UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM NEUROCIÊNCIAS

RELAÇÃO ENTRE A HIPÓXIA-ISQUEMIA PERINATAL E O PAPEL DA DOPAMINA NO DESENVOLVIMENTO DE CARACTERÍSTICAS RELACIONADAS AO TRANSTORNO DE DEFICIT DE ATENÇÃO/HIPERATIVIDADE: UMA ABORDAGEM TRANSLACIONAL

Patrícia Maidana Miguel

Porto Alegre
2019

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Patrícia Maidana Miguel

Tese apresentada como requisito parcial à obtenção do título de Doutor em Neurociências pelo Programa de Pós-graduação em Neurociências da Universidade Federal do Rio Grande do Sul.

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Uma vez que você para de aprender,
você começa a morrer

Albert Einstein

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Apresentação

Esta tese está organizada em 3 Partes, divididas da seguinte maneira:

Parte I: Lista de abreviaturas, Lista de tabelas, Resumo, Resumo em inglês (*abstract*), Introdução e Objetivos;

Parte II: Resultados em forma de Capítulos (Capítulos 1 a 5), onde em cada capítulo apresentamos um breve prefácio seguido de um artigo científico;

Parte III: Discussão, Conclusões, Perspectivas, Bibliografia e Anexos.

Os experimentos apresentados nos Capítulos 1 e 2 foram realizados no Departamento de Ciências Morfológicas localizado no Instituto de Ciências Básicas da Saúda da Universidade Federal do Rio Grande do Sul (UFRGS) com aprovação pelo Comitê de Ética de Uso de Animais desta universidade (n. 29750 – ANEXO 1). O experimento realizado no Capítulo 3 foi realizado na Unidade de Experimentação Animal do Hospital de Clínicas de Porto Alegre, onde também foi aprovado pelo Comitê de Ética desta instituição (n. 150566 – ANEXO 2). O experimento referente ao Capítulo 4 foi desenvolvido no *Douglas Mental Health University Institute* vinculado à McGill University (Canadá), sob orientação dos professores Michael Meaney e Patrícia Pelufo Silveira.

Lista de abreviaturas

5-CSRTT – *5-choice serial reaction time task*
APGAR – Índice de APGAR do recém-nascido
ASST – *Attentional set-shifting task*
BDNF – Fator neurotrófico derivado do encéfalo
COMT – Catecol O-Metiltransferase
CPF – Córtex pré-frontal
D1-D5 – Receptores dopaminérgicos
DA – Dopamina
DAT – Transportador de dopamina
DAT1 – Gene do transportador de dopamina
DCCS – *Dimensional Change Card Sort*
DSM – Manual Diagnóstico e Estatístico de Transtornos Mentais
ePRS – escore de “risco” poligênico baseado na expressão
GWAS – Estudos de associação ampla do genoma
HI – Hipóxia-isquemia
HICs – Condições hipóxico-isquêmicas perinatais
IA2BC – *Interminent access to 2-botttle choice*
IED – *Intra-/Extra-dimensional Set Shift*
KO – *Knock-out*
MFD – Metilfenidato
NAcc – Núcleo accumbens
NE – Noradrenalina
NET – Transportador de noradrenalina
PRS – Escore de risco poligênico
pTH – Tirosina hidroxilase fosforilada
SHR – *Spontaneously hypertensive rat*
SNC – Sistema nervoso central
SNP – Polimorfismos de nucleotídeo único
TDAH – Transtorno de deficit de atenção/hiperatividade

TH – Tirosina hidroxilase

VMAT – Transportador vesicular de monoaminas

VNTR – Número variável de repetições em série

VTA – Área tegmental ventral

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Resumo

O Transtorno de deficit de atenção/hiperatividade (TDAH) é uma desordem do neurodesenvolvimento ocasionada por fatores genéticos, ambientais ou pela interação entre esses fatores. A exposição a condições hipóxico-isquêmicas perinatais (HICs) é constantemente associada ao TDAH, mas pouco se sabe sobre os mecanismos envolvidos nessa relação e o efeito da interação entre esses fatores com o perfil genético dos indivíduos para o desenvolvimento do transtorno. Dessa forma, propusemos validar o uso do modelo de hipóxia-isquemia (HI) neonatal de Rice-Vannucci em ratos para o estudo do TDAH experimental, avaliando as validades aparente, de construto e preditiva (utilizando o fármaco metilfenidato, MFD). Em um estudo clínico, investigamos se o perfil genético funcionalmente associado a menor transmissão dopaminérgica no córtex pré-frontal (CPF) de crianças interage com a exposição a HICs alterando a flexibilidade cognitiva e a densidade da substância cinzenta cerebral. A validade aparente do modelo de HI para o estudo do TDAH foi confirmada pelos seguintes resultados: inflexibilidade cognitiva, deficit de memória de curta e longa duração em ratos jovens e comportamento alimentar desregulado e aumento no consumo do álcool na fase adulta. Alterações nos parâmetros da sinalização dopaminérgico no CPF dos ratos HI apoiaram a validade de construto desse modelo. O tratamento com MFD melhorou os deficit de flexibilidade cognitiva, de memória de longa duração, o comportamento alimentar desregulado e também diminuiu o consumo de álcool em ratos HI – embasando a validade preditiva deste modelo. Um aumento na expressão da enzima pTH (envolvida na síntese de dopamina) no CPF e dos níveis de BDNF maduro no hipocampo de ratos HI tratados com MFD podem ter colaborado para estas melhorias funcionais. Na pesquisa clínica, demonstramos que a exposição a HICs prejudicou a flexibilidade cognitiva apenas de crianças que apresentavam o perfil genético funcionalmente associado a maior recaptação dopaminérgica no CPF (relacionada à rede genética do gene *DAT1*). Ainda, a exposição a HICs alterou a relação entre os polimorfismos genéticos relacionados à rede do *DAT1* e a densidade de substância cinzenta em áreas envolvidas em funções executivas e integrativas. Dessa forma, os resultados pré-clínicos desta tese demonstraram que o modelo de HI de Rice-Vannucci apresenta validades aparente, de construto e preditiva para o estudo do TDAH, contribuindo para a área dos estudos

experimentais, mas também confirmando os estudos clínicos que apontam a relação entre a HI perinatal e o desenvolvimento do TDAH. No estudo com crianças, reportamos que o perfil genético associado com a recaptação dopaminérgica no CPF é um importante moderador dos efeitos das HICs nos deficit de função executiva e desenvolvimento encefálico. Assim, nossos resultados demonstraram que variações nos níveis de oxigenação perinatais podem contribuir para o desenvolvimento de características relacionadas ao TDAH, alertando sobre a importância de medidas preventivas que melhorem as condições intrauterinas e periparto para evitar distúrbios no neurodesenvolvimento.

Palavras-chave: Hipóxia-isquemia; Transtorno de deficit de atenção/hiperatividade; TDAH; dopamina; BDNF; flexibilidade cognitiva; DAT1.

Abstract

Attention-deficit/hyperactivity disorder (ADHD) is a neurodevelopmental disorder caused by genetic or environmental factors or by the interaction between these factors. Exposure to perinatal hypoxic-ischemic conditions (HICs) is constantly associated with ADHD in the literature but little is known about the mechanisms involved in this relationship and the effect of the interaction between these factors and the individual genetic profile for the development of the disorder. Thus, we proposed to validate the use of the Rice-Vannucci neonatal model of hypoxia-ischemia (HI) in rats as an alternative model for the preclinical ADHD study, evaluating the face validity, construct validity and predictive validity (using the drug methylphenidate, MPH) of this model. Additionally, in a clinical study we investigated whether the genetic profile functionally associated with lower dopaminergic transmission in the prefrontal cortex (PFC) of children interacts with exposure to HICs influencing cognitive flexibility and brain gray matter density. The face validity of the HI model for the ADHD study was confirmed by the following results: cognitive inflexibility, short- and long-term memory deficit in young rats and dysregulated eating behavior and increase in alcohol consumption in adult rats. Alterations in the dopaminergic signaling parameters in the PFC of HI rats supported the construct validity of this model. MPH treatment improved cognitive flexibility, long-term memory deficit, dysregulated eating behavior and also decreased alcohol consumption in HI rats – demonstrating the predictive validity of this model. An increase in the expression of the phosphorylated tyrosine hydroxylase enzyme (pTH; involved in dopamine synthesis) in the PFC and in mature BDNF levels in the hippocampus of HI rats treated with MPH may have contributed to these functional improvements. In the clinical study, we demonstrated that exposure to HICs impaired cognitive flexibility only in children with the genetic background functionally associated with increased dopamine reuptake in the PFC (related to the DAT1 gene network). Furthermore, exposure to HICs altered the relationship between the genetic polymorphisms related to the DAT1 network and gray matter density in areas involved in executive and integrative functions. Therefore, the preclinical results of this thesis have demonstrated that the Rice-Vannucci HI model presents face, construct and predictive validities for the ADHD study, contributing to the preclinical ADHD field, but also confirming the clinical studies that indicate the relationship between perinatal HI and

the development of ADHD. In the clinical study, we reported that the genetic profile associated with dopaminergic reuptake in the PFC is an important moderator of the effects of HICs on executive function deficit and brain development. In this way, our results indicated that variations in perinatal oxygenation levels may contribute to the development of ADHD-related characteristics, highlighting the significance of preventive measures to improve intrauterine and intrapartum health, avoiding disturbances in the neurodevelopment.

Keywords: hypoxia-ischemia; attention-deficit/hyperactivity disorder; ADHD; dopamine; BDNF; cognitive flexibility; DAT1.

1. INTRODUÇÃO

1.1 **TRANSTORNO DE DEFICIT DE ATENÇÃO/HIPERATIVIDADE (TDAH)**

O Transtorno de deficit de atenção/hiperatividade (TDAH) é a desordem do neurodesenvolvimento mais comumente diagnosticada na infância (Centers for Disease Control and Prevention, 2013), tendo como característica um padrão persistente de desatenção e/ou hiperatividade-impulsividade em um nível maior ao esperado naquele nível de desenvolvimento (American Psychiatric Association, 2013). Embora seja um transtorno típico da infância, este também é observado em adolescentes e adultos e já foi demonstrado que 60% das crianças diagnosticadas com TDAH persistirão com os sintomas ainda na idade adulta (Sibley *et al.*, 2017).

De acordo com as sete revisões sistemáticas conduzidas para se avaliar a prevalência mundial do TDAH, há uma estimativa de prevalência que varia entre 2,2 e 7,2% (Sayal *et al.*, 2018). Na intenção de minimizar as diferenças metodológicas utilizadas em diferentes países, a Organização Mundial da Saúde vem realizando a Pesquisa Mundial Sobre Saúde Mental para obter dados sobre prevalência e condições de transtornos mentais em adultos ao redor do mundo. Nesta pesquisa realizada em 20 países, foi demonstrado que a média de prevalência do TDAH em crianças é de 2,2%, mas teve uma faixa de prevalência muito ampla, variando de 0,1 a 8,1% (Fayyad *et al.*, 2017). Nos estudos realizados no Brasil, essa taxa também varia amplamente entre 5,8 e 17,1% (Rohde *et al.*, 2000; Vasconcelos *et al.*, 2003; Fontana *et al.*, 2007). Discrepâncias entre esses valores podem ter relação com o tipo de amostra, com os instrumentos e critérios diagnósticos adotados e com a fonte das informações obtidas no processo de avaliação diagnóstica (pacientes, pais e/ou professores) (Rohde *et al.*, 2000). De fato, já foi demonstrado que as taxas de prevalência tendem a ser maiores quando as amostras são provenientes de escolas ao invés da comunidade, como foi observado pelas altas taxas de 13% e 17,1% em escolares brasileiros (Vasconcelos *et al.*, 2003; Fontana *et al.*, 2007).

O diagnóstico do TDAH é realizado através de avaliação clínica, sendo utilizado como principal ferramenta o *Manual Diagnóstico e Estatístico de Transtornos Mentais* (American Psychiatric Association, 2013). Para o diagnóstico ser confirmado, os pacientes precisam reportar um mínimo de seis sintomas de desatenção (ex.: ter

dificuldade de sustentar a atenção em tarefas ou atividades lúdicas) ou de hiperatividade/impulsividade (ex.: dificuldade em esperar pela sua vez) com persistência nos sintomas por pelo menos seis meses e sendo aparentes em pelo menos dois ambientes diferentes. Dessa forma, três diferentes apresentações do TDAH podem ser distinguidas com este manual: tipo predominantemente desatento, predominantemente hiperativo-impulsivo e o tipo combinado (desatento com impulsividade/hiperatividade). Já foi demonstrado que o tipo combinado é o mais frequente, enquanto o tipo predominantemente hiperativo-impulsivo é o mais raro (Rohde e Jellinek, 2002; Fontana *et al.*, 2007).

Diferentes autores propõem que os sintomas clássicos do TDAH sejam decorrentes de um deficit primário das funções executivas (Barkley, 1997; Castellanos e Tannock, 2002). A função executiva pode ser definida como o produto da operação coordenada de vários processos com o intuito de atingir uma meta específica de maneira flexível. Dentre estes processos, podemos citar planejamento, flexibilidade cognitiva, controle inibitório e memória de trabalho (Castellanos e Tannock, 2002; Roth e Saykin, 2004). A memória de trabalho refere-se à manipulação ativa e "de cima para baixo" das informações armazenadas na memória de curto prazo. Esse tipo de memória desempenha um papel crítico na orientação do comportamento cotidiano e parece ser fundamental para regular o planejamento necessário para executar uma tarefa com objetivo específico (Kofler *et al.*, 2018). Relevante para o TDAH pediátrico, a memória de trabalho serve como uma interface entre o ambiente e a memória de longo prazo (Baddeley, 2007) e é fundamental para uma infinidade de habilidades de aprendizagem, incluindo anotações (Mcintyre, 2014), compreensão auditiva (Mcinnes *et al.*, 2003) e seguir instruções (Jaroslawska *et al.*, 2016). Já a flexibilidade cognitiva refere-se à capacidade de redirecionar a atenção para estímulos relevantes quando ocorrem apresentações de informações conflitantes, com o objetivo de atingir a meta específica estipulada (Jacques e Zelazo, 2005; Diamond, 2006).

Alguns testes são amplamente utilizados na clínica para a avaliação das funções executivas, sendo a versão padrão do *Dimensional Change Card Sort* (DCCS) capaz de detectar alterações nesses processos em crianças entre a faixa etária de 3 e 5 anos. Nesse teste, cartas que contenham duas dimensões diferentes – cores (vermelho ou

azul) ou formas (coelho ou barco) – são apresentadas às crianças. Em um primeiro momento, as crianças precisam classificar as cartas por cor, independente da forma apresentada. No entanto, em um segundo estágio a regra muda e as cartas precisam ser classificadas pela forma, ignorando a regra anterior que estava sendo utilizada (Zelazo, 2006). Testes mais complexos e específicos foram desenvolvidos, como o *Intra-/Extra-dimensional Set Shift (IED)* que avalia mudanças atencionais dentro de uma mesma dimensão (por exemplo, avaliando a performance em relação a diferentes cores) ou entre diferentes dimensões (da cor para a forma, como ocorre no DCCS). Como esse é um teste mais complexo, é adequado para crianças com uma idade superior aos 6 anos (Luciana e Nelson, 2002). E como esperado, crianças com TDAH já demonstraram pior performance nessas tarefas (Papazian *et al.*, 2009; Coghill *et al.*, 2014; Agha *et al.*, 2017).

Comorbidades, definido como a presença de dois ou mais diagnósticos no mesmo indivíduo, são frequentemente relatadas em pacientes com TDAH, sendo considerado mais a regra do que a exceção (Barkley, 2014). Dificuldades nos processos de aprendizagem são especialmente relatados, uma vez que a atenção seletiva é fundamental para que ocorra a aprendizagem. Foi demonstrado que crianças com TDAH apresentam uma chance três vezes maior de ter um transtorno de aprendizagem quando comparado a seus colegas que não apresentam TDAH (Dupaul *et al.*, 2013) e ainda que estes indivíduos apresentam uma maior probabilidade de repetência, suspensão e expulsão escolar (Rohde *et al.*, 1999). Nesses casos, acredita-se que a impulsividade desses indivíduos possa influenciar em possíveis desvios de conduta, bem como possam ter uma relação com comportamentos aditivos. Durante o período da adolescência e na fase adulta, já é bem conhecida a ocorrência de um risco maior para os portadores de TDAH apresentarem uso abusivo de substâncias psicoativas e transtornos alimentares que podem levar à obesidade (Lee *et al.*, 2011; Cortese e Tessari, 2017; Brunault *et al.*, 2019). Outros transtornos frequentemente reportados em indivíduos com TDAH incluem transtornos de sono, transtorno desafiante opositor e transtornos de ansiedade (Reale *et al.*, 2017). Como observado, o diagnóstico de TDAH tem um grande impacto na vida dos portadores, com os riscos se estendendo até a vida adulta. Uma vez que o TDAH é o

transtorno comportamental mais comum da infância, o mesmo é considerado um importante problema de saúde pública (Stephen Hinshaw & Katherine Ellison, 2016).

1.1.1 Etiologia do TDAH

O TDAH é conhecido pela sua etiologia multifatorial. Foi demonstrado que os fatores genéticos contribuem substancialmente na etiologia do transtorno (∼70–80%), embora os fatores ambientais também tenham bastante relevância, podendo contribuir em ∼20–30% dos casos (Biederman e Faraone, 2005). Ainda, complexas interações gene-ambiente podem contribuir para o desenvolvimento do transtorno (Palladino *et al.*, 2019).

1.1.1.1 Base genética do TDAH

Embora a taxa de hereditariedade do TDAH seja uma das mais altas entre os transtornos psiquiátricos (∼76%), apenas em 2019 foram descritos os primeiros 12 polimorfismos associados ao TDAH que atingiram significância estatística a nível do genoma amplo (Demontis *et al.*, 2019). Até então, o que a maioria dos estudos vinha demonstrando é que parece ocorrer uma interação entre múltiplas variações genéticas, cada uma com um pequeno efeito no risco para o desenvolvimento do transtorno (Thapar *et al.*, 2013). A grande maioria dos estudos de base genética no TDAH têm focado em genes associados com a sinalização dopaminérgica, uma vez que o sistema dopaminérgico apresenta um papel central na fisiopatologia do TDAH. Diferentes polimorfismos nos genes dos receptores dopaminérgicos, da enzima Catecol O-Metiltransferase (COMT – responsável por degradar catecolaminas), e do transportador de dopamina (DAT1 – que recapta a dopamina da fenda sináptica) - foram associados com o diagnóstico de TDAH em estudos de gene candidato (Gizer *et al.*, 2009).

O transportador de dopamina (DAT) é uma proteína transmembrana responsável pela recaptação da dopamina (DA) da fenda sináptica de volta para o neurônio pré-sináptico, encerrando dessa forma com a sinalização de DA (Giros e Caron, 1993). A importância do DAT é comprovada pelo fato de ele ser alvo de diferentes terapias farmacológicas que aumentam a transmissão de DA, como é o caso do Metilfenidato

(MFD) para o tratamento do TDAH (Minzenberg, 2012; Vaughan e Kratochvil, 2012). Por estes motivos, o gene do transportador de dopamina (DAT1/SLC6A3) tem recebido muita atenção como um potencial fator de risco para diferentes doenças psiquiátricas, incluindo o TDAH (Franke *et al.*, 2012). Diferentes polimorfismos no DAT1 já foram associados com o diagnóstico do TDAH, sendo esses polimorfismos tanto do tipo VNTR (número variável de repetições em série, do inglês *variable number of tandem repeats*) assim como SNP (polimorfismos de nucleotídeo único, do inglês *single nucleotide polymorphisms)* (Franke *et al.*, 2012).

Embora os estudos de genes candidatos sejam importantes para entender a contribuição de possíveis genes na susceptibilidade para determinadas doenças, eles têm a limitação de se ter um conhecimento prévio sobre o impacto funcional daquele gene na doença ou traço comportamental investigado. Nesse contexto, os estudos de associação ampla do genoma (GWAS, do inglês *genome-wide association study*) começaram a ser desenvolvidos, onde o genoma inteiro de pacientes com determinado fenótipo é comparado com o de indivíduos saudáveis, na busca de polimorfismos do tipo SNP que estejam significantemente associados aquele fenótipo. Assim, GWAS foram um importante avanço para a identificação de variações genéticas que influenciam determinado traço ou doença – possuindo vantagens em relação aos estudos que investigam variações específicas em genes pré-determinados (candidatos). O primeiro GWAS foi publicado em 2002, e desde então esse campo de investigação tem crescido exponencialmente (Ikegawa, 2012). A partir do GWAS, pode-se calcular o escore de risco poligênico (PRS, do inglês *polygenic risk score)* para determinada doença. Uma limitação que se apresenta é que as metodologias dos GWAS e PRS não consideram o fato de que os genes operam em redes, codificando para funções biológicas precisas e em tecidos específicos. Assim, novas abordagens que avaliam polimorfismos em genes que operam na mesma rede genética têm recebido atenção recentemente (Pergola *et al.*, 2017; Silveira *et al.*, 2017; Hari Dass *et al.*, 2019). Uma recente revisão compilou os avanços dos estudos genéticos no TDAH, onde demonstrou que os estudos de redes genéticas parecem promissores caminhos para desordens tão complexas como o TDAH (Hayman e Fernandez, 2018)

1.1.1.2 Fatores ambientais associados ao TDAH

Os fatores ambientais também possuem um papel importante na etiologia do TDAH, podendo afetar tanto de forma direta no desenvolvimento do transtorno, assim como por interações com o perfil genético dos indivíduos. Os fatores ambientais mais reportados ocorrem no período gestacional ou periparto, considerado um momento de grande vulnerabilidade ao encéfalo em pleno desenvolvimento (Sciberras *et al.*, 2017). O tabagismo durante a gestação tem recebido muito destaque como um importante fator ambiental associado ao desenvolvimento do TDAH na prole; dois recentes estudos de meta-análise confirmaram a associação entre esses dois fatores (Dong *et al.*, 2018; Huang *et al.*, 2018). Acredita-se que o tabaco afete diretamente a oxigenação fetal, uma vez que o monóxido de carbono presente no tabaco se liga com maior afinidade à hemoglobina do que o oxigênio, resultando em carboxiemoglobina - que já se mostrou estar presente em altos níveis tanto no sangue da mãe quanto do filho (Morrow *et al.*, 1988; Soothill *et al.*, 1996). Diminuição no aporte de oxigênio é um dos fatores mais danosos ao feto em desenvolvimento. Nesse contexto, pode ocorrer uma asfixia perinatal, que é definida quando há comprometimento nas trocas gasosas por diminuição de aporte de oxigênio (hipóxia) e/ou interrupção do fluxo sanguíneo (isquemia) (Herrera e Silver, 2016; Laptook, 2016). Desta forma, muitas vezes o termo hipóxia-isquemia (HI) é usado como um sinônimo para asfixia perinatal.

Ruptura uterina, descolamento de placenta e compressão/prolapso do cordão umbilical são alguns dos fatores que podem levar à HI perinatal (Herrera e Silver, 2016). Outros fatores que podem afetar a oxigenação/fluxo sanguíneo incluem o posicionamento do feto no momento do parto, sendo as posições pélvica e transversa as mais preocupantes, e a distocia fetal, uma condição onde ocorre dificuldade de expulsão do feto do meio intrauterino para o meio externo (Hofmeyr, 1992). A observação clínica do recém-nascido imediatamente após o nascimento é de suma importância para reconhecer se o neonato sofreu HI perinatal. Nesse sentido, o índice de APGAR é o primeiro exame realizado no recém-nascido, onde se avaliam cinco sinais físicos que refletem o seu estado clínico no 1º, 5º e 10º minuto após o nascimento. Os sinais avaliados são: coloração da pele, frequência cardíaca, irritabilidade reflexa, tônus

muscular e esforço respiratório. Para cada um dos cinco itens é atribuída uma nota de 0 a 2, onde no final são somadas as notas de todos os itens para se obter uma nota final de no máximo 10 pontos (American Academy of Pediatrics Committee On *et al.*, 2015) (Figura 1). O índice de APGAR quando inferido no primeiro minuto irá informar sobre variabilidade na oxigenação e nas trocas gasosas entre a mãe e o feto na hora do parto (Fahey e King, 2005; Omo-Aghoja, 2014). Dessa forma, índice de APGAR <7 no 1º minuto é um indicativo de asfixia perinatal, sendo um valor entre 0-3 considerado asfixia perinatal grave e entre 4-7, leve a moderada (ICD-10, 2016). Ainda, outros sinais como desconforto respiratório e a utilização de manobras de reanimação neonatal também são importantes indícios de danos intercorrentes na hora do parto (Berglund *et al.*, 2008).

Conforme exposto acima, podemos observar que diferentes fatores podem contribuir para o desencadeamento da HI perinatal e que muitas vezes esses fatores são inter-relacionados. Ainda, considerando que não existe um diagnóstico específico e claro para a HI perinatal, a análise conjunta do histórico clínico e das complicações do parto podem ser consideradas uma abordagem mais confiável no diagnóstico da HI perinatal.

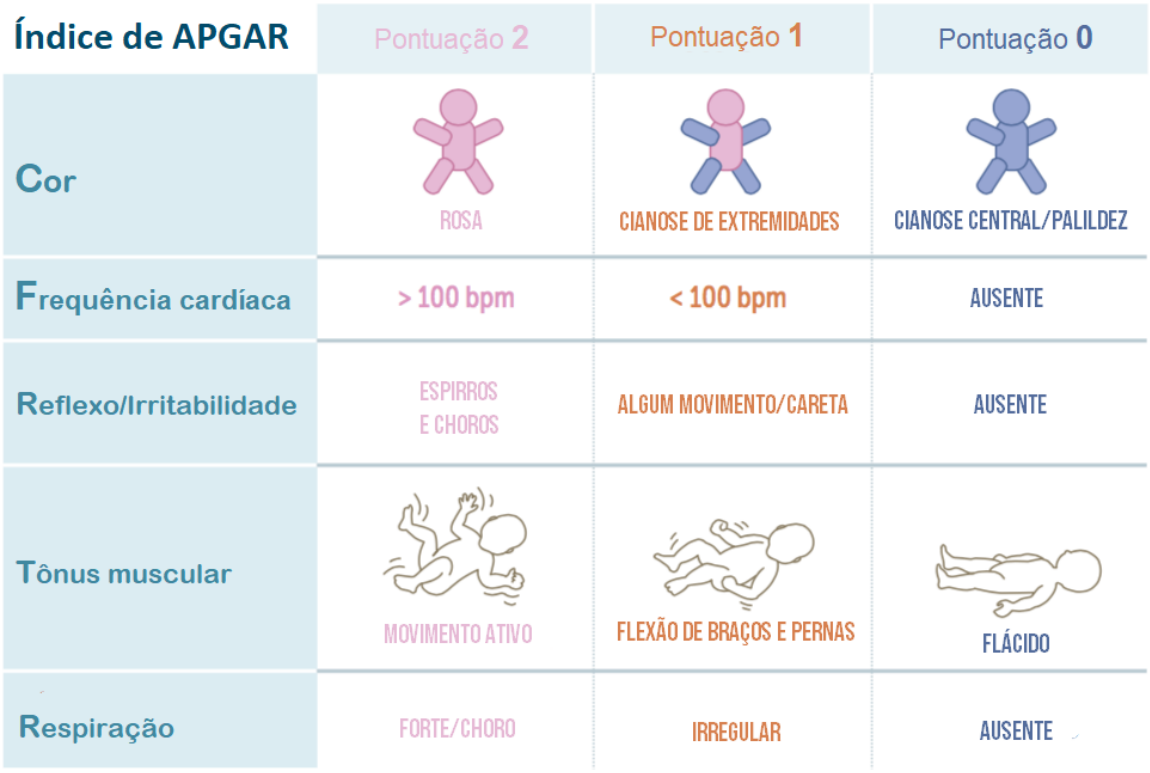

Figura 1: Índice de APGAR do recém-nascido. Figura adaptada do site Grepmed.

Dos neonatos sobreviventes à HI perinatal, uma grande parcela apresentará sequelas persistentes que serão notadas ao longo do desenvolvimento da criança. Por exemplo, Getahun e colaboradores demonstraram que crianças em idade escolar (entre 5 e 11 anos) que foram expostas a condições hipóxico-isquêmicas perinatais tiveram um risco aumentado para o diagnóstico de TDAH. Ainda, estes autores reportaram que algumas destas condições elevam o risco para o TDAH de forma independente, como a asfixia perinatal, a síndrome do desconforto respiratório neonatal e a pré-eclâmpsia – aumento em 26%, 47% e 34%, respectivamente (Getahun *et al.*, 2013). Uma meta-análise que incluiu dez estudos caso-controle (45.821 casos e 9.207.363 controles) confirmou a relação entre eventos hipóxico-isquêmicos perinatais e o diagnóstico de TDAH e também apontou a independência de alguns fatores nessa relação, como: pré-eclâmpsia, índice de APGAR <7 no 5º minuto, apresentação pélvica/transversa e compressões no cordão umbilical (prolapso/enrolado no pescoço) (Zhu *et al.*, 2016). Ainda, baixo índice de APGAR no 1º minuto também foi associado com um maior risco para o diagnóstico de TDAH (Sucksdorff *et al.*, 2018). Embora a associação entre a asfixia perinatal e o desenvolvimento de características relacionadas ao TDAH seja constantemente apontada na literatura, são escassos os estudos que investigam os mecanismos biológicos envolvidos nessa relação, tanto em pesquisa clínica quanto pré-clínica. Ainda, a maioria dos estudos avalia diferentes fatores associados à HI de forma isolada, apesar de se saber que esses fatores muitas vezes ocorrem de forma inter-relacionada, afetando de forma diferente o feto em desenvolvimento. Sendo assim, análises de índices cumulativos de múltiplos fatores associados à HI perinatal são propostos como uma melhor medida de complicações hipóxico-isquêmicas perinatais. Ademais, precisamos considerar que complexas interações entre a exposição a fatores ambientais e o perfil genético do individuo podem ser fundamentais para o desenvolvimento do transtorno.

1.1.2 Fisiopatologia do TDAH

Embora não exista um perfil fisiopatológico único no TDAH, os estudos confirmam um envolvimento central do córtex pré-frontal (CPF) nesse transtorno. O CPF continua a

se desenvolver até a idade adulta e é uma das últimas regiões cerebrais a amadurecer por completo (Gogtay *et al.*, 2004). Essa estrutura apresenta um papel fundamental na regulação das funções executivas, inibindo o processamento de estímulos irrelevantes (e aumentando o processamento daqueles importantes) assim como inibindo ações inapropriadas e/ou impulsivas (Arnsten e Pliszka, 2011). Dessa forma, alterações no CPF observadas nos portadores de TDAH parecem influenciar diretamente nos sintomas de desatenção e hiperatividade/impulsividade. Já foram demonstrados um menor volume e menor atividade do CPF em indivíduos com TDAH quando comparado a pessoas sadias (Rubia *et al.*, 1999; Seidman *et al.*, 2005; Castellanos *et al.*, 2008), assim como um atraso na maturação dessa estrutura (Shaw *et al.*, 2007). Os tratos que conectam o CPF a outras regiões encefálicas também se mostraram menos organizados e com menor conectividade funcional em indivíduos com TDAH (Casey *et al.*, 2007; Castellanos *et al.*, 2008; Makris *et al.*, 2008). Alterações também são geralmente reportadas em estruturas que apresentam conexão recíproca com o CPF, como os córtices associativos (parietal, temporal), o estriado e o cerebelo (Castellanos e Tannock, 2002; Cubillo *et al.*, 2012). Ainda, foi demonstrado que crianças com TDAH apresentam uma menor atividade no estriado ventral em resposta à recompensa, ao contrário do que é observado em crianças saudáveis (Scheres *et al.*, 2007; Plichta e Scheres, 2014), sendo sugerido que essa hiporresponsividade possa aumentar o comportamento de busca pela recompensa para assim compensar a menor ativação no estriado ventral (Scheres *et al.*, 2007).

As funções executivas regidas pelo CPF, assim como a resposta a recompensas no estriado ventral, são estritamente reguladas pela transmissão dopaminérgica nessas regiões. A dopamina (DA) é um neurotransmissor da classe das catecolaminas, sintetizada a partir do aminoácido tirosina pela ação sequencial de duas enzimas: tirosina hidroxilase (TH) e dopa-descarboxilase. Quando é liberada no meio extracelular, a DA pode se ligar a cinco diferentes tipos de receptores – subdivididos na família de receptores do tipo D1 (D1, D5) e D2 (D2, D3, D4). O transportador de dopamina (DAT) é encarregado de recaptar a DA do meio extracelular para o neurônio pré-sináptico, onde será reutilizada ou degradada (Beaulieu e Gainetdinov, 2011; Baik, 2013).

Existem quatro vias dopaminérgicas principais no encéfalo: a via mesocortical, mesolímbica, nigroestriatal e tuberoinfundibular. Os neurônios que compõem as três

primeiras vias se originam de regiões específicas do mesencéfalo: da substância negra e da área tegmental ventral (VTA). Foi demonstrado que esses neurônios dopaminérgicos mesencefálicos começam a ser identificados no dia embrionário 10.5 mas que eles apenas começam a produzir TH em torno do dia embrionário 12 em ratos, identificando seu fenótipo neuronal final (Puelles e Verney, 1998; Gates *et al.*, 2006).

Os neurônios com origem na VTA irão se projetar para regiões cerebrais distintas, formando as vias mesocortical e mesolímbica. A *via mesocortical* projeta seus neurônios para o CPF, onde a DA exercerá um papel fundamental no controle das funções executivas. A inervação dopaminérgica no CPF de ratos já é observada logo após o nascimento, mas ocorrem alterações tanto no calibre como na densidade das fibras nessa estrutura ao longo do desenvolvimento. Na área pré-límbica do CPF, a inervação dopaminérgica continua a se desenvolver até o dia 60 pós-natal (final da fase da adolescência), onde após esse período não foram detectadas alterações visíveis (Kalsbeek *et al.*, 1988; Caballero *et al.*, 2016). Também já foi demonstrado que os receptores dopaminérgicos do tipo D1 e D2 são distribuídos de forma diferente nos neurônios piramidais do CPF de ratos, com pouca sobreposição entre esses receptores (Vincent *et al.*, 1993; Gaspar *et al.*, 1995; Santana *et al.*, 2008).

Já na *via dopaminérgica mesolímbica*, o destino final dos neurônios do VTA é o núcleo accumbens (NAcc), uma região pertencente ao estriado ventral e relacionada com os processos de prazer, busca de recompensa e drogas de abuso. A segunda população de neurônios dopaminérgicos do mesencéfalo encontra-se na substância negra, projetando-se para o estriado dorsal (núcleo caudado e putâmen) e assim formando a *via nigroestriatal* – envolvida no controle motor (Figura 2) (Beaulieu e Gainetdinov, 2011).

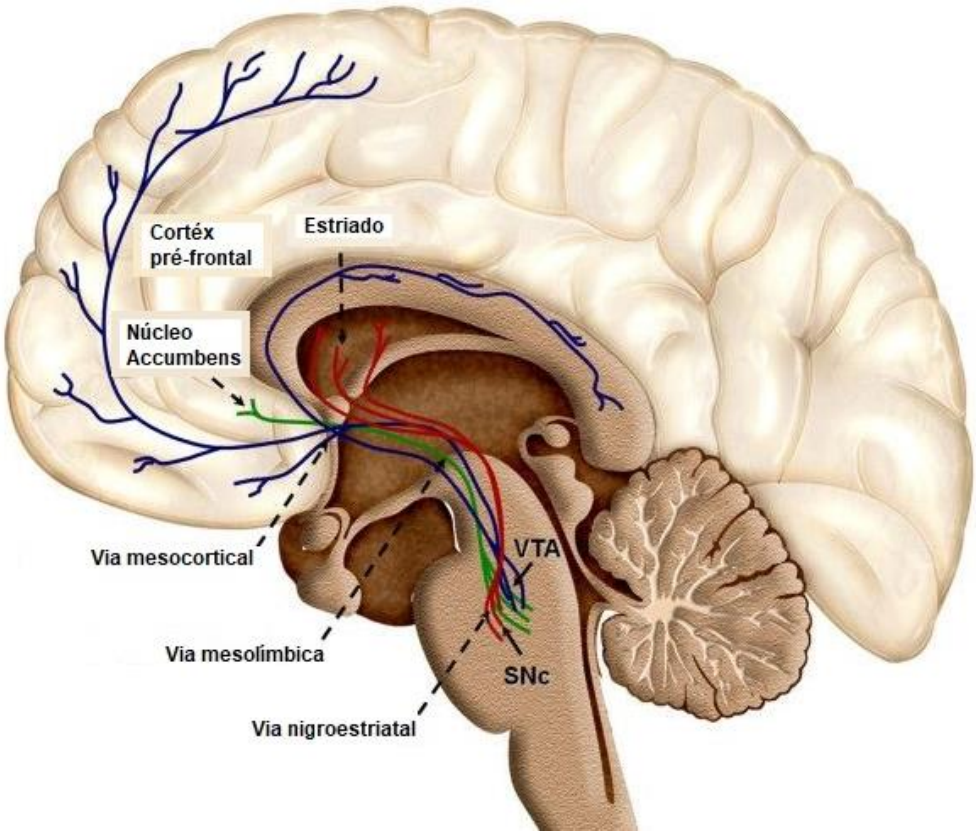

Figura 2: Principais vias dopaminérgicas encefálicas. Figura modificada de (Arias-Carrion *et al.*, 2010).

Acredita-se que pacientes com TDAH apresentem uma *hipofunção* da transmissão dopaminérgica nessas vias dopaminérgicas, interferindo assim nos processos relacionados às funções executivas e resposta a recompensas. No entanto, postula-se que o termo mais correto no caso do TDAH seria uma *disfunção* dopaminérgica, uma vez que existem estudos que reportam que a hiperatividade desses pacientes pode ser atribuída por uma maior transmissão dopaminérgica no estriado – em decorrência de uma falha na autorregulação da liberação de DA. A DA pode ser liberada por atividade tônica, onde ocorre uma pequena liberação de DA através de disparos espontâneos; ou por atividade fásica, que libera quantidades maiores de DA após um potencial de ação. Na atividade tônica, essa pequena liberação de DA será apenas capaz de alcançar receptores próximos localizados no neurônio pré-sináptico, os chamados autorreceptores inibitórios (especificamente do tipo D2). Quando ativados, esses receptores irão inibir a síntese e/ou liberação de DA em um processo de *feedback* negativo inibitório. Esse feedback inibitório realizado durante a atividade tônica irá

influenciar na quantidade de DA que será liberada na atividade fásica, após um estímulo relevante (Grace, 1991).

Em indivíduos com TDAH, o que se propõe é que ocorra uma menor atividade tônica no estriado, diminuindo o *feedback* inibitório, o que por sua vez permite uma liberação excessiva de DA durante a atividade fásica, após um estímulo relevante (Figura 3). Uma maior expressão de DAT em pacientes com TDAH dá suporte a essa teoria, uma vez que uma maior captação da DA na sinapse provavelmente resultará em redução da atividade dopaminérgica tônica (Krause *et al.*, 2000; Spencer *et al.*, 2013). Acredita-se, ainda, que essa menor atividade da fase tônica no estriado de pacientes com TDAH seja regulada pelas aferências corticais frontais – onde uma hipofunção dopaminérgica é reportada nesse transtorno (Grace, 1995; Levy e Swanson, 2001; Sharma e Couture, 2014).

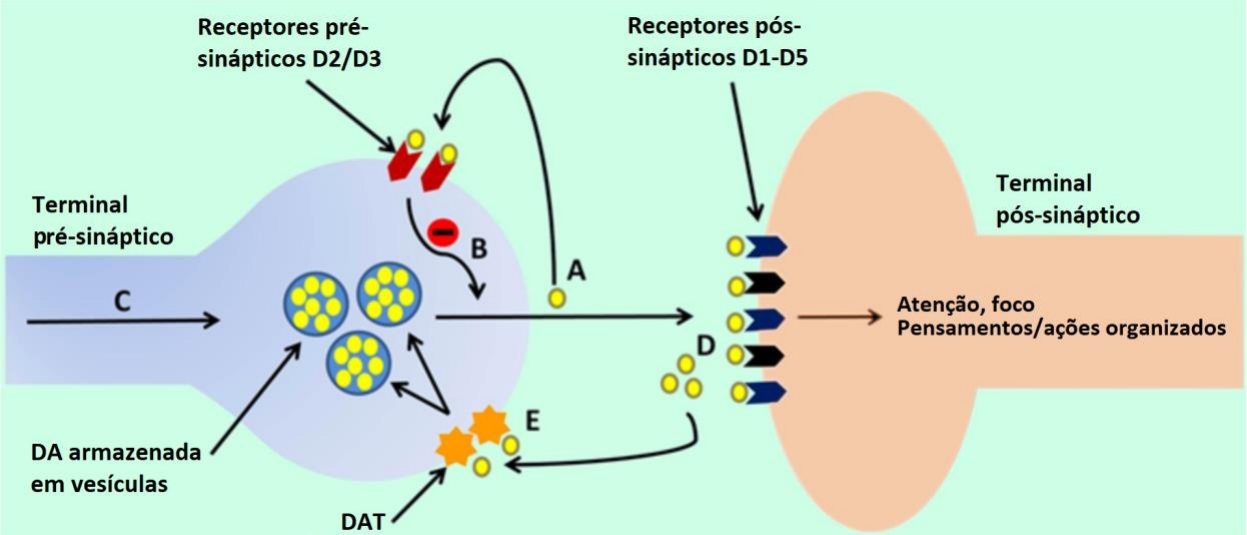

Figura 3: Mecanismo de integração da hipoatividade e hiperatividade da transmissão dopaminérgica proposta em pacientes com TDAH. Figura adaptada de (Sharma e Couture, 2014).

O CPF é uma região extremamente sensível às variações nos níveis de DA, sendo que baixos níveis estão relacionados com uma função executiva prejudicada e consequente pior desempenho cognitivo, mas altos níveis desse neurotransmissor também podem afetar negativamente o comportamento. Dessa forma, níveis ótimos de DA no CPF são necessários para um comportamento adequado, sendo reconhecido que os níveis de DA no PFC e o comportamento apresentam uma relação do tipo “curva em

U invertida" (Arnsten e Pliszka, 2011; Floresco, 2013) (Figura 4). É importante mencionar que, ainda que o papel da DA seja mais estudado nessa relação com o CPF e na fisiopatologia do TDAH, a noradrenalina (NE) também tem um importante papel para manter um bom funcionamento nessa estrutura (para revisão (Arnsten e Pliszka, 2011).

Figura 4: O CPF é uma região extremamente sensível ao seu ambiente neuroquímico, onde a liberação insuficiente ou excessiva de catecolaminas prejudica a função do CPF. As catecolaminas noradrenalina (NE) e dopamina (DA) são liberadas no PFC de acordo com o estado de excitação: muito pouco é liberado durante processos tediosos, uma quantidade moderada de liberação fásica para estímulos relevantes durante o estado de vigília (não estressante), e alta liberação tônica sob condições estressantes. Fonte: Modificado de (Arnsten e Pliszka, 2011).

Ainda, considerando que o TDAH é uma desordem caracterizada por atrasos no neurodesenvolvimento, postulou-se que alterações nos níveis de neurotrofinas possam estar contribuindo na fisiopatologia desse transtorno (Tsai, 2017). O fator neurotrófico derivado do encéfalo (BDNF, do inglês *brain-derived neurotrophic factor*) é uma neurotrofina crucial para o desenvolvimento encefálico, uma vez que ela é essencial nos processos de sobrevivência e crescimento neural. De fato, já foram observados menores níveis de BDNF em crianças com TDAH (Akay *et al.*, 2018), assim como variações genéticas no gene do BDNF também demonstraram associação com o TDAH (Kent *et al.*, 2005; Ozturk *et al.*, 2016).

1.1.3 Metilfenidato (MFD) como tratamento para o TDAH

O cloridrato de metilfenidato (MFD; nomenclatura IUPAC: metil 2-fenil-2-(2-piperidil) acetato) é o fármaco de escolha para o tratamento do TDAH, sendo classificado como um estimulante do sistema nervoso central (SNC). O MFD é vendido comercialmente sob os nomes de Ritalina®, Ritalina LA® e Concerta®, sendo os dois últimos considerados fármacos de liberação prolongada. A Ritalina apresenta uma meia vida de 2 a 4 horas, a Ritalina LA de até 8 horas, e o Concerta de 12 horas (Coghill *et al.*, 2013). Quando administrado por via oral, sua ação se inicia em 30 minutos, com um pico de concentração em 1 a 2 horas (Bennett *et al.*, 1999; Swanson e Volkow, 2002). A efetividade do tratamento com MFD em pacientes com TDAH é bem reconhecida, reduzindo expressivamente os sintomas relacionados ao transtorno (Greenhill *et al.*, 2002; Fredriksen *et al.*, 2014). Em pacientes adultos com TDAH, a eficácia terapêutica foi de 76% quando comparado aos pacientes que receberam placebo (19%) (Spencer *et al.*, 2005).

O principal mecanismo de ação do MFD é o bloqueio dos transportadores de dopamina (DAT) e de noradrenalina (NET), aumentando a disponibilidade dessas catecolaminas na fenda sináptica (Gatley *et al.*, 1996; Volkow *et al.*, 2005). Dessa forma, o MFD melhora as funções executivas e a eficiência da atividade do CPF em pacientes com TDAH (Bush *et al.*, 2008), mas também em indivíduos saudáveis (Elliott *et al.*, 1997; Mehta *et al.*, 2000; Linssen *et al.*, 2014). No entanto, sabendo da estreita relação dos níveis de catecolaminas e a função do CPF, é sugerido que doses excessivas de estimulantes podem interferir na função do CPF, causando prejuízos comportamentais (Figura 4) (Arnsten e Pliszka, 2011). Como tem se proposto uma diminuição da atividade tônica dopaminérgica no estriado de pacientes com TDAH, parece que o tratamento com MFD atua aumentando essa atividade tônica, que por conseguinte atenua a liberação excessiva de DA na atividade fásica (Vaidya e Lee, 2009). Ainda, foi demonstrado que o tratamento com MFD foi capaz de aumentar os níveis de BDNF em pacientes com TDAH, após 6 ou 8 semanas de tratamento (Akay *et al.*, 2018).

Em estudos avaliando o efeito do MFD a longo prazo, foi demonstrado uma normalização no volume da substância cinzenta no CPF em desenvolvimento dos

pacientes com TDAH (Shaw *et al.*, 2009; Schnoebelen *et al.*, 2010), assim como uma diminuição no risco desses indivíduos desenvolverem transtornos relacionados ao abuso de substâncias psicoativas (Biederman, 2003; Hammerness *et al.*, 2017). Dessa forma, o tratamento atua em mudanças neuroquímicas que podem normalizar a morfologia encefálica e assim diminuir o risco para problemas futuros ao longo do desenvolvimento do individuo.

1.1.4 Modelos animais para o estudo do TDAH

Estudos experimentais com modelos animais são necessários para se buscar entender a base neurobiológica por trás dos transtornos, assim como orientar para o desenvolvimento de terapias efetivas. Para um modelo ser reconhecido para o estudo de determinado transtorno, este precisará passar por uma série de validações. O modelo animal precisa mimetizar: 1) as características comportamentais do distúrbio humano (validade de face ou aparente), 2) a etiologia ou os mecanismos neurobiológicos hipotetizados ou estabelecidos para o transtorno (validade de construto) e 3) a resposta do tratamento farmacológico disponível na clínica (validade preditiva) (Willner, 1986; Van Der Staay *et al.*, 2009). Para o estudo do TDAH, o modelo mais utilizado na literatura é a linhagem SHR (do inglês, *spontaneously hypertensive rat),* um modelo genético desenvolvido inicialmente para estudar a hipertensão (Okamoto e Aoki, 1963). Demonstrou-se mais tarde que esse modelo apresentava hiperatividade espontânea (Schaefer *et al.*, 1978; Mccarty e Kopin, 1979) sendo por esse motivo amplamente utilizado como um modelo para TDAH. Apesar da validade aparente desse modelo, os estudos não confirmam uma validade preditiva completa quando o tratamento farmacológico para o TDAH é testado nesses animais (Wickens *et al.*, 2011). Para sanar esses questionamentos, um recente estudo de meta-análise agrupou 36 estudos que investigaram o efeito do MPH no comportamento de ratos SHR, e identificaram que o tratamento foi capaz de melhorar parâmetros atencionais, de memória e a impulsividade, mas não foi capaz de reduzir a hiperatividade desses ratos (Leffa *et al.*, 2018). Dessa forma, a busca por modelos alternativos ao SHR é necessária, e nesse ponto diferentes modelos têm sido propostos. Existem outros modelos geneticamente modificados, como

os camundongos *knock-out* para o DAT (DAT-KO) e modelos induzidos por exposição a substâncias lesivas ao SNC durante o período perinatal, como etanol, nicotina e 6-hidroxidopamina (Russell *et al.*, 2005; Russell, 2011; De La Pena *et al.*, 2018).

1.1.4.1 Hipóxia-isquemia (HI) neonatal como um modelo para o estudo do TDAH

Considerando que as condições hipóxico-isquêmicas perinatais estão relacionadas ao desenvolvimento de TDAH em humanos (Getahun *et al.*, 2013; Zhu *et al.*, 2016), modelos animais explorando essa relação também são propostos. Um modelo de exposição repetida à hipóxia no período neonatal em ratos resultou em um perfil hiperativo desses animais na fase adulta, mas sem causar deficit de atenção (Oorschot *et al.*, 2007; Oorschot *et al.*, 2013). Um segundo modelo proposto é o modelo de hipóxia-isquemia (HI) neonatal de Rice-Vannucci (Rice *et al.*, 1981; Vannucci e Vannucci, 2005), que consiste na oclusão unilateral permanente da artéria carótida comum (isquemia), seguido de exposição a um ambiente hipóxico (Figura 5). Esse procedimento é geralmente realizado no sétimo dia pós-natal em ratos, um período que corresponde ao desenvolvimento fetal humano entre as 32-36 semanas gestacionais (Patel *et al.*, 2014).

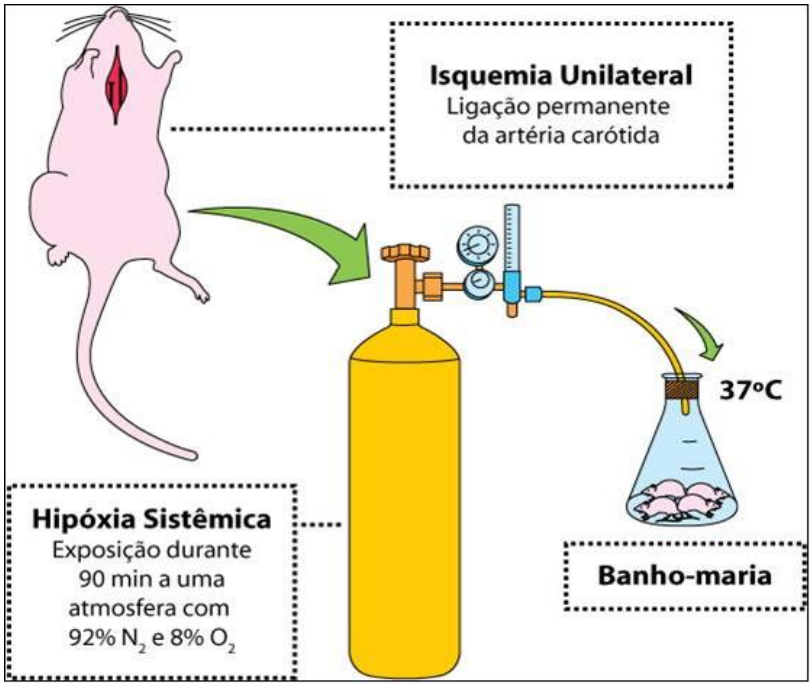

Figura 5: Procedimento de hipóxia-isquemia de Rice-Vannucci.

Utilizando esse modelo, foram observados prejuízos atencionais na fase adulta dos animais utilizando a tarefa *two-lever choice reaction time* (Ikeda *et al.*, 2001; Ikeda *et al.*, 2004; Mishima, K. *et al.*, 2004), assim como deficit de memória de trabalho e impulsividade (Smith, Hill*, et al.*, 2014). A tarefa *5-choice serial reaction time task* (5-CSRTT) é o paradigma mais reconhecido para a análise de atenção e impulsividade em roedores, e nessa tarefa foi demonstrado um menor percentual de respostas corretas por animais adultos que sofreram HI neonatal, utilizando um protocolo de aproximadamente um mês de treinamento (Smith, Alexander*, et al.*, 2014). Uma vez que o modelo de HI de Rice-Vannucci parece induzir deficit atencionais e cognitivos bem estabelecidos, nosso grupo de pesquisa começou a investigar mais profundamente a relação entre esse modelo e as características relacionadas ao TDAH, buscando as validades necessárias para que este possa ser apontado como um modelo animal alternativo para o estudo do TDAH. No primeiro trabalho, demonstramos que a HI neonatal ocasionou deficit de aprendizagem, deficit de atenção e falhas no controle inibitório – indicados por aumento de respostas prematuras e perseverativas – em animais adultos avaliados durante um treinamento extensivo por seis meses no 5-CSRTT. Ainda, esses comportamentos foram associados a uma atrofia cerebral geral no lado ipsilateral à lesão isquêmica (Miguel *et al.*, 2015). No trabalho seguinte, demonstramos deficit de função executiva também em animais adultos, evidenciados por uma inflexibilidade cognitiva na tarefa *attentional set-shifting*, uma tarefa análoga ao *Intra-/Extra-dimensional Set Shift (IED)* utilizada em humanos. Essa inflexibilidade cognitiva correlacionou-se com a atrofia no CPF, e ainda demonstramos redução na expressão de receptores dopaminérgicos do tipo D2 no hemisfério ipsilateral à lesão isquêmica também no CPF de animais HI (Miguel *et al.*, 2018). Dessa forma, estes estudos contribuíram para a comprovação da validade aparente e validade de construto do modelo de HI neonatal para o estudo do TDAH. No entanto, diversas questões continuam em aberto, como a análise dos comportamentos em animais mais jovens (uma vez que o TDAH tem uma incidência maior em crianças e adolescentes) e se comportamentos do tipo aditivos (geralmente associados a comorbidades no TDAH) também estão presentes em animais submetidos à HI neonatal. Adicionalmente, buscando as validações para o modelo animal, faz-se necessário considerar a validade

preditiva desse modelo, ou seja, analisar se o metilfenidato possui a capacidade de melhorar os deficit comportamentais no modelo de HI.

2. OBJETIVOS

2.1 OBJETIVO GERAL

O objetivo geral desta tese foi identificar as validades aparente, de construto e preditiva do modelo de hipóxia-isquemia neonatal em ratos para o estudo do TDAH experimental e também investigar o impacto de eventos hipóxico-isquêmicos perinatais no desenvolvimento de características relacionadas ao TDAH em crianças

2.2 OBJETIVOS ESPECÍFICOS

Capítulo 1: Analisar o impacto da HI neonatal sobre parâmetros comportamentais e bioquímicos relacionados ao TDAH em *ratos jovens* e o efeito do tratamento com Metilfenidato sobre esses parâmetros:

- A atividade locomotora
- A flexibilidade cognitiva
- O imunoconteúdo de diferentes parâmetros do metabolismo da dopamina no córtex pré-frontal: receptores D1 e D2 de dopamina, transportador de dopamina (DAT), enzima tirosina hidroxilase (TH), e sua forma fosforilada (pTH)

Capítulo 2: Analisar o impacto da HI neonatal sobre parâmetros comportamentais e bioquímicos relacionados ao TDAH em *ratos jovens* e o efeito do tratamento com Metilfenidato sobre esses parâmetros:

- A memória de reconhecimento
- A aprendizagem espacial, a memória de longa duração, a flexibilidade cognitiva e a memória de trabalho
- Os níveis de BDNF no hipocampo e córtex pré-frontal

Capítulo 3: Analisar o impacto da HI neonatal sobre parâmetros comportamentais relacionados ao TDAH em *ratos adultos* e o efeito do tratamento com Metilfenidato sobre esses parâmetros:

- O padrão de comportamento alimentar frente a uma ração padrão ou palatável
- A preferência pelo consumo de álcool em relação à água

Capítulo 4: Analisar o efeito da interação entre condições hipóxico–isquêmicas perinatais e o perfil genético que reflete a função da rede do *DAT1* no córtex pré-frontal de crianças, avaliando:

- A flexibilidade cognitiva
- A relação entre a densidade de substância cinzenta encefálica e os polimorfismos genéticos incluídos na rede do *DAT1*

Capítulo 5: Revisar as influências ambientais precoces que podem afetar o encéfalo em desenvolvimento, pontuando estudos de neuroimagem que demonstram essas associações, assim como novas perspectivas nos estudos de interação gene x ambiente

3. CAPÍTULO 1

Artigo: *Methylphenidate administration reverts attentional inflexibility in adolescent rats submitted to a model of neonatal hypoxia-ischemia: Predictive validity for ADHD study*

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No Capítulo 1 desta tese tivemos como objetivo avaliar características comportamentais relacionadas ao TDAH, como atividade locomotora e flexibilidade cognitiva, em ratos adolescentes que foram submetidos à HI neonatal. Diferentes parâmetros do metabolismo dopaminergico no CPF também foram avaliados após o tratamento com MFD: receptores D1 e D2, transportador de dopamina (DAT), enzima tirosina hidroxilase (TH) e sua forma fosforilada (pTH).

Os resultados deste trabalho demonstraram que ratos HI apresentaram inflexibilidade cognitiva que foi revertida pelo tratamento com MFD. No entanto, um aumento na locomoção foi gerado pelo tratamento com MFD nos animais HI. Ainda, diminuição da expressão de receptores D2 e do DAT e um aumento nos níveis da forma fosforilada da TH (pTH), possivelmente de forma compensatória, foram observados no CPF de ratos HI. Um efeito do tratamento com MFD ainda foi identificado para aumentar ainda mais a expressão da pTH em animais HI.

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Research paper

Methylphenidate administration reverts attentional inflexibility in adolescent rats submitted to a model of neonatal hypoxia-ischemia: Predictive validity for ADHD study

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ABSTRACT

Perinatal complications such as birth asphyxia were associated with a higher risk for Attention-Deficit/Hyperactivity Disorder (ADHD) in humans. Data from a rat model of neonatal hypoxia-ischemia (HI) have revealed inattention, impulsive behavior and dopamine (DA) disturbances in the prefrontal cortex (PFC), confirming the face validity and construct validity for ADHD study. However, the predictive validity (similar therapeutic efficacy of the pharmacological treatment available in the clinic) should be considered. Therefore, we aimed to investigate the effects of methylphenidate (MPH) - the treatment of choice for ADHD - on exploratory and attentional flexibility behaviors and DA-related proteins in the PFC of animals submitted to neonatal HI. Male Wistar rats were divided into four groups: control saline (CTS, $n = 12$), control MPH (CTMPH, $n = 12$), HI saline (HIS, $n = 13$) and HIMPH (n = 12). The HI procedure was conducted at postnatal day (PND) 7 and behavioral measures between PND 30–40, followed by protein analysis in the PFC. The MPH administration (2.5 mg/kg, i.p.) occurred 30 min prior each behavioral session and euthanasia for western blot analysis. We observed that the MPH increased the locomotor activity in the open field especially in HI rats. In the attentional-set shifting task, the MPH reversed the HI- induced attentional inflexibility, but impaired the task acquisition in control rats. Neonatal HI resulted in lower DA D2 receptors expression but also decreased DA transporter (responsible for DA reuptake) and increased pTH (phosphorylated-tyrosine hydroxylase) levels in the PFC, probably to compensate the dysfunctional DA transmission. This compensation was higher in the HIMPH group and it could explain the improvement in the attentional flexibility as well as the increased locomotor activity in this group. Taken this data together, we can assume the predictive validity of the HI model for the ADHD study concerning the impact of MPH treatment on attentional parameters.

Abbreviations: 5-CSRTT, 5-choice serial reaction time task; ADHD, attention-deficit/hyperactivity disorder; ASST, attentional set-shifting task; CTMPH, control treated with methylphenidate; CTS, control treated with saline; DA, dopamine; DAT, dopamine transporter; DAT1, dopamine transporter gene; EF, executive functions; HI, hypoxia-ischemia; HIMPH, hypoxia-ischemia treated with methylphenidate; HIS, hypoxia-ischemia treated with saline; I.P., intraperitoneally; IUGR, intrauterine growth restriction; MPH, methylphenidate; PFC, prefrontal cortex; PND, postnatal day; pTH, phosphorylated-tyrosine hydroxylase; SHR, spontaneously hypertensive rat; TH, tyrosine hydroxylase

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1. Introduction

Attention-Deficit/Hyperactivity Disorder (ADHD) is a neurodevelopmental disorder characterized by inattention and/or hyperactive-impulsive behaviors (American Psychiatric Association, 2013). It is the most commonly diagnosed neurobehavioral disorder of childhood (Centers for Disease Control and Prevention, 2013) and the ADHD worldwide prevalence rate has been maintained in the last three decades around 5% (Polanczyk et al., 2007, 2014). The ADHD etiology is complex, comprising genetic and environmental contribution (Biederman and Faraone, 2005). Among the environmental factors, perinatal complications such as birth asphyxia have been associated with a higher risk for ADHD (Ketzer et al., 2012; Mikkelsen et al., 2017).

The psychostimulant methylphenidate (MPH), commercially known as Ritalin®, is the first-line pharmacological treatment for ADHD patients (National Institute for Health and Care Excellence, 2018) and its effectiveness in reducing ADHD symptoms is strongly reported in the literature (Jadad et al., 1999; Oh et al., 2018; Sudnawa et al., 2018). Although it appears paradoxical, psychostimulants such as MPH are shown to decrease hyperactivity in ADHD patients, but this effect seems to be due to a focusing action of these drugs (Porrino et al., 1983; Swanson et al., 2002). MPH blocks the dopamine transporter (DAT), responsible for the dopamine (DA) reuptake from the synaptic cleft, then increasing the extracellular DA levels (Schmeichel and Berridge, 2013; Volkow et al., 1995a). This effective psychostimulant therapy reverses the dopaminergic dysfunction underlying the ADHD pathophysiology (Del Campo et al., 2011; Levy, 1991). When used at low and clinically relevant doses, MPH preferentially increases DA release in the prefrontal cortex (PFC) (Berridge et al., 2006; Devilbiss and Berridge, 2008; Schmeichel et al., 2013; Spencer et al., 2012). This brain area is the main region involved in executive functions (EF) - a set of skills necessary for the cognitive control of behavior, such as cognitive flexibility, planning, and inhibition (Barkley, 1997; Diamond, 2013). In ADHD patients, impairments in EF are commonly described (Holmes et al., 2010; Marzocchi et al., 2008; Reader et al., 1994) as well as abnormalities in the PFC (Cubillo et al., 2012; Proal et al., 2011; Rubia, 2018).

Attempting to better understand the neurobiological basis of the disorder, as well as to guide clinical drug development, several animal models to study ADHD have been proposed (Bari and Robbins, 2011; Russell, 2011). A reliable animal model should mimic the behavioral characteristics of the human disorder (face validity), its mechanisms (construct validity), and the pharmacological treatment response available in the clinic (predictive validity) (van der Staay et al., 2009; Willner, 1986). Considering that perinatal hypoxic-ischemic conditions are related to ADHD development in humans (Zhu et al., 2016), a neonatal model of anoxia has been appointed as an animal model for ADHD, but it failed to succeed on the validities modalities or does not have enough data available (Russell, 2011). Recently, our group has been examining ADHD-related outcomes in a rat model of neonatal hypoxia-ischemia (HI), a widely used model proposed by Levine (Levine, 1960) and modified by Rice and colleagues (Rice 3rd et al., 1981; Vannucci and Vannucci, 2005). Attentional impairments and inhibitory control failures (impulsivity and compulsivity observed in the 5-choice serial reaction time task/5-CSRTT) associated with a general brain atrophy in the ipsilateral side to the ischemic lesion were observed in adult animals (Miguel et al., 2015). Moreover, we demonstrated attentional inflexibility correlated to PFC atrophy and dopaminergic dysfunction in this structure also in adult animals that underwent neonatal HI (Miguel et al., 2018). Then we have already established the face validity and construct validity of the Levine-Vannucci model of HI as a possible rat model to study ADHD.

Therefore, intending to investigate the predictive validity of the HI animal model for the ADHD study (using the pharmacological treatment adopted for ADHD) the aim of this work was to evaluate the effects of MPH administration on ADHD-related outcomes: behavioral measures (exploratory and attentional flexibility) and dopaminergic system parameters in the PFC of animals submitted to neonatal HI. Our previous data reporting attentional inflexibility in HI animals was conducted in adult animals (Miguel et al., 2018); considering that ADHD is more prevalent in children and adolescents, we delineated the current study with young animals, starting the analysis on the 30th postnatal day (PND). We hypothesize that the MPH treatment will improve the behavioral deficits and dopaminergic transmission in the PFC of animals submitted to neonatal HI.

2. Materials and methods

2.1. Animals

Forty-nine male Wistar rats were used in this study and they were obtained from the Central Animal House of the Institute of Basic Health Sciences (Universidade Federal do Rio Grande do Sul). On the 7th PND, pups were randomly distributed into control and HI groups and then subdivided in saline and MPH treatment, resulting in four experimental groups: control treated with saline (CTS, n = 12), control treated with MPH (CTMPH, n = 12), HI treated with saline (HIS, n = 13) and HI treated with MPH (HIMPH, n = 12). Animals were maintained with their dams until PND 21 when they were weaned and housed in 2–3 per cage (Plexiglas cages). In all stages, they were maintained in a controlled room temperature (22–24 °C) on a 12:12 h light/dark cycle, with food and water available ad libitum until the PND 30, when a protocol of food restriction was started for the attentional set-shifting task. All procedures were approved by the Institutional Ethics Committee on Animal Use (No. 29750) and were in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023), the guide of the Federation of Brazilian Societies for Experimental Biology and the Arouca Law (n° 11.794/2008).

2.2. Hypoxia-ischemia

The HI procedure was conducted on the 7th PND of the rat, a period that is comparable to 32 to 36 weeks' gestational age of the human infant (Patel et al., 2014). Animals were anesthetized with halothane (2–4%) and an incision on the ventral surface of the neck was made to permit access to the right common carotid artery, that was permanently occluded with a surgical silk thread. Following a 2-h interval with their dams to recover, the pups were placed in chambers partially immersed in a 37 °C water bath, where they were exposed to a hypoxic atmosphere (8% oxygen and 92% nitrogen, 5 L/min) for 90 min. After the HI procedure, the animals returned immediately to maternal care. Control animals were submitted to sham surgery, i.e., animals received only anesthesia and neck incision (Miguel et al., 2018; Miguel et al., 2015).

2.3. MPH administration

Methylphenidate hydrochloride (MPH) (Ritalina®, Novartis, Brazil) was dissolved in saline solution (0.9% NaCl) and injected intraperitoneally (i.p.) at a volume of 1 mL/kg, 30 min prior to each behavioral session and 30 min prior to euthanasia. Saline injections consisted of only 0.9% NaCl solution administered i.p. in the same volume (1 mL/kg). The MPH dose of 2.5 mg/kg, adopted in this study, corresponds to a medium dose (Dafny and Yang, 2006) and it was effective in improving attentional deficits in the attentional-set shifting task in rats (Cao et al., 2012).

2.4. Open field test

The open field is one of the most widely used tasks to measure locomotor activity and spontaneous exploration of a novel environment

in animal studies (Seibenhener and Wooten, 2015). At the 30th PND, rats were exposed individually to a wood square arena (54 cm length, 38 cm width and 45 cm height) facing the corner wall of the apparatus and their free exploration was recorded for 5 min (Deniz et al., 2018). The software ANY-Maze video-tracking system 4.70 (Stoelting Co., Wood Dale, IL) was used to analyze the following parameters: total distance travelled, average speed, number of rotations of the animal's body (entire rotations of 360°), latency to the first entry into the central zone, number of entries into the central zone and total number of entries into the central and peripheral zone (Mestriner et al., 2013; Miguel et al., 2015). The number of rearings (a measure of vertical exploration) was evaluated by a blind researcher.

2.5. Attentional set-shifting task (ASST)

Immediately after the open field assessment (30th PND), animals started a food restriction protocol (12-15 g of food/rat/day) required to motivate the animal to eat the reward in the ASST. The food restriction started four days prior to the habituation on the ASST maze and at this period, daily weighing was conducted to certify that animals maintained a body weight at 85% to 90% of their free-feeding weight (Birrell and Brown, 2000; Miguel et al., 2018).

The attentional flexibility was measured using a maze-based set-shifting task that allows animals, in a sequence of trials, to turn left or right on a "T" maze to obtain a food reward. The apparatus consisted of a wood four-arm maze and each arm measures 55 cm long, 9.5 cm wide and 40 cm high. A bowl (7 cm in diameter and 4 cm in depth) was situated at the end of each arm, in which the food reward was provided. The four-arm maze was used only during the habituation process; during the turn bias and testing, it was made into a "T" maze by placing a wood block at the entrance of one of the arms (Floresco et al., 2006; Ragozzino et al., 2002).

2.6. Habituation

Two days prior to their initial exposure to the maze, each animal received four sweetened food rewards (Froot Loops, Kellogg's®) in their home cages, to avoid food neophobia. On the first day of habituation, two pieces (1/4 Froot Loops) of reward were placed in each arm and one piece in each bowl, and the rat was allowed to explore the apparatus and eat the froot loops for 15 min. If a rat did not consume the rewards in 15 min, the same habituation session was conducted in the following days. In the next habituation stage, the reward was provided only in the bowls. As soon as the rat consumed the reward on the bowl, it was picked up and placed in a different arm to habituate the animal to being handled in the maze after consuming the reward. The criterion to move to the next stage (the turn bias) was to consume all four pieces of reward at least four times in 15 min, in 2 consecutive days.

2.7. Turn bias

For assessing the turn bias, the cross maze was made into a "T" maze such that animals could only turn left or right to obtain the food reward – that was available in both left and right sides. This stage measures the animal natural turn preference for one of the sides. The rat was placed in the start arm and after it chose an arm and consumed the reward, it was picked up and placed again in the start arm until it chose the other arm and consumed the reward. After choosing both arms, the rat was returned to the holding cage and the visual cue (white stripes cardboard; 20 cm long, 9.4 cm wide and 1 cm thickness) was placed randomly in the right or left arms' floor when assessing the turn bias. This procedure was conducted to avoid neophobia to the visual cue during the test in the following day.

In the turn bias evaluation, in each trial the rat was placed in the start arm and allowed to consume the reward that was available in both sides but the turn that the rat made first during the initial choice of each trial was recorded and counted toward its turn bias. After consuming the reward in both sides, the rat was picked up and returned to the holding cage, the maze rebaited and a new trial started. Seven trials were applied and the direction (right or left) that the rat turned four or more times was considered its turn bias, i.e., the natural turn preference. The test started on the following day.

2.8. Test 1: egocentric response (Acquisition learning)

In the first day of testing, the animal was required to turn in the opposite direction as its turn bias to obtain the food reward, regardless of the presence of the visual cue placed in one of the choice arms. The experimenter room had at least one spatial cue in each wall and to discourage animals from using an allocentric spatial strategy, the maze was placed on a rotating platform that easily allows us to turn the maze and modify the position (south, east, west) of the start arm. We never started a rat on the north position during the tests and this position was used only for the probe trial. The order of the start position and the location of the visual cue for each trial were determined pseudorandomly and taken from a preset sequence that was identical for each animal.

In each trial, the rat was placed on the start arm and as soon as it consumed the reward or realized its mistake, it was picked up and placed back in the holding cage for approximately 10 s (the inter-trial interval). The criterion to complete this test was 10 correct consecutive choices and a probe trial was applied after the animal achieved this criterion. In the probe trial, the rat started from the north position and the visual cue was inserted in the opposite arm to the correct response. The test ended if the rat turned to the appropriate direction but if the rat made a mistake, the egocentric testing was continued until the rat made an additional five correct consecutive choices, and a subsequent probe trial was performed. For each rat, we analyzed the number of trials to reach the criterion, the number of errors, time to complete the test and number of probe trials.

2.9. Test 2: shift to visual-cue discrimination (Attentional flexibility)

In the second testing day, the strategy used previously changed and now the correct response was the arm that contained the visual cue, regardless of the side. Thus, this stage required attentional flexibility (Supplementary Video 1). The protocol was very similar to the test 1: the location of the cue and the start position were determined pseudorandomly, the criterion to complete the test was 10 correct consecutive responses and the same variables were analyzed.

Additionally, in Test 2, the errors were further broken down into three error subtypes: perseverative, regressive and never-reinforced. The perseverative and regressive errors were counted when a rat continued to make the same previous egocentric response on those trials that required the rat to turn to the opposite direction (with the visual cue). The difference is that in regressive errors, the animal started to use an alternative strategy, i.e., it made some correct answers to the opposite direction of the egocentric response. To discriminate these two types of errors, we separated the trials in blocks of 16 consecutive trials and perseverative errors were scored until less than six of them were made within a block (Brady and Floresco, 2015). Beginning with the next block and continuing through the end of the task, subsequent errors of this type were counted as regressive. Never-reinforced errors were scored when a rat entered the incorrect arm on trials where the visual cue was placed in the same arm that the rat had been trained to enter on the previous day. The combination of regressive and never-reinforced errors has been used as an index of the animals' ability to maintain a new strategy (Floresco et al., 2006) while perseverative errors occurring early in testing reflect an animal's inability to abandon the previous strategy (Floresco et al., 2008).

2.10. Tissue collection

At the end of the ASST (approximately PND 40), animals received rat chow ad libitum in their home cage. In the following day, they were euthanized by decapitation 30 min after the MPH or saline injection and the PFC was dissected out, instantaneously placed in liquid nitrogen and stored at −80 °C until western blot analysis.

2.11. Western blot

The Western Blot technique was applied to analyze the expression of different proteins involved in the dopaminergic signaling in the PFC region. Assessments of the DA transporter (DAT, responsible for the DA reuptake), D1 and D2 receptors, tyrosine hydroxylase (TH, the rate-limiting enzyme for DA synthesis) and phosphorylated-TH (pTH; Ser40) expression were conducted in both ipsilateral and contralateral hemispheres to the arterial occlusion. PFC samples were homogenized in cytosolic extraction buffer with protease (Complete, Roche) and phosphatase inhibitors (Phostop, Roche). The samples were then centrifuged at 3000 rpm (4 °C) for 10 min for cytosolic protein extraction (for TH and pTH) and thereafter at 13000 rpm (4 °C) for 30 min for purification of the cytosolic fraction (for DAT, D1, and D2). The supernatant of the centrifugation processes was used to quantify the total protein in the sample, using a BCA protein assay with bovine serum albumin as standard (Thermo Scientific). Aliquots containing 40 µg of protein were incubated with lithium dodecyl sulfate (LDS, Invitrogen) and dithiothreitol (DTT, Sigma-Aldrich), and protein denaturation occurred by boiling the samples at 99 °C for 3 min. They were then loaded on 4–12% polyacrylamide gradient gels (Invitrogen) and a standard molecular weight marker (Magic MarkerVR, Invitrogen) guided the right position of protein weights. Samples were submitted to electrophoresis, transferred to a nitrocellulose membrane (GE Healthcare) and blocked in Tris-buffered saline with 1% Tween-20 (Sigma) and 5% non-fat dry milk. The membranes were incubated overnight at 4 °C with the following primary antibodies: anti-DA transporter (Sigma-Aldrich, AB1591P, 1:500), anti-DA D1 receptor (Millipore, AB9141, 1:500), anti-DA D2 receptor (Millipore, AB5084P, 1:500), anti-TH (Millipore, AB152, 1:2000) and anti-pTH (Invitrogen, 1:1000). Secondary antibodies anti-mouse (Cell Signaling, 7076 s, 1:2000) or anti-rabbit (Cell Signaling, 7074 s, 1:2000) were incubated for 2 h at room temperature. The chemiluminescence signal was then detected using ECL (ECL Western Blotting Analysis System, GE healthcare, RNP2106) and the intensity of the bands was quantified by densitometry using Image J® software (National Institute of Health, USA). Results were expressed as the ratio between the protein of interest and ß-actin (Sigma-Aldrich, A4700, 1:1000) or α-tubulin (Sigma-Aldrich, 1:2000) on the same membrane.

2.12. Statistical analysis

Two-way ANOVA followed by Tukey's post-hoc, with lesion and treatment as factors, was used to analyze the open field test, ASST performance and protein quantification by western blot. All variables were expressed as mean ± standard error of the mean (SEM), and the results were considered significant when $p < .05$. The analyses were performed using the Statistica software package (StatSoft, Tulsa, OK, USA), version 10.

3. Results

Table 1 presents the statistical results concerning the main effects and interactions for all dependent variables considered in this study.

3.1. Open field

In the open field test, we observed a significant lesion effect and a trend toward the treatment effect for the variables total distance travelled (lesion $F(1,45) = 8.53$, $p = .005$; treatment $F(1,45) = 3.49$, $p = .06$), average speed (lesion $F(1,45) = 8.21$, $p = .006$; treatment $F(1,45) = 3.45$, $p = .06$) and rotations of the animal's body (lesion $F(1,45) = 10.46$, $p = .002$; treatment $F(1,45) = 3.39$, $p = .07$) in the total 5 min of exploration. The Tukey post hoc demonstrated that the HIMPH group travelled longer distances, in a higher speed, and rotated more than the CTS and CTMPH group (Fig. 1 A, B and C, respectively). Considering the total number of entries into the central and peripheral zone, we found a lesion ($F(1,45) = 7.82$, $p = .007$), treatment ($F(1,45) = 6.31$, $p = .01$) and a trend toward lesion x treatment effect ($F(1,45) = 3.41$, $p = .07$), indicating that the HIMPH group had a higher number of entries compared to all other groups (Fig. 1D). A treatment effect was observed for number of rearings ($F(1,45) = 5.64$, $p = .02$) and the post hoc pointed out that the HIMPH animals had an increase in the number of rearings compared to the HIS group (CTS: 49.58 ± 4.51, CTMPH: 52.33 ± 3.55, HIS: 40.23 ± 3.55, HIMPH: 57.83 ± 5.35).

Analyzing only the exploration on the periphery, the same general pattern was observed: a lesion effect for distance travelled ($F(1,45) = 6.21$, $p = .01$) and average speed ($F(1,45) = 6.94$, $p = .01$) demonstrating that the HIMPH had longer distance travelled and higher speed when compared to both control groups – CTS and CTMPH (Table 2). In the central zone, a lesion effect was again observed for the variable distance travelled ($F(1,45) = 4.06$, $p = .04$) with the post hoc showing a trend for the HIMPH group exploring more this area than the CTS group ($p = .08$). A significant lesion x treatment interaction was also found for the latency to the first entry in the central zone ($F(1,45) = 7.08$, $p = .01$) and the post hoc demonstrated that the HIMPH group had a tendency to decreased latency to entry in the center when compared to the HIS group ($p = .052$). Considering the number of entries into the central zone, a treatment effect was observed ($F(1,45) = 4.78$, $p = .03$) with the MPH administration increasing the number of entries (see Table 2).

3.2. Attentional set-shifting task

No differences were observed between groups in relation to the number of habituation days in the ASST, neither on the first habituation stage (lesion $F(1,45) = 0.83$, $p = .36$; treatment $F(1,45) = 0.83$, $p = .36$) nor the second habituation stage (lesion $F(1,45) = 0.004$, $p = .95$; treatment $F(1,45) = 1.18$, $p = .28$).

In the Test 1 we measured the egocentric response acquisition, i.e., the animal had to choose the opposite direction of its turn bias in the T maze, regardless of the visual cue. In this test, we found a lesion x treatment interaction for the number of trials ($F(1,45) = 10.79$, $p = .001$), number of errors ($F(1,45) = 10.05$, $p = .002$) and time to complete the task ($F(1,45) = 7.11$, $p = .01$). Surprisingly, the Tukey post hoc demonstrated that the CTMPH group had a higher number of trials and errors than CTS and HIMPH groups. The CTMPH animals also took longer to complete the task than the HIMPH group (Fig. 2A-C). A treatment effect was observed for the probe trial ($F(1,45) = 6.67$, $p = .01$) but no post hoc interaction was detected.

In the following day (Test 2), the visual-cue discrimination was assessed, requiring behavioral flexibility. Here, we observed a lesion effect ($F(1,45) = 5.06$, $p = .02$) and lesion x treatment interaction ($F(1,45) = 6.29$, $p = .01$) for number of trials and number of errors (lesion $F(1,45) = 4.22$, $p = .04$; lesion x treatment $F(1,45) = 5.81$, $p = .02$). Lesion ($F(1,45) = 6.48$, $p = .01$), treatment ($F(1,45) = 7.7$, $p = .007$) and lesion x treatment interaction ($F(1,45) = 5.64$, $p = .02$) was detected for time to complete the task. Different from the Test 1, the post hoc showed that the HIS group made a higher number of trials in a longer time when compared to all other groups. The total number of errors was also higher in the HIS group in relation to the CTS and HIMPH groups (Fig. 2D-F). No effect was observed for the number of probe trials on Test 2. Additionally, when segregated the different types of errors in this phase, we observed a trend toward a treatment effect (F

Table 1
Statistical results.

Dependent variables	Lesion effect	Treatment effect	Lesion × Treatment interaction
Open field – total exploration			
Distance travelled	$F(1,45) = 8.53$, $p = .005$, partial $\eta^2 = 0.15$	$F(1,45) = 3.49$, $p = .06$, partial $\eta^2 = 0.07$	$F(1,45) = 2.09$, $p = .15$, partial $\eta^2 = 0.04$
Average speed	$F(1,45) = 8.21$, $p = .006$, partial $\eta^2 = 0.15$	$F(1,45) = 3.45$, $p = .06$, partial $\eta^2 = 0.07$	$F(1,45) = 2.12$, $p = .15$, partial $\eta^2 = 0.04$
Number of rotations of the animal's body	$F(1,45) = 10.46$, $p = .002$, partial $\eta^2 = 0.18$	$F(1,45) = 3.39$, $p = .07$, partial $\eta^2 = 0.07$	$F(1,45) = 0.64$, $p = .42$, partial $\eta^2 = 0.01$
Number of entries into the central and peripheral zone	$F(1,45) = 7.82$, $p = .007$, partial $\eta^2 = 0.14$	$F(1,45) = 6.31$, $p = .01$, partial $\eta^2 = 0.12$	$F(1,45) = 3.41$, $p = .07$, partial $\eta^2 = 0.07$
Number of rearings	$F(1,45) = 0.20$, $p = .65$, partial $\eta^2 = 0.004$	$F(1,45) = 5.64$, $p = .02$, partial $\eta^2 = 0.11$	$F(1,45) = 3.00$, $p = .09$, partial $\eta^2 = 0.06$
Open field – central exploration			
Distance travelled	$F(1,45) = 4.06$, $p = .04$, partial $\eta^2 = 0.08$	$F(1,45) = 2.20$, $p = .14$, partial $\eta^2 = 0.04$	$F(1,45) = 0.38$, $p = .53$, partial $\eta^2 = 0.009$
Average speed	$F(1,45) = 3.16$, $p = .08$, partial $\eta^2 = 0.06$	$F(1,45) = 0.27$, $p = .60$, partial $\eta^2 = 0.006$	$F(1,45) = 3.13$, $p = .08$, partial $\eta^2 = 0.06$
Latency to the first entry	$F(1,45) = 0.77$, $p = .38$, partial $\eta^2 = 0.01$	$F(1,45) = 1.09$, $p = .30$, partial $\eta^2 = 0.02$	$F(1,45) = 7.08$, $p = .01$, partial $\eta^2 = 0.13$
Number of entries	$F(1,45) = 1.35$, $p = .25$, partial $\eta^2 = 0.02$	$F(1,45) = 4.78$, $p = .03$, partial $\eta^2 = 0.09$	$F(1,45) = 0.007$, $p = .93$, partial $\eta^2 = 0.0001$
Open field – peripheral exploration			
Distance travelled	$F(1,45) = 6.21$, $p = .01$, partial $\eta^2 = 0.12$	$F(1,45) = 2.29$, $p = .13$, partial $\eta^2 = 0.04$	$F(1,45) = 1.90$, $p = .17$, partial $\eta^2 = 0.04$
Average speed	$F(1,45) = 6.94$, $p = .01$, partial $\eta^2 = 0.13$	$F(1,45) = 3.18$, $p = .08$, partial $\eta^2 = 0.06$	$F(1,45) = 1.60$, $p = .21$, partial $\eta^2 = 0.03$
Number of entries	$F(1,45) = 1.72$, $p = .19$, partial $\eta^2 = 0.03$	$F(1,45) = 7.16$, $p = .01$, partial $\eta^2 = 0.13$	$F(1,45) = 0.005$, $p = .94$, partial $\eta^2 = 0.0001$
Attentional set-shifting task – Test 1			
Number of trials	$F(1,45) = 5.21$, $p = .02$, partial $\eta^2 = 0.10$	$F(1,45) = 1.31$, $p = .25$, partial $\eta^2 = 0.02$	$F(1,45) = 10.79$, $p = .001$, partial $\eta^2 = 0.19$
Number of errors	$F(1,45) = 1.07$, $p = .30$, partial $\eta^2 = 0.02$	$F(1,45) = 3.06$, $p = .08$, partial $\eta^2 = 0.06$	$F(1,45) = 10.05$, $p = .002$, partial $\eta^2 = 0.18$
Time to complete the test	$F(1,45) = 2.14$, $p = .15$, partial $\eta^2 = 0.04$	$F(1,45) = 0.23$, $p = .63$, partial $\eta^2 = 0.005$	$F(1,45) = 7.11$, $p = .01$, partial $\eta^2 = 0.13$
Probe trial	$F(1,45) = 0.34$, $p = .56$, partial $\eta^2 = 0.008$	$F(1,45) = 6.67$, $p = .01$, partial $\eta^2 = 0.12$	$F(1,45) = 0.34$, $p = .56$, partial $\eta^2 = 0.008$
Attentional set-shifting task – Test 2			
Number of trials	$F(1,45) = 5.06$, $p = .02$, partial $\eta^2 = 0.10$	$F(1,45) = 2.17$, $p = .14$, partial $\eta^2 = 0.04$	$F(1,45) = 6.29$, $p = .01$, partial $\eta^2 = 0.12$
Number of errors	$F(1,45) = 4.22$, $p = .04$, partial $\eta^2 = 0.08$	$F(1,45) = 1.95$, $p = .16$, partial $\eta^2 = 0.04$	$F(1,45) = 5.81$, $p = .02$, partial $\eta^2 = 0.11$
Time to complete the test	$F(1,45) = 6.48$, $p = .01$, partial $\eta^2 = 0.12$	$F(1,45) = 7.70$, $p = .007$, partial $\eta^2 = 0.14$	$F(1,45) = 5.64$, $p = .02$, partial $\eta^2 = 0.11$
Probe trial	$F(1,45) = 0.81$, $p = .37$, partial $\eta^2 = 0.01$	$F(1,45) = 0.81$, $p = .37$, partial $\eta^2 = 0.01$	$F(1,45) = 0.81$, $p = .37$, partial $\eta^2 = 0.01$
Perseverative errors	$F(1,45) = 0.17$, $p = .68$, partial $\eta^2 = 0.004$	$F(1,45) = 0.03$, $p = .85$, partial $\eta^2 = 0.001$	$F(1,45) = 1.11$, $p = .29$, partial $\eta^2 = 0.02$
Regressive errors	$F(1,45) = 1.35$, $p = .25$, partial $\eta^2 = 0.02$	$F(1,45) = 3.22$, $p = .07$, partial $\eta^2 = 0.06$	$F(1,45) = 2.03$, $p = .16$, partial $\eta^2 = 0.04$
Never-reinforced errors	$F(1,45) = 2.80$, $p = .10$, partial $\eta^2 = 0.05$	$F(1,45) = 1.66$, $p = .20$, partial $\eta^2 = 0.03$	$F(1,45) = 0.60$, $p = .44$, partial $\eta^2 = 0.01$
Protein quantification – ipsilateral side			
D1 receptor	$F(1,22) = 1.25$, $p = .27$, partial $\eta^2 = 0.05$	$F(1,22) = 0.29$, $p = .59$, partial $\eta^2 = 0.01$	$F(1,22) = 0.07$, $p = .78$, partial $\eta^2 = 0.003$
D2 receptor	$F(1,22) = 0.95$, $p = .33$, partial $\eta^2 = 0.04$	$F(1,22) = 1.22$, $p = .28$, partial $\eta^2 = 0.05$	$F(1,22) = 0.69$, $p = .41$, partial $\eta^2 = 0.03$
DAT	$F(1,22) = 1.21$, $p = .28$, partial $\eta^2 = 0.05$	$F(1,22) = 0.01$, $p = .90$, partial $\eta^2 = 0.001$	$F(1,22) = 0.01$, $p = .91$, partial $\eta^2 = 0.001$
TH	$F(1,24) = 0.009$, $p = .92$, partial $\eta^2 = 0.0003$	$F(1,24) = 0.89$, $p = .35$, partial $\eta^2 = 0.03$	$F(1,24) = 0.41$, $p = .52$, partial $\eta^2 = 0.01$
pTH	$F(1,24) = 10.85$, $p = .003$, partial $\eta^2 = 0.31$	$F(1,24) = 0.67$, $p = .42$, partial $\eta^2 = 0.02$	$F(1,24) = 0.26$, $p = .60$, partial $\eta^2 = 0.01$
Protein quantification – contralateral side			
D1 receptor	$F(1,16) = 1.26$, $p = .27$, partial $\eta^2 = 0.07$	$F(1,16) = 0.001$, $p = .97$, partial $\eta^2 = 0.00008$	$F(1,16) = 0.03$, $p = .86$, partial $\eta^2 = 0.002$
D2 receptor	$F(1,21) = 6.26$, $p = .02$, partial $\eta^2 = 0.23$	$F(1,21) = 1.19$, $p = .28$, partial $\eta^2 = 0.05$	$F(1,21) = 0.82$, $p = .37$, partial $\eta^2 = 0.03$
DAT	$F(1,21) = 4.90$, $p = .03$, partial $\eta^2 = 0.21$	$F(1,21) = 1.43$, $p = .24$, partial $\eta^2 = 0.07$	$F(1,21) = 0.39$, $p = .53$, partial $\eta^2 = 0.02$
TH	$F(1,22) = 0.006$, $p = .94$, partial $\eta^2 = 0.0002$	$F(1,22) = 0.04$, $p = .83$, partial $\eta^2 = 0.002$	$F(1,22) = 0.43$, $p = .51$, partial $\eta^2 = 0.02$
pTH	$F(1,22) = 5.20$, $p = .03$, partial $\eta^2 = 0.19$	$F(1,22) = 0.13$, $p = .71$, partial $\eta^2 = 0.006$	$F(1,22) = 0.18$, $p = .67$, partial $\eta^2 = 0.008$

All analyses were performed using Two-way ANOVA.

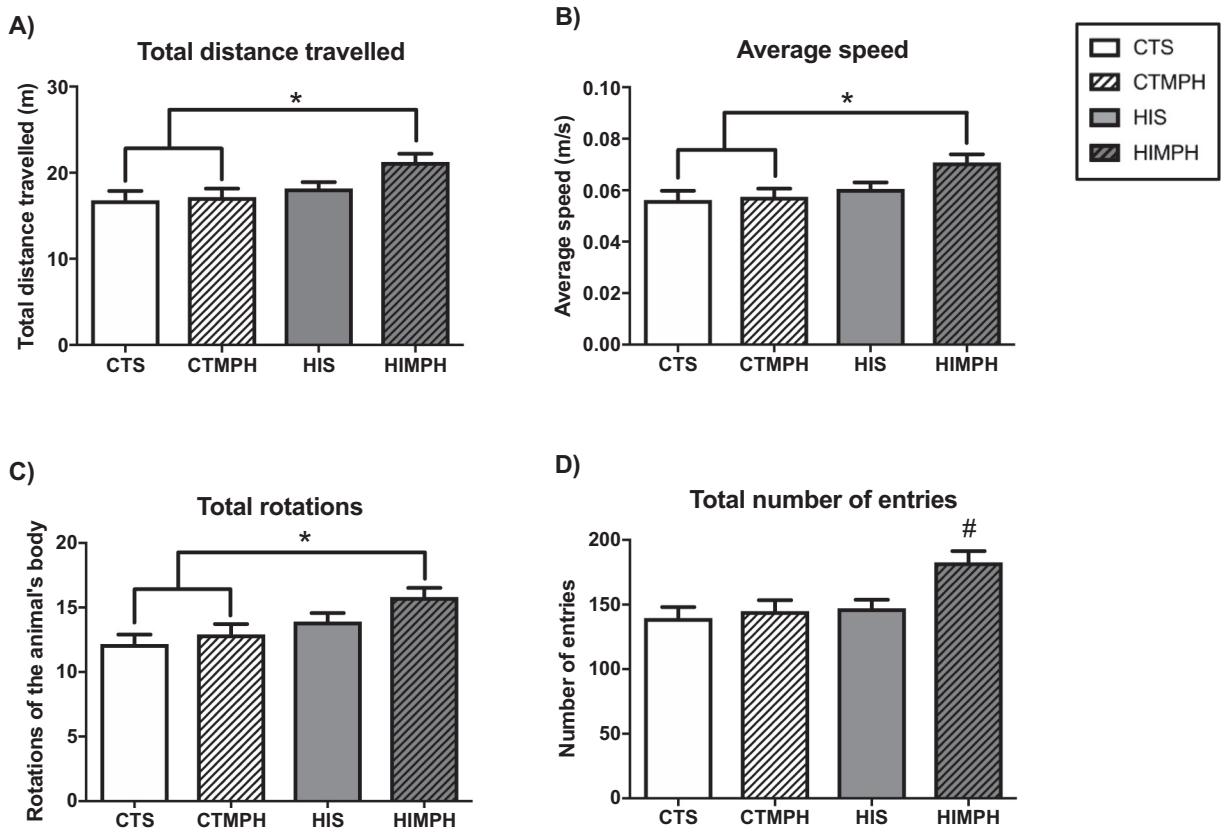

Fig. 1. Total exploratory behavior during a 5-min Open field test session. The following parameters are shown: (A) Total distance travelled, (B) Average speed, (C) Total rotations of the animal's body and (D) Total number of entries in the central and peripheral areas. Results are expressed as mean ± S.E.M. *Different from CTS and CTMPH. #Different from all other groups. Two-way ANOVA followed by Tukey's post hoc, $p < .05$. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. $n = 12–13$/group.

(1,45) = 3.22, $p = .07$) for regressive errors, without any effect for never-reinforced and perseverative errors (Fig. 3).

3.3. Western blotting

Two-way ANOVA showed a lesion effect for the D2 receptor (F (1,21) = 6.26, $p = .02$) and DAT expression (F(1,21) = 4.9, $p = .03$) only on the contralateral side to the ischemia, demonstrating that these proteins are decreased in the HI groups (Fig. 4B and C). The D1 receptor was not affected by lesion or treatment factors in both hemispheres (Fig. 4A). In the same way, the TH enzyme levels were not affected by any factor in both hemispheres (Fig. 5A). Nonetheless, we found a significant lesion effect for the pTH levels in both hemispheres: contralateral (F(1,22) = 5.2, p = .03) and ipsilateral (F(1,24) = 10.85, $p = .003$). The HI groups showed an increased expression of pTH levels and the post hoc demonstrated that the HIMPH group has higher expression levels compared to the CTS group in the ipsilateral hemisphere (Fig. 5B).

4. Discussion

In the present study, we aimed to investigate the effects of MPH administration on ADHD-like behaviors and dopaminergic signaling in the PFC of young animals that underwent neonatal HI. We observed that the MPH administration increased the locomotor activity in the open field especially in HI animals. Confirming our hypothesis the MPH reversed the attentional inflexibility caused by the neonatal HI but impaired the task acquisition in control rats in the ASST. Hypoxic-ischemic animals had lower DA D2 receptors and transporter (DAT) and higher pTH enzyme levels in the PFC, suggesting a disruption of the DA signaling in this brain region.

Table 2
Central and peripheral exploration in the Open field task.

	Central exploration				Peripheral exploration	
	Distance travelled (m)	Average speed (m/s)	Latency to first entry (s)	Number of entries	Distance travelled (m)	Average speed (m/s)
CTS	1.59 ± 0.24	0.11 ± 0.012	26.58 ± 6.88	11.66 ± 1.56	15.20 ± 1.03	0.053 ± 0.003
CTMPH	1.81 ± 0.24	0.091 ± 0.006	39.11 ± 8.73	15.16 ± 1.63	15.32 ± 0.81	0.055 ± 0.003
HIS	1.95 ± 0.29	0.11 ± 0.013	40.42 ± 9.75	13.46 ± 1.55	16.17 ± 0.67	0.057 ± 0.002
HIMPH	2.51 ± 0.26	0.13 ± 0.011	11.66 ± 3.82	17.25 ± 1.89	18.70 ± 0.95*	0.067 ± 0.003*

Results are expressed as mean ± S.E.M. Two-way ANOVA followed by Tukey's post hoc, p < .05. *HIMPH different from CTS and CTMPH groups. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n = 12–13/group.

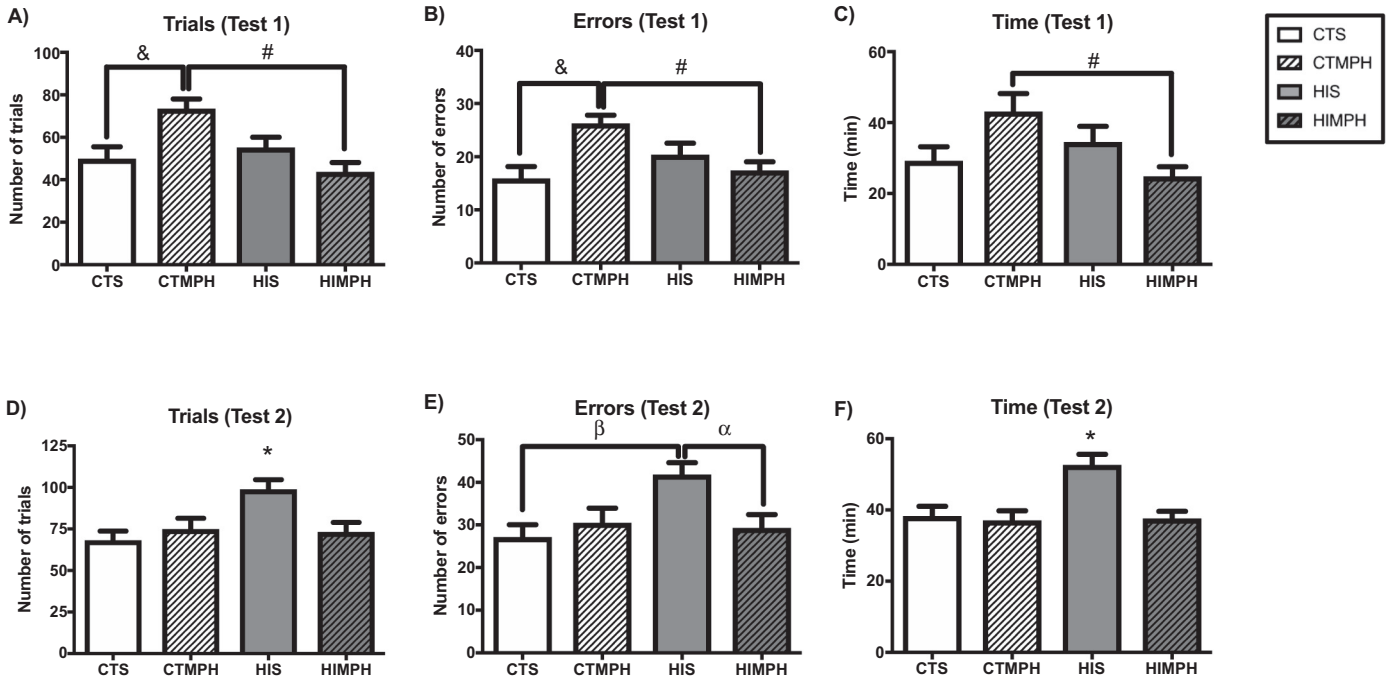

Fig. 2. Performance in the attentional set-shifting task. Measures of the egocentric response acquisition (Test 1) are demonstrated by (A) Number of trials to reach the criterion, (B) Number of errors and (C) Time to complete the test. The same variables are depicted for the Test 2 (D-F), in which a shift to visual-cue discrimination occurs, requiring animal's behavioral flexibility. Results are expressed as mean ± S.E.M. &CTMPH different from CTS; #CTMPH different from HIMPH; *HIS different from all other groups; ßHIS different from CTS; αHIS different from HIMPH. Two-way ANOVA followed by Tukey's post hoc, $p < .05$. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. $n = 12–13$/group.

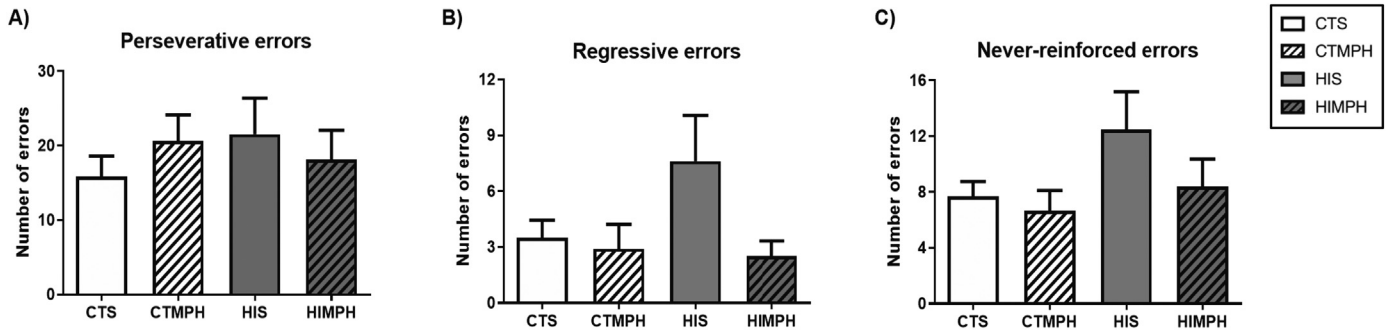

Fig. 3. Types of errors performed in the visual-cue discrimination (Test 2) of the attentional set-shifting task: (A) Perseverative, (B) Regressive and (C) Never-reinforced errors. Results are expressed as mean ± S.E.M. Two-way ANOVA followed by Tukey's post hoc, $p < .05$. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n = 12–13/group.

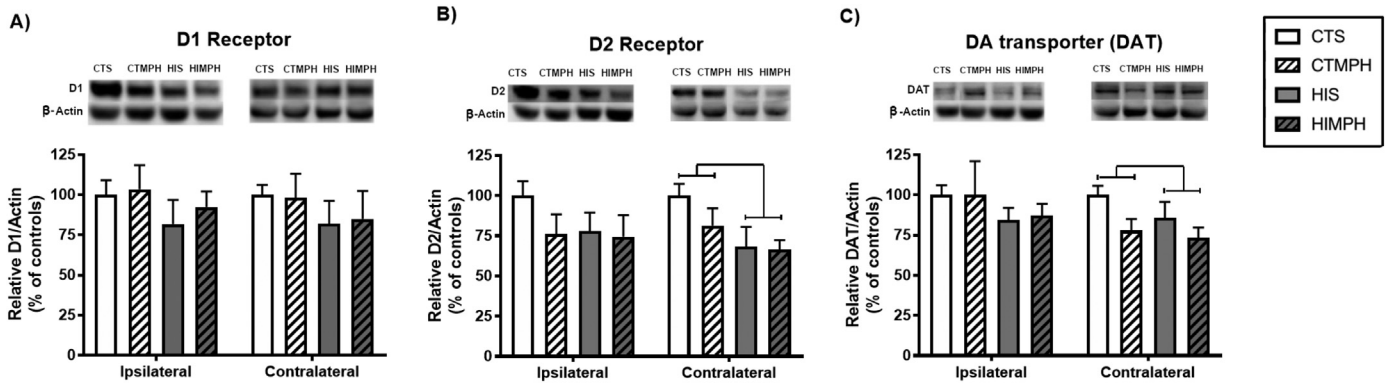

Fig. 4. Protein expression of DA receptors D1 (A) and D2 (B) and DA transporter (C) in the prefrontal cortex (PFC). Results are expressed as mean ± S.E.M. Two-way ANOVA followed by Tukey's post hoc, $p < .05$. Lesion effect was observed for D2 and DAT in the contralateral hemisphere. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. $n = 5–8$/group.

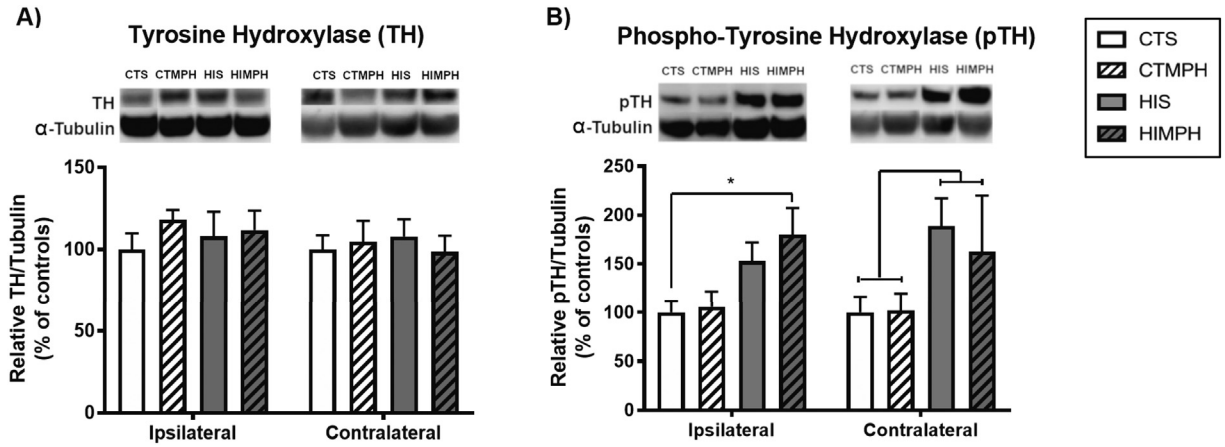

Fig. 5. Protein expression of tyrosine hydroxylase (TH) and phosphorylated-tyrosine hydroxylase (pTH) enzymes in the prefrontal cortex (PFC). Results are expressed as mean ± S.E.M. Two-way ANOVA followed by Tukey's post hoc, $p < .05$. Lesion effect was observed for pTH in both hemisphere. *HIMPH different from CTS group. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. $n = 6–7$/group.

4.1. MPH administration increased locomotor activity in adolescent HI animals

The present findings did not demonstrate a strong impact of the HI on exploratory activity in young rats, corroborating previous data in young HI animals (Carletti et al., 2012; Kim et al., 2017; Schuch et al., 2016b). However, hyperactivity has been a recurrent finding in adult rats submitted to neonatal HI (Deniz et al., 2018; Markostamou et al., 2016; Rojas et al., 2013; Sanches et al., 2015). The occurrence of divergent results in young and adult animals could be explained by the progressive brain damage that occurs following the neonatal HI, which was also associated with continuous cognitive impairment (Diaz et al., 2016; Mishima et al., 2004). Further studies should address this issue and evaluate locomotion in adult animals and using different MPH doses.

It is important to observe that only HI animals under MPH effect increased their locomotor activity in relation to both control groups. This result indicates that the MPH has a potentiating effect in hypoxic-ischemic rats, since the CTMPH group did not demonstrate this pattern of behavior. We should consider that the MPH is a psychostimulant drug in the same class as cocaine and amphetamine – drugs well-recognized to increase locomotion (McKinzie et al., 2002; Wellman et al., 2002). In a meta-analysis conducted by Askenasy (Askenasy et al., 2007) it was showed MPH-induced hyperactivity in 93% of the studies evaluating the MPH's acute locomotor effects in rats. We can infer that our findings in HIMPH group are in line with the literature data. It could be considered an unexpected effect because psychostimulants are used to treat hyperactivity in ADHD patients. However, it has been reported that the effects of stimulants depend on the behavior elicited by the environment. For example, dextroamphetamine use in children decreased activity during controlled classroom settings (with rules and planned activities); however, during physical education (where a free exploration is allowed), there was a significant drug-induced increase in motor activity (Porrino et al., 1983). Also, MPH treatment in ADHD children decreased expressively the activity in classroom but this effect was smaller in playground activities (Swanson et al., 2002). Taking together, these results suggest that the “calming effects” designated to psychostimulants are due to an improved focusing of activity rather than a decreased motor activity. This fact is recognized as “the paradoxical calming effect of psychostimulants” (Gainetdinov et al., 1999; Napolitano et al., 2010). Interestingly, the data of the present study are strictly in accordance with this statement since the open field is a new environment where a free exploration is allowed. Therefore, measures of the MPH effects in different contexts and with different stimulus should be cautiously interpreted.

The hyperactivity consequent to MPH treatment was also observed in the central zone of the open field apparatus. MPH administration increased the number of entries in the center in both groups and decreased the latency to the first entry in the central zone only in the HI group. An increased exploration in the central zone could be interpreted as a risk behavior since rodents display a natural aversion to open areas that ethologically mimic a situation of predator risk (Choleris et al., 2001; Prut and Belzung, 2003). Then, the increased locomotor activity of the HIMPH group observed even in the central area of the open field could enhance their exposure to danger.

A particular question is that the stimulant effect of MPH was observed especially in hypoxic-ischemic rats. This could be explained by the vulnerability of the striatum region to the Levine-Vannucci model of HI (Miguel et al., 2015; Schuch et al., 2016a) since that region has a fundamental role in motor activity (Grillner et al., 2005). Previous reports demonstrated loss of dopaminergic fibers (Park et al., 2013) and alteration in the DA receptors D1 and D2 (Filloux et al., 1996) in this model. In newborn piglets, the HI brain insult resulted in a transient increase in striatal DAT (Zhang et al., 2011). Thus, we can infer that alterations in the DA signaling in the striatum of HI animals would contribute for a higher response to MPH-induced hyperlocomotion, when compared to control animals. Interestingly, similar findings were observed in the recognized model of ADHD: the spontaneously hypertensive rat (SHR) respond more to the MPH administration (increasing locomotion) than their control group (Chelaru et al., 2012; Yetnikoff and Arvanitogiannis, 2013). Striatal dysfunction was also found in SHR animals such as polymorphisms on the DAT gene (DAT1) (Mill et al., 2005), higher DAT1 gene expression and higher striatal DAT density compared to control group (Roessner et al., 2010; Watanabe et al., 1997). Thus, the results observed in HI animals are in harmony with the findings from the most recognized animal model for ADHD, supporting the idea of the HI model as a potential option for the ADHD study.

4.2. Neonatal HI did not alter ASST acquisition learning (Test 1) but impaired attentional flexibility (Test 2)

The attentional set-shifting task (ASST) was used to measure executive function parameters such as learning process and attentional flexibility in rats. In Test 1, essentially involving learning skills (Ragozzino et al., 2002), we did not observe any impairment in hypoxic-ischemic animals. Conversely, in Test 2, HI animals demonstrated cognitive inflexibility. This result is in agreement with our previous

report showing that HI animals had no learning deficits in another ASST (using digging mediums and odors) despite the inflexibility identified in further stages of the task (Miguel et al., 2018). Such results were correlated to PFC atrophy found in HI animals. In agreement, other studies have revealed that lesions in the PFC in primates or rats do not affect initial discrimination learning, but profoundly impair the ability to inhibit an old strategy and utilize a new one (Brown and Bowman, 2002; Dias et al., 1996; Floresco et al., 2008; Ragozzino et al., 2002). In order to interpret the findings in the HI group, it should be considered that differential response to reward could influence on attention to task performance. Although there are no studies investigating directly food responses in the HI animal model, our previous report demonstrated that HI animals had perseverative responses in the 5-CSRTT that uses sweet pellet as reward and it could be an indication of higher wanting for the sweet food (Miguel et al., 2015). Then, this higher motivation for palatable food could improve acquisition in the first stage of ASST used in the present study. This behavior was already observed in rats that suffered intrauterine growth restriction (IUGR), which had higher dopamine response to sweet food resulting in better performance in the reversal learning of an ASST (Alves et al., 2015). The authors suggested that attention (salience) and wanting for the sweet food, a term called "incentive salience" (Berridge and Robinson, 1998; Wise, 2006), was greater in IUGR animals, causing them to become more focused on the task and, consequently, to perform better. In light of our objective of correlating the findings observed in HI rats with ADHD characteristics, clinical reports have shown a significant association between adult ADHD and obesity/overweight, eating disorders and bulimia nervosa (Cortese and Tessari, 2017; Nazar et al., 2016; Seitz et al., 2013). This idea could explain the standard performance in the acquisition phase in HI animals and reinforce our hypothesis of an association between HI and ADHD-related outcomes.

Attentional flexibility was measured in the ASST Test 2, when animals had to shift rule from the egocentric response to the visual-cue discrimination. A clear impairment in this test was observed in hypoxic-ischemic animals. Interestingly, this finding reveals that only highly cognitive demanding are compromised in this early age in HI animals since neither learning abilities nor locomotor activity were affected by the hypoxic-ischemic event. We have previously identified attentional inflexibility in adult animals that underwent neonatal HI (Miguel et al., 2018) and the present study demonstrated that these executive function impairments already exist in young animals (approximately 40 PND). This is interesting data considering that the ADHD prevalence is more common in children and adolescents.

4.3. MPH impaired acquisition learning in control animals (Test 1) but reversed HI-induced cognitive inflexibility (Test 2)

Our results regarding the Test 1 demonstrated that the MPH administration impaired the task rule acquisition only for control animals. Although this is not an expected result, current literature has described the ability of stimulants to induce cognitive deficits particularly depending on the dose used (Wood et al., 2014). MPH is the first pharmacological option for ADHD treatment and it is recognized to increase DA levels in the PFC (Berridge et al., 2006; Devilbiss and Berridge, 2008; Schmeichel et al., 2013; Spencer et al., 2012). As seen in humans, an increased in PFC DA levels was also observed in rats performing the initial rule acquisition in the ASST, demonstrating that this stage per se increased DA levels in the PFC (Stefani and Moghaddam, 2006). Thus, we can propose that the supplementary stimulation on DA pathway in control animals - by ASST and MPH treatment - could explain the learning impairment observed in CTMPH group. Accordingly, an "inverted-U" curve relationship has been proposed for PFC DA levels and cognition, where too little or too much DA levels have detrimental effects on performance (For review see (Cools and D'Esposito, 2011; Floresco, 2013).

Interestingly, the impairment seen in CTMPH group in Test 1 (rule acquisition) was not observed in Test 2 (attentional flexibility), indicating that higher DA levels for flexibility demands did not prejudice the animals' performance. In the same way, Stefani & Moghaddam (Stefani and Moghaddam, 2006) showed that higher PFC DA levels were associated with better performance during the rule shift in the ASST, indicating that the "inverted-U" shape is not applicable for attentional flexibility. In this highly cognitive demanding process, that requires not only rule acquisition but the inhibition of responding according to the previous rule, higher levels of DA facilitate performance, in a curvilinear manner (Floresco, 2013; Stefani and Moghaddam, 2006).

Hypoxic-ischemic animals demonstrated a profound impairment in the cognitive flexibility measures (Test 2) of the ASST and the MPH reversed these deficits. Such effect of MPH seems to be associated with a decrease of regressive and never-reinforced errors in HIS group. The combination of these types of errors has been considered as an index of the animals' ability to maintain a new rule strategy (Floresco et al., 2008; Floresco et al., 2006). Thus, HI animals showed difficulty to comprehend and sustain the new strategy and an MPH-induced improvement was reveal in these aspects. MPH is a drug well recognized to improve attentional deficits in ADHD patients (Sunohara et al., 1999; Yang et al., 2004) and we could translate the clinical features to our experimental model, demonstrating the predictive validity of the HI model to the ADHD study. Non-pharmacological strategies have been also adopted to treat the ADHD symptoms; for example, aerobic exercise resulted in benefits for cognitive function in children with ADHD (Ludyga et al., 2016, 2017, 2018). It has been well established that attentional flexibility is a process essentially dependent on the DA signaling in the PFC (Brozoski et al., 1979; Floresco, 2013). Aiming to interpret the current behavioral findings and to support the construct validity of the HI model, we measured different proteins involved in the dopaminergic signaling in the PFC region.

4.4. Dopaminergic system was disrupted in the PFC of HI animals

Different proteins responsible for an efficient DA transmission in the PFC were evaluated in both ipsilateral and contralateral hemisphere to the ischemic occlusion: 1) DA transporter (DAT, responsible for the DA reuptake), 2) DA receptor D1, 3) DA receptor D2, 4) tyrosine hydroxylase (TH, the rate-limiting enzyme for DA synthesis) and 5) phosphorylated-TH (pTH, the TH isoform phosphorylated on serine 40). Our findings demonstrate that HI animals had lower D2 and DAT levels in the contralateral PFC, when compared to control animals. These findings revealed a disruption of the DA signaling in the PFC of HI rats that are probably associated with the attentional inflexibility observed in HIS group.

The implication of D2 receptors for cognitive flexibility performance are documented in both experimental (Floresco et al., 2006) and clinical trials (Mehta et al., 2004; van Holstein et al., 2011). Previously, we had demonstrated decreased in PFC D2 receptors in further developmental stage (adulthood) of HI animals, that was also associated with attentional inflexibility (Miguel et al., 2018). In the present study, although the D2 receptor was altered by the neonatal HI, we did not observe alterations in D1 expression in both PFC hemispheres. It is known that D1 and D2 receptors are differently distributed in pyramidal neurons of the rat PFC, with little overlap between these receptors (Gaspar et al., 1995; Santana et al., 2008; Vincent et al., 1993). For cognitive flexibility, reports have identified that D1 stabilizes network activity, whereas D2 attenuates inhibitory influences, allowing PFC to process multiple stimuli. Thus, D2 activity places networks in a more labile state that facilitates flexible patterns of behavior, having then a key role in cognitive flexibility when compared to the D1 subtype (Durstewitz et al., 2000; Durstewitz et al., 2010; Floresco, 2013; Seamans and Yang, 2004). Therefore, we can infer that neonatal HI impaired cognitive flexibility by damaging neurons expressing D2 rather than D1 receptors.

DAT regulates the strength and duration of dopaminergic transmission and it is the main target for many psychostimulants that increase DA signaling (Schmeichel and Berridge, 2013; Volkow et al., 1995b). Interestingly, a DAT knock-out mice have been proposed as an ADHD model, showing spontaneous hyperactivity and impulsivity (Leo and Gainetdinov, 2013). Thus, the current findings, revealing an alteration in DAT expression in the contralateral PFC in HI animals, might be a contributing feature to the behavioral outcomes observed in this group.

Phosphorylated tyrosine hydroxylase (pTH) at Ser40 is positively related to the speed of dopamine synthesis (Dunkley et al., 2004) and HI animals had higher expression of pTH in both PFC hemispheres, indicating an increase in DA synthesis. Activation of dopamine D2 receptors results in selective inhibition of TH phosphorylation at Ser40 in the striatum (Lindgren et al., 2001) and D2 antagonists have opposite effects, increasing TH phosphorylation at Ser40 (Salvatore et al., 2000). We suggest that the upregulation observed in the pTH enzyme is a compensatory mechanism resulting from the downregulation of the D2 receptor in an attempt to improve DA signaling in this brain region. Although this is a molecular strategy that occurs to improve dysfunctional processes, it does not seem to be effective in HIS group, for its attentional flexibility deficits. Taking together, we demonstrated that neonatal HI had the ability to impact on DA system-related proteins in the PFC, giving additional support to the construct validity of the HI model for the ADHD study.

4.5. Methylphenidate increased pTH levels in the PFC of HI rats

MPH administration appears to be also related to compensatory upregulation of pTH enzyme in HI animals, that was higher in the HIMPH group in the ipsilateral PFC, when compared to the CTS group. Higher pTH enzyme levels indicate higher TH activity and probably higher DA levels in the PFC, that we could presume to be associated with the improved attentional flexibility observed in this group. MPH is well recognized to increase DA levels in the PFC (Berridge et al., 2006; Devilbiss and Berridge, 2008; Schmeichel et al., 2013; Spencer et al., 2012) and we assume that an increase in the DA signaling in the PFC of HIMPH animals was responsible for their attentional improvements. Although MPH administration was able to reverse attentional impairments caused by the neonatal HI, the drug did not impact on protein levels of DA receptors and DAT. Our treatment comprehended approximately 7 days of MPH administration and we propose that this short period was insufficient to alter brain protein expression.

The higher DA synthesis in HIMPH group could be also generalized to the striatum, a region that demonstrates higher levels of DAT (Piccini, 2003; Sesack et al., 1998) and consequently is more sensitive to the MPH effect. Therefore, higher DA levels in the HIMPH group might be also associated with the increased locomotor activity observed in the open field test.

In conclusion, our findings showed that the MPH administration reversed the impairments in attentional flexibility in adolescent rats that underwent neonatal HI. This result supports the predictive validity of the rat model of HI as an animal model to study ADHD. Alterations in proteins involved in the DA signaling in the PFC of hypoxic-ischemic rats also confirmed the construct validity of the Levine-Vannucci model of HI. Given that ADHD is the most diagnosed disorder in children and negatively impacting on their quality of life new experimental options to study the etiology, neurobiology and effective therapies are necessary and urgent.

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4. CAPÍTULO 2

Artigo: *Increased hippocampal BDNF levels and improved long-term memory in hypoxic-ischemic rats following methylphenidate administration – one more step toward the characterization of an alternative ADHD animal model*

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No Capítulo 2 o nosso foco de estudo permaneceu nas análises de características relacionadas ao TDAH em animais HI jovens. Sabendo que os deficit de atenção podem afetar diretamente a aprendizagem e a memória, propusemos avaliar especificamente estes parâmetros sob o efeito do tratamento com MFD. Ainda, mensuramos os níveis de BDNF maduro tanto no hipocampo quanto no CPF de animais HI após 15 dias de tratamento com MFD.

Animais HI apresentaram deficit de aprendizagem e de memória em todos os parâmetros avaliados (aprendizagem espacial, aprendizagem reversa, memoria de curta e longa duração); no entanto, o MFD foi capaz de melhorar apenas os deficit na memoria de longa duração dos animais HI. A melhora neste aspecto de memória esta possivelmente associada com o aumento do BDNF observado no hipocampo de animais HI após o tratamento com MFD.

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Increased hippocampal BDNF levels and improved long-term memory in hypoxic-ischemic rats following methylphenidate administration– one more step toward the characterization of an alternative ADHD animal model

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Increased hippocampal BDNF levels and improved long-term memory in hypoxic-ischemic rats following methylphenidate administration– one more step toward the characterization of an alternative ADHD animal model

Running head: MPH affects BDNF and long-term memory in HI rats

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Abstract

Background: Attention-deficit/hyperactivity disorder (ADHD) is characterized by inattention, hyperactivity and/or impulsivity that frequently disrupts learning and memory of affected individuals. Previously, we demonstrated that neonatal hypoxia-ischemia (HI) induced attentional deficits in rats and methylphenidate (MPH) administration, the treatment of choice for ADHD, reversed these deficits. However, the MPH effects on memory deficits after the HI procedure has not been evaluated yet. **Aims:** We aimed at analyzing learning and memory performance of young HI rats after MPH administration and correlate their performance with BDNF levels in the prefrontal cortex and hippocampus. **Methods:** Male Wistar rats were divided into four groups (n=11-13/group): control saline (CTS), control MPH (CTMPH), HI saline (HIS) and HIMPH. The HI procedure was conducted at postnatal day (PND) 7 and memory tasks between PND 30-45. MPH administration (2.5mg/kg, i.p.) occurred 30min prior to each behavioral session and daily, for 15 days, for the BDNF assay (n=5-7/group). **Results:** HI animals demonstrated learning and memory deficits in all parameters evaluated, in the Novel-object recognition (NOR) and Morris water maze (MWM) tasks. However, MPH was only able to improve long-term memory deficits observed in the MWM. Increased BDNF levels were found in the hippocampus of HI animals after MPH treatment, that can be related to their improvement in long-term memory.

Conclusions: Our results confirm that neonatal HI induces learning and memory deficits, providing additional support to our hypothesis that neonatal HI can result in ADHD-like characteristics in rats. Additionally, MPH administration had partial predictive validity for this model. Funding: CAPES, CNPq.

Keywords: hypoxia-ischemia; attention-deficit/hyperactivity disorder; brain-derived neurotrophic factor; water maze; novel-object recognition

Introduction

Methylphenidate (MPH) is the first choice drug for the treatment of children and adolescents with Attention-deficit/hyperactivity disorder (ADHD) (National Institute for Health and Care Excellence, 2018). Even though inattention and hyperactivity have been considered the main dysfunctions in ADHD patients, learning and memory deficits are frequently described, and are considered important co-morbidities (Andersen et al., 2013; Mangina and Beuzeron-Mangina, 2009). MPH action mechanism involves mainly the blockade of the dopamine (DA) transporter (DAT), reducing the clearance of this neurotransmitter from the synaptic cleft (Schmeichel and Berridge, 2013; Volkow et al., 1995). DA regulates activity in the prefrontal cortex (PFC) and hippocampus, as well as the communication between these structures (Birn et al., 2018; Li et al., 2003). Therefore, MPH treatment in ADHD patients has been associated to improvements in both executive functions impairments (mainly related to PFC function) (Kramer et al., 2001; Sunohara et al., 1999), and memory deficits dependent on the hippocampal activity (Bedard and Tannock, 2008; Rhodes et al., 2006; Verster et al., 2010). Based on the potential cognitive enhancement associated with this drug, it has been increasingly used by healthy students (Guthrie et al., 2003) but inconsistent findings are observed across individuals diagnosed or not with ADHD (for review (Cools and D'Esposito, 2011). For example, our group demonstrated that MPH

administration in control rats affected learning during an attentional set-shifting task (Miguel et al., 2019) and this finding agrees with the current literature which proposed that excessive DA activity in the PFC (which may occur by MPH administration) could disturbed cognition (Arnsten, 2011; Floresco, 2013).

DA stimulation regulates some neurotrophic factors in the brain, such as the brain-derived neurotrophic factor (BDNF) (Iwakura et al., 2008; Kuppers and Beyer, 2001; Williams and Undieh, 2009). For example, experimental findings demonstrated that DAT-knockout (DAT-KO) rodents had dysregulated BDNF expression in both frontal cortex and striatum (Fumagalli et al., 2003; Leo et al., 2018). This neurotrophin is involved in neuronal growth and survival, neurotransmitter modulation and neuronal plasticity – crucial for learning and memory (Bathina and Das, 2015). For this reason, decreased BDNF activity is hypothesized to be associated with the ADHD pathophysiology (Tsai, 2007; Tsai, 2017). Lower serum BDNF levels were observed in boys with ADHD-inattentive subtype when compared to healthy controls and these levels increased to a higher extent in the inattentive group after 8 weeks of MPH treatment (Akay et al., 2018). Amiri and colleagues also reported increased BDNF levels in ADHD subjects after 6 weeks of MPH treatment (Amiri et al., 2013), reinforcing the relationship between DA signaling and BDNF production.

ADHD etiology is complex, involving multiple genetic and environmental factors. Pregnancy and birth complications, such as perinatal hypoxia-ischemia (HI), are environmental conditions associated with an increased risk for ADHD (Millichap, 2008; Zhu et al., 2016). Neonatal HI has been extensively modeled in rodents aiming at determining underlying mechanisms and effectiveness of therapeutic interventions (Yager and Ashwal, 2009). We have shown that HI induction using the well-recognized model of Levine-Vannucci (Vannucci and Vannucci, 2005) was able to induce ADHD-related phenotypes in adult rats, such as attentional and executive function impairments (Miguel et al., 2018; Miguel et al., 2015). These behavioral deficits were associated with dopaminergic dysfunction in the PFC, as it occurs in patients with ADHD (Miguel et al., 2018). Recently, we also demonstrated that acute MPH administration reverses executive function impairments in adolescent rats submitted to neonatal HI. However, MPH effects concerning memory deficits dependent on the hippocampus have not been studied yet in the HI model.

Thus, we aimed to analyze the effects of MPH in control and hypoxic-ischemic rats using two different memory tasks, the novel-object recognition (NOR) and the Morris Water maze (MWM). The NOR task measures episodic, non-spatial memory, and does not involve primary reinforcement such as food or electric shocks (Ennaceur and Delacour, 1988) and the MWM evaluates spatial learning and memory using an aversive condition (Vorhees and

Williams, 2006). Additionally, BDNF levels were analyzed in both groups after MPH treatment - in the PFC and hippocampus - considering its importance in ADHD pathophysiology and neuronal plasticity. We hypothesized that MPH administration improves cognitive deficits resulting from neonatal HI via an increase in brain BDNF levels.

Materials and Methods

Animals

Male Wistar rats were obtained from the Central Animal House of the Institute of Basic Health Sciences (Universidade Federal do Rio Grande do Sul) and maintained in a controlled room temperature (22–24°C) on a 12:12h light/dark cycle, with food and water available *ad libitum*. On the 7th PND, pups were randomly distributed into control and HI groups and then subdivided in saline and MPH treatment, resulting in four experimental groups: control treated with saline (CTS, n=11), control treated with MPH (CTMPH, n=13), HI treated with saline (HIS, n=13) and HI treated with MPH (HIMPH, n=12). Female pups of the litters were used for another research project. Animals were maintained with their dams until PND 21 when they were weaned and housed in 2-3 per cage (Plexiglas cages). Another

set of animals (n=5-6/group), that did not undergo behavioral tasks, was used for BDNF immunoassay. All procedures were approved by the Institutional Ethics Committee on Animal Use (Nº 29750) and were in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023), the guide of the Federation of Brazilian Societies for Experimental Biology and the Arouca Law (Nº 11.794/2008).

Hypoxia-ischemia (HI)

The HI procedure was induced based on the protocol developed by Levine (Levine, 1960) and modified by Rice-Vannucci (Rice et al., 1981; Vannucci and Vannucci, 2005). At PND 7, rats were anesthetized with halothane (2– 4%) and an incision on the ventral surface of the neck was made to permit access to the right common carotid artery. After isolation of the artery from other surrounding anatomical structures, it was permanently occluded with a surgical silk thread. Following a 2-h interval with their dams to recover, the pups were placed in chambers partially immersed in a 37°C water bath, where they were exposed to a hypoxic atmosphere (8% oxygen and 92% nitrogen, 5 L/min) for 90 min. The animals returned immediately to maternal care after hypoxia. Control animals were submitted to sham surgery,

i.e., animals received only anesthesia and neck incision (Miguel et al., 2019; Miguel et al., 2018; Miguel et al., 2015).

MPH administration

Methylphenidate hydrochloride (MPH) (Novartis, Brazil) was dissolved in saline solution (0.9% NaCl) and injected intraperitoneally (dose of 2.5mg/kg, volume of 1 ml/kg) 30 minutes prior to each behavioral session (from PND30 to PND45). Control animals received equivalent volume of saline solution. The other set of animals (n=5-6/group) received the same daily treatment but did not undergo behavioral tasks – they were designated to biochemical analysis. The MPH dose of 2.5mg/kg, adopted in this study, corresponds to a medium dose (Dafny and Yang, 2006) and improved attentional deficits of HI animals in our previous study (Miguel et al., 2019).

Behavioral Analysis

The novel-object recognition test was performed on PND 31, and the Morris water maze tests from PND 32 to 37 (reference memory) and from PND 42 to 45 (working memory). All behavioral tasks were conducted from 1 p.m. to 5 p.m.

Novel-object Recognition (NOR)

The novel-object recognition task is widely used to evaluate learning and memory based on the natural tendency of rodents to interact with a novel object over a familiar one (Ennaceur and Delacour, 1988). On the day previous the NOR task (PND 30), animals were habituated to the apparatus (wood square arena: 54cm length, 38cm width and 45cm height) for 5 minutes. In the following day (PND 31), during the first session, the rats were placed in the apparatus with two similar objects (A and A') and the time exploring each object was recorded for a total of 5 minutes. The second session (test phase) was conducted after a 5 minutes interval, aiming to evaluate the short-term memory (Deniz et al., 2018; Pereira et al., 2008). Rats were replaced in the apparatus with a familiar (A) and a novel object (B) and the time exploring each object was recorded for 5 minutes. Object exploration was defined when the animal sniffed or touched the object with the paws; climbing onto the object without sniffing was not considered exploration (Klein et al., 2018; Pereira et al., 2008). An object

preference index was calculated using the test session data, consisting of the difference between the exploration of the new object and the familiar object, divided by the total time exploring both objects (B − A / B + A, where B is the new object and A is the familiar object) (Deniz et al., 2018; Pereira et al., 2008).

Morris Water maze (MWM)

The Morris Water Maze task was used to evaluate spatial learning, long-term reference memory, perseveration in the previous target (reversal learning) and working memory (Vorhees and Williams, 2006). Training in the MWM task started the day after the NOR task (PND 32). The maze was composed by a circular tank (117 cm diameter) virtually divided into 4 quadrants and filled with water at 22 ± 1°C. A transparent escape platform was 2 cm submerged beneath the water surface and the rats had to learn the platform position based on visual distal cues placed on the walls of the testing room. In each trial (maximum of 60 sec), the rat was placed in the water (facing the tank wall) in an established random position - that changed daily and was the same for all animals.

In the spatial learning training, the submerged platform remained at the same position in all daily sessions, and the latency to reach the platform was measured throughout the

sessions (5 sessions with 4 trials/session, 20min ITI). If a rat failed to find the platform in 60 sec, it was gently guided through the water and placed on the platform for 10 sec. After training, on the sixth day, the probe trial to assesses long-term reference memory was conducted. The platform was removed, and each rat was placed into the water on the opposite quadrant of the platform target area. The following parameters were measured during this probe trial (60 sec): the latency to the first target area crossover, the number of crossings on the target area, the time spent on the target and in the opposite quadrant (Deniz et al., 2018; Klein et al., 2018; Pereira et al., 2008).

The reversal learning phase was conducted four days after the probe trial. The location of the platform was switched to the opposite quadrant, measuring the animals' ability to extinguish their initial learning of the platform's position and search for a new goal position. It is known that rats rapidly switch their search strategies to the new goal on the first day of reversal testing – and perseverations to the old platform position may be seen on individual trials within this day (Vorhees and Williams, 2006). Therefore, we analyzed the time spent in the previous platform quadrant in 4 trials (5min ITI) on a single day. This testing day was the first session of the working memory protocol.

In the working memory protocol, the platform was reallocated daily, and the rats were subjected to 4 trials/day (with an ITI of 5min), during four consecutive days. The mean

latencies to find the platform on each trial were calculated for all testing days (Carletti et al., 2016; Pereira et al., 2008).

Mature BDNF assay

For mature BDNF analysis we used a different set of animals that did not perform any behavioral task. This procedure was conducted considering that behavioral tasks with long period of training or exercise can quickly alter BDNF levels (Hall et al., 2000; Silhol et al., 2007). At PND 30, animals (5-7/group) started the daily MPH or saline injections that were administered for over the same period of treatment from the animals that performed the behavioral tasks (15 days). Animals were euthanized by decapitation 30min after the last drug injection. The hippocampus and prefrontal cortex were quickly dissected out bilaterally, placed on liquid nitrogen and stored at -80°C until the biochemical assay.

Mature BDNF concentration was measured through the E-Max ELISA kit (Promega, USA), according to the manufacturer's instructions. Briefly, the samples of each rat were individually homogenized in lysis buffer (137mM NaCl, 20mM Tris-HCl (pH 8.0), Igepal (1%), glycerol (10%), 1mM phenylmethanesulfonyl fluoride (PMSF), 0.5mM sodium vanadate, 0.1mM EDTA, and 0.1mM EGTA) and centrifuged for 3min at 14,000 rpm at 4°C.

Supernatant was diluted (1:5 v/v) in sample buffer and incubated in 96-well flat-bottom plates previously coated with anti-BDNF monoclonal antibody, and blocked with Block & Sample buffer. After blocking, plates were incubated with polyclonal anti-human antibody for 2 h and horseradish peroxidase for 1 h. Colorimetric reaction with tetramethylbenzidine was quantified in a plate reader at 450 nm; the standard BDNF curve ranged from 0 to 500 pg/mL (Klein et al., 2018; Pereira et al., 2009).

Statistical analysis

Two-way ANOVA followed by Tukey's post hoc test, with lesion and treatment as factors, was used to analyze the NOR task, long-term and working memories in the MWM and the BDNF concentration. Reference training and reversal learning in the MWM were evaluated by repeated-measures ANOVA. All variables were expressed as mean±standard error of the mean (SEM), and the results were considered significant when $p<.05$. Data were analyzed using the IBM Statistical Package for the Social Sciences (SPSS) version 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Novel-object recognition test

Two-way ANOVA demonstrated significant main effects for *lesion* ($F(1,45)=11.19$, $p<.01$, partial $\eta^2=0.19$) and *treatment* ($F(1,45)=6.99$, $p<.05$, partial $\eta^2=0.13$) and a trend towards a *lesion*treatment* interaction effect ($F(1,45)=3.6$, $p=.06$, partial $\eta^2=0.07$) for the novel-object preference index. Tukey's post hoc indicated that the CTS group had higher index when compared to all other groups (Figure 1). These results indicate that HI animals, independent of the treatment, had impairments in the ability to discriminate the novel object. The preference index in the CTMPH group was similar to the HI animals, indicating that MPH administration impaired the discrimination of the new object in control rats.

[Please insert Figure 1 here]

Morris Water Maze

Spatial learning

Spatial learning was assessed in the water maze for five consecutive days, with the submerged platform in the same position. Repeated-measures ANOVA showed significant main effects for *lesion* ($F(1,45)=12.96$, $p<.01$, partial $\eta^2=0.22$) and *session* factors

(F(3.32,149.72)=26.86, p<.0001, partial η^2=0.37) considering the latency to find the platform. No *treatment* or *lesion*treatment*session* interaction effects were observed, indicating spatial learning impairment in hypoxic-ischemic rats that was not recovered by MPH administration. Additionally, when analyzing the learning curve of each group separately, we observed that CTMPH animals decreased their latency to find the platform from the second session onwards while the other groups decreased latency only from the fourth session onwards (Figure 2). These results suggest a better spatial learning by MPH administration in CT rats.

[Please insert Figure 2 here]

Long-term reference memory

In the probe trial, without the platform, two-way ANOVA demonstrated a *lesion* effect for the variables latency to cross the target (F(1,45)=9.87, p<.01, partial η^2=0.18) and number of crossings on the target (F(1,45)=6.58, p<.05, partial η^2=0.12), with no effect of *treatment* or *lesion*treatment* interaction effect. Tukey post hoc showed that HIS animals had higher latency to cross the target in comparison with CTS and CTMPH groups (Figure 3A). The HIS animals also made fewer number of crossings on the target when compared to

the CTS group (Figure 3B). These findings suggest partial recovery of memory deficits in HI animals by the MPH administration. No effect was observed for the time spent on the target area nor the time spent in the opposite quadrant (Fig. 3C-D).

[Please insert Figure 3 here]

Perseveration in the previous target (reversal learning)

The platform was relocated to the opposite quadrant aiming to investigate the behavioral flexibility of the animals to extinguish their initial learning of the platform's position. Within the first day of reversal learning (4 trials, 5min ITI), repeated-measures ANOVA demonstrated *lesion* ($F(1,45)=4.09$, $p<.05$, partial $\eta^2=0.08$) and *trial* ($F(2.03, 91.37)=17.86$, $p<.0001$, partial $\eta^2=0.28$) effects, indicating that hypoxic-ischemic animals perseverate more in the previous platform location over the trials. Comparing the performance within the 4 trials, we observed that the CTS group decreased their time spent on the previous platform quadrant from the second trial and the CTMPH group from the third trial onwards (Figure 4). On the contrary, both HIS and HIMPH groups did not significantly decrease the latency on the previous platform quadrant throughout the trials (Figure 4A), confirming their difficulty to abandon the previous platform position.

Working memory

To measure working memory capacity, the platform was relocated daily, and the latency to find the new location within 4 successive trials was assessed. Two-way ANOVA performed for each trial demonstrated *lesion* effect on the third ($F(1,45)=5.86$, $p<.05$, partial $\eta^2=0.11$) and fourth ($F(1,45)=8.10$, $p<.01$, partial $\eta^2=0.15$) trials but no *treatment* or *lesion*treatment* interaction effects were observed. These findings showed working memory impairments in HI animals that were not improved by the MPH administration (Figure 4B).

[Please insert Figure 4 here]

Mature BDNF assay

BDNF analyses were conducted in the PFC and hippocampus from both hemispheres – ipsilateral and contralateral to the ischemic lesion. Two-way ANOVA did not demonstrate any significant effect for the PFC in both hemispheres (Figure 5A). In the ipsilateral hippocampus, it was seen *lesion* ($F(1,23)=7.89$, $p<.01$, partial $\eta^2=0.25$) and *treatment* ($F(1,23)=7.59$, $p<.05$, partial $\eta^2=0.24$) effects. Tukey post hoc revealed that the HIMPH

group had an increase in BDNF levels in comparison to all other groups (Figure 5B). In the contralateral hippocampus, only a trend towards the *lesion* effect was observed ($F(1,23)=3.15$, $p=.08$, partial $\eta^2=0.12$) (Figure 5B).

[Please insert Figure 5 here]

Discussion

We investigated the relationship between neonatal hypoxia-ischemia and ADHD-like phenotypes in rats. In this context, the current study was proposed to examine the effects of MPH administration on learning and memory tasks performance and mature BDNF levels in the PFC and hippocampus of young animals that underwent neonatal HI. MPH administration improved long-term memory deficits observed in the MWM task and increased BDNF levels in the hippocampus of hypoxic-ischemic animals. However, MPH administration was not able to reverse learning and short-term memory deficits resulting from HI. In control animals, MPH had differential effects depending on the context of the task: impairments were observed in the NOR task while no effects or rather an improvement were observed in the MWM task.

MPH administration did not improve object recognition deficits of HI animals and disturbed the performance in CT rats

Supporting our hypothesis, findings in the NOR task demonstrated an impairment to discriminate novel and familiar objects by adolescent rats submitted to neonatal HI, as also seen in SHR rats, an animal model of ADHD (Pires et al., 2009). This result is in line with the current literature since impaired object recognition by the neonatal HI is a frequent finding (Deniz et al., 2018; Pereira et al., 2008; Rojas et al., 2013). Considering that we previously demonstrate alterations in DA signaling in the PFC of hypoxic-ischemic rats (Miguel et al., 2019; Miguel et al., 2018) and that there is evidence that optimal DA levels in the PFC are required for object recognition (Hotte et al., 2005; Nagai et al., 2007), we can suggest that the impairment observed in the object recognition may be associated with deregulated DA transmission. Then, we also hypothesized that administration of MPH (that increases DA levels) could improve object recognition in HI animals. Contrarily to the hypothesis, MPH administration did not reverse object recognition deficits of HI animals. The absence of a protective effect of MPH could be justified by the psychostimulant and locomotor effects of this drug (Askenasy et al., 2007). Corroborating this idea, the same MPH dose used in the current study induced hyperactivity in adolescent HI animals in the

open field task (Miguel et al., 2019). We believe that hyperactivity associated with MPH use may be responsible to an unfocused object exploration and consequently impaired object recognition memory.

Additionally, we observed that control rats under MPH effect had worse performance in object recognition test than control rats non-treated. Disruptive object recognition following MPH administration in control animals was already observed in several studies (Bouchatta et al., 2018; Chuhan and Taukulis, 2006; Heyser et al., 2013; Heyser et al., 2004). As suggested above, increased locomotor behavior after MPH administration could interfere on object recognition. We previously identified hyperlocomotion using the same-dose MPH injection (2.5mg/kg) adopted in the present study in adolescent control rats (PND30) which had an increased number of entries in the central area of the open field (Miguel et al., 2019). We also observed that MPH disturbed the acquisition learning of a task involving food reward, corroborating previous studies that reported cognitive impairments related to excessive PFC DA stimulation (as by the MPH administration) (Arnsten, 2011; Floresco, 2013). Thus, considering these unexpected findings, MPH effects under distinct doses and contexts should be carefully assessed and interpreted when evaluating the different studies.

MPH administration did not alleviate learning impairments of HI rats in the water maze task but improved spatial memory of CT rats

Differently from the NOR task, learning in the MWM involves an aversive condition in which animals must find a hidden platform (using spatial cues) to escape from the water. Our results demonstrated learning impairments in the acquisition phase of the MWM task resulting from neonatal HI, as observed in several previous studies (Deniz et al., 2018; Odorcyk et al., 2017; Pereira et al., 2008). This result is also in accordance with findings on SHR rats, which show deficits in spatial learning (Grunblatt et al., 2015; Liu et al., 2008) and with data showing learning deficits in ADHD patients (Andersen et al., 2013). MPH administration had no impact on these HI-induced learning deficits, although it improved the performance of control rats, confirming the well-described cognitive enhancement of the drug (Linssen et al., 2014). It should be noted that MPH administration had a positive effect for control rats in this task, differently from the negative result observed in the NOR task. We believe that MPH-induced hyperactivity, while disturbing object recognition, can favor the search for the escape platform in the MWM – involving higher motivation and focus. As the hippocampus is essential for the initial consolidation of explicit or declarative memory (Squire, 1992), and that large hippocampal atrophy has been linked to learning and memory deficits following the HI procedure (Miguel et al., 2015; Pereira et al., 2007), we postulate

that MPH benefits were not sufficient to counterbalance the learning impairments related to this extensive atrophy in HI animals.

Deficits in long-term reference memory are improved by methylphenidate injection in HI animals

After spatial learning, the probe trial was conducted (without the platform) to investigate the retention of long-term memory. As well as during learning training, hypoxic-ischemic animals performed worse than control rats, with a higher latency to cross the target and lower number of crossing on the target (Figure 3). This was an expected finding considering the learning training impairment observed in HI animals and their well-described hippocampal atrophy, as discussed in the previous section. In agreement to these findings, a meta-analysis showed long-term memory deficits in ADHD patients, and these deficits were associated to their learning deficit observed at the acquisition stage (Skodzik et al., 2016).

Although MPH administration did not impact on HI-learning deficits, it was effective to ameliorate the long-term memory of HI animals observed in the probe trial. The first point we should consider is that although HI animals had lower learning rates compared to controls, all groups decreased their latency to find the platform across sessions, indicating learning of

the platform position – necessary for later retention. Moreover, when comparing the learning curve of HIS and HIMPH groups (Figure 2), we observe that HIMPH had already better performance than the HIS groups on some points, although no statistical significance was found.

Memory formation is a process that has several stages (acquisition, consolidation and retrieval) and different signaling molecules and brain structures participate at each stage. For example, DA stimulation in the hippocampus proved to be essential for the persistence of new memories over time, but does not affect acquisition *per se* (Bethus et al., 2010). Thus, we believe that although the hippocampus of HI animals has an extensive atrophy that disrupts acquisition in both groups (treated or not with MPH), DA stimulation induced by MPH administration was able to improve the persistence of the new acquired memory in the HIMPH group. In agreement to our findings, others have also found that MPH administration did not alter memory acquisition in the MWM, but was able to enhance memory retention in SHR rats (Guo et al., 2012; Tian et al., 2009).

Moreover, we should consider that although the hippocampus is crucial for spatial memory formation, it does not store long-lasting memories, having the PFC an important role in this process (Leon et al., 2010; Maviel et al., 2004). We previously demonstrated that MPH administration in HI animals increased phosphorylated-tyrosine hydroxylase levels in

the PFC, indicating an increase in DA synthesis in this structure (Miguel et al., 2019). Thus, we can suggest that the MPH effects in structures other than the hippocampus in HI rats may be also associated with their improvement of long-term memory.

Methylphenidate did not improve behavioral inflexibility nor working memory deficits resulting from hypoxia-ischemia

Behavioral flexibility was measured by the animal's ability to extinguish their initial learning of the platform position. We observed that HI animals had difficulties to abandon the previous platform position and the MPH did not impact on animals' performance in both groups. Behavioral inflexibility is a major condition reported in ADHD patients and was also described in hypoxic-ischemic rats previously (Miguel et al., 2019; Miguel et al., 2018). Although MPH reversed attentional inflexibility of HI animals in our previous study (Miguel et al., 2019), the task used in that experiment was designed to capture specifically this type of PFC dependent behavior. Differently, the MWM is a task mainly designed to capture hippocampal-dependent functions.

Working memories abilities in the MWM were also disrupted in HI animals, as expected. Working memory deficits are a core symptom of ADHD patients (Kofler et al.,

2018), and serve as an interface between the environment and long-term memory (Baddeley, 2007). Besides, impairments in working memory in ADHD children are associated with difficulties to maintain goal-directed behaviors (Kofler et al., 2018). Our results demonstrating working memory impartments is an advantage of the HI model in relation to the SHR strain, that did not demonstrate an impairment of working memory (Sontag et al., 2013). As observed in acquisition learning and reversal learning in the MWM, MPH administration did not improve working memory deficits of HI rats. The overall results in the MWM task indicate that the positive MPH effects in HI animals were observed only on processes related to long-term memory storage, but not short-term memory processes.

Increased BDNF levels in the ipsilateral hippocampus were observed following MPH administration in animals submitted to neonatal HI

Our present findings demonstrate that systemic MPH administration (for 15 days) increased BDNF levels in the lesioned hippocampus of HI animals, and we suggest that this increase was related to the improved long-term memory in this group. Higher BDNF levels induced by MPH seem to be an attempt to recover the hippocampal dysfunction widely reported in HI animals (Miguel et al., 2015; Rojas et al., 2013). This dysfunction is not

necessarily related to lower BDNF levels, since HI-untreated animals have similar BDNF levels than control rats, but can be associated to other neurotrophic factors, such as NGF and NT-3 (Fantacci et al., 2013).

BDNF is known to induce long-term potentiation (LTP) in the hippocampus (Pang et al., 2004; Pastalkova et al., 2006), a form of synaptic plasticity that underlies long-term memory formation (Izquierdo and Medina, 1997; Morris, 2003). Thus, we assume that our regime of systemic MPH administration was able to increase DA levels in the hippocampus, that resulted in higher BDNF expression and increase LTP – which in turn improved long-term memory. DA stimulation, as the one induced by MPH treatment, was already shown to regulate BDNF expression (Iwakura et al., 2008; Kuppers and Beyer, 2001; Williams and Undieh, 2009). More specifically, when this stimulation occurred directly in the hippocampus (by DA receptors agonists or MPH) it increased hippocampal BDNF levels and LTP, that were related to persistence of long-term memory (Rossato et al., 2009; Rozas et al., 2015). Increased BDNF levels, but also other synaptic plasticity parameters, such as ultrastructural changes (longer active zone, thicker postsynaptic density and larger synaptic curvature), were also observed following systemic MPH administration in both control mice (Lee et al., 2012) or in the SHR strain (Kim et al., 2011; Tian et al., 2009). Another interesting point to be mentioned is that BDNF activity is hypothesized to be associated with ADHD

pathophysiology (Tsai, 2007; Tsai, 2017) and that 6 or 8 weeks of MPH treatment increased BDNF levels in ADHD patients (Akay et al., 2018; Amiri et al., 2013).

In conclusion, our findings indicated that MPH administration was able to improve HI-induced long-term memory deficits, which was probably associated with the increased BDNF levels observed in the lesioned hippocampus of HIMPH animals. Learning and short-term memory deficits were also observed in HI animals, but the MPH injection previous to the behavioral task was not sufficient to ameliorate these deficits. Altogether, these findings suggest that the neonatal HI model in rats induces learning and memory deficits that can be translated to the behavioral profile often observed in ADHD patients (face validity). However, the predictive validity of the MPH treatment in this model was not totally confirmed, since only one aspect of memory retention was improved by the drug. Further studies are needed to determine the underlying mechanisms involved in this specific MPH benefit in the HI model.

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Declaration of Conflicting Interests

The Authors declare that there is no conflict of interest.

Figure legends:

Figure 1: Preference index for the novel object in the Novel-object recognition (NOR) task. Results are expressed as mean ± S.E.M. Two-way ANOVA followed by Tukey's post hoc, $p<.05$. *CTS different from all other groups. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n=11-13/group.

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Figure 3: Performance during the probe trial to measure long-term reference memory in the MWM task. The following parameters are shown: A) Latency to the first cross on the target, B) Number of target crossings, C) Time spent on the target quadrant, and D) Time spent in

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Figure 4: Reversal learning (A) and working memory (B) performance in the MWM task. Results are expressed as mean ± S.E.M. Repeated-measures ANOVA (reversal learning) and Two-way ANOVA (working memory), followed by Tukey's post hoc, p<.05. *Difference between HI and CT groups over the trials. #CTS group decreased time on the previous target from the second trial and CTMPH group decreased latency from the third trial onwards (&). CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n=11-13/group.

Figure 5: Brain-derived neurotrophic factor (BDNF) levels in prefrontal cortex (PFC) (A) and hippocampus (B). Results are expressed as mean ± S.E.M. Two-way ANOVA followed by Tukey's post hoc, p<.05. *HIMPH different from all other groups in the right

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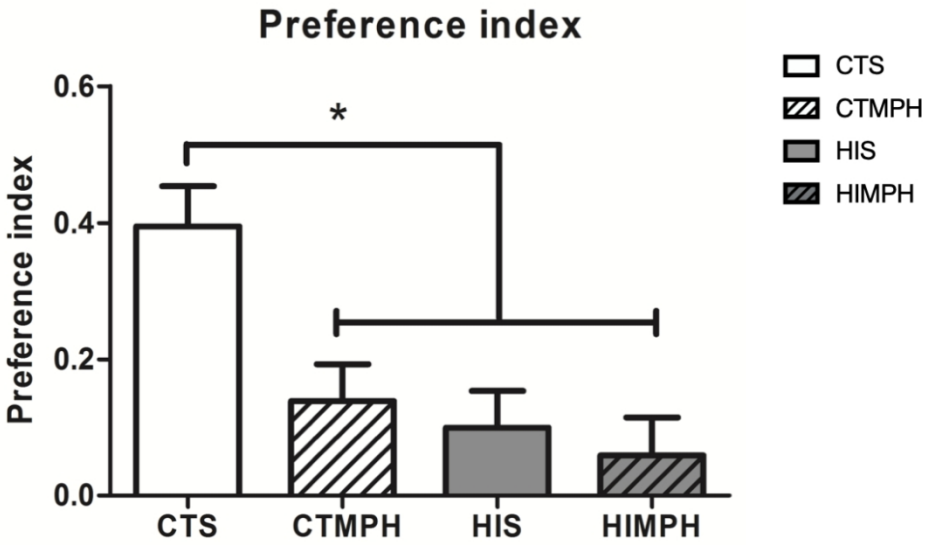

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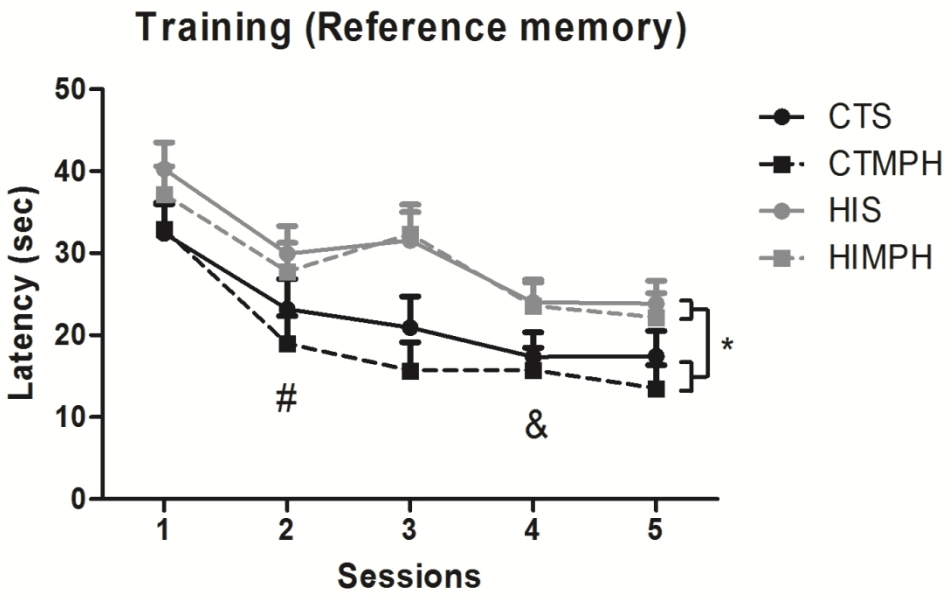

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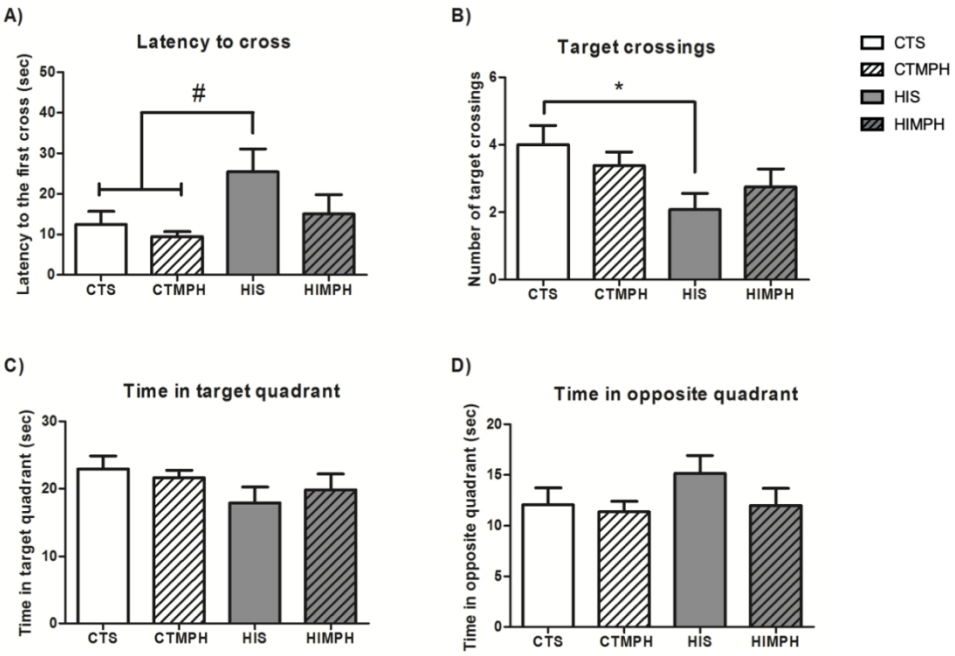

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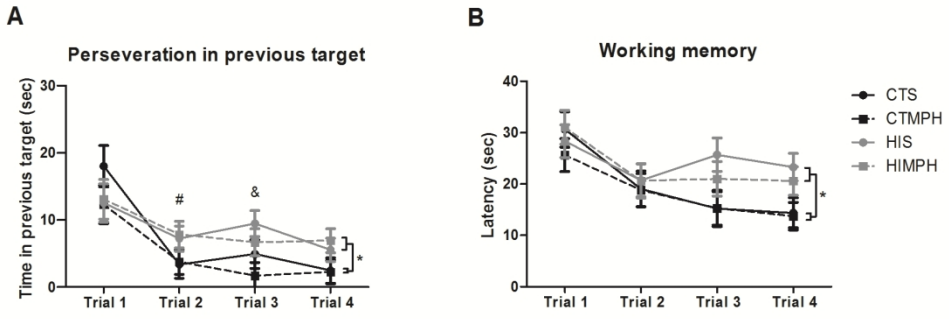

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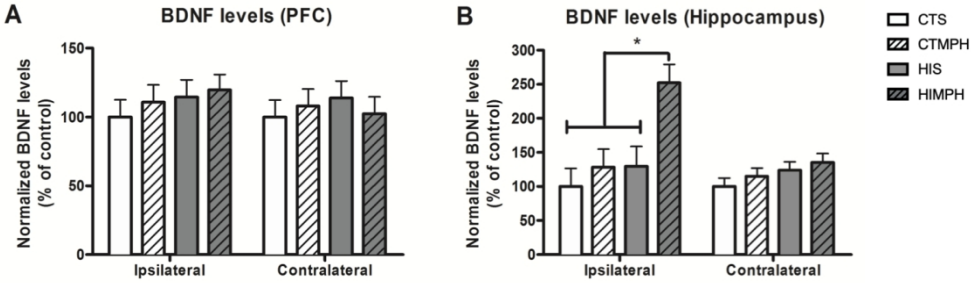

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321x108mm (127 x 127 DPI)

5. CAPÍTULO 3

Artigo: *Neonatal hypoxia-ischemia induces dysregulated feeding patterns and ethanol consumption that were improved by methylphenidate administration in rats*

Será submetido para publicação após sugestões da banca

O Capítulo 3 desta tese teve como objetivo avaliar comportamentos associados ao TDAH na fase adulta dos animais que foram submetidos à HI neonatal. Na clínica, o uso abusivo de substâncias e comportamentos alimentares desregulares são frequentes comorbidades relatadas no TDAH, especialmente nos períodos da adolescência e fase adulta. Assim, avaliamos o padrão de consumo alimentar (em relação a ração padrão ou palatável) e o consumo de álcool em ratos HI com ou sem o tratamento com MFD.

Os resultados deste estudo demonstraram que animais HI apresentaram um comportamento alimentar desregulado (em relação à ração padrão) após consumirem uma pequena porção da ração palatável, assim como também tiveram um aumento no consumo de álcool. O tratamento com MFD foi capaz de melhorar ambos comportamentos desregulados dos ratos HI.

Neonatal hypoxia-ischemia induces dysregulated feeding patterns and ethanol consumption that were improved by methylphenidate administration in rats

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Abstract

Higher levels of impulsivity, as observed in patients diagnosed with Attention-deficit/hyperactivity disorder (ADHD), can induce dysregulated behaviors such as binge eating and drug addiction. Perinatal complications including neonatal hypoxia-ischemia (HI) are involved in the ADHD etiology and we previously demonstrated that experimental HI resulted in ADHD-like behaviors in rats - impulsivity and attentional deficits. Additionally, methylphenidate (MPH) administration, the first therapeutic option for ADHD, reversed these deficits in HI animals. However, addictive-like behaviors following the HI procedure, and the MPH effects on these parameters have not been studied. We aimed at investigating the reward-based feeding behavior (using the BioDAQ system) and ethanol consumption (using the intermittent access 2-bottle choice, IA2BC), in adult rats submitted to neonatal HI and treated with MPH. Male Wistar rats were divided into four groups (n=10-12/group): control saline (CTS), CTMPH, HI saline (HIS) and HIMPH. The HI procedure was conducted at postnatal day (PND) 7 and behavioral analyses between PND 60–90, in which MPH (2.5 mg/kg, i.p.) was administered 30 min prior to each behavioral session. HI animals had a dysregulated feeding intake shortly after eating a small piece of a highly palatable diet, and MPH reversed this dysregulated pattern. However, when the palatable diet was freely available, MPH stimulated the intake of this diet, and this effect was potentialized in HI rats. Increased ethanol intake was observed in HI rats, and MPH administration reversed this behavior; contrarily, MPH treatment in control rats induced an increase in ethanol consumption. Considering that dysregulated feeding behaviors and drug addiction are common features in ADHD patients, these findings support the face validity of the HI model as a possible

ADHD animal model. Additionally, the predictive validity of the HI model was also confirmed, since MPH treatment was able to improve the dysregulated behaviors of HI rats.

Keywords: Attention-deficit/hyperactivity disorder; ADHD; perinatal complication; BioDAQ; intermittent access 2-bottle choice; IA2BC

Introduction

Impulsive behavior, which is broadly defined as the tendency to act prematurely without foresight (Dalley et al., 2011), is a core feature in patients diagnosed with Attention-deficit/hyperactivity disorder (ADHD). Higher levels of impulsivity can induce dysregulated behaviors such as binge eating and drug addiction. A significant association between ADHD and obesity was demonstrated by a meta-analysis (Cortese and Tessari, 2017) and this association was reported to be driven by food addiction and binge eating in ADHD patients, especially in adults (Brunault et al., 2019). In relation to substance abuse disorders, a large body of research have investigated its relationship with ADHD. A meta-analysis including prospective studies that followed children with and without ADHD into adolescence or adulthood demonstrated that children with ADHD were more likely to develop disorders of substance abuse/dependence (including nicotine, alcohol, marijuana, cocaine, and other substances) (Lee et al., 2011). Elevated levels of impulsivity mediate the association between childhood ADHD and alcohol problems in adulthood (Pedersen et al., 2016). Dopamine (DA) neurotransmission seems to be central in these relationships. ADHD neurobiology has been associated with lower levels of brain DA signaling (Del Campo et al., 2011); drug and palatable food intake are known to increase DA neurotransmission in the reward pathways, especially in the nucleus accumbens (NAc) (Gearhardt et al., 2011; Russo and Nestler, 2013). Thus, abnormal food intake or drug abuse would be an attempt to compensate for the decreased activation of the brain reward system, as a form of self-medication (Cortese and Vincenzi, 2012; Wilens et al., 2007).

ADHD treatment includes stimulant medications such as methylphenidate (MPH), which acts blocking the dopamine transporter (DAT), highly expressed in the striatum (a region that includes the NAc) (Rosa-Neto et al., 2005). With DAT blockade, more DA remains available on

the synaptic cleft, enhancing the dopaminergic activation. Indeed, MPH treatment was associated to lower rates of alcohol and drug use in ADHD youth when compared to ADHD-untreated or healthy controls, possibly via an increased DA signaling promoted by MPH (Hammerness et al., 2017). Despite this positive effect of the MPH, there is a large body of research that implicates MPH treatment in childhood as the causal link to later substance abuse disorders risk (Humphreys et al., 2013; Winhusen et al., 2011), through modifications in sensitivity to reward induced by the drug.

In relation to feeding behavior, there is a consensus that MPH treatment induces loss of appetite and growth stunting in humans (Gurbuz et al., 2016). Despite that, a study conducted by Davis and colleagues showed that although food-related behaviors (appetite, cravings and snack-food intake) diminished in response to MPH in normal weight individuals, this was not the case for obese males, which in fact had a small increase on all parameters after MPH challenge (Davis et al., 2012). Besides, MPH was able to increase the desire for food in food-deprived humans (Volkow et al., 2002b) and this was also observed in rats (Silveira et al., 2010). Taken together, these results indicate that MPH effects in relation to addictive behaviors could vary depending on the characteristics of the population as well as their physiological state.

ADHD etiology has a strong genetic component, but environmental conditions, especially those occurring during the perinatal period, have an important role on the disorder. For example, the association between perinatal hypoxia-ischemia (HI) and later ADHD diagnosis is evident in the literature (Zhu et al., 2016). We have confirmed this relationship in experimental studies, using a rat model of neonatal HI proposed by Levine (Levine, 1960) and modified by Rice and colleagues (Rice et al., 1981; Vannucci and Vannucci, 2005). Rats that underwent neonatal HI had attentional deficits, impulsive action and disturbances in the DA system (Miguel et al., 2018; Miguel et al.,

2015). MPH treatment was able to improve the attentional deficits in this model (Miguel et al., 2019). However, the analysis of addictive-like behaviors following the HI procedure was not explored in the literature, and this could provide a platform to the understanding of the ADHD-like characteristics observed in this model. Thus, we aimed at analyzing feeding behaviors (facing standard or highly palatable chow) and alcohol consumption in hypoxic-ischemic or control rats treated or not with MPH in adulthood. Considering the impulsivity trait and DA disturbances observed in hypoxic-ischemic animals, we hypothesized that these animals would have higher consumption of the palatable diet and alcohol, and these behaviors would be reversed with MPH treatment.

Materials and Methods

Animals

Male Wistar rats were obtained from the institutional breeding facility (CREAL, ICBS, UFRGS) and maintained at the university hospital animal research facility (UEA, CPE-HCPA) under standard conditions: controlled room temperature (22±2◦C), 12:12h light/dark cycle (lights on between 7:00 a.m. and 7:00 p.m.) and food and water available *ad libitum*. On the 7th postnatal day (PND), pups were randomly distributed into control and HI groups and then subdivided in saline and MPH treatment, resulting in four experimental groups: control treated with saline (CTS, n=12), control treated with MPH (CTMPH, n=12), HI treated with saline (HIS, n=12) and HI treated with MPH (HIMPH, n=12). Female pups of the litters were used for another research project. Animals were maintained with their dams until PND 21 when they were weaned and

housed in 2-3 per cage (Plexiglas cages: 49 cm x 34 cm x 16 cm) until the behavioral analysis in the adult phase (PND 60). The timeline of experimental procedures is shown in Figure 1.

All procedures were approved by the Institutional Ethics Committee on Animal Use (UFRGS 29750; GPPG/HCPA 15-0566) and were in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023), the guide of the Federation of Brazilian Societies for Experimental Biology and the Arouca Law (Nº 11.794/2008).

Hypoxia-ischemia (HI)

The HI procedure was conducted on the 7th PND, using the protocol developed by Levine (Levine, 1960) and modified by Rice and colleagues (Rice et al., 1981; Vannucci and Vannucci, 2005). Rats were anesthetized with isoflurane (4-5% for induction and 1.5-2% for maintenance) and an incision on the ventral surface of the neck was made to permit access to the right common carotid artery. After isolation of the artery from other surrounding anatomical structures, it was permanently occluded with a surgical silk thread. Following a 2-h interval with their dams to recover, the pups were placed in chambers partially immersed in a 37°C water bath, where they were exposed to a hypoxic atmosphere (8% oxygen and 92% nitrogen, 5 L/min) for 90 min. The animals returned immediately to maternal care after hypoxia. Control animals were submitted to sham surgery, i.e., animals received only anesthesia and neck incision (Miguel et al., 2019; Miguel et al., 2018; Miguel et al., 2015).

MPH administration

Methylphenidate hydrochloride (MPH) (Novartis, Brazil) treatment started on PND 60, concomitantly with the beginning of the behavioral analysis. MPH was dissolved in saline solution (0.9% NaCl) and injected intraperitoneally (dose 2.5mg/kg, volume 1 ml/kg), once a day, at the end of the light cycle. Control animals received an equivalent volume of saline solution. The MPH dose of 2.5mg/kg, adopted in this study, corresponds to a medium dose (Dafny and Yang, 2006) and was able to improve attentional deficits of HI animals in our previous study (Miguel et al., 2019).

Feeding behavior (BioDAQ® Food Intake Monitor)

After reaching 60 days of life, rats were transferred into cages equipped with a BioDAQ® food intake monitoring system (Research Diets), which can provide detailed feeding behavior data, such as total food intake and meal patterns. The BioDAQ® uses a food hopper mounted on an electronic strain gauge-based load cell connected to a computer for data transmission. The food hopper is weighed 50 times per sec (accurate to 0.01 g) and the mean and standard deviation (S.D.) of food consumption over approximately 1 sec is calculated by the computer software. Feeding is signaled by a change in the food hopper weight (defined as S.D. > 2000 mg), caused by the animal eating. There were two kinds of events recorded in BioDAQ®: feeding bouts and meals. A bout is an episode of uninterrupted feeding and the end of the event is signaled when the hopper was left undisturbed for 5 s (defined as a S.D. <2000 mg). A meal is defined as a difference in hopper weight of >0.1 g, separated from other feeding bouts within a range of 15 min (Eckel et al., 1998; Surina-Baumgartner et al., 1995). The duration of the feeding event, its start date and time and the

amount eaten is recorded and exported to the computer (Dalle Molle et al., 2015; Laureano et al., 2019).

Rats were individually housed for the feeding behavior analysis, which lasted 6 days. In the habituation phase (first four days), rats were given access to standard rat chow (2.95 kcal/g, 22% protein, 4,5% fat, 54% carbohydrate in each kg; NUVILAB®) on both food hoppers from the cage. In the fifth and sixth days on the BioDAQ, one of the food hoppers was provided with palatable diet (4.82 kcal/g, 14% protein, 34% fat, 30% carbohydrate in each kg, whose 20% were sucrose; Prag Soluções Biociências®) while the other hopper remained filled with standard chow to evaluate food preferences. These days were considered the first and second exposure days to the highly palatable diet. The palatable diet position was swapped between days to avoid side preference. For the analyses of the standard chow intake, the sum of both hoppers was used, and for the subsequent analysis of food preferences, the food hoppers were analyzed separately. Additionally, the preference index for the palatable diet was calculated by dividing the caloric intake from the palatable diet by total calorie intake (Dalle Molle et al., 2015; Laureano et al., 2019).

Animals were weighted daily before MPH or saline administration, that occurred at the end of the light cycle. This period was chosen to capture the drug effect in the most active phase of the rats. At the time that animals were removed from the cage, the diets were replenished and the cages and BioDAQ® system were cleaned and maintained. The feeding data was analyzed in two different periods of the day, at 2h and 20h after drug administration to evaluate both acute and protracted effects of the drug. A small piece of the palatable diet was given to the animals in their home cage previous to their transference to the BioDAQ system, and also 2 days prior to the food

preferences experiment, to avoid neophobia during the experiment. In the feeding analyses, the total consumption in grams was adjusted by the animal's body weight.

Ethanol consumption (Intermittent access to ethanol in 2-bottle choice procedure; IA2BC)

At the end of the feeding behavior analysis, animals were transferred from the BioDAQ® to standard rat cages for the analysis of ethanol consumption, that started two days later. We used the intermittent access to ethanol 20% in 2-bottle choice procedure (IA2BC), as described in Carnicella et al. (Carnicella et al., 2014). The IA2BC drinking session occurred on alternate days, in which animals were isolated and had 24h-concurrent access to two bottles, one with ethanolic solution (20% in tap water, v/v) and another with tap water. The MPH or saline administration occurred only in the drinking sessions; in the withdrawal period, animals were regrouped (2-3/cage) with their familiar animals to avoid prolonged social isolation, a condition known to increase ethanol intake in the IA2BC protocol (Chappell et al., 2013). The drinking sessions begun right after drug administration, at the end of the light cycle. Rat chow was available during the sessions and both bottles, chow and animals were weighted before and after drinking sessions to calculate the consumptions and body weight gain. In each session, the position of the bottles was alternated to control for side preferences. A total of 12 sessions were carried out and in the last three sessions (sessions 10-12), the water and ethanol consumption were also measured 2h after drug administration to capture possible MPH acute effects. The following variables were analyzed throughout the 12 sessions: 1) ethanol intake (ml/kg), 2) water intake (ml/kg), 3) total fluid intake (ml/kg), 4) ethanol preference (%), 5) food intake (g/kg), and 6) body weight gain (g).

Statistical analysis

Repeated-measures ANOVA followed by Tukey's post hoc, with lesion and treatment as factors, was used to analyze the consumption during the habituation to the BioDAQ, food preference and ethanol parameters throughout the 12 sessions (IA2BC protocol). Two-way ANOVA was conducted to analyze feeding behavior at each habituation day, the mean of all measures in the last 6 sessions of the IA2BC protocol, and the mean ethanol and water consumption in the first 2h of exposure (only in the last 3 sessions). All variables were expressed as mean±standard error of the mean (SEM), and the results were considered statistically significant when $p<.05$. Data were analyzed using the IBM Statistical Package for the Social Sciences (SPSS) version 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Feeding behavior

Habituation to the BioDAQ

Standard chow intake was analyzed throughout the first 4 days of exposure to the BioDAQ, considering sessions of 2h or 20h after drug administration. In the first 2 days, animals did not have any interference in their home cage. In the last 2 days, a small piece of palatable diet was given to animals to avoid neophobia in the following days (Figure 1).

In relation to the 2h analyses, a *day* main effect was observed in all feeding parameters. Tukey post hoc showed a statistically significant increase in total consumption, number of bouts, number of meals and meal size in the HIS group only, that happened on days 3 and 4 in comparison

to day 1 (Figure 2). When analyzing each day separately, trends for *treatment* main effect on day 3 for number of bouts ($F(1,43)= 2.89$, $p=0.09$, partial $\eta^2=0.06$), number of meals ($F(1,43)=3.73$, $p=0.06$, partial $\eta^2=0.08$) and bout size ($F(1,43)= 4.03$, $p=0.05$, partial $\eta^2=0.08$) were observed. This suggests a tendency for a decrease in the number of bouts and meals with MPH treatment (Figure 2B and C), as a consequence of an increase in bout size (CTS: 1.06±0.19, CTMPH: 1.43±0.19, HIS:1.1±0.19, HIMPH: 1.5±0.16). On day 4, statistically significant *lesion* effects were observed for total consumption ($F(1,43)=4.5$, $p<0.05$, partial $\eta^2=0.09$) and bout number ($F(1,43)=5.18$, $p<0.05$, partial $\eta^2=0.10$). Trends for a *treatment* main effect were also found on these measures (total consumption: ($F(1,43)=3.93$, $p=0.05$, partial $\eta^2=0.08$; bout number: ($F(1,43)=2.98$, $p=0.09$, partial $\eta^2=0.06$). The post hoc analysis indicated that the HIS group consumed more rat chow in the first 2h in relation to both control groups; in relation to the HIMPH group, the difference did not reach statistical significance ($p=0.06$) (Figure 2A). HIS rats had higher number of bouts in relation to the CTMPH group, and a trend was seen in comparison to the CTS group ($p=0.06$) (Figure 2B).

Analysis of the consumption over 20h also shows an effect of the *day* for all variables, indicating that all animals increased the consumption throughout the days. For total chow intake (adjusted by body weight), *day*lesion* ($F(3,129)=3.52$, $p<0.05$, partial $\eta^2=0.07$) and *day*lesion*treatment* were observed ($F(3,129)=2.78$, $p<0.05$, partial $\eta^2=0.01$) and for number of bouts, *day*lesion* interaction effect ($F(3,129)=5$, $p<0.01$, partial $\eta^2=0.1$). The Tukey post hoc indicated that both hypoxic-ischemic and the CTMPH groups increased chow intake, number of bouts and meal size on days 3 and 4, when compared to day 1. This difference was only observed on day 4 in the CTS group, suggesting a more stable pattern of eating in this group (Supplementary Figure 1). Two-way ANOVA was also performed within each day to investigate possible punctual

group differences. For number of bouts, a *lesion* effect was observed ($F(1,43)= 4.23$, $p<0.05$, partial $\eta^2=0.09$) on day 4, in which HI animals had more bouts when compared to controls (Supplementary Figure 1B). In relation to meal size, a *lesion*treatment* interaction effect was observed on day 2 ($F(1,43)= 5.45$, $p<0.05$, partial $\eta^2=0.11$), and the post hoc indicated a trend for a larger meal size in the HIMPH group compared to the HIS group ($p=0.07$; Supplementary Figure 1D).

Palatable diet consumption

After the habituation period, one of the food hoppers was filled with a highly palatable chow (during 2 exposure days), while the other had standard chow to measure food preference. Animals ate very little or nothing from the standard diet chow during this period; no differences between groups were observed in any of the standard chow intake measures.

Analyzing the palatable diet consumption between days 1 and 2 of exposure (sessions of 2h), repeated-measures ANOVA showed a *day* main effect and *day*treatment* interaction effects for the measures: total consumption in grams (*day* $F(1,43)=4.8$, $p<0.05$, partial $\eta^2=0.1$; *day*treatment* $F(1,43)=12.24$, $p<0.01$, partial $\eta^2=0.22$), number of bouts (*day* $F(1,43)=21.57$, $p<0.001$, partial $\eta^2=0.33$; *day*treatment* $F(1,43)=6.4$, $p<0.05$, partial $\eta^2=0.13$), meal size (*day* $F(1,43)=10.44$, $p<0.01$, partial $\eta^2=0.19$; *day*treatment* $F(1,43)=17.82$, $p<0.001$, partial $\eta^2=0.29$), and total caloric consumption (*day* $F(1,43)=7.24$, $p<0.05$, partial $\eta^2=0.14$; *day*treatment* $F(1,43)=12.24$, $p<0.01$, partial $\eta^2=0.22$). In the first 2h following MPH injection and diet exposure the HIMPH group consumed more palatable diet than CTS rats (Figure 3A) and comparing the difference between day 1 and 2 within groups, HIMPH was the only group that significantly decrease diet consumption (Figure 3A), number of bouts (Figure 3B) and total caloric consumption

(Figure 3D) on day 2 compared to day 1. For meal size, both CTMPH and HIMPH groups decrease meal size on day 2 compared to day 1, and the CTMPH group also had a larger meal portion in relation to the CTS group on day 1 (Figure 3C). *Day*treatment* interaction effect was observed for bout size ($F(1,43)=4.2$, $p<0.05$, partial $\eta^2=0.08$) and *day*lesion*treatment* interaction effect was seen for number of meals ($F(1,43)=4.47$, $p<0.05$, partial $\eta^2=0.09$), but no differences were indicated by the post hoc analysis. A *day* main effect was observed for palatable diet preference ($F(1,43)=11.97$, $p<0.01$, partial $\eta^2=0.21$) and the post hoc indicated a trend ($p=0.06$) for an increase in preference on day 2 only for the CTS group, indicating that all other groups had higher preference since day 1.

The feeding behavior was also analyzed over 20h after the drug administration. Here, repeated-measures ANOVA demonstrated *day*treatment* interaction effects for total consumption of the palatable diet in grams ($F(1,43)=4.19$, $p<0.05$, partial $\eta^2=0.08$), as well as meal size ($F(1,43)=9.63$, $p<0.01$, partial $\eta^2=0.18$). Tukey post hoc indicated that HIMPH animals ate larger meals in relation to CTS group on day 1 (Supplementary Figure 2C). A *Day*treatment* interaction effect ($F(1,43)=4.76$, $p=0.03$, partial $\eta^2=0.10$), as well as *lesion* ($F(1,43)=4.66$, $p<0.05$, partial $\eta^2=0.09$) and *day* ($F(1,43)=5.07$, $p<0.05$, partial $\eta^2=0.10$) main effects were also statistically significant for total caloric consumption in kilocalories (considering palatable diet plus standard diet).The post hoc pointed out a higher caloric intake in the HIMPH group in relation to the CTS group on day 1 (Supplementary Figure 2D). A *Lesion*treatment* interaction effect was found for bout size ($F(1,43)=4.11$, $p<0.05$, partial $\eta^2=0.08$), and a *day* main effect was seen for palatable food preference ($F(1,43)=9.19$, $p<0.01$, partial $\eta^2=0.17$), but no differences were detected in these variables in the post hoc analysis.

Ethanol consumption

Ethanol consumption and related measures were analyzed using the IA2BC procedure during 12 sessions of 24h/each. Repeated-measures ANOVA was conducted to identify differences over the 12 sessions and considering the higher intake variability in the first 6 sessions, the mean of the last 6 sessions (sessions 7-12) of each variable was analyzed by two-way ANOVA. For water intake, repeated-measures ANOVA demonstrated significant *session*lesion*treatment* interaction effect ($F(6.68,274.26)=2.49$, $p<0.05$, partial $\eta^2=0.05$) and *lesion* main effect ($F(1,41)= 5.42$, $p<0.05$, partial $\eta^2=0.11$) but no difference was observed on the post hoc (Figure 5A). The mean intake in the last 6 sessions (sessions 7-12) had effects for *lesion* main effect ($F(1,41)=4.33$, $p=0.04$, partial $\eta^2=0.09$) and a trend for *lesion*treatment* interaction effects ($F(1,41)=3.86$, $p=0.05$, partial $\eta^2=0.08$); the Tukey post hoc test indicated that the HIS group ingested less water than CTS rats (Figure 4B). For ethanol consumption (Figure 4C), *session* ($F(5.85,240.07)=3.83$, $p<0.01$, partial $\eta^2=0.08$) and a trend for *lesion* main effects were observed over the sessions ($F(1,41)=3.93$, $p=0.054$, partial $\eta^2=0.08$). In the mean ethanol intake, *lesion* main effect was again observed ($F(1,41)=4.56$, $p<0.05$, partial $\eta^2=0.10$), and the post hoc indicated a trend for the HIS group consuming more ethanol than the CTS group ($p=0.09$; Figure 4D). Analyzing preference for ethanol, i.e., alcohol solution consumed in relation to the total fluid intake, significant main effects for *session* ($F(6.44,264.04)=3.20$, $p<0.01$, partial $\eta^2=0.07$), *lesion* ($F(1,41)=6.83$, $p<0.05$, partial $\eta^2=0.14$) and a trend for *lesion*treatment* interaction effect were seen ($F(1,41)= 3.57$, $p=0.06$, partial $\eta^2=0.08$) (Figure 4E). *Lesion* main effect ($F(1,41)=7.19$, $p<0.05$, partial $\eta^2=0.18$) and *lesion*treatment* interaction effect ($F(1,41)=5.04$, $p<0.05$, partial $\eta^2=0.11$) were confirmed in the mean preference analysis, and the post hoc pointed out that the HIS group had a higher preference

for ethanol in relation to the CTS group (Figure 4F). Overall, we can observe that MPH had different effects in control vs. hypoxic-ischemic animals, increasing water intake and decreasing alcohol preference in the HIMPH group, but having the opposite effect on CTMPH rats (Figure 4B and 4F).

Session and *session*lesion*treatment* were statistically significant effects for total fluid intake (session: $F(6.16,252.68)=2.17$, $p<0.05$, partial $\eta^2=0.05$; *session*lesion*treatment:* $F(6.16,252.68)=2.53$, $p<0.05$, partial $\eta^2=0.05$) and rat chow intake (session: $F(5.3,217.38)=6.25$ $p<0.001$, partial $\eta^2=0.13$; *session*lesion*treatment:* $F(5.3,217.38)=2.14$, $p=0.05$, partial $\eta^2=0.05$) (Figure 5A and 5C). The post hoc showed that the HIMPH group increased total fluid intake in sessions 3 and 5 in comparison to session 1 (Figure 5A), and less chow intake was also observed in this group on session 1 compared to sessions 3 to 12 (Figure 5C). This suggests that HIMPH animals had lower intake in general in the first sessions. When averaging sessions 7-12, no statistically significant effect was observed for fluid and chow intake, suggesting that MPH effects in HI animals were only observed at the beginning of the protocol (Figures 5B and 5D). Body weight was affected by *session* ($F(3.24,132.85)=79.61$, $p<0.001$, partial $\eta^2=0.66$) and *lesion* factors ($F(1,41)=19.66$, $p<0.001$, partial $\eta^2=0.32$), showing that animals gained weight throughout sessions, but HI animals had lower body weight than controls (Figure 5E). Although no statistical significance was detected in the post hoc, we observe that the HIMPH was the only group with decreasing body weight in the first sessions, as a consequence from their decreased fluid and chow intake (Figure 5E). In the average of sessions 7-12, a *lesion* main effect was observed ($F(1,41)=19.85$, $p<0.001$, partial $\eta^2=0.32$). Tukey's test showed that the CTMPH group had higher body weight in relation to both HI groups; contrarily, HIMPH rats had lower body weight in

relation to both CT groups, once more demonstrating that the MPH treatment affected CT and HI animals differently (Figure 5F).

Ethanol and water consumption were also evaluated 2h after drug administration in sessions 10-12 (last 3 sessions). Mean consumption was analyzed by two-way ANOVA, that showed no statistically significant differences between the groups for water intake (Figure 6A). However, a *lesion* main effect is observed for ethanol consumption ($F(1,41)=4.72$, $p<0.05$, partial $\eta^2=0.10$), indicating that HI animals consumed more ethanol in the first 2h of alcohol exposure (Figure 6B), in a very similar pattern to that observed in 24h-sessions (Figure 6D). For total fluid intake and ethanol preference, no statistically significant differences were found, although we can observe that HIS animals have already a tendency to increase their preference for ethanol in the first 2h of exposure (Figure 6C).

Discussion

The current study was delineated to investigate ADHD-related outcomes, such as addictive-like behaviors, and the possible effect of MPH in adult rats submitted to neonatal hypoxia-ischemia. Our main findings showed that HI animals increased standard chow intake shortly after eating a small piece of a palatable diet, and MPH administration was able to revert this behavior in HIMPH rats. When palatable food was freely available, MPH treatment induced an increase in palatable food intake, having a higher effect in hypoxic-ischemic animals. Increased ethanol intake was observed in HI-untreated rats, and MPH administration decreased ethanol intake in the HI group. Contrarily, MPH treatment induced an increase in ethanol consumption in control rats.

HI animals showed a dysregulated feeding pattern shortly after eating a highly palatable food sample, and MPH administration reversed this behavior

During the habituation phase on the BioDAQ apparatus, the animals had only standard chow in the food hoppers. However, on days 3 and 4, they received a small piece of the highly palatable food into the cage for habituation to the new diet. Intriguingly, after eating this small piece of palatable food, HIS animals increased their standard chow intake, especially in the subsequent 2h (Figure 2). This finding suggests that HIS rats had a differential response to the pleasurable sensation associated with highly palatable food intake, hence increasing general food intake. This differential response to a pleasurable stimulus could be a result of variances in the hedonic response. In fact, we cannot directly infer alterations in the hedonic response in HI animals, since there is no study that evaluated it yet; however, some of our previous studies indicate that HI animals have a higher incentive salience for rewarding stimuli. For example, higher perseverative responses to receive a sweet pellet in adult HI animals was observed (Miguel et al., 2015). We have also described normal cognitive learning when tasks involved sweet reward, despite the substantial cognitive deficits associated with this model (Miguel et al., 2019; Miguel et al., 2018). Additionally, we cannot exclude the possibility that these animals persisted eating the standard diet by a failure in inhibitory control processes or as a way to maintain a certain level of DA stimulation (Fogel et al., 2019). Both inhibitory control failure as well as disruption in parameters of DA transmission were already observed in this model, supporting these possibilities (Filloux et al., 1996; Ikeda et al., 2004; Miguel et al., 2019; Miguel et al., 2018; Miguel et al., 2015). Considering that dysregulated eating patterns are frequently associated with ADHD in

humans (Brunault et al., 2019; Cortese and Tessari, 2017), the current findings support our hypothesis that neonatal HI results in ADHD-phenotypes in adult rats, corroborating our range of studies indicating the association between these two conditions (Miguel et al., 2019; Miguel et al., 2018; Miguel et al., 2015).

Our findings showed that MPH administration was able to repair the dysregulated behavior of HI rats, since the HIMPH group did not have altered feeding pattern over the days, behaving similarly to controls. Moreover, we found that MPH administration has a tendency to induce an increase in standard chow bout size after a small portion of palatable diet on day 3, consequently down-regulating the number of bouts and meals. As day 3 was the animals' first contact with palatable food, we infer that MPH increased the animal's focus on feeding at that given episode, increasing the bout size with uninterrupted bites. However, differently from the pattern observed in the HIS group, animals treated with MPH did not increase the total amount of food consumed, inducing in fact a lower total intake in treated animals on this day (Figure 2A). Thus, we postulate that MPH administration, while improving the focus on the behavior as mentioned above, also allowed the animals to interrupt the consumption when realizing that the food hopper had only standard diet, as opposed to what was observed in HIS rats. Some of the findings observed in the 2h-sessions were maintained over the 20h-sessions, such as the effect of MPH increasing focus in feeding and leading to larger meal portions in HIMPH animals on day 3. However, higher number of bouts in hypoxic-ischemic animals, independent of the treatment, were observed on day 4, suggesting that the MPH effect is more restricted to the drug half-life (approximately 3h).

Increased palatable food intake was observed in both control and hypoxic-ischemic rats following MPH administration

In the food preference analyses, one of the food hoppers was filled up with a palatable diet, that remained freely available, and the feeding activity was analyzed over 2 or 20h after drug administration (in 2 exposure days). MPH administration induced an increase in the consumption of the palatable diet on day 1, and this behavior was observed especially in the first two hours of diet exposure (Figure 3). As discussed previously in relation to the standard diet, we suggest that the MPH administration increased the focus on feeding behavior. But contrarily to the observed with the standard diet, the animals remained focused on eating when palatable diet is available, eating larger amounts of this food.

MPH is a stimulant drug that amplifies the dopaminergic transmission in the mesolimbic pathway (Del Campo et al., 2011; Volkow et al., 2002a), similarly to what occurs facing natural rewards such as food, especially those highly palatable. It is possible that the combination of these two factors (MPH plus palatable food) induces a hyperstimulation of the mesolimbic pathway, leading animals to consumed higher amounts of palatable food consumption. Several factors suggest that overconsumption of this food in our model is associated with a higher motivation for ingestion rather than higher hedonic sensation associated with the intake. Firstly, after an intense debate in the literature about the DA role in pleasure versus motivational aspects related to rewards, it has been suggested that stimulation of the mesolimbic pathway affects incentive salience properties of the rewarding stimulus rather than the pleasurable experience itself (for review, see (Adinoff, 2004). Secondly, Peciña and colleagues demonstrated that knockout rats for the dopamine transporter (DAT) - and consequently with higher levels of synaptic DA - attributed greater value to a sweet reward, increasing its consumption, but did not increase the hedonic "liking" reactions associated to the consumption (Pecina et al., 2003). Considering that MPH

increases DA transmission by inactivating DAT function, our findings could be considered as in line with those reported by Peciña. At first, these findings of MPH increasing consumption seem intriguing considering that the MPH is known to suppress appetite and has been suggested for obesity treatment (Albayrak et al., 2011; Leddy et al., 2004). However, a very interesting review on this topic suggests that "the effect that stimulants have for enhancing reward could lead to inappropriate use or potentiate addictive behavior or compulsions such as binge eating", which is one of the reasons why stimulants are not appropriate for obesity treatment and supporting our findings. MPH increases the desire for food in food-deprived humans (Volkow et al., 2002b) and rats (Silveira et al., 2010), and more fascinating, although MPH decreased food-related behaviors in normal weight individuals, it increases these behaviors (appetite, cravings and snack-food intake) in obese individuals (Davis et al., 2012).

Although MPH increased palatable food consumption in all treated animals, this effect was higher in HI animals. We can suggest that neonatal HI altered brain dopaminergic signaling, hence making these animals more responsive to the effects of MPH. In fact, we previously demonstrated that MPH has a potentiation effect in HI rats, robustly increasing locomotion only in this group (Miguel et al., 2019). It is important to note that hypoxia-ischemia itself did not increase palatable intake, in relation to the control group, when the diet was freely available. The lack of differences was intriguing considering that this group was more responsive to the presentation of palatable food during the habituation phase. However, in the analysis of preference for the palatable diet (in comparison to standard chow), we observed that only the CTS group had a trend ($p=0.06$) to increase the preference for palatable diet between the first and second days, indicating that MPH groups and also the HIS group had already a higher preference for the palatable diet on the first day. This finding was an indicative that in fact the HIS group has a different behavior when facing

the highly palatable diet. The lesion effect itself was seemingly not captured in other feeding parameters because of the short period of exposure to the freely available palatable diet (2 days). In this protocol, only MPH had the ability to substantially increase the consumption beyond the animals' natural capacity. Possibly a prolonged exposure to the palatable diet could inform better about feeding behavior over the days. Another point that should be mentioned is that homeostatic signals are also important, and interact with hedonic signals to induce feeding (Lutter and Nestler, 2009). For example, we observed that HIMPH rats significantly increased the consumption in the first day, and consequently ate less in the second day. This indicates that the caloric intake at one point in time interferes with subsequent feeding behavior. In the same way, HIS animals had a higher standard chow intake in the last habituation day (day 4), and this difference may influence the subsequent food intake measures.

Methylphenidate attenuated the higher ethanol intake in hypoxic-ischemic animals, but stimulated the intake in control animals

Ethanol intake was measured over 12 intercalate sessions, and our results demonstrated that adult animals submitted to neonatal HI increased their ethanol intake and preference for ethanol compared to control rats treated with saline. Considering that substance abuse disorders are a common co-morbidity in ADHD patients, we reinforce that the neonatal HI model induces ADHD-related outcomes in rats, as indicated previously (Miguel et al., 2019; Miguel et al., 2018; Miguel et al., 2015). Impulsivity has been linked to drug addiction in humans, and higher levels of impulsivity were also found in adult rats that underwent neonatal HI (Miguel et al., 2015), suggesting that this behavioral phenotype could be associated with higher ethanol intake in these

animals. Impairments in mesolimbic DA signaling induce substance abuse disorders, to compensate this deficit (Lindgren et al., 2018). In HI animals, dysregulated DA parameters were observed in the striatum (Filloux et al., 1996; Park et al., 2013), a region constituted by the NAc which receives DA projections coming from the ventral tegmental area (VTA). The VTA DA neurons also innervate the PFC (Russo and Nestler, 2013), a structure that displays impairments in DA signaling as a consequence of HI exposure (Miguel et al., 2019; Miguel et al., 2018). We suggest that failures in DA transmission in HI animals may cause an increased ethanol intake in this group. Supporting this idea, increased DA signaling induced by MPH administration was able to decrease the ethanol consumption in the HIMPH group. In agreement with our findings, a clinical trial conducted by Hammerness and colleagues (Hammerness et al., 2017) showed that MPH treatment in ADHD adolescent patients significantly reduced the rates of alcohol and drug use in comparison with ADHD-untreated or healthy controls.

Another point that should be mentioned is that excessive use of alcohol and other substances is frequently considered a habitual behavior, resulting from a loss of flexible control over drug use (Barker and Taylor, 2014; Corbit and Janak, 2016). Cognitive flexibility is part of the executive functions, i.e., higher order brain functions highly dependent on DA transmission in the PFC (Ott and Nieder, 2019). Interestingly, we recently showed cognitive inflexibility associated with PFC DA dysregulation in adolescent HI rats, that was reversed by MPH administration (Miguel et al., 2019). We can suggest that lower cognitive flexibility in HI rats may induce a higher ethanol intake, and MPH-induced improved cognitive flexibility may explain the lower ethanol intake in HIMPH rats. Giving support to our findings, Shnitko and colleagues demonstrated that pre-existing low cognitive flexibility in rhesus monkey was predictive of future classification as a heavy alcohol drinker (Shnitko et al., 2019).

Contrarily to the effects observed in HI rats, MPH administration in control animals resulted in increased ethanol consumption. This detrimental effect caused by MPH was not an unexpected result, since behavioral impairments, such as learning and memory deficits, were already observed in CT rats when administered with this same MPH dose (Miguel et al., 2019; Miguel et al., unpublished). Excessive DA transmission in the PFC, as well as lower DA levels, have been linked to cognitive impairments, and this has been recognized as the "inverted-U" curve relationship between PFC DA levels and cognition (Cools and D'Esposito, 2011; Floresco, 2013). Thus, our findings showing a differential response to MPH in control or hypoxic-ischemic animals highlight the importance of avoiding an indiscriminate use of MPH by healthy individuals.

Hypoxic-ischemic animals under the MPH effect had lower fluid and food intake in the first sessions of the IA2BC protocol and consequently this group decreased weight gain in the first sessions (Figure 6). This finding demonstrates a different habituation to the novel environment in the HIMPH group, which could be associated with higher locomotor activity following the MPH administration (Miguel et al., 2019). In the fourth session, this group normalized their behavior, being similar to the other groups, but they always had lower body weight than their control (HIS), the group which had higher caloric intake from the ethanol. Contrarily, CTMPH had the highest body weight and this group consumed higher amounts of ethanol than their control (CTS). Overall, these results sustain the assumption that MPH treatment can affect CT or HI animals very differently.

In conclusion, the findings demonstrated that neonatal HI induces ADHD-related outcomes in rats, such as dysregulated feeding activity and ethanol intake in adulthood, providing additional support to the face validity of the HI model as a possible ADHD experimental model. MPH administration was able to improve these behaviors in HI animals, confirming also the predictive

validity of this model. Additionally, MPH administration induced higher palatable diet intake in both CT and HI groups, but this effect was higher in HI animals, probably by a higher attributed value to the palatable diet in this group. Then, the current results added important new findings to the ADHD field, both to the experimental and clinical aspects, supporting that perinatal hypoxia-ischemia may substantially disrupt the developing brain.

Acknowledgment

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Figures

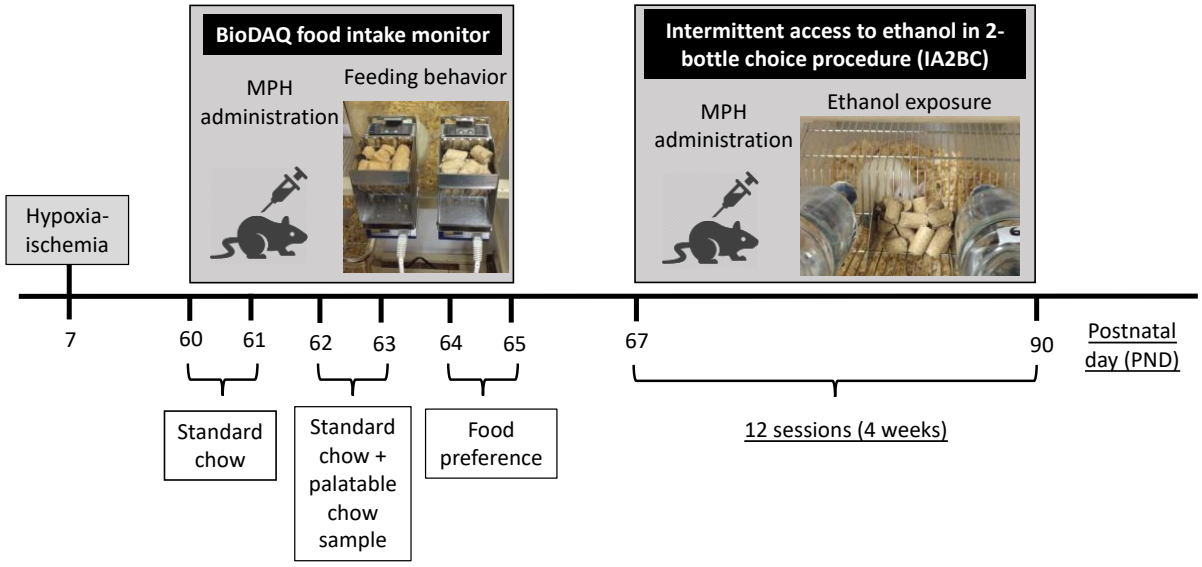

Figure 1: Timeline of experimental procedures.

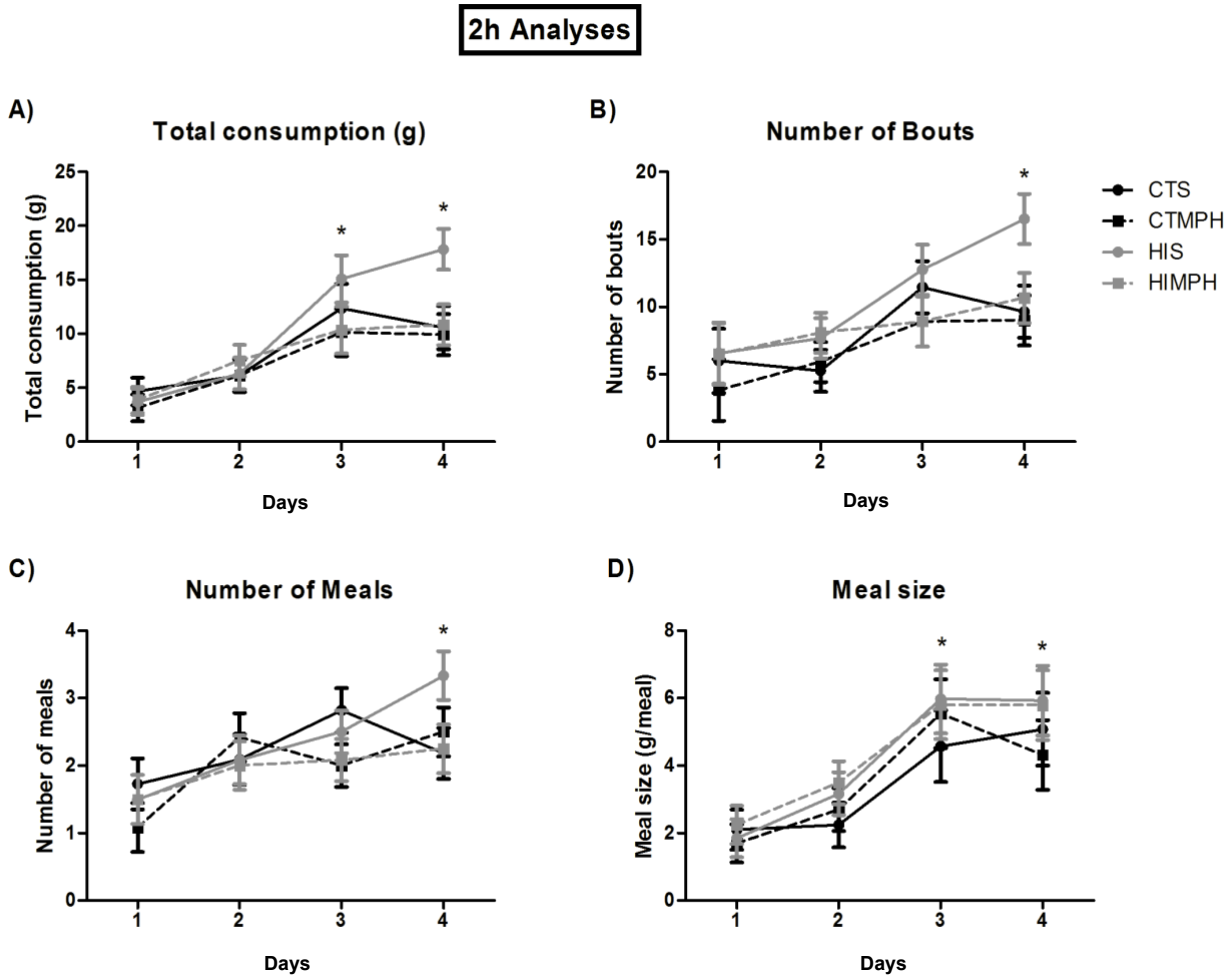

Figure 2. Feeding activity parameters in relation to standard rat chow in sessions of 2h in the first 4 days in the BioDAQ. Results are expressed as mean ± S.E.M. Repeated-measures ANOVA throughout the days and two-way ANOVA within each day, followed by Tukey's post hoc, $p<.05$. *difference in relation to the first day in the HIS group. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n=11-12/group.

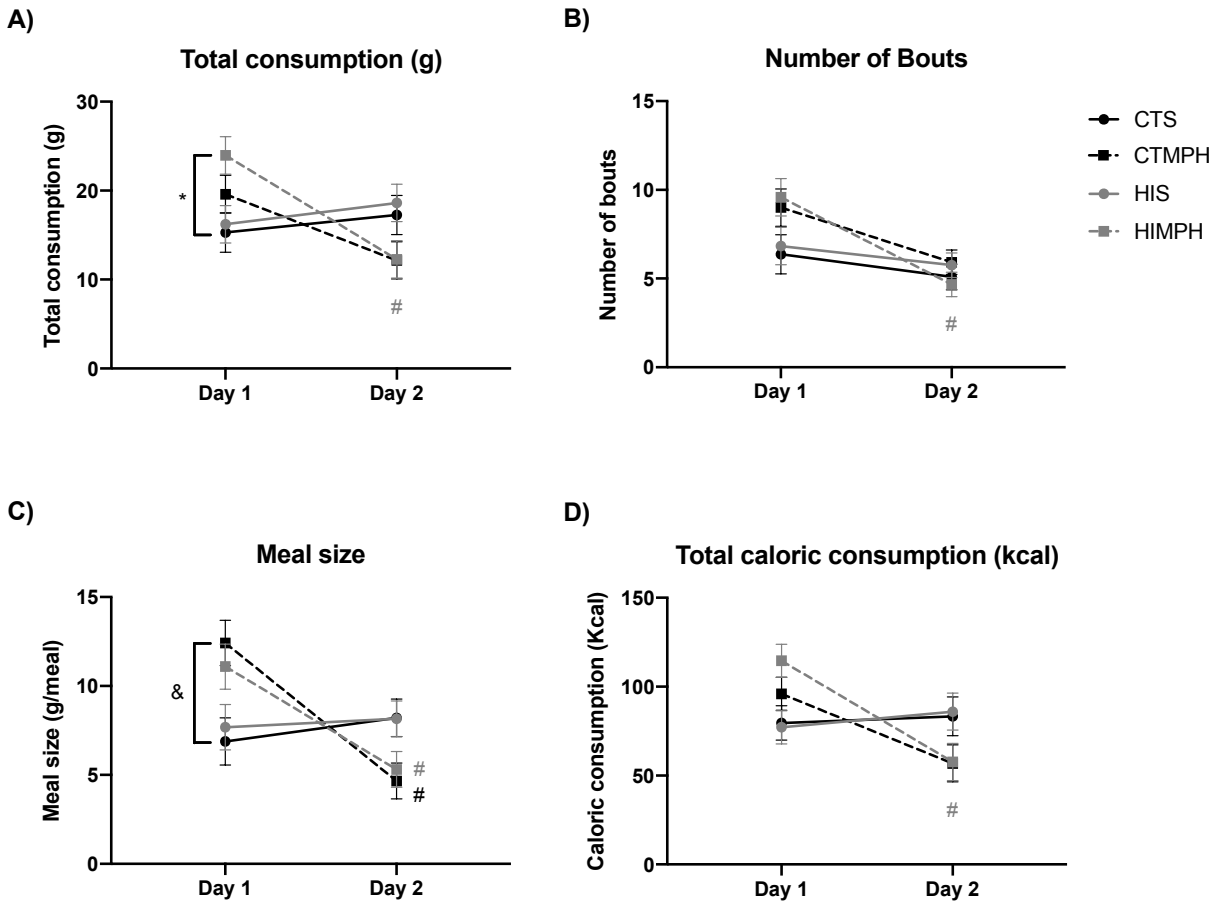

Figure 3. Feeding activity parameters in relation to the palatable diet in sessions of 2h in 2 days of exposure. Results are expressed as mean ± S.E.M. Repeated-measures ANOVA followed by Tukey's post hoc, $p<.05$. *HIMPH different from the CTS group in the first day, #(gray) difference between days in the HIMPH group, #(black) difference between days in the CTMPH group, &CTMPH different from the CTS in the first day. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n=11-12/group.

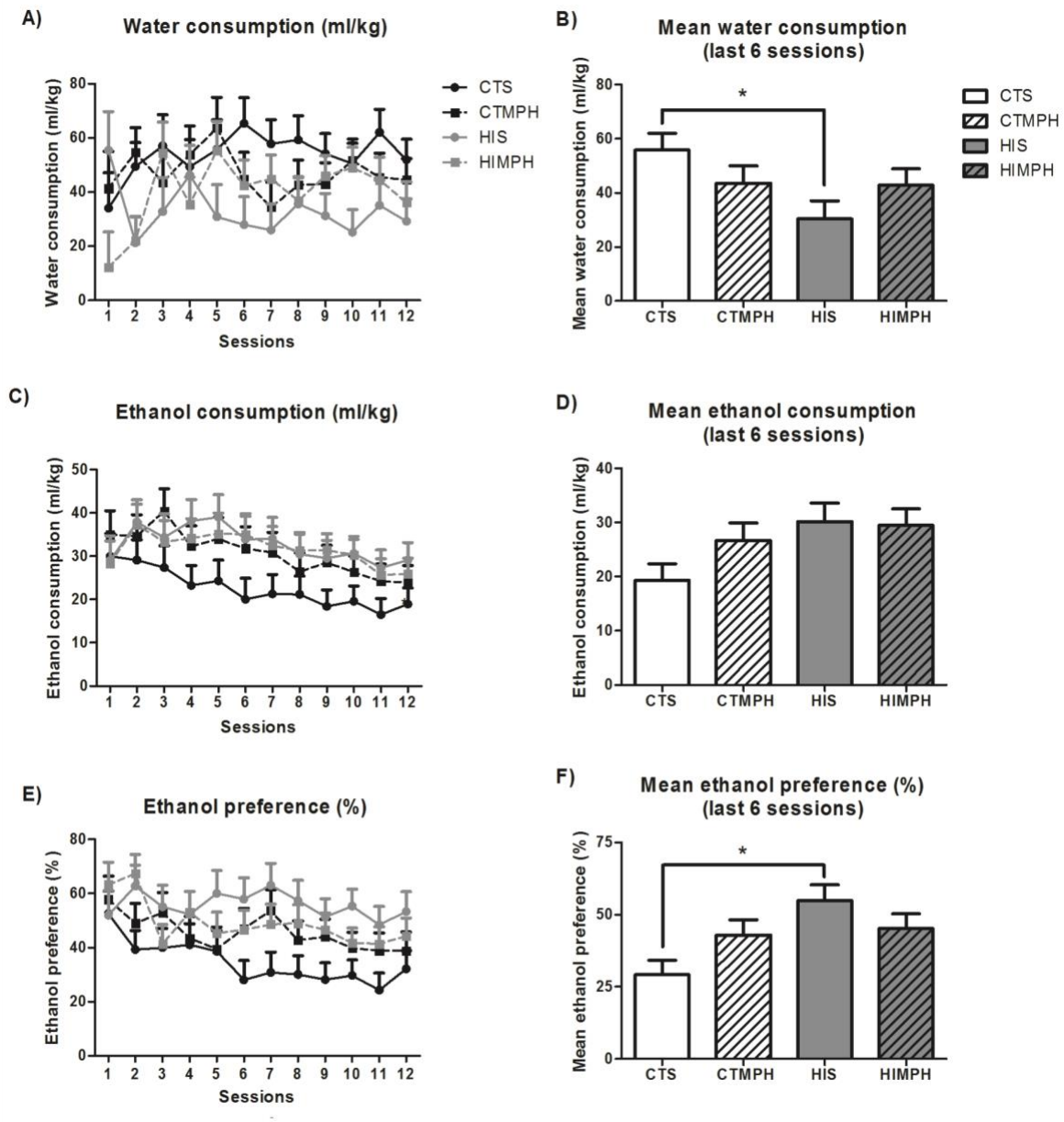

Figure 4. Water and ethanol consumption, as well as the ethanol preference, over the 12 sessions of the IA2BC procedure (A, C, E) or the mean of the last 6 sessions of each measure (B, D, F). Results are expressed as mean ± S.E.M. Repeated-measures ANOVA or two-way ANOVA, followed by Tukey's post hoc, $p<.05$. *HIS different from the CTS group. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n=11-12/group.

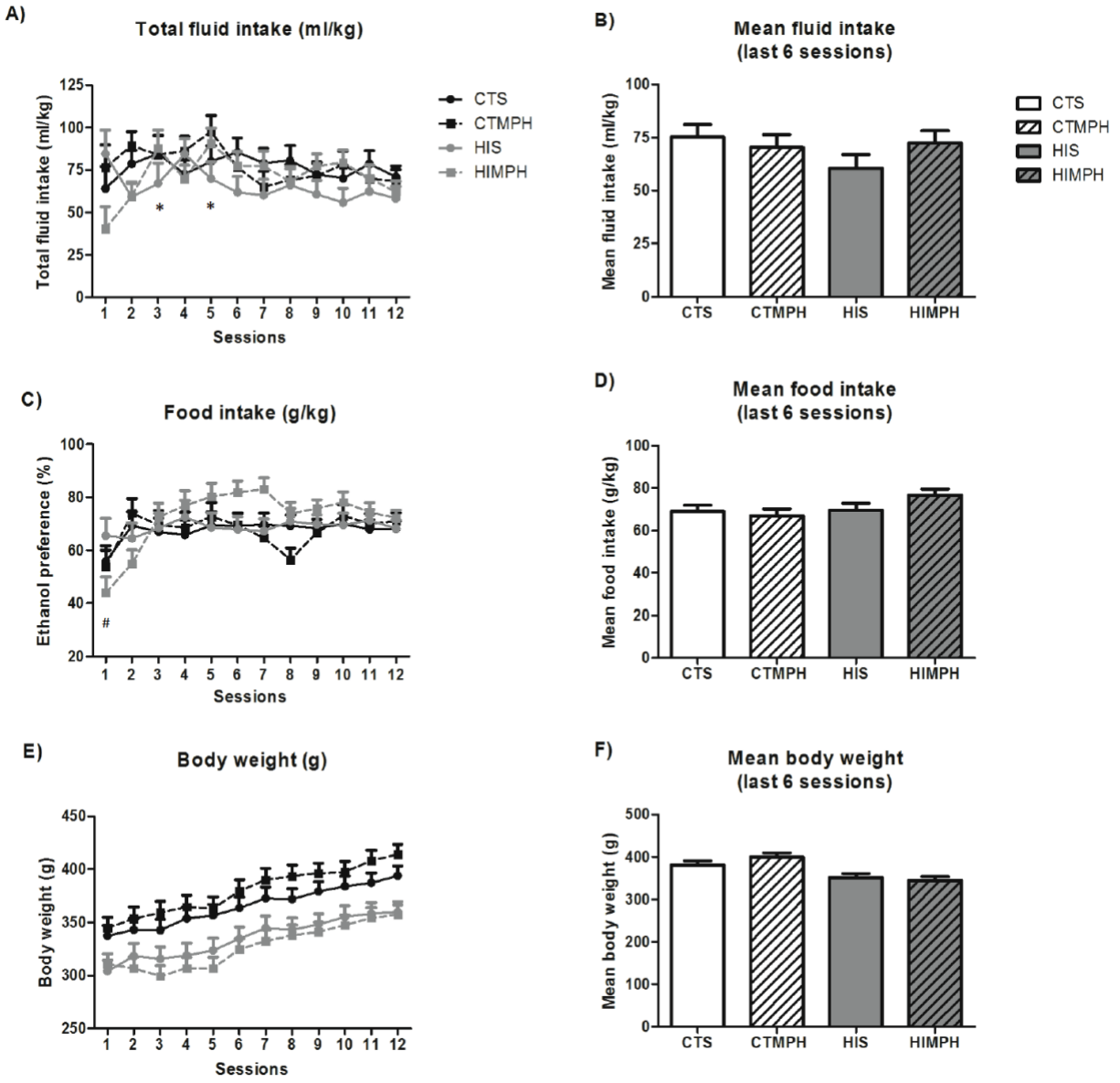

Figure 5. Total fluid and food intake, as well as the body weight measurement, over the 12 sessions of the IA2BC procedure (A, C, E) or the mean of the last 6 sessions of each measure (B, D, F). Results are expressed as mean ± S.E.M. Repeated-measures ANOVA or two-way ANOVA, followed by Tukey's post hoc, $p<.05$. *difference in relation to the first session, in the CTMPH group; #difference in relation to sessions 3 to 12, in the CTMPH group. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n=11-12/group.

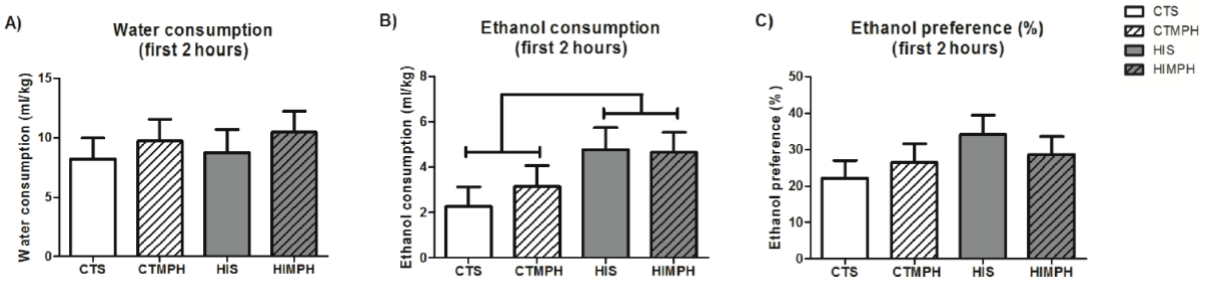

Figure 6. Mean of the water (A) and ethanol consumption (B), as well as the ethanol preference (C) in the first 2 hours after drug administration. Results are expressed as mean ± S.E.M. Two-way ANOVA, followed by Tukey's post hoc, $p<.05$. Lesion effect was observed for ethanol consumption. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n=11-12/group.

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Supplementary material

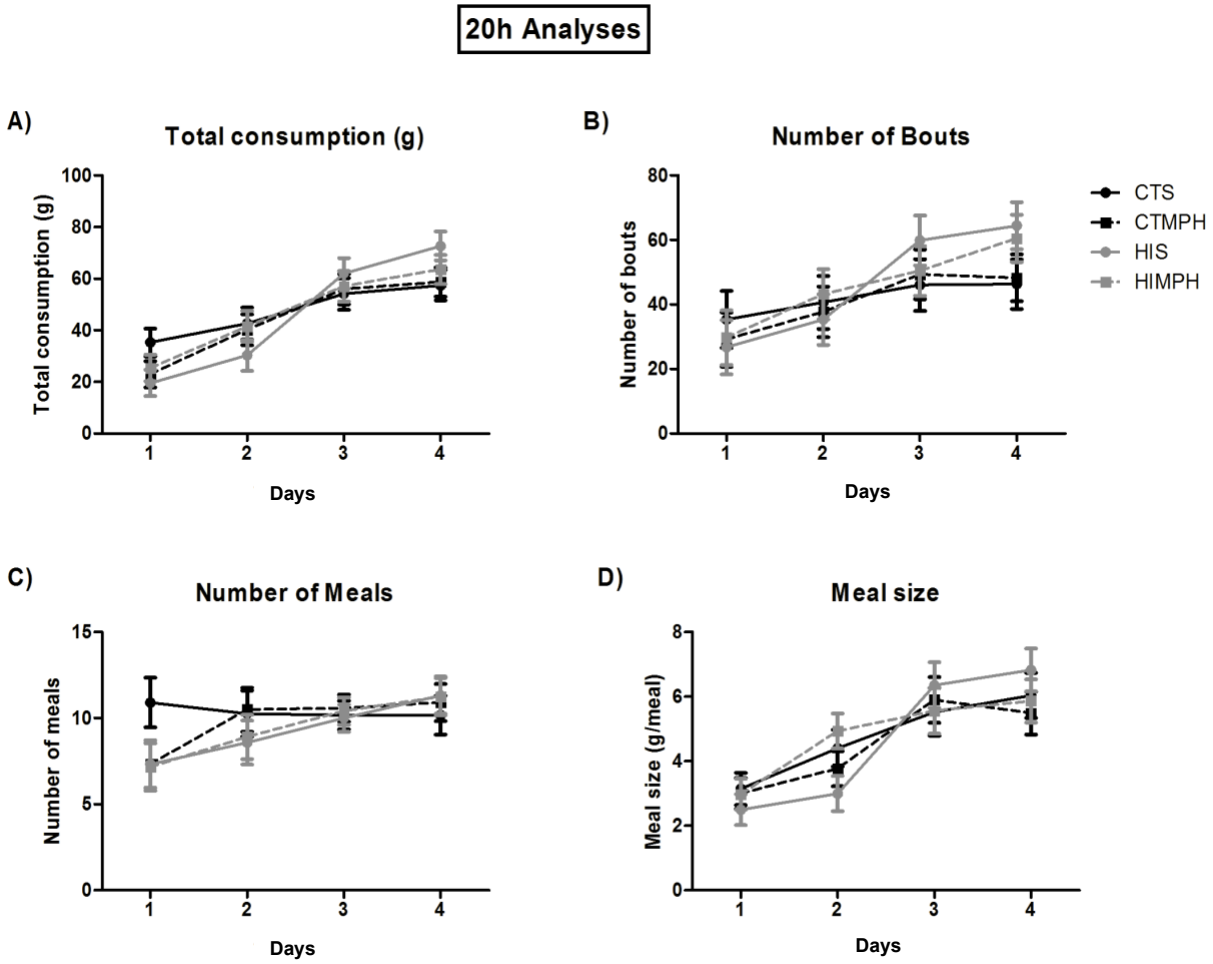

Figure S1. Feeding activity parameters in relation to standard rat chow in sessions of 20h in the first 4 days in the BioDAQ. Results are expressed as mean ± S.E.M. Repeated-measures ANOVA throughout the days and two-way ANOVA within each day, followed by Tukey's post hoc, $p<.05$. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n=11-12/group.

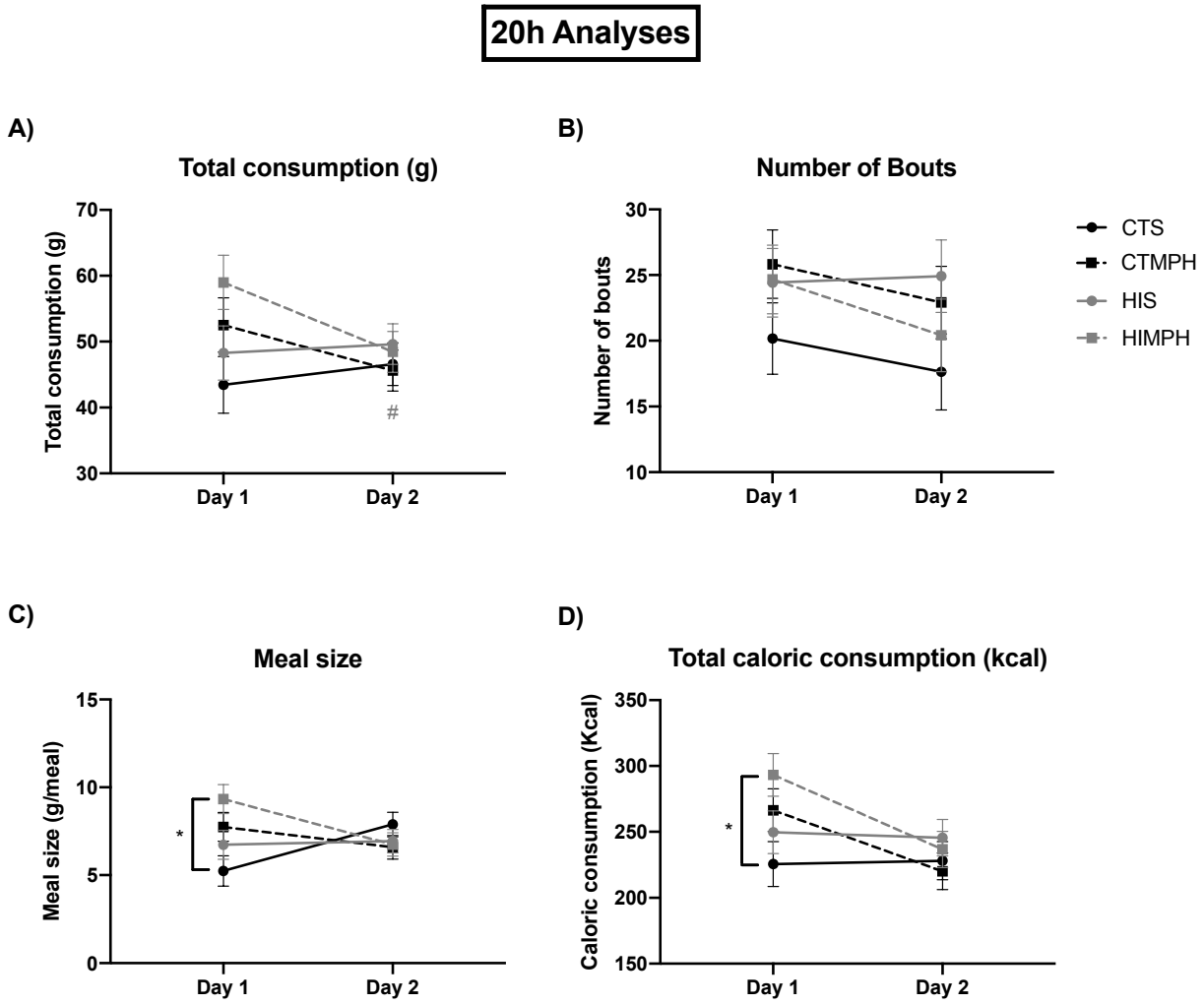

Figure S2. Feeding activity parameters in relation to the palatable diet in sessions of 20h in 2 days of exposure. Results are expressed as mean ± S.E.M. Repeated-measures ANOVA followed by Tukey's post hoc, $p<.05$. *difference between the HIMPH and the CTS group in the first day. CTS: control treated with saline; CTMPH: control treated with methylphenidate; HIS: hypoxia-ischemia treated with saline; HIMPH: hypoxia-ischemia treated with methylphenidate. n=11-12/group.

6. CAPÍTULO 4

Artigo: *Prefrontal cortex dopamine transporter gene network moderates the effect of perinatal hypoxic-ischemic conditions on cognitive flexibility and brain gray matter density in children*

Publicado na revista ***Biological Psychiatry***

O Capítulo 4 desta tese foi desenvolvido na Universidade McGill (Canadá) durante um período de doutorado sanduíche no exterior.

Considerando que tanto fatores genéticos, ambientais ou interações entre estes fatores podem afetar o neurodesenvolvimento, nosso objetivo foi avaliar o efeito da interação entre o perfil genético individual e a exposição a condições hipóxico-isquêmicas perinatais (HICs) em desfechos de flexibilidade cognitiva e densidade de substância cinzenta cerebral em crianças de duas coortes étnicas distintas. Para a avaliação da exposição às HICs, foi criado um escore cumulativo de múltiplos fatores associados à HI perinatal; para o componente genético, empregamos a metodologia do estudo de redes genéticas e criamos um escore de "risco" poligênico baseado na expressão (ePRS) dos genes que são co-expressos com o gene *DAT1* no CPF.

Em ambas as coortes, demonstramos que a exposição a vários HICs perinatais prejudicou a flexibilidade cognitiva apenas de crianças que tinham um ePRS alto para a rede do *DAT1*, o que pode sugerir uma alta funcionalidade dessa rede e menor sinalização dopaminérgica no CPF. Ainda, demonstramos que os polimorfismos do tipo SNP incluídos no escore ePRS modularam a relação entre a exposição aos HICs e a densidade de substância cinzenta em áreas envolvidas nas funções executivas (regiões corticais) e integrativas (tálamo e putâmen).

Biological Psychiatry

Archival Report

Prefrontal Cortex Dopamine Transporter Gene Network Moderates the Effect of Perinatal Hypoxic-Ischemic Conditions on Cognitive Flexibility and Brain Gray Matter Density in Children

Patrícia Maidana Miguel, Lenir Orlandi Pereira, Barbara Barth, Euclides José de Mendonça Filho, Irina Pokhvisneva, Thao T.T. Nguyen, Elika Garg, Bruna Regis Razzolini, Dawn Xin Ping Koh, Heather Gallant, Roberto Britto Sassi, Geoffrey B.C. Hall, Kieran John O'Donnell, Michael J. Meaney, and Patrícia Pelufo Silveira

ABSTRACT

BACKGROUND: Genetic polymorphisms of the dopamine transporter gene (*DAT1*) and perinatal complications associated with poor oxygenation are risk factors for attentional problems in childhood and may show interactive effects.

METHODS: We created a novel expression-based polygenic risk score (ePRS) reflecting variations in the function of the *DAT1* gene network (ePRS-*DAT1*) in the prefrontal cortex and explored the effects of its interaction with perinatal hypoxic-ischemic–associated conditions on cognitive flexibility and brain gray matter density in healthy children from two birth cohorts—MAVAN from Canada (n = 139 boys and girls) and GUSTO from Singapore (n = 312 boys and girls).

RESULTS: A history of exposure to several perinatal hypoxic-ischemic–associated conditions was associated with impaired cognitive flexibility only in the high-ePRS group, suggesting that variation in the prefrontal cortex expression of genes involved in dopamine reuptake is associated with differences in this behavior. Interestingly, this result was observed in both ethnically distinct birth cohorts. Additionally, parallel independent component analysis (MAVAN cohort, n = 40 children) demonstrated relationships between single nucleotide polymorphism–based ePRS and gray matter density in areas involved in executive (cortical regions) and integrative (bilateral thalamus and putamen) functions, and these relationships differ in children from high and low exposure to hypoxic-ischemic–associated conditions.

CONCLUSIONS: These findings reveal that the impact of conditions associated with hypoxia-ischemia on brain development and executive functions is moderated by genotypes associated with dopamine signaling in the prefrontal cortex. We discuss the potential impact of innovative genomic and environmental measures for the identification of children at high risk for impaired executive functions.

Keywords: ADHD, Cognitive flexibility, *DAT1*, Dopamine transporter gene, Hypoxic-ischemic conditions, Parallel independent component analysis

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The dopamine transporter (DAT) is a transmembrane protein responsible for the reuptake of dopamine (DA) from the synaptic cleft into the presynaptic neuron, which thereby terminates DA signaling (1,2). The DAT regulates the strength and duration of dopaminergic transmission, a role that is revealed by the effects of many DAT-targeted pharmacological therapies, such as methylphenidate, that improve DA dysfunction in attention-deficit/hyperactivity disorder (ADHD) (3–5). Although DAT is abundant in the striatum and sparse in the prefrontal cortex (PFC) (6,7), several studies show that low doses of methylphenidate (dosages that are usually more effective in treating attentional impairments than hyperactivity) preferentially increase DA release in the PFC (8–11). The PFC is highly involved in executive functions—a set of cognitive processes comprising cognitive flexibility and planning—that are typically impaired in children with ADHD (12–14).

The DAT gene (*DAT1*, also known as *SLC6A3*), which is located in chromosome 5p15.3, is one of the most studied genes in ADHD (15). A 40-bp variable number of tandem repeats polymorphism and single nucleotide polymorphisms

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(SNPs), for example rs2652511, were identified as risk factors for ADHD (16–18). Although we acknowledge the contribution of studies focusing on a single candidate polymorphism, genes act not in isolation but in concert with other genes in molecular pathways. The principle of gene networks considers that gene expression is coregulated by other genes, and consequently genes involved in the same network are expected to have similar expression profiles (19). Analyzing genomic data through gene sets defined by functional pathways represents a potentially powerful and biologically oriented link between genotypes and phenotypes (20).

Although genetic factors contribute substantially to the etiology of ADHD, there is considerable evidence for the influence of environmental factors (21). Getahun *et al.* (22) identified a direct relationship between hypoxic-ischemic–associated conditions (HICs) in utero and the later development of ADHD. Preeclampsia, Apgar score <7 at 1 or 5 minutes, breech or transverse presentations, prolapsed or nuchal cord, and elective cesarean births were all significantly associated with ADHD (23,24). Using an animal model of perinatal hypoxia-ischemia, we demonstrated cognitive inflexibility using the attentional set-shifting task, a task comparable to the Intra-Extra/Dimensional (IED) set shift in humans. Additionally, we showed that the cognitive inflexibility in this model was correlated to PFC atrophy and dopaminergic dysregulation also in the PFC, reflecting the profile reported in ADHD individuals (25).

Our rodent study suggests that perinatal hypoxia-ischemia associates with both impaired cognitive flexibility and altered PFC DA signaling. Considering that the literature has linked *DAT1* polymorphisms and ADHD, we hypothesized that a genomic measure based on a PFC-specific *DAT1* gene network would moderate the impact of perinatal HICs on cognitive flexibility in children. To test this hypothesis, we constructed an expression-based polygenic risk score (ePRS) that reflects the function of a PFC *DAT1* gene network (ePRS-*DAT1*/PFC) and analyzed its interaction with perinatal HICs on cognitive flexibility performance and brain gray matter density in healthy children.

METHODS AND MATERIALS

Participants

We used data from two prospective birth cohorts, one based in Canada [Maternal Adversity, Vulnerability and Neurodevelopment (MAVAN) (26)] and the other from Singapore [Growing Up in Singapore Towards Healthy Outcomes (GUSTO) (27)].

Main Cohort (MAVAN). MAVAN is a community-based, birth-cohort study of Canadian mothers and their offspring. Pregnant women aged 18 years and above were recruited in Montréal (Quebec) and Hamilton (Ontario), Canada. Approval for the MAVAN project was obtained from McGill University, Université de Montréal, Royal Victoria Hospital, Jewish General Hospital, Centre hospitalier de l'Université de Montréal, Hôpital Maisonneuve-Rosemount, St Joseph's Hospital, and McMaster University. A total of 139 children of both sexes had complete data (birth records, genotype, and cognitive flexibility task at 6 years of age) and were included in the study. Of this sample, 40 participants had brain magnetic resonance imaging (MRI) used for the parallel independent component analysis (p-ICA) (see the subsection "Parallel Independent Component Analysis").

Replication Cohort (GUSTO). Pregnant women $\geq$18 years of age were recruited at the National University Hospital and KK Women's and Children's Hospital in Singapore. The cohort included women of Chinese, Malay, or Indian ethnicity with homogeneous parental ethnic background that allowed us to extend the analysis to include Southeast Asian ethnic groups (28). The study was approved by the National Healthcare Group Domain Specific Review Board and the Sing Health Centralized Institutional Review Board. Informed consent was obtained from each participating adult. A total of 312 children had complete data (birth records, genotype, and cognitive flexibility task at 4.5 years of age) and were included in the study.

PFC *DAT1* Coexpressed Genes and ePRS

The ePRS was created considering genes coexpressed with the DA transporter gene (ePRS-*DAT1*) in the PFC, according to the protocol previously described by Silveira *et al.* (28,29) (Supplemental Figure S1). The genetic score was created using data from the 1) GeneNetwork (http://genenetwork.org), 2) BrainSpan (http://www.brainspan.org), 3) National Center for Biotechnology Information Variation Viewer (https://www.ncbi.nlm.nih.gov/variation/view), and 4) Genotype-Tissue Expression (GTEx) (https://www.gtexportal.org/home). A full explanation and the final list of coexpressed genes included in the ePRS (Supplemental Table S1) are described in the Supplement. The final score in both cohorts was categorized into "low ePRS" or "high ePRS" using a median split for the behavioral analysis.

Perinatal HICs Score

We aggregated information reported by Getahun *et al.* (22) and Linnet *et al.* (30) and compiled by Smith *et al.* (31), which provided the list of nine variables to consider in the score: 1) Apgar score at 1 minute <7 (32), 2) respiratory distress, 3) fetal dystocia, 4) occurrence of umbilical cord prolapse, 5) placental abruption, 6) breech or transverse birth presentation, 7) neonatal resuscitation, 8) maternal smoking during pregnancy, and 9) birth weight ratio (observed birth weight/mean populational weight adjusted by sex and gestational age) (33,34). Each one of these variables was categorized into absent or present condition (Supplemental Table S2).

A principal component analysis was performed on these variables using tetrachoric correlations, extracting one component. Two variables (maternal smoking during pregnancy and birth weight ratio) did not exhibit significant component loadings and were excluded. We reapplied a principal component analysis to the remaining variables to compute the perinatal HICs score according to the method proposed by Distefano *et al.* (35). This method aims to maximize validity by producing scores that are highly correlated with the underlying component in order to obtain unbiased

estimates (35). A higher absolute value of the loading is indicative of a larger contribution of the corresponding variable to the component score (see Supplemental Table S2 and Supplemental Figure S2).

The HICs score was either used as a continuous variable (for the behavioral outcomes) or categorized into low or high HICs score groups using a median split (for the p-ICA). Low HICs score indicates minimal exposure to HICs in the perinatal period, and a high HICs score suggests a history of exposure to several HICs.

Behavioral Outcomes

IED Set Shift. The IED set shift task comprises rule acquisition and reversal throughout nine stages with increasing difficulty. There are two dimensions used in the task (color-filled shapes and white lines), and in the first seven stages, shape remains the relevant dimension. An extra-dimensional shift occurs in stage 8, where the white lines are the relevant dimension for a correct response (36) (Supplemental Figure S3A). This task is part of the CANTAB battery and was performed by MAVAN children at 72 months of age. We focused specifically on stage 8 (extra-dimensional shift), which measures cognitive flexibility.

Dimensional Change Card Sort. The Dimensional Change Card Sort (DCCS), like the IED task, measures the ability to shift between two dimensions, but it is more readily completed by younger children. For this reason, we used this task to assess the cognitive flexibility of children at 54 months of age in the GUSTO cohort. In the standard version of the DCCS task, children are shown cards with two dimensions: different colors (red vs. blue) and shapes (rabbit vs. boat). In the first stage (preswitch), children must sort the cards by the color dimension independently of the shape presented on the cards. In the following stage (postswitch), the rule changes and children must sort the cards by the shape dimension and ignore the first rule, requiring attentional flexibility (37) (Supplemental Figure S3B).

Gray Matter Density

Parallel Independent Component Analysis. Structural MRI acquisition and data preparation were conducted prior to the p-ICA (see the Supplement). A multivariate p-ICA was applied to identify relationships between clusters of interrelated SNPs and brain gray matter information in a data-driven manner (38). We sought to find the relationship between the SNP-based ePRS-*DAT1* (or genotype × GTEx gene expression slope at each SNP comprised by the ePRS-*DAT1*) and the voxel-based gray matter in the whole brain (full description in the Supplement), instead of investigating the relationship between the crude genotype and the gray matter-voxel-based measures. The groups for comparison (20 children with high HIC score and 20 children with low HIC score) were defined by the perinatal environment aggregated with population stratification (ethnicity) for adjustment. Loading coefficients, which describe the presence of the identified component across participants (39), were extracted for each component, modality, and participant. The mean participant-specific loading coefficients of these components between children from high- and low-HICs-score groups was compared using Student's *t* test.

Validation of the Prefrontal *DAT1* Coexpression Network

Gene Expression Levels at Different Time Points. We used BrainSpan data to analyze the correlation between the expression levels of all genes included in the ePRS-*DAT1* in the human PFC at different time points: perinatal, childhood, and adulthood. Thus, we can investigate whether the same pattern of coexpression is maintained through the life course. The analyses were performed in R (https://www.r-project.org) using the heatmaply package (40).

Gene Ontology Enrichment Analysis. Enrichment analysis for functional ontologies of the genes included in ePRS-*DAT1* was performed using Metacore (https://portal.genego.com).

Protein–Protein Interaction Network Analysis. The STRING database (https://string-db.org) was used to analyze functional interactions between the corresponding protein from our list of *DAT1* coexpressed genes (269 genes) and the same top 269 genes associated with SNPs from the genome-wide association study (GWAS) for ADHD (41). We compared the mean number of interactions of the top 20 most interactive proteins of each dataset (ePRS-*DAT1* and GWAS-ADHD).

Comparison With Another PFC ePRS. As the creation of the ePRS-*DAT1* was highly informed by the main action of a pharmacological agent (e.g., methylphenidate), we created a control PFC ePRS with the same premise and using the same methods. The control ePRS had the serotonin transporter solute carrier family 6 member 4 (*SLC6A4*) as the target (ePRS-*SLC6A4*) considering the action of serotonin reuptake inhibitors (e.g., fluoxetine). This choice is interesting because methylphenidate also acts on the serotonin transporter, but with much less affinity than that for the DAT (42,43).

Statistical Analysis

Data were analyzed using the SPSS version 20.0 (SPSS Inc., Chicago, IL) and R. Significance levels for all measures were set at $\alpha = .05$. Student's *t* test was performed to compare 1) the mean number of protein interactions between the ePRS and GWAS-ADHD, 2) the continuous data of the sample baseline characteristics between low and high ePRS, and 3) the mean subject-specific loading coefficients between high- and low-HICs-score groups (p-ICA). χ^2 tests were performed to analyze the categorical variables of the sample baseline characteristics. Linear regressions were used to examine the effect of interaction between the genetic score (median split: low and high ePRSs) and the perinatal HICs (continuous variable: HICs) on the behavioral outcomes (IED and DCCS tasks). The ePRSs and HICs scores were included as main factors along with covariates of sex and population stratification. Additionally, the preswitch performance was included as covariate for the DCCS task. Simple

slope analyses were conducted to analyze the post hoc differences.

RESULTS

Behavioral Outcomes

In both MAVAN and GUSTO datasets, children with high and low genetic scores on ePRS-*DAT1* do not differ in the main confounding variables, which were chosen based on the literature (see Table 1). We considered well-established variables that affect child neurodevelopment as possible confounders: birth weight and gestational age (44–46), maternal age (47), socioeconomic status (48), and maternal education level (49).

IED Task (Stage 8, Extra-Dimensional Shift). In the IED task, we observed a significant ePRS × HICs interaction in predicting the latency to respond (β = 32489.1, $p < .001$) at stage 8 of the task. The simple slopes analysis showed that the high-ePRS group demonstrated worse outcomes (higher latency to respond) as HICs score increased (β = 29002.6, $p < .05$) (Figure 1A). No significant interactions were seen for number of trials (β = 3.92, p = .14) or errors (β = 1.89, p = .29). Results for other IED stages are shown in Supplemental Table S3.

DCCS Task (Postswitch Phase). We then replicated the ePRS-*DAT1*/PFC × HICs interaction effect in the GUSTO cohort. The significant ePRS-*DAT1* × HICs score interaction was observed for total accuracy ($\beta = -.46$, $p < .05$) and number of commission errors (β = .44, $p < .05$) in the postswitch phase. Higher HICs was associated with lower accuracy ($\beta = -.50$, $p < .001$) and higher commission errors (β = .44, $p < .01$) only in the high-ePRS group (Figure 1B and 1C, respectively). The adjusted/unadjusted analysis for IED stage 8 in MAVAN and DCCS in GUSTO for the main effect model and for the model including the interaction term are described in Supplemental Table S4.

Specificity of the *DAT1* Gene Network. We then analyzed the specificity of our findings in relation to the *DAT1* gene network. We used the same ePRS bioinformatic process (Supplemental Figure S1) to create an ePRS from genes that are coexpressed with the *SLC6A4* gene in the PFC. Despite the fact that ePRS-*SLC6A4* also formed a cohesive gene network (Supplemental Figure S4), there were no significant interactions between this genetic score and the HICs score on IED outcomes (number of trials, $\hat{\beta} = -1074.52$, p = .11; number of errors, $\hat{\beta} = -576.50$, p = .20; and latency, $\hat{\beta}$ = 87428.9, p = .97).

Gray Matter Density

The p-ICA identified highly significant relationships between regional gray matter volume and SNP-based ePRS-*DAT1* data on 1) the genetic component 2 and MRI component 1 ($r = -.77$, $p = 5.953 \times 10^{-9}$); 2) genetic component 5 and MRI component 7 (r = .69, $p = 7.1487 \times 10^{-7}$); and 3) genetic component 12 and MRI component 4 ($r = -.61$, $p = 2.1291 \times 10^{-5}$). When comparing the mean loading coefficients of these components between children from high- and low-HICs-score groups by Student's t test, we found statistically significant differences in the pair genetic component 2 and MRI component 1 (Figure 2), suggesting that the relationship between ePRS-*DAT1* and gray matter volume in these brain regions is moderated by the neonatal environmental condition. Genetic component 5 was also significantly different between the groups. The pair MRI component 7 did not reach significance (p = .069), although it is clear on Figure 2 that groups have opposite directions in loading coefficients. For the other relationship between genetic component 12 and MRI component 4, no differences between groups were observed.

To define the significant SNPs in each component, we used a threshold of higher than 2.5 and lower than −2.5. In component 2, we found 78 significant SNPs, and the enrichment analysis demonstrated that these SNPs are involved especially in positive regulation of long-term synaptic potentiation (false discovery rate [FDR]–adjusted $q = 3.635 \times 10^{-6}$), astrocyte activation (FDR-adjusted $q = 6.529 \times 10^{-6}$), dopaminergic transmission (FDR-adjusted $q = 2.710 \times 10^{-5}$), and gamma-aminobutyric acidergic transmission (FDR-adjusted $q = 1.867 \times 10^{-6}$). Additionally, these SNPs were enriched for diseases including anxiety disorders (FDR-adjusted $q = 9.243 \times 10^{-5}$), schizophrenia (FDR-adjusted $q = 3.269 \times 10^{-3}$), and dementia (FDR-adjusted $q = 9.835 \times 10^{-3}$). This group of SNPs was related to differential gray matter density in areas of the putamen and thalamus (MRI component 1). In

Table 1. Description of the Baseline Characteristics of the MAVAN and GUSTO Samples

	MAVAN			GUSTO		
Sample Characteristic	Low ePRS, n = 67	High ePRS, n = 72	p Value	Low ePRS, n = 150	High ePRS, n = 162	p Value
Male Participants, n (%)	38 (56.7)	35 (48.6)	.339	62 (41.3)	82 (50.6)	.100
Maternal Age at Birth, Years	31.25 ± 5.18	31.82 ± 4.19	.075	31.45 ± 5.21	31.62 ± 5.18	.772
Full Weeks of Gestation	39.09 ± 1.28	38.78 ± 1.14	.248	38.60 ± 1.22	38.33 ± 1.33	.324
Birth Weight, Grams	3404.68 ± 429.13	3366.11 ± 440.65	.732	3131.65 ± 411.48	3067.46 ± 420.42	.741
HICs Score	0.04 ± 1.07	−0.14 ± 0.81	.179	0.04 ± 1.04	−0.04 ± 0.96	.473
Low SES[a], n (%)	14 (23.3)	8 (14.3)	.214	28 (19.4)	21 (13.6)	.176

Data are expressed as mean ± SD unless otherwise noted.

Differences between low- and high-ePRS groups were not significant for all variables shown (Student's t test for means and χ^2 test for percentages).

ePRS, expression-based polygenic risk score; GUSTO, Growing Up in Singapore Towards Healthy Outcomes; HICs, hypoxic-ischemic–associated conditions; MAVAN, Maternal Adversity, Vulnerability and Neurodevelopment study; SES, socioeconomic status.

[a]Low SES in MAVAN: Maternal education attained high school or less, or monthly income under low bound from the cutoff proposed by Statistics Canada (71). Low SES in GUSTO: Maternal education attained primary school or monthly income <$2000.

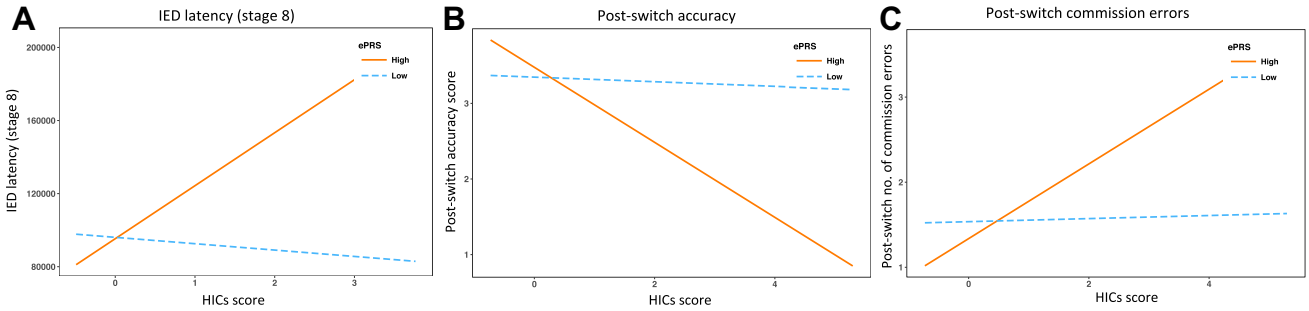

Figure 1. Cognitive flexibility performance **(A)** in the Intra-Extra/Dimensional (IED) set shift task in children in the Maternal Adversity, Vulnerability and Neurodevelopment study and **(B and C)** in the Dimensional Change Card Sort task in children in the Growing Up in Singapore Towards Healthy Outcomes study. **(A)** A higher hypoxic-ischemic–associated conditions (HICs) score was associated to longer latency to respond only in the high expression-based polygenic risk score (ePRS) group in the IED task. **(B and C)** A higher HICs score was associated with lower accuracy and higher number of commission errors only in the high-ePRS group in the Dimensional Change Card Sort task. Analysis comprised linear regression followed by simple slope analysis.

genetic component 5, we found 77 significant SNPs that were involved in nervous system development (FDR-adjusted $q = 7.617 \times 10^{-4}$), neurogenesis (FDR-adjusted $q = 7.365 \times 10^{-4}$), and neuron migration (FDR-adjusted $q = 6.755 \times 10^{-4}$). This component was related to differential gray matter in cortical regions (MRI component 7).

Validation of the *DAT1* Coexpression Network

We used BrainSpan data to correlate the PFC expression levels of all genes included in the ePRS during the perinatal period. We observed two large clusters of highly coexpressed genes specifically at this developmental period (Figure 3A). These findings confirmed the coexpression network from the genes included in the ePRS-*DAT1* in the PFC. For the developmental trajectory analysis, we kept the same order of the genes that composed the perinatal correlation matrix to visualize whether the clusters would be consistent throughout development. We observed that the general pattern of coexpression was generally maintained during the life course (Figure 3B and 3C). We analyzed *DAT1* expression by age in different human brain regions using Human Brain Transcriptome data (http://hbatlas.org) (50) and observed stable gene expression throughout development, with very similar levels of expression in the neocortex and striatum (Supplemental Figure S5).

The gene ontology enrichment analysis showed several statistically significant enrichment processes, functions, and cellular localizations, and we focused on the top 10 significant

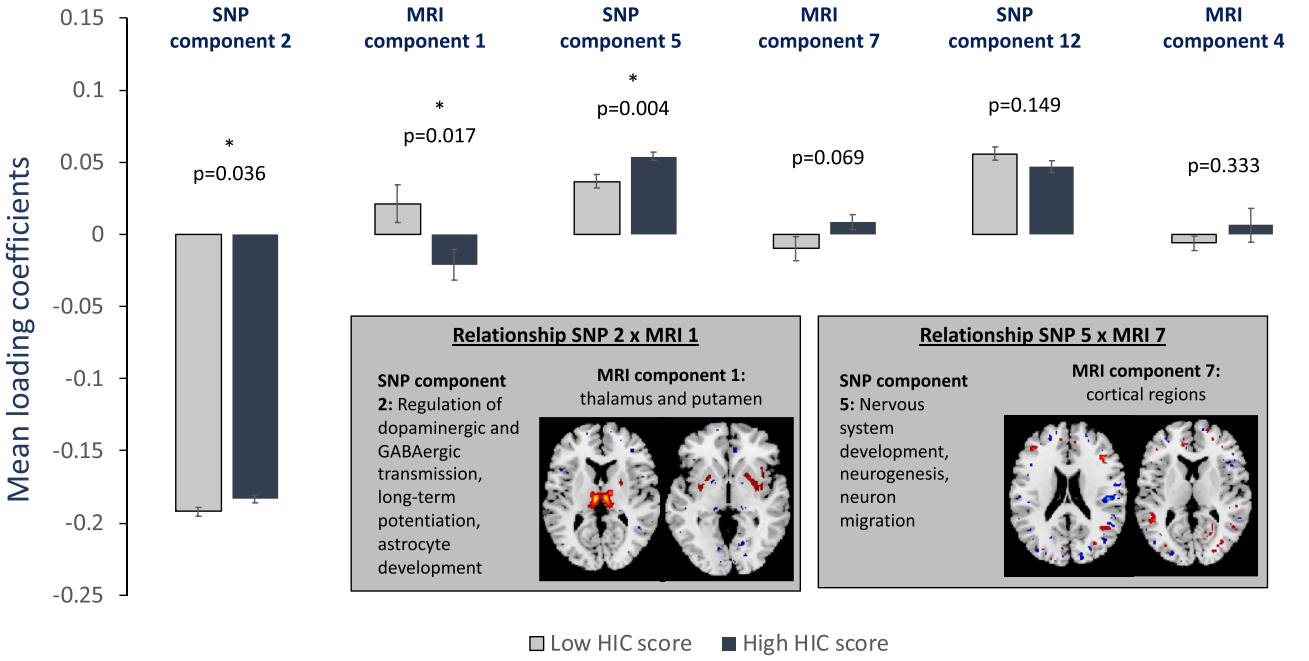

Figure 2. A bar plot of the mean loading coefficients of brain magnetic resonance imaging (MRI) component and genetic component. Student's *t* test was performed to compare loading coefficients means between groups. *Group differences among children with low and high hypoxic-ischemic–associated condition (HIC) scores. GABAergic, gamma-aminobutyric acidergic; SNP, single nucleotide polymorphism.

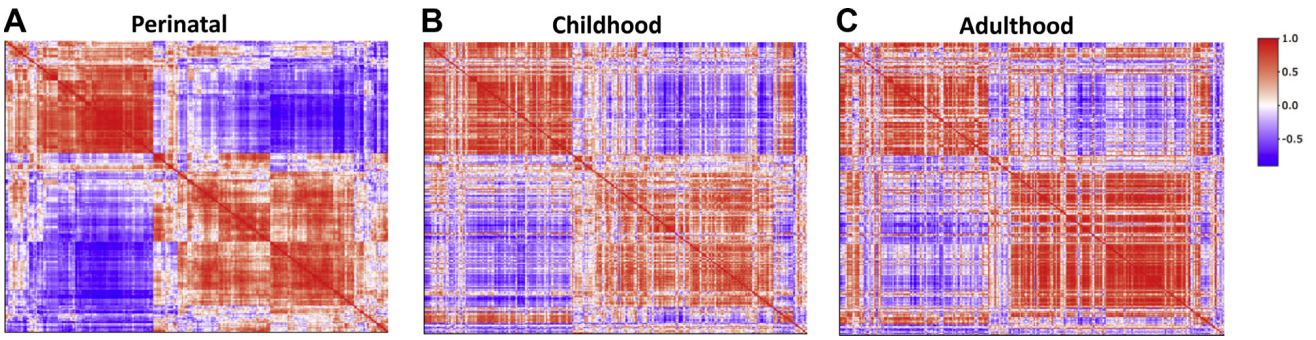

Figure 3. Heat map of *DAT1*-related genes (expression levels) correlation throughout development in the human prefrontal cortex. Genes from the same expression quantification tend to cluster together and could be visualized in red (positive correlation). A blue pattern indicates a negative correlation. In this analysis, the arrangement in clusters considering similar gene expression are shown in the **(A)** perinatal, **(B)** childhood, and **(C)** adulthood stages. The perinatal period ranges from 24 weeks post conception to 4 months of age (n = 5); childhood is defined as 1–12 years of age (n = 8) and adulthood as 20–40 years of age (n = 7).

results (Supplemental Figure S6). This analysis revealed gene ontology processes involving neurodevelopmental processes and cell signaling, among others. Molecular functions were enriched for protein binding (FDR-adjusted $q = 8.71 \times 10^{-11}$) and transmembrane receptor tyrosine kinase activity (FDR-adjusted $q = 1.28 \times 10^{-\times}$). Gene ontology localizations were enriched for extracellular space, cytoplasm, adherens junction, and cell junction.

The protein–protein network analysis is depicted in Figure 4, demonstrating the protein network resulting from the ePRS-*DAT1* (Figure 4A), and the top genes (comparable size) from the 2017 ADHD GWAS (41) (Figure 4B). Analyzing the top 20 most interactive proteins, we found a significantly higher number of interactions in our *DAT1* network compared with those in the GWAS-ADHD dataset ($p < .0001$, mean *DAT1* = 11.65 $\pm$ 5.41, mean GWAS-ADHD = 2.8 $\pm$ 1.64), suggesting that the ePRS-*DAT1* represents a more cohesive gene network.

DISCUSSION

We used a novel informatics approach to show that the association between HICs and executive function in childhood is moderated by genetic variants in a PFC-specific *DAT1* gene network. A composite measure of perinatal HICs was related to cognitive flexibility only for children with a genetic background reflecting higher PFC activity of the DAT machinery (high ePRS-*DAT1*). This result was observed in two ethnically distinct birth cohorts: MAVAN (Caucasians from Canada) and GUSTO (Southeast Asians from Singapore). The SNP-based ePRS-*DAT1* also moderated the relation between perinatal HICs and gray matter density in areas involved in executive (cortical regions) and integrative (bilateral thalamus and putamen) functions.

The DA system is implicated in the regulation of cognitive flexibility. In clinical trials, systemic administration of an antagonist of the DA receptor D_2 impaired the attentional set-shifting performance (51), whereas methylphenidate administration improved performance (52). One of the trigger points for DA system dysfunction seems to be the DAT, and several studies reveal associations between polymorphisms in the *DAT1* gene and a higher risk for attentional problems (17,18,53–55). Our findings extend studies focusing on single variants to show that a *DAT1* PFC expression–based gene network moderates the impact of perinatal conditions known to increase the risk for ADHD on executive function in childhood. These findings are consistent with the position that the analysis of gene sets defined by functional pathways is a promising approach for investigating the relationship between genotypes and phenotypes (20).

We validated our *DAT1* network using several approaches. We used databases that included gene expression levels in human PFC to demonstrate that a high proportion of the *DAT1*-related genes have similar expression levels in the perinatal period, confirming that the coexpression patterns within this gene network go beyond the coexpression with *DAT1* only, but they form several clusters of coexpressed genes. Clusters of coexpressed genes are generally maintained throughout development, suggesting that this network is also operative at later ages. Protein network analysis resulting from the ePRS-*DAT1* shows that the *DAT1* network represents a more cohesive protein network with significantly more connections than the protein network resulting from the same number of top genes from the most recent GWAS for ADHD (41). An ePRS based on PFC *SLC6A4* coexpression produced a gene network that was not associated with the cognitive flexibility performance in children, emphasizing the specificity of the ePRS technique.

The ePRS method is a robust approach that goes beyond finding associations between scattered genetic variants and phenotypes and captures information about the whole gene network, and its function, in specific brain regions (28,29). Our enrichment analysis for the *DAT1* network included nervous system development, which is in agreement with the choice of genes overexpressed during fetal and early postnatal periods when we filtered using BrainSpan data. Enrichment for many extracellular localizations, but also the cytoplasmic part, adherens, and cell junction, is aligned with the reported function of DAT mediating the transport of extracellular DA to the intracellular space (56,57). Multiple intracellular and extracellular signaling pathways have been implicated in the regulation of DAT function, and its expression is modulated through internalization and recycling from the cell surface (58).

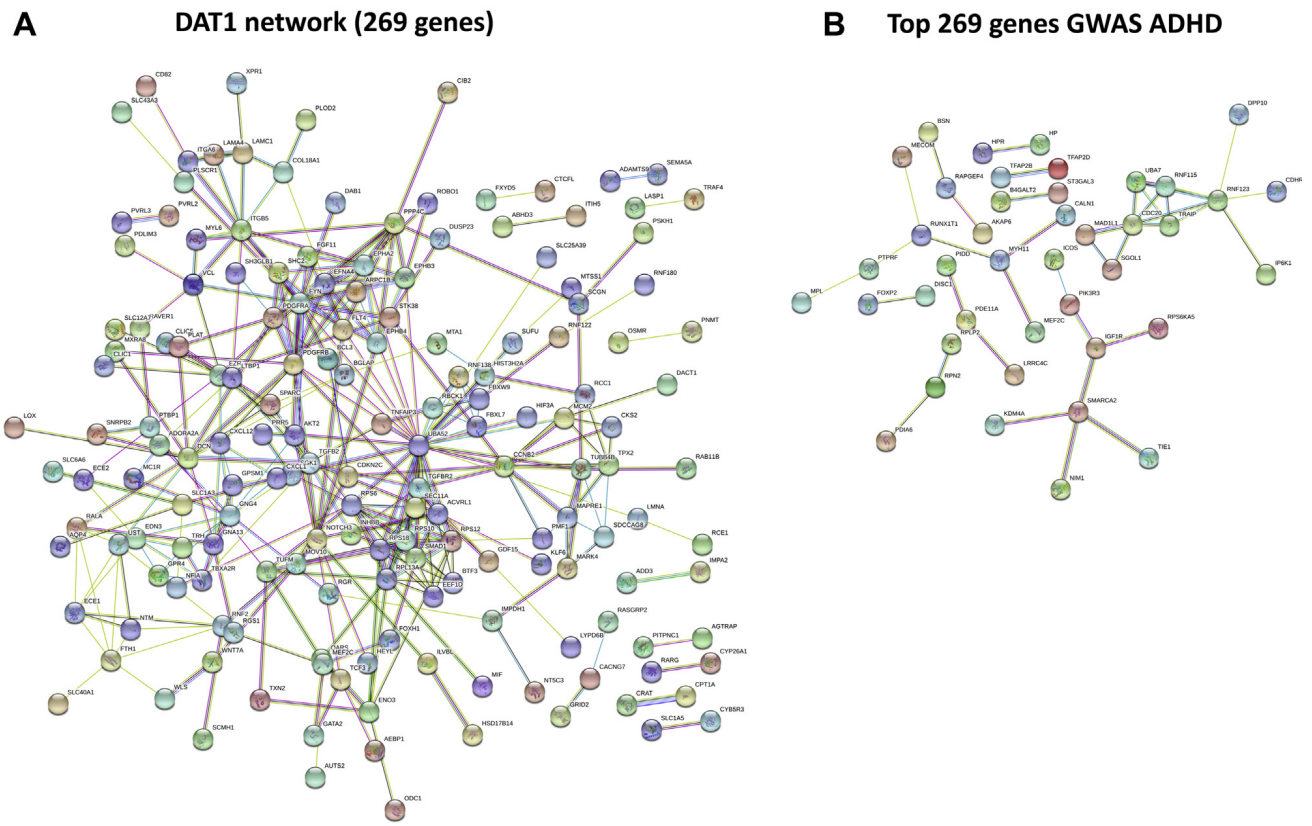

Figure 4. Protein networks **(A)** from the genes featured in the expression-based polygenic risk score reflecting variations in the function of the *DAT1* gene network and **(B)** a comparable number of the top genes from the attention-deficit/hyperactivity disorder (ADHD) genome-wide association study (GWAS) (41). The expression-based polygenic risk score reflecting variations in the function of the *DAT1* gene network contains proteins with a higher number of connections ($p < .0001$; Student's *t* test).

Moreover, DAT is the main target site for psychostimulant drugs, and thus several binding functions observed in the enrichment analysis provide a valid representation of the *DAT1* gene network function.

Several factors, such as birth asphyxia, preeclampsia, respiratory distress syndrome, low Apgar score, and prolapsed or nuchal cord, were significantly associated with ADHD (22–24). Considering that different, intercorrelated conditions could influence perinatal oxygenation levels, we aimed to create a novel cumulate index of perinatal adversity, the HICs score. Smith *et al.* (31) have proposed that accounting for multiple ischemia-hypoxia–related obstetric complications during pregnancy and birth may provide a more accurate measure of ischemia-hypoxia exposure in community-based samples. These authors recommend creating a weighted summary score of perinatal risk factors, allowing for the severity of ischemia-hypoxia exposure to be measured on a continuum, an approach we adopted for the current study. Other studies had used a similar approach, and investigators observed that perinatal health risk increased inattention and hyperactivity or impulsivity later in life (59–61). A strength of our prospective study is that we used birth cohorts with extensive and detailed data including gestational and birth records to create a reliable perinatal score without reliance on self-report retrospective questionnaires.

Differential exposure to perinatal HICs modifies the relationships between the SNP-based ePRS-*DAT1* and gray matter volume in areas involved in information processing (cortical regions, thalamus, and putamen). Our previous work using the rat model of hypoxia-ischemia demonstrates smaller total brain and gray matter volumes in areas such as the cerebral cortex and striatum, in addition to attentional impairments observed in adult animals (62). Experimental studies indicate that perinatal hypoxia-ischemia induces lasting changes in dopaminergic neurotransmission depending on the severity and duration of the hypoxic insult (25). Neuropathological and in vivo imaging studies in humans indicate that dopamine synthesis capacity is reduced in participants exposed to high HICs, and this is positively related to brain atrophy (63,64). Such reduced dopamine synthesis capacity could even worsen the strength and duration of dopamine transmission in general and especially in the high-ePRS-*DAT1* group. In clinical studies, smaller thalamus and frontal and parietal cortices were associated with lower attentional and executive functions in adolescents and adults born preterm or with low birth weight (65–67). Additionally, frontostriatothalamic circuitry has been implicated in ADHD pathophysiology in several studies (68–70). Considering that, we can infer that differences in gray matter density of the described structures appear to contribute to the impaired cognitive

flexibility in our study, with perinatal HICs modulating the relationship between the genetic background and gray matter volumes.

One limitation of the study is the smaller sample size for the neuroimaging analysis; hence, these results require replication. Independent replication and the use of a falsification approach (another pathway) are strengths of this study, which with other efforts such as preregistration can help avoid type I errors in future studies using our methodology.

We demonstrated that the gene network associated with PFC DAT interacts with the history of exposure to perinatal HICs, impairing cognitive flexibility and modifying the relationship between genetics and gray matter density. DA neurotransmission in the PFC is an essential moderator of the effects of perinatal adversity on attentional outcomes and brain development and define children's endophenotype and risk for attentional disturbances. We innovate by proposing new ways of integrating genotype data and perinatal history of HICs to predict cognitive flexibility in community cohorts, which may inform practices for early detection of vulnerability to poor academic performance. The proposed research approach could be important for the study of other DA-related gene networks and psychiatric disorders (71). Considering that we cannot modify our genetic predisposition for certain traits or disease, we highlight the significance of preventive measures to improve intrauterine and intrapartum health, avoiding disturbances in the fragile fetal developing brain.

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7. CAPÍTULO 5

Artigo: *Early environmental influences on the development of children's brain structure and function*

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Finalizamos os trabalhos desta tese revisando as principais influências ambientais precoces (período pré-natal, periparto e pós-natal) que podem afetar o encéfalo em desenvolvimento, incluindo a HI perinatal como uma destas influências ambientais adversas. Novas ferramentas tanto nos estudos de neuroimagem quanto nos estudos genéticos estão nos permitindo elucidar os mecanismos envolvidos na relação entre a exposição aos eventos adversos precoces e as alterações encefálicas e funcionais.

Early environmental influences on the development of children's brain structure and function

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ABBREVIATION

BDNF	Brain-derived neurotrophic factor

The developing brain in utero and during the first years of life is highly vulnerable to environmental influences. Experiences occurring during this period permanently modify brain structure and function through epigenetic modifications (alterations of the DNA structure and chromatin function) and consequently affect the susceptibility to mental disorders. In this review, we describe evidence linking adverse environmental variation during early life (from the fetal period to childhood) and long-term changes in brain volume, microstructure, and connectivity, especially in amygdala and hippocampal regions. We also describe genetic variations that moderate the impact of adverse environmental conditions on child neurodevelopment, such as polymorphisms in brain-derived neurotrophic factor and catechol-O-methyltransferase genes, as well as genetic pathways related to glutamate and monoaminergic signaling. Lastly, we have depicted positive early life experiences that could benefit childhood neurodevelopment and reverse some detrimental effects of adversity in the offspring.

Experiences during early development have powerful effects on brain function, leading to individual differences that could contribute to behavioral dysfunction and risk for chronic diseases over the lifetime. Gene x environment interactions account for a large portion of the variance in these individual differences, mainly through alterations of the DNA structure and chromatin function that affect gene expression. These physical modifications to the DNA molecule and chromatin function – or epigenetic changes – regulate the operation of the genome and can have an impact on the development of brain structure and function, especially during early life.[1] DNA methylation marks in children have been proposed as a mechanism for the enduring effects of the early environment, and contain a 'molecular memory' of the individual early environment. In neonates, regions of the methylome that are highly variable across individuals, termed variably methylated regions, are explained by the genotype alone in 25 percent of cases. The best explanation for 75 percent of variably methylated regions is the interaction of genotype with different in utero environments, including maternal smoking, maternal depression, maternal body mass index, infant birthweight, gestational age, and birth order.[2]

In this review, we describe evidence linking environmental variation during early life (from the fetal period to childhood) to long-term changes in brain function and behavior, as well as putative mechanisms underlying these processes (Fig. 1). Early positive conditions that can possibly revert detrimental outcomes are also described. We summarize important new findings from the last 10 years including the terms 'early adversity', 'early environment', 'brain function', and 'brain structure' and the well-described early adversities subtopics, such as 'birthweight' and 'prenatal anxiety', according to previous reviews in this topic.[3]

DEFINING EARLY LIFE ADVERSITIES – EXAMPLES OF INFLUENCES ON BRAIN DEVELOPMENT AND FUNCTION

Poor fetal growth has been largely used as a marker of an adverse intrauterine environment and extensively studied when researching prenatal influences on brain development and behavior. Fetal growth is influenced by maternal, placental, and genetic factors. The mother's age, socio-economic status, maternal health, substance use, and nutrition are considered maternal influences. Placental dysfunction leading to a poor supply of nutrients and oxygen to the fetus and fetal malformations are also important factors that affect fetal growth. Poor intrauterine growth is associated with developmental delays and increased risk for many different mental health problems. A meta-analysis demonstrated that extremely low birthweight was associated with

inattention, hyperactivity, and internalizing problems in childhood and adolescence, that converge to higher rates of social problems, depression, and anxiety in adulthood.[4] The moderator role of sex is seen in many of these effects, although the literature on sex differences as a function of prenatal adversity is not consistent. As an example, being born small for gestational age, with a small head circumference, or with a low ponderal index were all associated with attention difficulties in females, but not in males,[5] however mixed findings are reported.[6] A meta-analysis including studies reporting brain volumes using magnetic resonance imaging (MRI) demonstrated that low birthweight/prematurity is related with an overall reduction in brain volume in childhood and adolescence, that was implicated in decreased general cognitive functioning.[7]

Although not unlikely, there is generally no evidence that long-term changes are persistent. There is a well-established correlation between early life adversity and increased risk for psychopathology, and this increased risk is persistent throughout life. It is possible that the neuroanatomical differences seen in early life on neuroimaging are related to differential levels of function at the cellular or molecular level that are, indeed, persistent. These, rather than large anatomical changes, are likely responsible for psychopathology.

Many prenatal factors that affect fetal development do not necessarily cause growth restriction. For example, substance abuse has a well-known impact on neurodevelopment.[8] Nicotine is the most prevalent substance used during pregnancy, and several studies have showed that toxins present in tobacco can cross the placental barrier. Intrauterine exposure to tobacco dampens the relative gene expression of selected fetal brain regulatory genes responsible for brain growth, myelination, and neuronal migration,[9]

What this paper adds

- Prenatal, peripartum, and postnatal adversities influence child behavior and neurodevelopment.
- Exposure to environmental enrichment and positive influences may revert these effects.
- Putative mechanisms involve alterations in neurotrophic factors and neurotransmitter systems.
- New tools/big data improved the understanding on how early adversity alters neurodevelopment.
- This permits better translation/application of the findings from animal models to humans.

altering brain structure and function. The long lasting behavioral impairments associated with prenatal smoking include reductions in cognitive and motor functioning, impaired mental development and risk for bipolar disorder, depression, and addiction in the offspring.[10] Attention-deficit/hyperactivity disorder is also more prevalent in children exposed prenatally to tobacco.[11]

Beyond physical states, the effects of psychological stress and altered maternal mental health during pregnancy on the offspring are extensively documented in the literature. Exposure to death of a family member, catastrophic disasters (earthquakes or terrorist attacks), and chronic stressors (homelessness, poverty/crime, unemployment, crowding, racism or discrimination) during gestation were observed to affect pregnancy duration and birthweight.[12] Prenatal maternal anxiety is associated with a downregulation of placental enzyme 11β-hydroxysteroid dehydrogenase type 2, which catalyzes rapid inactivation of glucocorticoids in the placenta, therefore increasing fetal exposure to maternal cortisol.[13] Prenatal exposure to maternal stress was observed to affect offspring neurodevelopment, neurocognitive function, cerebral processing, functional and structural brain connectivity involving amygdala and prefrontal

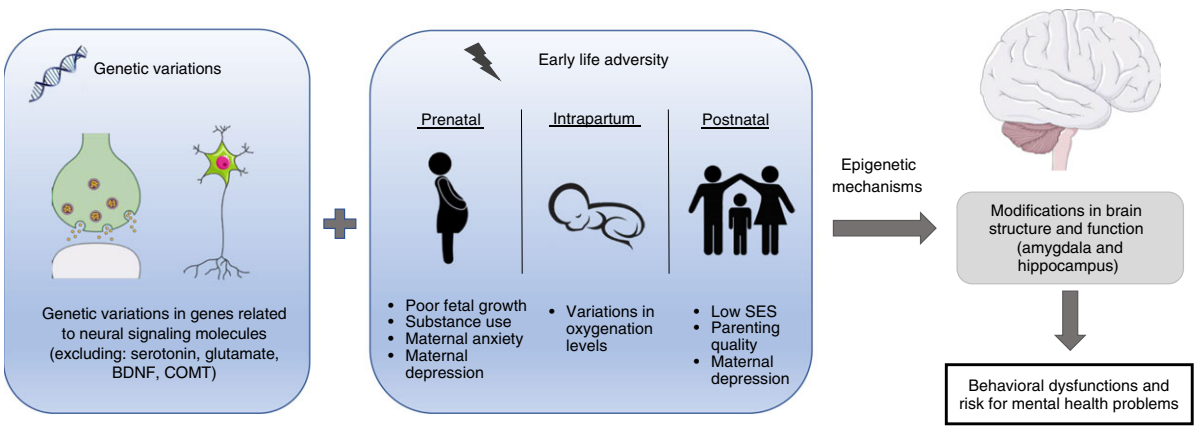

Figure 1: Genetic variations in genes related to neural signaling molecules (e.g. serotonin, glutamate, BDNF, and COMT) interacts with the exposure to early life adversity events and affects child neurodevelopment, mainly through epigenetic mechanisms. Modifications are observed in brain structure and function, especially in the hippocampus and areas related to emotional regulation such as the amygdala. These brain changes could affect socio-emotional outcomes, behavioral dysfunctions, and the risk for mental health problems. BDNF, brain-derived neurotrophic factor; COMT, catechol-O-methyltransferase; SES, socio-economic status. [Colour figure can be viewed at wileyonlinelibrary.com].

cortex, and hypothalamic-pituitary-adrenal axis. All these changes increase the risk for behavioral and mental health problems later in life. A recent systematic review of neuroimaging studies in this topic identified 10 research articles, three of them studies from our group. The findings suggest reduced volume and/or thickness in frontal, temporal, and limbic areas (by MRI) and increased frontal activation (by electroencephalography studies) in children whose mothers had higher prenatal anxiety.[14] Moreover, greater functional connectivity between amygdala and the left temporal cortex and insula was reported, suggesting an acceleration of the connectivity pattern similar to that observed in adolescents and adults with major depressive disorder.[15]

More acute, peripartum adverse events are also known to markedly affect neurodevelopmental outcomes. From these, perinatal hypoxia-ischemia/hypoxic-ischemic encephalopathy stands out, as it is the most common cause of morbidity and mortality in human neonates. The diagnostic criteria for neonatal hypoxia-ischemia are based on a set of markers: 5-minute Apgar score of less than 5, need for delivery room intubation or cardiopulmonary resuscitation, umbilical cord arterial pH less than 7, and abnormal neurological signs, such as hypotonic muscles or lack of sucking reflex. Among the surviving individuals, 5 percent to 10 percent demonstrate persistent motor deficits and 20 percent to 50 percent exhibit sensory or cognitive abnormalities that persist to adolescence.[16] However, subtle cognitive impairments, including hyperactivity, inattention, and poorer cognitive ability, can be found in the absence of neuromotor impairment. A meta-analysis including 45 821 individuals with attention-deficit/hyperactivity disorder and 9 207 363 controls suggests that conditions such as preeclampsia, Apgar score lower than 7 at 5 minutes, breech/transverse presentations, and prolapsed/nuchal cord – all of which involve some sort of poor oxygenation during delivery – are significantly associated with attention-deficit/hyperactivity disorder.[17] The dopaminergic system seems to be one of the brain systems most affected by perinatal hypoxia-ischemia.[18] Adopting the Rice-Vannucci rat model of perinatal hypoxia-ischemia, our group has shown attention deficits and impulsivity associated with brain atrophy in different regions in adult rats.[19] More recently, attentional inflexibility was also observed after neonatal hypoxia-ischemia and related to atrophy severity and dopaminergic disturbances in the prefrontal cortex,[20] reflecting the clinical associations between perinatal variations in oxygenation and the development of attention-deficit/hyperactivity disorder later in life.

The quality of the postnatal period is also well known to have a profound impact on a wide range of neurodevelopmental outcomes. Exposure to traumatic events such as child maltreatment, bullying, terrorism, exposure to war, and violence are associated with higher rates of posttraumatic stress symptoms, depression, anxiety, and substance use disorders. Exposure to childhood trauma activates the stress response systems and dysregulates serotonin (5-hydroxytryptamine; 5-HT) transmission that can adversely impact brain development. Smaller cerebral, cerebellar, prefrontal cortex, and corpus callosum volumes were reported in maltreated young people as well as reduced hippocampal activity.[21] Similarly, orphanage rearing is associated with diminished autonomic and hypothalamic-pituitary-adrenal axis responses to psychosocial stress, enlarged amygdala volumes, and accelerated amygdala-medial prefrontal cortex connectivity mediated by cortisol.[22]

In rodent studies, naturally occurring variations on maternal care during the first days of life were associated with long-term alterations in stress responsiveness and hippocampal morphology and function.[1] These effects are mediated by epigenetic changes at the hippocampal glucocorticoids receptor gene promoter of the offspring, through acetylation of histones H3K9 and DNA methylation preventing the transcription factor NGFI-A binding to the exon 1_7.[23] In humans, childhood maltreatment was similarly associated with decreased hippocampal glucocorticoids receptor expression, with increased DNA methylation and decreased NGFI-A binding to the exon 1_F (homolog of the rat exon 1_7).[24]

The quality of the early environment can be considerably affected by disturbances of maternal mental health, which reduces maternal sensitivity and engagement. Indeed, children of mothers presenting depressive symptomatology since birth demonstrate larger left and right amygdala volumes and increased levels of glucocorticoids, with a significant positive correlation between maternal depressive scores and children's amygdala volumes.[25] Increased postnatal maternal depressive symptoms are associated with higher right amygdala fractional anisotropy, a measure of microstructural integrity, especially in females.[26] Maternal sensitivity levels correlated with connectivity between the hippocampus and areas related to emotional regulation and socio-emotional functioning.[27] Early childhood maternal support is related to increased hippocampal volume growth across school age and early adolescence, and this growth trajectory is associated with later emotional conditions.[28]

EXPLORING THE MECHANISMS LINKING EARLY LIFE ADVERSITY TO CHILDHOOD NEURODEVELOPMENT

Studies using single candidate genes have proposed that specific neural signaling molecules are potentially involved in the effects of early life adversity on child brain structure and function (see Fig. 1). For example, the brain-derived neurotrophic factor (BDNF), encoded by the BDNF gene, is a neurotrophin closely linked to synaptic plasticity throughout the central nervous system. Reports have demonstrated that different BDNF genotypes moderate the impact of environmental conditions on child neurodevelopment. Variations on the Val66Met (rs6265) single nucleotide polymorphism moderate attention problems in adopted young people depending on the duration of institutional care (orphanage rearing),[29] and interacts with the history of child maltreatment to affect the risk for depressive disorders and hippocampal size in adults.[30] The Met allele presence interacted with harsh intrusive

parenting behavior to predict oppositional defiant disorder and callous-unemotional behaviors in 3-year-old children.[31] Moreover, it was shown that the effect of antenatal maternal anxiety on neonates' DNA methylation varies as a function of BDNF genotype, with a greater influence of antenatal maternal anxiety on the neonatal epigenome among Met/Met compared to Val/Val carriers.[32] The volume of the right amygdala shows association with higher numbers of methylated cytosine-phosphate-guanine sites in the Met/Met group, and the left hippocampal volume shows higher numbers of methylated cytosine-phosphate-guanine sites in the Val/Val group,[32] suggesting that the effects of gene by environment interactions are brain region- and thus function-specific.

Another example is the genetic variation on the catechol-O-methyltransferase gene, which regulates catecholamine signaling in the prefrontal cortex. Genetic variants in the catechol-O-methyltransferase gene determine the intensity and neuroanatomical localization of the effects of antenatal maternal anxiety on neonatal cortical morphology.[33] Childhood adversity was associated with higher reward sensitivity in adults genotyped as Met/Met for the catechol-O-methyltransferase Val(158)Met polymorphism.[34] Met homozygotes for the Val(158)Met polymorphism who had greater levels of early life adversity demonstrated progressively smaller cortisol responses in stressful events in adulthood.[35] As another example, a polymorphism in the promoter region of the serotonin-transporter gene, referred to as 5-HTTLPR, is classically linked to psychopathology. The 5-HTTLPR genotype interacted with maternal childhood adversity to predict negative emotionality/behavioral dysregulation for the child.[36]

However, single candidate gene approaches have their limitations, especially considering that the neurobiological alterations that enhance vulnerability for psychopathology derive from small effects in multiple genomic variants that converge to influence common biological systems. Methods of genomic risk profiling, or polygenic risk scores, summarize the genetic risk of an individual for a given condition, by summing the influence of several alleles.[37] Indeed, the relationship between antenatal maternal depression and amygdala development was recently shown to be different, according to the infant polygenic risk scores for major depression disorder.[38] The authors explored the candidate biological processes associated with genes involved in the polygenic risk scores for major depression disorder that might mediate the influence of antenatal maternal depressive symptoms on fetal brain development. 'Glutamate receptor activity' was associated with the genetic pathway of the metabotropic glutamate receptor group III, as expected, and the 'snap receptor complex' function, linked to signaling mediated by serotonin (5-HT_2 subtype) and oxytocin receptors. These findings are interesting not only because of the well-known implication of glutamatergic synaptic transmission in the etiology of depression,[39] but also because altered glutamatergic signaling is a common factor linking other types of early life adversity discussed here, such as perinatal hypoxia,[40] maternal substance use,[41] and prenatal stress[42] in animal models. It is particularly exciting that recent findings have shown that pharmacological activation of metabotropic glutamate receptors attenuates the oxidative stress induced by perinatal hypoxia,[40] inducing neuroprotective results that are already described in major depression,[43] with potential for reversibility of the effects discussed here.

The snap receptor complex is an essential component of neural connectivity and neurotransmitter release and was the primary biological function associated with 5-HT2-type receptor- and oxytocin receptor-mediated signaling pathways, identified as candidate moderators of the effect of antenatal maternal depressive symptoms on cortical thickness.[38] Serotonin is known to affect the development of brain pathways and networks[44] and, as mentioned previously, childhood trauma dysregulates serotonin neurotransmission.[21] We recently created a hippocampal-specific, expression-based polygenic score reflecting variations in the function of the serotonin transporter gene network, using a novel process for genome profiling.[45] We showed that the serotonin transporter gene expression-based polygenic score significantly interacts with a cumulative prenatal adversity score on measures of childhood cognitive-emotional problems, suggesting again that serotonin function is involved in the long-term effects of prenatal adversity on child socio-emotional outcomes.[45]

POSSIBLE FACTORS FOR REVERSIBILITY

In contrast to the above, positive early life experiences also benefit childhood neurodevelopment. For instance, clinical studies suggest that physical activity during gestation is associated with a reduced risk of preterm birth and birth-related complications[46] as well as improving offspring neurodevelopment at 12 months (measured by Battelle's Development Inventory score).[47] In preclinical studies, maternal exercise during pregnancy was associated with increased BDNF levels and absolute numbers of neuronal and non-neuronal cells in the hippocampus of the adult rat offspring, that was associated with better cognitive performance (habituation behavior and spatial learning).[48] Pregnancy swimming also increased hippocampal BDNF levels in the rat offspring, which contributed for smaller lesions, preservation of white matter tracts, improved delay in reflex maturation as well as spatial memory deficits in rats that suffered neonatal hypoxia-ischemia.[49]

Experimental environmental enrichment has a series of beneficial effects associated with neuroplasticity mechanisms, increasing hippocampal volume, and enhancing dorsal dentate gyrus-specific differences in gene expression.[50] Environmental enrichment after prenatal stress decreases depressive-like behaviors and fear, and improves cognitive deficits, as well as increases spine density, granular cells, and BDNF in the hippocampus of the offspring.[51] Environmental enrichment also improves learning and memory deficits associated with neonatal hypoxia-ischemia, through an increased dendritic spine density in the hippocampus of adult rats.[52]

Emerging evidence suggests that long-chain polyunsaturated fatty acids intake may attenuate maternal psychosocial stress during pregnancy.[53] Prenatal docosahexaenoic acid supplementation, an unsaturated omega–3 fatty acid, is associated with better birth outcomes and modulation of the cortisol response to a stressor in infants living in a low socio-economic status environment.[54] In rodents, gestational docosahexaenoic acid supplementation prevents prenatal stress-induced impairment of learning and memory and normalizes the biomarkers of oxidative damage and apoptosis in the offspring's hippocampus.[55] The supplementation with n-3 polyunsaturated fats directly to the offspring exposed to prenatal adversity also seems to be associated with less brain oxidative damage in rodents.[56] In humans, spontaneous intake of n-3 polyunsaturated fats in childhood is associated with a more adaptive behavioral profile after the exposure to prenatal adversity. For instance, we have shown that children exposed to poor fetal growth have better inhibitory control when consuming n-3 polyunsaturated fats,[57] reflected in patterns of prefrontal cortex activation in functional MRI in adolescents.[58]

Infants born to mothers who report high prenatal stress and low partner support exhibit higher cortisol reactivity relative to those whose mothers report high support, suggesting that partner support may buffer the impact of prenatal stress on infant cortisol reactivity.[59] Moreover, a positive antenatal mental health is associated with better offspring's cognitive, language, and parentally rated competences at 12 months, 18 months, and 24 months of age.[60] All these positive environmental experiences mentioned in this section could counterbalance the detrimental effects of early life adversities, making individuals resilient to brain alterations and development of later psychopathology. This topic is now extensively discussed in the field of mental health.[61,62]

CONCLUSION

In conclusion, exposure to early life adversity is associated with modifications in volume, microstructure, and connectivity in specific brain regions including the amygdala and the hippocampus. These differences affect socio-emotional outcomes in childhood and the risk for psychopathology later in life. It is important to consider that although there is a large bulk of evidence suggesting relationships between variations in the early life environment and long-term effects on brain and development, some negative findings were also reported.[63,64] Inconsistent effects in structural MRI studies may partially be due to the lack of control for later comorbid psychiatric disorders. It is often difficult to determine whether the brain volumetric changes are due to the exposure to early adversity, to the associated psychiatric condition, or an interaction between both factors.[65] For example, smaller hippocampal volumes were observed in individuals exposed to early maltreatment only in cases that were followed by post-traumatic stress disorder.[64,66] Thus, these factors should be considered with caution when comparing findings from different studies.

In the last few years, new tools and big data have improved our ability to better understand the relationships between early adversity and alterations in brain development and function, permitting better translation of the findings from animal models to humans, especially with the use of new genetic approaches like the ones described here. Epigenetic modifications seem to be an important mechanism that explains these associations; however, the cross-sectional nature of most epigenetic studies and the tissue specificity of the epigenetic changes are still challenges faced by the scientists devoted to investigating epigenetic markers as moderators of the effects of early life adversity on brain development and behavior.[67,68] Awareness about the mechanisms and potential factors that modify these associations opens a venue for the development of preventive and therapeutic measures.

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8. DISCUSSÃO

Nesta tese buscamos investigar o impacto da exposição a condições hipóxico-isquêmicas perinatais (HICs) no desenvolvimento de características relacionadas ao Transtorno de deficit de atenção/hiperatividade (TDAH), tanto em roedores quanto em crianças. Para isso, utilizamos um modelo animal proposto por Rice-Vannucci que mimetiza o dano causado pela hipóxia-isquemia em crianças no período perinatal, buscando validar esse modelo como uma possível opção para o estudo experimental do TDAH. Um estudo clínico também foi realizado, com o intuito de avaliar o efeito da exposição às HICs em crianças de acordo com um perfil genético que reflete a função do maquinário associado à receptação de dopamina no córtex pré-frontal. Por fim, revisamos os estudos da literatura que relatam o impacto de diferentes influências ambientais precoces no encéfalo em desenvolvimento (incluindo a relação entre HI e características relacionadas ao TDAH) e como as novas ferramentas de análises que melhoraram a compreensão sobre como essas adversidades alteram o neurodesenvolvimento. Nos próximos parágrafos, será apresentada uma discussão em relação aos principais resultados provenientes desses estudos, com um enfoque nas implicações e avanços decorrentes desses achados.

Validade aparente do modelo de HI para o estudo do TDAH

A hipóxia-isquemia neonatal induz deficit na função executiva em animais jovens

Primeiramente, nosso objetivo foi investigar se a HI neonatal gera comportamentos similares aos observados no TDAH em ratos adolescentes, entre os 30 e 45 dias de vida, uma vez que todos os achados prévios demonstrando essa relação foram observados em animais adultos (Ikeda *et al.*, 2001; Ikeda *et al.*, 2004; Mishima, Kenichi *et al.*, 2004; Smith, Alexander*, et al.*, 2014; Smith, Hill*, et al.*, 2014; Miguel *et al.*, 2015; Miguel *et al.*, 2018). Nossos resultados demonstraram os seguintes deficit cognitivos em ratos jovens que foram submetidos à HI neonatal: inflexibilidade cognitiva, deficit na memória de reconhecimento, na aprendizagem espacial, na memória de trabalho e na memória de longa duração. No entanto, não foi demonstrada hiperatividade e nem deficit de aprendizagem para executar uma tarefa que envolvia recompensa

alimentar (Capítulos 1 e 2). Estes resultados demonstrando prejuízos comportamentais já aparentes em animais jovens fortalecem a validade aparente do modelo de HI de Rice-Vannucci para o estudo experimental do TDAH, uma vez que a incidência do transtorno é maior em crianças e adolescentes.

Apesar da gama de achados em relação aos processos executivos, não foram observadas alterações significativas na locomoção desses animais. Embora pareça um consenso que a atividade locomotora não seja afetada em animais HI jovens (Carletti *et al.*, 2012; Schuch *et al.*, 2016; Kim *et al.*, 2017), uma variedade de trabalhos demonstram hiperatividade em animais HI na fase adulta (Rojas *et al.*, 2013; Sanches *et al.*, 2015; Markostamou *et al.*, 2016; Deniz *et al.*, 2018). Essa divergência nos resultados pode ser explicada pelo perfil progressivo da lesão que ocorre nesse modelo (Mishima, Kenichi *et al.*, 2004; Diaz *et al.*, 2016); desta forma as regiões envolvidas com o controle motor, como o estriado, podem estar mais afetadas em uma fase mais tardia do desenvolvimento. Um estudo de meta-análise integrando todos os trabalhos que avaliam a locomoção após o modelo de HI seria uma proposta interessante para ser executada, com a intenção de inferirmos a real contribuição do modelo para este aspecto do comportamento.

Uma questão importante é que o modelo de exposição repetida à hipóxia no período neonatal em ratos apresenta achados opostos ao modelo de HI de Rice-Vannucci: a hiperatividade é bem reportada, mas não o deficit de atenção (Oorschot *et al.*, 2007; Oorschot *et al.*, 2013). O modelo de hipóxia repetida é realizado em um período mais precoce, do dia 1 ao 3 pós-natal, o que corresponde ao período entre 24 e 26 semanas gestacionais em humanos, sendo por isso considerado um modelo que simula prematuridade extrema. Neste modelo, a lesão afeta principalmente a substância branca, ao contrário do modelo de HI que ocorre no 7º dia e gera uma extensa atrofia cortical (Chang *et al.*, 2013; Miguel *et al.*, 2015). Dessa forma, observamos que estes modelos causam lesões cerebrais com perfis diferentes e por esse motivo diferentes desfechos comportamentais são observados.

Um achado intrigante do nosso trabalho foi que, apesar de todos os deficit cognitivos encontrados, os animais HI não apresentarem deficit de aprendizagem quando a tarefa envolvia alimento palatável como reforço positivo (tarefa *attentional set-*

shifting, Capítulo 1 da tese). Este resultado parece indicar que esses animais têm uma resposta diferencial à recompensa, os levando a ter maior motivação e atenção para executar a tarefa. Como já foi demonstrado que pacientes com TDAH apresentam respostas diferenciais frente a recompensas (Scheres *et al.*, 2007) e um risco maior para desenvolverem transtornos alimentares e de uso de substâncias quando adultos (Lee *et al.*, 2011; Pedersen *et al.*, 2016; Brunault *et al.*, 2019), buscamos avaliar especificamente esses comportamentos na fase adulta dos animais HI.

A hipóxia-isquemia neonatal gera comportamentos alimentares desregulados e aumento no consumo de álcool em ratos adultos

No Capítulo 3 da tese investigamos o perfil de comportamento alimentar frente a uma ração padrão ou palatável, assim como a preferência pelo consumo de álcool em relação à água. Os resultados demonstraram que animais HI apresentam um comportamento alimentar desregulado, aumentando o consumo de ração padrão, logo após ingerirem uma pequena porção de alimento palatável. Ainda, esses animais apresentaram um maior consumo voluntário de álcool quando comparado aos animais controles. Em conjunto, esses achados nos permitem inferir que a HI neonatal pode alterar a via mesolímbica dopaminérgica, associada com a regulação desses comportamentos, levando os animais a terem uma resposta diferencial frente a um alimento palatável e também ingerindo mais álcool. De fato, outros autores já demonstraram alterações em parâmetros da sinalização dopaminérgica no estriado de ratos HI (Filloux et al., 1996; Park et al., 2013), uma região que inclui o NAcc. Para comprovar a alteração nessa via, estudos futuros empregando técnicas como a cronoamperometria poderiam nos informar sobre o perfil de liberação de DA no NAcc após o consumo do alimento palatável ou do álcool em ratos HI ou controles, de forma semelhante ao que foi realizado no trabalho de Laureano e colaboradores (Laureano *et al.*, 2019).

É importante considerar que o perfil de comportamento alimentar desregulado dos ratos HI foi observado em relação à alimentação padrão - mas após os animais consumirem uma pequena porção da ração palatável. Quando o alimento palatável foi

disposto em abundância para os animais, não foram observadas diferenças significativas entre ratos HI e controles, provavelmente em decorrência da alta palatabilidade do alimento e consequente aumento do consumo em todos os grupos. No entanto, o nosso protocolo que consistia da exposição à ração palatável por 2 dias, indicou uma tendência para o grupo CT ter menor preferência por essa ração no primeiro dia, sugerindo que o grupo HI já apresenta uma maior preferência pela ração palatável desde as primeiras exposições.

Um segundo ponto a ser considerado neste estudo é que tomamos o cuidado de não usarmos sacarose ou adoçante junto à solução de álcool, para conseguirmos distinguir entre o efeito do estímulo prazeroso e o viciante/aditivo, uma vez que já foi demonstrado que esses compostos produzem padrões de ativação cerebral similares aos de drogas de abuso (Spangler *et al.*, 2004; Lenoir *et al.*, 2007). A adição de sacarose/adoçante junto à solução alcoólica é uma prática recorrente nos estudos com roedores, uma vez que soluções com álcool diluídas apenas em água não são atrativas aos ratos (Samson, 1986; Samson *et al.*, 1999). No entanto, o que se sabe na clínica é que, na maioria das vezes, os indivíduos adictos já não sentem mais prazer em ingerir aquela substância, mas o fazem pelas respostas recompensadoras mais tardias da mesma (Lamb *et al.*, 1991; Berridge, 2007). Por esses motivos, nós optamos por utilizar um protocolo bem estabelecido na literatura (protocolo IA2BC) que induz os animais a ingerirem mais álcool pela exposição intermitente às soluções alcoólicas – não necessitando da mistura com sacarose/adoçantes (Simms *et al.*, 2008; Carnicella *et al.*, 2014). Desta maneira, nosso desenho experimental permitiu demonstrar que os ratos HI apresentam uma resposta alterada tanto em relação a um alimento palatável assim como nos efeitos produzidos pelo álcool. Com isso, podemos sugerir que o modelo de HI neonatal de Rice-Vannucci altera a sinalização dopaminérgica de forma persistente, contribuindo para comportamentos desajustados frente a alimentos palatáveis ou drogas de abuso na fase adulta. Como estes comportamentos são caraterísticas frequentes em pacientes com TDAH na fase adulta (Lee *et al.*, 2011; Cortese e Tessari, 2017; Brunault *et al.*, 2019), nossos resultados fortalecem a relação entre HI perinatal e o desenvolvimento de características relacionadas ao TDAH, colaborando para a validade aparente do modelo de HI para o estudo do TDAH em roedores.

No geral, os resultados em conjunto desta tese que apoiam a validade aparente do modelo de HI de Rice-Vannucci para o estudo do TDAH foram os seguintes: inflexibilidade cognitiva, deficit na memória de reconhecimento, na aprendizagem espacial, na memória de trabalho e na memória de longa duração – todos em ratos HI jovens, assim como um comportamento alimentar desregulado e um aumento no consumo do álcool em ratos HI adultos.

Validade de construto do modelo de HI para o estudo do TDAH

A hipóxia-isquemia neonatal altera a sinalização dopaminérgica no CPF de ratos jovens

Um segundo objetivo do nosso estudo foi analisar a validade de construto do modelo de HI para o estudo do TDAH, ou seja, investigar se os mecanismos neurobiológicos envolvidos no TDAH também estão presentes no modelo de HI neonatal. Nossos resultados demonstraram que no CPF de ratos jovens que sofreram HI ocorreu uma diminuição dos receptores D2 e do DAT, assim como um aumento no imunoconteúdo da enzima tirosina hidroxilase na sua forma fosforilada (pTH), a qual indica uma maior síntese de DA. Esse aumento na síntese de DA pode ser um mecanismo compensatório pela diminuição da recaptação de DA pelo DAT ou pela menor efetividade na transmissão sináptica dopaminérgica em decorrência da menor expressão de receptores D2. No entanto, o mecanismo inverso também pode estar ocorrendo: uma *downregulation* do D2 e do DAT pela alta síntese de DA (Figura 6). Análises futuras investigando outros marcadores do metabolismo dopaminérgico, como as enzimas degradadoras COMT e monoamina oxidase (MAO), o transportador NET e o transportador vesicular de monoaminas (VMAT) poderiam agregar importantes informações para interpretarmos os resultados deste estudo em um contexto mais amplo. Apesar de não sabermos qual etapa ocorre primeiro – diminuição do D2 e DAT ou aumento de pTH – sabemos que essas alterações implicam em transmissão dopaminérgica ineficiente no CPF de animais HI. Essas alterações podem sugerir importantes mecanismos subjacentes aos prejuízos comportamentais observados em animais HI (Capítulos 1, 2 e 3). Em trabalhos anteriores, nosso grupo já havia

demonstrado diminuição da expressão de receptores D2 no CPF de ratos HI na fase adulta (Miguel *et al.*, 2018), o que indica que as alterações reportadas na fase jovem podem ser persistentes. Ainda, o tamanho da atrofia no CPF de ratos HI adultos foi positivamente correlacionado com o número de tentativas e de erros na tarefa *attentional set shifting*; ou seja, quanto maior a atrofia na CPF, pior o desempenho na tarefa. Desta forma, os resultados encontrados no presente estudo confirmam trabalhos anteriores do nosso grupo que já demonstraram o impacto da HI neonatal no desenvolvimento do CPF. Já que o envolvimento do CPF e alterações na sinalização de DA nessa estrutura têm sido apontadas como um mecanismo central na fisiopatologia do TDAH (Levy e Swanson, 2001), nossos achados agregam embasamento para a validação de construto do modelo de Rice-Vannuci para o estudo do TDAH em pesquisa pré-clínica.

A neurotrofina BDNF também parece ter um papel na fisiopatologia do TDAH, uma vez que o BDNF é essencial nos processos de sobrevivência e crescimento neural – necessários para um desenvolvimento encefálico normal (Tsai, 2007; 2017). No entanto, nossos resultados demonstraram níveis inalterados de BDNF tanto no CPF como no hipocampo de animais HI jovens (Capítulo 2), o que pode ser resultado das variações cíclicas que ocorrem nos níveis de BDNF no modelo de HI (Pereira *et al.*, 2009; Deniz *et al.*, 2018).

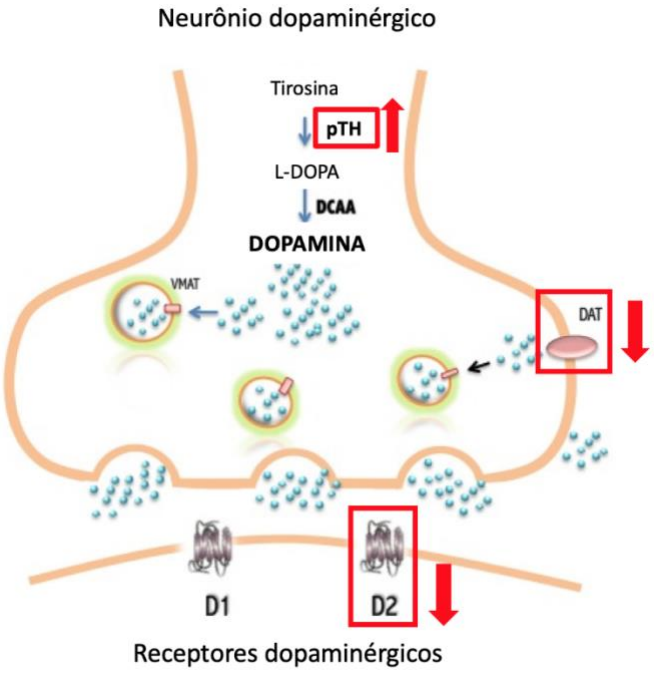

Figura 6: Alterações encontradas no CPF de ratos adolescentes submetidos à hipóxia-isquemia neonatal. Uma diminuição nos receptores dopaminérgicos do tipo D2 e no transportador de dopamina (DAT) foram observadas, assim como um aumento na enzima tirosina hidroxilase na sua forma fosforilada (pTH).

Validade preditiva do modelo de HI para o estudo do TDAH

Um terceiro objetivo do nosso trabalho na busca dos critérios de validação do modelo de HI em ratos incluiu a análise do efeito do tratamento com metilfenidato (MFD) nas características comportamentais relacionadas ao TDAH (i.e., validade preditiva). Os resultados demonstraram que ratos HI jovens sob o efeito do MFD (administrado em uma dose de 2.5mg/kg, i.p., 30 min antes das análises) apresentaram melhoras nos deficit de flexibilidade cognitiva (tarefa *attentional set-shifting*) e de memória de longa duração (teste labirinto aquático de Morris). No entanto, o MFD não teve nenhum efeito sobre os deficit na memória de reconhecimento (tarefa de reconhecimento de objetos) e nem nos demais parâmetros avaliados no labirinto aquático de Morris: aprendizagem espacial, memória de trabalho e aprendizagem reversa. Ainda, ratos HI que receberam MFD demonstraram uma maior atividade exploratória/locomotora no teste do campo aberto (Resultados apresentados nos Capítulos 1 e 2). A seguir, interpretaremos como o MFD pode estar atuando em cada um dos aspectos observados nos testes comportamentais.

O tratamento com Metilfenidato melhora a flexibilidade cognitiva e a memória de longa duração em ratos HI jovens

Flexibilidade cognitiva é um processo das funções executivas altamente dependente da sinalização dopaminérgica no CPF (Arnsten, 2011; Cools e D'esposito, 2011; Floresco, 2013). Nossos resultados demonstraram que a HI neonatal prejudicou a flexibilidade dos ratos adolescentes e que o tratamento com MFD reverteu esse deficit. Uma vez que a sinalização dopaminérgica no CPF de ratos HI se apresentou alterada, acreditamos que o aumento na transmissão de DA nessa estrutura pelo MFD tenha contribuído para a melhora comportamental observada nos ratos HI tratados. Assim

como nos nossos achados, estudos utilizando a linhagem SHR também já demonstraram melhoras na flexibilidade cognitiva após o tratamento com MFD (Kantak *et al.*, 2008; Cao *et al.*, 2012).

O segundo comportamento que foi melhorado pelo tratamento com MFD foi o deficit de memória de longa duração no labirinto aquático de Morris, embora todos os outros parâmetros avaliados nesta tarefa não tenham sido favorecidos pela droga. O labirinto aquático é uma tarefa que exige localização espacial, sendo altamente dependente do hipocampo (Vorhees e Williams, 2006). Considerando que o hipocampo é uma das estruturas mais afetadas pela HI (Miguel *et al.*, 2015), acreditamos que o MFD não tenha conseguido atuar de forma efetiva nessa estrutura e, portanto, não melhorando a maioria dos deficit cognitivos observados nos ratos HI. O único parâmetro que apresentou melhora pós o tratamento com MFD nessa tarefa foi a memória de longa duração, e duas hipóteses foram sugeridas para esse resultado:

1. O aumento da transmissão de DA no hipocampo pelo MFD foi capaz de contribuir para a consolidação da memória, uma vez que este é um processo onde a DA tem um papel essencial (mas não na aquisição da memória em si) (Bethus *et al.*, 2010);
2. Embora o hipocampo seja crucial para a formação da memória espacial, ele não armazena memórias duradouras, tendo o CPF um papel importante nesse processo (Maviel *et al.*, 2004; Leon *et al.*, 2010).

Dessa maneira, acreditamos que o aumento de DA induzido pelo MFD tanto no CPF como no hipocampo de ratos HI tenha sido eficiente especificamente para este aspecto da memória. Colaborando com os dados do nosso modelo de HI, estudos utilizando a linhagem SHR também já demonstraram que o MFD não teve efeito sobre a aquisição da memória no labirinto aquático, mas sim nos processos de retenção da mesma (Tian *et al.*, 2009; Guo *et al.*, 2012).

Considerando que tanto os processos de inflexibilidade cognitiva como deficit de memória de longa duração são alterados em pacientes com TDAH e mostram benefícios após o tratamento com MFD (Pietrzak *et al.*, 2006; Skodzik *et al.*, 2016), nossos resultados mostrando deficit nesses parâmetros em ratos HI e melhora após tratamento com MFD sustentam a validação preditiva do modelo experimental de HI para o estudo do TDAH. No entanto, precisamos considerar que a grande atrofia no hipocampo dos

animais HI pode ser vista como um ponto negativo desse modelo, uma vez que esta não é uma característica de pacientes com TDAH e que o MFD parece não atuar de forma efetiva em tarefas altamente dependentes dessa estrutura. Ainda assim, os benefícios do MFD foram observados na persistência da memória aprendida. Além dos pontos abordados, ressaltamos o fato de que nossas interpretações foram baseadas no estudo que utilizou apenas uma dose do fármaco (2.5mg/kg) e trabalhos futuros focados em avaliações dose-resposta seriam uma importante abordagem no modelo de HI neonatal.

O tratamento com Metilfenidato aumenta os níveis de pTH no CPF e de BDNF no hipocampo de ratos HI: contribuição para a melhora comportamental

Com a intenção de entender os mecanismos inerentes à ação do MFD no comportamento de ratos expostos à HI, investigamos os mesmos parâmetros analisados para a validade de construto após um tratamento de 15 dias com MFD. Demonstramos que o MFD não alterou a expressão dos receptores dopaminérgicos e nem do DAT no CPF de ratos HI. No entanto, o efeito compensatório do aumento da pTH observado nos ratos HI foi ainda maior naqueles que receberam tratamento com MFD, o que sugere uma maior atividade dessa enzima e possível aumento nos níveis de DA (Capítulo 1). Desta maneira, parece que o MFD não só bloqueou os recaptadores das monoaminas no CPF dos ratos HI, mas também colaborou para o processo compensatório de aumento de síntese de DA nessa estrutura, o que pode ter implicações diretas tanto na melhora da flexibilidade cognitiva como na retenção da memória observados no grupo HI tratado com MFD.

Além disso, observamos um aumento nos níveis do BDNF no hipocampo de ratos HI que receberam tratamento com MFD, também no hemisfério ipsilateral à lesão isquêmica (Capítulo 2). O aumento nos níveis dessa neurotrofina está intimamente relacionado com a plasticidade sináptica necessária para os processos de aprendizagem e memória. Sendo assim, podemos sugerir que o aumento nos níveis de BDNF no hipocampo de ratos HI tratados com MFD pode estar contribuindo para as melhorias funcionais, especialmente para o processo de retenção de memória. De forma interessante, alguns estudos já demonstraram um aumento nos níveis de BDNF no soro

de pacientes com TDAH após um tratamento com MFD por mais de um mês (Amiri *et al.*, 2013; Akay *et al.*, 2018), dando um suporte ainda maior para os nossos achados em modelo animal.

O tratamento com Metilfenidato induz hiperatividade em animais HI

Um outro resultado que iremos discutir em conjunto é o efeito do MFD na locomoção no campo aberto e no teste de reconhecimento de objetos. Observamos que animais HI sob o efeito do MFD aumentaram a atividade locomotora no campo aberto, indicando que o MFD *contribuiu* para a hiperatividade desses animais. Um aumento da locomoção em ratos após administração de MFD é bem descrito na literatura (Askenasy *et al.*, 2007) mas não corrobora com o perfil comportamental que é observado na clínica, uma vez que o MFD diminui a hiperatividade de indivíduos com TDAHO que tem se proposto é que o efeito dos estimulantes depende dos estímulos provocados pelo ambiente, ou seja: quando o indivíduo está exposto a uma situação que exige atenção, como em uma sala de aula, este irá focar a sua atenção naquela atividade e assim diminuir a atividade motora. No entanto, quando o sujeito está exposto a uma situação que demanda maior exploração/atividade, o foco será nesse comportamento e assim um aumento de atividade locomotora pode ser observado (Porrino *et al.*, 1983; Swanson e Volkow, 2002).

Trazendo essa ideia para os experimentos com roedores, o que podemos sugerir é que quando o animal é exposto a um meio que contenha estímulos relevantes, o MFD geralmente facilita os processos de foco e atenção, contribuindo para um melhor desempenho no caso de uma tarefa cognitiva. No entanto, quando os animais são expostos a um aparato amplo sem nenhum estímulo relevante, como é o caso do campo aberto, a tendência dos animais é explorar aquele novo ambiente e o MFD pode agir exatamente nesse aspecto, aumentando o foco neste comportamento exploratório. Um recente estudo que contribui para esta hipótese é a meta-análise realizada por Leffa e colaboradores (2019), onde os autores concluíram que a administração de MFD em ratos SHR melhora os aspectos atencionais, de memória e de impulsividade de uma maneira dose-dependente, mas não apresenta efeitos na locomoção nas doses baixa e média e

aumenta a locomoção dos animais em doses altas. Em consonância, nossos resultados se assemelham aos achados encontrados no modelo SHR, o mais reconhecido para o estudo do TDAH atualmente.

No teste de reconhecimento de objetos, demonstramos um deficit no reconhecimento do objeto novo nos animais HI, sem melhora pela administração do MFD. Uma vez que esse teste é realizado em um aparato de campo aberto com livre exploração, nós supomos que a hiperatividade dos animais HI tratados com MFD tenha se sobressaído em relação ao foco nos objetos presentes no aparato. Uma hipótese é de que o aumento na hiperatividade desses animais tenha ocorrido pela alta estimulação dopaminérgica no estriado, uma região que apresenta uma maior densidade de DAT (Sesack *et al.*, 1998; Piccini, 2003) e consequentemente é mais sensível ao efeito do MFD. Sabendo que o teste de reconhecimento de objetos também depende da função do estriado (Qiao *et al.*, 2017), a alta estimulação dessa estrutura pode estar afetando o foco na exploração dos objetos. Análises mais detalhadas do comportamento dos animais neste teste, como locomoção especificamente nos dias de treino e teste, precisam ser ainda realizadas para podermos interpretar melhor estes resultados.

O tratamento com Metilfenidato reverte o comportamento alimentar desregulado e diminui o consumo de álcool em ratos HI

Em relação aos efeitos do MFD nos padrões de comportamento alimentar e consumo de álcool em ratos HI adultos, demonstramos uma reversão do comportamento alimentar desregulado em relação à ração padrão e uma diminuição do consumo de álcool após o tratamento com MFD nos ratos HI. Sabendo que o estriado é uma estrutura central na regulação desses comportamentos pela via mesolímbica e que apresenta grande quantidade de DAT (Sesack *et al.*, 1998; Piccini, 2003), sugerimos que a droga atue diretamente nesta estrutura, melhorando a transmissão dopaminérgica na via mesolímbica e assim diminuindo a expressão desses comportamentos desregulados. O CPF também apresenta um papel importante na regulação desses comportamentos, uma vez que a tomada de decisão e a flexibilidade comportamental são decisivas para a ação acontecer. Por exemplo, tem se postulado que o uso excessivo de álcool (e outras

drogas) pode ser considerado um comportamento habitual ou inflexível, resultado da perda de controle flexível sobre o uso da droga (Barker e Taylor, 2014; Corbit e Janak, 2016). De forma interessante, nossos resultados em ratos HI jovens demonstraram uma inflexibilidade comportamental nesse grupo e o tratamento com MFD melhorou este deficit. Dessa forma, podemos sugerir que a melhora na flexibilidade comportamental pelo MFD nos ratos HI pode ter contribuído para a diminuição no consumo do álcool nestes animais.

Nossos achados estão de acordo com pesquisas clínicas que já demonstraram que pacientes com TDAH em tratamento com MFD apresentaram taxas menores de uso de álcool e outras drogas em relação aos indivíduos com TDAH sem tratamento (Hammerness *et al.*, 2017). O tratamento com MFD também se mostrou benéfico para diminuir o índice de massa corporal em crianças com TDAH com sobrepeso/obesidade (Mellstrom *et al.*, 2018) e também para melhorar a alimentação compulsiva de indivíduos com bulimia nervosa (Schweickert *et al.*, 1997; Sokol *et al.*, 1999). Sendo assim, os efeitos do MFD nos comportamentos relacionados ao TDAH no nosso modelo animal estão de acordo com os achados em pesquisa clínica, dando um suporte adicional para a validade preditiva do modelo de HI. Esses resultados ainda evidenciam que embora o tratamento com MFD seja prescrito para melhora dos sintomas clássicos do TDAH, seus efeitos podem melhorar diretamente outros aspectos que interferem na qualidade de vida desses pacientes.

Em um segundo momento da análise do comportamento alimentar, a ração palatável ficou disponível à vontade aos animais, e observamos que os ratos que estavam sob efeito do MFD aumentaram o consumo total da ração palatável. Em um primeiro momento, estes resultados parecem intrigantes considerando que o MFD é reconhecido por diminuir o apetite; no entanto, podemos interpretar este resultado como um aumento do foco no comportamento alimentar para aquele estímulo que gerou sensação de prazer. Trazendo para o presente contexto a ideia de que o efeito dos estimulantes depende dos estímulos do ambiente, podemos inferir que as pistas ambientais induziram um maior consumo do alimento altamente palatável, e o MFD agiu intensificando o foco nesse comportamento. Um dado interessante é que quando comparamos o efeito do MFD nos ratos HI com o de ratos controles, percebemos que o

efeito do MFD nos ratos HI foi ainda maior, sugerindo que o valor atribuído à recompensa associada ao alimento palatável pode ser maior nos animais do grupo HI. Análises subsequentes avaliando os efeitos do MFD após vários dias de exposição ao alimento palatável seriam uma abordagem importante para distinguirmos o efeito da novidade daquele relacionado à adição a esse tipo de alimento.

O Metilfenidato prejudica o desempenho de ratos controles em alguns parâmetros comportamentais

Embora a análise do efeito do MFD em animais controle não tenha sido o foco principal do nosso estudo, incluímos o grupo controle tratado com MFD em todas as análises para compararmos o efeito da droga nos dois diferentes grupos (CT ou HI). Nossos resultados indicaram que animais CT jovens que receberam MFD tiveram um melhor desempenho cognitivo apenas na aprendizagem espacial no labirinto aquático de Morris, onde esse grupo aprendeu mais rápido o local da plataforma ao longo dos dias. No entanto, o MFD prejudicou o desempenho dos animais CT jovens na aprendizagem inicial da tarefa *attentional set shifting* e na discriminação do novo objeto na tarefa de reconhecimento de objetos (Capítulos 1 e 2). Além disso, observamos que os ratos CT adultos tratados com MFD apresentaram um aumento no consumo de álcool em relação aos ratos CT que receberam salina (Capítulo 3). Analisando em conjunto, podemos observar que o tratamento com MFD induziu uma série de comportamentos desfavoráveis para os animais CT e estes resultados podem ter relação com a bem estabelecida teoria da relação do tipo “curva em U invertida” entre os níveis de DA no CPF e o comportamento (Arnsten e Pliszka, 2011). Dessa maneira, acreditamos que a administração de MFD em animais que já apresentavam níveis ótimos de DA no CPF (animais CT) resultou em uma superestimulação do sistema dopaminérgico nessa região, levando a comportamentos desajustados os quais reportamos nessa seção.

Apesar dos efeitos terapêuticos evidentes do MPH para pacientes com TDAH, alguns efeitos colaterais são bem descritos, como: euforia, agitação, insônia, diminuição do apetite e cefaleia (Ahmann *et al.*, 1993). Assim, esses efeitos colaterais podem desfavorecer a adesão ao tratamento. Além disso, pouco se sabe sobre os efeitos a

longo prazo do tratamento diário com MFD. Em estudos pré-clínicos, já foi demonstrado que a administração crônica de MFD ocasionou efeitos persistentes no sistema dopaminérgico (Moll *et al.*, 2001), deficit de memória e aprendizado, morte de neurônios e astrócitos e excitotoxicidade (Scherer *et al.*, 2010; Schmitz *et al.*, 2016; Schmitz *et al.*, 2017), assim como aumento dos comportamentos do tipo depressivo e ansioso (Carlezon *et al.*, 2003).

Esses achados levantam uma importante questão em relação ao uso indevido do MFD por indivíduos saudáveis que buscam uma maior atenção e foco nos estudos para assim melhorar o rendimento escolar, acadêmico ou em concursos. O mais preocupante é que o uso indevido dessa droga por indivíduos que não apresentam o TDAH é uma condição que vem aumentando exponencialmente na nossa sociedade (Guthrie *et al.*, 2003; Teter *et al.*, 2003). Sendo assim, estudos investigando os efeitos a longo prazo do tratamento com MFD tanto na população com TDAH quanto em indivíduos saudáveis são extremamente necessários para alertar a população sobre os seus efeitos.

Rede genética do *DAT1* modera o efeito da exposição a condições hipóxico–isquêmicas perinatais em características associadas ao TDAH em crianças

No Capítulo 4 do nosso trabalho realizamos um estudo clínico de avaliação da interação rede genética x ambiente, levando em consideração que tanto condições genéticas quanto a exposição a fatores adversos ambientais como a HI perinatal podem influenciar no neurodesenvolvimento. Para o componente genético, empregamos a metodologia do estudo de redes genéticas e criamos um escore de “risco” poligênico baseado na expressão (ePRS) dos genes que são co-expressos com o gene *DAT1* no CPF. Dessa forma, conseguimos inferir através do perfil genético a funcionalidade biológica desta rede tão importante para o controle da sinalização de DA no CPF, e que é um dos principais alvos da ação do MFD. Em relação à avaliação da exposição a condições hipóxico-isquêmicas perinatais (HICs), criamos um escore cumulativo de múltiplos fatores associados à HI perinatal, tais como: tabagismo materno durante a gestação, descolamento placentário, apresentação pélvica/transversa, distocia fetal, ocorrência de prolapso de cordão umbilical, APGAR <7 no 1º minuto, baixo peso ao

nascer, distúrbio respiratório e necessidade de reanimação neonatal. Após termos o índice genético e a condição ambiental para cada criança, avaliamos a interação entre esses fatores para o desfecho comportamental (flexibilidade cognitiva) e densidade de substância cinzenta em diferentes regiões cerebrais, utilizando duas coortes étnicas distintas. Em ambas as coortes, demonstramos que a exposição a vários HICs perinatais prejudicou a flexibilidade cognitiva apenas de crianças que tinham um ePRS alto para a rede do *DAT1*, o que pode sugerir uma alta funcionalidade dessa rede e menor sinalização dopaminérgica no CPF. Ainda, utilizamos uma nova abordagem conhecida como *Parallel independent component analysis* (p-ICA) para demonstrar que os polimorfismos do tipo SNP incluídos no escore ePRS ponderados pela sua associação com expressão gênica modularam a relação entre a exposição aos HICs e a densidade de substância cinzenta em áreas envolvidas nas funções executivas (regiões corticais) e integrativas (tálamo e putamen). Considerando que essas regiões estão envolvidas nos processos de função executiva, inferimos que as diferenças na densidade da substância cinzenta nestas estruturas contribuem para a inflexibilidade cognitiva observada em crianças expostas aos HICs e de acordo com o perfil genético relacionado à rede do DAT.

Precisamos mencionar que as crianças incluídas no nosso estudo são crianças saudáveis, sem o diagnóstico para o TDAH. No entanto, sabendo que deficit de função executiva são frequentemente reportados em pacientes com TDAH, podemos inferir que estas crianças podem apresentar um risco maior para desenvolver o transtorno. Ainda, mesmo que estes indivíduos não cheguem a ser diagnosticados com o TDAH, os prejuízos relacionados à inflexibilidade cognitiva podem acarretar em dificuldades em atividades cotidianas e uma vulnerabilidade maior para o uso de substâncias na adolescência ou fase adulta, uma vez que a inflexibilidade comportamental está intimamente relacionada aos transtornos relacionados ao uso de substâncias (Barker & Taylor, 2014; Corbit & Janak, 2016).

Este estudo gerou importantes contribuições tanto para o campo genético assim como para os estudos de fatores ambientais, onde demonstramos que análises mais informativas e complexas podem ser importantes para capturar o efeito de fatores adversos em crianças saudáveis, e informar sobre diferenças individuais que

potencialmente possam estar associadas a doenças mais tarde na vida. Comprovamos que o método ePRS é uma abordagem promissora, que vai além de encontrar associação entre variantes genéticas dispersas e fenótipos, mas que captura informações sobre toda a rede genética e sua função em regiões específicas do encéfalo. Ainda, demonstramos que a neurotransmissão dopaminérgica no CPF é um importante moderador dos efeitos das HICs nos deficit de função executiva e desenvolvimento encefálico, o que confirma diversos achados discutidos acima no modelo animal, reafirmando a importância desse modelo para o estudo do TDAH em humanos. Considerando que não podemos modificar nossa predisposição genética para determinadas características ou doenças, destacamos a importância de medidas preventivas para melhorar a saúde intrauterina e intraparto, evitando assim distúrbios no desenvolvimento fetal, infantil e a longo prazo.

Novas ferramentas de análises permitem uma melhor compreensão de como as adversidades precoces alteram o neurodesenvolvimento

Por fim, fizemos uma revisão abordando os principais fatores ambientais precoces (período pré-natal, periparto e pós-natal) que podem impactar diretamente o encéfalo em desenvolvimento. Dentre os fatores pré-natais, destacamos a restrição de crescimento intrauterino (que apresenta diferentes causas), o uso de substâncias pela mãe e a presença de fatores estressantes ou transtornos psiquiátricos durante a gestação. No período periparto, a HI perinatal é a causa mais comum de morbidade e mortalidade em neonatos, podendo levar a deficit motores severos até comprometimentos cognitivos leves, como deficit de atenção. Ainda, o período pós-natal precoce também apresenta grande influência no encéfalo em desenvolvimento, onde condições como maus tratos, violência e estresse psicológico podem ter impactos negativos e duradouros.

Embora estes fatores sejam bastante associados com desfechos negativos para o encéfalo em desenvolvimento, sabe-se que alguns indivíduos mesmo sendo expostos a estas condições não apresentam modificações funcionais/estruturais encefálicas e nem alterações comportamentais. Desta maneira, a interação entre estes fatores ambientais e o perfil genético precisa ser levada em consideração. Estudos já

demostraram que diferentes variações genéticas do gene do BDNF, da COMT e do receptor de serotonina moderam o impacto das condições ambientais no desenvolvimento da criança. Avançando nessa área, pesquisadores evidenciaram que a depressão materna afeta de forma diferente o desenvolvimento da amígdala em crianças que apresentam maior risco genético para transtorno depressivo (Qiu *et al.*, 2017). Ainda, foi demonstrado que o efeito da exposição a adversidades pré-natais em desfechos cognitivo-emocionais em crianças foi moderado pelo ePRS para a rede genética do transportador de serotonina no hipocampo; ou seja, quanto maior o número de adversidades pré-natais, maiores os problemas cognitivos-emocionais apenas em crianças com um alto ePRS-transportador de serotonina (hipocampo) (Silveira *et al.*, 2017). Desta forma, novas ferramentas melhoraram a nossa capacidade de entender as relações entre as adversidades precoces e as alterações no desenvolvimento e função do encéfalo, permitindo uma melhor empregabilidade dos resultados obtidos em modelos animais para os estudos em humanos, especialmente com o uso de novas abordagens genéticas.

Todo o desenvolvimento desta tese baseou-se neste princípio, uma vez que os nossos achados experimentais nos guiaram para as investigações em humanos, onde empregamos técnicas modernas para avaliarmos funções específicas que podem estar impactando nos desfechos em questão. Em suma, os resultados experimentais demonstraram que o modelo de HI neonatal afetou o sistema dopaminérgico e por consequência deficit comportamentais associados ao TDAH foram observados. Desta forma, investigamos se a exposição a HICs poderia interagir com um perfil genético funcionalmente associado à transmissão dopaminérgica no CPF, impactando de forma diferente no desenvolvimento de características relacionadas ao TDAH em crianças.

9. CONCLUSÕES

Os resultados da parte experimental desta tese demonstraram que o modelo de HI de Rice-Vannucci apresenta validade aparente, de construto e preditiva para o estudo do TDAH em roedores. Os seguintes resultados apoiaram essas validações:

Validade aparente	Validade de construto	Validade preditiva (tratamento com MFD)
Inflexibilidade cognitiva em ratos jovens	Desregulação na transmissão dopaminérgica no CPF de ratos jovens	Reversão dos deficit de flexibilidade cognitiva em ratos jovens
Deficit de aprendizagem e de memória de curta e longa duração em ratos jovens		Melhora na memória de longa duração em ratos jovens
Comportamento alimentar desregulado na fase adulta		Reversão do comportamento alimentar desregulado na fase adulta
Aumento no consumo do álcool na fase adulta		Diminuição no consumo de álcool na fase adulta

No estudo clínico, demonstramos que o perfil genético associado com a recaptação dopaminérgica no CPF é um importante moderador dos efeitos das HICs nos deficit de função executiva e desenvolvimento encefálico.

Na revisão da literatura, descrevemos as principais influências ambientais precoces que podem afetar o encéfalo em desenvolvimento, incluindo a HI perinatal, assim como as novas ferramentas em estudos genéticos e de neuroimagem que estão permitindo elucidar os mecanismos biológicos subjacentes a essas alterações encefálicas.

Com a realização desta tese, apresentamos diferentes alternativas para o estudo do TDAH tanto em pesquisa experimental quanto em pesquisa clínica. Sabendo que o TDAH é a desordem mais diagnosticada na infância, com consequências que podem perdurar por toda a vida, estudos investigando novas abordagens para a pesquisa desse transtorno são extremamente necessários. Nossos dados ainda demonstraram que o período perinatal é altamente vulnerável às adversidades do ambiente, onde variações nos níveis de oxigenação podem contribuir para o desenvolvimento do TDAH. Assim,

medidas preventivas que melhorem as condições intrauterinas e periparto são importantes para evitar tais adversidades ao feto.

10. PERSPECTIVAS

As perspectivas para este trabalho são as seguintes:

- Investigar os efeitos do MFD no modelo de HI neonatal utilizando diferentes doses do fármaco;
- Avaliar os diferentes parâmetros de sinalização dopaminérgica realizados neste estudo também na região do NAcc;
- Mensurar especificamente as vias envolvidas nas respostas diferenciais em relação ao MFD em ratos HI e CT – por exemplo, a análise da liberação de dopamina no CPF e no NAcc por cronoamperometria;
- Avaliar outros possíveis marcadores de plasticidade sináptica que podem ser alterados pelo MFD, tais como: sinaptofisina, PSD-95 e o receptor de BDNF TrkB;
- Utilizar um protocolo mais longo de exposição ao alimento palatável para assim conseguirmos distinguir o efeito relacionado à novidade daquele relacionado à adição ao alimento palatável;
- Avaliar a resposta hedônica ao alimento palatável;
- Avaliar o efeito do MFD nos diferentes grupos no teste de Preferência Condicionada de Lugar para mensurar os efeitos reforçadores dessa droga.

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12. ANEXOS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 29750
Título: CARACTERÍSTICAS DO TRANSTORNO DE DÉFICIT DE ATENÇÃO/HIPERATIVIDADE EM UM MODELO DE HIPÓXIA-ISQUEMIA NEONATAL E O POSSÍVEL EFEITO DO TRATAMENTO COM METILFENIDATO

Vigência: 01/10/2015 à 30/09/2019

Pesquisadores:

Equipe UFRGS:

LENIR ORLANDI PEREIRA SILVA - coordenador desde 01/10/2015
LISIANE BIZARRO ARAUJO - pesquisador desde 01/10/2015
PATRICIA PELUFO SILVEIRA - pesquisador desde 01/10/2015
SILVIA BARBOSA - Técnico de Laboratório desde 01/10/2015
Jaqueline Vieira Carletti - Aluno de Doutorado desde 01/10/2015
Daniela Pereira Laureano - Aluno de Doutorado desde 01/10/2015
Márcio Bonesso Alves - Aluno de Doutorado desde 01/10/2015
Bruna Ferrary Deniz - Aluno de Doutorado desde 01/10/2015
PATRÍCIA MAIDANA MIGUEL - Aluno de Doutorado desde 01/10/2015
Ramiro Diaz - Aluno de Doutorado desde 01/10/2015
Heloisa Deola Confortim - Aluno de Doutorado desde 01/10/2015

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 26/10/2015 - Sala 323 do Anexo I do Prédio da Reitoria - Campus Centro da UFRGS- Bairro Farroupilha - Porto Alegre - RS, em seus aspectos éticos e metodológicos, para a utilização de aprovação de 264 Recém Nascidos ratos Wistar machos, 48 ratos Wistar machos adultos e 48 ratas Wistar fêmeas adultas provenientes do CREAL, de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.

Porto Alegre, Quinta-Feira, 5 de Novembro de 2015

Cristiane Matte

CRISTIANE MATTE
Coordenador da comissão de ética

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
GRUPO DE PESQUISA E PÓS-GRADUAÇÃO

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

A Comissão de Ética no Uso de Animais (CEUA/HCPA) analisou o projeto:

Projeto: 150566
Data da Versão do Projeto: 04/02/2016

Pesquisadores:
PATRICIA PELUFO SILVEIRA
PATRÍCIA MAIDANA MIGUEL
MARCIO BONESSO ALVES
DANIELA PEREIRA LAUREANO

Título: Características do Transtorno de déficit de atenção/hiperatividade em um modelo de Hipóxia-isquemia neonatal e o possível efeito do tratamento com Metilfenidato

Este projeto foi APROVADO em seus aspectos éticos e metodológicos de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08/10/2008, que estabelece procedimentos para o uso científico de animais.

- Os membros da CEUA/HCPA não participaram do processo de avaliação de projetos onde constam como pesquisadores.
- Toda e qualquer alteração do Projeto deverá ser comunicada à CEUA/HCPA.
- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao CEUA/HCPA.

Porto Alegre, 05 de abril de 2016.

Biol. Michael Everton Andrades
Coordenador CEUA/HCPA