UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

EFEITO DA ESTIMULAÇÃO TRANSCRANIANA POR CORRENTE CONTÍNUA EM UM MODELO ANIMAL DO TRANSTORNO DO DÉFICIT DE ATENÇÃO E HIPERATIVIDADE

DOUGLAS TEIXEIRA LEFFA

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“Se enxerguei mais longe, foi porque me apoiei nos ombros de gigantes.”

- Isaac Newton

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SUMÁRIO

RESUMO

O Transtorno de Déficit de Atenção e Hiperatividade (TDAH) é um transtorno prevalente do neurodesenvolvimento cerebral que se caracteriza por sintomas inapropriados para o desenvolvimento nas dimensões de desatenção, hiperatividade-impulsividade, ou ambas. Sua fisiopatologia, apesar de não totalmente elucidada, aparenta envolver um déficit em sistemas de neurotransmissão dopaminérgica. Além disso, fatores inflamatórios e oxidativos têm sido propostos como coadjuvantes neste processo patológico. O tratamento do TDAH envolve principalmente o uso de fármacos estimulantes. Tais fármacos, apesar de efetivos, apresentam inúmeros efeitos adversos que reduzem sua aderência a longo prazo, o que estimula a busca por novas estratégias terapêuticas. A estimulação transcraniana por corrente contínua (ETCC) é uma ferramenta de neuromodulação não-invasiva que promove a modulação da atividade neuronal espontânea. A ETCC tem sido proposta como uma estratégia terapêutica em diferentes transtornos neuropsiquiátricos, no entanto o entendimento de seus efeitos em diferentes estruturas do sistema nervoso central ainda é escasso. Neste sentido, o uso de modelos animais é de suma importância para a melhor compreensão do mecanismo de ação da ETCC em doenças neuropsiquiátricas. Nesta tese, avaliamos o potencial terapêutico da ETCC no modelo animal de TDAH mais comumente utilizado, os Ratos Espontaneamente Hipertensos (SHR). SHR adultos e os seus controles, os Ratos Wistar Kyoto, foram expostos a 20 min de ETCC diária por um período de 8 dias, utilizando uma corrente constante de 0,5 mA de intensidade, ou a uma estimulação *sham*. Os efeitos do tratamento foram avaliados por meio de testes comportamentais. A hiperatividade foi avaliada utilizando-se o teste do campo aberto, a memória de trabalho foi avaliada utilizando-se o teste do labirinto em Y, e a memória de curta e longa duração foram avaliadas com o uso do teste do reconhecimento de objetos. Além disso, objetivamos avançar o conhecimento dos mecanismos de ação da ETCC por meio da mensuração de marcadores

bioquímicos relacionados com o transtorno. Avaliamos os níveis de dopamina, fator neurotrófico derivado do cérebro, interleucinas (fator de necrose tumoral alpha, interleucina 1β e interleucina 10), e parâmetros oxidativos (níveis de 2′-7′-diclorofluoresceína diacetato e glutationa, e atividade das enzimas glutationa peroxidase, superóxido dismutase e catalase). As avaliações bioquímicas foram realizadas em córtex pré-frontal, córtex (demais regiões), hipocampo e estriado. Animais SHR expostos a um tratamento com ETCC apresentaram melhora da memória de curta duração e memória de longa duração quando comparados a animais expostos à ETCC *sham*. Do ponto de vista bioquímico, demonstramos que a ETCC induziu aumento dos níveis de dopamina no hipocampo e estriado de ambas as linhagens, aumento da produção de espécies reativas de oxigênio no hipocampo dos SHR e redução da resposta inflamatória em ratos controle. Demonstramos também, pela primeira vez, que os SHR se caracterizam por apresentam um perfil pró-oxidativo em estruturas cerebrais quando comparado aos controles. Além disso, esta linhagem apresenta níveis reduzidos de interleucinas pró-inflamatórias, indicando um déficit basal. Esses resultados caracterizam a ETCC como uma alternativa promissora para o tratamento de déficits mnemônicos em pacientes com TDAH, e apontam o aumento do aporte dopaminérgico em hipocampo como possível mecanismo envolvido nessa melhora.

ABSTRACT

Attention-deficit/hyperactivity disorder (ADHD) is a prevalent neurodevelopmental disorder characterized by age-inappropriate symptoms of inattention, hyperactivity-impulsivity, or both. The pathophysiology of ADHD, although not entirely known, involves impaired activation of dopaminergic neurotransmission systems. Besides that, inflammatory and oxidative parameters have been proposed as important players in the pathologic process. The treatment of ADHD involves mainly the use of stimulant medication. Although effective, stimulants induce a wide range of adverse effects that reduce the long-term adherence. Therefore, there has been a constant search for new therapeutic strategies. Transcranial direct current stimulation (tDCS) is a non-invasive neuromodulatory tool that modulates spontaneous neuronal activity. TDCS has been proposed as a therapeutic strategy in different neuropsychiatric disorders. However, knowledge regarding its effects in distinct regions of the central nervous system is still scarce. In this sense, the use of animals models is essential in order to better understand the mechanisms of action of tDCS in neuropsychiatric disorders. In this thesis, we evaluated the therapeutic potential of tDCS in the most commonly used animal model of ADHD, the Spontaneous Hypertensive Rats (SHR). Adult SHR and their control, the Wistar Kyoto Rats, were exposed to 20 min daily sessions of tDCS over 8 days, using a constant current with an intensity of 0.5 mA, or to a *sham* stimulation. The effects of treatment were evaluated using behavioral tests. Hyperactivity was evaluated using the open field test, working memory was evaluated using the Y-maze test, long and short-term memories were evaluated using the object recognition test. Besides that, we aimed at advancing the knowledge regarding the mechanisms of action of tDCS by measuring biochemical markers related to the disorder. We measured the levels of dopamine, brain derived neurotrophic factor, interleukins (tumor necrosis factor alpha, interleukin 1β and interleukin 10), and oxidative parameters (levels of 2'-7'-dichlorodihydrofluorescein diacetate

and glutathione, the activities of glutathione peroxidase, superoxide dismutase and catalase). Biochemical measurements were performed in the prefrontal cortex, cortex (remaining regions), hippocampus and striatum. SHR treated with tDCS presented an improvement in short-term memory and long-term memory when compared to the *sham* group. In relation to the biochemical parameters, we showed that tDCS induced an increase in dopamine levels in the hippocampus and striatum of both strains. TDCS also induced an increase in the production of oxygen reactive species in the hippocampus of SHR, and a reduction in the inflammatory response in the controls rats. We also showed, for the first time, that the SHR are characterized by a pro-oxidative profile in brain regions when compared to the control strain. In addition, the SHR presented reduced levels of pro-inflammatory interleukins, indicating a basal deficit. Those results indicate that tDCS may be a promising alternative for treating mnemonic deficits in patients with ADHD, and also that an increase in dopaminergic inputs to the hippocampus may be a possible mechanisms involved in this improvement.

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LISTA DE ABREVIATURAS E SIGLAS

AMPA	Alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiónico
BDNF	Fator neurotrófico derivado do cérebro
CPF	Córtex pré-frontal
DA	Dopamina
DAT	Transportador de dopamina
ETCC	Estimulação transcraniana por corrente contínua
IL	Interleucina
LTD	Depressão de longa duração
LTP	Potenciação de longa duração
MAO-B	Monoamino oxidase B
NMDA	N-metil-D-aspartato
SHR	Ratos espontaneamente hipertensos
TDAH	Transtorno do déficit de atenção e hiperatividade
TNF-α	Fator de necrose tumoral alpha
WKY	Ratos Wistar Kyoto

I. INTRODUÇÃO

1. INTRODUÇÃO

O Transtorno de Déficit de Atenção/Hiperatividade (TDAH) é um transtorno do desenvolvimento cerebral que se caracteriza por sintomas inapropriados de desatenção, hiperatividade-impulsividade, ou ambos [1]. Sua prevalência é de cerca de 5,29% em indivíduos com menos de 18 anos [2], e estudos longitudinais demonstraram que há uma persistência de até 70% na idade adulta [3]. O diagnóstico de TDAH está relacionado com inúmeros desfechos negativos relacionados a contextos sociais, acadêmicos e ocupacionais em crianças e adultos, tendo impacto direto na qualidade de vida dos pacientes acometidos [4].

O TDAH se caracteriza por uma alta herdabilidade de aproximadamente 70% [5]. Os pacientes acometidos pelo transtorno apresentam déficits em uma ampla gama de domínios cognitivos [1]. Esses déficits, por sua vez, são acompanhados por alterações estruturais e funcionais em circuitos cerebrais relacionados a processos de atenção, controle de impulsos, entre outros [1]. Sua fisiopatologia, apesar de não totalmente compreendida, aparenta estar relacionada a um hipofuncionamento do sistema dopaminérgico [6]. Nesse sentido, os fármacos estimulantes, que atuam aumentando os níveis de dopamina (DA) na fenda sináptica, se apresentam como a estratégia terapêutica mais efetiva [7, 8]. Apesar de efetivos, os fármacos estimulantes induzem diversos efeitos colaterais, reduzindo sua aderência a longo prazo [9]. Deste modo, há uma constante busca por tratamentos que sejam efetivos e que apresentem um perfil de efeitos colaterais mais vantajoso.

A estimulação transcraniana por corrente contínua (ETCC) é uma ferramenta de neuromodulação não-invasiva que consiste em aplicar uma corrente elétrica de baixa voltagem sob áreas corticais com o objetivo de modular a atividade neuronal espontânea [10]. Essa ferramenta tem sido utilizada em um número crescente de ensaios clínicos randomizados, apresentando-se como um potencial tratamento para diversas doenças

neuropsiquiátricas [11]. No TDAH, estudos pilotos demonstraram potencial eficácia da ETCC para controle de sintomas de desatenção e impulsividade. No entanto, ensaios clínicos randomizados com delineamentos e tamanho amostral mais adequados ainda são necessários para comprovar a eficácia e a segurança dessa técnica nesta população.

Modelos animais são sabidamente essenciais para a investigação do mecanismo de ação de estratégias terapêuticas farmacológicas e não-farmacológicas [12]. De modo curioso, enquanto a investigação da eficácia da ETCC por meio de ensaios clínicos randomizados avança a passos largos, ainda há escassez de estudos básicos relacionados ao tema. Dois fatores possivelmente relacionados a esta aparente discrepância são: (1) ETCC apresenta perfil de efeitos adversos bastante favorável, estimulando o delineamento de novos ensaios clínicos randomizados; e (2) a aparente dificuldade na adaptação da ETCC para uso em roedores sem alterar em demasia o potencial translacional do estudo. Desse modo, estudos em ciência básica que façam uso de modelos animais são de suma importância para o avanço da ETCC como uma alternativa terapêutica para pacientes com TDAH.

Os ratos espontaneamente hipertensos (SHR) são, atualmente, o modelo animal do TDAH mais comumente utilizado na literatura. Os SHR apresentam inúmeras alterações comportamentais compatíveis com as observadas em pacientes com TDAH, as quais são, em grande parte, revertidas com o uso de fármacos estimulantes [13, 14]. Além disso, essa linhagem se caracteriza por uma aparente hipoativação de vias dopaminérgicas, caracterizando-se como um modelo animal apropriado ao estudo do TDAH [13, 14]. Deste modo, neste trabalho objetivamos avaliar o potencial terapêutico da ETCC nos SHR comparados ao seu controle, os Ratos Wistar Kyoto (WKY), por meio de testes comportamentais. Os comportamentos avaliados foram: hiperatividade; memória de trabalho; memória de curta duração; e memória de longa duração. Além disso, objetivamos explorar possíveis mecanismos de ação da ETCC nos SHR por meio de ensaios bioquímicos. As

avaliações bioquímicas envolveram fatores inflamatórios, fatores oxidativos, níveis de DA e fator neurotrófico derivado do cérebro (BDNF).

II. REVISÃO DA LITERATURA

2.1. ESTRATÉGIA PARA LOCALIZAÇÃO DE INFORMAÇÕES

Na revisão da literatura, buscou-se abordar o TDAH, SHR (modelo animal mais utilizado no estudo do TDAH), a ETCC (estratégia terapêutica testada na tese), memória, inflamação e estresse oxidativo. Para isso, as seguintes bases de dados foram consultadas: MEDLINE, LILACS e SciELO. As buscas foram realizadas utilizando diferentes combinações dos seguintes termos: *ADHD, SHR, tDCS, memory, inflammation, oxidative stress*. As combinações de busca, assim como os resultados obtidos, podem ser visualizados na Figura 1.

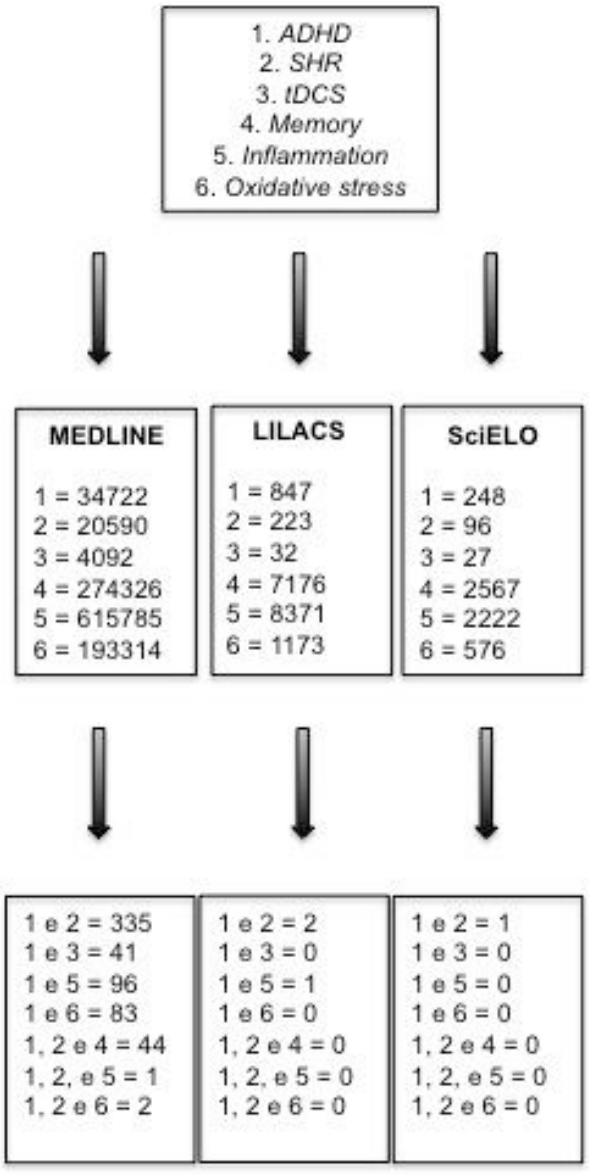

Figura 1. Estratégia de busca e resultados nas diferentes bases de dados.

2.2. TRANSTORNO DO DÉFICIT DE ATENÇÃO E HIPERATIVIDADE

O TDAH é atualmente compreendido como um transtorno neuropsiquiátrico do desenvolvimento cerebral que se caracteriza por sintomas de desatenção, hiperatividade-impulsividade ou ambos. Acredita-se que a primeira descrição da síndrome seja de 1775, feita pelo médico alemão Melchior Adam Weikard em seu livro intitulado "*Der PhilosophischeArzt*" [15]. Em 1798, o médico escocês Alexander Crichton, interessado na origem dos transtornos mentais, publicou um livro intitulado "*An inquiry into the nature and origin of mental derangement: comprehending a concise system of the physiology and pathology of the human mind and a history of the passions and their effects"*. No segundo capítulo desse livro ("*On Attention and its Diseases*"), Weikard descreve uma síndrome de desatenção que torna o indivíduo "incapaz de atentar constantemente a qualquer objeto de educação" [16]. De modo interessante, Weikard ressalta em sua publicação que existe uma variabilidade esperada na capacidade atencional de indivíduos saudáveis, e que tal estado mental também apresenta uma variação temporal em um mesmo indivíduo. Desse modo, ele enfatiza que uma desatenção aparente não se correlaciona necessariamente com um estado patológico. Acredita-se que Weikard tenha apresentado uma descrição do que hoje é considerado o subtipo predominantemente desatento do TDAH [17].

De forma mais anedótica, em 1844, alguns dos principais sintomas que atualmente compõem o espectro do TDAH foram descritos por um médico chamado Heinrich Hoffmann, que viria a fundar o primeiro hospital psiquiátrico de Frankfurt, em um livro infantil intitulado "*Struwwelpeter*". Nesse livro, Hoffmann conta a história do "Inquieto Phil" ("*Fidgety Phil*") um menino que não conseguia ficar sentado quieto à mesa, que se contorcia, pulava da cadeira e ficava se movendo constantemente para frente e para trás, não

conseguindo se ater à atividade em questão: o jantar (figura 2). Acredita-se que essa e outras histórias descritas por Hoffmann em seu livro foram baseadas em sua experiência clínica com crianças portadoras do que hoje se caracteriza como TDAH [18].

Figura 2. Ilustrações representando o "Inquieto Phil" no livro infantil "*Struwwelpeter"* de 1844. Figura adaptada de: https://germanstories.vcu.edu/struwwel/philipp_e.html (acesso dia 24/09/2018).

Em 1902, o médico pediatra inglês George Frederick Still apresentou uma série de três palestras para o *Royal College of Physicians*, em Londres, intitulada "Sobre algumas condições psíquicas anormais em crianças", que foram publicadas no mesmo ano no *The Lancet* [19]. Still descreveu 43 pacientes de sua prática clínica que apresentavam sérios problemas em manter a atenção e que, por muitas vezes, eram: hiperativos; agressivos; resistentes a imposições disciplinares; excessivamente emotivos; com pouca capacidade de inibição comportamental; e inconsequentes de seus atos [20]. Segundo Still, a gratificação imediata era uma característica marcante nesta população e ele acreditava que os pacientes sofriam de "defeitos no controle moral".

Em 1932, os médicos alemães Franz Kramer e Hans Pollnow descrevem uma síndrome hipercinética da infância caracterizada especialmente por uma inquietação motora. Tais crianças não conseguiam ficar quietas por muito tempo, se aborrecendo quando impedidas de agir de acordo com seus impulsos motores. Elas seriam incapazes de se concentrar em tarefas difíceis e não apresentavam perseverança em suas atividades [16]. O

cérebro começou então a ser o principal foco de estudo nas pesquisas relacionadas a esse tema, que começou a ser descrito como "disfunção cerebral mínima". Tal denominação se deu especialmente após uma epidemia de encefalite resultar em um grande número de crianças com a presença de sintomas comportamentais [21]. Tais pesquisas culminaram, em 1980, com a terceira edição do Manual Diagnóstico e Estatístico de Transtornos Mentais descrevendo pela primeira vez os critérios diagnósticos operacionais do transtorno.

Todos essas descrições clínicas apresentadas em diferentes períodos do desenvolvimento da psiquiatria moderna corroboram o conceito atualmente mais aceito na literatura internacional: TDAH é um transtorno neuropsiquiátrico com características sintomatológicas definidas, e não um constructo advindo de imposições da vida moderna.

2.2.1. EPIDEMIOLOGIA

O TDAH é um transtorno neuropsiquiátrico de alta prevalência. Uma meta-análise publicada em 2007 incluiu 102 estudos com um total de 171.756 indivíduos menores de 18 anos, e encontrou uma prevalência de 5,29% [2]. Em uma análise multivariada, três fatores estavam significativamente relacionados à variabilidade da estimativa entre os estudos: a escolha dos critérios diagnósticos; a fonte das informações; e a inclusão da necessidade de comprometimento funcional para o diagnóstico [2]. Além disso, não há evidência de aumento na prevalência do TDAH em crianças e adolescentes nas últimas três décadas após o controle para essas três medidas metodológicas [22]. Em adultos, o TDAH persiste em até de 70% dos casos [3]. Assim, na idade adulta, duas meta-análises indicaram uma prevalência ao redor de 2.5% [1, 23]. Um estudo realizado pela Organização Mundial de Saúde encontrou uma prevalência média em adultos de 2.8% nos 30 países pesquisados [24].

2.2.2. IMPACTO PARA A SAÚDE

O TDAH impacta a qualidade de vida e o bem-estar em contextos sociais, acadêmicos e ocupacionais de crianças, adolescentes e adultos (figura 3). O transtorno está correlacionado a diversas comorbidades psiquiátricas, disfunções psicológicas e comportamentos de risco. Estudos observacionais têm demonstrado que pacientes com TDAH possuem maior risco de desenvolver transtorno por uso de substâncias [25] e de apresentar comportamento criminoso [26]. Além disso, o TDAH está correlacionado ao aumento do risco da ocorrência de acidentes [27]. Acidentes graves de trânsito são mais comuns em pacientes com TDAH quando comparados a indivíduos sem o transtorno [28]. Desse modo, um estudo de coorte que acompanhou 1,92 milhões de pessoas, sendo 32.061 com diagnóstico de TDAH, encontrou um aumento significativo na taxa de mortalidade nessa população [29]. Tais resultados se devem principalmente a mortes por causas não-naturais, especialmente acidentes [29]. Desfechos adversos na adolescência e na vida adulta incluem baixo desempenho acadêmico e vocacional [30], obesidade [31], desemprego, menor status socioeconômico [32], desregulação emocional [33] e gestação na adolescência [34]. Além disso, foi observado que pacientes com TDAH apresentam maior número de tentativas de suicídio quando comparados à população geral [35]. Em resumo, o TDAH representa um impacto importante na qualidade de vida dos pacientes, comparável a outras doenças psiquiátricas graves [4].

Devido a sua prevalência e seu impacto para a saúde, TDAH gera custos enormes diretos e indiretos para o sistema de saúde de países ao redor do mundo, incluindo o Brasil. Estima-se que o Brasil tenha um prejuízo de cerca de R$ 1,841 bilhões por ano devido às consequências de não tratar adequadamente indivíduos com TDAH apenas na faixa de 5 a 19 anos de idade [36]. Um estudo prévio patrocinado pelo CNPq e Ministério da Saúde documentou que o tratamento do transtorno com metilfenidato de liberação imediata, a

medicação mais barata disponível, é custo-efetivo no Brasil [37]. Apesar disso, estima-se que apenas 16,2-19,9% dos indivíduos com TDAH estavam recebendo tratamento de primeira linha em 2009-2010 [38], configurando uma alta prevalência de pacientes não tratados em nosso país.

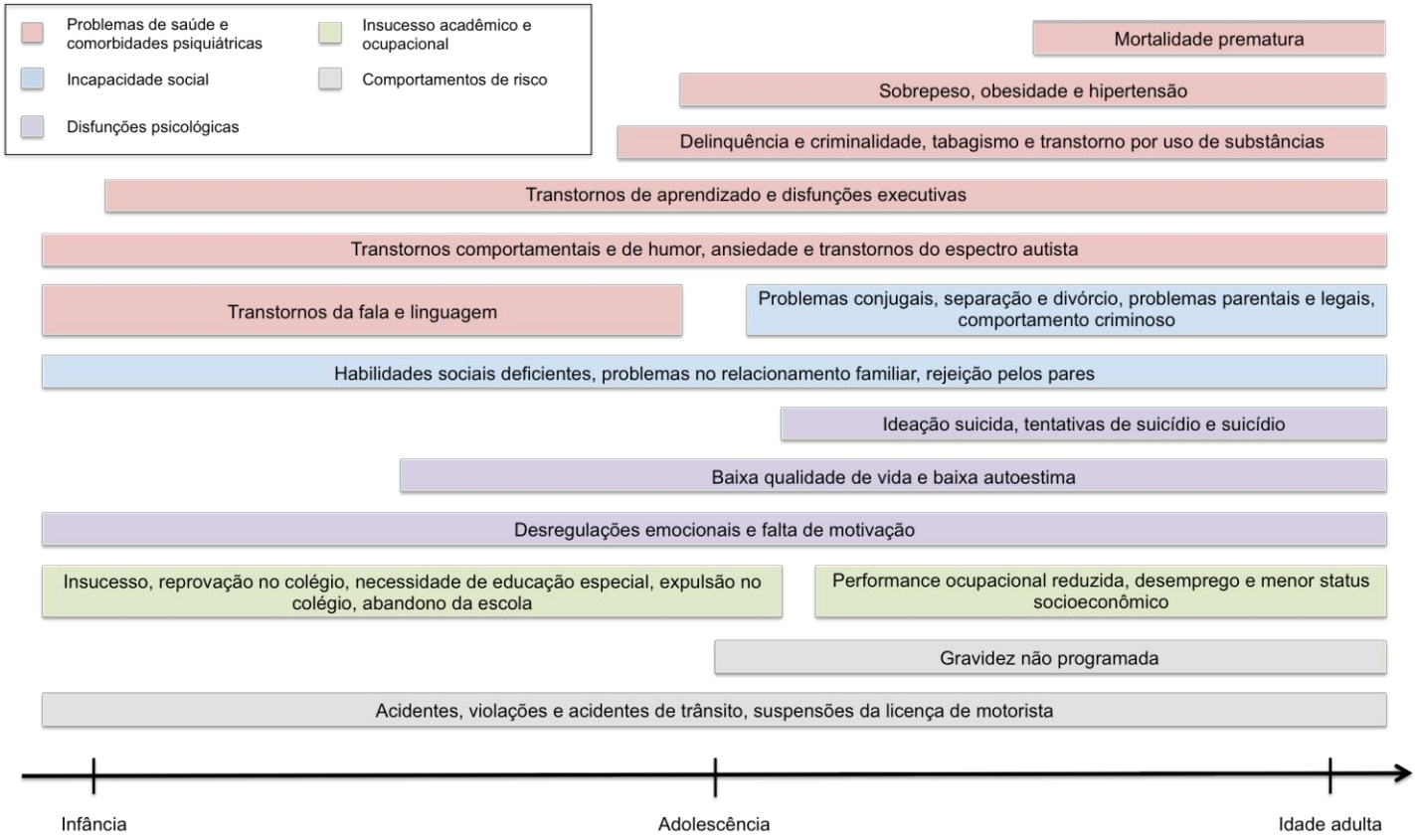

Figura 3. Impacto do transtorno do déficit de atenção e hiperatividade na qualidade de vida e bem-estar na infância, adolescência e idade adulta. Figura adaptada pelo autor de Faraone, Asherson [1].

2.2.3. FISIOPATOLOGIA

O TDAH é um transtorno com alta predisposição genética. Estudos realizados em gêmeos demonstraram que há no TDAH uma heritabilidade de cerca de 70-80% em crianças

e adultos [5, 39]. Apesar de ser um diagnóstico categórico, diversas linhas de evidência têm apontado que o transtorno é melhor caracterizado como um estado extremo de traços quantitativos normalmente presentes na população [40]. Nesse sentido, aproximadamente 40% da heritabilidade do TDAH pode ser atribuída a inúmeras variantes genéticas comuns [41]. Uma meta-análise recente de estudos de varredura genômica identificou, pela primeira vez, 12 genes significativamente associados ao TDAH [42]. Os genes identificados aparentam estar relacionados a processos de formação sináptica, aprendizado, linguagem, e homeostase de neurotransmissores [42]. Os achados desse estudo vêm ao encontro de estudos prévios que investigaram o papel de genes candidatos na fisiopatologia do TDAH. Tais estudos reportam alterações principalmente em sistemas de transmissão dopaminérgica [6]. Nesse sentido, há evidências que demonstram menor atividade dopaminérgica em regiões cerebrais de pacientes com TDAH quando comparados a indivíduos sem o transtorno [43]. O papel desse sistema no TDAH também se evidencia ao se observar que o tratamento medicamentoso mais eficaz é o uso de fármacos agonistas dopaminérgicos, tais como o metilfenidato. O metilfenidato é um bloqueador do transportador de dopamina (DAT), aumentando os níveis extracelulares deste neurotransmissor [44].

Uma das teorias mais aceitas para explicar a fisiopatologia do TDAH relaciona uma diminuição na transmissão dopaminérgica a déficits em múltiplos domínios cognitivos. Entre estes estão sistemas executivos, como memória de trabalho verbal e viso-espacial, controle inibitório, vigilância e planejamento [45]. Pacientes com TDAH apresentam também déficits em sistemas de recompensa [46], linguagem [47] e controle motor [48]. Há uma grande variabilidade relacionada aos domínios cognitivos acometidos em pacientes com TDAH, sendo que grande maioria dos indivíduos apresenta déficits em um ou mais domínios [49].

Além de déficits em uma grande variedade de domínios cognitivos, pacientes com TDAH apresentam alterações anatômicas e funcionais em diversas regiões cerebrais quando

comparados a indivíduos sem o transtorno. Pacientes com TDAH aparentam ter uma redução de cerca de 3% na substância cinzenta e de 2,5% no volume cerebral total comparados aos controles [50]. Em relação a estruturas subcorticais, uma recente meta-análise realizada com mais de 1.700 pacientes e 1.500 controles encontrou reduções volumétricas no núcleo accumbens, amígdala, caudado, hipocampo, e putamen em pacientes com o transtorno [51]. Tais alterações estruturais estão possivelmente relacionadas a mecanismos de desenvolvimento cerebral. Além de alterações morfológicas, pacientes com o transtorno também apresentam alterações funcionais em regiões cerebrais. Meta-análises de estudos de ressonância magnética funcional encontraram redução da atividade em circuitos corticais e subcorticais (figura 4) envolvidos com funções executivas, componentes afetivos e sistemas atencionais [52, 53].

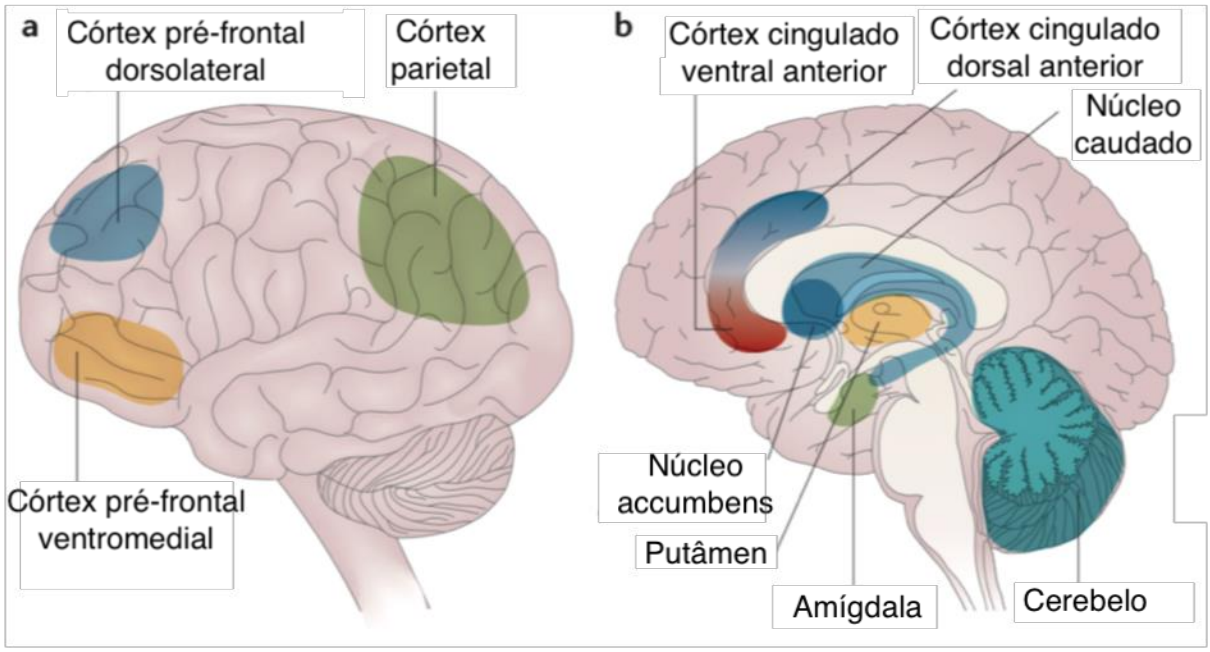

Figura 4. Estruturas corticais e subcorticais envolvidas no transtorno do déficit de atenção e hiperatividade. (a) O córtex pré-frontal dorsolateral está envolvido na memória de trabalho; o córtex pré-frontal ventromedial na tomada de decisões e planejamento; e o córtex parietal na orientação da atenção. (b) O córtex cingulado ventral anterior e o córtex cingulado dorsal anterior estão envolvidos nos componentes afetivos e cognitivos do controle executivo.

Estudos de neuroimagem demonstram alteração na ativação de tais estruturas, juntamente com os núcleos da base, amígdala e cerebelo. Figura adaptada de Faraone, Asherson [1].

Em resumo, a fisiopatologia do TDAH ainda não é totalmente compreendida, mas tem sido demonstrado que há uma grande predisposição genética relacionada principalmente a genes envolvidos na plasticidade e desenvolvimento cerebral [42]. A apresentação clinica do TDAH é bastante heterogênea e os pacientes podem apresentar déficits em diferentes domínios cognitivos [1]. Os déficits em domínios cognitivos estão associados a alterações morfológicas e funcionais em áreas cerebrais envolvidas principalmente com atividade executiva, atenção e controle inibitório [53]. Uma das hipóteses mais aceitas atualmente indica que há um déficit no aporte dopaminérgico em pacientes com o transtorno.

2.2.3.1. INFLAMAÇÃO E ESTRESSE OXIDATIVO

Entre os mecanismos fisiopatológicos possivelmente relacionados ao TDAH, tem havido um interesse crescente em sistemas de inflamação e estresse oxidativo. Nos últimos anos, diversos estudos demonstraram que doenças psiquiátricas estão relacionadas a um estado pró-inflamatório no organismo, especialmente no tecido cerebral. Em pacientes com depressão, por exemplo, meta-análises sugerem que há um aumento nos níveis periféricos de diversas interleucinas pró-inflamatórias [54], e que a mensuração dos níveis de citocinas poderia auxiliar na identificação de indivíduos com o transtorno [55]. Além disso, tem sido demonstrado que o tratamento com fármacos antidepressivos reduz o nível sérico de diversos fatores pró-inflamatórios séricos, tais como a interleucina (IL)-6, fator de necrose tumoral alpha (TNF-alpha) e IL-10 [56, 57]. Uma associação entre alterações em sistemas inflamatórios e oxidativos também é observada na esquizofrenia [58], no transtorno bipolar [59] e no transtorno do estresse pós-traumático [60]. Essa associação entre um estado pró-inflamatório e doenças psiquiátricas aparenta possuir uma etiologia multivariada. Entre os

possíveis mecanismos responsáveis estão: ativação glial [61]; dano neuronal e neurodegeneração [62]; redução do aporte neurotrófico [63]; alterações no metabolismo de neurotransmissores [64]; e dano na barreira hematoencefálica [65].

A evidência da relação entre TDAH e processos inflamatórios/oxidativos provém de duas linhas principais: estudos observacionais demonstrando forte comorbidade do TDAH com doenças inflamatórias e autoimunes, e estudos avaliando biomarcadores séricos. Meta-análises de estudos observacionais, realizadas com mais de 61 mil crianças, têm indicado que o TDAH está relacionado a uma maior probabilidade de diagnóstico de doenças alérgicas tais como asma, rinite alérgica, dermatite atópica e conjuntivite alérgica [66, 67]. Um estudo de coorte prospectivo realizado com mais de 23 mil pacientes demonstrou que uma história pessoal e materna de doenças autoimunes também está associada a um maior número de diagnósticos de TDAH [68]. A maior ocorrência de doenças inflamatórias e autoimunes em pacientes com o transtorno pode sugerir uma diversa gama de mecanismos fisiopatológicos como, por exemplo, alterações na resposta imune, predisposição genética e influências ambientais. A comorbidade do TDAH com doenças inflamatórias também pode explicar a associação da doença com o uso de paracetamol durante o período gestacional [69], fármaco que é por vezes utilizado para controle álgico em tais condições.

Os estudos em pacientes com TDAH com medidas de biomarcadores séricos não demonstraram evidências conclusivas, especialmente devido a tamanhos amostrais pequenos e grande heterogeneidade entre os biomarcadores estudados. Um estudo de coorte prospectivo realizado com mais de 1.500 recém-nascidos prematuros e com baixo peso ao nascer mensurou 25 proteínas séricas relacionadas à atividade inflamatória e encontrou que crianças com aumento nos marcadores inflamatórios durante as duas primeiras semanas pós-natal apresentaram maior probabilidade de problemas atencionais aos 24 meses de vida [70].

Além disso, infecções neonatais, sabidamente associadas a uma resposta inflamatória e inflamação sistêmica durante o primeiro mês de vida, aumentam o risco de TDAH [71, 72].

2.2.3. TRATAMENTO

O tratamento do TDAH envolve abordagens farmacológicas e não-farmacológicas. A sua escolha é guiada pela gravidade dos sintomas, presença de comorbidades e período do dia no qual o alívio dos sintomas é necessário. Intervenções farmacológicas consistem em fármacos estimulantes, os mais utilizados, e não-estimulantes, sendo que meta-análises de ensaios clínicos randomizados demonstraram que ambos são efetivos na redução dos sintomas em crianças e adultos [7, 8]. Pacientes em tratamento apresentam uma redução de 45% no número de visitas à emergência [27] e uma redução de cerca de 40% no número de acidentes com veículos automotores [73]. Além disso, estudo prévio demonstrou que pacientes em tratamento apresentam uma performance significativamente melhor em exames de admissão para ensino superior [74].

Apesar de efetivo, o tratamento farmacológico apresenta importantes limitações ao seu uso. Por exemplo, existe uma variabilidade substancial na resposta à medicação e cerca de 30% dos pacientes não apresentam resposta clínica [75]. Estudos realizados na comunidade demonstraram que o uso consistente da medicação ocorre apenas por 2 a 5 meses na maior parte dos pacientes [76, 77]. Após 2 anos, apenas 50% dos pacientes são aderentes ao tratamento e, após 5 anos, esse número cai para 36% [9]. Pacientes com TDAH apresentam baixa aderência ao tratamento medicamentoso, possivelmente relacionado à ocorrência de efeitos colaterais comuns tais como insônia, perda do apetite, dor abdominal, disforia, e irritabilidade [78-80]. Uma pesquisa com 325 pacientes encontrou uma prevalência de 48% de efeitos colaterais, sendo 21% considerados muito ou extremamente inconvenientes [81]. O tratamento farmacológico com estimulantes também está relacionado

a aumento da pressão arterial e alterações do ritmo cardíaco [82]. Extensa literatura aponta que fármacos estimulantes apresentam potencial de abuso. Em estudo realizado com mais de 300 pacientes com TDAH, 25% reportaram que já utilizaram a medicação pelos seus efeitos psicotrópicos estimulantes e 29% já ofereceu ou vendeu sua medicação para outras pessoas [83]. Em geral, o abuso e o uso de fármacos estimulantes sem prescrição médica ocorre com uma prevalência de até 35%, representando um risco tanto para os pacientes quanto para os usuários sem o transtorno [84]. De forma ainda mais preocupante, nos últimos anos têm-se observado um aumento desproporcional na prescrição de estimulantes. No Brasil, houve um aumento de mais de 700% na venda desses fármacos nos últimos anos [85]. Tais dados se associam a um lucro da indústria farmacêutica que quintuplicou apenas nos últimos cinco anos [86].

Em suma, o tratamento farmacológico é capaz de reduzir os sintomas do TDAH e melhorar a qualidade de vida das pessoas acometidas e, portanto, considera-se uma área estratégica para investimentos em saúde pública. No entanto, a variabilidade na resposta clínica, baixa aderência ao tratamento, efeitos adversos associados aos fármacos e o risco de abuso são importantes limitações ao seu uso. Dessa forma, há uma busca contínua por novas intervenções não-farmacológicas efetivas.

2.2.4. MODELOS ANIMAIS – RATOS ESPONTANEAMENTE HIPERTENSOS

Modelos animais são considerados ferramentas essenciais para o entendimento de mecanismos patológicos e para o desenvolvimento de novas alternativas terapêuticas para doenças neuropsiquiátricas [12]. Desse modo, a ausência de bons modelos animais tem sido apontada como um importante fator no baixo número de novos tratamentos desenvolvidos pela indústria farmacêutica para doenças neuropsiquiátricas nos últimos anos [12]. Para que

seja considerado adequado, um modelo animal deve apresentar validade de face, validade preditiva e validade de constructo [87]. A validade de face corresponde às similaridades na apresentação clínica entre o modelo animal e pacientes com o transtorno. A validade preditiva diz respeito a como o modelo animal responde a um tratamento comprovadamente eficaz para o transtorno. Já a validade de construto corresponde às similaridades entre os mecanismos fisiopatológicos observados no modelo animal e nos pacientes com o transtorno.

Entre os modelos animais de TDAH, os SHR são considerados os mais adequados e, portanto, são os mais utilizados na literatura [13, 14]. Os SHR são uma linhagem provinda da seleção artificial de ratos Wistar. Essa linhagem se originou do cruzamento entre os animais Wistar de uma mesma ninhada que possuíam maior pressão arterial, sendo realizado durante diversas gerações [88]. Os SHR foram primariamente desenvolvidos para o estudo da hipertensão arterial, no entanto foi observado que estes animais apresentavam alterações comportamentais que viriam a ser compatíveis com as encontradas em pacientes com TDAH. Desse modo, se tem demonstrado que os SHR apresentam hiperatividade [89, 90], déficits atencionais mensurados com o uso de diferentes paradigmas comportamentais [91-94], maior impulsividade [95, 96], menor tolerância para recompensas maiores e demoradas em relação a recompensas menores e imediatas [97], menor memória de trabalho [94], menor memória viso-espacial de longa duração [98, 99], menor aprendizado motor [100], e maior preferência a substâncias psicotrópicas tais como agonistas canabinóides [101, 102] e cocaína [103, 104]. Por esses motivos, considera-se que os SHR apresentam boa validade de face.

Em relação à validade preditiva, há uma ampla gama de estudos demonstrando a efetividade de um tratamento com metilfenidato para melhora dos sintomas comportamentais dos SHR. Um tratamento com metilfenidato é capaz de reverter o déficit atencional [92, 105, 106], reduzir o comportamento impulsivo [92, 107] e aumentar a capacidade mnemônica dos SHR [98, 108, 109]. Em relação à hiperatividade, há divergências na literatura sobre os

efeitos do tratamento com este fármaco. Enquanto alguns autores reportaram redução da atividade locomotora [106, 110, 111], outros demonstraram aumento da hiperatividade [112-114]. Desse modo, mais estudos ainda são necessários para que se confirme a validade preditiva dos SHR como um modelo de TDAH.

Muitos autores argumentam que a validade de construto é a mais desafiadora, principalmente devido às grandes diferenças na complexidade do sistema nervoso central entre humanos e roedores. Além disso, apesar dos grandes avanços no estudo da fisiopatologia do TDAH nos últimos anos, não há uma teoria que seja capaz de unificar todos os achados reportados em estudos clínicos. E, como previamente mencionado, sabe-se que a apresentação fenotípica do transtorno é bastante heterogênea, o que possivelmente se reflete em mecanismos fisiopatológicos também heterogêneos. Apesar de tais dificuldades, estudos realizados com os SHR foram capazes de demonstrar mecanismos fisiopatológicos potencialmente correlacionados às alterações comportamentais dessa linhagem. As evidências para validade de construto dos SHR provém principalmente das seguintes linhas de investigação: (1) neurotransmissão dopaminérgica; (2) neurotransmissão glutamatérgica e noradrenérgica; e (3) análise genética.

Diversos estudos foram desenvolvidos com o intuito de investigar a neurotransmissão dopaminérgica nos SHR. Isso se deve principalmente ao fato de que há evidências de déficits dopaminérgicos em pacientes com TDAH [43], sendo o principal tratamento farmacológico para o transtorno, o metilfenidato, um agonista dopaminérgico. Tem sido demonstrado, por exemplo, que os SHR apresentam menor liberação de DA na fenda sináptica em resposta à despolarização neuronal causada tanto por estímulos elétricos quanto por exposições a altas concentrações extracelulares de potássio (K^+), quando comparados aos WKY [115, 116]. Evidências indicam que este déficit na liberação de DA está relacionado a redução desse neurotransmissor em vesículas intracelulares [117]. Essa redução, por sua vez, poderia estar

sendo desencadeada por uma alteração na atividade da enzima monoamino oxidase B (MAO-B), a qual catalisa a deaminação da DA [118], produzindo espécies reativas de oxigênio [119]. Há relatos na literatura que demonstram aumento da atividade desta enzima em tronco encefálico e medula espinhal dos SHR [120], reforçando essa hipótese. Nesse sentido, Boix, Qiao [121] observaram melhora da impulsividade após administração de fármaco inibidor da MAO-B nos SHR, indicando que um aumento da atividade dessa enzima pode estar relacionado com as alterações comportamentais observadas na linhagem.

Outra linha de investigação, que busca explicar a neurotransmissão dopaminérgica pouco efetiva nessa linhagem, estuda o papel dos DAT. Essa é uma proteína essencial na neurotransmissão dopaminérgica, visto que a captação da DA é realizada em sua maior parte pela atividade desse transportador [122]. Uma das hipóteses para o déficit na neurotransmissão dopaminérgica nos SHR é uma maior atividade do DAT na fenda sináptica. Diversos estudos identificaram que SHR adultos apresentam maior expressão de DAT no córtex pré-frontal e estriado quando comparados aos WKY, com aumento da captação de DA nessas regiões [123-126]. Também já foi demonstrado que a maior expressão de DAT é normalizada após um tratamento com metilfenidato [125], e também com cafeína [123]. Desta forma, a DA liberada na fenda sináptica após um potencial de ação pode não estar se ligando a seus receptores pós-sinápticos devido a uma rápida remoção pela alta atividade do DAT. De modo interessante, observou-se que SHR jovens (3-6 semanas pós-natal) apresentam maior liberação de DA por neurônios cujos corpos celulares se encontram na substância negra [127], e menor quantidade de DAT no estriado [128] e mesencéfalo [129] quando comparados aos WKY. Dessa forma, hipotetiza-se que o aumento de DAT nos SHR adultos é um mecanismo compensatório devido ao excesso de DA no circuito mesoestriatal durante o desenvolvimento do sistema nervoso central, resultante de um hipofuncionamento dos receptores deste neurotransmissor.

Além do sistema dopaminérgico, diversos outros sistemas de neurotransmissores parecem estar alterados nos SHR, especialmente os sistemas glutamatérgico e noradrenérgico. Em relação à neurotransmissão glutamatérgica, foi detectada uma redução na transmissão sináptica mediada por receptores alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiónico (AMPA) em neurônios piramidais do córtex pré-frontal de SHR, o que foi relacionado a redução na expressão de subunidades deste receptor na superfície neuronal [130]. Além disso, a atividade sináptica foi restaurada pela administração de metilfenidato [130]. Lehohla, Kellaway [131] encontrou menor captação de cálcio após a estimulação de receptores N-metil-D-aspartato (NMDA) em SHR, sugerindo concentrações intracelulares de cálcio mais elevadas nessa linhagem. Alterações na transmissão glutamatérgica em SHR também foram sugeridas devido a uma menor atividade sináptica, caracterizada por potenciais pós-sinápticos excitatórios, em sinapses hipocampais de SHR [132]. O tratamento com metilfenidato aumentou as concentrações tônicas extracelulares de glutamato nos SHR, corroborando alterações na neurotransmissão glutamatérgica nessa linhagem [133]. Em relação à norepinefrina, nos SHR foi observado um aumento na liberação desse neurotransmissor no córtex pré-frontal após estimulação com glutamato [134]. Foi sugerido que esse efeito seja mediado por receptores AMPA [135] e não por receptores NMDA [134]. Além disso, há também um aumento da atividade do transportador de norepinefrina no córtex orbitofrontal [136], resultando em uma redução dos níveis sinápticos desse neurotransmissor. O tratamento com metilfenidato foi capaz de normalizar a atividade deste transportador [136]. Em suma, apesar da DA ser o neurotransmissor mais implicado nas alterações comportamentais observadas nos SHR, disfunções em outros sistemas de neurotransmissores estão sendo estudados, podendo estas serem resultado da interação dos diferentes circuitos de neurotransmissão no sistema nervoso central.

Tendo-se em vista a alta heritabilidade no TDAH, estudos genéticos também foram conduzidos com os SHR buscando-se maior compreensão da fisiopatologia do transtorno. Além disso, alterações genéticas nessa linhagem são esperadas, tendo-se em vista que os animais são derivados de seleção artificial a partir dos WKY. Uma análise de expressão gênica realizada por Dela Pena, Dela Pena [137], identificou aumento da expressão de 21 genes e redução da expressão de 36 genes no córtex pré-frontal dos SHR em relação aos WKY e ratos Wistar, sendo a maior parte dos genes envolvidos com processos de transmissão sináptica e resposta imune [137]. De modo semelhante, a expressão de genes relacionados com sistemas de neurotransmissores foi avaliada por Santoro, Santos [138] nos SHR e ratos Wistar. Nesse estudo, 84 genes foram avaliados, sendo que quatro deles (Gad2, Chrnb4, Slc5a7, e Qrfpr) se mostraram menos expressos no córtex pré-frontal do modelo animal de TDAH. Além da análise de expressão gênica, uma análise de genes candidatos dopaminérgicos foi realizada, demonstrando polimorfismos no gene do DAT1, sem polimorfismos nos genes que expressam os receptores de DA [139]. As alterações em expressão gênica também estão acompanhadas por diferenças na expressão de diversas proteínas envolvidas no metabolismo energético, citoesqueleto, mielinização e função de neurotransmissores [140]. Tais diferenças foram encontradas no córtex pré-frontal (CPF) e estriado [140].

De modo geral, os SHR apresentam boa validade de face, preditiva e de construto (figura 5). Essa linhagem apresenta inúmeras anormalidades comportamentais compatíveis com as observadas em pacientes com TDAH, sendo que há uma melhora de tais comportamentos com o uso de fármacos sabidamente eficazes no transtorno. Apesar da fisiopatologia do TDAH ainda ser pouco compreendida, acredita-se que o transtorno se deva principalmente a um déficit na neurotransmissão dopaminérgica. Desse modo, os SHR apresentam uma transmissão dopaminérgica pouco efetiva e um maior número de DAT

expressos em diferentes regiões cerebrais. Há diversos genes que são mais ou menos expressos no SHR, indicando essa linhagem como o modelo animal de TDAH de maior validade.

Figura 5. Validade de face, preditiva e de construto nos Ratos Espontaneamente Hipertensos. MDF=metilfenidato; DA=dopamina; DAT=transportador de dopamina. Fonte: autor.

2.3. ESTIMULAÇÃO TRANSCRANIANA POR CORRENTE CONTÍNUA

A ETCC é uma técnica neuromodulatória na qual uma corrente elétrica contínua e de baixa intensidade é aplicada sobre áreas corticais com o objeto de facilitar ou inibir a atividade neuronal espontânea [10]. Os mecanismos de ação responsáveis pelo seu efeito têm sido amplamente explorados por meio de estudos em modelos animais, modelos computacionais e culturas celulares [141, 142].

Acredita-se que o efeito primário da ETCC esteja relacionado a uma modulação do potencial de repouso neuronal, sem a indução de potenciais de ação [143, 144]. As mudanças no potencial de repouso resultantes da ETCC podem levar a uma estimulação ou uma inibição neuronal. A corrente elétrica é transmitida por dois eletrodos, o ânodo e o cátodo (figura 6). O ânodo possui uma carga positiva em relação ao cátodo. A estimulação com o eletrodo anodal aumenta a atividade cortical, enquanto a estimulação com o eletrodo catodal tem o efeito oposto [143]. Os modelos atualmente propostos sugerem que as alterações na polarização da membrana neuronal são o resultado da atração ou repulsão de íons de sódio e potássio, especialmente no meio extracelular [145]. No entanto, é importante salientar que o efeito da corrente elétrica no cérebro pode ser influenciado por inúmeros fatores, tais como a distância e orientação dos axônios ou corpos celulares dos neurônios em relação ao campo elétrico [146].

Apesar de não induzir a propagação de potenciais de ação em neurônios, diversos estudos têm demonstrado que a ETCC resulta em alterações funcionais e morfológicas de longa duração no sistema nervoso central. Por exemplo, o uso da ETCC tem sido proposto como um indutor da potenciação e da depressão de longa duração (LTP e LTD, respectivamente), ambos envolvidos na plasticidade cerebral [147]. As formas mais comuns de LTP e LTD são mediadas por receptores NMDA, os quais são ativados pelo glutamato. Foi demonstrado, por exemplo, que o bloqueio de receptores NMDA inibe os efeitos da ETCC [145, 148]. Além disso, estudos eletrofisiológicos demonstraram que a ETCC é capaz de potencializar a LTP em fatias hipocampais [147].

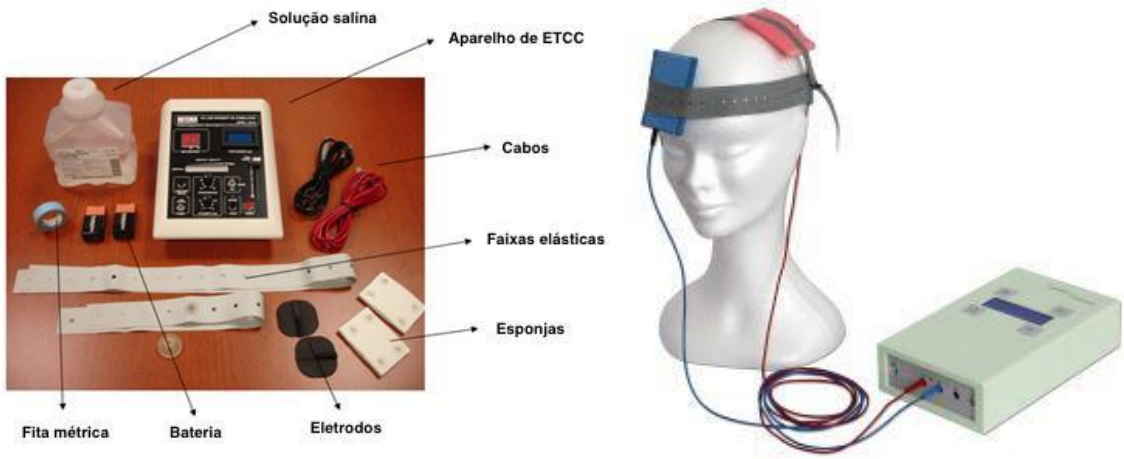

Figura 6. Exemplo de aparelho de estimulação transcraniana por corrente contínua utilizado em estudos clínicos. Figura adaptada de DaSilva, Volz [149] e https://hearingthevoice.org/2016/05/20/transcranial-direct-current-stimulation-over-hyped-or-under-studied-by-peter-moseley/ (acesso dia 24/09/2018).

A ETCC é uma técnica de baixo custo e complexidade, que tem sido repetidamente demonstrada como segura. Eventos adversos moderados reportados incluem queimaduras na pele devido ao contato ineficiente com o eletrodo, enquanto eventos adversos menores incluem cefaleia e fatiga após a estimulação e sensação de queimação durante o procedimento [10]. Até o presente momento, o uso da ETCC convencional em estudos clínicos não produziu relatos de eventos adversos graves em 33.200 sessões realizadas em mais de 1.000 sujeitos de pesquisa [11].

Em pacientes com TDAH, ensaios clínicos randomizados fase II acessando a eficácia da ETCC foram conduzidos utilizando-se metodologias heterogêneas e obtiveram resultados positivos e negativos [150-158]. A tabela 1 traz um resumo de tais estudos. Dessa forma, ainda há a necessidade de se conduzir ensaios clínicos randomizados com delineamentos mais adequados e com um número maior de pacientes para que o potencial terapêutico da

ETCC seja avaliado de forma adequada. Em relação a modelos animais, não há na literatura nenhum estudo prévio avaliando os efeitos da ETCC em modelos animais do TDAH.

Tabela 1. Estudos randomizados prévios conduzidos por outros grupos de pesquisa utilizando a estimulação transcraniana por corrente contínua em pacientes com transtorno do déficit de atenção e hiperatividade.

Autor e ano	Tamanho amostral	Desenho do estudo	Montagem da ETCC	Parâmetros de estimulação	Desfechos	Resultados	Limitações
Cosmo et al., 2015 [150]	60 adultos	Paralelo, duplo mascaramento	Ânodo sob o CPFDL esquerdo	1 mA, uma sessão	*Go/no go task*	Sem diferença no controle inibitório	Estimulação sob o CPFDL esquerdo, apenas uma sessão
Breitling et al., 2016 [151]	21 adolescentes; 21 controles	Paralelo, mascaramento único	Ânodo sob o giro frontal inferior	1 mA, uma sessão	*Flanker task*	Sem diferença no controle inibitório	Mascaramento único, estimulação sob o giro frontal inferior, apenas uma sessão
Soltaninejad et al., 2016 [152]	20 adolescentes	Cruzado, mascaramento único	Ânodo sob o CPFDL esquerdo	1,5 mA, uma sessão	*Go/no go task; Stroop task*	Aumento da proporção de respostas corretas (teste *go/no-go*)	Mascaramento único, estimulação sob o CPFDL esquerdo, apenas uma sessão, cruzado com *washout* de 72h
Nejati et al., 2017 [153]	25 adolescentes	Cruzado, duplo mascaramento	Ânodo sob o CPFDL esquerdo	1 mA, uma sessão	*Go/no go task; Stroop task; N-back task; Wisconsin card sorting task (WCST)*	Melhora da memória de trabalho (WCST)	Estimulação sob o CPFDL esquerdo, apenas uma sessão, cruzado com *washout* de 72h
Sotnikova et al., 2017 [154]	16 adolescentes	Cruzado, duplo mascaramento	Ânodo sob o CPFDL esquerdo	1 mA, uma sessão	*N-back task; fMRI*	Sem diferença na memória de trabalho. Aumento da atividade no CPFDL esquerdo	Estimulação sob o CPFDL esquerdo, apenas uma sessão, cruzado com *washout* de 2 semanas
Soff et al, 2017 [155]	15 adolescentes	Cruzado, duplo mascaramento	Ânodo sob o CPFDL esquerdo	1 mA, 5 sessões	*Checklist de sintomas do TDAH (FBB-TDAH)*	Melhora da desatenção	Estimulação sob o CPFDL esquerdo, cruzado com *washout* de 2 semanas
Jacoby et al., 2018 [156]	20 adultos; 15 controles	Cruzado, mascaramento único	Ânodo sob o CPFDL esquerdo e direito	1,8 mA, uma sessão	*MOXO Continuous Performance Test (CPT)*	Sem diferença na atenção	Mascaramento único, apenas uma sessão, cruzado com *washout* de 1 semana
Allenby et al., 2018 [157]	37 adultos	Cruzado, duplo mascaramento	Ânodo sob o CPFDL esquerdo	2 mA, 3 sessões	*CPT; Stop signal task (SST)*	Redução da impulsividade (CPT), sem diferença no SST	Estimulação sob o CPFDL esquerdo, apenas 3 sessões, cruzado com *washout* de 2 semanas

CPFDL = Córtex pré-frontal dorsolateral

III. MARCO CONCEITUAL

3.1. MARCO CONCEITUAL DO TDAH

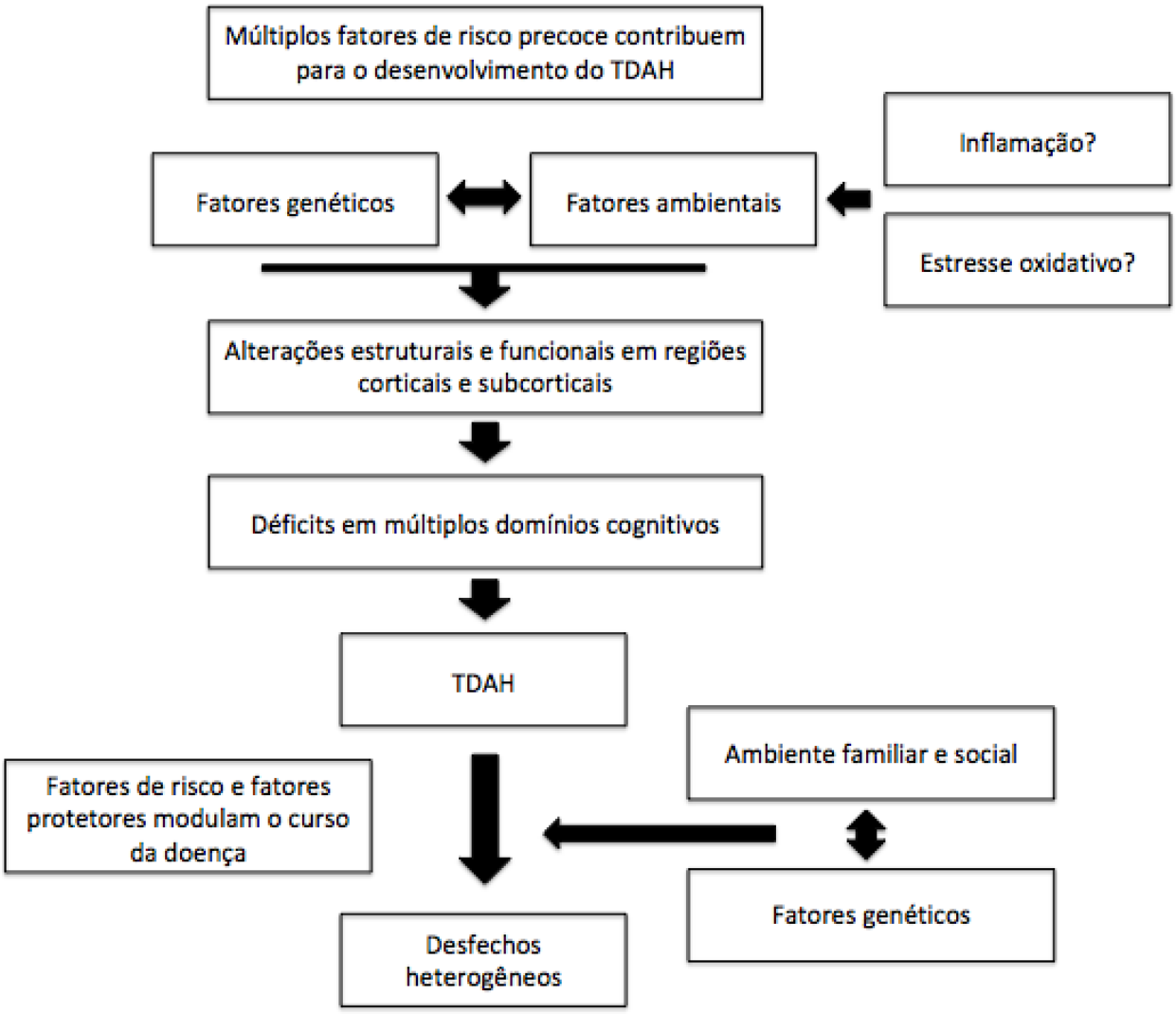

Figura 7. Marco conceitual do TDAH. Figura adaptada pelo autor de Thapar and Cooper [159].

IV. JUSTIFICATIVA

4.1 JUSTIFICATIVA

Tendo-se em vista que (1) TDAH é um transtorno prevalente do desenvolvimento cerebral, (2) o diagnóstico de TDAH é relacionado a diversos desfechos negativos na infância, adolescência e vida adulta – incluindo aumento da mortalidade, e (3) os tratamentos farmacológicos atualmente utilizados na prática clínica se caracterizam por baixa aderência em longo prazo, principalmente devido a efeitos adversos, há necessidade de estudos que busquem desenvolver alternativas terapêuticas eficazes, de baixo custo e com poucos efeitos adversos para pacientes com TDAH. Nesse sentido, a ETCC tem sido proposta como uma alternativa promissora para diversos transtornos neuropsiquiátricos devido a seu mecanismo de ação envolver a modulação do potencial de repouso neuronal em áreas cerebrais específicas. Estudos-piloto têm identificado a ETCC como um tratamento efetivo para o TDAH, apesar de, em sua maioria, terem sido conduzidos com um pequeno tamanho amostral e uma probabilidade razoável de erros sistemáticos. Apesar de promissor, o desenvolvimento dessa ferramenta para aplicação na prática clínica ainda depende de um melhor entendimento de seus mecanismos de ação e da realização de ensaios clínicos randomizados com melhor delineamento. Um melhor entendimento dos mecanismos de ação da ETCC está diretamente relacionado ao seu estudo em modelos animais, essenciais para o desenvolvimento de novas estratégias terapêuticas. No TDAH, os SHR são o modelo animal mais aceito pela comunidade científica internacional por este apresentar validade de face, preditiva e de constructo. Por esses motivos, justifica-se os uso dos SHR para melhor compreensão dos possíveis efeitos comportamentais da ETCC, e exploração dos mecanismos potencialmente relacionados a esses efeitos no TDAH.

V. OBJETIVOS

5. OBJETIVOS

5.1. Objetivo geral

O objetivo geral deste trabalho foi avaliar os efeitos da exposição repetida à ETCC em parâmetros comportamentais e neuroquímicos de animais SHR, um modelo animal de TDAH.

5.2. Objetivos específicos

5.2.1. Artigo I

- Investigar os efeitos da ETCC na locomoção em animais SHR e WKY;
- Investigar os efeitos da ETCC e memória de curta duração em animais SHR e WKY;
- Avaliar o efeito da ETCC em níveis de dopamina em diferentes regiões cerebrais em animais SHR e WKY;
- Avaliar o efeito da ETCC em níveis de BNDF em diferentes regiões cerebrais em animais SHR e WKY.

5.2.2. Artigo II

- Avaliar parâmetros de estresse oxidativo (níveis de 2′-7′-diclorofluoresceína diacetato e glutationa, e atividade das enzimas glutationa peroxidase, superóxido dismutase e catalase) em animais SHR e WKY;
- Avaliar parâmetros inflamatórios (TNF-α, IL-1β e IL- 10) em animais SHR e WKY.

5.2.3. Artigo III

- Investigar os efeitos da ETCC na memória de trabalho e memória de longa duração em animais SHR e WKY;
- Avaliar os efeitos da ETCC em parâmetros de estresse oxidativo (níveis de 2′-7′-diclorofluoresceína diacetato e glutationa, e atividade das enzimas glutationa peroxidase, superóxido dismutase e catalase) e parâmetros inflamatórios (TNF-α, IL-1β e IL- 10) em animais SHR e WKY.

5.2.4. Artigo IV

- Revisar as evidências sugerindo um papel da inflamação na fisiopatologia do TDAH.

VI. REFERÊNCIAS BIBLIOGRÁFICAS

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VII. ARTIGOS CIENTÍFICOS

Transcranial direct current stimulation improves short-term memory in an animal model of attention-deficit/hyperactivity disorder

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Transcranial direct current stimulation improves short-term memory in an animal model of Attention-Deficit/Hyperactivity Disorder

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Abstract

Attention deficit hyperactivity disorder (ADHD) is characterized by impairing levels of hyperactivity, impulsivity and inattention. However, different meta-analyses have reported disruptions in short and long-term memory in ADHD patients. Previous studies indicate that mnemonic dysfunctions might be the result of deficits in attentional circuits, probably due to ineffective dopaminergic modulation of hippocampal synaptic plasticity. In this study we aimed to evaluate the potential therapeutic effects of a neuromodulatory technique, transcranial direct current stimulation (tDCS), in short-term memory (STM) deficits presented by the spontaneous hypertensive rats (SHR), the most widely used animal model of ADHD. Adult male SHR and Wistar Kyoto rats (WKY) were subjected to a constant electrical current of 0.5 mA intensity applied on the frontal cortex for 20 min/day during 8 days. STM was evaluated with an object recognition test conducted in an open field. Exploration time and locomotion were recorded, and brain regions were dissected to determine dopamine and BDNF levels. SHR spent less time exploring the new object when compared to WKY, and tDCS improved object recognition deficits in SHR without affecting WKY performance. Locomotor activity was higher in SHR and it was not affected by tDCS. After stimulation, dopamine levels were increased in the hippocampus and striatum of both strains, while BDNF levels were increased only in the striatum of WKY. These findings suggest that tDCS on the frontal cortex might be able to improve STM deficits present in SHR, which is potentially related to dopaminergic neurotransmission in the hippocampus and striatum of those animals.

Keywords: ADHD; tDCS; SHR; memory; dopamine.

1. Introduction

Attention deficit hyperactivity disorder (ADHD) is a disorder characterized by hyperactivity, impulsivity and inattention (Biederman and Faraone, 2002). Its pathophysiology, although not fully understood, involves alterations in the dopaminergic transmission, mainly in the mesocorticolimbic system (Biederman and Faraone, 2002), together with reduced activation in fronto-basal ganglia networks (Hart et al., 2013).

Different meta-analyses have reported that ADHD patients have disruptions in short and long-term memory (Hervey et al., 2004; Skodzik et al., 2013). Studies have not identified hippocampal structural or functional changes in those patients (Ellison-Wright et al., 2008; Valera et al., 2007), even though this region is one of the most important for declarative memory consolidation (Kandel et al., 2014). Since the encoding of information is dependent on the activity of attentional circuits (Kentros et al., 2004; Muzzio et al., 2009), long-term deficits in ADHD might be related to attentional deficits (Skodzik et al., 2013). The influence of attention on the encoding of information is probably due to dopaminergic modulation of hippocampal synaptic plasticity (Kentros et al., 2004; Li et al., 2003; O'Carroll et al., 2006; Rossato et al., 2009), suggesting an involvement of the dopaminergic system in ADHD mnemonic problems.

The spontaneous hypertensive rats (SHR) are the most widely accepted animal model of ADHD (Sagvolden et al., 2005). They present dysregulations in fronto-basal ganglia connectivity that seems to be related to abnormal dopaminergic signaling (Sagvolden et al., 2005). These animals also present deficits in short and long-term memory (Meneses et al., 2011; Pandolfo et al., 2013; Pires et al., 2009; Prediger et al., 2005a, 2005b), both improved with modulation of dopaminergic systems. Mnemonic deficits in SHR support this lineage as a suitable model in the study of memory disruptions found in ADHD patients.

Transcranial direct current stimulation (tDCS) is a technique that consists of applying a weak, constant, low intensity electric current between two electrodes over the scalp in order to modulate cortical excitability (Nitsche and Paulus, 2000). Anodal stimulation increases neuronal activity while cathodal stimulation inhibits it (Nitsche and Paulus, 2000). Many researchers have applied tDCS on the prefrontal cortex (PFC) in humans, and this approach seems to influence a wide array of brain functions, like working (Fregni et al., 2005) and declarative memory (Manenti et al., 2013). Concerning ADHD, however, we are not aware of previous studies examining the effects of tDCS on cognitive functions.

Bearing in mind that ADHD pathophysiology may involve inadequate modulation of frontal circuits, and that tDCS is able to modulate cortical excitability, we investigated the effects of tDCS in an animal model of ADHD, the SHR rats. The aim of the present study was to evaluate whether repeated tDCS applications over the frontal cortex might modify aberrant short-term memory (STM) (Pires et al., 2009) and hyperlocomotion (Sagvolden et al., 2005) seem in SHR. Brain-derived neurotrophic factor (BDNF) and dopamine (DA) levels were quantified due to its importance for the encoding of information (Leal et al., 2014; Rossato et al., 2009). We expected an improvement in STM and a reduction in the locomotion, together with an increase in BDNF and DA mainly in the hippocampus.

2. Experimental procedures

2.1 Animals

Adults (60 days old) male WKY (n= 20) and SHR (n=28) rats, weighing 220-350 g, from our own colony were used. They were kept in groups of five animals per cage, maintained in a room under controlled temperature (22±2), on a standard 12-hour light/dark cycle. Animals had access to water and chow *ad libitum*. All experiments and procedures were approved by the Institutional Committee for Animal Care and Use (GPPG-HCPA

protocol No. 14-0103) and performed in accordance with the Guide for the Care and Use of Laboratory Animals 8th edition (2011). The maintenance of the animals followed the law 11.794 (Brazil), which establishes procedures for the scientific use of animals. The experiment used the number of animals necessary to produce reliable scientific data.

2.2 Experimental Design

Rats were habituated to the maintenance room for 1 week before the experiment started. After this period, animals from each lineage (WKY and SHR rats) were randomly allocated in three experimental groups: for the control group (C, n=6-9), rats had no intervention; for the tDCS sham group (tS, n=6-9), animals were restrained during 20 minutes, electrodes were positioned on the head but the stimulator was turned off; and for the tDCS active group (tA, n= 7-10), animals were restrained, electrodes were positioned on the head and the stimulator was turned on for 20 minutes.

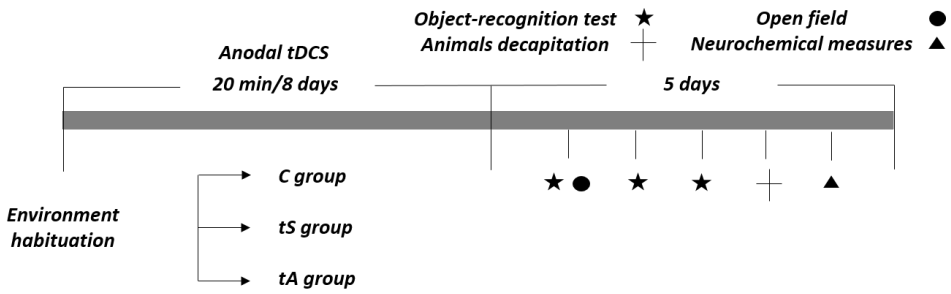


Fig. 1. Experimental design.

2.3 Transcranial direct current stimulation (tDCS)

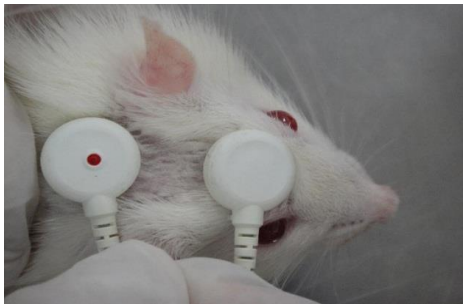
Animals from tA group were subjected to a constant electrical current applied by a battery-driven stimulator designed for continuous application of low currents to small mammals. ECG electrodes (1.5 cm^2) with a conductive adhesive hydrogel were used. Before

application, the animals had their heads shaved for better adherence. The electrodes were placed against the skin, fixed with adhesive tape and covered with a protective mesh to prevent removal. This protocol was in accordance to previous animal studies from our group [160, 161].

The center of the anodal electrode was placed in the midpoint of the lateral angle of the eyes in order to stimulate the frontal cortex, and the cathodal electrode was positioned from the neck to shoulder areas, as described by Takano, Yokawa [162]. A constant current of 0.5 mA intensity was applied for 20 min/day during 8 days. Since a current density higher than 142.9 A/m^2 is associated with brain lesions [163], we used stimulation parameters that resulted in a current density of 33.4 A/m^2. The intensity and period of stimulation were used in previous animal studies [160, 161] and produced behavioral and neurochemical effects in rats.

In order to deliver the current, animals had to be immobilized with the use of a cloth for the total time of stimulation. Animals from tS group were subjected to sham stimulation where electrodes were placed in the same sites as for real stimulation, however they were not connected to the battery. The animals were also immobilized in order to reproduce tA group's procedure. Since animals from tA and tS groups were immobilized during the stimulation and this could interfere with the behavioral measures [164], we used a control group C who were only manipulated for 2 min daily in order to prevent a greater stress response during the behavioral tests. 24 hours after the last stimulation, animals were subjected to the behavioral tests.

Panel A



Panel B

Fig. 2. Setup for the tDCS treatment. A) Location of electrodes on the rat scalp during tDCS sessions. The center of the anodal electrode was placed in the middle region between the animal's eyes, a region right above the frontal cortex. The cathodal electrode had its center positioned between the ears of the animal. B) Experimental setup for tDCS treatment. Animals were immobilized, and the tDCS-stimulator was placed onto the ventral thorax and held by a corset. Afterward, rats received a 20-min electrical stimulation session.

2.4 Object recognition test

The object recognition test was used to measure short-term memory. It was conducted in an open field made of white-painted wood measuring 60x40x50 cm. The floor was divided into 12 squares of 13x13 cm delimited by dark lines. The protocol is based on the differential exploration of familiar and new objects by rats (as described by Ennaceur, Cavoy [165]). It consists of three phases: habituation, sample and discrimination [109]. The habituation phase was done 24 hours after the last stimulation. In this phase the animals were allowed to freely

explore the open field on two consecutive days during 10 min in each day. In the sample phase, 24 hours later, two identical objects (O1 and O2) in the shape of a cube were positioned in the open field, 15 cm away from the walls and approximately 20 cm from each other. The animals were allowed to explore the objects for 3 min. After this time, the animals were returned for their home cage and the apparatus was cleaned. In the discrimination phase, after a delay period of 30 min, an identical copy of the familiar object (O3) was positioned in the open field together with a new T-shaped object (T), in the same locations previously occupied by O1 and O2. The rats were allowed to freely explore the object for another 3 min. The T-shaped object was placed in different sides of the open field for each trial to avoid bias. The experiment was conducted in a sound-attenuated room under low-intensity light (12 lx).

Exploration of an object was defined as directing the nose to the object at a distance of equal to or less than 2 cm or touching it with the nose. Data was analyzed as total time exploring the identical objects (O1+O2) in the sample phase, and discrimination index, defined by the difference in exploration time between the novel and the familiar objects, divided by the total time exploring these two objects in the discrimination phase (T-O3)/(T+O3). Discrimination index was used as a measure of short-term memory. The objects were constructed using plastic LEGO blocks (São Paulo, SP, Brazil), and their shapes were based on a previous study [109].

2.5 Spontaneous locomotor activity

Exploratory and locomotor activities were assessed during the first day of habituation for the object recognition test. The number of transitions between squares was analyzed during 10 min. The animal was recorded as entering a new area when all four paws crossed to an adjacent square. The apparatus was cleaned between trials.

2.6 Dopamine and BDNF measurements

Animals were killed 24 hours after the discrimination phase of the object recognition test, 4 days after the last stimulation, and the hippocampus, striatum, PFC and brainstem were collected and frozen at −80°C until time of testing. The PFC, brainstem and striatum were evaluated due to its participation in the mesocorticolimbic system [166], and in the hippocampus due to its role in declarative memory formation [167]. DA levels were determined by ELISA kit, according to the manufacturer's instructions (MyBioSources #MBS7214676). BDNF levels were determined by sandwich-ELISA using monoclonal antibodies specific for BDNF (R&D Systems, Minneapolis, United States #DY248). Total protein was measured by Bradford's method using bovine serum albumin as standard.

2.7 Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). The statistical evaluation of the results was carried out using two-way analysis of variance (ANOVA) with strain and treatment as independent variables. Post-hoc comparisons were performed with Bonferroni test after significant ANOVAs. Correlations were analyzed with Person *r* correlation coefficient. P values less than 0.05 was reported as statistically significant. SPSS 19.0 for Windows was used for statistical analysis.

3. Results

3.1 Object recognition test

We tested the effects of tDCS on STM of adult male WKY and SHR rats measured in the object recognition test. Figure 3 shows the results of the sample and discrimination phase. Two-way ANOVA analysis revealed no significant effect on investigation time for strain [F (2, 42) = 3.145, p=0.08], treatment [F (2, 42) = 0.142, p=0.86] or interaction between strain and treatment [F (2, 42) = 0.762, p=0.47]. In the discrimination phase, a significant effect on discrimination index for interaction between strain and treatment [F (2, 42) = 4.263,

p=0.021], and strain [F (2, 42) = 4.067, p=0.05] was observed, but not for treatment [F (2, 42) = 2.878, p=0.06]. Subsequent Bonferroni´s post-hoc test indicated that SHR control have a lower discrimination index than the WKY from the same treatment ($p<0.05$). SHR subjected to tDCS treatment seems to have an increased discrimination index when compared to both SHR control and SHR tDCS sham ($p<0.001$ and $p<0.05$, respectively). Difference between SHR rats from sham tDCS group and WKY rats from the same group almost reach significance, with a p=0.052.

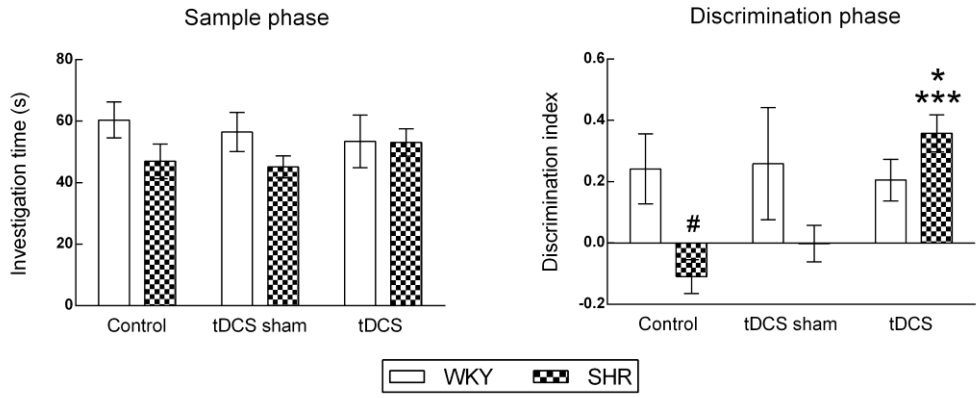


Fig. 3. Effects of tDCS on the performance of adult spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY) in the object recognition test. After habituation in an open field for two days, animals were exposed to two identical objects (O1 and O2, cube-shaped) during the sample phase and 30 min after to a familiar (O3, cube-shaped) and a new object (T, T-shaped) in the discrimination phase. Investigation time (s) was calculated by the sum of time that the animals spent investigating O1 and O2. Discrimination index was calculated by the time that animals spent investigating (T-O3)/(T+O3). Bars represent the means ± SEM of animals grouped according to treatment and strain (n = 6-10). * $p<0.05$ compared to sham from the same lineage. *** $p<0.001$ compared to control from the same lineage. # $p<0.05$ compared to WKY from the same treatment (Bonferroni´s post-hoc test).

3.2 Effects of tDCS on the spontaneous locomotor activity of adult male WKY and SHR rats

Locomotor activity (Fig. 4) was evaluated during first day of habituation for the object recognition test. Two-way ANOVA analysis revealed significant effect for strain [$F(2, 42) = 27.110$, $p<0.0001$] and no significant effect for treatment [$F(2, 42) = 0.696$, $p=0.50$] or interaction between strain and treatment [$F(2, 42) = 0.340$, $p=0.71$]. Subsequent Bonferroni´s post-hoc test indicated that SHR rats from all groups (control, tDCS sham and tDCS) crossed significantly more squares than the WKY from the same treatment ($p<0.01$, $p<0.001$ and $p<0.05$, respectively).

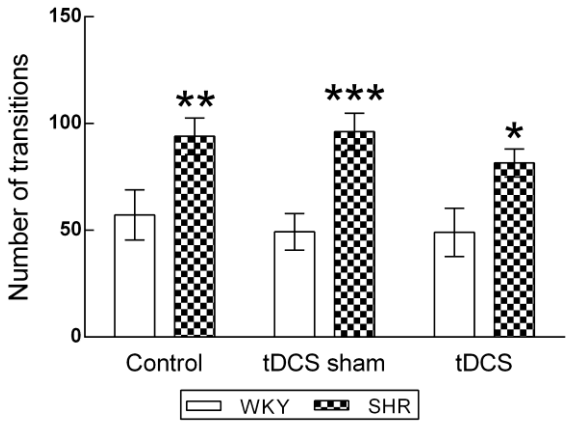


Fig. 4. Effects of tDCS on the number of transitions of adult spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY) in the open field (10 min). Bars represent the means ± SEM of animals grouped according to treatment and strain (n = 6-10). * $p<0.05$ compared to WKY from the same treatment. ** $p<0.01$ compared to WKY from the same treatment. *** $p<0.001$ compared to WKY from the same treatment (Bonferroni´s post-hoc test).

3.3 Dopamine levels

DA levels were quantified due to its important role in ADHD [166] and memory formation [168-170]. Figure 5 shows the effect of tDCS on DA levels. Two-way ANOVA analysis revealed significant differences in the hippocampus and striatum, but not in the PFC or brainstem. In the hippocampus there was a significant effect for treatment [$F(2, 36) = 7.608$, $p=0.002$], and no significant effect for the interaction between treatment and strain [$F(2, 36) = 1.273$, $p=0.292$] or strain [$F(2, 36) = 2.042$, $p=0.162$]. Post-hoc analysis showed that WKY rats from tDCS group have increased DA levels compared to WKY from control group ($p<0.05$, Bonferroni´s *post-hoc* test). Also, SHR rats from tDCS group have increased DA levels compared to SHR from tDCS sham group ($p<0.05$, Bonferroni´s *post-hoc* test).

In the striatum there was a significant effect for treatment [$F(2, 36) = 18.198$, $p<0.001$], and no significant effect for the interaction between treatment and strain [$F(2, 36) = 0.222$, $p=0.802$] or strain [$F(2, 36) = 1.052$, $p=0.312$]. WKY rats from tDCS group have increased DA levels compared to WKY from control and tDCS sham groups ($p<0.01$ for both, Bonferroni´s *post-hoc* test). SHR rats from tDCS group have increased DA levels compared to SHR from control and tDCS sham groups ($p<0.001$ for both, Bonferroni´s post-hoc test).

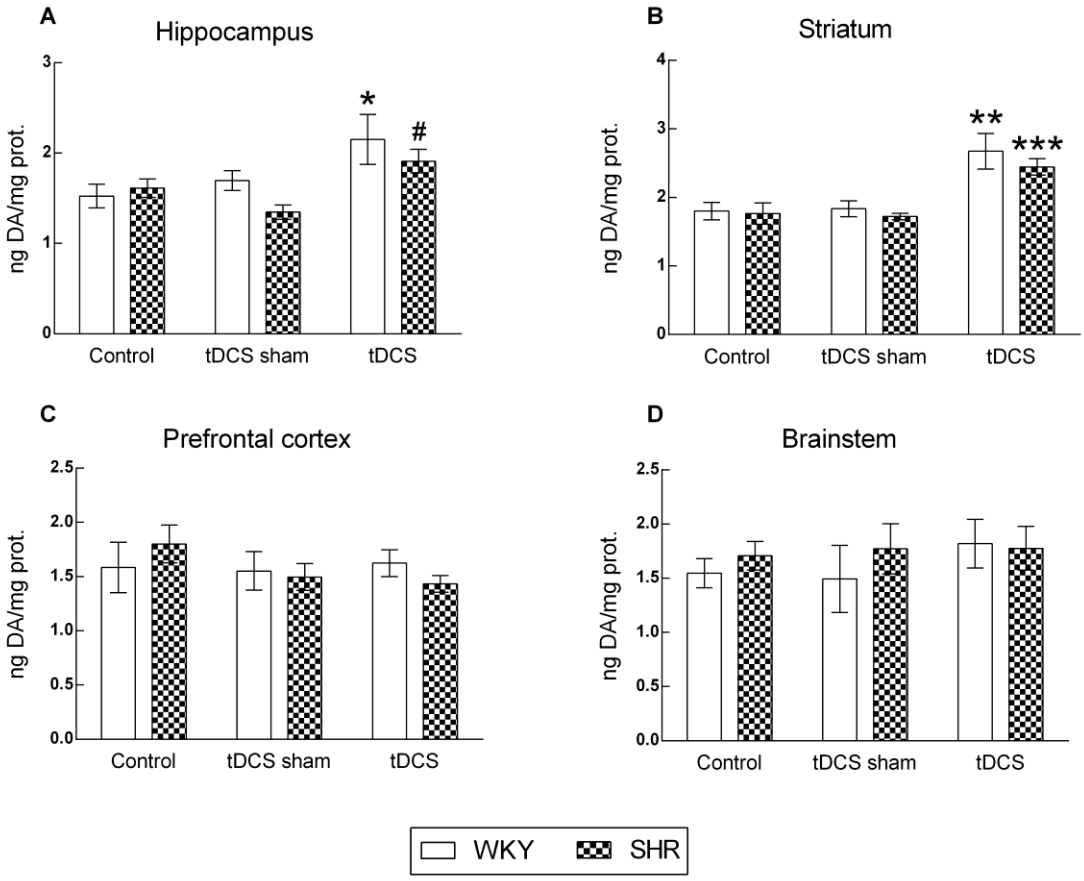


Fig. 5. Effects of tDCS on DA levels in the hippocampus (A), striatum (B), PFC (C) and brainstem (D) of adult spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY). The number of animals analyzed was 6 in the WKY control group, 5 in both the WKY tDCS sham and tDCS groups, 9 in the SHR control group, 7 in the SHR tDCS sham group and 10 in the SHR tDCS group. Bars represent the means ± SEM of animals grouped according to treatment and strain (n = 5-10). * $p<0.05$ compared to control from the same lineage. # $p<0.05$ compared to sham from the same lineage. ** $p<0.01$ compared to control and tDCS sham from the same lineage. *** $p<0.001$ compared to control and tDCS sham from the same lineage (Bonferroni´s post-hoc test).

3.4 BDNF levels

Since BDNF is an important regulator of synaptic plasticity and memory formation [171], its levels were quantified. As shown in figure 6, two-way ANOVA analysis revealed a significant difference in the striatum, but not in the hippocampus, PFC or brainstem. In the striatum there was a significant effect for the interaction between treatment and strain [$F(2, 36) = 12.604$, $p<0.001$], treatment [$F(2, 36) = 7.350$, $p=0.002$] and strain [$F(2, 36) = 5.397$, $p=0.026$]. WKY rats from tDCS group have increased levels of BDNF in the striatum when compared to WKY from control and tDCS sham groups ($p<0.001$ for both, Bonferroni´s post-hoc test), and increased levels of BDNF when compared to SHR from the same group ($p<0.001$, Bonferroni´s post-hoc test).

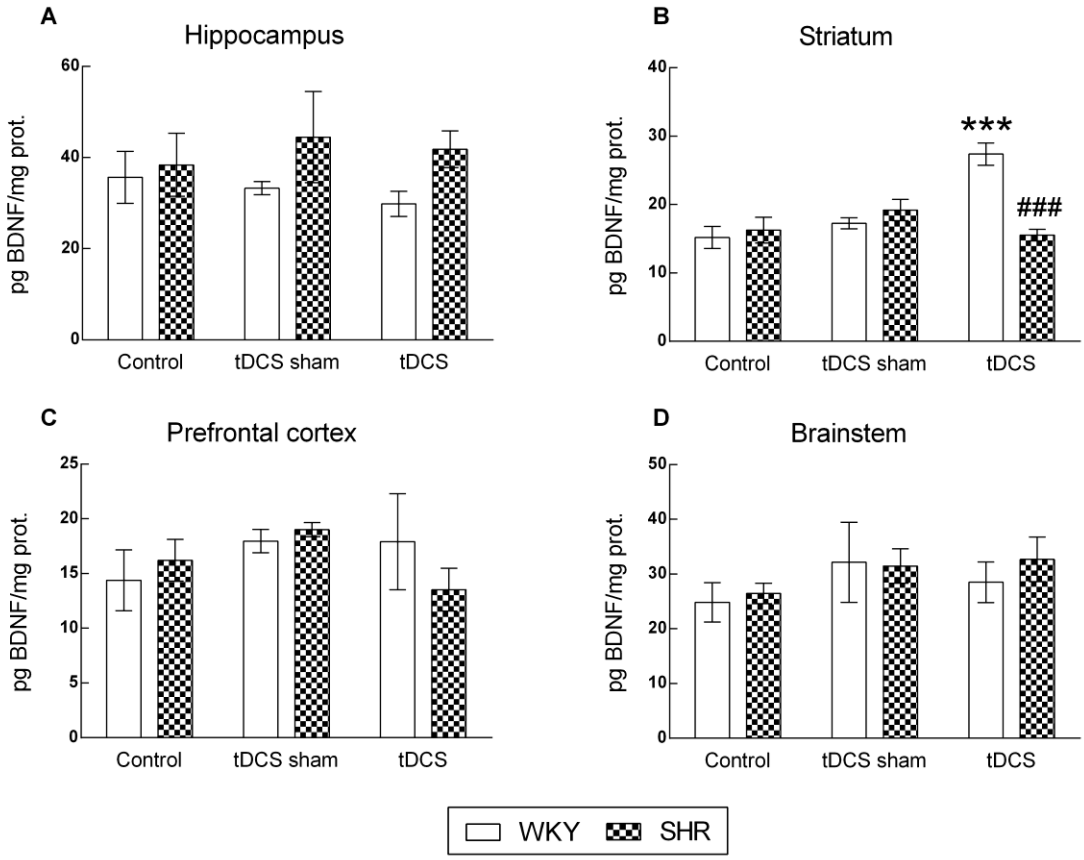


Fig. 6. Effects of tDCS on the BDNF levels in the hippocampus (A), striatum (B), PFC (C) and brainstem (D) of adult spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY). The number of animals analyzed was 6 in the WKY control group, 5 in both the

WKY tDCS sham and tDCS groups, 9 in the SHR control group, 7 in the SHR tDCS sham group and 10 in the SHR tDCS group. Bars represent the means ± SEM of animals grouped according to treatment and strain (n = 5-10). *** p<0.001 compared to control from the same lineage. ### p<0.001 compared to WKY from the same treatment (Bonferroni´s post-hoc test).

3.5 Correlations

In order to evaluate a possible relation between DA and BDNF levels in the hippocampus and discrimination index from the object recognition test, correlations between the values were performed. Values from the 3 treatments were analyzed together for each strain. As shown in figure 7, DA levels correlated positively with discrimination index in the SHR (r = 0.44, p = 0.024), but not in the WKY (r = -0.21, p = 0.41). BDNF levels did not correlate with discrimination index neither in SHR (r = 0.12, p = 0.54) nor in WKY (r = -0.038, p = 0.88).

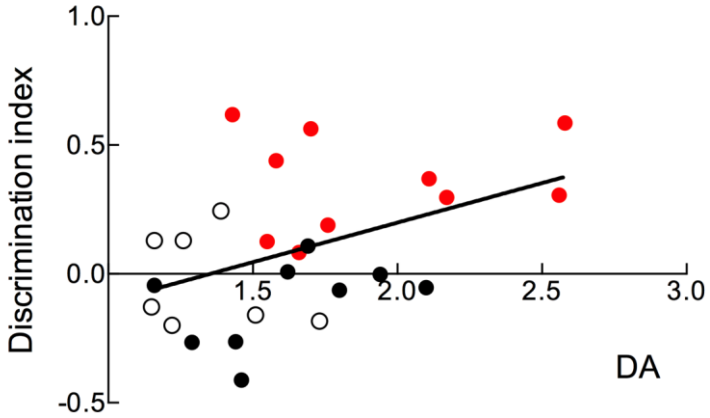


Fig. 7. Correlations between discrimination index and dopamine levels (DA) in the hippocampus of adult spontaneously hypertensive rats (r = 0.44, p = 0.024). Values from the 3 treatments (control, tDCS sham and tDCS) were analyzed together.

4. Discussion

In this study about the effects of tDCS in the SHR, an animal model of ADHD, the most significant positive result was that this treatment was able to revert STM deficits presented in the SHR. These same deficits had already been previously published by Pires, Pamplona [109], which, additionally, showed that a systemic unique administration of methylphenidate [MFD, a catecholamine reuptake inhibitor used as first choice in the treatment of ADHD [172]] improves STM function in SHR. In addition, STM deficits presented by SHR have been observed using different behavioral tests [123, 173]. STM is a memory system responsible for information maintenance while the long-term formation is not yet completed [174]. Nowadays, it is known to be a parallel system with long-term memory (LTM), and not a pre step of it [174]. It has been shown that SHR present deficits in both types of memory, with improvements after modulation of dopaminergic system [99, 109]. Different evidences have pointed out that tDCS might be able to improve cognitive functions in healthy humans [175] and in healthy animal models [176], but it had no observed effects on our control strain, the WKY. However, since previous studies in healthy subjects assessed mainly working memory and long-term memory, it could be the case that short-term memory is differently influenced by tDCS. It is important to bear in mind that the object recognition test also might not be sensible enough to detect memory improvements in healthy controls. We chose to use animals entering adult life (60 days old), and it is known that they begin displaying vascular changes after this period [177]. However, their degree of cognitive impairment seems not to change as they pass from adolescence to adulthood [123, 178].

Another important finding of this study was that tDCS increased DA levels in the hippocampus and striatum of SHR. As mentioned above, dopaminergic system is broadly involved in the pathology of ADHD [166]. The mesocorticolimbic dopaminergic system is involved in both emotional and cognitive functions. It is important in reward pathways [179] and also in executive functions like attention, planning and behavioral flexibility [180].

Rossato, Bevilaqua [168] described that DA in the hippocampus is necessary for the maintenance of LTM storage in a BDNF-dependent way. In a similar way, it was reported that STM is also dependent on dopaminergic inputs to the hippocampus [181]. Overall, the importance of dopaminergic circuits for memory formation is corroborated by diverse studies [169, 170, 182], and it was also shown that a systemic and a prelimbic administration with a D1-receptor antagonist impaired discrimination index in a dose-dependent way in the object recognition test [183]. DA seems to facilitate the induction of activity-dependent increases in glutamatergic transmission, or long-term potentiation (LTP), in hippocampal synapses [169], which is known to be part of the molecular processes involved in memory formation [167]. Interestingly, modulation of LTP at rat hippocampal synapses after DCS has been shown using brain slices [184], which suggests a mechanism for memory improvement. DA release in the hippocampus, after the stimulation, may be modulating LTP and, consequently, memory formation. It has been suggested that DA modulation is the result of attentional processes [169, 182, 185], which could explain memory deficits in ADHD, having in mind that the disorder is characterized by lack of attention [185]. Even though the increase in DA levels found in the hippocampus of SHR might be related to improvement in STM, basal DA levels did not differ between control groups. Also, WKY presented an increase in DA levels in the hippocampus without any change in STM. In conclusion, these findings do not present an evidence for a causal relationship between STM improvement and DA increased levels, indicating that STM improvement in the SHR probably involves other neurotransmitters and/or pathways than just DA, or that the behavior test used had no sufficient sensitivity to detect an eventual difference in the WKY, as mentioned before.

DA levels were increased also in the striatum of both strains. This finding is particularly relevant since it has been repeatedly shown that Transcranial Magnetic Stimulation (TMS), which is another non-invasive neuromodulatory technique, is able to

increase DA levels in the striatum of rats when applied in the frontal lobes [186-189]. With tDCS, Tanaka, Takano [190] also observed an increase in extracellular level of DA in the striatum of rats. Besides that, Takano, Yokawa [162] showed that tDCS application on the frontal cortex of rats is able to increase neuronal activation in the same structure, which could be related to dopaminergic modulation. Our results support the hypothesis that tDCS over the frontal cortex might influence dopaminergic transmission in the striatum. Surprisingly, we did not find differences in DA levels among controls WKY and SHR even though studies have found dysregulations in the dopaminergic system of SHR when compared to other lineages [13]. In the literature, the most consistent alterations involve mainly DA transporter and DA receptor D1, both found to be hyper expressed in striatum and PFC of SHR [191, 192]. Although expression of receptors and transporters may not be directly translated into neurotransmitter levels when quantified by ELISA, this apparent conflicting result can also be explained by different experimental setups or age of the animals.

In this study we showed that tDCS was able to increase BDNF levels in the striatum of WKY. However, there is no effect of tDCS on the other brain structures, and no difference in basal levels between SHR and WKY. It has been shown that DA regulates BDNF expression in the striatum [193], and since BDNF is released in an activity-dependent manner [194], increased neuronal activation in the striatum with tDCS [162] may explain the result found in the WKY. Curiously, the SHR did not present the same improvement, which could be the result of the abnormal dopaminergic neurotransmission seen in this strain. The findings of the current study are consistent with the literature in respect of increased locomotor activity seen in the SHR when analyzed in the open field test [90]. Nevertheless, tDCS was not able to change the aberrant hyper locomotion observed in SHR.

As far as we know, we are the first to examine tDCS effects in an animal model of ADHD, and the major strength of this study is its novelty and originality. Nevertheless, there

are some limitations that must be pointed. First of all, in order to apply the tDCS, animals had to be immobilized during 20 min daily for 8 days, and it has been shown that this protocol can alter different brain functions [195]. In order to control for this intervention we used a sham group that was restrained the same way as the active group, and a control group that was not subjected to manipulations. Another factor that must be stressed is that the locomotor activity and the discrimination phase of the object recognition test were assessed 1 and 3 days after the last stimulation, respectively. Besides that, neurochemical analyses were done 4 days after the last stimulation. Even though this delay period might have influenced results of the measurements that were done, we have evidence that tDCS have effects for at least 3 weeks after the last stimulation [196], so we believe that our design was not a confounder in the analysis.

While we evaluated DA and BDNF levels in striatum and hippocampus using the whole structures, it is known that both of them have anatomical and functional distinctions. For instance, dorsomedial and ventral parts of the striatum are more related to cognitive and emotional circuits, respectively, while dorsolateral parts are more concerned with motor functions [197, 198]. In addition, the ventral hippocampus is more associated with emotional aspects of memory and cognition, whereas the dorsal aspect appears to be more important for encoding of spatial memory [199, 200]. The absence of these anatomical differentiation in structures should be taken in consideration while interpreting the results. Also, it is important to note that the increase in DA levels in both structures was found in total tissue, which do not necessarily corresponds linearly to DA extracellular concentration. Moreover, the expression of receptors and transporters in the cell membrane seems to be more important than the extracellular or intracellular neurotransmitter content in order to influence synaptic connectivity. For instance, Pandolfo showed that SHR presents increased dopamine transporter density and increased dopamine uptake in frontocortical and striatal terminals

when compared to WKY, which seems to be related to deficits in attention and spatial recognition. Furthermore, decreased performance presented by SHR in the object recognition test do not encompass STM deficits throughout its range, and additional behavioral tests to assess STM in different context are important to evaluate tDCS effects. The fact that tDCS might be affecting other factors influencing their performance in this test, like sensory integrative systems [201], should also be considered. It is true that in this paper we have not administered MFD together with the stimulation. This would be the subject of an entirely worthwhile research project, since it has been shown that in depression there seems to be a synergic effect of tDCS and sertraline [202], which could also be the case in ADHD.

In conclusion, this paper has presented a new approach for the treatment of memory deficits in an animal model of ADHD, the SHR. In short, the results of this study support the hypothesis that tDCS might have a beneficial effect on STM deficits in ADHD. Besides, it has pointed out a possible influence of dopaminergic modulation on the observed effects. This research provides a framework for the exploration of the behavioral and neurochemical effects of tDCS in an animal model of ADHD. Further research to determine the mechanisms underlying the effects shown in this study could benefit both our understanding of the pathophysiology of ADHD and the neurobiological substrates of tDCS, and also may help to develop a new therapeutic tool to memory deficits in ADHD.

Conflicts of interest

L. A. Rohde was on the speakers' bureau/advisory board and/or acted as consultant for Eli-Lilly, Janssen-Cilag, Novartis and Shire in the past 3 years. The ADHD and Juvenile Bipolar Disorder Outpatient Programs chaired by L. A. Rohde received unrestricted educational and research support from the following pharmaceutical companies in the past 3 years: Eli-Lilly, Janssen-Cilag, Novartis and Shire. He receives authorship royalties from

Oxford Press and ArtMed. He has also received travel awards for taking part in the 2014 APA meeting from Shire. E. H. Grevet was on the speakers' bureau for Novartis and Shire in the past 3 years. He has also received travel awards for taking part in the 2014 ADHD World Federation meeting from Shire. The other authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Contributors

Mr. Leffa designed the study, managed literature searches, performed the tDCS stimulation, behavior experiments and statistical analyses, participated in the interpretation of data, and wrote the first draft of the manuscript. Dr. Torres and Dr. Rohde contributed to study design, literature searches, statistical analysis, interpretation of data, and to writing the article. Dr. Grevet participated in the study design, data interpretation and in writing the manuscript. Dr. Caumo and Dr. Onofre de Souza collaborated in the writing of the manuscript. Dr. de Souza, Dr. Medeiros, Ms. Scarabelot, and Ms. de Oliveira contributed to tDCS stimulation, behavior and neurochemical experiments, and interpretation of data. All authors contributed to and have approved the final manuscript.

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Study sponsors had no role in study design, in the collection, analysis and interpretation of data, in the writing of the report, and in the decision to submit the paper for publication.

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Increased oxidative parameters and decreased cytokine levels in an animal model of attention-deficit/hyperactivity disorder

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Increased oxidative parameters and decreased cytokine levels in an animal model of attention-deficit/hyperactivity disorder

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Abstract

Attention-deficit/hyperactivity disorder (ADHD) is a highly heterogeneous disorder characterized by impairing levels of hyperactivity, impulsivity and inattention. Oxidative and inflammatory parameters have been recognized among its multiple predisposing pathways, and clinical studies indicate that ADHD patients have increased oxidative stress. In this study, we aimed to evaluate oxidative (DCFH oxidation, glutathione levels, glutathione peroxidase, catalase and superoxide dismutase activities) and inflammatory (TNF-α, IL-1β and IL-10) parameters in the most widely accepted animal model of ADHD, the spontaneously hypertensive rats (SHR). Prefrontal cortex, cortex (remaining regions), striatum and hippocampus of adult male SHR and Wistar Kyoto rats were studied. SHR presented increased reactive oxygen species (ROS) production in the cortex, striatum and hippocampus. In SHR, glutathione peroxidase activity was decreased in the prefrontal cortex and hippocampus. TNF-α levels were reduced in the prefrontal cortex, cortex (remaining regions), hippocampus and striatum of SHR. Besides, IL-1β and IL-10 levels were decreased in the cortex of the ADHD model. Results indicate that SHR presented an oxidative profile that is characterized by an increase in ROS production without an effective antioxidant counterbalance. In addition, this strain showed a decrease in cytokine levels, mainly TNF-α, indicating a basal deficit. These results may present a new approach to the cognitive disturbances seen in the SHR.

Keywords: ADHD; SHR; Oxidative stress; Cytokines

1. Introduction

Attention-deficit/hyperactivity disorder (ADHD) is characterized by impairing levels of hyperactivity, impulsivity and inattention [1]. Its prevalence is estimated to be 5.3% in children and adolescents [2] and 2.5% in adults [3]. Although a common disorder, its pathophysiology is not completely understood. Studies on neuroimaging, cognition and biochemical assessment have been delineating ADHD as a heterogeneous disorder with a multifactorial causation [1].

Among the multiple pathways predisposing to ADHD phenotype, oxidative parameters have been increasingly investigated. There is a growing body of literature indicating that an increase in oxidative markers might be related with the pathophysiology of psychiatric disorders as cause and/or consequence of abnormal brain signaling [4]. In ADHD, a recent meta-analysis including six studies with a total of 231 patients showed that this population have increased oxidative status, supposedly leading to increase oxidative damage [5].

Patient´s cytokine profile has also been implicated in psychiatric disorders. Studies have shown increased levels of pro-inflammatory cytokines in depression [6], schizophrenia [7], bipolar disorder [8], and post-traumatic stress disorder [9]. In ADHD, their role is still less clear. It has been shown that elevations in inflammatory markers in preterm infants are associated with attention problems at the age of 2 years [10]. Also, Donfrancesco, Nativio [11] found increased levels of interleukin (IL)-6 and IL-10 in ADHD children who were positive to Purkinje cell antibodies. On the other hand, Oades, Dauvermann [12] observed no clear differences in cytokine levels between ADHD children and controls.

The spontaneously hypertensive rats (SHR) are the most widely accepted animal model of ADHD, presenting symptoms of inattention, hyperactivity and impulsivity [13].

They are derived from progenitor Wistar Kyoto rats (WKY), thus presenting a common background with this strain [14]. Bearing in mind the relation between oxidative stress and cytokine profile with psychiatric disorders, the aim of the present study was to evaluate: (1) pro-inflammatory response [tumor necrosis factor alpha (TNF-α) and IL-1β levels]; (2) anti-inflammatory response (IL-10 levels); (3) intracellular reactive oxygen species (ROS) production (DCFH oxidation); (4) antioxidant molecule [glutathione (GSH) levels]; and (5) antioxidant enzymatic defense activities [glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) activities] in the SHR. We expected increased oxidative stress and cytokine levels in the animal model of ADHD.

2. Materials and methods

2.1. Animals

Adults (90 days old) male SHR (n=7) and their control, the WKY (n=6), from our own colony were used. Animals were kept in groups of four animal per cage, maintained in a room under controlled temperature (22±2°C), on a standard 12 h light/dark cycle, and had access to water and chow ad libitum. Animals were killed by decapitation and the prefrontal cortex, cortex (remaining regions), striatum and hippocampus were dissected. All the procedures were approved by the Institutional Committee for Animal Care and Use (GPPG-HCPA protocol n. 14-0103) and performed in accordance with the Guide for the Care and Use of Laboratory Animals 8th edition (2011). The maintenance of the animals followed the law 11.794 (Brazil), which establishes procedures for the scientific use of animals. Vigorous attempts were made to minimize animal suffering and decrease external sources of pain and discomfort, as well as to use the minimum number of animals necessary to produce reliable scientific data.

2.2. Oxidative measurements

2.2.1. DCFH oxidation

ROS production was detected using 2′-7′-dichorofluorescein diacetate (DCFH-DA). Cerebral tissues were treated with DCFH-DA at a concentration of 10 μM for 30 min at 37°C. Following DCFH-DA exposure, the homogenates were placed into phosphate-buffered saline with 0.2% Triton X-100. Fluorescence was measured in a plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm. Results were expressed as percentages relative to WKY.

2.2.2. Glutathione levels

GSH levels were assessed as described previously [15]. Cerebral tissues were homogenized in a sodium phosphate buffer with 140-mM KCl and were diluted with a 100-mM sodium phosphate buffer (pH 8.0) containing 5-mM EDTA, and the protein was precipitated with 1.7% meta-phosphoric acid. The supernatant was assayed with o-phthaldialdehyde (at a concentration of 1 mg/ml methanol) at 22°C for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard GSH solutions at concentrations ranging from 0 to 500 μM. Results were expressed in nmol/mg protein.

2.2.3. Glutathione peroxidase activity

GPx activity was measured using the RANSEL kit from Randox (Autrim, UK). The GPx activity in tissue homogenate was assessed by measuring the absorption of NADPH at 340 nm. Results were expressed as UI/mg protein.

2.2.4. Superoxide dismutase activity

SOD activity was determined using the RANSOD kit from Randox (Autrim, UK). SOD activity in tissue homogenate was assayed spectrophotometrically at 505 nm. Results were expressed as UI/mg protein.

2.2.5. Catalase activity

CAT activity was assayed by the method described previously [16]. The decrease absorbance at 240 nm was measured in tissue homogenate suspended in a reaction medium containing 20 mM H2O2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and 50 μg protein. Results were expressed as UI/mg protein.

2.3. Cytokine measurements

TNF-α, IL-1β and IL-10 levels were measured in an extracellular medium using commercial ELISA kits from Peprotech for TNF-α, and from Thermo Fisher Scientific for IL-1β and IL-10. The average minimum sensitivity of the ELISA kit detection is 0.4 ng/ml of cytokines. Results were expressed as pg/mg tissue.

2.4. Statistical analyzes

Data were expressed as mean ± standard error of the mean (SEM). All statistical analyzes were carried out using Student´s t test. All data were normally distributed. P values less than 0.05 were reported as statistically significant. P values less than 0.07 were reported as trends. SPSS 19.0 for Windows was used for statistical analyzes.

3. Results

3.1. Oxidative parameters

3.1.1. DFCH oxidation

Intracellular ROS production in SHR (n=7) and WKY (n=6) was measured with DCFH oxidation. Results are presented in Fig. 1. SHR presented increased DCFH oxidation in the cortex ($t(11)=2.8$, $p=0.01$), striatum ($t(11)=3.51$, $p=0.004$), and hippocampus ($t(11)=2.97$, $p=0.01$), and no difference in the prefrontal cortex ($t(11)=1.23$, $p=0.24$).

3.1.2. Glutathione levels

The antioxidant molecule GSH was measured in SHR (n=7) and WKY (n=6). As shown in Fig 1, there was no difference in GSH levels between SHR and WKY in the prefrontal cortex ($t(11)=1.29$, $p=0.22$), cortex ($t(11)=0.85$, $p=0.41$), striatum ($t(11)=0.02$, $p=0.97$), and hippocampus ($t(11)=1.17$, $p=0.26$).

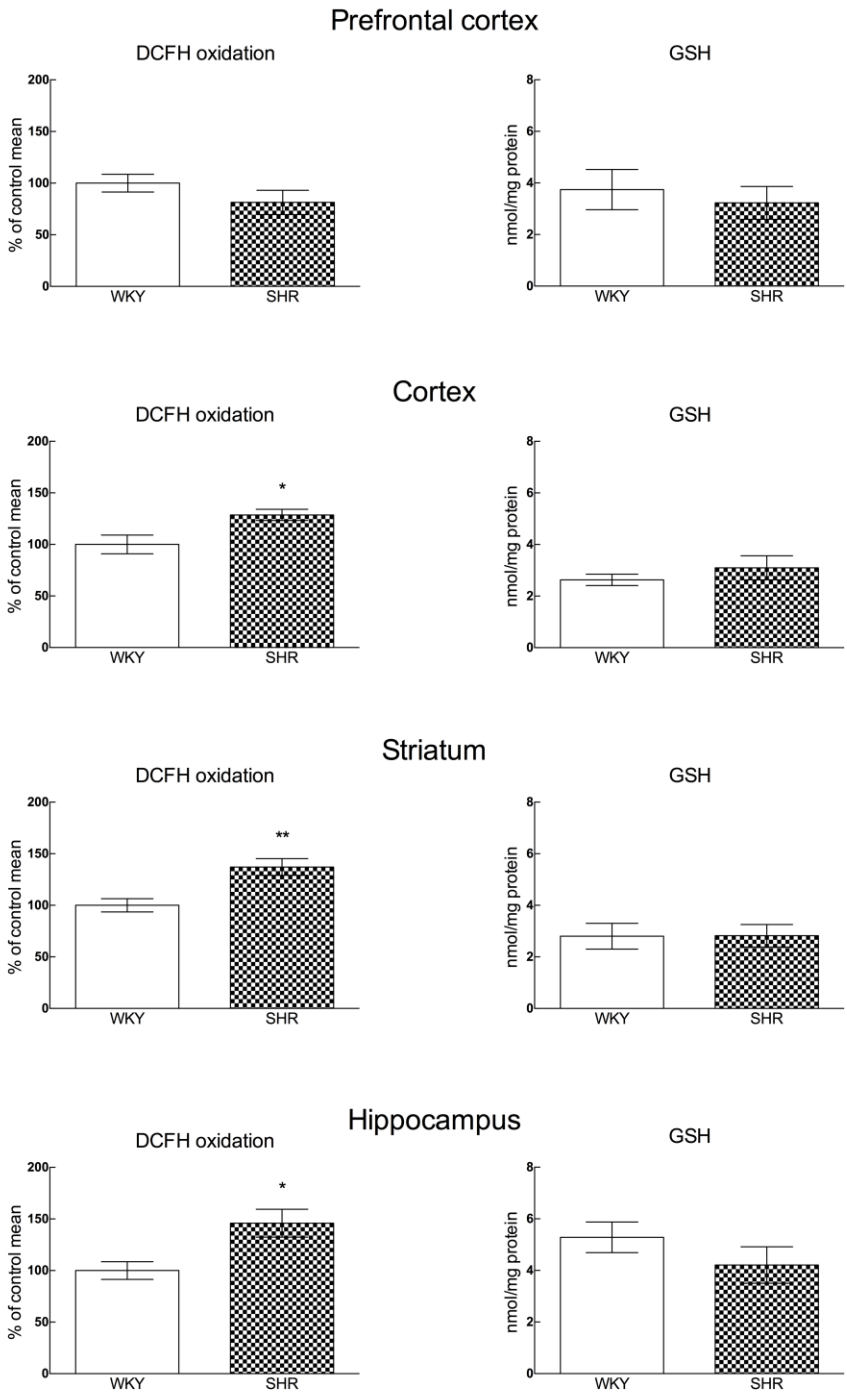

Fig. 1. DCFH oxidation and glutathione (GSH) levels in adult spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY). Bars represent the means ± SEM of animals grouped according to strain (SHR n=7 and WKY n=6). *p<0.05 compared to WKY; **p<0.01 compared to WKY (Student´s t test).

3.1.3. Antioxidant enzymatic defense activities

The activity of three main antioxidant enzymes, GPx, SOD and CAT, was measured in SHR (n=7) and WKY (n=6). Results are presented in Fig. 2. SHR presented decreased GPx activity in the prefrontal cortex ($t(11)=2.51$, $p=0.02$), and hippocampus ($t(11)=3.04$, $p=0.01$), with no difference in the cortex ($t(11)=0.37$, $p=0.71$), or striatum ($t(11)=1.09$, $p=0.29$). There was no difference between strain in SOD activity in the prefrontal cortex ($t(11)=1.11$, $p=0.29$), cortex ($t(11)=0.28$, $p=0.78$), striatum ($t(11)=0.05$, $p=0.95$), and hippocampus ($t(11)=0.01$, $p=0.99$). There was also no difference in CAT activity in the prefrontal cortex ($t(11)=0.03$, $p=0.97$), cortex ($t(11)=0.20$, $p=0.83$), striatum ($t(11)=0.03$, $p=0.97$), and hippocampus ($t(11)=0.71$, $p=0.48$).

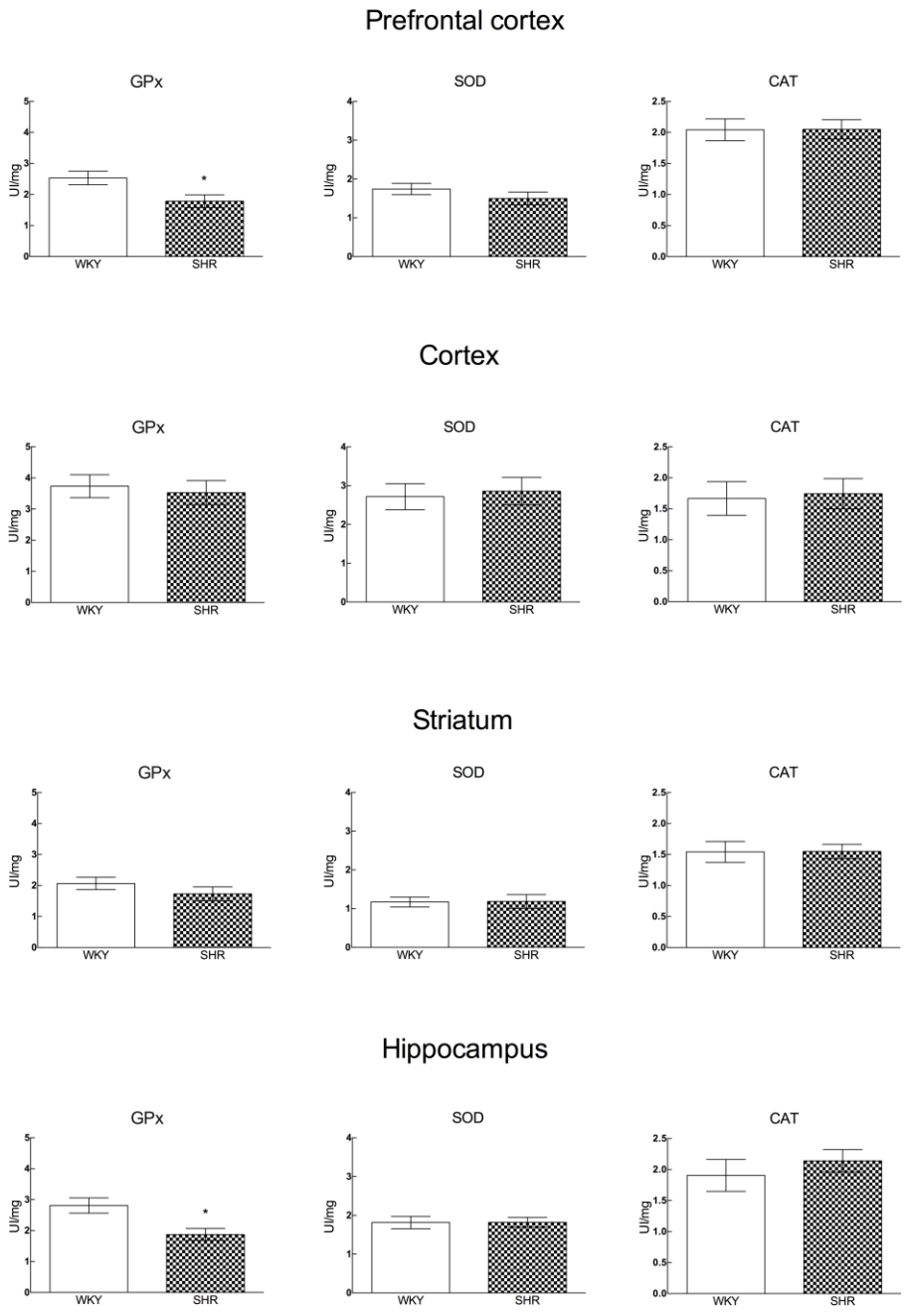

Fig. 2. Glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) activities in adult spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY). Bars represent the means ± SEM of animals grouped according to strain (SHR n=7 and WKY n=6). *p<0.05 compared to WKY (Student´s t test).

3.2. Cytokine levels

TNF-α, IL-1β and IL-10 were measured in the prefrontal cortex, cortex, striatum and hippocampus of SHR (n=7) and WKY (n=6). Fig. 3 shows the results for cytokine levels. SHR presented reduced TNF-α levels in the prefrontal cortex ($t(11)=3.15$, $p=0.009$), cortex ($t(11)=2.34$, $p=0.03$), and hippocampus ($t(11)=2.77$, $p=0.01$), with a trend to reduction in the striatum ($t(11)=2.15$, $p=0.057$). In relation to IL-1β, SHR presented a trend to reduction in the cortex ($t(11)=2.13$, $p=0.056$), and no difference in the prefrontal cortex ($t(11)=1.73$, $p=0.11$), striatum ($t(11)=1.13$, $p=0.27$), or hippocampus ($t(11)=0.52$, $p=0.3$). The SHR showed reduced levels of IL-10 in the cortex ($t(11)=2.37$, $p=0.03$), and no difference in the prefrontal cortex ($t(11)=0.87$, $p=0.39$), striatum ($t(11)=0.42$, $p=0.67$), or hippocampus ($t(11)=0.94$, $p=0.36$).

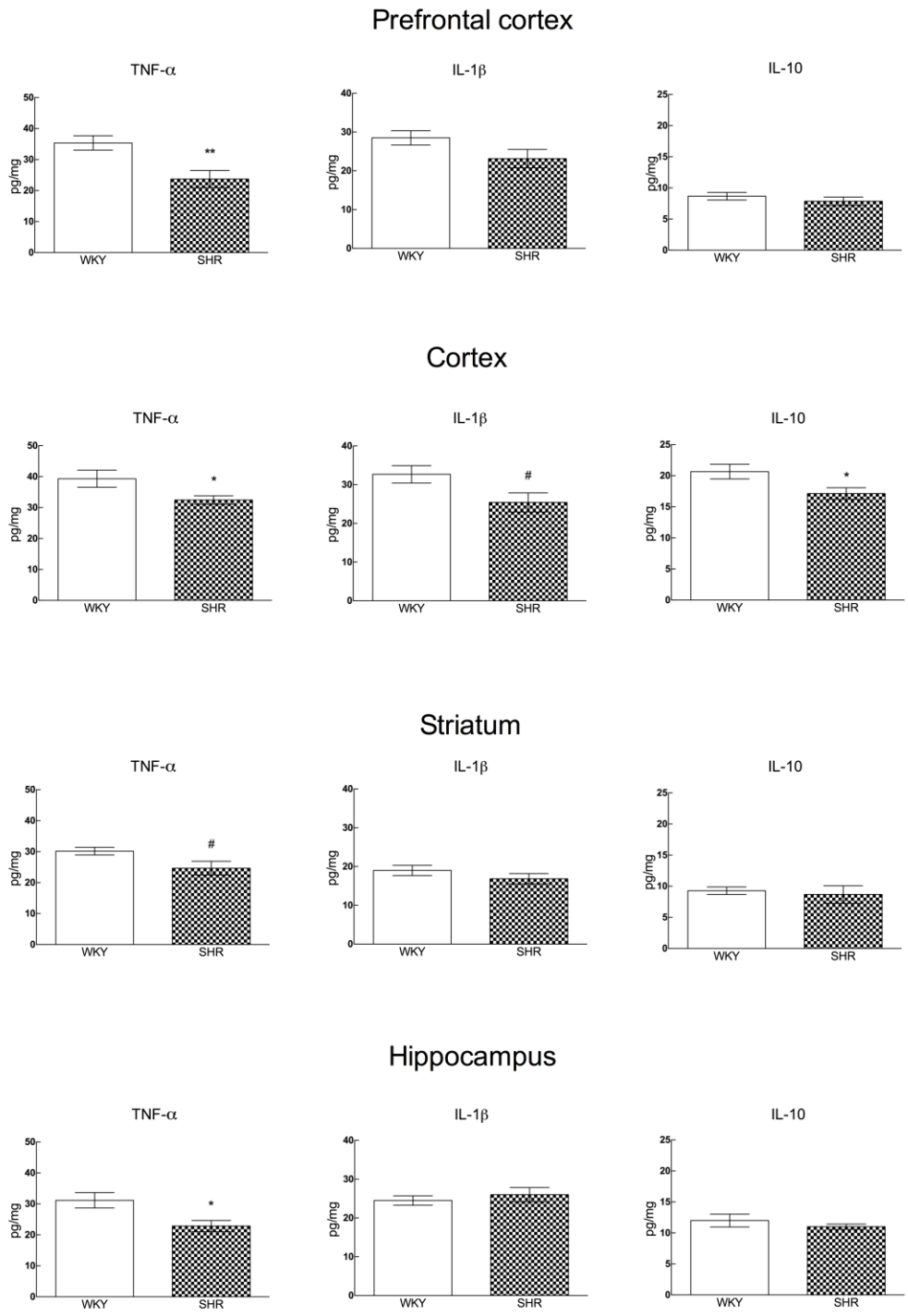

Fig. 3. TNF-α, IL-1β and IL-10 levels in adult spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY). Bars represent the means ± SEM of animals grouped according to strain (SHR n=7 and WKY n=6). *p<0.05 compared to WKY; **p<0.01 compared to WKY; #p<0.07 compared to WKY (Student´s t test).

4. Discussion

In this study we aimed at measuring cytokines and oxidative stress markers levels in an animal model of ADHD. Results indicate that the SHR present disruptions in cytokine and oxidative profile when compared to the control strain. They showed increased ROS production in distinct brain regions and, contrary to our hypothesis, presented reduced cytokine levels in the cortex. Moreover, a more widespread TNF-α difference was found in their brain. While the oxidative profile reasserts the SHR´s role as an animal model of ADHD, the cytokine profile might offer a new point of view on the pathophysiology of the disorder.

Oxidative stress is a process that occurs when excessive amounts of free radicals overwhelm the antioxidant defenses of the body. ROS is the major class of free radicals, and about 90% of ROS production occurs in the mitochondrial respiratory chain during oxygen metabolism [17]. An increase in ROS is usually accompanied by an increase in the so-called antioxidants [18], and the imbalance between ROS overproduction and antioxidant defense reflects oxidative stress, which can disrupts biological functions by causing damage to biomolecules such as DNA, lipids, carbohydrates and proteins [18, 19]. The antioxidant defense system of the brain includes non-enzymatic (like GSH) and enzymatic (SOD, CAT and GPx) components.

As previously mentioned, it has been shown that different psychiatric disorders seems to have its pathophysiology associated with cell damage caused by an increased oxidative/nitrosative stress [4]. This possible relation may be the product of an intrinsic brain sensitivity to oxidative damage [20]. There are various reasons for this susceptibility: (1) brain´s high oxygen consumption; (2) the presence of excitotoxic amino acids [21]; (3) its

humble antioxidant defenses [22]; (4) the presence of ions capable of catalyzing free radical reactions, such as copper and iron [23]; (5) its lipid rich environment, which is prone to oxidation [23]. Oxidative stress seems to represent a common final pathway that ties together different pathogenic mechanisms involved in the disruptions of brain circuits in psychiatric disorders. For instance, both in schizophrenia [24], bipolar disorder [25], and depression a whole range of predisposing factors, like environmental, genetic and medical factors, are able to activate oxidative pathways leading to cell damage [26].

Results of this study indicate that the SHR present increase ROS generation in the cortex, striatum and hippocampus. Besides, SHR had a decrease in GPx activity in the prefrontal cortex and hippocampus, but no difference in GSH levels, SOD and CAT activities. SHR increased ROS levels in distinct brain structures, without a physiologically expected antioxidant response, is in agreement with a meta-analysis that showed high oxidative stress in ADHD patients [5]. The same report found no difference in antioxidant production between patients and controls, suggesting that the inability to regulate ROS levels might be the result of neurochemical abnormalities in the brains of both ADHD patients and SHR. In ADHD, oxidative stress might also represent a common final pathway from distinct pathophysiological mechanisms. It is known that ADHD has a heritability of about 70%, however environmental factors also play an important role in disease expression [1]. Among those, severe early deprivation and stress are the most likely to have a causal relationship [27, 28]. Other environment factors that may be related to ADHD are maternal smoking and alcohol use during pregnancy, premature birth, low birth weight and exposure to environmental toxins such as lead and organophosphate pesticides [27, 29]. Interestingly, some of these environmental factors are also correlated with an increase in ROS production. Evidence from basic and clinical research have been demonstrating how early stress may lead to oxidative damage in the brain (reviewed by Schiavone, Jaquet [30]. Moreover, oxidative

stress in the infant of smoking mothers was already shown to be higher than from non-smoking mothers [31]. Preterm neonates also appear to have increased markers of oxidative stress when compared to full-term neonates [32]. Lead [33] and organophosphate pesticides [34] exposure might also induce oxidative stress. To sum up, convergent evidence suggests that ADHD predisposing factors may be at least partially responsible for the increased number of ROS found in patients and, possibly, in the animal model.

Furthermore, monoamine oxidase (MAO) enzyme family is another mitochondrial source of ROS, and its activity might be linked with SHR´s oxidative profile. These enzymes catalyze the metabolism of monoamines in the brain including dopamine, norepinephrine and serotonin, generating free radicals during their activity [35]. Their role in dopamine and norepinephrine metabolism has already called the attention of the ADHD community. For instance, a meta-analysis of peripheral biomarkers in ADHD patients found MAO to be significantly associated with the disorder and also related with drug response and symptom severity [36]. In fact, the effects of MAO inhibitor were already accessed in small clinical trials with promising results [37-39], suggesting that the ADHD phenotype might be linked with impairment in MAO´s ability to metabolize catecholamines. In the SHR, MAO activity in the brainstem, medulla oblongata and pons were increased in relation to WKY [40], and its inhibition seems to improve behavioral outcomes in this strain [41]. Combined data points to impaired MAO activity as a promising candidate for the markedly altered oxidative profile seen in ADHD patients and also replicated in the SHR.

The SHR presented decreased level of TNF-α in the prefrontal cortex, cortex and hippocampus, with a trend to reduction in the striatum. The cortex of SHR presented also decreased IL-10 and IL-1β levels, while IL-1β almost reached significance. Cytokines are a broad class of proteins secreted mainly by astrocytes and microglia, which has a wide range of actions in the central nervous system [42]. TNF-α and IL-1β are considered pro-

inflammatory proteins, while IL-10 is the main anti-inflammatory protein. It has been well known that cytokine release occurs with microglia and astrocyte activation, which can lead to neuronal damage [42]. Besides, different psychiatric and neurological disorders seems to be associated with increased levels of pro-inflammatory proteins, indicating a neuroinflammatory status in those conditions [43]. In our study, contrary to our hypothesis, SHR showed decreased levels of interleukins when compared to the control strain. While TNF-α production was reduced throughout the brain, the cortex presented a more preeminent cytokine deficit.

Besides being involved in pathological conditions when present in high levels, cytokines seems to be present also in the healthy brain, suggesting a physiological role in the body [44]. In relation to brain function, TNF-α is the most studied one. TNF-α knockout mice display cognitive disturbances [45], and polymorphisms in the TNF-α gene might influence executive functions in humans [46]. Moreover, TNF-α can be released in response to neuronal activity [47]. Albensi and Mattson [48] showed impairment in the hippocampus of mice lacking TNF receptors in long-term depression. TNF-α modulation of cognitive functions seems to be a product of its ability to control the strength of glutamatergic synapses, controlling both presynaptic glutamate release and postsynaptic AMPA receptors response (for a review, see Santello and Volterra [49]. Then, we can speculate that the SHR have a basal deficit of interleukins, mainly TNF-α, which could be involved in its cognitive disturbances.

In this paper we presented evidence corroborating the SHR as an animal model of ADHD due to biochemical similarities. We also reported a new approach to explore ADHD phenotype in this strain, which is a possible disturbance in the brain cytokine environment. As previously mentioned, disturbances in oxidative and inflammatory parameters have been proposed as mediators of neuronal circuits dysregulations seen in different neuropsychiatric

disorders. In spite of the new insights offered by our results, it is important to bear in mind that our study has important limitations. Results obtained using the SHR should always be interpreted cautiously due to its increased blood pressure. Ideally, these results should be replicated using adolescent animals, known to have ADHD-like phenotype without being hypertensive [50]. In addition, sample size used might decrease power to some statistical analysis, and more research needs to be done in order to better characterize which factors are likely involved in the disturbances presented. While MAO activity might be a candidate, empirical data should be obtained in order to test this hypothesis. Another worthwhile research project would be the characterization of the TNF-α deficit in the SHR and its possible relation with cognitive deficits.

5. Conclusion

In conclusion, there is considerable evidence in the literature indicating that inflammatory and oxidative parameters may be correlated with distinct pathological brain conditions. However, in the main animal model of ADHD, the SHR, data are still lacking. Our results imply that the SHR present an oxidative profile that is characterized by an increase in ROS production without an antioxidant counterbalance. Convergent risk factor may be responsible for this finding. Besides, this strain showed cytokine reduction when compared to the control strain, indicating a basal deficit. Having in mind the pleiotropic effects of cytokines, and that TNF-α seems to be especially important in cognitive circuits; these results may present a new approach to the cognitive disturbances seen in the SHR. To sum up, it is possible to suggest that our results may contribute to a better understanding of ADHD pathophysiology, and also to the search for new therapeutic approaches.

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Conflicts of interest

Luis Augusto Paim Rohde has been a member of the speakers' bureau/advisory board and/or acted as a consultant for Eli-Lilly, Janssen-Cilag, Medice, Novartis and Shire in the last three years. He receives authorship royalties from Oxford Press and ArtMed. He has also received travel awards from Shire for his participation of the 2014 APA and 2015 WFADHD meetings. The ADHD and Juvenile Bipolar Disorder Outpatient Programs chaired by him received unrestricted educational and research support from the following pharmaceutical companies in the last three years: Eli-Lilly, Janssen-Cilag, Novartis, and Shire. Eugenio Horacio Grevet was on the speakers' bureau/advisory board for Novartis and Shire in the past 3 years. He has also received travel awards for taking part in the 2015 WFADHD meeting from Shire. The other authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Transcranial direct current stimulation improves long-term memory deficits in an animal model of attention-deficit/hyperactivity disorder and modulates oxidative and inflammatory parameters

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Transcranial direct current stimulation improves long-term memory deficits in an animal model of attention-deficit/hyperactivity disorder and modulates oxidative and inflammatory parameters

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Abstract

Background: Transcranial direct current stimulation (tDCS) is a technique that modulates neuronal activity and has been proposed as a potential therapeutic tool for attention-deficit/hyperactivity disorder (ADHD) symptoms. Although pilot studies have shown evidence of efficacy, its mechanism of action remains unclear.

Objective/Hypothesis: We evaluated the effects of tDCS on behavioral (working and long-term memory) and neurochemical (oxidative and inflammatory parameters) outcomes related to ADHD pathophysiology. We used the most widely accepted animal model of ADHD: spontaneously hypertensive rats (SHR). The selected behavioral outcomes have been shown to be altered in both ADHD patients and animal models, and were chosen for their relation to the proposed mechanistic action of tDCS.

Methods: Adult male SHR and their control, the Wistar Kyoto rats (WKY), were subjected to 20 min of bicephalic tDCS or sham stimulation for 8 consecutive days. Working memory, long-term memory, and neurochemical outcomes were evaluated.

Results: TDCS improved long-term memory deficits presented by the SHR. No change in working memory performance was observed. In the hippocampus, tDCS increased both the production of reactive oxygen species in SHR and the levels of the antioxidant molecule glutathione in both strains. TDCS also modulated inflammatory response in the brains of WKY by downregulating pro-inflammatory cytokines.

Conclusion: TDCS had significant effects that were specific for strain, type of behavioral and neurochemical outcomes. The long-term memory improvement in the SHR may point to a possible therapeutic role of tDCS in ADHD that does not seem to be mediated by inflammatory markers. Additionally, the anti- inflammatory effects observed in the brain of WKY after tDCS needs to be further explored.

1. Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a heterogeneous neuropsychiatric disorder characterized by impaired levels of hyperactivity, impulsivity and inattention (1). Working memory (WM) deficits are widely considered a primary phenotype (2), and long-term memory (LTM) impairments are also characteristic of this population (3). Imaging studies indicate that ADHD symptoms are related to abnormal neuronal activity in several brain regions, including the prefrontal cortex (PFC), striatum, hippocampus and cerebellum (1, 4). Stimulant medications, such as methylphenidate, are recommended as first-line pharmacological therapy (5). Although effective (6), long-term pharmacologic therapy repeatedly suffers from non-adherence and treatment discontinuation among patients (7, 8), therefore supporting a continuous search for non-pharmacological interventions.

Transcranial direct current stimulation (tDCS) is a neuromodulatory tool in which a low-intensity electrical current is applied over the scalp in order to modulate neuronal activity (9, 10). Anodal stimulation increases cortical excitability, while cathodal stimulation decreases it (9). The combination of tDCS with pharmacological interventions in humans has demonstrated that the effects of tDCS are partially modulated by glutamatergic receptors (10, 11). The role of tDCS as an adjuvant therapy for a range of neuropsychiatric disorders has been extensively studied over the last years in several clinical trials (12). In patients with ADHD, tDCS was shown to improve clinical symptoms (13-16), thus characterizing it as a possible non-pharmacological treatment for this disorder. Although clinical trials have demonstrated the potential of tDCS for reducing ADHD symptoms, the mechanisms of action are still unclear.

Animal studies have suggested that changes in membrane polarization induced by tDCS are the result of attraction and repulsion of sodium and potassium ions, especially in the extracellular medium (17, 18). It is important to stress that neurons are not the only brain

cells sensible to electrical fields, and glial cells are also susceptible to tDCS effects (17, 19). In this sense, tDCS appears to modulate inflammatory pathways in animal models, possibly due to the activation of glial cells (20). The modulation of neuronal spontaneous discharge rate is able to induce long-term functional and morphological adaptations in brain pathways (17). However, how it translates into behavioral and neurochemical effects is still mostly unknown.

We therefore aimed to evaluate the effects of tDCS in an animal model of ADHD, and possible neurochemical substrates related to the effect. We chose the Spontaneously Hypertensive Rats (SHR) since this is considered the best animal model of ADHD, presenting face, construct and predictive validity (21). We measured WM and LTM as behavioral outcomes. WM deficits have been previously shown in the SHR when tested in the y-maze test (22, 23). In addition, short-term memory deficits were observed using the object recognition test (24, 25), strengthening the validity of the animal model. In this sense, WM and LTM were evaluated using the y-maze and the object recognition test, respectively. We also measured key markers of brain oxidative and inflammatory pathways (26, 27), which are known to be altered in the ADHD patients (28) and in the SHR rats (29), as the neurochemical mechanistic outcomes. The neurochemical markers were measured in the PFC, cortex (remaining regions), striatum and hippocampus, all regions previously shown to be affected in the SHR (29). We expected tDCS to improve WM and LTM, and decrease oxidative parameters in the SHR. Results from neurochemical analyses will be used in order to explore possible mechanisms of action of tDCS.

2. Material and methods

2.1. Animals

90-day-old adult male SHR and their control, the Wistar Kyoto Rats (WKY), weighing 299±25.9g and 284.6±28.6g respectively (mean ± standard deviation), from Institutional colony were used. Adult animals were chosen since our tDCS apparatus has an electrode size and produces a current density adapted for this age. A total of 15 SHR (7 in sham and 8 in active stimulation) and 15 WKY (7 in sham and 8 in active stimulation) were used for the behavioral tests. From those, 13 SHR (6 in sham and 7 in active stimulation) and 13 WKY (6 in sham and 7 in active stimulation) were randomly selected for the neurochemical analysis. Animals were kept in groups of four per cage under controlled temperature (22±2°C), and kept on a standard 12 h light/dark cycle (lights on at 7 AM), with access to water and chow *ad libitum* for the duration of the experiment. All procedures were approved by the Institutional Committee for Animal Care and Use (GPPG-HCPA protocol no. 14-0103) and performed in accordance with the Guide for the Care and Use of Laboratory Animals 8th edition (2011). The maintenance of the animals followed the law 11.794 (Brazil), which establishes procedures for the scientific use of animals. The experiment used the number of animals necessary to produce reliable scientific data. All the behavioral tests were conducted at the same time (9 AM) by the same researcher.

2.2. Experimental design

Rats were habituated to the maintenance room for 2 weeks before the experiment started. After this period, animals from each strain (SHR and WKY) were randomly assigned to active or sham treatment. Treatment was conducted for 8 consecutive days as described in section 2.3. Behavioral tests were conducted 24 hour after the last stimulation session (see Fig 1 for the experimental design). During the behavioral tests, animals were randomly selected for outcome assessments, which were conducted by a blinded investigator. Twenty-four hours after the last behavioral test, the rats were killed by decapitation and the PFC,

cortex (remaining regions), striatum and hippocampus were dissected for neurochemical analysis.

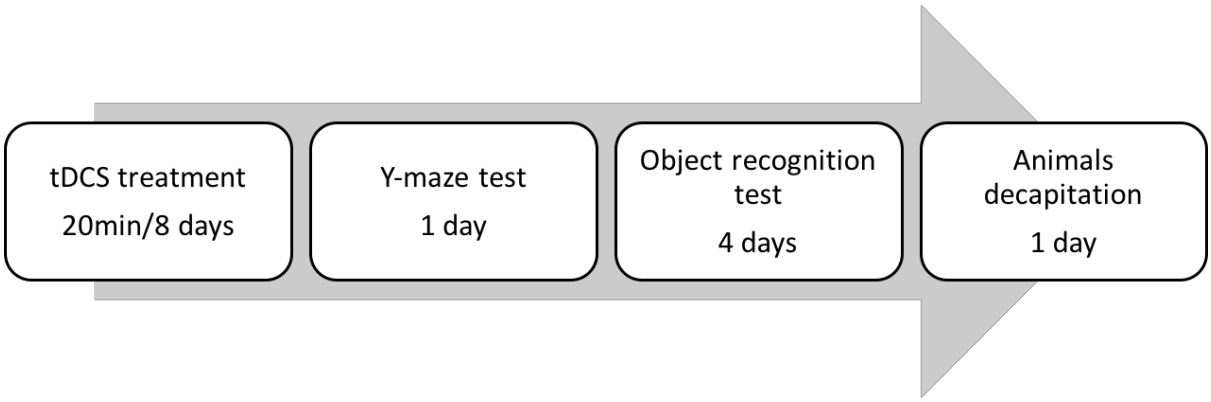

Fig 1. Experimental design.

2.3. Transcranial direct current stimulation

Animals from the active bicephalic tDCS group were subjected to a constant current of 0.5 mA intensity applied for 20 min/day over 8 days. The stimulation was performed at the same time (11 AM) and by the same researcher every day. Adapted ECG electrodes (1.5 cm^2) with a conductive hydrogel were fixed to their heads with adhesive tape to prevent detachment and connected to a battery-driven stimulator to deliver a constant electrical current. The animals' heads were shaved for better adherence. The center of the anodal electrode was placed on the midpoint of the lateral angle of the eyes to stimulate the frontal cortex, and the cathodal electrode was positioned between the neck and shoulder area. This placement was designed to mimic tDCS protocols in ADHD. These protocols are characterized by the placement of the anodal electrode in distinct regions of the PFC, as over the right dorsolateral PFC (13), the left dorsolateral PFC (14-16, 30, 31), or the right inferior frontal gyros (32). The objective of this placement was the stimulation of brain regions that, in rats, have similar functions from those of the human PFC (33). We have used a current density of 33.3 A/m^2, as a current higher than 142.9 A/m^2 was reported to induce tissue

damage (34). This design was previously applied by our research group to demonstrate behavioral and neurochemical changes in models of ADHD (25), chronic pain (35-39), and food craving (40). In order to deliver the current, animals had to be immobilized using a cloth for the total time of stimulation. Animals in the sham group were submitted to the same procedures, but the electrodes were not connected to the battery.

2.4. Behavioral tests

2.4.1. Y-maze test

WM was evaluated by the y-maze test. This test was adapted from its previously description (41). The apparatus has three grey arms disposed at 120^{o} angle from each other. Each arm is 45 cm long, 10 cm wide and 20 cm high. Rats were placed at the end of an arm and allowed to freely explore the maze for 8 min. The sequence of arm entries was recorded, with an arm entry defined as the entry of all four paws into one arm. Correct alternations were defined as the consecutive entry into three different arms, which has been positively correlated with WM performance in previous studies (42, 43). The maximum number of correct alternations was the total number of arms entered minus 2. The percentage of correct alternations was calculated as: correct alternations x 100 / maximum number of correct alternations.

2.4.2. Object recognition test

The object recognition test was used to measure LTM. It was conducted in an open field made of white-painted wood measuring 40 cm long, 50 cm wide and 60 cm high. The floor was divided into 12 squares of 13 x 13 cm delimited by dark lines. The experiment was conducted in a sound-attenuated room under low-intensity light (12 lx). The protocol is based

on the differential exploration of familiar and new objects by rats, as described by Ennaceur, Cavoy (44). It consists of three phases: habituation, sample, and discrimination. In the habituation phase, animals could freely explore the open field on two consecutive days for 10 min each day. In the sample phase, which took place 24 h latter, two identical cube-shaped objects (O1 and O2) were positioned in the open field, 15 cm away from the walls and approximately 20 cm from each other. The animals were allowed to freely explore the objects for 5 min. After this time, animals were returned to their home cage and the apparatus was cleaned. In the discrimination phase, after a delay period of 24 h, an identical copy of the familiar object (O3) was positioned in the open field together with a new T-shaped object (T) in the same locations previously occupied by O1 and O2. Rats were allowed to freely explore the objects for another 5 min. The T-shaped object was placed in different sides of the open field in each trial to avoid bias. Object exploration was defined as directing the nose less than or equal to 2 cm from, or touching it directly with the nose. Data were analyzed using the total time exploring the identical objects (O1+O2) in the sample phase, and the discrimination index, defined by the difference in exploration time between the novel and the familiar objects divided by the total time exploring these two objects [(T-O3)/(T+O3)], in the discrimination phase. Discrimination index was used as a measure of LTM. The objects were constructed using plastic LEGO blocks (São Paulo, SP, Brazil), and their shapes were based on previous studies (24, 25).

2.5. Cellular redox parameters

Our group has described disturbances in oxidative parameters in the SHR in a previous paper (29), where a more detailed methodological description of the measurements can be found. In summary, intracellular reactive oxygen species (ROS) production was measured using 2′-7′-dichorofluorescein diacetate (DCFH), with increased DCFH oxidation

indicating increased ROS production. Mean values obtained from the control group (WKY sham) were arbitrarily considered as 100%, and results were expressed as percentages relative to the mean of WKY sham. The levels of the antioxidant molecule glutathione (GSH) were detected using a fluorescence method, and the results were expressed in nmol/mg protein. The activity of three main antioxidant enzymes was also evaluated. Glutathione peroxidase (GPx) and superoxide dismutase (SOD) were measured using commercial kits, and catalase (CAT) was measured by colorimetric assay. Results were expressed in UI/mg protein. Cellular redox measurements were conducted in the PFC, cortex (remaining regions), striatum and hippocampus.

2.6. Cytokine measurements

Our group described alterations in the inflammatory profile of SHR in a previous paper (29), where a more detailed methodological description of the measurements can be found. Levels of the pro-inflammatory mediator tumor necrosis factor alpha (TNF-α) and interleukin (IL) 1 beta, and the anti-inflammatory cytokine IL-10 were measured using ELISA commercial kits. Results were expressed as pg/mg tissue. Cytokine measurements were conducted in the PFC, cortex (remaining regions), striatum and hippocampus.

2.7. Statistical analysis

Data were expressed as mean ± standard deviation. Normality was evaluated using histograms and quantile plots, and statistical analysis was conducted using two-way analysis of variance (ANOVA) with strain and treatment as independent variables. The main effects of strain, treatment, and the interaction between strain and treatment were described, and a *p*-value less than 0.05 was considered as statistically significant. Effect sizes are reported as partial η^2. Values of 0.01, 0.09 and 0.25 were considered as small, moderate and large effect

sizes, respectively (45). There was no control for multiple comparisons. Therefore, up to 4.8 statistically significant tests ($p<0.05$) would be expected due to chance alone in the neurochemical analysis.

3. Results

3.1. Y-maze test

The effects of tDCS in WM performance in SHR and WKY were evaluated using the y-maze test. Fig 2A shows the total number of arm entries and the percentage of correct alternations. SHR presented increased number of entries when compared to WKY (main effect of strain, $F_{(1, 26)}$=7.89, p=0.009, η^2=0.233). There was no effect of tDCS treatment (interaction between independent variables, $F_{(1, 26)}$=1.166, p=0.2901, η^2=0.043; main effect of treatment, $F_{(1, 26)}$=0.5436, p=0.4676, η^2=0.02). There were no statistically significant differences in the percentage of correct alternations (interaction between independent variables, $F_{(1, 26)}$=0.2656, p=0.6107, η^2=0.01; main effect of treatment, $F_{(1, 26)}$=0.341, p=0.5643, η^2=0.013; main effect of strain, $F_{(1, 26)}$=2.212, p=0.1490, η^2=0.078).

3.2. Object recognition test

The object recognition test measured tDCS effects on LTM in SHR and WKY. Fig 2B shows the results from the sample and discrimination phases. In the sample phase, the SHR had shorter investigation time when compared to the WKY (main effect of strain, $F_{(1, 26)}$=12.98, p=0.001, η^2=0.333), with no effect of tDCS (interaction between independent variables, $F_{(1, 26)}$=0.2195, p=0.6433, η^2=0.008; main effect of treatment, $F_{(1, 26)}$=0.8305, p=0.3705, η^2=0.031). In the discrimination phase, on the other hand, there was a significant interaction effect between tDCS and strain ($F_{(1, 26)}$=6.67, p=0.01, η^2=0.204), thus showing

that tDCS reversed the LTM relative deficits in SHR. Results from the open fields conducted during the habituation phase are available upon request.

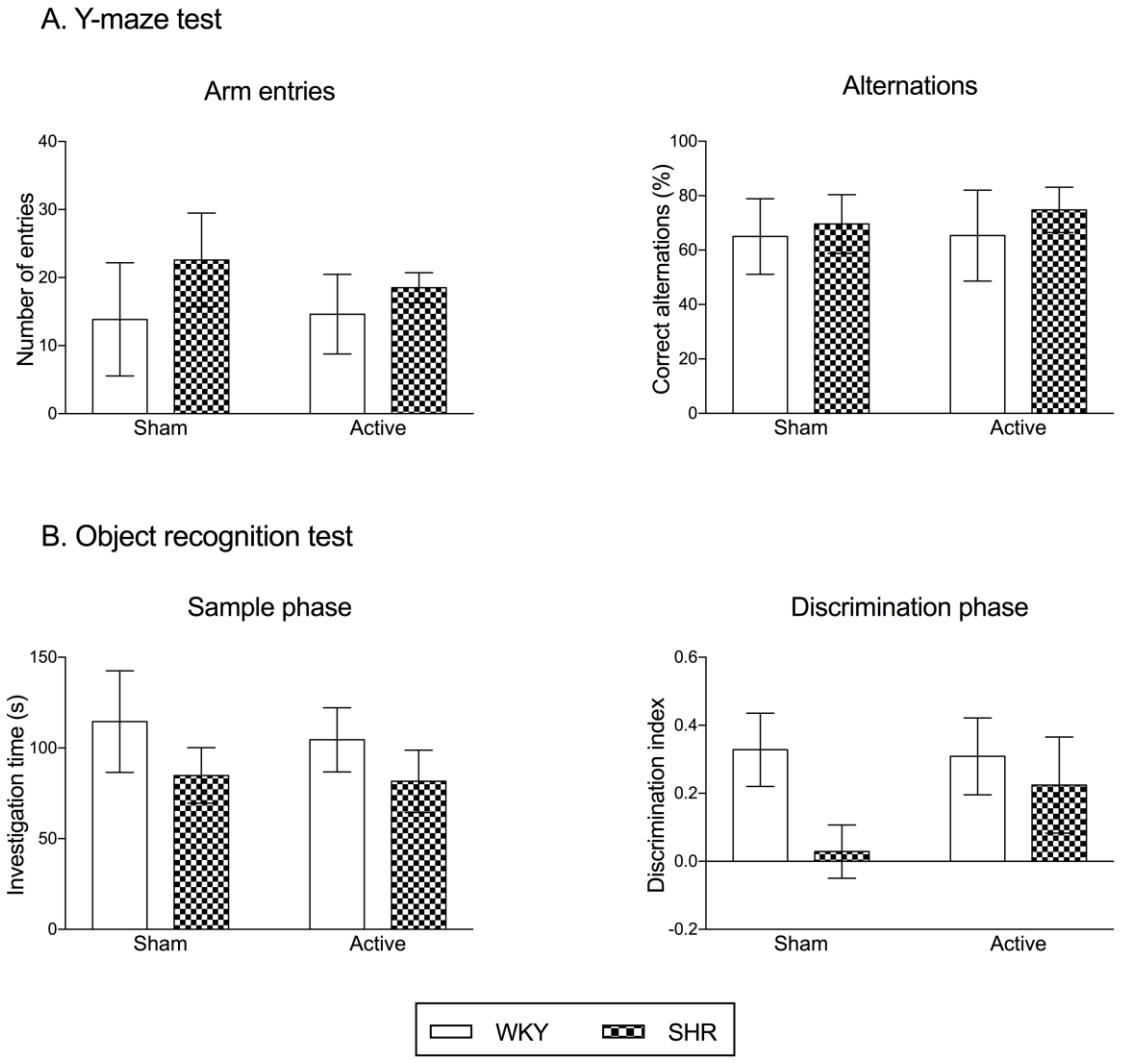

Fig 2. Effects of tDCS on the performance of the spontaneously hypertensive rats (SHR, n=7 for sham and n=8 for active) and Wistar Kyoto rats (WKY, n=7 for sham and n=8 for active) in the y-maze test (A) and object recognition test (B). Bars represent the mean ± standard deviation of animals grouped according to treatment and strain. Data were analyzed using a two-way analysis of variance with treatment and strain as independent variables.

3.4. Cellular redox parameters

Intracellular ROS production was measured with DCFH oxidation. Table 1 shows the results for the four structures evaluated. In the cortex and striatum, SHR rats presented increased ROS production when compared to WKY (main effect of strain, $F_{(1, 22)}$=24.96, p<0.0001, η^2=0.532 and $F_{(1, 22)}$=5.769, p=0.0252, η^2=0.208, respectively). In the hippocampus, tDCS increased ROS production in the SHR rats (interaction between independent variables, $F_{(1, 22)}$=5.628, p=0.0268, η^2=0.204). No changes were observed in the PFC of the evaluated groups (p>0.05).

Table 1 shows the results for the antioxidant molecule GSH and for the activity of the main antioxidant enzymes. In the hippocampus, tDCS treatment was able to increase GSH levels in both strains (main effect of treatment, $F_{(1, 22)}$=5.639, p=0.0267, η^2=0.204). In the cortex, SHR presented increased levels of GSH when compared to WKY (main effect of strain, $F_{(1, 22)}$=7.114, p=0.0141, η^2=0.244). No differences in GSH levels were found in the PFC or striatum (p>0.05). The activity of GPx was reduced in the SHR in the PFC, striatum and hippocampus (main effect of strain, $F_{(1, 22)}$=4.44, p=0.0467, η^2=0.168; $F_{(1, 22)}$=7.114, p=0.0141, η^2=0.244; and $F_{(1, 22)}$=6.125, p=0.0215, η^2=0.218, respectively). There were no differences in the cortex of the evaluated groups (p>0.05). In addition, there were no differences in SOD and CAT activities in any structures evaluated (p>0.05).

Table 1
Effects of tDCS on oxidative parameters in the prefrontal cortex (PFC), cortex (remaining regions), striatum and hippocampus of spontaneously hypertensive rats (SHR, n = 6 for sham and n = 7 for active) and Wistar Kyoto rats (WKY, n = 6 for sham and n = 7 for active). DCFH (2′-7′-dichorofluorescein diacetate) values were expressed as percentage relative to the mean of WKY sham; GSH (glutathione) content was expressed in nmol/mg protein; GPx (glutathione peroxidase), SOD (superoxide dismutase) and CAT (catalase) activities were expressed in UI/mg protein. Data were analyzed using a two-way analysis of variance with treatment and strain as independent variables. The interaction between independent variables (interaction), main effect of treatment and main effect of strain are presented. For each group, mean ± standard deviation is described. Results with a p-value less than 0.05 were considered as statistically significant and were highlighted in bold.

Structure and measurement	Interaction	Main effect of treatment	Main effect of strain	WKY sham	WKY active	SHR sham	SHR active
PFC							
DCFH	$F(1, 22) = 0.0367$ $p = 0.8497$	$F(1, 22) = 0.5181$ $p = 0.4792$	$F(1, 22) = 0.0528$ $p = 0.8203$	100 ± 29.38	91.59 ± 25.08	96.11 ± 24.47	91.23 ± 12.96
GSH	$F(1, 22) = 0.668$ $p = 0.4225$	$F(1, 22) = 0.01561$ $p = 0.9017$	$F(1, 22) = 0.1512$ $p = 0.7011$	3.43 ± 0.97	3.83 ± 1.31	3.61 ± 1.10	3.32 ± 0.85
GPx	$F(1, 22) = 0.3075$ $p = 0.5848$	$F(1, 22) = 0.00025$ $p = 0.9875$	**$F(1, 22) = 4.44$ $p = 0.0467$**	2.40 ± 0.74	2.52 ± 0.43	2.05 ± 0.55	1.92 ± 0.55
SOD	$F(1, 22) = 0.0839$ $p = 0.7748$	$F(1, 22) = 0.04784$ $p = 0.8289$	$F(1, 22) = 0.0839$ $p = 0.7748$	1.70 ± 0.62	1.71 ± 0.43	1.81 ± 0.60	1.71 ± 0.38
CAT	$F(1, 22) = 0.0233$ $p = 0.8799$	$F(1, 22) = 0.3498$ $p = 0.5603$	$F(1, 22) = 0.0604$ $p = 0.8081$	2.25 ± 0.44	2.17 ± 0.44	2.23 ± 0.53	2.10 ± 0.40
Cortex							
DCFH	$F(1, 22) = 3.608$ $p = 0.0707$	$F(1, 22) = 3.084$ $p = 0.0930$	**$F(1, 22) = 24.96$ $p < 0.0001$**	100 ± 23.78	101.18 ± 17.81	157.17 ± 27.99	126.86 ± 13.67
GSH	$F(1, 22) = 2.17$ $p = 0.1549$	$F(1, 22) = 0.03924$ $p = 0.8448$	**$F(1, 22) = 7.114$ $p = 0.0141$**	2.43 ± 0.71	2.11 ± 0.38	2.73 ± 0.54	3.15 ± 0.81
GPx	$F(1, 22) = 0.0621$ $p = 0.8054$	$F(1, 22) = 0.1421$ $p = 0.7098$	$F(1, 22) = 0.1696$ $p = 0.6845$	3.40 ± 1.22	3.14 ± 0.81	3.46 ± 0.90	3.41 ± 1.18
SOD	$F(1, 22) = 1.139$ $p = 0.2974$	$F(1, 22) = 0.951$ $p = 0.3401$	$F(1, 22) = 0.951$ $p = 0.3401$	2.93 ± 0.73	2.30 ± 0.92	2.30 ± 0.89	2.32 ± 0.55
CAT	$F(1, 22) = 0.2577$ $p = 0.6168$	$F(1, 22) = 2.046$ $p = 0.1666$	$F(1, 22) = 0.0854$ $p = 0.7728$	1.8 ± 0.30	2.01 ± 0.61	1.75 ± 0.69	2.20 ± 0.65
Striatum							
DCFH	$F(1, 22) = 0.4601$ $p = 0.5046$	$F(1, 22) = 2.475$ $p = 0.1300$	**$F(1, 22) = 5.769$ $p = 0.0252$**	100 ± 20.05	124.52 ± 26.17	133.54 ± 33.88	143.29 ± 28.86
GSH	$F(1, 22) = 1.751$ $p = 0.1993$	$F(1, 22) = 0.01464$ $p = 0.9048$	$F(1, 22) = 1.133$ $p = 0.2987$	2.39 ± 1.03	2.88 ± 0.62	3.36 ± 1.31	2.77 ± 1.10
GPx	$F(1, 22) = 0.3175$ $p = 0.5788$	$F(1, 22) = 0.2509$ $p = 0.6214$	**$F(1, 22) = 7.114$ $p = 0.0141$**	2.11 ± 0.61	2.12 ± 0.45	1.71 ± 0.34	1.51 ± 0.48
SOD	$F(1, 22) = 0.2902$ $p = 0.5955$	$F(1, 22) = 0.2902$ $p = 0.5955$	$F(1, 22) = 1.58$ $p = 0.2219$	1.05 ± 0.44	1.20 ± 0.22	1.30 ± 0.42	1.30 ± 0.31
CAT	$F(1, 22) = 1.031$ $p = 0.3208$	$F(1, 22) = 0.1488$ $p = 0.7034$	$F(1, 22) = 0.4692$ $p = 0.5005$	1.78 ± 0.30	1.57 ± 0.33	1.73 ± 0.44	1.82 ± 0.43
Hippocampus							
DCFH	**$F(1, 22) = 5.628$ $p = 0.0268$**	**$F(1, 22) = 5.857$ $p = 0.0242$**	**$F(1, 22) = 5.795$ $p = 0.0249$**	100 ± 24.30	100.56 ± 35.66	100.41 ± 24.37	156.95 ± 32.22
GSH	$F(1, 22) = 0.5225$ $p = 0.4774$	**$F(1, 22) = 5.639$ $p = 0.0267$**	$F(1, 22) = 1.091$ $p = 0.3075$	4.57 ± 0.96	6.54 ± 1.36	4.36 ± 2.24	5.41 ± 1.66
GPx	$F(1, 22) = 0.2563$ $p = 0.6177$	$F(1, 22) = 0.001324$ $p = 0.9713$	**$F(1, 22) = 6.125$ $p = 0.0215$**	2.86 ± 0.79	2.99 ± 0.66	2.34 ± 0.68	2.20 ± 0.54
SOD	$F(1, 22) = 0.02624$ $p = 0.8728$	$F(1, 22) = 0.2754$ $p = 0.6050$	$F(1, 22) = 0.0050$ $p = 0.9438$	1.71 ± 0.68	1.84 ± 0.39	1.73 ± 0.41	1.80 ± 0.32
CAT	$F(1, 22) = 0.03677$ $p = 0.8497$	$F(1, 22) = 0.002563$ $p = 0.9601$	$F(1, 22) = 0.1341$ $p = 0.7177$	2.17 ± 0.56	2.20 ± 0.59	2.29 ± 0.57	2.24 ± 0.51

3.5. Cytokine measurements

TNF-α, IL-1β and IL-10 were measured in the PFC, cortex, striatum and hippocampus, and the results are presented in Table 2. Stimulation with tDCS in the WKY was able to decrease TNF-α levels in the PFC, striatum and hippocampus (interaction between treatment and strain, $F_{(1, 22)}$=8.079, p=0.0095, η^2=0.269; $F_{(1, 22)}$=8.818, p=0.0071, η^2=0.286; and $F_{(1, 22)}$=8.721, p=0.0074, η^2=0.284, respectively). In the cortex, tDCS increased TNF-α in the SHR rats (interaction between treatment and strain, $F_{(1, 22)}$=5.011, p=0.0356,

η^2=0.186). Treatment with tDCS decreased IL-1β levels in the WKY in the PFC, cortex, striatum and hippocampus (interaction between treatment and strain, $F_{(1, 22)}$=4.727, p=0.0407, η^2=0.177; $F_{(1, 22)}$=5.861, p=0.0242, η^2=0.21; $F_{(1, 22)}$=7.655, p=0.0113, η^2=0.258; and $F_{(1, 22)}$=7.238, p=0.0134, η^2=0.248, respectively). No differences were found in IL-10 levels (p>0.05).

Table 2
Effects of tDCS on inflammatory parameters in the prefrontal cortex (PFC), cortex (remaining regions), striatum and hippocampus of spontaneously hypertensive rats (SHR, n = 6 for sham and n = 7 for active) and Wistar Kyoto rats (WKY, n = 6 for sham and n = 7 for active). TNF-α (tumor necrosis factor alpha), IL-1β (interleukin 1 beta) and IL-10 (interleukin 10) values were expressed as pg/mg tissue. Data were analyzed using a two-way analysis of variance with treatment and strain as independent variables. The interaction between independent variables (interaction), main effect of treatment and main effect of strain are presented. For each group, mean ± standard deviation is described. Results with a p-value less than 0.05 were considered as statistically significant and were highlighted in bold.

Structure and measurement	Interaction	Main effect of treatment	Main effect of strain	WKY sham	WKY active	SHR sham	SHR active
PFC							
TNF-α	**$F(1, 22) = 8.079$ $p = 0.0095$**	$F(1, 22) = 3.259$ $p = 0.0847$	$F(1, 22) = 0.06234$ $p = 0.8052$	32.66 ± 6.80	21.14 ± 6.76	25.00 ± 4.69	27.57 ± 6.55
IL-1β	**$F(1, 22) = 4.727$ $p = 0.0407$**	$F(1, 22) = 1.505$ $p = 0.2329$	$F(1, 22) = 3.017$ $p = 0.0964$	28.66 ± 3.20	22.00 ± 6.70	21.00 ± 5.58	22.85 ± 3.38
IL-10	$F(1, 22) = 1.756$ $p = 0.1987$	$F(1, 22) = 0.6248$ $p = 0.4377$	$F(1, 22) = 0.394$ $p = 0.5367$	6.83 ± 3.06	9.00 ± 2.16	8.83 ± 2.31	8.28 ± 2.81
Cortex							
TNF-α	**$F(1, 22) = 5.011$ $p = 0.0356$**	$F(1, 22) = 1.572$ $p = 0.2231$	$F(1, 22) = 4.102$ $p = 0.0551$	34.16 ± 4.16	31.85 ± 4.45	33.66 ± 6.02	41.85 ± 8.11
IL-1β	**$F(1, 22) = 5.861$ $p = 0.0242$**	**$F(1, 22) = 6.351$ $p = 0.0195$**	$F(1, 22) = 2.419$ $p = 0.1341$	39.33 ± 8.18	26.28 ± 7.29	28.83 ± 6.64	28.57 ± 4.39
IL-10	$F(1, 22) = 1.381$ $p = 0.2526$	$F(1, 22) = 0$ $p > 0.9999$	$F(1, 22) = 0.8979$ $p = 0.3536$	17.66 ± 4.13	19.14 ± 1.57	20.33 ± 2.58	18.85 ± 3.89
Striatum							
TNF-α	**$F(1, 22) = 8.818$ $p = 0.0071$**	**$F(1, 22) = 5.782$ $p = 0.0250$**	$F(1, 22) = 0.05372$ $p = 0.8189$	29.00 ± 3.91	20.16 ± 3.54	24.50 ± 4.68	25.42 ± 4.46
IL-1β	**$F(1, 22) = 7.655$ $p = 0.0113$**	$F(1, 22) = 1.211$ $p = 0.2831$	**$F(1, 22) = 7.655$ $p = 0.0113$**	20.16 ± 2.31	15.85 ± 3.18	14.00 ± 2.28	15.85 ± 3.23
IL-10	$F(1, 22) = 0.352$ $p = 0.5590$	$F(1, 22) = 1.238$ $p = 0.2779$	$F(1, 22) = 0.198$ $p = 0.6607$	8.50 ± 1.87	8.00 ± 3.41	9.50 ± 2.16	7.85 ± 1.86
Hippocampus							
TNF-α	**$F(1, 22) = 8.721$ $p = 0.0074$**	**$F(1, 22) = 5.759$ $p = 0.0253$**	$F(1, 22) = 0.5044$ $p = 0.4850$	28.66 ± 3.61	20.14 ± 3.97	22.83 ± 3.37	23.71 ± 4.88
IL-1β	**$F(1, 22) = 7.238$ $p = 0.0134$**	$F(1, 22) = 2.014$ $p = 0.1698$	$F(1, 22) = 1.357$ $p = 0.2565$	26.50 ± 3.27	19.57 ± 3.50	20.00 ± 4.85	22.14 ± 5.14
IL-10	$F(1, 22) = 0.07261$ $p = 0.7901$	$F(1, 22) = 0.1591$ $p = 0.6939$	$F(1, 22) = 0.01406$ $p = 0.9067$	12.00 ± 4.05	12.14 ± 1.95	11.83 ± 3.18	12.57 ± 1.71

4. Discussion

In this study, we aimed at measuring behavioral and neurochemical outcomes related to ADHD pathophysiology after a treatment with a neuromodulatory technique, tDCS, in an animal model of ADHD. The results of this study show that the SHR presented LTM deficits in the object recognition test, and tDCS was able to restore LTM performance in the SHR to levels of control rats (WKY). WM did not differ between the ADHD model and the control rats, and tDCS had no effect in this learning task. Regarding the neurochemical outcomes, tDCS induced ROS production in the hippocampus of SHR and increased GSH levels in the hippocampus of both strains. Moreover, an anti-inflammatory effect was observed in the brain of WKY after treatment.

TDCS improved LTM deficits presented by the SHR in the object recognition task. Long and short-term memory impairments have been previously identified in the SHR using distinct behavioral approaches (25, 46, 47). In addition, our group had already reported that tDCS was able to increase short-term memory in SHR, probably by modulating dopaminergic inputs to the hippocampus (25). The benefits of tDCS for memory impairments have also been demonstrated in animal models of traumatic brain injury (48), Alzheimer´s disease (49), and chronic nicotine consumption (50). The mechanism of memory enhancement with tDCS appears to be related to the induction of hippocampal plasticity. LTP is an activity-dependent synaptic enhancement that is believed to be the biochemical substrate of memory consolidation in the hippocampus (51). Rohan, Carhuatanta (52) demonstrated that an *in vivo* stimulation with tDCS in rats was able to induce a LTP increment in hippocampal slices, and that the effect was dose-related. Similar results were found in mice by Podda, Cocco (53), thus implying that memory enhancement with tDCS could be the result of LTP induction in the hippocampus.

The SHR presented similar WM performance to WKY in the y-maze task, and no effect of tDCS was detected. The y-maze task is based on the premise that, since rats have a natural tendency to explore new environments, WM would be necessary to remember which arm they had previously accessed in order to allow an alternate choice in the future (43). WM impairment in the SHR using this test has been previously described (22, 23), although some reports have also shown no differences when compared to WKY (42, 54). These divergences could indicate that this kind of impairment is not a main finding in the SHR. Nevertheless, since distinct behavioral approaches have observed WM deficit in the SHR (55-57), the y-maze test might not the most sensitive approach to detect it.

SHR presented a tDCS-dependent increase in ROS production in the hippocampus. Oxidative stress is a process that occurs when the cellular antioxidant defenses are not able to

couple with excessive amounts of free radicals. Also, about 90% of ROS production occurs in the mitochondrial respiratory chain during oxygen metabolism (58). Therefore, an increase in hippocampal neuronal activity following treatment with tDCS could be associated with more oxygen metabolism and, consequently, more ROS production. Another possible explanation is that the increased ROS production observed in the hippocampus is a consequence of a high dopaminergic input. The monoamine oxidase enzyme catalyzes the metabolism of monoamines in the brain, including dopamine, and generates free radicals during this activity (59). Since an enhanced dopaminergic input to the hippocampus have been observed in the SHR after a treatment with tDCS (25), the breakdown of excessive amounts of dopamine would potentially induce ROS production.

Both SHR and WKY presented high levels of GSH in the hippocampus after tDCS stimulation. A physiologically expected reaction for increased ROS production is an up-regulation of antioxidant defenses (60). It can thus be suggested that the physiologic expected response characterized by a GSH up-regulation was a response to increased ROS production in the hippocampus. However, while GSH up-regulation could prevent increased ROS production in the WKY, in the SHR this was not sufficient. The impaired response in the SHR may be the result of their baseline inability to couple with oxidative stress (29).

TDCS treatment reduced TNF-α and IL-1β protein levels in the WKY rats. This finding was unexpected and suggests the modulation of inflammatory response by tDCS in the control strain. Treatment reduced TNF-α levels in the PFC, striatum and hippocampus of WKY, while IL-1β was reduced in the four structures evaluated in the control strain. The maintenance of the effect across structures decreases the probability that the findings are false-positive results. Previous studies in rats have shown a decrease in IL-1β in the spinal cord and reduced TNF-α levels in the hippocampus after a treatment with tDCS (35, 38). However, the modulation of neuroinflammatory pathways by tDCS is still not completely

understood. For instance, it has been described that tDCS might activate glial cells (20), thus implying a pro-inflammatory effect of the stimulation. It has also been suggested that the effects on inflammation might be dependent on the stimulation parameters (61). Central and peripheral neuromodulatory techniques like electro acupuncture (62), vagus nerve stimulation (63), epidural motor cortex stimulation (64) and deep brain stimulation (65) have been shown to induce anti-inflammatory effects in pathological conditions, thus giving some support to the role of tDCS in it.

As far as we know, we are the first to demonstrate LTM improvement in an animal model of ADHD with tDCS. Besides that, enhanced hippocampal activity may be a possible neurochemical substrate for the behavior observed. Although the raise in ROS production can presume hippocampal activity, more evidence is needed in order to strength this hypothesis. Moreover, our results should be interpreted in the light of some important limitations. Although the SHR are the most widely used animal model of ADHD, its validity is not a consensus, and suggestions that it is not a representative animal model for ADHD have been reported (66, 67). Even though tDCS has been shown to induce functional improvements in healthy subjects (68-70), our control strain did not present behavioral changes. In addition, although we used the WKY as the strain control, the selection of an appropriate reference strain is a measure of debate in the literature (71). The WKY rats have even been proposed as an animal model of the predominantly inattentive subtype of ADHD (71), which would be a possible explanation for the effects detected in our study. In relation to the object recognition test, similar (72) or even superior (73) SHR performance has been previously reported. In this regard, Pires, Pamplona (24) have demonstrated that the SHR are able to discriminate highly distinct objects, but are unable to discriminate a pair of objects with subtle structural differences, thus, indicating that the presence of impairment may depend on the difficulty level of the test. Regarding the statistical analysis, we did not control for multiple

comparisons and therefore our results should be viewed as exploratory. If our results were corrected for multiple comparisons using the Bonferroni correction, only the increased oxidative stress in the cortex of SHR would maintain significance. Future studies should replicate our main results using neurochemical markers as primary outcomes. We have not performed a sample size calculation, and thus our study may be underpowered to detect a treatment effect. In fact, a post-hoc analysis showed that our non-significant behavioral tests had a power that ranged from 7,4% to 29%, while in our non-significant neurochemical tests it ranged from 5% to 49%. It should be stressed that the benefits of a post-hoc power analysis for the interpretation of negative results is questioned in the literature, since it will always lead to a low value (74, 75). It should also be stressed that the neurochemical analysis was done four days after the last tDCS session, reinforcing the long-lasting effects of the treatment.

5. Conclusion

To sum up, this paper has demonstrated that a treatment with tDCS can modulate behavioral, redox and inflammatory parameters in an animal model of ADHD. The LTM improvement observed in the SHR may point to a possible therapeutic role of tDCS in this disorder. Besides, tDCS induced increased ROS production in the hippocampus of SHR, which could be related to an enhanced synaptic activity without a proper antioxidant physiologically expected response. Further research is needed in order to determine the mechanisms underlying the observed effects, mainly the anti-inflammatory effects in the control strain. Therefore, this paper provides a framework for the exploration of a possible new treatment for ADHD and for the understanding of the behavioral and neurochemical effects of tDCS.

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Conflicts of interest

Luis Augusto Paim Rohde has been a member of the speakers' bureau/advisory board and/or acted as a consultant for Eli-Lilly, Janssen-Cilag, Medice, Novartis and Shire in the last three years. He receives authorship royalties from Oxford Press and ArtMed. He has also received travel awards from Shire for his participation of the 2015 WFADHD and 2016 AACAP meetings. The ADHD and Juvenile Bipolar Disorder Outpatient Programs chaired by him received unrestricted educational and research support from the following pharmaceutical companies in the last three years: Eli-Lilly, Janssen-Cilag, Novartis, and Shire.

A review on the role of inflammation in attention-deficit/hyperactivity disorder

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A review on the role of inflammation in Attention-Deficit/Hyperactivity Disorder

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Abstract

Attention-deficit/hyperactivity disorder (ADHD) is a prevalent neurodevelopmental condition that impairs quality of life in social, academic and occupational contexts for both children and adults. Although a strong neurobiological basis has been demonstrated, the pathophysiology of ADHD is still poorly understood. Among the proposed mechanisms are glial activation, neuronal damage and degeneration, increased oxidative stress, reduced neurotrophic support, altered neurotransmitter metabolism, and blood-brain barrier disruption. In this way, a potential role of inflammation has been increasingly researched. However, evidence for the involvement of inflammation in ADHD is still scarce and comes mainly from: (1) observational studies showing a strong comorbidity of ADHD with inflammatory and autoimmune disorders; (2) studies evaluating serum inflammatory markers; and (3) genetic studies. A co-occurrence of ADHD with inflammatory disorders has been demonstrated in a large number of subjects, suggesting a range of underlying mechanisms, as an altered immune response, common genetics and environmental links. The evaluation of serum inflammatory markers has provided mixed results, likely due to small sample sizes and high heterogeneity between biomarkers. However, there is evidence that increased inflammation during early development may be a risk factor for ADHD symptoms. Although genetic studies have demonstrated a potential role for inflammation in this disorder, there is no clear evidence. To sum up, inflammation may be an important mechanism in ADHD pathophysiology, but more studies are still needed for a more precise conclusion.

Key-words: ADHD, inflammation, neuronal damage, biomarkers

Epidemiology

Attention-deficit/hyperactivity disorder (ADHD) is a neurodevelopmental disorder characterized by impairing symptom of inattention and/or hyperactivity-impulsivity (1). A meta-analysis published in 2007 has demonstrated that the worldwide prevalence of ADHD in children and adolescents is of 5.3% (95% CI: 5.01-5.56) (2). In adults, ADHD has been shown to persist in about 50-70% of cases depending on methodological issues in the studies (3, 4). Accordingly, a meta-analysis has shown a prevalence of about 2.4% in adulthood (5). Concurring with those results, a recent study conducted by the World Health Organization has found a prevalence of 2.5% in adults in the 30 countries evaluated (6).

Impairments

ADHD impairs quality of life and well-being in social, academic and occupational contexts for both children and adults. The pathology is correlated with a wide range of psychiatry comorbidities, psychological dysfunctions and risk behaviors. Observational studies conducted in large populations have demonstrated that an ADHD diagnosis is a risk factor for substance use disorder (7), criminal behavior (8), and increase the risk of physical injuries (9). A cohort study that followed 1,92 million people, 32,061 having a diagnosis of ADHD, found a significant increase in mortality rate in this population. The increase in mortality was mainly related to death caused by unnatural factors, especially accidents (10). Adverse outcomes in adolescence and adulthood include academic and vocational underachievement (11), obesity (12), unemployment, low economic status (13), emotional dysregulation (14), and pregnancy in adolescence (15). It has been shown that ADHD patients present higher suicide attempts when compared to the general population (16). To sum up, ADHD has an important impact in quality of life, comparable to other severe psychiatry disorders (17).

Diagnosis

The diagnosis of ADHD is based on the assessment of inattentive or hyperactive-impulsive symptoms. According to the Diagnostic and Statistical Manual of Mental Disorders – 5th edition (DSM-5), the diagnosis of ADHD should be performed when there is clear evidence that the symptoms impair quality of life in social, academic or occupational performance (1). In addition, the symptoms should be present in two or more different settings, and their onset should be prior to 12 years of age. The diagnosis relies on the reporting of patients or other informants (relatives, teachers), and the clinical interview remains the gold standard.

The diagnosis of ADHD involves cultural and ethnical differences related to the attitudes towards the disease. For instance, it has been reported that African Americans youth have more ADHD symptom when compared to Caucasians, but were diagnosed two-thirds as often (18). This differences could be explained by parents beliefs about ADHD and the lack of access to treatment (18). Currently, there is no biomarker for the diagnosis of ADHD, and the reliance only on a clinical interview may be an important factor predisposing negative cultural views on the disorder, and also uncertainty concerning the validity of the diagnosis (19).

Neurobiological basis

The neurobiological basis of ADHD has been confirmed through genetic and neuroimaging studies (5). Twin studies have shown that ADHD has a strong heritability of 70-80% in both children and adults (20). According to genome-wide association studies, approximately 40% of the heritability is associated to common genetic variants (21). In addition, candidate gene studies have shown mainly the influence of genes involved in the

monoamine neurotransmitter system (22). A recent genome-wide association meta-analysis conducted with 20,183 ADHD patients and 35,191 controls identified 12 independent loci exceeding genome-wide significance (Demontis, D, et al., personal communication, 2018). The biological role of the identified loci appears to be related to neural developmental and plasticity, neuronal wiring, dopamine levels in the synapses, intellectual disability, and the development of speech and learning (Demontis, D, et al., personal communication, 2018). In relation to environmental factors, severe early maternal deprivation appears to have a causal role (22). However, other environmental factors such as maternal smoking and alcohol use, low birth weight, premature birth and exposure to environmental toxins have been also associated with ADHD symptoms (23, 24).

ADHD is characterized by structural and functional dysfunctions in a wide range of cortical and subcortical regions. A recent mega-analysis performed in a sample of 1,713 patients with ADHD and 1,529 controls has demonstrated a volume reduction in the nucleus accumbens, amygdala, caudate, hippocampus and putamen in patients with the disorder (25). A meta-analysis of functional magnetic studies including approximately 200 ADHD patients and 200 controls has demonstrated a reduction in the activity of distinct cortical regions during tests requiring attention and impulsivity control (26). Although a wide literature has repeatedly shown that ADHD has a strong neurobiological basis, it pathophysiology is still poorly understood. It is believed that several factors, including genetic and environmental factors, interact during development giving rise to ADHD symptomatology.

The role of inflammation

Among the neuropathological mechanisms believed to be involved in ADHD, there has been a growing interest in the immune system. Over the last years, an increasing body of evidence has supported the role of inflammation in neuropsychiatric disorders. A strong

association between altered inflammatory mechanisms and neuropsychiatric disorders has been provided for depression (27), schizophrenia (28), bipolar disorder (29), and post-traumatic stress disorder (30) through systematic reviews and meta-analyses. It has been hypothesized that inflammatory mechanisms are related to the physiopathology of neuropsychiatric disorders through several mechanisms. Among them are glial activation (31), neuronal damage and degeneration (32), increased oxidative stress (33), reduced neurotrophic support (34), altered neurotransmitter metabolism (35), and blood-brain barrier disruption (36). For ADHD, evidence supporting a role of inflammatory mechanisms comes from three main lines: comorbidity with inflammatory and autoimmune disorders, biochemical markers, and genetic studies. In this sense, the aim of this review is to present a summary of the existing literature regarding the role of inflammation in the pathophysiology of ADHD.

Comorbidity with inflammatory and autoimmune disorders

Observational data from a large number of subjects show a strong association between ADHD and inflammatory and autoimmune disorders. Miyazaki, Koyama (37) performed a systematic review and meta-analysis with more than 61,000 children (about 8,000 ADHD patients), in order to evaluate an association between ADHD and allergies. They found that ADHD patients were more likely to have asthma, allergic rhinitis, atopic dermatitis and allergic conjunctivitis in comparison to the non-ADHD subjects from the population. Similar results were found by Schans, Cicek (38), performing a systematic review and meta-analysis examining the co-occurrence of atopy and ADHD. They found a higher presence of asthma, eczema and rhinitis in ADHD patients when compared to a control population. A prospective cohort study performed with more than 23,000 patients demonstrated that a personal and a maternal history of autoimmune disease were also

associated with an increased risk of ADHD (39). Among the autoimmune diseases were thyrotoxicosis, type 1 diabetes, autoimmune hepatitis, psoriasis, and ankylosing spondylitis. Cross-sectional studies have also identified a higher prevalence of psoriasis in ADHD patients (40).

The higher co-occurrence of ADHD with inflammatory and autoimmune disorders may suggest a range of underlying mechanisms, including an altered immune response, common genetics and environmental links. It has also been suggested that increased cytokine release due to an inflammatory process may affect the prefrontal cortex functioning (41). In addition, ADHD abnormalities may be the result of an exaggerated central nervous system inflammatory response in the fetus caused by maternal inflammation, such as in allergy or autoimmune diseases. The prevalent comorbidity between ADHD and inflammatory disorders may also explain the association found between ADHD diagnosis and the use of acetaminophen during pregnancy (42). To sum up, although observational data support the co-occurrence of ADHD and inflammatory and autoimmune disorders, the studies conducted so far have not identified which factors may have a causal role in this comorbidity. For that, well designed prospective studies are still needed.

Biochemical Markers

Studies searching for inflammatory biomarkers in ADHD patients have not provided conclusive findings, likely due to small sample sizes and high heterogeneity among biomarkers. Inflammatory markers tested in this population include specific antibodies, cytokines, and neurotrophic factors. Passarelli, Donfrancesco (43) evaluated the role of antibodies against Purkinje cells as a possible marker of an immune response in ADHD patients. These specific antibodies were chosen since a role of the cerebellum in the pathophysiology of ADHD has been previously proposed (5). The authors found a

significantly higher immunoreactivity against anti-Purkinje cell antibodies in ADHD patients when compared to controls, suggesting an involvement of the autoimmune system in the disorder. Those results have been replicated in a following study (44), and the authors also demonstrated that ADHD patients had increased serum levels of interleukin 6 (IL-6) and IL-10. Increased levels of antibasal ganglia antibodies (45) and antibodies against the dopamine transporter (46) have also been detected in ADHD, supporting the role of the immune system in the disorder.

Several cytokines have been researched as possible neurochemical markers of ADHD. As previously mentioned, there is a high heterogeneity among the biomarkers tested, which makes the interpretations of the findings more challenging. Evaluation of pro and anti-inflammatory cytokines, and the cytokine-related neurotrophin S100B have been performed by Oades, Dauvermann (47) in the serum of ADHD patients. There was no major imbalance in the levels of inflammatory markers between ADHD patients and controls, and no differences in the levels of S100B. In a second study, however, the total serum S100B levels were positively associated with reduced symptoms in patients (48). Serum IL-6 and tumor necrosis factor alpha (TNF-a) were evaluated in a different study, and no significant results were found (49).

Although the previously mentioned studies did not show significant main findings related to inflammatory markers, a cohort study performed with more than 1,500 premature and low birth weight newborns measured 25 inflammation-related proteins in the serum, and found that children who had elevated concentrations of inflammation-related proteins during the first two postnatal weeks were more likely to have attention problems at 24 months (50). In addition, neonatal infections - which are associated with inflammatory responses - and systemic inflammation during the first postnatal month increased the risk of ADHD (51, 52). To sum up, the evaluation of serum levels of inflammatory markers in ADHD patients has

provided mixed results, likely due to small sample sizes and high heterogeneity between biomarkers. However, there is evidence that increased inflammation during early development may be a risk factor for ADHD symptoms.

Genetic studies

There has been evidence from genetic studies that polymorphisms in genes related to inflammatory pathways play a role in ADHD. Smith, Anastopoulos (53) performed a study evaluating a set of 164 single nucleotide polymorphisms (SNPs) from 31 candidate genes in a total of 398 subjects. They found that two SNPs in a cytokine related gene, the ciliary neurotrophic factor receptor (CNTFR), were associated with ADHD inattentive symptom severity. A population-based association study performed in 546 ADHD patients and 546 controls has also identified an association between the CNTFR and ADHD in both adults and children. An association of ADHD with genes of the major histocompatibility complex has been reported (54), supporting the role of inflammation and autoimmunity in the disorder. However, those findings were not replicated in a recent genome-wide association meta-analysis (Demontis, D, et al., personal communication, 2018).

A genome-wide association study performed by Zayats, Athanasiu (55) in 478 ADHD patients and 880 controls found no SNPs at the significance threshold. However, a pathway analyses found an association with SNPs involved in the regulation of gene expression, cell adhesion and inflammation. A study performed by de Jong, Newhouse (56) investigated a genomic overlap between ADHD and other psychiatric disorders in 318 individual, 93 with a diagnosis of ADHD. They found a similar genetic signature between ADHD and depression in genes related to inflammation. Segman, Meltzer (57) investigated the role of the IL-1 receptor antagonist gene variable number tandem repeat polymorphism in the risk of ADHD. The IL-1 was chosen since it has been shown to modulate catecholaminergic transmission in

mice (58). They evaluated a sample of 86 children of ADHD and found an association of the 4-repeat allele with an increased risk for ADHD, and an association with the 2-repeat allele with a decreased risk. However, the same results were not replicated in a larger sample (59).

Although a good number of genetic studies provide evidence on the role of inflammation in ADHD, it is important to consider that there is a high variation between the methodologies performed. In addition, there is no consensus on which inflammatory-related genes predispose to ADHD. A possible explanation for that is the fact that ADHD is a disorder with a highly heterogeneous genetic makeup and clinical presentation. Therefore, studies focusing on a more homogeneous population may be more likely to be conclusive.

Conclusions

There has been a growing interest in inflammation as a predisposing mechanism in psychiatric disorders. In ADHD, evidence from comorbidity with inflammatory and autoimmune disorders, serum biomarkers and genetic studies can be found. The high co-occurrence between ADHD and inflammatory disorder suggests a range of underlying mechanisms, including altered immune response and common genetic and environmental links. Biomarkers measured in ADHD patients have provided unclear results. However, increased inflammation during early development appears to be related to an ADHD phenotype. ADHD is a highly hereditary disorder, therefore it might be expected that polymorphisms in inflammatory-related genes are present in patients. Although some studies have found this association, there is no consensus on which genes are affected. To sum up, there have been indications for a role of the immune system in the pathophysiology of ADHD. However, well designed studies are still needed to confirm this hypothesis.

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Disclosure Statement

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VIII. CONSIDERAÇÕES FINAIS

8. CONSIDERAÇÕES FINAIS

Considerando que o TDAH é um transtorno prevalente e com alto impacto na saúde e qualidade de vida, e que há uma busca contínua por novas intervenções terapêuticas que sejam eficazes, baratas e com poucos efeitos adversos, nesse trabalho buscamos avaliar os efeitos comportamentais da ETCC, uma estratégia terapêutica promissora para transtornos neuropsiquiátricos, em um modelo animal do TDAH. O uso de modelos animais adequados é essencial para que se compreenda a fisiopatologia do transtorno e para que se desenvolva novas alternativas terapêuticas.

Nesse trabalho, nós demonstramos que um protocolo de ETCC com duração de 8 dias é capaz de melhorar a memória de curta e longa duração nos ratos SHR quando comparados aos seus controles, os ratos WKY. A memória de curta duração é um sistema responsável pelo armazenamento de informações por um período de minutos a poucas horas, fase em que a memória de longa duração ainda não está consolidada [174]. Ao contrário da memória de longa duração, a memória de curta duração não envolve a expressão de genes ou a síntese de novas proteínas [174]. Diversas linhas de evidências apontam a plasticidade sináptica hipocampal na forma de LTP como o substrato bioquímico que dá origem à memória de longa duração [203]. Nesse sentido, Rohan, Carhuatanta [147] demonstraram que a ETCC em ratos *in vivo* é capaz de induzir um aumento da LTP em fatia hipocampais, sendo este efeito dose-dependente. Resultados semelhantes foram encontrados por Podda, Cocco [204]. Dessa forma, sugerimos que a melhora na memória de longa duração observada nos SHR possa estar relacionada a um aumento da LTP no hipocampo desses animais.

Os achados bioquímicos evidenciados nesse trabalho colaboram com o entendimento do mecanismo de ação da ETCC nos SHR. Primeiramente, observamos aumento dos níveis de DA em hipocampo e estriado de ambas as linhagens, após serem submetidos ao protocolo

de ETCC. Rossato, Bevilaqua [168] demonstraram que, em ratos, o aporte dopaminérgico no hipocampo é necessário para a manutenção da LTP. De modo similar, esse neurotransmissor também é importante para a formação da memória de curta duração [181]. A DA é capaz de modular a transmissão glutamatérgica em sinapses hipocampais, influenciando assim a indução de LTP [169]. Desse modo, acreditamos que a melhora da memória de curta e de longa duração observada nos SHR com o uso da ETCC possa estar correlacionada a um aumento do aporte de DA, especialmente em hipocampo.

Nesse trabalho observamos também um aumento na produção de espécies reativas de oxigênio no hipocampo dos SHR após o tratamento com ETCC. Cerca de 90% da produção de espécies reativas de oxigênio ocorre na cadeia respiratória mitocondrial durante o processo de metabolização do oxigênio [205]. Portanto, um aumento da atividade neuronal nessa estrutura após o tratamento pode estar associado a um maior metabolismo de oxigênio e, consequentemente, maior produção de espécies reativas de oxigênio. Outra possível explicação para tal achado é que o aumento de espécies reativas de oxigênio no hipocampo é uma consequência do maior aporte de DA nessa estrutura. A metabolização de monoaminas no cérebro, incluindo DA, é realizada pela enzima monoamino oxidase, que gera radicais livres durante sua atividade [206]. Uma resposta fisiológica ao aumento da geração de radicais livres é uma maior produção de espécies antioxidantes, como por exemplo o tripeptídeo GSH [207]. Em nosso estudo observamos que ambas as linhagens apresentaram aumento dos níveis da GSH no hipocampo após ETCC. Podemos sugerir, portanto, que o aumento de GSH é uma resposta esperada a uma maior produção de espécies reativas de oxigênio. No entanto, enquanto esse aumento foi capaz de evitar um excesso de espécies reativas de oxigênio nos ratos WKY, o mesmo não aconteceu nos SHR.

Nessa tese também observamos, pela primeira vez, que os SHR apresentam um perfil oxidativo basal caracterizado por um maior número de espécies reativas de oxigênio em

diferentes estruturas cerebrais comparados aos ratos WKY. Mais especificamente, encontramos um aumento das espécies reativas de oxigênio no córtex, estriado e hipocampo dos SHR quando comparado aos controles. Evidências apontam que um estado pró-oxidativo está presente em distintos transtornos neuropsiquiátricos [208]. Acredita-se que o estresse oxidativo represente uma via final comum que conecta diferentes mecanismos patológicos envolvidos em transtornos neuropsiquiátricos. O aumento da espécies reativas de oxigênio observado nos SHR, juntamente com sua resposta antioxidante deficiente, está de acordo com uma meta-análise realizada em pacientes com TDAH [209]. Além disso, também demonstramos que essa linhagem apresenta níveis reduzidos de diferentes interleucinas pró-inflamatórias em estruturas cerebrais.

Em suma, a ETCC foi capaz de melhorar os déficits em memória de curta e longa duração apresentados pelos SHR. Hipotetizamos que tais efeitos comportamentais estejam relacionados a um maior aporte dopaminérgico no hipocampo. Esses resultados caracterizam a ETCC como uma alternativa promissora para o tratamento de déficits mnemônicos em pacientes com TDAH, estimulando o delineamento de ensaios clínicos randomizados que possam averiguar esse efeito na prática clínica.

IX. PERSPECTIVAS FUTURAS

9. PERPECTIVAS FUTURAS

Acreditamos que nossos resultados sejam capazes de expandir um frutífero campo de pesquisa que envolve a ETCC no TDAH. Como perspectivas futuras podemos incluir a investigação mais pormenorizada dos mecanismos de ação envolvidos nos efeitos comportamentais da ETCC nos ratos SHR. Uma abordagem promissora é administração de um antagonista dopaminérgico em regiões cerebrais específicas (no hipocampo e estriado, por exemplo), com o objetivo de observar se há uma reversão do efeito benéfico da ETCC. Caso os efeitos da ETCC sejam de fato desencadeados por um aumento do aporte de DA, supõe-se que o antagonismo de receptores dopaminérgicos reverta tais efeitos. De modo mais específico, essa mesma estratégia poderia incluir o antagonismo dos diferentes subtipos de receptores de DA (D1 e D2, por exemplo).

Em relação a estudos clínicos, nossos resultados ressaltam o promissor potencial terapêutico da ETCC para pacientes com TDAH principalmente no que se refere a déficits mnemônicos. Desse modo, uma abordagem interessante seria a confecção de um ensaio clínico randomizado em pacientes com TDAH que avalie os efeitos da ETCC na memória de curta e longa duração.

X. ANEXOS E/OU APÊNDICES

10.1. ARTIGOS PUBLICADOS DURANTE O PERÍODO DE DOUTORAMENTO CUJOS TEMAS SE RELACIONAM A ESTA TESE, MAS NÃO FORAM INCLUÍDOS NO CORPO PRINCIPAL

Positive effects of transcranial direct current stimulation in adult patients with attention-deficit/hyperactivity disorder - A pilot randomized controlled study.

Publicado no periódico Psychiatry Research

Positive effects of transcranial direct current stimulation in adult patients with attention-deficit/hyperactivity disorder – A pilot randomized controlled study

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ABSTRACT

Almost 30% of adult patients with attention-deficit/hyperactivity disorder (ADHD) do not respond or tolerate standard pharmacological interventions. Few clinical investigations addressed the efficacy and tolerability of transcranial direct current stimulation (tDCS), a non-invasive neuromodulatory technique, in the disorder. We performed a double-blind, sham-controlled randomized clinical trial in 17 patients with ADHD. The set up for tDCS was the following: 2 mA/20 min/day for 5 days with the anode over the right dorsolateral prefrontal cortex and cathode over the left dorsolateral prefrontal cortex. ADHD symptoms were measured by the Adult ADHD Self-Report Scale (ASRS) and impairment with the Sheehan Disability Scale (SDS) in four different time points after stimulation. Participants achieved significant lower ASRS inattention and SDS scores after active tDCS in comparison with sham stimulation group. In addition, we detected a trend for a lower ASRS total score in the active tDCS group. Follow up data analysis revealed a positive interaction between time and treatment in both ASRS inattention, SDS and ASRS total scores. Short-term application of tDCS in adult patients with ADHD improved their symptoms, and this improvement persisted after the end of the stimulation. Future studies with larger sample sizes are needed.

Behavioral effects of methylphenidate in an animal model of attention-deficit/hyperactivity disorder, the spontaneously hypertensive rats: a systematic review and meta-analysis protocol

Publicado no periódico BMJ Open Science

BMJ Open Science

Behavioural effects of methylphenidate in an animal model of attention-deficit/hyperactivity disorder, the spontaneously hypertensive rats: a systematic review and meta-analysis protocol

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ABSTRACT

Introduction Attention-deficit/hyperactivity disorder (ADHD) is a prevalent condition related to several negative outcomes. Although a strong neurobiological basis has been demonstrated, its pathophysiology is still poorly understood. In this sense, proper animal models are needed to further unravel the neurobiological basis of ADHD. The spontaneously hypertensive rats (SHRs) are the most commonly used animal model of ADHD. However, its validity, and especially its predictive validity, has been questioned. The current protocol discloses the background, aims and methods of a systematic review and meta-analysis of studies reporting the behavioural effects of methylphenidate (MPH), the most commonly prescribed treatment for ADHD, in the SHR.

Methods and analysis The following behavioural outcomes will be evaluated: locomotion, attention, impulsivity and memory. In addition, we plan to evaluate how predefined covariates influence the effects of MPH using meta-regression and sensitivity analyses.

Ethics and dissemination Data will be reported following PRISMA guidelines.

INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is a prevalent neurodevelopmental disorder characterised by impairing levels of hyperactivity, impulsivity and inattention.[1] Observational studies have shown that ADHD is related to several negative outcomes, including, among others, decreased quality of life,[2] increased number of suicide attempts,[3] less socioeconomic status[4] and increased mortality due to accidents.[5]

A strong neurobiological basis has been repeatedly demonstrated in ADHD. Twin studies showed that ADHD has a heritability of about 70%–80%,[6] and significant genome-wide hits have been reported, especially in neurodevelopmental processes that are relevant to ADHD.[7] A recent meta-analysis performed with more than 1500 patients with ADHD found a reduced volume in the nucleus accumbens, amygdala, caudate, hippocampus and putamen in patients with the disorder.[8] In addition, a meta-analysis of functional MRI studies demonstrated reduced activation in distinct cortical region during attention and impulsivity tests.[9] Even though molecular genetics and neuroimaging studies have been improving our understanding of the disorder, the pathophysiology of ADHD is still poorly understood.

Animal models are considered a fundamental tool to unravel the neurobiological factors associated with neuropsychiatric disorders.[10] More importantly, the absence of proper animal models has been proposed as a main factor driving the slow advancement of new treatments for neuropsychiatric disorders.[10] An animal model should present predictive, face and construct validity in order to be considered a proper model of a disease.[11] In ADHD, the spontaneously hypertensive rats (SHRs) are widely considered the most appropriate model.[12 13] Evidence has shown that the SHR presents face validity,[12 13] which corresponds to the extent of similarities between the animal model and the disorder. The evaluation of construct validity, which represents the resemblance between the aetiological processes in patients and animal model, is still a challenge because the pathophysiology of ADHD is mostly unknown. In addition, there is no consensus on the predictive and construct validity of the SHR as an animal model of ADHD.

Construct validity depends on the understanding of the pathophysiology of the

Systematic review and meta-analysis of the behavioral effects of methylphenidate in the spontaneously hypertensive rat model of attention-deficit/hyperactivity disorder

Submetido para o periódico Neuroscience & Biobehavioral Reviews

Systematic review and meta-analysis of the behavioral effects of methylphenidate in the spontaneously hypertensive rat model of attention-deficit/hyperactivity disorder

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ABSTRACT

The spontaneously hypertensive rats (SHR) are the most widely used model for ADHD. While face and construct validity are consolidated, questions remain about the predictive validity of the SHR model. We aim at summarizing the evidence for the predictive validity of SHR by evaluating its ability to respond to methylphenidate (MPH), the most well documented treatment for ADHD. A systematic review was carried out to identify studies evaluating MPH effects on SHR behavior. Studies (n=36) were grouped into locomotion, attention, impulsivity or memory, and a meta-analysis was performed. Meta-regression, sensitivity, heterogeneity, and publication bias analyses were also conducted. MPH increased attentional and mnemonic performances in the SHR model and decreased impulsivity in a dose-dependent manner. However, MPH did not reduce hyperactivity in low and medium doses, while increased locomotor activity in high doses. Thus, since the paradoxical effect of stimulant in reducing hyperactivity was not observed in the SHR model, our study does not fully support the predictive validity of SHR, questioning their validity as an animal model for ADHD.

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Adenosine A_{2A} Receptors in the Rat Prelimbic Medial Prefrontal Cortex Control Delay-Based Cost-Benefit Decision Making

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Adenosine A_{2A} Receptors in the Rat Prelimbic Medial Prefrontal Cortex Control Delay-Based Cost-Benefit Decision Making

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Adenosine A_{2A} receptors (A_{2A}Rs) were recently described to control synaptic plasticity and network activity in the prefrontal cortex (PFC). We now probed the role of these PFC A_{2A}R by evaluating the behavioral performance (locomotor activity, anxiety-related behavior, cost-benefit decision making and working memory) of rats upon downregulation of A_{2A}R selectively in the prelimbic medial PFC (PLmPFC) via viral small hairpin RNA targeting the A_{2A}R (shA_{2A}R). The most evident alteration observed in shA_{2A}R-treated rats, when compared to sh-control (shCTRL)-treated rats, was a decrease in the choice of the large reward upon an imposed delay of 15 s assessed in a T-maze-based cost-benefit decision-making paradigm, suggestive of impulsive decision making. Spontaneous locomotion in the open field was not altered, suggesting no changes in exploratory behavior. Furthermore, rats treated with shA_{2A}R in the PLmPFC also displayed a tendency for higher anxiety levels in the elevated plus maze (less entries in the open arms), but not in the open field test (time spent in the center was not affected). Finally, working memory performance was not significantly altered, as revealed by the spontaneous alternation in the Y-maze test and the latency to reach the platform in the repeated trial Morris water maze. These findings constitute the first direct demonstration of a role of PFC A_{2A}R in the control of behavior in physiological conditions, showing their major contribution for the control of delay-based cost-benefit decisions.

Keywords: adenosine A_{2A} receptors, impulsive choice, prefrontal cortex (PFC), anxiety, working memory, cost-benefit decision making

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INTRODUCTION

Adenosine A_{2A} receptors (A_{2A}Rs) are mostly known to control long-term synaptic plasticity throughout the brain (reviewed in Cunha, 2016), namely in the prefrontal cortex (PFC) where they facilitate long-term potentiation (LTP) in excitatory synapses onto fast spiking interneurons and control network activity (Kerkhofs et al., 2018). The PFC mediates cognitive and executive functions including working memory, attention and inhibitory control

Caffeine and cannabinoid receptors modulate impulsive behavior in an animal model of Attentional Deficit and Hyperactivity Disorder

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Caffeine and cannabinoid receptors modulate impulsive behavior in an animal model of Attentional Deficit and Hyperactivity Disorder

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ABSTRACT

Attention deficit and hyperactivity disorder (ADHD) is characterized by impaired levels of hyperactivity, impulsivity and inattention. Adenosine and endocannabinoid systems tightly interact in the modulation of dopamine signaling, involved in the neurobiology of ADHD. In this study, we evaluated the modulating effects of the cannabinoid and adenosine systems in a tolerance to delay of reward task using the most widely used animal model of ADHD. Spontaneous Hypertensive Rats (SHR) and Wistar-Kyoto (WKY) rats were treated chronically or acutely with caffeine, a non-selective adenosine receptor antagonist, or acutely with a cannabinoid agonist (WIN55212-2, WIN) or antagonist (AM251). Subsequently, animals were tested in the tolerance to delay of reward task, in which they had to choose between a small, but immediate, or a large, but delayed, reward. Treatment with WIN decreased while treatment with AM251 increased the choices of the large reward, selectively in SHR rats, indicating a CB1 receptor-mediated increase of impulsive behavior. An acute pretreatment with caffeine blocked WIN effects. Conversely, a chronic treatment with caffeine increased the impulsive phenotype and potentiated the WIN effects. The results indicate that both cannabinoid and adenosine receptors modulate impulsive behavior in SHR: the antagonism of cannabinoid receptors might be effective in reducing impulsive symptoms present in ADHD; in addition, caffeine showed the opposite effects on impulsive behavior depending on the length of treatment. These observations are of particular importance to consider when therapeutic manipulation of CB1 receptors is applied to ADHD patients who consume coffee.

10.2. ARTIGOS PUBLICADOS DURANTE O PERÍODO DE DOUTORAMENTO CUJOS TEMAS NÃO SE RELACIONAM DIRETAMENTE A ESTA TESE

Preclinical to Clinical Translation of Studies of Transcranial Direct-Current Stimulation in the Treatment of Epilepsy: A Systematic Review.

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SYSTEMATIC REVIEW
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Preclinical to Clinical Translation of Studies of Transcranial Direct-Current Stimulation in the Treatment of Epilepsy: A Systematic Review

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Epilepsy is a chronic brain syndrome characterized by recurrent seizures resulting from excessive neuronal discharges. Despite the development of various new antiepileptic drugs, many patients are refractory to treatment and report side effects. Non-invasive methods of brain stimulation, such as transcranial direct current stimulation (tDCS), have been tested as alternative approaches to directly modulate the excitability of epileptogenic neural circuits. Although some pilot and initial clinical studies have shown positive results, there is still uncertainty regarding the next steps of investigation in this field. Therefore, we reviewed preclinical and clinical studies using the following framework: (1) preclinical studies that have been successfully translated to clinical studies, (2) preclinical studies that have failed to be translated to clinical studies, and (3) clinical findings that were not previously tested in preclinical studies. We searched PubMed, Web of Science, Embase, and SciELO (2002–2017) using the keywords "tDCS," "epilepsy," "clinical trials," and "animal models." Our initial search resulted in 64 articles. After applying inclusion and exclusion criteria, we screened 17 full-text articles to extract findings about the efficacy of tDCS, with respect to the therapeutic framework used and the resulting reduction in seizures and epileptiform patterns. We found that few preclinical findings have been translated into clinical research (number of sessions and effects on seizure frequency) and that most findings have not been tested clinically (effects of tDCS on status epilepticus and absence epilepsy, neuroprotective effects in the hippocampus, and combined use with specific medications). Finally, considering that clinical studies on tDCS have been conducted for several epileptic syndromes, most were not previously tested in preclinical studies (Rasmussen's encephalitis, drug resistant epilepsy, and hippocampal sclerosis-induced epilepsy). Overall, most studies report

Systemic Inflammation as a Driver of Brain Injury: the Astrocyte as an Emerging Player.

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Systemic Inflammation as a Driver of Brain Injury: the Astrocyte as an Emerging Player

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ABSTRACT

Severe systemic inflammation has strong effects on brain functions, promoting permanent neurocognitive dysfunction and high mortality rates. Additionally, hippocampal damage seems to be directly involved in this process and astrocytes play an important role in neuroinflammation and in the neuroimmune response. However, the contribution of the astrocytes to the pathology of acute brain dysfunction is not well understood. Recently, our group established a protocol for obtaining astrocyte cultures from mature brain to allow the characterization of these cells and their functions under pathologic conditions. The present study was designed to characterize astrocyte function after acute systemic inflammation induced by cecal ligation and perforation (CLP). Hippocampal astrocyte cultures from CLP animals presented increased levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-18, and cyclooxygenase-2 and decreased levels of IL-10. This proinflammatory profile was accompanied by an increase in Toll-like receptor (TLR)2 mRNA expression levels and no change either in TLR4 or in vascular endothelial growth factor (VEGF) gene expression. These alterations were associated with increased expressions of p21, nuclear factor kappa B (NFκB), and inducible nitric oxide synthase (iNOS) in astrocytes from CLP animals. The same parameters were also evaluated in whole hippocampal tissue, but differences in this profile were found compared to hippocampal astrocyte cultures from CLP, reflecting an interaction between other central nervous system cell types, which may mask specific astrocytic changes. These results improve our understanding of the mechanisms by which astrocytes react against systemic inflammation, and suggest these cells to be potential targets for therapeutic modulation.

Olfactory bulbectomy in mice triggers transient and long-lasting behavioral impairments and biochemical hippocampal disturbances.

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Olfactory bulbectomy in mice triggers transient and long-lasting behavioral impairments and biochemical hippocampal disturbances

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ABSTRACT

Major depressive disorder (MDD) is a neuropsychiatric disease that is associated with profound disturbances in affected individuals. Elucidating the pathophysiology of MDD has been frustratingly slow, especially concerning the neurochemical events and brain regions associated with disease progression. Thus, we evaluated the time-course (up to 8 weeks) behavioral and biochemical effects in mice that underwent to a bilateral olfactory bulbectomy (OBX), which is used to modeling depressive-like behavior in rodents. Similar to the symptoms in patients with MDD, OBX induced long-lasting (e.g., impairment of habituation to novelty, hyperactivity and an anxiety-like phenotype) and transient (e.g., loss of self-care and motivational behavior) behavioral effects. Moreover, OBX temporarily impaired hippocampal synaptosomal mitochondria, in a manner that would be associated with hippocampal-related synaptotoxicity. Finally, long-lasting pro-oxidative (i.e., increased levels of reactive oxygen species and nitric oxide and decreased glutathione levels) and pro-inflammatory (i.e., increased levels of pro-inflammatory cytokines IL-1, IL-6, TNF-α and decreased anti-inflammatory cytokine IL-10 levels) effects were induced in the hippocampus by OBX. Additionally, these parameters were transiently affected in the posterior and frontal cortices. This study is the first to suggest that the transient and long-lasting behavioral effects from OBX strongly correlate with mitochondrial, oxidative and inflammatory parameters in the hippocampus; furthermore, these effects show a weak correlation with these parameters in the cortex. Our findings highlight the underlying mechanisms involved in the biochemical time course of events related to depressive behavior.