UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:

NEUROCIÊNCIAS

Efeitos do Enriquecimento Ambiental sobre o Comportamento e a Densidade de Espinhos Dendríticos no Hipocampo de Ratos Submetidos à Hipóxia-Isquemia Neonatal

Joseane Jiménez Rojas

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Dissertação apresentada ao Programa de Pós-Graduação como requisito parcial para a obtenção do título de Mestre em Ciências Biológicas: Neurociências.

Orientadora: Prof. Dra. Lenir Orlandi Pereira Silva

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“Nosotros, los de entonces, ya no somos los mismos”

Pablo Neruda

Para as pessoas que mais amo no mundo, Dilce Annelise e José Orlando, José Waldomiro e Edson Vinícius.

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LISTA DE ABREVIATURAS

AMPA	A-amino-3-hidroxi-5-metil-4-isoxasol-propionato
ATP	Adenosina Trifosfato
Ca^{2+}	Íon cálcio
CA1	Subcampo 1 do Corno de Amon
CA3	Subcampo 3 do Corno de Amon
EA	Enriquecimento Ambiental
HI	Hipóxia-isquemia
N_2	Nitrogênio
NMDA	N-metil-D-aspartato
O_2	Oxigênio Molecular

RESUMO

A hipóxia-isquemia (HI) é a principal causa de mortalidade no período perinatal e, nos sobreviventes, a incidência de seqüelas neurológicas é elevada. O encéfalo imaturo, altamente susceptível ao insulto hipóxico-isquêmico, é vulnerável a estímulos ambientais tais como o enriquecimento ambiental (EA). O objetivo deste estudo foi investigar o desempenho comportamental no teste do campo-aberto, reconhecimento de objetos, esquiva-inibitória e no rota-rod, bem como a densidade de espinhos dendríticos no hipocampo, utilizando o método de Golgi, em ratos submetidos à HI e expostos ao EA (1h/dia, 6 dias/semana, 9 semanas). Ratos de 7 dias de idade foram submetidos ao procedimento de HI e divididos em 4 grupos experimentais: controle mantido em ambiente padrão (CTAP), controle em ambiente enriquecido (CTAE), HI em ambiente padrão (HIAP) e HI em ambiente enriquecido (HIAE). Parâmetros comportamentais e morfológicos foram avaliados após 9 semanas de estimulação ambiental. Os dados indicaram que a memória de reconhecimento de objetos foi prejudicada em ratos HI adultos e recuperada após a estimulação pelo ambiente enriquecido; no teste de esquiva-inibitória os animais apresentaramum prejuízo na memória aversiva em animais HI, independentemente do ambiente. Surpreendentemente, no teste do campo-aberto, um maior número de *crossings* foi identificado nos grupos HI no primeiro minuto quando comparados aos grupos controle. No teste de rota-rod não foram detectadas diferenças entre animais controle e animais HI. Resultados morfológicos demonstraram uma diminuição na densidade de espinhos dendríticos no hipocampo de animais HI, com recuperação pelo EA. A densidade de espinhos dendríticos do hemisfério esquerdo (contralateral à oclusão arterial) obteve os melhores resultados, indicando uma recuperação total

do dano hipóxico-isquêmico pelo EA. Os dados dos espinhos dendríticos do hemisfério direito indicaram uma recuperação parcial pela estimulação ambiental nos animais HI. Concluindo, o enriquecimento ambiental foi efetivo na recuperação do déficit comportamental e da densidade de espinhos dendríticos nos neurônios hipocampais conseqüente à hipóxia-isquemia neonatal em ratos.

Palavras-chave: Hipóxia-isquemia, enriquecimento ambiental, neuroproteção.

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 responsive to environmental stimuli, as environmental enrichment (EE). The aim of this study was to investigate behavioral performance in the open field apparatus, objects recognition, inhibitory avoidance and in the Rota-rod apparatus, and dendritic spines density in the hippocampus, using the Golgi technique, in rats submitted to the HI and exposed to EE (1h/day, 6 days/week, 9 weeks). Seven-days old rats were submitted to the HI procedure and divided in 4 groups: control in standard conditions (CTSE), control in enriched environment (CTEE), HI in standard conditions (HISE) and HI in enriched environment (HIEE). Behavioral and morphological parameters were evaluated after 9 weeks of environmental stimulation. Data indicated that object-recognition memory was impaired in HI adult rats and recovered after stimulation by the EE; in the inhibitory avoidance task was demonstrated aversive memory impairment in HI animals, independent of the environment. Interestingly, in the open field task, significant more crossing responses were identified in HI groups, in the first minute, comparing to control groups. No differences between control and HI adult animals were detected in the rota-rod test. Morphological results demonstrated a decreased spines density in the hippocampus of the HI animals, with recovery by the EE. Dendritic spines density from left hemisphere (contralateral to arterial occlusion) obtained the better results, indicating a total recovery effect of the EE on HI damage. Data of dendritic spines from right hemisphere indicated a partial recovery by the environmental stimulation on HI animals. Concluding, environmental enrichment was

effective in recovery behavioral impairment and dendritic spine density in hippocampal neurons, consequent to neonatal hypoxia-ischemia in rats.

Keywords: Hypoxia-ischemia, environmental enrichment, neuroprotection.

1 INTRODUÇÃO

1.1 Hipóxia-Isquemia

A hipóxia é a principal causa de morbidade e mortalidade no período neonatal e a causa mais importante de dano neurológico ao recém-nascido, ocorrendo em aproximadamente 6 por 1000 nascidos vivos a termo (FERRIERO, 2004). Estimativas da Organização Mundial da Saúde (OMS) indicam que dos 37% dos casos de mortalidade infantil antes dos 5 anos de idade, 23% deles estão relacionados à asfixia perinatal (DURAN *et al.*, 2007), sendo que mais de 25% dos sobreviventes poderão exibir incapacidades neuropsicológicas permanentes, incluindo retardo mental, paralisia cerebral, epilepsia ou dificuldade de aprendizagem (VANNUCCI e HAGBERG, 2004; WEITZDOERFER *et al*., 2004).

Estudos prévios têm indicado que o encéfalo imaturo dos recém-nacidos é altamente suscetível à hipóxia-isquemia (HI) quando comparado a encéfalos adultos (RICE *et al.*, 1981). A vulnerabilidade aumentada ao dano hipóxico-isquêmico em neonatos pode ser atribuída a muitas razões, incluindo um risco maior de fracasso energético, a imaturidade da barreira hemato-encefálica e substância branca, uma alta suscetibilidade à excitotoxicidade glutamatérgica (McDONALD *et al.,* 1990), baixa expressão de transportadores de glutamato (VANUCCI e HAGBERG, 2004), bem como outros mecanismos ainda desconhecidos. A asfixia fetal ocorre primariamente como resultado de troca placentária prejudicada, sendo que o fluxo sangüíneo uterino prejudicado, a hipóxia materna, a insuficiência placentária e a compressão do cordão umbilical, entre outras causas, podem interferir com a transferência de substratos (WILLIAMS e LUCCI, 1990; JENSEN, 2002).

A encefalopatia hipóxico-isquêmica está intimamente relacionada à asfixia perinatal. Qualquer processo mórbido que envolva a presença de hipoxemia, isquemia e acidose no feto, seja no período anterior ao parto ou durante o mesmo, pode desencadear a cascata de alterações que culmina na lesão do sistema nervoso central (GUINSBURG, 2002). O dano cerebral hipóxico-isquêmico é um processo evolutivo, o qual se inicia durante o insulto e estende-se no período de recuperação após a lesão por reperfusão (SHERMAN, 1998). A redução do fluxo sangüíneo cerebral desencadeia eventos tóxicos interligados, tais como, a falência energética, a despolarização das membranas, a liberação excessiva de aminoácidos excitatórios, o acúmulo de radicais livres e a apoptose, contribuindo para a disfunção celular e a morte neuronal após insultos hipóxico-isquêmicos (VEXLER e FERRIERO, 2001; HOSSAIN, 2005).

Em relação às respostas celulares, a redução do fluxo sanguíneo cerebral inicia uma cascata de eventos bioquímicos deletérios que duram horas ou dias. A depleção do oxigênio impossibilita a fosforilação oxidativa e ocorre uma mudança para o metabolismo anaeróbico. Este é um estado de energia ineficiente que resulta na rápida depleção de reservas de fosfato de alta energia, incluindo a molécula de adenosina trifosfato (ATP). A redução da síntese de ATP resultante da hipóxia acentuada altera o equilíbrio iônico através da membrana celular (GOLAN e HULEIHEL, 2006; PERLMAN, 2006). Transcorridos segundos do insulto isquêmico, a atividade elétrica normal encefálica cessa como resultado da ativação dos canais de K^+ da membrana e da hiperpolarização generalizada. A hiperpolarização pode ser devido à abertura de canais de K^+ como resposta às mudanças agudas nas

concentrações locais de ATP, H^+ ou Ca^{2+}. Essa resposta, supostamente de proteção, contudo, não preserva os níveis de fosfato de alta energia nos tecidos, como as concentrações de fosfocreatina (PCr), assim, o ATP diminui passados minutos do início da isquemia. A queda na PO_2 durante a isquemia leva a uma produção aumentada de ácido láctico já que as células sofrem uma mudança de dependência do metabolismo aeróbico a uma dependência da glicólise. A acidose láctica resultante diminui o pH do tecido isquêmico de valores normais (7,3) a valores isquêmicos oscilando entre 6,8 e 6,2, dependendo, em parte, das quantidades pré-isquêmicas disponíveis de glicose para conversão em ácido láctico. Adicionalmente, o efluxo de K^+ de neurônios despolarizados resulta em elevações da concentração extracelular de K^+ e despolarização celular massiva, um estado conhecido como depressão generalizada, a qual pode se propagar por todo o tecido encefálico (SIEGEL *et al*., 2006). A falência na bomba de íons transcelulares resulta no acúmulo de cálcio (Ca^{2+}) extracelular, enquanto que o sódio (Na^+) entra na célula carregando água (edema citotóxico). As mudanças nos íons extracelulares causadas pela hipóxia-isquemia estão demonstradas na Figura 1.

Conseqüentemente, a despolarização da membrana resulta em uma liberação de neurotransmissores excitatórios, especificamente o glutamato dos terminais axônicos. A Figura 2 ilustra a complexidade do sistema glutamatérgico. A transmissão do glutamato é estreitamente regulada por inibição retrógrada da recaptação pré-sináptica, bem como por diversos transportadores de glutamato localizados primariamente nas células gliais. A sinalização glutamatérgica ocorre tanto em densidades pré-sinápticas quanto pós-sinápticas, atuando sobre receptores de glutamato que podem ser

caracterizados como ionotrópicos ou metabotrópicos. Os três receptores ionotrópicos excitatórios são AMPA (a-amino-3-hidroxi-5-metil-4-isoxazol propionato), NMDA (N-metil-D-aspartato) e cainato. O único receptor ionotrópico inibitório é a 4-aminopiridina. Os receptores do glutamato metabotrópicos (mGluRs) são proteínas G acopladas e estão divididos em três grupos com base no efetor acoplado e na sensibilidade do ligando: Grupo I (mGluR1 a-d, mGluR5 a-b), Grupo II (mGluR2/3) e Grupo III (mGluR4, mGluR6-8) (KELMENDI *et al.,* 2006).

Nos casos de isquemia, o glutamato, ativa os receptores NMDA (N-metil-D-aspartato), AMPA (a-amino-3-hidroxi-5-metil-4-isoxazol propionato) e cainato, resultando no influxo de Na^{+} e Ca^{2+} aos neurônios pós-sinápticos. O aumento de Ca^{2+} intracelular induz à produção do radical livre óxido nítrico. O efeito combinado de falência de energia celular, acidose, liberação de glutamato, aumento de Ca^{2+} intracelular e neurotoxicidade por óxido nítrico altera os componentes celulares essenciais, levando à morte neuronal (PERLMAN, 2004; SHALAK e PERLMAN, 2004; HOSSAIN, 2005; PERLMAN, 2006).

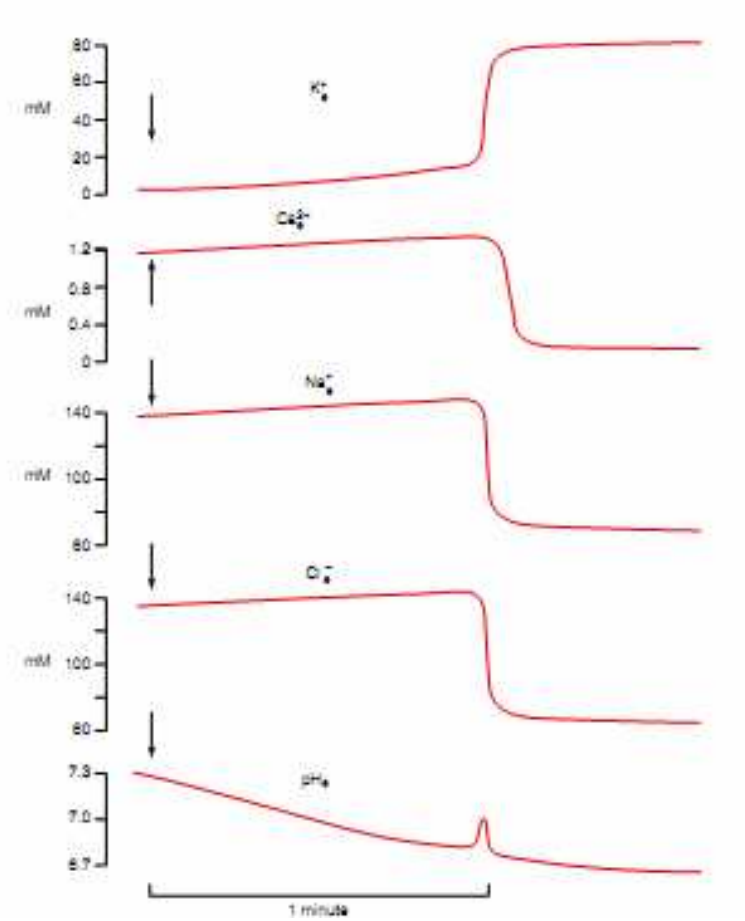

FIGURA 1 – Alterações iônicas extracelulares após isquemia. Mudanças nas concentrações iônicas extracelulares após isquemia. O pH extracelular começa a diminuir imediatamente após o início da isquemia. Esta mudança está acompanhada por rápidos aumentos nas concentrações extracelulares de K^+, Cl^- e Na^+. Após aproximadamente 1 minuto de isquemia, uma dramática mudança iônica acontece, com K abandonando as células e Ca^{2+}, Cl^- e Na^+ abandonando o espaço extracelular (modificado de SIEGEL *et al*., 2006).

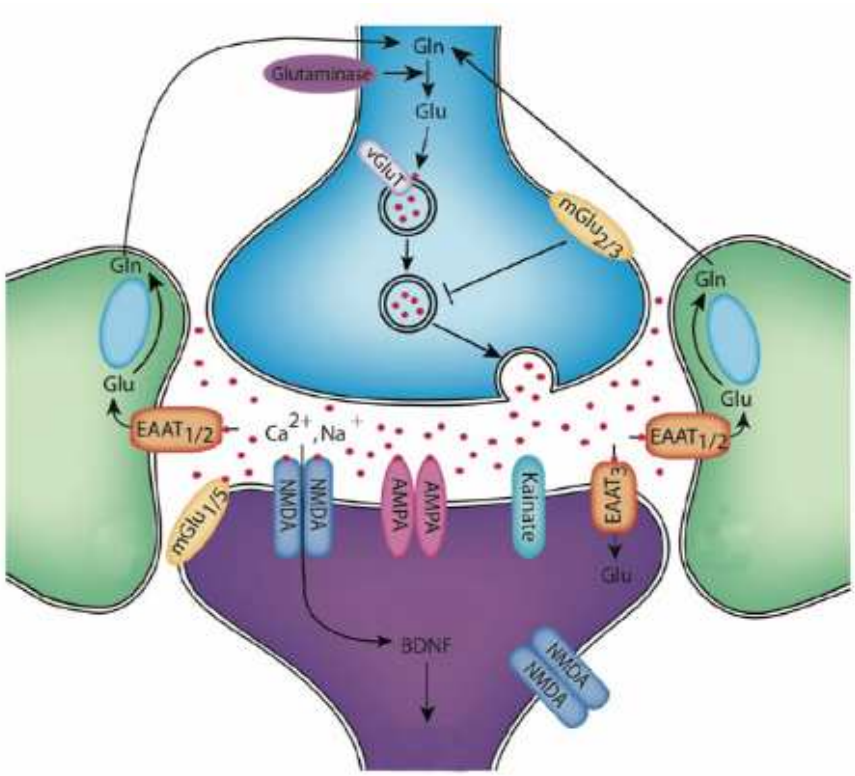

FIGURA 2 – Esquema do funcionamento da sinapse glutamatérgica (modificado de KELMENDI *et al*., 2006).

Os mecanismos de morte neuronal em animais e humanos após a hipóxia-isquemia incluem necrose e apoptose. A necrose decorre de uma agressão intensa à célula, com diminuição da atividade mitocondrial total das mitocôndrias, falência completa das bombas iônicas, edema celular, ruptura das organelas, lise das células, vazamento do citoplasma no meio extracelular e presença de resposta inflamatória intensa com fagocitose. Trata-se de um processo passivo de morte celular (SHERMAN, 1998; PERLMAN, 2006). A apoptose decorre da agressão lenta à célula, com inibição parcial da fosforilação oxidativa, redução do tamanho da célula e ruptura do DNA (Figura 3). A apoptose é um processo de “suicídio” ativo celular, desencadeado pela ativação de reações químicas em cascata que induzem a morte celular. A

necrose celular é irreversível sob determinadas circunstâncias; já a apoptose pode ser revertida. A Figura 3 demonstra de forma esquemática os eventos que ocorrem na hipóxia-isquemia, como as mudanças eletrolíticas, a liberação de substâncias tóxicas, a ativação de enzimas, entre outras, os quais culminam com a morte celular, seja por necrose ou apoptose. Assim, em geral, no centro do tecido lesado forma-se uma região necrótica e, ao redor, há uma área em que a evolução pode ser tanto um processo regenerativo quanto a morte definitiva. Trata-se da chamada “zona de penumbra”, à qual vem se atribuindo importância crescente nas intervenções terapêuticas (SHERMAN, 1998; PERLMAN, 2006).

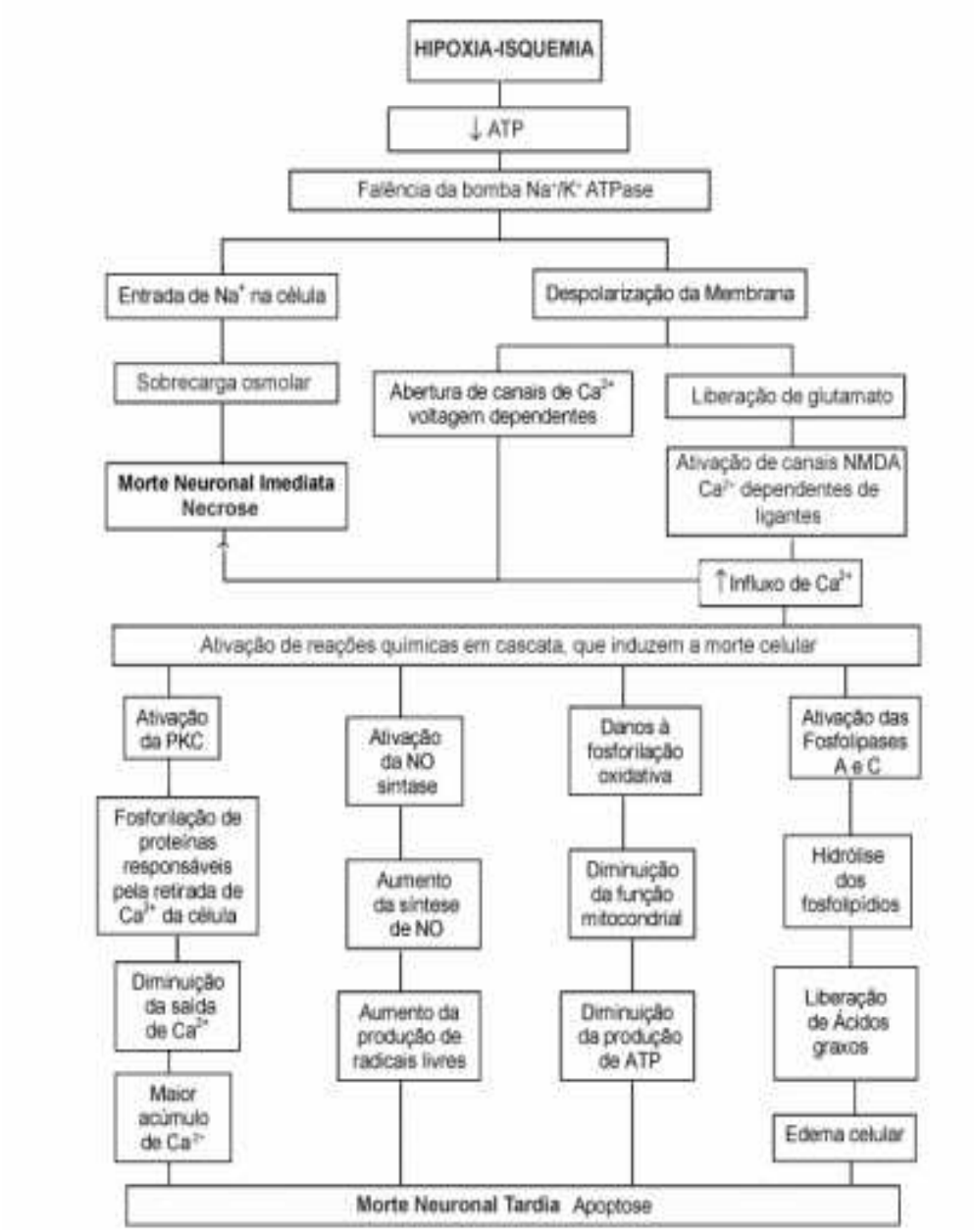

FIGURA 3 – Esquema ilustrativo da fisiopatologia da encefalopatia hipóxico-isquemica (modificado de ARAÚJO *et al.*, 2008).

A maioria dos episódios de hipóxia-isquemia severa causa lesões variadas a estruturas encefálicas específicas. Tais regiões incluem o hipocampo, os núcleos da base, o tálamo e a substância branca periventricular e subcortical (VANNUCCI, 2000). A justaposição de populações neuronais relativamente resistentes e relativamente vulneráveis ao insulto hipóxico-

isquêmico dentro de uma mesma distribuição vascular sugere a contribuição de fatores de vulnerabilidade tissular intrínsecos. Por exemplo, neurônios piramidais da região CA1 hipocampal morrem após 5-10 minutos de hipóxia-isquemia, enquanto que neurônios do giro denteado são preservados (Figura 4). Populações de neurônios que são seletivamente vulneráveis à isquemia incluem neurônios piramidais corticais, células de Purkinje do cerebelo, neurônios piramidais da região CA1 hipocampal e subpopulações da amígdala, estriato, tálamo e núcleos da medula espinhal (SIEGEL *et al.*, 2006).

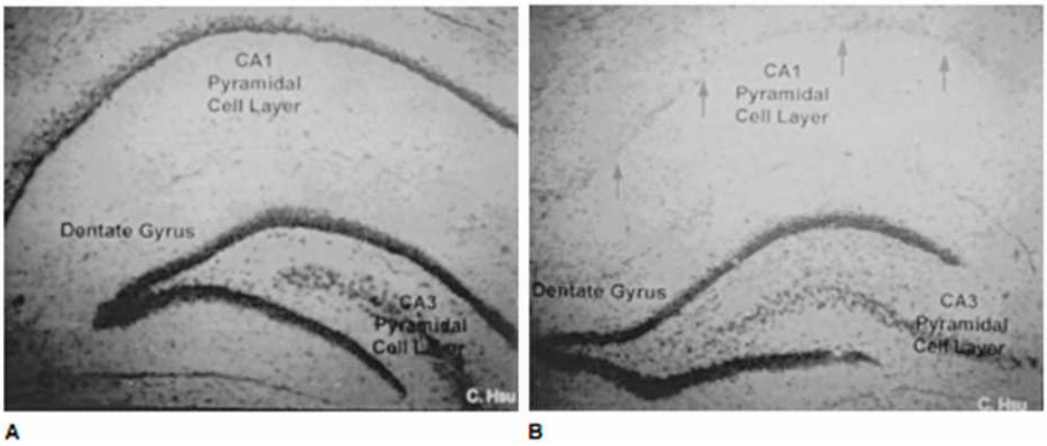

FIGURA 4 – Hipocampo de rato mostrando populações neuronais que são seletivamente vulneráveis ao dano isquêmico. (A) controle, (B) isquemia. Um curto período de isquemia causa quase que a completa perda de neurônios na região CA1 (setas) do hipocampo, enquanto que neurônios da região CA3 são quase que completamente poupados (modificado de SIEGEL *et al.*, 2006).

1.2 Modelos animais de hipóxia-isquemia

Os objetivos dos modelos animais de um modo geral são: contribuir para o conhecimento dos mecanismos de uma determinada injúria; melhorar a

compreensão sobre a evolução de tal injúria e os seus desfechos e fornecer um modelo no qual se desenvolvam e se testem estratégias terapêuticas. Ratos e camundongos são os animais mais comumente usados em modelos de asfixia perinatal (YAGER, 2004).

No intuito de reproduzir com êxito o insulto hipóxico-isquêmico ocorrido em humanos, o modelo experimental mais comumente utilizado em neonatos é o proposto por Rice *et al*., baseado no procedimento de Levine em ratos adultos (LEVINE, 1960; RICE *et al.*, 1981; ROOHEY *et al.*, 1997; VANNUCCI e VANUCCI, 1997; VANNUCCI *et al.*, 1999; VANNUCCI e VANNUCCI, 2005). O modelo de Levine e Rice consiste no dano cerebral hipóxico-isquêmico unilateral em ratos com 7 dias pós-natal, obtido pela associação da oclusão unilateral da artéria carótida comum, com subseqüente exposição a ambiente hipóxico (Figura 5). O motivo pelo qual a lesão é provocada precisamente com 7 dias é que o encéfalo de ratos nessa idade tem sido histologicamente comparado ao encéfalo em desenvolvimento de neonatos (SANDERS *et al.*, 2005). As lesões podem ser encontradas no hemisfério ipsilateral à oclusão da artéria carótida nas regiões do córtex cerebral, substância branca periventricular e subcortical, estriado (núcleos da base) e hipocampo, de forma mais pronunciada, bem como no hemisfério contralateral.

Neste modelo, as vantagens vão além das mudanças histopatológicas consistentes e previsíveis: o animal é de fácil manuseio, os estudos são facilmente desempenhados, o custo financeiro é modesto e sua reprodutibilidade é alta (RAJU, 1992; JANSEN e LOW, 1996; VANUCCI *et al.*, 1999; VANUCCI e VANUCCI, 2005). Uma variedade de outros protocolos para

indução da hipóxia perinatal também têm sido propostos, porém menos estudados (WEITZDOERFER *et al.*, 2004; JENSEN *et al.*, 1992).

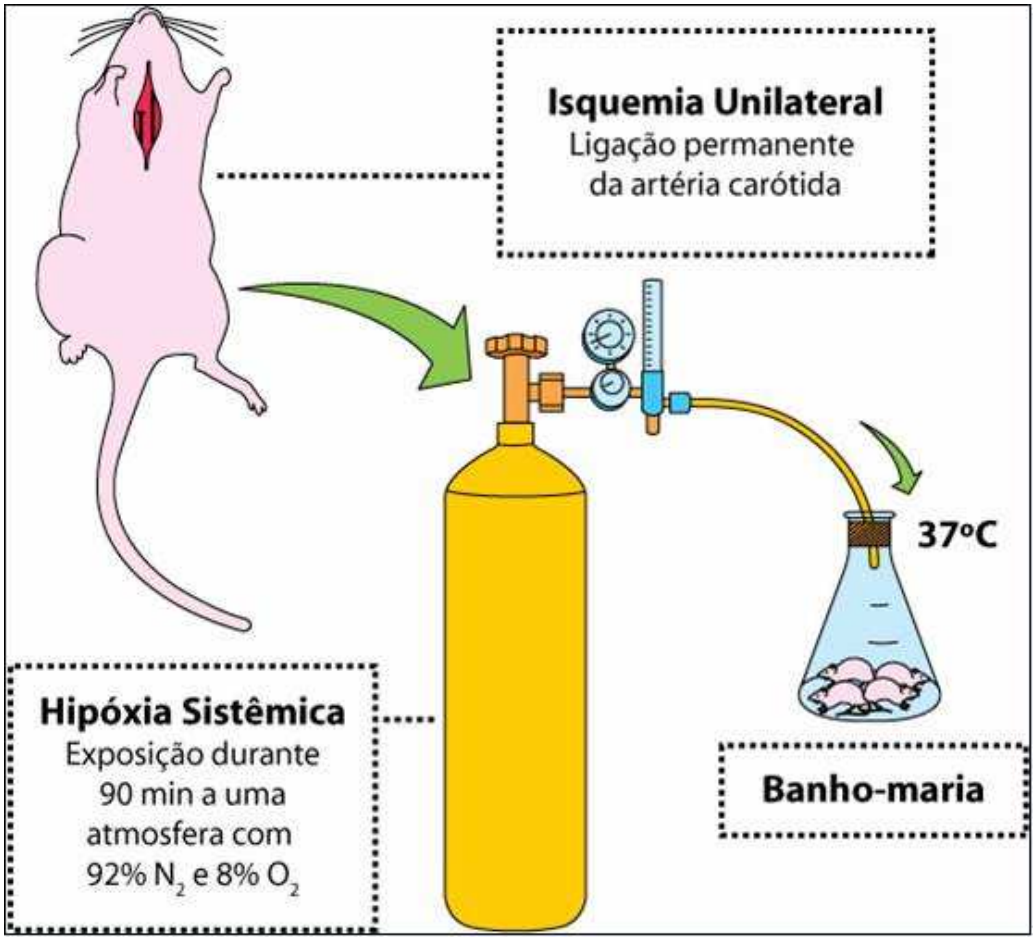

FIGURA 5 – Procedimento modificado de Levine utilizando a combinação de hipóxia e isquemia.

Estudos realizados previamente, inclusive em nosso grupo de pesquisa, demonstraram que ratos com esse tipo de lesão apresentam déficits cognitivos na memória de referência, espacial, de trabalho e aversiva (YOUNG *et al.,* 1986*;* JANSEN e LOW, 1996; IKEDA *et al.*, 2001; ALSCHER, 2002; ARTENI *et al*., 2003; RODRIGUES *et al*., 2004; PEREIRA *et al*., 2007; CHÁVEZ *et al.*,

2008; DE PAULA *et al*., 2009). O prejuízo cognitivo em animais submetidos ao modelo de HI de Levine-Rice é bastante evidente e, provavelmente, por este motivo, bastante estudado. Porém, além dos déficits cognitivos, já foram descritos também déficits sensório-motores em roedores submetidos à HI neonatal (JANSEN e LOW, 1996; BONA *et al.*, 1997). Lubics e colaboradores (2005) demonstraram em uma série de testes motores em animais submetidos à HI neonatal que, embora a maioria dos ratos HI tenha alcançado um desempenho pior quando comparados aos animais controles na maioria dos testes (teste de preensão, escada horizontal e rota-rod), após 5 semanas a atividade motora aumentou significativamente no teste do campo-aberto. Outro estudo demonstrou que a força muscular e a coordenação motora, avaliadas nos testes de preensão, de colocação da pata e rota-rod, bem como os reflexos para diferentes estímulos sensoriais, estão severamente afetados em animais submetidos à HI neonatal (SPANDOU *et al.*, 2005). De forma semelhante, um trabalho avaliando a execução do teste de *skilled forelimb* e dos movimentos digitais demonstrou que a hipóxia-isquemia neonatal influencia negativamente o desempenho dos animais (ANDREWS *et al.,* 2008). Embora exista um maior número de trabalhos destacando os déficits cognitivos dos animais submetidos à HI, também existe, contudo em menor quantidade, estudos que demonstram a existência de déficits motores nestes animais.

Ademais, existem trabalhos demonstrando as alterações morfológicas encefálicas que acompanham a hipóxia-isquemia, como atrofia do hipocampo, córtex sensório-motor e estriado (JANSEN e LOW, 1996; RODRIGUES *et al.*, 2004; PEREIRA *et al.,* 2007; DE PAULA *et al*., 2009), morte celular dos neurônios piramidais da região CA1 do hipocampo (PULSINELLI *et al*., 1982;

KIRINO e SANO, 1984), além de alterações da densidade de espinhos dendríticos e dano à arborização dendrítica apical hipocampal (OBEIDAT *et al*., 2000; HASBANI *et al.,* 2001; RUAN *et al.,* 2009).

1.3 Enriquecimento ambiental

As estratégias para reduzir a extensão da área comprometida após eventos hipóxicos e/ou isquêmicos são chamadas de estratégias neuroprotetoras. A neuroproteção pode ser definida como uma intervenção, que tenta resgatar a área de penumbra, ou seja, uma área de hipoperfusão ainda viável circundante ao infarto. Entre as diversas formas de neuroproteção se encontra o enriquecimento ambiental (EA) (ROSENZWEIG e BENNETT, 1996).

Sabe-se que o EA pode proteger o encéfalo em desenvolvimento de roedores das conseqüências adversas do isolamento social e cognitivo (ROSENZWEIG e BENNETT, 1996). O enriquecimento tipicamente expõe os animais a uma combinação de estímulos sociais, cognitivos e físicos (Figura 6) fornecidos por gaiolas, brinquedos e rodas. Comparativamente aos grupos controle, os córtices de ratos mantidos em condições de enriquecimento ambiental evidenciam várias alterações morfológicas, incluindo aumento da espessura cortical, aumento da densidade de espinhos dendríticos e de arborização dendrítica, além de aumento do tamanho do soma neuronal (DIAMOND *et al.*, 1964; DIAMOND, 1967; GLOBUS *et al.*, 1973; GREENOUGH e VOLKMAR, 1973). O enriquecimento iniciado na idade jovem tem também demonstrado afetar a morfologia da região CA1 e o giro denteado, bem como incrementar os níveis de sinaptofisina no hipocampo (FAHERTY *et al.*, 2003;

LAMBERT *et al.*, 2005). Além disso, roedores mantidos sob enriquecimento tiveram melhor desempenho, quando comparados a grupos controle isolados e socializados, em numerosos testes de aprendizado e memória (GREENOUGH *et al.*, 1972; WILLIAMS *et al.*, 2001).

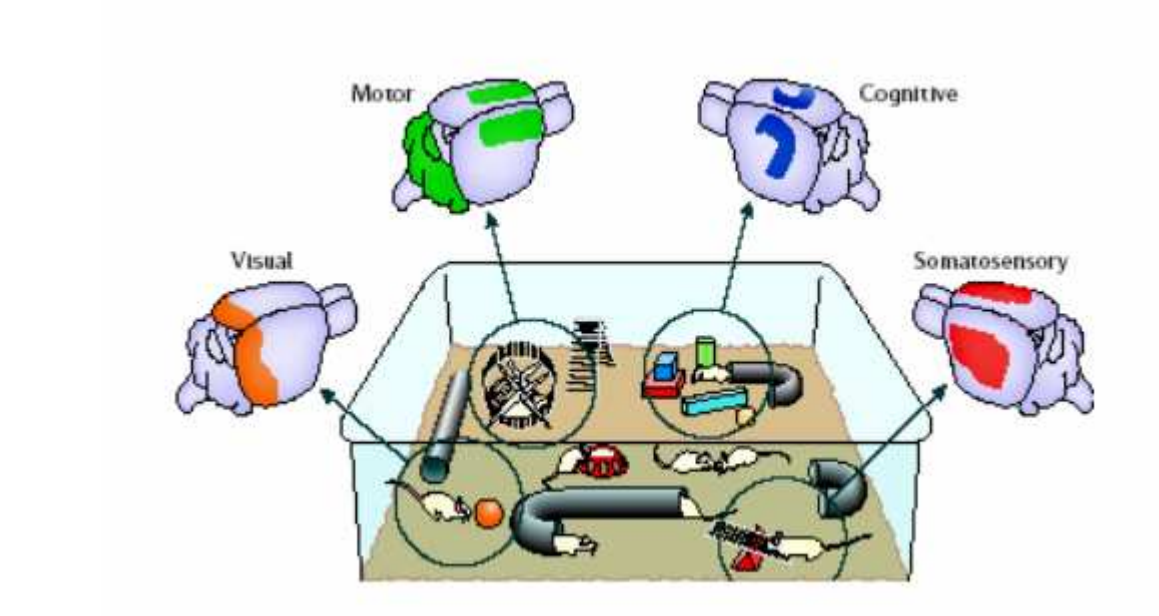

FIGURA 6 – Esquema ilustrativo dos efeitos visuais, motores, cognitivos e somatosensoriais do Enriquecimento Ambiental (modificado de NITHIANANTHARAJAH e HANNAN, 2006).

Muitos estudos vêm sendo desenvolvidos com a temática da encefalopatia hipóxico-isquêmica, suas conseqüências e possíveis tratamentos, tendo em vista o importante dano neurológico causado ao recém-nascido e que resultam em severas morbidades no desenvolvimento. O mesmo não acontece com o EA, estudos que dizem respeito a esse tema ainda são escassos, embora a neurorreabilitação não farmacológica venha tomando cada vez mais espaço na terapêutica contemporânea. Estudos anteriores como o de Pereira e colaboradores, em 2007, demonstraram que os danos

encefálicos em nível macroscópico de animais adultos (hipocampo e córtex) não são amenizados com a exposição diária ao EA (1h/dia/9 semanas). No entanto, nesse mesmo estudo, constatou-se um melhor desempenho dos animais adultos expostos ao ambiente enriquecido em alguns testes cognitivos, como o teste do labirinto aquático de Morris. Em outro trabalho (Pereira *et al*., 2008), contudo com animais adolescentes, o EA permanente foi eficaz em reverter o déficit na memória de reconhecimento de objetos, porém também sem evitar a atrofia do hipocampo e estriado. Isto posto, pode-se considerar a hipótese de que os danos em nível microscópico sim podem ser amenizados através do EA, bem como os déficits motores e cognitivos causados pela Hipóxia-isquemia. O estudo da densidade de espinhos dendríticos em neurônios hipocampais pode servir para identificar alterações plásticas possivelmente associadas à recuperação funcional previamente identificada.

2 OBJETIVOS

2.1 Objetivo Geral

Investigar parâmetros de função motora e aprendizagem, associando-os à densidade dos espinhos dendríticos de neurônios do hipocampo de ratos submetidos à hipóxia-isquemia neonatal e estimulados em ambiente enriquecido.

2.2 Objetivos Específicos

1. Avaliar o comportamento de ratos submetidos à HI e estimulados por EA utilizando a tarefa de esquiva inibitória, campo aberto, reconhecimento de objetos e rota rod.

2. Analisar a densidade dos espinhos dendríticos na região CA1 do hipocampo dorsal de ratos submetidos à HI e estimulados por EA, utilizando-se da Técnica de Golgi.

3 ARTIGO

“Effects of daily environmental enrichment on behavior and dendritic spine density in hippocampus following neonatal hypoxia-ischemia in the rat”

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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 responsive to environmental stimuli, as environmental enrichment (EE). The aim of this study was to investigate behavioral performance in the open field apparatus, objects recognition, inhibitory avoidance and in the Rota-rod apparatus, and dendritic spines density in the hippocampus, using the Golgi technique, in rats submitted to the HI and exposed to EE (1h/day, 6 days/week, 9 weeks). Seven-days old rats were submitted to the HI procedure and divided in 4 groups: control in standard conditions (CTSE), control in enriched environment (CTEE), HI in standard conditions (HISE) and HI in enriched environment (HIEE). Behavioral and morphological parameters were evaluated after 9 weeks of environmental stimulation. Data indicated that object-recognition memory was impaired in HI adult rats and recovered after stimulation by the EE; in the inhibitory avoidance task was demonstrated aversive memory impairment in HI animals, independent of the environment. Interestingly, in the open field task, significant more crossing responses were identified in HI groups, in the first minute, comparing to control groups. No differences between control and HI adult animals were detected in the rota-rod test. Morphological results demonstrated a decreased spines density in the hippocampus of the HI animals, with recovery by the EE. Dendritic spines density from left hemisphere (contralateral to arterial occlusion) obtained the better results, indicating a total recovery effect of the EE on HI damage. Data of dendritic spines from right hemisphere indicated a partial recovery by the environmental stimulation on HI animals. Concluding,

environmental enrichment was effective in recovery behavioral impairment and dendritic spine density in hippocampal neurons, consequent to neonatal hypoxia-ischemia in rats.

1. Introduction

Hypoxic and ischemic complications during the pre and perinatal period are common causes of neonatal brain damage which are frequently associated with neurodevelopmental disabilities (Trollmann e Gassmann, 2009), including cerebral palsy, epilepsy and a range of cognitive impairments as well as learning and memory problems (Koelfen *et al*., 1995; Delsing *et al*., 2001). These sequelae are the result of injury to a range of brain structures, including hippocampus (Kadam *et al*., 2009).

Levine e Rice method (Levine, 1960; Rice *et* al., 1981) is an animal model which has been widely used to study neonatal encephalic hypoxia-ischemia (HI) in rodents. It has been well established that neonatal HI induces significant long-term behavioral impairment on spatial memory in water maze task in adult rats (Ikeda *et al.*, 2001; Arteni *et al*., 2003; Pereira *et al*., 2007), on aversive memory in inhibitory avoidance task (Arteni *et al*., 2003; Young *et al., 1986*), on working memory (Arteni *et al*., 2003; Pereira *et al*., 2007; Chávez *et al.*, 2008; de Paula *et al*., 2009) and in sensorimotor tasks (Jansen and Low, 1996; Bona *et al.*, 1997). Considering morphological data, studies have shown neuronal death of hippocampal CA1 neurons (Pulsinelli *et* al., 1982; Kirino and Sano, 1984), alterations of hippocampal dendritic spine density (Ruan *et al.,* 2009) and brain atrophy, specifically hippocampus, sensorimotor cortex and striatum (de Paula *et al.,* 2009; Jansen and Low; 1996, Rodrigues *et al*., 2004).

It is known that the immature brain is benefited to environmental stimuli (Meaney e Aitken, 1985), therefore the possibility that EE would be a neuroprotective strategy is acceptable. This therapeutic strategy involves a combination of social interaction, physical exercise and exposure to learning tasks, (Krech *et al*., 1960, Harburger *et al.*, 2007) producing interesting results. Regarding to cognitive alterations, it was found that EE improved learning, working and reference spatial memory in rats (Kempermann *et al*., 1997; Nilsson *et al*., 1999; Van Praag *et al*., 2000; Bindu *et al*., 2005; Leggio *et al.,* 2005; Pereira *et al*., 2007). Morphologically, several hippocampal aspects are enhanced by EE (Van Praag *et al.*, 2000), such as neurogenesis (Kempermann *et al.,* 1997; Kempermann, 2002), cortical thickness, dendritic branching (Greenough *et al.*, 1973), dendritic spine growth (Diamond *et al*., 1976, Nakamura *et al.*, 1999) and synaptophysin levels (Frick and Fernadez, 2003).

Our previous results with EE following neonatal hypoxia-ischemia demonstrated that stimulation by daily enrichment recovered spatial memory deficits but it had no effect in the atrophy neither in the hippocampus nor in the cerebral cortex in adult rats (Pereira *et al*., 2007). In another study, we demonstrated that early housing animals in environmental enrichment caused memory recovery in object recognition and a partial improvement in the working memory spatial task in adolescent females and male rats after neonatal HI; however, again no effects of enrichment were revealed in adult rats ‘considering extension of tissue atrophy of hippocampus and striatum consequent to hypoxia-ischemia (Pereira *et al*., 2008).

In spite of the fact that enrichment has resulted in a clear protective effects on cognitive performance in rodents after the hypoxic-ischemic episode,

it has no evidence of microscopic alterations in the hippocampal neurons of these animals, as seen in Pereira and coworkers (2007, 2008). Taking these considerations into account, the present study was designed to investigate: 1) behavioral performance in the open field apparatus, objects recognition, passive avoidance and in the Rota-rod apparatus, and 2) dendritic spines density in the dorsal hippocampus, using the Golgi technique, in rats submitted to the hypoxia-ischemia and exposed to the environmental enrichment. We hypothesize that cognitive impairment as well as the morphological damage caused by encephalic hypoxia-ischemia will be reverted or alleviated by the environmental enrichment exposure.

2. Materials and methods

2.1 Animals

Male Wistar rats were obtained from the Central Animal House of the Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul. They were maintained in a temperature-controlled room (approximately 22℃), on a 12/12 light/dark cycle and with food and water *ad libitum*. At postnatal day 7, after surgery, animals were randomly divided into four experimental groups: control maintained in standard environmental (CTSE); control exposed to environmental enrichment (CTEE); submitted to hypoxia-ischemia and maintained in standard environmental (HISE); submitted to hypoxia-ischemia exposed to environmental enrichment (HIEE).

For the behavioral analysis, were used 8 to 10 animals per group. For the morphological analysis, in each experimental group the dendritic spine density

was obtained from other 5 rats, not submitted to behavioral tests. Progenitor female and female pup rats were used in other parallel experiments in the laboratory. A time line of experimental events is presented in Fig.1.

All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals adopted by national Institute 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 2008247.

2.2 Hypoxia-ischemia

The Levine method (1960) as modified by Rice *et al.* (1981) was utilized in this study to produce unilateral brain injury in neonate rats. At postnatal day 7, animals were anesthetized with halothane 2-4% as the laboratory routine and submitted to surgical procedure. An incision was made in the ventral surface of the neck; the right common carotid artery was assessed, isolated from the adjacent structures, and permanently occluded with 4.0 surgical silk thread. Finalized this procedure, animals were then maintained in controlled temperature to recover for 15 minutes and returned to their dams. After a period of 2.5 hours, in groups of 5 animals, pups were exposed to 90 minutes to hypoxic atmosphere (8% oxygen and 92% nitrogen, 5 L/minute flow) in a 1500 mL chamber partially immersed in a 37℃ water bath in order to maintain body temperature of lactating rats in the physiological limits. Controls animals were sham-operated, they were submitted to manipulation, anesthesia and neck incision, but did not receive arterial occlusion or hypoxic atmosphere exposition.

Following the HI procedure, animals were returned to their respective home cages.

2.3 Environmental Enrichment

Environmental enrichment procedure used in this study was the same proposed by Pereira and coworkers (2007, 2009). A daily enrichment began when rats reached 22 days old and continued during 9 weeks, 6 days per week, 1 hour per day, in groups of 7-10 animals. The enriched environment consisted of a large cage (40 x 60 x 90cm) with three floors, ramps, running wheel and several objects with different shapes and textures, modeled as previously described (Wildman and Rosselini, 1990; Diamond, 2001). Objects in the cage 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 enrichment period (Pereira *et al.,* 2007, 2009).

2.4 Behavioral Tests

Each rat was submitted to a series of behavioral tests: Open Field, Novel Object Recognition, Passive Avoidance Task and the Rota-rod Test. These tests were carried out in consecutive days, starting 24 h after the end of environmental enrichment period.

2.4.1 OPEN FIELD

The open field apparatus consisted of a wooden chamber (55 x 44 x 50 cm) with a dark gray floor divided into 12 fields. The box was placed in a quiet room, with the same illumination utilized in the others tests. Rats were placed in

a corner square of the chamber and the latency to leave this first square was recorded. Subsequent rearings and crossings responses (in the 1st minute and total time) were recorded (Netto *et al.,* 1985). Animals were observed individually for 5 minutes.

2.4.2 NOVEL OBJECT RECOGNITION

The novel object recognition task measures a form of declarative memory (Clark *et al.,* 2005). In the first phase of the test, each animal was confronted with two different objects, placed in an open-field box, and the time of object exploration is registered in five minutes. Following this phase, the rodent was removed from the open-field box to a other separate box for a period of five minutes. In the second phase, each animal was exposed to two objects placed in the same open-field box: one familiar object, used in the first phase, and one novel object. The time spent exploring the novel object and the familiar object was measured (Benice e Raber, 2005). Besides, it was calculated a discrimination index in the test session (second), as follows: the difference in exploration time divided by the total time spent exploring the two objects (B − A / B + A, where B is the new object and A is the familiar object) (Zou *et al.,* 2006).

2.4.3 INHIBITORY AVOIDANCE

An acrylic box was used (50 x 25 x 25 cm), with the left-most 7 cm of the box floor was occupied by a 3 cm high platform. The box floor was a grid of parallel stainless steel bars (1.5 mm-diameter) spaced 1 cm apart. Animals were gently placed on the platform and their latencies to step down placing their

four paws on the grid were measured with an automatic device. On stepping down, they received a 0.5 mA; 60 Hz scrambled foot shock for 2 s, and were withdrawn from the box. Animals were tested for retention 24 h later. Test session was procedurally similar to the training one except that foot was omitted; step-down latency in test was used as an index of retention (Netto *et al.,* 1985; Arteni *et al.,* 2003).

2.4.4 ROTA-ROD TEST

The effect of motor coordination was assessed using a Rota-rod apparatus (Insight) (Capasso, 1996; Liu *et al.*, 2007). Animals were exposed to one habituation session during 3 min in the apparatus on slow velocity (20 rpm). In the test session, 24 h later, animal's motor ability was evaluated. The rota-rod test was performed by placing rats on rotating drums (3 cm diameter) and measuring the time each animal was able to maintain its balance on the rod. The speed of the rota-rod accelerated from 16 to 40 rpm over a 5 min period. It was recorded the latency of the first downfall, number of falls (maximum 3) and time of permanence in the apparatus (Takao *et al.*, 2010).

2.5 Golgi Method

Two days after the environmental enrichment, the animals were deeply anesthetized with ketamine and xylazine and transcardially perfused with fixative solutions. The "single-section" Golgi method procedure followed the same methodology published in details for Castilhos and coworkers (2006). Brains were fixed with 4% paraformaldehyde and 1.5% picric acid in 0.1 M phosphate buffer (pH 7.4), sectioned coronally (200µm 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 at least 48 hours, 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).

2.6 Microscopic Analysis

The microscopic analysis was performed using a camera lucida (1000x) coupled to an optic microscope (Olympus BX-41, Japan) unforbidding design specimens visualized on microscope providing high precision, providing simultaneous visualization of the sample and the area designed. In all animals was studied the region corresponding to the dorsal CA1 hippocampus, ipsilateral and contralateral to arterial occlusion, more specifically the lacunoso - molecular layer, due these cells are more vulnerable to the ischemic insult (Ito *et al.,* 1975; Pulsinelli e*t al.,* 1982). The location of the dorsal CA1 was based in the Paxinos and Watson Atlas of Stereotaxic Coordinates (2004). For each CA1 pyramidal cell the morphologic analysis included the dendritic spines number in 20 μm of the selected most lateral tertiary dendrites on the apical tree. From each rat, ten different dendrites were analyzed, five per hemisphere, one per neuron. Consequently, dendritic spines data were obtained from a total of 50 different dendrites in each experimental group. Drawings were scanned and had their lengths measured using an image analysis system (Image J 1.44, National Institutes of Health, USA). Spine density was defined as the number of spines divided per unit of dendritic length (Rasia-Filho *et al*., 2004). For

statistical comparisons, the means of each rat were used after performing an initial nested analysis of variance (ANOVA) test.

2.7 Statistical analysis

Statistical analyses were conducted using two-way analysis of variance (ANOVA) with lesion and environment as independent variables. Analyses were followed by post hoc Duncan's test for multiple comparisons, when necessary and the data were expressed as means ± S.E.M.. Non-parametric data (latency to step-down platform in the inhibitory avoidance) are expressed as median latencies of each group and interquartile ranges; their statistical differences in each session were assessed by Kruskall–Wallis one-way analysis of variance. Probability values less than 5% were considered significant. All statistical analysis was performed using the Statistica® software package running on a compatible personal computer.

3. Results

3.1 Open-field

Analysis showed that there is not effect considering lesion ($F(1,31) = 1.97$, $p = 0.17$) or environment ($F(1,31) = 0.21$, $p = 0.65$) referent to the latency to leave the first square. With respect to the number of crossings, interestingly, in the first minute there was effect of lesion ($F(1,31) = 21.95$, $p<0.0001$) but not on the environment ($F(1,31) = 1.72$, $p = 0.20$), the Duncan post-hoc analysis indicated that HISE and HIEE groups presented more crossings responses compared to the control animals. Considering the number of crossings in total

time, ANOVA followed by Duncan not showed significance to lesion ($F(1,31) = 1.50$, $p = 0.23$) or environment factors ($F(1,31) = 0.22$, $p = 0.64$). Regarding to the rearing responses, there was not effect from the lesion ($F(1,31) = 0.87$, $p = 0.36$) or environment ($F(1,31) = 1.15$, $p = 0.29$). The results are illustrated in the table 1.

3.2 Novel Object Recognition

Hypoxia-ischemia resulted in memory deficits in the novel-object recognition task in rats. Two-way ANOVA indicated significant differences in relation to lesion ($F(1,32) = 15.45$, $p<0.001$) and environment factors ($F(1,32) = 9.7514$, $p = 0.004$), considering novel-object preference index. Duncan's test for multiple comparisons revealed that hypoxic-ischemic animals maintained in standard environment had lower preference index when compared to all other groups. Interestingly, there was no difference between HI enriched and control groups (Fig. 2).

In the first session of the task, ANOVA indicated a difference in time exploration of object A in relation to lesion ($F(1,32) = 9.76$, $p = 0.004$) and Duncan's test indicated that HISE and HIEE groups spent lower time exploring object A comparing to CTSE group. Similarly, ANOVA followed Duncan's test revealed that there was a lower exploration of object B in the HISE and HIEE groups when compared with CTEE group (data not shown).

3.3 Inhibitory Avoidance

It was demonstrated a memory impairment in HI animals, independent of the environment. Kruskal-Wallis one way analysis of variance showed no

differences on the latencies to step down the platform among all the groups in the training session (H = 4.12, p = 0.25), although there was a significant difference (H = 14.76, p = 0.002) in test session performance (Fig. 3). Mann-Whitney U-test demonstrated a lesion effect, environment independent, since hypoxic-ischemic animals presented lower latencies to step down the platform than control animals.

3.4 Rota-rod

There were no significant differences between the hypoxic-ischemic groups and the control groups on the rota-rod test (Table 2). Considering the latency of the first downfall, both parameters, neither lesion ($F(1,32) = 1.29$, $p = 0.26$) and nor environment ($F(1,32) = 1.04$, $p = 0.32$) resulted in significant effect. Taking into consideration the number of falls, the results not also demonstrated significance: lesion ($F(1,32) = 1.27$, $p = 0.27$); environment ($F(1,32) = 1.27$, $p = 0.27$). Making an evaluation for the maximum time of permanence in apparatus, no differences was found on lesion ($F(1,32) = 1.10$, $p = 0.75$) and environment parameters ($F(1,32) = 1.14$, $p = 0.29$).

3.5 Dendritic spines density

Results demonstrated a decreased spines density in the hippocampus of the HI animals, with recovery by the environmental enrichment. Considering dendritic spines density from left hemisphere (contralateral to the lesion), two-way ANOVA identified effect referent to the lesion ($F(1,16) = 39.340$, $p < 0.01$) and environment ($F(1,16) = 13.256$, $p < 0.01$). Duncan's test for multiple comparisons demonstrated that hypoxic-ischemic animals maintained in

standard environment had lower dendritic spines densities when compared to all other groups ($p < 0.01$) (Fig.4). Control enriched animals had the highest densities when compared with the other three groups ($p < 0.01$ in all cases). Hypoxic-ischemic enriched animals had increased spine density, compared with HISE group, and equal level of spine density, compared with CTSE group. These findings indicated a total recovery effect of the environmental enrichment, consequent to hypoxic-ischemic damage.

Dendritic spines from right hemisphere (ipsilateral to the lesion) showed effect referent to the lesion ($F (1,16) = 55.104$, $p < 0.01$) and environment ($F (1,16) = 20.680$, $p < 0.01$). Duncan's test for multiple comparisons demonstrated that hypoxic-ischemic animals maintained in standard environment had lower dendritic spines densities when compared with CTSE and CTEE ($p < 0.01$ in both groups). Enriched HI group had no significant differences when compared with the HISE and CTSE groups ($p = 0.3$ and $p = 0.059$, respectively). This result indicated a partial recovery by the environmental stimulation on HI animals.

4. Discussion

This study investigated the effects of daily environmental enrichment (1 h/day for 9 weeks) on the behavioral performance in the open field apparatus, objects recognition, passive avoidance and in the Rota-rod apparatus and on the number of the dendritic spine in the hippocampus caused by a hypoxic-ischemic event to the neonatal rats. Confirming the working hypothesis, exposure to the enriched environment reversed cognitive impairment and this finding was associated with increased spine density in hippocampus of the rats

submitted to neonatal HI. Previous works of our research group demonstrated that cognitive deficits following hypoxia-ischemia were reversed after environmental enrichment exposure, but not the morphological damage measured by hippocampal volume (Pereira *et al*., 2007).

In this work it was also studied the animals behavior in the open-field test. Interestingly, significant more crossing responses were identified in HI groups, in the first minute, comparing to control groups. It is known that in hypoxia-ischemia, one of regions more affected is the hippocampus, and several authors have reported that complete or partial hippocampal injury resulted in hyperactivity in an open-field test (Valle and Gorzalka, 1980; Gray and McNaughton, 1983; Lipska *et al*., 1991; Shen *et al*., 1991*;* Cassel *et al*., 1998, Coutureau *et al*., 2000; Brotto *et al*., 2000). Some authors have studied the connection between hyperactivity and brain ischemia. This hyperactivity occurred immediately after occlusion, consequent to hippocampal CA1 neuronal death (Wang and Corbett, 1990; Kuroiwa *et al*., 1991; Mileson and Schwartz, 1991, Araki *et al.*, 1998). The increased locomotor activity has been associated with a reduction in the animal potential to form spatial maps (Wang and Corbett, 1990). It has been described that several motor functions has relation with the dopaminergic systems (Araki *et al.*, 1998). D_1 and D_2 dopamine receptors act synergistically in the hyperactivity seen in locomotor activity (Ichihara *et al.,* 1993) and there are reports showing a relation between cerebral ischemia and disturbances in dopaminergic transmission (Maggin *et al*., 1997). Araki and coworkers (1998) identified that dopamine D_2 receptor antagonists decreased the ischemia-induced hyperactivity significantly. The extensive pyramidal cell damage in the CA1 region of the hippocampus in case of bilateral carotid artery

in gerbils was associated with increases in locomotor activity (Mileson e Schwartz, 1991). In this study, additionally, environmental enrichment was ineffective in reduce hyperactivity characteristics in HI animals.

The present data stated that object-recognition memory was impaired in HI adult rats and recovered after stimulation in enriched environment. Even if daily enrichment has started two weeks after the HI event, an important memory deficit was prevented in rats submitted to neonatal insult. We had shown in another study that object-recognition memory was recovered in HI adolescent rats after an early permanent enrichment period (Pereira *et al*., 2008). Interestingly, the present finding indicate an extension of therapeutic window for functional prevention consequent to neonatal HI event, corroborating our previous results on spatial memory in water maze task (Pereira *et al*, 2007).

In the inhibitory avoidance task was demonstrated a memory impairment in HI animals, independent of the environment, on the latencies to step down the platform in the test performance. This result could be justified due to fact that HI injury has damaged brain regions important in acquisition and expression of aversive memory, for example, the amygdala (Coleman-Mesches and Mc Gaugh, 1995; Arteni *et al.,* 2003*;* Baker and Kim, 2004). However, it is important to mention the fact that in this work it was not evaluated morphological characteristics of other structures beyond the hippocampus.

The rat model of HI is known to result in wide cerebral atrophy, but, unlike to human neonates, rats that underwent neonatal HI do not show gross motor deficits: they apparently move like control animals and do not display evident postural and locomotor abnormalities (Jansen and Low, 1996). However, a few studies have reported that hypoxic-ischemic animals display

long term deficits in motor coordination tasks such as rota-rod test (Jansen and Low, 1996; Bona *et al.*, 1997; Spandou *et al.*, 2005). Although there are contradictions between results obtained by different investigators, similarly to our observation in the rota-rod test, Balduini and coworkers (2000) and Lubics and coworkers (2005), found no differences between control and HI adult animals in the rota-rod test. Since the rota-rod test is useful to evaluate balance and coordination, we can suppose that gross locomotor activity, in adult animals, is not impaired by neonatal HI. However, it was reasonable to consider the presence of fine motor deficits, not identified in the rota-rod task. In addition, it is known that the neonatal brain has an expressive level of plasticity (Balduini *et al.*, 2000; De Paula *et al.,* 2009) and this capacity could be responsible by a spontaneous recovery on sensorymotor deficits.

In previous studies of our group evolved HI rats, in spite of cognition improvement, housing in enriched environments did not cause benefits over the extension of in hippocampal and striatal damage (Pereira *et al*., 2007, Pereira *et al*., 2008). It was observed that hippocampal volume of HI animals was not recovered by the environmental enrichment. Several authors affirm that EE promotes neuronal protection increasing levels of trophic factors, neurogenesis, dendritic branching, synaptogenesis, number of dendritic spines and enhanced cell survivor (Van Praag et *al*., 2000; Leggio *et al*., 2005). Then, in the present study it was evaluated the density of dendritic spines in tertiary dendrite of pyramidal neurons on hippocampal CA1. Our initial hypothesis was that there are other morphological targets for EE effects, such as increased dendritic arborization and number of dendritic spines, rather than lesion volume. Such forms of neuronal plasticity could be responsible for the functional recovery of

EE and they were considered in this study. Confirm our hypothesis; present findings demonstrated a decreased spines density in the hippocampus of the HI animals, with recovery by the environmental enrichment. Dendritic spines density from left hemisphere (contralateral to arterial occlusion) obtained the better results, indicating a total recovery effect of the environmental enrichment on hypoxic-ischemic damage. The results of dendritic spines from right hemisphere (ipsilateral to the lesion) indicated a partial recovery by the environmental stimulation on HI animals. To our knowledge, this is the first work showing the EE effect on morphological brain damage after a neonatal HI event. In studies evaluating effects of enrichment in adult rats submitted to brain ischemia, some data demonstrated plastic effects on neural tissue such as: increase in number, size and shape of dendritic spines (Johansson and Belichenko, 2002; Leggio *et al.*, 2005), increase dendritic branching and size of synaptic contact (Johansson and Belichenko, 2002), decrease infarct volume, normalized astrocyte-to-neurons ratios and increase number of putative neural stem cells, astrocytes and oligodendrocyte progenitors (Leggio *et al.*, 2005).

Certainly, there is a complex group of molecular events associated to neuroprotective effect following EE stimulation. It was proposed that the development of dendritic spines occurs concurrently with the growth of the presynaptic elements, suggesting that cell-cell interaction and extrinsic cues likely induce the formation of dendritic spines (Lipman and Dunaevsky, 2005). Fischer and coworkers (1998) identified several molecular families, including receptors, scaffolding proteins, and regulators of the cytoskeleton, implicated in spine number and shape regulation, maybe one of these families can be the responsible for the increase spine density. Besides, it was suggested two

models of morphologic plasticity in dendritic spines, at the first, the formation of new spines is initiated by long-term potentiation-like stimulation operating through *N* - methyl - D - aspartate receptors, the second is that spine morphology at established synapses is stabilized by AMPA (α - amino - 3 - hydroxyl - 5 - methyl - 4 - isoxazole propionic acid) receptor activation (Fischer *et al*., 1998).

The results of the present study corroborate previous results have shown that brain lesions can induce neuronal changes in the ipsilateral and contralateral hemisphere that may vary with the type and size of lesion and have a different time course (Cheng *et al*., 1997; Nicolelis, 1997; Shimada *et al*., 1997). Probably, the highest number of spines in the contralateral hemisphere (left) is due to more intracortical connections in the enriched group, like proposed by Johansson and Belichenko (2002). Johansson and Belichenko (2002) with Lucifer yellow, indicated a change in membrane permeability in the contralateral cortex to the infarct, this finding might be related to the changes in dendritic spine density observed in both brain hemispheres in our work.

In summary, environmental enrichment was effective in recovery behavioral impairment consequent to neonatal hypoxia-ischemia in rats. Interestingly, it was also indentified an enhanced dendritic spine density in hippocampal neurons of hypoxic-ischemic rats stimulated in enriched environment. These findings present a clear neuronal morphology-function relationship, showing that the structural changes in dendritic spines are responsible, at least in part, by the functional effects of the EE, in a model of hypoxia-ischemia neonatal. Besides, this study had new contributions on the

potential of EE as a non-invasive rehabilitation strategy to ameliorate deficits in the development of the CNS and to treat neurological disorders.

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Figures and Legends

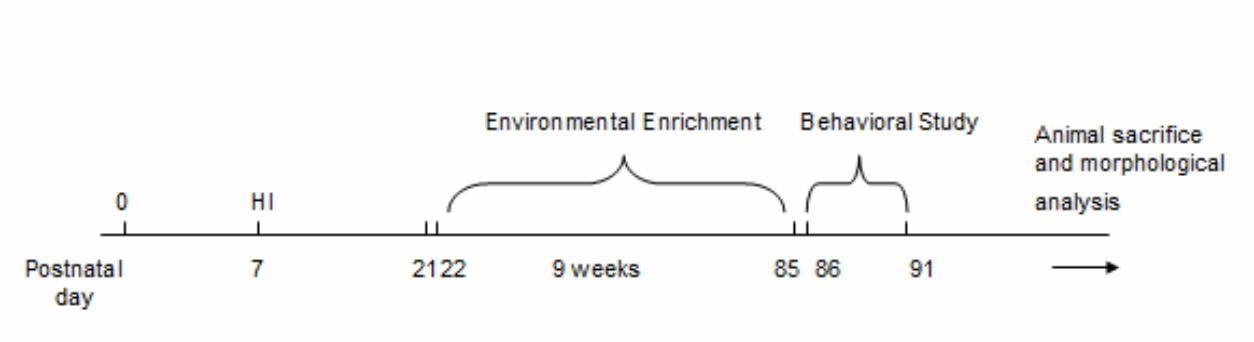

Figure 1. Time line of experimental procedures. HI: Hypoxic-ischemic event.

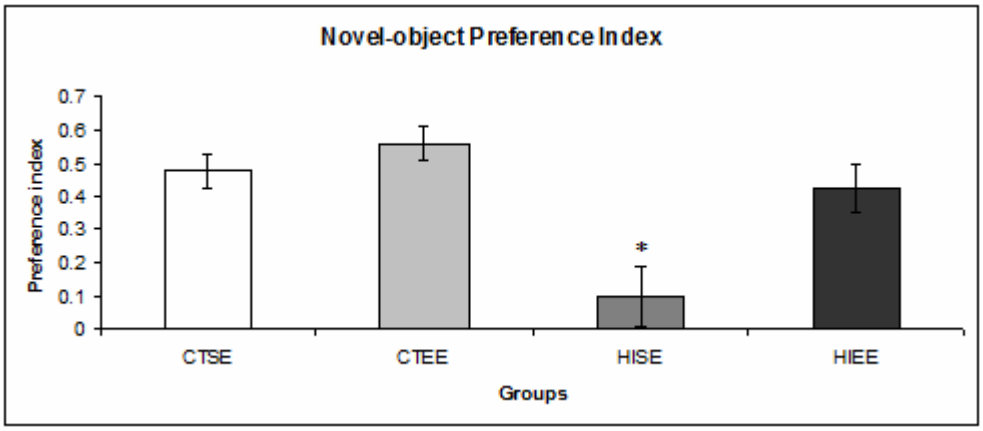

Figure 2. Novel-object recognition memory. * Difference of HISE group compared to all other groups; ANOVA followed by Duncan's test, $p<0.05$. Bars represent the mean ± SD.

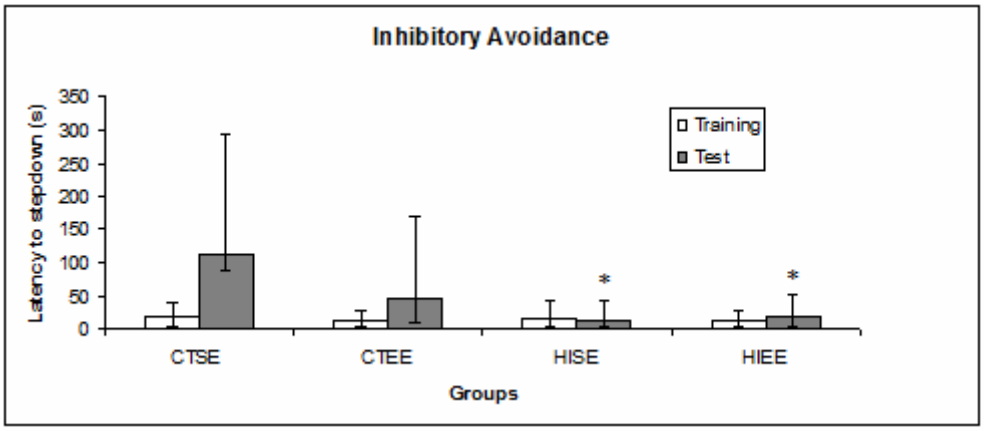

Figure 3. Inhibitory avoidance task. Bars represent median and interquartile range of groups latencies to step down the platform in the training and test session. *p<0.05, Kruskal-Wallis one-way analysis followed by Mann-Whitney: different from CT groups.

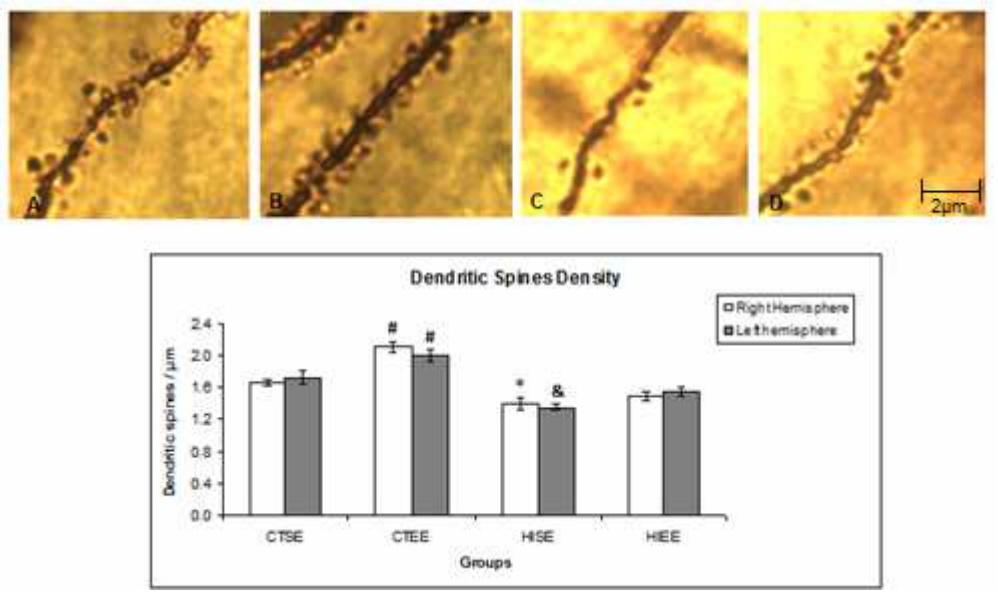

Figure 4. A - D: Illustrative images referent to tertiary branches from CA1 pyramidal neurons: **A** - CTSE group; **B** - CTEE group; **C** - HISE group and **D** - HIEE group. **E**: Dendritic spines densities per hemisphere (spines/µm). * # & p<.05, ANOVA followed Duncan's test: Left hemisphere, & HISE different from other groups. # CTEE different from other groups. Right hemisphere, * HISE different from control groups. # CTEE different from all other groups. Bars represent the mean ± SD.

Table and Legend

Table 1 Open-field test. Mean ± S. E. M. of absolutes values.

Group	*Crossings 1st minute*	*Total crossings*	*Latency*	*Rearings*
CTSE	17.6 ± 1.75	75.7 ± 4.36	7.2 ± 0.84	47.8 ± 1.84
CTEE	18.5 ± 1.13	74.4 ± 6.69	7.1 ± 1.17	37.1 ± 4.33
HISE	25.87 ± 2.95*	85.625 ± 8.65	6.125 ± 1.10	37.625 ± 4.14
HIEE	30.71 ± 3.04*	80.714 ± 6.46	5.287 ± 0.77	40.28 ± 4.28

* $p < 0.05$, Duncan's post-hoc. Differences in relation to CTSE and CTEE

Table 2. Rota-rod test. Mean ± S. E. M. of values.

Group	Number of falls	Latency of the 1st downfall	Maximum time of permanence
CTSE	3	119.1 ± 12.88	199.1 ± 11.94
CTEE	3	108.8 ± 17.8	173.5 ± 20.3
HISE	3	106.75 ± 18.32	188.25 ± 17.66
HIEE	2.87 ± 1.12	81.875 ± 20.13	171.75 ± 27.67

4 CONSIDERAÇÕES FINAIS

Ao finalizar este trabalho que buscou investigar parâmetros de função motora e aprendizagem, associando-os à densidade dos espinhos dendríticos de neurônios do hipocampo de ratos submetidos à hipóxia-isquemia neonatal e estimulados em ambiente enriquecido, chegou-se às seguintes conclusões:

-Houve uma maior atividade dos animais submetidos à HI quando comparados com os animais controle no campo-aberto, o que indica uma hiperatividade causada pela lesão, a qual não foi revertida pelo EA;

- A hipóxia-isquemia resultou em déficits de memória no teste de reconhecimento de objetos nos ratos, além disso, animais HI mantidos em ambiente padrão obtiveram menor índice de exploração quando comparados a animais de todos os outros grupos;

- Foi demonstrada uma deterioração da memória aversiva em animais hipóxico-isquêmicos, independente do ambiente ao qual foram expostos;

- Animais hipóxico-isquêmicos e animais controle não apresentaram déficits motores no teste do rota-rod;

- Na avaliação da densidade de espinhos dendríticos no hipocampo dos animais, os resultados demonstraram uma diminuição da densidade na região CA1 de animais hipóxico-isquêmicos, a qual foi revertida pelo enriquecimento ambiental.

Sendo assim, evidenciou-se neste estudo que o enriquecimento ambiental, além de gerar recuperação em variáveis comportamentais, é eficaz em recuperar as alterações estruturais nos neurônios do hipocampo. Estes achados somam-se aos previamente publicados, indicando que a estimulação em ambiente enriquecido pode ser uma importante ferramenta na reabilitação neurofuncional, podendo exercer significância clínica.

5 PERSPECTIVAS

Frente aos resultados encontrados e somando-os aos trabalhos anteriores aliando hipóxia-isquemia neonatal e enriquecimento ambiental, ficam as seguintes perspectivas:

- Seguir a avaliação morfológica com o intuito de avaliar outras variáveis que possam ser influenciadas pelo enriquecimento ambiental, tais como a arborização dendrítica;

- Avaliar parâmetros bioquímicos que possam vir a servir como suporte na avaliação da relação entre o enriquecimento ambiental e a melhoria nos parâmetros funcionais da hipóxia-isquemia neonatal;

- Mensurar os níveis de sinaptofisina no hipocampo dos animais submetidos a enriquecimento ambiental, a fim de estabelecer uma relação entre ambos, já que, como mencionado na discussão deste trabalho, a sinaptofisina é uma importante proteína envolvida na neurotransmissão.

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