UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL-UFRGS

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

DEPARTAMENTO DE FISIOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: FISIOLOGIA

Tese de Doutorado

Efeitos do Enriquecimento Ambiental no Período Gestacional e Lactacional em Ratos Wistar Submetidos a Hipóxia-Isquemia Neonatal

Aluna: Luz Elena Durán Carabali

Orientador: Prof. Dr. Carlos Alexandre Netto

Co-Orientador: Prof. Dra. Aline de Souza Pagnussat

Porto Alegre, 2019

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

Efeitos do Enriquecimento Ambiental no Período Gestacional e Lactacional em Ratos Wistar Submetidos a Hipóxia-Isquemia Neonatal

Aluna: Luz Elena Durán Carabali
Orientador: Prof. Dr. Carlos Alexandre Netto
Co-Orientador: Prof. Dra. Aline de Souza Pagnussat

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

Porto Alegre, 2019

"Quando não podemos mais mudar uma situação,
somos desafiados a mudar a nós mesmos".

Viktor Frankl.

AGRADECIMENTOS

A conclusão desta Tese não teria sido possível sem o apoio, o esforço, a dedicação e o voto de confiança que recebi por muitas pessoas.

Em primeiro lugar, agradeço a YHWH pelas oportunidades que me trouxeram até aqui e me permitiram concluir um sonho. Agradeço a minha família, em especial a minha tia e a minha prima, pelo amor, pelo apoio e pelo incentivo, apesar da distância. A minha mãe (*In memorian*), pelo exemplo de caráter, perseverança e dedicação. Suas palavras e ensinamentos formaram o meu caráter e me tornaram quem sou hoje.

Ao professor Dr. Carlos Alexandre Netto, que desde o início acreditou e apostou no meu projeto. Agradeço por ser um exemplo de Mestre e pesquisador, mas principalmente por permitir que eu aprendesse com meus erros e meus acertos, pela acolhida e pelo carinho nos diversos momentos da minha vida acadêmica e pessoal.

À professora Dra. Aline de Souza Pagnussat, pela oportunidade oferecida para desenvolver o meu projeto de doutorado no Brasil ao me indicar para o professor Netto. Pelo incentivo para que eu seguisse em frente e por estar sempre disposta a me apoiar durante este processo.

Ao Dr. Samuel Greggio e à Dr. Gianina T. Venturin do Instituto do Cérebro do Rio Grande do Sul (InsCer), assim como ao professor Dr. Eduardo Zimmer, pela parceria e pela troca de experiências e de conhecimento.

À professora Dra. Carla Dalmaz e aos alunos do laboratório em neurobiologia do estresse, em especial à Danussa Arcego, pela ajuda, pelo incentivo e pelas assertivas contribuições durante o desenvolvimento deste projeto.

A todos os colegas do laboratório de Isquemia e Neuroproteção (lab.35), por sempre estarem dispostos a me ajudar, além da grata e divertida convivência. Aos alunos de iniciação cientifica, pelo comprometimento e responsabilidade.

Aos colegas, amigos e quase irmãos – Eduardo Sanches, Marilia Marques e Felipe Odorcyk: Obrigada pelos laços de amizade que jamais serão desfeitos, pela generosidade, pelo carinho, pelo incentivo, pelos ensinamentos, pelas contribuições cientificas e, sem dúvida, por acreditar em mim quando eu achei difícil acreditar em mim mesma.

À equipe de Pesquisa em Genética Médica, pela flexibilidade nos horários, pela compreensão e pelo companheirismo, especialmente à Claudia Vanzella, à Larissa Pozzebon e à Marina Zambrano, pela amizade, pela confiança e pelo incentivo.

À UFRGS, ao Instituto de Ciências Básicas da Saúde e ao Laboratório de Isquemia Cerebral e Neuroproteção, pela estrutura disponibilizada para a realização desta Tese. Aos funcionários do biotério, em especial à Fabiola, à Leticia, ao Everton, à Claudia e à Cibele, por sempre estarem dispostos a me ajudar e por contribuírem para que os trabalhos em andamento disponham das melhores condições.

Ao CNPq, pela bolsa de estudos concedida neste período.

Aos amigos de Shabat Team, por se tornarem tão importantes quanto minha própria família. Por suas palavras, por seu apoio e amor incondicional, pelos momentos de desabafo e de risadas, mas principalmente por me lembrarem que YHVH tem o controle e tem um momento apropriado para todo.

Ao meu esposo Oscar, por dizer algumas vezes o que eu realmente precisava ouvir, em vez do que eu queria ouvir; por fazer eu me sentir tão amada e por estar sempre presente quando necessito. Por ser meu amigo e meu parceiro de travessia, e por ser a pessoa capaz de mostrar um outro lado a considerar na vida, me tornando uma pessoa mais feliz.

A minha filha Emma, que nasceu durante esta jornada para encher meus dias de amor e de felicidade por meio de sua ternura, sua inocência e suas incansáveis descobertas.

SUMÁRIO

LISTA DE ABREVIATURAS

µPet	Microtomografia por Emissão De Pósitrons
18F	Flúor-18
18F-FDG	18F-Fluordesoxiglicose
ACOG	American College of Obstetrician and Gynecology
AE	Ambiente Enriquecido
AKT	Proteína Cinase B
ANOVA	Análise de Variância
AP	Ambiente Padrão
ATP	Trifosfato de Adenosina
BDNF	Fator Neurotrófico Derivado do Encéfalo
BHE	Barreira Hematoencefálica
Ca^{++}	Cálcio
CO_2	Dióxido de Carbono
DG	Dia Gestacional
DNA	Ácido Desoxirribonucleico
DPN	Dia Pós-natal
EPM	Erro Padrão Médio
FOV	Field-Of-View
GFAP	Proteína Ácida Fibrilar Glial
GLUT	Transportadores de Glicose
H^+	Hidrogênio
HI	Hipóxia-Isquemia
IGF-1	Fator de Crescimento Semelhante a Insulina
InsCer	Instituto do Cerebro
Na^+	Sódio
Na+/K+-ATPase	Bomba Sódio e Potássio ATPase
NeuN	Antígeno Nuclear Neuronal
NMDA	N-Metil-D-Aspartato
NO	Óxido Nítrico
O_2	Oxigênio

OLS	Oligodendrócitos
PARP	Poli (ADP-Ribose) Polimerase
PBS	Tampão Fosfato-Salino
PBS-Tx	Tampão Fosfato Salino Com Triton X-100
PFA	Paraformaldeido
Pré-OLS	Progenitora De Oligodendrócitos
PUCRS	Pontifícia Universidade Católica do Rio Grande do Sul
SNC	Sistema Nervoso Central
SUV	Standard Uptake Value
TCE	Traumatismo Crânio-encefálico
Trkb	Receptor de Tropomiosina Quinase B
UFRGS	Universidade Federal Do Rio Grande Do Sul
VEGF	Fator De Crescimento Vascular Endotelial

LISTA DE FIGURAS

Capítulo 1

Capítulo 2

Capítulo 3

Capítulo 4

LISTA DE TABELAS

Capítulo 1

Capítulo 2

Capítulo 3

RESUMO

O aumento da taxa de sobrevivência de recém-nascidos muito prematuros está associado à maior incidência de dano cerebral e a patologias neurológicas, como a hipóxia-isquemia neonatal (HI). A HI afeta 2/1000 nascidos vivos e é a principal causa de mortalidade e morbidade infantil, sendo considerada um problema de saúde pública devido a seu impacto ao longo do ciclo vital e a alta demanda de suporte social e familiar necessária. As alterações no metabolismo energético decorrentes da HI desencadeiam uma cascata de eventos moleculares implicados no dano cerebral e nos déficits sensoriomotores, cognitivos e comportamentais subsequentes. O ambiente enriquecido (AE) é uma estratégia experimental caraterizada por oferecer um maior número de estímulos cognitivos, sociais e motores, apresentando efeitos benéficos em uma variedade de doenças e lesões ao sistema nervoso central e tornando o cérebro mais resiliente. Entretanto, os efeitos do AE em períodos críticos do desenvolvimento e/ou maturação encefálica, bem como os mecanismos biológicos envolvidos quando associado à HI, ainda não são totalmente entendidos. Desse modo, esta Tese teve como objetivo avaliar os efeitos do AE no período gestacional e lactacional sobre os prejuízos induzidos pela HI, quando realizada no terceiro dia pós-natal do rato. Para isso, ratas Wistar prenhas foram alocadas no AE ou em condições padrão durante a gestação. Após o nascimento, as ninhadas foram mantidas nas mesmas condições de moradia do período gestacional ou realocadas em um novo ambiente durante a lactação. Observou-se que os animais submetidos à HI e expostos ao AE na gestação, na lactação ou em ambos os períodos do desenvolvimento apresentaram redução na morte celular por apoptose na fase aguda da lesão, melhor desempenho funcional quando comparados ao grupo controle, assim como redução na expressão de sinaptofisina, proteína relacionada à plasticidade sináptica, no hipometabolismo encefálico e na perda tecidual na idade adulta. Esses achados podem ser decorrentes da ativação de vias relacionadas com sobrevivência celular como AKT (48h pós-lesão), bem como do aumento na expressão de fatores de crescimento, aumento no imunoconteúdo do receptor TrkB e modulação na resposta astrocitária na idade adulta. Além disso, os animais apresentaram uma resposta sexo-específica à estimulação ambiental, na qual ratos machos exibiram efeitos positivos tanto no período gestacional como lactacional, enquanto ratas fêmeas pareceram ser mais beneficiadas pelo enriquecimento pós-natal precoce. Os resultados obtidos permitem concluir que o AE tem efeitos tanto preventivos como terapêuticos ao atenuar o grau de comprometimento neurológico induzido pela HI neonatal. Estes achados ressaltam a importância de uma maior compreensão sobre os mecanismos envolvidos nos efeitos benéficos do AE, bem como a influência das condições ambientais no prognóstico a curto e longo prazo do recém-nascido exposto a eventos cerebrais adversos como a hipóxia isquemia.

Palavras chaves: prematuridade, hipóxia-isquemia neonatal, enriquecimento ambiental, neurodesenvolvimento, dimorfismo sexual.

ABSTRACT

The increasing survival rates of very preterm newborns is associated to brain damage and several neurological conditions, such as neonatal hypoxia-ischemia (HI). HI affects 2/1000 live births and it is the main cause of mortality and morbidity in infants, being considered a public health problem for its great impact throughout the life cycle and increased family and social support requirements. Changes in the energy metabolism as consequence of HI insult trigger a cascade of molecular events implicated in brain damage and subsequent sensorimotor, cognitive and behavioral deficits. Environmental enrichment (EE) is an experimental strategy that aims to promote greater cognitive, social and motor stimuli, showing beneficial effects in a variety of central nervous system diseases and injuries, thus making brain more resilient. However, the effects of EE during critical neurodevelopmental periods or encephalic maturation, as well as the biological mechanisms involved when related to HI are not yet understood. The aim of this Thesis is to evaluate the EE effects in gestational and lactation period on the HI-induced damage, on the third postnatal day. Thereby, pregnant Wistar rats were allocated in EE or standard conditions during gestation. After birth, the litters were kept in the same housing condition or reallocated to a new environment during lactation. It was observed that animals submitted to HI and exposed to EE on gestation, lactation and the combination of both neurodevelopmental periods showed a reduction in cell death by apoptosis in acute stage of the injury and had greater functional recovery when compared to control group. In addition, EE animals showed a reduction in the expression of synaptopysin, protein related to synaptic plasticity, in encephalic hypometabolism and in tissue loss at adulthood. These findings can be due to activation of pathways related to cell survival, such as AKT (48h after the insult), up-regulation of VEGF and IGF-1 levels, as well as the increase in the immunocontent of TrkB receptor and modulation in astrocyte reaction at adulthood. Moreover, a sex-specific response was observed, in which male rats exhibited positive effects both at gestational and lactation period, while females seem to be more benefited by early postnatal enrichment. Data here presented allow the conclusion that EE has preventive and therapeutic effects attenuating the neurological impairments caused by neonatal HI. These results highlight the relevance of a better understanding of the mechanism responsible for protective EE effects, as well as the environmental influences on short and long-term prognosis of neonate suffering from brain injury such as hypoxia-ischemia.

Key words: prematurity, neonatal hypoxia-ischemia, enriched environment, neurodevelopment, sexual dimorphism

APRESENTAÇÃO

Esta Tese é constituída por:

1. Introdução: embasamento teórico para a compreensão da proposta de trabalho e das hipóteses.

2. Objetivos: descrição dos objetivos geral e específicos a serem desenvolvidos ao longo dos capítulos.

3. Abordagem Metodológica: descrição geral do modelo experimental e dos métodos utilizados.

4. Resultados, constituído pelos capítulos 1, 2, 3, 4 e 5.

    - Capítulo 1, artigo publicado na revista Neuroscience: *Longer hypoxia-ischemia periods to neonatal rats causes motor impairments and muscular changes;*
    - Capítulo 2, artigo publicado na revista Molecular Neurobiology: *Prenatal and early postnatal environmental enrichment reduce acute cell death and prevent neurodevelopment and memory impairments in rats submitted to neonatal hypoxia ischemia;*
    - Capítulo 3, artigo publicado na revista Behavioral Brain Research: *Preventive and therapeutic effects of environmental enrichment in Wistar rats submitted to neonatal hypoxia-ischemia;*
    - Capítulo 4, short communication publicada na revista Behavioral Brain Research: *Enriched experiences during pregnancy and*

lactation period protect against motor impairments induced by neonatal hypoxia-ischemia

- Capítulo 5, O enriquecimento ambiental precoce reduz parcialmente o hipometabolismo encefálico e modifica a estrutura da rede metabólica em ratos submetidos à hipóxia-isquemia neonatal: Resultados parciais.

5. Discussão: interpretação dos resultados apresentados e sua contextualização.
6. Conclusões e perspectivas.
7. Referências bibliográficas.

1. INTRODUÇÃO

1.1 Prematuridade

O nascimento prematuro é considerado um problema de saúde pública, sendo a principal causa de mortalidade em crianças menores de cinco anos (Blencowe et al., 2013) e de morbidade infantil (Abajobir et al., 2017). A prematuridade é definida como o nascimento do feto humano antes de 37 semanas de gestação, ou antes de 259 dias após a data do último período menstrual (World Health Organization, 2012). De acordo com a idade gestacional, o *American College of Obstetrician and Gynecology* (ACOG) classifica o recém-nascido em: prematuro extremo (< 28 semanas), muito imaturo (entre 28 e 32 semanas), prematuro moderado (entre 32 e 37 semanas), a termo precoce (entre 37 e 38 semanas), a termo (entre 39 e 40 semanas), a termo tardio (entre 41-42 semanas) e pós-termo (> 43 semanas) (ACOG, 2013; Quinn et al., 2016).

Atualmente, cerca de 15 milhões de recém-nascidos nascem antes de 37 semanas de gestação no mundo, perfazendo uma taxa de 11.1% de todos os nascimentos (Blencowe et al., 2012; Suff et al., 2018). Avanços no serviço de neonatologia nas últimas décadas têm melhorado a taxa de sobrevivência, particularmente em prematuros extremos, entre as 24ª e 28ª semanas de gestação e peso inferior a 1500 gramas; porém tal sucesso traz consigo aumento da incidência de sequelas neurológicas como paralisia cerebral, epilepsia e déficit de aprendizagem (Volpe, 2009a, 2009b; Frey and Klebanoff, 2016). Dados da Organização Mundial da Saúde revelam que 60% dos nascimentos pré-termo ocorrem na África e no sul da Ásia, contudo, no *ranking* mundial de prematuridade países como a Índia, a China e os Estados Unidos ocupam os

primeiros lugares. O Brasil ocupa a décima posição, com cerca de 300 mil nascimentos prematuros por ano (Blencowe et al., 2013).

Estima-se que nos países em desenvolvimento 12% dos bebês nascem antes do tempo previsto e 90% daqueles que têm prematuridade extrema morrem em poucos dias. Entretanto, nos países desenvolvidos essa taxa de mortalidade cai para menos de 10% dos casos. Esta situação tem sido relacionada a diferenças na qualidade da atenção médica, no acesso aos serviços de saúde especializada e nas intervenções preventivas para melhorar a qualidade de vida da gestante (World Health Organization, 2012).

O nascimento prematuro pode ser espontâneo, em 75% dos casos, ou por causas iatrogênicas (Kramer et al., 2012). Apesar da patogênese do nascimento pré-termo ser pouco compreendida, as evidências científicas indicam a prematuridade como uma síndrome complexa, com múltiplas causas possíveis (Suff et al., 2018). Entre os fatores de risco associados podemos citar: idade materna (menor de 17 anos e maior de 40 anos), índice de massa corporal (muito alto ou muito baixo), intervalo reduzido entre uma e outra gravidez, gravidez múltipla, infecções das vias urinárias, hipertensão arterial, diabetes, consumo de tabaco ou álcool, disfunção placentária, estresse e nível socioeconômico desfavorável (Goldenberg et al., 2009; Gravett et al., 2010; Muglia and Katz, 2010; Angelidou et al., 2012; Kramer et al., 2012).

A classificação de prematuridade do ACOG considera tanto a apresentação clínica como os mecanismos fisiológicos desta condição. A primeira refere-se ao parto prematuro, espontâneo ou induzido, e a segunda subdivide o nascimento

pré-termo em causas infecciosas e/ou inflamatórias, por vasculopatias ou estresse (Kramer et al., 2012).

É importante salientar que as consequências da prematuridade têm impacto tanto no período neonatal como ao longo do ciclo vital. Diferentes estudos têm mostrado um incremento na taxa de complicações a curto prazo, como hemorragia encefálica periventricular, hipoglicemia, asfixia perinatal e dificuldade respiratória (Escobar et al., 2006; Femitha and Bhat, 2012). Chama atenção de que em recém-nascidos prematuros a incidência de eventos hipóxico isquêmicos atinge entre 12 e 14% dos casos, enquanto no recém-nascido a termo, essa taxa se reduz a 1 e 2% dos nascimentos (Blencowe et al., 2012).

No longo prazo, a prematuridade está associada a alterações no desenvolvimento e no desempenho neurológico, como atraso no desenvolvimento psicomotor, paralisia cerebral, autismo, déficit de aprendizado, deficiência nas funções executivas, doença pulmonar crônica, doenças cardiovasculares, déficit visual e auditivo, déficit de atenção e hiperatividade, ansiedade e depressão (Volpe, 2009b; Teune et al., 2011; Angelidou et al., 2012; Roggero et al., 2013). Assim, é de fundamental relevância o estudo de condições como a prematuridade e algumas de suas complicações primárias, como a hipóxia-isquemia neonatal nessa faixa de desenvolvimento, a fim de avançar a compreensão da lesão cerebral, das alterações funcionais associadas e da capacidade de resiliência e recuperação dos sujeitos o que poderá facilitar novas abordagens terapêuticas, tanto no contexto experimental como clínico (O'Shea, 2002; Volpe, 2005; Lai and Yang, 2011).

1.2 Hipóxia-Isquemia Neonatal (HI)

A **Hipóxia-Isquemia** neonatal (HI) é uma das principais causas de óbito e de lesões ao Sistema Nervoso Central (SNC) no período perinatal (Verklan, 2009), com incidência de 1,8 a 6 casos/ 1000 nascidos vivos (Shevell et al., 2001). Disfunções circulatórias e/ou complicações obstétricas como a hipóxia materna, o descolamento prematuro da placenta e a compressão do cordão umbilical podem interferir na troca de gases entre a placenta e o feto, levando a um déficit no aporte de oxigênio (O_2), excesso de dióxido de carbono (CO_2) e uma diminuição do aporte sanguíneo (isquemia) causando a hipóxia-isquemia neonatal (Berger and Garnier, 1999; Back et al., 2001; Ferriero, 2004).

Complicações sistêmicas da asfixia neonatal frequentemente incluem alterações cardiovasculares, respiratórias, metabólicas e renais, sendo, o SNC o mais comprometido (Berger and Garnier, 1999). As alterações metabólicas decorrentes do insulto (HI) podem levar a modificações bioquímicas e fisiológicas que se traduzem em manifestações clínicas secundárias ao comprometimento fisiológico ou estrutural (McKenna et al., 2015). A HI tem sido correlacionada a 25% dos casos de deficiências físicas e cognitivas em crianças (Kurinczuk et al., 2010) e vinculada ao comprometimento das capacidades neurológicas e/ou a patologias como a paralisia cerebral, a epilepsia e retardos no desenvolvimento psicomotor e de aprendizado (Vannucci and Hagberg, 2004; Khwaja and Volpe, 2008). As implicações das sequelas neurológicas associadas à HI são consideradas um problema de saúde pública, devido ao grande impacto ao longo do ciclo vital e ao amplo suporte familiar e social necessário (McKenna et al., 2015).

As características neuropatológicas da HI são complexas e variam dependendo da idade gestacional e pós-natal do neonato, da natureza e da duração do insulto (Roelfsema et al., 2005; Yang and Lai, 2011; Bock et al., 2014), ainda que o encéfalo do neonato tenha uma maior tolerância anaeróbica quando comparado ao do adulto (Towfighi et al., 1997; Perlman, 2001; George et al., 2004). As alterações no metabolismo energético cerebral têm grande relevância devido aos períodos de vulnerabilidade celular e tecidual em que ocorre o insulto (Yang and Lai, 2011; Bock et al., 2014). No período prenatal/neonatal ocorre o desenvolvimento e/ou a maturação de diferentes estruturas cerebrais como o hipocampo, o sistema límbico e o neocórtex (Yager and Ashwal, 2009), havendo intensa sinaptogênese e alta taxa de diferenciação de algumas células, como por exemplo, os oligodendrócitos (Back et al., 2002; Back, 2006).

Como apresentado na Figura 1, os achados neuropatológicos da HI variam de acordo com a maturidade do feto, a natureza e a extensão da lesão, podendo resultar em lesões teciduais com características macroscópicas diferentes como morte seletiva neuronal, lesão cerebral para-sagital, leucomalácia periventricular, hemorragia intra ou periventricular e isquemia focal e/ou multifocal (Khwaja and Volpe, 2008; Silbereis et al., 2010).

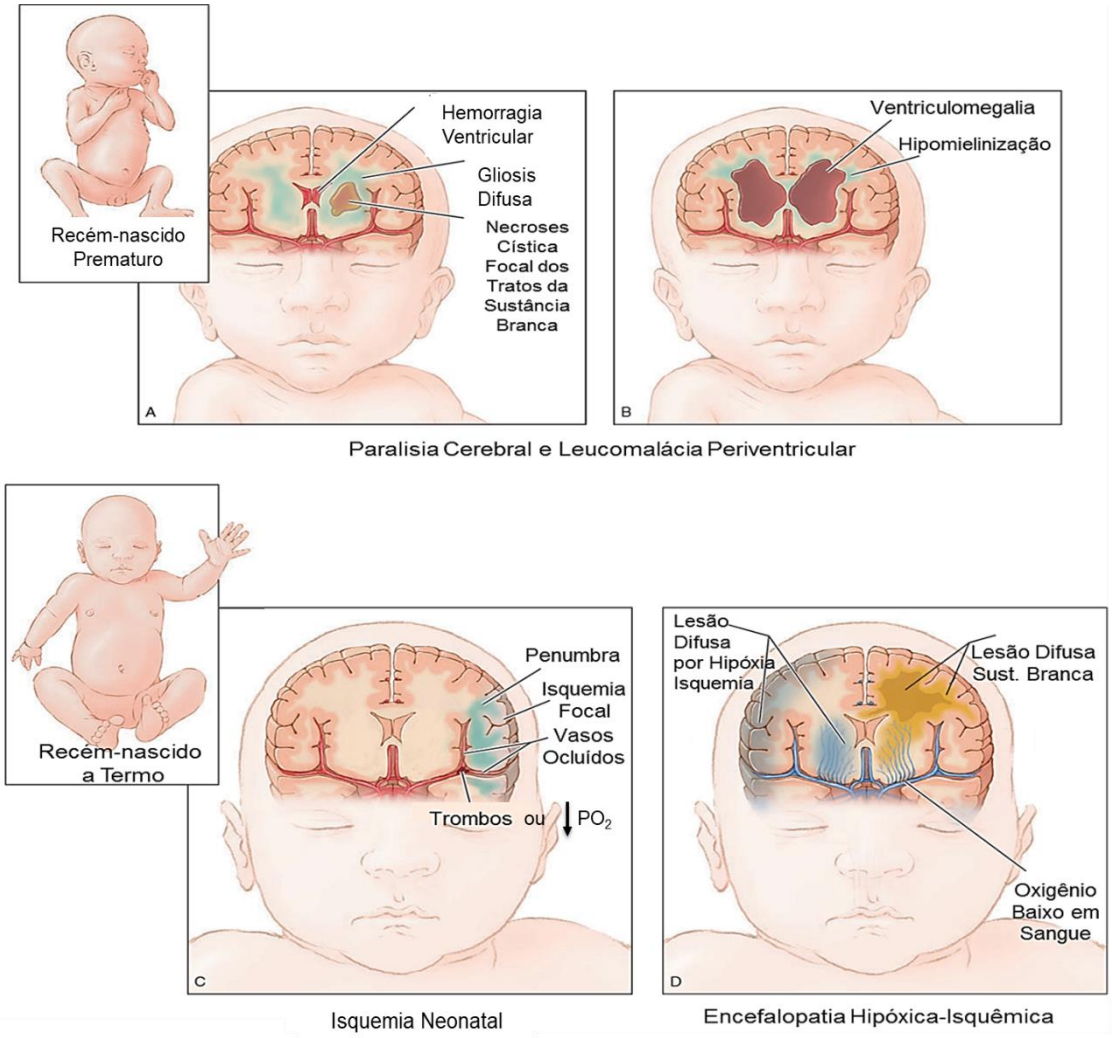


Figura 1: Ilustração de lesões cerebrais que comumente afetam recém-nascidos prematuros (A,B) e crianças nascidas a termo (C,D) [Adaptado de (Silbereis et al., 2010)].

Os mecanismos fisiopatológicos da HI são divididos em três fases: primária, secundária e terciária (Figura 2), cujas manifestações clínicas progridem após um período de horas a dias, e podem expressar tanto o metabolismo encefálico quanto a cascata de eventos neuroquímicos responsáveis pela lesão cerebral (Hassell et al., 2015).

Fase primária:

A falha energética primária é caracterizada pela redução do fluxo sanguíneo e pela queda do suprimento de glicose e oxigênio. A redução das moléculas fosforiladas de alta energia, como o ATP e a fosfocreatina, favorece o metabolismo anaeróbico; essa mudança metabólica promove o acúmulo de lactato e íons hidrogênio (H^+), que progressivamente comprometem o potencial de regulação vascular e favorecem a acidose do tecido (Johnston et al., 2001a, 2001b).

Estudos pré-clínicos têm mostrado que a diminuição de substratos energéticos após a lesão compromete a atividade da bomba Na+/K+ ATPase, resultando no acúmulo intracelular de sódio (Na^+), cálcio (Ca^{++}) e água (edema tóxico), seguido da despolarização excessiva da membrana e liberação de neurotransmissores excitatórios, especialmente glutamato (Ferriero, 2001; Back et al., 2002; McLean and Ferriero, 2004). O aumento nas concentrações de glutamato extracelular e a ativação de seus receptores, principalmente do subtipo N-metil-D-aspartato (NMDA), desencadeiam uma cascata “excitotóxico-oxidativa” que repercute na estabilidade sináptica, na plasticidade neuronal dependente da atividade e na excitabilidade do hipocampo, do córtex cerebral e de outras estruturas cerebrais (i.e. estriado e cerebelo) relacionadas com aprendizado e a memória, processos nos quais os receptores ionotrópicos de glutamato têm uma importante participação (Hagberg et al., 2002).

Os efeitos deletérios do aumento do Ca^{++} livre no citosol pela ativação excessiva dos receptores de NMDA, despolarização da membrana e liberação de Ca^{++} dos estoques intracelulares incluem alteração na respiração mitocondrial

pela ativação da enzima óxido nítrico sintase neuronal, que leva à liberação do radical óxido nítrico (NO) e degradação de lipídios, proteínas e DNA celular pela ativação de fosfolipases, proteases e nucleases, respectivamente (Hagberg et al., 2002; Cross et al., 2010). Sinais liberados pelas mitocôndrias danificadas podem estimular a liberação do citocromo C ao citosol, favorecendo a ativação das caspases 9 e 3 (enzimas ativadas durante a HI) que dão início à apoptose neuronal (morte celular programada) desde que o fornecimento de energia persista. A exaustão do suprimento energético e os efeitos combinados da acidose, da excitoxicidade, da neurotoxicidade por óxido nítrico (NO) e do acúmulo de Ca^{++}, são elementos importantes que levam à necrose celular (Hagberg et al., 2009). A intensidade da morte celular observada dependerá extensão da lesão e do estágio de maturidade das células afetadas (Yang and Lai, 2011).

Ao ser reestabelecido o fluxo sanguíneo (reperfusão), uma segunda onda de dano celular acontece. Durante esse período, o oxigênio volta a estar disponível para as células e causa exacerbação na produção de radicais livres, como o NO, bem como reações inflamatórias mediadas pela liberação de interleucinas e lesão da unidade microvascular (Khwaja and Volpe, 2008). Assim, danos à membrana celular podem agravar os eventos negativos através da liberação aumentada de radicais livres, que são capazes de atuar indiretamente na sinalização redox e ativar a apoptose. Ao mesmo tempo, os leucócitos podem ligar-se ao endotélio de pequenos capilares obstruindo-os e levando a piora da isquemia, o que exacerba o dano tecidual (Berger and Garnier, 1999).

Fase Secundária

A falha energética secundária acontece entre 6 a 24 horas após a lesão e é caracterizada pelo início das convulsões. Essa fase difere da primária porque o declínio nos níveis de fosfocreatina e de ATP não são acompanhados de acidose (Lorek et al., 1994). Sua patogênese envolve edema tóxico, acúmulo de citocinas, falha mitocondrial, apoptose e níveis alterados de fatores de crescimento e de síntese proteica (Hassell et al., 2015).

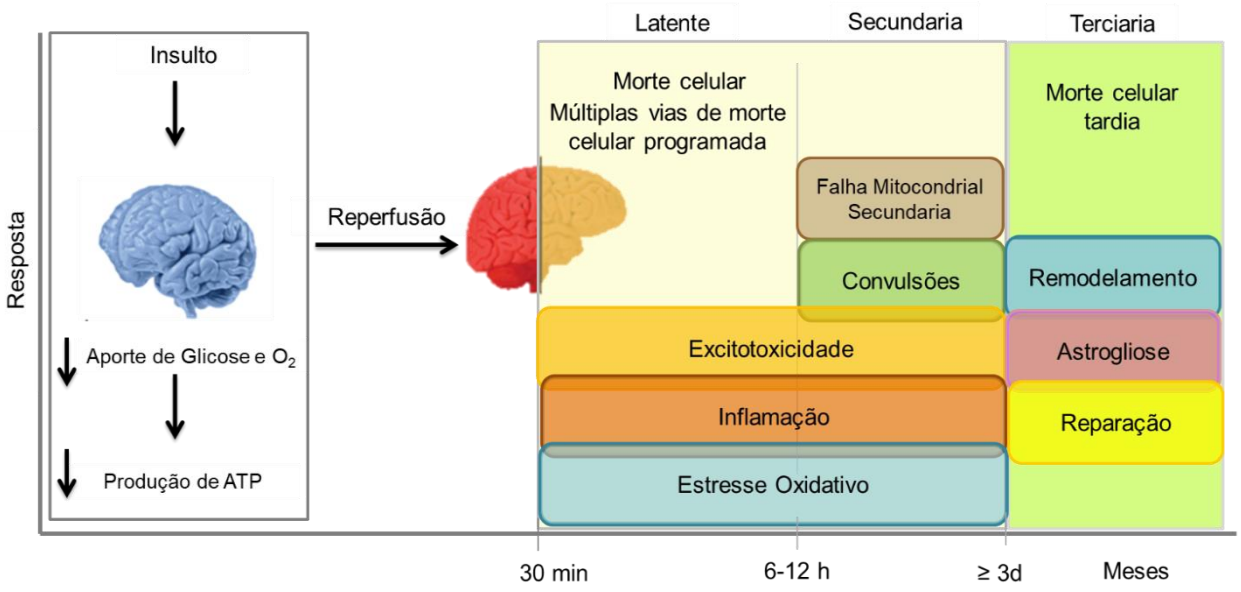


Figura 2: Esquema geral das características fisiopatológicas da resposta ao evento hipóxico-isquêmico [Adaptado de (Douglas-Escobar and Weiss, 2015)].

Fase Terciária

A lesão cerebral terciária refere-se a processos patológicos ativos que ocorrem por semanas, meses e anos após o insulto hipóxico-isquêmico. Os mecanismos de dano persistente envolvem gliose, ativação dos receptores inflamatórios e modificações epigenéticas (Hassell et al., 2015).

A ativação dos astrócitos tem como objetivo preservar o tecido neuronal e prevenir um maior dano na função do SNC, pois estes regulam a concentração de íons e neurotransmissores no meio extracelular, previnem a inflamação excessiva e apresentam um efeito neuroprotetor ao inibir a atividade da microglia e favorecer a liberação de fatores neurotróficos importantes na reparação e proliferação celular (Villapol et al., 2008; Sonnewald, 2014). Entretanto, em estágios avançados da lesão, os astrócitos podem formar cicatrizes durante o rearranjo celular e molecular do tecido (Sofroniew, 2009; Anderova et al., 2011). A barreira física criada pela cicatriz glial pode dificultar os axônios de atingir seu comprimento total e impedir que novos contatos axonais aconteçam, afetando o processo normal de mielinização e a comunicação axônio-glia (Franklin and Ffrench-Constant, 2008). As células da micróglia estão presentes em grande número na substância branca periventricular em desenvolvimento (Burda and Sofroniew, 2014) e em volta dos axônios em degeneração. Além disso, a micróglia é responsável por fagocitar às células em necrose e apoptose e a sua ativação excessiva pode afetar o funcionamento do tecido nervoso e parece estar relacionada aos distúrbios de longo prazo observados nos bebês prematuros (Zhu et al., 2014).

1.3 Metabolismo Encefálico

O metabolismo encefálico apresenta grandes variações durante o desenvolvimento, apesar dos níveis circulantes de glicose se manterem sem muitas alterações (Brekke et al., 2014). A taxa metabólica na substância cinzenta do neonato é muito menor que a do adulto, embora possa ser maior na substância branca. Esta diferença parece ser responsável pela capacidade do cérebro imaturo de suportar períodos prolongados de hipóxia (McKenna et al., 2012).

Em condições fisiológicas, a glicose é essencialmente o único substrato energético para o encéfalo adulto. Em contrapartida, o consumo encefálico de glicose em roedores neonatos é de aproximadamente 50% do encéfalo adulto, sendo utilizadas outras fontes energéticas como os corpos cetônicos, acetato e lactato (Brekke et al., 2014). Estima-se que o metabolismo dos corpos cetônicos possa fornecer 30% da energia total em roedores durante o período de amamentação e 48% da demanda energética em ratos no DPN 16, sendo um dos principais substratos nesse período, enquanto o consumo de glicose é de apenas 12% no DPN 7 e 28% no DPN 14, quando comparado com os níveis de ratos adultos (Vannucci et al., 1994; Brekke et al., 2014).

O consumo encefálico de glicose durante o desenvolvimento está diretamente relacionado com os níveis de transportadores de glicose (GLUT), sendo este um fator limitante na taxa de utilização deste substrato energético no período pós-natal precoce. Entre os principais transportadores estão o GLUT1, presente nas células gliais e na barreira hematoencefálica (BHE) e o GLUT3 encontrado predominantemente nos neurônios (Vannucci et al., 1994). A baixa

utilização de glicose no período neonatal coincide também com baixa atividade das enzimas da via glicolítica e do ciclo do ácido tricarboxílico (comumente referido como ciclo de Krebs) (Brekke et al., 2014).

Durante a lactação o consumo de leite com alto conteúdo de gordura, assim como de corpos cetônicos derivados dos lipídeos, suprem grande parte das necessidades energéticas do cérebro em desenvolvimento (Nehlig, 2004), Isso pode estar relacionado com os transportadores de monocarboxilato (MCT), como o MCT-1, o qual está mais expresso na BHE e a nas células glias do cérebro neonatal com relação à idade adulta (Vannucci and Simpson, 2003).

Como mencionado anteriormente, a HI causa uma diminuição no suprimento de oxigênio e glicose ao cérebro, prejudicando o metabolismo oxidativo mitocondrial e os níveis de energia na célula. Alterações agudas no metabolismo energético e sua desregulação prolongada durante o período neonatal podem aumentar a vulnerabilidade encefálica e comprometer a maturação e o desenvolvimento de processos essenciais no SNC, tendo em consideração que o aumento do consumo de glicose durante o período pós-natal está correlacionado com a aquisição de competências neurológicas ao nível funcional. Assim, modificações no metabolismo encefálico podem ser os responsáveis pelos diferentes graus de incapacidade após a lesão (Nehlig, 2004; McKenna et al., 2015).

No modelo experimental de HI neonatal (vide abaixo) os níveis de glicose após o insulto são inicialmente mais baixos no hemisfério ipsilateral à oclusão da carótida, porém estes valores aumentam acima dos níveis basais em ambos hemisférios na primeira hora após a HI, assim 30 minutos após a lesão há um

aumento de 26% nos níveis de glicose no hemisfério ipsilateral e de 44% no contralateral, indicando uma reperfusão rápida e possivelmente um aumento na captação de glicose como mecanismo compensatório (Brekke et al., 2014). Além disso, os níveis de transportadores de glicose são afetados pela HI, com aumento da expressão do GLUT1 tanto no hemisfério ipsi como contralateral à lesão, com níveis ainda mais elevados 24 horas após a HI no hemisfério ipsilateral. A expressão de GLUT3 (transportador neuronal) apresenta um aumento nas regiões perilesionais, mas é reduzida rapidamente nas áreas mais afetadas como o córtex e o hipocampo (Morken et al., 2014). Esses achados experimentais estão em concordância com dados reportados em infantes a termo acometidos pela HI, os quais tem mostrado uma correlação inversa entre a severidade da lesão e o metabolismo de glicose (Thorngren-jerneck et al., 2001). É importante salientar que embora o fluxo sanguíneo cerebral não esteja reduzido no hemisfério contralateral neste modelo, a exposição dos animais a condições hipóxicas poderia explicar várias alterações no metabolismo encefálico no hemisfério contralateral nas primeiras 48 horas após HI. Isso torna ainda mais interessante o estudo do metabolismo da glicose no cérebro em desenvolvimento e as possíveis associações com os prejuízos induzidos por lesões neonatais (Brekke et al., 2017).

A técnica de microtomografia por emissão de pósitrons (μPET) com o radiofármaco 18F-fluordesoxiglicose (18F-FDG) tem sido amplamente utilizada para investigar o metabolismo cerebral em pacientes com alterações do SNC; e recentemente seu uso foi estendido a animais de pequeno porte. Essa metodologia é análoga àquela disponível clinicamente, facilitando assim a translação dos resultados experimentais (Zanirati et al., 2018).

1.4 Modelo Experimental da Hipóxia-Isquemia

A hipóxia-isquemia é uma condição patológica complexa, que envolve diversos mecanismos fisiopatológicos, tornando relevante o uso de modelos experimentais para a compreensão dos diversos eventos moleculares e celulares pós-lesão e para a avaliação de novas estratégias preventivas e/ou terapêuticas. Com esse intuito, ratos e camundongos são os animais mais comumente utilizados (Yager, 2004; Rumajogee et al., 2016).

O modelo experimental de hipóxia-isquemia desenvolvido por Levine em 1960 e posteriormente modificado por Rice e colaboradores (1981) tem sido utilizado com sucesso no intuito de reproduzir o insulto hipóxico-isquêmico neonatal ocorrido em humanos (Figura 3). O modelo consiste na indução da isquemia, gerada pela oclusão unilateral de uma das artérias carótidas comuns (carótida direita, utilizada na presente Tese), seguida pela exposição a uma atmosfera hipóxica dentro de uma câmara (kitasato), a qual fornece 8% de O_2 e 92 % de N_2, com fluxo de 5 L/min, simulando a combinação de hipoxemia e isquemia encontrada em neonatos humanos após um quadro de asfixia (Levine, 1960; Rice et al., 1981). Este modelo é vantajoso por apresentar alta reprodutibilidade, baixo custo e baixa mortalidade, além de produzir dano em 90% dos animais e permitir a recuperação do fluxo sanguíneo mesmo com a oclusão da artéria carótida (Vannucci. and Vannucci., 2005; Weis et al., 2011).

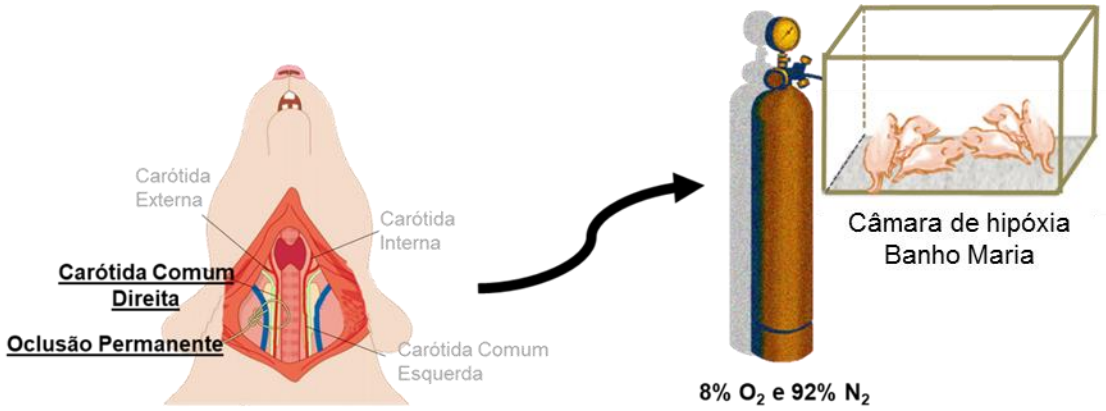


Figura 3: Desenho esquemático do modelo de HI Rice-Vannuci. Fonte do autor.

O modelo de HI foi descrito por Rice-Vannucci em animais no sétimo dia pós-natal (DPN 7), quando o desenvolvimento encefálico do rato é semelhante ao do recém-nascido a termo. Essa idade é caraterizada por um pico de crescimento encefálico e gliogênese; aumento da densidade axonal e dendrítica; maior maturação de oligodendrócitos; e a consolidação do sistema imunitário, sendo equivalentes aos processos encontrados em humanos entre a 36ª e 40ª semana de gestação (Semple et al., 2013). Uma variante desse modelo caracteriza-se por realizar a HI no terceiro dia pós-natal (DPN 3), com o intuito de entender os efeitos da lesão em recém-nascidos prematuros, uma vez que nesta idade os ratos exibem maturação encefálica semelhante àquela observada em humanos com 24-28 semanas de gestação (Sizonenko et al., 2003, 2005; Van De Looij et al., 2011). Apesar da diferença de idade, ambos protocolos causam déficits funcionais e lesões no hemisfério ipsilateral à artéria carótida ocluída em regiões como o córtex cerebral e o hipocampo, sendo o hemisfério contralateral muito pouco, ou mesmo nada, afetado morfologicamente (Pereira et al., 2007; Arteni et al., 2010). A HI no DPN 3 causa ventriculomegalia e lesiona preferencialmente áreas mielinizadas e corticais, como o corpo caloso e o córtex parietal, devido à vulnerabilidade na maturação-dependente da linhagem

progenitora de oligodendrócitos (pré-OLS) caraterística desse período de desenvolvimento (Cai and Rhodes, 2001; Sizonenko et al., 2003; Stadlin et al., 2003), o que confirma a sua semelhança com o padrão de lesão observado em humanos prematuros após um evento hipóxico-isquêmico. Os oligodendrócitos (OLS) são células responsáveis pela mielinização do SNC, pelo desenvolvimento trófico axonal, pela sobrevivência e pelo funcionamento neural. Por isso, a sua ausência ou diminuição durante o desenvolvimento acarreta deficiências de mielinização, podendo ser esta uma das causas dos déficits comportamentais apresentados pelos animais (Back et al., 2001; Sizonenko et al., 2005; Back, 2006).

Estudos prévios demonstram que a HI nesta idade causa redução no volume hemisférico em regiões como o corpo caloso (estrutura altamente mielinizada e fundamental aos processos cognitivos), associada a prejuízos de memória aversiva, espacial e de trabalho (Huang et al., 2009; Sanches et al., 2013a, 2013b; Alexander et al., 2014). Alterações motoras têm sido observadas em animais submetidos a HI no DPN 3 quando expostos a concentrações de oxigênio de 6% ou quando associado a estímulos inflamatórios (Girard et al., 2009; Misumi et al., 2016). Estudos são necessários para determinar se a exposição a um período de hipóxia mais elevado, com uma concentração de oxigênio de 8% (comumente utilizada no modelo de HI), causa as alterações sensoriomotoras observadas clinicamente em recém-nascidos pré-termo.

A HI no DPN 3 prejudica o processo de mielinização aumentando a astrogliose e a microgliose durante a fase inicial (72 horas) e a cicatriz glial (44 dias após o insulto) (Cai et al., 2006). Além disso, as espécies reativas de oxigênio/nitrogênio e as citocinas pró-inflamatórias têm sido relacionadas a um

dano de início tardio (ao redor de 5 semanas) após o insulto HI (Cai et al., 2006), indicando que a lesão muda ao longo do tempo e pode ter impacto variável nas diferentes etapas do ciclo vital (Mishima et al., 2005; Tsuji et al., 2010).

Apesar da alta incidência da HI neonatal e dos esforços de pesquisadores de ciências básicas e clínicas, estratégias terapêuticas efetivas para reduzir a severidade da lesão e o impacto funcional ainda são escassas. A hipotermia durante as primeiras horas de vida é o tratamento padrão para os recém-nascidos com uma lesão hipóxico-isquêmica entre moderada e grave. Apesar dessa alternativa terapêutica melhorar o prognóstico funcional dos neonatos, seus efeitos são parciais e seu uso é limitado a recém-nascidos a termo (Davidson et al., 2015). O recém-nascido prematuro que sofre com a HI apresenta acometimento sistêmico em diferentes graus de severidade, motivo pelo qual o enfoque terapêutico se torna complexo e o neonato necessita de atenção para distintas manifestações clínicas (Nguyen et al., 2013). Estratégias não-farmacológicas como a manipulação ambiental parecem ser uma alternativa promissora devido ao seu potencial de promover mudanças estruturais e funcionais no SNC, o que favorece a preservação cerebral e a recuperação funcional após uma lesão (Nithianantharajah and Hannan, 2006).

1.5 Ambiente Enriquecido

O **Ambiente Enriquecido** (AE) é definido como a exposição de animais de laboratório a ambientes complexos que permitem atividade exploratória e oferecem possibilidades contínuas de aprendizagem, quando comparado às condições-padrão de moradia (Krech et al., 1962; Diamond, 2001; Nithianantharajah and Hannan, 2006; Rosenzweig, 2007; McDonald et al., 2018; Kempermann, 2019).

Esse paradigma tem sua origem no trabalho de Donald Hebb (1947), que mostrou que ratas mantidas em espaços abertos que facilitavam a exploração voluntária, apresentavam mais habilidades cognitivas do que animais mantidos em condições padrão de laboratório (Hebb, 1947). O termo AE foi introduzido na comunidade científica por Rosenzweig e colaboradores no ano de 1962, quando demonstraram experimentalmente os efeitos da exposição a ambientes diversos (enriquecidos versus empobrecidos). (Krech et al., 1962). Estudos posteriores enfatizaram que a exposição a experiências enriquecidas são necessárias para o bem-estar do animal, para facilitar a expressão do comportamento natural, para melhorar a função biológica do sistema nervoso e para desenvolver habilidades de resolução de problemas em animais de laboratório (Rosenzweig, 2007).

O AE caracteriza-se por gaiolas grandes que contêm rodas, túneis, escadas, abrigos e brinquedos com diversas formas, tamanhos e texturas, que são reorganizados e substituídos regularmente, a fim de fornecer maior estimulação sensorial, cognitiva, motora e social nos animais (Petrosini et al., 2009). Além disso, os animais são mantidos em grupos grandes facilitando a exposição a estímulos olfativos, visuais e auditivos de outros animais, assim

como comportamentos cooperativos e lúdicos (Mering and Jolkkonen, 2015). Algumas hipóteses têm sido levantadas para a ação do AE no SNC (Figura 4); dentre elas podemos citar a aceleração da maturação neural, a neuroproteção e a plasticidade dependente de experiência (Nithianantharajah and Hannan, 2006; Simonetti et al., 2009).

A literatura recente destaca que o AE tem a capacidade de modular estrutural e funcionalmente o SNC, de facilitar processos de recuperação após insultos cerebrais e de prevenir ou reduzir as sequelas associadas a diversos distúrbios neurológicos, quando estudados em modelos animais (Petrosini et al., 2009; Alwis and Rajan, 2014; Mering and Jolkkonen, 2015). No modelo de HI, o AE diminui parcialmente o atraso no desenvolvimento neurológico (Horvath et al., 2013; Schuch et al., 2016a), melhora a memória espacial e declarativa (Pereira et al., 2007, 2008; Rojas et al., 2013; Galeano et al., 2015) e reverte os prejuízos da função sensório-motora dos animais (Seo et al., 2013a; Marques et al., 2014; Schuch et al., 2016b; Meireles et al., 2017). No entanto, o AE apresenta efeitos limitados em relação a preservação tecidual de estruturas como o corpo caloso, o córtex, o hipocampo e o estriado (Pereira et al., 2007, 2008), sendo possível que os principais efeitos de experiências enriquecidas estejam associados a modificações plásticas que favorecem a reorganização das áreas lesionadas e a otimização das regiões cerebrais não comprometidas pelo insulto neonatal (Mering and Jolkkonen, 2015; McDonald et al., 2018).

Como mencionado anteriormente, o evento hipóxico-isquêmico inicia uma cascata de eventos moleculares e celulares associados com excitotoxicidade, estresse oxidativo e inflamação como um dos principais mecanismos responsáveis pela morte celular após lesão. Diversos protocolos usando AE no

período pós-desmame reportam efeitos benéficos sobre vários desses fatores, tais como: redução da excitotoxicidade induzida por Ca^{++} (Iuvone et al., 1996), preservação da atividade da bomba Na^{+}/K^{+} ATPase (Rojas et al., 2015), redução de radicais livres derivados do complexo derivado da NADPH oxidase-2 (Zhang et al., 2017) e normalização da atividade de enzimas antioxidantes como superóxido dismutase (Pereira et al., 2009). Além disso, o AE também exerce um papel protetor sobre o aumento da vascularização cerebral (Yu et al., 2014) e a manutenção da integridade da barreira hematoencefálica (BHE), impedindo que células inflamatórias periféricas penetrem no parênquima cerebral (Diaz et al., 2016).

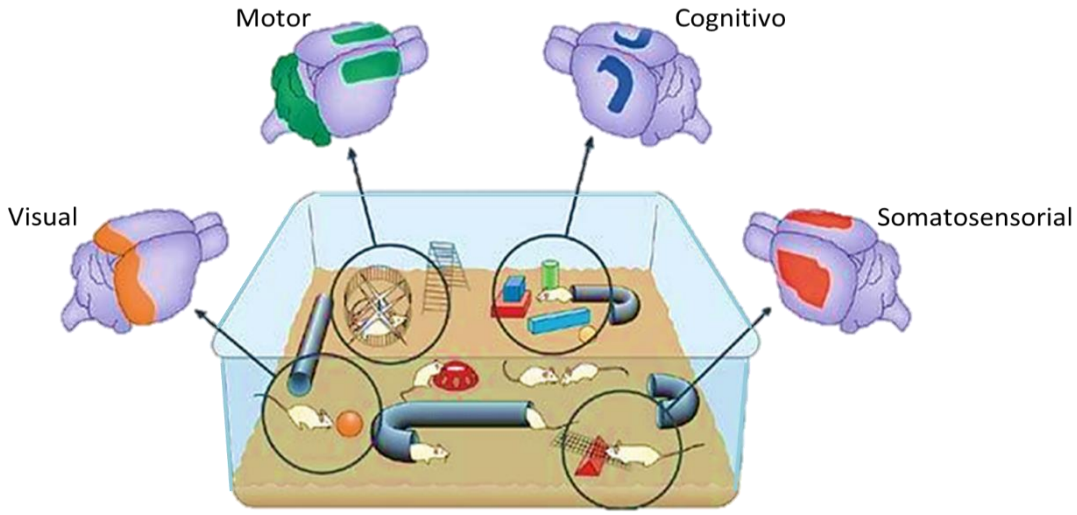


Figura 4: Desenho esquemático do Ambiente Enriquecido e regiões cerebrais ativadas pelo enriquecimento [Adaptado de (Nithianantharajah and Hannan, 2006)].

1.6 Efeitos do Ambiente Enriquecido no Período Gestacional

O dano encefálico e o comprometimento funcional após a HI resultam do equilíbrio entre os mecanismos prejudiciais (morte celular, inflamação, estresse oxidativo) e as respostas de proteção endógena do SNC (Hassell et al., 2015). Atualmente, estratégias terapêuticas focadas em potencializar as respostas de neuroproteção endógena tem-se demostrado atraentes, dado que promovem menos alterações na homeostase fisiológica, apresentam menos efeitos colaterais e podem embasar tratamentos mais efetivos em resposta a estímulos nocivos (Hassell et al., 2015). Estudos recentes também sugerem que o período gestacional favorece processos de proteção endógena ao SNC em desenvolvimento da prole, sendo esse um período crítico do desenvolvimento onde estímulos ambientes podem ser usados como prevenção primária de eventos celulares após insultos cerebrais e para reduzir as deficiências funcionais (Jiang et al., 2014; Bale, 2015; Hassell et al., 2015; Netto et al., 2018).

As condições maternas influenciam profundamente o desenvolvimento adequado do feto e podem promover resiliência precoce a condições adversas de saúde, como sugerido no Paradigma das Origens desenvolvimentistas da saúde e da doença (Gluckman et al., 2008; Heindel and Vandenberg, 2015). Em contrapartida ao amplo número de pesquisas avaliando os efeitos do ambiente gestacional adverso sobre a prole, existem poucos estudos que têm feito referência ao impacto de contextos positivos, como o ambiente enriquecido sobre a interação mãe-filho e do cérebro imaturo com seu entorno. O conceito de programação precoce indica que um insulto, ou um estímulo, durante períodos críticos do desenvolvimento pode ter efeitos negativos, ou positivos, a longo prazo tanto na estrutura como na função do organismo (Lucas, 2005). Este

processo pode ocorrer como resultado de fatores ambientais sugerindo, por exemplo, que a plasticidade cerebral pode ser incrementada durante a interação com diferentes estímulos e permitir adaptações individuais do SNC baseadas na experiência, ainda no período pré-natal (Baroncelli et al., 2009; Horvath et al., 2015; Sale, 2018).

Outro conceito que abrange a plasticidade dependente da experiência é o de reserva cognitiva, o qual postula que os sujeitos podem desenvolver mudanças estruturais e funcionais no cérebro capazes de amenizar os efeitos de eventos negativos, reduzir o risco de maiores deficiências ou desenvolver a capacidade de absorver danos maiores antes de atingir o limiar para a expressão clínica ou funcional. Muito embora o conceito tenha sido postulado no estudo do declínio cognitivo da maturidade e do envelhecimento, ele também pode ser evocado em períodos precoces do desenvolvimento. Tal reserva varia entre os indivíduos e pode ser inata ou modulada durante o ciclo vital por fatores ambientais que contribuem para a construção de circuitos neuronais mais eficientes e flexíveis, podendo ser avaliada em nível comportamental e indicando a existência de certa resiliência cerebral (Petrosini et al., 2009).

Em 1993, Satz mencionou que existem dois tipos de reserva cognitiva: uma reserva estrutural passiva e uma funcional ativa. A primeira está baseada no potencial de proteção segundo características anatômicas tais como tamanho do cérebro, número de neurônios, densidade e tamanho dos espinhos dendríticos. A segunda trata da eficiência existente nos circuitos neuronais, que são reforçados pelo uso repetitivo e permitem o processamento, armazenamento e recuperação da informação. O desenvolvimento destas reservas está relacionado a fatores ativos nas primeiras fases da vida e à interação materna e

ou social as quais os indivíduos estão expostos continuamente (Satz, 1993). Além disso, existem fatores epigenéticos que indicam como os fatores ambientais interagem ao nível do sistema nervoso para promover a maturação e/ou a reorganização dependente da experiência ao modificar a programação genética do crescimento das áreas cerebrais e dos circuitos neuronais importantes na regulação do comportamento e da função (Bock et al., 2014; Matas et al., 2016).

Estudos pré-clínicos utilizando AE prévio ou durante a gestação têm mostrado que a prole de mães expostas ao protocolo de enriquecimento ambiental apresenta modificações ou caraterísticas benéficas como: aceleração da maturação e funcionalidade de circuitos sensoriais (Cancedda et al., 2004; Sale et al., 2004, 2007; Cárdenas et al., 2015), melhora do aprendizado e da memória (Cutuli et al., 2015; Zuena et al., 2016), melhora na função sensório - motora (Maruoka et al., 2009; Caporali et al., 2014; Zuena et al., 2016), redução da ativação do eixo hipotalâmico-pituitário–adrenal (Welberg et al., 2006), diminuição da expressão de receptores de glicocorticóides (Connors et al., 2014), redução do dano hipocampal induzido por estresse pré-natal (Zheng et al., 2013), modulação de comportamentos do tipo ansioso (Dell and Rose, 1987; Rosenfeld and Weller, 2012; Zuena et al., 2016), modulação da proliferação celular (Maruoka et al., 2009) e redução da metilação global do DNA (Mychasiuk et al., 2012). Apesar dos achados acima mencionados destacarem o papel do AE durante a gestação sobre o SNC do recém-nascido e sobre sua capacidade de responder a diferentes estímulos, os efeitos do AE sobre os déficits induzidos pela HI neonatal na prole ainda não foram estudados.

1.7 Efeitos do Ambiente Enriquecido no Período Lactacional

Estratégias terapêuticas durante o período de recuperação pós-lesão são utilizadas com o objetivo de promover um melhor prognostico funcional dos sujeitos (Nithianantharajah and Hannan, 2006). Pesquisas nas quais o AE é utilizado como estratégia de intervenção experimental antes ou após um evento hipóxico-isquêmico, frequentemente utilizam o período após o desmame, a fim de não interferir com o cuidado maternal e devido à capacidade dos animais de se movimentar de maneira voluntária, aumentando a exploração e a interação com o ambiente (Adriani et al., 2006; Pereira et al., 2007; Komitova et al., 2013).

O primeiro estudo investigando os efeitos do AE após a HI por meio do modelo de Rice-Vannucci foi publicado pelo grupo de pesquisa de Isquemia Cerebral da Universidade Federal do Rio grande do Sul (UFRGS), demostrou que a exposição diária (1 hora / durante 9 semanas) ao AE reverte os déficits de memória espacial quando avaliado no labirinto aquático de Morris, sem evidência de preservação tecidual no córtex nem no hipocampo (Pereira et al., 2007). Estudos posteriores usando modelos semelhantes confirmaram que o AE aumenta a função social, motora e cognitiva dos animais (Adriani et al., 2006; Pereira et al., 2008; Zhang et al., 2016) e causa alterações estruturais e moleculares relacionadas à plasticidade sináptica (Salmaso et al., 2012; Komitova et al., 2013; Griva et al., 2017).

Experiências enriquecidas durante a amamentação são associadas ao cuidado materno e a aspectos como temperatura, estimulação tátil e suporte nutricional (Di Segni et al., 2017). O cuidado maternal tem uma grande influência no desenvolvimento do cérebro da prole, sendo responsável por modificações

epigenéticas, regulação do crescimento físico, maturação neural, organização cerebral e desempenho comportamental (Liu et al., 2000; Kappeler and Meaney, 2010). No entanto, estudos recentes envolvendo AE durante a lactação e HI neonatal não avaliavam o comportamento materno como um mecanismo relacionado aos efeitos benéficos do AE (Diaz et al., 2016; Schuch et al., 2016a). Modificações comportamentais em parâmetros relacionados ao neurodesenvolvimento e a função cognitiva dos animais têm sido descritas, sugerindo que o AE no período prévio ao desmame pode ser uma estratégia promissora (Pereira et al., 2008; Horvath et al., 2013; Kiss et al., 2013; Schuch et al., 2016a, 2016b). No entanto, os efeitos protetores do AE durante a lactação sobre o modelo de HI neonatal no DPN 3 ainda não foram abordados.

1.8 Dimorfismo Sexual

Recentemente o **Dimorfismo Sexual** tem sido objeto de diversas pesquisas devido à sua relevância para o estudo do SNC, devido que machos e fêmeas têm diferentes respostas comportamentais, morfológicas e neuroquímicas tanto em condições fisiológicas como em diversas patologias (Cahill and Aswad, 2015). Apesar das evidências clínicas e pré-clínicas sobre as respostas distintas entre sexos a fatores extrínsecos como estímulos ambientais, administração de fármacos, nutrição, entre outros, com frequência os estudos são apresentados sem estratificação sexual durante as análises e as fêmeas eram em grande parte excluídas, com exceção das pesquisas acerca de comportamentos reprodutivos (Flórez-Vargas et al., 2016).

Doenças como Alzheimer (Barnes et al., 2005), esquizofrenia (Lindamer et al., 1999) e depressão (Gorman and Read, 2006) apresentam maior incidência em mulheres do que em homens. Por outro lado, os homens são mais propensos a serem diagnosticados com perturbações do desenvolvimento neurológico (Gualtieri et al., 1985; Lauterbach et al., 2001; Donders and Hoffman, 2002; Rutter et al., 2003) e a apresentar maior suscetibilidade a insultos graves após a HI, tendo seus déficits cognitivos e comportamentais exacerbados (Hindmarsh et al., 2000; Kesler et al., 2008; Kent et al., 2012; Peacock et al., 2012). A influência do sexo sobre lesões do SNC não é observada somente na idade adulta, onde o efeito hormonal seria esperado. A paralisia cerebral e a deficiência intelectual por exemplo, são mais comuns em homens (Johnston and Hagberg, 2007). Estes ainda mostram maior incidência de prematuridade, anóxia, hemorragia intraventricular e morte por prematuridade (Lauterbach et al., 2001; Mayoral et al., 2009; Raz et al., 2010; Peacock et al., 2012).

Um estudo de revisão sobre o dimorfismo sexual em parâmetros comportamentais e neuroquímicos das ratas, os autores relatam que dentre 443 artigos publicados entre outubro de 2010 e 2011, apenas 17% empregaram ratas fêmeas, 11% usaram ambos os sexos, e a maioria (72%) utilizou apenas ratos machos (Simpson and Kelly, 2011). Outros achados comportamentais destacam o fato de as fêmeas serem mais ativas que os machos no campo aberto e no labirinto em cruz elevado e apresentarem taxas inferiores de aprendizado no labirinto aquático (Simpson and Kelly, 2011).

Evidências recentes sobre HI neonatal têm demonstrado que há diferenças entre os sexos tanto na extensão da lesão quanto na resposta a diferentes tratamentos (para revisão ver: (Netto et al., 2017; Charriaut-Marlangue et al.,

2018). É interessante notar que a expressão e a atividade de enzimas mitocondriais parece ser o ponto central do dimorfismo sexual após isquemia e hipóxia-isquemia neonatal (Dukhande et al., 2009; Weis et al., 2012). A lesão isquêmica produz efeitos diferentes sobre a função mitocondrial em machos e fêmeas a curto prazo, que envolve a morte celular e vias neuroinflamatórias distintas, levando a déficits motores e cognitivos variados na idade adulta (Demarest et al., 2016a, 2016b). Em termos gerais, a apoptose é mais robusta no sexo feminino, enquanto a necrose é a via mais importante no sexo masculino (Netto et al., 2017). Além disso, os machos apresentam um aumento na ativação da microglia e de respostas inflamatórias periféricas em relação as fêmeas, o que aprofunda a gravidade da lesão (Jin et al., 2010; Patel et al., 2013). Contudo, os mecanismos biológicos subjacentes a essas diferenças sexo-específicas não estão totalmente esclarecidos e certamente demandam mais investigação experimental. Atualmente, torna-se cada vez mais importante obter informações sobre a influência do sexo em diferentes modelos de lesão encefálica assim como na resposta do SNC a diferentes estratégias terapêuticas, visto que mecanismos fisiopatológicos distintos podem implicar em estratégias diferenciadas, a fim de promover uma maior proteção e/ou recuperação.

Zuena e colaboradores (2016) reportaram que o AE no período pós-natal também apresenta uma resposta sexo-específica, possivelmente mediada por modificações no cuidado materno. Seus resultados revelaram um aumento no comportamento do tipo ansioso apenas em machos, e uma melhora na capacidade de aprendizado apenas nas fêmeas (Zuena et al., 2016). Sob condições de lesão no SNC, a exposição ao AE também mostrou respostas dependentes do sexo. No modelo de traumatismo crânio-encefálico (TCE), por

exemplo, as ratas fêmeas apresentaram melhor resposta à estimulação ambiental quando comparadas aos machos, em parâmetros relacionados com a função do sistema dopaminérgico (Wagner et al., 2005). Em animais submetidos à HI, o enriquecimento durante 22 dias (iniciado 24 horas depois da lesão) melhorou o desempenho de ratos de ambos sexos na tarefa de reconhecimento de objetos; enquanto a memória de trabalho no labirinto aquático revelou efeitos benéficos da estimulação ambiental apenas em fêmeas (Pereira et al., 2008).

1.9 Hipótese de Trabalho

Considerando os efeitos positivos da exposição ao ambiente enriquecido no modelo de hipóxia-isquemia neonatal, a hipótese da presente Tese é que o enriquecimento ambiental durante o período gestacional e/ou lactacional promove alterações encefálicas que podem reduzir os prejuízos comportamentais, moleculares, metabólicos e morfológicos da HI, quando realizada no terceiro dia pós-natal do rato, mimetizando condições de prematuridade no humano. Também testamos a hipótese de que o ambiente enriquecido durante esses períodos críticos do neurodesenvolvimento elicita mecanismos neuroprotetores de maneira sexo-específica.

2. OBJETIVOS

2.1 Objetivo Geral

Avaliar os efeitos da exposição ao ambiente enriquecido no período gestacional e lactacional sobre os prejuízos comportamentais, moleculares, celulares e morfológicos induzidos pela HI, no terceiro dia pós-natal do rato.

2.2 Objetivos Específicos

- Comparar diferentes tempos de exposição à hipóxia (120, 180 e 210 minutos) em animais submetidos ao modelo de hipóxia-isquemia (HI) no terceiro dia pós-natal (DPN3), através da avaliação da função sensório-motora, do volume de preservação tecidual no estriado e da área de seção transversal do musculo esquelético na idade adulta, a fim de padronizar o grau de lesão responsável por mimetizar os prejuízos motores observados em neonatos humanos nascidos prematuramente;

- Determinar os efeitos da exposição continua ao ambiente enriquecido durante os períodos gestacional e lactacional em ratos submetidos à hipóxia-isquemia no terceiro dia pós-natal (DPN3) sobre parâmetros celulares relacionados à morte celular por apoptose, sobrevivência celular, reatividade astrocitária e a expressão de fatores de crescimento e neurotróficos, durante a fase aguda da lesão (48h pós-HI). Além disso, avaliar os efeitos do enriquecimento ambiental no comportamento maternal, na atividade reflexa da prole, na função cognitiva dos animais e no volume de lesão na idade adulta (DPN60);

- Verificar na idade adulta os efeitos do ambiente enriquecido no período gestacional e lactacional sobre o comportamento motor dos animais submetidos à hipóxia isquemia;

- Avaliar a possível influência do dimorfismo sexual sobre parâmetros funcionais, expressão de fatores de crescimento e neurotróficos, reatividade astrocitária e volume de lesão, quando avaliados na idade adulta (DPN60). Além disso, verificar se os efeitos do ambiente enriquecido são dependentes da estimulação durante a gestação, a lactação ou a combinação de ambos os períodos, em animais submetidos à hipóxia-isquemia (HI) no dia pós-natal 3;

- Caracterizar o metabolismo cerebral *in vivo* dos animais submetidos à HI e ao ambiente enriquecido no período gestacional e lactacional por meio de microtomografia por emissão de pósitrons (μPET) e estabelecer possíveis associações com parâmetros funcionais e histológicos.

3. METODOLOGIA

3.1 Aspetos Éticos

Os procedimentos realizados na presente estão de acordo com a lei nº 11,794 de 8 de outubro de 2008, diretrizes do CONCEA e os Princípios Internacionais Orientadores para a Pesquisa Biomédica Envolvendo Animais (*Council for International Organizations of Medical Sciences* [CIOMS] NIH publication 85-23, 1985).

Além disso, foram providos todos os cuidados necessários antes e após a cirurgia, e os protocolos experimentais utilizados neste estudo foram aprovados pela Comissão de Ética no Uso de Animais (CEUA) da Universidade Federal do Rio Grande do Sul, sob o número 28641.

3.2 Animais

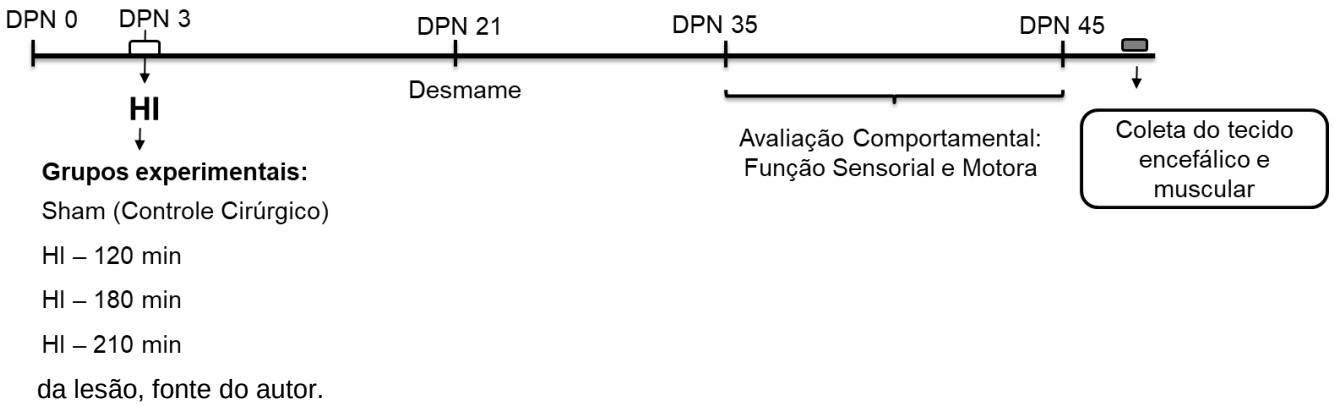
Ratos Wistar foram obtidos do Biotério do Departamento de Bioquímica da Universidade Federal do Rio Grande do Sul. Os animais foram mantidos com alimentação padrão e água *ad libitum*, e ciclos de 12h claro e escuro em salas climatizadas (22ºC ± 2ºC).

Padronização do grau de lesão

A fim de determinar o grau de lesão responsável por afetar a função sensoriomotora dos animais, foram comparados diferentes tempos de exposição à hipóxia. Para isso, filhotes de ambos os sexos foram submetidos ao modelo de hipóxia-isquemia (descrito posteriormente) no terceiro dia pós-natal. Após o procedimento cirúrgico os animais foram aleatoriamente expostos a um ambiente hipóxico durante 120, 180 ou 210 minutos. A partir do DPN 35 a função sensorial e motora dos animais foi avaliada, por meio dos testes de preensão,

remoção de adesivo, cilindro e escada horizontal (Figura 5). Uma vez que os testes comportamentais foram completados, os encéfalos foram removidos e os músculos bíceps braquial e tibial anterior esquerdo (lado contralateral a oclusão a carótida) foram coletados para posterior análise. Uma descrição detalhada encontra-se no capítulo 1 da seção de resultados.

Figura 5: Representação esquemática do desenho experimental usado durante a padronização da lesão, fonte do autor.



Exposição ao ambiente enriquecido

Durante a execução dos experimentos utilizando o ambiente enriquecido foi levado em consideração o protocolo de acasalamento reportado previamente por Rosenfeld e Welle (2012). Assim, um grupo de 6 ou 8 fêmeas Wistar virgens com idade superior a 60 dias foram mantidas em caixas convencionais e a fase de estro foi monitorada pelo esfregaço vaginal (Rosenfeld and Weller, 2012). As fêmeas foram divididas aleatoriamente em dois grupos experimentais: ambiente enriquecido (AE) ou ambiente padrão (AP-controle). Após três dias de aclimatação e quando a fêmea atingiu a fase de proestro, um macho foi introduzido na gaiola por uma noite. As ratas prenhes foram identificadas no dia seguinte pela presença de espermatozóides no esfregaço vaginal, o que foi

confirmado posteriormente pelo ganho de peso. Esse dia foi definido como o primeiro dia de gestação (DG 1).

As fêmeas permaneceram juntas nas gaiolas até o vigésimo dia gestacional. Após, foram mantidas em caixas isoladas até o momento do parto, dia considerado pós-natal zero (DPN0) e foram utilizados entre 8 e 10 filhotes de ambos sexos por ninhada. O procedimento cirúrgico do modelo de HI foi feito no terceiro dia pós-natal. Entre os DPN 0 e o desmame (DPN 23) os filhotes ficaram com as suas respectivas mães, a fim de evitar os efeitos negativos da separação materna. O desempenho funcional dos animais foi avaliado em dois momentos: durante a lactação, por meio dos reflexos de neurodesenvolvimento, e na idade adulta, através de testes cognitivos e sensoriomotores.

3.3 Procedimento Cirúrgico

O modelo adaptado de Rice-Vannucci foi utilizado para induzir a HI. Para isto, no DPN 3 (Sanches et al., 2013), os filhotes de todos os grupos experimentais foram anestesiados com isoflurano (4% para indução e 1,5 % para manutenção) e submetidos a uma incisão na linha média da face anterior da região cervical; a artéria carótida comum direita foi identificada, isolada de estruturas adjacentes e ocluída com fio cirúrgico de seda 4.0 (isquemia). A assepsia da região cervical pré e pós- cirurgia foi feita com álcool iodado. Finalizado este procedimento, os animais permaneceram em recuperação sob temperatura controlada durante 15 minutos antes de serem devolvidos às caixas moradia, onde permaneceram por um intervalo de 2 horas junto às mães. Após esse tempo, grupos de 6 a 8 animais foram gentilmente colocados em uma câmara específica de controle de O_2 e expostos à atmosfera hipóxica (8% de O_2

e 92 % de N_2, com fluxo de 5 L/min) em temperatura controlada de 37ºC (Figura 3).

Como descrito anteriormente, durante o experimento de padronização do grau de lesão, os animais foram submetidos a diferentes tempos de exposição à hipóxia, sendo mantidos por 120, 180 ou 210 minutos sob condições hipóxicas. Os animais do grupo controle (*sham*), foram submetidos à anestesia e ao procedimento cirúrgico, porém sem a oclusão permanente da carótida, além, de serem mantidos sob condições atmosféricas, após o período de recuperação. Ao final da hipóxia, os animais foram retirados da câmara e devolvidos à sua caixa de origem junto às mães. Após a padronização, o período de 180 min foi utilizado nos experimentos envolvendo ambiente enriquecido, dado que este tempo causa uma lesão encefálica entre moderada e severa, além de mimetizar os prejuízos morfológicos e funcionais observados em humanos nascidos prematuramente, como detalhado no Capítulo 1 da seção de resultados.

3.4 Condições de Moradia

As ratas destinadas ao grupo de ambiente enriquecido foram alocadas em gaiolas de 1,2m x 1m x 1m que possuíam duas regiões diferentes para a colocação de comida e água, facilitando o acesso a estes locais e aumentando o interesse dos animais pela exploração ambiental. As gaiolas tinham rampas e túneis, a fim de estimular a exploração tridimensional do ambiente. Além disso, foram disponibilizados brinquedos, correntes para escalar e rodas de corrida no seu interior (Welberg et al., 2006; Li et al., 2012). As gaiolas de enriquecimento foram mantidas no mesmo biotério junto aos demais animais sob as mesmas condições-padrão e os objetos foram trocados duas vezes por semana.

Os animais do grupo controle ficaram em uma caixa convencional padrão do biotério sem nenhum tipo de brinquedo. Todas as ratas gestantes ficaram em seus respectivos grupos durante toda a gravidez. No DPN 1, os ratos foram aleatoriamente distribuídos em seus grupos experimentais, onde foram mantidos às mesmas condições durante a gestação ou foram realocados em um novo ambiente com suas respectivas mães até o desmame (DPN 23). Assim, quatro grupos foram estabelecidos considerando as condições de moradia:

1. Animais mantidos no ambiente padrão (AP) desde a gestação até o desmame, denominado como **AP-AP**. Este grupo serviu como grupo controle.
2. Animais alocados no ambiente padrão durante a gestação e realocados no período pós-natal a condições enriquecidas até o desmame, denominado como **AP-AE**. Este grupo foi usado para avaliar o efeito do ambiente enriquecido nos animais quando expostos durante o período pós-natal precoce.
3. Animais alocados no ambiente enriquecido durante a gestação e realocados no período pós-natal a condições padrão até o desmame, denominado **AE-AP**. Este grupo permitiu avaliar os efeitos do ambiente enriquecido durante o período gestacional.
4. Animais mantidos em condições enriquecidas desde a gestação até o desmame, denominados como **AE-AE**. Este grupo foi incluído para avaliar os efeitos da combinação de EE em ambos estágios do neurodesenvolvimento.

Para os grupos alocados no AE durante a lactação, duas ninhadas foram mantidas juntas com o propósito de aumentar a interação social entre as mães.

Já no grupo controle, foi mantida uma ninhada por caixa durante esse período. As características gerais do desenho experimental utilizando o ambiente enriquecido durante a gestação e lactação são apresentadas na Figura 6. No entanto, uma descrição detalhada encontra-se no respectivo capítulo da seção de resultados. Após o desmame todos os animais foram mantidos em condições padrão e separados por sexo.

3.5 Interação Mãe-Filhote

O comportamento materno durante o período lactacional foi observado na primeira semana pós-natal, em três sessões diárias (ciclo diurno às 10 horas e às 16 horas e ciclo noturno às 20 horas). Para as ninhadas alocadas no AE, foi realizada uma marcação na prole, a fim de identificar as ninhadas e observar se cada genitora estava cuidando do seu próprio filhote. Cada sessão incluía 15 observações a cada 3 min dos seguintes comportamentos:

- Comportamento dirigido ao filhote: composto de transporte do filhote, *licking/grooming*, e *nursing* nas posturas cifótica, coberta (na qual a mãe deita sobre os filhotes) e passiva (onde a mãe fica de costas ou de lado para os filhotes mamarem).
- Localização do filhote dentro ou fora do ninho (Welberg et al., 2006).

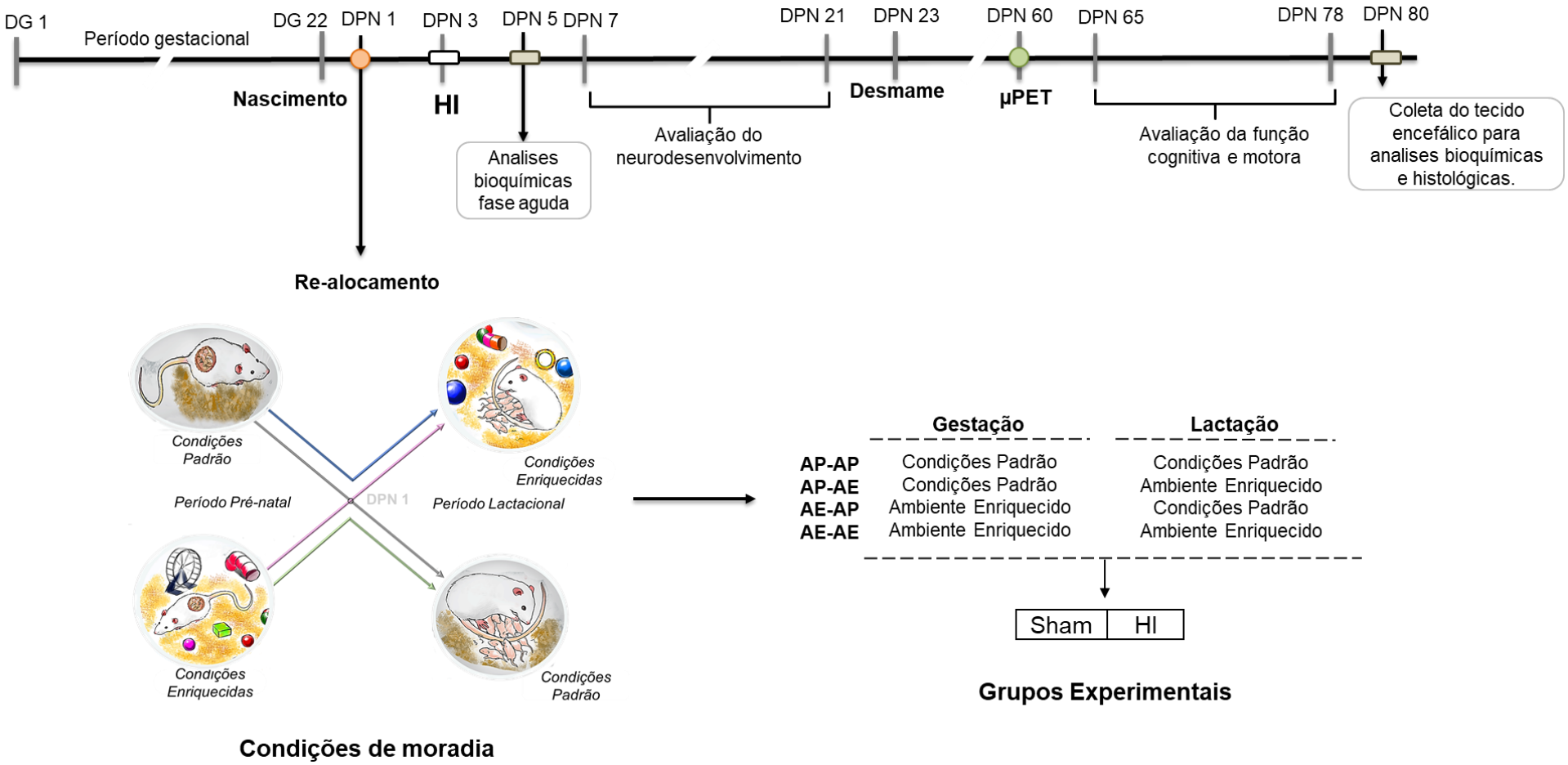


Figura 6: Representação esquemática do desenho experimental usando ambiente enriquecido durante a gestação e/ou lactação. No primeiro dia pós-natal foi realizada a realocação dos animais em um dos seguintes quatro grupos: AP-AP (linha cinza), AP-AE (linha azul), AE-AP (linha verde) e AE-AE (linha rosa) dos animais, segundo as condições de moradia durante o período gestacional ou lactacional, tanto para os animais HI como para os *Sham*. Fonte do autor.

3.6 Análise Comportamental

A avaliação comportamental dos animais foi realizada por meio dos seguintes testes:

3.6.1 **Geotaxia negativa** (DPN 7,14 e 21)

O animal foi submetido a um plano inclinado de 30º com a face virada em direção ao solo. Foi medida a latência do animal em adotar uma posição contrária, ou seja, virar-se 180º (direção cefálica contrária ao solo). O tempo máximo do teste foi de 60 segundos (Sanches et al., 2012).

3.6.2 **Reflexo de endireitamento postural** (DPN 7,14 e 21)

O animal foi colocado em decúbito ventral em uma superfície, e logo foi permitido que ele assumisse a posição de decúbito dorsal. Foi medida a latência do animal para assumir tal postura, sendo estabelecido um tempo máximo de 15 segundos (Sanches et al., 2012).

3.6.3 **Teste olfatório** (DPN 7,14 e 21)

O teste foi realizado em uma caixa acrílica retangular transparente, onde o animal foi exposto de um dos lados à maravalha (cama dos animais) da caixa moradia e do outro à maravalha limpa. O tempo máximo destinado ao teste foi de 180 segundos. A latência para se direcionar à maravalha da caixa-moradia pela primeira vez foi registrado (Sanches et al., 2017).

3.6.4 **Teste de remoção de adesivo** (DPN 35)

O teste foi utilizado para avaliar a função somatosensorial dos animais. Uma fita adesiva de 1cm de diâmetro foi colocada na pata dianteira dos animais, seguido da exploração espontânea em uma caixa de acrílico (largura: 100 cm,

altura: 30 cm) até a total remoção da fita. Os animais foram treinados 72 horas antes do teste comportamental e a posição da fita adesiva foi alternada. No dia do teste, a latência requerida para a remoção da fita adesiva da pata dianteira esquerda (lado contralateral à oclusão da carótida) foi registrada primeiro, seguida pela pata direita. O tempo máximo estabelecido para a execução da atividade foi de 120 segundos para cada pata (Beray-Berthat et al., 2010).

3.6.5 **Teste de preensão** (Idade adulta)

O teste de preensão foi utilizado para avaliar a força de preensão dos animais com as patas dianteiras. Para isso, utilizou-se o dinamômetro TEC-04422 (Instrutherm DD-500, Brasil). O animal foi segurado pelo corpo, e as patas dianteiras foram colocadas na barra de suporte, permitindo o movimento de preensão. Uma vez nessa posição, o animal foi gentilmente puxado pela cauda, em direção horizontal até se liberar do suporte. Cada animal realizou o movimento de preensão três vezes, com um intervalo de 5 minutos entre eles. A força final registrada corresponde à média dos *trials* em quilogramas (Wood et al., 1996).

3.6.6 **Teste do cilindro** (Idade adulta)

O teste do cilindro avaliou a assimetria locomotora durante a realização de uma tarefa de exploração e suporte corporal. O animal foi colocado dentro de um cilindro de vidro transparente de 20 cm de diâmetro e 40 cm de altura. Após, foi realizada a filmagem de cada animal, individualmente, durante 3 minutos. Logo foi quantificado o número de apoios com as patas dianteiras na parede do cilindro. O uso do membro anterior é definido através da colocação de toda a palma na parede o que indica a sua utilização para o apoio do corpo

(Pagnussat et al., 2012).

3.6.7 **Escada horizontal** (Idade adulta)

O teste foi empregado para avaliar a coordenação e a habilidade na execução da marcha. Consistiu em duas paredes de acrílico transparente (1 m de largura por 20 cm de altura) e traves de metal (3 mm de diâmetro cada) que foram inseridas inferiormente entre as paredes com uma distância mínima de 1cm entre elas. A escada permaneceu elevada 30 cm do solo, existindo um refúgio escuro para o animal ao final da sua extensão. A largura do aparato foi ajustada ao tamanho do animal, permanecendo cerca de 1 cm mais larga que o mesmo, de modo a evitar que o animal caminhe em torno de si ou vire-se no sentido contrário ao teste (Metz and Whishaw, 2002). A dificuldade do teste foi determinada pelo padrão de colocação das traves, podendo ser regular ou irregular. No padrão regular as traves foram espaçadas em intervalos de dois centímetros, sendo utilizado nas sessões de aclimatação. Enquanto no padrão irregular, utilizado nas avaliações, as traves foram espaçadas em intervalos de um a cinco centímetros. Para o teste os animais foram colocados em uma extremidade da escada e tiveram a possibilidade de andar em direção à outra extremidade da escada durante três vezes. A trajetória dos animais pelo aparato foi filmada utilizando uma câmera digital (Sony, DCR-SR47, USA) e o número de erros e acertos de posicionamento dos membros posteriores foi quantificado. A porcentagem de erro foi calculada de acordo com a equação [(número de erros / número de passos) x 100]. Foi considerado como escore final a média dos três *trials* (Marques et al., 2014).

3.6.8 **Campo aberto** (Idade adulta)

Os animais foram colocados na arena circular do campo aberto, dividida em duas subáreas (central e periférica) e cercada por uma parede de 40 cm de altura. O piso da arena foi dividido em linhas e raios concêntricos semelhantes, de maneira a formar 8 regiões periféricas e 4 regiões centrais possuindo relativamente a mesma área. Cada animal foi observado durante 5 minutos, sendo registrado o número de cruzamentos (número de linhas atravessadas), a exploração vertical (levantar e sustentar o corpo em duas patas) e a relação entre as áreas centrais e periféricas percorridas (Arteni et al., 2010).

3.6.9 **Labirinto aquático de Morris** (Idade adulta)

Este teste avaliou a função cognitiva dos animais e foi realizado em um tanque circular em uma sala com pistas visuais nas paredes da sala. O tanque consiste em 200 cm de diâmetro, sendo 40 cm de profundidade cobertos por água (temperatura de ± 23ºC). Uma plataforma com 10 cm de diâmetro (da mesma cor do tanque) encontrava-se submersa 2 cm abaixo da superfície da água. O tanque foi dividido virtualmente em 4 quadrantes de onde os animais iniciaram as tentativas de localizar a plataforma: N (norte), S (sul), L (leste) e O (oeste). A memória espacial dos animais foi avaliada por meio do protocolo da memória de referência (Netto et al., 1993).

Protocolo de Memória de Referência

A posição da plataforma permaneceu no mesmo local durante todo o período de treino. Os animais realizaram quatro *trials* por dia, durante 5 dias

consecutivos. Vinte e quatro horas após a fase de treino, uma sessão de 60 segundos, sem a plataforma foi realizada para avaliar a memória de longo prazo para a posição da plataforma. Foram analisados a latência para cruzar pela primeira vez a zona da plataforma, o tempo gasto no quadrante, a velocidade e a distância percorrida. Os animais foram filmados e os dados registrados pelo software ANY-MAZE (Netto et al., 1993; Sanches et al., 2017).

3.7 Análise de Proteínas por Western Blot

Para a quantificação da expressão das proteínas de interesse, foi realizada a técnica de *Western Blot*. Os animais foram submetidos à eutanásia por decapitação e, posteriormente, os encéfalos foram rapidamente removidos e crioprotegidos.

A detecção da quantidade de proteínas nas amostras de hipocampo e de córtex foi realizada através do protocolo previamente descrito por Arcego e colaboradores (Arcego et al., 2016). Foram utilizados anticorpos primários específicos para as proteínas anti-caspase 3, anti- Poli (ADP-ribose) polimerase (PARP-1), anti- proteína cinase B (AKT), anti- fator de crescimento semelhante a insulina (IGF-1), anti- fator de crescimento vascular endotelial (VEGF), anti- proteína ácida fibrilar glial (GFAP), anti- fator neurotrófico derivado do encéfalo (BDNF), anti- receptores de tropomiosina quinase B (TrkB), e anti-sinaptofisina. Anticorpos secundários específicos (anti-camundongo ou anti-coelho, conforme o anticorpo primário utilizado) ligados à peroxidase foram incubados com a proteína ligada ao anticorpo primário na sequência. O sinal foi detectado com o Kit ECL de quimioluminescência (GE Life Sciences), os imunoblots foram quantificados por escaneamento das membranas no equipamento ImageQuant

LAS4000 (GE Healthcare Life Sciences – Reino Unido) e as densidades ópticas foram determinadas pelo software Image Studio Lite V5.0 (LI-COR Biosciences - EUA).

3.8 Análise Histológica

3.8.1 Perfusão transcardíaca

Os animais foram anestesiados com uma dose letal, via intraperitoneal de tiopental sódico (100 mg/kg) associado a lidocaína (5mg/kg) e submetidos à perfusão transcardíaca com solução salina (0,9%), seguido de uma solução de paraformaldeído (PFA 4%). Os encéfalos foram removidos e mantidos na mesma solução contendo paraformaldeído para pós-fixação. Para a análise histológica, os encéfalos foram crioprotegidos com solução de sacarose 15% e 30% durante dois dias e depois congelados e mantidos a -80ºC até a análise.

3.8.2 Volume da lesão encefálica

Para as análises do tecido encefálico, foram feitos cortes coronais em criostato (CM1850, Leica, São Paulo-SP, Brazil) de 20 ou 30μm, com um intervalo de 200 ou 300μm, e corados com hematoxilina e eosina (Sigma-Aldrich. St Louis. MO. EUA). As áreas de interesse (hemisfério, córtex, estriado, hipocampo e corpo caloso) foram delineadas segundo o atlas de Paxinos & Watson (1997) e o volume das estruturas foi calculado de acordo com a equação (∑ das áreas x intervalo da seção dos cortes). As imagens das áreas foram capturadas com uma câmera de vídeo acoplada a um microscópio (Nikon),

utilizando-se o software Image-J. A razão volumétrica foi usando a equação [(Volume ipsilateral (mm^3) / Volume contralateral (mm^3)].

3.8.3 Morfometria da fibra muscular

O tecido muscular esquelético foi cortado no criostato em secções transversais consecutivas (20µm de espessura), que foram posteriormente coradas com hematoxilina e eosina. Para a análise, um número de oito imagens aleatórias dos músculos bíceps braquial e tibial anterior foram capturadas e digitalizadas inicialmente em aumento de 20x. A área da seção transversal das fibras (AST) foi estimada utilizando o Software Image Pro Plus. A distribuição de frequência foi realizada considerando o diâmetro das fibras musculares em intervalos de $30\mu^2$. (Marques et al., 2014).

3.9 Imunofluorescência

A técnica de imunofluorescência foi realizada utilizando secções coronais dos encéfalos. Resumidamente, os cortes dos encéfalos em espessura de 20 ou 30 µm foram fixados em PFA (Reagen, Brasil) 4%, lavados em PBS e, após, bloqueados por 30 minutos em uma solução contendo 3% de soro normal de cabra (NGS) (Sigma-Aldrich, USA) diluído em PBS Triton X-100 à 0,3% (PBS-Tx) em temperatura ambiente (Nicola et al., 2016). Em seguida, as secções foram incubadas durante a noite com os anticorpos primários anti-GFAP (1:200, Sigma-Aldrich), para a identificação de astrócitos e anti-NeuN para identificação de neurônios (1:500, Sigma-Aldrich), diluídos em PBS-Tx e NGS e mantidas em câmara fria (4ºC).

No dia seguinte, as secções foram lavadas em PBS e incubadas com

os anticorpos secundários fluorescentes compatíveis (Alexa-Fluor, Molecular Probes), diluídos em PBS-Tx e NGS por 2 horas em uma sala escura (temperatura ambiente), seguida de lavagem em PBS. Após as lâminas foram cobertas com meio de montagem PVA-DABCO (Fluka Analytical) e lamínulas. Para o controle negativo, alguns cortes foram incubados apenas com os anticorpos secundários. Todas as amostras foram processadas concomitantemente e incubadas durante o mesmo tempo médio. Decorrido o procedimento acima descrito, foi utilizado o microscópio confocal (Olympus FV1000) para a visualização das marcações fluorescentes e captura das imagens (Nicola et al., 2016).

3.10 Análise Estatística

A análise estatística dos dados foi realizada utilizando o *software* SPSS para Windows, versão 21. A distribuição normal dos dados foi estabelecida por meio do teste de Shapiro-Wilk. Para os dados paramétricos foi realizada a análise de variância (ANOVA) considerando os desfechos como fatores fixos (condição de moradia, lesão e sexo dos animais), seguido do teste de comparações múltiplas quando diferenças significativas foram encontradas. Os dados não paramétricos foram analisados por Kruskall-Wallis seguido de Mann-Whitney para múltiplas comparações. Os dados foram expressos como média ± erro padrão médio (EPM). O valor de significância aceito foi de ≤ 0.05. A Tabela 1, resume a distribuição dos dados segundo o desfecho, assim como as análises realizadas em cada um dos experimentos. Uma descrição detalhada da análise

estatística utilizada em cada experimento encontra-se no respectivo capítulo da seção de resultados.

Tabela 1: Síntese das análises estatísticas realizadas de acordo com o capítulo.

	Tipo de dados _ Desfecho		**Analise(s) _ Fator(es)**
Capítulo 1	Dados paramétricos:	Testes comportamentais Volume encefálico Morfometria muscular	Anova de uma via: grupo Anova de medidas repetidas: dia e grupo
Capítulo 2	Dados não paramétricos:	Testes do Neurodesenvolvimento Volume do córtex e hipocampo	Friedman test e Kruskal-Wallis
	Dados paramétricos:	Comportamento maternal Labirinto aquático de Morris Analise bioquímicos	Anova de medidas repetidas: dia e condição de moradia Anova de medidas repetidas: dia, condições de moradia e lesão Anova de duas vias: condições de moradia e lesão
Capítulo 3	Dados paramétricos:	Labirinto aquático de Morris Analise bioquímicos Volume encefálico	Modelo linear generalizado: condições de moradia, lesão e sexo Distribuição Poisson Distribuição linear
	Dados não paramétricos:	Campo aberto	Anova de medidas repetidas: dia, condições de moradia, lesão e sexo
Capítulo 4	Dados paramétricos:	Testes comportamentais Volume encefálico	Anova de duas vias: Condições de moradia e lesão
Capítulo 5	Dados paramétricos	Testes comportamentais Volume do estriado e córtex	Anova de duas vias: Condições de moradia e lesão

4. RESULTADOS

4.1 CAPÍTULO 1

Longer Hypoxia–Ischemia Periods to Neonatal Rats Causes Motor Impairments and Muscular Changes

Neuroscience 340 (2017) 291–298 (Impact factor: 3.382)

Neuroscience 340 (2017) 291–298

LONGER HYPOXIA–ISCHEMIA PERIODS TO NEONATAL RATS CAUSES MOTOR IMPAIRMENTS AND MUSCULAR CHANGES

L. E. DURÁN-CARABALI,[a*] E. F. SANCHES,[b] M. R. MARQUES,[b] D. ARISTIMUNHA,[b] A. PAGNUSSAT[c] AND C. A. NETTO[a,b]

[a] *Post-graduation Program of Physiology, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil*

[b] *Post-graduation Program of Neuroscience, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre (UFRGS), RS, Brazil*

[c] *Rehabilitation Sciences Graduate Program, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Brazil*

Abstract—Prematurity and hypoxia–ischemia (HI) can lead to movement disorders in infants. Considering that mild-moderate HI induced at postnatal day (PND) 3 has failed to produce motor disabilities similar to those seen in preterm newborns, the main goal of the present study was to verify whether longer hypoxia periods would mimic motor function impairment, brain and muscle morphological alterations. Forty-nine Wistar rat pups of both sexes were randomly assigned to surgical control (CG) and HI groups. HI animals were submitted to the Levine-Rice model at PND 3, and exposed to 120 (HI-120′), 180 (HI-180′) or 210 (HI-210′) minutes of hypoxia (FiO_2: 0.08). Sensorimotor function was assessed as from PND 35–45, by means of grasping strength, adhesive removal, cylinder and ladder walking tests. Histological staining was used to quantify the striatal volume and the cross-sectional area (CSA) of skeletal muscles. Cylinder and adhesive removal test evidenced that HI-180′ and HI-210′ groups had asymmetrical use of the forepaws when compared to controls. HI animals showed a decrease in the step placement quality and an increase in step errors when compared to CG ($P \leqslant 0.05$). Reduction in striatal volume correlates with behavioral assessment, HI-180′ and HI-210′ groups presented lower biceps brachii and tibialis anterior CSA. These results show that rats exposed to longer hypoxic periods at PND3 have encephalic and sensorimotor impairments that mimic those observed in preterm infants. Morphological changes in muscle tissue evidence a new pathophysiological characteristic of the HI model that might be of relevance for the study of sensorimotor deficits.

*Corresponding author. Address: Post-graduation Program of Physiology, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos 2600, anexo. CEP 90035-003, Porto Alegre, RS, Brazil.

E-mail address: luzeled67@gmail.com (L. E. Durán-Carabali).

Abbreviations: CG, control group; CSA, cross-sectional area; FiO_2, fraction of inspired oxygen; HI, hypoxia ischemia; PND, postnatal day.

http://dx.doi.org/10.1016/j.neuroscience.2016.10.068
0306-4522/

Key words: neonatal hypoxia ischemia, motor impairment, striatum, skeletal muscle.

INTRODUCTION

Epidemiological studies estimate that 15 million newborns are born prematurely (Blencowe et al., 2012) and prematurity is known to be the major cause of neonatal death, and the second cause of mortality in children younger than 5 years old (Mehren, 2012; Blencowe et al., 2013). Similarly, neonatal hypoxia ischemia (HI) occurs in 1.8–6 per 1000 births and is responsible for 25% of morbidity in infants (Shevell et al., 2001).

The continuous improvement of health services associated to technological advance have led to increased survival rates of children under conditions of prematurity and hypoxia–ischemia (Blencowe et al., 2013). There has been augmentation of disabilities in the infant period that contribute to learning deficits, and to motor and cognitive impairments (Shevell et al., 2001). Such disabilities are probably related to pathologies like epilepsy, psychomotor developmental delay, autism and cerebral palsy (Khwaja and Volpe, 2008). Therefore, the implications of the neurological sequelae associated with prematurity and HI are considered a public health problem (McKenna et al., 2015) because of their great impact throughout the life cycle and the extensive family and social support requirements (Glass et al., 2015).

The Levine-Rice model in rodent has been used to mimic hypoxic ischemic (HI induced at postnatal day 7, PND7) encephalopathy and data produced have provided information on the pathophysiological mechanisms and functional outcomes after perinatal asphyxia and postnatal hypoxia (Arteni et al., 2010; Weis et al., 2012; Sanches et al., 2013a; Alexander et al., 2014). In order to model HI in preterm infants, the experimental protocol combines the permanent carotid artery ligation with hypoxic exposure on the third postnatal day (PND3) (Stadlin et al., 2003; Sizonenko et al., 2008; Sanches et al., 2013b). This adaptation of the original model was introduced because rat brain development at PND3 corresponds to that of a human fetus with 24–28 weeks of gestation (Sizonenko et al., 2005).

The striatum is an important brain structure related to movement control (Jamon, 2006) and is one of the main areas affected by infant prematurity (Volpe, 2009). Experimental studies have reported severity-dependent volu-

metric loss associated with movement disorders (Rice et al., 1981; Hobbs and Oorschot, 2008). The musculoskeletal system also shows HI-associated changes, such as reduction in muscle volume and decreases of fiber cross-sectional area (CSA) after hypoxia (Hoppeler and Vogt, 2001).

It was recently shown that HI at PND3, with hypoxia duration of 90 min and FiO_2 of 0.08, causes volumetric reduction of the affected brain hemisphere, the hippocampus and cortex, as well as decreases white matter, as evaluated by the corpus callosum thickness (Sanches et al., 2015). However, this and other studies using hypoxic periods lower than 120 min at PND3 have failed to demonstrate any motor function impairment (Alexander et al., 2014; Sanches et al., 2015). Girard and colleagues (2009) showed motor function and encephalic morphological impairments when the surgical procedure was performed at PND1 and was followed by longer hypoxia periods or by intrauterine infection (Girard et al., 2009). In addition, a recent study performing HI at PND3, with FiO_2 of 0.06 observed coordination dysfunction in the injured animals (Misumi et al., 2016).

Considering the clinical relevance of sensorimotor deficits in long-term functionality and the lack of motor impairments following HI at PND 3 with FiO_2 of 0.08, the present study was designed to investigate whether longer periods (greater than 90 min) of hypoxia exposure at PND 3 would affect sensorimotor function and muscle morphology. The working hypothesis is that longer hypoxia exposure at PND3 would cause significant motor impairment, and that motor status would correlate with brain and muscle morphological changes.

EXPERIMENTAL PROCEDURES

Pregnant Wistar rats were provided by the Biochemistry Department of Universidade Federal do Rio Grande do Sul in the last week of gestation period. Dams and pups were housed in standard conditions (22 °C ± 2 °C and 12 h of light/dark cycle). Water and food were provided *ad libitum* during all the procedures. Hypoxia–ischemia was performed at PND 3 and offspring was weaned at PND 21. After the weaning, a total of 49 animals were maintained in groups of 4 animals per cage. The animal care followed the recommendations of the Brazilian Society for Neuroscience and The guidelines for the Care and Use of Laboratory Animals (publication n° 85–23, revised 1985). All procedures were approved by the University Ethics Committee (Under protocol number 28641). Fig. 1 shows the summary of the experimental design.

Hypoxia–ischemia procedure

Pups of both sexes at PND 3 were randomly assigned to control (CG) or hypoxia- ischemia (HI) groups. Animals of the HI group were submitted to Levine-Rice model and the surgical procedure was performed as previously described (Sanches et al., 2013a). Briefly, animals were anesthetized with 4% halothane, the right common carotid was isolated and permanently occluded with 4.0 surgical silk. The rats were kept in a warm cage during 10–15 min to post-surgical recovery. Later, offspring was returned to their respective dam for 2 h. After that, animals were exposed to 120 (HI-120′, n = 11), 180 (HI-180′, n = 14) or 210 (HI-210′, n = 10) minutes of hypoxia (FiO_2 = 0.08 at 37 °C) into a glass-made hypoxia chamber. For the CG (n = 14), the artery occlusion was not performed and rats were kept in atmospheric conditions (FiO_2 = 0.21).

Sensorimotor function assessment

Sensorimotor tests were conducted between PND 35 and PND 45 (10–14 animals per group) and were performed in the following order: grasping strength, adhesive removal, cylinder and ladder walking tests, with one-day intervals in between them.

Grasping strength. In order to determine the grasping strength of the forepaws, a digital force gauge instrument (TEC-04422 Instrutherm DD-500, Brazil) was used. The forepaws were put on the handle-rod allowing the grip movement. The animal was gently pulled away in the horizontal direction by the tail until releasing the rod. The force produced was recorded in kilograms. Three trials were run and the mean value for each animal was calculated (Wood et al., 1996).

Adhesive removal test. The adhesive removal test was used to measure the somatosensory function (Beray-Berthat et al., 2010). An adhesive tape of 1 cm × 1 cm was put on the forepaw palm and the animal was positioned into a clean transparent box (Diameter: 100 cm. High: 30 cm) until the removal of the adhesive tape. During the training period (72 h before the probe), the position of the adhesive tape (right or left forepaw) was alternated. Randomly half of the animals performed the test on the right forepaw and the other half with the left forepaw. Five minutes later, the other side was trained. On the probe day, the left forepaw (the limb contralateral to the injured brain hemisphere) was assessed first, followed by the right forepaw. The latency to remove the adhesive tape was recorded. The maximal time for adhesive removal was set as 120 s for each paw.

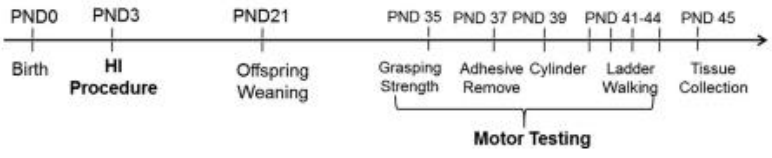


Fig. 1. Experimental design. PND: Postnatal day, HI. Hypoxia ischemia.

Cylinder test. The asymmetrical use of the forelimb was assessed by means of the cylinder test. A transparent cylinder (Diameter: 20 cm. Height: 40 cm) was placed on a glass table and a mirror from below was used for video register. Animals were put into the cylinder for

three minutes and the number of wall-touches with forepaws was scored. Animals that did fewer than 12 touches were excluded from the statistical analysis. The asymmetry percentage was determined by the equation $\left[\frac{\text{contralateral injury side}}{\Sigma\text{both forelimbs}} * 100\right]$ (Pagnussat et al., 2012).

Ladder walking test. Coordination and gait skills were assessed in the ladder walking apparatus. It consisted of two transparent acrylic walls (Diameter: 1 m. High: 20 cm) with metal rungs (Diameter: 3 mm) inserted into the walls. The ladder remained 30 cm above the ground and a dark cage was located at the end of the apparatus (Marques et al., 2014). All animals were trained for two days using a regular pattern (keeping the same distance between rungs). Along each training session, the rats walked across the ladder three times and they could explore the refuge cage for 10 s.

On the third day, the walking of the animals was evaluated using an irregular pattern, that is, the rungs were spaced at intervals of 1–3 cm (Mestriner et al., 2011; Antonow-Schlorke et al., 2013). The test was video-recorded from an inferior view. The paw placement on the metal rungs was rated from 0 to 6. Zero was scored for a total miss and six was scored for a totally correct placement (Metz and Whishaw, 2002). The number of limb placement errors and the number of steps with each paw were counted and the percentage of each limb was calculated according to the equation $\left[\frac{\text{number of errors}}{\text{number of steps}} * 100\right]$. An error was considered when the animal slipped the paw or failed to place it on the rung. The final score corresponded to the mean of the three trials (Antonow-Schlorke et al., 2013).

Histology. Rats (5–6 per group) were randomly chosen for histology. They received a lethal dose of thiopental sodium (Thiopentax, Cristália®, 100 mg/kg) associated with lidocaine (5 mg/kg) at the end of behavioral assessment. Then, they were perfused with 9% saline solution followed by 4% formaldehyde solution through the left cardiac ventricle. The brains were removed from the skull and the left biceps brachii and tibialis anterior muscle (contralateral to the injured brain hemisphere) were dissected out to remove the adjacent connective tissue. Both structures were deposited in fixative solution (4% formaldehyde solution) and cryoprotected with sucrose (30%).

The brains were sliced in serial 30-μm thickness on a cryostat (Leyca). One slice in every 10th was collected in gelatin-coated slides and stained with cresyl violet, as previously described (Sanches et al., 2013b). The brain region of interest (striatum +1.70 to −1.30 from Bregma) was identified using the rat brain atlas (Paxinos and Watson, 1998). All measurements were calculated in software from the NIH-ImageJ and the Cavalieri method was used to measure the structural volume. Volume ratio was calculated using the equation $\frac{\text{Ipsilateral volume}(mm^3)}{\text{contralateral volume}(mm^3)}$.

The skeletal muscle tissue was transversely sectioned in consecutive 30-μm slices and stained with hematoxylin and eosin (Sigma–Aldrich. St Louis. MO. USA) (Zaccagnini et al., 2007). Images of biceps brachii and tibialis anterior muscle (40×) were captured using Nykon E600 microscope (Japan). For analysis, eight randomly images were selected from one slice, and the fiber cross-sectional area (CSA) was estimated with the software Image Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA).

Statistical analysis

The software SPSS for Windows (version 21) was used. Data normality distribution was confirmed by Shapiro–Wilk test and the variance equality by the Levene's test. Differences among groups were determined by a one-way ANOVA followed by Duncan's *post hoc test*. Differences between contra and ipsilateral sides to the carotid occlusion were assessed by student's t-tests; correlations were calculated by the Pearson correlation coefficients. P-values $\leqslant 0.05$ were considered significant and data are expressed as mean ± standard error (S.E).

RESULTS

Grasping strength

There was no significant reduction of forepaws strength in the HI animals when compared to control group (Control: 174.33 ± 12.22; HI-120′: 155.09 ± 14.18; HI-180′: 134.07 ± 10.01 and HI-210′: 158.7 ± 13.00. $F_{(3.45)} = 2.084$, $P > 0.05$). This outcome suggests that HI at PND3 did not affect the global strength as evaluated by means of the grasping strength test.

Adhesive removal test

An increase in the latency to remove the adhesive from the contralateral forepaw (left) was found in all the HI groups when compared to the ipsilateral side. (HI-120′:$t_{(8)} = 3.733$, $P \leqslant 0.01$; HI-180′:$t_{(10)} = 3.632$, $P \leqslant 0.01$ and HI-210′:$t_{(8)} = 3.733$, $P \leqslant 0.01$). Also, somatosensory asymmetry was observed in the HI-180′ and HI-210′ group during adhesive removal tests when compared to CG ($F_{(3,36)} = 3.01$, $P \leqslant 0.05$) (Fig. 2a). As expected, there were no differences in the ipsilateral (right) forepaw somatosensory function between HI and CG animals. These results indicate that HI animals have a delay to recognize and remove the adhesive, pointing to somatosensory deficits.

Cylinder test

The asymmetrical use of forepaws in the HI-180′ and HI-210′ groups was observed ($F_{(3,45)} = 4.139$, $P \leqslant 0.01$), while no differences in the ipsilateral forepaw function was found (Fig. 2b). Moreover, the number of wall-touches with the contralateral forepaw (left forepaw) was significantly decreased in all HI groups when compared to the ipsilateral side (HI-120′: $t_{(10)} = 2.662$, $P \leqslant 0.05$; HI-180′: $t_{(13)} = 3.721$, $P \leqslant 0.01$ and HI-210′: $t_{(9)} = 2.611$, $P \leqslant 0.05$). These suggest a disuse of the limb contralateral to the injured brain hemisphere.

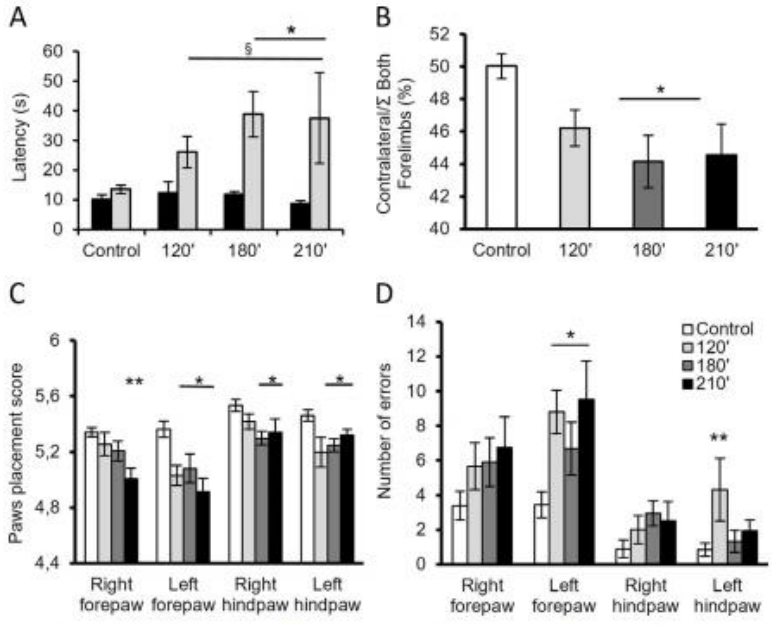


Fig. 2. Somatosensory and motor function assessment: Adhesive removal test: Ipsilateral (black) and contralateral (white) to the injured side (A). Data expressed as mean ± S.E of the animal latency. Cylinder test. Data expressed as mean ± S.E of the percentage use of contralateral forepaw (B). Ladder walking test: Step placement quality (C) and number of step errors. (D) Data expressed as mean ± S.E of foot placement score and percentages of limb placement errors per number of paw steps, respectively. Behavioral data analyzed by one-way ANOVA. *Differences from the control group. **Differences from the control group and HI groups and § Differences between contra and ipsilateral side, $P \leqslant 0.05$.

Ladder walking test

HI groups showed significant reductions in step placement quality when compared to CG (Fig. 2c). Ipsilateral forepaw performance was decreased in HI-210′ compared to CG and other HI groups ($F_{(3,41)} = 4.849$, $P \leqslant 0.01$). The contralateral forepaw scores were lower for all HI groups when compared to CG ($F_{(3,41)} = 5.820$, $P \leqslant 0.01$). Rat's performance with hind paws was decreased in HI-180′and HI-210′ compared to CG ($F_{(3,41)} = 3.330$, $P \leqslant 0.05$). Considering the number of limb placement errors per number of paw steps (Fig. 2d), all HI groups showed an increase in the step errors with the contralateral (left) forepaws ($F_{(3,41)} = 4.190$, $P \leqslant 0.01$). However, only the HI-120′ showed differences in the number of errors with left hind paw when compared to CG and HI groups ($F_{(3,41)} = 2.768$, $P \leqslant 0.05$). Altogether, these results demonstrate an impairment of global movement quality after HI at PND3.

Histological analysis of striatum

All HI groups had a volume reduction in the ipsilateral striatum as compared to the CG ($F_{(3,25)} = 4.588$, $P \leqslant 0.01$ – Table 1 and Fig. 3a) irrespective of the duration of HI. In addition, significant reductions were seen between ipsilateral and contralateral hemispheres (volume ratio) when compared to CG ($F_{(3,25)} = 7.206$, $P \leqslant 0.01$ – Fig. 3b). Striatal volume was positively correlated with the number of touches and symmetry percentage in the cylinder test ($R = 0.42$, $P \leqslant 0.05$ and $R = 0.56$, $P \leqslant 0.01$ respectively). Also, the striatal volume was positively correlated with the quality of the step placement with the left paws on the Ladder walk test (forepaw: $R = 0.48$, $P \leqslant 0.05$ and hind paw: $R = 0.55$, $P \leqslant 0.01$). These data confirm the negative impact of HI on striatal tissue loss and highlights the relevance of this brain structure in motor function.

Skeletal muscle analysis

The cross-sectional area (CSA) in biceps brachii and tibialis anterior muscle revealed significant differences among groups ($F_{(3,20)} = 4.857$, $P \leqslant 0.01$ and $F_{(3,20)} = 5.604$, $P \leqslant 0.01$ respectively). HI-180′ and HI-210′ groups showed a significant decrease in the CSA of biceps brachii when compared to CG and HI-120′ (Fig. 4a and e). When analyzing fibers of distinct diameters, significant differences were observed in fibers within 0–30 μm^2 ($F_{(3,20)} = 3.462$, $P \leqslant 0.05$), 30–60 μm^2 ($F_{(3,20)} = 4.030$, $P \leqslant 0.05$), 60–90 μm^2 ($F_{(3,20)} = 1.084$, $P > 0.05$), 90–120 μm^2 ($F_{(3,20)} = 4.559$, $P \leqslant 0.05$) and 120–150 μm^2 ($F_{(3,20)} = 3.462$, $P \leqslant 0.05$). *Post-hoc* tests revealed that HI-180′ and HI-210′ had a higher number of small fibers when compared to CG and HI-120′ group. On the other hand, the CG and HI-120′ had a greater number of large-size fibers (Fig. 4c).

As regards the tibialis anterior muscle, all HI groups have a significant reduction in the CSA when compared to CG (Fig. 4b). In addition, significant differences between distinct fiber diameters were found: 0–30 μm^2 ($F_{(3,20)} = 2.402$, $P > 0.05$), 30–60 μm^2 ($F_{(3,20)} = 2.279$, $P > 0.05$), 90–120 μm^2 ($F_{(3,20)} = 3.252$, $P \leqslant 0.05$), 120–150 μm^2 ($F_{(3,20)} = 4.346$, $P \leqslant 0.05$), 150–180 μm^2 ($F_{(3,20)} = 2.217$, $P > 0.05$), 180–210 μm^2 ($F_{(3,20)} = 4.273$, $P \leqslant 0.05$) and 210–240 μm^2 ($F_{(3,20)} = 3.754$, $P \leqslant 0.05$). These data revealed that HI animals have a predominance of small-size fibers when compared to controls (Fig. 4d), what could indicate muscular atrophy following HI. In addition, a positive correlation between muscle fiber size and the striatal volume ratio was observed (Biceps Brachii Muscle: $R = 0.63$, $P \leqslant 0.05$ and Tibialis Anterior Muscle: $R = 0.81$, $P \leqslant 0.01$), but no correlation appeared with performance in functional tests.

Table 1. Striatum volume: Ipsilateral (right) and contralateral (left) Striatum volume (mm^3). Data expressed as mean ± S.E.

	Control	HI-120′	HI-180′	HI-210′	*p*-Value
Striatum volume					
Right	297.90 ± 82.10	74.26 ± 32.20*	51.91 ± 47.18*	25.60 ± 20.97*	⩽0.05
Left	310.89 ± 80.64	167.56 ± 37.78	217.08 ± 99.19	35.78 ± 16.34	>0.05

* Differences from the control group, $P < 0.05$.

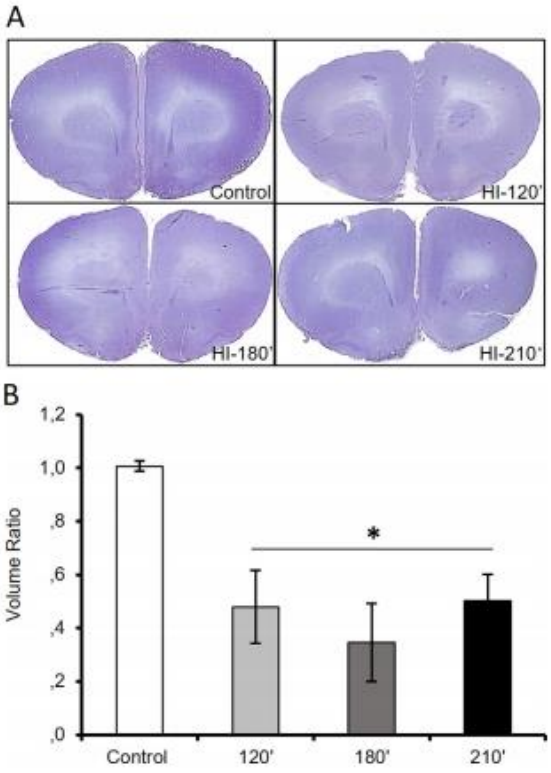


Fig. 3. Histological analysis of the Striatum: (A) Representative cresyl-violet-stained coronal brain sections at the levels of striatum (Bregma 1.60 mm) at PND 45. (B) Striatum volume ratio. Data analyzed by one-way ANOVA and expressed as mean ± S.E. *Differences from the control group. $P < 0.05$.

DISCUSSION

The present study was conducted to test whether longer periods of hypoxia exposure at PND 3 would affect sensorimotor function and muscle morphology. Confirming the working hypothesis: (a) 180 and 210 min of hypoxia exposure at PND 3 caused significant reduction of movement quality of the limb contralateral to the brain insult; (b) the motor status correlated with striatal tissue loss; (c) the HI insult caused a reduction of the cross sectional area of studied skeletal muscle, with predominance of small size fibers.

Animals of HI-180′ and HI-210′ groups showed a marked reduction in the use of the contralateral limb and a latency increase in the adhesive removal test, evidencing the sensorimotor asymmetry produced by this model. The analysis of walking skills performed by the ladder walking test showed a reduction in the placement score both the ipsilateral (HI-180′ and HI-210′) and the contralateral limbs (all HI animals) with subsequent increase in the number of errors. A possible explanation for these results is that post-insult morphological alterations lead to modifications in the input and output projections from and to the striatum, as well as the interaction of the striatum with the corticospinal and the corticobulbar tracts (Clowry et al., 2014). These would promote a delay in the error feedback control, affecting the quality of movement and the capability to reduce the number of errors through the sensory and motor information (Smith et al., 2000). Furthermore, the motor skills of contralateral and ipsilateral limbs could be affected in different degrees (major and minor compromise, respectively) in injuries that compromise only one brain hemisphere (Hicks and D'Amato, 1975; Hobbs and Oorschot, 2008; Clowry et al., 2014). The results indicate that experimental hypoxia periods of 180 min or longer, at PND 3 shall be used in order to model sensorimotor impairments associated with human prematurity and hypoxia–ischemia.

The analysis of the striatum showed a significant volume loss (approximately 50%) in the lesioned hemisphere in all animals exposed to the HI procedure. The striatum is an important brain structure that modulates the response of other motor areas, such as the cerebral cortex (Jamon, 2006). Towfighi and coworkers (1997) reported that animals exposed to 4 h of hypoxia at PND3 showed mild histological damage in the striatum 24 and 72 h post-insult (Towfighi et al., 1997), however these differences might increase after injury progression (Martin et al., 1997; Zhu et al., 2005). Thus, our results confirm the long-term impact of the HI insult on striatal tissue damage.

As expected, there were positive correlations between striatum volume ratio and the motor deficits observed. Experimental evidence indicates that the striatum network is established during the early postnatal period and is responsible for the movement initiation and control (Piek et al., 2008). Also, the maturation of locomotor structures and motor pathways occurs in parallel to striatal network activity (Dehorter, 2011). Thus, changes in the neuronal connectivity of motor structures, as well as aberrant plasticity after severe brain damage have been associated with secondary deficiencies that can disturb functional recovery (Kolb and Gibb, 2007; Clowry et al., 2014). Hobb and coworkers (2008) demonstrated motor deficits in the left forepaw associated with mild to moderate injury of the striatal medium-spiny neuron after HI at PND7 without remarkable histological consequences (Hobbs and Oorschot, 2008), these data highlight the need of longer hypoxic exposure at PND 3 to

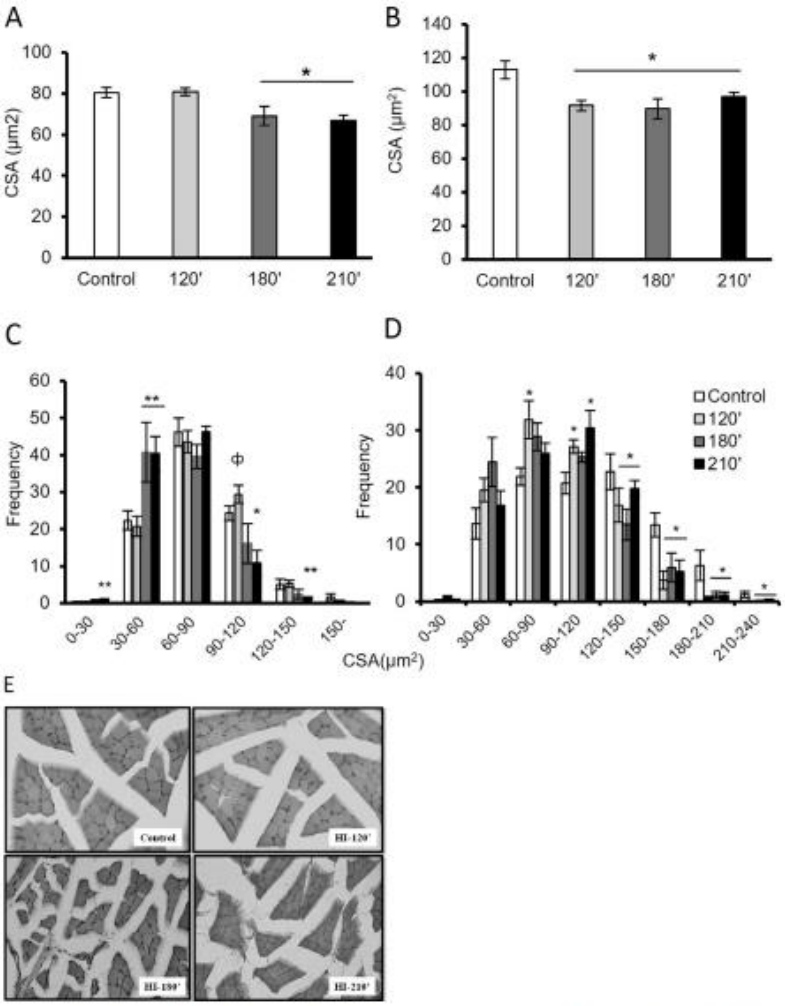


Fig. 4. Histological analysis of skeletal muscle: Cross-sectional area (CSA) of Biceps Brachii (A) and Tibialis Anterior fiber (B). Frequency histogram of Biceps Brachii (C) and Tibialis Anterior (D) according to the fiber size. Representative digitalized images (40×) of 30-µm transverse section of brachial biceps muscle (E). Data analyzed by one-way ANOVA and expressed as mean ± S.E. *Differences from the control group, $P \leqslant 0.05$ **Differences from the control group and HI-120′ and Φ Differences from the other HI groups without differences from CG, $P \leqslant 0.05$.

promote a striatal lesion with comparable severity to that observed at PND7.

Previous studies using the rat HI model at PND 3 and FIO2 of 0.08 did not show motor impairments in behavioral tests, like the open field (Sanches et al., 2015), cylinder (Sanches et al., 2013a) and rotarod tests (Alexander et al., 2014). However, Quairiaux and coworkers (2010) running HI at PND 3 and FiO_2 of 0,06 evidenced an early functional deficit associated to a reduction in the somatosensory-evoked potential when the all large whiskers are stimulated unilaterally. The same research group also reported a potential functional recovery in the somatosensory network at PND 21 (Quairiaux et al., 2010). Moreover, a recent work using a similar HI protocol showed coordination dysfunction in the injured animals (Misumi et al., 2016). These results point to effects of hypoxia duration and oxygen concentration in somatosensory function.

In addition to the neuronal control mediated by cerebral structures, the effective motor function requires an integrated participation of the musculoskeletal system to allow for a better motor performance in different environments and behavioral situations (Jamon, 2006). To our knowledge, this is the first study showing a significant reduction in the fiber cross-sectional area of the left forelimb and hind limb muscle (biceps brachii and tibialis anterior) and a positive correlation between the striatal injury and muscle fiber reduction. HI animals showed a reduction in the CSA, with a predominance of small-size fibers when compared to CG, indicating that muscular atrophy might follow HI. In agreement with present results, acute hypoxia in human causes muscle structural changes such as reduction in muscle volume and a decrease in the CSA (Hoppeler and Vogt, 2001); as a matter of fact, children with cerebral palsy may exhibit smaller muscle CSA in the affected limbs (Elder et al., 2003).

The morphofunctional correlation between the striatum and behavioral performance would have enormous clinical impact on patients with motor alterations associated with prematurity and HI. A higher compromise on the contralateral hemibody could affect the functionality on the ipsilateral side, possibly due to balance dysfunction. Moreover, a delay between the corporal adjustment and the voluntary movement could be a limiting factor for the movement efficiency (Hobbs and Oorschot, 2008).

Givon (2009), in a review of muscle weakness in the cerebral palsy condition, mentioned the relationship between the CSA and motor function, and pointed that factors such as motor unit recruitment, selective control and agonist–antagonist co-contraction are important characteristics of movement performance (Givon, 2009). However, results here presented did not show any correlation with functional tests. A possible explanation is that experimental neonatal HI did not visibly affect muscle activation, gross control movement and anti-gravitational control. The morphological changes observed in the skeletal muscle might indicate hypoxia compensatory mechanisms on metabolism of peripheral tissues, so promoting redistribution of blood flow to enhance brain metabolic support (Gunn & Bennet, 2009). The reduction in

muscle CSA could be also associated with changes in protein synthesis (Hoppeler and Vogt, 2001), or represent an adaptive response to minimize peripheral metabolic demands in order to protect cell viability and contractile function (Clanton & Klawitter, 2001).

On the other hand, the modification of skeletal muscle architecture could be related to some degree of denervation, remodeling (Scherbakov et al., 2013) or to disuse of the limb contralateral to the injury (Edgerton et al., 2002; Mohagheghi et al., 2007). Clinical studies have described the secondary effects of stroke in muscle tissue (English et al., 2010) indicating that muscle tissue changes during early and sub-acute phases could be related to uncoupled synaptic transmission of the muscle innervation (Scherbakov et al., 2013). In addition, muscle atrophy has been associated with fiber adaptation to motor disabilities caused by the brain injury and to the loss of sarcomeres (Mohagheghi et al., 2007). Thus, further studies are needed to identify whether neonatal HI could affect muscular metabolic parameters and contractile capacity.

CONCLUSION

The present study shows that rats exposed to 180 min, or longer periods, of hypoxia ischemia at PND 3 with FiO_2 of 0.08 present brain lesion and sensorimotor impairments that mimic those observed in preterm infants. A reduction in fiber cross-sectional area of biceps brachii and tibialis anterior muscles following longer HI periods was also reported. Therefore, our results reveal a new pathophysiologic aspect of the HI model and its possible clinical relevance for the study of sensorimotor deficits.

CONFLICT OF INTEREST

The authors have no conflict of interest.

Acknowledgments—This study was partly supported by financial resources from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

REFERENCES

Alexander M, Garbus H, Smith AL, Rosenkrantz TS, Fitch RH (2014) Behavioral and histological outcomes following neonatal HI injury in a preterm (P3) and term (P7) rodent model. Behav Brain Res 259:85–96.

Antonow-Schlorke I, Ehrhardt J, Knieling M (2013) Modification of the ladder rung walking task – new options for analysis of skilled movements. Stroke Res Treat 2013:1–11.

Arteni NS, Pereira LO, Rodrigues AL, Lavinsky D, Achaval ME, Netto CA (2010) Lateralized and sex-dependent behavioral and morphological effects of unilateral neonatal cerebral hypoxia-ischemia in the rat. Behav Brain Res 210:92–98.

Beray-Berthat V, Delifer C, Besson VC, Girgis H, Coqueran B, Plotkine M, Marchand-Leroux C, Margaill I (2010) Long-term histological and behavioural characterisation of a collagenase-induced model of intracerebral haemorrhage in rats. J Neurosci Methods 191:180–190.

Blencowe H, Cousens S, Oestergaard MZ, Chou D, Moller AB, Narwal R, Adler A, Vera Garcia C, Rohde S, Say L, Lawn JE (2012) National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. Lancet 379:2162–2172.

Blencowe H, Cousens S, Chou D, Oestergaard M, Say L, Moller A-B, Kinney M, Lawn J (2013) Born too soon: the global epidemiology of 15 million preterm births. Reprod Health 10:1–14.

Clanton TL, Klawitter PF (2001) Adaptive responses of skeletal muscle to intermittent hypoxia: the known and the unknown. J Appl Physiol 90:2476–2487.

Clowry GJ, Basuodan R, Chan F (2014) What are the best animal models for testing early intervention in cerebral palsy? Front Neurol 5:1–17.

Dehorter N (2011) Onset of pup locomotion coincides with loss of NR2C/D-mediated cortico-striatal EPSCs and dampening of striatal network immature activity. Front Cell Neurosci 5:1–14.

Edgerton VR, Roy RR, Allen DL, Monti RJ (2002) Adaptations in skeletal muscle disuse or decreased-use atrophy. Am J Phys Med Rehabil 81:S127–S147.

Elder G, Kirk J, Steward G, Cook K, Weir D, Marshal A, Leahey L (2003) Contributing factors to muscle weakness in children with cerebral palsy. Dev Med Child Neurol 45:542–550.

English C, McLennan H, Thoirs K, Coates A, Bernhardt J (2010) Loss of skeletal muscle mass after stroke: A systematic review. Int J Stroke 5:395–402.

Girard S, Kadhim H, Beaudet N, Sarret P, Sébire G (2009) Developmental motor deficits induced by combined fetal exposure to lipopolysaccharide and early neonatal hypoxia/ischemia: A novel animal model for cerebral palsy in very premature infants. Neuroscience 158:673–682.

Givon U (2009) Muscle weakness in cerebral palsy. Acta Orthop Traumatol Turc 2:87–93.

Glass HC, Costarino AT, Stayer SA, Brett CM, Cladis F, Davis PJ (2015) Outcomes for extremely premature infants. Anesth Analg 120:1337–1351.

Gunn AJ, Bennet L (2009) Fetal hypoxia insults and patterns of brain injury: insights from animal models. Clin Perinatol 36:579–593.

Hicks SP, D'Amato CJ (1975) Motor sensory cortex corticospinal system and developing locomotion and placing in rats. Am J Anat 143:1–41.

Hobbs CE, Oorschot DE (2008) Neonatal rat hypoxia-ischemia: long-term rescue of striatal neurons and motor skills by combined antioxidant-hypothermia treatment. Brain Pathol 18:443–454.

Hoppeler H, Vogt M (2001) Muscle tissue adaptations to hypoxia. J Exp Biol 204:3133–3139.

Jamon M (2006) The early development of motor control in neonate rat. Comptes Rendus – Palevol 5:657–666.

Khwaja O, Volpe JJ (2008) Pathogenesis of cerebral white matter injury of prematurity. Arch Dis Child – Fetal Neonatal Ed 93: F153–F161.

Kolb B, Gibb R (2007) Brain plasticity and recovery from early cortical injury. Dev Psychobiol 49:107–118.

Marques MR, Stigger F, Segabinazi E, Augustin OA, Barbosa S, Piazza FV, Achaval M, Marcuzzo S (2014) Beneficial effects of early environmental enrichment on motor development and spinal cord plasticity in a rat model of cerebral palsy. Behav Brain Res 263:149–157.

Martin LJ, Brambrink AM, Lehmann C, Portera-Cailliau C, Koehler R, Rothstein J, Traystman RJ, Lee Martin J, Brambrink AM, Lehmann C, Portera-Cailliau C, Koehler R, Rothstein J, Traystman RJ (1997) Hypoxia-ischemia causes abnormalities in glutamate transporters and death of astroglia and neurons in newborn striatum. Ann Neurol 42:335–348.

McKenna MC, Scafidi S, Robertson CL (2015) Metabolic alterations in developing brain after injury: knowns and unknowns. Neurochem Res 40:2527–2543.

Mehren E (2012) Born too soon. J Perinatol 13:393–396.

Mestriner RG, Pagnussat AS, Boisserand LSB, Valentim L, Netto CA (2011) Skilled reaching training promotes astroglial changes and facilitated sensorimotor recovery after collagenase-induced intracerebral hemorrhage. Exp Neurol 227:53–61.

Metz GA, Whishaw IQ (2002) Cortical and subcortical lesions impair skilled walking in the ladder rung walking test: a new task to evaluate fore- and hindlimb stepping, placing, and co-ordination. J Neurosci Methods 115:169–179.

Misumi S, Ueda Y, Nishigaki R, Suzuki M, Ishida A, Jung C-G, Hida H (2016) Dysfunction in motor coordination in neonatal white matter injury model without apparent neuron loss. Cell Transplant 25:1381–1393.

Mohagheghi AA, Khan T, Meadows TH, Giannikas K, Baltzopoulos V, Maganaris CN (2007) Differences in gastrocnemius muscle architecture between the paretic and non-paretic legs in children with hemiplegic cerebral palsy. Clin Biomech 22:718–724.

Pagnussat AS, Simao F, Anastacio JR, Mestriner RG, Michaelsen SM, Castro CC, Salbego C, Netto CA (2012) Effects of skilled and unskilled training on functional recovery and brain plasticity after focal ischemia in adult rats. Brain Res 1486:53–61.

Paxinos G, Watson C (1998) The rat brain. In: Stereotaxic coordinates (Press A, ed)., fourth ed., United States of American.

Piek JP, Dawson L, Smith LM, Gasson N (2008) The role of early fine and gross motor development on later motor and cognitive ability. Hum Mov Sci 27:668–681.

Quairiaux C, Sizonenko SV, Mégevand P, Michel CM, Kiss JZ (2010) Functional deficit and recovery of developing sensorimotor networks following neonatal hypoxic-ischemic injury in the rat. Cereb Cortex 20:2080–2091.

Rice JE, Vannucci RC, Brierley JB (1981) The influence of immaturity on hypoxic-ischemic brain damage in the rat. Ann Neurol 9:131–141.

Sanches EF, Arteni NS, Nicola F, Boisserand L, Willborn S, Netto CA (2013a) Early hypoxia-ischemia causes hemisphere and sex-dependent cognitive impairment and histological damage. Neuroscience 237:208–215.

Sanches EF, Arteni NS, Scherer EB, Kolling J, Nicola F, Willborn S, Wyse ATS, Netto CA (2013b) Are the consequences of neonatal hypoxia-ischemia dependent on animals' sex and brain lateralization? Brain Res 1507:105–114.

Sanches EF, Arteni N, Nicola F, Aristimunha D, Netto CA (2015) Sexual dimorphism and brain lateralization impact behavioral and histological outcomes following hypoxia–ischemia in P3 and P7 rats. Neuroscience 290:581–593.

Scherbakov N, Von Haehling S, Anker SD, Dirnagl U, Doehner W (2013) Stroke induced Sarcopenia: muscle wasting and disability after stroke. Int J Cardiol 170:89–94.

Shevell MI, Majnemer A, Rosenbaum P, Abrahamowicz M (2001) Etiologic determination of childhood developmental delay. Brain Dev 23:228–235.

Sizonenko SV, Kiss JZ, Inder T, Gluckman PD, Williams CE (2005) Distinctive neuropathologic alterations in the deep layers of the parietal cortex after moderate ischemic-hypoxic injury in the P3 immature rat brain. Pediatr Res 57:865–872.

Sizonenko SV, Camm EJ, Dayer A, Kiss JZ (2008) Glial responses to neonatal hypoxic-ischemic injury in the rat cerebral cortex. Int J Dev Neurosci 26:37–45.

Smith M, Brandt J, Shadmehr R (2000) Motor disorder in Huntington's disease begins as a dysfunction in error feedback control. Nature 403:544–549.

Stadlin A, James A, Fiscus R, Wong YF, Rogers M, Haines C (2003) Development of a postnatal 3-day-old rat model of mild hypoxic-ischemic brain injury. Brain Res 993:101–110.

Towfighi J, Mauger D, Vannucci RC, Vannucci SJ (1997) Influence of age on the cerebral lesions in an immature rat model of cerebral hypoxia-ischemia: a light microscopic study. Brain Res Dev Brain Res 100:149–160.

Volpe JJ (2009) The Encephalopathy of prematurity-brain injury and impaired brain development inextricably intertwined. Semin Pediatr Neurol 16:167–178.

Weis SN, Pettenuzzo LF, Krolow R, Valentim LM, Mota CS, Dalmaz C, Wyse ATS, Netto CA (2012) Neonatal hypoxia-ischemia induces sex-related changes in rat brain mitochondria. Mitochondrion 12:271–279.

Wood NI, Sopesen BV, Roberts JC, Pambakian P, Rothaul AL, Hunter AJ, Hamilton TC (1996) Motor dysfunction in a photothrombotic focal ischaemia model. Behav Brain Res 78:113–120.

Zaccagnini G, Martelli F, Magenta A, Cencioni C, Fasanaro P, Nicoletti C, Biglioli P, Pelicci PG, Capogrossi MC (2007) P66ShcA and oxidative stress modulate myogenic differentiation and skeletal muscle regeneration after hind limb ischemia. J Biol Chem 282:31453–31459.

Zhu C, Wang X, Xu F, Bahr BA, Shibata M, Uchiyama Y, Hagberg H, Blomgren K (2005) The influence of age on apoptotic and other mechanisms of cell death after cerebral hypoxia-ischemia. Cell Death Differ 12:162–176.

(Accepted 29 October 2016)
(Available online 5 November 2016)

Principais Achados do Capítulo 1

Entre os resultados obtidos neste capítulo, destacamos que:

1. Períodos mais prolongados de hipóxia são necessários para promover déficits motores em animais submetidos a HI no DPN 3, a fim de mimetizar alguns dos desfechos observados em crianças nascidas prematuramente;

2. O volume de lesão do estriado apresenta uma correlação positiva com os prejuízos motores induzidos pela HI;

3. Modificações morfológicas no músculo esqueléticos apresentam uma nova caraterística nos efeitos induzidos pela exposição prolongada a hipóxia no modelo da HI.

4.2 CAPÍTULO 2

Prenatal and Early Postnatal Environmental Enrichment Reduce Acute Cell Death and Prevent Neurodevelopment and Memory Impairments in Rats Submitted to Neonatal Hypoxia Ischemia

Molecular Neurobiology 2018;55(5):3627-3641 (Impact factor: 5.076)

Mol Neurobiol
DOI 10.1007/s12035-017-0604-5

Prenatal and Early Postnatal Environmental Enrichment Reduce Acute Cell Death and Prevent Neurodevelopment and Memory Impairments in Rats Submitted to Neonatal Hypoxia Ischemia

L. E. Durán-Carabali[1] · D. M. Arcego[2] · F. K. Odorcyk[1] · L. Reichert[3] · J. L. Cordeiro[4] · E. F. Sanches[2] · L. D. Freitas[2] · C. Dalmaz[2,3] · A. Pagnussat[5] · C. A. Netto[1,2,4]

Received: 12 December 2016 / Accepted: 8 May 2017

Abstract Environmental enrichment (EE) is an experimental strategy to attenuate the negative effects of different neurological conditions including neonatal hypoxia ischemia encephalopathy (HIE). The aim of the present study was to investigate the influence of prenatal and early postnatal EE in animals submitted to neonatal HIE model at postnatal day (PND) 3. Wistar rats were housed in EE or standard conditions (SC) during pregnancy and lactation periods. Pups of both sexes were assigned to one of four experimental groups, considering the early environmental conditions and the injury: SC-Sham, SC-HIE, EE-sham, and EE-HIE. The offspring were euthanized at two different time points: 48 h after HIE for biochemical analyses or at PND 67 for histological analyses. Behavioral tests were performed at PND 7, 14, 21, and 60. Offspring from EE mothers had better performance in neurodevelopmental and spatial memory tests when compared to the SC groups. HIE animals showed a reduction of IGF-1 and VEGF in the parietal cortex, but no differences in BDNF and TrkB levels were found. EE-HIE animals showed reduction in cell death, lower astrocyte reactivity, and an increase in AKTp levels in the hippocampus and parietal cortex. In addition, the EE was also able to prevent the hippocampus tissue loss. Altogether, present findings point to the protective potential of the prenatal and early postnatal EE in attenuating molecular and histological damage, as well as the neurodevelopmental impairments and the cognitive deficit, caused by HIE insult at PND 3.

Keywords Prematurity · Gestation · Pre-weaning period · Environmental stimulation · Cell death · Neurodevelopment · Memory

Electronic supplementary material The online version of this article (doi:10.1007/s12035-017-0604-5) contains supplementary material, which is available to authorized users.

✉ L. E. Durán-Carabali
luzeled67@gmail.com

[1] Post-graduation Program of Physiology, Institute of Health Science, Universidade Federal do Rio Grande do Sul (UFRGS), Rua Ramiro Barcelos 2600, anexo, Porto Alegre, RS CEP 90035-003, Brazil

[2] Department of Biochemistry, Institute of Health Science, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil

[3] Neurorehabilitation and Neural Repair Research Group, Pontificia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, RS, Brazil

[4] Post-graduation Program of Neuroscience, Institute of Health Science|, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil

[5] Rehabilitation Sciences Graduate Program, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, Brazil

Abbreviations

AKT	Protein kinase B
AKTp	Phosphorylate protein kinase B
BBB	Blood-brain barrier
ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
Caspases	Cysteinyl-aspartate proteases
SC	Standard condition
EE	Environmental enrichment
EPM	Elevate plus maze
FIO_2	Fraction of inspired oxygen
GD	Gestational day
GFAP	Glial fibrillary acid protein
HIE	Hypoxia ischemia encephalopathy
IGF-1	Insulin-like growth factor 1
PARP-1	Poly (ADP-ribose) polymerase-1

Published online: 18 May 2017

PND	Postnatal day
TBS	Tris buffer saline
TrkB	Tropomyosin receptor kinase B
T-TBS	Tween tris buffer saline
VEGF	Vascular endothelial growth factor

Introduction

Neonatal hypoxia ischemia encephalopathy (HIE) is one of the most common neurological complications in preterm infants, and it has been associated with clinical manifestations like neurodevelopmental delays, behavioral, cognitive deficits, and sensorimotor impairments [1, 2]. The energy metabolism has a key role in cellular and tissue vulnerability [3]; therefore, the reduction of oxygen and glucose supply consequent to the HIE insult triggers a cascade of neurochemical events implicated in cellular dysfunction and death [4]. The expression of cleaved caspase-3 and of cleaved poly (ADP-ribose) polymerase-1 (PARP-1) is increased after brain injury. These proteins participate in the downstream events that culminate in cell death via the apoptotic pathway during the primary and the secondary energy failure [5–8]. Following HIE, astrocytes become reactive, hypertrophic, hyperplasic, and migrate to the injury site to regulate ion concentrations in the extracellular environment [9, 10]. However, this astrocytic reaction can exacerbate the production of pro-inflammatory signals leading to a positive feedback in the apoptosis of neuronal cells [11]. Astrocytes can also inhibit the maturation of oligodendrocytes [12] and the axonal regeneration through scar formation, which has been associated with functional deficits [13].

Pre- and postnatal periods represent distinct critical windows to encephalic maturation of structures such as the hypothalamus, cerebral cortex, and hippocampus [14], which are sensitive to environmental stimuli [15]. The intrauterine milieu during pregnancy can affect physiological and metabolic functions of the offspring, and the ongoing organogenesis, through a variety of inter-related mechanisms including maternal nutrition and maternal environmental exposure [16, 17]. The neuronal function is susceptible to the in utero programming and the beneficial, or negative, effect of prenatal condition in promoting offspring's resilience or vulnerability under stress situations, brain insults, and adult diseases are well-recognized [18]. Furthermore, the influence of birth and weaning experiences are relevant to the adequate maturation process, organization, and function of the developing brain [19].

Environmental enrichment (EE) combines voluntary physical activity, novelty exposure, social interaction, and sensory and cognitive stimulation [20]. In addition, it is a well-accepted strategy to attenuate the negative effects of different neurological conditions even during early development such as acute stress [21], intrauterine asphyxia [22], and gestational inflammation [23]. The EE increases the expression of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) [24, 25], insulin-growth factor 1 (IGFI) [26, 27], and vascular endothelial growth factors (VEGF) [28, 29] in both the hippocampus as the cerebral cortex, all of which have remarkable action in growth, maturation, plasticity, and preservation of neuronal tissue [30, 31]. Neurotrophic factors-mediated signaling involves the serine-threonine kinase, AKT, also named protein kinase B. Its active form is responsible for the suppression of apoptosis, contributing to tissue preservation after brain injury [32, 33]. Thus, endogenous neuroprotective mechanisms as well as antenatal treatments have been proposed as possible prophylactic approaches after HIE in an attempt to minimize the damage and to promote recovery [4, 34].

The Rice-Vanucci model has allowed a better comprehension of the pathophysiology of HIE condition and the effects of distinct therapeutic strategies. Previous reports have documented that animals submitted to neonatal HIE at postnatal day (PND) 7 (period used to mimic a term infant) and exposed to EE during the post-weaning period improve spatial, working [35, 36], and declarative memories [36, 37], possibly through the increase of spine density in the hippocampus [37]. The EE also leads to a recovery of the Na+, K + −ATPase activity [36]; reduces the oxidative stress biomarkers levels [38]; and improves motor function [39, 40]. However, little is known about the effects of early EE (during lactation) in animals bearing neonatal brain injury. It was shown a partial recovery of the brain atrophy, improvement of neurodevelopmental reflexes [41], and mitigation of the blood-brain barrier dysfunction [42]. On the other hand, the Rice-Vanucci model at PND 3 is an adaptation of the original procedure in order to mimic brain development of preterm newborns with 24–28 weeks of gestation [43] Nevertheless, the effects of the EE in animals submitted to HIE at this early postnatal age are not present in literature, although it is conceivable that age might influence insult severity and the efficacy of therapeutic strategies [44].

To date, literature has highlighted the biochemical, morphological, and behavioral impact of prenatal EE on the fetus and the long-term resilience mechanisms observed in the offspring [23, 45–47]. Factors such as the maternal milieu influence, developmental and maturational brain periods, early programming, and epigenetic and translational mechanisms [18] have been considered the main aspects involved in the positive effects of gestational environment stimulation.

Due to the EE neuroprotective effects following HIE, the current study sought to determine the effects of environmental stimulation during prenatal and lactational periods on the early expression of hippocampal and parietal cortex levels of (a) apoptotic biomarkers, (b) astrocytic reactivity, (c) growth and neurotrophic factors, and (d) AKT protein expression in rats receiving neonatal HIE at PND 3. Furthermore, the preventive effect of EE on HIE-induced brain tissue loss and on neurodevelopmental delay and spatial memory impairment was also investigated.

Experimental Procedures

The experimental procedures were approved by the Ethics committee of the Universidade Federal do Rio Grande do Sul (no 28641). Animal care followed the guidelines of the Brazilian Society of Science in Laboratory Animals—Law no 11.794; and the recommendation of the Council for International Organizations of Medical Sciences (CIOMS - Publication 85-23, 1985).

Animals

Wistar rats were housed in the colony of the Biochemistry Department, at room temperature of 22 °C ± 2 and under 12 light/dark cycle (lights on at 7:00 am). Water and food were provided ad libitum. Nulliparous female ($n = 12$), at 60 days of age, were randomly assigned to the standard (SC) or environmental enrichment (EE) group. As previously described [48], after 1 week of habituation, vaginal smear was monitored daily. When proestrus stage was observed, a same-strain male was introduced in the standard cage for one night. Pregnancy was inferred by the presence of sperm in the vaginal smear (first gestational day-GD1) and it was confirmed by the weight gain. At GD20, the pregnant rats were placed in individual standard cages without being disturbed until the parturition, denominated as postnatal day 0 (PND 0). The animals returned to their previous environment at PND 1. The hypoxia ischemia model was performed on the offspring at PND 3 and the weaning was at PND 23. Figure 1b, c summarizes the timeline of experiments.

Housing Conditions

Gestational Environment

Pregnant rats of the EE group ($n = 6$) were placed together in an enriched cage (height 1.2 m, width 80 cm, length 1 m). The EE contained plastic toys, a running wheel, ramps, plastic tubing, balls, and contrasting visual stimuli (Fig. 1a). The objects were changed twice per week, and food and water were moved to a different location to promote additional exploration. In the SC group ($n = 6$), three animals per cage were housed in standard condition cages (height 16 cm, width 41 cm, length 34 cm). The cages were provided with wood shaving bedding and a minimum of external stimuli [21].

Postnatal Environment

The pups were cared by their own mother from PND 1 until weaning under the same gestational environment (SC or EE). To offer social interaction, two litters (dam and litters) of the EE group were kept together [49, 50]. For the SC group, one

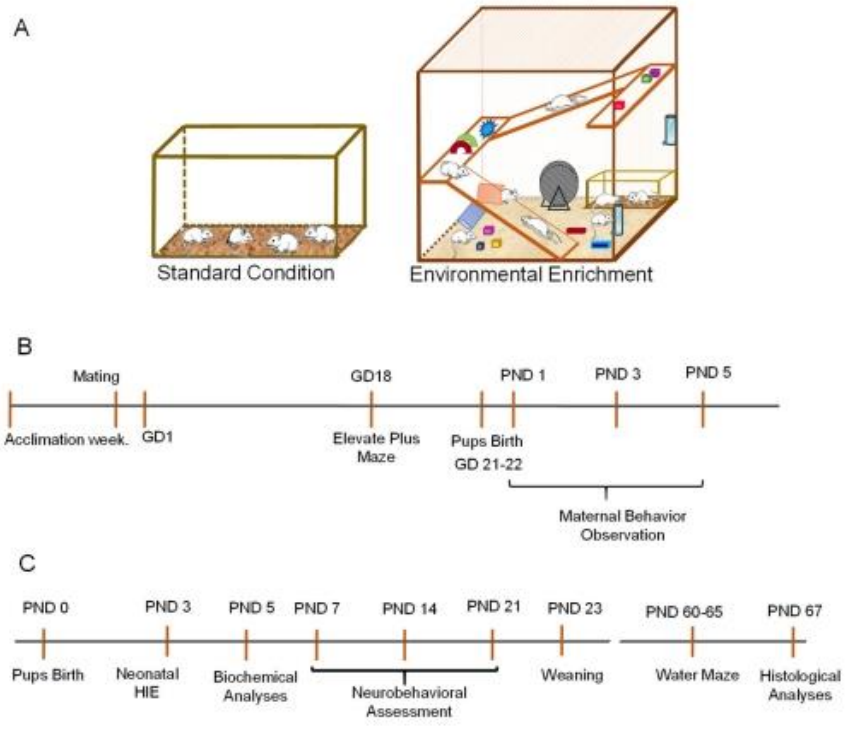


Fig. 1 Illustration of the housing conditions (**a**). Experimental timeline of: mother rats during pregnancy and first days after parturition (**b**) and pups during postnatal period (**c**). *GD* gestational day, *HIE* hypoxia ischemia encephalopathy, *PND* postnatal day

litter per cage was kept under standard condition. After weaning, the offspring was separated by sex and housed in the standard conditions (four animals per cage).

Neonatal Hypoxic Ischemic Encephalopathy

At PND 3, two and three siblings per litter were randomly assigned to surgery control (sham) or the Rice-Vanucci model. Under isoflurane anesthesia (4% induction and 1.5% maintenance), a longitudinal neckline incision was performed and permanent occlusion of the right carotid artery was made. During 2 h, the animals were located in a standard cage with their mother for post-surgery recovery. After this period, the HIE animals were placed in a hypoxia chamber (FIO_2 0.08 and 37 °C) for 180 min.

For the sham group, the pups were anesthetized, the right carotid artery was isolated without occlusion, and they were kept under atmospheric condition. Once the hypoxia exposure was completed, all the animals were returned to their biological mother and respective environment. Thereby, four groups were established considering the early environmental conditions and the injury: animals exposed to standard conditions without HIE (SC-Sham), animals exposed to standard conditions and submitted to the HIE procedure (SC-HIE), animals exposed to environmental enrichment without HIE (EE-sham), and animals exposed to environmental enrichment and submitted to the HIE procedure (EE-HIE).

Biochemical Analysis

Tissue Preparation

Animals were killed by decapitation 48 h after HIE (n = 5–8 animals per group). The brain was quickly dissected out and the right parietal cortex and right hippocampus were collected on a bed of ice. The tissue was stored at −80 °C until biochemical analysis. These encephalic structures were selected due to their significant susceptibility to gestational stimulation and HIE [51, 52].

Western Blot

The parietal cortex and hippocampus were homogenized in ice-cold lysis buffer pH 7.9 (10 mM KCl, 10 mM Hepes, 0.6 mM EDTA, 1% NP 40, and 1% protease inhibitor cocktail). Protein concentration was determined using the BCA protein assay following the manufacturer's specifications (Thermo Scientific, USA). Forty micrograms/lane of total protein was put onto NuPAGE® 4–12% Bis-Tris Gels to electrophoresis. Proteins were then transferred to nitrocellulose membranes (XCellSureLock® Mini-Cell, Invitrogen) using a transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol, and 0.25% sodium dodecyl sulfate). Membranes were blocked with tris buffer saline and 0.1% Tween 20 (T-TBS) plus 5% fat-free milk powder for 2 h (for anti-phospho-AKT (Ser473, 1:1000, Cell Signaling), the membranes were previously blocked with T-TBS plus 5% of bovine serum albumin), and incubated overnight at 4 °C with one of the following primary antibodies: anti-IGF-I (1:1000, Millipore), anti-VEGF (1:200, Santa Cruz), anti-BDNF (1:500, Abcam), anti-caspase 3 (1:1000, Millipore), anti-PARP-1 (1:100-, Cell signaling), anti-GFAP (1:1000, Thermo Fisher), anti-TrkB (1:1000, Millipore), and anti-β-actin (1:3500, Millipore).

Following three washings with T-TBS for 5 min, the membranes were incubated for 2 h with peroxidase-conjugated secondary antibody (anti-rabbit or anti-mouse 1:1000) and then washed again with T-TBS solution (four times per 5 min each). Specific bands were visualized by enhance chemiluminescence (Amersham, Oakville, Ontario) and detected using a GE-LAS 4000 digital image reader (GE Healthcare Life Sciences). The protein expression was quantified by means of Image Studio Lite (version 5.2), and the results are indicated as the ratio of intensity of protein of interest and β-actin from the same membrane [53]. No differences between the groups were observed for β-actin.

Behavioral Assessments

To evaluate the influence of the environment stimulation, the mother rats were evaluated twice: at gestational day 19 and during the first 5 days after parturition. In addition, the offspring was evaluated before the weaning and during adulthood by means of neurodevelopmental and spatial memory tests.

Elevated Plus Maze During Pregnancy

Evidence has indicated that EE could influence anxiety-like behavior [54]. In order to evaluate this behavior during the gestational period, the female rats were tested at gestational day (GD) 19 in the elevated plus maze (EPM). The four-arm apparatus was elevated 70 cm above the floor. Two arms are closed (50 × 10 × 15 cm) and the other ones remained open (50 × 10 cm). Before the testing, animals were acclimatized during 20 min in the experimental room. Then, each rat was placed in the center of the apparatus facing one enclosed arm. The number of entries and the time spent in each arm were recorded during 5 min. An arm entry was considered when the four paws were placed into an arm [55].

Maternal Behavior

To determine possible modifications in the mother-pup relationship induced by the housing conditions, maternal behavior was observed during PND 1 to PND 5 in three daily sessions (light cycle 10 am and 16 pm and dark cycle 20 pm). The

observation during the dark cycle was performed under red illumination. Each session included 15 observations every 3 min, for a total of 225 observations per dam. Behavioral responses were licking and grooming, arched-back nursing, blanket nursing, passive nursing, and mother off pups [47].

Offspring Neurodevelopmental Assessment

Neurodevelopmental assessment was conducted during lactation by means of righting reflex, negative geotaxis, and olfactory discrimination task at PND 7, 14, and 21. All the tests were performed between 9:30 and 12:00 a.m. by an evaluator—blind to the experimental condition of the animal. The maximum latency for each test was assigned when the animal did not complete the activity.

Righting Reflex Test The righting reflex test allows to assess motor and coordination function. Each animal was put on supine position and the latency required to reach the prone position with its four paws on the floor was recorded [46].

Negative Geotaxis The purpose of this test is to evaluate vestibular and proprioception function. The animal was placed on an incline board of 45° with its head down. The latency to turn around 180° and put its face up and its body in longitudinal axis was recorded. The maximum period for this test was 60 s [56].

Olfactory Discrimination Using a clean standard cage (16 × 41 × 34 cm), fresh bedding was spread over one side and soiled bedding (from the home cage of the rat) was put on the other one. A distance around 7 cm was used for each bedding. The animal was located on the center of the cage with the head towards the wall. The latency required to go to its home bedding was recorded. The cage was cleaned up with a 70% ethanol solution after each pup's test and the maximum latency for this test was 180 s [57].

Spatial Memory at Adulthood At PND 60, the spatial memory was tested in the Morris water maze. The apparatus consisted of a circular black pool (diameter of 200 cm) filled to a depth of 40 cm with room-temperature water. A platform was put 2 cm below the water level and kept in the same position during the training period. The maze was divided into four quadrants that were denominated as N, S, W, and E. In addition, visual cues were placed around the experimental room. During five consecutive days, each rat performed four trails and had 60 s to find the platform, when this time was exceeded the animal was located on the platform for 15–20 s to help it to identify the wall cues. On the sixth day, the platform was removed and the probe trial was performed. Each rat was subjected to one trial during 60 s. The variables recorded using the ANY-Maze software were latency for the first crossing of the platform region, total distance traveled, average speed, time spent in the target quadrant, time spent in non-target quadrants, and the distance traveled to the first entry to the platform zone. In addition, the target quadrant ratio was calculated ((time spent in the target quadrant/(time spent in non-target quadrants + time in target quadrant)).

Histological Analysis

After behavioral testing at PND 67, a number of five–six rats per group were randomly selected for histological analyses. The rats were anesthetized with a lethal dose of thiopental sodium (Thiopentax, Cristália®, 100 mg/kg) and were perfused with 9% saline solution followed by 4% formaldehyde solution through the left cardiac ventricle. The brains were removed from the skull, deposited in formaldehyde, and cryoprotected with sucrose solution (30%). The brains were cut on a cryostat (Leyca) in serial 30-μm slices. Each 10th section was put on gelatin-coated slides and stained with hematoxylin and eosin. (Sigma-Aldrich. St Louis, MO, USA). The cortex and the hippocampus were identified using the rat brain atlas [58] and the Cavalieri method was used for structural volume measurements. The values were acquired using the NIH-ImageJ software and the volume ratio was calculated by the equation $\frac{\text{Ipsilateral volume (mm3)}}{\text{Contralateral volume (mm3)}}$.

Statistical Analysis

The statistical analysis was performed by means of the software IBM-SPSS-version 21. For parametric data (maternal behavior, biochemical analyses and spatial memory), the general linear models for univariate dependent variable was used. Groups' differences according housing condition were compared using repeated measures ANOVA or by Student's *t* test. Differences considering housing condition and surgical procedure were identified by means of two-way ANOVA followed by multiple comparisons test. In the case of non-parametric data (neurobehavioral tests and histological analysis), the Friedman test was used to analyze changes over time, and the Kruskal-Wallis followed by Dunn test was used to determine differences between groups. Behavioral and biochemical data are expressed as means ± standard error (S.E.) and, histological data are expressed as median and interquartile intervals (25, 75). Significance was defined as $P \leq 0.05$.

Results

Elevated Plus Maze in Pregnant Rats

Independent-sample *t* tests revealed no significant differences between pregnant rats housed in standard or

enrichment conditions regarding the number of entries or time spent in the open or closed arm (number of entries/time spent (seconds): open arms: SC 5.33 ± 1.63/56.00 ± 18.6. EE 4.67 ± 0.95/46.33 ± 11.12. Closed arms: SC 9.50 ± 0.76/197.66 ± 1.63. EE 9.16 ± 0.60 / 188.33 ± 18.04). These data suggest that the environmental conditions here used do not alter anxiety levels during pregnancy.

Maternal Behavior

There were no significant differences in the frequency of maternal behavior on licking and grooming, arched-back nursing, and blanket nursing between animals housed in SC and EE ($P > 0.05$, Fig. 2a–c). However, dams of EE spent more time in the passive nursing and showed less episodes away from the nest when compared to the SC at PND5 as indicated in the Fig. 2d, e ($F = 7.40$, $P = 0.03$, and $F = 5.56$, $P = 0.05$, respectively). In addition, an interesting cooperative behavior was observed among mothers in the EE condition, with one staying in the nest, while the other one would stay away from the pups. This behavior could indicate a maternal cooperation to increase the pup's care or avoid that the offspring remained alone.

Biochemical Analysis

Expression of Pro-apoptotic Biomarkers

No significant differences were observed in the expression of total caspase-3 nor total PARP-1 in the hippocampus and parietal cortex ipsilateral to the carotid occlusion 48 h after HIE ($P > 0.05$, data not shown). Considering cleaved caspase-3, a significant interaction between HIE and housing conditions was found ($F_{(1,22)} = 4.746$, $P = 0.04$), indicating that HIE-induced an increase in the immunocontent of this pro-apoptotic protein in the hippocampus and the EE was able to prevented it, as shown in Fig. 3a. However, this difference was not observed in the parietal cortex ($P > 0.05$, Fig. 3b). EE treatment also reduced the expression of cleaved PARP-1 on the hippocampus ($F_{(1,25)} = 6.266$, $P = 0.02$) and parietal cortex ($F_{(1,22)} = 10.165$, $P = 0.005$) when compared to SC (Fig. 3c, d). These outcomes suggest that cell death mediated by caspase-dependent and caspase-independent pathways exhibit a different pattern of expression following HIE condition, and that HIE-induced cleaved-capase-3 increase was counteracted by EE. On the other hand, presented results suggest that EE reduces the cleaved PARP-1-dependent cell death in both non-injured and injured animals.

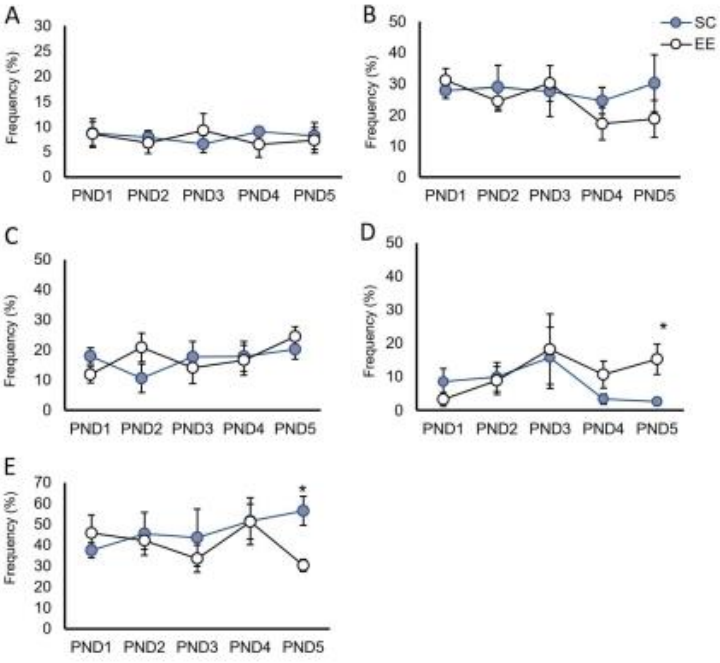


Fig. 2 Maternal behavior: **a** licking and grooming, **b** arched-back nursing, **c** blanket nursing, **d** passive nursing, and **e** mother off nest. Data analyzed by means of repeated measures ANOVA. *Asterisk* significant differences considering experimental groups and time after parturition. $n = 6$ animals per group. Significance was accepted when $p \leq 0.05$

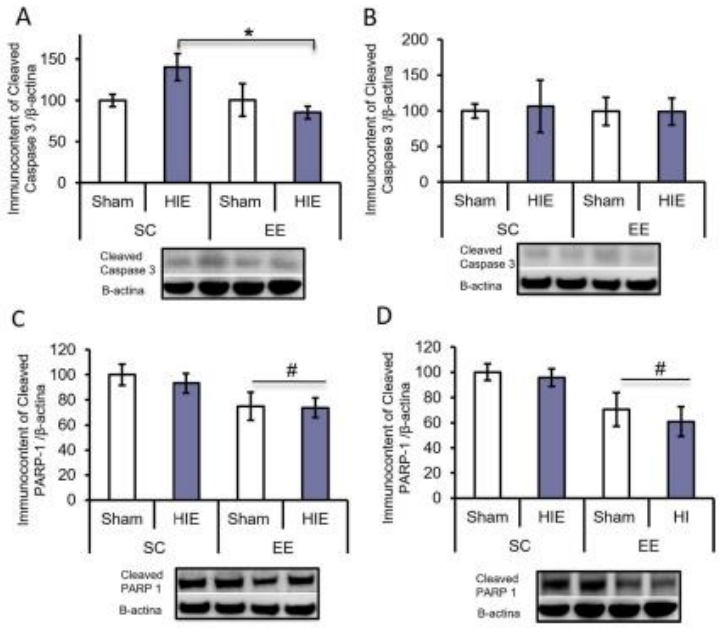


Fig. 3 Pro-apoptotic biomarkers assessed 48 h after neonatal HIE: immunocontent of cleaved caspase-3 on the hippocampus (**a**) and parietal cortex (**b**). Expression of cleaved PARP-1 on the hippocampus (**c**) and parietal cortex (**d**). Data analyzed by two-way ANOVA. *Asterisk* significant differences considering HIE and treatment (prenatal housing condition) effects, *number sign* significant effect of prenatal housing condition. $n = 5–7$ animals per group. Significance was accepted when $p \leq 0.05$

Expression of Growth and Neurotrophic Factors

Figures 4b, d show a significant reduction of the IGF-1 ($F_{(1,21)} = 9.190$, $P = 0.008$) and VEGF ($F_{(1,21)} = 4.838$, $P = 0.04$) immunocontent in the parietal cortex of HIE animals, independently of the prenatal housing condition; however, no difference was detected in the hippocampus ($P > 0.05$, Fig. 4a, c). There were no BDNF and tropomyosin receptor kinase B (TrkB) expression differences among groups ($P > 0.05$, Fig. 4e–h).

Phosphorylation of AKT

It has been reported that IGF1, VEGF, and BDNF binding to TrkB activates AKT, which is a crucial signaling cascade to promote cell survival [59]. Thus, the phosphorylated AKT isoform was evaluated. Two-way ANOVA revealed an increase in this protein in the EE group when compared to SC groups, in both the hippocampus ($F_{(1,27)} = 11.454$, $P = 0.002$) and the parietal cortex ($F_{(1,23)} = 5.917$, $P = 0.03$) of the offspring, as shown in Fig. 5a, b. These data suggest that prenatal EE facilitates the activation of the AKT pathway, reducing cell death by apoptosis.

Expression of GFAP

As observed in Fig. 5c, HIE animals presented increased glial fibrillary acid protein (GFAP) in the hippocampus 48 h after HIE insult ($F_{(1,22)} = 6.259$, $P = 0.02$). Nevertheless, animals from EE groups had a significant decrease on the expression of GFAP ($F_{(1,22)} = 6.481$, $P = 0.02$), in such a way that EE partially counteracted this HIE effect. An interaction between HIE and housing conditions ($F_{(1,22)} = 7.873$, $P = 0.01$) was found in the parietal cortex, since EE was able to prevent the increase of GFAP immunocontent that was observed in the SC-HIE group (Fig. 5d) ($F_{(1,22)} = 7.873$, $P = 0.01$). These findings suggest that prenatal EE reduces the early astrocyte overreaction after neonatal HIE.

Offspring Neurodevelopment Assessment

Over time, all animals showed a significant reduction in the time required to perform the neurobehavioral tests ($P < 0.05$). SC-HIE animals showed higher latency during the righting reflex at PND7 (Fig. 6a) when compared to EE-HIE and sham groups ($X^2 = 9.348$, $P = 0.025$). This difference was not observed at PND 14 and PND 21 ($P > 0.05$). Animals of the EE group (EE-Sham and EE-HIE) had significantly better performance in negative geotaxis and olfactory discrimination tests at PND 14 when compared to SC, as shown in Fig. 6b ($X^2 = 9.776$, $P = 0.021$). In addition, the Dunn test by Kruskal-Wallis showed a significant increase in the latency of the SC-HIE group in the olfactory discrimination tests, which was prevented by EE at PND 14 ($X^2 = 7.819$, $P = 0.05$—Fig. 6c). The animals' performance indicated that prenatal and early postnatal EE were able to prevent the

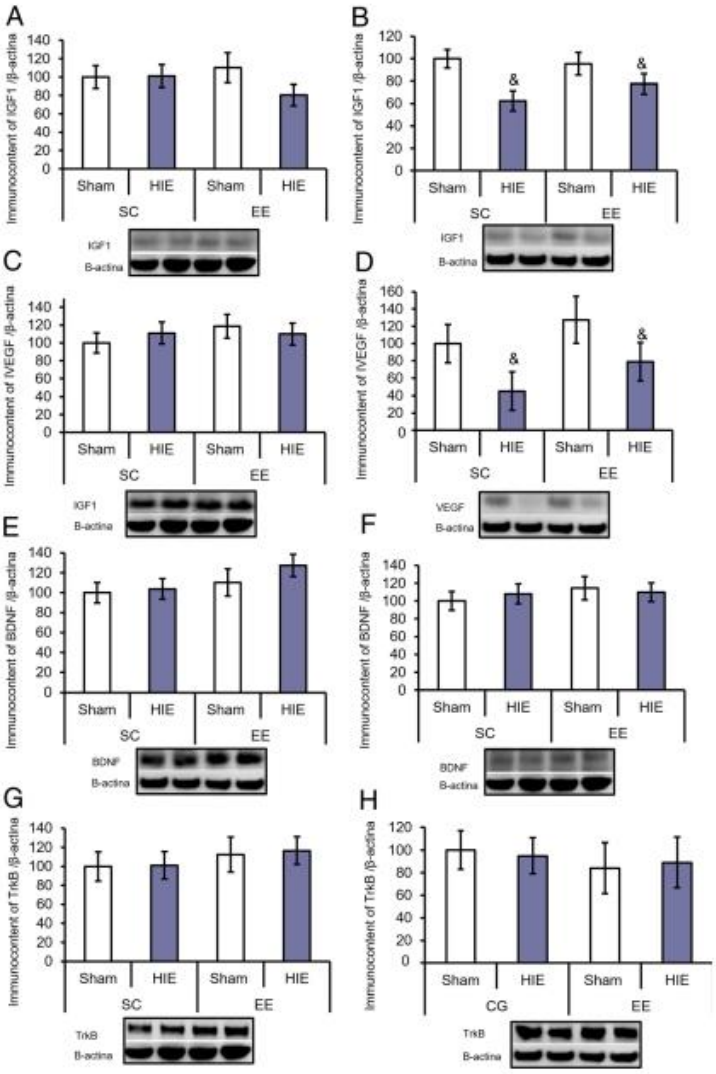


Fig. 4 Expression of growth and neurotrophic factors 48 h after HIE in the hippocampus (*left*) and cerebral cortex (*right*). **a–b** insulin-like growth factor 1 (IGF1); **c–d** vascular endothelial growth factor (VEGF); **e–f** brain-derived neurotrophic factor (BDNF); and **g–h** tropomyosin receptor kinase B (TrkB). Data analyzed by two-way ANOVA. *Ampersand* significant HIE effect when compared to their respective sham groups. $n = 5–7$ animals per group. Significance was accepted when $p \leq 0.05$

neurobehavioral delay induced by HIE and to promote the acceleration of neurodevelopmental reflexes.

Spatial Memory at Adulthood

Reduction of the latency to find the platform over the training days was observed in all the experimental groups ($F_{(4)} = 35.941$, $P = 0.000$). As shown in Fig. 6d, no significant differences were observed in first day of the spatial memory training. However, on the second day, EE animals showed a better performance during the task when compared to SC animals ($F_{(3,43)} = 13.007$, $P = 0.00$). In the third and fourth days, the SC-HIE group had higher latency when compared to the EE group and Sham animals, respectively (third day: $F_{(3,43)} = 6.769$, $P = 0.01$ and fourth day: $F_{(3,43)} = 2.280$, $P = 0.05$). In the fifth day, animals of the SC-HIE group showed the worst performance in comparison with the other groups, whereas EE-HIE had a similar latency to SC-Sham animals ($F_{(3,43)} = 3.553$, $P = 0.02$). During the probe trial, HIE rats had significantly higher latency to cross the platform region ($F_{(1,43)} = 6.324$, $P = 0.016$) when compared to Sham animals (Fig. 6e). No significant differences were observed in the total distance traveled, distance traveled to the first entry to the platform zone, and average speed ($P > 0.05$—supplementary Table 1), suggesting that HIE and EE did not alter locomotion of the animals. The target quadrant ratio showed that HIE animals spent less time in the target quadrant when compared to Sham animals ($F_{(1,43)} = 14.778$, $P = 0.001$—Fig. 6f). However,

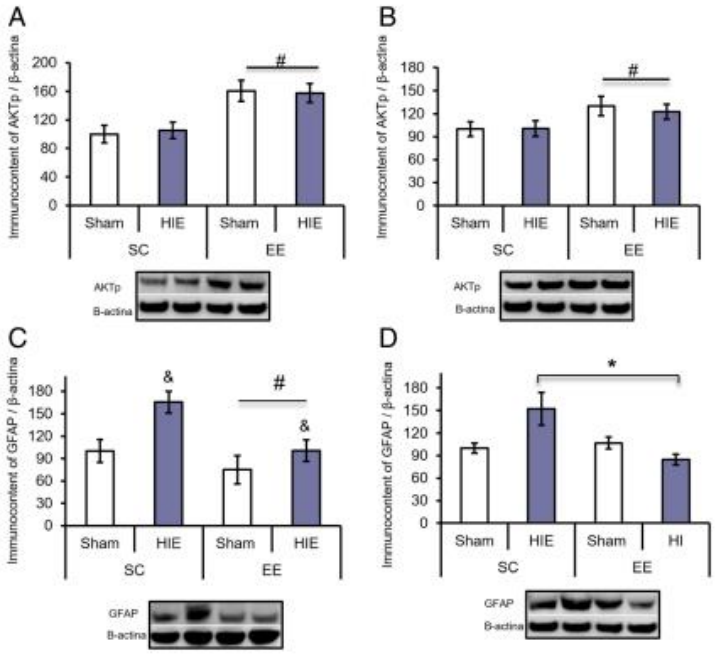


Fig. 5 Expression of phosphorylated AKT 48 h after HIE in the hippocampus (**a**) and in the cerebral cortex (**b**). Expression of GFAP 48 h after HIE in the hippocampus (**c**) and cerebral cortex (**d**). *Ampersand* significant HIE effect when compared to their respective sham, *number sign* effect of prenatal housing condition. Data analyzed by two-way ANOVA. *Asterisk* significant differences considering HIE and treatment (prenatal housing condition) effects. $n = 5–7$ animals per group. Significance was accepted when $p \leq 0.05$

animals exposed to EE increased the time spent in this quadrant ($F_{(1,43)} = 5.627$, $P = 0.023$). These data evidence that EE partially prevented the spatial memory impairment caused by the HIE and improve performance of non-lesioned animals. Representative plots of the probe trial are shown in Fig. 6g.

Histological Analysis

The SC-HIE group had a volume reduction of the hippocampus, as compared to the sham animals. In addition, EE was able to prevent such volume loss in EE-HIE animals ($X^2 = 8.874$, $P = 0.031$—Fig. 7b). No significant differences were observed in the volume ratio of the cerebral cortex between the animals ($X^2 = 5.850$, $P = 0.119$—Fig. 7c). The outcomes revealed that EE conditions prevented the tissue loss in the hippocampus after neonatal brain insult.

Discussion

The present study assessed early molecular changes in the hippocampus and parietal cerebral cortex of pups subjected to HIE, from mothers submitted or not to gestational environmental enrichment. Results showed that environmental stimulation during pregnancy and the first postnatal days (PND 1–5) is able to (a) reduce the levels of proteins involved in early cell death by apoptosis, (b) increase the activation of the AKT pathway in offspring, (c) attenuate the increase of GFAP levels, (d) promote a better performance in the neurodevelopmental tests, (e) prevent the spatial memory deficit of offspring from EE mothers, and (f) prevent the hippocampus tissue loss after HIE. Considering the public health burden [60] of neonatal hypoxia ischemia encephalopathy and premature birth and the need to define neuroprotective strategies to mitigate the severity of brain injury and its negative impacts, here presented results show that exposure to EE during gestation and the early postnatal period is a relevant experimental behavioral strategy for its demonstrated preventive effects.

Preclinical studies point to the influences of environmental conditions in female anxiety-like behavior and its possible effects on the offspring [61]. In the present study, no significant differences between EE and SC were observed during the plus maze test, being these results in agreement with previous reports [54, 62]. Sparling and coworkers found that animals housed in social colonies had an increase in the time spent on the closed arm after 10 min of exploration [63]. Their results may well indicate that the duration of the plus maze test could be a relevant factor to evidence behavioral differences when the housing conditions are taken into account.

Maternal care has a pivotal role in brain development of the offspring [64]. For this reason, some authors have indicated that the enrichment effects could be associated with modifications in the mother-pup relationship [47, 65]. The maternal behavior towards offspring was evaluated during the first postnatal week, and we did not observe significant differences in parameters such as licking/grooming, arched and blanked

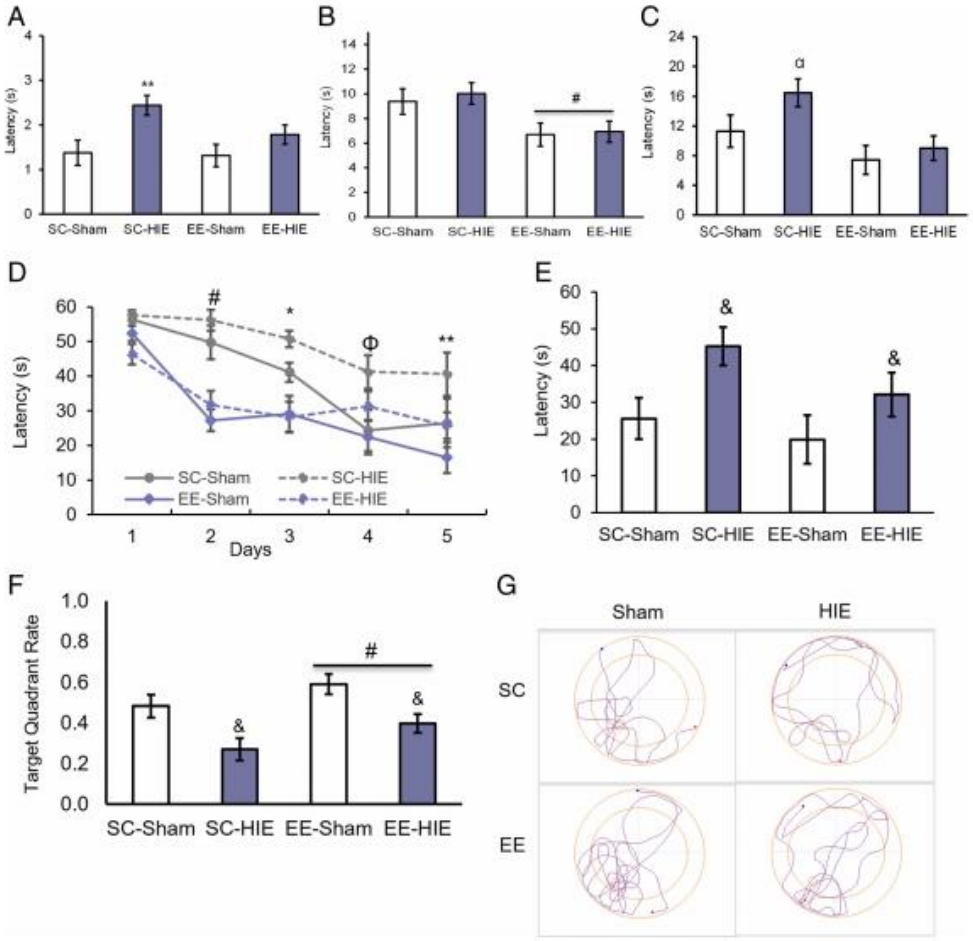


Fig. 6 Behavioral assessment: Righting reflex at PND 7 (**a**), negative geotaxis at PND 14 (**b**), and olfactory discrimination test at PND 14 (**c**). Data analyzed by Kruskal-Wallis and its corresponding pairwise test. Performance during the reference memory training at PND 60 (**d**) latency to cross the platform region during the probe trial in the Morris water maze test (**e**). Target quadrant rate (**f**) and representative plots get during the probe trial (**g**). Data analyzed by means of repeated measures and two-way ANOVA. *Double asterisk* significant differences of SC-HIE when compared to the other experimental groups, *alpha sign* significant differences of SC-HI when compared to EE groups, *number sign* significant effects of housing condition, *asterisk* significant differences of EE-HIE when compared to SC-HIE, *phi* significant differences of SC-HIE to the sham groups, *ampersand* significant HIE effect when compared to their respective sham. n = 10–12 animals per group. Significance was accepted when $p \leq 0.05$

nursing. Nevertheless, EE dams spent less time out of the nest, dedicated more time to passive nursing, and showed a cooperative behavior during the pup's care. In fact, the offspring reared in EE conditions experienced a more continuous physical contact due to the presence of another adult female, as already pointed by Sale and coworkers (2004). Considering reports from the literature, data regarding maternal behavior in EE conditions are inconclusive, since some authors indicated an increase [47, 66] and others report a reduction [21, 48], or no significant changes in pups care, when compared to the control group [67]. Although the influence of EE on maternal care ought not to be disregarded, present results of licking/grooming and arched-back did not show significant differences among groups, suggesting that other factors are responsible for the EE effects observed in the present study.

Maturation of the central nervous system can be assessed through functional performance such as neurological reflexes [39]. Thus, the EE was able to prevent the neurodevelopmental delay caused by HIE and the early EE condition induced a long-term effect on the reference memory of the stimulated animals. These data confirm that EE improves performance on righting reflex, negative geotaxis, olfactory discrimination tests during the pre-weaning period [22, 41] as well as the performance in the water maze test at adulthood [35].

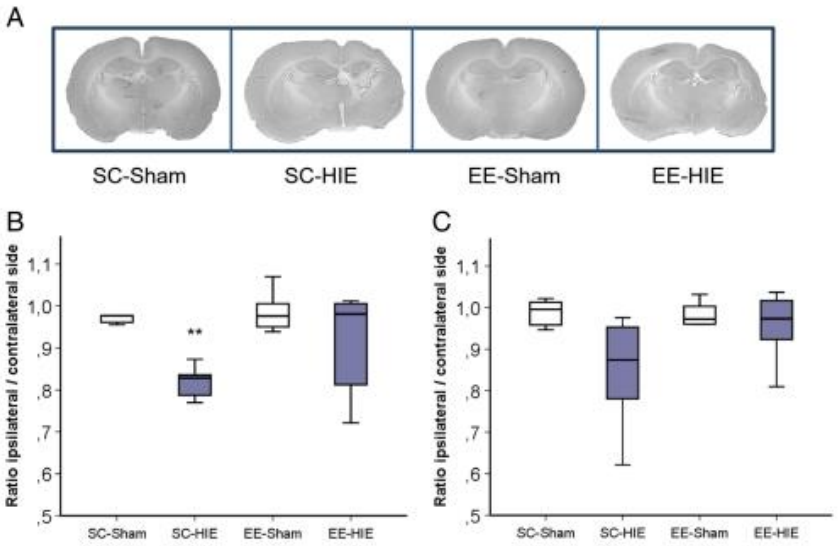


Fig. 7 Representative cresyl-violet-stained of coronal brain section at PND 67 (**a**). Ratio volume loss in the hippocampus (**b**) and in the cerebral cortex (**c**). Data analyzed by Kruskal-Wallis and its corresponding pairwise test. n = 5–6 animals per group. Significance was accepted when $p \leq 0.05$

Growth and neurotrophic factors promote cellular survival, enhance maturation processes, facilitate potentiation of cerebral networks, induce synaptic plasticity, and improve functional performance in rodents raised in an enriching environment [25, 68]. The expression of IGF-1 and VEGF in the hippocampus showed no significant differences in the period analyzed. However, the parietal cortex of HIE animals showed a decrease in the immunocontent of the abovementioned growth factors. Experimental models after brain insults have revealed increases in IGF-1 and VEGF levels to stimulate angiogenesis, promote cell viability, induce axonal growth, and facilitate functional recovery [69, 70]. However, Zhao and coworkers (2006) suggested that, under inflammatory conditions, the initial increases of IGF-1 could be sequestered by binding proteins causing a decrease in IGF-1 levels [71]. On the other hand, a dual function of the VEGF has been described after ischemia, since it enhances the blood-brain barrier (BBB) permeability and contributes to the subsequent BBB disruption; it may also exacerbate microvascular perfusion and induce cell loss [68]. Thus, the reduction observed in the HIE animals may suggest a distinct temporal course of the IGF-1 and VEGF according to the encephalic region. It is not possible at the moment to know if this decrease could be a compensatory mechanism to mitigate the negative effects associated with the brain damage severity.

The immature brain shows transient increases of IGF-1 and VEGF expressions during early and late development in different encephalic regions [72–74]. Our results show no EE effect on IGF-1 levels, what is in accordance with a previous study reporting no significant differences in IGF-1 immunoreactivity in the visual cortex of animals exposed to early postnatal EE until PND 15 [75]; however, others have suggested that EE induces two distinct peaks of IGF-1 that are necessary for visual cortex development [76]. Taking into account that we have evaluated other encephalic areas, the hippocampus and parietal cortex, in animals exposed to preweaning EE, further studies are clearly needed to determine the effects of EE protocols on the expression of IGF-1 and VEGF.

BDNF and its TrkB receptor are relevant to protect the immature brain against HIE [69] and facilitate the activity-dependent plasticity in EE animals [21, 70]. At postnatal day 5, no significant differences in BDNF and TrkB levels were identified in the brain structures examined. Previous studies evaluating BDNF levels in EE animals at different postnatal ages showed similar results to those found in this work, because significant differences were only reported from the second postnatal week [77, 78] or at adulthood [79]. Therefore, animal interaction with the postnatal environment may have a greater impact on growth and neurotrophic factors later on.

AKT is an important protein kinase that, when activated, suppresses apoptotic responses and reduces cell death after tissue damage [80]. The phosphorylated AKT isoform inhibits a group of cysteinyl-aspartate proteases (caspases) that are responsible for activating and cleaving other proteins that participate in the cell death process, such as PARP-1, which is also considered a useful marker of apoptosis [81]. Previous studies identified that neuronal death is increased during the first 72 h after HIE injury both by caspase-3-dependent as well as caspase-3-independent mechanisms [44, 82]. Our findings evidenced that EE animals present higher levels of phosphorylated AKT, with lower expression of cleaved caspase-3 and PARP-1 in the hippocampus and parietal cortex, which suggests that EE was able to reduce the cell death in the treated

animals. It is possible that reduction in the immunocontent of cleaved caspase-3 observed in the present study could be associated with lower BBB permeability, recovery of the Na+/K+ ATPase dysfunction, suppression of excitatory receptors, and reduction of excitotoxicity as previously described [37, 38, 42]. In addition, our results suggest that HIE-induced cell death can present a different temporal course for caspase-independent and caspase-dependent pathways, since no significant difference was observed in cleaved PARP-1. However, it has been shown that environmental stimulation after weaning promotes neuronal survival during the postnatal period, as a consequence of the inhibition of spontaneous and developmental apoptosis [83]. This fact could be associated with the reduction of cleaved PARP-1 in EE animals, which suggests that the prenatal EE protocol was able to facilitate a similar response in this pro-apoptotic protein. The outcomes observed in growth and neurotrophic factors in the hippocampus and parietal cortex at PND 5 do not exclude a possible previous activation of signaling pathways related to cell survival. To our knowledge, this is the first work indicating that prenatal associated to early postnatal EE could modulate the apoptotic processes downstream of the AKT pathway, revealing a novel mechanism for activating cell survival after neonatal HIE at PND3.

Another important molecular change by which EE could reduce the deleterious effects of the HIE insult is through a reduction in the GFAP levels both in the hippocampus and parietal cortex. The GFAP is a gold standard astrocyte marker and this cell has a critical role in the maturation and function of the developing brain. In response to different degrees of damage severity, astrocytes show a high susceptibility, being primary responders to injury [13]. In addition, astrocytes stimulate the proliferation and interaction of several cells to make the glia scar, which act like a mechanical barrier to the neuronal and non-neuronal cell function [13, 84]. Our data suggest that under the same brain insult, EE is able to change astrocyte reactivity and consequently decrease the brain damage. This event has the potential to mitigate the neurodevelopmental delay, as well as the cognitive deficits as observed after HIE. Furthermore, the EE-induced molecular changes observed during the acute period after HIE insult were able to reduce the tissue loss in the hippocampus at adulthood, a period in which the extension of the brain damage is clearly delimited.

Conclusion

Results here presented provide evidence that prenatal and early postnatal environmental enrichment reduce the HIE-induced apoptosis and astrocytic reaction, possibly through the upregulation of the AKT pathway. In addition, EE promoted hippocampus tissue preservation and prevented neurodevelopmental delays and cognitive deficits following HIE at PND3. Altogether, the exposure to early EE is able to protect developing brain structures of the detrimental damage caused by HIE under experimental conditions that mimic premature birth.

Acknowledgments This study was partly supported by financial resources from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). The authors also thank the staff of the Reproduction and Laboratory Animal Research Center from Biochemistry Department–UFRGS.

Compliance with Ethical Standards The experimental procedures were approved by the Ethics committee of the Universidade Federal do Rio Grande do Sul (no 28641). Animal care followed the guidelines of the Brazilian Society of Science in Laboratory Animals—Law no 11.794; and the recommendation of the Council for International Organizations of Medical Sciences (CIOMS - Publication 85-23, 1985).

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

References

1. Volpe JJ (2005) Encephalopathy of prematurity includes neuronal abnormalities. Pediatrics 116:221–225. doi:10.1542/peds.2005-0191
2. Roggero P, Gianni ML, Garbarino F, Mosca F (2013) Consequences of prematurity on adult morbidities. Eur J Intern Med 24:624–626. doi:10.1016/j.ejim.2013.01.011
3. Ness JK, Romanko MJ, Rothstein RP, et al (2001) Perinatal hypoxia-ischemia induces apoptotic and excitotoxic death of periventricular white matter oligodendrocyte progenitors. In: Dev. Neurosci. pp 203–208
4. Hassell KJ, Ezzati M, Alonso-Alconada D et al (2015) New horizons for newborn brain protection: enhancing endogenous neuroprotection. Arch Dis Child - Fetal Neonatal Ed 100:F541–F552. doi:10.1136/archdischild-2014-306284
5. Hill CA, Fitch RH (2012) Sex differences in mechanisms and outcome of neonatal hypoxia-ischemia in rodent models: implications for sex-specific neuroprotection in clinical neonatal practice. Neurol Res Int 2012:12–14. doi:10.1155/2012/867531
6. Renolleau S, Fau S, Charriaut-marlangue C, et al (2007) Gender-related differences in apoptotic pathways after neonatal cerebral ischemia. Neuroscientist XX:1–7. doi: 10.1177/1073858407308889
7. Joly L-M, Mucignat V, Mariani J et al (2004) Caspase inhibition after neonatal ischemia in the rat brain. J Cereb Blood Flow Metab 24:124–131. doi:10.1097/01.WCB.0000100061.36077.5F
8. Zhu C, Wang X, Huang Z et al (2007) Apoptosis-inducing factor is a major contributor to neuronal loss induced by neonatal cerebral hypoxia-ischemia. Cell Death Differ 14:775–784. doi:10.1038/sj.cdd.4402053
9. Brekke E, Morken TS, Sonnewald U (2015) Glucose metabolism and astrocyte-neuron interactions in the neonatal brain. Neurochem Int 82:33–41. doi:10.1016/j.neuint.2015.02.002
10. Freeman MR (2010) Specification and morphogenesis of astrocytes. Science 330:774–778. doi:10.1126/science.1190928

11. Liu F, McCullough LD (2013) Inflammatory responses in hypoxic ischemic encephalopathy. Acta Pharmacol Sin 34:1121–1130. doi:10.1038/aps.2013.89
12. Mallard C, Davidson JO, Tan S et al (2013) Astrocytes and microglia in acute cerebral injury underlying cerebral palsy associated with preterm birth. Pediatr Res 75:1–7. doi:10.1038/pr.2013.188
13. Burda JE, Sofroniew MV (2014) Reactive gliosis and the multicellular response to CNS damage and disease. Neuron 81:229–248. doi:10.1016/j.neuron.2013.12.034
14. Yager JY, Ashwal S (2009) Animal models of perinatal hypoxic-ischemic brain damage. Pediatr Neurol 40:156–167. doi:10.1016/j.pediatrneurol.2008.10.025
15. Coutellier L, Würbel H (2009) Early environmental cues affect object recognition memory in adult female but not male C57BL/6 mice. Behav Brain Res 203:312–315. doi:10.1016/j.bbr.2009.05.001
16. Sale A, Berardi N, Maffei L (2014) Environment and brain plasticity: towards an endogenous pharmacotherapy. Physiol Rev 94:189–234. doi:10.1152/physrev.00036.2012
17. Jiang X, West AA, Caudill MA (2014) Maternal choline supplementation: a nutritional approach for improving offspring health? Trends Endocrinol Metab 25:263–273. doi:10.1016/j.tem.2014.02.001
18. Bale TL (2015) Epigenetic and transgenerational reprogramming of brain development. Nat Rev Neurosci 1–13. doi: 10.1038/nrn3818
19. Baroncelli L, Braschi C, Spolidoro M et al (2009) Nurturing brain plasticity: impact of environmental enrichment. Cell Death Differ 17:1092–1103. doi:10.1038/cdd.2009.193
20. Nithianantharajah J, Hannan AJ (2006) Enriched environments, experience-dependent plasticity and disorders of the nervous system. Nat Rev Neurosci 7:697–709. doi:10.1038/nrn1970
21. Welberg L, Thrivikraman KV, Plotsky PM (2006) Combined pre- and postnatal environmental enrichment programs the HPA axis differentially in male and female rats. Psychoneuroendocrinology 31:553–564. doi:10.1016/j.psyneuen.2005.11.011
22. Kiss P, Vadasz G, Kiss-Illes B et al (2013) Environmental enrichment decreases asphyxia-induced neurobehavioral developmental delay in neonatal rats. Int J Mol Sci 14:22258–22273. doi:10.3390/ijms141122258
23. Connors EJ, Shaik a N, Migliore MM, Kentner a C (2014) Environmental enrichment mitigates the sex-specific effects of gestational inflammation on social engagement and the hypothalamic pituitary adrenal axis-feedback system. Brain Behav Immun 42:178–190. doi:10.1016/j.bbi.2014.06.020
24. Kazlauckas V, Pagnussat N, Mioranzza S et al (2011) Enriched environment effects on behavior, memory and BDNF in low and high exploratory mice. Physiol Behav 102:475–480. doi:10.1016/j.physbeh.2010.12.025
25. Novkovic T, Mittmann T, Manahan-Vaughan D (2015) BDNF contributes to the facilitation of hippocampal synaptic plasticity and learning enabled by environmental enrichment. Hippocampus 25:1–15. doi:10.1002/hipo.22342
26. Sale A, Cenni MC, Clussi F et al (2007) Maternal enrichment during pregnancy accelerates retinal development of the fetus. PLoS One 2:1–8. doi:10.1371/journal.pone.0001160
27. Maya-Vetencourt JF, Baroncelli L, Viegi A et al (2012) IGF-1 restores visual cortex plasticity in adult life by reducing local GABA levels. Neural Plast. doi:10.1155/2012/250421
28. Ortuzar N, Rico-Barrio I, Bengoetxea H et al (2013) VEGF reverts the cognitive impairment induced by a focal traumatic brain injury during the development of rats raised under environmental enrichment. Behav Brain Res 246:36–46. doi:10.1016/j.bbr.2013.02.036
29. During MJ, Cao L (2006) VEGF, a mediator of the effect of experience on hippocampal neurogenesis. Curr Alzheimer Res 3:29–33. doi:10.2174/156720506775697133
30. Koskela M, Bäck S, Võikar V et al (2016) Update of neurotrophic factors in neurobiology of addiction and future directions. Neurobiol Dis. doi:10.1016/j.nbd.2016.05.010
31. Gonzalez A, Moya-Alvarado G, Gonzalez-Billaut C, Bronfman F (2016) Cellular and molecular mechanisms regulating neuronal growth by brain-derived neurotrophic factor (BDNF). Cytoskeleton 0:1–17. doi:10.1002/cm.21312
32. Li L, Qu Y, Mao M et al (2008) The involvement of phosphoinositid 3-kinase/Akt pathway in the activation of hypoxia-inducible factor-1α in the developing rat brain after hypoxia-ischemia. Brain Res 1197:152–158. doi:10.1016/j.brainres.2007.12.059
33. Zhang L, Li P-P, Feng X et al (2003) Sex-related differences in neuronal cell survival and signaling in rats. Neurosci Lett 337:65–68. doi:10.1016/S03
34. Faustino JV, Wang X, Johnson CE et al (2011) Microglial cells contribute to endogenous brain defenses after acute neonatal focal stroke. J Neurosci 31:12992–13001. doi:10.1523/JNEUROSCI.2102-11.2011
35. Pereira LO, Arteni NS, Petersen RC et al (2007) Effects of daily environmental enrichment on memory deficits and brain injury following neonatal hypoxia-ischemia in the rat. Neurobiol Learn Mem 87:101–108. doi:10.1016/j.nlm.2006.07.003
36. Rojas JJ, Deniz BF, Schuch CP et al (2015) Environmental stimulation improves performance in the ox-maze task and recovers Na+, K+–ATPase activity in the hippocampus of hypoxic–ischemic rats. Neuroscience 291:118–127. doi:10.1016/j.neuroscience.2015.01.017
37. Rojas JJ, Deniz BF, Miguel PM et al (2013) Effects of daily environmental enrichment on behavior and dendritic spine density in hippocampus following neonatal hypoxia-ischemia in the rat. Exp Neurol 241:25–33. doi:10.1016/j.expneurol.2012.11.026
38. Pereira LO, Nabinger PM, Strapasson ACP et al (2009) Long-term effects of environmental stimulation following hypoxia-ischemia on the oxidative state and BDNF levels in rat hippocampus and frontal cortex. Brain Res 1247:188–195. doi:10.1016/j.brainres.2008.10.017
39. Horvath G, Dora Reglődi, Farkas J, et al (2015) Perinatal positive and negative influences on the early neurobehavioral reflex and motor development. In: Marta C. Antonelli (ed) Perinat. Program. Neurodev., Springer. New York Heidelberg Dordrecht London, pp 149–167
40. Marques MR, Stigger F, Segabinazi E et al (2014) Beneficial effects of early environmental enrichment on motor development and spinal cord plasticity in a rat model of cerebral palsy. Behav Brain Res 263:149–157. doi:10.1016/j.bbr.2014.01.007
41. Schuch CP, Diaz R, Deckmann I et al (2016) Early environmental enrichment affects neurobehavioral development and prevents brain damage in rats submitted to neonatal hypoxia-ischemia. Neurosci Lett 617:101–107. doi:10.1016/j.neulet.2016.02.015
42. Diaz R, Maidana Miguel P, Ferrary Deniz B et al (2016) Environmental enrichment attenuates the blood brain barrier dysfunction induced by the neonatal hypoxia-ischemia. Int J Dev Neurosci 53:35–45. doi:10.1016/j.ijdevneu.2016.06.006
43. Sizonenko SV, Sirimanne E, Mayall Y et al (2003) Selective cortical alteration after hypoxic-ischemic injury in the very immature rat brain. Pediatr Res 54:263–269. doi:10.1203/01.PDR.0000072517.01207.87
44. Towfighi J, Mauger D, Vannucci RC, Vannucci SJ (1997) Influence of age on the cerebral lesions in an immature rat model of cerebral

hypoxia-ischemia: a light microscopic study. Brain Res Dev Brain Res 100:149–160
45. Branchi I (2009) The mouse communal nest: investigating the epigenetic influences of the early social environment on brain and behavior development. Neurosci Biobehav Rev 33:551–559. doi:10.1016/j.neubiorev.2008.03.011
46. Cárdenas L, García-García F, Santiago-Roque I et al (2015) Enriched environment restricted to gestation accelerates the development of sensory and motor circuits in the rat pup. Int J Dev Neurosci 41:68–73. doi:10.1016/j.ijdevneu.2014.11.008
47. Sale A, Putignano E, Cancedda L et al (2004) Enriched environment and acceleration of visual system development. Neuropharmacology 47:649–660. doi:10.1016/j.neuropharm.2004.07.008
48. Rosenfeld A, Weller A (2012) Behavioral effects of environmental enrichment during gestation in WKY and Wistar rats. Behav Brain Res 233:245–255. doi:10.1016/j.bbr.2012.05.006
49. Simonetti T, Lee H, Bourke M et al (2009) Enrichment from birth accelerates the functional and cellular development of a motor control area in the mouse. PLoS One. doi:10.1371/journal.pone.0006780
50. Ivinskis a, Homewood J (1980) Effects of preweaning environmental enrichment on later problem-solving behavior in rats. Anim Learn Behav 8:336–340. doi:10.3758/BF03199614
51. Mychasiuk R, Zahir S, Schmold N et al (2012) Parental enrichment and offspring development: modifications to brain, behavior and the epigenome. Behav Brain Res 228:294–298. doi:10.1016/j.bbr.2011.11.036
52. Nakajima W, Ishida A, Lange MS et al (2000) Apoptosis has a prolonged role in the neurodegeneration after hypoxic ischemia in the newborn rat. J Neurosci 20:7994–8004
53. Arcego DM, Krolow R, Lampert C et al (2016) Early life adversities or high fat diet intake reduce cognitive function and alter BDNF signaling in adult rats: interplay of these factors changes these effects. Int J Dev Neurosci 50:16–25. doi:10.1016/j.ijdevneu.2016.03.001
54. Mann PE, Gervais KJ (2011) Environmental enrichment delays pup-induced maternal behavior in rats. Dev Psychobiol 53:371–382. doi:10.1002/dev.20526
55. Sanches EF, Arteni NS, Nicola F et al (2013) Early hypoxia-ischemia causes hemisphere and sex-dependent cognitive impairment and histological damage. Neuroscience 237:208–215. doi:10.1016/j.neuroscience.2013.01.066
56. Sanches EF, Arteni NS, Spindler C et al (2012) Effects of pre- and postnatal protein malnutrition in hypoxic-ischemic rats. Brain Res 1438:85–92. doi:10.1016/j.brainres.2011.12.024
57. Favero AM, Weis SN, Zeni G et al (2006) Diphenyl diselenide changes behavior in female pups. Neurotoxicol Teratol 28:607–616. doi:10.1016/j.ntt.2006.08.003
58. Paxinos G, Watson C (1998) The rat brain—in stereotaxic coordinates, Fourth. United States of American
59. Kim GS, Cho S, Nelson JW et al (2014) TrkB agonist antibody pretreatment enhances neuronal survival and long-term sensory motor function following hypoxic ischemic injury in neonatal rats. PLoS One 9:1–9. doi:10.1371/journal.pone.0088962
60. McKenna MC, Scafidi S, Robertson CL (2015) Metabolic alterations in developing brain after injury: knowns and unknowns. Neurochem Res 40:2527–2543. doi:10.1007/s11064-015-1600-7
61. Gapp K, von Ziegler L, Tweedie-Cullen RY, Mansuy IM (2014) Early life epigenetic programming and transmission of stress-induced traits in mammals: how and when can environmental factors influence traits and their transgenerational inheritance? BioEssays 36:491–502. doi:10.1002/bies.201300116
62. Hoffmann LC, Schütte SRM, Koch M, Schwabe K (2009) Effect of "enriched environment" during development on adult rat behavior and response to the dopamine receptor agonist apomorphine. Neuroscience 158:1589–1598. doi:10.1016/j.neuroscience.2008.11.035
63. Sparling JE, Mahoney M, Baker S, Bielajew C (2010) The effects of gestational and postpartum environmental enrichment on the mother rat: a preliminary investigation. Behav Brain Res 208:213–223. doi:10.1016/j.bbr.2009.11.041
64. Kappeler L, Meaney MJ (2010) Epigenetics and parental effects. BioEssays 32:818–827. doi:10.1002/bies.201000015
65. Li KA, Lund ET, Voigt J-PW (2016) The impact of early postnatal environmental enrichment on maternal care and offspring behaviour following weaning. Behav Process 122:51–58. doi:10.1016/j.beproc.2015.11.008
66. Cutuli D, Caporali P, Gelfo F et al (2015) Pre-reproductive maternal enrichment influences rat maternal care and offspring developmental trajectories: behavioral performances and neuroplasticity correlates. Front Behav Neurosci 9:1–18. doi:10.3389/fnbeh.2015.00066
67. Connors E, Migliore M, Pillsbury S et al (2014) Environmental enrichment models a naturalistic form of maternal separation and shapes the anxiety response patterns of offspring. Psychoneuroendocrinology 52C:153–167. doi:10.1016/j.psyneuen.2014.10.021
68. Zhang ZG, Zhang L, Jiang Q et al (2000) VEGF enhances angiogenesis and promotes blood-brain barrier leakage in the ischemic brain. J Clin Invest 106:829–838. doi:10.1172/JCI9369
69. Carmeliet P (2003) Blood vessels and nerves: common signals, pathways and diseases. Nat Rev Genet 4:710–720. doi:10.1038/nrg1158
70. Trejo JL, Carro E, Torres-Aleman I (2001) Circulating insulin-like growth factor I mediates exercise-induced increases in the number of new neurons in the adult hippocampus. J Neurosci 21:1628–1634
71. Zhao W, Xie W, Xiao Q et al (2006) Protective effects of an anti-inflammatory cytokine, interleukin-4, on motoneuron toxicity induced by activated microglia. J Neurochem 99:1176–1187. doi:10.1111/j.1471-4159.2006.04172.x
72. Bondy C a (1991) Transient IGF-I gene expression during the maturation of functionally related central projection neurons. J Neurosci 11:3442–3455
73. Fernandez AM, Torres-Alemán I (2012) The many faces of insulin-like peptide signalling in the brain. Nat Rev Neurosci 13:225–239. doi:10.1038/nrn3209
74. Ward MC, Cunningham AM (2015) Developmental expression of vascular endothelial growth factor receptor 3 and vascular endothelial growth factor C in forebrain. Neuroscience 303:544–557. doi:10.1016/j.neuroscience.2015.04.063
75. Ciucci F, Putignano E, Baroncelli L et al (2007) Insulin-like growth factor 1 (IGF-1) mediates the effects of enriched environment (EE) on visual cortical development. PLoS One. doi:10.1371/journal.pone.0000475
76. Baroncelli L, Cenni MC, Melani R et al (2017) Early IGF-1 primes visual cortex maturation and accelerates developmental switch between NKCC1 and KCC2 chloride transporters in enriched animals. Neuropharmacology 113:167–177. doi:10.1016/j.neuropharm.2016.02.034
77. Caporali P, Cutuli D, Gelfo F et al (2014) Pre-reproductive maternal enrichment influences offspring developmental trajectories: motor behavior and neurotrophin expression. Front Behav Neurosci 8:195. doi:10.3389/fnbeh.2014.00195
78. Cancedda L, Putignano E, Sale A et al (2004) Acceleration of visual system development by environmental enrichment. J Neurosci 24:4840–4848. doi:10.1523/JNEUROSCI.0845-04.2004
79. Baldini S, Restani L, Baroncelli L et al (2013) Enriched early life experiences reduce adult anxiety-like behavior in rats: a role for

insulin-like growth factor 1. J Neurosci 33:11715–11723. doi:10.1523/JNEUROSCI.3541-12.2013
80. Kim AH, Khursigara GUS, Sun X et al (2001) Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. Mol Cell Biol 21:893–901. doi:10.1128/MCB.21.3.893
81. Chaitanya GV, Steven AJ, Babu PP (2010) PARP-1 cleavage fragments: signatures of cell-death proteases in neurodegeneration. Cell Commun Signal 8:31. doi:10.1186/1478-811X-8-31
82. Koike M, Shibata M, Tadakoshi M et al (2008) Inhibition of autophagy prevents hippocampal pyramidal neuron death after hypoxic-ischemic injury. Am J Pathol 172:454–469. doi:10.2353/ajpath.2008.070876
83. Young D, Lawlor P a, Leone P et al (1999) Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective. Nat Med 5:448–453. doi:10.1038/7449
84. Sizonenko SV, Kiss JZ, Inder T et al (2005) Distinctive neuropathologic alterations in the deep layers of the parietal cortex after moderate ischemic-hypoxic injury in the P3 immature rat brain. Pediatr Res 57:865–872. doi:10.1203/01.PDR.0000157673.36848.67

Supplementary Table 1

Table 1: Probe trial in the Morris Water Maze on adult rats exposed to gestational and early postnatal EE

Variable	SC-Sham	SC-HIE	EE-Sham	EE-HIE
Total Distance Travelled (m)	0.06 ± (0.0047)	0.05 ± (0.0071)	0.06 ± (0.0045)	0.11 ± (0.0415)
Average Speed (m/s)	0.0009 ± (0.0001)	0.001 ± (0.00000)	0.0011 ± (0.00009)	0.0009 ± (0.00007)
Time Spent in the Target Quadrant (s)	28.50 ± (3.70)	15.93[&] ± (3.74)	35.23[#] ± (2.83)	23.93[&#] ± (2.76)
Time Spent in Non-Target Quadrant (s)	30.57 ± (3.64)	43.33[&] ± (3.88)	24.52[#] ± (2.88)	36.87[&#] ± (2.17)
Distance Travelled to first entry to the Platform Zone (m)	0.028 ± (0.0052)	0.03 ± (0.0095)	0.03 ± (0.0045)	0.04 ± (0.0059)
Latency (s)	29.44 ± (6.80)	45.20[&] ± (6.72)	19.9 ± (5.53)	34.07[&] ± (4.91)

SC: Standard Condition. EE: Environmental Enrichment. The values are expressed as mean ± S.E.M. Significance: P= 0.05
[&] Significant HIE effect when compared to their respective sham.
[#] Effect of prenatal and early postnatal housing condition.

Principais Achados do Capítulo 2

Entre os resultados obtidos neste capítulo, destacamos que:

1. O ambiente enriquecido gestacional não induziu diferenças significativas no comportamento maternal, comumente associadas aos efeitos positivos na prole (e.g. licking grooming, postura cifótica e postura coberta);
2. O ambiente enriquecido atenuou a morte celular por apoptose e a reatividade astrocitária ocasionada pela hipóxia isquemia, na fase aguda (48h após o insulto);
3. A hipóxia isquemia reduz a expressão de fatores de crescimento no córtex parietal, 48h após a lesão, independentemente das condições de moradia dos animais;
4. O ambiente enriquecido favoreceu vias relacionadas a sobrevivência celular, como a da Proteína Cinase B – AKT - e otimizou o desempenho funcional dos animais;
5. A perda tecidual no hipocampo após a HI foi atenuada pelo ambiente enriquecido na gestação e lactação.

4.3 CAPÍTULO 3

Preventive and therapeutic effects of environmental enrichment in Wistar rats submitted to neonatal hypoxia-ischemia

Behavioral Brain Research, Vol. 359, 1 February 2019, Pages 485-497

(Impact factor: 3.173)

Behavioural Brain Research 359 (2019) 485–497

Contents lists available at ScienceDirect

Behavioural Brain Research

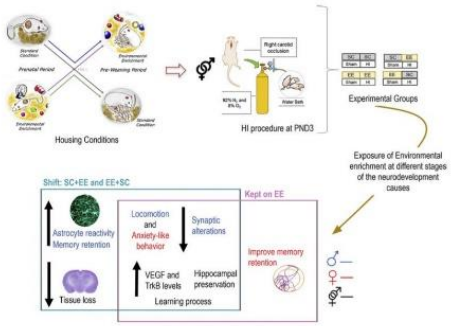
journal homepage: www.elsevier.com/locate/bbr

Preventive and therapeutic effects of environmental enrichment in Wistar rats submitted to neonatal hypoxia-ischemia

L.E Durán-Carabali[a,*], D.M. Arcego[b], E.F. Sanches[b], F.K. Odorcyk[a], M.R. Marques[c], A. Tosta[b], L. Reichert[d], A.S. Carvalho[b], C. Dalmaz[b,c], C.A. Netto[a,b,c]

[a] Post-Graduation Program of Physiology, Instituto de Ciências Básicas da Saúde (ICBS), Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
[b] Department of Biochemistry, Instituto de Ciências Básicas da Saúde (ICBS), Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
[c] Post-Graduation Program of Neuroscience, Instituto de Ciências Básicas da Saúde (ICBS), Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
[d] Neurorehabilitation and Neural Repair Research Group, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, RS, Brazil

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Environmental enrichment
Hippocampus
Hypoxia-Ischemia
Neuroprotection
Immature brain

ABSTRACT

Environmental enrichment (EE) at early stages of neurodevelopment attenuates HI-induced behavioral, histological and cellular damage. However, the effects of EE exposure during gestational or early postnatal period and the possible influence of sexual dimorphism on EE protection are not fully understood. Present study evaluated the effects of pre-natal and postnatal EE, as well as their combination, in male and female rats submitted to neonatal HI at postnatal day (PND) 3. Wistar rats were housed in EE or in standard condition (SC) during all pregnancy. At PND1, the litters were randomly allocated to the same prenatal environment during lactation (SC + SC or EE + EE) or housed in a new environment until weaning (SC + EE or EE + SC). Behavioral tasks were performed from PND 60-75. Then, animals were euthanized for biochemical and histological analysis. Prenatal and early postnatal EE alone improved performance of HI males in the Water Maze spatial memory task, while HI females were most benefited from early postnatal stimulation. Moreover, EE attenuated HI-induced lower anxiety-like behavior in rats of both sexes and decreased hyperlocomotion in HI females. Hippocampus tissue preservation and higher VEGF and TrkB levels were observed in all HI groups exposed to EE. Interestingly, HI males exposed to prenatal or postnatal EE alone exhibited higher GFAP levels and additional tissue preservation. Therefore, both prenatal and early postnatal environmental enrichment cause attenuation of HI-

* Corresponding author at: Post-graduation Program of Physiology, Instituto de Ciências Básicas da Saúde (ICBS), Universidade Federal do Rio Grande do Sul (UFRGS), Rua Ramiro Barcelos 2600, anexo., CEP 90035-003, Porto Alegre, RS, Brazil.
E-mail address: luzeled67@gmail.com (L.E. Durán-Carabali).

https://doi.org/10.1016/j.bbr.2018.11.036
Received 22 August 2018; Received in revised form 14 November 2018; Accepted 24 November 2018
Available online 26 November 2018
0166-4328/

induced impairments, revealing their preventive and therapeutic actions, possibly due to VEGF and astrocyte activity; some of these effects are sex-specific.

1. Introduction

Neonatal encephalic damage caused by glucose and oxygen deprivation affects up to 26 per 1000 newborns [1]. Conditions such as premature birth enhance the vulnerability to hypoxic-ischemic (HI) insults, increasing the incidence of motor and sensory deficits, cognitive impairment and emotional disorders [2]. The Rice-Vannucci experimental animal model performed at postnatal day three (PND 3) is commonly used to mimic HI insult in prematurity conditions observed in humans, since the rat brain development at this period corresponds to the human newborn brain at 24–28 weeks of gestation [3]. This model has provided information on the susceptibility of encephalic structures, such as cortex, periventricular white matter, striatum and hippocampus according to the brain maturation, as well as the underlying sensorimotor and cognitive dysfunctions [4,5].

It was recently demonstrated that neurodevelopmental disorders in preterm infants are associated with neurological changes starting during the gestational period [6]. Moreover, the antenatal period has been suggested as an emerging target for neuroprotective interventions in order to reduce the detrimental effects of complications subsequent to early delivery [7,8]. Behavioral, morphological and molecular consequences of HI insults can be attenuated by the interaction between the animal and the environment, considering the high plasticity of the immature brain and the relevance of external stimuli across the life cycle [4,9]. Environmental enrichment (EE) is an experimental paradigm used to promote experience-dependent plasticity by a combination of sensory, cognitive and motor stimulations [10]. The beneficial effects of EE after weaning and at adulthood have been well documented [10,11] and a growing body of evidence has shown that EE protocols cause epigenetic, transgenerational and early postnatal alterations [12–15].

Environmental stimulation during prenatal and early postnatal periods accelerates neurodevelopment, improves memory, reduces stress levels, increases dendritic spine density, promotes neurogenesis and cell proliferation, modifies the sensory and motor circuits as well as increases neurotrophic and growth factors levels [16–22]. The long-term effects of prenatal EE can be influenced by postnatal conditions and vice versa, confirming the close relationship between them [23,24] and supporting the need for studies aiming to differentiate the intrauterine from extra-uterine influences on response to brain injuries [6].

There are a few studies investigating how EE could modulate brain responses following neonatal HI. Animals submitted to HI at PND 7 (protocol used to mimic term infant brain development) and exposed after weaning to EE for nine weeks, 1 h per day, have shown positive outcomes against HI impairments in cellular, morphological and functional parameters. EE prevented Na^+/K^+_ ATPase dysfunction [25], decreased oxidative stress and increased tissue preservation [26] and reduced cognitive and motor deficits [27,28]. Furthermore, HI animals housed in an enriched environment until weaning showed decreased blood-brain barrier damage [29], reduced brain atrophy and accelerated neurodevelopmental reflexes as compared to HI rats not exposed to EE [18].

Sexual dimorphism is a major variable in neonatal HI, altering aspects such as susceptibility to brain damage, behavioral responses and treatment efficacy [30]. In addition, sex modulates several aspects of brain functionality, such as memory, synaptic transmission, gene expression and homeostatic mechanisms [31,32]. However, little is known regarding the effects of the combination of pre and early postnatal EE in animals submitted to HI considering differences between

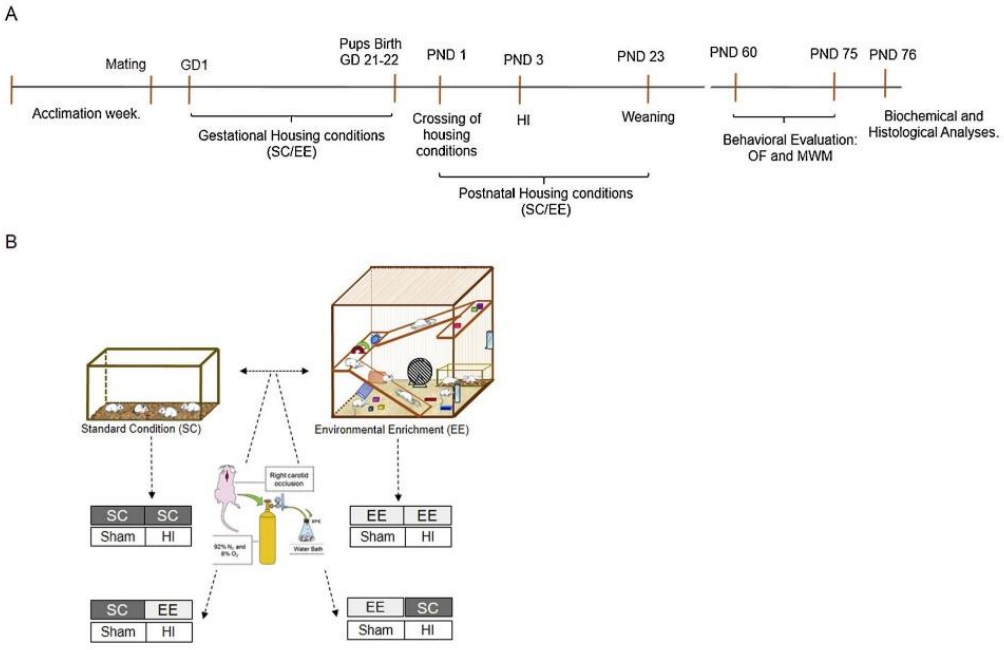


Fig. 1. (A) Experimental timeline during gestational and postnatal periods; (B) Illustration of the housing conditions and experimental groups. GD: Gestational Day. HI: Hypoxia-Ischemia. PND: Postnatal Day. OF: Open Field. MWM: Morris Water Maze.

females and males.

Recently, we reported that prenatal and early postnatal EE was able to reduce cell death and astrocyte reactivity, to increase phosphorylated protein kinase B (AKTp) levels, to prevent hippocampus tissue loss and to improve cognitive function in animals submitted to HI at PND 3 (protocol used to mimic the preterm condition) [4]. However, such study did not allow us to identify whether protective effects occurred exclusively during the prenatal period, after animal´s birth (during the early postnatal period), or as a consequence of EE exposure at both developmental periods. Moreover, animals of both sexes were mixed, and a possible effect of sexual dimorphism in HI rats exposed to EE was not explored.

The aims of the present study were to investigate how prenatal and early postnatal EE alone, as well as their combination, influence: a) anxiety-like behavior, locomotion, and cognitive performance at adulthood; b) the expression of growth and neurotrophic factors, the astrocytic reactivity and plasticity biomarkers in the hippocampus, and c) brain tissue damage in rats submitted to neonatal hypoxia-ischemia. The working hypothesis is that exposure to enriched environment during prenatal or early postnatal period promotes sex-specific responses in the offspring and is able to attenuate HI damage in a sex-dependent manner. Furthermore, we predict that the combination of enriched environment during both stages of neurodevelopment, pre- and postnatal, will produce a cumulative effect and cause greater neuroprotection against neonatal HI.

2. Materials and methods

2.1. Animals

A total of 24 nulliparous Wistar rats were randomly assigned to standard condition (SC, n = 12) or environmental enrichment (EE, n = 12, later described) and were mated with a same–strain male during the proestrus cycle. The first gestational day (GD1) was confirmed by the presence of sperm in the vaginal smear and weight gain was assessed weekly during pregnancy. At GD-21, pregnant rats were kept in individual cages until the parturition, defined as postnatal day 0 (PND 0). Pups were submitted to neonatal hypoxia-ischemia protocol at PND 3 and weaned at PND 23 [4]. The timeline of experiments is summarized in Fig. 1A.

All experimental procedures were performed in accordance with the recommendations of the Council for International Organizations of Medical Sciences (CIOMS - Publication 85-23, 1985) and the Brazilian Society of Science in Laboratory Animals - Law nº 11.794. Ethical approval was obtained by the review board of the Universidade Federal do Rio Grande do Sul protocol # 28,641.

2.2. Environmental conditions

At PND 1, litters (dam and pups) were randomly kept in the same prenatal housing environment or allocated to a new environment. Thereby, four groups were established considering housing conditions: animals allocated to standard conditions (SC) or environmental enrichment (EE), from gestation to the weaning period (**SC + SC** or **EE + EE**) and animals kept in SC during pregnancy and then reared into EE until weaning (**SC + EE**), or vice versa (**EE + SC**). The SC + SC group served as control, EE + SC and SC + EE groups were used in order to examine the isolated effect of prenatal or postnatal EE and the EE + EE group was included to evaluate the combination EE effects at both stages of the neurodevelopment. During the early postnatal period, female and male pups were allocated together with their respective dams. Eight to ten pups per litter were used in the study. After weaning, litters were split by sex and kept in the standard conditions (4–5 rats per cage).

Animals in the **environmental enrichment condition** were housed in cages measuring 120 × 80 x 100 cm (height x width x length) including a running wheel, plastic toys, balls, plastic tubing, contrasting visual stimuli and ramps. The objects were rearranged every day and new objects were introduced twice a week; water and food dispensers were also repositioned to promote additional exploration. In addition, to facilitate social interaction, groups of six rats were kept together during pregnancy and two litters in the early postnatal period [33]. Rats in the **standard condition** were housed in cages measuring 16 × 41 x 34 cm (height x width x length), provided with wood shaving bedding and minimum stimuli. Groups of four rats were kept together during pregnancy and there was only one litter per cage at the early postnatal period.

2.3. Neonatal hypoxia ischemia

The Rice-Vannucci model was adapted in order to investigate the effects of HI in very immature animals. This modified protocol is performed at PND 3, since rats at this age exhibit brain maturation similar to that observed in infants with 24–28 gestational weeks [3,34,35]; the original HI model is performed at PND 7 and mimic brain development to term human babies [36]. Despite the age difference, both protocols cause functional HI-induced deficits and affect structures such as hippocampus and cortex. HI at PND 3 preferentially produces brain damage to white matter regions (such as corpus callosum), ventricle size enlargement and hypometilation, confirming its similarity with the lesion pattern observed in preterm humans after a hypoxic-ischemic event [5,37].

To reduce a possible "*litter effect*", two or three siblings per dam and housing condition were randomly assigned to surgery control (sham) or neonatal hypoxia-ischemia (HI). PND 3 pups (males and females) were anesthetized (Isoflurane 4–5% for induction and 1.5–2% for maintenance) and underwent right common carotid occlusion, as previously described [38]. After two hours of post-surgery recovery, pups were placed in a hypoxia chamber with fraction inspired of oxygen (FIO_2: 0.08), at 37 °C for 180 min. Sham animals underwent carotid artery isolation without occlusion and were kept under atmospheric conditions (FIO_2: 0.21). At the end of hypoxic period, all pups were returned to their dam and housing condition.

Thereby, offspring was assigned by sex into eight groups considering the housing condition and the injury: offspring born to mothers allocated in SC during pregnancy and that remained in SC until weaning, that were submitted to the HI procedure (**SC + SC HI**, n = 24) or not (**SC + SC Sham**, n = 22); offspring born to mothers allocated in SC during pregnancy and then housed in EE until weaning, that were submitted to the HI procedure (**SC + EE HI**, n = 29) or not (**SC + EE Sham**, n = 24); offspring born to mothers allocated in EE during pregnancy and then housed in SC until the weaning, that were submitted to the HI procedure (**EE + SC HI**, n = 24) or not (**EE + SC Sham**, n = 22), and offspring born to mothers allocated in EE during pregnancy and that remained in EE until weaning, that were submitted to the HI procedure (**EE + EE HI**, n = 27) or not (**EE + EE Sham**, n = 26). The HI mortality rate was less than 4%, with no effect of housing environmental conditions.

2.4. Behavioral assessment

The behavior of animals was assessed at two points: at PND 62 and, from PND 65 to 71. The exploratory abilities were evaluated in the Open Field test and the cognitive function in the Morris Water Maze. The number of animals per group used in the study was: **SC + SC HI** (12 females and 12 males), **SC + SC Sham** (12 females and 10 males); **SC + EE HI** (16 females and 13 males), **SC + EE Sham** (13 females and 11 males); **EE + SC HI** (12 females and 12 males), **EE + SC Sham** (12 females and 10 males) and **EE + EE HI** (15 females and 12 males) and **EE + EE Sham** (15 females and 11 males).

2.4.1. Morris water maze

The spatial memory protocol was performed as previously described [38]. Briefly, each rat performed four daily trials, for five consecutive days, to find a hidden platform that was kept in the same position during the training days. The interval between trials was 10 min and the maximum time to find the platform was 60 s for each animal. If the animal failed to reach the platform it was gently placed on it for 15 s. In the probe test, the platform was removed and each rat had a single 60-second trial. The latency to cross the platform area for the first time, the total distance traveled, the average speed and the target quadrant ratio (time spent in the target quadrant / (time spent in non-target quadrants + time in target quadrant) were recorded using ANY-Maze software.

2.4.2. Open field (OF)

The circular open field arena (100 cm diameter x 30 cm high wall) contained two sub-areas (eight peripheral and four central regions). Animals were placed in the center of the arena and the latency to leave the initial position and the number of crossings (peripheral or central) was recorded for 5 min. The peripheral crossings were used to assess locomotion and the total crossings were considered as a measure of general motor activity. Furthermore, the frequency of central crossings was taken as anxiety-like behavior, that is, a reduced percentage of central crossings is interpreted as anxiety response [39].

2.5. Biochemical analysis

Following behavioral testing (at PND 76), six to eight animals per group were decapitated and brain structures were collected. Brains were dissected out and the right hippocampus (ipsilateral to the carotid occlusion) was collected and stored at -80 °C. Western blot analysis was performed as previously described [40]. Briefly, tissue was homogenized and the protein concentration determined. Then, an equal protein concentration (40 µg of total protein/lane) was loaded into NuPAGE® 4–12% Bis-Tris Gels. After electrophoresis, proteins were transferred to nitrocellulose membranes and the blots were blocked for 2 h and incubated overnight at 4 °C with one of the primary antibodies: anti-insulin-like growth factor 1 - IGF1 (1:1000, Millipore), anti- vascular endothelial growth factor - VEGF (1:200, Santa Cruz), anti-brain-derived neurotrophic factor - BDNF (1:500, Abcam), anti- tropomyosin receptor kinase B - TrkB (1:1000, Millipore), anti-synaptophysin (1:1000, Dako), anti- glial fibrillary acidic protein - GFAP (1:1000, ThermoFisher) and anti-β-actin (1:3500, Milipore). Membranes were washed in T-TBS (5 min/each) and incubated with peroxidase-conjugated secondary antibody (anti-rabbit or anti-mouse in a concentration of 1:1000) for 2 h at room temperature (22 °C ± 2). The enhanced chemiluminescence plus GE-LAS 4000 digital image reader (GE Healthcare Life Sciences) was used for protein detection. Data were quantified as the ratio between optical density of the interest protein and β-actin in the same blot. Results are expressed as percentage of the

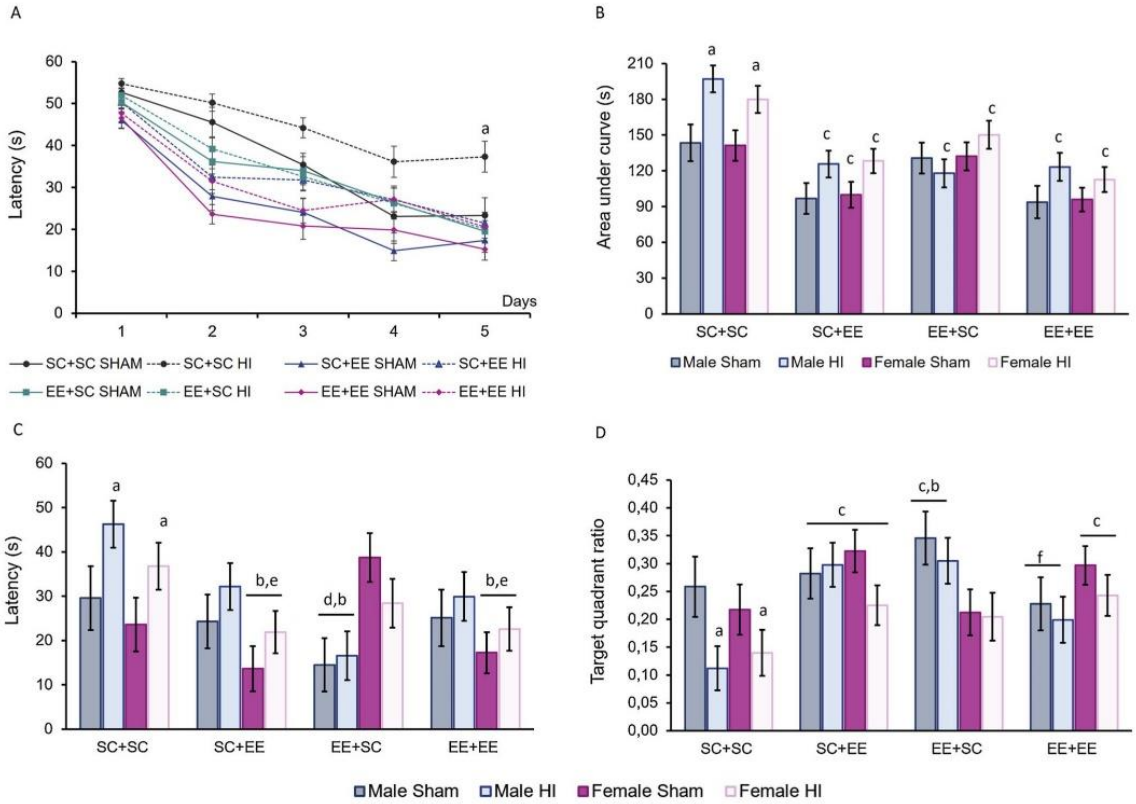


Fig. 2. Morris Water Maze (MWM) Task: (A) Performance of the rats during the five training days. Data analyzed by repeated measures ANOVA; (B) General performance of the animals in the MWM expressed as the area under the learning curve; (C) Latency to cross the platform region during the probe trial in the MWM test session; (D) Target quadrant ratio. Data were analyzed by the generalized linear model using linear distribution. SC: Standard Condition. EE: Environmental Enrichment. [a] Difference of HI animals from their respective Sham. [b] Difference between males and females of the same housing condition and surgical procedure. [c] Difference from control group (SC + SC) of the same surgical procedure [d] Difference of the EE + SC animals from the other experimental groups belonging to the same gender. [e] Difference of animals exposed to early postnatal EE when compared to postnatal SC groups. [f] Differences between EE + EE and EE + SC males animals. Significance was accepted when $p \leq 0.05$.

control group (SC + SC Sham). The number of animals per group used for biochemical analysis in this study was: SC + SC HI (6 females and 6 males), SC + SC Sham (6 females and 5 males); SC + EE HI (6 females and 6 males), SC + EE Sham (7 females and 5 males); EE + SC HI (7 females and 6 males), EE + SC Sham (6 females and 5 males) and EE + EE HI (8 females and 6 males), EE + EE Sham (7 females and 5 males).

2.6. Histology and immunofluorescence analysis

At PND 76, animals were anesthetized (100 mg/kg of sodium thiopental, Thiopentax, Cristália®) and transcardially perfused with 0.9% saline solution and 4% formaldehyde solution. Brains were removed from the skull and placed in formaldehyde. Then, the brains were placed in sucrose solution (15%, followed by 30%) for cryoprotection and sliced in a cryostat (Leica) at 30 µm thickness. Every tenth section was mounted on a gelatin-coated slide and stained using hematoxylin and eosin (Sigma-Aldrich. St Louis. MO.USA). The volumes of hemisphere, cortex, hippocampus and corpus callosum were estimated using NIH-ImageJ software following the equation : Σ *area x (n° of slices) x (interslice interval)*. Data are expressed as the volume ratio between the ipsilateral hemisphere and the contralateral hemisphere to the carotid occlusion [41]. The number of animals per group used for histological analysis was: SC + SC HI (6 females and 6 males), SC + SC Sham (6 females and 5 males); SC + EE HI (7 females and 6 males), SC + EE Sham (6 females and 5 males); EE + SC HI (5 females and 6 males), EE + SC Sham (6 females and 5 males) and EE + EE HI (6 females and 6 males) and EE + EE Sham (8 females and 5 males).

Tissue slices were washed three times (5 min/each) in PBS at room temperature, permeabilized in 0.25% PBS-Triton X-100 and then blocked with 1% albumin for 30 min. After this process, the slices were incubated overnight at 4 °C with primary antibodies anti-GFAP (mouse IgG, 1:200, Sigma-Aldrich) and anti-NeuN (rabbit IgG, 1:200) to identify astrocytes and neurons, respectively. Following PBS washes, secondary antibodies anti-mouse Alexa 488 and anti-rabbit Alexa 555 (1:500, Molecular Probes, Invitrogen) were incubated at room temperature for 1 h. Slices were mounted on Fluoroshield with DAPI (Sigma-Aldrich) and coverslipped. A tissue slice without primary antibodies was used as negative control. Hippocampus images (20x) were captured using Nykon E600 microscope-(Japan). A region of interest (ROI–19,454 µm^2) was drawn on each slice and the staining intensities were quantified in the CA1 area using ImageJ software. Data are expressed as the mean of integrated density value/µm^2 [42].

2.7. Statistics

Statistical analysis was performed by the Generalized Linear Model run in SPSS-21 for Windows. Housing condition, injury and sex were considered as fixed factors and the litter was used as covariate factor (for possible non-independence of behavior among littermates). Discrete data (number of total and central crossing in the open field) were modeled with Poisson distribution and biochemical and histological data were analyzed using linear distribution. For assessing water maze data during training days, repeated measures ANOVA was used. Once a main effect or an interaction between factors was found, the *post-hoc* test Holm-Sidak was used to compare differences among groups. Correlations among behavioral and biochemical parameters were calculated by the Pearson correlation coefficients. Significance was accepted whenever $P \leq 0.05$. Data are expressed as means ± standard error (S.E).

Significant differences considered relevant and discussed were: a) Differences between HI animals and their respective control group (Sham); b) Differences between Sham animals of the same sex; c) Differences between HI animals of the same sex; d) Differences between males and females exposed to the same housing condition and injury procedure.

3. Results

3.1. Behavioral assessment

3.1.1. Morris water maze

Morris Water maze (MWM) test was carried out to assess spatial learning and memory functions. Repeated measures ANOVA test revealed an interaction between injury and training days ($F_{(4)} = 2.370$, $P = 0.05$) as well as an interaction between environmental conditions and training days ($F_{(12)} = 3.684$, $P < 0.001$), with no effects of sex ($P > 0.05$). All animals improved their performances over days; however, HI animals of SC + SC group had increased latencies to find the platform when compared to sham SC + SC animals, from the fourth day until the end of training. As shown in Fig. 2A, on the last day of

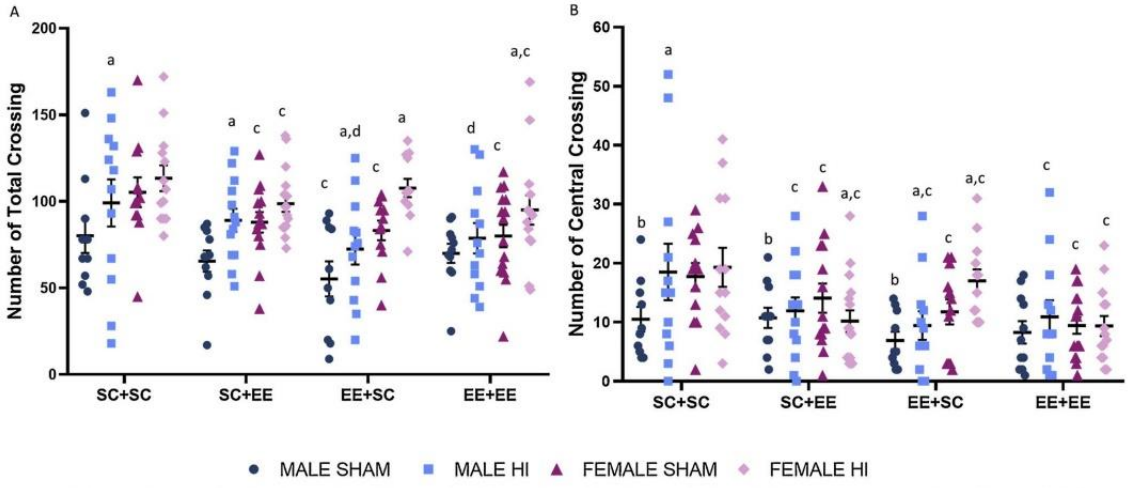


Fig. 3. Open Field Task: (A) Number of total crossings; (B). Number of central crossing. SC: Standard Condition. EE: Environmental Enrichment. [a] Difference of HI animals from their respective Sham. [b] Difference between males and females of the same housing condition and surgical procedure. [c] Difference from control group (SC + SC) of the same surgical procedure. [d] Difference of animals exposed to prenatal EE when compared to prenatal SC groups. Data analyzed by the generalized linear model. Significance was accepted when $p \leq 0.05$.

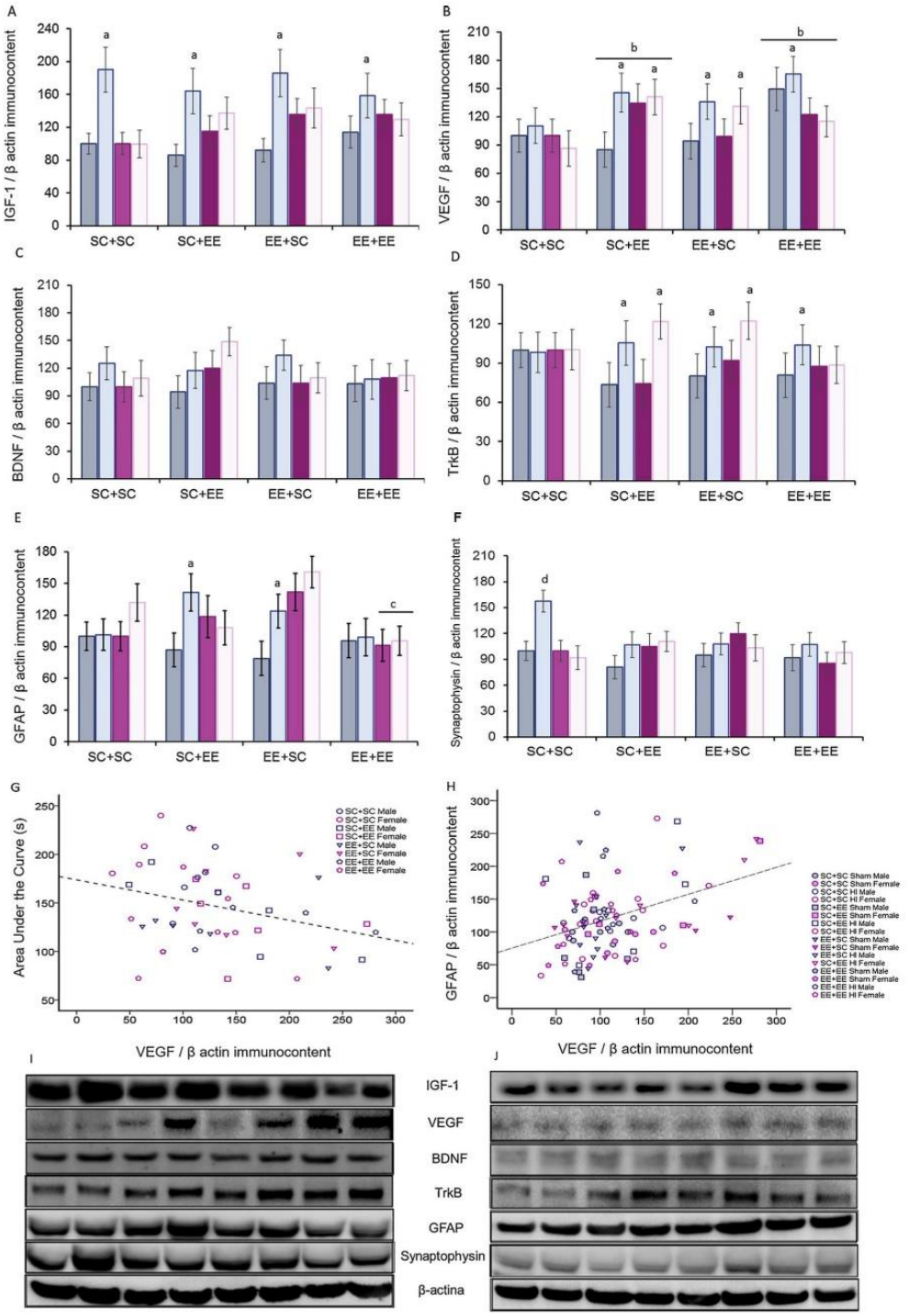


Fig. 4. Immunocontent of hippocampus protein at adulthood in animals receiving hypoxia-ischemia and exposed to environmental enrichment: (A): Insulin-like growth factor 1 (IGF1); (B): Vascular endothelial growth factor (VEGF); (C): Brain-derived neurotrophic factor (BDNF); (D): Tropomyosin receptor kinase B (TrkB); (E): Glial fibrillary acidic protein (GFAP); (F): synaptophysin; (G) Correlation between VEGF and the area under curve during MWM training in HI animals; (H): Correlation between the immunocontent of VEGF and GFAP; (I): Representative blots of male animals (J) Representative blots of female rats. [a] Difference of HI animals from their respective Sham. [b] Difference of animals exposed to early postnatal EE when compared to SC + SC c Difference of EE + EE group from the EE + SC animals of the same sex. [d] Difference from the other experimental groups. n = 5–8 animals per group. Significance was accepted when $p \leq 0.05$.

training all HI enriched groups had better performances in comparison to HI SC + SC group ($P < 0.001$) with no significant between enriched groups (SC + EE, EE + SC and EE + EE – $P > 0.05$).

In order to analyze animals' general performance in MWM during the training days, the areas under the groups' learning curves were compared. There was an interaction between housing condition and injury ($X^2_{(3)} = 8.435$, $P < 0.05$); SC + SC HI had the worst performance (greater area under the curve) than all other groups (shown in Fig. 2B). Confirming the pattern in Fig. 2A, HI-induced learning impairment was prevented and/or reversed by prenatal and/or postnatal environmental enrichment, respectively. Moreover, enriched experiences improved the spatial memory performance of rats, regardless of sex.

There was an injury effect in the latency to cross the platform area for the first time in the probe trial ($X^2_{(1)} = 4.202$, $P < 0.05$, Fig. 2C); pairwise comparisons revealed that HI animals of SC + SC group (female and male) had higher latencies to find the platform when compared to SC + SC sham. An interaction between housing condition and sex was also observed ($X^2_{(3)} = 17.490$, $P < 0.01$): males from EE + SC group had lower latencies to cross the platform area for the first time, in comparison to the other environmental conditions, and to the females from the respective group (EE + SC, $P < 0.05$). In addition, EE + EE and SC + EE females had lower latencies compared to their respective males and from females of the EE + SC and SC + SC groups ($P < 0.05$).

A similar pattern was seen in the target quadrant analysis during the probe trial. HI animals of SC + SC group spent less time in the target quadrant than sham SC + SC animals, independently of the sex (Injury effect: $X^2_{(1)} = 6.689$, $P < 0.01$). The interaction between housing condition and sex ($X^2_{(3)} = 8.666$, $P < 0.05$) showed that males from EE + SC and SC + EE groups spent more time in the target quadrant than the SC + SC animals. EE + SC males showed also significant differences from EE + EE group, increasing the time spent in the target quadrant ($P < 0.05$). As observed in Fig. 2D, females from SC + EE and EE + EE group had increased times in the target quadrant ratio compared to the females from SC + SC group, with no differences from animals of EE + SC group. Altogether, these data indicate that prenatal and early postnatal environmental enrichment were able to influence memory retention in a sex-specific manner, in both injured and non-injured animals, in which males exposed to prenatal or postnatal EE alone (EE + SC or SC + EE) showed better performances, while females were mainly benefited by postnatal EE. No differences were found in the total distance traveled nor swimming average speed among groups ($P > 0.05$, data not shown), confirming that swimming ability was not modified by the housing condition nor by the HI insult.

3.1.2. Open field test

The open field test was used to evaluate animal's general motor activity. As depicted in the Fig. 3A, the analysis of total crossings showed an interaction between housing condition, injury and sex ($X^2_{(3)} = 10.081$, $P < 0.05$ and $X^2_{(3)} = 11.447$, $P < 0.01$, respectively), with control HI males (SC + SC group) showing increased locomotion in comparison to sham SC + SC animals ($P < 0.001$). Interestingly, HI-induced hyper locomotion was only reverted in HI males of EE + EE group, which did not show differences from its respective sham group (EE + EE sham). No significant differences were observed when comparing females of the SC + SC HI and SC + SC Sham groups ($P > 0.05$). Nevertheless, an enrichment effect was observed, in which HI females from the SC + EE and EE + EE groups showed a reduction in the frequency of total crossings with respect to HI SC + SC group ($P < 0.05$). Surprisingly, HI females exposed to prenatal enrichment (EE + SC and EE + EE) had an increase in motor activity as compared to their respective sham groups ($P < 0.01$). Data here reported suggesting that environmental enrichment mainly at the early postnatal period could attenuate the hyperlocomotion associated with neonatal HI both in females and males.

The number of central crossings was used as an index of anxiety-like behavior in the open field. There was an interaction between environmental conditions, injury and sex ($X^2_{(3)} = 7.656$, $P = 0.05$, Fig. 3B): HI males of SC + SC group showed higher exploration in unprotected areas when compared to sham SC + SC ($P < 0.001$), interpreted as a decrease of anxiety or increase of impulsivity; in turn, HI females from SC + SC group did not show significant differences from the sham SC + SC group ($P > 0.05$). The increase of HI-induce number of central crossing was prevented (prenatal) or reverted (early postnatal period) by enrichment, being that all HI animals exposed to EE at some stage of neurodevelopment showed a reduction in the frequency of crossings in comparison to the SC + SC HI group ($P < 0.001$). These results indicate that EE exposure at some stage of neurodevelopment leads to a more natural behavior in female and male rats, increasing the exploration in protected areas and reducing impulsivity.

3.2. Biochemical analysis

As shown in Fig. 4A, growth factors analysis in the right hippocampus (ipsilateral to the carotid occlusion) showed an interaction between injury and sex ($X^2_{(1)} = 12.737$, $P < 0.001$) with a significant increase in the IGF-1 immunocontent in HI males. No significant effect of housing condition was observed. As regards to VEGF expression, an effect of housing was observed ($X^2_{(3)} = 9.532$, $P < 0.05$); animals exposed to early postnatal EE (SC + EE and EE + EE) had an increase in the VEGF immunocontent as compared to the SC + SC group, with no

Table 1
Cerebral volumes ratio at adulthood of animals exposed HI and EE protocol.

Structure	Groups	Male		Female	
		Sham Mean ± (S.E)	HI Mean ± (S.E)	Sham Mean ± (S.E)	HI Mean ± (S.E)
Hemisphere	SC + SC	1.0130 ± (0.076)	0.6613 ± (0.062)[a]	1.0098 ± (0.069)	0.8836 ± (0.057)
	SC + EE	1.0221 ± (0.097)	0.9270 ± (0.087)	1.0157 ± (0.073)	0.9834 ± (0.073)
	EE + SC	1.0006 ± (0.097)	0.9774 ± (0.087)	0.9438 ± (0.087)	0.8878 ± (0.087)
	EE + EE	1.0180 ± (0.097)	0.7073 ± (0.087)[a]	1.0013 ± (0.069)	0.08971 ± (0.087)
Cortex	SC + SC	1.0000 ± (0.087)	0.6012 ± (0.063)[a]	0.9881 ± (0.080)	0.8524 ± (0.065)[a]
	SC + EE	0.9745 ± (0.097)	0.8557 ± (0.087)	0.9888 ± (0.074)	0.9618 ± (0.073)
	EE + SC	0.9924 ± (0.097)	0.9572 ± (0.087)	0.9071 ± (0.087)	0.8091 ± (0.087)
	EE + EE	0.9801 ± (0.097)	0.6400 ± (0.087)[a]	0.9530 ± (0.069)	0.8625 ± (0.087)
Corpus Callosum	SC + SC	0.9712 ± (0.098)	0.6409 ± (0.071)[a]	1.0248 ± (0.089)	0.8913 ± (0.073)
	SC + EE	1.0907 ± (0.110)	0.8787 ± (0.098)	1.0290 ± (0.083)	1.0491 ± (0.083)
	EE + SC	0.9480 ± (0.110)	1.0001 ± (0.098)	1.0066 ± (0.098)	0.9474 ± (0.098)
	EE + EE	1.0524 ± (0.110)	0.6927 ± (0.098)[a]	1.0647 ± (0.078)	0.9823 ± (0.098)

SC: Standard Condition. EE: Environmental Enrichment. The values are expressed as mean ± S.E.M. Significance: $P \leq 0.05$.
[a] Significant difference of HI animals when compared to their respectively Sham group.

differences from EE + SC group ($P > 0.05$). In addition, an injury effect was observed ($X^2_{(1)} = 3.805$, $P = 0.05$) as indicated in Fig. 4B. Pairwise comparison revealed that enriched HI groups (SC + EE, EE + SC and EE + EE) had an increase in VEGF levels when compared to SC + SC HI group.

Interestingly, there was a negative correlation between the area under the curve during the water maze training and VEGF levels in HI animals (Pearson's correlation of 0.295, $P = 0.036$, Fig. 4G); animals with better MWM performance exhibited increased VEGF levels. The correlation suggests that the long-term increase of VEGF levels elicited by gestational and early postnatal EE is possibly related to the functional recovery following neonatal HI.

There were no significant differences among groups in the immunocontent of BDNF ($P > 0.05$, Fig. 4C), although TrkB receptor levels were increased in animals submitted to the neonatal HI, being more evident in animals exposed to prenatal or postnatal EE alone (EE + SC and SC + EE groups) (Injury effect: $X^2_{(1)} = 6.212$, $P < 0.05$ - Fig. 4D). GFAP expression was influenced by injury ($X^2_{(1)} = 4.824$, $P < 0.05$). Although pairwise comparisons showed no differences between HI SC + SC and Sham SC + SC group, as indicated in Fig. 4E, it was observed that isolated exposure to EE during prenatal and postnatal periods increased GFAP levels in HI males from EE + SC and SC + EE groups as compared to respective sham groups. Interestingly, a positive correlation was also found between GFAP and VEGF levels (Pearson's correlation of 0.404, $P = 0.001$, Fig. 4H), suggesting an association between this growth factor and astrocyte reactivity in the beneficial effects derived from the enriched condition.

Synaptophysin immunocontent was quantified in order to evaluate the long-term effect of EE on synaptic plasticity. Surprisingly, neonatal HI caused an increase in the expression of synaptophysin only in males from the SC + SC group (injury and sex interaction: $X^2_{(1)} = 4.312$, $P < 0.05$, Fig. 4F) compared to the female SC + SC HI and enriched groups (EE + SC, SC + EE and EE + EE).

3.3. Histology and immunofluorescence analysis

HI caused a reduction in the volume of the right hemisphere, cortex and corpus callosum ($X^2_{(1)} = 7.677$, $P < 0.01$; $X^2_{(1)} = 13.338$, $P < 0.001$ and $X^2_{(1)} = 8.316$, $P < 0.001$, respectively). However, HI rats exposed to prenatal or early postnatal enriched condition alone (SC + EE and EE + SC) showed significant tissue preservation (Table 1). This effect was not observed in animals kept in SC or EE from gestation to the weaning period. As expected, no significant differences were observed in volume ratios between sham groups.

Fig. 5B shows that injury and sex affected hippocampus volume ratio ($X^2_{(1)} = 4.315$, $P < 0.05$); male rats were the most affected by HI, in which the reduction of the right hippocampus (ipsilateral side to the carotid occlusion) was greater as compared to HI females. In addition, an interaction between housing condition and injury was observed ($X^2_{(3)} = 7.942$, $P < 0.05$): SC + SC HI animals exhibited greater tissue loss compared to animals exposed to EE at any stage of the neurodevelopment. These results showed an effect of sexual dimorphism associated with HI effects and indicate that prenatal and early postnatal EE were able to contribute to the encephalic partial tissue preservation following HI. Also, our results reinforce the hippocampus vulnerability to HI as well as the positive effects of enriched environment. Surprisingly, NeuN immunolabelling in the CA1 region did not evidence any differences among groups ($P > 0.05$, data not shown). A representative image of immunofluorescence performed in the CA1 region of the hippocampus is depicted in Fig. 6.

4. Discussion

The current study sought to investigate the effects of environmental enrichment (EE) exposure during prenatal or early postnatal period, as well as their combination, in rats submitted to neonatal hypoxia-ischemia (HI) at postnatal day 3. It was shown that the effects of EE on behavioral performance, molecular responses and tissue damage were highly influenced by sexual dimorphism: e.g, HI-induced hyperlocomotion was reduced in enriched females, HI males from EE + SC and SC + EE groups showed better spatial cognitive function, higher GFAP levels and greater tissue preservation. Interestingly, prenatal or early postnatal EE long-term effects on functional, morphological and molecular parameters last until adulthood. The negative correlations

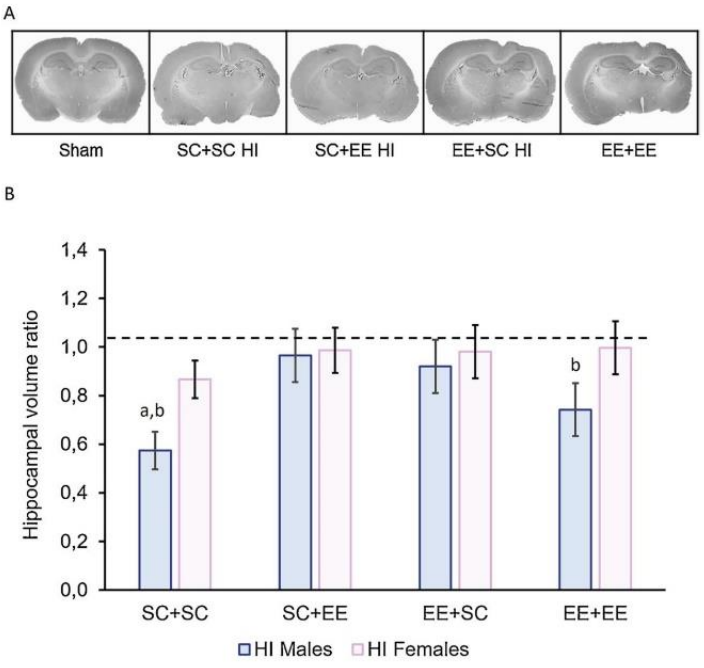


Fig. 5. Histological analysis of the hippocampus (A) Representative hematoxylin and eosin stained, coronal brain section at the level of the male hippocampus (Bregma -3.30 mm) at adulthood; (B): Hippocampal volume ratio (calculated by dividing the ipsilateral volume to the carotid occlusion by the contralateral volume) [a] Difference of the SC + SC HI from all other experimental groups. [b] Difference between males and females of the same housing condition and surgical procedure. Sham groups are represented as dashed line. n = 5–8 animals per group. Significance was accepted when $p \leq 0.05$.

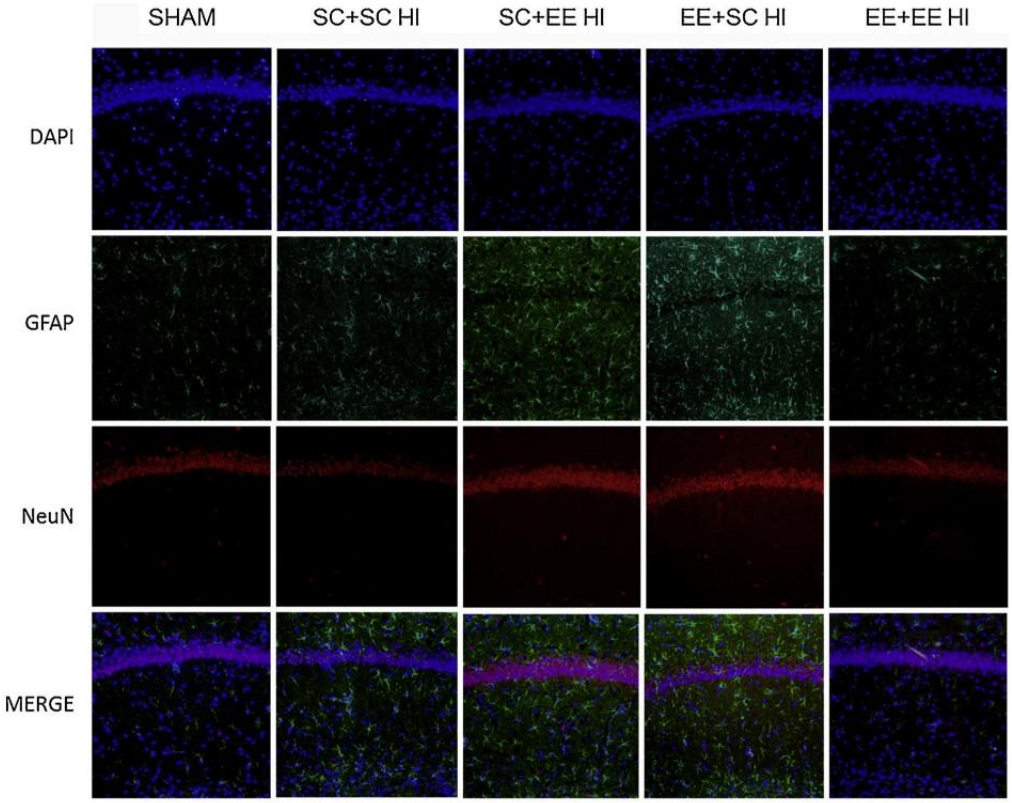


Fig. 6. Representative immunolabelling in the Hippocampus (CA1).

between VEGF levels with cognitive performance in the water maze, as well as with GFAP levels, suggest that this neurotrophic factor and astrocyte activity could be implicated in the functional recovery promoted by enrichment. Surprisingly, the combination of prenatal and early postnatal EE did not produce any additive behavioral, molecular and morphological effects, as hypothesized.

4.1. Environmental enrichment reverses cognitive deficits after neonatal HI and causes sex-specific responses

Morris water maze is a well-accepted test to evaluate memory capabilities. HI-induced memory deficits in this task are consistently reported and these deficits were prevented by the EE protocol here proposed. In fact, female and male HI rats exposed to EE at both stages of neurodevelopment showed a pattern similar to SC + SC sham animals during the learning phase. Interestingly, during the probe test HI males exposed to prenatal or early postnatal EE alone showed better memory retention, and HI females allocated to early postnatal enriched condition had an increase in spatial memory abilities after neonatal HI, especially when time spent in the target quadrant was analyzed. Koo and colleagues (2003), using a cross-housing study during prenatal and postnatal periods, observed that environmental stimulation improves animals' performance in Y-maze and Water maze tests, an effect possibly associated with neurogenesis and synaptic changes [24].

In agreement with present observations, non-additive effects have been reported in animals exposed to the EE for long-term periods [24]. Modifications in the early life environment have a major impact on brain development and in behavioral performance at adulthood. Continuous manipulation of female rats during prenatal and postnatal periods could influence maternal behavior and alter offspring's response to an enriched environment. However, most studies involving neonatal HI and early EE do not evaluate maternal care behavior. A single report using a protocol similar to the one here employed did not reveal significant differences in licking/grooming behaviors and arched-back posture in the dams [4]. Such behaviors are considered as the main responsible for epigenetic modifications, as well as molecular and metabolic regulations mediating experience-dependent neural plasticity in the hippocampus and cognitive function in adulthood [23,43]. Furthermore, the cognitive function preservation observed in animals exposed to prenatal or early postnatal EE alone, as well as the non-additive EE effect in the control and HI animals from EE + EE group, are probably related to the novelty aspect of complex stimuli during critical windows of brain development [15]. Some authors highlight that stimulus withdrawal or regular periods of EE optimize cellular proliferation and neuronal circuit activation as a consequence of experience-dependent response. Conversely, continued EE exposure could reduce the efficiency of environmental stimulation [44,45].

The above mentioned aspects do not exclude the possibility that EE could improve the animal`s awareness of its surroundings, enhancing the focus on the exposed task, making it more "flexible" in order to solve problems and to reduce thigmotaxis often observed in HI animals [46]. Data here presented are also in accordance with previous reports showing sexual-dimorphism in memory function tests [30,47–49] and sex-specific responses to environmental stimuli [50].

4.2. Environmental enrichment at early stages of the neurodevelopment attenuates HI-induced motor impairments and anxiety-like behavior

In the current study, HI offspring exposed to environmental enrichment at the early postnatal period showed a reduction in the

locomotion and general motor activity both in female and male rats. In addition, enriched experiences at any neurodevelopment stage modulated anxiety-like behavior (expressed as higher exploration in protected areas in the open field). Particularly, HI males exhibit higher motor activity and increased exploration of the unprotected areas. To this point, sex-specific responses induced by neonatal HI are still conflicting in the literature; some studies reported increase locomotion in males [48] or in both sexes [5,51]. Other studies indicated increased anxious behavior in female [5,52] or male rats suffering HI [53]. These discrepancies could be related to differences in hypoxia exposure protocols and the reduced number of studies evaluating sexual-dimorphism in very immature animals [30].

Enriched animals showed a partial protective effect of environmental stimulation in locomotion and anxiety. Some authors report decreased activity, as well as increased anxiety-like behavior in enriched males [39,54,55] and females at adulthood [54,56]. As mentioned above, evidence regarding sexual dimorphism in rats exposed to enriched conditions during gestation or the early postnatal period, considering parameters such as locomotion and anxiety-like behavior still remains inconclusive. This fact arises from the variability of the protocols used, the lack of studies using both sexes in a single experiment and the age of the animals at the point of behavioral testing.

4.3. Environmental enrichment upregulates neurotrophic receptors and growth factors in HI rats

Prenatal and early postnatal periods are sensitive brain development windows to the environmental influence. The attenuation of functional deficits, especially those related to cognitive impairment, could be also associated with the expression of neurotrophic and growth factors. It is shown that enriched HI animals had an increase in VEGF and TrkB expression; with TrkB levels increased in groups exposed to prenatal and postnatal EE alone. TrkB is known to be a high affinity receptor for BDNF; its expression starts during the embryonic period and it is found in most neurons in the central nervous system [57]. Furthermore, its interaction with BDNF is important in the activity-dependent plasticity and protection against brain insults [58]. Previous studies have shown that deprivation of habitual running leads to decreased TrkB expression in the hippocampus [59]. In addition, experimental research using TrkB blockers in juvenile rats kept in an enriched condition showed an increase in the expression of this receptor, facilitating BDNF binding and reverting cognitive impairments [60].

IGF-1 is a well-known growth factor involved in cognitive function and its up regulation is considered a feasible mechanism involved in the positive effects of EE at different periods of neurodevelopment [20,61] and of post-injury recovery [62,63]. Our results showed a significant increase of hippocampus IGF-1 in male rats following HI, independently of the housing conditions. Previous reports indicate that higher IGF-1 levels are a compensatory response to brain injury [62]. Results here presented also suggest a sex-specific response to neonatal brain damage, however, the relationship between protective growth factor mechanisms and sexual dimorphism in neonatal HI are not understood. It is not possible, at the moment, to know if the increase in IGF-1 levels is modulating cognitive performance in HI males; however, it does not exclude that EE-induced IGF-1 up-regulation occurs in other brain areas or in the contralateral hemisphere, and that could result in functional recovery. Further studies are needed to determine whether enriched experiences lead to changes in the expression of IGF-1 and to establish an association with behavioral function and sex-specific responses in the immature brain suffering HI.

An increase in VEGF levels was observed in all enriched animals during adulthood, with this growth factor showing a negative correlation with the learning curve in the water maze, i.e., higher levels of this protein are associated with better cognitive abilities. This is in accordance with literature mentioning that high VEGF levels could be necessary to facilitate growth, cell viability and functional preservation [64,65]. Recently, studies point VEGF as a promising therapeutic agent for enhancing neuronal plasticity and improving memory deficits [66]. In addition, it has been reported that VEFG is required in mediating the improvement of cognition and promoting hippocampus-dependent memory by increasing synaptic strength on mature neurons, even under some degree of brain damage [67–69]. Therefore, the beneficial effect of enrichment protocols could involve multiple neurogenesis-independent pathways such as the upregulation of neurotrophic and growth factors [70,71].

4.4. Prenatal and early postnatal EE increase astrocyte reactivity in a sex-specific manner

Surprisingly, the exposure to prenatal or early postnatal EE alone caused an increase in GFAP protein expression in male rats. In response to brain injuries, high levels of GFAP has been frequently associated with a mechanical barrier, interfering negatively in CNS cells function [72]. However, a subset of recent data implies that both enriched environment and physical exercise increase astrocyte proliferation that has positive effects on neuronal activity, contributing to improve neurological function following brain insults [73,74] and to modulate synapse remodeling in the lesion area [72]. Sexual dimorphism observed in astrocyte reactivity could be related to the brain damage severity, in which male rats are more vulnerable to the effects of neonatal HI as females. Astrocytes reactivity play an important role in the injury cascade, post-injury recovery [30,75,76] and could be associated with physiological differences related to nervous tissue maturation during early postnatal period and hormonal influence [77]. The hippocampus GFAP levels are increased in males in the pre-weaning period, followed by a slow stabilization until the third month of life [77]. However, the impact of environmental stimulation during prenatal or early postnatal periods in GFAP expression after neonatal HI had not been previously reported. Further research is necessary to understand the mechanisms related to the cellular and morphological remodeling involve astrocyte reactivity in a sex-specific manner.

4.5. Enriched conditions could prevent hippocampus aberrant plasticity after neonatal HI

Brain response to HI might result in aberrant neurogenesis and synaptogenesis in the hippocampus, what contribute to cognitive decline [78,79]. Our data evidence a sex-specific increase in synaptophysin levels in HI males from SC + SC group; this was inhibited in all HI groups exposed to enriched conditions. Literature suggests that reductions in hippocampal plasticity in lesioned animals could be a neuroprotective mechanism and attenuate the deleterious effects of brain damage [80]. Synapse formation exceeds pruning at early ages, however, it is followed by a decline from the late childhood to the adult age, resulting in the selection, maturation, formation and synapse stabilization of neuronal circuits [81,82]. Males require higher synaptic remodeling due to increased number of synapses in comparison to females [83]. The sexually dimorphic results are in agreement with previous reports describing higher levels of synaptophysin in males that could also indicate changes in synaptic pruning [81,84], such increase was prevented by EE at early stages of the neurodevelopment.

4.6. Prenatal and early postnatal EE promote higher tissue preservation after neonatal HI

Neonatal HI causes brain damage both in female and male animals [38,50]. Our results showed that HI male SC + SC group exhibited greater encephalic tissue loss than females, supporting the idea of sex-specific vulnerability to HI-related brain injury in males [85,86]. This might be associated with sex differences in expression and activity of mitochondrial enzymes that participate in distinct cell death pathways;

in general terms, apoptosis is more robust in females, while necrosis is in males [30]. In addition, males showed an increase in microglia activation and peripheral inflammatory responses than females, what deepens injury severity [87]. It is important to note that minor histological damage is also responsible for behavioral deficits [88,89] as observed in HI females of SC + SC group. Previous reports using early EE in animals submitted to neonatal HI observed that enriched conditions cause partial tissue loss preservation, especially in the hippocampus [4,18]. However, there are few studies exploring morphological and cellular sex-differences that could contribute to explain conflicting results, in which tissue volume was not altered by environmental stimulation [27,50].

Curiously, animals exposed to prenatal or early postnatal EE alone showed additional tissue preservation in the right hemisphere, cortex and corpus callosum (ipsilateral side of the carotid occlusion). The increase of tissue preservation after brain insults could facilitate compensatory plasticity mechanisms in non-injured areas, which is relevant to the subsequent brain function [90], helping to explain the functional outcomes here observed. Gestational and pre-weaning periods correspond to different cellular, metabolic, functional and environmental demands to the offspring [15,91]. We propose that EE requires alternation between stimulation and resting periods in order to promote brain re-organization by experience-induced modifications in the neuronal network after a novel and challenging environment. Thereby, further studies are required to confirm this assumption, which could be relevant in neurorehabilitation programs focused on prematurity and/or neonatal brain insults.

4.7. Prenatal and early postnatal EE could be relevant clinical interventions

Our findings offer preclinical evidence of positive effects of enriched environment and the importance of gestational and early interventions to improve brain health recovery following injury. Current reports agree that enriched environment could be translated into clinical practice, including protocols providing diverse stimuli both in daily activities as well as in rehabilitation settings [92]. Enrichment paradigm could be used in pregnant women, in order to promote mother-fetus wellness as a preventive strategy, and also applied to infant population in an attempt to improve functional recovery from HI-related brain damage. Clinical trials are needed to evaluate interventions focused on enriched environment and to determine if they could be used as adjuvant treatments for children suffering neonatal HI [8,93].

5. Conclusion

Present study highlights the preventive (gestational) and therapeutic (early postnatal period) effects of enrichment protocols against hypoxia-ischemia injury in the very immature rat. Our data indicate that both prenatal and early postnatal environmental enrichment cause similar attenuation of HI-induced behavioral impairments assessed in spatial memory, motor activity and anxiety-like behavior, as well as morphological preservation, especially in the hippocampus. Molecular mechanisms related to prenatal and early postnatal EE are not clearly delimited. However, these seem to involve up-regulation of VEGF and TrkB levels, in both sexes, and increased astrocyte reactivity in male rats. Interestingly, a sex-specific response is observed, in which male rats exhibit positive effects at both studied neurodevelopmental stages, while female seem to be most benefited by early postnatal enrichment. Further studies are needed to uncover the molecular processes responsible for the dual neuroprotective effect in immature animals suffering HI, as well as to explore the impact of intermittent or continuous stimulation during offspring's neurodevelopment.

Conflict of interest

The authors have no conflicts of interest.

Funding sources

This study was supported by the brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Acknowledgment

The authors thank the staff of Reproduction and Laboratory Animal Research Center from Biochemistry Department UFRGS.

References

[1] J.J. Kurinczuk, M. White-Koning, N. Badawi, Epidemiology of neonatal encephalopathy and hypoxic-ischaemic encephalopathy, Early Hum. Dev. 86 (2010) 329–338, https://doi.org/10.1016/j.earlhumdev.2010.05.010.
[2] K.R. Gopagondanahalli, J. Li, M.C. Fahey, R.W. Hunt, G. Jenkin, S.L. Miller, A. Malhotra, Preterm hypoxic–ischemic encephalopathy, Front. Pediatr. 4 (2016) 1–10, https://doi.org/10.3389/fped.2016.00114.
[3] Y. Van De Looij, A. Chatagner, P.S. Hüppi, R. Gruetter, S.V. Sizonenko, Longitudinal MR assessment of hypoxic ischemic injury in the immature rat brain, Magn. Reson. Med. 65 (2011) 305–312, https://doi.org/10.1002/mrm.22617.
[4] L. Durán-Carabali, D. Arcego, F. Odorcyk, L. Reichert, J. Cordeiro, E. Sanches, L. Freitas, C. Dalmaz, A. Pagnussat, C. Netto, Prenatal and early postnatal environmental enrichment reduce acute cell death and prevent neurodevelopment and memory impairments in rats submitted to neonatal hypoxia ischemia, Mol. Neurobiol. (2017), https://doi.org/10.1007/s12035-017-0604-5.
[5] E.F. Sanches, N. Arteni, F. Nicola, D. Aristimunha, Ca. Netto, Sexual dimorphism and brain lateralization impact behavioral and histological outcomes following hypoxia–ischemia in P3 and P7 rats, Neuroscience 290 (2015) 581–593, https://doi.org/10.1016/j.neuroscience.2014.12.074.
[6] M.E. Thomason, D. Scheinost, J.H. Manning, L.E. Grove, J. Hect, N. Marshall, E. Hernandez-Andrade, S. Berman, A. Pappas, L. Yeo, S.S. Hassan, R.T. Constable, L.R. Ment, R. Romero, Weak functional connectivity in the human fetal brain prior to preterm birth, Sci. Rep. 7 (2017) 39286, https://doi.org/10.1038/srep39286.
[7] K.J. Hassell, M. Ezzati, D. Alonso-Alconada, D.J. Hausenloy, N.J. Robertson, New horizons for newborn brain protection: enhancing endogenous neuroprotection, Arch. Dis. Child. - Fetal Neonatal Ed. 100 (2015) F541–F552, https://doi.org/10.1136/archdischild-2014-306284.
[8] C. Netto, E. Sanches, F. Odorcyk, L. Duran-Carabali, S. Sizonenko, Pregnancy as a valuable period for preventing hypoxia-ischemia brain damage, Int. J. Dev. Neurosci. 18 (2018), https://doi.org/10.1016/j.ijdevneu.2018.06.004.
[9] E. Rocha-Ferreira, M. Hristova, E. Rocha-Ferreira, M. Hristova, Plasticity in the neonatal brain following hypoxic-ischaemic injury, Neural Plast. 2016 (2016) 1–16, https://doi.org/10.1155/2016/4901014.
[10] J. Nithianantharajah, A.J. Hannan, Enriched environments, experience-dependent plasticity and disorders of the nervous system, Nat. Rev. Neurosci. 7 (2006) 697–709, https://doi.org/10.1038/nrn1970.
[11] H. van Praag, G. Kempermann, F.H. Gage, Neural consequences of environmental enrichment, Nat. Rev. Neurosci. 1 (2000) 191–198, https://doi.org/10.1038/35044558.
[12] P. Caporali, D. Cutuli, F. Gelfo, D. Laricchiuta, F. Foti, P. De Bartolo, L. Mancini, F. Angelucci, L. Petrosini, Pre-reproductive maternal enrichment influences offspring developmental trajectories: motor behavior and neurotrophin expression, Front. Behav. Neurosci. 8 (2014) 195, https://doi.org/10.3389/fnbeh.2014.00195.
[13] T.L. Bale, Epigenetic and transgenerational reprogramming of brain development, Nat. Rev. Neurosci. 16.6 (2015) 1–13, https://doi.org/10.1038/nrn3818.
[14] J.A. Arai, L.A. Feig, Long-lasting and transgenerational effects of an environmental enrichment on memory formation, Brain Res. Bull. 85 (2011) 30–35, https://doi.org/10.1016/j.brainresbull.2010.11.003.
[15] A. Sale, A systematic look at environmental modulation and its impact in brain development, Trends Neurosci. 41 (2018) 4–17, https://doi.org/10.1016/j.tins.2017.10.004.
[16] I. Welberg, K.V. Thrivikraman, P.M. Plotsky, Combined pre- and postnatal environmental enrichment programs the HPA axis differentially in male and female rats, Psychoneuroendocrinology 31 (2006) 553–564, https://doi.org/10.1016/j.psyneuen.2005.11.011.
[17] I. Cancedda, E. Putignano, A. Sale, A. Viegi, N. Berardi, L. Maffei, Acceleration of visual system development by environmental enrichment, J. Neurosci. 24 (2004) 4840–4848, https://doi.org/10.1523/JNEUROSCI.0845-04.2004.
[18] C.P. Schuch, R. Diaz, I. Deckmann, J.J. Rojas, B.F. Deniz, L.O. Pereira, Early environmental enrichment affects neurobehavioral development and prevents brain damage in rats submitted to neonatal hypoxia-ischemia, Neurosci. Lett. 617 (2016) 101–107, https://doi.org/10.1016/j.neulet.2016.02.015.
[19] E. Connors, M. Migliore, S. Pillsbury, A. Shaik, A. Kentner, Environmental enrichment models a naturalistic form of maternal separation and shapes the anxiety response patterns of offspring, Psychoneuroendocrinology 52C (2014) 153–167, https://doi.org/10.1016/j.psyneuen.2014.10.021.
[20] S. Baldini, L. Restani, L. Baroncelli, M. Coltelli, R. Franco, M.C. Cenni, L. Maffei, N. Berardi, Enriched early life experiences reduce adult anxiety-like behavior in

rats: a role for insulin-like growth factor 1, J. Neurosci. 33 (2013) 11715–11723, https://doi.org/10.1523/JNEUROSCI.3541-12.2013.
[21] P. Kiss, G. Vadasz, B. Kiss-Illes, G. Horvath, A. Tamas, D. Reglodi, M. Koppan, Environmental enrichment decreases asphyxia-induced neurobehavioral developmental delay in neonatal rats, Int. J. Mol. Sci. 14 (2013) 22258–22273, https://doi.org/10.3390/ijms141122258.
[22] Z. Zhang, H. Zhang, B. Du, Z. Chen, Neonatal handling and environmental enrichment increase the expression of GAP-43 in the hippocampus and promote cognitive abilities in prenatally stressed rat offspring, Neurosci. Lett. 522 (2012) 1–5, https://doi.org/10.1016/j.neulet.2012.05.039.
[23] D. Liu, J. Diorio, J.C. Day, D.D. Francis, M.J. Meaney, Maternal care, hippocampal synaptogenesis and cognitive development in rats, Nat. Neurosci. 3 (2000) 799–806, https://doi.org/10.1038/77702.
[24] J.W. Koo, C.H. Park, S.H. Choi, N.J. Kim, H. Kim, C. Choe, Y. Suh, N. Creative, S. National, Postnatal environment can counteract prenatal effects on cognitive ability, cell proliferation, and synaptic protein expression, FASEB J. (2003), https://doi.org/10.1096/fj.02-1032fje.
[25] J.J. Rojas, B.F. Deniz, P.M. Miguel, R. Diaz, É.D.E.S. Hermel, M. Achaval, C.A. Netto, L.O. Pereira, Effects of daily environmental enrichment on behavior and dendritic spine density in hippocampus following neonatal hypoxia-ischemia in the rat, Exp. Neurol. 241 (2013) 25–33, https://doi.org/10.1016/j.expneurol.2012.11.026.
[26] L.O. Pereira, P.M. Nabinger, A.C.P. Strapasson, P. Nardin, C.A.S. Gonçalves, I.R. Siqueira, C.A. Netto, Long-term effects of environmental stimulation following hypoxia-ischemia on the oxidative state and BDNF levels in rat hippocampus and frontal cortex, Brain Res. 1247 (2009) 188–195, https://doi.org/10.1016/j.brainres.2008.10.017.
[27] L.O. Pereira, N.S. Arteni, R.C. Petersen, A.P. da Rocha, M. Achaval, C.A. Netto, Effects of daily environmental enrichment on memory deficits and brain injury following neonatal hypoxia-ischemia in the rat, Neurobiol. Learn. Mem. 87 (2007) 101–108, https://doi.org/10.1016/j.nlm.2006.07.003.
[28] J.J. Rojas, B.F. Deniz, C.P. Schuch, J.V. Carletti, I. Deckmann, R. Diaz, C. Matté, T.M. dos Santos, A.T. Wyse, Ca. Netto, L.O. Pereira, Environmental stimulation improves performance in the ox-maze task and recovers Na+,K+-ATPase activity in the hippocampus of hypoxic–ischemic rats, Neuroscience 291 (2015) 118–127, https://doi.org/10.1016/j.neuroscience.2015.01.017.
[29] R. Diaz, P. Maidana Miguel, B. Ferrary Deniz, H.D. Confortim, S. Barbosa, M.C. Padilha Mendonça, M.A. da Cruz-Höfling, L. Orlandi Pereira, Environmental enrichment attenuates the blood brain barrier dysfunction induced by the neonatal hypoxia-ischemia, Int. J. Dev. Neurosci. 53 (2016) 35–45, https://doi.org/10.1016/j.ijdevneu.2016.06.006.
[30] C.A. Netto, E. Sanches, F.K. Odorcyk, L.E. Duran-Carabali, S.N. Weis, Sex-dependent consequences of neonatal brain hypoxia-ischemia in the rat, J. Neurosci. Res. 95 (2017) 409–421, https://doi.org/10.1002/jnr.23828.
[31] L. Cahill, D. Aswad, Neuroview sex influences on the brain: an issue whose time has come, Neuron 88 (2015) 1084–1085, https://doi.org/10.1016/j.neuron.2015.11.021.
[32] E. Jazin, L. Cahill, Sex differences in molecular neuroscience: from fruit flies to humans, Nat. Rev. Neurosci. 11 (2010) 9–17, https://doi.org/10.1038/nrn2754.
[33] T. Simonetti, H. Lee, M. Bourke, Ca. Leamey, A. Sawatari, Enrichment from birth accelerates the functional and cellular development of a motor control area in the mouse, PLoS One 4 (2009), https://doi.org/10.1371/journal.pone.0006780.
[34] S.V. Sizonenko, J.Z. Kiss, T. Inder, P.D. Gluckman, C.E. Williams, Distinctive neuropathologic alterations in the deep layers of the parietal cortex after moderate ischemic-hypoxic injury in the P3 immature rat brain, Pediatr. Res. 57 (2005) 865–872, https://doi.org/10.1203/01.PDR.0000157673.36848.67.
[35] A. Stadlin, A. James, R. Fiscus, Y.F. Wong, M. Rogers, C. Haines, Development of a postnatal 3-day-old rat model of mild hypoxic-ischemic brain injury, Brain Res. 993 (2003) 101–110, https://doi.org/10.1016/j.brainres.2003.08.058.
[36] R.C. Vannucci, Hypoxic-ischemic encephalopathy, Am. J. Perinatol. 17 (2018) 113–120 (accessed July 27, 2015)((n.d.)), http://cat.inist.fr/?aModele=afficheN&cpsidt=1517440.
[37] M. Alexander, H. Garbus, A.L. Smith, T.S. Rosenkrantz, R.H. Fitch, Behavioral and histological outcomes following neonatal HI injury in a preterm (P3) and term (P7) rodent model, Behav. Brain Res. 259 (2014) 85–96, https://doi.org/10.1016/j.bbr.2013.10.038.
[38] E.F. Sanches, N.S. Arteni, F. Nicola, L. Boisserand, S. Willborn, C. Netto, Early hypoxia-ischemia causes hemisphere and sex-dependent cognitive impairment and histological damage, Neuroscience 237 (2013) 208–215, https://doi.org/10.1016/j.neuroscience.2013.01.066.
[39] A.R. Zuena, M. Zinni, C. Giuli, C. Cinque, G.S. Alemà, A. Giuliani, A. Catalani, P. Casolini, R. Cozzolino, Maternal exposure to environmental enrichment before and during gestation influences behaviour of rat offspring in a sex-specific manner, Physiol. Behav. 163 (2016) 274–287, https://doi.org/10.1016/j.physbeh.2016.05.010.
[40] D.M. Arcego, R. Krolow, C. Lampert, A.P. Toniazzo, C. Berlitz, C. Lazzaretti, F. Schmitz, A.F. Rodrigues, A.T.S. Wyse, C. Dalmaz, Early life adversities or high fat diet intake reduce cognitive function and alter BDNF signaling in adult rats: interplay of these factors changes these effects, Int. J. Dev. Neurosci. 50 (2016) 16–25, https://doi.org/10.1016/j.ijdevneu.2016.03.001.
[41] F.K. Odorcyk, E.F. Sanches, F.C. Nicola, J. Moraes, L.F. Pettenuzzo, J. Kolling, C. Siebert, A. Longoni, E.L. Konrath, A. Wyse, C.A. Netto, Administration of Huperzia quadrifariata extract, a cholinesterase inhibitory alkaloid mixture, has neuroprotective effects in a rat model of cerebral hypoxia-ischemia, Neurochem. Res. 0 (2016) 1–11, https://doi.org/10.1007/s11064-016-2107-6.
[42] E. Sanches, L. Durán-Carabali, A. Tosta, F. Nicola, F. Schmitz, A. Rodrigues, C. Siebert, A. Wyse, C. Netto, Pregnancy swimming causes short- and long-term neuroprotection against hypoxia-ischemia in very immature rats, Pediatr Res. 31 (2017), https://doi.org/10.1038/pr.2017.110.
[43] L. Kappeler, M.J. Meaney, Epigenetics and parental effects, BioEssays 32 (2010) 818–827, https://doi.org/10.1002/bies.201000015.
[44] Y. Hase, L. Craggs, M. Hase, W. Stevenson, J. Slade, D. Lopez, R. Mehta, A. Chen, D. Liang, A. Oakley, M. Ihara, K. Horsburgh, R.N. Kalaria, Effects of environmental enrichment on white matter glial responses in a mouse model of chronic cerebral hypoperfusion, J. Neuroinflamm. 14 (2017) 81, https://doi.org/10.1186/s12974-017-0850-5.
[45] G. Kempermann, F.H. Gage, Experience-dependent regulation of adult hippocampal neurogenesis: effects of long-term stimulation and stimulus withdrawal, Hippocampus 9 (1999) 321–332, https://doi.org/10.1002/(SICI)1098-1063(1999)9:3<321::AID-HIPO11>3.0.CO;2-C.
[46] C.V. Vorhees, M.T. Williams, Forms of learning and memory, Nat. Protoc. 1 (2006) 848–858, https://doi.org/10.1038/nprot.2006.116.Morris.
[47] A.L. Smith, M. Alexander, T.S. Rosenkrantz, M.L. Sadek, R.H. Fitch, Sex differences in behavioral outcome following neonatal hypoxia ischemia: insights from a clinical meta-analysis and a rodent model of induced hypoxic ischemic brain injury, Exp. Neurol. 254 (2014) 54–67, https://doi.org/10.1016/j.expneurol.2014.01.003.
[48] N.S. Arteni, L.O. Pereira, A.L. Rodrigues, D. Lavinsky, M.E. Achaval, C.A. Netto, Lateralized and sex-dependent behavioral and morphological effects of unilateral neonatal cerebral hypoxia-ischemia in the rat, Behav. Brain Res. 210 (2010) 92–98, https://doi.org/10.1016/j.bbr.2010.02.015.
[49] R.J. Keeley, A.V. Tyndall, G.A. Scott, D.M. Saucier, Sex difference in cue strategy in a modified version of the morris water task: correlations between brain and behaviour, PLoS One 8 (2013) 1–9, https://doi.org/10.1371/journal.pone.0069727.
[50] L.O. Pereira, a.C.P. Strapasson, P.M. Nabinger, M. Achaval, C.A. Netto, Early enriched housing results in partial recovery of memory deficits in female, but not in male, rats after neonatal hypoxia-ischemia, Brain Res. 1218 (2008) 257–266, https://doi.org/10.1016/j.brainres.2008.04.010.
[51] E.F. Sanches, N.S. Arteni, C. Spindler, F. Moysés, I.R. Siqueira, M.L. Perry, C.A. Netto, Effects of pre- and postnatal protein malnutrition in hypoxic-ischemic rats, Brain Res. 1438 (2012) 85–92, https://doi.org/10.1016/j.brainres.2011.12.024.
[52] S. Ashwal, N. Ghosh, C.I. Turenius, M. Dulcich, B. Tone, Christopher M. Denham, R. Hartman, E.Y. Snyder, A. Obenaus, The reparative effects of neural stem cells in neonatal hypoxic-schemic injury are not influenced by host gender, Pediatr. Res. 75 (2014) 603–611, https://doi.org/10.1038/pr.2014.7.The 2014.
[53] J. Waddell, M. Hanscoma, N.S. Edwards, M.C. McKenna, M.M. McCarthy, Sex differences in cell genesis, hippocampal volume and behavioral outcomes in a rat model of neonatal HI, Exp. Neurol. 275 (2016) 285–295, https://doi.org/10.1016/j.expneurol.2015.09.003.
[54] A. Rosenfeld, A. Weller, Behavioral effects of environmental enrichment during gestation in WKY and Wistar rats, Behav. Brain Res. 233 (2012) 245–255, https://doi.org/10.1016/j.bbr.2012.05.006.
[55] I. Branchi, E. Alleva, Communal nesting, an early social enrichment, increases the adult anxiety-like response and shapes the role of social context in modulating the emotional behavior, Behav. Brain Res. 172 (2006) 299–306, https://doi.org/10.1016/j.bbr.2006.05.019.
[56] T. Maruoka, I. Kodomari, R. Yamauchi, E. Wada, K. Wada, Maternal enrichment affects prenatal hippocampal proliferation and open-field behaviors in female offspring mice, Neurosci. Lett. 454 (2009) 28–32, https://doi.org/10.1016/j.neulet.2009.02.052.
[57] R. Klein, V. Nanduri, S. Jing, F. Lamballe, P. Tapley, S. Bryant, C. Cordon-Cardo, K.R. Jones, L.F. Reichardt, M. Barbacid, The trkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3, Cell 66 (1991) 395–403, https://doi.org/10.1016/0092-8674(91)90628-C.
[58] J.J. Volpe, Perinatal brain injury: from pathogenesis to neuroprotection, Ment. Retard. Dev. Disabil. Res. Rev. 7 (2001) 56–64, https://doi.org/10.1002/1098-2779(200102)7:1<56::AID-MRDD1008>3.0.CO;2-A.
[59] J. Widenfalk, L. Olson, P. Thorén, Deprived of habitual running, rats downregulate BDNF and TrkB messages in the brain, Neurosci. Res. 34 (1999) 125–132, https://doi.org/10.1016/S0168-0102(99)00051-6.
[60] H. Bengoetxea, I. Rico-Barrio, N. Ortuzar, A. Murueta-Goyena, J.V. Lafuente, Environmental enrichment reverses tyrosine kinase inhibitor-mediated impairment through BDNF-TrkB pathway, Mol. Neurobiol. (2017) 1–17, https://doi.org/10.1007/s12035-017-0716-y.
[61] F. Ciucci, E. Putignano, L. Baroncelli, S. Landi, N. Berardi, L. Maffei, Insulin-like growth factor 1 (IGF-1) mediates the effects of enriched environment (EE) on visual cortical development, PLoS One 2 (2007), https://doi.org/10.1371/journal.pone.0000475.
[62] M. Wadowska, J. Woods, M. Rogozinska, T.L. Briones, Neuroprotective effects of enriched environment housing after transient global cerebral ischaemia are associated with the upregulation of insulin-like growth factor-1 signalling, Neuropathol. Appl. Neurobiol. 41 (2015) 544–556, https://doi.org/10.1111/nan.12146.
[63] K. Gustafson, H. Hagberg, B.Å. Bengtsson, C. Brantsing, J. Isgaard, Possible protective role of growth hormone in hypoxia-ischemia in neonatal rats, Pediatr. Res. 45 (1999) 318–323, https://doi.org/10.1203/00006450-199903000-00005.
[64] P. Carmeliet, Blood vessels and nerves: common signals, pathways and diseases, Nat. Rev. Genet. 4 (2003) 710–720, https://doi.org/10.1038/nrg1158.
[65] H.P. Gerber, K.J. Hillan, A.M. Ryan, J. Kowalski, G.A. Keller, L. Rangell, B.D. Wright, F. Radtke, M. Aguet, N. Ferrara, VEGF is required for growth and survival in neonatal mice, Development 126 (1999) 1149–1159 http://www.ncbi.nlm.nih.gov/pubmed/10021335.
[66] K.R. Howell, J. Armstrong, Vascular endothelial growth factor (VEGF) in

neurodevelopmental disorders, Curr. Behav. Neurosci. Rep. (2017) 1–10, https://doi.org/10.1007/s40473-017-0130-9.
[67] K. Plaschke, J. Staub, E. Ernst, H.H. Marti, VEGF overexpression improves mice cognitive abilities after unilateral common carotid artery occlusion, Exp. Neurol. 214 (2008) 285–292, https://doi.org/10.1016/j.expneurol.2008.08.014.
[68] N. Ortuzar, I. Rico-Barrio, H. Bengoetxea, E.G. Argando??a, J.V. Lafuente, VEGF reverts the cognitive impairment induced by a focal traumatic brain injury during the development of rats raised under environmental enrichment, Behav. Brain Res. 246 (2013) 36–46, https://doi.org/10.1016/j.bbr.2013.02.036.
[69] N. Ortuzar, E.G. Argandoña, H. Bengoetxea, J.V. Lafuente, Combination of intracortically administered VEGF and environmental enrichment enhances brain protection in developing rats, J. Neural Transm. 118 (2011) 135–144, https://doi.org/10.1007/s00702-010-0496-2.
[70] T. Licht, I. Goshen, A. Avital, T. Kreisel, S. Zubedat, R. Eavri, M. Segal, R. Yirmiya, E. Keshet, Reversible modulations of neuronal plasticity by VEGF, Proc. Natl. Acad. Sci. 108 (2011) 5081–5086, https://doi.org/10.1073/pnas.1007640108.
[71] D. Meshi, M.R. Drew, M. Saxe, M.S. Ansorge, D. David, L. Santarelli, C. Malapani, H. Moore, R. Hen, Hippocampal neurogenesis is not required for behavioral effects of environmental enrichment, Nat. Neurosci. 9 (2006) 729–731, https://doi.org/10.1038/nn1696.
[72] J.E. Burda, M.V. Sofroniew, Reactive gliosis and the multicellular response to CNS damage and disease, Neuron 81 (2014) 229–248, https://doi.org/10.1016/j.neuron.2013.12.034.
[73] X. Chen, X. Zhang, W. Liao, Q. Wan, Effect of physical and social components of enriched environment on astrocytes proliferation in rats after cerebral ischemia/reperfusion injury, Neurochem. Res. 42 (2017) 1308–1316, https://doi.org/10.1007/s11064-016-2172-x.
[74] S. Keiner, F. Wurm, A. Kunze, O.W. Witte, C. Redecker, Rehabilitative therapies differentially alter proliferation and survival of glial cell populations in the perilesional zone of cortical infarcts, Glia 56 (2008) 516–527, https://doi.org/10.1002/glia.20632.
[75] V. Chanana, A. Tumturk, D. Kintner, E. Udho, P. Ferrazzano, P. Cengiz, Sex differences in mouse hippocampal astrocytes after &em&in-vitro&/em& ischemia, J. Vis. Exp. (2016), https://doi.org/10.3791/53695.
[76] T.S. Morken, E.E. Brekke, A. Håberg, M. Widerøe, A.-M.A. Brubakk, U. Sonnewald, Altered astrocyte-neuronal interactions after hypoxia-ischemia in the neonatal brain in female and male rats, Stroke 45 (2014) 2777–2785, https://doi.org/10.1161/STROKEAHA.114.005341.
[77] A. Catalani, M. Sabbatini, C. Consoli, C. Cinque, D. Tomassoni, E. Azmitia, L. Angelucci, F. Amenta, Glial fibrillary acidic protein immunoreactive astrocytes in developing rat hippocampus, Mech. Ageing Dev. 123 (2002) 481–490, https://doi.org/10.1016/S0047-6374(01)00356-6.
[78] R.J. Lichtenwalner, J.M. Parent, Adult neurogenesis and the ischemic forebrain, J. Cereb. Blood Flow Metab. 26 (2006) 1–20, https://doi.org/10.1038/sj.jcbfm.9600170.
[79] L.-L. Zhu, T. Zhao, H.-S. Li, H. Zhao, L.-Y. Wu, A.-S. Ding, W.-H. Fan, M. Fan, Neurogenesis in the adult rat brain after intermittent hypoxia, Brain Res. 1055 (2005) 1–6, https://doi.org/10.1016/j.brainres.2005.04.075.
[80] J.J. Hu, X.L. Yang, W. Di Luo, S. Han, J. Yin, W.H. Liu, X.H. He, B.W. Peng, Bumetanide reduce the seizure susceptibility induced by pentylenetetrazol via inhibition of aberrant hippocampal neurogenesis in neonatal rats after hypoxia-ischemia, Brain Res. Bull. 130 (2017) 188–199, https://doi.org/10.1016/j.brainresbull.2017.01.022.
[81] G. Tang, K. Gudsnuk, S.H. Kuo, M.L. Cotrina, G. Rosoklija, A. Sosunov, M.S. Sonders, E. Kanter, C. Castagna, A. Yamamoto, Z. Yue, O. Arancio, B.S. Peterson, F. Champagne, A.J. Dwork, J. Goldman, D. Sulzer, Loss of mTOR-dependent macroautophagy causes autistic-like synaptic pruning deficits, Neuron 83 (2014) 1131–1143, https://doi.org/10.1016/j.neuron.2014.07.040.
[82] M.M. Riccomagno, A.L. Kolodkin, Sculpting neural circuits by axon and dendrite pruning, Annu. Rev. Cell Dev. Biol. 31 (2015) 779–805, https://doi.org/10.1146/annurev-cellbio-100913-013038.
[83] S.L. Andersen, M.H. Teicher, Stress, sensitive periods and maturational events in adolescent depression, Trends Neurosci. 31 (2008) 183–191, https://doi.org/10.1016/j.tins.2008.01.004.
[84] M. Olczak, M. Duszczyk, P. Mierzejewski, T. Bobrowicz, M.D. Majewska, Neonatal administration of thimerosal causes persistent changes in Mu opioid receptors in the rat brain, Neurochem. Res. 35 (2010) 1840–1847, https://doi.org/10.1007/s11064-010-0250-z.
[85] C. Zhu, F. Xu, X. Wang, M. Shibata, Y. Uchiyama, K. Blomgren, H. Hagberg, Different apoptotic mechanisms are activated in male and female brains after neonatal hypoxia-ischaemia, J. Neurochem. 96 (2006) 1016–1027, https://doi.org/10.1111/j.1471-4159.2005.03639.x.
[86] S.R. Mayoral, G. Omar, A.A. Penn, Sex differences in a hypoxia model of preterm brain damage, Pediatr. Res. 66 (2009) 248–253, https://doi.org/10.1203/PDR.0b013e3181b1bc34.
[87] M. a Mirza, R. Ritzel, Y. Xu, L.D. McCullough, F. Liu, Sexually dimorphic outcomes and inflammatory responses in hypoxic-ischemic encephalopathy, J. Neuroinflamm. 12 (2015) 32, https://doi.org/10.1186/s12974-015-0251-6.
[88] Z. Huang, J. Liu, P.Y. Cheung, C. Chen, Long-term cognitive impairment and myelination deficiency in a rat model of perinatal hypoxic-ischemic brain injury, Brain Res. 1301 (2009) 100–109, https://doi.org/10.1016/j.brainres.2009.09.006.
[89] C. Quairiaux, S.V. Sizonenko, P. Mégevand, C.M. Michel, J.Z. Kiss, Functional deficit and recovery of developing sensorimotor networks following neonatal hypoxic-ischemic injury in the rat, Cereb. Cortex 20 (2010) 2080–2091, https://doi.org/10.1093/cercor/bhp281.
[90] K. Keyvani, T. Schallert, Plasticity-associated molecular and structural events in the injured brain, J. Neuropathol. Exp. Neurol. 61 (2002) 831–840 http://www.ncbi.nlm.nih.gov/pubmed/12387449.
[91] A. Sale, N. Berardi, L. Maffei, Environment and brain plasticity: towards an endogenous pharmacotherapy, Physiol. Rev. 94 (2014) 189–234, https://doi.org/10.1152/physrev.00036.2012.
[92] S. Mering, J. Jolkkonen, Proper housing conditions in experimental stroke studies-special emphasis on environmental enrichment, Front. Neurosci. 9 (2015) 1–8, https://doi.org/10.3389/fnins.2015.00106.
[93] M.W. McDonald, K.S. Hayward, I.C.M. Rosbergen, M.S. Jeffers, D. Corbett, Is environmental enrichment ready for clinical application in human post-stroke rehabilitation? Front. Behav. Neurosci. 12 (2018) 1–16, https://doi.org/10.3389/fnbeh.2018.00135.

Principais Achados do Capítulo 3

Entre os resultados obtidos neste capítulo, destacamos que:

1. O ambiente enriquecido reduziu o déficit de aprendizado espacial e o comportamento de tipo ansioso/ impulsividade nos animais submetidos a hipóxia isquemia, de ambos os sexos;
2. A estimulação durante a gestação reduziu a hiperlocomoção nos machos;
3. Os efeitos benéficos induzidos pelo ambiente enriquecido podem estar relacionados com um aumento na expressão de fatores neurotróficos como VEGF e IGF-1, bem como na expressão do receptor TrkB;
4. A perda tecidual no hipocampo após a hipóxia isquemia foi prevenida (gestação) ou revertida (lactação) pelo ambiente enriquecido nos machos;
5. A combinação de ambos os períodos de estimulação (gestacional e lactacional) não causou um efeito aditivo nos parâmetros avaliados.

4.4 CAPÍTULO 4

Enriched experiences during pregnancy and lactation protects against motor impairments induced by neonatal hypoxia-ischemia

Short communication publicada no journal Behavioral Brain Research

(Impact factor: 3.173)

Behavioural Brain Research
Available online 28 March 2019
In Press, Accepted Manuscript

Short communication

Enriched experience during pregnancy and lactation protects against motor impairments induced by neonatal hypoxia-ischemia

Durán-Carabali, LE [a], Sanches, EF [b], Reichert, L [c], Netto, CA [a, b]

[a] Post-graduation Program of Physiology, Instituto de Ciências Básicas da Saúde (ICBS), Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
[b] Department of Biochemistry, Instituto de Ciências Básicas da Saúde (ICBS), Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
[c] Neurorehabilitation and Neural Repair Research Group, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, RS, Brazil

Received 26 November 2018, Revised 26 March 2019, Accepted 27 March 2019, Available online 28 March 2019.

Abstract

Neonatal hypoxia-ischemia (HI) is responsible for movement disorders in preterm infants. Non-pharmacological strategies, such as environmental enrichment (EE) during adulthood, have shown positive effects on promoting sensorimotor recovery after HI. However, little is known about the effects of perinatal EE on sensorimotor function following HI. In present study, we investigated the hypothesis that enriched experiences during pregnancy and lactation would reduce motor impairments caused by a model of neonatal HI in rats. At postnatal day (PND) 3, Wistar pups of both sexes were subject to the modified Rice-Vannucci model. Motor function was evaluated from PND 60 to PND 64. HI caused a reduction in the forepaws strength and worsening of movement quality in the right forepaw. These effects were attenuated in animals receiving prenatal or lactational EE, which showed better performance when compared to the control group. Moreover, enriched experiences during lactation reversed HI-induced asymmetric use of the forepaws and the trend to increased paw errors in a walking test. Lower scores were found in the contralateral forepaw placement in HI animals, except when EE was provided at both stages of neurodevelopment. These results indicate that enriched experiences reduce motor impairments, *i.e*, measured in force, asymmetry and coordination domains, and that EE during lactation is more effective in promoting post-injury recovery. These data support that early therapeutic interventions might enhance functional reorganization at a period of high brain plasticity and that enriched-like experience might be encouraged in pediatric rehabilitation programs, in order to reduce long-term movement disorders after neonatal brain insults.

Neonatal hypoxia-ischemia (HI) is a major cause of several neurological sequelae in preterm newborns[1] of which movement disorders impair functional performance throughout the life span[2]. Experimental models of HI, such as the one described by Rice-Vannucci, have been widely used to study the pathophysiological mechanisms associated with neonatal brain injury and to investigate the effectiveness of therapeutic interventions[3]. Cellular and functional impairments caused by HI in very immature rats[4], at third postnatal day (PND3), have produced motor impairments that are similar to those observed in extremely preterm children[5,6].

Environmental enrichment (EE) paradigm is characterized by a combination of physical activity as well as sensory, social and cognitive stimuli; it has positive effects in promoting sensorimotor recovery after brain insults[7]. Recently, it has been reported the protective role of prenatal and lactational EE in reducing brain damage and cognitive deficits[8,9]. Nevertheless, little is known about the effects of early EE on sensorimotor deficits, especially when enriched experiences are provided during pregnancy. In the present study, we evaluated whether the exposure to an enriched environment during pregnancy and lactation would be able to mitigate motor impairments caused by neonatal HI induce at PND3.

Female Wistar rats were allocated to standard conditions (SC/n=8) or enriched environment (EE/n=8) during pregnancy. As shown in Figure 1, after pups' birth (PND1), litters were randomly assigned to a new housing condition from lactation until weaning (**SC-EE** or **EE-SC**) or were kept in the same housing from gestation until weaning (**SC-SC** or **EE-EE**). After weaning, all rats were housed in standard conditions, divided by sex

in standard home cages (height: 16cm, width: 41cm and length: 34cm). At PND 3, pups of both sexes were subjected to neonatal HI[10]. Briefly, a cervical incision was performed and the right common carotid artery was exposed and ligated with suture thread, under isoflurane anesthesia (4-5% for induction and 1.5-2% for maintenance). After two hours, pups were exposed to 8% O_2 and 92% N_2 for 2.5h. The surgical control (*sham*) group was subjected to anesthesia and cervical incision with neither carotid ligation nor subsequent hypoxia[10]. All protocols were conducted according to the National Institutes of Health Guide for The Care and Use of Laboratory Animals and were approved by Institutional Ethical committee (under protocol # 28641).

As previously described[9], environmental enrichment conditions included large cages (height: 120cm, width: 80cm and length: 100cm), ramps, running wheel and plastic toys of different textures. Objects were changed twice per week and were reorganized every day. In addition, water and food were provided *ad libitum*. Eight animals per cage were housed during pregnancy and two litters per cage were kept together during the lactation period, in order to increase social interaction among animals. Standard conditions included wood shaving bedding and minimum stimuli inside the cages. Four female rats were allocated together during pregnancy and litters (dam and pups) were kept in a single cage until weaning[9].

Motor function was assessed from PND 60 to PND 64, as shown in Figure 1. Grasping strength test was used to determine the digital force. Animal's forepaws were put on the handle and then a gently pull in the horizontal direction was performed. The force generated by the animal until release was recorded. A mean of three trials was used and data are presented in kilograms[6]. Cylinder test was performed to assess forelimb

asymmetry. Animals were put into an acrylic transparent cylinder for three minutes and the numbers of touches with the forepaws were recorded. Asymmetry percentage was calculated using the following equation: contralateral forepaw/∑Forepaws[10]. Gait abilities and motor coordination were evaluated through the ladder walking test. Animals were stimulated to walk across the ladder for three trials. During the training period (two consecutive days), a regular rung distribution was used on the apparatus. On the third day, the rungs were spaced at irregular intervals of 1-3 cm and the probe trial was video-recorded. Paw placement was scored using the Metz and Whishaw classification and the final value corresponded to the mean of the three trials[11]. The percentage of errors was calculated by the mean of the total number of errors divided by the number of steps with each paw, as previously described[6]. An error was considered when the animal slipped the paw or failed to place it on the rung. The number of animals used for behavioral assessment was: SC-SC Sham (n=10), SC-SC HI (n=13); SC-EE Sham (n=12), SC-EE HI (n=14); EE-SC Sham (n=13), EE-SC HI (n=11); EE-EE Sham (n=10) and EE-EE HI (n=11). Five to seven rats per group were randomly chosen for histological analyses. Brain slices were stained with hematoxylin and eosin, cerebral cortex and striatum volumes (from coordinates +1.70 to -1.30 from Bregma level) were estimated by *ImageJ software* and the volume ratio among ipsilateral/contralateral side to carotid occlusion was calculated[6]. Normality of data distribution was confirmed by Shapiro–Wilk test. Two-way ANOVA with housing conditions and injury as factors was performed, followed by Duncan's *post hoc* test. P-values ≤ 0.05 were considered significant; data are expressed as mean ± S.E.M.

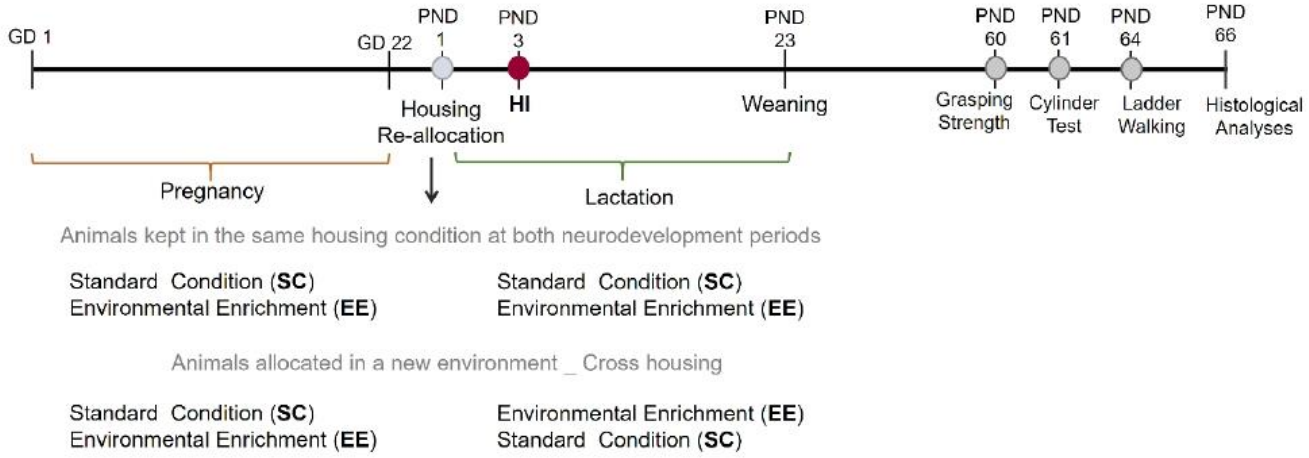


Figure 1: Summary of experimental design. GD: Gestational Day; PND: Postnatal day; HI: Hypoxia-Ischemia.

There was an effect of housing condition ($F_{(3,91)}$=6.242, P=0.001) in the grasping strength test, indicating a reduction in the forepaws strength in the SC-SC group compared to enriched groups (Figure 2A). No significant differences were observed between HI and Sham animals ($F_{(1,91)}$=0.116 P>0.05). As depicted in Figure 2B, an injury effect was observed ($F_{(1,92)}$=4.443, P=0.005) in the cylinder test. Pairwise comparison revealed that HI animals from SC-SC and EE-SC group showed an asymmetric use of the forepaws as compared to their respective sham groups. This injury effect was not observed in animals exposed to postnatal EE (SC-EE and EE-EE).

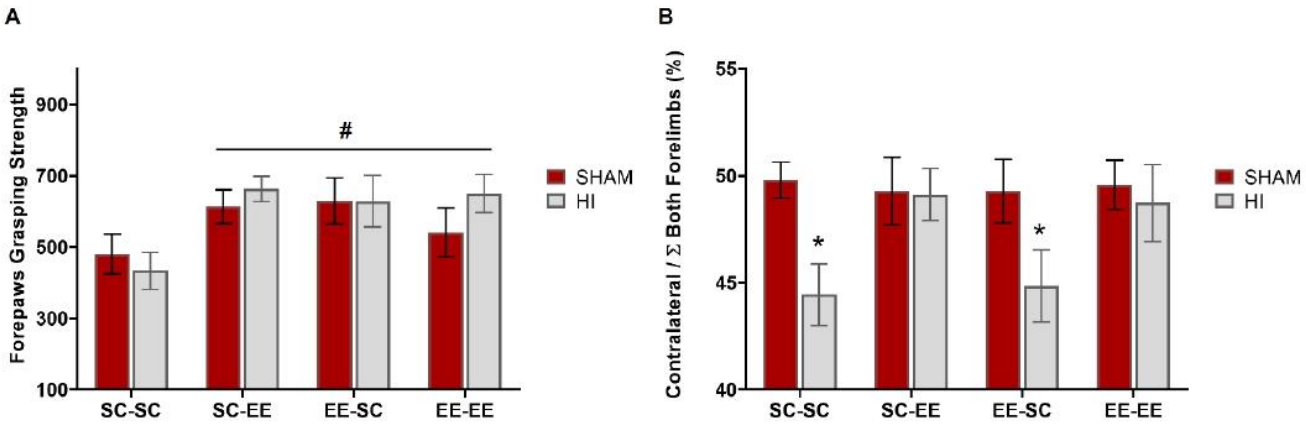


Figure 2. Strength and asymmetry evaluation: **A**. Grasping strength test. **B.** Cylinder test. # Environmental enrichment effect. * Differences between HI and Sham. Data expressed as mean ± S.E.M

In the ladder walking test, the SC-SC group showed lower placement quality score of the paws compared to enriched groups (SC-EE, EE-SC and EE-EE) ($F_{(3,93)}$=6.406, P=0.001, Figure 3A). In addition, an injury effect was observed ($F_{(1,93)}$=3.850 P=0.05); pairwise comparison revealed that SC-SC HI group had a reduction in right forepaw placement score when compared to SC-SC sham. No differences were found between HI enriched animals and their respective sham groups, indicating a protective effect of both prenatal and postnatal EE. Considering the left forepaw use (contralateral side to the carotid occlusion), HI animals exhibited a decrease in the quality score ($F_{(1,93)}$=4.701, P=0.03), however, such effect was not observed in the EE-EE HI group (Figure 3B), suggesting that prolonged exposure to EE would be necessary to reduce HI-induced contralateral impairment. No significant differences were found in the hind paws scores (P>0.05, data not shown). There was an interaction between housing conditions and injury ($F_{(3,93)}$=3.804, P=0.05, Figure 3C); HI animals kept in SC during lactation period (SC-SC and EE-SC) had more errors per number of steps in the right forepaw compared

to all other experimental groups. No significant differences were observed in the number of errors with the left forepaw (Figure 3D) nor with the hind paws (P>0.05).

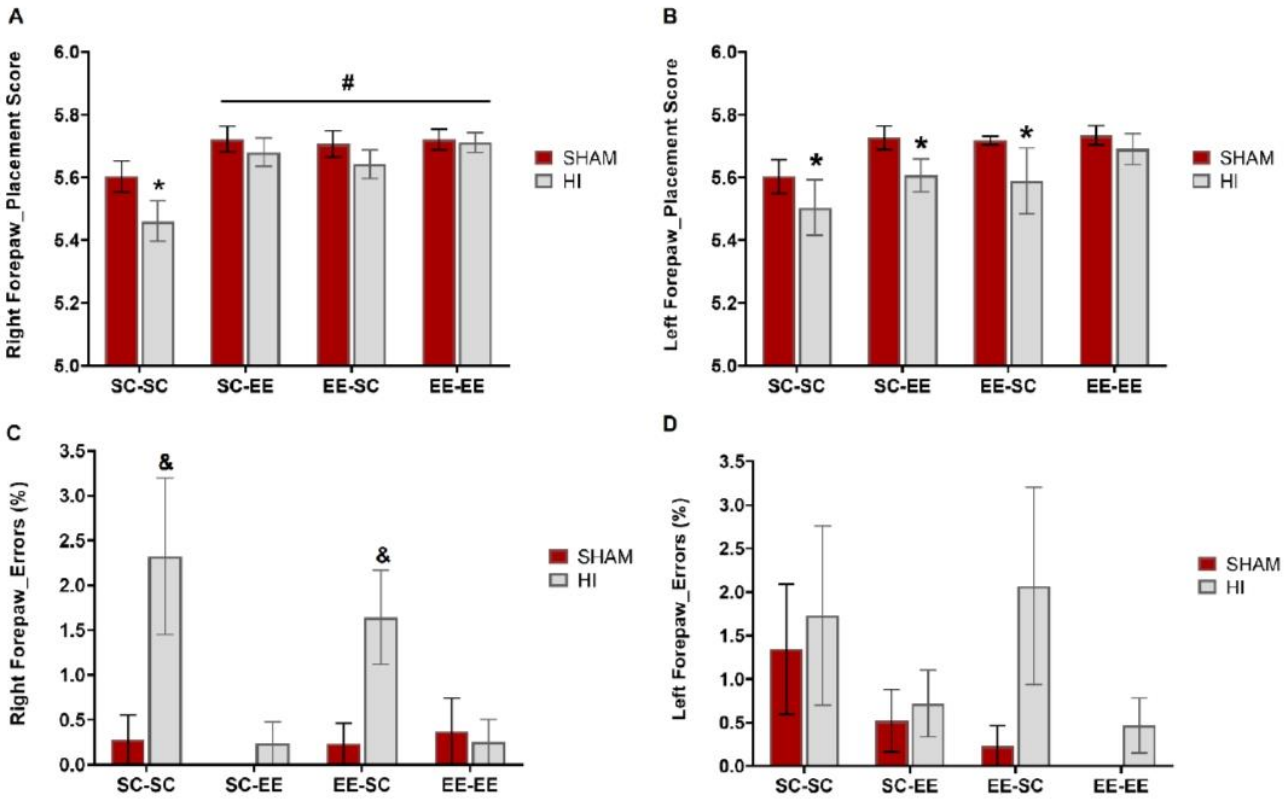


Figure 3. Ladder walking test_ Step Quality: **A**. Right forepaw, **B**. Left forepaw. Step Errors: **C**. Right forepaw, **D**. Left forepaw # Environmental enrichment effect. *Differences between HI and Sham. & Difference from the other experimental groups. Data expressed as mean ± S.E.M

Cerebral cortex and striatum volume ratios revealed an injury effect ($F_{(1,48)}$=5.123, P=0.029 and $F_{(1,48)}$=10.572, P=0.002, respectively), indicating that SC-SC HI animals have decreased volume structures compared to SC-SC sham group (Figure 4). No

significant differences were observed in HI animals exposed to enriched environment, compared to their respective control group, in the structure's volumes analyzed.

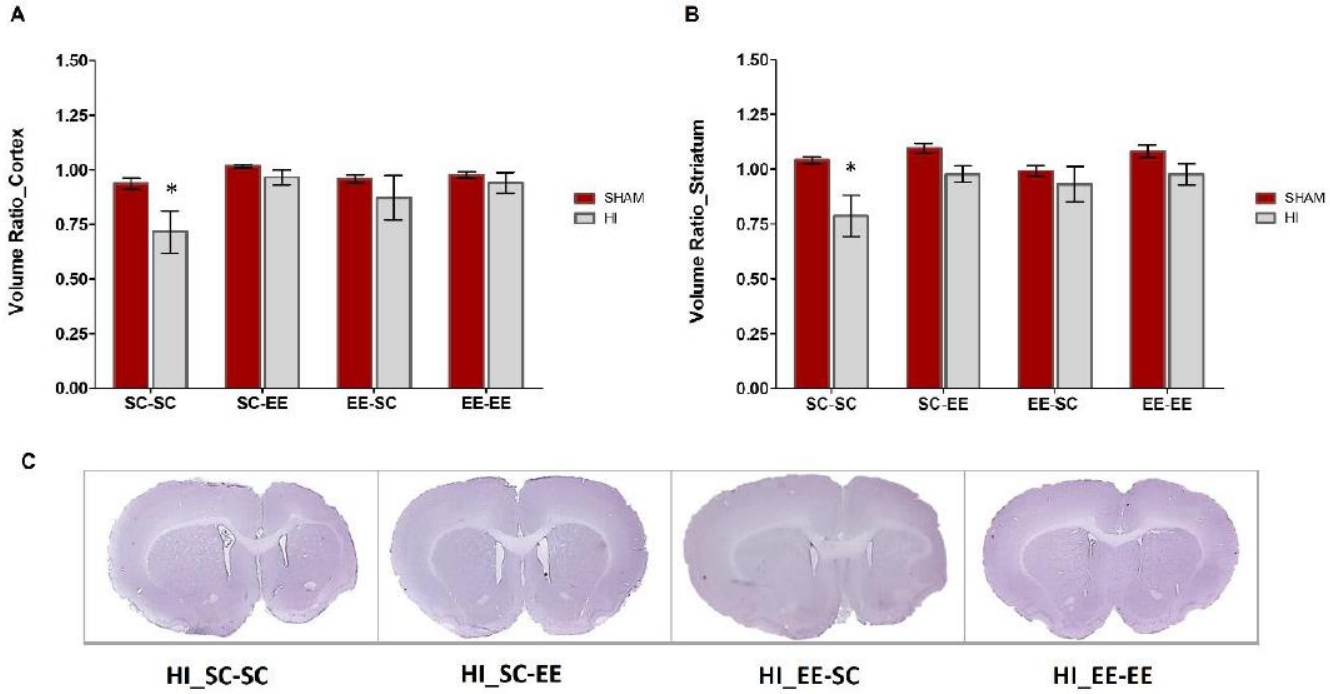


Figure 4. Histological Analyses; **A.** Volume ratio of the cerebral cortex, **B.** Volume ratio of the striatum, **C.** Representative hematoxylin and eosin stained, coronal brain sections of HI animals. * Differences between HI and Sham. Data expressed as mean ± S.E.M

Previous reports of our research group have focused on the effects of early EE (gestational and lactation periods) over HI-induced cell death and survival pathways, the response of protective endogenous molecules, such as growth and neurotrophic factors, as well as the cognitive impairment and sex-specific responses[9,12]. Here, we show that enriched experiences during pregnancy and suckling are able to reduce HI-induced motor impairments in rats submitted to neonatal hypoxia-ischemia. Nevertheless, beneficial effects were mainly observed in animals exposed to enriched conditions during lactation,

when sensoriomotor domains such as forelimbs symmetry, motor coordination and gait abilities were evaluated at adulthood. SC-SC HI animals showed a decrease in the use of the contralateral forepaw in the cylinder test, evidencing a movement asymmetry induced by neonatal HI. Moreover, HI causes a reduction in the quality score in the right (ipsilateral to carotid occlusion) and left (contralateral) forepaws, as well as increased number of step errors. Data are in agreement with previous reports from our research group and others, indicating that hypoxic-ischemic events cause motor impairment, which can limit movement efficiency and compromise functional performance in over demanding tasks[6].

In the present study, EE increased the forepaws grasping strength compared to SC group. This finding is in agreement with previous reports indicating that bilateral grip strength could be influenced by maternal experiences[13] and improved by postnatal EE[14,15]. Asymmetric forelimb-use induced by neonatal HI was reversed in animals housed in enriched conditions during lactation. Nevertheless, prenatal EE did not attenuate this motor impairment. The ladder walking test is sensitive to reveal long-term and subtle impairments in the fore and hind limbs, especially when forebrain control is required[11,16]. In this test, SC-SC HI animals had the worst performance compared to other groups, in agreement with previous studies[17,16]. Moreover, these rats showed both ipsi- and contralateral forepaws impairment, confirming that the general movement efficiency can be affected even after unilateral brain damage[3]. Interestingly, the literature is scarce regarding the influence of gestational EE in motor behavior of animals submitted to neonatal HI. In this study, EE-SC HI animals had a decrease in the ipsilateral forepaw use and more foot faults than rats exposed to enriched experiences from the birth until

the weaning (SC-EE or EE-EE). Previous studies using gestational interventions showed that maternal voluntary physical activity and EE are able to attenuate motor deficits following CNS insults[18,19]. Nevertheless, aspects such as different injury models and less sensitive behavioral tests are likely to be responsible for the discrepancies observed among these studies. In agreement with literature, animals exposed to EE after weaning showed a reduction in sensorimotor asymmetry caused by the HI insult[16,20].

The lactation period is characterized by a progressive increase in physical voluntary movements, enhanced environmental exploration and interaction with stimuli[21]. In addition, motor circuits and networks responsible for movement initiation are established after birth in rodents[22]. Thereby, components such as increased maturation of sensoriomotor system and neuroplastic mechanisms associated with action-dependent organization could be relevant promoting functional brain re-organization during post-injury recovery process. Moreover, these processes could facilitate a better use of the impaired paws (left ones, in this case), improving the forelimbs movement quality[22]. There was a decrease in the volume of cerebral cortex and striatum in the SC-SC HI animals compared to their respective sham group. Such HI-induced tissue loss was mitigated in animals exposed to gestational and lactation EE, which could be associated with the EE capacity to modulate brain injury severity[9]. It is interesting to note that after neonatal brain insults tissue preservation *per se* (as observed in animals exposed to prenatal EE) does not seem to be a determinant to attenuate functional deficits, suggesting that anatomical maturation and neuronal connections tuning could contribute to the subsequent execution of complex motor patterns[22,23].

Although promising, the interpretation of positive EE effects should be cautious. As a matter of fact, standard laboratory rodent housing conditions in many animal facilities does not resemble the naturalistic rodent environment, since rodents are exposed to many different stimuli and experiences outdoor[24]. In addition, some modification reducing environment deprivation such as that observed on control groups could induce positive effects on the animal responses to CNS injuries or functional tasks. We suggest that further studies should review the environmental conditions in control groups, in order to improve the animals' wellbeing and to allow a more natural expression of animal behavior, increasing the scientific validity of EE effects[25]. Similarly, hospitals and care institution settings could also be considered human impoverished environments[24]. Thus, it is feasible that programs based on the concept of enriched environmental experiences could translate aspects of the EE paradigm through multimodal interventions in order to improve the sensory environment for at risk infants (e.g premature babies)[22,23].

Concluding, current findings demonstrate that the enriched experience reduced motor impairments following neonatal HI, being the exposure to complex environment during lactation more effective to cause post-injury recovery, as evaluated by functional motor domains. In addition, we suggest that enriched-like experiences should be encouraged in pediatric rehabilitation programs, for they could help to reduce long-term movement disorders in newborns suffering from neonatal brain insults.

Conflict of interest

The authors have no conflicts of interest.

Acknowledgment

The authors thank the staff of Reproduction and Laboratory Animal Research Center from Biochemistry Department UFRGS.

Funding Sources

This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

References

[1] M.I. Shevell, A. Majnemer, P. Rosenbaum, M. Abrahamowicz, Etiologic determination of childhood developmental delay, Brain Dev. 23 (2001) 228–235. doi:10.1016/S0387-7604(01)00212-1.

[2] M.C. McKenna, S. Scafidi, C.L. Robertson, Metabolic alterations in developing brain after injury: knowns and unknowns, Neurochem. Res. 40 (2015) 2527–2543. doi:10.1007/s11064-015-1600-7.

[3] G.J. Clowry, R. Basuodan, F. Chan, What are the Best Animal Models for Testing Early Intervention in Cerebral Palsy?, Front. Neurol. 5 (2014) 1–17. doi:10.3389/fneur.2014.00258.

[4] S. V. Sizonenko, E. Sirimanne, Y. Mayall, P.D. Gluckman, T. Inder, C. Williams, Selective cortical alteration after hypoxic-ischemic injury in the very immature rat brain, Pediatr. Res. 54 (2003) 263–269.

doi:10.1203/01.PDR.0000072517.01207.87.

[5] S. Misumi, Y. Ueda, R. Nishigaki, M. Suzuki, A. Ishida, C.-G. Jung, H. Hida, Dysfunction in Motor Coordination in Neonatal White Matter Injury Model without Apparent Neuron Loss., Cell Transplant. 25 (2016) 1381–1393. doi:10.3727/096368915X689893.

[6] L.E. Durán-Carabali, E.F. Sanches, M.R. Marques, D. Aristimunha, A. Pagnussat, C.A. Netto, Longer hypoxia–ischemia periods to neonatal rats causes motor impairments and muscular changes, Neuroscience. 340 (2017) 291–298. doi:10.1016/j.neuroscience.2016.10.068.

[7] S. Mering, J. Jolkkonen, Proper housing conditions in experimental stroke studies-special emphasis on environmental enrichment, Front. Neurosci. 9 (2015) 1–8. doi:10.3389/fnins.2015.00106.

[8] C. Netto, E. Sanches, F. Odorcyk, L. Duran-Carabali, S. Sizonenko, Pregnancy as a valuable period for preventing hypoxia-ischemia brain damage, Int. J. Dev. Neurosci. 18 (2018). doi:10.1016/j.ijdevneu.2018.06.004.

[9] L.. Durán-Carabali, D.. Arcego, F.. Odorcyk, L. Reichert, J.. Cordeiro, E.. Sanches, L.. Freitas, C. Dalmaz, A. Pagnussat, C.. Netto, Prenatal and Early Postnatal Environmental Enrichment Reduce Acute Cell Death and Prevent Neurodevelopment and Memory Impairments in Rats Submitted to Neonatal Hypoxia Ischemia, Mol. Neurobiol. (2017). doi:10.1007/s12035-017-0604-5.

[10] E.F. Sanches, N.S. Arteni, F. Nicola, L. Boisserand, S. Willborn, C.. Netto, Early

hypoxia-ischemia causes hemisphere and sex-dependent cognitive impairment and histological damage, Neuroscience. 237 (2013) 208–215. doi:10.1016/j.neuroscience.2013.01.066.

[11] G.A. Metz, I.Q. Whishaw, Cortical and subcortical lesions impair skilled walking in the ladder rung walking test : a new task to evaluate fore- and hindlimb stepping, placing, and co-ordination, J. Neurosci. Methods. 115 (2002) 169–179.

[12] L.E. Durán-Carabali, D.M. Arcego, E.F. Sanches, F.K. Odorcyk, M.R. Marques, A. Tosta, L. Reichert, A.S. Carvalho, C. Dalmaz, C.A. Netto, Preventive and therapeutic effects of environmental enrichment in Wistar rats submitted to neonatal hypoxia-ischemia, Behav. Brain Res. 359 (2019) 485–497. doi:10.1016/j.bbr.2018.11.036.

[13] P. Caporali, D. Cutuli, F. Gelfo, D. Laricchiuta, F. Foti, P. De Bartolo, L. Mancini, F. Angelucci, L. Petrosini, Pre-reproductive maternal enrichment influences offspring developmental trajectories: motor behavior and neurotrophin expression., Front. Behav. Neurosci. 8 (2014) 195. doi:10.3389/fnbeh.2014.00195.

[14] J.H. Seo, J.H. Yu, H. Suh, M.S. Kim, S.R. Cho, Fibroblast Growth Factor-2 Induced by Enriched Environment Enhances Angiogenesis and Motor Function in Chronic Hypoxic-Ischemic Brain Injury, PLoS One. 8 (2013). doi:10.1371/journal.pone.0074405.

[15] B. Mattsson, J.C. Sørensen, J. Zimmer, B.B. Johansson, Neural grafting to experimental neocortical infarcts improves behavioral outcome and reduces

thalamic atrophy in rats housed in enriched but not in standard environments, Stroke. 28 (1997) 1225–1232. doi:10.1161/01.STR.28.6.1225.

[16] C.P. Schuch, M.S. Jeffers, S. Antonescu, C. Nguemeni, M. Gomez-Smith, L.O. Pereira, C.M. Morshead, D. Corbett, Enriched rehabilitation promotes motor recovery in rats exposed to neonatal hypoxia-ischemia, Behav. Brain Res. 304 (2016) 42–50. doi:10.1016/j.bbr.2016.02.010.

[17] A.L.F. Meireles, M.R. Marques, E. Segabinazi, C. Spindler, F. V. Piazza, G.S. Salvalaggio, O.A. Augustin, M. Achaval, S. Marcuzzo, Association of environmental enrichment and locomotor stimulation in a rodent model of cerebral palsy: Insights of biological mechanisms, Brain Res. Bull. 128 (2017) 58–67. doi:10.1016/j.brainresbull.2016.12.001.

[18] J. Fragoso, A. de O. Lira, G.S. Chagas, C.C. Lucena, Cavalcanti, R. Beserra, G. de Santana-Muniz, A. Bento-Santos, G. Martins, L. Pirola, R. da S. Aragão, C.G. Leandro, Maternal voluntary physical activity attenuates delayed neurodevelopment in malnourished rats, Exp. Physiol. 102 (2017) 1486–1499. doi:10.1113/ep086400.

[19] M.R. Marques, F. Stigger, E. Segabinazi, O.A. Augustin, S. Barbosa, F.V. Piazza, M. Achaval, S. Marcuzzo, Beneficial effects of early environmental enrichment on motor development and spinal cord plasticity in a rat model of cerebral palsy, Behav. Brain Res. 263 (2014) 149–157. doi:10.1016/j.bbr.2014.01.007.

[20] S.R. Cho, H. Suh, J.H. Yu, H.H. Kim, J.H. Seo, C.H. Seo, Astroglial activation by an enriched environment after transplantation of mesenchymal stem cells

enhances angiogenesis after hypoxic-ischemic brain injury, Int. J. Mol. Sci. 17 (2016). doi:10.3390/ijms17091550.

[21] A. Sale, N. Berardi, L. Maffei, Environment and brain plasticity: towards an endogenous pharmacotherapy., Physiol. Rev. 94 (2014) 189–234. doi:10.1152/physrev.00036.2012.

[22] M. Jamon, The early development of motor control in neonate rat, Comptes Rendus - Palevol. 5 (2006) 657–666. doi:10.1016/j.crpv.2005.11.018.

[23] N. Dehorter, L. Vinay, C. Hammond, Y. Ben-Ari, Timing of developmental sequences in different brain structures: Physiological and pathological implications, Eur. J. Neurosci. 35 (2012) 1846–1856. doi:10.1111/j.1460-9568.2012.08152.x.

[24] M.W. McDonald, K.S. Hayward, I.C.M. Rosbergen, M.S. Jeffers, D. Corbett, Is Environmental Enrichment Ready for Clinical Application in Human Post-stroke Rehabilitation?, Front. Behav. Neurosci. 12 (2018) 1–16. doi:10.3389/fnbeh.2018.00135.

[25] K. Bayne, Environmental enrichment and mouse models: Current perspectives, Anim. Model. Exp. Med. 1 (2018) 82–90. doi:10.1002/ame2.12015.

Principais Achados do Capítulo 4

Entre os resultados obtidos neste capítulo, destacamos que:

1. A hipóxia isquemia não alterou a força das patas anteriores nos animais, porém reduz o uso da pata anterior contralateral a lesão, alterou a coordenação motora bem como o padrão de marcha dos animais;

2. A exposição ao ambiente enriquecido na lactação favoreceu um melhor desempenho motor nos animais, revertendo os prejuízos induzidos pela hipóxia isquemia;

3. O ambiente enriquecido durante a gestação não mostrou efeitos positivos em prevenir o comprometimento funcional na simetria e na marcha dos animais após a hipóxia isquemia;

4. O ambiente enriquecido causou uma preservação tecidual em áreas relacionadas ao controle motor como o córtex e o estriado.

4.5 CAPÍTULO 5

O enriquecimento ambiental precoce reduz parcialmente o hipometabolismo encefálico e modifica a estrutura da rede metabólica em ratos submetidos à hipóxia-isquemia neonatal: Resultados preliminares

4.5.1 Introdução

A hipóxia-isquemia neonatal é caracterizada pela presença de déficits motores, sensoriais, comportamentais e cognitivos, que são dependentes da severidade do insulto e podem estar associados a alterações metabólicas (Morken et al., 2014). A glicose é o principal substrato energético para o cérebro adulto. No entanto, ao nascimento a taxa de consumo da glicose é inferior a 30% quando comparada à do adulto e ao longo do desenvolvimento (entre a puberdade e a idade adulta) apresenta mudanças, entendidas como níveis mais altos ou mais baixos, que refletem indiretamente os períodos de maturação do SNC, e processos de plasticidade como a proliferação e a poda sináptica (Chugani, 2018; Rudolph, 2018).

Resultados experimentais indicam que após um evento hipóxico-isquêmico, há oscilações dos níveis encefálicos da glicose, sendo estes elevados entre 6 e 12 horas pós-lesão, com uma posterior redução até 48 e 72 horas (Thorngren-Jerneck et al., 2001), sugerindo que o hipometabolismo tardio está estritamente relacionado à morte celular e consequentemente ao grau de lesão (Marik et al., 2009). A resposta celular após a HI neonatal está determinada pela disponibilidade e utilização de outros substratos energéticos tais como o acetato, o lactato e os corpos cetônicos, os quais são críticos para o metabolismo energético do encéfalo em desenvolvimento e podem ser essenciais para reduzir a severidade da lesão e os subsequentes prejuízos funcionais.

O imageamento metabólico refere-se à visualização, não-invasiva e em tempo real de eventos bioquímicos complexos como o consumo de glicose. Para tal, equipamentos específicos foram adaptados para detectar marcadores

biológicos em animais de pequeno porte (James and Gambhir, 2012). A tomografia por emissão de pósitrons (μPET) permite determinar o consumo de glicose através do radiofármaco 18F-fluordesoxiglicose (18F-FDG/ t 1/2 = 109,8 min) e possibilita a realização de estudos sem alterações do microambiente celular. Outra vantagem da técnica refere-se à oportunidade de analisar as interações entre diversas áreas de interesse e o seu metabolismo encefálico, através de uma representação matemática conhecida como rede metabólica (Baptista et al., 2015; James and Gambhir, 2012),

Até o momento, o único estudo utilizando a técnica de μPET em animais expostos ao ambiente enriquecido, reportou que camundongos saudáveis, expostos à estimulação ambiental na idade adulta e durante oito semanas, apresentaram níveis mais baixos de captação do radiofármaco para os transportadores de dopamina no estriado quando comparado com os animais controle (Kim et al., 2016). Assim, o objetivo do presente experimento foi investigar os efeitos do ambiente enriquecido durante o período gestacional e lactacional em ratos submetidos à HI neonatal no DPN3 no metabolismo encefálico, bem como a sua associação com a funções cognitivas e parâmetros histológicos avaliados na idade adulta.

4.5.2 Metodologia

Ratas fêmeas Wistar foram alocadas ao ambiente padrão (AP) ou enriquecido (AE) durante a gestação. Após o nascimento (DPN 1), as ninhadas foram aleatoriamente designadas a uma nova condição de moradia ou foram mantidas nas mesmas condições ambientais até o desmame, estabelecendo assim, quatro grupos segundo as condições de moradia em ambos os períodos do desenvolvimento (período gestacional – período lactacional): AP-AP, AP-AE, AE-AP e AE-AE. No DPN 3, os filhotes foram submetidos ao modelo de HI conforme descrito anteriormente. Na idade adulta (DNP 60), o metabolismo encefálico dos animais foi mensurado por meio da técnica de microtomografia por μPET. A memória espacial dos animais foi avaliada através do labirinto aquático de Morris a partir do DPN 65 até o DPN 71. Uma vez finalizada a avalição funcional, os animais foram submetidos a eutanásia e os encéfalos foram coletados para a análise histológica (Figura 7). O detalhamento das condições de moradia, procedimento cirúrgico, avaliação comportamental e do volume de lesão estão descritos na seção Metodologia (Item 3) da presente Tese.

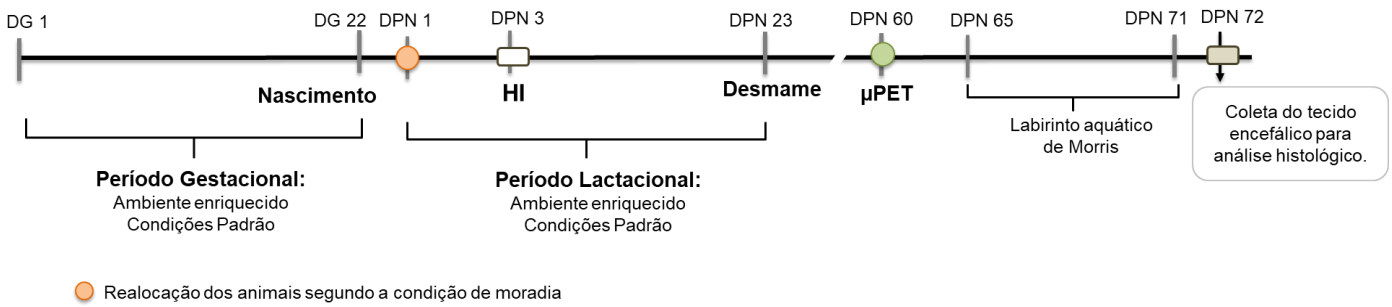


Figura 7: Representação esquemática do desenho experimental para a análise do metabolismo encefálico.

Para este experimento foram utilizados apenas animais machos, pois estes apresentaram uma maior vulnerabilidade à HI e melhor resposta à estimulação ambiental em ambos os períodos do neurodesenvolvimento, como descrito no Capítulo 4. Além disso, foram considerados aspectos logísticos relacionados ao número de animais a serem escaneados (22 animais por dia) no Instituto do Cérebro (InsCer) da Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS). Para completar o número de animais foi necessário realizar o experimento em quatro levas, incluindo a cada vez 2-3 animais por grupo.

Escaneamento por Microtomografia de Emissão de Pósitrons

a. Produção do rádio-fármaco

O Flúor-18 (18F) é um radioisótopo com meia-vida de 109,8 minutos, produzido através de um acelerador circular de partículas PET trace. Foi utilizado um alvo líquido, composto de água enriquecida com oxigênio-18 que, por meio da da reação nuclear 18O(p,n)18F, produziu o flúor nucleofílico. A síntese de 18F-Fluordesoxiglicose (18F-FDG) foi realizada no módulo automatizado *FASTlab*. Basicamente, a água contendo 18F passa através de uma coluna de troca iônica onde o radioisótopo fica retido. Em seguida, este é diluído em solução aquosa de acetonitrila contendo carbonato de potássio e Kryptofix 2.2.2, obtendo o fluoreto de potássio complexado ao Kryptofix 2.2.2. A molécula precursora, triflato de manose em acetonitrila, foi adicionada junto ao complexo formado na segunda etapa da síntese. Esta mistura foi aquecida a 125°C por aproximadamente 2 minutos para facilitar a reação de substituição. A última etapa da síntese foi a hidrólise básica dos grupos protetores acetil, com hidróxido de sódio. Finalmente, o produto passou por uma série de colunas de purificação,

ajuste de pH e esterilização (Yu, 2006). Para a definição dos critérios de qualidade do produto final, utilizou-se como compêndio oficial a versão mais atual da Farmacopéia Americana.

b. Escaneamento

Os animais foram transportados, em caixas padrão até o biotério do Centro de Pesquisa Pré-Clínica do InsCer, na PUCRS, um dia antes do escaneamento no DPN 60. Para o transporte foi usado um veículo climatizado a uma temperatura de 22ºC e foi tomado o devido cuidado para evitar os horários de trânsito intenso, a fim reduzir ao máximo o estresse dos animais.

No dia do escaneamento, os animais foram então anestesiados com isoflurano e injetados com 18F-FDG num volume de 0,01 ml/g (ratos adultos: 1 mCi, i.v.), posteriormente, os animais permaneceram isolados e conscientes por 45 minutos, tempo suficiente para a captação cerebral de 18F-FDG. Para o escaneamento no TriumphTM µPET (LabPET-4, TriFoil Imaging, Northridge, CA, USA), o roedor foi novamente anestesiado com isoflurano e posicionado na câmara de imageamento permanentemente aquecida. Os animais foram escaneados por 30 minutos, com a região cerebral posicionada no *field-of-view* (FOV) do µPET. Ao término dos escaneamentos, o rato foi retirado do aparelho e permaneceu dentro de sua caixa moradia, sobre uma placa de aquecimento, até sua completa recuperação. O algoritmo de reconstrução para o processamento das imagens de µPET foi o OSEM-3D e a captação de FDG cerebral foi quantificada através do software PMOD v3.5 e *Fusion Toolbox* (PMOD Technologies, Zurich, Switzerland) (Baptista et al., 2015). Um molde da área de interesse para imagens obtidas por ressonância magnética foi usado

para sobrepor as imagens previamente normalizadas e registradas por µPET. Os valores de captação de glicose foram normalizados segundo a dose injetada de 18F-FDG e o peso corporal do animal. O resultado obtido foi expresso como o valor padrão de captação (SUVs SUV- por seu nome em inglês *standard uptake value*), dessa forma, foi calculada a média do SUVs de 58 regiões cerebrais (considerando quando necessário cada hemisfério)(Nonose et al., 2018). Após o escaneamento os animais foram devolvidos ao Departamento de Bioquímica da UFRGS

Análise de redes metabólicas

A rede metabólica é uma ferramenta matemática para a representação de um sistema complexo e é definida por uma coleção de nodos (vértices) e ligações (arestas) entre pares de nodos. Os nodos geralmente representam regiões cerebrais, enquanto as ligações (*links*), indicam se há acoplamento entre os nodos, i.e. regiões anatômicas podem estar conectadas (interação linear) e não ligadas (interação não-linear) (Rubinov and Sporns, 2010).

No delineamento das redes metabólicas foi o utilizado o método de *boostrap,* a partir dos dados de SUV obtidos por cada grupo experimental. A base da técnica é a obtenção de um "novo" conjunto de dados, por reamostragem do conjunto de dados originais, isso permite obter estimativas robustas da variabilidade da distribuição gerada pelas iterações de reamostragem (Martinez-Espinosa et al., 2006). Assim, as redes metabólicas foram construídas através do cálculo dos coeficientes de correlação de Pearson baseados em 1000 amostras de *bootstrap.* Cada amostra de *bootstrap* foi corrigida para comparações múltiplas usando FDR (do inglês, false Discovery

rate) (p <0,001). A densidade de medidas teóricas do gráfico foi calculada para avaliação quantitativa da rede. A densidade é a medida proporcional às bordas de conexão na rede. Quanto maior o valor da densidade, maior o número de ligações entre nodos, ou seja, maior a sincronicidade encefálica.

Análises Estatísticas

Foi verificada a normalidade dos resultados pelo teste de Shapiro-Wilk. Os dados paramétricos foram submetidos a análise de variância (ANOVA) de duas vias, tendo como fatores fixos as condições de moradia e a lesão, seguido do *post-hoc* Duncan para múltiplas comparações. As latências obtidas durante o treinamento no labirinto aquático de Morris foram analisadas por meio de ANOVA de medidas repetidas. Correlações entre os parâmetros metabólicos e histológicos foram calculadas através do coeficiente de correlação de Pearson. A significância aceita foi de P≤ 0.05, os testes foram realizados utilizando o *software* SPSS versão 21. As diferenças que foram consideradas relevantes foram:

- Entre os animais *sham* e HI pertencentes ao mesmo grupo experimental, conforme as condições de moradia
- Entre os animais *sham*
- Entre os animais HI

4.5.3 Resultados

Avaliação da memória espacial no Labirinto aquático de Morris

A ANOVA de medidas repetidas, evidenciou uma interação entre os fatores "dia de treinamento" e "condições de moradia", assim como entre os fatores "dia de treinamento" e "lesão" ($F_{(3,112)}$= 5.734 P= 0.018 e $F_{(1,112)}$= 3.648 P= 0.015, respetivamente): no primeiro e segundo dia de treinamento não houve diferenças significativas entre os grupos. No terceiro dia de treinamento os animais do grupo HI_AP-AP apresentaram uma maior latência para encontrar a plataforma quando comparado com os animais HI expostos ao AE durante o período gestacional (HI_AE-AP e HI_AE-AE). No quarto dia, o grupo HI_AP-AP foi diferente do seu respectivo *sham*. Como observado na Figura 2a, no último dia do treinamento os animais HI que nunca foram expostos ao enriquecimento ambiental apresentaram um pior desempenho quando comparado com quaisquer outro grupo experimental. Os dados sugerem que o prejuízo cognitivo causado pela lesão neonatal foi prevenido/revertido pelo ambiente enriquecido, salientando que os animais expostos à estimulação apresentaram comportamento semelhante aos dos animais não lesados. Resultado semelhante foi observado na área sob a curva ($F_{(3,112)}$= 3.506 P= 0.02), no qual os animais do grupo HI_AP-AP apresentaram uma maior área, indicando déficits na memória espacial, dado que os animais requeriam de mais tempo para localizar a plataforma a longo do treinamento quando comparado com os outros grupos experimentais (Figura 2b).

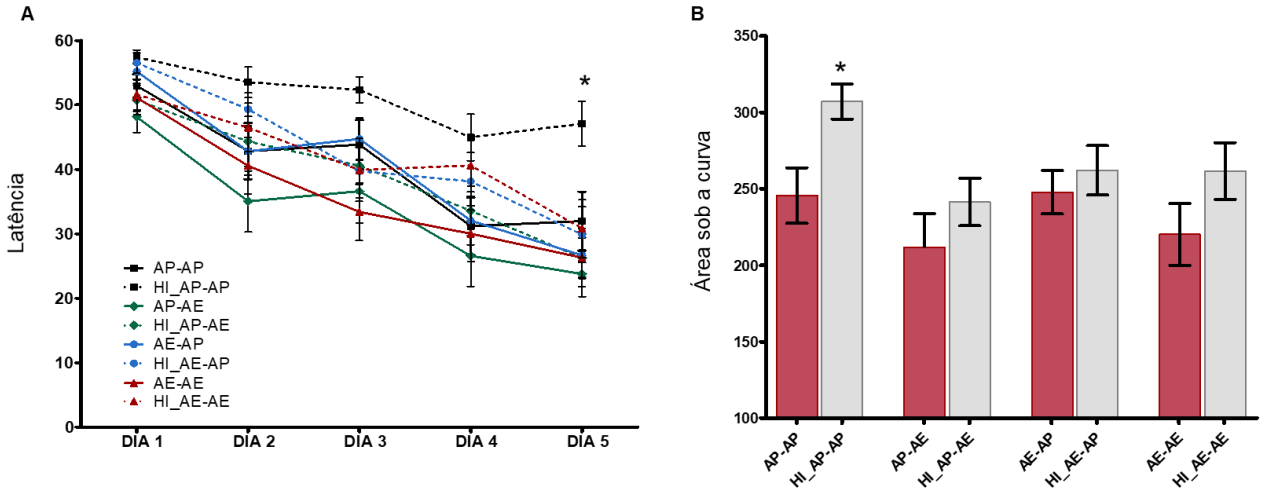


Figura 8: Labirinto aquático de Morris: A. Latência durante os dias de treino. B. Desempenho geral do aprendizado espacial considerando a área sob a curva. Os dados são expressos em média ± EPM. *Diferentes dos outros grupos experimentais. n= 12-18 animais por grupo, valor considerado significativo P≤ 0.05).

Captação encefálica de 18F-FDG

A análise do nível de captação encefálica de glicose considerando as 58 áreas analisadas (somatória dos valores de SUV de todas as áreas), mostrou que os animais do grupo HI_AP-AP apresentaram menor metabolismo encefálico quando comparados aos animais expostos ao AE em ambos os períodos do desenvolvimento e que tinham sido submetidos ao modelo de HI ($F_{(1,69)}$= 4.133 P= 0.046 – Figura 9a,c). Ao analisar os níveis de captação de glicose do córtex (somatória dos valores de SUV para as diferentes regiões corticais – Figura 9e), do estriado (Figura 9g) e o do hipocampo (somatória dos valores de SUV da região dorsal anterior e posterior –Figura 9I), a partir da relação entre os lados ipsilateral e contralateral à lesão, houve interação estatística entre as condições de moradia e a lesão ($F_{(1,69)}$= 5.202, P= 0.003; $F_{(1,69)}$= 5.286, P= 0.003 e $F_{(1,69)}$= 6.274, P= 0.001, respetivamente), indicando uma redução do metabolismo no grupo HI_AP-AP quando comparado aos outros

grupos experimentais. Na tabela 2, apresentam-se as diferencias estatísticas encontradas nas diversas regiões analisadas, usando a relação entre o lado ipsilateral e o contralateral à lesão.

Volume de lesão

A ANOVA de duas vias, usando a relação entre o lado ipsilateral e contralateral à lesão evidenciou uma interação entre os fatores “condição de moradia” e “lesão” para o volume do hemisfério ($F_{(3,69)}$= 3.594, P= 0.018 –Figura 9d), córtex cerebral ($F_{(3,69)}$= 3.402, P= 0.023- Figura 9f), estriado ($F_{(3,69)}$= 3.268, P= 0.027- Figura 9h) e hipocampo ($F_{(3,69)}$= 3.842, P= 0.014 – Figura 9j), mostrando uma diminuição no volume encefálico das estruturas mencionadas nos animais HI_AP-AP. Esse efeito foi prevenido/revertido pelo ambiente enriquecido gestacional e lactacional, cujos animais apresentaram maior preservação tecidual, especialmente quando os animais foram expostos ao enriquecimento em um dos períodos estudados, sendo o volume encefálico semelhante ao dos seus respectivos controles (*sham).* Ainda que tenha havido um efeito positivo do ambiente enriquecido, os animais do grupo HI_AE-AE mostraram uma redução no volume do hemisfério, córtex e estriado (Figura 9d, f, h, respectivamente) quando comparados com seu controle (AE-AE), indicando uma possível atenuação dos efeitos da estimulação quando os animais são expostos de maneira continua ao enriquecimento.

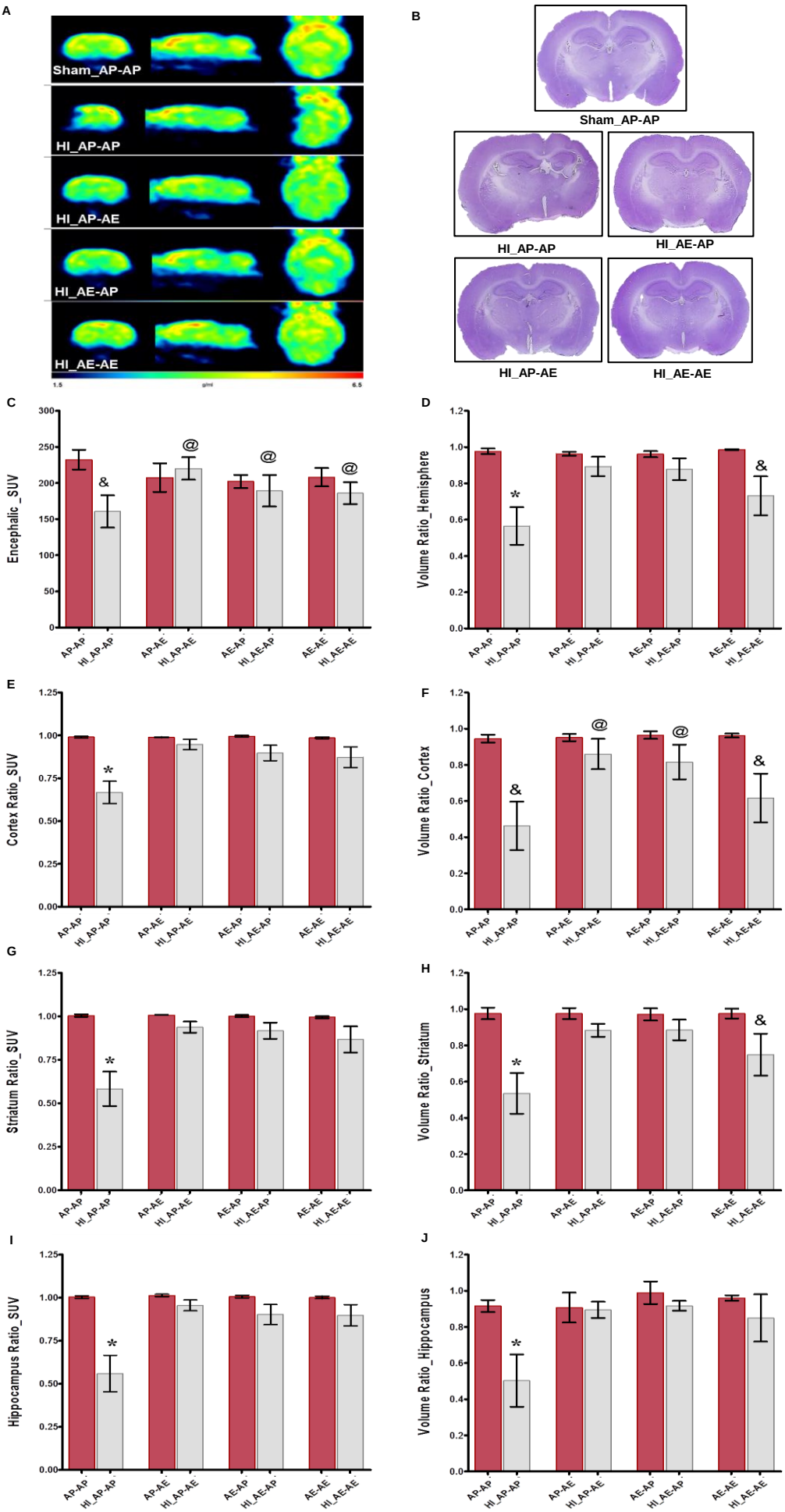

A
Sham_AP-AP
HI_AP-AP
HI_AP-AE
HI_AE-AP
HI_AE-AE
1.5
g/ml
6.5
B
Sham_AP-AP
HI_AP-AP
HI_AE-AP
HI_AP-AE
HI_AE-AE
C
Encephalic_SUV
D
Volume Ratio_Hemisphere
E
Cortex Ratio_SUV
F
Volume Ratio_Cortex
G
Striatum Ratio_SUV
H
Volume Ratio_Striatum
I
Hippocampus Ratio_SUV
J
Volume Ratio_Hippocampus
AP-AP
HI_AP-AP
AP-AE
HI_AP-AE
AE-AP
HI_AE-AP
AE-AE
HI_AE-AE

Figura 9: Níveis do valor de captação de glicose (SUV) e volume de lesão, avaliados na idade adulta: **A**. Imagem representativa de da captação de 18F-FDG nos animais submetidos à HI por cada condição de moradia em relação ao grupo controle (*Sham*_AP-AP). Ilustrado em vistas coronal (esquerda), sagital (média) e transversal (direita); **B.** Imagem representativa do volume de lesão dos animais em relação ao grupo controle; **C.** Valores obtidos para o encéfalo total, a partir da somatória das áreas cerebrais analisadas; **D** Razão do volume de lesão do hemisfério. Razão dos valores de SUV entre o lado ipsi e contralateral a lesão do (direita) e razão do volume de lesão (esquerda) das estruturas: **E-F**; córtex cerebral; **G-H** estriado e **I-J.** Hipocampo**.** Os dados foram expressos em média ± EPM. & Diferente do seu respectivo *sham*, @ Diferente do grupo HI_AP-AP, *Diferente dos outros grupos experimentais. N= 7-10 animais por grupo, valor considerado significativo P≤ 0.05).

Ao considerar a razão entre o lado ipsilateral e contralateral à lesão dos níveis do valor de captação de glicose (SUV) e do volume de lesão, houve uma correlação considerada forte (entre 0.7 e 0.9) entre ambos desfechos para o córtex cerebral (Correlação de Pearson: 0.918 P=0.0001, Figura 10a), estriado Correlação de Pearson: 0.701 P= 0.0001, Figura 10b) e hipocampo (Correlação de Pearson: 0.841 P= 0.0001, Figura 10c) indicando que quando há maior metabolismo encefálico, há maior preservação tecidual (correlação positiva).

Também analisamos a rede metabólica aos 57 dias pós- HI (DPN 60), a fim de avaliar o potencial de remodelação da rede em animais submetidos a uma lesão encefálica neonatal e a influência do ambiente enriquecido no período gestacional e lactacional. O ANOVA de duas vias usando a densidade da rede mostrou uma interação entre as condições de moradia e a lesão ($F_{(3,79)}$= 1,329, P= 0.001) indicando que os animais sham dos grupos AP-AE e AE-AE apresentaram diferenças significativas (menor e maior número de conexões na

rede metabólica, respectivamente) quando comparados com os grupos AP-AP e AE-AP.

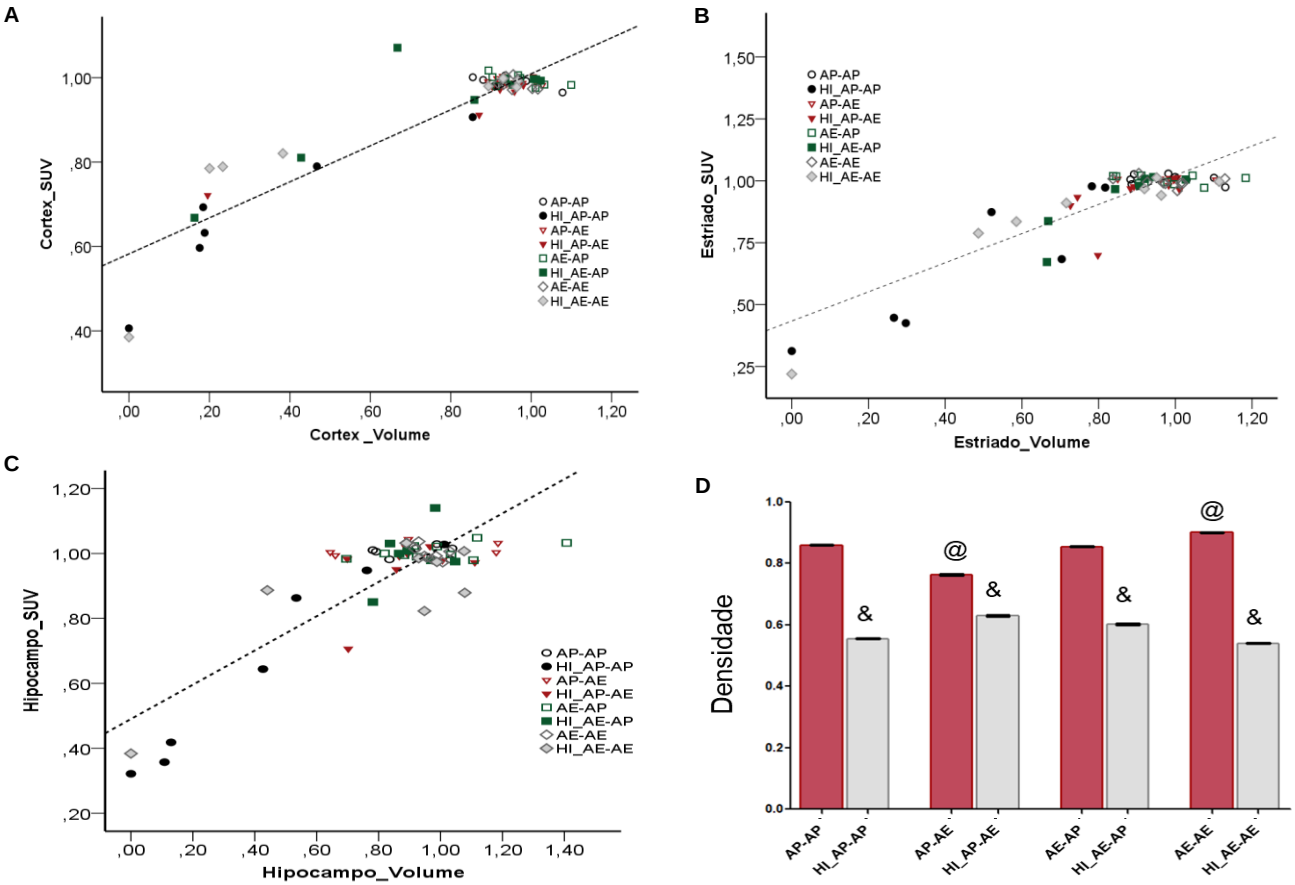


Figura 10: Metabolismo encefálico e o volume da lesão**.** Correlação entre a relação do lado ipsilateral e contralateral à lesão da captação de 18F-FDG e o volume de lesão do: **A.** Cortex cerebral; **B.** estriado e **C.** hipocampo. **D**. Avaliação da densidade de conexão da rede metabólica. Os dados são expressos em média ± EPM. SUV- por seu nome em inglês standard uptake value. & Diferente do seu respectivo sham, @ Diferente do grupo HI_AP-AP. 1000 bootstrap para cada grupo experimental, valor considerado significativo P≤ 0.05).

Por sua vez, todos os animais HI apresentaram uma hiposincronicidade metabólica quando comparados a seus respectivos grupos controle (*sham*). No entanto, os animais HI e expostos ao ambiente enriquecido tiveram um maior sincronismo quando comparado com o grupo HI_AP-AP. Os resultados confirmam que lesões neonatais causam alterações no metabolismo encefálico

que perduram até a idade adulta e evidencia o efeito parcial do AE para atenuar essa resposta (Figura 10d, 11).

Tabela 2: Razão dos níveis de captação de glicose nas diversas regiões encefálicas. Os dados são expressos em média ± EPM. [&]Diferente do seu respectivo *sham*, [@]Diferente do grupo HI_AP-AP, *Diferente dos outros grupos experimentais. N= 7-10 animais por grupo, valor considerado significativo P≤ 0.05). Uma redução no metabolismo da glicose foi observada em estruturas como o núcleo acumbens, a amigdala, o córtex, o hipocampo, o hipotálamo, a área ventral tegmental e o tálamo nos animais do grupo HI_AP-AP, cujo efeito foi revertido parcialmente pelo enriquecimento no período gestacional e lactacional.

Região Encefálica	AP-AP		HI_AP-AP		AP-AE		HI_AP-AE		AE-AP		HI_AE-AP		AE-AE		HI_AE-AE	
	Média	± EPM	Média	± EPM	Média	± EPM	Média	± EPM	Média	± EPM	Média	± EPM	Média	± EPM	Média	± EPM
Núcleo Accumbens	1,03	0,01	0,73	0,05*	0,99	0,01	0,94	0,04	1,02	0,02	0,93	0,03	0,98	0,01	0,86	0,05&
Amigdala	1,01	0,01	0,59	0,10*	1,00	0,01	0,92	0,04	1,00	0,01	0,86	0,05	1,01	0,02	0,83	0,07&
Córtex Auditivo	0,97	0,01	0,42	0,08*	0,98	0,01	0,87	0,06	0,94	0,01	0,77	0,08&	0,98	0,01	0,78	0,08&
Córtex Cingulado	1,00	0,01	0,88	0,05	1,01	0,01	1,00	0,01@	1,01	0,01	0,99	0,01@	0,99	0,01	0,95	0,05
Córtex Entorrinal	0,96	0,01	0,61	0,08*	0,99	0,01	0,90	0,04	0,96	0,01	0,87	0,06	0,97	0,01	0,86	0,06
Córtex Frontal	1,02	0,01	0,80	0,07&	0,99	0,02	0,99	0,03@	1,03	0,01	0,95	0,03@	0,99	0,01	0,94	0,07@
Córtex Insular	0,94	0,01	0,57	0,09*	0,94	0,01	0,87	0,03	0,97	0,01	0,86	0,06	0,93	0,01	0,84	0,07
Córtex Medial Pré-frontal	1,01	0,01	0,92	0,03&	1,01	0,01	0,98	0,01	1,00	0,00	0,97	0,01	1,01	0,01	0,92	0,04&
Córtex Motor	1,00	0,01	0,72	0,09*	1,01	0,01	1,00	0,03	1,02	0,01	0,96	0,04	1,01	0,01	0,88	0,07
Córtex Orbitofrontal	0,98	0,01	0,84	0,05*	0,96	0,01	0,95	0,02@	1,00	0,01	0,94	0,01@	0,96	0,01	0,90	0,05*
Córtex Parietal	1,00	0,02	0,44	0,09*	1,00	0,01	0,92	0,06	1,01	0,01	0,81	0,09&	1,00	0,01	0,79	0,08&
Córtex Retroesplenial	1,01	0,01	0,74	0,06*	1,02	0,01	0,99	0,02	1,00	0,01	0,94	0,04	1,02	0,01	0,93	0,03
Córtex Somatossensorial	0,99	0,01	0,49	0,10*	0,98	0,01	0,93	0,06	0,99	0,01	0,83	0,08	0,98	0,01	0,80	0,08
Córtex Visual	1,01	0,01	0,47	0,08*	0,99	0,01	0,91	0,05	0,99	0,01	0,80	0,09&	1,00	0,01	0,81	0,08&
Hipocampo dorsal anterior	0,99	0,01	0,57	0,10*	1,02	0,01	0,96	0,03	1,00	0,01	0,92	0,06	1,01	0,01	0,92	0,06
Hipocampo Posterior	1,01	0,01	0,55	0,11*	1,01	0,01	0,95	0,03	1,02	0,01	0,88	0,06	1,00	0,01	0,88	0,06
Hipotálamo	1,01	0,01	0,83	0,06*	1,01	0,01	0,98	0,01	1,01	0,01	0,95	0,02	1,00	0,01	0,96	0,03
Olfatório	0,98	0,02	0,81	0,04*	0,96	0,01	0,92	0,03	0,98	0,01	0,92	0,02	0,98	0,01	0,91	0,03&
Colículo Superior	1,04	0,02	0,81	0,07&	1,02	0,01	0,96	0,03	1,02	0,02	0,96	0,03	1,01	0,01	0,94	0,05
Colículo Inferior	1,03	0,03	0,86	0,07&	1,04	0,01	0,96	0,03	1,02	0,02	0,97	0,03	1,03	0,02	1,00	0,04@
Mesencéfalo	1,01	0,01	0,87	0,07	1,01	0,01	1,00	0,01	1,00	0,01	1,01	0,02	0,99	0,01	0,97	0,03
Área ventral Tegmental	0,99	0,02	0,87	0,06*	0,99	0,01	1,00	0,02	0,99	0,01	0,98	0,02	0,96	0,01	1,00	0,02
Cerebelo_Sust Cinzenta	0,96	0,01	0,95	0,02	0,97	0,01	0,98	0,01	0,96	0,01	0,99	0,01	0,97	0,01	1,00	0,02
Cerebelo_ Sust Branca	0,97	0,01	0,98	0,02	0,99	0,01	0,99	0,01	0,98	0,01	1,01	0,01	0,98	0,01	1,01	0,01
Tálamo	1,00	0,01	0,70	0,09*	0,99	0,01	0,97	0,01	1,00	0,01	0,98	0,02	0,99	0,01	0,93	0,05
Portal da Hipófise	0,01	0,00	0,01	0,00	0,01	0,00	0,01	0,00	0,01	0,00	0,01	0,00	0,01	0,00	0,01	0,00
Fluxo Sanguíneo Cerebelo	0,02	0,00	0,02	0,00	0,02	0,00	0,02	0,00	0,02	0,00	0,02	0,00	0,02	0,00	0,02	0,00
Canal Central-Periaquedutal	0,02	0,00	0,02	0,00	0,02	0,00	0,02	0,00	0,02	0,00	0,02	0,00	0,02	0,00	0,02	0,00
Ponte	0,01	0,00	0,02	0,00	0,01	0,00	0,01	0,00	0,01	0,00	0,01	0,00	0,01	0,00	0,02	0,00
Septo	0,02	0,00	0,02	0,00	0,02	0,00	0,02	0,00	0,02	0,00	0,02	0,00	0,02	0,00	0,02	0,00
Medula	0,01	0,00	0,02	0,00	0,01	0,00	0,01	0,00	0,01	0,00	0,01	0,00	0,01	0,00	0,02	0,00

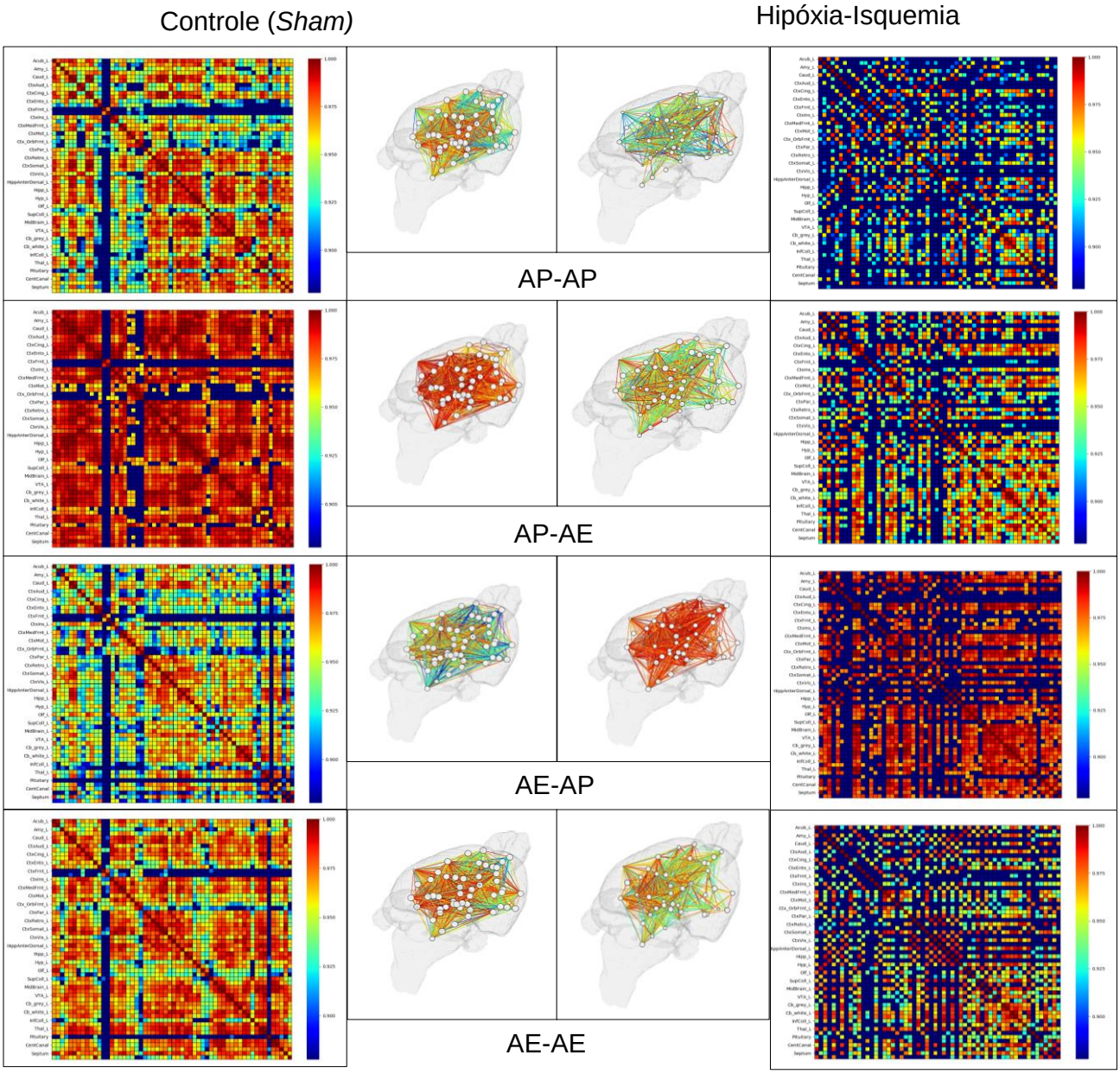


Figura 11: Alterações no metabolismo encefálico. Representação da rede metabólica dos grupos experimentais por meio de matrizes de correlação entre as regiões cerebrais (mencionadas na tabela 2) e redes metabólicas sobrepostas no cérebro 3D (imagem central).

Resultados referentes à proliferação celulares, assim como a avaliação de possíveis associações entre parâmetros histológicos, comportamentais e o nível de captação de glicose, serão inseridos no conjunto de dados iniciais apresentados neste capítulo.

5. Discussão

A Hipóxia-isquemia neonatal é uma das principais causas de dano ao SNC em desenvolvimento, levando a comprometimentos funcionais a curto e a longo prazo (Volpe, 2009a). O aumento significativo na incidência da HI sob condições de prematuridade, reforça a importância de pesquisas focadas na compreensão da fisiopatologia da HI e das consequências da lesão encefálica no recém-nascido (Blencowe et al., 2012).

Ao longo do ciclo vital há uma estreita relação entre as experiências físicas, sociais e ambientais dos indivíduos e a plasticidade neuronal. Contudo, essa relação é mais evidente durante os primeiros períodos do neurodesenvolvimento, como o período gestacional e pós-natal precoce (lactação), devido à presença de um maior número de processos relacionados à maturação, à organização e ao rearranjo do SNC aos níveis estrutural e funcional (Sale, 2018). As alterações aí observadas são regidas por fatores intracelulares e extracelulares responsáveis por modular respostas metabólicas e celulares complexas, que podem estar associadas à plasticidade dependente da experiência e/ou à resiliência endógena, o que facilita processos de reparo após condições patológicas como a HI neonatal (Diamond, 2001; Katušić, 2011; Dennis et al., 2013; Hassell et al., 2015).

De maneira sucinta, nesta Tese demostramos que o enriquecimento ambiental durante a gestação e a lactação impactam de forma distinta, porém decisiva, os efeitos comportamentais, moleculares, metabólicas e histológicas causados pela hipóxia-isquemia neonatal, realizada no terceiro dia pós-natal. Os animais desprovidos de qualquer tipo de estimulação (HI_AP-AP) apresentaram maior prejuízo na função motora e cognitiva, na morte celular e na expressão de proteínas relacionadas à plasticidade sináptica, bem como no hipometabolismo

e a perda tecidual; todos estes efeitos foram prevenidos/revertidos total ou parcialmente pela exposição ao ambiente enriquecido.

Padronização da severidade da lesão

Resultados experimentais usando HI no DPN 3 (período utilizado para mimetizar a lesão no neonato humano nascido prematuramente), evidenciaram prejuízos na função cognitiva dos animais, porém não foram observados déficits sensoriomotores, apesar de desordens do movimento serem frequentemente descritas em crianças expostas a um evento hipóxico-isquêmico (Volpe, 2005). Com o objetivo de padronizar o grau de lesão responsável por causar déficits cognitivos e motores nos animas, os resultados obtidos deram origem ao primeiro trabalho desta Tese (Capítulo 1).

Verificamos o efeito da exposição a diferentes tempos de hipóxia e demonstramos que períodos superiores a 180 minutos causam uma lesão encefálica de grau moderado a severo, responsável pela assimetria no uso das patas anteriores, pela redução na qualidade do movimento da pata ipsilateral e contralateral à lesão e pela perda tecidual em estruturas associadas com o controle motor, como o estriado. O estriado é uma importante estrutura cerebral responsável por modular a resposta de outras áreas motoras, como o córtex (Jamon, 2006); assim, modificações na conectividade neural dessas estruturas após a lesão podem causar desordens do movimento secundarias à lesão (Kolb and Gibb, 2007; Clowry et al., 2014).

A função motora requer tanto um adequado controle neural como a participação integrada do sistema musculoesquelético para um melhor desempenho em diferentes entornos (Jamon, 2006). Neste trabalho foi

observado uma redução na área de seção transversal dos músculo bíceps e tibial anterior, descrevendo pela primeira vez uma característica fisiopatológica do modelo da HI relacionada a parâmetros motores. A alteração morfológica do musculo esquelético e a presença de prejuízos na função motora podem estar relacionadas a algum grau de desnervação, desacoplamento na transmissão sináptica, remodelação da fibra muscular ou atrofia por desuso (Edgerton et al., 2002; Mohagheghi et al., 2007; English et al., 2010; Scherbakov et al., 2013). Além disso, pode indicar mecanismos compensatórios da hipóxia no metabolismo tecidual periférico, ao promover uma redistruibuição do fluxo sanguíneo e aumentar o suporte metabólico cerebral, assim como respostas adaptativas para proteger a viabilidade celular e a função contrátil (Clanton and Klawitter, 2001; Gunn and Bennet, 2009).

Os resultados deste trabalho destacam a necessidade da exposição a períodos mais prolongados de hipóxia, em animais submetidos à HI no terceiro dia pós-natal para promover déficits comparáveis àqueles observados nos animais quando o modelo é realizado no sétimo dia pós-natal e mimetizar alguns dos desfechos observados em infantes nascidos prematuramente. Consequentemente, o período de 180 minutos de hipóxia foi utilizado nos experimentos seguintes, com o objetivo de avaliar os efeitos do enriquecimento ambiental no período gestacional e lactacional sobre as consequências de HI por meio da avaliação de parâmetros comportamentais, moleculares, metabólicos e histológicos.

O ambiente enriquecido precoce favorece a recuperação funcional dos animais submetidos à HI

Os testes comportamentais envolvendo tarefas sensório-motoras e cognitivas são uma importante ferramenta para quantificar os danos causados ao SNC pela HI (Van De Looij et al., 2011). O ambiente enriquecido durante a gestação e a lactação foi capaz de prevenir/reverter o atraso no neurodesenvolvimento causado pela lesão, sendo a atividade reflexa um indicador de maturação do SNC. Os animais expostos ao ambiente enriquecido apresentaram uma melhora nos testes de endireitamento, geotaxia negativa e discriminação olfatória nos DPN 7 e 14 (Capítulo 2). Em concordância com a literatura, não foram observadas diferenças significativas na atividade reflexa dos animais em períodos posteriores do neurodesenvolvimento (DPN 21). Esse resultado pode indicar algum grau de recuperação funcional ou maior atividade física voluntária (Lubics et al., 2005).

O labirinto aquático de Morris é um dos testes mais empregados para a avaliação da função cognitiva em roedores (Vorhees and Williams, 2006). Os déficits induzidos pela HI, consistentemente descritos na literatura (Sanches et al., 2013b, 2015; Alexander et al., 2014), foram atenuados pelo protocolo de AE proposto. De fato, os animais estimulados em ambos os períodos do desenvolvimento e submetidos a HI apresentaram um desempenho semelhante ao do grupo controle e passaram mais tempo no quadrante alvo, durante o teste, indicando uma melhora tanto no aprendizado como na memória espacial (Capítulos 2,4).

O teste do campo aberto é utilizado rotineiramente para analisar atividade espontânea de exploração dos animais em um ambiente novo (Iuvone et al., 1996). Embora existam dados conflitantes sobre o prejuízo causado pela HI no desempenho da tarefa, em termos gerais a HI é reconhecida por causar aumento nas respostas de exploração do aparato (Arteni et al., 2003, 2010; Stadlin et al., 2003; Lubics et al., 2005; Tai et al., 2009). Contudo, foi observado que o AE, especialmente durante a lactação, atenua o aumento na locomoção associado à HI neonatal. Além disso, os resultados desse trabalho indicam que a exposição ao AE em ambos os períodos estimula um comportamento mais natural nos animais, aumentando a exploração em áreas protegidas e reduzindo a impulsividade (Capítulo 4).

De maneira interessante, o AE durante o período gestacional não se mostrou efetivo para prevenir a assimetria de uso dos membros anteriores ou para prevenir a redução na qualidade do passo durante a marcha, quando avaliados no teste do cilindro e no teste de escada horizontal, respectivamente. No entanto, esses déficits sensório-motores foram atenuados pelo AE durante a lactação (Capítulo 3), sendo relacionados a um aumento na exploração do ambiente e a uma maior interação com os estímulos (Sale et al., 2014), o que poderia levar a um maior uso dos membros e consequentemente otimizar processos de iniciação do movimento e de controle motor, visto que que os circuitos cerebrais relacionados são estabelecidos após o nascimento (Piek et al., 2008).

Experiências enriquecidas reduzem a morte celular após a HI

A exposição ao AE antes (pré-natal) ou após (lactacional) o insulto neonatal causa modificações celulares que podem embasar as respostas comportamentais positivas observadas e que não estão diretamente relacionadas a alterações com o cuidado maternal, como descrito no Capítulo 2. A fase aguda da lesão caracteriza-se por um aumento da morte celular durante as primeiras horas após a HI, mediada por mecanismos dependentes e independentes de caspase 3 (Towfighi et al., 1997; Koike et al., 2008). Proteínas quinases como a AKT são responsáveis por reduzir a perda celular, por meio da regulação negativa de respostas pró-apoptóticas (Kim et al., 2001). A avaliação de marcadores relacionados à morte e à sobrevivência celular 48 horas após a HI mostrou que os animais expostos ao AE tinham maiores níveis de AKT fosforilada (isoforma ativa) e uma menor expressão de caspase-3 clivada e PARP-1 no hipocampo e córtex parietal. Estes dados indicaram que o enriquecimento ambiental durante a gestação e os primeiros dias pós-natais (PND 1 – PND 5) foi capaz de reduzir o dano celular dos animais (Capítulo 2).

O ambiente enriquecido durante a gestação e a lactação favorece a neuroproteção endógena

Fatores de crescimento e fatores neurotróficos promovem um aumento na sobrevivência celular, aprimoram processos de maturação, potencializam circuitos/redes cerebrais, induzem plasticidade sináptica e melhoram o desempenho funcional de roedores expostos ao AE (Zhang et al., 2000; Reichardt, 2006; Rojas et al., 2015). Neste trabalho, a expressão de IGF-1 e VEGF no hipocampo não revelou diferenças significativas quando avaliadas 48

horas após a lesão. No córtex parietal dos animais submetidos a HI foi observada uma redução na expressão dos fatores acima mencionados, independentemente da condição de moradia (Capítulo 2). A redução nos níveis de IGF-1 pode estar relacionada a processos inflamatórios, onde essa diminuição é favorecida pelas proteínas de ligação (Zhao et al., 2006). Por sua vez, a diminuição nos níveis de VEGF pode indicar mecanismos compensatórios a fim de evitar um maior dano à BHE, e consequente exacerbação da perfusão microvascular e indução de maior perda celular (Zhang et al., 2000).

Na idade adulta, foi observada uma elevação na expressão do IGF-1 no hipocampo ipsilateral à lesão dos animais submetidos a HI, sugerindo uma resposta compensatória, a fim de favorecer os processos de recuperação pós-lesão (Wadowska et al., 2015). Por sua vez, um aumento nos níveis de VEGF foi observado nos animais expostos ao ambiente enriquecido, mostrando uma correlação negativa com o labirinto aquático de Morris (Capítulo 4), no qual altos níveis de VEGF foram associados com um melhor desempenho cognitivo (i.e. menor latência para achar a plataforma). O VEGF tem sido descrito como um agente terapêutico promissor frente a lesões do SNC por participar de processos de memória dependentes do hipocampo, ao aumentar a viabilidade celular e a plasticidade neuronal (Plaschke et al., 2008; Ortuzar et al., 2013). Assim, uma regulação positiva de ambos fatores de crescimento poderia ser responsável pelos efeitos positivos do AE durante o período gestacional e lactacional.

O receptor TrkB é conhecido pela alta afinidade por BDNF; sua expressão começa durante o período embrionário e é encontrado na maioria dos neurônios do SNC (Klein et al., 1991). Sua interação com o BDNF é importante na plasticidade dependente de atividade e na proteção contra danos

cerebrais (Volpe, 2001; Carmeliet, 2003). No DPN 5 (48h após a HI) não foram identificadas diferenças significativas nos níveis do BDNF nem de TrkB em hipocampo e córtex parietal (Capítulo 2). Estudos prévios avaliando o BDNF no período pós-natal precoce têm relatado achados semelhantes aos deste trabalho, sendo identificadas diferenças significativas após a segunda semana pós-natal (Cancedda et al., 2004; Caporali et al., 2014), sugerindo que a interação dos animais com o ambiente é um fator relevante para aumentar a expressão desse fator neurotrófico. Na idade adulta, foi observado um aumento nos níveis de TrKB nos grupos expostos ao AE durante a gestação e a lactação sem diferenças significativas no BDNF (Capítulo 4). Esses achados poderiam estar em concordância com os de Bengoetxea e colaboradores (2017), que relataram que animais expostos ao AE apresentam reversão nos déficits cognitivos, mediada pelo aumento na expressão do TrkB, facilitando a subsequente ligação do BDNF com seu receptor (Bengoetxea et al., 2017).

Os astrócitos apresentam respostas diferentes após HI em animais expostos ao ambiente enriquecido

Outra mudança celular induzida pelo AE durante a gestação e a lactação que pode ser responsável por minimizar os efeitos deletérios do HI neonatal, é a expressão dos astrócitos. A GFAP é um biomarcador padrão das células astrocitárias; elas têm um papel crítico na maturação e função do encéfalo em desenvolvimento. Em resposta a diferentes graus de lesão, os astrócitos apresentam uma alta suscetibilidade, sendo as primeiras células a responderem a insultos cerebrais (Burda and Sofroniew, 2014), além de aumentar a proliferação e a interação de várias células, favorecem a formação da cicatriz glial, que age como uma barreira mecânica à função celular neural (Sofroniew,

2009). Os resultados apresentados nesta Tese, sugerem que o AE modifica a resposta astrocitária de acordo ao estágio da lesão, que pode estar associado a uma função dupla dos astrócitos. Como mostrado no Capítulo 2, durante a fase aguda da lesão, o AE foi capaz de reduzir os níveis de GFAP, tanto no hipocampo quanto no córtex parietal, o que pode estar relacionado a um menor dano encefálico após o insulto nos animais expostos ao enriquecimento, fato que demandaria uma menor reatividade dos astrócitos. No entanto, na idade adulta foi observado um aumento na reatividade astrocitária nos animais expostos a AE durante o período gestacional ou lactacional, mostrando também uma correlação positiva com os níveis de VEGF (Capítulo 3). Esse achado encontra-se em concordância com dados recentes da literatura, onde diversos autores têm demostrado que o AE aumenta a proliferação dos astrocitos, os quais contribuem para a remodelação da atividade neural e das sinapses na área da lesão, favorecendo dessa forma a fase de reparo (Keiner et al., 2008; Miller et al., 2017).

O ambiente enriquecido atenua mecanismos associados à plasticidade após HI neonatal

Estudos em modelos de hipóxia/isquemia têm demonstrado que, em lesões ao SNC, processos associados a neurogênese e sinaptogênese podem favorecer a formação de conexões sinápticas inadequadas (denominada plasticidade aberrante), responsáveis por interferir na interação de células neurais e não-neurais em regiões cerebrais como o hipocampo, o que contribui para os prejuízos na função cognitiva dos animais (Zhu et al., 2005; Lichtenwalner and Parent, 2006; Hu et al., 2017). Além disso, tem sido salientado que experiências enriquecidas nem sempre desencadeiam

modificações na plasticidade sináptica (Komitova et al., 2013; Meireles et al., 2017), o que poderia estar relacionado a mecanismos compensatórios a fim de reduzir modificações aberrantes nos diversos circuitos neuronais, atenuar o dano encefálico e reduzir os déficits funcionais.

Como mencionado no Capítulo 4 da presente Tese, houve um aumento na expressão da sinaptofisina (biomarcador pré-sináptico) nos animais HI mantidos em condições padrão em ambos os períodos do neurodesenvolvimento (HI_AP-AP), sem alterações nos animais expostos ao ambiente enriquecido, fato que poderia estar relacionado a efeitos compensatórios e/ou neuroprotetores como descrito anteriormente. Além disso, não foram observadas diferenças significativas no número de neurônios na região CA1 do hipocampo. Meshi e colaboradores (2006) sugeriram que os efeitos benéficos do enriquecimento não são dependentes da neurogênese (Meshi et al., 2006); deste modo, é possível sugerir que os efeitos benéficos observados em parâmetros comportamentais nos animais expostos ao AE, podem ser consequência da otimização de processos que auxiliam na modelagem das conexões cerebrais, como a poda sináptica e o fortalecimento de conectividades sinápticas (Nithianantharajah and Hannan, 2006; Rocha-Ferreira et al., 2016).

***O enriquecimento ambiental durante a gestação e a lactação tem efeito parcial sobre o hipometabolismo encefálico* induzido pela HI**

No presente estudo, os animais foram avaliados usando escaneamento por microtomografia por emissão de pósitrons na idade adulta. Já é conhecido que a HI ocasiona um hipometabolismo cerebral de glicose que apresenta uma estreita relação com a severidade da lesão (Thorngren-Jerneck et al., 2001;

Morken et al., 2014). Os resultados desta Tese também mostraram uma redução na capatação do radiofármaco 18F-FDG nos animais HI_AP-AP em várias regiões encefálicas, como o hipocampo, o córtex, o estriado, o tálamo, a amigdala, entre outros (Tabela 2 - Capítulo 5). Essas regiões estão relacionadas com a fisiopatologia da HI e têm importância para as funções cognitivas e motoras e para o comportamento emocional dos animais. No presente trabalho foi observado que o AE durante a gestação e a lactação foi capaz de prevenir/reverter as alterações no metabolismo encefálico. Esta resposta metabólica pode estar associada à diferenciação no uso dos substratos energéticos no período neonatal, assim como ao grau de maturação dos receptores glutamatérgicos e dos transportadores de glicose, o que pode determinar o padrão de lesão após HI (Brekke et al., 2017). Até onde se sabe, não há estudos sobre a influência do AE no metabolismo encefálico de animais submetidos à HI, especialmente quando a estimulação ambiental é fornecida em períodos críticos do neurodesenvolvimento. São necessários estudos longitudinais focados em avaliar as alterações no metabolismo encefálico *in-vivo*, nos animais expostos ao AE para estabelecer possíveis associações com parâmetros morfológicos e comportamentais.

O metabolismo da glicose cerebral analisado por µPET é uma medida estática amplamente utilizada. No entanto, baseado no princípio de que existe acoplamento entre a atividade cerebral e o metabolismo da glicose e que isto permite a identificação de conexões entre as diversas regiões cerebrais, o valor de captação de glicose usando 18F-FDG também pode ser usado para a identificação de redes metabólicas (Zimmer et al., 2017). Como descrito no Capítulo 5, foi observada uma diminuição na sincronia da rede metabólica nos

animais submetidos à HI quando comparados a seus respectivos controles (*sham)*. Embora haja persistência do efeito da lesão, os animais expostos ao AE apresentaram proteção parcial, quando comparados ao grupo HI_AP-AP, indicando o efeito do estimulação ambiental, minimizando as alterações no acoplamento entre regiões cerebrais. Estes achados iniciais sobre o efeito do AE no metabolismo da glicose, precisam ser confirmados e aprofundado para determinar as caraterísticas das conexões e redes metabólicas na fisiopatologia da HI neonatal e para precisar a efetividade de estratégias preventivas e/ou terapêuticas.

Alterações no volume encefálico após a HI neonatal são atenuadas pelo ambiente enriquecido precoce

Modificações celulares induzidas pelo AE expressam a capacidade intrínseca do SNC de compensar danos estruturais e facilitar a recuperação funcional pós-lesão. A HI neonatal no DPN 3 causou reduções significativas no volume do hipocampo, do córtex, do estriado e do corpo caloso ipsilateral à lesão nos animais não estimulados (HI_AP-AP) (Capítulos 1,2 e 4). Essa perda tecidual foi semelhante à mencionada em relatos prévios da literatura e está associada aos prejuízos comportamentais causados pela HI (Sanches et al., 2013a, 2013b; Alexander et al., 2014). No entanto, o enriquecimento ambiental no período gestacional e a lactacional foi capaz de preservar um maior volume de tecido encefálico, apesar da vulnerabilidade dessas regiões à HI (Capítulo 2 e 4). Isso facilita que mecanismos compensatórios não fiquem limitados à otimização dos circuitos neuronais nas regiões cerebrais não lesadas e permite a reorganização celular do tecido cerebral acometido pela lesão (Grefkes and Ward, 2014). Contudo, os achados histológicos relacionando AE e HI ainda são

controversos, visto que alguns autores reportam proteção parcial ou inexistente (Pereira et al., 2007, 2008, Rojas et al., 2013, 2015; Griva et al., 2017); por tanto as diferenças podem estar relacionadas com o período de início do enriquecimento e precisam ser melhor esclarecidas.

Animais expostos ao ambiente enriquecido apresentam respostas sexo-especificas associadas à HI

Confirmando a hipótese desta Tese, os efeitos do enriquecimento ambiental sobre o desempenho comportamental, as respostas moleculares e o dano tecidual apresentaram respostas sexo-especificas (Capítulo 4). Os machos do grupo HI_AP-AP exibiram uma maior perda tecidual comparado às fêmeas e aos machos HI expostos ao AE em ambos os períodos do neurodesenvolvimento. Esses resultados ratificam a preservação tecidual induzida pelo o enriquecimento, bem como a vulnerabilidade sexo-específica para a lesão cerebral neonatal (Mayoral et al., 2009), e que pode estar relacionada a diferenças na expressão e atividade mitocondrial e à sua participação nas diferentes vias de morte celular (Zhu et al., 2006).

O maior dano tecidual observado nos machos HI_AP-AP poderia explicar também as diferenças entre sexos na expressão de biomarcadores como sinaptofisina, GFAP e IGF-1. Neste trabalho foi observado aumento na expressão da sinaptofisina nos machos HI_AP-AP. Embora estudos prévios indiquem que os machos requeiram mais remodelagem sináptica devido ao maior número de sinapses em comparação as fêmeas, alterações nos processos de poda sináptica podem levar à persistência de níveis elevados de sinaptofisina inclusive na idade adulta (Andersen and Teicher, 2008; Tang et al., 2014). O

aumento na expressão do IGF-1 nos machos HI, independentemente das condições de moradia, sugere uma resposta à lesão cerebral neonatal ou mecanismos moduladores que levam a uma recuperação funcional associada à relação entre o papel protetor dos fatores de crescimento e o dimorfismo sexual na HI neonatal. Na resposta astrocitária observamos que uma única exposição ao AE durante o período gestacional ou lactacional, aumenta os níveis de GFAP nos animais submetidos à HI, o que pode indicar modulações distintas entre machos e fêmeas baseadas na experiências, onde podem estar implicados processos relacionado à maturação do tecido nervoso, à expressão das células gliais no início do período pós-natal e à influência hormonal (Catalani et al., 2002; Chanana et al., 2016).

O espectro comportamental nos domínios cognitivo, sensorial, motor e social é resultado de processos dinâmicos e complexos que podem ser modificados tanto por fatores intrínsecos quanto extrínsecos, como o sexo, experiências prévias ou insultos cerebrais (Cahill and Aswad, 2015). Embora animais de ambos os sexos apresentem respostas positivas quanto ao AE na atividade locomotora e no comportamento associado a ansiedade e/o impulsividade, os machos expostos ao AE durante a gestação ou a lactação apresentaram menor latência para achar a plataforma e passaram mais tempo no quadrante alvo, durante teste de labirinto aquático de Morris, enquanto a melhora nas habilidades de memória espacial foram observadas nas fêmeas alocadas no AE durante a lactação. Esses dados estão de acordo com relatos anteriores mostrando o dimorfismo sexual exerce papel determinante em testes de memória e em respostas específicas a estímulos ambientais (Keeley et al., 2013; Smith et al., 2014). Apesar das diferenças mencionadas acima, as

respostas sexo-específicas induzidas pela HI neonatal e o AE ainda são conflitantes na literatura devido às discrepâncias nos protocolos utilizados e especialmente ao reduzido número de estudos avaliando o dimorfismo sexual em animais muito imaturos, assim como os efeitos de experiências enriquecidas em períodos críticos do neurodesenvolvimento (Netto et al., 2017, 2018).

A novidade e a periodização dos estímulos podem ser fatores chave para os efeitos do ambiente enriquecido

Na prática clínica, os neonatos acometidos com lesões encefálicas frequentemente são encaminhados de maneira precoce a programas de estimulação sensorial como alternativa terapêutica, a fim de aprimorar o prognóstico funcional a longo prazo das crianças. Nesta Tese (Capítulo 4), foram avaliados o efeito isolado do ambiente enriquecido gestacional, lactacional e a combinação de ambos os períodos, com o objetivo de investigar o papel preventivo e/ou protetor das experiências enriquecidas, além de avaliar se estímulos prolongados poderiam tornar o cérebro mais resiliente contra um evento hipóxico–isquêmico. Os resultados evidenciaram o efeito benéfico do AE tanto no período gestacional como lactacional, porém não foi observada uma maior proteção (soma dos efeitos) quando os animais eram mantidos em condições enriquecidas desde a gestação até o desmame. De acordo com os achados deste trabalho, efeitos não aditivos têm sido relatados em animais expostos ao AE por longos períodos (Koo et al., 2003). Alguns autores têm proposto que a redução ou periodização dos estímulos permite uma melhor ativação dos circuitos neuronais, evitando uma atenuação nas respostas dependentes da experiência devido a uma adaptação aos estímulos, decorrente de presença continua dos mesmos (Kempermann and Gage, 1999). Nesse

sentido, propomos que a exposição ao AE requer alternâncias entre estímulos e períodos de repouso, a fim de permitir uma reorganização das redes neuronais após a exposição a um ambiente novo e desafiador. Sem dúvida, mais estudos são necessários para confirmar essa suposição, o que poderia ser relevante em programas de reabilitação neurológica focados em prematuridade e/ou insultos cerebrais neonatais.

As experiências ambientais são um fator determinante no prognostico funcional após HI neonatal

A Figura 12 apresenta, de maneira sucinta, os efeitos descrito na literatura (incluindo os dados referente a presente Tese) sobre o ambiente enriquecido em animais submetidos a eventos hipóxico-isquêmicos. A imagem destaca o potencial preventivo e terapêutico de experiências enriquecidas em diversos períodos do neurodesenvolvimento como o período pré-natal, lactacional e adolescência/idade ao bloquear os principais eventos celulares induzidos pela HI e potencializar mecanismos responsáveis por favorecer a recuperação após a o insulto neonatal.

A proteção induzida pelo AE no modelo de HI está relacionada com a diminuição do influxo de cálcio no interior da célula (Iuvone et al., 1996; Komitova et al., 2013) e a preservação da atividade da Na^+ / K^+ ATPase no hemisfério contralateral à lesão (Rojas et al., 2015), causando uma redução do dano por excitotoxicidade glutamatérgica. A HI desencadeia diferentes tipos de morte celular, dependendo da gravidade do insulto inicial. No modelo de HI neonatal, o AE reduz os níveis de marcadores apoptóticos, utilizados como indicadores de maior preservação celular 48h após a lesão (Durán-Carabali et al., 2017). O

estresse oxidativo é outro mecanismo fisiopatológico associado na HI neonatal, porém a exposição do AE no período juvenil melhorou a atividade anti-oxidante, embora não haja uma redução na formação de espécies reativas de oxigênio (Pereira et al., 2009; Rojas et al., 2015), o que pode estar relacionado à imaturidade do sistema antioxidante (Morgane et al., 2002). A manutenção da BHE é outro dos mecanismos pelos quais o AE diminui os processos inflamatórios, já que a BHE previne a entrada de células periféricas no tecido encefálico (Diaz et al., 2016) e a sua integridade reduz a exacerbação na reatividade das células gliais após o insulto encefálico (Diaz et al., 2016; Durán-Carabali et al., 2017).

No esquema geral sobre os efeitos do AE (Figura 12), foi usado o termo reabilitação para indicar as alterações celulares e anatômicas induzidas pelo AE e que podem ajudar a entender algumas das respostas positivas observada nos roedores expostos ao enriquecimento. Entre as modificações morfológicas têm sido descrito maior proliferação celular (Salmaso et al., 2012), aumento no número de espinhos dendríticos (Rojas et al., 2013) bem como potenciação de longo prazo (Akers et al., 2006). Além disso, o AE favorece a reatividade astrocitária (Durán-Carabali et al., 2019) e aumenta os níveis de fatores de crescimento (Seo et al., 2013b; Griva et al., 2017; Durán-Carabali et al., 2019). Cabe salientar que características intrínsecas e extrínsecas dos animais, tais como, idade, sexo, tratamentos concomitantes, período de início, intensidade e duração da estimulação, são responsáveis por modular a resposta do AE e pelo prognostico funcional a longo e curto prazo dos sujeitos acometidos pela HI neonatal e devem ser levados em conta durante a interpretação e comparação dos resultados.

O tópico proposto nesta Tese traz novas e importantes questões sobre a influência ambiental precoce no prognóstico a curto e longo prazo do recém-nascido exposto a eventos cerebrais adversos como a hipóxia isquemia. As condições ambientais podem ser um aspecto crítico na função do SNC e têm sido negligenciadas em unidades hospitalares, programas comunitários e em políticas públicas de saúde (Mering and Jolkkonen, 2015). Assim como em modelos animais, o ambiente natural para os seres humanos pode ser enriquecido, no entanto, o ambiente de hospitais e outras instituições de cuidados têm sido muitas vezes considerados empobrecidos (McDonald et al., 2018). Atualmente, existe um esforço global para minimizar o impacto negativo dos insultos cerebrais neonatais na população (McKenna et al., 2015). Mais estudos pré-clínicos e clínicos são necessários para entender os mecanismos pelos quais o AE induz efeitos benéficos. Porém é relevante que o conhecimento atualmente disponível seja considerado no desenvolvimento de um novo enfoque terapêutico e no desenvolvimento de estratégias de saúde pública.

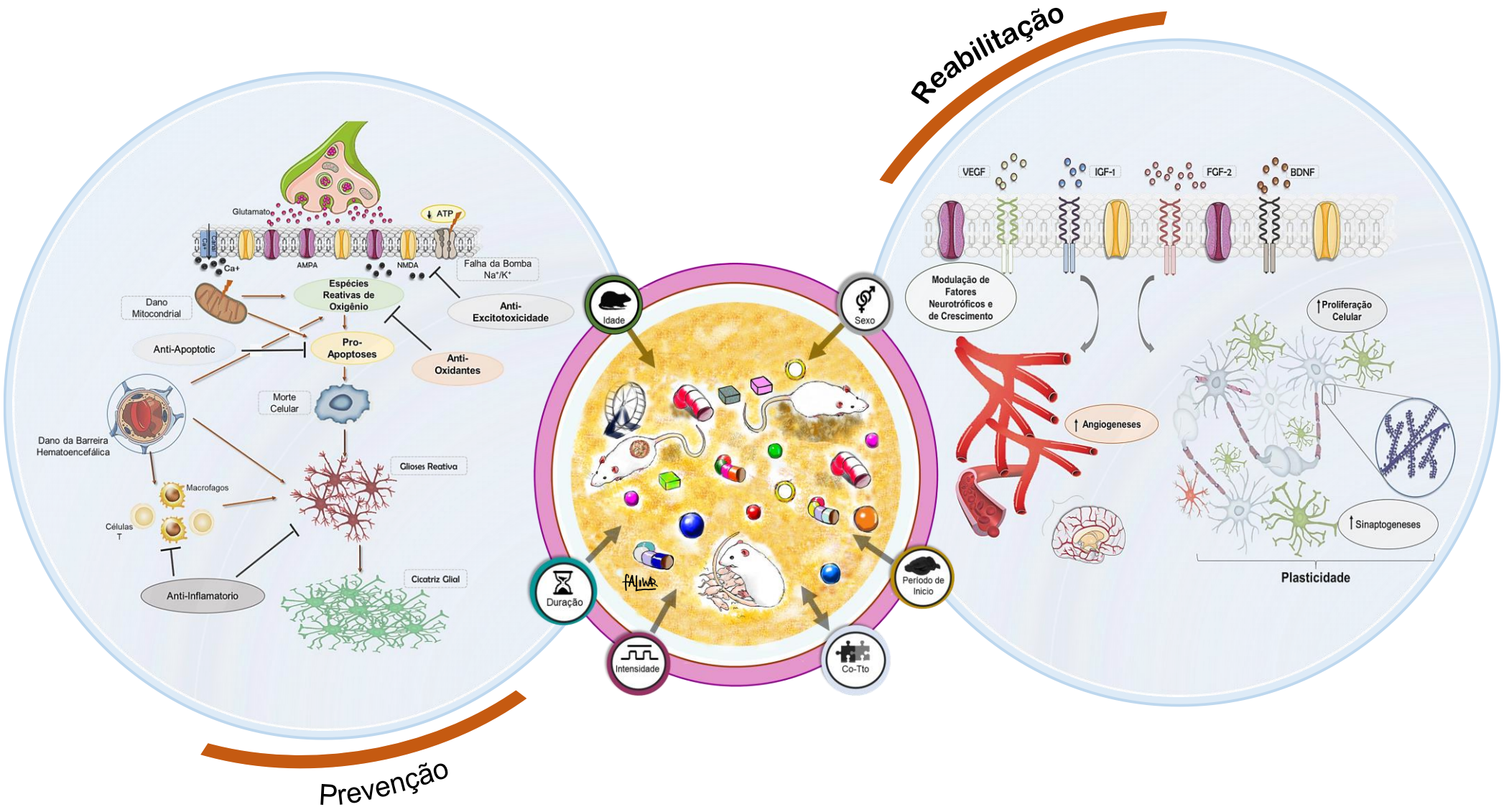


Figura 12: Esquema geral sobre o potencial preventivo e reabilitador do AE na HI neonatal (Grupo de isquemia da UFRGS. Dados não publicados)

6. Conclusões e Perspectivas

6.1 Conclusões

Os resultados obtidos permitem concluir que:

- A exposição a períodos prolongados de hipóxia (iguais ou superior a 180 minutos) causa uma lesão encefálica entre moderada-severa responsável por prejuízos na função sensório-motora dos animais e na morfologia de estruturas relacionadas ao controle motor como o estriado e tecido musculoesquelético, em animais submetidos ao modelo de HI no DPN 3;

- O ambiente enriquecido durante o período gestacional e lactacional reduz a morte celular por apoptose e a reatividade astrocitária, através da regulação positiva da via da AKT. Além disso, atenuou a perda tecidual, o atraso no neurodesenvolvimento e os déficits cognitivos dos animais;

- O ambiente enriquecido em períodos críticos do neurodesenvolvimento, mostrou efeitos positivos na redução dos prejuízos motores em relação a força, a simetria dos membros e a qualidade do movimento, salientando que a exposição a ambientes complexos durante a lactação é mais eficaz para induzir recuperação funcional após HI;

- O AE durante a gestação e lactação causam resultados semelhantes na atenuação do comprometimento na memória espacial, atividade motora e comportamento do tipo ansioso, bem como na preservação tecidual, especialmente no hipocampo. Essas respostas parecem ser mediadas por um aumento nos níveis de VEGF e TrkB, em ambos sexos, e aumento

da reatividade astrocitária nos machos;

- Observou-se uma resposta sexo-especifica, na qual os ratos machos exibiram efeitos positivos à exposição ao AE nos dois períodos estudados, enquanto as fêmeas mostraram ser mais beneficiadas pelo enriquecimento durante a lactação.

- Resultados preliminares indicam que a HI neonatal causou uma redução no metabolismo encefálico dos animais, além de uma hiposincronicidade na rede metabólica. Ambos efeitos foram prevenidos e/ou atenuados pelo AE.

6.2 Perspectivas

- Caracterizar longitudinalmente o metabolismo encefálico *in vivo* dos animais, por meio da técnica de microtomografia por emissão de pósitrons (μPET/CT), com uso do radiofármaco 18F- fluordesoxiglicose (18F-FDG) para quantificar o uso de glicose durante o período pós-natal precoce nos ratos submetidos a HI;

- Avaliar a resposta sináptica excitatória na região CA1 do hipocampo por meio da avaliação das correntes pós-sinápticas excitatórias espontâneas e de miniatura fazendo uso da técnica de *whole-cell patch clamp* (modo *voltage-clamp*).

7. Referências

Abajobir AA et al. (2017) Global, regional, and national disability-adjusted life-years (DALYs) for 333 diseases and injuries and healthy life expectancy (HALE) for 195 countries and territories, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet.

ACOG (2013) Definition of term pregnancy. Obs Gynecol.

Adriani W, Giannakopoulou D, Bokulic Z, Jernej B, Alleva E, Laviola G (2006) Response to novelty, social and self-control behaviors, in rats exposed to neonatal anoxia: Modulatory effects of an enriched environment. Psychopharmacology (Berl) 184:155–165.

Akers KG, Nakazawa M, Romeo RD, Connor JA, McEwen BS, Tang AC (2006) Early life modulators and predictors of adult synaptic plasticity. Eur J Neurosci 24:547–554.

Alexander M, Garbus H, Smith AL, Rosenkrantz TS, Fitch RH (2014) Behavioral and histological outcomes following neonatal HI injury in a preterm (P3) and term (P7) rodent model. Behav Brain Res 259:85–96 Available at: http://dx.doi.org/10.1016/j.bbr.2013.10.038.

Alwis DS, Rajan R (2014) Environmental enrichment and the sensory brain: the role of enrichment in remediating brain injury. Front Syst Neurosci 8:1–20 Available at: http://journal.frontiersin.org/article/10.3389/fnsys.2014.00156/abstract.

Anderova M, Vorisek I, Pivonkova H, Benesova J, Vargova L, Cicanic M, Chvatal A, Sykova E (2011) Cell death/proliferation and alterations in glial morphology contribute to changes in diffusivity in the rat hippocampus after hypoxia-ischemia. J Cereb Blood Flow Metab 31:894–907 Available at:

http://dx.doi.org/10.1038/jcbfm.2010.168.

Andersen SL, Teicher MH (2008) Stress, sensitive periods and maturational events in adolescent depression. Trends Neurosci 31:183–191.

Angelidou A, Asadi S, Alysandratos K-D, Karagkouni A, Kourembanas S, Theoharides TC (2012) Perinatal stress, brain inflammation and risk of autism-Review and proposal. BMC Pediatr 12:89 Available at: http://www.biomedcentral.com/1471-2431/12/89.

Arcego DM, Krolow R, Lampert C, Toniazzo AP, Berlitz C, Lazzaretti C, Schmitz F, Rodrigues AF, Wyse ATS, Dalmaz C (2016) Early life adversities or high fat diet intake reduce cognitive function and alter BDNF signaling in adult rats: Interplay of these factors changes these effects. Int J Dev Neurosci 50:16–25 Available at: http://dx.doi.org/10.1016/j.ijdevneu.2016.03.001.

Arteni NS, Pereira LO, Rodrigues AL, Lavinsky D, Achaval ME, Netto CA (2010) Lateralized and sex-dependent behavioral and morphological effects of unilateral neonatal cerebral hypoxia-ischemia in the rat. Behav Brain Res 210:92–98 Available at: http://dx.doi.org/10.1016/j.bbr.2010.02.015.

Arteni NS, Salgueiro J, Torres I, Achaval M, Netto CA (2003) Neonatal cerebral hypoxia-ischemia causes lateralized memory impairments in the adult rat. Brain Res 973:171–178.

Back SA (2006) Perinatal white matter injury: The changing spectrum of pathology and emerging insights into pathogenetic mechanisms. Ment Retard Dev Disabil Res Rev.

Back S a, Luo NL, Borenstein NS, Levine JM, Volpe JJ, Kinney HC (2001) Late

oligodendrocyte progenitors coincide with the developmental window of vulnerability for human perinatal white matter injury. J Neurosci 21:1302–1312.

Back SA, Han BH, Luo NL, Chricton CA, Xanthoudakis S, Tam J, Arvin KL, Holtzman DM (2002) Selective vulnerability of late oligodendrocyte progenitors to hypoxia-ischemia. J Neurosci 22:455–463.

Bale TL (2015) Epigenetic and transgenerational reprogramming of brain development. Nat Rev Neurosci 16.6:1–13 Available at: http://www.nature.com/doifinder/10.1038/nrn3818.

Baptista PPA, Saur L, Bagatini PB, Greggio S, Venturin GT, Vaz SP, Ferreira KDR, Junqueira JS, Lara DR, DaCosta JC, Jeckel CMM, Mestriner RG, Xavier LL (2015) Antidepressant Effects of Ketamine Are Not Related to (18)F-FDG Metabolism or Tyrosine Hydroxylase Immunoreactivity in the Ventral Tegmental Area of Wistar Rats. Neurochem Res.

Barnes LL, Wilson RS, Bienias JL, Schneider JA, Evans DA, Bennett DA (2005) Sex differences in the clinical manifestations of Alzheimer disease pathology. Arch Gen Psychiatry 62:685–691.

Baroncelli L, Braschi C, Spolidoro M, Begenisic T, Sale A, Maffei L (2009) Nurturing brain plasticity : impact of environmental enrichment. Cell Death Differ 17:1092–1103 Available at: http://dx.doi.org/10.1038/cdd.2009.193.

Bengoetxea H, Rico-Barrio I, Ortuzar N, Murueta-Goyena A, Lafuente J V. (2017) Environmental Enrichment Reverses Tyrosine Kinase Inhibitor-Mediated Impairment Through BDNF-TrkB Pathway. Mol Neurobiol:1–17.

Beray-Berthat V, Delifer C, Besson VC, Girgis H, Coqueran B, Plotkine M, Marchand-Leroux C, Margaill I (2010) Long-term histological and behavioural characterisation of a collagenase-induced model of intracerebral haemorrhage in rats. J Neurosci Methods 191:180–190 Available at: http://dx.doi.org/10.1016/j.jneumeth.2010.06.025.

Berger R, Garnier Y (1999) Pathophysiology of perinatal brain damage. Brain Res Brain Res Rev 30:107–134.

Blencowe H, Cousens S, Chou D, Oestergaard M, Say L, Moller A-B, Kinney M, Lawn J (2013) Born too soon: the global epidemiology of 15 million preterm births. Reprod Health 10:1–14 Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3828585&tool=pmcentrez&rendertype=abstract.

Blencowe H, Cousens S, Oestergaard MZ, Chou D, Moller AB, Narwal R, Adler A, Vera Garcia C, Rohde S, Say L, Lawn JE (2012) National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: A systematic analysis and implications. Lancet 379:2162–2172 Available at: http://dx.doi.org/10.1016/S0140-6736(12)60820-4.

Bock J, Rether K, Gröger N, Xie L, Braun K (2014) Perinatal programming of emotional brain circuits : an integrative view from systems to molecules. Front Neurosci 8:1–16.

Brekke E, Berger HR, Widerøe M, Sonnewald U, Morken TS (2017) Glucose and Intermediary Metabolism and Astrocyte–Neuron Interactions Following Neonatal Hypoxia–Ischemia in Rat. Neurochem Res 42:115–132.

Brekke EMF, Morken TS, Widerøe M, Håberg AK, Brubakk AM, Sonnewald U (2014) The pentose phosphate pathway and pyruvate carboxylation after neonatal hypoxic-ischemic brain injury. J Cereb Blood Flow Metab 34:724–734 Available at: http://dx.doi.org/10.1016/j.neuint.2015.02.002.

Burda JE, Sofroniew M V. (2014) Reactive gliosis and the multicellular response to CNS damage and disease. Neuron 81:229–248 Available at: http://dx.doi.org/10.1016/j.neuron.2013.12.034.

Cahill L, Aswad D (2015) NeuroView Sex Influences on the Brain: An Issue Whose Time Has Come. Neuron 88:1084–1085 Available at: http://dx.doi.org/10.1016/j.neuron.2015.11.021.

Cai Z, Lin S, Fan LW, Pang Y, Rhodes PG (2006) Minocycline alleviates hypoxic-ischemic injury to developing oligodendrocytes in the neonatal rat brain. Neuroscience 137:425–435.

Cai Z, Rhodes PG (2001) Intrauterine hypoxia-ischemia alters expression of the NMDA receptor in the young rat brain. Neurochem Res 26:487–495.

Cancedda L, Putignano E, Sale A, Viegi A, Berardi N, Maffei L (2004) Acceleration of visual system development by environmental enrichment. J Neurosci 24:4840–4848.

Caporali P, Cutuli D, Gelfo F, Laricchiuta D, Foti F, De Bartolo P, Mancini L, Angelucci F, Petrosini L (2014) Pre-reproductive maternal enrichment influences offspring developmental trajectories: motor behavior and neurotrophin expression. Front Behav Neurosci 8:195 Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4038762&tool=pmcentrez&rendertype=abstract.

Cárdenas L, García-García F, Santiago-Roque I, Martínez AJ, Coria-Ávila G a., Corona-Morales A a. (2015) Enriched environment restricted to gestation accelerates the development of sensory and motor circuits in the rat pup. Int J Dev Neurosci 41:68–73 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0736574815000027.

Carmeliet P (2003) Blood vessels and nerves: common signals, pathways and diseases. Nat Rev Genet 4:710–720 Available at: http://dx.doi.org/10.1038/nrg1158.

Catalani A, Sabbatini M, Consoli C, Cinque C, Tomassoni D, Azmitia E, Angelucci L, Amenta F (2002) Glial fibrillary acidic protein immunoreactive astrocytes in developing rat hippocampus. Mech Ageing Dev 123:481–490.

Chanana V, Tumturk A, Kintner D, Udho E, Ferrazzano P, Cengiz P (2016) Sex Differences in Mouse Hippocampal Astrocytes after In-Vitro Ischemia. J Vis Exp.

Charriaut-Marlangue C, Besson VC, Baud O (2018) Sexually dimorphic outcomes after neonatal stroke and hypoxia-ischemia. Int J Mol Sci 19.

Chugani HT (2018) Imaging Brain Metabolism in the Newborn. J Child Neurol 33:851–860.

Clanton TL, Klawitter PF (2001) Adaptive responses of skeletal muscle to intermittent hypoxia: the known and the unknown. J Appl Physiol 90:2476–2487.

Clowry GJ, Basuodan R, Chan F (2014) What are the Best Animal Models for Testing Early Intervention in Cerebral Palsy? Front Neurol 5:1–17 Available

at: http://journal.frontiersin.org/article/10.3389/fneur.2014.00258/abstract.

Connors EJ, Shaik a. N, Migliore MM, Kentner a. C (2014) Environmental enrichment mitigates the sex-specific effects of gestational inflammation on social engagement and the hypothalamic pituitary adrenal axis-feedback system. Brain Behav Immun 42:178–190 Available at: http://dx.doi.org/10.1016/j.bbi.2014.06.020.

Cross JL, Meloni BP, Bakker AJ, Lee S, Knuckey NW (2010) Modes of neuronal calcium entry and homeostasis following cerebral ischemia. Int J Alzheimers Dis.

Cutuli D, Caporali P, Gelfo F, Angelucci F, Laricchiuta D, Foti F, De Bartolo P, Bisicchia E, Molinari M, Farioli Vecchioli S, Petrosini L (2015) Pre-reproductive maternal enrichment influences rat maternal care and offspring developmental trajectories: behavioral performances and neuroplasticity correlates. Front Behav Neurosci 9:1–18 Available at: http://journal.frontiersin.org/Article/10.3389/fnbeh.2015.00066/abstract.

Davidson JO, Wassink G, van den Heuij LG, Bennet L, Gunn AJ (2015) Therapeutic Hypothermia for Neonatal Hypoxic–Ischemic Encephalopathy – Where to from Here? Front Neurol 6 Available at: http://journal.frontiersin.org/Article/10.3389/fneur.2015.00198/abstract.

Dell P a, Rose FD (1987) Transfer of effects from environmentally enriched and impoverished female rats to future offspring. Physiol Behav 39:187–190.

Demarest T., Schuh R., Waddell J, McKenna M., Fiskum G (2016a) Sex dependent mitochondrial respiratory impairment and oxidative stress in a rat model of neonatal hypoxic-ischemic encephalopathy. J Neurochem:n/a-

n/a Available at: http://doi.wiley.com/10.1111/jnc.13590.

Demarest T., Waite E., Kristian T, Puche A., Waddell J, McKenna M., Fiskum G (2016b) Sex-dependent mitophagy and neuronal death following rat neonatal hypoxia–ischemia. Neuroscience 335:103–113.

Dennis M, Spiegler BJ, Juranek JJ, Bigler ED, Snead OC, Fletcher JM (2013) Age, plasticity, and homeostasis in childhood brain disorders. Neurosci Biobehav Rev 37:2760–2773 Available at: http://dx.doi.org/10.1016/j.neubiorev.2013.09.010.

Diamond MC (2001) Response of the brain to enrichment. An Acad Bras Cienc 73:210–220.

Di Segni M, Andolina D, Ventura R (2017) Long-term effects of early environment on the brain: Lesson from rodent models. Semin Cell Dev Biol Available at: http://dx.doi.org/10.1016/j.semcdb.2017.09.039.

Diaz R, Maidana Miguel P, Ferrary Deniz B, Confortim HD, Barbosa S, Padilha Mendonça MC, da Cruz-Höfling MA, Orlandi Pereira L (2016) Environmental enrichment attenuates the blood brain barrier dysfunction induced by the neonatal hypoxia-ischemia. Int J Dev Neurosci 53:35–45 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0736574816300557.

Donders J, Hoffman NM (2002) Gender differences in learning and memory after pediatric traumatic brain injury. Neuropsychology 16:491–499.

Douglas-Escobar M, Weiss MD (2015) Hypoxic-Ischemic Encephalopathy A Review for the Clinician. JAMA Pediatr 169:397–403.

Dukhande V V., Isaac AO, Chatterji T, Lai JCK (2009) Reduced glutathione regenerating enzymes undergo developmental decline and sexual dimorphism in the rat cerebral cortex. Brain Res 1286:19–24.

Durán-Carabali L., Arcego D., Odorcyk F., Reichert L, Cordeiro J., Sanches E., Freitas L., Dalmaz C, Pagnussat A, Netto C. (2017) Prenatal and Early Postnatal Environmental Enrichment Reduce Acute Cell Death and Prevent Neurodevelopment and Memory Impairments in Rats Submitted to Neonatal Hypoxia Ischemia. Mol Neurobiol Available at: http://link.springer.com/10.1007/s12035-017-0604-5.

Durán-Carabali LE, Arcego DM, Sanches EF, Odorcyk FK, Marques MR, Tosta A, Reichert L, Carvalho AS, Dalmaz C, Netto CA (2019) Preventive and therapeutic effects of environmental enrichment in Wistar rats submitted to neonatal hypoxia-ischemia. Behav Brain Res 359:485–497 Available at: https://doi.org/10.1016/j.bbr.2018.11.036.

Edgerton VR, Roy RR, Allen DL, Monti RJ (2002) Adaptations in skeletal muscle disuse or decreased-use atrophy. Am J Phys Med Rehabil 81:S127-47 Available at: http://www.ncbi.nlm.nih.gov/pubmed/12409818.

English C, McLennan H, Thoirs K, Coates A, Bernhardt J (2010) Loss of skeletal muscle mass after stroke: A systematic review. Int J Stroke 5:395–402.

Escobar GJ, Clark RH, Greene JD (2006) Short-term outcomes of infants born at 35 and 36 weeks gestation: We need to ask more questions. In: Seminars in Perinatology.

Femitha P, Bhat BV (2012) Early neonatal outcome in late preterms. Indian J

Pediatr.

Ferriero DM (2001) Oxidant mechanisms in neonatal hypoxia-ischemia. Dev Neurosci 23:198–202.

Ferriero DM (2004) Neonatal brain injury. N Engl J Med 351:1985–1995.

Flórez-Vargas O, Brass A, Karystianis G, Bramhall M, Stevens R, Cruickshank S, Nenadic G (2016) Bias in the reporting of sex and age in biomedical research on mouse models. Elife.

Franklin RJM, Ffrench-Constant C (2008) Remyelination in the CNS: From biology to therapy. Nat Rev Neurosci.

Frey HA, Klebanoff MA (2016) The epidemiology, etiology, and costs of preterm birth. Semin Fetal Neonatal Med 21:68–73.

Galeano P, Blanco E, Logica Tornatore TMA, Romero JI, Holubiec MI, RodrÃ-guez de Fonseca F, Capani F (2015) Life-long environmental enrichment counteracts spatial learning, reference and working memory deficits in middle-aged rats subjected to perinatal asphyxia. Front Behav Neurosci 8:1–12 Available at: http://journal.frontiersin.org/article/10.3389/fnbeh.2014.00406/abstract.

George S, Gunn AJ, Westgate JA, Brabyn C, Guan J, Bennet L, Gunn AJ, Westgate JA (2004) Fetal heart rate variability and brain stem injury after asphyxia in preterm fetal sheep. :925–933.

Girard S, Kadhim H, Beaudet N, Sarret P, Sébire G (2009) Developmental motor deficits induced by combined fetal exposure to lipopolysaccharide and early neonatal hypoxia/ischemia: A novel animal model for cerebral

palsy in very premature infants. Neuroscience 158:673–682 Available at: http://dx.doi.org/10.1016/j.neuroscience.2008.10.032.

Gluckman PD, Hanson MA, Cooper C, Thornburg KL (2008) In utero and early-life conditions and adult health and disease. N Engl J Med 359:1523–1524; author reply 1524.

Goldenberg RL, Culhane JF, Iams JD, Romero R (2009) Preterm Birth 1: Epidemiology and Causes of Preterm Birth. Obstet Anesth Dig.

Gorman BK, Read JG (2006) Gender Disparities in Adult Health: An Examination of Three Measures of Morbidity. J Health Soc Behav 47:95–110.

Gravett MG, Rubens CE, Nunes TM (2010) Global report on preterm birth and stillbirth (2 of 7): Discovery science. BMC Pregnancy Childbirth.

Grefkes C, Ward NS (2014) Cortical reorganization after stroke: How much and how functional? Neuroscientist 20:56–70.

Griva M, Lagoudaki R, Touloumi O, Nousiopoulou E, Karalis F, Georgiou T, Kokaraki G, Simeonidou C, Tata DA, Spandou E (2017) Long-term effects of enriched environment following neonatal hypoxia-ischemia on behavior, BDNF and synaptophysin levels in rat hippocampus: Effect of combined treatment with G-CSF. Brain Res 1667:55–67 Available at: http://dx.doi.org/10.1016/j.brainres.2017.05.004.

Gualtieri CT, Ondrusek MG, Finley C (1985) Attention deficit disorders in adults. Clin Neuropharmacol 8:343–356.

Gunn AJ, Bennet L (2009) Fetal Hypoxia Insults and Patterns of Brain Injury:

Insights from Animal Models. Clin Perinatol 36:579–593.

Hagberg H, Mallard C, Rousset Catherine CI, Wang X (2009) Apoptotic mechanisms in the immature Brain: Involvement of mitochondria. J Child Neurol.

Hagberg H, Peebles D, Mallard C (2002) Models of white matter injury: Comparison of infectious, hypoxic-Ischemic, and excitotoxic insults. Ment Retard Dev Disabil Res Rev 8:30–38.

Hassell KJ, Ezzati M, Alonso-Alconada D, Hausenloy DJ, Robertson NJ (2015) New horizons for newborn brain protection: enhancing endogenous neuroprotection. Arch Dis Child - Fetal Neonatal Ed 100:F541–F552 Available at: http://fn.bmj.com/content/100/6/F541.abstract.

Hebb D. (1947) The effects of early experience on problem solving at maturity. Am Psychol 2:306–307.

Heindel JJ, Vandenberg LN (2015) Developmental origins of health and disease: A paradigm for understanding disease cause and prevention. Curr Opin Pediatr.

Hindmarsh G, O'Callaghan M, Mohay H, Rogers Y (2000) Gender differences in cognitive abilities at 2 years in ELBW infants. Early Hum Dev 60:115–122 Available at: http://www.sciencedirect.com/science/article/pii/S0378378200001055.

Horvath G, Dora Reglődi, Farkas J, Vadasz G, Mammel B, Kvarik T, Bodzai G, Kiss-Illes B, Farkas D, Matkovits A, Manavalan S, Gaszner B, Tamas A, Kiss P, Abstract (2015) Perinatal positive and negative influences on the

early neurobehavioral reflex and motor development. In: Perinatal Programming of Neurodevelopment, Springer. (Marta C. Antonelli, ed), pp 149–167. New York Heidelberg Dordrecht London. Available at: http://link.springer.com/10.1007/978-1-4939-1372-5.

Horvath G, Reglodi D, Vadasz G, Farkas J, Kiss P (2013) Exposure to enriched environment decreases neurobehavioral deficits induced by neonatal glutamate toxicity. Int J Mol Sci 14:19054–19066.

Hu JJ, Yang XL, Luo W Di, Han S, Yin J, Liu WH, He XH, Peng BW (2017) Bumetanide reduce the seizure susceptibility induced by pentylenetetrazol via inhibition of aberrant hippocampal neurogenesis in neonatal rats after hypoxia-ischemia. Brain Res Bull 130:188–199 Available at: http://dx.doi.org/10.1016/j.brainresbull.2017.01.022.

Huang Z, Liu J, Cheung PY, Chen C (2009) Long-term cognitive impairment and myelination deficiency in a rat model of perinatal hypoxic-ischemic brain injury. Brain Res 1301:100–109 Available at: http://dx.doi.org/10.1016/j.brainres.2009.09.006.

Iuvone L, Geloso MC, Dell'Anna E (1996) Changes in Open Field Behavior, Spatial Memory, and Hippocampal Parvalbumin Immunoreactivity Following Enrichment in Rats Exposed to Neonatal Anoxia. Exp Neurol 139:25–33 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0014488696900771.

James ML, Gambhir SS (2012) A Molecular Imaging Primer: Modalities, Imaging Agents, and Applications. Physiol Rev 92:897–965.

Jamon M (2006) The early development of motor control in neonate rat.

Comptes Rendus - Palevol 5:657–666.

Jiang X, West AA, Caudill MA (2014) Maternal choline supplementation: A nutritional approach for improving offspring health? Trends Endocrinol Metab 25:263–273 Available at: http://dx.doi.org/10.1016/j.tem.2014.02.001.

Jin R, Yang G, Li G (2010) Inflammatory mechanisms in ischemic stroke: role of inflammatory cells. J Leukoc Biol 87:779–789.

Johnston M., Trescher W., Ishida A, Nakajima W (2001a) Neurobiology of hypoxic-ischemic injury in the developing brain. Pediatr Res 49:735–741.

Johnston M., Trescher W., Ishida A, Nakajima W, Zipursky A (2001b) The Developing Nervous System: A Series of Review Articles: Neurobiology of Hypoxic-Ischemic Injury in the Developing Brain. Pediatr Res 49:735–741.

Johnston M V., Hagberg H (2007) Sex and the pathogenesis of cerebral palsy. Dev Med Child Neurol 49:74–78.

Kappeler L, Meaney MJ (2010) Epigenetics and parental effects. BioEssays 32:818–827.

Katušić A (2011) Early brain injury and plasticity: Reorganization and functional Recovery. Transl Neurosci 2:33–42 Available at: http://www.springerlink.com/index/10.2478/s13380-011-0006-5.

Keeley RJ, Tyndall A V., Scott GA, Saucier DM (2013) Sex Difference in Cue Strategy in a Modified Version of the Morris Water Task: Correlations between Brain and Behaviour. PLoS One 8:1–9.

Keiner S, Wurm F, Kunze A, Witte OW, Redecker C (2008) Rehabilitative

therapies differentially alter proliferation and survival of glial cell populations in the perilesional zone of cortical infarcts. Glia 56:516–527.

Kempermann G (2019) Environmental enrichment, new neurons and the neurobiology of individuality. Nat Rev Neurosci Available at: http://www.nature.com/articles/s41583-019-0120-x.

Kempermann G, Gage FH (1999) Experience-dependent regulation of adult hippocampal neurogenesis: Effects of long-term stimulation and stimulus withdrawal. Hippocampus 9:321–332.

Kent AL, Wright IMR, Abdel-Latif ME (2012) Mortality and Adverse Neurologic Outcomes Are Greater in Preterm Male Infants. Pediatrics 129:124–131 Available at: http://pediatrics.aappublications.org/cgi/doi/10.1542/peds.2011-1578.

Kesler SR, Reiss AL, Vohr B, Watson C, Schneider KC, Katz KH, Maller-Kesselman J, Silbereis J, Constable RT, Makuch RW, Ment LR (2008) Brain Volume Reductions within Multiple Cognitive Systems in Male Preterm Children at Age Twelve. J Pediatr 152.

Khwaja O, Volpe JJ (2008) Pathogenesis of cerebral white matter injury of prematurity. Arch Dis Child - Fetal Neonatal Ed 93:F153–F161 Available at: http://fn.bmj.com/content/93/2/F153.abstract.

Kim AH, Khursigara GUS, Sun X, Franke TF, Chao M V (2001) Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. Mol Cell Biol 21:893–901.

Kim MS, Yu JH, Kim CH, Choi JY, Seo JH, Lee MY, Yi CH, Choi TH, Ryu YH,

Lee JE, Lee BH, Kim H, Cho SR (2016) Environmental enrichment enhances synaptic plasticity by internalization of striatal dopamine transporters. J Cereb Blood Flow Metab.

Kiss P, Vadasz G, Kiss-Illes B, Horvath G, Tamas A, Reglodi D, Koppan M (2013) Environmental enrichment decreases asphyxia-induced neurobehavioral developmental delay in neonatal rats. Int J Mol Sci 14:22258–22273.

Klein R, Nanduri V, Jing S, Lamballe F, Tapley P, Bryant S, Cordon-Cardo C, Jones KR, Reichardt LF, Barbacid M (1991) The trkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. Cell 66:395–403 Available at: https://www.scopus.com/inward/record.uri?eid=2-s2.0-0025873435&partnerID=40&md5=83c39c51f40a446c2629844413f25ae3.

Koike M, Shibata M, Tadakoshi M, Gotoh K, Komatsu M, Waguri S, Kawahara N, Kuida K, Nagata S, Kominami E, Tanaka K, Uchiyama Y (2008) Inhibition of autophagy prevents hippocampal pyramidal neuron death after hypoxic-ischemic injury. Am J Pathol 172:454–469 Available at: http://dx.doi.org/10.2353/ajpath.2008.070876.

Kolb B, Gibb R (2007) Brain Plasticity and Recovery from Early Cortical Injury. Dev Psychobiol 49:107–118 Available at: http://onlinelibrary.wiley.com/doi/10.1002/dev.20200/abstract%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/17299788.

Komitova M, Xenos D, Salmaso N, May Tran K, Brand T, Schwartz ML, Ment L, Vaccarino FM (2013) Hypoxia-Induced Developmental Delays of Inhibitory

Interneurons Are Reversed by Environmental Enrichment in the Postnatal Mouse Forebrain. J Neurosci 33:13375–13387 Available at: http://www.jneurosci.org/cgi/doi/10.1523/JNEUROSCI.5286-12.2013.

Koo JW, Park CH, Choi SH, Kim NJ, Kim H, Choe C, Suh Y, Creative N, National S (2003) Postnatal environment can counteract prenatal effects on cognitive ability, cell proliferation, and synaptic protein expression. FASEB J.

Kramer MS, Papageorghiou A, Culhane J, Bhutta Z, Goldenberg RL, Gravett M, Iams JD, Conde-Agudelo A, Waller S, Barros F, Knight H, Villar J (2012) Challenges in defining and classifying the preterm birth syndrome. Am J Obstet Gynecol 206:108–112.

Krech D, Rosenzweig MR, Bennett EL (1962) Relations between chemistry and problem-solving among rats raised i n enriched and impoverished environments. J Comp Physiol Psychol 55:801.

Kurinczuk JJ, White-Koning M, Badawi N (2010) Epidemiology of neonatal encephalopathy and hypoxic-ischaemic encephalopathy. Early Hum Dev 86:329–338 Available at: http://dx.doi.org/10.1016/j.earlhumdev.2010.05.010.

Lai M-C, Yang S-N (2011) PerinatalHypoxic-Ischemic Encephalopathy. J Biomed Biotechnol 15:138–146.

Lauterbach MD, Raz S, Sander CJ (2001) Neonatal hypoxic risk in preterm birth infants: The influence of sex and severity of respiratory distress on cognitive recovery. Neuropsychology 15:411–420.

Levine S (1960) Anoxic-Ischemic Encephalopathy in Rats. Am J Pathol 36:1–17.

Li M, Wang M, Ding S, Li C, Luo X (2012) Environmental Enrichment during Gestation Improves Behavior Consequences and Synaptic Plasticity in Hippocampus of Prenatal-Stressed Offspring Rats. Acta Histochem Cytochem 45:157–166.

Lichtenwalner RJ, Parent JM (2006) Adult neurogenesis and the ischemic forebrain. J Cereb Blood Flow Metab 26:1–20.

Lindamer LA, Lohr JB, Harris MJ, McAdams LA, Jeste D V (1999) Gender-related clinical differences in older patients with schizophrenia. J Clin Psychiatry 60:61–69.

Liu D, Diorio J, Day JC, Francis DD, Meaney MJ (2000) Maternal care, hippocampal synaptogenesis and cognitive development in rats. Nat Neurosci 3:799–806.

Lorek A, Takei Y, Cady EB, Wyatt JS, Penrice J, Edwards AD, Peebles D, Wylezinska M, Owen-Reece H, Kirkbride V, Cooper CE, Aldridge RF, Roth SC, Brown G, Delpy DT, Reynolds EO (1994) Delayed (“secondary”) cerebral energy failure after acute hypoxia-ischemia in the newborn piglet: Continuous 48-hour studies by phosphorus magnetic resonance spectroscopy. Pediatr Res.

Lubics A, Reglodi D, Tamás A, Kiss P, Szalai M, Szalontay L, Lengvári I (2005) Neurological reflexes and early motor behavior in rats subjected to neonatal hypoxic-ischemic injury. Behav Brain Res 157:157–165.

Lucas A (2005) Long-term programming effects of early nutrition — implications for the preterm infant. J Perinatol 25:S2–S6.

Marik J, Ogasawara A, Martin-McNulty B, Ross J, Flores JE, Gill HS, Tinianow JN, Vanderbilt AN, Nishimura M, Peale F, Pastuskovas C, Greve JM, van Bruggen N, Williams SP (2009) PET of Glial Metabolism Using 2-18F-Fluoroacetate. J Nucl Med 50:982–990.

Marques MR, Stigger F, Segabinazi E, Augustin OA, Barbosa S, Piazza FV, Achaval M, Marcuzzo S (2014) Beneficial effects of early environmental enrichment on motor development and spinal cord plasticity in a rat model of cerebral palsy. Behav Brain Res 263:149–157 Available at: http://dx.doi.org/10.1016/j.bbr.2014.01.007.

Martinez-Espinosa M, Sandanielo VLM, Louzada-Neto F (2006) O MÉTODO DE BOOTSTRAP PARA O ESTUDO DE DADOS DE FADIGA DOS MATERIAIS. Rev Mat Estat 24(2):37–50.

Maruoka T, Kodomari I, Yamauchi R, Wada E, Wada K (2009) Maternal enrichment affects prenatal hippocampal proliferation and open-field behaviors in female offspring mice. Neurosci Lett 454:28–32.

Matas E, Bock J, Braun K (2016) The Impact of Parent-Infant Interaction on Epigenetic Plasticity Mediating Synaptic Adaptations in the Infant Brain. Psychopathology 49:201–210.

Mayoral SR, Omar G, Penn AA (2009) Sex differences in a hypoxia model of preterm brain damage. Pediatr Res 66:248–253.

McDonald MW, Hayward KS, Rosbergen ICM, Jeffers MS, Corbett D (2018) Is

Environmental Enrichment Ready for Clinical Application in Human Post-stroke Rehabilitation? Front Behav Neurosci 12:1–16 Available at: https://www.frontiersin.org/article/10.3389/fnbeh.2018.00135/full.

McKenna M., Dienel G., Sonnewald U, Waagepetersen S., Schousboe A (2012) Chapter 11 - Energy Metabolism of the Brain. In: Basic Neurochemistry. Principles of Molecular, Cellular, and Medical Neurobiology, pp 200–231.

McKenna MC, Scafidi S, Robertson CL (2015) Metabolic alterations in developing brain after injury: knowns and unknowns. Neurochem Res 40:2527–2543.

McLean C, Ferriero D (2004) Mechanisms of hypoxic-ischemic injury in the term infant. Semin Perinatol 28:425–432.

Meireles ALF, Marques MR, Segabinazi E, Spindler C, Piazza F V., Salvalaggio GS, Augustin OA, Achaval M, Marcuzzo S (2017) Association of environmental enrichment and locomotor stimulation in a rodent model of cerebral palsy: Insights of biological mechanisms. Brain Res Bull 128:58–67 Available at: http://dx.doi.org/10.1016/j.brainresbull.2016.12.001.

Mering S, Jolkkonen J (2015) Proper housing conditions in experimental stroke studies-special emphasis on environmental enrichment. Front Neurosci 9:1–8.

Meshi D, Drew MR, Saxe M, Ansorge MS, David D, Santarelli L, Malapani C, Moore H, Hen R (2006) Hippocampal neurogenesis is not required for behavioral effects of environmental enrichment. Nat Neurosci 9:729–731 Available at: http://www.nature.com/doifinder/10.1038/nn1696.

Metz GA, Whishaw IQ (2002) Cortical and subcortical lesions impair skilled walking in the ladder rung walking test : a new task to evaluate fore- and hindlimb stepping, placing, and co-ordination. J Neurosci Methods 115:169–179.

Miller LR, Marks C, Becker JB, Hurn PD, Chen WJ, Woodruff T, McCarthy MM, Sohrabji F, Schiebinger L, Lee Wetherington C, Makris S, Arnold AP, Einstein G, Miller VM, Sandberg K, Maier S, Cornelison TL, Clayton JA (2017) Considering sex as a biological variable in preclinical research. FASEB J 31:29–34.

Mishima K, Ikeda T, Aoo N, Takai N, Takahashi S, Egashira N, Ikenoue T, Iwasaki K, Fujiwara M (2005) Hypoxia-ischemic insult in neonatal rats induced slowly progressive brain damage related to memory impairment. Neurosci Lett 376:194–199.

Misumi S, Ueda Y, Nishigaki R, Suzuki M, Ishida A, Jung C-G, Hida H (2016) Dysfunction in Motor Coordination in Neonatal White Matter Injury Model without Apparent Neuron Loss. Cell Transplant 25:1381–1393 Available at: http://www.ncbi.nlm.nih.gov/pubmed/26564423.

Mohagheghi AA, Khan T, Meadows TH, Giannikas K, Baltzopoulos V, Maganaris CN (2007) Differences in gastrocnemius muscle architecture between the paretic and non-paretic legs in children with hemiplegic cerebral palsy. Clin Biomech 22:718–724.

Morgane PJ, Mokler DJ, Galler JR (2002) Effects of prenatal protein malnutrition on the hippocampal formation. Neurosci Biobehav Rev 26:471–483.

Morken TS, Brekke E. E, Håberg A, Widerøe M, Brubakk A-MA., Sonnewald U (2014) Altered astrocyte-neuronal interactions after hypoxia-ischemia in the neonatal brain in female and male rats. Stroke 45:2777–2785.

Muglia LJ, Katz M (2010) The enigma of spontaneous preterm birth. N Engl J Med:529–535.

Mychasiuk R, Zahir S, Schmold N, Ilnytskyy S, Kovalchuk O, Gibb R (2012) Parental enrichment and offspring development: Modifications to brain, behavior and the epigenome. Behav Brain Res 228:294–298 Available at: http://dx.doi.org/10.1016/j.bbr.2011.11.036.

Nehlig A (2004) Brain uptake and metabolism of ketone bodies in animal models. Prostaglandins Leukot Essent Fat Acids 70:265–275.

Netto CA, Hodges H, Sinden JD, LE Peillet E, Kershaw T, Sowinski P, Meldrum BS, Gray JA (1993) Effects of fetal hippocampal field grafts on ischaemic-induced deficits in spatial navigation in the water maze. Neuroscience 54:69–92.

Netto CA, Sanches E, Odorcyk FK, Duran-Carabali LE, Weis SN (2017) Sex-dependent consequences of neonatal brain hypoxia-ischemia in the rat. J Neurosci Res 95:409–421.

Netto C, Sanches E, Odorcyk F, Duran-Carabali L, Sizonenko S (2018) Pregnancy as a valuable period for preventing hypoxia-ischemia brain damage. Int J Dev Neurosci 18.

Nguyen T-MN, Crowther CA, Wilkinson D, Bain E (2013) Magnesium sulphate for women at term for neuroprotection of the fetus. In: Cochrane Database

of Systematic Reviews.

Nicola FC, Rodrigues LP, Crestani T, Quintiliano K, Sanches EF, Willborn S, Aristimunha D, Boisserand L, Pranke P, Netto CA (2016) Human dental pulp stem cells transplantation combined with treadmill training in rats after traumatic spinal cord injury. Brazilian J Med Biol Res = Rev Bras Pesqui medicas e Biol 49:e5319.

Nithianantharajah J, Hannan AJ (2006) Enriched environments, experience-dependent plasticity and disorders of the nervous system. Nat Rev Neurosci 7:697–709 Available at: http://www.ncbi.nlm.nih.gov/sites/entrez?Db=pubmed&DbFrom=pubmed&Cmd=Link&LinkName=pubmed_pubmed&LinkReadableName=Related Articles&IdsFromResult=16924259&ordinalpos=3&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum.

Nonose Y, Gewehr PE, Almeida RF, da Silva JS, Bellaver B, Martins LAM, Zimmer ER, Greggio S, Venturin GT, Da Costa JC, Quincozes-Santos A, Pellerin L, de Souza DO, de Assis AM (2018) Cortical Bilateral Adaptations in Rats Submitted to Focal Cerebral Ischemia: Emphasis on Glial Metabolism. Mol Neurobiol 55:2025–2041.

O'Shea TM (2002) Cerebral palsy in very preterm infants: New epidemiological insights. Ment Retard Dev Disabil Res Rev.

Ortuzar N, Rico-Barrio I, Bengoetxea H, Argando??a EG, Lafuente J V. (2013) VEGF reverts the cognitive impairment induced by a focal traumatic brain injury during the development of rats raised under environmental enrichment. Behav Brain Res 246:36–46.

Pagnussat AS, Simao F, Anastacio JR, Mestriner RG, Michaelsen SM, Castro CC, Salbego C, Netto CA (2012) Effects of skilled and unskilled training on functional recovery and brain plasticity after focal ischemia in adult rats. Brain Res 1486:53–61 Available at: http://dx.doi.org/10.1016/j.brainres.2012.09.019.

Patel AR, Ritzel R, McCullough LD, Liu F (2013) Microglia and ischemic stroke: A double-edged sword. Int J Physiol Pathophysiol Pharmacol 5:73–90.

Peacock JL, Marston L, Marlow N, Calvert SA, Greenough A (2012) Neonatal and infant outcome in boys and girls born very prematurely. Pediatr Res 71:305–310.

Pereira LO, Arteni NS, Petersen RC, da Rocha AP, Achaval M, Netto CA (2007) Effects of daily environmental enrichment on memory deficits and brain injury following neonatal hypoxia-ischemia in the rat. Neurobiol Learn Mem 87:101–108.

Pereira LO, Nabinger PM, Strapasson ACP, Nardin P, Gonçalves CAS, Siqueira IR, Netto CA (2009) Long-term effects of environmental stimulation following hypoxia-ischemia on the oxidative state and BDNF levels in rat hippocampus and frontal cortex. Brain Res 1247:188–195 Available at: http://dx.doi.org/10.1016/j.brainres.2008.10.017.

Pereira LO, Strapasson a. CP, Nabinger PM, Achaval M, Netto CA (2008) Early enriched housing results in partial recovery of memory deficits in female, but not in male, rats after neonatal hypoxia-ischemia. Brain Res 1218:257–266.

Perlman JM (2001) Neurobehavioral deficits in premature graduates of

intensive care--potential medical and neonatal environmental risk factors. Pediatrics 108:1339–1348 Available at: http://www.ncbi.nlm.nih.gov/pubmed/11731657.

Petrosini L, De Bartolo P, Foti F, Gelfo F, Cutuli D, Leggio MG, Mandolesi L (2009) On whether the environmental enrichment may provide cognitive and brain reserves. Brain Res Rev 61:221–239.

Piek JP, Dawson L, Smith LM, Gasson N (2008) The role of early fine and gross motor development on later motor and cognitive ability. Hum Mov Sci 27:668–681 Available at: http://dx.doi.org/10.1016/j.humov.2007.11.002.

Plaschke K, Staub J, Ernst E, Marti HH (2008) VEGF overexpression improves mice cognitive abilities after unilateral common carotid artery occlusion. Exp Neurol 214:285–292 Available at: http://dx.doi.org/10.1016/j.expneurol.2008.08.014.

Quinn JA, Munoz FM, Gonik B, Frau L, Cutland C, Mallett-Moore T, Kissou A, Wittke F, Das M, Nunes T, Pye S, Watson W, Ramos AMA, Cordero JF, Huang WT, Kochhar S, Buttery J (2016) Preterm birth: Case definition & guidelines for data collection, analysis, and presentation of immunisation safety data. Vaccine.

Raz S, Debastos AK, Newman JB, Batton D (2010) Extreme prematurity and neuropsychological outcome in the preschool years. J Int Neuropsychol Soc 16:169–179 Available at: http://www.ncbi.nlm.nih.gov/pubmed/19900351.

Reichardt LF (2006) Neurotrophin-regulated signalling pathways. Philos Trans R Soc B Biol Sci 361:1545–1564 Available at:

http://rstb.royalsocietypublishing.org/cgi/doi/10.1098/rstb.2006.1894.

Rice JE, Vannucci RC, Brierley JB (1981) The influence of immaturity on hypoxic-ischemic brain damage in the rat. Ann Neurol 9:131–141 Available at: http://www.ncbi.nlm.nih.gov/pubmed/7235629.

Rocha-Ferreira E, Hristova M, Rocha-Ferreira E, Hristova M (2016) Plasticity in the Neonatal Brain following Hypoxic-Ischaemic Injury. Neural Plast 2016:1–16 Available at: http://www.hindawi.com/journals/np/2016/4901014/.

Roelfsema V, Gunn AJ, Fraser M, Quaedackers JS, Bennet L (2005) Cortisol and ACTH responses to severe asphyxia in preterm fetal sheep. Exp Physiol 90.4:545–555.

Roggero P, Giannì ML, Garbarino F, Mosca F (2013) Consequences of prematurity on adult morbidities. Eur J Intern Med 24:624–626 Available at: http://dx.doi.org/10.1016/j.ejim.2013.01.011.

Rojas JJ, Deniz BF, Miguel PM, Diaz R, Hermel ÉDES, Achaval M, Netto CA, Pereira LO (2013) Effects of daily environmental enrichment on behavior and dendritic spine density in hippocampus following neonatal hypoxia-ischemia in the rat. Exp Neurol 241:25–33 Available at: http://dx.doi.org/10.1016/j.expneurol.2012.11.026.

Rojas JJ, Deniz BF, Schuch CP, Carletti JV, Deckmann I, Diaz R, Matté C, dos Santos TM, Wyse AT, Netto C a., Pereira LO (2015) Environmental stimulation improves performance in the ox-maze task and recovers Na+,K+-ATPase activity in the hippocampus of hypoxic–ischemic rats. Neuroscience 291:118–127 Available at:

http://linkinghub.elsevier.com/retrieve/pii/S0306452215000652.

Rosenfeld A, Weller A (2012) Behavioral effects of environmental enrichment during gestation in WKY and Wistar rats. Behav Brain Res 233:245–255 Available at: http://dx.doi.org/10.1016/j.bbr.2012.05.006.

Rosenzweig MR (2007) Chapter 4 Modification of Brain Circuits through Experience. In: Neural Plasticity and Memory.

Rubinov M, Sporns O (2010) Complex network measures of brain connectivity: Uses and interpretations. Neuroimage 52:1059–1069 Available at: http://dx.doi.org/10.1016/j.neuroimage.2009.10.003.

Rudolph A (2018) Cerebral glucose deficiency versus oxygen deficiency in neonatal encephalopathy. J Neonatal Perinat Med 11(2):115–120.

Rumajogee P, Bregman T, Miller SP, Yager JY, Fehlings MG (2016) Rodent hypoxia-ischemia models for cerebral palsy research: A systematic review. Front Neurol 7.

Rutter M, Caspi A, Moffitt TE (2003) Using sex differences in psychopathology to study causal mechanisms: Unifying issues and research strategies. J Child Psychol Psychiatry Allied Discip 44:1092–1115.

Sale A (2018) A Systematic Look at Environmental Modulation and Its Impact in Brain Development. Trends Neurosci 41:4–17 Available at: http://dx.doi.org/10.1016/j.tins.2017.10.004.

Sale A, Berardi N, Maffei L (2014) Environment and brain plasticity: towards an endogenous pharmacotherapy. Physiol Rev 94:189–234 Available at: http://www.ncbi.nlm.nih.gov/pubmed/24382886.

Sale A, Cenni MC, Clussi F, Putignano E, Chierzi S, Maffei L (2007) Maternal enrichment during pregnancy accelerates retinal development of the fetus. PLoS One 2:1–8.

Sale A, Putignano E, Cancedda L, Landi S, Cirulli F, Berardi N, Maffei L (2004) Enriched environment and acceleration of visual system development. Neuropharmacology 47:649–660.

Salmaso N, Silbereis J, Komitova M, Mitchell P, Chapman K, Ment LR, Schwartz ML, Vaccarino FM (2012) Environmental Enrichment Increases the GFAP+ Stem Cell Pool and Reverses Hypoxia-Induced Cognitive Deficits in Juvenile Mice. J Neurosci 32:8930–8939 Available at: http://www.jneurosci.org/cgi/doi/10.1523/JNEUROSCI.1398-12.2012.

Sanches E., Arteni N., Scherer E., Kolling J, Nicola F, Willborn S, Wyse AT., Netto C. (2013a) Are the consequences of neonatal hypoxia-ischemia dependent on animals' sex and brain lateralization? Brain Res 1507:105–114 Available at: http://dx.doi.org/10.1016/j.brainres.2013.02.040.

Sanches E, Durán-Carabali L, Tosta A, Nicola F, Schmitz F, Rodrigues A, Siebert C, Wyse A, Netto, C A (2017) Pregnancy swimming causes short- and long-term neuroprotection against hypoxia-ischemia in very immature rats. Pediatr Res 31.

Sanches EF, Arteni N, Nicola F, Aristimunha D, Netto C a. (2015) Sexual dimorphism and brain lateralization impact behavioral and histological outcomes following hypoxia–ischemia in P3 and P7 rats. Neuroscience 290:581–593 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0306452215000494.

Sanches EF, Arteni NS, Nicola F, Boisserand L, Willborn S, Netto C. (2013b) Early hypoxia-ischemia causes hemisphere and sex-dependent cognitive impairment and histological damage. Neuroscience 237:208–215 Available at: http://dx.doi.org/10.1016/j.neuroscience.2013.01.066.

Sanches EF, Arteni NS, Spindler C, Moysés F, Siqueira IR, Perry ML, Netto CA (2012) Effects of pre- and postnatal protein malnutrition in hypoxic-ischemic rats. Brain Res 1438:85–92 Available at: http://dx.doi.org/10.1016/j.brainres.2011.12.024.

Satz P (1993) Brain Reserve Capacity on Symptom Onset After Brain Injury: A Formulation and Review of Evidence for Threshold Theory. Neuropsychology.

Scherbakov N, Von Haehling S, Anker SD, Dirnagl U, Doehner W (2013) Stroke induced Sarcopenia: Muscle wasting and disability after stroke. Int J Cardiol 170:89–94 Available at: http://dx.doi.org/10.1016/j.ijcard.2013.10.031.

Schuch CP, Diaz R, Deckmann I, Rojas JJ, Deniz BF, Pereira LO (2016a) Early environmental enrichment affects neurobehavioral development and prevents brain damage in rats submitted to neonatal hypoxia-ischemia. Neurosci Lett 617:101–107 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0304394016300830.

Schuch CP, Jeffers MS, Antonescu S, Nguemeni C, Gomez-Smith M, Pereira LO, Morshead CM, Corbett D (2016b) Enriched rehabilitation promotes motor recovery in rats exposed to neonatal hypoxia-ischemia. Behav Brain Res 304:42–50 Available at: http://dx.doi.org/10.1016/j.bbr.2016.02.010.

Semple BD, Blomgren K, Gimlin K, Ferriero DM, Noble-Haeusslein LJ (2013) Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. Prog Neurobiol 106–107:1–16 Available at: http://dx.doi.org/10.1016/j.pneurobio.2013.04.001.

Seo JH, Kim H, Park ES, Lee JE, Kim DW, Kim HO, Im SH, Yu JH, Kim JY, Lee M-Y, Kim CH, Cho S-R (2013a) Environmental enrichment synergistically improves functional recovery by transplanted adipose stem cells in chronic hypoxic-ischemic brain injury. Cell Transplant 22:1553–1568 Available at: http://www.ncbi.nlm.nih.gov/pubmed/23394350.

Seo JH, Yu JH, Suh H, Kim MS, Cho SR (2013b) Fibroblast Growth Factor-2 Induced by Enriched Environment Enhances Angiogenesis and Motor Function in Chronic Hypoxic-Ischemic Brain Injury. PLoS One 8.

Shevell MI, Majnemer A, Rosenbaum P, Abrahamowicz M (2001) Etiologic determination of childhood developmental delay. Brain Dev 23:228–235.

Silbereis JC, Huang EJ, Back SA, Rowitch DH (2010) Towards improved animal models of neonatal white matter injury associated with cerebral palsy. Dis Model Mech 3:678–688.

Simonetti T, Lee H, Bourke M, Leamey C a., Sawatari A (2009) Enrichment from birth accelerates the functional and cellular development of a motor control area in the mouse. PLoS One 4.

Simpson J, Kelly JP (2011) The impact of environmental enrichment in laboratory rats-Behavioural and neurochemical aspects. Behav Brain Res 222:246–264 Available at: http://dx.doi.org/10.1016/j.bbr.2011.04.002.

Sizonenko S V., Kiss JZ, Inder T, Gluckman PD, Williams CE (2005) Distinctive neuropathologic alterations in the deep layers of the parietal cortex after moderate ischemic-hypoxic injury in the P3 immature rat brain. Pediatr Res 57:865–872.

Sizonenko S V., Sirimanne E, Mayall Y, Gluckman PD, Inder T, Williams C (2003) Selective cortical alteration after hypoxic-ischemic injury in the very immature rat brain. Pediatr Res 54:263–269.

Smith AL, Alexander M, Rosenkrantz TS, Sadek ML, Fitch RH (2014) Sex differences in behavioral outcome following neonatal hypoxia ischemia: Insights from a clinical meta-analysis and a rodent model of induced hypoxic ischemic brain injury. Exp Neurol 254:54–67 Available at: http://dx.doi.org/10.1016/j.expneurol.2014.01.003.

Sofroniew M V. (2009) Molecular dissection of reactive astrogliosis and glial scar formation. Trends Neurosci 32:638–647 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0166223609001532.

Sonnewald U (2014) Altered astrocyte-neuronal interactions after hypoxia-ischemia in the neonatal brain in female and male rats. Stroke.

Stadlin A, James A, Fiscus R, Wong YF, Rogers M, Haines C (2003) Development of a postnatal 3-day-old rat model of mild hypoxic-ischemic brain injury. Brain Res 993:101–110.

Suff N, Story L, Shennan A (2018) The prediction of preterm delivery: What is new? Semin Fetal Neonatal Med.

Tai W, Burke KA, Dominguez JF, Gundamraj L, Turman JE (2009) Growth

deficits in a postnatal day 3 rat model of hypoxic-ischemic brain injury. Behav Brain Res 202:40–49.

Tang G, Gudsnuk K, Kuo SH, Cotrina ML, Rosoklija G, Sosunov A, Sonders MS, Kanter E, Castagna C, Yamamoto A, Yue Z, Arancio O, Peterson BS, Champagne F, Dwork AJ, Goldman J, Sulzer D (2014) Loss of mTOR-Dependent Macroautophagy Causes Autistic-like Synaptic Pruning Deficits. Neuron 83:1131–1143 Available at: http://dx.doi.org/10.1016/j.neuron.2014.07.040.

Teune MJ, Bakhuizen S, Bannerman CG, Opmeer BC, Van Kaam AH, Van Wassenaer AG, Morris JM, Mol BWJ (2011) A systematic review of severe morbidity in infants born late preterm. Am J Obstet Gynecol 205:374.e1-374.e9 Available at: http://dx.doi.org/10.1016/j.ajog.2011.07.015.

Thorngren-Jerneck K, Ley D, Hellström-Westas L, Hernandez-Andrade E, Lingman G, Ohlsson T, Oskarsson G, Pesonen E, Sandell A, Strand SE, Werner O, Maršal K (2001) Reduced postnatal cerebral glucose metabolism measured by PET after asphyxia in near term fetal lambs. In: Journal of Neuroscience Research, pp 844–850.

Thorngren-jerneck K, Ohlsson T, Sandell A, Erlandsson K, Strand S, Ryding E, Svenningsen NW (2001) Cerebral Glucose Metabolism Measured by Positron Emission Tomography in Term Newborn Infants with Hypoxic Ischemic Encephalopathy. 49:495–501.

Towfighi J, Mauger D, Vannucci RC, Vannucci SJ (1997) Influence of age on the cerebral lesions in an immature rat model of cerebral hypoxia-ischemia: a light microscopic study. Brain Res Dev Brain Res 100:149–160.

Tsuji M, Aoo N, Harada K, Sakamoto Y, Akitake Y, Irie K, Mishima K, Ikeda T, Fujiwara M (2010) Sex differences in the benefits of rehabilitative training during adolescence following neonatal hypoxia-ischemia in rats. Exp Neurol 226:285–292.

Van De Looij Y, Chatagner A, Hüppi PS, Gruetter R, Sizonenko S V. (2011) Longitudinal MR assessment of hypoxic ischemic injury in the immature rat brain. Magn Reson Med 65:305–312.

Vannucci. RC, Vannucci. SJ (2005) Perinatal hypoxic-ischemic brain damage: evolution of an animal model. Dev Neurosci 27.2-4:81–86.

Vannucci RC, Yager JY, Vannucci SJ (1994) Cerebral glucose and energy utilization during the evolution of hypoxic- ischemic brain damage in the immature rat. J Cereb Blood Flow Metab 14:279–288.

Vannucci SJ, Hagberg H (2004) Hypoxia-ischemia in the immature brain. J Exp Biol 207:3149–3154.

Vannucci SJ, Simpson IA (2003) Developmental switch in brain nutrient transporter expression in the rat. Am J Physiol - Endocrinol Metab 285:E1127–E1134 Available at: http://ajpendo.physiology.org/lookup/doi/10.1152/ajpendo.00187.2003.

Verklan T. (2009) The chilling details: Hypoxic-ischemic encephalopathy. J Perinat Neonatal Nurs 23:59–68 Available at: http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L354402520%5Cnhttp://sfx.library.uu.nl/utrecht?sid=EMBASE&issn=08932190&id=doi:&atitle=The+chilling+details%3A+Hypoxic-ischemic+encephalopathy&stitle=J.+Perinat.+Neonatal+Nurs.&title.

Villapol S, Gelot A, Renolleau S, Charriaut-Marlangue C (2008) Astrocyte responses after neonatal ischemia: the yin and the yang. Neuroscientist 14:339–344.

Volpe JJ (2001) Perinatal brain injury: From pathogenesis to neuroprotection. Ment Retard Dev Disabil Res Rev 7:56–64.

Volpe JJ (2005) Encephalopathy of prematurity includes neuronal abnormalities. Pediatrics 116:221–225.

Volpe JJ (2009a) Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. Lancet Neurol 8:110–124 Available at: http://dx.doi.org/10.1016/S1474-4422(08)70294-1.

Volpe JJ (2009b) The Encephalopathy of Prematurity-Brain Injury and Impaired Brain Development Inextricably Intertwined. Semin Pediatr Neurol 16:167–178 Available at: http://dx.doi.org/10.1016/j.spen.2009.09.005.

Vorhees C V, Williams MT (2006) Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nat Protoc 1:848–858.

Wadowska M, Woods J, Rogozinska M, Briones TL (2015) Neuroprotective effects of enriched environment housing after transient global cerebral ischaemia are associated with the upregulation of insulin-like growth factor-1 signalling. Neuropathol Appl Neurobiol 41:544–556.

Wagner AK, Chen X, Kline AE, Li Y, Zafonte RD, Dixon CE (2005) Gender and environmental enrichment impact dopamine transporter expression after experimental traumatic brain injury. Exp Neurol 195:475–483.

Weis SN et al. (2011) Early biochemical effects after unilateral hypoxia-

ischemia in the immature rat brain. Int J Dev Neurosci 29:115–120 Available at: http://dx.doi.org/10.1016/j.ijdevneu.2010.12.005.

Weis SN, Pettenuzzo LF, Krolow R, Valentim LM, Mota CS, Dalmaz C, Wyse ATS, Netto CA (2012) Neonatal hypoxia-ischemia induces sex-related changes in rat brain mitochondria. Mitochondrion 12:271–279 Available at: http://dx.doi.org/10.1016/j.mito.2011.10.002.

Welberg L, Thrivikraman K V., Plotsky PM (2006) Combined pre- and postnatal environmental enrichment programs the HPA axis differentially in male and female rats. Psychoneuroendocrinology 31:553–564.

Wood NI, Sopesen B V., Roberts JC, Pambakian P, Rothaul AL, Hunter AJ, Hamilton TC (1996) Motor dysfunction in a photothrombotic focal ischaemia model. Behav Brain Res 78:113–120.

World Health Organization (2012) Born Too Soon: The global Action Report on Preterm Birth. J Perinatol 13:393–396.

Yager JY (2004) Animal models of hypoxic-ischemic brain damage in the newborn. Semin Pediatr Neurol 11:31–46.

Yager JY, Ashwal S (2009) Animal models of perinatal hypoxic-ischemic brain damage. Pediatr Neurol 40:156–167 Available at: http://dx.doi.org/10.1016/j.pediatrneurol.2008.10.025.

Yang SN, Lai MC (2011) Perinatal hypoxic-ischemic encephalopathy. J Biomed Biotechnol.

Yu K, Wu Y, Zhang Q, Xie H, Liu G, Guo Z, Li F, Jia J, Kuang S, Hu R (2014) Enriched environment induces angiogenesis and improves neural function

outcomes in rat stroke model. J Neurol Sci 347:275–280.

Yu S (2006) Review of F-FDG Synthesis and Quality Control. Biomed Imaging Interv J 2:e57.

Zanirati G, Azevedo PN, Venturin GT, Greggio S, Alcará AM, Zimmer ER, Feltes PK, DaCosta JC (2018) Depression comorbidity in epileptic rats is related to brain glucose hypometabolism and hypersynchronicity in the metabolic network architecture. Epilepsia 59:923–934.

Zhang M, Wu J, Huo L, Luo L, Song X, Fan F, Lu Y, Liang D (2016) Environmental Enrichment Prevent the Juvenile Hypoxia-Induced Developmental Loss of Parvalbumin-Immunoreactive Cells in the Prefrontal Cortex and Neurobehavioral Alterations Through Inhibition of NADPH Oxidase-2-Derived Oxidative Stress. Mol Neurobiol 53:7341–7350.

Zhang X, Chen XP, Lin J Bin, Xiong Y, Liao WJ, Wan Q (2017) Effect of enriched environment on angiogenesis and neurological functions in rats with focal cerebral ischemia. Brain Res 1655:176–185.

Zhang ZG, Zhang L, Jiang Q, Zhang R, Davies K, Powers C, Van Bruggen N, Chopp M (2000) VEGF enhances angiogenesis and promotes blood-brain barrier leakage in the ischemic brain. J Clin Invest 106:829–838.

Zhao W, Xie W, Xiao Q, Beers DR, Appel SH (2006) Protective effects of an anti-inflammatory cytokine, interleukin-4, on motoneuron toxicity induced by activated microglia. J Neurochem 99:1176–1187.

Zheng H, Dong Y, Xu Z, Crosby G, Culley DJ, Zhang Y, Xie Z (2013) Sevoflurane anesthesia in pregnant mice induces neurotoxicity in fetal and

offspring mice. Anesthesiology 29:997–1003.

Zhu C, Xu F, Wang X, Shibata M, Uchiyama Y, Blomgren K, Hagberg H (2006) Different apoptotic mechanisms are activated in male and female brains after neonatal hypoxia-ischaemia. J Neurochem 96:1016–1027.

Zhu L-L, Zhao T, Li H-S, Zhao H, Wu L-Y, Ding A-S, Fan W-H, Fan M (2005) Neurogenesis in the adult rat brain after intermittent hypoxia. Brain Res 1055:1–6.

Zhu LH, Bai X, Zhang N, Wang SY, Li W, Jiang L (2014) Improvement of human umbilical cord mesenchymal stem cell transplantation on glial cell and behavioral function in a neonatal model of periventricular white matter damage. Brain Res.

Zimmer ER, Parent MJ, Souza DG, Leuzy A, Lecrux C, Kim HI, Gauthier S, Pellerin L, Hamel E, Rosa-Neto P (2017) [18F]FDG PET signal is driven by astroglial glutamate transport. Nat Neurosci 20:393–395.

Zuena AR, Zinni M, Giuli C, Cinque C, Alemà GS, Giuliani A, Catalani A, Casolini P, Cozzolino R (2016) Maternal exposure to environmental enrichment before and during gestation influences behaviour of rat offspring in a sex-specific manner. Physiol Behav 163:274–287.