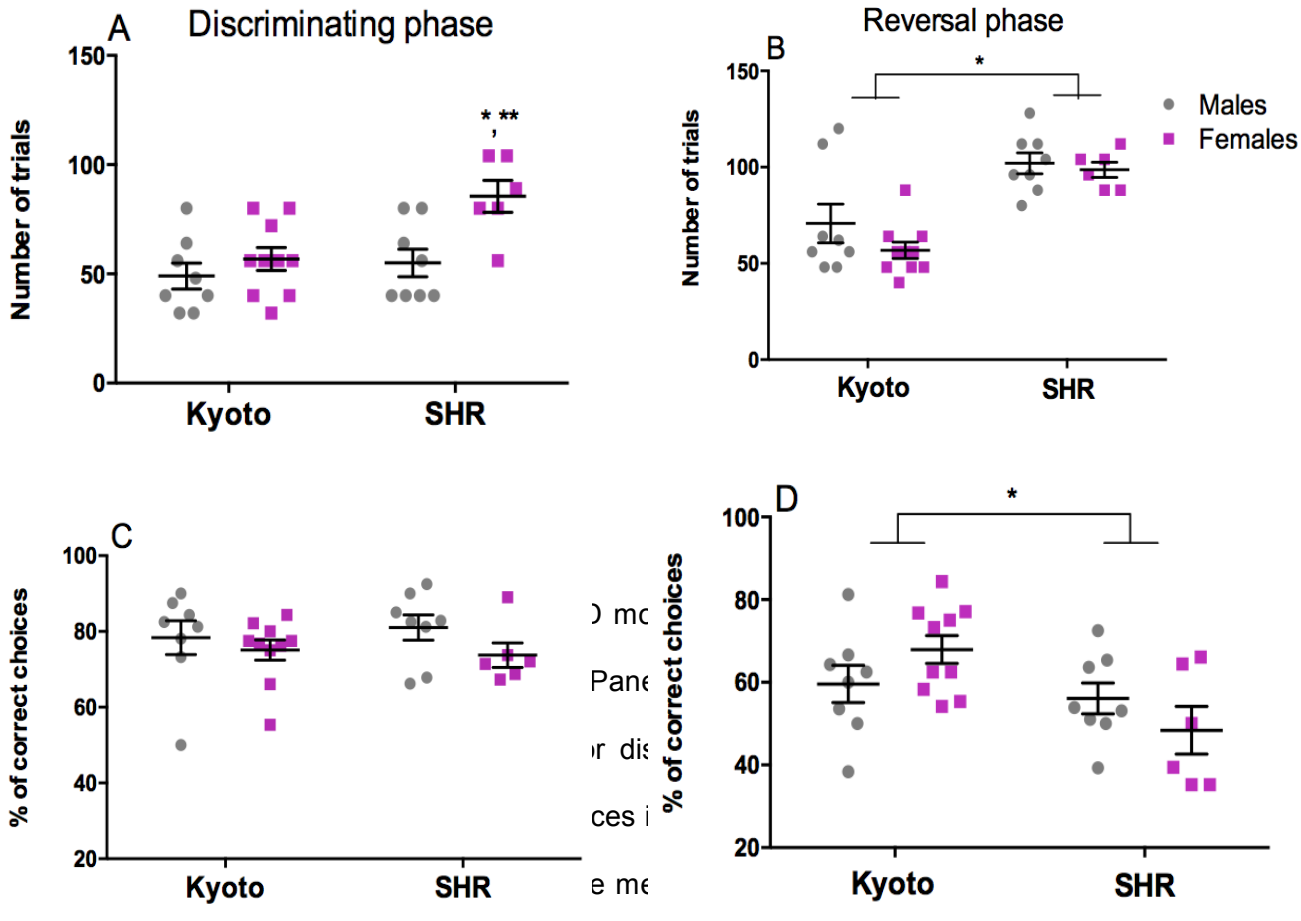

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Fig. 3

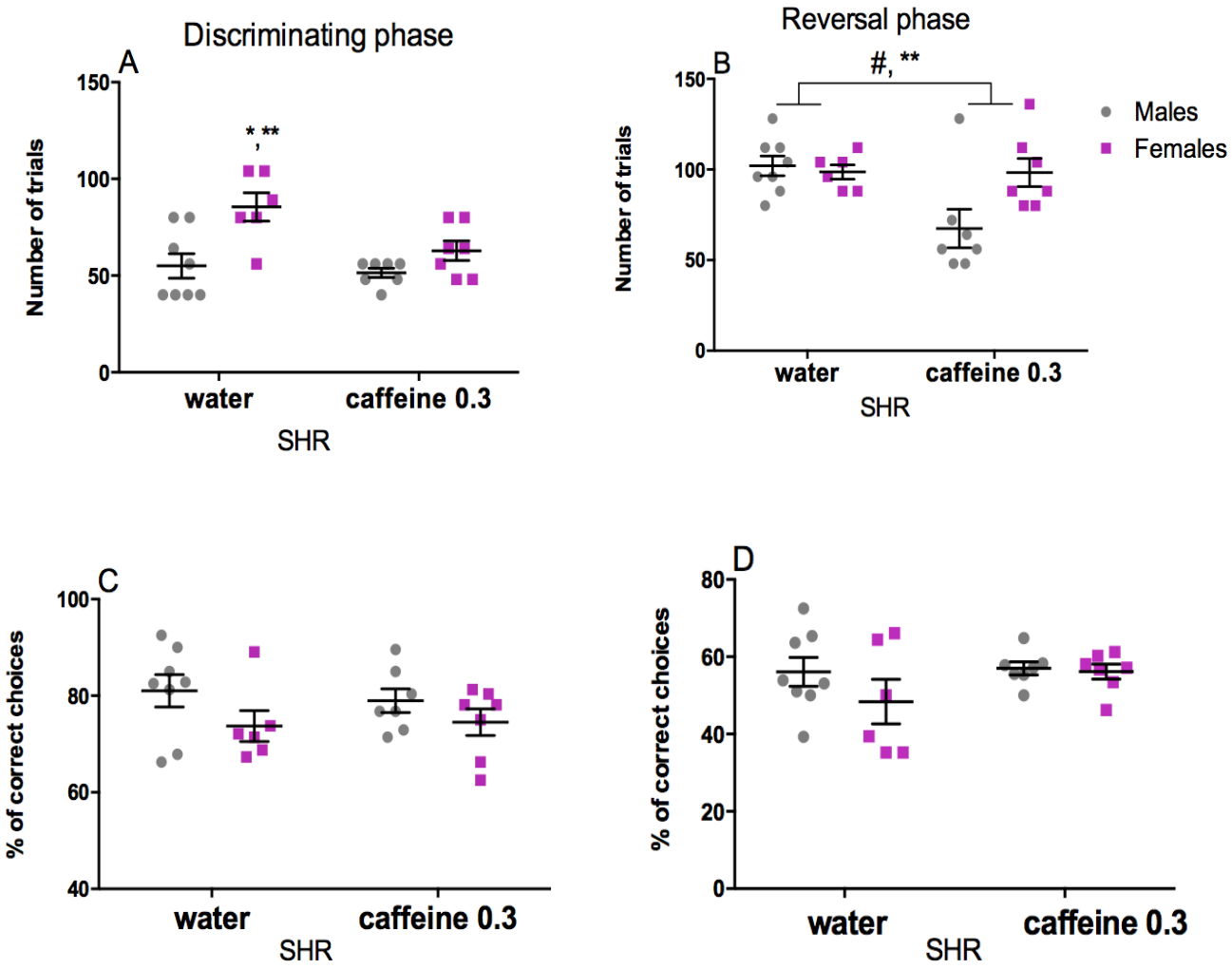


Figure 3- Performance of ADHD model (SHR) from both sexes after receiving caffeine (0.3 g/L) treatment in the decision-making behavior evaluated in the Dig task. Panels show the total number of trials required to complete the task and percentage of right choices. A - number of trials for discriminating phase; B – number of trials for reversal phase; C – percentage of right choices in the discriminating phase; D - percentage of right choices in the reversal phase. Data are means ± S.E.M (n = 6-8, SHR water and n = 7 SHR treated with caffeine). Two-way ANOVA followed by Bonferroni´s post hoc test. $^{\#}P < 0.05$; interaction; $^{*}P < 0.05$; sex effect; $^{**}P < 0.05$;

Fig. 4

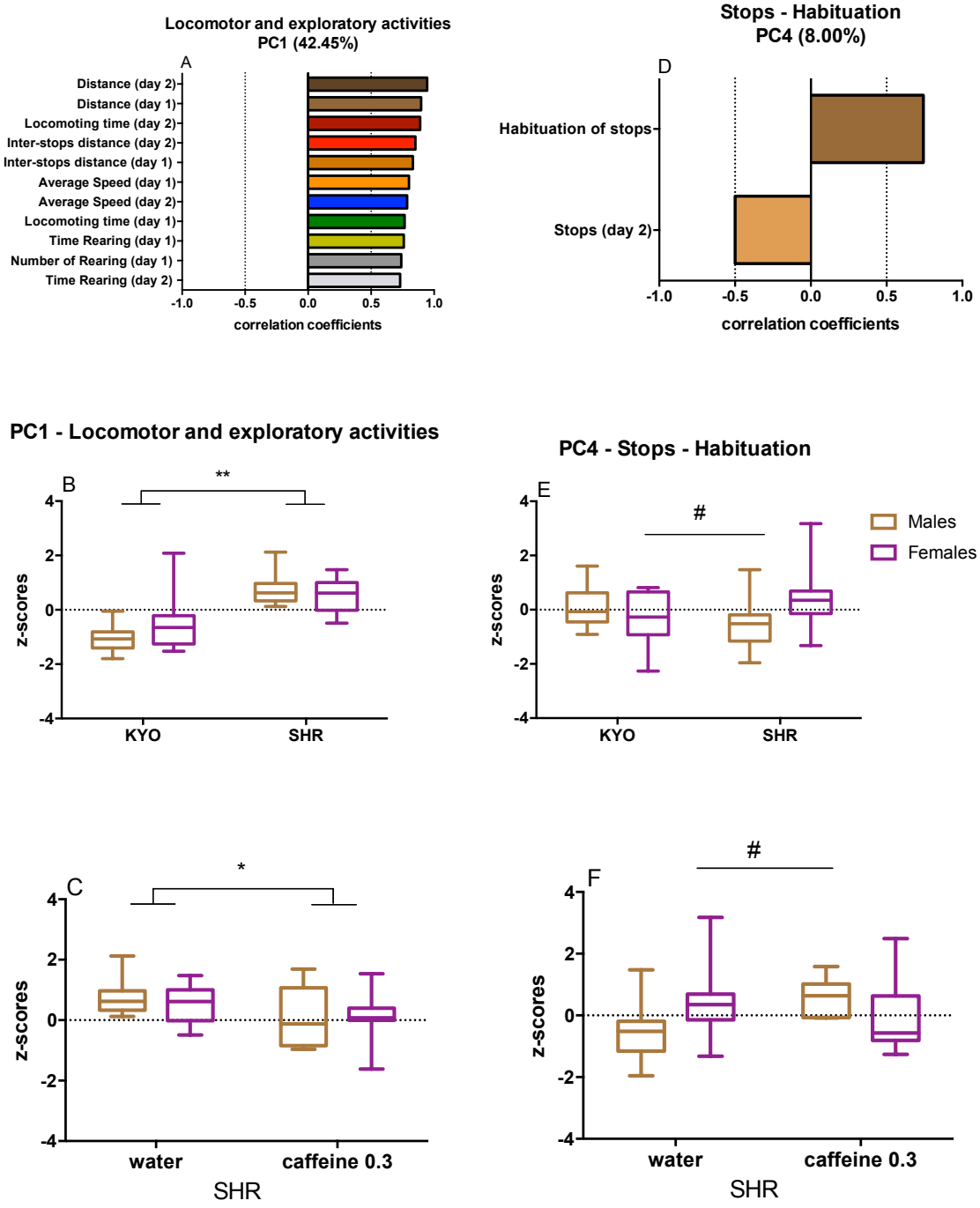

Locomotor and exploratory activities
PC1 (42.45%)
A
Distance (day 2)
Distance (day 1)
Locomoting time (day 2)
Inter-stops distance (day 2)
Inter-stops distance (day 1)
Average Speed (day 1)
Average Speed (day 2)
Locomoting time (day 1)
Time Rearing (day 1)
Number of Rearing (day 1)
Time Rearing (day 2)
-1.0
-0.5
0.0
0.5
1.0
correlation coefficients
Stops - Habituation
PC4 (8.00%)
D
Habituation of stops
Stops (day 2)
-1.0
-0.5
0.0
0.5
1.0
correlation coefficients
PC1 - Locomotor and exploratory activities
B
**
z-scores
4
2
0
-2
-4
KYO
SHR
PC4 - Stops - Habituation
E
#
Males
Females
z-scores
4
2
0
-2
-4
KYO
SHR
C
*
z-scores
4
2
0
-2
-4
water
caffeine 0.3
SHR
#
F
z-scores
4
2
0
-2
-4
water
caffeine 0.3
SHR

Figure 4 - Evaluation of locomotor/exploratory activities and habituation of Kyoto and ADHD model (SHR) from both sexes. PC1 was identified as locomotor/exploratory activities (A) and PC4 corresponds to habituation of stops (D). PC1 - Analysis from male and female Kyoto and SHR (B) and male and female SHR receiving water or caffeine 0.3 g/L (C). PC4 - Analysis from male and female Kyoto and SHR (E) and male and female SHR receiving water or caffeine 0.3 g/L (F). The z-scores axis represents the median of data population (zero value) for each PC and interquartile ranges. Thus, animals displaying positive z-scores for PC1 presented greater locomotor/exploratory activities than animals displaying negative z-scores, while positive z-scores for PC4 indicates increased habituation of stops when compared to negative z-scores. Comparison between KYO and SHR with respect to the PC1 and PC4 z-scores are presented in “B” and “E”, respectively, while comparison between SHR receiving water or caffeine PC1 and PC4 z-scores are presented in “C” and “F”, respectively. Data are expressed as median:interquartile range (n = 9-10 animals/group; strain analysis) and (n = 6-11 animals/group; treatment analysis). Two-way ANOVA. *P < 0.05, treatment effect; **P < 0.0001, strain effect; $^{\#}$P < 0.01, interaction.

Fig. 5

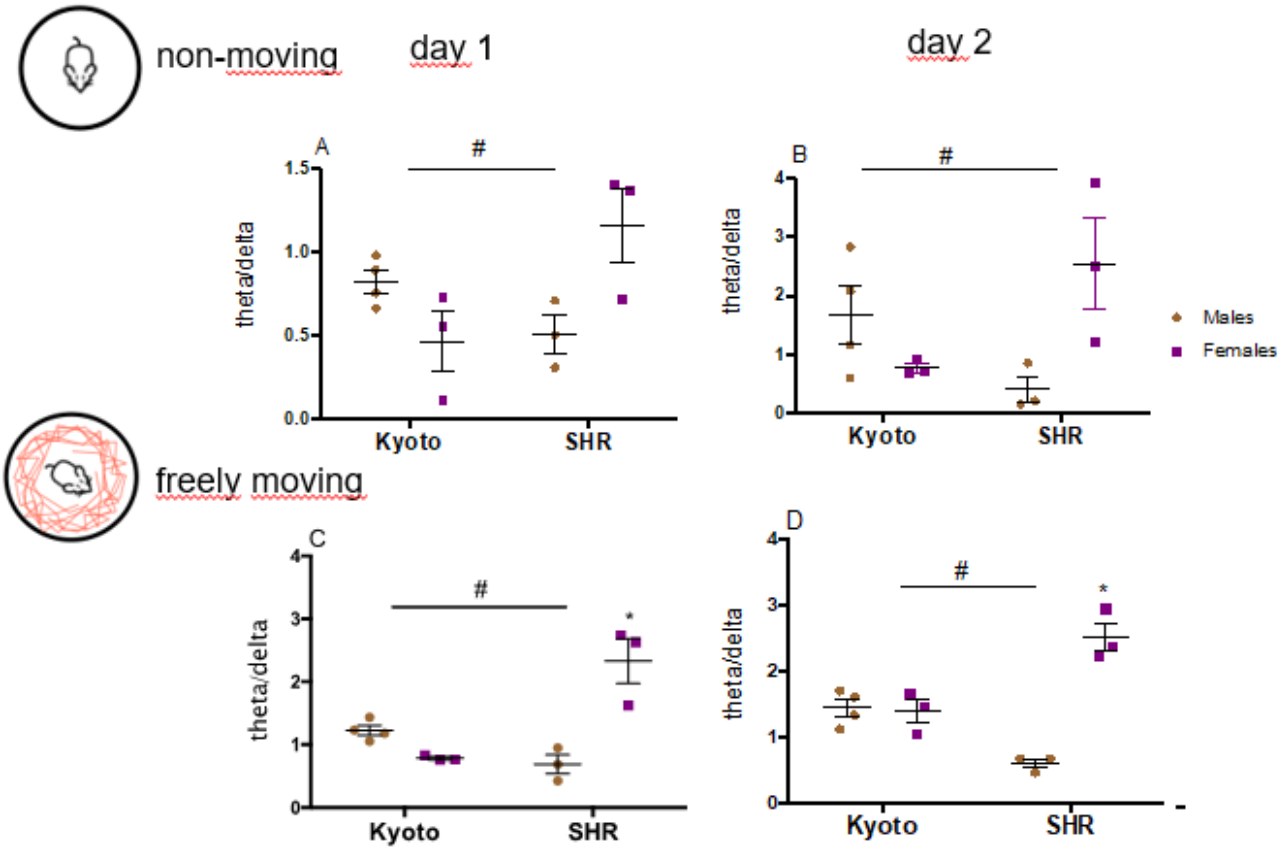


Figure 5 - Comparisons of electroencephalogram of theta/delta ratio between Kyoto and ADHD model (SHR) from both sexes receiving drinking water (A-D). Ratio EEG power of the frontal cortex electrode was obtained in both open field sessions (day 1 and 2) during resting state (non moving in the arena) and locomotor activity (freely moving in the arena) (n = 3-4 animals/group). Two-way ANOVA followed by Bonferroni´s post hoc test. $^{\#}P < 0.05$, interaction between strain and sex.

Fig. 6

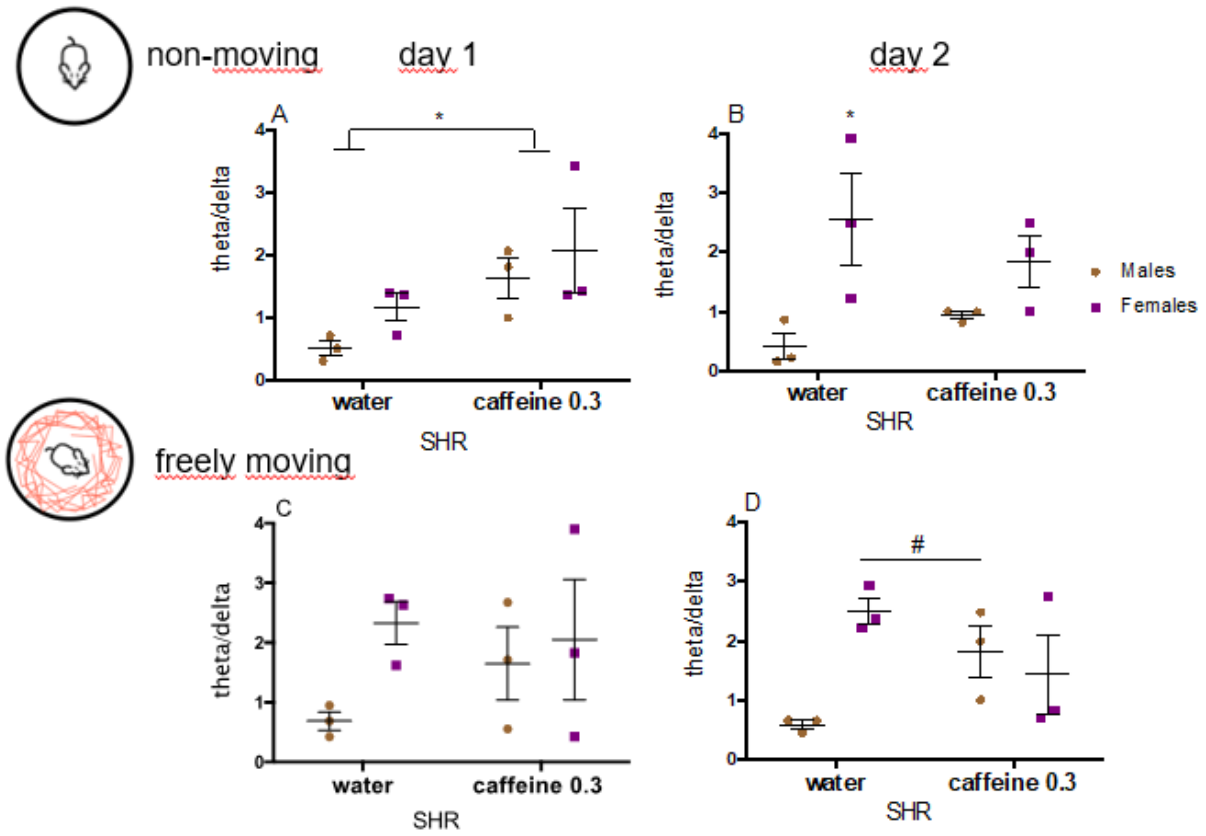


Figure 6 - Comparisons of electroencephalogram of theta/delta ratio between ADHD model (SHR) from both sexes receiving drinking water or caffeine 0.3 g/L (A-D). Ratio EEG power of the frontal cortex electrode was obtained in both open field sessions (day 1 and 2) during resting state (non-moving in the arena) and locomotor activity (freely moving in the arena) (n = 3-4 animals/group). Two-way ANOVA followed by Bonferroni´s post hoc test. $^{\#}P < 0.05$, interaction between sex and treatment.

Fig. 7

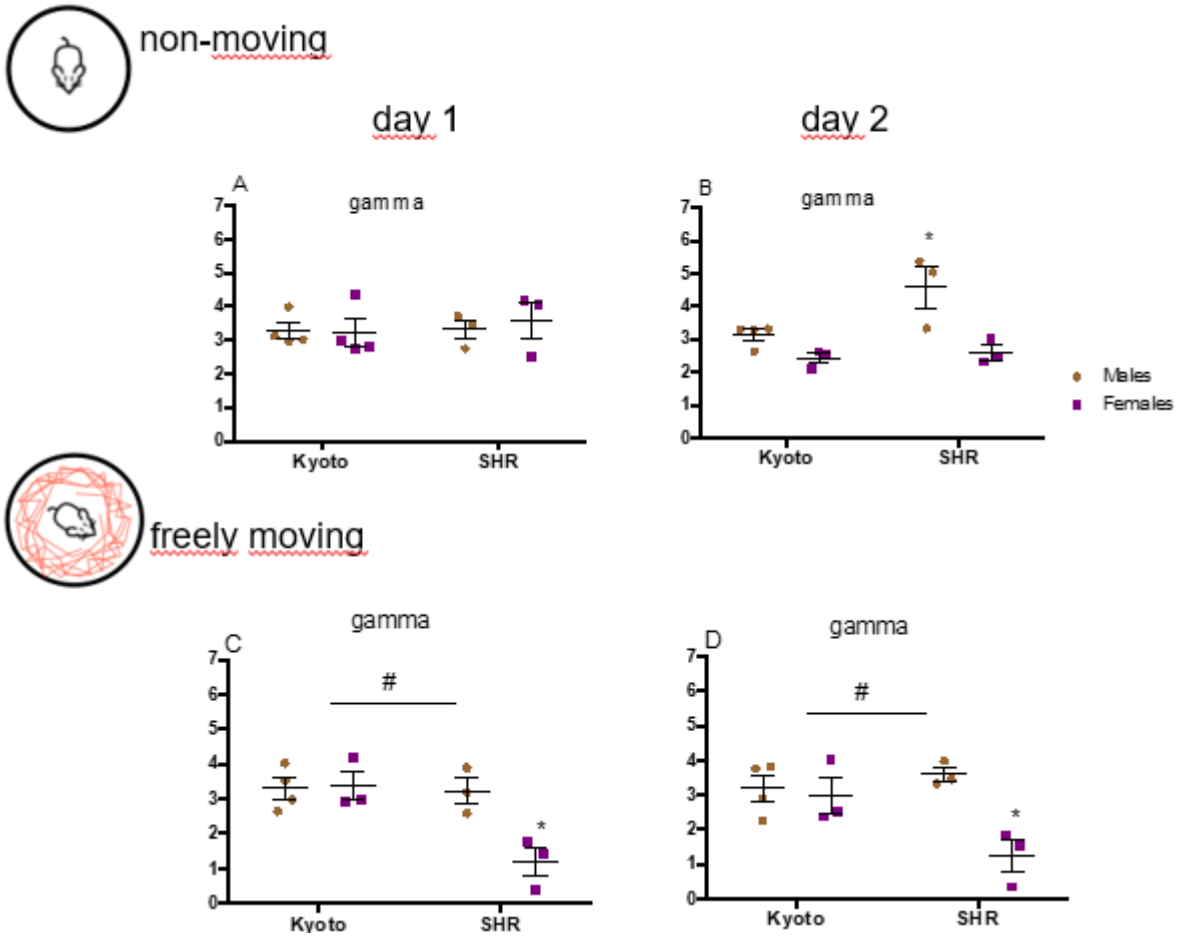


Figure 7 - Comparisons of electroencephalogram in gamma waves between Kyoto and ADHD model (SHR) receiving drinking water (A-D). Ratio EEG power of the frontal cortex electrode was obtained in both open field sessions (day 1 and 2) during resting state (non-moving in the arena) and locomotior activity (freely moving in the arena) (n = 3-4 animals/group). Two-way ANOVA followed by Bonferroni´s post hoc test. $^{\#}P < 0.05$, interaction between strain and sex. $^{*}P < 0.05$, sex effect.

Fig. 8

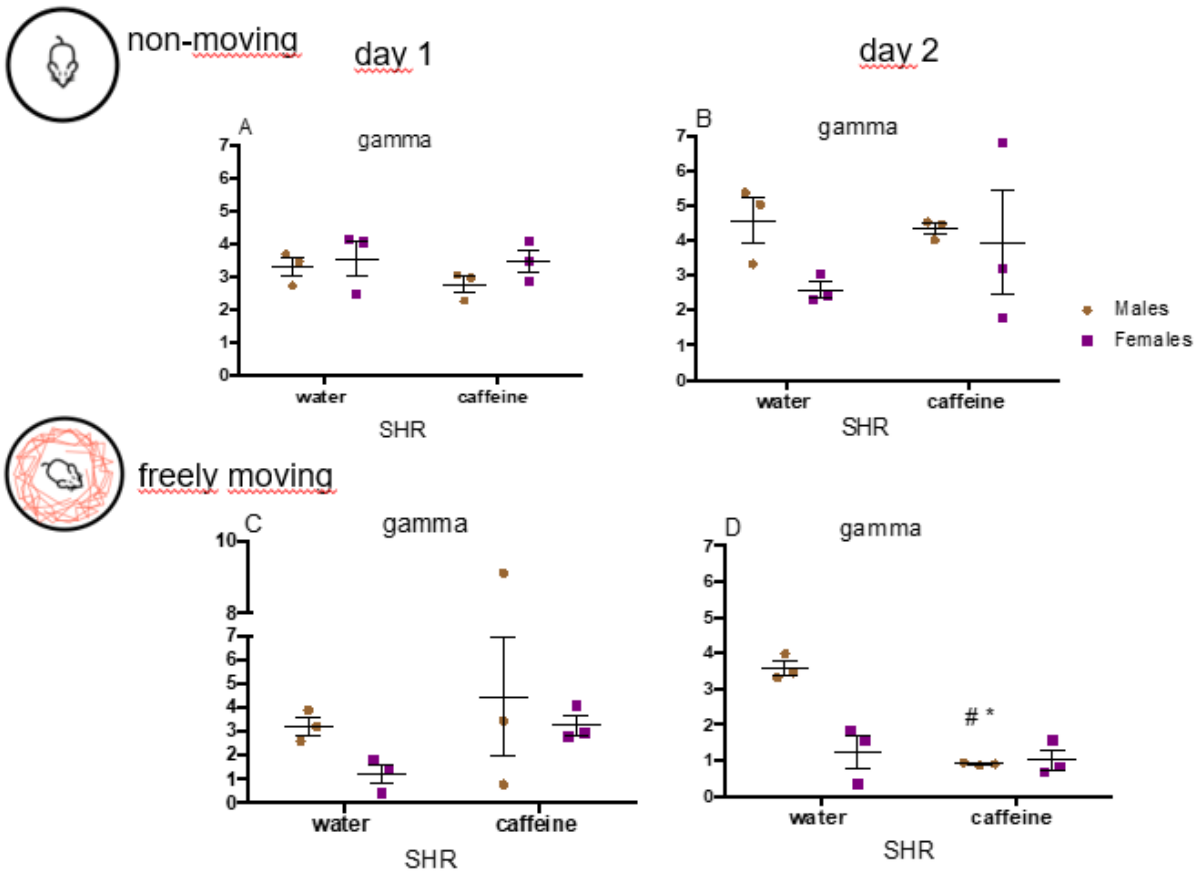


Figure 8 – Comparisons of electroencephalogram in gamma waves between ADHD models (SHR) from both sexes receiving drinking water or caffeine 0.3 g/L (A-D). Ratio EEG power of the frontal cortex electrode was obtained in both open field sessions (day 1 and 2) during resting state (non-moving in the arena) and locomotion (freely moving in the arena) (n = 3-4 animals/group). Two-way ANOVA followed by Bonferroni´s post hoc test. $^{\#}P < 0.05$, interaction between sex and treatment. $^{*}P < 0.05$, sex effect.

Fig. 9

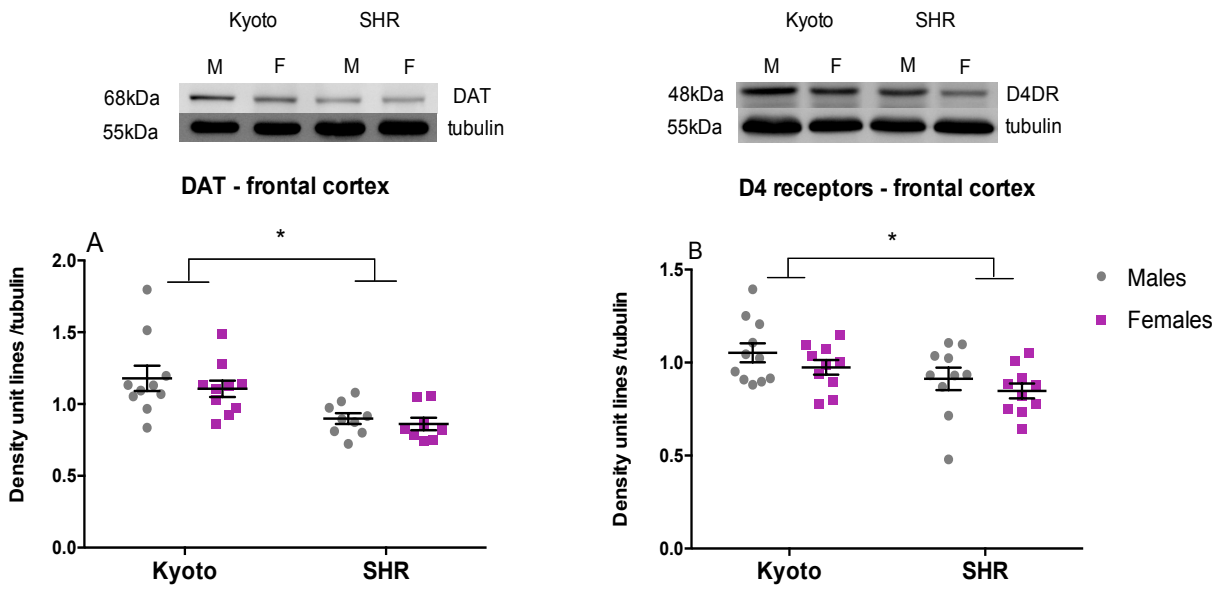


Figure 9 - Dopamine Transporter (DAT) and Dopamine D4 receptors (D4DR) immunocontent in the frontal cortex of Kyoto and ADHD model (SHR) from both sexes. A - DAT immunocontent; B – D4DR immunocontent. Data are represented as means ± S.E.M (n = 8-13 animals per group) of density unit lines (normalized by the β-tubulin immunocontent). At the top of the figures are the representative bands for all proteins. *P < 0.05, Two-way ANOVA, strain differences.

Fig. 10

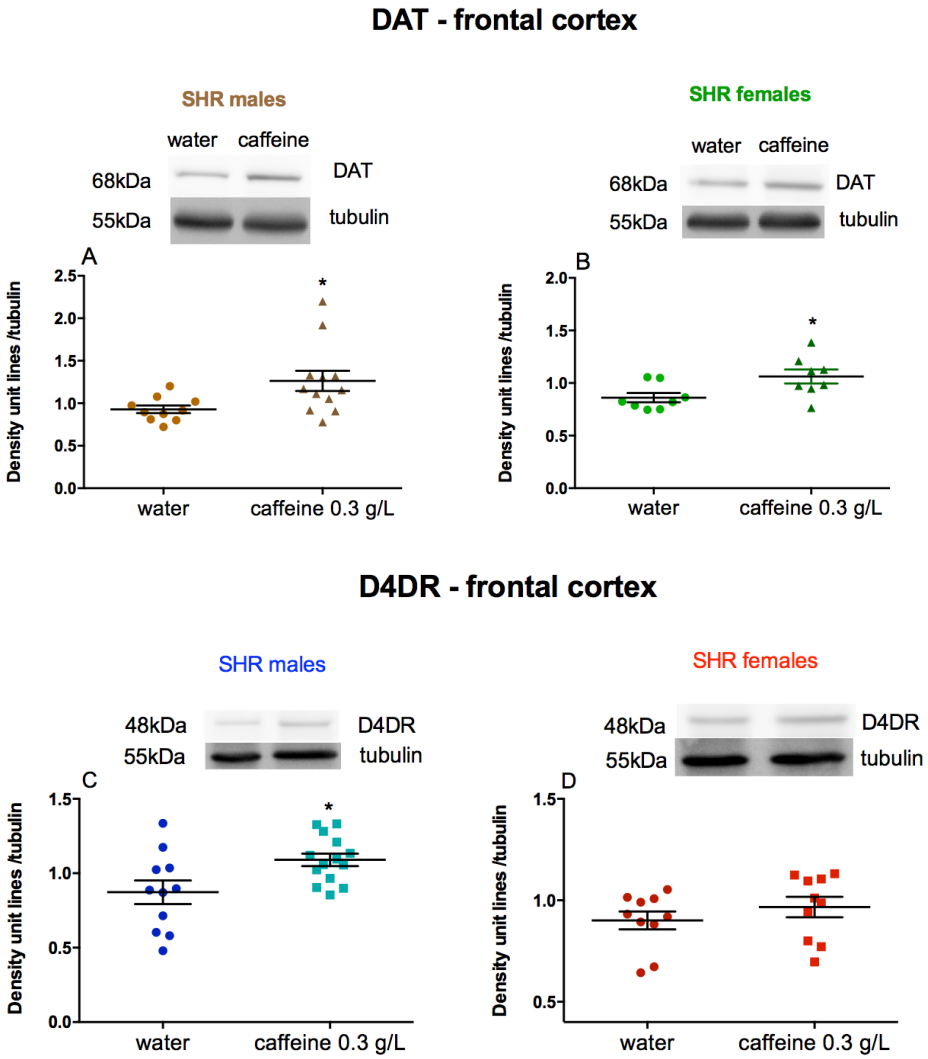
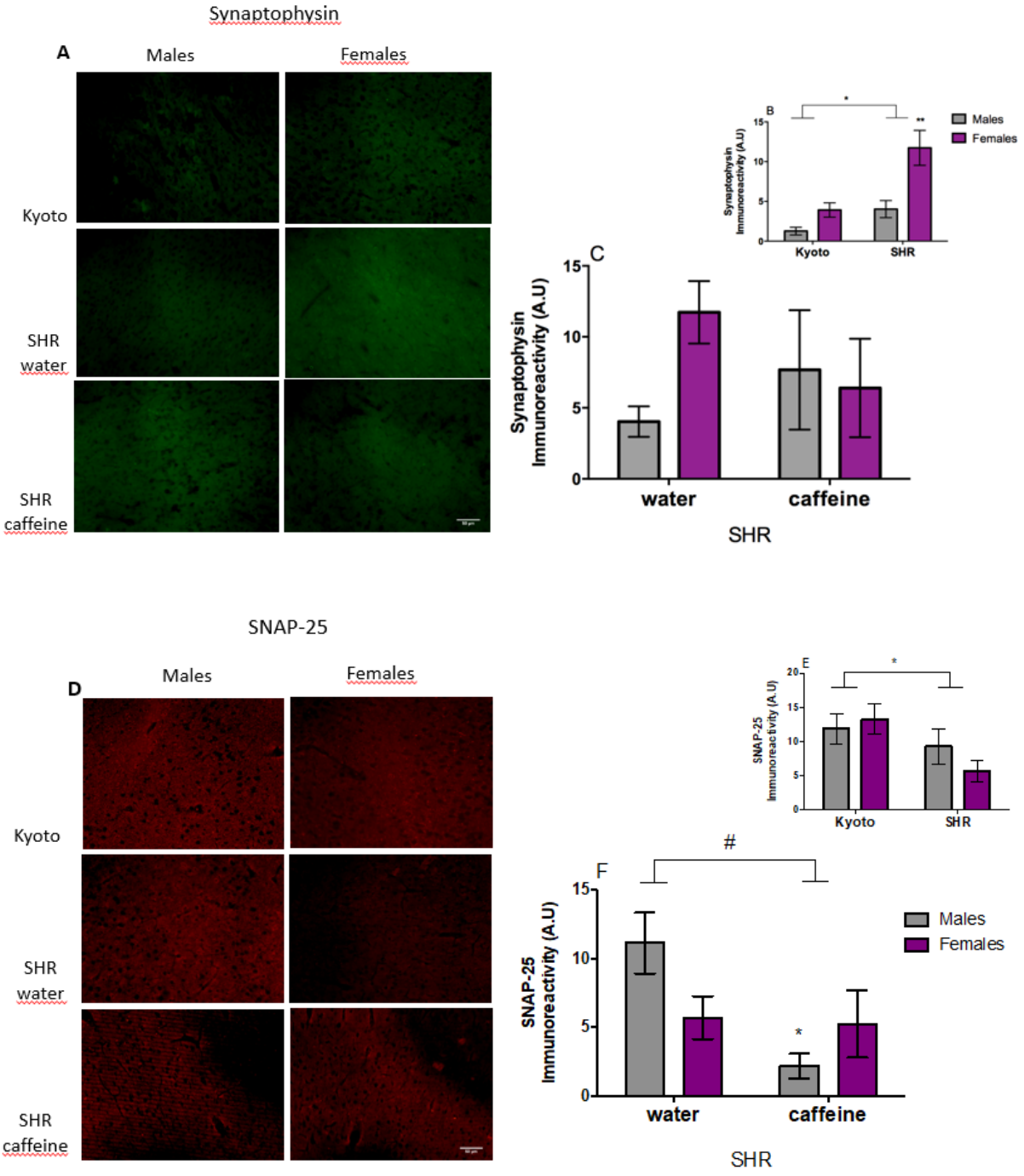


Figure 10 – Caffeine effects on DAT and D4DR immunocontent in the frontal cortex from both sexes of ADHD model.DAT levels in the frontal cortex of males (A) and females (B) from ADHD model (SHR) after receiving drinking water or caffeine (0.3 g/L) from PND 15 up to PND 59. Data are represented as means ± S.E.M (n = 8-12 animals per group). D4DR levels in the frontal cortex of males (C) and females (D) from ADHD model (SHR) after receiving drinking water or caffeine (0.3 g/L) from PND 15 up to PND 59. Data are represented as means ± S.E.M (n = 10-14 animals per group). At the top of the figures are the representative bands for all proteins. Density unit lines (normalized by β-tubulin immunocontent). *P < 0.05, Unpaired t - test.

Fig. 11


Synaptophysin
A
Males
Females
Kyoto
SHR water
SHR caffeine
B
Males
Females
Synaptophysin Immunoreactivity (A.U)
Kyoto
SHR
C
Synaptophysin Immunoreactivity (A.U)
water
caffeine
SHR
SNAP-25
D
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SHR water
SHR caffeine
E
SNAP-25 Immunoreactivity (A.U)
Kyoto
SHR
F
SNAP-25 Immunoreactivity (A.U)
Males
Females
water
caffeine
SHR

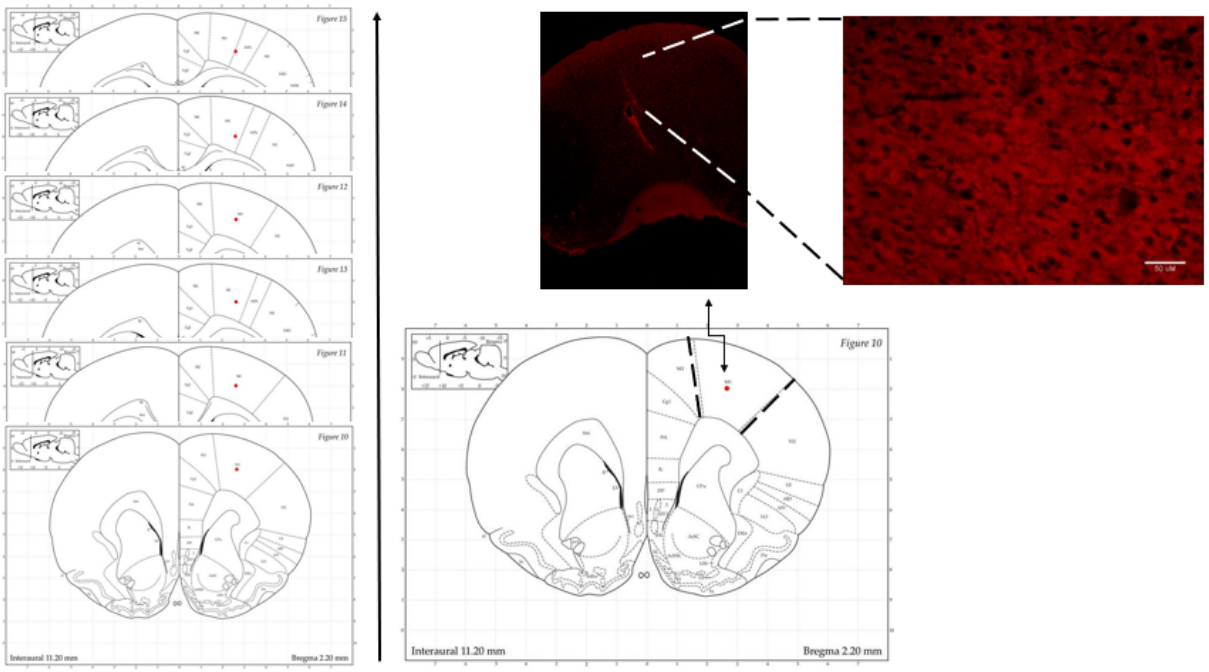

Figure 11 – Coronal sections from frontal cortex of Kyoto and ADHD model (SHR) receiving water or caffeine (0.3 g/L) immunostained for synaptophysin and SNAP-25.

A and D - Representative panels from coronal sections (30 µm) from frontal cortex of Kyoto and SHR from both sexes receiving water or caffeine (0.3 g/L) immunostained for synaptophysin in green (A) and SNAP-25 in red (D); B – insert graphic showing strain and sex differences for synaptophysin quantification. Data are means ± S.E.M. of fluorescence arbitrary units (A.U.) of 4-8 slices (180 µm interval) from 3-4 animals per group. Two-way ANOVA; $^{*}P<0.05$, strain effect; $^{**}P < 0.05$, sex effect; C – graphic showing sex and treatment differences. Data are present as means ± S.E.M. of fluorescence arbitrary units (A.U.) of 4-8 slices (180 µm interval) from 3-4 animals per group.

E – insert graphic showing strain and sex differences for SNAP-25 quantification. Data are means ± S.E.M. of fluorescence arbitrary units (A.U.) of 4-8 slices (180 µm interval) from 4-6 animals per group. Two-way ANOVA; $^{*}P<0.05$, strain effect; F – graphic showing sex and treatment differences. Data are present as means ± S.E.M. of fluorescence arbitrary units (A.U.) of 4-8 slices (180 µm interval) from 5-6 animals per group. Two-way ANOVA followed by Bonferroni´s post hoc test; $^{\#}P < 0.05$, interaction; $^{*}P < 0.05$, treatment effect;

Supplementary figures

Fig. S1

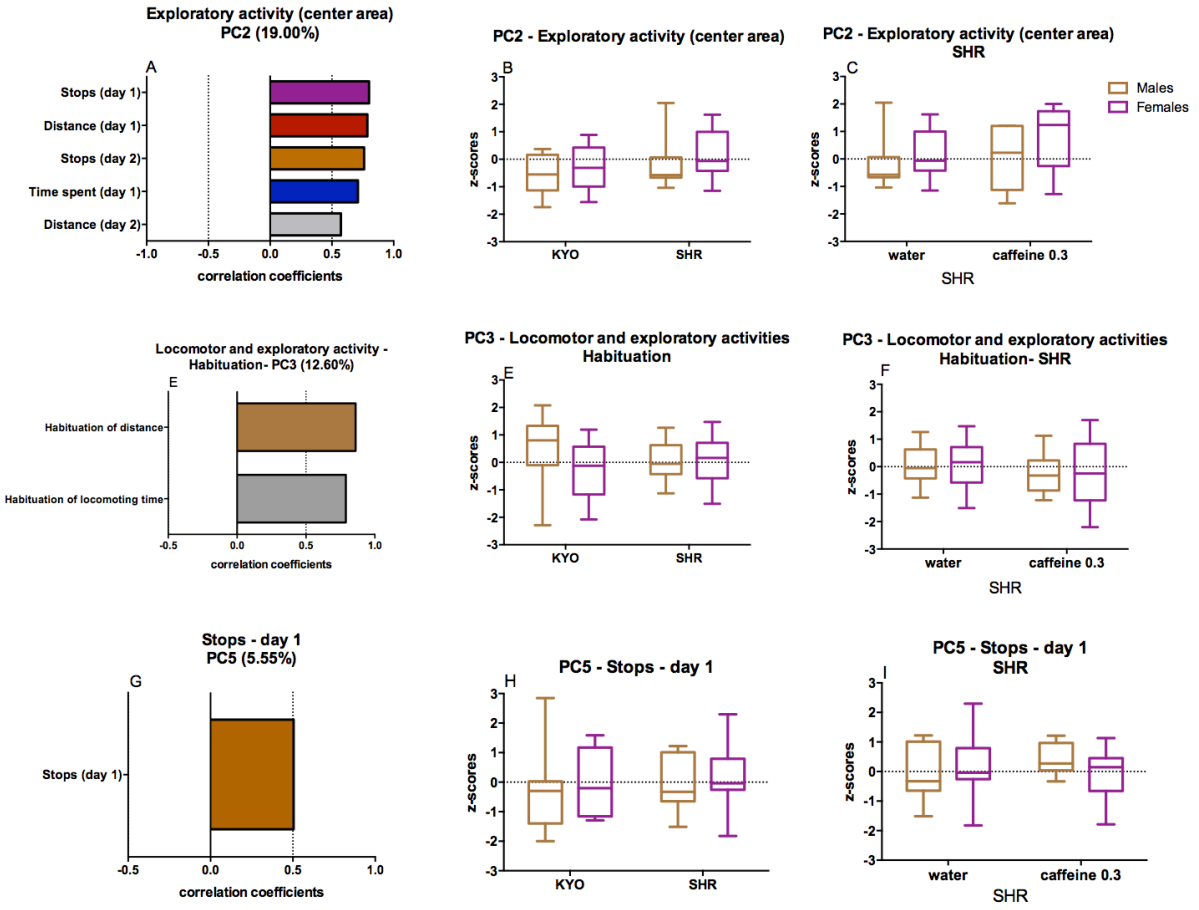

Figure S1 – Evaluation of locomotor/exploratory activities at the center area, habituation and stops in the open field arena. Principal component analysis (PC) of locomotor/exploratory activities at the center area (PC2 - A), habituation (PC3 - D) and Stops (PC5 – G). Dashed lines (values 0.5 and -0.5 in the Y-axis) indicate the cutoff points (i.e., the variables which presented correlation values > -0.5 and < 0.5 are not represented in the graphs).

PC2 - Locomotor/exploratory activities at the center area of male and female Kyoto and SHR (B) and male and female SHR receiving water or caffeine 0.3 g/L (C). PC3 – Habituation of male and female Kyoto and SHR (E) and male and female SHR receiving water or caffeine 0.3 g/L (F). PC5 – Stops of male and female Kyoto and SHR (H) and male and female SHR receiving water or caffeine 0.3 g/L (I). The z-scores axis represents the median of data

population (zero value) for each PC and interquartile ranges (e.g., 3, 2, 1, -1, -2, -3). Data are expressed as median: interquartile range (n = 9-10 animals/group; strain analysis) and (n = 6-11 animals/group; treatment analysis).

Fig. S2.

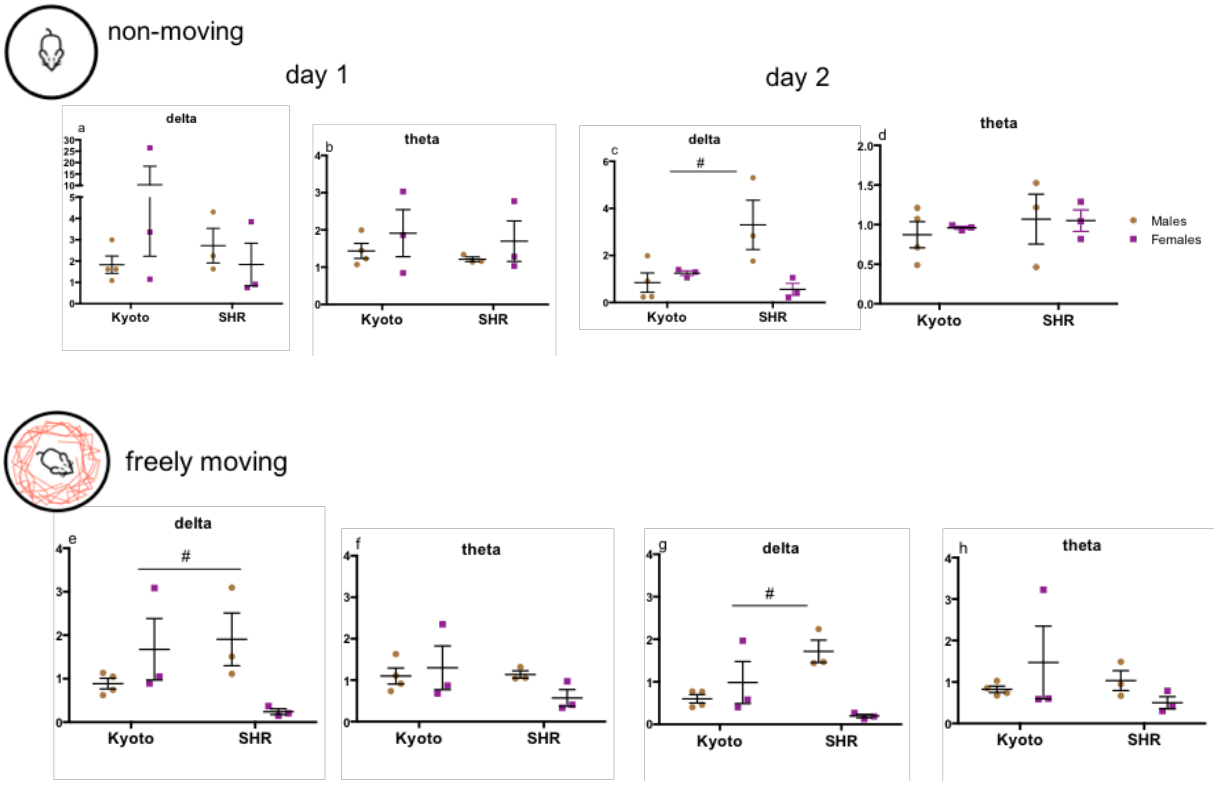

Figure S2 – Quantitative analysis of electroencephalogram of delta and theta waves of Kyoto and ADHD model (SHR) from both sexes. Ratio EEG power of the frontal cortex electrode was obtained in both open field sessions (day 1 and 2) during resting state (non moving in the arena) and locomotor activity (freely moving in the arena) (n = 3-4 animals/group). Two-way ANOVA followed by Bonferroni´s post hoc test. $^{\#}P < 0.05$, interaction between strain and sex.

Fig. S3

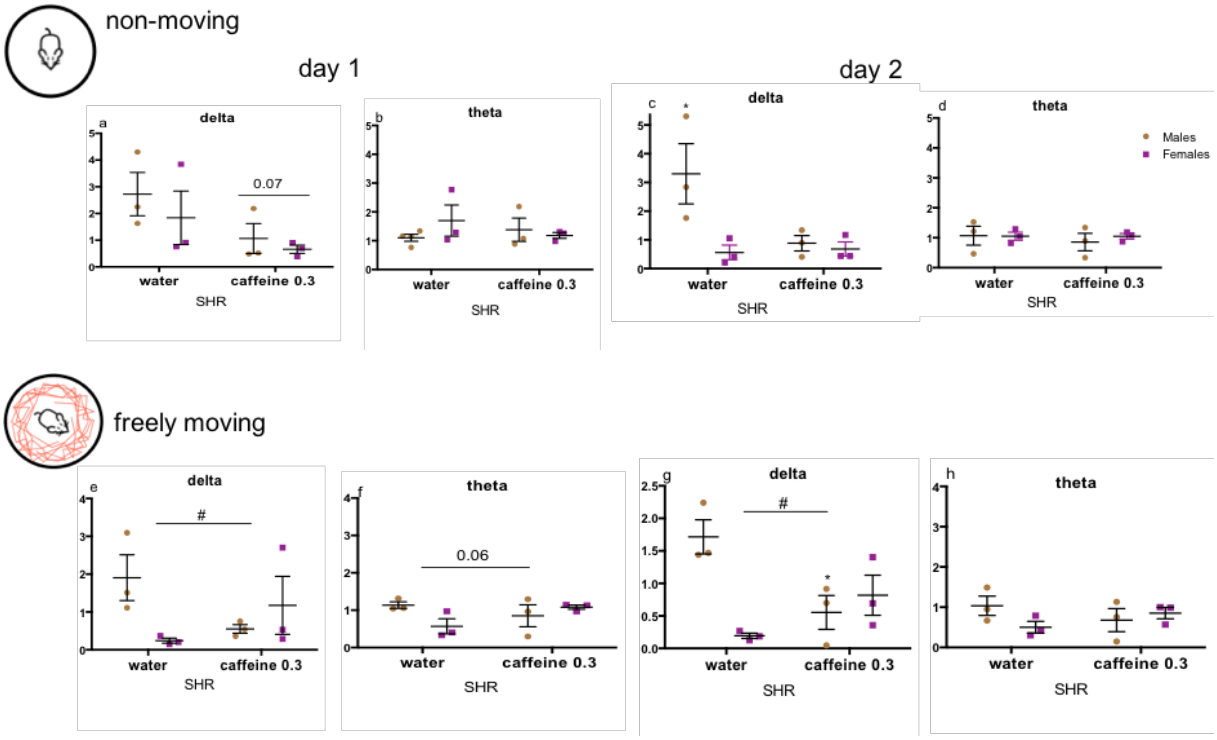

Figure S3 – Quantitative analysis of electroencephalogram of delta and theta waves of ADHD model (SHR) from both sexes receiving drinking water or caffeine (0.3 g/L). Ratio EEG power of the frontal cortex electrode was obtained in both open field sessions (day 1 and 2) during resting state (non moving in the arena) and locomotor activity (freely moving in the arena) (n = 3-4 animals/group). Two-way ANOVA followed by Bonferroni´s post hoc test. *P < 0.05, sex effect. P = 0.0560, interaction between sex and treatment.

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PARTE III

3. DISCUSSÃO

3.1 Dimorfismo sexual nos achados comportamentais

O Transtorno de déficit de Atenção e Hiperatividade (TDAH) é uma desordem neurocomportamental do desenvolvimento bem estudada dentro da medicina. Atualmente sabe-se da forte influência do componente genético, principalmente os polimorfismos encontrados no sistema catecolaminérgico. No entanto, a falta de conhecimento sobre a sua etiologia continua sendo ainda um grande desafio, visto que o diagnóstico continua essencialmente clínico. Adicionalmente, apesar de ser evidente no decorrer da infância a proporção de meninos mais diagnosticados do que as meninas, pouco ainda foi explorado sobre as causas das diferenças de gênero na sua sintomatologia.

Dessa forma, a proposta deste trabalho foi avaliar aspectos comportamentais que pudessem ser correlacionados com os principais sintomas do transtorno, e paralelamente analisar as possíveis alterações em parâmetros neuroquímicos e funcionais contemplando as diferenças de sexo. Neste trabalho também foi investigado os efeitos da cafeína, o psicoestimulante mais consumido mundialmente, sobre as alterações comportamentais, neuroquímicas e funcionais observadas no modelo animal do TDAH com ênfase também no dimorfismo sexual.

A hiperatividade é um dos sintomas mais marcantes na infância dentro da tríade sintomática do TDAH. No primeiro trabalho, avaliamos a atividade locomotora dos animais na tarefa de campo aberto durante dois dias consecutivos e em duas fases distintas, no final da infância (DPN 28-29) e da adolescência (DPN 49-50). Durante esta tarefa os animais SHR de ambos os sexos apresentaram um aumento da locomoção quando comparados à linhagem controle, corroborando com

evidências prévias de que este modelo animal apresenta hiperatividade na locomoção (Langen et al., 2011; Russel, 2011; Bayless et al., 2015; Yang et al., 2015; Botanas et al., 2016), mas sem uma diferença significativa no que diz respeito ao sexo. Além de avaliar o perfil locomotor, o teste de campo aberto a partir de duas exposições por dois dias consecutivos pode ser utilizado para analisar o aprendizado não-associativo (Réus et al., 2008). Neste contexto, observamos que os machos e as fêmeas habituam ao aparato ao final da infância, o que não aconteceu no final da adolescência/início da vida adulta. De fato, talvez não tenha sido possível identificar diferenças entre os sexos porque inclusive as fêmeas da linhagem controle Kyoto também não habituaram, o que pode ser um padrão da linhagem.

Por outro lado, é importante ressaltar que foi avaliada somente a distância total percorrida na arena de campo aberto, ou seja, uma única variável dentro de um conjunto de parâmetros que representaria o fenótipo comportamental de hiperatividade. Portanto, neste primeiro trabalho não foi possível identificarmos na infância e adolescência uma diferença de sexo na hiperatividade. Entretanto, no segundo trabalho em que foram analisados mais variáveis na tarefa do campo aberto, e por meio de uma análise estatística de componentes principais (PC), foi possível observar diferenças entre os sexos nos animais SHR adolescentes. Embora ambos os sexos apresentaram uma hiperatividade quando um conjunto de variáveis foram analisadas de forma conjunta (distância percorrida, velocidade média, tempo de locomoção, distância entre as paradas, elevações verticais). A principal diferença entre os sexos foi documentada para a razão do número de paradas (nº de paradas do primeiro dia/ segundo dia) e maior número de paradas no segundo dia da exploração ao campo aberto (figura suplementar 1), em que os ratos machos apresentaram um padrão de hiperatividade maior do que as fêmeas SHR da mesma

idade. Estas observações sugerem que os machos SHR são ligeiramente mais hiperativos que as fêmeas, pois não habituam no número de paradas e apresentam maior número de paradas que as fêmeas SHR.

Este padrão comportamental foi diferente do observado no final da adolescência, pois ambos os sexos não habituaram ao aparato, demonstrando um prejuízo no aprendizado não-associativo para ambos os sexos. Sabe-se que a pré-adolescência é um período em que ocorre um segundo pico de rearranjos neuronais, com um excesso de sinapses e receptores seguidos por uma eliminação competitiva (Andersen, 2003). Além disso, a maturidade da cognição parece ocorrer paralelamente a essa eliminação competitiva (Casey et al., 2000), e com isso, machos e fêmeas SHR parecem ter um atraso ou um defeito durante o amadurecimento e eliminação de sinapses e receptores, levando a um prejuízo do aprendizado não-associativo no final da adolescência.

Na sequência do primeiro trabalho, também analisamos os déficits cognitivos por meio da tarefa de reconhecimento de objetos e do labirinto em Y. Nossa proposta foi verificar a influência do sexo neste modelo do TDAH, e, observamos que tanto os machos quanto as fêmeas demonstraram um prejuízo similar na memória de reconhecimento, como já previamente demonstrado para essa linhagem de animais em estudos com machos e fêmeas de diferentes idades, analisados separadamente (Prediger et al., 2005b; Pires et al., 2009; 2010). Além da memória de reconhecimento, procuramos investigar também a memória espacial de curta-duração por meio da análise da exploração do terceiro braço no teste do labirinto em Y. Ambas as linhagens não apresentaram um desempenho satisfatório na tarefa, pois os animais não exploraram mais o terceiro braço, eles exploraram igualmente os três braços na sessão de teste. Porém, as fêmeas SHR obtiveram um pior

desempenho, pois além de explorarem igualmente os dois braços ainda exploraram significativamente menos o terceiro braço. De acordo com estudo prévio, os animais SHR parecem ter um déficit cognitivo na memória espacial seletiva, precisando de mais sessões de treino para melhorarem seu desempenho quando comparado aos seus controles (Prediger et al., 2005a). Isto também é particularmente evidente para as fêmeas, pois foi observado que elas precisam de mais dias de treinamento para adquirirem um melhor desempenho (Bucci et al., 2008a; Bucci et al 2008b); neste caso é sugerido que as fêmeas têm um processo de aprendizado mais lento quando comparadas aos machos da mesma linhagem, corroborando com evidências prévias (Sagvolden et al., 1996; Berger, 1998; Pires et al., 2010).

A partir dos resultados obtidos no primeiro trabalho, cujo foco dentro dos aspectos cognitivos no modelo do TDAH foi mais direcionado aos estudos da memória episódica, na sequência do segundo trabalho optou-se por estudar outros aspectos da cognição que dependem do funcionamento do córtex frontal. A avaliação da tomada de decisão em roedores tem sido extensivamente estudada por meio de tarefas operantes que utilizam o paradigma de escolha comportamental (Fantino, 1998; Williams, 1994). As tarefas operantes valem-se de variações para os diferentes paradigmas de escolha, que abrangem múltiplas modalidades, de tátil a visual para auditivo ou olfativo (Iversen, 2008; Kaiser et al., 2006). A tarefa de cavar (dig task) é uma tarefa simples de discriminação de odores pelos ratos para encontrarem a recompensa, cujo uso de pistas olfatórias auxilia na avaliação do desempenho da tarefa (Martens et al., 2012; 2013). Portanto, esta tarefa avalia princípios básicos de aprendizagem, atenção, tomada de decisão e mudança de estratégia, por meio da discriminação de dois odores diferentes e qualifica principalmente danos relacionados a região pré-frontal do córtex, uma das regiões

cerebrais que apresenta anormalidades anatômicas e funcionais no TDAH (Greven et al., 2015). Embora a tarefa de cavar pode fornecer resultados comportamentais que vão além da aprendizagem e tomada de decisão, neste estudo avaliamos o número de sessões necessárias para completar cada fase e a porcentagem de escolhas corretas em cada fase. Com o objetivo de documentarmos um prejuízo no desempenho na tarefa, e não um comprometimento, optou-se por não estabelecer um teto para os animais SHR para a execução das duas fases (discriminação e reversa). Desta forma, a continuidade das sessões para os animais SHR - independentemente do número de sessões feitas pelos animais controle Kyoto - nos permitiu documentar a finalização de cada fase, a fim de comprovarmos que o modelo animal do TDAH apresenta um atraso no desempenho da tarefa e, não necessariamente, um prejuízo mais global. A fase de discriminação nos permitiu avaliar a aprendizagem de uma discriminação, uma vez que dois odores diferentes foram apresentados a todos os animais. Ao avaliar o número de sessões, observamos que as fêmeas SHR precisaram de mais sessões para completar a tarefa na fase de discriminação do que os machos SHR. De uma certa forma, esse resultado corrobora com os dados anteriores em que as fêmeas SHR parecem apresentar prejuízos cognitivos mais exacerbados que os machos SHR. Conforme mencionado anteriormente, as fêmeas parecem ter um aprendizado mais lento e precisam de mais sessões para melhorar seu desempenho quando comparadas com os machos da mesma linhagem (Bucci et al, 2008a; Bucci et al., 2008b). Em um estudo recente com meninas e meninos submetidos a uma tarefa que utilizou recompensas, as meninas apresentaram um atraso quando comparado com os meninos bem como um maior prejuízo na memória de trabalho, o que pode contribuir para essa aprendizagem mais lenta (Patros, 2017). Além disso, como as

meninas são mais diagnosticadas com o subtipo desatento (Wess, 2003; Rucklidge, 2010), o prejuízo no desempenho deste tipo de tarefa também pode envolver déficits de atenção.

Na fase de reversão da tarefa de cavar, a recompensa está no outro odor (diferente do da discriminação) e isto nos permitiu avaliar a adaptação dos animais quanto a sua capacidade de mudança de estratégia e novo aprendizado. Nesta etapa os animais SHR de ambos os sexos apresentaram prejuízo no desempenho e uma menor porcentagem de escolhas certas quando comparados aos animais controle Kyoto. Esses resultados revelam que o pior desempenho dos animais SHR em relação a mudança de estratégia e um prejuízo na aquisição de novos aprendizados, é independente do sexo. De acordo com o estudo de Birrel & Brown (2000), lesões na região frontal foram capazes de promover prejuízos nas respostas em tarefa similar a do nosso estudo, e ainda com o dobro de erros nas opções de escolha.

Neste trabalho, realizamos um conjunto de análises comportamentais e podemos inferir que machos e fêmeas da linhagem SHR têm comportamentos ligeiramente diferentes em alguns parâmetros motores e cognitivos. Apesar de ambos os sexos apresentarem hiperatividade, essa característica tornou-se mais acentuada nos machos. Embora ambos os sexos apresentaram um comprometimento cognitivo, tanto no campo aberto quanto no reconhecimento de objetos, as fêmeas demonstraram um prejuízo cognitivo mais pronunciado na memória de trabalho espacial e na tarefa de tomada de decisão.

3.2 O efeito da administração de cafeína nas alterações comportamentais, funcionais e neuroquímicas em ambos os sexos do modelo do TDAH

Neste trabalho, propusemos um tratamento alternativo com administração crônica de cafeína (0,3 g/L), que corresponde a um consumo moderado em humanos (Fredholm et al., 1999).

De acordo com os dados obtidos no primeiro trabalho, o tratamento com cafeína teve efeitos distintos conforme o sexo e a idade em que o tratamento foi administrado. A cafeína não causou efeitos significativos sobre a locomoção dos animais SHR quando administrada somente durante a infância (até DPN 29), enquanto que o tratamento que se estendeu até o final da adolescência foi capaz de exacerbar a hiperatividade somente nas fêmeas SHR. Em análises similares a cafeína não exerceu efeitos significativos na locomoção dos SHR machos adolescentes (Pandolfo et al., 2013) e fêmeas púberes (Pires et al., 2010). Diferentemente dos trabalhos referenciados, vale ressaltar que o nosso desenho experimental permitiu que os animais de ambos os sexos e mesma idade fossem avaliados simultaneamente.

Uma vez que no primeiro trabalho desta tese o parâmetro analisado foi somente a distância total percorrida na arena de campo aberto, nossos dados inicialmente sugeriram que as fêmeas pareciam ser mais sensíveis ao tratamento de cafeína administrado até o final da adolescência. De fato, inicialmente observamos que a cafeína foi capaz de alterar a locomoção de maneira dependente do sexo, exacerbando a locomoção somente nas fêmeas tratadas desde a infância até a adolescência e isso pode ser atribuído a faixa etária e a dose administrada.

Entretanto, no segundo trabalho por meio de uma maior abrangência de parâmetros comportamentais para verificar a atividade exploratória e locomotora, as quais foram agrupadas e analisadas pela estatística de componentes principais, foi possível uma nova interpretação dos resultados. Foi possível observar que nos

parâmetros mais globais de atividade locomotora e exploratória a cafeína foi capaz de atenuar a hiperatividade em ambos os sexos. A cafeína também foi efetiva em prevenir os parâmetros de hiperatividade mais exacerbados apresentados exclusivamente nos machos SHR, que foram a falta de habituação ao número de paradas. Considerando esses achados, podemos inferir que o tratamento de cafeína pode ser útil no tratamento de déficits no aprendizado não-associativo e no manejo de distúrbios motores apresentados pelo modelo animal do TDAH.

No primeiro trabalho, utilizando dois esquemas de administração, foi interessante observar que o tratamento com cafeína só durante a infância foi tão eficaz quanto o tratamento contínuo até a adolescência em reverter o prejuízo cognitivo da memória de reconhecimento em ambos os sexos. Apesar das diferenças entre doses e cronogramas de tratamento utilizados neste trabalho, esses resultados sobre a memória de reconhecimento estão em concordância com outros trabalhos que não avaliaram simultaneamente machos e fêmeas SHR (Prediger et al., 2005a; Pandolfo et al., 2013; Pires et al., 2010). Como a cafeína administrada somente durante a infância foi capaz de melhorar o prejuízo apresentado pelos animais SHR adolescentes, é possível que a cafeína possa modificar de forma permanente alguns processos de plasticidade sináptica que permitam que seu efeito preventivo perdure até a adolescência, pelo menos neste tipo de memória. Entretanto, neste trabalho não foram investigados parâmetros neuroquímicos e funcionais nesta fase (final da infância) visto que os efeitos benéficos da cafeína sobre outras tarefas de memória foram mais contundentes no tratamento contínuo.

Quando analisada a memória espacial de trabalho, observamos uma melhora que foi seletiva para as fêmeas SHR tratadas com cafeína, justamente a população que apresentou o pior desempenho na tarefa. Além disso, podemos inferir que o

tratamento crônico com cafeína foi capaz de ter efeitos distintos na tarefa de labirinto em Y, pois sua ingesta somente durante a infância teve efeitos sobre o comportamento de inspeção, caracterizado pelo aumento do número de entradas no braço novo, enquanto a ingesta contínua (desde a infância até o final da adolescência) influenciou o comportamento inquisitivo, avaliado pelo tempo gasto no braço novo com as fêmeas SHR as únicas beneficiadas pelo tratamento. Em outro estudo cafeína administrada por via intraperitoneal e em períodos pré-estabelecidos (antes, durante e após o treinamento) foram beneficiadas por um pré-tratamento com cafeína em relação a memória espacial (Prediger et al., 2005a). No nosso trabalho a administração de cafeína foi feita por via oral desde o décimo quinto dia pós-natal e testamos os animais ainda durante a adolescência. Em outro modelo utilizado para os estudos sobre o TDAH, os ratos que receberam 6-hidroxidopamina (6-OHDA) apresentaram melhora na memória espacial quando tratados com cafeína pela água de beber (1 g/L) e durante o período pré-púbere (DPN 24- DPN 38) (Callabero et al., 2011), similar ao nosso trabalho, embora nós observamos um efeito benéfico com uma menor dose (0,3 g/L). Esses resultados para a memória espacial corroboram com outros achados comportamentais desta tese, onde fêmeas SHR demonstram um pior desempenho em relação aos machos SHR e, ao mesmo, tempo, apresentaram uma melhora mais pronunciada após o tratamento com cafeína em relação aos demais grupos, evidenciando a importância de se investigar os efeitos considerando a diferença entre os sexos (Andersen, 2000; Arnett et al., 2015).

A cafeína melhorou o desempenho da tarefa de tomada de decisão - Dig task - nas fêmeas SHR, pois houve uma diminuição significativa no número de sessões necessárias para atingirem o critério para terminarem a fase de discriminação da

tarefa. Curiosamente, a cafeína não se manteve eficaz em reverter o prejuízo nas fêmeas SHR na fase reversa, mas foi efetiva para os machos SHR, os quais só apresentaram prejuízo somente nesta fase. Portanto, a cafeína melhorou a fase de aprendizado para as fêmeas SHR, mas seu efeito não foi sustentado para uma melhora da tomada de decisão, mudança de estratégia e aprendizado novo. É plausível que as diferenças de esquema de tratamento iniciando na infância sejam capazes de promover alterações plásticas que perduram até o final da adolescência.

As respostas aos tratamentos farmacológicos podem ser diferentes quando administradas durante a adolescência, em comparação com os adultos e crianças em desenvolvimento (Casey et al., 2000). Para o metilfenidato, por exemplo, já foram descritas diferenças em relação a idade nos animais SHR em que os adolescentes foram mais sensíveis a doses mais baixas do que os adultos (Barron et al., 2009). Os tratamentos com metilfenidato também podem causar uma sensibilização para adultos já previamente tratados durante a fase da adolescência (Barron et al., 2009; Baskin et al., 2015; Somkuwar et al., 2015).

3.3 Achados Neuroquímicos

O TDAH é considerado um transtorno do desenvolvimento e isso torna fundamental a investigação de proteínas sinápticas e receptores para neurotransmissores envolvidos nesta fase de intensa plasticidade. Visto que o fator neurotrófico derivado do encéfalo (do inglês, *brain derived neurotrophic fator*, BDNF) está intimamente relacionado a processos de sobrevivência, crescimento, plasticidade sináptica e diferenciação de neurônios (Skaper, 2008; Numakawa et al., 2010; Balaratnasingam et al., 2012; Tsai et al., 2016) e o seu papel fundamental no desenvolvimento encefálico (Tsai et al., 2003), ele tem surgido como um potencial candidato a participar da patofisiologia do TDAH. As associações entre

polimorfismos no gene do BDNF já foram relatadas em pacientes com o transtorno (Kent et al., 2005; Lanktree et al., 2008). Já foi relatado que o perfil dos níveis cerebrais de BDNF, principalmente nas regiões do hipocampo e córtex, são similares aos níveis de BDNF no soro durante o desenvolvimento (Karege et al., 2002). Neste trabalho, nós verificamos um aumento do imunoconteúdo de BDNF na região do hipocampo de ambos os sexos dos animais SHR. Em consonância com nossos resultados, crianças diagnosticadas com TDAH revelou apresentaram um aumento nos níveis plasmáticos de BDNF (Shim et al., 2008), enquanto que em adultos houve uma diminuição desses níveis (Corominas-Roso et al., 2013). Poucos estudos investigaram os níveis de BDNF e de proteínas relacionadas a sua sinalização no modelo animal do TDAH. Em um estudo com animais SHR adolescentes não foram encontradas alterações nos níveis desta neurotrofina (Kim at el., 2016), enquanto que em outro trabalho, a expressão de BDNF e do seu receptor TrkB encontraram-se diminuídas na região do hipocampo (Jeong et al., 2014). Nossos resultados, demonstraram um aumento do BDNF e da forma truncada de seu receptor TrkB, em ambos os sexos dos animais SHR. A hiperatividade e o déficit cognitivo apresentado pelos animais SHR podem estar associados com aumentos dos níveis de BDNF e ambas as formas do seu receptor TrkB, sugerindo uma ativação desta via nos animais modelo do TDAH. É importante ressaltar que, apesar do BDNF ser crucial no início do desenvolvimento (Skaper et al, 2008; Yang et al., 2009), uma ativação exacerbada da sua via pode ter como consequência uma sinalização aberrante com uma significativa diminuição da arborização dendrítica e ramificação axonal (Danzer et al., 2002).

O tratamento com cafeína promoveu efeitos distintos de acordo com o sexo em relação aos níveis do BDNF e as duas formas do seu receptor, TrkB-FL e TrkB-T

no hipocampo. O tratamento crônico foi capaz de diminuir os níveis de BDNF somente no hipocampo dos machos SHR. Já foi observada a mesma diminuição nos níveis de BDNF pelo metilfenidato em adolescentes SHR (Fumagalli et al., 2010), mesmo que os psicoestimulantes costumam aumentar os seus níveis em adultos (Lanktree et al., 2008; Banerjee et al., 2006; Meredith et al., 2001). Uma vez que o período da infância e adolescência compreende uma fase de intensa plasticidade sináptica, já foi relatada que a exposição aos psicoestimulantes pode promover efeitos opostos em relação aos observados na idade adulta (Andersen, 2006; Bolaños et al., 2003; Dow-Edwards et al., 2001). Numa comparação entre ratos adolescentes e adultos tratados com metilfenidato, houve revelou uma diminuição na atividade do BDNF/TrkB nos ratos adolescentes (Wetzell et al., 2014).

Apesar do tratamento com cafeína não alterar os níveis das formas fosforiladas do receptor TrkB e do fator de transcrição CREB, o tratamento foi capaz de diminuir a isoforma truncada do receptor TrkB (TrkB-T) em ambos os sexos dos animais SHR, os quais coincidentemente também apresentaram uma melhora na memória de reconhecimento. O tratamento com cafeína também diminuiu os níveis da forma nativa TrkB-FL somente nas fêmeas, as quais demonstraram benefícios em relação a memória espacial. De acordo com alguns estudos, um desequilíbrio da razão TrkB-FL/TrkB-T e/ou uma sinalização aberrante do receptor TrkB estão intimamente relacionados aos transtornos neuropsiquiátricos (Danelon et al., 2016; Gomes et al., 2012; Gupta et al., 2013).

Como a etiologia do TDAH ainda não está totalmente elucidada, os estudos genéticos se concentraram predominantemente em investigar os sistemas de neurotransmissores monoaminérgicos, principalmente o dopaminérgico, com atenção especial para o transportador de dopamina (DAT) e os receptores de

dopamina do tipo D4 (Faraone et al., 2010; Tsai, 2016). Visto que o comportamento de tomada de decisão afeta principalmente áreas córtico-estriatais (Montague and Berns, 2002; Adriani et al., 2006) avaliamos, nesta tese, o imunoconteúdo dos transportadores de dopamina (DAT) e seu receptor D4 (DRD4) nessas duas regiões. No estriado não encontramos modificações significativas (dados não mostrados), mas as análises bioquímicas por western blotting revelaram uma diminuição do transportador DAT e do receptor dopaminérgico DRD4 no córtex frontal de ambos os sexos dos animais SHR. Já foi demonstrada uma relação entre a expressão do gene para o DAT e o risco para o desenvolvimento do transtorno (Chen, 2003; Li, 2006). Especificamente em relação aos modelos animais para o TDAH, estudos têm revelado que uma hipofunção causada por um bloqueio do gene do DAT gera um fenótipo com falta de atenção, hiperatividade e impulsividade, similar aos sintomas apresentados pelos pacientes acometidos pelo transtorno (Mereu et al., 2017). Foi relatada também uma diminuição na densidade do DAT acompanhada por uma menor liberação de dopamina antes de um tratamento com metilfenidato em ratos jovens SHR, sugerindo que esses níveis mais baixos de DAT possam ser um mecanismo compensatório devido a diminuição da liberação de dopamina (Simchon et al., 2010). Esses achados corroboram com os nossos resultados, onde os níveis de DAT encontraram-se diminuídos no córtex pré-frontal de ambos os sexos dos animais SHR, quando comparados aos controles Kyoto. Ainda de acordo com os nossos achados, Léo e colaboradores (2003) encontraram uma diminuição significativa dos níveis DAT nos animais SHR, que foi mais acentuada após o dia pós-natal 49. Ainda de acordo com achados prévios, observamos uma diminuição do imunoconteúdo do receptor de dopamina D4 (DRD4) em ambos os sexos dos animais SHR, sendo que em ratos SHR machos já havia sido encontrado uma

diminuição na expressão e na síntese proteica do DRD4 na região pré-frontal (Li et al., 2007). Outros estudos encontraram resultados opostos, ou seja, aumento tanto dos níveis de proteína quanto do mRNA desse transportador, mas localizados nos terminais nervosos onde os receptores de dopamina D4 são menos abundantes (Pandolfo, 2013; JiChao, 2017).

Com o intuito de relacionar os achados comportamentais deste trabalho com as anormalidades funcionais encontradas, também avaliamos as proteínas sinápticas sinaptofisina e SNAP-25, uma vez que polimorfismos destas proteínas já foram relacionados aos sintomas do TDAH (Guan et al., 2009; Choi et al., 2007; Faraone et al., 2005; Liu et al., 2013; Liu et al., 2017). No nosso trabalho, observamos uma redução da SNAP-25 em ambos os sexos dos animais SHR, com uma diminuição mais pronunciada nas fêmeas, as quais demonstraram um aumento robusto na sinaptofisina. A sinaptofisina é considerada um marcador do desenvolvimento ontogenético (Knaus et al., 1986) e já foram encontradas diferenças entre os sexos durante o desenvolvimento do córtex frontal de ratos (Drzewiecki et al., 2016). Da mesma forma, a SNAP-25 também tem um papel fundamental na regulação armazenamento e liberação de neurotransmissores no compartimento pré-sináptico (Söllner et al., 1993). Este desequilíbrio entre os níveis de sinaptofisina (marcador de vesículas sinápticas) que se encontram aumentados e a diminuição da SNAP-25 (marcador de terminais nervosos) observado apenas nas fêmeas SHR, poderia contribuir para uma transmissão sináptica aberrante e/ou um atraso na maturação do córtex frontal.

Em relação a cafeína, o tratamento crônico foi capaz de aumentar os níveis de DAT em ambos os sexos dos SHR, “normalizando” esses níveis em ambos os sexos. Como um antagonista não seletivo A_1R e $A_{2A}R$, a cafeína não teve impacto

evidente nos níveis de dopamina (Acquas et al., 2002; De Luca et al., 2007). De fato, os sistemas dopaminérgico e adenosinérgico são intimamente interligados através de uma interação funcional e molecular bem estabelecida entre os receptores de adenosina A_1/A_{2A} estriatais e dopamina D1/D2 (Ferré et al., 1997; 2008; Fuxe et al., 2003). É previsível que devido a esta interação e os estudos epidemiológicos com consumo de café diminuindo o risco para o desenvolvimento da Doença de Parkinson, a cafeína e posteriormente os antagonistas seletivos dos receptores A_{2A} foram considerados promissores para o tratamento desta doença (revisado em Chen, 2014; Prediger et al., 2010). Diferentemente do estriado, ainda são escassos estudos mais contundentes sobre uma interação funcional e/ou molecular entre os receptores de adenosina e dopamina no córtex frontal, bem como outras regiões encefálicas. Recentemente, do ponto de vista funcional foi descrita uma interação sinérgica (ao invés da antagônica, observada no estriado) entre A_{2A}R-D2R, na neurotransmissão glutamatérgica no córtex pré-frontal (Real et al., 2018), e em estudos comportamentais o bloqueio dos receptores A_{2A} reverteu as alterações promovidas pelos dos antagonistas dopaminérgicos em processos de tomada de decisão (Mott et al., 2009; Worden et al., 2009).

Diferentemente do que mostramos para o tratamento com cafeína, alguns estudos revelaram uma diminuição da função de DAT no córtex pré-frontal em animais SHR após a interrupção do tratamento com metilfenidato (Roessner et al., 2010; Harvey et al., 2011). No que se refere ao receptor D4, observamos um efeito da cafeína sendo dependente do sexo, aumentando o imunoconteúdo desse receptor somente no córtex frontal dos machos SHR. Como resultados anteriores indicaram um comprometimento do armazenamento de dopamina pelas vesículas de ratos SHR e uma diminuição da liberação de dopamina (Russel, 1998), inferimos

que esses níveis mais baixos de DAT e DRD4 poderiam ser um mecanismo adaptativo em virtude da menor liberação de dopamina (já demonstrada em ratos SHR), levando a uma diminuição dos seus efeitos inibitórios e, consequentemente, poderia contribuir para a hiperatividade observada por estes animais. Como o tratamento de cafeína crônica foi eficiente na normalização dos níveis de DAT em ambos os sexos, e nos níveis do receptor D4 nos machos SHR, isto poderia ser associado a reversão da hiperatividade nos animais SHR. Já foi observado que o antagonismo dos receptores D4 previne a hiperatividade em um outro modelo animal do TDAH, os ratos tratados com 6-OHDA (Avale et al., 2004).

Em relação às proteínas sinápticas, enquanto o tratamento com cafeína foi capaz de diminuir drasticamente os níveis de SNAP-25 nos machos SHR, nas fêmeas a cafeína não alterou os níveis dessas proteínas. Em doses semelhantes, mas com diferentes esquemas de tratamento, já foi demonstrado que a cafeína diminuiu os níveis de SNAP-25 durante o desenvolvimento cerebral de ratos Wistar machos e a hiperlocomoção (Ardais et al., 2014; 2016). Portanto, a atenuação na hiperatividade pela cafeína parece não estar estritamente relacionada às mudanças nos níveis SNAP-25 no córtex frontal dos ratos SHR.

3.4 Achados neurofuncionais

Os resultados das análises quantitativas de eletroencefalograma nos pacientes diagnosticados com TDAH já foram documentadas (Barry et al., 2009a) e, os estudos não demonstraram uma convergência no que diz respeito a atividade das ondas delta e teta (Bresnahan et al., 1999; Buyck and Wiersema 2014; Clarke et al., 2005; 2006; Kitsune et al., 2015; Koehler et al., 2009; Skirrow et al., 2015; Shephard et al., 2018). Apesar disso, têm-se tentado utilizar o EEG a fim de caracterizar os

padrões dessas ondas dentre os diferentes subtipos do transtorno (Aldemir, et al., 2017; Dupuy, et al.,2017), e até mesmo, para fins diagnósticos (Lenartowicz e Loo, 2014).

A partir de dados obtidos por meio do EEG, já foi relatado que a atividade das oscilações delta pode representar um dano cerebral ou condições patológicas (Spironelli et al., 2011). Neste trabalho, observamos um aumento no poder de atividade das ondas delta nos machos SHR, enquanto que as fêmeas demonstraram uma diminuição durante a fase de habituação. Esses resultados se invertem em relação a atividade locomotora, os machos SHR demonstraram uma diminuição enquanto que as fêmeas um aumento do poder da atividade das ondas delta. Algumas interpretações e associações com os achados comportamentais podem ser feitas a partir destes dados funcionais. Primeiramente, esse aumento em delta observado nos machos SHR está de acordo com resultados prévios (Vorobyov et al., 2011), podendo ser interpretado como uma desconexão cortical progressiva, visto que é documentado que a atividade dessas ondas lentas se modifica no decorrer da idade, atingindo um pico durante a infância e declinando acentuadamente durante a adolescência e início da vida adulta, como um processo natural da maturação cerebral (Clarke et al., 2001; Darchia et al., 2007). Com isso, podemos sugerir que pode estar ocorrendo um atraso na maturação do córtex frontal dos SHR de ambos os sexos, mesmo que seja relativamente bem conhecido que as fêmeas de várias espécies de mamíferos sempre apresentarão os níveis de atividade das ondas lentas mais elevados (Ehlers et al., 1993; Knyazev, 2007). Como o aumento da atividade de ondas lentas (delta) está frequentemente associado à falta de controle inibitório sobre o comportamento, e a potência de ondas gama tem se mostrado mais alta em ratos que apresentam maior atividade (Molina et al., 2014),

provavelmente ambas as alterações em delta e gama podem estar contribuindo para hiperatividade ligeiramente mais acentuada nos machos SHR.

Em indivíduos saudáveis, as ondas delta apresentaram uma associação negativa com a conectividade da rede neural em modo padrão, do inglês "*default mode network"* (DMN), a qual se torna desativada durante o desempenho de tarefas (Hlinka et al., 2010). Traduzindo para o nosso modelo, essas reduções nas ondas delta observadas para as fêmeas poderiam estar associadas a uma conectividade funcional atípica dentro da DMN, o que resulta em problemas de atenção conforme já relatado anteriormente para as crianças com TDAH (Barber et al., 2015; Castellanos e Proal, 2012; Sonuga-Barke e Castellanos, 2007; Uddin et al., 2008).

As alterações que observamos no imunoconteúdo de DAT e DRD4 podem estar influenciando as oscilações no EEG encontradas para ambos os sexos do modelo do TDAH. De fato, a hipoatividade no sistema dopaminérgico altera as ondas delta (Knyazev, 2007), e já foi documentado que a liberação de dopamina no córtex pré-frontal medial altera a co-modulação da amplitude de fase de oscilações delta e teta (Andino-Pavlovsky et al., 2017). Associações significativas entre os polimorfismos genéticos para DRD4 e DAT com alterações na atividade das ondas gama também foram encontradas (Demiralp et al., 2007; Yordanova et al., 2001). Na maioria das regiões corticais, as ondas gama predominam desde a infância, coincidindo com eventos de plasticidade sináptica (Uhlhaas et al., 2008), e depois diminuem durante a adolescência, voltando a aumentar no início da idade adulta. Nesse período, a dopamina exerce um maior controle na razão excitação/inibição, contribuindo para a adequação das respostas comportamentais (Furth et al., 2013; O'Donnell, 2010). No nosso trabalho, observamos uma diminuição das ondas gama nas fêmeas SHR, enquanto os machos SHR apresentaram aumento nestas ondas concomitante a uma

hiperatividade ligeiramente maior que as fêmeas. Conforme mencionado anteriormente, essas diferenças observadas corroboram com o já documentado aumento do poder gama no córtex pré-frontal de roedores que apresentaram hieeratividade (Molina et al., 2014). Além disso, observamos que as fêmeas do modelo de TDAH apresentaram um aumento da relação delta/teta e diminuição do poder da atividade de delta e gama. Já foi demonstrado em meninas do subtipo combinado um aumento da atividade de delta e uma redução da atividade de gama (Dupuy et al., 2014). Considerando que as ondas gama podem estar envolvidas no processamento cognitivo e atenção, podemos inferir que uma redução na atividade dessas ondas pode contribuir para o déficit observado no TDAH (Barry et al., 2010). Embora o EEG não tenha sido registrado durante uma tarefa que necessite mais habilidades cognitivas, não se pode descartar que uma diminuição da atividade das ondas gama podem estar envolvidas com um pior desempenho das fêmeas SHR na tarefa de tomada de decisão (Furth et al., 2013). Estudos com camundongos heterozigotos para o DAT (+/-) revelaram uma susceptibilidade dependente do sexo em relação ao hipofuncionamento do sistema dopaminérgico, pois foram relatadas melhoras em prejuízos cognitivos durante a idade adulta de camundongos adolescentes machos DAT +/-, mas não nas fêmeas (Mereu et al., 2017).

Em relação ao efeito do tratamento crônico de cafeína, observamos que os machos foram os mais influenciados pelo tratamento, provavelmente porque as fêmeas do modelo do TDAH parecem ter atingido um limiar nas ondas delta que se tornou difícil observar qualquer impacto da cafeína. De uma forma geral, a cafeína tem essa habilidade de reduzir o poder de ondas delta, devido ao seu clássico efeito psicoestimulante e aumento no estado de alerta, já visto também sobre a atividade das ondas teta e alfa em crianças com TDAH (Barry et al., 2005; 2009b). De certa

forma, o efeito da cafeína sobre o EEG parece seguir um mesmo padrão, o de diminuir o efeito das diferentes ondas, apesar de ser bem estabelecido o seu efeito em aumentar a excitabilidade (Barry et al., 2005; 2009b; 2012). A cafeína diminuiu a atividade das ondas delta e gama, e, foi capaz de atenuar a diminuição dos receptores D4DR apenas em machos do modelo de TDAH. Considerando que essa diminuição na expressão do receptor dopaminérgico D4DR no córtex frontal pode promover uma menor influência dopaminérgica, esse efeito da cafeína em neutralizar a hiperatividade sugere a participação dos receptores D4DR. A cafeína também foi capaz de diminuir o poder de delta tanto em repouso quanto durante a atividade locomotora, além de diminuir as ondas gama.

Considerando nossos resultados sobre as alterações neuroquímicas e funcionais observadas para os animais modelo do TDAH, foi possível observar algumas semelhanças com achados clínicos em pacientes portadores do transtorno. Com isso, foi possível investigar de uma forma um pouco mais aprofundada os mecanismos envolvidos nos efeitos benéficos da cafeína (já documentados no modelo do TDAH) devido a sua capacidade de restaurar alguns padrões do funcionamento do córtex frontal.

4. CONCLUSÃO

Durante as últimas décadas, os olhares se voltaram para o Transtorno de déficit de atenção e hiperatividade por ser um dos transtornos mais prevalentes durante a infância e, aos poucos, sua neurobiologia vem sendo esclarecida. Muitas vezes os sintomas são confundidos com outros transtornos dentro da Psiquiatria, fazendo com que o TDAH seja subdiagnosticado e por consequência, não tratado corretamente. Em adição, o não tratamento acaba gerando um impacto na vida familiar, onde é visto como um fator que dificulta o convívio, no ambiente acadêmico e profissional de quem é acometido por ele.

Com este trabalho, foi possível estabelecer uma visão mais detalhada deste transtorno, desde a infância até o final da adolescência, evidenciando suas principais modificações ao longo da vida. Durante o desenvolvimento desta tese, realizamos um conjunto de análises comportamentais e podemos inferir que machos e fêmeas da linhagem SHR têm comportamentos similares em diversos parâmetros, no entanto, apesar de pertenceram à mesma linhagem, a SHR, se tornou evidente a influência do sexo no desempenho das tarefas, enfatizando a relevância de se analisar os sexos separadamente. Com esses resultados, ressaltamos o quão importante é considerar o gênero no momento do diagnóstico, principalmente para entendermos as diferenças na prevalência deste transtorno psiquiátrico.

Considerando nossos resultados sobre as alterações neuroquímicas e funcionais observadas para os animais modelo do TDAH, foi possível observar algumas semelhanças com achados clínicos em pacientes portadores do transtorno.

Sendo assim, as alterações que relatamos em proteínas sinápticas, fatores neurotróficos, receptores e transportadores podem contribuir para futuras pesquisas com o intuito de desvendar as causas do TDAH e, assim, facilitar a compreensão dos mecanismos envolvidos nas causas deste transtorno.

Atualmente, o tratamento padrão para o TDAH é o metilfenidato (Ritalina®), no entanto, são observados diversos efeitos colaterais e, mais importante, algumas pessoas não respondem ao tratamento padrão. Dessa forma, torna-se fundamental a busca por novos alvos terapêuticos e neste trabalho foi possível investigar, de uma forma um pouco mais aprofundada, os efeitos benéficos da cafeína em um modelo de animais que mimetizam os sintomas do TDAH. Portanto, a administração de cafeína demonstrou ser promissora como alternativa/adjuvante no tratamento do TDAH e, até mesmo, contribuindo para o uso mais racional de medicamentos controlados como o metilfenidato.

5. PERSPECTIVAS

- Avaliar a influência do sexo e os efeitos da cafeína em testes comportamentais que explorem outras características do TDAH, como por exemplo, a impulsividade.

- Analisar o imunoconteúdo de outros receptores dopaminérgicos, principalmente aqueles que formam dímeros com os receptores de adenosina (D1, D2) nos animais do modelo do TDAH, e também explorar a via da noradrenalina.

- Investigar o efeito do tratamento com cafeína na atividade de outros tipos de oscilações cerebrais, entre elas as ondas mais rápidas como ondas Beta e Alfa, dos animais modelo do TDAH.

- Avaliar a influência do tratamento com cafeína e da diferença de sexo na atividade neuronal através da técnica de tomografia por emissão de pósitrons (PET).

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