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

ENRIQUECIMENTO AMBIENTAL COMO ESTRATÉGIA NEUROPROTETORA EM RATOS SUBMETIDOS À HIPÓXIA-ISQUEMIA NEONATAL

TESE DE DOUTORADO

JOSEANE JIMÉNEZ ROJAS

Porto Alegre, 2015

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Tese de doutorado apresentada
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Dedicatória

Dedico esta tese ao meu melhor amigo de toda a vida, meu pai, José Orlando, a quem tantas vezes pedi para que me desse forças para terminar esta etapa.
Pai, em mim o senhor é eterno, nos meus filhos e nos filhos dos meus filhos, pelo teu exemplo e os teus valores.

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“Caminante, son tus huellas el camino y nada más

Caminante no hay camino, se hace camino al andar

Al andar se hace el camino y al volver la vista atrás

Se ve la senda que nunca se ha de volver a pisar

Caminante no hay camino sino estelas en la mar.”

Joan Manuel Serrat

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Artigo 1

LISTA DE ABREVIATURAS

AMPA: α-amino-3–hidroxi-5-metil-4-isoxazol-propionato

ANOVA: Análise da variância

AOI: Área de interesse (imunofluorescência)

ATP: Trifosfato de adenosina

BDNF: Fator neurotrófico derivado do encéfalo

CA1: Região do corno de Amón (hipocampo)

CAT: Catalase

CTAE: Grupo controle exposto a ambiente enriquecido

CTAP: Grupo controle exposto a ambiente padrão

CTEE: Grupo controle exposto a ambiente enriquecido

CTSE: Grupo controle exposto a ambiente padrão

DNA: Ácido desoxirribonucléico

EA: Enriquecimento ambiental

EE: Enriquecimento ambiental

EDTA: Ácido etilenodiamino tetra-acético

ER: Retículo endoplasmático

EROs: Espécies reativas de oxigênio

ERNs:Espécies reativas de nitrogênio

GFAP: Proteína glial fribrilar ácida

GPx: Glutationa peroxidase

HEPES: Ácido N-(2-hidroxietil)piperazina-N'-2-etanossulfónico

HI: Hipóxia-isquemia

HIAP: Grupo submetido à hipóxia-isquemia mantido em ambiente padrão

HIAE: Grupo submetido à hipóxia-isquemia exposto a ambiente enriquecido

H_2O_2: Peróxido de Hidrogênio

mGlu: Receptor metabotrópico de glutamato;

NMDA: *N*-metil-*D*-Aspartato;

NADPH: Nicotinamida adenina dinueleotídeo fosfato

NO: Óxido Nítrico

NOS: Óxido Nítrico sintase

NOO^-: Peroxinitrito

O_2^-: Íon superóxido

OH^-: Radical hidroxil

PBS: Tampão fosfato salino

PFA: Paraformolaldeído

PND: Dia pós-natal

SOD: Superóxido dismutase

VDCC: Canais de cálcio dependentes de voltagem.

RESUMO

A hipóxia-isquemia (HI) é a principal causa de mortalidade no período perinatal e, nos sobreviventes, a incidência de comorbidades neurológicas é elevada. O encéfalo imaturo, altamente susceptível ao insulto hipóxico-isquêmico, é bastante sensível a estímulos ambientais tais como o enriquecimento ambiental (EA). Os objetivos deste estudo foram: 1) investigar o desempenho comportamental em um novo teste de memória e aprendizagem, o Ox-maze; 2) analisar a atividade das enzimas Na^+,K^+-ATPase, catalase (CAT) e glutationaperoxidase (GPx) no hipocampo; 3) caracterizar os neurônios piramidais da região CA1 hipocampal quanto à arborização dendrítica; 4) analisar alterações astrocíticas e sinápticas pela avaliação da imunoreatividade das proteínas GFAP e sinaptofisina usando a técnica de imunofluorescência e, 5) quantificar a densidade celular por meio de cortes semifinos da região CA1 do hipocampo de animais hipóxico-isquêmicos expostos a um ambiente enriquecido. Ratos com sete dias de idade foram divididos em quatro grupos e submetidos ou não ao procedimento cirúrgico de acordo com o grupo experimental ao qual pertenciam: controle mantido em ambiente padrão (CTAP), controle em ambiente enriquecido (CTAE), HI em ambiente padrão (HIAP) e HI em ambiente enriquecido (HIAE). Passado o período de EA (1h/dia, 6 dias/semana, 9 semanas iniciando após o desmame), os parâmetros mencionados foram avaliados nos animais. Os dados indicaram que a HI causou um prejuízo na memória e no aprendizado no teste do “OX-maze”, o qual foi revertido pelo efeito do ambiente enriquecido. A HI causou diminuição da atividade enzimática da Na^+,K^+-ATPase no hipocampo contralateral, assim como uma redução na imunorreatividade à sinaptofisina e nadensidade neuronal, sendo

que o EA foi efetivo na recuperação da atividade da enzima Na^+,K^+-ATPase e dos níveis de sinaptofisina no hipocampo contralateral à lesão.

As atividades de CAT e GPX não foram alteradas pela HI em nenhum dos grupos avaliados, mesmo resultado encontrado nas análises de GFAP e de padrão de arborização dendrítica. Por fim, neste estudo foi observado o importante efeito lesivo causado pela HI neonatal e o papel do EA como estratégia neuroprotetora na recuperação funcional, na atividade da Na^+,K^+-ATPase e na expressão de sinaptofisina. Este estudo traz avanços em busca dos mecanismos pelos quais a melhora funcional ocorre em animais HI expostos ao EA, mas pode-se verificar que não fica totalmente esclarecido como esta estratégia atua. Outros estudos são necessários para a identificação de possíveis mecanismos que atuem como mediadores da resposta funcional do EA após um evento isquêmico.

Palavras-chave: Hipóxia-isquemia, estimulação ambiental, plasticidade, densidade celular, área CA1 hipocampal, sinaptofisina, GFAP, Na^+,K^+-ATPase, enzimas antioxidantes.

ABSTRACT

Hypoxia-ischemia (HI) is the main mortality cause in perinatal period and, in survivors, the incidence of neurological disabilities is elevated. The immature brain, highly susceptible to hypoxic-ischemic insult, is sensible to environmental stimuli, as environmental enrichment (EE). The aims of this study were to investigate: 1) behavioral performance in a new memory and learning task, the ox-maze task; 2) evaluate Na^+,K^+-ATPase, catalase (CAT) and glutathione peroxidase (GPx) activities in the hippocampus; 3) characterizes dendritic arbor in pyramidal neurons from CA1 region from hippocampus; 4) analyze alterations in hippocampal synaptophysin and GFAP immmunoreactivity and, 5) analyze neuronal density alterations in hippocampus of hypoxic-ischemic rats exposed to enriched environment. Seven-day-old rats were divided into four groups: controlmaintained in standard environment (CTSE), control submitted to EE (CTEE), HI in standard environment (HISE) and HI in EE (HIEE). Past the end of EE period (1 hour/day, 6 days/week, 9 weeks), mentioned parameters were evaluated in animals. Present results indicate learning and memory in the “OX-maze” task were impaired in HI rats and this effect was recovered after EE. On the contralateral hemisphere, HI caused a decrease in Na^+,K^+-ATPase activity that was recovered by EE. Results also indicate that HI damage decreases hippocampal synaptophysin immunoreactivity and neuronal density, moreover EE was effective in recovering synaptophysin levels on contralateral to the lesion hippocampus. The activities of GPx and CAT were not changed by HI in any group evaluated, some result founded on GFAP immunoreactivity and dendritic arborization characterization analysis. In conclusion, the important effect of HI

lesion and the role of EE like neuroprotective strategy on functional impairment and on Na^+,K^+-ATPase activity and synaptophysin immunoreactivity was proven. Although this study have important advances in search of mechanisms by which the functional enhancement occurs in the animals submitted to HI and exposed to EE, it can be seen that it is not completely clear how this approach works. Further studies are needed to identify possible mechanisms that act as mediators of EE functional response after an ischemic event.

Key-words: Neonatal hypoxia-ischemia, environmental stimulation, plasticity, neuronal density, CA1 activity, antioxidant enzymes. hippocampal area, synaptophysin, GFAP, Na^+,K^+-ATPase.

1. INTRODUÇÃO

1.1 Hipóxia-Isquemia neonatal

Nos últimos anos a temática da encefalopatia hipóxico-isquêmica vem despertando interesse tanto científico quanto social, uma vez que ela é considerada a principal causa de dano encefálico em neonatos (Ferriero, 2004). Sua incidência é de 1 a 1,5% dos nascidos vivos em países desenvolvidos (Hagmann*et al*., 2011), embora sua prevalência seja ainda mais significativa em países em vias de desenvolvimento, como o Brasil (Cruz e Ceccon, 2010). Na Índia, país que compartilha características epidemiológicas com o Brasil, a prevalência relatada foi de 5% nos pacientes neonatos, sendo que a incidência de encefalopatia é de 50-60% em crianças com asfixia perinatal grave (MillionDeathStudyCollaborators*et al*., 2010). Entre as crianças com hipóxia-isquemia (HI) encefálica grave, 50% morrem, enquanto o restante desenvolve déficits neurológicos. Nos casos de HI moderada, de 10 a 20% das crianças morrem, sendo que 30 a 40% desenvolvem anormalidades neurológicas (García-Alix*et al*., 2009). Consequências em longo prazo, incluindo paralisia cerebral, déficits cognitivos, transtornos convulsivos, déficits intelectuais e problemas comportamentais costumam ser devastadoras e prejudicam expressivamente as atividades da vida diária (Weitzdoerfer*et al*., 2004; Vannucci e Hagberg, 2004;Volpe, 2008;Martinez-Biarge*et al*. 2012; Tata *et al*., 2015).

1.1.1 Fisiopatologia da Hipóxia-Isquemia neonatal

A encefalopatia hipóxico-isquêmica pode ser consequência de condições patológicas maternas prévias ao parto, obstétricas e/ou do próprio concepto devido à falta de oxigênio, completa ou parcial, nos tecidos corporais (hipoxemia) e à isquemia, a qual é definida como a diminuição da perfusão sanguínea encefálica (Boskabadi *et al*., 2015). Alterações metabólicas decorrentes de tais eventos, que induzem alterações bioquímicas, biofísicas e fisiológicas, caracterizam a encefalopatia hipóxico-isquêmica (Rotta, 2002).

Os modelos animais são bastante utilizados quando se busca conhecer mais profundamente os mecanismos de dano, compreendendo sua evolução e seus desfechos, além de servir como modelo de desenvolvimento de novas estratégias terapêuticas. É importante salientar que, ainda assim, nenhum modelo animal replica todos os aspectos das condições em seres humanos. Ainda que existam limitações, os modelos animais fornecem um meio viável para o conhecimento das desordens do sistema nervoso (Wilson, 2015). Visando mimetizar o insulto hipóxico-isquêmico ocorrido em humanos, o modelo proposto por Rice e colaboradores (1981), adaptado do procedimento de Levine (1960) em ratos adultos, é muito comumente utilizado (Levine, 1960; Rice *et al*., 1981; Vanucci e Vanucci, 1997; Vanucci e Vanucci, 2005).

Numerosos estudos utilizando o modelo de Levine-Rice demonstraram que animais com esse tipo de lesão apresentam déficits cognitivos envolvendo a memória de referência, espacial, de trabalho e aversiva (Ikeda *et al*, 2001; Alsher, 2002; Arteni *et al*., 2003; Pereira *et al*., 2007; Carletti *et al*., 2012; Rojas *et al*., 2013). Ademais dos déficits cognitivos, roedores submetidos à HI

neonatal já tiveram descritos déficits sensório-motores no teste do rota-rod (Jansen e Low, 1996), teste de reflexo postural, teste de erros por passo, teste de preensãoe no teste de posicionamento das patas (Bona *et al*., 1997; Drunalini Perera *et al*., 2014). As alterações comportamentais causadas pelo modelo de Levine-Rice estão frequentemente associadas a mudanças morfológicas, muitas delas descritas no hipocampo, como por exemplo, morte de neurônios piramidais da região CA1 (Kirino e Sano, 1984; Pulsinelli *et al*., 1982), diminuição da densidade de espinhos dendríticos e da arborização dendrítica (Ruan*et al*., 2009; Rojas *et al*., 2013), atrofia do hipocampo, córtex sensório-motor e estriado (Jansen e Low, 1996; Rodrigues *et al*., 2004; Pereira *et al*., 2007).

A patogênese da lesão encefálica perinatal é bastante complexa, envolvendo múltiplas vias e mecanismos de lesão (Damman *et al*., 2011). Sabe-se que múltiplos eventos deletérios interligados estão envolvidos na patogênese do dano hipóxico-isquêmico, tais como a falência energética, a excitotoxicidade, o estresse oxidativo e a inflamação, os quais ocorrem simultaneamente e contribuem para a disfunção e a morte neuronal (Vexler e Ferriero, 2001; Hossain, 2005; Liu e McCullough *et al*., 2013; Chen *et al*., 2015) (Figura 1).

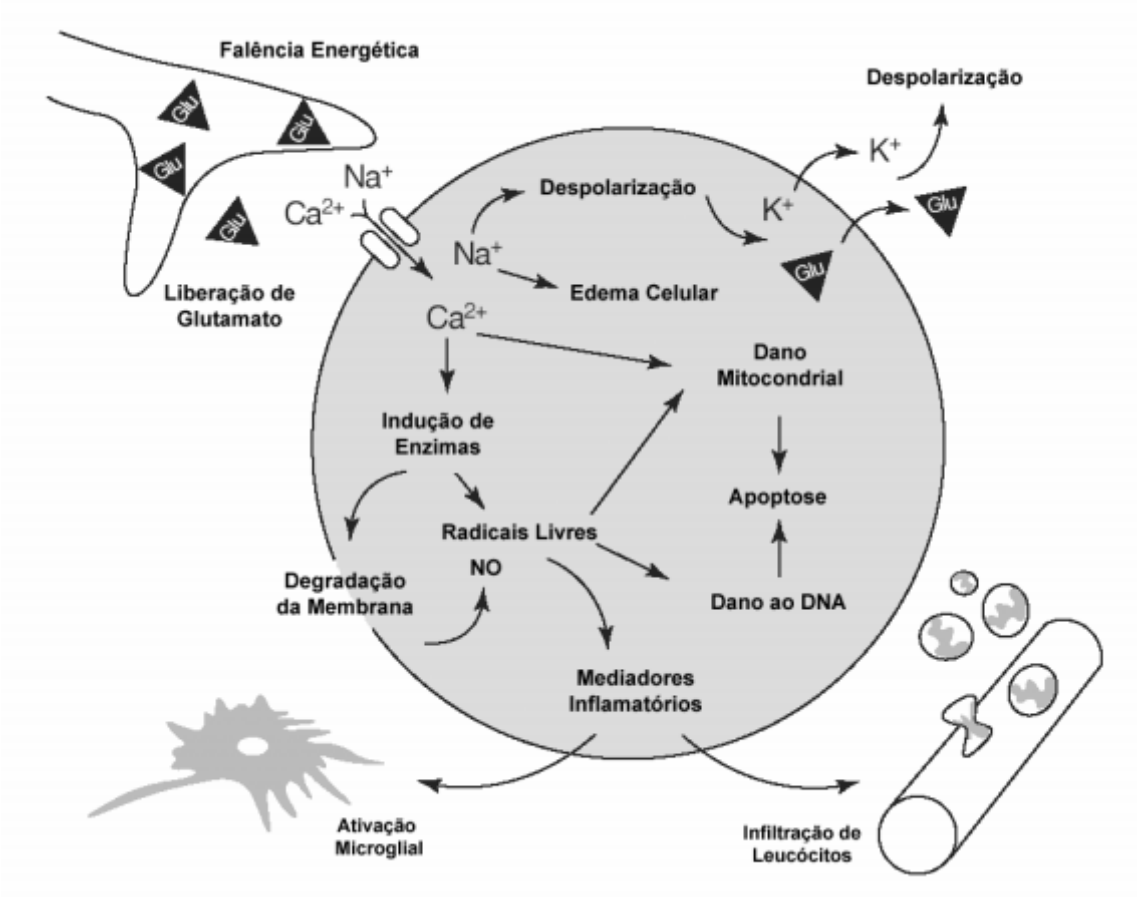

Figura 1. Visão geral dos mecanismos fisiopatológicos ocorrendo na hipóxia-isquemia (modificado de Dirnagl *et al*., 1999).

A redução do fluxo sanguíneo cerebral desencadeia uma série de eventos bioquímicos deletérios que podem durar de horas a semanas (Perlman, 2006). A depleção do oxigênio impossibilita a fosforilação oxidativa, ocorrendo uma mudança para o metabolismo anaeróbio, estado energético ineficiente que resulta na rápida diminuição das reservas de fosfato de alta energia, incluindo a adenosina trifosfato (ATP). Essa diminuição de ATP ocasiona uma modificação no equilíbrio iônico através das membranas celulares (Perlman, 2006; Golan e Huleihel, 2006). Com a exaustão das reservas energéticas durante a hipóxia, ocorre falência nos mecanismos de manutenção dos potenciais de membrana normais (Busl e Greer, 2010). A glicólise anaeróbia é insuficiente para manter

a produção de ATP, não havendo, então, energia suficiente para manter as funções encefálicas, ocorrendo desativação das bombas iônicas, despolarização das membranas e abertura dos canais iônicos dependentes de voltagem (Dugan e Shoi, 1999). Acontece, assim, uma diminuição da atividade da bomba de Na^+,K^+-ATPase que resulta em edema celular e ação citotóxica. (Desclou*x et al*., 2015). A enzima Na^+,K^+-ATPase é de extrema importância para a sobrevivência neural, pois sua principal função é a manutenção do gradiente iônico necessário para a excitabilidade neuronal (Xiao*et al*., 2002). Declínios na sua expressão são indicativo de distúrbios na homeostase iônica que podem conduzir à morte neuronal (Siesjo, 1988).

Outro processo implicado no dano neuronal pós HI é a excitotoxicidade, na maior parte das vezes consequente à despolarização da membrana, resultando em liberação de neurotransmissores excitatórios, mais especificamente o glutamato (Folletet al., 2004; Volpe, 2005).O glutamato, por sua vez, ativa os receptores NMDA (N-metil-D-aspartato), AMPA (a-amino-3-hidroxi-5-metil-4-isoxazol propionato) e cainato(Perlman, 2004; Hossain, 2005; Perlman, 2006). Falhas na recaptação de glutamato combinadas com excessiva liberação deste neurotransmissor conduzem a um acúmulo do mesmona fenda sináptica,superativação dos receptores e entrada massiva de cálcio para o meio intracelular (Lau e Tymianski, 2010). O aumento da concentração de cálcio intracelular desencadeia a ativação de uma série de enzimas que conduzem ao dano celular por numerosos mecanismos, incluindo formação de espécies reativas de oxigênio (EROs) e de nitrogênio (ERNs), destruição de membranas e danos às organelas e ao DNA (Sattler e Tymianski, 2000). É importante mencionar que o encéfalo imaturo dos neonatos é

particularmente vulnerável à excitotoxicidade glutamatérgica (Rice et al., 1981). Tal característica se deve ao fato de que durante o desenvolvimento encefálico existe maior densidade de receptores NMDA (McDonaldet al., 1988), que são facilmente ativados devido a sua alta permeabilidade ao cálcio e maior capacidade de resposta à ativação pela glicina em comparação com outros receptores (Gurdet al., 2002; Hossain, 2005; Johnston, 2005).

Como já mencionado, a ação de aminoácidos excitatórios, como o glutamato, é mediada pelo NMDA, o qual parece ser essencial para os mecanismos de lesão cerebral hipóxico-isquêmica, uma vez que apresenta sítios modulatórios que exercem um influxo altamente regulado de cálcio pelos canais iônicos (Du Plessis e Johnston, 1997). A atividade do complexo canal iônico-receptor NMDA pode ser modulada pelo íon magnésio (Mg^{2+}) devido a sua capacidade de bloquear o canal iônico, ao qual o receptor NMDA está acoplado (Nowak*et al*., 1984). Porém, também foi descrito que níveis excitotóxicos de glutamato causam uma remoção deste bloqueio, provavelmente como consequência da despolarização mediada pelos receptores AMPA (Clerc *et al*., 2013).

Todo o exposto é crucial para favorecer a plasticidade sináptica e maturação encefálica, mas por outro lado rende neurônios mais suscetíveis ao dano excitotóxico (Descloux *et al*., 2015). Um bom marcador quando se deseja avaliar a plasticidade sináptica é a sinaptofisina, uma proteína vesicular pré-sináptica presente nas terminações nervosas e usada amplamente na identificação dos locais de sinapses (Walaas *et al*., 1988).

Outro importante mecanismo fisiopatológico envolvido é o estresse oxidativo, processo este muito relacionado à excitotoxidade, uma vez que o

influxo excessivo de cálcio pode ativar diferentes enzimas envolvidas na produção de EROs, como a óxido nítrico sintetase ou a xantina e a NADPH (dinucleotídeo de adenina nicotinamida fosfato) oxidase (Tataranno *et al*., 2015). A disfunção da fosforilação oxidativa mitocondrial é a principal causa de formação excessiva de espécies reativas de O_2 e H^+ [superóxido (O_2^-), peróxido de hidrogênio (H_2O_2), óxido nítrico (NO), radical hidroxil (OH^-) e peroxinitrito (NOO^-)] (Descloux *et al*., 2015). As espécies reativas de oxigênio são extremamente tóxicas para a sobrevivência celular, uma vez que os sistemas de defesa naturais se encontram sobrecarregados na HI (Pereira *et al*., 2009). O acúmulo de EROs desencadeia uma série de eventos citotóxicos, incluindo nitrosilação e oxidação proteica, peroxidação lipídica e dano ao DNA (Southorn e Powis, 1988). Um importante mecanismo de defesa é a dismutação do superóxido (O_2^-) para formar peróxido de hidrogênio (H_2O_2) e O_2 através da enzima superóxido dismutase (SOD), bem como a transformação de H_2O_2 a H_2O pela enzima glutationaperoxidase (GPx) ou a O_2 e H_2O pela enzima catalase (CAT), prevenindo assim a formação do radical hidroxil (OH). Tanto GPx quanto CAT exercem um papel antioxidante fudnamental, atuando junto à SOD na proteção antioxidante celular (Southorn e Powis, 1988; Halliwell e Gutteridge, 1990; McCord, 1993). É importante destacar que a SOD é efetiva particularmente quando acompanhada de mudanças também nas atividades das enzimas catalase e glutationaperoxidase (Halliwell, 2001) (Figura 2).

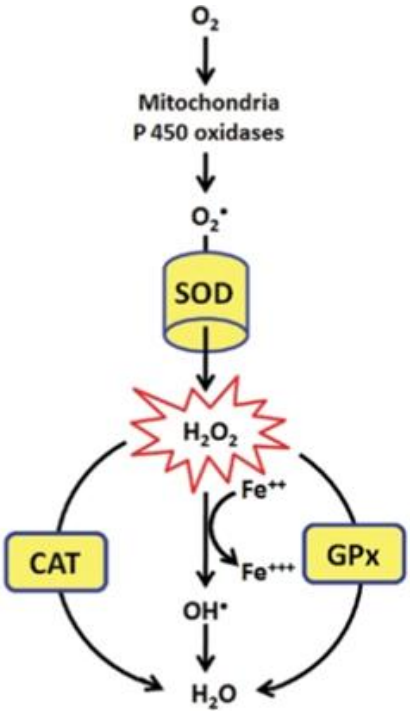

Figura 2. Atividade das enzimas catalase e glutationa peroxidase (Pandey e Risvi, 2010).

Ademais, a HI ocasiona um aumento na permeabilidade da barreira hematoencefálica devido à ativação da inflamação por meio da liberação de citocinas e quimiocinas (a partir de astrócitos reativos, microglia ativada, neurônios e células endoteliais) que estão envolvidas no recrutamento leucocitário ao local da lesão (Pun *et al*., 2009; Baburamani *et al*., 2012).

Sabe-se também que os astrócitos se tornam reativos após um evento isquêmico (Theodosis *et al*., 2008). Essa astrogliose reativa ocorre em paralelo com alterações neuronais (Spolidoro *et al*., 2008), prejudicando a recuperação funcional (Roy Choudhury *et al*, 2014). Sizonenko e colaboradores (2008) relataram alteração da arquitetura glial e proliferação aguda de astrócitos após a HI. A proteína fibrilar glial ácida (GFAP) é expressa em astrócitos maduros e sua detecção pode ser utilizada como um dos principais marcadores astrocíticos no SNC (Taft *et al*., 2005).

Os eventos lesivos mencionadosque ocorrem após a HI acabam conduzindo à morte neuronal. Os mecanismos de morte neuronal após a HI incluem necrose e apoptose (Northington *et al*., 2011). A morte celular por necrose representa o mecanismo precoce de morte no contexto da isquemia encefálica e da excitotoxicidade, uma vez que está diretamente associada com o influxo massivo de sódio e cálcio ao citoplasma neuronal (Northington *et al*., 2011). A necrose desempenha um papel central nas lesões encefálicas agudas, como tem sido demonstrado em muitos modelos animais adultos (Mehta *et al*., 2007), mas também em modelos de HI neonatal (Nakajima *et al*, 2000; Northington *et al*., 2001; Carloni *et al*, 2007). A morte celular por necrose, caracterizada por edema celular que pode levar à lise da célula, é visualizada em uma fase precoce após o insulto, em até 30 minutos, sendo desencadeada pela falência celular energética abrupta com diminuição notável de ATP (Neumar *et al*., 2001). Microscopicamente, a necrose se caracteriza por dilatação das organelas, vacuolização do citoplasma e fragmentação nuclear (Clarke, 1990; Puyal *et al*., 2013). Características apoptóticas também são observadas em danos encefálicos cujo mecanismo fisiopatológico envolve a excitotoxicidade, como são a isquemia cerebral e a asfixia neonatal (Northington *et al*., 2011; Puval *et al*., 2013), inclusive em casos de HI encefálica em humanos (Tanigushi *et al*., 2007). Como já está bem descrito na literatura, a apoptose é um mecanismo de morte mais tardio e programado (Thornton *et al*., 2012).

Normalmente, a HI grave causa lesões encefálicas em regiões que compreendem porções específicas do hipocampo, núcleos da base, tálamo, tronco encefálico e substância branca periventricular e subcortical em roedores

(Vannucci, 2000). Alterações em neurônios e células gliais causadas pelo infarto em um território vascular específico são características da lesão cerebral isquêmica multifocal/focal (Volpe, 2000). No entanto, a justaposição de populações neuronais relativamente resistentes ou vulneráveis ao dano hipóxico-isquêmico dentro de um mesmo território vascular sugere a existência de fatores de vulnerabilidade específicos. Os neurônios piramidais região CA1 hipocampal, células de Purkinje do cerebelo e subpopulações da amígdala, e estriado, são conhecidamente mais vulneráveis ao insulto hipóxico-isquêmico (Siegel *et al*., 2006).

1.2 Enriquecimento ambiental

As estratégias que buscam reduzir os efeitos deletérios após lesões encefálicas são conhecidas como estratégias neuroprotetoras. A proteção neural contra qualquer dano causado por qualquer tipo de trauma, por exemplo, acidente vascular encefálico ou doenças neurodegenerativas, tem sido foco de muitas pesquisas na atualidade (Burnstock, 2015). A neuroproteção não necessariamente há de ser farmacológica, definindo-se como uma intervenção que busca, no caso da HI neonatal, resgatar a área de hipoperfusão circundante à área isquêmica, ainda viável, assim como estimular a plasticidade em regiões não diretamente acometidas, como, por exemplo, o encéfalo contralateral à lesão (Rosenzweig e Bennett, 1996). Algumas formas de neuroproteção não farmacológica se encontram em estudo, como são a hipotermia (Davidson *et al*., 2015; Kasdorf *et al*., 2015), o exercício físico (Tsuji

et al., 2010; Marcelino *et al*., 2015) e o enriquecimento ambiental (EA), forma de neuroproteção abordada nesta tese.

O objetivo principal do EA é proteger o encéfalode roedores, ainda em desenvolvimento, das consequências adversas do isolamento social (Rosenzweig e Bennett, 1996). Ademais, sabe-se que o EA é capaz de amenizar desordens causadas em fases precoces do desenvolvimento como o estresse pré-natal e a exposição a drogas como a cocaína e a metais pesados (Ahmadalipour *et al*., 2015). Esta estratégia é descrita como a combinação de estímulos inanimados e sociais proporcionando aos animais várias interações, sejam elas cognitivas, motoras ou sensoriais com o ambiente (Rosenzweig *et al*., 1978). Hebb, em 1949, foi o primeiro a descrever o EA utilizando animais de laboratório e, desde então, vários estudos tem demonstrado a utilidade do EA para o estudo de diferentes transtornos encefálicos como doença de Alzheimer (Jankowsky *et al*., 2005, Pietropaolo *et al*., 2014), doença de Parkinson (Bezard *et al*., 2013) e doença de Huntington (Nithianantharajah *et al*. 2008; Mo *et al*., 2015). Experimentalmente, o EA é basicamente composto por animais que vivem juntos em grandes gaiolas com objetos de diferentes tamanhos, formas e texturas, os quais são alternados periodicamente a fim de estimular a curiosidade e a exploração (Nithianantharajah e Hannan, 2006; Sozda *et al*., 2010; Simpson e Kelly, 2011).

Estudos demonstram que o EA está associado a uma série de alterações anatômicas e histológicas (Rosenzweig e Bennet, 1996), como estimulação da neurogênese (Van Praag *et al*., 2000; Kempermann, 2002), aumento da espessura cortical e da arborização dendrítica em neurônios piramidais da região CA1 hipocampal (Greenough *et al*., 1973), aumento dos

níveis de sinaptofisina no hipocampo (Lambert *et al*., 2005; Marquez *et al*., 2014) e aumento da densidade de espinhos dendríticos e mudanças na morfologia dos espinhos em hipocampo (Diamond *et al*., 1976; Leggio *et al*., 2005; Rojas *et al*., 2013). Além disso, sabe-se que o hipocampo é uma das áreas encefálicas mais suscetíveis aos efeitos benéficos da exposição ao enriquecimento ambiental (Meaney e Aitken, 1985), como já mencionado anteriormente. Ante ao exposto, o EA apresenta efeitos benéficos em distintos modelos experimentais, e em diferentes faixas etárias nos animais testados. No entanto, uma vez que não há dúvidas quanto à eficácia do EA como recurso não farmacológico de neuroproteção ou de reabilitação, a extensão da "janela" terapêutica (tempo de duração) para a aplicação do EA ainda é algo que precisa ser investigado.

Apesar do comprovado papel neuroprotetor do enriquecimento ambiental, estudos associando-o à encefalopatia hipóxico-isquêmica ainda são escassos. Primeiramente foi demonstrado que a atrofia de hipocampo causada pela HI não é revertida pela exposição a dois diferentes protocolos de ambientes enriquecidos, embora os déficits na memória espacial tenham apresentado reduções (Pereira *et al*., 2007; Pereira *et al*., 2008). Em um estudo subsequente, foi identificado um déficit na memória declarativa, avaliada no teste de reconhecimento de objetos, nos animais hipóxico-isquêmicos; tal prejuízo foi revertido pela exposição ao ambiente enriquecido (Rojas *et al*., 2013). Neste mesmo estudo, foi avaliada a densidade de espinhos dendríticos na região CA1 hipocampal, a qual estava diminuída pela HI e tal dano foi revertido pelo EA (Rojas *et al*., 2013).

Os estudos verificando os efeitos do enriquecimento ambiental sobre a HI neonatal ainda são insuficientes e se restringem aos realizados pelo nosso grupo de pesquisa (Pereira *et al*., 2007, 2008 e 2009; Rojas *et al*., 2013 e 2015). Esse fato causa certa estranheza uma vez que a incidência e a morbi-mortalidade dessa doença é bastante elevada, deixando crianças incapacitadas por toda a vida. Por outro lado, também se sabe que a estimulação em crianças pode exercer uma função importante na recuperação de funções sensório-motoras e na plasticidade encefálica. É importante aprofundar os conhecimentos quanto aos possíveis mecanismos envolvidos na melhora tanto funcional quanto histológica provocada pelo EA, buscando assim,por meio de um modelo experimental, prover resultados que sirvam como justificativa para a aplicação clínica desta terapia neuroprotetora. Nos capítulos seguintes desta tese (mais especificamente nos dois artigos) se encontra exposto muito do que foi citado nesta introdução com o objetivo de prover uma melhor compreensão para aqueles que tiverem acesso exclusivamente aos artigos de forma isolada.

2. OBJETIVOS

2.1 Objetivo Geral

O objetivo geral deste trabalho foi verificar os efeitos terapêuticos do enriquecimento ambiental na memória espacial e em parâmetros bioquímicos e histológicosdo hipocampo de ratos submetidos ao procedimento de hipóxia-isquemia encefálica neonatal.

2.2 Objetivos Específicos

Artigo 1:

-Caracterizar o desempenho de ratos Wistar em um novo teste de memória *("*OX-maze*"*) verificando os efeitos da HI neonatal e do enriquecimento ambiental;

-Estudar os efeitos do EA sobre a atividade das enzimas Na^+,K^+-ATPase, catalase e glutationaperoxidase, avaliadas por meio de ensaios bioquímicos, no hipocampo de ratos submetidos ao procedimento de HI neonatal.

Artigo 2:

- Analisar alterações astrocíticas e sinápticas pela avaliação da porcentagem de área ocupada pela marcação da imunoreatividade das proteínas GFAP e sinaptofisina utilizando-nos da técnica de imunofluorescência, verificando os efeitos da HI neonatal e do enriquecimento ambiental;

- Analisar alterações na densidade celular pela quantificação neuronal em cortes semifinos da região CA1 do hipocampo de animais hipóxico-isquêmicos expostos a ambientes enriquecidos utilizando microscopia óptica;

- Caracterizar o padrão de arborização dendríticautilizando a técnica de Golgi em neurônios piramidais da região CA1 hipocampal de animais submetidos à HI neonatal e expostos aoEA.

3. ARTIGO 1

Environmental stimulation improves performance in the ox-maze task and recovers Na^+,K^+-ATPase activity in the hippocampus of hypoxic-ischemic rats.

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ENVIRONMENTAL STIMULATION IMPROVES PERFORMANCE IN THE OX-MAZE TASK AND RECOVERS Na^+,K^+-ATPASE ACTIVITY IN THE HIPPOCAMPUS OF HYPOXIC–ISCHEMIC RATS

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Abstract—In animal models, environmental enrichment (EE) has been found to be an efficient treatment for alleviating the consequences of neonatal hypoxia-ischemia (HI). However the potential for this therapeutic strategy and the mechanisms involved are not yet clear. The aim of present study is to investigate behavioral performance in the ox-maze test and Na^+,K^+-ATPase, catalase (CAT) and glutathione peroxidase (GPx) activities in the hippocampus of rats that suffered neonatal HI and were stimulated in an enriched environment. Seven-day-old rats were submitted to the HI procedure and divided into four groups: control maintained in standard environment (CTSE), control submitted to EE (CTEE), HI in standard environment (HISE) and HI in EE (HIEE). Animals were stimulated with EE for 9 weeks (1 h/day for 6 days/week) and then behavioral and biochemical parameters were evaluated. Present results indicate learning and memory in the ox-maze task were impaired in HI rats and this effect was recovered after EE. Hypoxic–ischemic event did not alter the Na^+,K^+-ATPase activity in the right hippocampus (ipsilateral to arterial occlusion). However, on the contralateral hemisphere, HI caused a decrease in this enzyme activity that was recovered by EE. The activities of GPx and CAT were not changed by HI in any group evaluated. In conclusion, EE was effective in recovering learning and memory impairment in the ox-maze task and Na^+,K^+-ATPase activity in the hippocampus caused by HI. The present data provide further support for the therapeutic potential of environmental stimulation after neonatal HI in rats.

Key words: hypoxic–ischemic event, enriched environment, neuroprotection, antioxidant enzymes, Na^+,K^+-ATPase, ox-maze.

INTRODUCTION

Neonatal hypoxia-ischemia (HI) is the main cause of mortality and neurologic morbidity in children, affecting about 1–3 newborns per 1000 live births (Shankaran et al., 2012; White et al., 2012). About half of HI events result in death, and 25% of the survivors remain with neurological disabilities, including cerebral palsy, cognitive and learning impairments and sensory deficits (Volpe, 2001; Low, 2004; Bass et al., 2004; Nelson and Lynch, 2004; Martinez-Biarge et al., 2012).

Several studies have shown neuropathology of HI involves multiple pathways, including glutamatergic excitotoxicity, inflammation and oxidative stress, which lead to cell damage and death (Siesjö, 1988; Pimentel et al., 2011); such pathological findings are observed on both ipsilateral and contralateral hemispheres (Kabakus et al., 2005; Kadam and Dudek, 2007; Kojima et al., 2013). It is important to note that the newborn immature brain is particularly susceptible to oxidative damage due to its limited antioxidant capacity (Carloni et al., 2008). In a previous study, an increase in the superoxide dismutase (SOD) activity was identified in the ipsilateral hippocampus of adult HI rats (Pereira et al., 2009) and, acutely, 2 h after HI in the ipsilateral cortex and hippocampus, with no effects on catalase (CAT) and glutathione peroxidase (GPx) activities (Weis et al., 2011). Additionally, it has also been described the Na^+,K^+-ATPase activity can be affected by oxidative stress (Wyse et al., 2002; Jaiswal et al., 2014) and, consequently, its function is impaired in animals suffering from neonatal hypoxia. Rosenkrantz and coworkers (1996) found a decrease in brain tissue Na^+,K^+-ATPase of piglets submitted to 1 h of hypoxia and Park and co-workers (2001) demonstrated HI decreased the activity of this enzyme in the cerebral cortex of newborn piglets. In adult rats, a recovery of enzyme inhibition in the hippocampus was associated with neuroprotection induced by brain ischemic preconditioning, indicating the fundamental role of

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Abbreviations: ANOVA, analysis of variance; CAT, catalase; CTEE, control exposed to environmental enrichment; CTSE, control maintained in standard environment; EDTA, ethylenediaminetetraacetic acid; EE, environmental enrichment; GPx, glutathione peroxidase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HI, hypoxia-ischemia; HIEE, hypoxia-ischemia exposed to environmental enrichment; HISE, hypoxia-ischemia maintained in standard environment; SOD, superoxide dismutase.

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Na^+,K^+-ATPase in cellular viability (Wyse et al., 2000). It has also been reported that that the Na^+,K^+-ATPase activity plays a role in neuronal plasticity and memory processes (Wyse et al., 2004; Moseley et al., 2007). Interestingly, Na^+,K^+-ATPase activity inhibition was found in the striatum and cortex of adult rats that had suffered neonatal HI (Carletti et al., 2012). In regard to cognition, several reports indicated consistent long-term impairments of spatial memory (Ikeda et al., 2001; Arteni et al., 2003; Pereira et al., 2007) and aversive memory (Young et al., 1986; Arteni et al., 2003; Carletti et al., 2012; Rojas et al., 2013) in adult rats submitted to neonatal HI.

Environmental enrichment (EE) is a paradigm recognized as a possible behavioral neuroprotective strategy for brain damage caused by different pathologies such as chronic cerebral hypoperfusion (Cechetti et al., 2012), stroke (Wang et al., 2008) and traumatic event (Monaco et al., 2013; Bondi et al., 2014). It consists of an association of social interaction, physical exercise and exposure to learning tasks (Harburger et al., 2007; Johnson et al., 2013). The relevance of this combination in EE paradigm has been investigated. Sozda and colleagues (2010) had evaluated whether a single element of EE paradigm is sufficient to reach functional and histological protection after traumatic brain injury in rats. Such authors concluded that even if each individual component of the enriched environment be important, typical EE results in better recovery of rats submitted to brain trauma.

It has previously been reported that stimulation offered by late enrichment (starting 2 weeks after neonatal HI) recovers spatial memory deficits, with no effect on hippocampus or cerebral cortex atrophy in rats (Pereira et al., 2007). Another study showed early exposure to enriched environment was associated with memory recovery in the novel object recognition task and amelioration in spatial memory in adolescent female and male rats after neonatal HI (Pereira et al., 2008). Despite its cognitive effect, enrichment was not able to prevent hippocampus and striatum tissue atrophy due to hypoxia-ischemia-induced damage (Pereira et al., 2008). However, morphological EE effects might occur since late enrichment was able to preserve hippocampal dendritic spine density loss after neonatal HI (Rojas et al., 2013). Taken together, these findings provide evidence for the value of EE as a treatment for alleviating the consequences of hypoxic-ischemic event but the potential and the mechanisms involved in such effects are not yet clear.

Therefore, this study was carried out to examine the effects of environmental stimulation in rats submitted to neonatal HI evaluating behavioral performance in the ox-maze and biochemical parameters (Na^+,K^+-ATPase, CAT and GPx activities) in the hippocampus.

EXPERIMENTAL PROCEDURES

Animals

Pregnant Wistar rats were obtained from Centro de Reprodução e Experimentação de Animais de Laboratório from the Universidade Federal do Rio Grande do Sul, Brazil. They were maintained in a temperature-controlled room (approximately 22 °C), on a 12/12 light/dark cycle with food and water *ad libitum*. After delivery, at postnatal day 7, male rats were randomly divided into four experimental groups: (a) control maintained in standard environment (CTSE); (b) control exposed to EE (CTEE); (c) submitted to HI and maintained in standard environment (HISE); (d) submitted to HI exposed to EE (HIEE). Forty-six animals were used for behavioral study (CTSE, $n = 11$; CTEE, $n = 11$; HISE, $n = 13$; HIEE, $n = 11$) and another 23 rats for biochemical analysis (six animals for CTSE, CTEE and HISE groups and five animals for the HIEE group), totaling 69 animals utilized in this study. The time line containing all experimental events is depicted in Fig. 1.

All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals adopted by The National Institutes of Health (USA) and with the Federation of Brazilian Societies for Experimental Biology. This project was approved by the Ethics in Research Committee at the Universidade Federal do Rio Grande do Sul (number 22045).

Hypoxia–ischemia

The HI model, described by Levine (1960) and modified by Rice et al. (1981), also known as the Vanucci model (Vannucci and Vannucci, 1997; Vannucci et al., 1999; Vannucci and Vannucci, 2005), was utilized in this study to produce unilateral brain injury to neonatal rats. At postnatal day 7, rats were anesthetized with halothane 2–4% and submitted to the HI procedure. Briefly, occlusion of the right common carotid artery was performed through a neck incision. Animals were maintained in controlled temperature to recover for 15 min and returned to their dams. After a period of approximately 2.5 h, pups were exposed to hypoxic atmosphere (8% oxygen and 92% nitrogen, 5 L/minute flow) for 90 min in a chamber partially immersed in a 37 °C water bath in order to maintain body temperature. Control animals were sham-operated, i.e., they received manipulation, anesthesia and neck incision, but no arterial occlusion or hypoxic atmosphere exposition. This procedure intends to prevent different maternal care and/or rejection of HI pups. Following the HI procedure, animals were returned to their respective home cages where they were maintained until weaning, at postnatal day 21 (Arteni et al., 2003; Pereira et al., 2007; Rojas et al., 2013).

EE

EE procedure used in this study was previously described by Pereira and co-workers (2007, 2009) and Rojas et al. (2013). Starting at postnatal day 22, animals were stimulated in the enriched environment for 9 weeks, 6 days/week, 1 h/day, in groups of 7–10 animals (randomly assigned). The apparatus of EE consisted of a large cage (40 × 60 × 90 cm) with three floors, ramps, running wheel and several objects with different shapes and textures, which were changed once a week. Rats from CTSE and HISE groups (non-enriched) were removed from their home cages to another standard cage during the

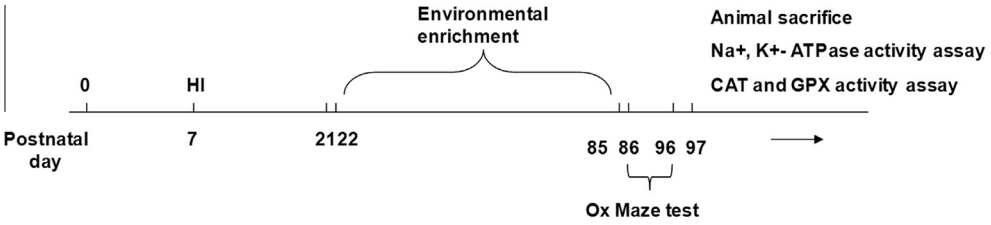

Fig. 1. Time line of experimental procedures. HI: hypoxic–ischemic event.

enrichment period (Pereira et al., 2007, 2009; Rojas et al., 2013).

Ox-maze test

The ox-maze is a new learning and memory task (Wood et al., 2011). In this paper an adapted and simplified version of the original apparatus was used. The test was carried out one day after the end of the EE period. The apparatus consisted of a black square acrylic box (60 cm × 60 cm × 30 cm high) in which the floor was marked with grid lines, producing 12-cm × 12-cm squares. Four black acrylic blocks (10 cm × 10 cm × 10 cm high) were put into a square box. Each block side had a hole (2 cm diameter × 2 cm deep) and one of four symbols (O, X, =, ||), as presented in Fig. 2.

Two days before testing, animals received daily Fruit loops (Kellogg's pellets of wheat and corn starch and sucrose) as a form of taste habituation. The test was performed with animals submitted to food restriction; 24 h before testing started, the lab chow offered was reduced to about 90% of the normal amount (Silveira et al., 2000). Body weight was also measured two days before the behavioral study and no differences were identified between groups. Rats were placed at the center of the maze and allowed to explore it for 10 min maximum, or until they had reached four rewards. One Fruit loop (reward) was located only in the rewarded symbol (O) and it was the same for each rat every day. During the course of the experiment (10 days), block positions were changed every day (clockwise). Variables recorded were: time to find the first reward (latency) and time to complete task (maximum time = 10 min), as well as the number of correct (reach the reward) and incorrect nose pokes into each hole of every block (including the return to the reward hole previously visited). Two researchers blinded to experimental conditions ran the test. The maze and blocks were carefully wiped between trials with 20% ethyl alcohol to minimize olfactory cues.

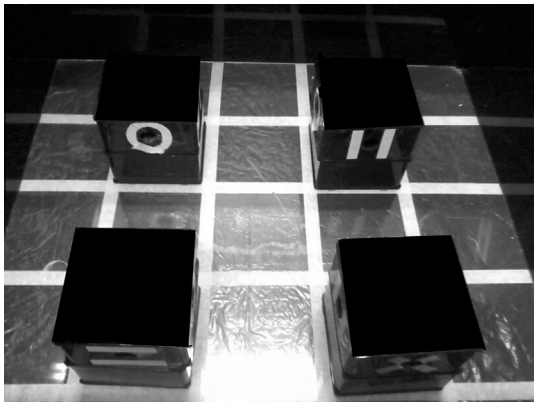

Fig. 2. Ox-maze apparatus.

Tissue preparation

As for biochemical assays animals were euthanized 24 h after the last behavioral testing by decapitation. The right and left hippocampi (six animals for CTSE, CTEE and HISE groups and five animals for the HIEE group) were dissected out and instantaneously placed in liquid nitrogen and stored at −70 °C. Hippocampus samples were homogenized in 10 volumes (1:10, w/v) of 0.32 M sucrose solution containing 5.0 mM HEPES and 0.1 mM EDTA, pH 7.4.

Na^+,K^+-ATPase activity assay

The reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris–HCl, pH 7.4, in a final volume of 200 μL. The reaction was initiated by ATP addition. Controls were treated under the same conditions with the addition of 1.0 mM ouabain. the Na^+,K^+-ATPase activity was calculated by the difference between two assays, as described by Wyse and coworkers (1998). Released inorganic phosphate (Pi) was measured by the Chan and colleagues (1986) method. Specific enzyme activity was expressed as nmol Pi released per min per mg of protein.

CAT assay

The CAT activity was assayed using a double-beam spectrophotometer with temperature control (Hitachi U-2001). This method is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer pH 7.0, and 0.1–0.3 mg protein/mL (Aebi, 1984; Matté et al., 2007; Matté et al., 2009). One CAT unit is defined as 1 mmol of H_2O_2 consumed per minute and the specific activity is represented as CAT units/mg protein.

GPx assay

The GPx activity was measured using tert-butyl-hydroperoxide as substrate. NADPH disappearance was monitored spectrophotometrically at 340 nm. The medium contained 2 mM glutathione, 0.15 U/mL

glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide and 0.1 mM NADPH (Paglia and Valentine, 1967; Wendel, 1981). One GPx unit is defined as 1 μmol of NADPH consumed per minute and the specific activity is represented as units per milligram of protein.

Statistical analysis

Results from the Ox-maze were analyzed by repeated measures of analysis of variance (ANOVA) to evaluate behavioral changes over 10 testing days. Means of each variable in all days in behavioral study and biochemical results were analyzed by a two-way ANOVA, with *lesion* and *environment* as the independent variables. All analyses were followed by *post hoc* Tukey's test for multiple comparisons whenever necessary. Data were expressed as mean ± S.E.M. Probability values less than 5% were considered significant. All statistical analysis was performed using the Statistica® software package.

RESULTS

Ox-maze test

Neonatal HI impaired performance in the ox-maze, was reversed by the environmental stimulation. A repeated measures ANOVA for latency to find the 1st reward revealed main effects of *day* ($F(9,351) = 111.37$; $p < 0.05$), *lesion* × *environment* interaction ($F(1,39) = 4.82$; $p < 0.05$) and *day* × *lesion* × *environment* interaction ($F(9,351) = 2.11$; $p < 0.05$). Tukey's *post hoc* indicated the HISE group took more time to reach reward than CTSE animals on the 2nd and 3rd testing days; also a better performance to find the 1st reward, comparing with the 1st day, was achieved starting on the 2nd testing day for CTSE and HIEE groups, on the 3rd testing day for the CTEE group and, later, on the 4th testing day for the HISE group (Fig. 3A). Means of latencies to find the 1st reward in all sessions were evaluated by a two-way ANOVA and a significant main effect for *lesion* × *environment* interaction was observed ($F(1,39) = 4.81$; $p < 0.03$). The HISE group took more time to find the 1st reward comparing with CTSE and HIEE groups (Fig. 3B).

Considering the number of correct nose pokes, a repeated measure analysis demonstrated significant effects of *day* [$F(9,351) = 101.54$; $p < 0.05$], *lesion* ($F(1,39) = 4.73$; $p < 0.05$), as well as *lesion* × *environment* interaction ($F(1,39) = 4.11$; $p < 0.05$). Post-hoc test indicated the HISE group had a smaller number of correct nose pokes on the 3th testing day comparing with CTSE and CTEE groups. A later improvement in executing correct nose pokes, comparing with 1st testing day, was observed in the HISE group (Fig. 4A). When evaluating the mean of correct nose pokes during all testing days, a two-way ANOVA presented a significant lesion × environment interaction [$F(1,39) = 4.1$; $p < 0.05$) with HISE presenting fewer correct options, comparing with all other groups (Fig. 4B).

With respect to time to complete task, data analysis demonstrated significant main effects of *day* ($F(9,351) = 136.62$; $p < 0.05$) and tendencies of *lesion* ($F(1,39) = 3.5$; $p = 0.06$) and *lesion* × *environment* interaction ($F(1,39) = 3.6$; $p < 0.07$). Following the same pattern, the HISE group presented a better performance, decreasing total time to complete task, on the 5th testing day, comparing with the 1st day; other groups had decreasing total time earlier, on the 3rd testing day (Fig. 5A). Evaluating means of total time to complete task during all testing, a strong tendency indicated HISE spent more time in the ox-maze apparatus, comparing with CTSE ($p = 0.06$), CTEE ($p = 0.08$) and HIEE groups ($p = 0.08$) (Fig. 5B). Taken together this data analysis indicated a cognitive impairment in HI animals which recovered by environmental stimulation. Such a finding is also supported by the fact the HIEE group had demonstrated

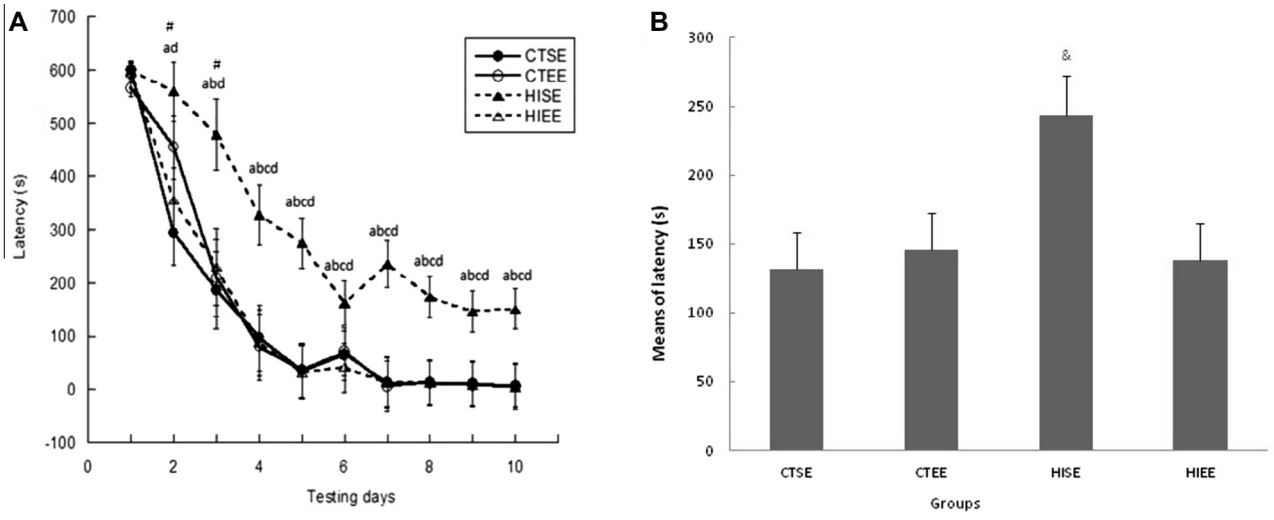

Fig. 3. Latency (s) to find the first reward in ox-maze task, conducted 9 weeks after HI procedure. (A) Data are mean ± S.E.M., day by day for 10 testing days. #HISE is different from CTSE on 2nd and 3rd days. Differences from the 1st day: (a) CTSE group; (b) CTEE group; (c) HISE group and (d) HIEE group (repeated measures ANOVA followed by Tukey's test, $p < 0.05$). (B) Data are mean ± S.E.M., considering means of total testing period. &HISE is different from HIEE and CTSE groups (two-way ANOVA followed by Tukey's test, $p < 0.05$).

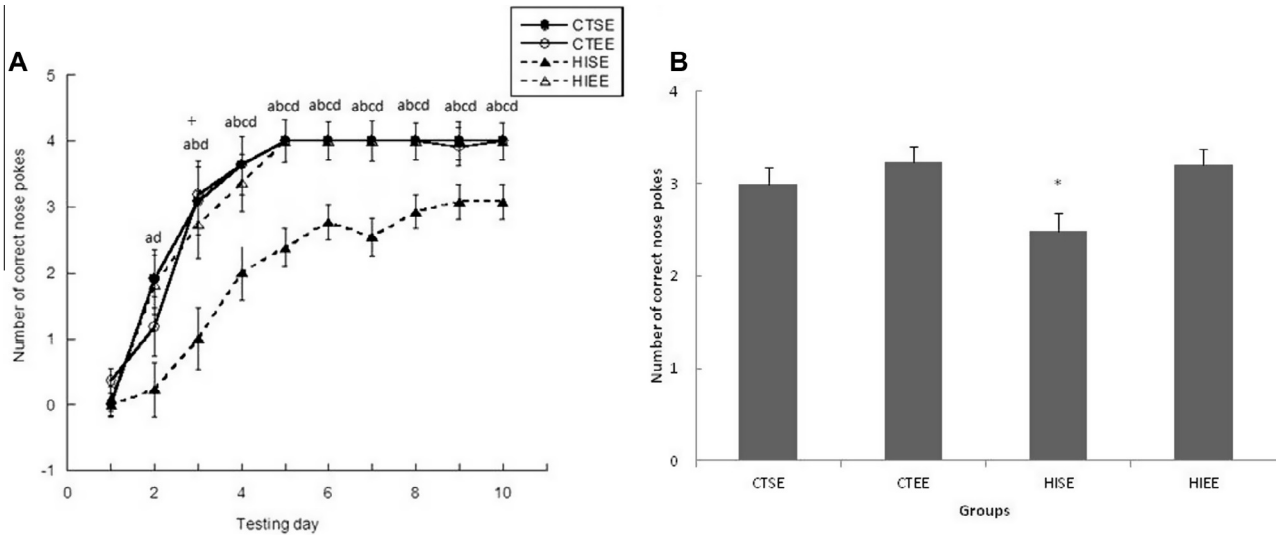

Fig. 4. Number of correct choices (nose pokes) in ox-maze task, conducted 9 weeks after HI procedure (maximum = 4). (A) Data are mean ± S.E.M., day by day for 10 testing days. $^{+}$HISE is different from control groups on the 3rd day. Differences from 1st day: (a) CTSE group; (b) CTEE group; (c) HISE group and (d) HIEE group (repeated measures ANOVA followed by Tukey's test, $p < 0.05$). (B) Data are mean ± S.E.M., considering means of total testing period. *The HISE group is different from all other groups (two-way ANOVA followed by Tukey's test, $p < 0.05$).

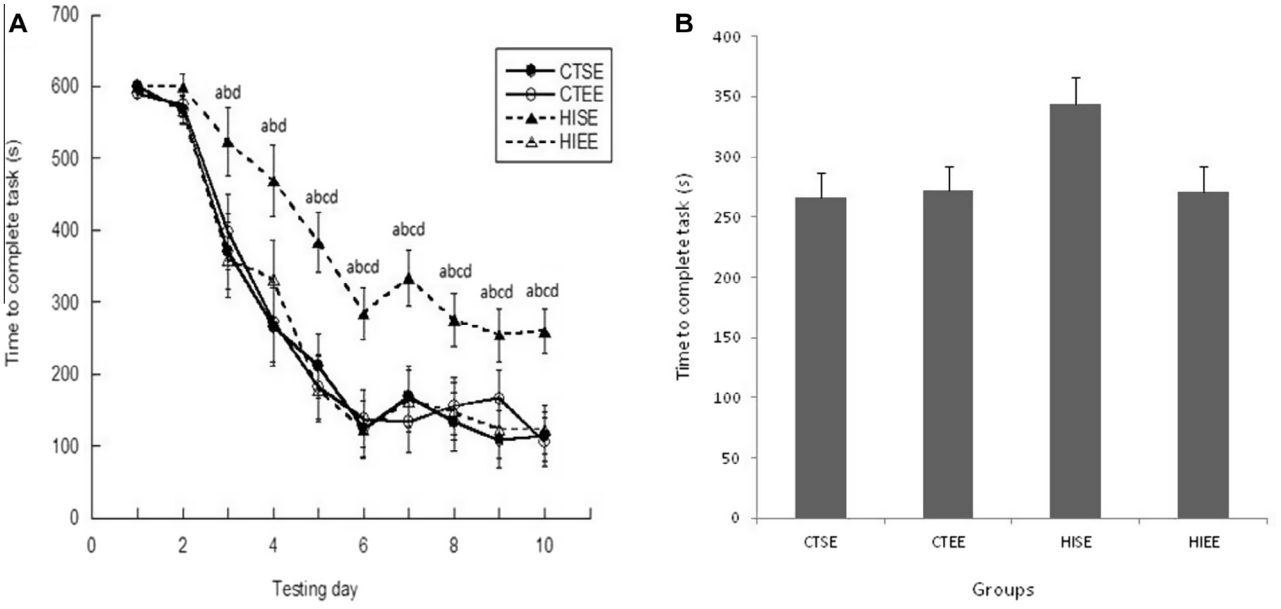

Fig. 5. Time to complete each session in ox-maze task, conducted 9 weeks after HI procedure (maximum = 600 s). (A) Data are mean ± S.E.M., day by day for 10 testing days. Differences from the 1st day: (a) CTSE group; (b) CTEE group; (c) HISE group and (d) HIEE group (repeated measures ANOVA followed by Tukey's test, $p < 0.05$). (B) Data are mean ± S.E.M., considering means of total testing period (two-way ANOVA followed by Tukey's test, $p < 0.05$).

during the entire behavioral testing performance similar to that of CT groups.

Na^+,K^+-ATPase activity

A two-way ANOVA showed significant differences for *lesion* ($F(1,19) = 29.72$; $p < 0.05$) and *environment* ($F(1,47) = 4.97$; $p < 0.05$) factors, as well as for the interaction between them ($F(1,19) = 8.23$; $p < 0.05$) on the Na^+,K^+-ATPase activity in the left hippocampus (contralateral to arterial occlusion). Enzyme activity was inhibited in the HISE group, as compared to all other groups, an effect that was prevented in HIEE rats. There was no effect in the right hippocampus (ipsilateral to arterial occlusion) neither for *lesion* ($F(1,19) = 1.58$; $p > 0.05$) nor *environment* ($F(1,19) = 3.37$; $p > 0.05$) (Fig. 6).

CAT activity

The CAT activity was also evaluated in the right and left hippocampi. A two-way ANOVA revealed a significant effect of *lesion* in the left hippocampus, contralateral to arterial occlusion ($F(1,19) = 10.33$; $p < 0.05$), with no

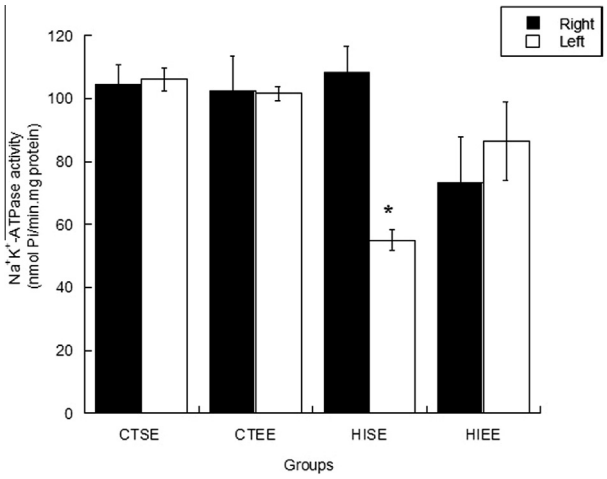

Fig. 6. Na^+,K^+-ATPase activity in the left (contralateral) and right (ipsilateral) hippocampi. Data are mean ± S.E.M. *The HISE group is different from all other groups (left hippocampus). ANOVA followed by Tukey's test, $p < 0.05$.

effect of *environment* factor in this hemisphere ($F(1,19) = 0.006$; $p > 0.05$). No effect on the CAT activity was found in the ipsilateral hippocampus. An ANOVA of the CAT activity in the right hippocampus revealed that neither hypoxic-ischemic event ($F(1,19) = 0.21$, $p > 0.05$) nor EE ($F(1,19) = 0.09$, $p > 0.05$) resulted in significant differences (Table 1).

GPx activity

The GPx activity in the right hippocampus was not affected by *lesion* ($F(1,19) = 1.57$; $p > 0.05$) or *environment* factors ($F(1,19) = 0.96$; $p > 0.05$). Also, in the left hippocampus a significant effect on *lesion* ($F(1,19) = 1.57$; $p > 0.05$) or *environment* factors ($F(1,19) = 0.96$; $p > 0.05$) was not found (Table 1).

DISCUSSION

This study investigated the effects of daily EE (for 9 weeks, 6 days/week, 1 h/day) in rats that suffered a neonatal hypoxic-ischemic event on behavioral performance in the ox-maze test, as well as on the Na^+,K^+-ATPase, CAT and GPx activities in the hippocampus. Results demonstrate that neonatal HI caused a memory deficit, which was recovered by EE. Confirming our hypothesis, HI resulted in a decreased Na^+,K^+- ATPase activity in the hippocampus and environmental stimulation also reversed such effect.

Several studies have investigated neuroprotective effects of EE in different models of brain injury and some of these have adopted an abbreviated protocol of stimulation with 1, 2, 4 or 6 h/day (Gaulke et al., 2005; Pereira et al., 2007; de Witt et al., 2011). De Witt and colleagues (2011) showed that 2 and 4 h of EE were ineffective in recovery functional deficits after traumatic brain injury but they identified protective effects with 6 h. It was important to consider that this model of traumatic injury utilized adult animals, contrary to the current study. Even if more extensive damage is frequently identified after neonatal HI, young rats may spend more time exploring the enriched environment. In the present study, although 1 h of daily enrichment has started two weeks after HI event, an important deficit was prevented in rats submitted to neonatal lesion, corroborating with previous findings that show an extension of the therapeutic window for functional prevention of neonatal HI effects (Pereira et al., 2007; Rojas et al., 2013). The Ox-maze was originally designed for brain training of the R6/2 mouse model of Huntington's disease and as a cognitive task which requires visual discrimination and spatial memory to find a food reward, without an intense aversive component, as found in other tests, such as the water maze and the inhibitory avoidance (Wood et al., 2011). In the present study, hypoxic-ischemic animals showed higher latencies to find the first reward, took more time to complete the task and made fewer correct choices. Previous studies have already indicated HI causes cognitive deficits, particularly in memory processes involving hippocampal circuitry (Pereira et al., 2007, 2009; Cengiz et al., 2011; Zhao et al., 2013); this functional effect is associated with significant hippocampal atrophy in rats submitted to neonatal HI (Pereira et al., 2007, 2008; Rojas et al., 2013). It is then reasonable to suggest a worse performance in the ox-maze is consequent to hippocampal damage, although olfactory discrimination ability could also be involved in the performance of rats in the ox-maze. Interestingly Hwang and coworkers (2004) indicated neurons in the main olfactory bulb of the gerbil are relatively resistant to ischemic damage and Ikonomidou and colleagues (1989) demonstrated 63% of rats submitted to HI had olfactory tubercle damage, only on the ipsilateral side. Despite being an adaptation of the original test, this is the first study demonstrating impaired cognition consequent to brain ischemia on the ox-maze.

To assess possible mechanisms implicated in this behavioral effect of EE, the Na^+,K^+-ATPase activity was investigated. This enzyme is important for neural function and survival and its function is necessary to maintain the ionic gradient for neuronal excitability

Table 1. Catalase (CAT) and glutathione peroxidase (GPx) activity in the hippocampus.

Group	Right		Left	
	CAT (U/mg protein)	GPx (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)
CTSE	3.09 ± 0.16	43.11 ± 1.78	3.55 ± 0.17	41.74 ± 1.06
CTEE	2.99 ± 0.07	41.65 ± 0.77	3.68 ± 0.1	44.83 ± 1.15
HISE	3.13 ± 0.15	42.9 ± 4.08	3.22 ± 0.16	43.2 ± 1.93
HIEE	3.12 ± 0.3	53.39 ± 9.17	3.07 ± 0.13	39.82 ± 1.17

(Xiao et al., 2002). Decreased Na^+,K^+-ATPase expression in the hippocampus suggests a disturbance of ion homeostasis (Siesjö, 1988) which can cause cell death. In addition, several studies have strongly suggested Na^+,K^+-ATPase plays an important role in spatial memory mechanisms (Wyse et al., 2004; dos Reis-Lunardelli et al., 2007; Heo et al., 2012; Jaques et al., 2013). Learning and memory deficits were observed in the alfa 2 and alfa 3 heterozygous mice, which presented deficiency in Na^+,K^+-ATPase α isoform genes (Moseley et al., 2007). These authors proposed that reduced alfa 3 Na^+,K^+-ATPase isoform expression causes chronic depolarization of the neuron, reducing neural activity and then leading to hippocampal NMDA receptor expression. Such proposal indicates a direct relationship between Na^+,K^+-ATPase activity, glutamatergic system and, consequently, cognitive functions, as spatial learning. In the present study, in the right hippocampus (ipsilateral to arterial occlusion), data analysis did not identify differences between groups, however a decrease of the Na^+,K^+-ATPase activity was found in non-stimulated HI rats on the contralateral hemisphere, compared to HI enriched and control groups. Thus, EE was effective in reversing enzyme function impairment. Corroborating that, a previous study indicated that EE induced prevention on tissue vulnerability, marked by SOD and BDNF levels (Pereira et al., 2009). It might be that the present findings on the Na^+,K^+-ATPase activity in the contralateral hippocampus can be a late biochemical consequence of HI injury on nervous tissue. This is based on findings that brain damage following neonatal hypoxic–ischemic event is progressive and causes a late impairment of brain function (Geddes et al., 2001; Mishima et al., 2005). Interestingly, Kojima and co-workers (2013) propose that progressive neuronal damage occurs in the contralateral cerebral cortex of mature rats after neonatal HI insult. In addition, Kadam and colleagues (2007) found that damage to gray and white matter was identified in HI rats, with mossy fiber sprouting identified in the atrophied ipsilateral hippocampus and also found in contralateral hippocampi. Also, apoptotic neurons and infarcted tissue in the contralateral hemisphere has been described (Kabakus et al., 2005).

Several studies have investigated acute effects of neonatal HI (Dafre et al., 2003; Weis et al., 2011; Zhang et al., 2014). Weis and colleagues (2011) evaluated ipsilateral hemisphere 0, 1 and 2 h after HI. They showed an early decrease of the Na^+,K^+-ATPase activity (at 0 and 1 h), as well as a late increase in the SOD activity (1 and 2 h after HI) in the hippocampus. In the present work, Na^+,K^+-ATPase assays were run approximately 3 months after the lesion and Na^+,K^+-ATPase disturbance was not found on the ipsilateral hippocampus . The data suggest the unaltered Na^+,K^+-ATPase activity in the ipsilateral hippocampus may be a result of EE reversing an early decrease in the Na^+,K^+-ATPase activity.

We also investigated whether CAT and GPx activities have been disturbed after HI and EE procedures. Interestingly our results showed there are no differences between HI and control groups in the GPx and CAT activity, in the hippocampus. Correlating with that our previous study demonstrated EE prevented SOD activity effects in the hippocampus consequent to neonatal HI (Pereira et al., 2009): increased SOD activity in the ipsilateral hippocampus was identified in the HI non-stimulated group and EE was effective in reversing this finding. It is well established that antioxidant capacity of tissue is very important for endogenous defense against free-radical-induced injury (Halliwell and Gutteridge, 1990) and SOD, CAT and GPx play crucial roles as free radical scavengers (Lin et al., 2011). An important defense mechanism is the dismutation of superoxide ($O_2^{\cdot-}$) to form hydrogen peroxide (H_2O_2) and O_2 by SODs, as well as the transformation of H_2O_2 to H_2O by GPx or to O_2 and H_2O by CAT, preventing the formation of the hydroxyl radical ($^*OH$). GPx and CAT play an essential role in scavenging hydrogen peroxide and act jointly with SOD for antioxidant protection (Southorn and Powis, 1988; Halliwell and Gutteridge, 1990; McCord, 1993) considering that the antioxidant role of SOD is effective particularly when it is accompanied by changes in CAT and GPx activities (Halliwell, 2001). Since increased SOD activity is identified only in HI rats maintained in standard environment (Pereira et al., 2009) and this increase is not accompanied by CAT and GPx increased activities, it confirms that oxidative stress participates in neural damage after neonatal HI and that EE seems to protect the long-term vulnerability of neural tissue.

The present study also aimed to correlate cognitive performance in the ox-maze with Na^+,K^+-ATPase, CAT and GPx activities in the hippocampus. Considering that both hemispheres play a role in cognitive tasks, we can suggest Na^+,K^+-ATPase inhibition in the contralateral hippocampus might be implicated in the worse performance of hypoxic-ischemic rats maintained in standard environment in the ox-maze test and that recovery of the enzyme activity can be partially responsible for the cognitive effect of enrichment in HI rats. Considering that oxidative stress influences the Na^+,K^+-ATPase activity (Jaiswal et al., 2014), CAT and GPx activities were also investigated. Given present data, we suggest this effect on the Na^+,K^+-ATPase activity is not directly related with CAT and GPx activities, however more studies are needed to understand this relationship.

In summary, a clear protective role of EE was observed on performance in the adapted ox-maze task after neonatal hypoxic-ischemic event. This effect is associated to Na^+,K^+-ATPase activity recovery in the hippocampus, which might explain, at least in part, such functional EE effect. Thus, present data provide further evidence for the therapeutic potential of environmental stimulation on neonatal HI in the rat and suggest a possible mechanism evolve.

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4. ARTIGO 2

Environmental enrichment enhanced synaptophysin levels but not GFAP and dendritic arborization after neonatal hypoxia-ischemia in rats.

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Environmental enrichment enhanced synaptophysin levels but not GFAP and dendritic arborization after neonatal hypoxia-ischemia in rats

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ABSTRACT

Hypoxic-ischemic encephalopathy is an important cause of mortality in newborns and is responsible for significant morbidity at long term, involving impairment in cognitive and motor functions and limitation of daily actions and general adaptation to social life. In animal models, environmental enrichment (EE) has been found to be a strategy for alleviating the consequences of neonatal hypoxia-ischemia (HI). Conversely, the potential for this therapeutic approach and the mechanisms involved are not yet clear. This study aimed to investigate some parameters such as dendritic arborization, synaptophysin and GFAP immunoreactivity and neuronal number in the hippocampus of rats submitted to neonatal HI and stimulated in an enriched environment. HI procedure occurs at post-natal day 7, when rats were divided into 4 experimental groups: control maintained in standard environment (CTSE), control submitted to EE (CTEE), HI in standard environment (HISE) and HI in EE (HIEE). Environmental enrichment was maintained for 9 weeks, 6 days/week, 1 hour/day; after this period of stimulation, morphological analyses were performed. Results indicated that hypoxic-ischemic damage is significantly harmful as regards to synaptophysin and cell quantification, and that the EE was effective in recovering synaptophysin immunoreactivity in the left hippocampus. No difference was found on the dendritic arborization and GFAP immunoreactivity between groups. Concluding, neonatal hypoxia-ischemia decreased synaptophysin imunoreactivity and neuronal density in the hippocampus and environmental stimulation was effective in preserving synaptophysin expression, without effect on neuronal loss consequent to HI.

Key-words: Perinatal asphyxia, environmental stimulation, plasticity, cell density, brain.

Abbreviations: EE: Environmental enrichment; HI: hypoxia-ischemia; GFAP: Glial fibrilar acidic protein; CTSE: control maintained in standard environment; CTEE: control submitted to environmental enrichment; HISE: hypoxia-ischemia maintained in standard environment; HIEE: hypoxia-ischemia submitted to environmental enrichment.

INTRODUCTION

Environmental enrichment (EE) was described as the combination of complex inanimate and social stimulation providing to the animals multiple sensory, cognitive and motor interactions with the environment (Rosenzweig *et al*. 1978, Nithianantharajah & Hannan, 2006, Van Praag *et al*., 2000). This protocol of stimulation has been associated with several anatomical and histological changes such as improved neurogenesis (Van Praag *et al*., 2000, Kempermann, 2002), increased cortical thickness and dendritic branching in the pyramidal neurons of hippocampal CA1 region (Greenough *et al*., 1973), improved synaptophysin levels in the hippocampus (Lambert *et al.*, 2005, Frick and Fernadez, 2003, Marquez *et al*., 2014) and dendritic spine growth and changes in spine morphology (Diamond *et al*., 1976, Nakamura *et al*., 1999, Leggio *et al*., 2005, Rojas *et al*., 2013). Considering these protective effects of

EE, such strategy has been extensively adopted as a therapeutic intervention in neurodegenerative diseases such as Alzheimer's (Jankowsky *et al*., 2005, Pietropaolo *et al*., 2014), Parkinson's (Bezard *et al*., 2013), Huntington's (Nithianantharajah *et al*. 2008, Mo *et al*., 2015), traumatic brain injury (*Hamm et al.* 1996; Hoffman *et al.* 2008, Ortuzar *et al.* 2009, Matter *et al*., 2011) and neonatal hypoxia-ischemia (Pereira *et al*., 2007, 2008 and 2009, Rojas *et al.,* 2013 and 2015).

Previous data from our research group with EE after neonatal hypoxia-ischemia (HI) evidenced that stimulation provided by late enrichment was associated with important memory improve (Pereira *et al*., 2007, Pereira *et al*., 2008, Rojas *et al.,* 2013 and Rojas *et al*., 2015). This functional effect was associated with hippocampal dendritic spine density preservation without effect on brain atrophy (Pereira *et al.,* 2007, Pereira *et al*., 2008, Rojas *et al.,* 2013). Our most recent study demonstrated that the EE reversed memory deficits in the ox-maze task and the Na^+,K^+-ATPase inhibition in the hippocampus caused by HI (Rojas *et al.,* 2015), this maybe can be one of mechanisms involved in the functional beneficial effects of EE.

Neonatal hypoxia-ischemia is an important public health problem, since a significant proportion (25%) of children with this injury develops sensory, motor, and learning disabilities (Kurinczuk *et al.,* 2010). Experimentally, the Levine and Rice method, also called Vannucci model (Levine, 1960, Rice *et al*., 1981), was frequently adopted to study functional, morphological and biochemical aspects related to this brain disease and also to investigate therapeutic strategies (Arteni *et al*., 2003, Ikeda, 2008, Miguel *et al*., 2015, Carletti *et al*., 2015). The

hippocampus is the main vulnerable area to ischemic events, and in this model of HI was described important neural changes such as atrophy (De Paula *et al*., 2009, Rodrigues *et al.*, 2004), pyramidal neuronal death in the CA1 region *(*Pulsinelli *et al*., 1982, Kirino and Sano, 1984*),* decrease of the dendritic arborization and spine density (Ruan *et al.,* 2009, Rojas *et al*., 2013) and astrocytic activation (Briones *et al*., 2006, Donega *et al*., 2014, Choudhury *et al*., 2014, Sampedro-Piquero *et al*., 2015).

There are only few studies demonstrating the relationship between HI and environmental stimulation and, to our knowledge, all these are from our research group. Besides environmental enrichment has been recognized as a potential therapeutic target for neonatal hypoxia-ischemia, there is a lack of knowledge about plasticity and morphological targets in this area. Thus, the aim of this study was to investigate whether EE contributes to the improvement of damage caused by the hypoxic-ischemic lesion. For this purpose, we evaluated synaptophysin and Glial fibrilar acidic protein (GFAP) quantification using immunofluorescence assay; effects of EE exposition on cellular loss by quantification in semithin sections and pyramidal neurons characterization (dendritic branching and intersections between dendrites) through the Golgi technique.

MATERIALS AND METHODS

Animals

Pregnant Wistar rats were obtained from the Central Animal House of the Institute of Basic Health Sciences (Universidade Federal do Rio Grande do Sul – Brazil). The offsprings were kept with their mothers until 21 days. All animals were housed in a temperature and humidity-controlled room (22 °C to 24 °C) with a 12-h light/dark cycle and had free access to standard food and water. Only male rats were used in the present study. All procedures of this study were approved by the local animal ethics commission ("Comissão de Ética no Uso de Animais/Universidade Federal do Rio Grande do Sul- CEUA/UFRGS") under the number 22045, and were in accordance with the Guide for the Care and Use of Laboratory Animals adopted by The National Institutes of Health (USA), the Federation of Brazilian Societies for Experimental Biology, the National Council of Animal Control Experimentation (CONCEA) and the Arouca law: 11.794/2008. We additionally attest that all efforts were made to minimize the number of animals used and their suffering.

Experimental design

At postnatal day 7, rats were subjected to hypoxia-ischemia model. From this point, the rats were randomly assigned into the follow experimental groups: (1) control maintained in standard environment (CTSE); (2) control exposed to EE (CTEE); (3) submitted to HI model and maintained in standard environment

(HISE) and (4) submitted to HI exposed to EE (HIEE). Sixteen animals were used for dendritic arborization analysis (4 animals per group), another sixteen rats for neuronal counting (4 animals per group) and twenty two rats for GFAP and synaptophysin immunofluorescence assay (CTSE and HIEE, n=6; CTEE and HISE, n=5), totaling 54 animals utilized in this study. The time line containing all experimental events is exhibited in the Figure 1.

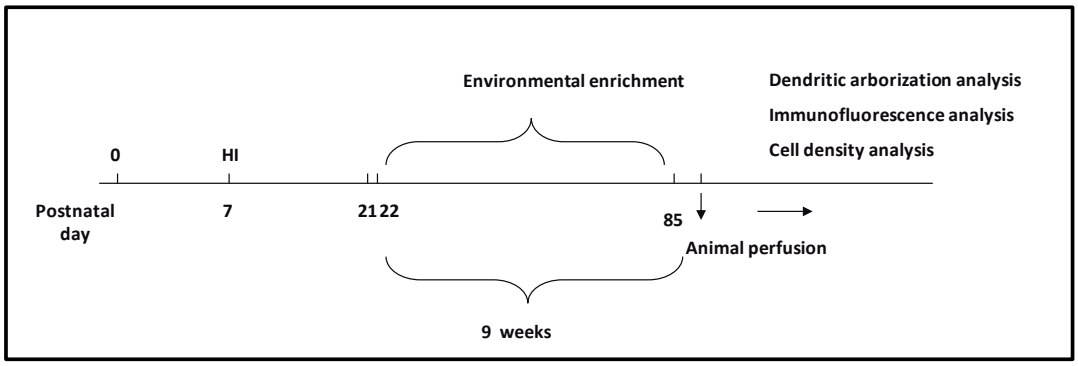


Fig. 1. Time line of experimental procedures. HI: Hypoxic-ischemic event.

Hypoxia-ischemia procedure

The HI model, described by Levine (Levine, 1960) and modified by Rice et al. (1981), was utilized to generate unilateral brain injury to neonatal rats. At PND 7, the pups were anesthetized with halothane (2 -4%), and a longitudinal midline incision was made in the neck. The right common carotid artery was located, separated from surrounding tissue, and completely occluded with surgical silk threads. The rats were returned to dams after recovering from anesthesia under a warming lamp. Two hours after recovery, the animals were placed in a chamber partially immersed in a 37 °C water bath, in which they received hypoxic atmosphere (8% oxygen and 92% nitrogen, 5 L/min) for 90

min. Control animals underwent the surgery procedure, excepting artery occlusion and they were not suffer the hypoxia. All pups were returned to their mothers, where they remained housed until weaning on PND 21. (Pereira *et al*., 2007 and 2008, Hill *et al*., 2011, Carletti *et al.,* 2012, Rojas *et al*., 2013 and 2015, Miguel *et al*., 2015).

Environmental enrichment

Environmental enrichment procedure used in this study is the same described by Pereira and coworkers (2007, 2009) and Rojas and coworkers (2013, 2015). At PND 21, rats were separated from their mothers and at PND 22 enrichment sessions began. Rats were stimulated in the enriched environment for 9 weeks, 6 days/week, 1 h/day. For EE housing, 7 to 10 animals (randomly assigned) were housed in big cages (40 x 60 x 90cm) containing three floors, ramps, running wheels, tubs and differently shape and texture objects (balls, toys, sponges) fully substituted once a week. Animals from non-enriched groups (CTSE and HISE) were removed from their home cages to another standard cage during the enrichment period (Pereira *et al*., 2007 and 2009, Rojas *et al*., 2013 and 2015).

GFAP and synaptophysin immunofluorescence

Past the end of EE, animals destined to the immunofluorescence analysis were anesthetized using ketamine (80 mg/kg) and xylazine (10mg/kg) i.p. and transcardially perfused using fixative solutions (0.9% saline followed by 4% paraformaldehyde), their brains were then removed and maintained in

paraformaldehyde solution. Brains were cryoprotected with a 30% sucrose solution and then frozen in isopentane and liquid nitrogen. Coronal 40 µm thickness sections were obtained using a cryostat (Leica, Germany). For the immunohistochemical investigation, coronal sections from 5 to 6 rats per group were stained for the astrocytic marker rabbit anti-glial fibrillary acidic protein (GFAP, 1:3000, Dako) and for the pre-synaptic marker synaptophysin (Syn, 1:3000, Dako). Secondary antibodies were goat anti-rabbit IgG Alexa 488 (1:500, Dako) and goat anti-mouse IgG Alexa 568 (1:500, Dako). Briefly, sections were fixed in 4% PFA, washed in PBS and blocked for 30 min with 3% normal goat serum (Sigma-Aldrich) in PBS with 0.4% Triton-X (PBS-Tx) at room temperature. Then, sections were incubated overnight with primary antibody at 4 °C with PBS-Tx with 3% NGS. In the next day sections were washed in PBS and incubated with secondary fluorescence antibody for 2 h at room temperature in a dark chamber, washed in PBS, mounted and cover slipped with antifading mounting medium PVA-DABCO (Fluka Analytical). Primary antibodies were omitted in negative controls for immunofluorescence stains. The samples were processed at the same time and incubated within the same medium during the same period (Cechetti *et al*., 2012, Piazza *et al.,* 2014).

Quantification of synaptophysin and GFAP

A laser scanning microscope was used to visualize fluorescent dyes (excitation wavelengths of 488 and 568 nm) in two randomized areas (40 × magnifications) within the CA1 subfield of hippocampus. All conditions and magnifications were kept constant during the capture process. Image J 1.44 software was used to

measure the intensities of the fluorescent signals in areas labeled for GFAP and synaptophysin after background correction. Each analysis was performed using 8 images per animal from both hemispheres (5 to 6 animals per group). The images were turned into binary images (black and white, 8 bits) and a single threshold value was established for both GFAP and synaptophysin staining, this value was kept constant for all animal groups. Three randomized squares measuring 1,270 µm2 and named areas of interest (AOIs) were overlaid on each image. The average value was used to measure the total percentage area (%) occupied by astrocytes and pre-synaptical vesicles, according to the procedure previously described. The observer was blind to animal experimental groups during image analysis (Piazza *et al.*, 2014, Lovatel *et al*., 2014).

Cellular number

Sixteen animals destined to the cellular number analysis were anesthetized using ketamine (80 mg/kg) and xylazine (10mg/kg) i.p. and transcardially perfused using fixative solution containing 2.5% glutaraldehyde (Sigma Chemicals Co., St Louis, MO, USA) and 4% paraformaldehyde (Reagen, Brazil) in 0.1 M phosphate buffer (PB), pH 7.4, at room temperature. Then, brains were removed from the skull and reserved in the same fixative solution. Coronal sections of the brain (1000 µm) were obtained using a vibratome and postfixed, in the same fixative solution for at least 1 hour. Hippocampal sections were washed in phosphate solution and postfixed, for 1 hour, in 1% osmium tetroxide (Sigma Chemicals Co., USA) at room temperature, dehydrated in a graded series of alcohol and propylene oxide (Electron Microscopy Science, USA),

embedded in resin (Durcupan, ACM-Fluka, Switzerland) and polymerized at 60° C for 72 hours. Semithin sections (1 µm) were obtained using an ultramicrotome (M T 600-XL, RMC, Tucson, USA) with a glass knife and stained with 1% toluidine blue. This procedure allowed the identification of hippocampus CA1 region pyramidal neurons (from bregma -2.30 mm to -3.60mm in accordance to Paxinos and Watson, 1986; Rodrigo et al., 1996). Images of these cells were captured and digitalized using a Nikon Eclipse E-600 microscope (Tokio, Japan, magnification: 1000x) coupled to a Pro-Series High Performance CCD camera. Only cells with viable nuclei and evident nucleolus were counted for cellular density estimation in five fields in each section (five in each hemisphere) in both hemispheres (Malyzs *et al*., 2010).Twenty-five images per hemisphere per animal were analyzed for quantification.

Golgi technique

Two days after the end of EE period, animals were anesthetized using ketamine (80 mg/kg) and xylazine (10mg/kg) i.p. and transcardially perfused using fixative solutions. This procedure followed the same methodology published in detail by De Castilhos and colleagues (2006) and previously used by our researcher group (Rojas *et al*., 2013). Brains were fixed with 4% paraformaldehyde and 1.5% picric acid in 0.1 M phosphate buffer (pH 7.4), coronally sectioned (200 µm thick) using a Vibratome (Leica, Germany) and impregnated in 1.5% silver nitrate following 3% potassium dichromate (Merck, Germany). After remaining in the dark for 48 h, the cover lips were removed and the sections were rinsed in distilled water, dehydrated, cleared with xylene, mounted on

slides and covered with non acidic synthetic balsam and cover slips (Rasia-Filho*et al*, 2004).

Microscopic analysis of dendritic arborization

Pyramidal neurons from the CA1 hippocampal region were analyzed in all animals. The location of the CA1 of dorsal hippocampus was based on previous descriptions and microscopic images were compared with the atlas (Paxinos and Watson, 2004). The neurons from the hippocampus were recognized by their characteristic triangular soma shape and visible main apical dendrite (Ranjan *et al*., 2014). The following criteria were used to select pyramidal neurons for this analysis: (1) visible neuronal cell bodies in the CA1 area with characteristic pyramidal appearance, according with Lorente de Nó (1934); (2) full impregnation of the entire neurons; (3) relative isolation from neighboring impregnated neurons (Brusco *et al*., 2008, Rojas *et al*., 2013, Ranjan *et al*., 2014). Four neurons per rat and four animals per group were used, consequently, dendritic arborization data were obtained from a total of 64 neurons (16 neurons per group). This choice was based in previous studies which adopted similar number of neurons evaluated (Puskas *et al*., 2014; Ranjan *et al*., 2014; Navarro e Mandyam, 2015).Only neurons from the left hemisphere (contralateral to injury) were analyzed because of the extensive hippocampal atrophy in the ipsilateral side. The microscopic analysis was performed using a camera lucida (1000 ×) coupled to an optic microscope and the draws were performed manually by only one of the authors who was blinded. For each neuron the morphological analysis included the following

parameters: (1) total dendritic branching in each arborization level which was determined by the order of centrifugal identification from the soma (being primary or secondary dendrites); then, dendrites originating from the cell soma were defined as primary and all dendrites originating from the primary were defined as secondary dendrites (Jaworski *et al*., 2005, Mavroudis *et al*., 2014); and (2) number of intersections between dendrites (points of dendritic branching) (Ristanovic *et al*., 2006, Markham *et al*., 2013). Means were calculated per animal and per group.

Statistical analysis

Two-way analysis of variance (ANOVA) was performed, with *lesion* and *environment* as independent variables. All analyses were followed by *post hoc* Tukey's test for multiple comparisons whenever necessary. Data were expressed as mean ± S.E.M. and results were considered significant when $p \leq 0.05$. All statistical analysis was performed using the Statistica® software package.

RESULTS

All analyzes were carried out from samples of the same region of the hippocampus (CA1region), which is identified in figure 2.

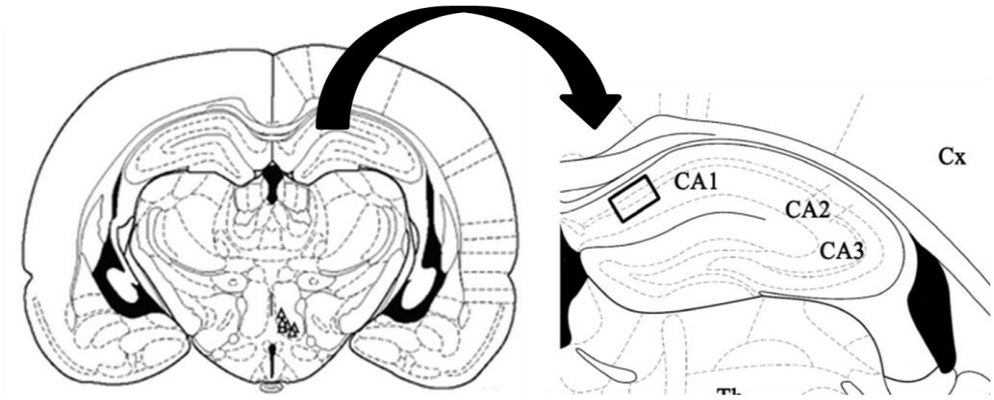
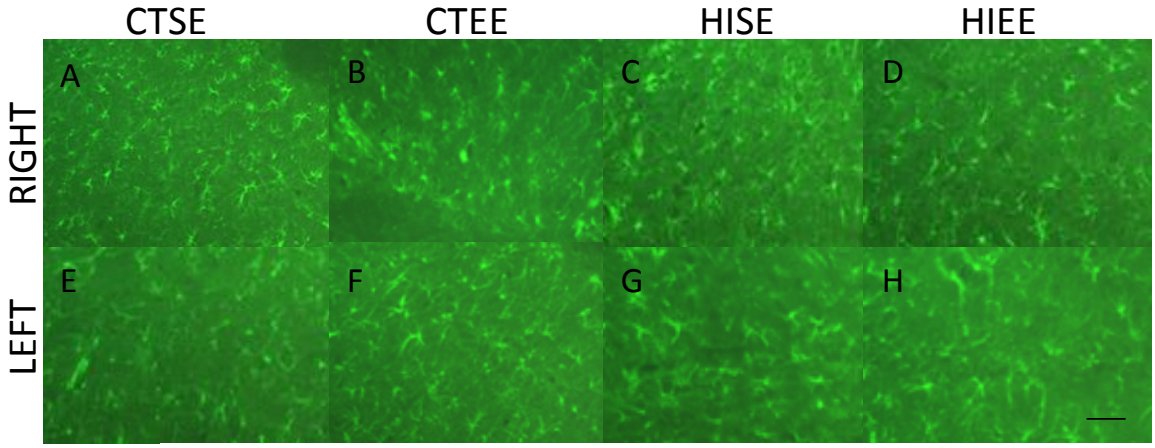


Fig. 2. CA1 region of the hippocampus (from bregma -2.30 mm to -3.60 mm). Images adapted from Paxinos and Watson Atlas (2004).

GFAP

The GFAP immunofluorescence in the hippocampus is showed in the figure 3. Percentage of occupied area by astrocytes in the right hemisphere was not affected by lesion [$F(1,18) = 1.11$; $p<0.05$] or environment [$F(1,18) = 0.49$; $p<0.05$] factors. Also, in the left hippocampus was not found significant effect on lesion [$F(1,18) = 0.31$; $p<0.05$] or environment [$F(1,18) = 1.53$; $p<0.05$].



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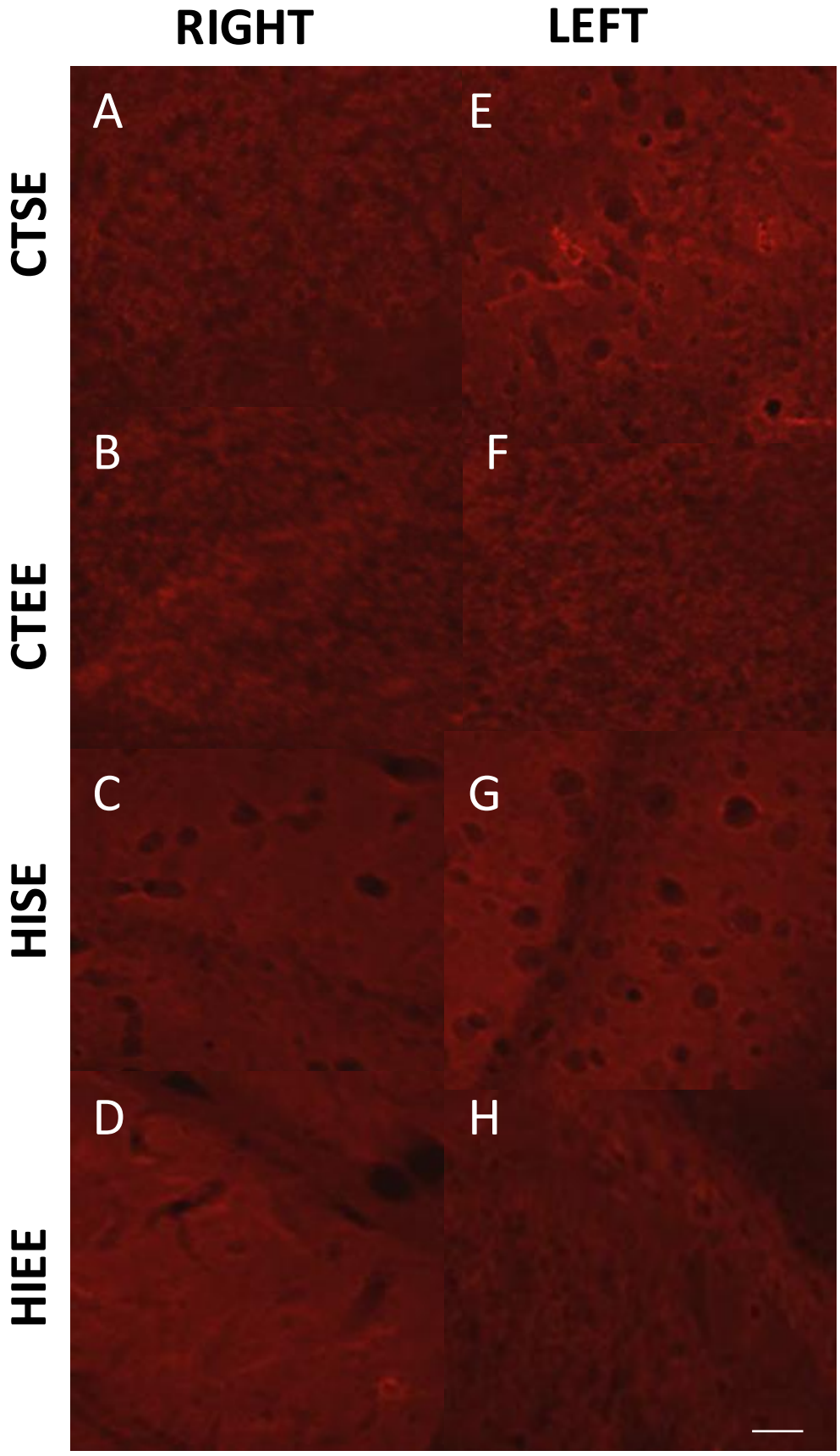
Percentage of GFAP positive astrocytes (Mean ± S.E.M.)		
Group	***Right hemisphere***	**Left hemisphere**
CTSE	53.66 ± 1.33	49 ± 1.5
CTEE	53.81 ± 1.85	53 ± 2.38
HISE	53.21 ± 1.59	49 ± 3.3
HIEE	50.66 ± 1.94	50.5 ± 1.66

Fig. 3. Percentage of GFAP positive astrocytes in the CA region from right and left hippocampus. A-D: Representative photomicrographs of CA1 region in right hippocampus; A - CTSE group; B- CTEE group; C - HISE group and D - HIEE group; E-F: Representative photomicrographs of CA1 region in left hippocampus; E - CTSE group; F - CTEE group; G - HISE group and H - HIEE group; I: Graphic representation of percentage of GFAP positive astrocytes in CA region from right and left hippocampus. Mean ± S.E.M. of values. Magnification of 40x. CTSE: control group in standard environment; CTEE: control group exposed to enriched environment; HISE: hypoxic-ischemic animals in standard environment; HIEE: hypoxic-ischemic animals exposed to enriched environment. Scale bar: 50µm. Atlas images adapted from Paxinos and Watson (2004).

Synaptophysin

Two-way ANOVA indicated a main effect of lesion [$F(1,18)=41.76$; $p<0.05$]. Considering the percentage of occupied area by synaptophysin staining in the right hippocampus (ipsilateral), the Tukey's post hoc test revealed an decrease

in the % of occupied area by synaptophysin in the HI groups, compared to the control groups ($p \leq 0.05$). On the left hemisphere (contralateral to the HI injury) was observed an effect of HI [$F(1,18) = 9.82$; $p<0.05$] and EE [$F(1,18) = 12.35$; $p<0.05$]. Post hoc test showed that hypoxic-ischemic animals maintained in standard environment had lower percentage occupied area by synaptophysin when compared with CTEE and HIEE groups ($p=0.001$ and $p =0.03$, respectively). These results demonstrate that HI caused a decrease of synaptophysin expression in the hippocampus and that EE reversed this effect in the contralateral hippocampus (Fig. 4 and 5).


RIGHT
LEFT
CTSE
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HISE
HIEE
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E
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Fig. 4. Occupied area by synaptophysin in the CA region from right and left hippocampus. A-D: Representative photomicrographs of right hippocampus; A - CTSE group; B - CTEE group; C - HISE group and D - HIEE group; E-F: Representative photomicrographs of left hippocampus; E - CTSE group; F - CTEE group; G - HISE group and H - HIEE group; CTSE: control group in standard environment; CTEE: control group exposed to enriched environment; HISE: hypoxic-ischemic animals in standard environment; HIEE: hypoxic-ischemic animals exposed to enriched environment. Scale bar: 50µm.

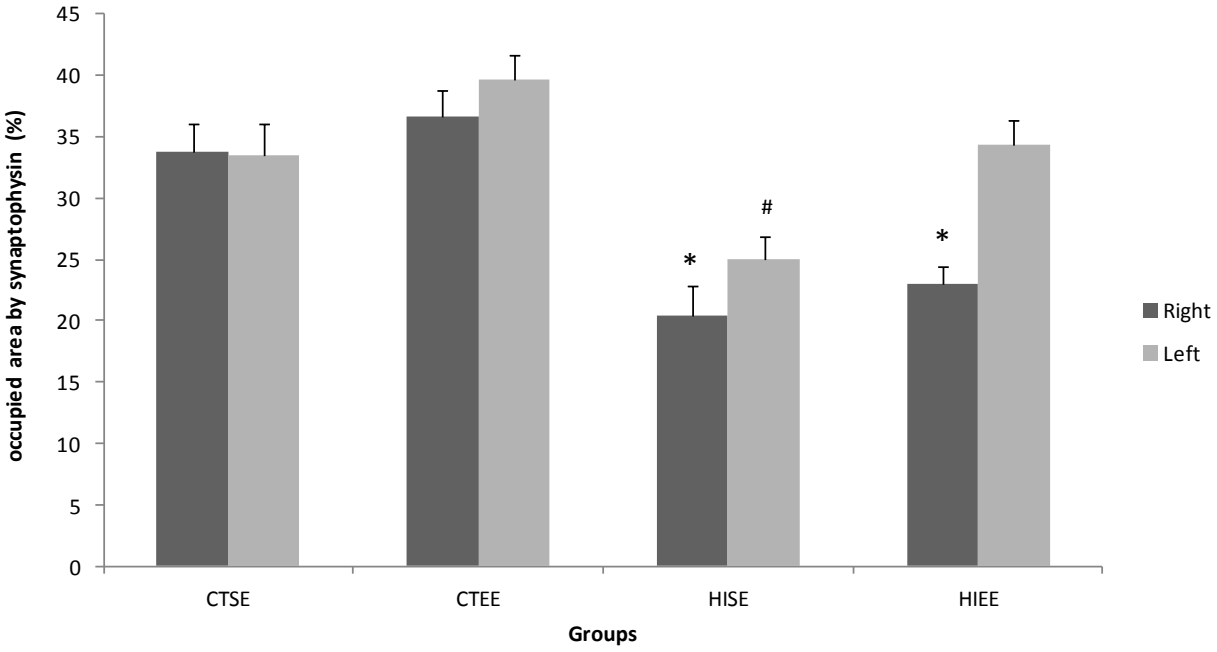


Fig. 5. Graphic representation of percentage of occupied area by synaptophysin in the CA region from right and left hippocampus. Bars represent mean ± S.E.M. of synaptophsysin area occupied per hemisphere; all analyses were performed comparing each hemisphere separately. *HISE and HIEE groups are significantly different from control groups in right hemisphere; #HISE is significantly different from CTEE and HIEE groups in left hemisphere. ANOVA followed by Tukey's test, $p \leq 0.05$. Magnification of 40x. CTSE: control group in standard environment; CTEE: control group exposed to enriched environment; HISE: hypoxic-ischemic animals in standard environment; HIEE: hypoxic-ischemic animals exposed to enriched environment.

Cell Counting

Cell number was estimated in the CA1 region of the hippocampus. Two-way ANOVA presented a main effect of *lesion* in the right [$F(1,12) = 37.01$; $p<0.05$] and left hippocampus [$F(1,12)= 26.01$; $p<0.05$] (ipsilateral and contralateral to the lesion, respectively). Tukey's post hoc test revealed that the hypoxic-ischemic animals had lower cell density than both control groups ($p \leq 0.005$ in all groups). There was no significant effect on the environment factor (fig. 6).

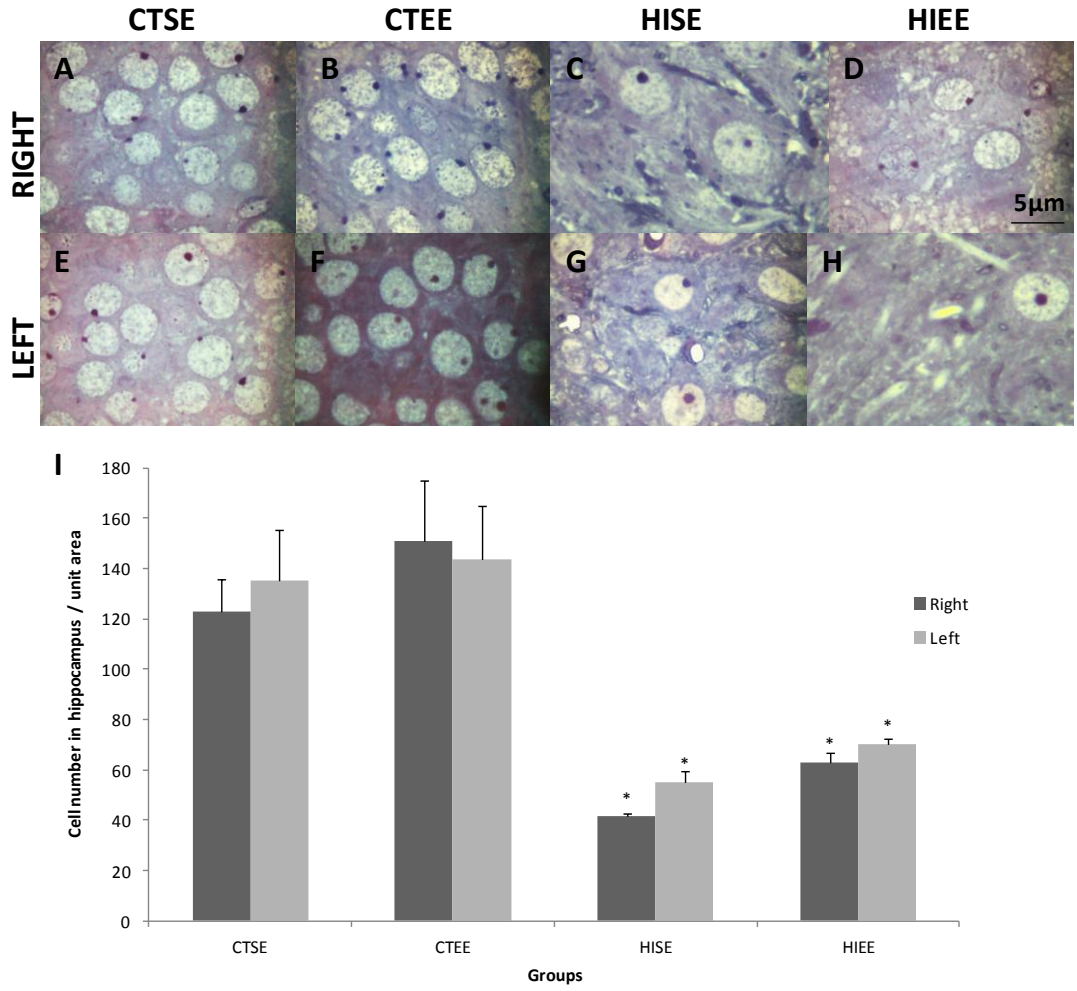
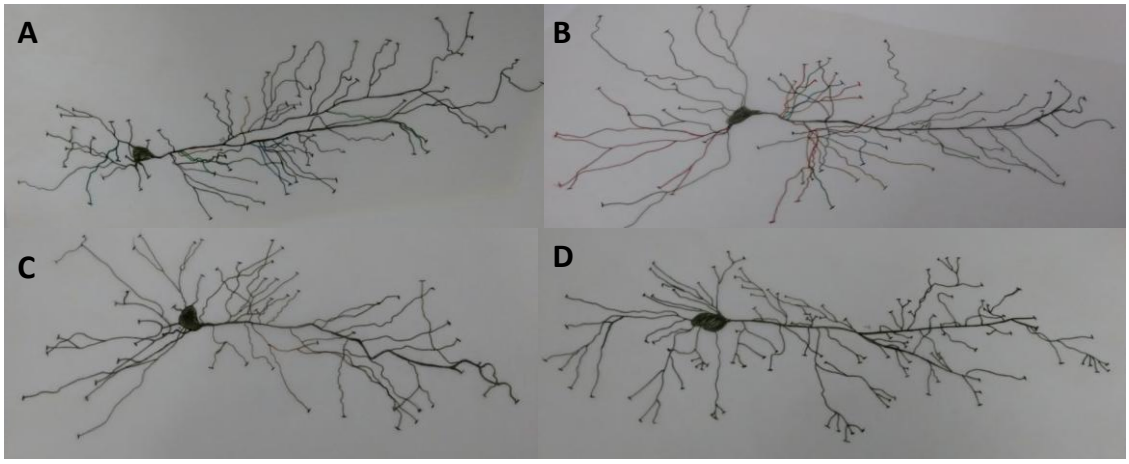


Fig 6. Number of cells per unit area in the CA region from hippocampus (pyramidal stratum,from bregma -2.30 mm to -3.60mm in accordance to Paxinos and Watson,

1986). A-D: Representative photomicrographs of CA1 region in right hippocampus; A - CTSE group; B - CTEE group; C - HISE group and D - HIEE group; E-F: Representative photomicrographs of CA1 region in left hippocampus; E - CTSE group; F - CTEE group; G - HISE group and H - HIEE group; I: Graphic representation of number of cells per unit area in CA region from hippocampus. *Difference from control groups. Results are expressed as mean ± S.E.M. ANOVA followed by Tukey's test, $p \leq 0.05$. Magnification of 1000x.CTSE: control group in standard environment; CTEE: control group exposed to enriched environment; HISE: hypoxic-ischemic animals in standard environment; HIEE: hypoxic-ischemic animals exposed to enriched environment. Atlas images adapted from Paxinos and Watson (2004).

Dendritic arborization

There were no significant differences between the hypoxic-ischemic group and control ones on dendritic arborization (fig 7). There were no effects, whatsoever, in any of the variables analyzed: primary dendrites, neither lesion [$F(1,12)=0.098$; $p>0.05$] nor environment [$F(1,12)=1.798$; $p>0.05$] effects; secondary dendrites, neither lesion [$F(1,16)=0.7079$; $p>0.05$] nor environment [$F(1,12)=0.0024$; $p>0.05$] effects; and intersections between dendrites, neither lesion [$F(1,12)=0.0039$; $p>0.05$] nor environment [$F(1,12)=0.189$; $p>0.05$] effects.



E

Group	Primary arborization	Secondary arborization	Intersections between dendrites
CTSE	6.41 ± 0.39	26.91 ± 2.26	87.75 ± 8.55
CTEE	6.12 ± 0.31	26.75 ± 0.85	77.25 ± 10.44
HISE	6.72 ± 0.30	23.29 ± 6.63	73.12 ± 10.88
HIEE	5.62 ± 0.14	23.83 ± 3.23	93.25 ± 13.95

Fig 7. Dendritic arborization in pyramidal neurons from CA1 region from left hippocampus. A-D: Drawings of pyramidal neurons showing examples of studied dendritic branches. A - CTSE group; B - CTEE group; C - HISE group and D - HIEE group; E: Total number of primary and secondary dendrites and number of intersections between dendrites. Mean ± S.E.M. of values. CTSE: control group in standard environment; CTEE: control group exposed to enriched environment; HISE: hypoxic-ischemic animals in standard environment; HIEE: hypoxic-ischemic animals exposed to enriched environment. There was no significant difference between groups.

DISCUSSION

Brain insult caused by the neonatal hypoxic-ischemic event is well recognized in the literature, evidenced by wide tissue injury in the territory irrigated by occluded carotid artery, particularly hippocampus; and as observed in our study, this brain damage has as consequence cognitive, motor and learning disabilities throughout the life (Levine, 1960, Rice *et al*., 1981, Vannucci and Vannucci, 2005, Pereira *et al*., 2007). The present study investigated the effects of daily environmental enrichment (9 weeks, 6 days/week, 1 h/day) in rats submitted to neonatal HI. Synaptophysin and GFAP levels, neuronal quantification and

dendritic arborization in the CA1 region from the hippocampus were evaluated. The results demonstrate neonatal HI caused a decreased neuronal number and synaptophysin expression. The EE only reversed the decreased synaptophysin expression in the left hippocampus without effect on other parameters.

The effects and possible mechanisms by which the EE improves memory and reduces the damage caused by neonatal hypoxia-ischemia have been studied. We previously showed that daily EE (9 weeks, 6 days/week, 1 h/day) recovered behavioral impairment on object recognition task and preserved dendritic spine density in the hippocampus (Rojas *et al.,* 2013); and, more recently, an evident protective role of EE was observed on performance in the ox-maze task, associated to the Na^+,K^+-ATPase activity recovery in the hippocampus of hypoxic-ischemic animals (Rojas *et al*., 2015).

In the present study to assess the synaptic activity in CA region from hippocampus, synaptophysin immunoistochemistry was performed. Synaptophysin is a presynaptic vesicular protein localized in nerve terminals and useful in identification of axonal nerve terminals and synapses (Walaas *et al,* 1988). Overexpression of synaptophysin results in increased neurotransmitter liberation (Alder *et al*., 1995). It is known that the neonatal hypoxia-ischemia leads to the synaptophysin decrease (Tuor*et al*., 2001, Xiao *et al*., 2008). And also studies using the EE as a neuroprotective strategy found increased synaptophysin expression indicating that EE can increase synaptogenesis (Birch *et al*., 2013, Dorfman*et al*., 2014, Marques *et al*., 2014). Confirming our working hypothesis, synaptophysin decrease in the hippocampus after HI insult was shown in both hemispheres. Interestingly, EE reversed this effect in the contralateral hippocampus, indicating a partial

recovery of the enrichment on the hypoxic-ischemic damage. As regards the right hippocampus (ipsilateral to arterial occlusion) results confirmed lower synaptophysin immunoreactivity in rats submitted to HI. These results are in agreement with anterior studies showing that unilateral brain insults induce changes in both ipsilateral and contralateral hemispheres (Cheng *et al*., 1997, Nicolelis, 1997 and Shimada *et al*., 1997). Taken out previous data (Rojas et al., 2013) this protective effect on synaptophysin expression can explain, at least in part, previous the increase in dendritic spine density after EE, which probably was associated with spinogenesis and consequently synaptogenesis.

To detect the astrocytes function, GFAP expression was also investigated in this study. The existence of reactive astrocytes is one of the most important changes observed subsequent to injury to the CNS. This cells show improved gene expression, with increased expression of GFAP (Pekny and Pekna, 2004) and both neuroprotective and neurotoxic effects of this reaction after ischemia are known (Pekny *et al*., 2014). When a stroke occurs, reactive astrogliosis with a compact glial scar formation may exert a protective role on surrounding healthy brain, or otherwise this scar can preventing axonal regeneration, prejudicing functional recovery processes (Choudhury *et al*., 2014). Neuroprotective strategies such as EE usually are associated with increase in volume and number astrocytic (Szeligo and Leblond, 1977, Sirevaag and Greenough, 1991, Briones *et al*., 2006, Williamson *et al*., 2012, Sampedro-Piquero, *et al.*, 2014) and probably this increase is involved in the functional effects of EE (Viola *et al*., 2009). Contrasting with previous findings, we found no changes in the GFAP immunoreactivity in the CA1 region from the hippocampus after EE. This result corroborate with the results found by

Sirevaag and colleagues (1991) and more recently by Viola and colleagues (2009). Maybe the discrepancies among these results are due to differences in EE protocols, once multiple protocols of EE have been reported including differences regarding physical exercise, time to exposition and age on beginning of exposure. In addition, we should consider that our evaluation is a late measure since is essential to study possible astrocytic alterations in different moments of the development, also observing early effects of the neonatal HI and of the environmental stimulation.

We also investigated cellular density with the main objective of verify cell death in CA1 area. Glutamatergic excitotoxicity, inflammation and oxidative stress are well known to be central cell damage and death mechanisms in cerebral ischemia and hypoxia-ischemia (Siesjö, 1988, Pimentel *et al*., 2011, Puyal *et al.*, 2013). Several studies have demonstrated that there is a progressive injury in nervous tissue due to apoptosis (McLean and Ferriero, 2004, Hossain, 2008) and necrosis (Carloni *et al*., 2007), both may expand for days after injury, resulting in wide cellular loss and extensive atrophy in some structures such as hippocampus, striatum and cortex (Pereira *et al*., 2007, Pereira *et al*., 2008). Corroborating these, in our study was observed a significant reduction of the neuronal number in HI animals in both hemispheres, independent of environment. This data contrary our initial hypothesis, once is known that at the cellular level EE promotes formation of new cells (Kempermann *et al*., 1997) and reduces apoptotic cell death in rat hippocampus (Young *et al*., 1999). Interestingly, Rodrigues and colleagues (2004) demonstrated a complete reversal of morphological damage to the hippocampus by an early protocol of tactile stimulation (PND 8 to 21) in HI rats. Considering that here the

environmental stimulation happening only 14 days after the HI episode, this fact may explain such distinct results.

Another evaluated parameter was dendritic arborization since pathological alterations in the dendritic structure are an early characteristic of brain damage in hypoxia-ischemia (Wen *et al*., 2013). Transitory ischemic episodes can stimulate rapid morphological changes in neurons as well as widespread beading and distension of dendrites, besides the selective deterioration of dendrites for the period of early ischemia probably serves as a precursor to neuronal cell death (Ikonomidou *et al*., 1989, Hori and Carpenter, 1994, Matesic and Lin, 1994). It is well established that pyramidal neurons of the CA1 region of the hippocampus usually die several days after a transient ischemic experience (Kirino, 1982), however exhibit extensive beading of dendrites and collapse of the dendritic cytoskeleton few hours after the ischemic damage (Hsu and Buzsaki, 1993, Hori and Carpenter, 1994, Matesic and Lin, 1994). On the other hand, EE paradigm is extensively used to study experience-dependent brain plasticity. Beauquies and coworkers (2010) demonstrate that a short exposure to EE was sufficient to enhance dendritic arborization and spine dendritic density of pyramidal CA1 neurons in diabetic rats. Biernaskie and Corbett (2001) using an animal model of focal ischemia found enhanced dendritic complexity and length in the basilar dendritic arbor in the ischemic animals exposed to EE combined with a task-specific rehabilitative therapy. Counteracting these studies, data obtained from Pascual and Bustamante (2013) indicated that the EE did not modify the Purkinje cell dendritic branching. The present results demonstrated no differences between groups on dendritic branching. A limitation of this study is the extensive damage on the ipsilateral

hippocampus, then data analyzes had been carried out in the hemisphere contralateral to the ischemia. Considering that in an anterior study we found preservation of the dendritic spine density on both hemispheres after environmental stimulation (Rojas *et al*., 2013), we can propose that the main morphological adaptation is on the dendritic spine plasticity, rather than on the general dendritic morphology. Another possibility is the evaluation of both, basilar and apical dendritic arbor, since previous studies demonstrate that apical arbor is not affected by hypoxic-ischemic damage (Biernaskie and Corbett, 2001). Obviously there are many open questions with respect to how neuronal changes in HI animals exposed to EE are modulated.

CONCLUSION

Concluding, a protective role of environmental enrichment was observed on decreased synaptophysin expression after neonatal hypoxia-ischemia, however no enrichment effects were revealed in neuronal loss consequent to HI. Even though our results do not fully confirm the working hypothesis, we demonstrate the important effect of HI lesion on the synaptophysin immunoreactivity and neuronal number. More studies are necessary to explain the functional recovery observed in animals exposed to EE, supporting our initial hypothesis that environmental enrichment could be an effective experimental strategy of neuroprotection after brain insult.

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5. DISCUSSÃO

A principal proposta desta tese foi avaliar de forma mais profunda o papel neuroprotetor do enriquecimento ambiental em casos de HI neonatal, buscando alicerces para a sua aplicação clínica, uma vez que a estimulação em crianças poderia exercer um papel importante na recuperação de funções sensório-motoras e na plasticidade encefálica. Estudos prévios já demonstraram o efeito benéfico do EA tanto na reversão de um déficit cognitivo de memória quanto na diminuição da densidade de espinhos dendríticos causados pela HI neonatal (Rojas *et al*., 2013), não ficando totalmente claraa base morfológica que permitiu que essas melhorias acontecessem. Tendo em vista o exposto, considerando a importância epidemiológica da HI neonatal e levando em conta o potencial terapêutico do EA, o objetivo principal desta tese foi verificar alguns parâmetros considerados importantes para a compreensão de como essa estratégia terapêutica atua beneficamente nestes casos. São eles: a) o desempenho de ratos Wistar em um teste cognitivo, o “Ox-maze”; b) a atividade das enzimas Na^+,K^+-ATPase, CAT e GPx no hipocampo; c) a imunorreatividade das proteínas GFAP e sinaptofisina no hipocampo; d) a densidade neuronal na região CA1 do hipocampo e e) a arborização dendrítica em neurônios piramidais da região CA1 hipocampal.

O primeiro artigo se refere à investigação do desempenho de ratos no teste do “Ox-maze” e na atividade das enzimas Na^+,K^+-ATPase, CAT e GPx no hipocampo de animais submetidos à HI e expostos ao EA. Os resultados demonstraram que a HI neonatal causa um importante déficit de memória, o qual é revertido pelo EA. Confirmando nossa hipótese, a HI resultou em um declínio da atividade da Na^+,K^+-ATPase no hipocampo, o qual também foi revertido pela estimulação ambiental.

O teste do “Ox-maze” é um novo teste comportamental com o diferencial de não apresentar um componente aversivo como outros testes correlatos, tais como o labirinto aquático e a esquiva inibitória (Wood *et al*., 2011). Este é o primeiro estudo demonstrando o efeito da HI no “Ox-maze”, com uma adaptação do teste original, utilizado por Wood e colaboradores (2011).O “Ox-maze” é um teste que envolve discriminação visual (pistas proximais), aprendizado e memória (Wood *et al*., 2011). Sabe-se que o hipocampo é uma estrutura crítica em testes de comportamento que envolvem pistas visuais, já que ratos com disfunções hipocampais costumam ter dificuldade neste tipo de tarefas de memória (Kim e Lee, 2012). O hipocampo é responsável pelo direcionamento até a pista e a posterior integração da informação, atuando junto ao estriado dorso medial na seleção da ação a ser realizada (Kimchi e Laubach, 2009; Braun e Hauber, 2011). A literatura sugere que estas estruturas são funcionalmente importantes na aquisição da memória espacial e na flexibilidade do comportamento, embora as evidências fisiológicas ainda sejam escassas (Brown, *et al*., 2012, Delcasso *et al*., 2014). No nosso estudo, os animais HI apresentaram um claro prejuízo cognitivo quando comparados aos animais controle neste teste. Como já mencionado, são conhecidos os danos cognitivos causados pela HI, particularmente em processos que envolvem estruturas hipocampais (Pereira *et al*., 2007, 2009; Cengiz *et al*., 2011; Zhao *et a*l., 2013) e acredita-se que este efeito funcional esteja associado ao extenso dano estrutural desta estrutura em ratos submetidos à HI neonatal (Pereira *et al*., 2007, 2008; Rojas *et a*l., 2013).

A fim de correlacionar os possíveis mecanismos implicados neste efeito comportamental do EA, a atividade da enzima Na^+,K^+-ATPase foi investigada, uma vez que muitos estudos afirmam que ela está envolvida em mecanismos de memória espacial (Wyse *et al*., 2004; dos Reis-Lunardelli *et al*., 2007; Moseley *et a*l., 2007; Heo *et a*l., 2012; Jaques *et al*., 2013). No nosso estudo, embora não houvesse diferença neste parâmetro entre os grupos no hipocampo direito, no hipocampo esquerdo (contralateral à lesão) o EA foi efetivo ao reverter a disfunção enzimática,causada pela HI, na vida adulta dos animais. Tal resultado corrobora nosso estudo prévio que também demonstrou efeitos crônicos da HI neonatal no tecido nervoso; níveis aumentados de SOD e de fator neurotrófico derivado do encéfalo (BDNF) no hipocampo de ratos adultos submetidos à HI foram identificados, sendo este efeito recuperado pelo EA (Pereira *et al*., 2009). Tal achado poderia indicar que a atividade da Na^+,K^+-ATPase no hipocampo contralateral pode ser uma consequência tardia da lesão hipóxico-isquêmica. Existem dados, como o de Geddes e colaboradores (2001), utilizando o mesmo modelo animal empregado pelo nosso grupo, que demonstram que a atrofia encefálica é progressiva ao longo de semanas. Mishima e colaboradores (2005) também demonstraram que o dano encefálico pós-HI é progressivo e causa prejuízos tardios na função encefálica, evoluindo até a 17ª semana após a lesão e afetando significativamente o hemisfério contralateral. Outro estudo, utilizando o mesmo modelo de oclusão arterial unilateral, avaliou a atividade das enzimas Na^+,K^+-ATPase e SOD em diferentes momentos após a lesão, evidenciando que há uma diminuição precoce da atividade da enzima Na^+,K^+-ATPase, bem como um aumento tardio na atividade da SOD no hipocampo (Weiss *et al*., 2011). Embora no nosso

estudo tenhamos utilizado o mesmo modelo experimental de lesão dos trabalhos acima mencionados, o objetivo aqui foi investigar parâmetros tardios relacionados com a disfunção bioquímica e morfológica do tecido nervoso. Tal aspecto também é fundamentado pela necessidade de um intervalo de tempo para incluirmos a intervenção terapêutica pelo EA, mantido desde o desmame até a vida adulta. Neste estudo, as análises foram realizadas aproximadamente 3 meses após a cirurgia de indução de HI, o que nos leva a acreditar que essa seja a causa de não haver alteração no hipocampo direito, sugerindo que isso poderia ser resultado de uma reversão pelo EA de um aumento anterior precoce na atividade da Na^+,K^+-ATPase.

A importância da capacidade antioxidante dos tecidos na defesa endógena contra o dano causado pela presença de radicais livres (Halliwell e Gutteridge, 1990) é amplamente conhecida. Tanto GPx quanto CAT exercem um papel essencial ao sequestrar peróxido de hidrogênio, atuando junto à SOD na proteção antioxidante (Southorn e Powis, 1988; Halliwell e Gutteridge, 1990; McCord, 1993). A função antioxidante da SOD é efetiva particularmente quando acompanhada de mudanças também nas atividades da CAT e da GPx (Halliwell, 2001). De forma interessante, nossos resultados demonstram que não há diferenças entre os grupos controle e HIs na avaliação desses parâmetros. Os resultados atuais podem ser comparados uma correlação com estudo anterior onde se demonstrou um aumento da atividade da SOD no hipocampo ipsilateral em animais HI não estimulados, que foi revertido pelo EA (Pereira *et al*., 2009). Então, considerando que o aumento da SOD é identificado somente em animais HI mantidos em ambiente padrão (Pereira *et al.*, 2009) e que este aumento não está acompanhado de aumentos nas

atividades da CAT e GPx, confirma-se a hipótese da participação do estresse oxidativo no dano neural após a HI neonatal e que o EA é capaz de proteger a longo prazo a vulnerabilidade hipocampal.

De um modo geral, essa primeira parte da tese demonstrou um claro papel neuroprotetor do EA no desempenho dos animais no “Ox-maze” após a HI neonatal. Este efeito talvez esteja associado, ao menos em parte, à recuperação da atividade da enzima Na^+,K^+-ATPase. Portanto, esses dados proporcionam mais evidências para o potencial terapêutico da estimulação ambiental em ratos submetidos ao procedimento de HI neonatal e sugerem um possível mecanismo de neuroproteção (por meio da enzima Na^+,K^+-ATPase) envolvido.

No segundo artigo, utilizando o mesmo modelo experimental anterior, avaliamos os níveis de sinaptofisina e GFAP, densidade neuronal e arborização dendrítica na região CA1 do hipocampo. Os resultados demonstraram que a lesão hipóxico-isquêmica causa um declínio na densidade neuronal e na expressão de sinaptofisina. Já o EA foi parcialmente efetivo na recuperação dos níveis de sinaptofisina em animais HI.

Estudos demonstraram que o dano hipóxico-isquêmico causa uma diminuição dos níveis de sinaptofisina na região adjacente ao infarto e na região CA3 hipocampal em ratos (Tuor *et al*., 2001; Xiao *et al*., 2008). Confirmando nossa hipótese de trabalho, foi observada uma diminuição da expressão de sinaptofisina nos dois hemisférios hipocampais em ratos adultos após o insulto hipóxico-isquêmico neonatal. Ademais, a exposição ao ambiente enriquecido foi capaz de recuperar a expressão de sinaptofisina no hipocampo

esquerdo (contralateral à obstrução arterial). OEA já havia sido utilizado como estratégia neuroprotetora em outros tipos de lesão, demonstrando um aumento significativo na expressão de sinaptofisina, indicando o papel do ambiente enriquecido na sinaptogênese em estruturas como o giro denteado, retina e medula espinhal (Birch *et al*., 2013; Dorfman *et al*., 2014; Marques *et al*., 2014). Em conjunto, esses resultados podem se relacionar com nossos achados prévios onde foi identificado reversão da diminuição da densidade de espinhos dendríticos em animais hipóxico-isquêmicos expostos ao EA (Rojas *et al*., 2013), uma vez que o aumento na expressão de sinaptofisina poderia ser explicado pela espinogênese, e consequentemente, sinaptogênese. Não há como afirmar que todo espinho efetivamente formará uma nova sinapse, no entanto, não se pode excluir essa possibilidade, visto que os espinhos dendríticos são sítios receptivos para a maioria das sinapses excitatórias no sistema nervoso (Tada e Sheng, 2006), constituindo um excelente modelo de estudo para sinaptogênese e plasticidade sináptica em curto e longo prazo (Yuste e Bonhoeffer, 2004).

A presença de astrócitos reativos é um dos mais importantes marcos subsequentes ao dano no sistema nervoso central. Além das alterações morfológicas, como a hipertrofia dos processos celulares, que acontecem nessas células, há um aumento da expressão de GFAP (Pekny e Pekna, 2004; Chen *et al*., 2015). Aumentos da imunoreatividade ao GFAP na região CA1 hipocampal após lesões isquêmicas estão diretamente associados com a extensão e a maturidade da necrose neuronal (Stoll *et al,* 1998).As estratégias neuroprotetoras como o EA normalmente também estão associadas a aumentos no volume e no número de astrócitos (Briones *et al*., 2006;

Williamson *et al*., 2012; Sampedro-Piquero *et al*., 2014) e provavelmente este aumento esteja relacionado às consequências funcionais do EA (Viola *et a*l., 2009). Em nosso estudo não encontramos diferenças na imunorreatividade do GFAP na região CA1 do hipocampo, corroborando alguns resultados anteriores (Sirevaag e Greenough, 1991; Viola *et a*l., 2009). Salientamos que em outros estudos que encontraram resultados semelhantes aos nossos, foi observado aumento significativo na ramificação dos processos astrocíticos, bem como um aumento no número e comprimento dos processos principais que se estendem em uma orientação paralela às fibras nervosas CA1. Isto poderia proporcionar uma morfologia mais estrelada aos astrócitos, o que poderia estar relacionado com o aumento da densidade sináptica hipocampal observada em outros estudos (Viola *et al*., 2009). Esta questão fica ainda não respondida, e, talvez, estudos em diferentes fases do desenvolvimento e observando a morfologia astrocítica precisam ser desenvolvidos para conhecer a resposta astrocitária à HI bem como os possíveis efeitos da estimulação por EA, já quemudanças estruturais em redes astrocitárias são parte integrante dos processos de plasticidade que ocorrem no cérebro.

Estudos reportaram que a injúria no tecido nervoso é progressiva, seja devido à apoptose (McLean e Ferriero, 2004; Hossain, 2008) ou à necrose (Carloni *et al*., 2007), resultando em perda celular considerável e atrofia extensa em algumas estruturas neurais, como o hipocampo (Pereira *et al*., 2007; Pereira *et al*., 2008). Em nosso estudo foi observada uma significativa diminuição na densidade neuronal no hipocampo de animais hipóxico-isquêmicos em ambos os hemisférios, independente do ambiente ao qual foram expostos. Estes dados contrariam parcialmente a nossa hipótese inicial,

uma vez que se sabe que o EA promove a formação de novas células (Kempermann *et al*., 1997) e reduz a morte celular por apoptose no hipocampo de ratos (Young *et al*., 1999). Um dado interessante é que Rodrigues e colaboradores (2004) demonstraram uma completa reversão do dano morfológico no hipocampo utilizando um protocolo de estimulação tátil precoce (iniciando no 8º dia pós-natal) em animais submetidos à HI. No nosso trabalho o EA teve início mais tardiamente (aos 21 dias de vida), talvez esse fato possa explicar os resultados divergentes, podendo ser levado em consideração em estudos futuros.

Alterações na estrutura dendrítica são uma característica precoce do dano encefálico observado na HI neonatal (Wen *et al*., 2013). Por outro lado, a exposição a ambientes enriquecidos é amplamente utilizada no estudo da plasticidade celular dependente da experiência. Estudos como o de Beauquies e colegas (2010) e Biernaskie e Corbett (2001) demonstraram os efeitos benéficos da exposição ao EA na arborização dendrítica e na densidade de espinhos dendríticos nos neurônios piramidais da região CA1 hipocampal de ratos diabéticos e isquêmicos, respectivamente. No entanto, contrariando estes estudos, Pascual e Bustamante (2013) indicaram que o EA não modifica de forma significativa a arborização dendrítica das células de Purkinje do cerebelo; tal resultado é análogo ao nosso, já que tampouco encontramos diferenças entre os grupos nesta análise. Destaco aqui a oclusão arterial foi realizada no hemisfério direito e as análises de arborização dendrítica foram realizadas somente no hemisfério contralateral (esquerdo). Tal fato se deve à presença de cistos porencefálicos, que são lesões cavitárias focais (Ten*et al*., 2004), nos encéfalos dos animais submetidos à HI, as quais não permitiram a visualização

de neurônios que cumprissem com os requisitos para participação da análise. Talvez este seja o motivo da inexistência de diferenças entre os grupos. Salientamos que outros efeitos morfológicos não são aqui excluídos, visto que em um estudo anterior, ao avaliar a densidade de espinhos dendríticos, tenhamos encontrado efeitos significativos da lesão em ambos os hemisférios (Rojas *et al*., 2013).Uma possibilidade em aberto é a avaliação tanto da árvore basal quanto apical feita nesse estudo, uma vez que estudos anteriores demonstraram que a árvore apical não é tão severamente afetada pelo dano hipóxico-isquêmico (Biernaskie e Corbett, 2001).

Frente a isso é bastante evidente que muitas questões permanecem em aberto com respeito a como são moduladas as alterações neuronais em animais HI expostos a ambientes enriquecidos, já que as mudanças comportamentais induzidas pelo EA devem estar relacionadas a estas alterações funcionais. Os resultados descritos nesta tese suportam a utilização do EA como paradigma terapêutico no tratamento da HI neonatal. No entanto, embora os resultados encontrados até aqui pelo nosso grupo de pesquisa sejam promissores quanto à melhoria histopatológica e cognitiva fornecida pelo EA , ainda resta muito trabalho a ser feito para compreender como o EA exerce esses efeitos. Também deve ser considerado que o modelo experimental de EA em animais ainda não foi padronizado, com condições de habitação, estímulos ambientais, número de animais por gaiola, idade dos animais no início do enriquecimento, assim como a duração do enriquecimento variando muito entre os diferentes grupos de estudo. Todavia, essas são variáveis fáceis de controlar, não sendo um fator que impeça a utilização desta terapia, já que as vantagens de sua utilização extrapolam em muito as suas limitações. O EA

é uma estratégia terapêutica plausível de ser realizada, uma vez que não exige infraestruturas complexas e nem grandes dispêndios financeiros, sendo acessível a populações que normalmente não teriam acesso a outros meios de tratamento.

6. CONCLUSÕES

Os dados obtidos permitem concluir que:

- A HI neonatal resultou em déficit funcional observado no teste do Ox-maze e tal foi revertido pela exposição ao EA;

- O EA foi capaz de reverter a diminuição da atividade da enzima Na^+,K^+-ATPase em animais submetidos à HI;

- O efeito lesivo da HI neonatal foi confirmado com a diminuição da imunorreatividade à sinaptofisina, sendo revertida em animais expostos ao EA;

- Foi demonstrada uma clara diminuição da densidade neuronal em animais submetidos ao procedimento de hipóxia-isquemia neonatal, independente do ambiente ao qual os animais foram mantidos;

- Quanto à imunorreatividade à GFAP no hipocampo, não houve diferença entre os grupos HI e controle, submetidos ou não ao EA;

- Quanto à avaliação dos ramos primários e secundários da arborização dendrítica no hipocampo, não houve diferença entre os animais HI e controle, submetidos ou não ao EA;

Sendo assim, evidenciou-se neste estudo que o EA, além de gerar recuperação funcional, é eficaz na recuperação de alterações bioquímicas e histológicas nos neurônios do hipocampo. Estes achados somam-se aos previamente publicados, indicando que a estimulação em EA constitui uma importante abordagem na reabilitação neurofuncional, podendo exercer importante significância clínica.

7. PERSPECTIVAS

Com base nos dados e conclusões obtidas, este estudo nos deixa como perspectivas:

- Investigar outras variáveis morfológicas que possam ser influenciadas pelo enriquecimento ambiental precoce, tais como, morfologia dos espinhos dendríticos;

- Avaliar parâmetros bioquímicos como peroxidação lipídica, carbonilação, níveis de glutationa reduzida e oxidada, S100B, bem como BDNF;

- Investigar alguns parâmetros já avaliados, como enzimas antioxidantes, GFAP e sinaptofisina, com diferentes protocolos de enriquecimento ambiental, com início de estimulação mais precoce.

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ANEXO:

Prezado Pesquisador LENIR ORLANDI PEREIRA SILVA,

Informamos que o projeto de pesquisa Enriquecimento ambiental como estratégia neuroprotetora em ratos submetidos à hipóxia-isquemia, encaminhado para análise em 22/12/2011, foi aprovado pelo Comissão de Ética no Uso de Animais com o seguinte parecer:

Projeto: 22045 - Enriquecimento ambiental como estratégia neuroprotetora em ratos submetidos à hipóxia-isquemia

O pesquisador informa que os animais apresentam uma rápida recuperação, sem interferência no cuidado maternal e/ou limitação para a amamentação, e por isso não se faz rotineiramente analgesia. A CEUA considera que esta justificativa não exclui a necessidade de analgesia após o procedimento cirúrgico. Recomenda-se também que o pesquisador reveja a estatisicanao paramétrica proposta.

CEUA/UFRGS

Parecer Reunião 23/04/2012

A diligência foi respondida adequadamente. Encaminhe-se para aprovação. Serão utilizados 96 ratos machos wistar.

CEUA/UFRGS

Atenciosamente,

Comissão de Ética no Uso de Animais