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

O DESENVOLVIMENTO EM FOCO: ENRIQUECIMENTO AMBIENTAL COMO ESTRATÉGIA DE REABILITAÇÃO EM ROEDORES SUBMETIDOS À HIPÓXIA-ISQUEMIA NEONATAL E ESTIMULAÇÃO PRECOCE EM CRIANÇAS EM SITUAÇÃO DE VULNERABILIDADE SOCIAL

CLARISSA PEDRINI SCHUCH

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“Navegar é preciso, viver não é preciso”

Fernando Pessoa

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Sumário

Apresentação

Esta tese está organizada em tópicos: *Introdução, Objetivos, Capítulos* (1 a 3 referentes aos artigos científicos), *Discussão, Conclusão, Perspectivas e Bibliografia.*

A *Introdução* apresenta o embasamento teórico necessário para a compreensão da proposta do trabalho. A seção *Objetivos* expõe as metas que serão apresentadas ao longo de cada capítulo. E, os *Capítulos* contêm os artigos científicos desenvolvidos no período do doutoramento. O experimento apresentado no Capítulo 1 foi desenvolvido no Departamento de Ciências Morfológicas localizado no Instituto de Ciências Básicas da Saúda da Universidade Federal do Rio Grande do Sul (UFRGS) com aprovação pelo Comitê de Ética de Uso de Animais desta universidade (n. 23260 – ANEXO 1). O experimento referente ao Capítulo 2 foi desenvolvido na Faculdade de Medicina da Universidade de Ottawa no Canadá em colaboração com o professor Dr. Dale Corbett. Todos os procedimentos realizados foram aprovados pelo Comitê de Cuidados Animais da Universidade de Ottawa. Os achados descritos no Capítulo 3 foram provenientes da parceria entre a UFRGS, representada pela professora Drª Lenir Orlandi Pereira Silva, com a Secretaria Municipal da Educação e com o Programa Primeira Infância Melhor (PIM-PIÁ) de Porto Alegre-RS. As avaliações realizadas neste terceiro projeto foram aprovadas pelo Comitê de Ética em Pesquisa da UFRGS (n. 22522– ANEXO 2) e pelo Departamento de Pesquisa do programa Primeira Infância Melhor (ANEXO 3). A seção *Discussão* abrange uma interpretação dos resultados obtidos relativos aos três capítulos acima. Os tópicos *Conclusões* e *Perspectivas* contêm as conclusões gerais da tese, bem como, a possibilidade de futuras pesquisas que poderão dar continuidade a este trabalho. O tópico *Bibliografia* lista as referências bibliográficas citadas nas seções *Introdução* e *Discussão.*

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Lista de abreviaturas

AHEMD Escala do Potencial do Ambiente Domiciliar para o Desenvolvimento Motor

AIMS Escala Motora Infantil Alberta

CsA ciclosporina A

EA Enriquecimento ambiental

HI Hipóxia-isquemia

PEDI Inventário de avaliação pediátrica

PIM Primeira Infância Melhor

pTPM poro de transição de permeabilidade mitocondrial

TPM transição de permeabilidade mitocondrial

Resumo

O objetivo desta tese foi verificar os efeitos terapêuticos do enriquecimento ambiental (EA) na recuperação motora, funcional e tecidual de roedores expostos à hipóxia-isquemia (HI) neonatal. Outro objetivo foi acompanhar e descrever o desenvolvimento motor de crianças de 0 a 2 anos de idade junto ao programa Primeira Infância Melhor (PIM) em Porto Alegre-RS. *No Capítulo 1* encontra-se o estudo em que foi avaliado o efeito do EA precoce sobre o aparecimento de sinais e reflexos neurológicos em animais submetidos à HI neonatal. No 7º dia pós-natal, ratos Wistar machos e fêmeas foram submetidos ao modelo de Rice-Vannucci de HI neonatal, onde a artéria carótida comum esquerda foi permanentemente ocluída e na sequência os animais foram expostos a uma atmosfera hipóxica (8% de oxigênio) durante 90 minutos. Um dia pós o procedimento de HI, os roedores foram alojados em gaiolas enriquecidas ou em gaiolas convencionais (ambiente padrão). Os sinais e reflexos neurológicos foram avaliados 24 horas antes da indução da HI, 24 horas após a HI e a cada 3 dias (11º, 14º, 17º) até o 20º dia pós-natal, quando os animais foram eutanaziados para posterior avaliação da extensão da lesão encefálica no estriado, corpo caloso e neocórtex. Os resultados apresentados no *Capítulo 1* mostraram que independente da lesão hipóxico-isquêmica, a exposição ao EA precoce (8º ao 20º dia pós-natal) antecipou o aparecimento dos reflexos neurológicos (abertura dos olhos, desdobramento das orelhas, erupção do incisivo, reflexos de contração das pálpebras e das orelhas). Ademais, o EA foi capaz de prevenir atrofia do corpo caloso ipsilateral à lesão e no neocortex contralateral à lesão. No estudo apresentado no *Capítulo 2* avaliou-se o efeito, pré (17º dia pós-natal) e pós (49º dia pós-natal) tratamento combinado de ciclosporina A (CsA) e reabilitação (EA e tarefa de alcance), na recuperação funcional de roedores submetidos à HI neonatal nos testes do cilindro, escada horizontal, campo aberto, reconhecimento de objetos e staircase. Ratos Sprague Dawley machos e fêmeas foram submetidos à HI (conforme descrito acima) e dez dias pós a lesão foram avaliados nos testes do cilindro, escada horizontal, campo aberto e reconhecimento de objetos. Os roedores apresentaram prejuízo motor no campo aberto (menor número de cruzamentos no primeiro minuto) e na escada horizontal (maior número de erros), contudo não houve comprometimento da função cognitiva. No 21º dia pós-natal os animais receberam a implantação subcutânea de uma bomba osmótica para liberação da CsA (420 mg/mL) ou veículo. E então, foram separados por sexo e alojados em gaiolas enriquecidas ou em gaiolas convencionais. Os animais enriquecidos também foram expostos a um treino de habilidade de alcance tendo como alvo a pata anterior afetada durante 4h/dia, 6 dias/semana por 4 semanas (21º ao 49º dia pós-natal). Ao final das 4 semanas de terapia combinada os animais foram reavaliados nas funções motora e cognitiva. Os resultados mostraram apenas o efeito da reabilitação motora, que por sua vez levou ao aumento da atividade exploratória no campo aberto, diminuição do número de erros na escada horizontal e melhor desempenho no teste do staircase. O componente farmacológico, CsA, não causou nenhum efeito sobre a recuperação dos roedores. A terapia combinada não recuperou a atrofia do hipocampo, córtex e hemisfério cerebral ipisilateral à lesão. E no estudo do *Capítulo 3* objetivamos descrever o desenvolvimento motor de crianças de 0 a 2 anos de idade junto ao programa PIM em Porto Alegre-RS. Tal programa tem como alvo o desenvolvimento pleno das capacidades físicas, intelectuais, sociais e emocionais da criança dentro do seu ambiente familiar. As crianças avaliadas apresentaram desenvolvimento motor normal (apenas risco de atraso). As famílias avaliadas apresentavam baixo nível socioeconômico e ofereciam poucas oportunidades de estimulação no ambiente domiciliar. Concluímos que a estimulação ambiental apresentou efeitos benéficos sobre os aspectos do desenvolvimento e recuperação funcional nos roedores submetidos à HI neonatal e o programa PIM tem um grande potencial de utilização para estimulação de crianças em situação de vulnerabilidade social.

Abstract

The aim of this study was to verify therapeutic effects of environmental enrichment (EE) on motor, functional and tissue recovery of rodents exposed to neonatal hypoxia-ischemia (HI). Besides, describe the motor development in children aged 0 to 2 years old attended by Better Early Childhood Program in Porto Alegre-RS, Brazil. In *Chapter 1*, the study aimed to evaluate the early housing in EE on maturation of physical characteristics and neurological reflexes in rats submitted to neonatal HI. At postnatal day 7, male and female Wistar rats were used to produce Rice-Vannucci model of unilateral brain injury where the left common carotid artery was occluded then pups were placed in a hypoxic chamber (O_2 level at 8%) for 90 minutes. Rodents were housed in EE cages or in standard cages. We evaluated the maturation of physical characteristics and neurological reflexes from the day preceding the HI induction postnatal day 6 until postnatal day 20. Morphological analysis included the evaluation of striatal, corpus callosum and neocortex volume. Our results demonstrated that HI had no effect on neurological parameters evaluated in neonate rats. But we demonstrated a clear effect of early EE on sensorimotor development through earlier appearance of opening, eye reflex and incisor eruption were identified in early stimulated rats. Also, brain tissue was preserved in ipsilateral corpus callosum and contralateral neocortex after early environmental stimulation. The experiment presented in *Chapter 2* investigated pre- and post- combinational therapy of cyclosporine A (CsA) and motor rehabilitation effects on motor function and cognition tests in rats submitted to neonatal HI. Male and female Sprague Dawley rats were submitted to neonatal HI model (as described above) and, ten days after HI surgery, rat pups were evaluated in motor function and cognition through cylinder, ladder-rung walking, open field and novel object recognition. Results showed no cognitive deficit but motor function impairment in open field and ladder rung walking test in rats submitted to HI. At postnatal day 21, all HI pups were implanted subcutaneously on flank with osmotic pumps delivering CsA (420 mg/mL) or vehicle. Then, animals were housed in either standard home cages or enriched environment cages. In addition to being housed in EE, enriched groups were exposed to rehabilitative reach training 4h/day, 6 days/week for 4 weeks (PND 21 until PND 49). At the end of 4 weeks of combined therapy (EE, reaching and CsA) rats were re-evaluated in same motor and cognitive tasks (cylinder, ladder walking, open field, novel object recognition, and staircase task). Rehabilitation appeared to be the most significant component of the combined therapy and was responsible for recovery of motor function as demonstrated in ladder rung walking, open field and staircase performance. Drug component, CsA, had no effects on behavioral outcomes. The combined therapy had no effect on hippocampal, cortical and hemispheric tissue atrophy. And the study of Chapter 3 aimed describe motor development of children from 0 to 2 years old inserted into Better Early Childhood Program (BECP) in south of Brazil. This program has the main goal to develop activities that cover physical, psychological, intellectual and social abilities inside the familiar context. Our results demonstrated that infants had typical motor development (only risk of delay). Evaluated families were considered at social-environmental risk and home affordances were limited. These findings can indicate a risk condition on infant development. We may conclude that environmental enrichment improved neurobehavioral development and functional recovery in rats submitted to neonatal HI, and the BECP has a great potential to stimulate children at social-environmental risk.

1. INTRODUÇÃO

1.1 O papel do ambiente e da estimulação em roedores

Estudos em roedores têm demonstrado que a exposição a distintos estímulos durante as primeiras semanas de vida provocam alterações persistentes em uma ampla variedade de processos fisiológicos e comportamentais os quais persistem na idade adulta (Pham et al., 1999; Rodrigues et al., 2004; Pereira et al., 2008). Assim como nos humanos, as experiências precoces dos roedores podem influenciar seu comportamento futuro em diversas tarefas. O desenvolvimento neurocomportamental dos roedores também segue uma sequência de aparecimento dos reflexos e maturação das habilidades motoras, considerado um sinal de amadurecimento encefálico (Fox, 1985; Heyser, 2003). Desta forma, o ambiente é um fator determinante desde o nascimento até o início da vida adulta, quando o sistema nervoso está mais suscetível às adaptações (Fernández-Teruel et al., 2002; Nunes et al., 2003). Por esta razão, as pesquisas sobre enriquecimento ambiental (EA) investigam o impacto da condição do ambiente sobre as adaptações comportamentais, morfológicas e funcionais em diferentes modelos experimentais. A primeira descrição dos efeitos do EA foi realizada pelo neurocientista Donald Hebb no final dos anos 40 (Hebb, 1947). O pesquisador observou que ratos expostos a um ambiente rico em novidade e complexidade apresentavam melhor desempenho em atividades de aprendizado e memória quando comparados com os roedores mantidos em condições padrão de laboratório. A partir de então inúmeros estudos, mesmo com protocolos variados de EA, descreveram os efeitos cognitivos (Pereira et al., 2007, 2008; Rojas et al., 2013; Van Praag et al., 2000), morfológicos (Rojas et al., 2013; Leggio et al., 2005; Bruel-Jungerman et al., 2005; Nakamura et al., 1999) e moleculares (Lambert et al., 2005; Puurunen et al., 2001; Ickes et al., 2000) no encéfalo de roedores.

O paradigma do EA envolve a exposição continuada a uma combinação de estímulos sociais, cognitivos e físicos na caixa-moradia (Nithianantharajah e Hannan, 2006; Will et al., 2004; Van Praag et al., 2000). Para isto as caixas-moradia são maiores, comparadas às caixas-padrão de laboratório, e permitem maior número de animais de modo a estimular a interação social. O ambiente também é enriquecido através de uma variedade de brinquedos e objetos de diferentes formas e texturas que permitem atividade cognitiva. Ainda, a atividade física voluntária é incentivada através da presença de rodas e rampas dentro da caixa-moradia. Portanto, três aspectos são chave para os efeitos benéficos do EA: o primeiro refere-se à complexidade ambiental, onde os brinquedos e objetos devem fornecer uma gama de oportunidades para o estímulo visual, somatossensorial e auditivo, o segundo aspecto é o fator novidade do ambiente, conseguido através da mudança e realocação dos objetos na caixa-moradia, que proporcionam a estimulação cognitiva adicional, e o terceiro fator é a interação social.

A motivação dos estudos com estimulação em roedores se dá pelo fato de que esta prática, em humanos, já é utilizada (Berk, 2006; Bonnier, 2008). Porém, os mecanismos neurobiológicos do estímulo precoce ao desenvolvimento ainda não são bem conhecidos. O fato importante é que o EA ocasiona consequências morfofuncionais tanto no encéfalo sadio quanto em situações patológicas e, por isso, pode ser utilizado como uma estratégia de neuroproteção e/ou de reabilitação. Há diversos relatos na literatura em relação às modificações encefálicas ocasionadas pela estimulação através do EA em roedores sadios. Podemos citar o córtex cerebral mais espesso e pesado (Diamont et al., 1967, 1976), com maior número de células gliais (especialmente oligodendrócitos), com corpos neuronais maiores, aumento da arborização dendrítica (Ip et al., 2002), com maior número de sinapses

(Rosenzweig e Bennett, 1996), aumento da densidade de vesículas sinápticas (Nakamura et al., 1999) e aumento da densidade de espinhos dendríticos (Kolb et al., 1998). Da mesma forma, diversos estudos mostram os efeitos benéficos do EA em situações patológicas como: na isquemia focal (Puurunen et al., 2001; Biernaskie e Corbett, 2001) e global (Belayev et al., 2003), no traumatismo crânio-encefálico (Johnson et al., 2013; de Witt et al., 2011; Matter et al., 2011), em situação de baixo cuidado materno (Bredy et al., 2003), na exposição a agentes tóxicos (Horvath et al., 2013; Schneider et al., 2001), na doença de Parkinson (Jadavji et al., 2006) e Alzheimer (Jankowsky et al., 2005). Também em distúrbios do desenvolvimento, tem sido estudado o papel protetor do EA, com na anóxia e hipóxia-isquemia neonatal (Rojas et al., 2015, 2013; Marques et al., 2014; Adriani et al., 2006; Iuvone et al., 1996). Portanto, o EA reproduz efeitos positivos nos mais variados modelos e em diferentes faixas etárias. Porém ainda pouco se discute sobre a extensão da janela terapêutica para a aplicação EA como recurso não farmacológico de neuroproteção ou de reabilitação. Alguns estudos já demonstram que tanto as abordagens farmacológicas quanto as não farmacológicas devem iniciar precocemente para a maior eficácia (Dixon et al., 2003; Kline et al., 2001, 2004; Kokiko e Hamm, 2007).

Sendo assim, o melhor entendimento sobre a contribuição da estimulação através do ambiente na recuperação de desordens do sistema nervoso central pode auxiliar na prevenção ou na redução dos danos provocados por algumas patologias.

1.2 Encefalopatia Hipóxico-Isquêmica

1.2.1 Mecanismos neuropatológicos

Apesar dos grandes avanços na obstetrícia e na neonatalogia, a encefalopatia neonatal ainda contribui para os altos índices de morbidade e mortalidade de

crianças recém-nascidas em todo o mundo (White et al., 2012; Rees et al., 2011). De acordo com os dados da Organização Mundial da Saúde as três principais causas de morte dos recém-nascidos são: infecções, prematuridade e asfixia perinatal (Kurinczuk et al., 2010; Flores-Compadre et al., 2013). A encefalopatia hipóxico-isquêmica é uma manifestação neurológica da asfixia perinatal, ocasionando graves consequências como paralisia cerebral, epilepsia, além de transtornos do desenvolvimento, cognitivo e comportamental ou, até mesmo, a morte (Lindström et al., 2006; Martinez-Biarge et al. 2012).

A hipóxia-isquemia (HI) caracteriza-se pela redução da oferta de oxigênio e hipoperfusão dos tecidos. O encéfalo exige um alto consumo de oxigênio e glicose para seu funcionamento, entretanto possui baixa capacidade de reserva energética. Desta forma, um dano no aporte sanguíneo cerebral restringe a chegada dos principais substratos e consequentemente afeta a função encefálica. Essa disfunção energética é marcada por uma despolarização neuronal, liberação excessiva e falha na recaptação do neurotransmissor glutamato, que promove abertura dos canais de cálcio e consequente aumento de sua concentração no meio intracelular. Na sequência ocorre perda da função mitocondrial e produção excessiva de espécies reativas de oxigênio, depleção dos níveis de enzimas antioxidantes, produção de ácido araquidônico e mediadores inflamatórios, além da ativação de segundos-mensageiros envolvidos na sinalização da morte celular programada (Figura 1). Portanto, de uma forma geral, os processos bioquímicos envolvidos na fisiopatologia da HI neonatal são: a excitotoxicidade glutamatérgica, o estresse oxidativo e o processo infamatório (Mclean e Ferriero, 2004; Distefano e Praticò, 2010).

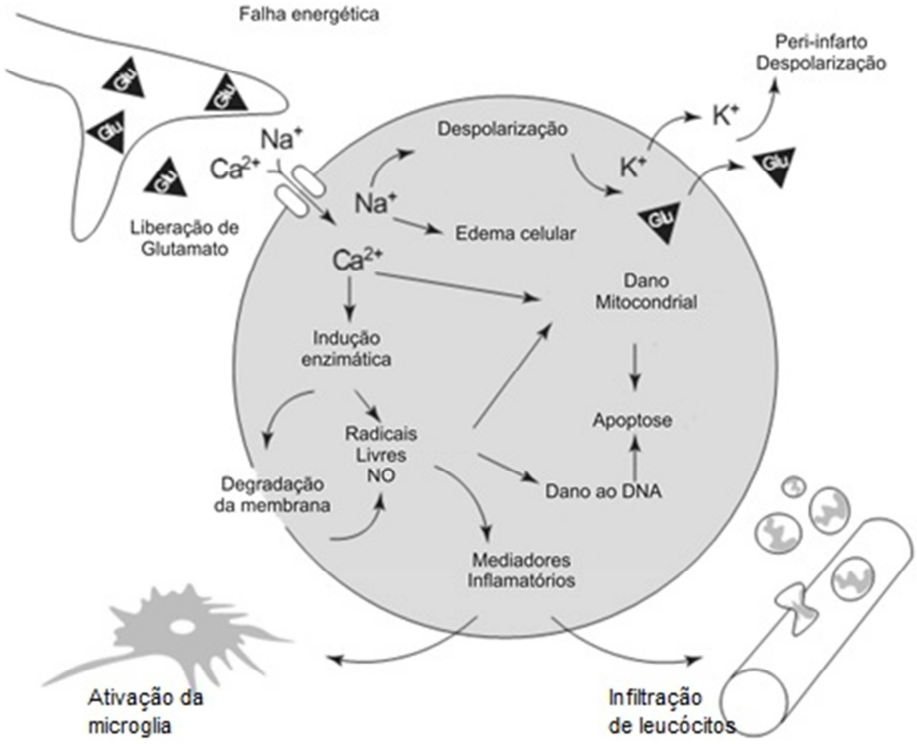

Figura 1: Mecanismo fisiopatológico simplificado da hipóxia-isquemia (adaptado de Dirnagl et al., 1999).

Dada a complexidade dos eventos bioquímicos e moleculares relacionados à HI, surgiu a necessidade do desenvolvimento de modelos experimentais que simulassem as condições patológicas da encefalopatia hipóxico-isquêmica para melhor entender os mecanismos de lesão e sua evolução além do aprimoramento de estratégias terapêuticas.

1.2.2 Modelo Experimental

Em roedores, o modelo de Rice-Vannucci é o mais utilizado para reproduzir o insulto hipóxico-isquêmico neonatal (Rice et al., 1981; Vannucci e Vannucci, 1997). O procedimento experimental é realizado no sétimo dia pós-natal dos ratos por ser histologicamente correspondente, em nível de desenvolvimento, ao encéfalo dos humanos recém-nascidos (Vannucci e Vannucci, 1997). Este modelo consiste na combinação da isquemia, através da oclusão unilateral da artéria carótida comum,

seguida da exposição a uma atmosfera hipóxica com concentração de apenas 8% de oxigênio. O dano encefálico localiza-se principalmente no hemisfério ipsilateral à isquemia, porém, também há relatos de mudanças no hemisfério contralateral à oclusão (Jansen e Low, 1996b; Kadam e Dudek, 2007). As áreas frequentemente afetadas são córtex sensoriomotor, estriado, hipocampo, substância branca periventricular e subcortical (Pereira et al., 2007; Arteni et al., 2003; Vannucci e Vannucci, 1997) (Figura 2). Estudos prévios do nosso laboratório de pesquisa demostraram déficits cognitivos decorrentes da HI neonatal nas memórias declarativa (Rojas et al., 2013; Pereira et al., 2008), aversiva (Rojas et al., 2013; Carletti et al., 2012; Arteni et al., 2003) e espacial (Rojas et al., 2015; Pereira et al., 2007, 2008). Outros dados da literatura também reportam déficits motores após a HI (Lubics et al., 2005; Jansen e Low, 1996a,b; Barth e Stanfield, 1990; Felt et al., 2002), porém não há consenso entre os achados relacionados à função motora e, tais déficits parecem não ser tão evidentes.

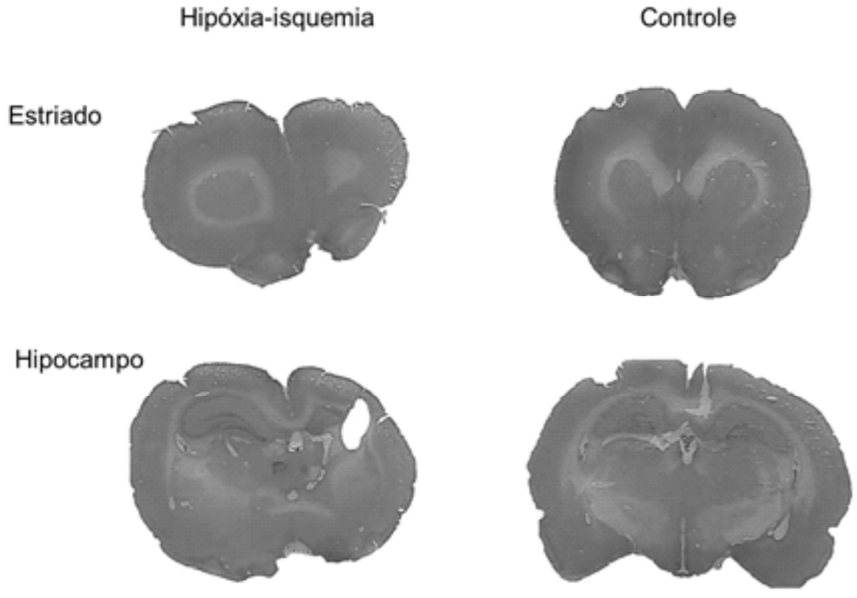

Figura 2: Cortes encefálicos coronais demonstrando as áreas afetadas pela hipóxia-isquemia à direita. Dano e atrofia do estriado e hipocampo quando comparado ao controle (hematoxilina-eosina).

Portanto, o modelo experimental de HI neonatal de Rice-Vannucci reproduz os danos encefálicos e as manifestações comportamentais de maneira similar ao que se observa em crianças que sofreram a mesma condição clínica. Consequentemente, os modelos experimentais são úteis por confirmarem estudos em humanos e proporcionarem o desenvolvimento de intervenções terapêuticas apropriadas. As pesquisas atuais buscam estratégias de prevenção e reabilitação farmacológicas e não farmacológicas para reparar ou minimizar os danos pós HI neonatal.

1.2.3 Intervenções terapêuticas

As manipulações ambientais e comportamentais, tais como a estimulação tátil e o enriquecimento ambiental (EA) são frequentemente utilizados como estratégia não-farmacológica de neuroproteção em ratos submetidos à HI neonatal. Já foram relatados efeitos benéficos do EA no déficit de memória (Rojas et al., 2013; Pereira et al. 2007, 2008; Chou et al., 2001; Rodrigues et al., 2004) e recuperação nos danos morfológicos como a preservação do volume e da densidade dos espinhos dendríticos no hipocampo (Rojas et al., 2013; Rodrigues et al., 2004). Dos estudos supracitados, apenas o de Pereira e colaboradores (2008) e o de Rodrigues e colaboradores (2004) realizaram os protocolos de estimulação a partir do 8° dia pós-natal, ou seja, aplicaram um tratamento de início precoce no modelo de HI neonatal.

Diversas abordagens farmacológicas vêm sendo testadas na recuperação dos danos ocasionados pela HI neonatal, dentre elas podemos citar o uso de antagonista de citocinas pró-inflamatórias, estabilizadores de membrana, inibidores de radicais livres ou de liberação de glutamato, antagonistas dos canais de cálcio, antagonistas de NMDA e agentes anti-apoptóticos (Bonnier, 2008). E mais

recentemente tem sido demostrado que o imunossupressor ciclosporina A (CsA) pode ter um importante papel na proliferação e migração de precursores neurais para o local da lesão isquêmica em ratos adultos (Erlandsson et al., 2011; Leger et al., 2011). A CsA atua bloqueando especificamente o poro de transição de permeabilidade mitocondrial (pTPM). A transição de permeabilidade mitocondrial (TPM) é decorrente de situações de estresse oxidativo associadas a altas concentrações de cálcio na matriz mitocondrial. A TPM caracteriza-se pela permeabilidade progressiva da membrana da mitocôndria interna, que leva à formação de edema e consequentemente morte celular (apoptose e necrose). A CsA possui, portanto, ação inibitória sobre a abertura do pTPM atuando especificamente sobre a Ciclofilina D, que está presente na matriz mitocondrial e é um importante regulador do poro (Halestrap et al., 1998; Hwang et al., 2010). Em resumo, as alterações em decorrência da abertura do pTPM podem ser evitadas através da CsA, minimizando a morte celular e aumentando a sobrevivência das células na área da lesão encefálica (Sachewsky et al., 2014; Walsh et al., 1992).

Há poucos estudos publicados sobre os efeitos da CsA na HI neonatal (Hwang et al., 2010; Leger et al., 2011; Nakai et al., 2004; Puka-Sundvall et al., 2001). Os efeitos neuroprotetores da CsA já foram demonstrados *in vitro* (Gogvadze e Richter 1993; Seaton et al., 1998) e *in vivo* em modelo de isquemia em roedores adultos (Yu et al., 2004; Erlandsson et al., 2011; Hunt et al., 2010). Outros estudos já demonstraram que a CsA também atua sobre as células precursoras neurais no sistema nervoso central de ratos adultos sem efeitos na diferenciação mas com aumento na sobrevivência das células (Hunt et al., 2010) e com melhoras funcionais em animais submetidos à isquemia focal (Erlandsson et al., 2011).

Sabe-se que os insultos isquêmicos tanto no encéfalo adulto quanto no encéfalo imaturo estimulam a proliferação de precursores na zona subventricular, no entanto, não há dados suficientes que mostram a geração significativa de novos neurônios e oligodendrócitos para reparar e regenerar o encéfalo danificado (Yang e Levinson 2006; McQuillen et al., 2003). Juntos, estes achados sugerem que os alvos intracelulares da CsA podem fornecer novas estratégias terapêuticas para a recuperação dos danos morfológicos e comportamentais causados pela HI neonatal.

1.3 O papel do ambiente e da estimulação em crianças

O desenvolvimento infantil é um processo complexo e contínuo por meio do qual a criança adquire capacidades em um contexto neuropsicomotor, ou seja, se desenvolve nos aspectos cognitivo, motor, emocional e social. De maneira mais notável, nos dois primeiros anos de vida, ocorre uma progressão dos marcos motores (reflexos e reações), bem como aquisição de habilidades específicas (Berk, 2006; Mancini et al., 2002). Os reflexos são respostas automáticas a um estímulo e constituem a base para aquisição de habilidades motoras complexas. A atividade reflexa é a primeira forma de integração entre o ser humano e o ambiente; e o processo de inibição da atividade reflexa é a chave para o aparecimento de movimentos voluntários. As habilidades e experiências fornecerão a base para os refinamentos do repertório motor em períodos subsequentes, resultantes da maturação encefálica e da intensa exploração corporal e ambiental por parte da criança (Clark e Metcalfe, 2001). Portanto, o desenvolvimento é altamente influenciado por uma interação de fatores, de modo que a riqueza do contexto e a quantidade e qualidade de experiências em diversas tarefas promovem o estabelecimento de novos comportamentos (Andrade et al., 2005; Almeida et al.,

2006, Maria-Mengel e Linhares, 2007). Um ambiente favorável facilita o desenvolvimento integral, uma vez que possibilita a interação da criança com seus pais e/ou cuidadores e o meio em que estão inseridas (Silva et al., 2006, Santos et al., 2009). As primeiras percepções das crianças em relação ao ambiente são provenientes da exploração e movimentação. É desta forma que as habilidades motoras e sociais, noções sobre objetos, espaço e tempo são adquiridas. Por outro lado, limitações do ambiente restringirão as possibilidades de exploração e interação da criança podendo desencadear um prejuízo no desenvolvimento global. Alguns estudos têm demonstrado uma forte associação entre a qualidade da estimulação no ambiente doméstico e o desenvolvimento físico, cognitivo e psicossocial infantil (Maria-Mengel e Linhares, 2007; Pilzl e Schermann, 2007). Além dos fatores relacionados ao ambiente físico, o grau de escolaridade dos pais, a renda e relações familiares também podem influenciar o desenvolvimento infantil (Mancini 2004; Pilzl e Schermann, 2007; Saccani et al., 2013). Consequentemente, ambientes desfavoráveis podem levar ao desenvolvimento inadequado das crianças (Andrade et al., 2005; Maria-Mengel e Linhares, 2007). A influência do ambiente sobre o desenvolvimento infantil é amplamente discutida, porém poucos estudos investigam o papel da qualidade do ambiente no desenvolvimento motor e social de crianças nos primeiros anos de vida. Conhecer o impacto dos diferentes contextos e a identificação dos fatores de risco presente nesses ambientes permite a definição de estratégias para prevenção de atrasos no desenvolvimento.

A definição de estimulação de acordo com as *Diretrizes educacionais sobre estimulação precoce* (1995) é: “Conjunto dinâmico de atividades e recursos humanos e ambientais incentivadores que são destinados a proporcionar a criança, nos primeiros anos de vida, experiências significativas para alcançar pleno

desenvolvimento no seu processo evolutivo". Desta forma, programas de estimulação têm sido desenvolvidos em vários países na tentativa de minimizar deficiências e melhorar as capacidades em crianças com ou em risco de desordens do desenvolvimento neuropsicomotor (Bonnier 2008, Als et al., 2004; Darrah et al., 2001). Esses programas de estimulação foram desenvolvidos para três públicos-alvo: crianças em situação de risco biológico, como resultado da prematuridade ou baixo peso ao nascer; crianças com doenças que induzem o atraso do desenvolvimento (e.g. síndrome de Down); e crianças que vivem em ambientes com baixo nível socioeconômico e estimulação limitada em casa. É neste último contexto que o programa Primeira Infância Melhor (PIM) atua, integrando a política de governo do Estado do Rio Grande do Sul sob a coordenação da Secretaria da Saúde. O programa tem ação socioeducativa voltada às famílias com crianças de zero a seis anos de idade e gestantes que se encontram em situação de vulnerabilidade social. O programa PIM orienta a maneira como os pais, as famílias e outros cuidadores devem se relacionar, criar vínculo e estimular suas crianças no ambiente que estão inseridas, otimizando a utilização de recursos como espaço físico e atividades como aliados no processo do desenvolvimento neuropsicomotor. Assim, cria-se um meio em que a ligação emocional, que tem função biológica protetora, se fortaleça e organize as redes neurais que refletirão nas tarefas de aprendizado, comportamento e saúde ao longo da vida (Mccain e Mustard, 1999). O PIM baseia-se em um programa cubano chamado "Educa a tu Hijo", este programa foi inicialmente aplicado na zona rural com o propósito de preparar as crianças no ingresso à escola. Seu conteúdo original abrange cinco áreas principais: comunicativa, afetiva, intelectual, desenvolvimento motor e a formação de hábitos. Adaptado para a população do Estado do Rio Grande do Sul, em 3 de julho de 2006

o PIM foi instituído pela Lei Estadual 12.544/06, a qual decretou que o programa está voltado para o desenvolvimento pleno das capacidades físicas, intelectuais, sociais e emocionais da criança, tendo como eixo de sustentação a Comunidade, a Família e a Intersetorialidade. O objetivo do Programa PIM é orientar as famílias para que possam promover o desenvolvimento integral de seus filhos desde a gestação até os 6 anos de idade. Os visitadores capacitados tem o papel orientar a estimulação da criança em cada fase evolutiva. Sendo assim o visitador deve ter criatividade para usar materiais disponíveis no domicílio para estimular as crianças dentro da realidade da família atendida. Portanto, identificar os fatores de risco e as oportunidades de estimulação do ambiente no qual a criança está inserida é um desafio para que sejam aplicadas orientações e medidas preventivas em relação a um possível atraso no desenvolvimento neuropsicomotor.

As crianças que vivem em países em desenvolvimento estão expostas a múltiplos riscos como, por exemplo, nascerem de gestações desfavoráveis e/ou incompletas e viverem em condições socioeconômicas adversas. A exposição a estes fatores negativos faz com que essas crianças tenham maior chance de apresentar atrasos em seu crescimento e desenvolvimento. Por essa razão, a influência dos fatores risco biológicos e ambientais no desenvolvimento infantil tem sido objeto de inúmeros estudos. Logo, destaca-se a importância de investigar o papel da estimulação através do ambiente tanto para o desenvolvimento e aprimoramento de estratégias de reabilitação para crianças em risco biológico (e.g. sequelas neurológicas pós asfixia perinatal, nesta tese representada pelo modelo experimental de hipóxia isquemia neonatal) quanto para crianças que se encontram em situação de vulnerabilidade social (risco socioambiental).

2. OBJETIVOS

2.1 Objetivo Geral

O objetivo geral deste estudo foi:

Verificar os efeitos terapêuticos do enriquecimento ambiental como estratégia de reabilitação em roedores submetidos à hipóxia-isquemia neonatal e da estimulação precoce em crianças.

2.2 Objetivos Específicos

(1) Verificar os efeitos terapêuticos do enriquecimento ambiental precoce sobre os **aspectos do desenvolvimento** (maturação das características físicas e reflexos neurológicos) e mensurar o volume do estriado, corpo caloso e neocórtex de ratos submetidos à HI neonatal (Capítulo 1).

(2) Avaliar pré- e pós-**tratamento combinado de ciclosporina A** e **reabilitação,** a recuperação funcional nos testes do cilindro, escada horizontal, campo aberto, reconhecimento de objetos e staircase em ratos submetidos à HI neonatal. Bem como avaliar o efeito do tratamento combinado no volume do hemisfério cerebral, córtex e hipocampo (Capítulo 2).

(3) Acompanhar e **descrever o desenvolvimento motor de crianças** de 0 a 2 anos de idade junto ao programa Primeira Infância Melhor (PIM) em Porto Alegre-RS através da Escala Motora Infantil Alberta (Alberta Infant Motor Scale-AIMS). Relacionar os fatores de risco sociais e o desenvolvimento motor através da Escala do Potencial do Ambiente Domiciliar para o Desenvolvimento Motor (Affordance in the home environment motor development- AHEMD). E por fim, verificar a independência funcional das crianças atendidas no PIM através do Inventário de Avaliação Pediátrica de Incapacidade (Pediatric Evaluation of Disability Inventory-PEDI) (Capítulo 3).

3. CAPÍTULO 1

Artigo: Early environmental enrichment affects neurobehavioral development and prevents brain damage in rats submitted to neonatal hypoxia–ischemia

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Research paper

Early environmental enrichment affects neurobehavioral development and prevents brain damage in rats submitted to neonatal hypoxia-ischemia

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HIGHLIGHTS

- Early environmental enrichment has affected developmental milestones.
- Daily performance of reflexes was earlier improved in enriched rats.
- Hypoxia-ischemia caused atrophy of striatum, corpus callosum and neocortex.
- Early stimulation prevented the tissue damage on corpus callosum and neocortex.
- Hypoxia-ischemia did not affect the sensorimotor development in neonate rats.

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ABSTRACT

Our previous results demonstrated improved cognition in adolescent rats housed in environmental enrichment (EE) that underwent neonatal hypoxia-ischemia (HI). The aim of this study was to investigate the effects of early EE on neurobehavioral development and brain damage in rats submitted to neonatal HI. Wistar rats were submitted to the HI procedure on the 7th postnatal day (PND) and housed in an enriched environment (8th–20th PND). The maturation of physical characteristics and the neurological reflexes were evaluated and the volume of striatum, corpus callosum and neocortex was measured. Data analysis demonstrated a clear effect of EE on neurobehavioral development; also, daily performance was improved in enriched rats on righting, negative geotaxis and cliff aversion reflex. HI caused a transient motor deficit on gait latency. Brain atrophy was found in HI animals and this damage was partially prevented by the EE. In conclusion, early EE stimulated neurobehavioral development in neonate rats and also protects the neocortex and the corpus callosum from atrophy following HI. These findings reinforce the potential of EE as a strategy for rehabilitation following neonatal HI and provide scientific support to the use of this therapeutic strategy in the treatment of neonatal brain injuries in humans.

1. Introduction

The observation of children through reflex assessment and motor developmental milestones are tools used to evaluate neurobehavioral development which relates to neurological status assessed by the maturation of motor coordination and cognitive aspects [1,2]. It has been also established that impaired development may represent a predictive factor of behavioral modifications in adulthood [3,4] and through the evaluation of neonatal reflexes, it is possible to identify the persistence or absence of reflexes and detect developmental delay. Reflexes are automatic responses to a stimulation form and constitute the basis for motor skills. The primitive reflex activity is the first form of integration between human beings and the environment; and based on the dynamical systems theory, the motor development is the product of the refining and remodeling pre-existing patterns and it is dependent of the interaction of neural maturation and self-organizing properties of

Abbreviations: HI, hypoxia-ischemia; EE, environmental enrichment; SE, standard environment.

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the sensorimotor system [5,6]. Likewise in humans, rat development is considered a sign of brain maturation [7] and follows a sequence of the appearance of reflexes and maturation of motor skills [3]. Consequently, there are many factors that may damage normal development in both humans and rodents such as visual and hearing impairment, growth disorders resulting from perinatal and postnatal asphyxia, and environmental factors [8–13].

Perinatal asphyxia represents a major cause of brain damage in term newborn infants and is frequently associated with neurodevelopmental disabilities [14,15]. Experimental models of hypoxia-ischemia (HI) aim to reproduce neuropathological and functional characteristics found in humans such as white matter damage and neuronal loss, motor and cognitive deficits [16–19].

Currently, a non-pharmacological therapy has been used for brain damage using stimulation through interactions with the environment known as environmental enrichment (EE) [20–22]. Some studies have demonstrated that late enrichment (starting two weeks after the neonatal HI) improves memory and preserves dendritic spine density in the hippocampus of rats submitted to HI [23,24]. Only one study evaluated the impact of early EE housing in rats submitted to HI; the authors found improvement in working memory with no effects on tissue atrophy in the hippocampus and striatum [25]. We hypothesized that if EE recovers functional deficits such as learning and memory, it could also act on the onset of neurobehavioral developmental milestones. This issue deserves attention, especially because, in clinical cases, early intervention is critical for successful therapy and leads to improvement in cognitive and motor outcomes in cases of perinatal disorders [2,19]. The novelty of this study lies on investigating the effects of early enrichment on neurological reflexes following HI. Thus, the present study aimed to evaluate the role of early EE as a therapeutic intervention in rats submitted to neonatal HI. We investigated: (a) the maturation of physical characteristics and neurological reflexes; and (b) the volume of the striatum, corpus callosum and neocortex of rats undergoing neonatal HI followed by an early EE protocol.

2. Materials and methods

2.1. Animals

Pregnant Wistar rats obtained from the Central Animal House of the Institute of Basic Health Sciences were maintained under standard laboratory conditions. Wistar pup rats from 6 litters were randomly assigned to four groups: control maintained in a standard environment (CTSE: n = 6 males and 5 females); CT maintained in EE (CTEE: n = 6 males and 6 females); HI maintained in SE (HISE: n = 5 males and 6 females); and HI maintained in EE (HIEE: n = 6 males and 6 females). It has already been reported that sex does not influence the appearance of reflexes or the extent of brain damage after HI; therefore, male and female rats were randomly distributed among groups [15,25]. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institute of Health (USA) and with the Federation of Brazilian Societies for Experimental Biology. This project was approved by the Ethics Committee at the Universidade Federal do Rio Grande do Sul (n. 23260).

2.2. Hypoxia-ischemia

On postnatal day (PND) 7, to produce unilateral brain injury, we used the Rice-Vannucci model [16,26], which consists of a permanent left common carotid artery occlusion associated with hypoxia (O_2 level at 8%) for 90 min [17].

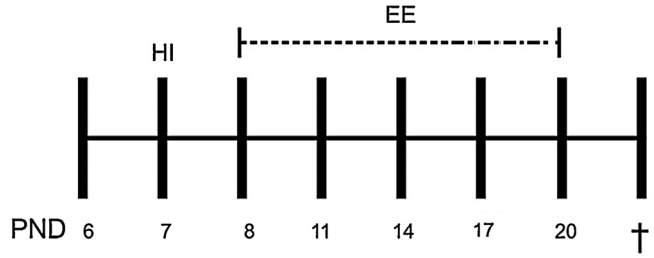

Fig. 1. Timeline of experimental procedures. Examinations of neurobehavioral development began 24 h before hypoxic-ischemic insult, 24 h after and were carried out every 3 days until PND 20. One day following the behavioral testing, animals were killed for morphological analysis (†).

2.3. Environmental enrichment

The early EE procedure used in this study was previously described by Pereira et al. [25]. The early continuous housing consists of free exploration by the dam and pups into the EE cage. The enriched environment consisted of a cage (40 × 40 × 20 cm) with three floors, a ramp, and a running wheel. So in volume the EE cage was larger than standard cages. We randomly used 7–10 objects with different shapes and textures (Lego pieces, soft toys, hairbrush, baby rattle, plastic toys, and rough objects). Objects were changed once a week to keep the novelty factor. Stimulated CT and HI-animals with your dams were housed in groups of 8 per cage, from PND 8–20, in EE cages, while non-stimulated litters were housed in standard cages.

2.4. Neurobehavioral development evaluation

Neurobehavioral development was assessed 24 h before and 24hr after HI and also on the PNDs 11, 14, 17 and 20 (Fig. 1). Physical characteristics such as eye opening, incisor tooth eruption and ear unfolding were observed and the day of appearance was recorded [18,27]. Also, the following signs and reflexes were tested: (1) *Righting reflex*: rats were placed in the supine position and time to turn over to the prone position was recorded [18]; (2) *Negative geotaxis*: animals were placed head down on an inclined board (45°) of 30 cm. The day on which they began to turn around and climb up the board with their forelimbs reaching the upper board was observed [28]. In cases where the animal did not turn around and climb up the board within 30 s, the maximal time was assigned. Time to reach the upper board's end was registered daily from the first negative geotaxis appearance day; (3) *Sensory reflexes*: the ears and eyelids were gently touched with a cotton swab and first day ear twitch reflex and eyelid contraction was recorded [18,28]; (4) *Limb placement*: each animal was suspended and the back of its forepaw and hindpaw were approached to the edge of the bench in order to touch it. The first day on which the rat lifted and placed its paws on Table was recorded [18]; (5) *Limb grasp*: the fore- and hindlimbs were touched with a thin rod, and the first day of grasping onto the rod was recorded [18]; (6) *Gait*: animals were placed in the center of a circle of paper (13 cm in diameter) and the day when they began to move off the circle with both forelimbs was recorded [18]. The maximum time was assigned (30 s) when the animal failed to leave the circle. Time to move off the circle was recorded daily from the first gait appearance day; (7) *Auditory startle*: startle response to a clapping sound was observed [18]; (8) *Cliff aversion reflex*: animals were placed with their forepaws overhanging the board's edge. Time required to turn more than 90° from the edge was recorded within a maximum observation time of 30 s [18].

Table 1
Average days ± S.E.M. of appearance of physical and neurological signs.

Sign	Days of appearance			
	CTSE	CTEE	HISE	HIEE
Eye opening	15.5 ± 0.4	14.0 ± 0.0[$]	15.7 ± 0.4	14.2 ± 0.8[$]
Eyelid reflex	15.5 ± 0.4	14.0 ± 0.0[$]	15.7 ± 0.4	14.2 ± 0.8[$]
Gait	8.58 ± 0.4	9.5 ± 0.4	9.0 ± 0.8	9.3 ± 0.7
Ear unfolding	14.0 ± 0.0	12.2 ± 0.4[$]	14.0 ± 0.0	11.7 ± 0.4[$]
Negative geotaxis	8.5 ± 0.5	8.6 ± 0.5	9.0 ± 0.5	8.3 ± 0.6
Incisor tooth eruption	12.5 ± 0.4	11.0 ± 0.0[$]	12.5 ± 0.4	11.0 ± 0.0[$]
Ear twitch reflex	23.0 ± 0.0	22.0 ± 0.5	21.2 ± 0.9	20.0 ± 0.9[#]
Auditory startle	14.7 ± 0.3	15.0 ± 0.4	14.0 ± 0.0	14.4 ± 0.3
Forelimb placing (right)	6.3 ± 0.2	6.1 ± 0.1	6.8 ± 0.6	6.4 ± 0.2
Forelimb placing (left)	6.1 ± 0.1	6.3 ± 0.2	6.1 ± 0.1	6.1 ± 0.1
Hindlimb placing (right)	6.4 ± 0.4	6.1 ± 0.1	6.1 ± 0.1	6.6 ± 0.4
Hindlimb placing (left)	6.0 ± 0.0	6.1 ± 0.1	6.6 ± 0.6	6.3 ± 0.2
Forelimb grasp (right)	6.0 ± 0.0	6.0 ± 0.0	6.3 ± 0.2	6.1 ± 0.1
Forelimb grasp (left)	6.0 ± 0.0	6.3 ± 0.2	6.0 ± 0.0	7.1 ± 0.7
Hindlimb grasp (right)	6.1 ± 0.1	6.7 ± 0.4	6.5 ± 0.2	6.9 ± 0.2
Hindlimb grasp (left)	6.3 ± 0.2	6.6 ± 0.2	7.8 ± 0.7	7.7 ± 0.5

Notes: [$]Difference from CTSE and HISE; [#]difference from all groups. ANOVA followed by Tukey's test, $p < 0.05$.

2.5. Morphological analysis

On the PND 21, rats were euthanized by transcardiac perfusion (n = 5/group). Brains were maintained in paraformaldehyde solution, and then cryoprotected and sectioned. Coronal sections (25 μm) with a 100 μm interval were stained with cresyl-violet. The neocortex was defined as comprising the primary motor cortex, the secondary motor cortex and the primary somatosensory cortex; such structures are related to somatosensory function and correlate with limbs cortical map representations [29,30]. Brain areas of each structure were measured using ImageJ program (NIH, USA) between Bregma 2.76 mm and −1.56 mm [31]. Tissue volumes were calculated according to the Cavalieri Method [16,32,33].

2.6. Statistical analysis

Neurobehavioral development data were analyzed using two-way repeated-measures analysis of variance (ANOVA) and independent samples *t*-tests (Bonferroni correction) for multiple comparisons. Two-way ANOVA was performed for the appearance of physical/neurological signs and morphological analysis followed by independent *t*-tests or Tukey post hoc test for multiple comparisons, when necessary. Significance was set at $p \leq 0.05$ for all analyses, and values are expressed as mean ± SEM.

3. Results

3.1. Neurobehavioral development evaluation

Table 1 summarizes the findings of appearance of physical and neurological signs. Eye opening, eyelid reflex, ear unfolding, incisor tooth eruption and ear twitch reflex were performed in shorter time by enriched animals. However, no differences were observed between groups in fore- and hindlimbs placement and grasping, gait, negative geotaxis and auditory startle. There was no effect of the lesion on the reflexes evaluated, except ear twitch reflex.

Repeated measure ANOVA indicated a main effect of environment ($F(1,46) = 13.1$, $p < 0.05$) and days ($F(5,46) = 62.1$, $p < 0.05$) on the latency to perform righting reflex. Fig. 2A shows the performance of all experimental conditions across time. Once there was a significant main effect for environment, we collapsed groups across standard environment and enriched environment animals. Unpaired Student's *t*-test showed that the EE group had a lower latency to perform righting reflex compared to the SE group at PND 8, 11, 14 and 17 (Fig. 2B). Such results showed that even without a lesion effect, early stimulation reduced the latency to perform righting reflex; in other words, EE acted on neurological maturation of this reflex.

With regard to latency to perform negative geotaxis reflex, a significant days*environment interaction ($F(5,46) = 3.1$, $p < 0.05$), environment effect ($F(5,46) = 5.4$, $p < 0.05$), and an effect of days ($F(5,46) = 115.9$, $p < 0.05$) were observed (Fig. 2C). Collapsing SE and EE animals, unpaired Student's *t*-test analysis showed that the EE group had a lower latency to turn around and climb up the board compared to SE group at PND 11 (Fig. 2D). We may confirm that independent of the lesion, EE promoted an earlier development of negative geotaxis.

Repeated measures ANOVA for cliff aversion reflex demonstrated an effect of day ($F(5,46) = 40.7$, $p < 0.05$) and days*environment interaction ($F(5,46) = 3.5$, $p < 0.05$). Fig. 2E shows the performance of all experimental conditions across time. We collapsed groups across SE and EE animals. Unpaired Student's *t*-test showed that the EE group improved their time to turn away from the edge compared to SE group at PND 14 and there is a tendency to perform better at PND 11 ($p = 0.051$; Fig. 2F).

Repeated measures ANOVA identified a significant main effect of days*lesion interaction ($F(5,46) = 2.4$, $p < 0.05$) and days ($F(5,46) = 214.4$, $p < 0.05$) on gait latency (Fig. 2G). Once there was a significant effect for days*lesion interaction, we collapsed groups across HI and CT animals. Unpaired Student's *t*-test showed that the CT group had a lower latency to moved off the circle than the HI group at PND 14 (Fig. 2H).

3.2. Striatum, corpus callosum and neocortex volume

Two-way ANOVA indicated a significant effect of lesion on the volume of the left (ipsilateral to arterial occlusion) striatum ($F(1,20) = 30.4$; $p < 0.05$). The HI group had a smaller left striatum volume compared to the CT animals. No effect was observed on the right striatum (Fig. 3A).

Also, there was a main effect of lesion ($F(1,20) = 27.0$; $p < 0.05$) and environment ($F(1,20) = 15.0$; $p < 0.05$) on the left corpus callosum volume. The volume of left corpus callosum was smaller in the HI group than the CT group and we observed that ipsilateral corpus callosum volume in the EE animals was bigger than the SE animals, indicating that EE was able to prevent ipsilateral corpus callosum atrophy (Fig. 3B). In the contralateral side, data analysis indicated a significant effect of lesion ($F(1,20) = 16.5$; $p < 0.05$) and environment ($F(1,24) = 11.9$; $p < 0.05$) on the right corpus callosum volume (Fig. 3B). Again, the HI group had a smaller volume of right corpus callosum than CT group and enrichment was able to prevent corpus callosum atrophy on the contralateral hemisphere.

Two-way ANOVA identified a main effect of lesion ($F(1,20) = 37.8$; $p < 0.05$) on the left neocortex volume. The HI animals had a smaller left neocortex volume than the CT group (Fig. 3C). ANOVA showed lesion*environment interaction ($F(1,20) = 7.1$; $p < 0.05$) on the right neocortex volume (contralateral). The Tukey test revealed that HISE animals had smaller right neocortex volume compared to HIEE (Fig. 3C). As observed in the corpus callosum, EE seems to protect the nervous tissue of the HI injury.

4. Discussion

In the present study, it was demonstrated that exposure to early EE positively affected some aspects of neurobehavioral development and physical maturation such as eye opening, eyelid reflex, ear unfolding, incisor tooth eruption and ear twitch reflex in rats that suffered neonatal HI. Also, daily performance was improved in

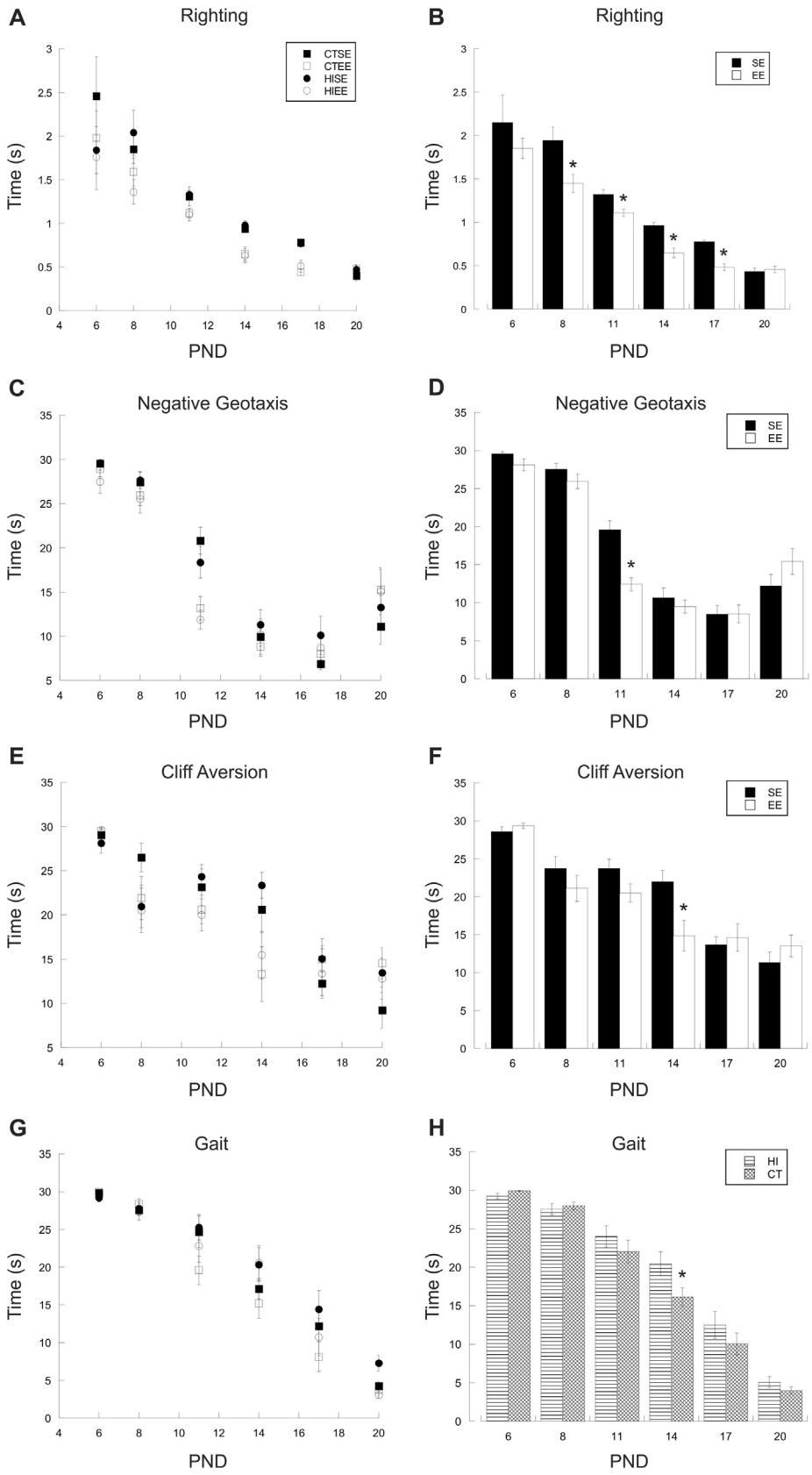

Fig. 2. Daily performance of all experimental conditions of righting (A), negative geotaxis (C), cliff aversion (E) and gait (G). Environment and day effect for righting, negative geotaxis, cliff aversion and days*lesion effect for gait. (B, D, F) Unpaired Student's *t*-test analysis collapsing standard environment (SE) and enriched environment (EE). EE condition demonstrated significant improvements over animals in the SE conditions (*$p < 0.05$). (H) Unpaired Student's *t*-test analysis collapsing control (CT) and hypoxia-ischemia (HI) groups. CT condition demonstrated significant improvements over animals in the SE conditions (*$p < 0.05$).

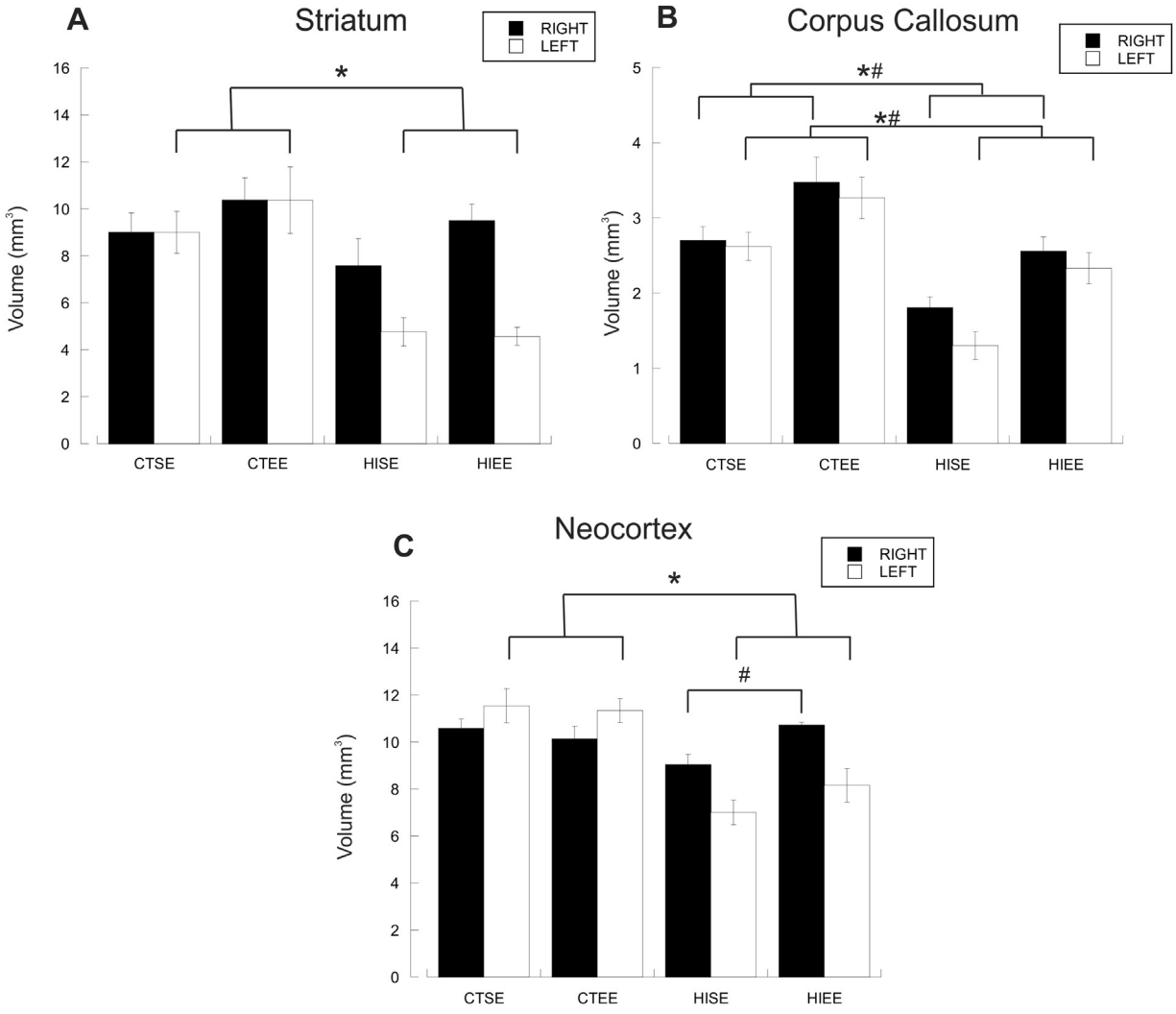

Fig. 3. Volume of brain structures: striatum (A), corpus callosum (B) and neocortex (C). Each bar represents the mean ± SEM; black bar to the right hemisphere (contralateral to arterial occlusion) and white bar to the left hemisphere (ipsilateral to arterial occlusion). (A) Striatum volume of all experimental conditions where Two-way ANOVA indicated a significant effect of lesion to left striatum. *significant difference between HI and CT groups ($p < 0.05$). (B) Two-way ANOVA indicated a significant effect of lesion and environment for left and right corpus callosum volume. *significant difference between HI and CT groups ($p < 0.05$); #significant difference between SE and EE groups ($p < 0.05$); (C) Neocortex volume of all experimental conditions where, Two-way ANOVA indicated a significant interaction of lesion*environment for right neocortex volume and lesion effect for left neocortex volume. #significant difference between HISE and HIEE groups ($p < 0.05$. Tukey test). *significant difference between HI and CT groups ($p < 0.05$, Unpaired *t*-test).

enriched rats on righting, negative geotaxis and cliff aversion reflex. HI caused a transient motor deficit on gait latency. Moreover, environmental stimulation partially prevented the brain from tissue damage.

The appearance of reflexes is influenced by various factors, such as, maternal care [34], malnutrition [35], and neonatal HI [17,18,36]. We highlighted the importance of developmental assessment in rats because short-term neurofunctional outcome has been correlated with long-term functional deficits [17,37,38]. Also in humans, the absence or persistence of neonatal reflexes is predictive of the extent of later functional impairments in patients underwent severe asphyxia [2,17,39]. The present study adds to the current literature the positive effects of early enrichment on neurobehavioral development in rats submitted to the HI.

An important benefit of the EE was demonstrated on neurological maturation through the reflexes and physical parameters, along with an improvement of neurobehavioral skills. Also, our data analysis revealed that animals subjected to EE have enhanced their performance on righting, negative geotaxis and cliff aversion reflex. Previous studies have reported that EE could diminish the developmental delay or lead to earlier development in some reflexes [28,36] and improved a wide variety of cognitive and behavioral outcomes [19,24,25,40] in HI experimental models. The positive effect of early EE protocol can be consequence of the dam's behavior that could stimulate neurobehavioral development of pups by means of dam's sounds and activity in EE cage, however more studies are needed to understand this relationship. In the clinic, intervention for high-risk children, through enriched early experience, can enhance the brain's development [41]. Systematic reviews reported the positive effects of early intervention programs on cognitive outcomes but do not have a clear beneficial effect on motor development [42,43]. In accordance with that, our previous data demonstrated that early EE improved spatial and recognition memory in young rats [25]. In the present study no substantial functional deficits were identified in the HI rats. However, animals stimulated in the EE had enhanced neurobehavioral development, compared with rats maintained in standard condition since in the impoverished environment the pups were less active and had no access to any stimulus objects. Sensory and motor abilities are crucial to normal development in humans and animals [3,17,39]. For example, negative geotaxis in rodents is essential for adaptation to the environment [44] and the absence

or persistence in reflexes development may interfere with newborn perception and reaction to the external environment. Our data indicated that HI caused a transient deficit in gait latency but did not affect the neurobehavioral development in neonatal rats, suggesting spontaneous recovery following injury. This is a suitable interpretation given that sensorimotor responses are vital for neonatal survival. Corroborating this, Golan and Huleihel [44] emphasized that newborn rodents submitted to HI complete their development evaluation tasks, despite the delay in reaching completion. Spontaneous recovery was also demonstrated in other studies with HI [14,15]. Taken together, results related to neurobehavioral developmental milestones in rats submitted to neonatal HI are controversial. Regarding to reflexes and motor developmental parameters, only minor differences between injured and uninjured animals were observed in the present study. There are several lines of evidence that the Rice-Vannucci model is a useful animal model of neonatal hypoxic-ischemic encephalopathy. This experimental model is extensively used to reproduce cognitive and also motor deficits [17–19,37,45–47]. However the inconsistency of the outcomes related to motor function indicate that this is not the most appropriate model to investigate motor behavior. In the present study, possibly, the inefficiency of HI model in generate damage in gross motor function affected the identification of neurodevelopmental milestones since most of them depend strongly of this function. To better understand and identify motor behavioral capacities, some studies have combining other insults with HI or anoxia. Interestingly the association of a paradigm of the prenatal inflammation (with injection of lipopolysaccharide in pregnant rats) with HI was able to generate significant and sustained motor deficits observed in open field and rotarod tasks [44] and an association of maternal exposure to lipopolysaccharide, perinatal anoxia and sensorimotor restriction of the pups resulted in motor deficits in rotarod, horizontal ladder and narrow suspended bar [48].

Morphological outcomes on Rice-Vannucci HI model [16,26] have been reported in several brain areas [18,23,37,46]. One study demonstrated recovery of morphological damage to the hippocampus after tactile stimulation in hypoxic-ischemic rats [32]. Here, we showed that HI damaged the striatum, corpus callosum and neocortex. Moreover, early enrichment was able to prevent volume loss in the ipsilateral corpus callosum and interestingly, in the contralateral neocortex. These data suggested some reorganization in undamaged hemisphere following EE, corroborating the findings of with Jansen and Low [37]. Volume preservation by EE might be a consequence of dendritic arborization in the lesioned area.

5. Conclusion

Early EE has stimulated the neurological maturation of some reflexes and has minimized sensorimotor and cognitive deficits in neonatal rats. The early initiation of EE also protects the neocortex and the corpus callosum of rats from atrophy following HI. Our present data reinforce the potential of early EE stimulation as a strategy for rehabilitation following neonatal HI, but also as normal stimulation procedure and give scientific support to the use of this therapeutic strategy in the treatment of neonatal brain injuries in humans.

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4. CAPÍTULO 2

Artigo: Enriched rehabilitation promotes motor recovery in rats exposed to neonatal hypoxia-ischemia

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Research report

Enriched rehabilitation promotes motor recovery in rats exposed to neonatal hypoxia-ischemia

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HIGHLIGHTS

- A combination therapy of cyclosporine A and enriched rehabilitation is evaluated.
- Enriched rehabilitation promotes motor recovery.
- Sensitive behavioural tests can detect early impairments in hypoxic-ischemic rats.
- CsA given 2 weeks after HI is not therapeutically efficacious.

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ABSTRACT

Despite continuous improvement in neonatology there is no clinically effective treatment for perinatal hypoxia ischemia (HI). Therefore, development of a new therapeutic intervention to minimize the resulting neurological consequences is urgently needed. The immature brain is highly responsive to environmental stimuli, such as environmental enrichment but a more effective paradigm is enriched rehabilitation (ER), which combines environmental enrichment with daily reach training. Another neurorestorative strategy to promote tissue repair and functional recovery is cyclosporine A (CsA). However, potential benefits of CsA after neonatal HI have yet to be investigated. The aim of this study was to investigate the effects of a combinational therapy of CsA and ER in attempts to promote cognitive and motor recovery in a rat model of perinatal hypoxic-ischemic injury. Seven-day old rats were submitted to the HI procedure and divided into 4 groups: CsA + Rehabilitation; CsA + NoRehabilitation; Vehicle + Rehabilitation; Vehicle + NoRehabilitation. Behavioural parameters were evaluated pre (experiment 1) and post 4 weeks of combinational therapy (experiment 2). Results of experiment 1 demonstrated reduced open field activity of HI animals and increased foot faults relative to shams in the ladder rung walking test. In experiment 2, we showed that ER facilitated acquisition of a staircase skilled-reaching task, increased number of zone crosses in open-field exploration and enhanced coordinated limb use during locomotion on the ladder rung task. There were no evident deficits in novel object recognition testing. Delayed administration of CsA, had no effect on functional recovery after neonatal HI. There was a significant reduction of cortical and hemispherical volume and hippocampal area, ipsilateral to arterial occlusion in HI animals; combinational therapy had no effect on these morphological measurements. In conclusion, the present study demonstrated that ER, but not CsA was the main contributor to enhanced recovery of motor ability after neonatal HI.

Abbreviations: HI, hypoxia-ischemia; ER, enriched rehabilitation; CsA, cyclosporine A; PND, postnatal day.

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1. Introduction

Perinatal hypoxia ischemia (HI) is one of the most common causes of mortality and morbidity in children [1,2]. Despite improvement in neonatal care there is no clinically effective treatment for this disorder. Therefore, development of a new therapeutic intervention to minimize the resulting neurological consequences of cerebral palsy, mental retardation and learning disabilities [3–5] is urgently needed.

The Rice-Vannucci model is widely used to study neonatal encephalic HI in rodents [6]. The main brain structures affected in this model are cerebral cortex, hippocampus, striatum and thalamus, mostly confined to the hemisphere ipsilateral to arterial occlusion [7,8]. As in humans, rats that experience cerebral HI have motor and cognitive deficits [9–14]. However, there is some inconsistency regarding motor impairments after HI lesion; some authors have found no impairment in forelimb use or motor coordination in adult rats exposed to neonatal HI [12,15,16].

Environmental enrichment has been used as a strategy to enhance neuroplasticity and to promote recovery of function following different types of brain injury such as stroke and HI [7,17–21]. Enriched housing provides sensory, cognitive and motor stimulation as well as social interaction by exposing groups of animals to a variety of objects such as ramps, toys, and other novel objects. Several studies have demonstrated that environmental enrichment can attenuate learning and memory deficits in HI rats [7,14,15,22]. However, motor impairments are not always rescued by exposure to an enriched environment. For example, some authors found no improvement on rotarod [15,19] and foot fault tests [23] thereby emphasizing the importance of conducting comprehensive test batteries. A more effective paradigm, especially for restoring upper limb function, is enriched rehabilitation (ER), which combines environmental enrichment with daily reach training [24]. Enriched rehabilitation improves forelimb and hindlimb motor function following both focal ischemic injury [24–26] as well as hemorrhagic stroke [27,28] in adult animals.

Another approach to enhance recovery is using drugs with pleiotropic actions such as Cyclosporine A (CsA) that has documented neurorestorative effects in adult animals with ischemic brain injury. This immunosuppressive drug enhances the activation of endogenous precursors to promote tissue repair that is correlated with functional recovery [29,30]. Additionally, CsA alters mitochondrial membrane permeability and transition pores so as to reduce oxidative damage [31,32] and recent data suggest that it reduces lipid peroxidation, apoptosis and neuroinflammation in a young rat model of closed head injury [33]. However, potential benefits of CsA after neonatal HI have yet to be investigated [34].

An emerging consensus is that interventions targeting single mechanisms are not successful in treating stroke and related neurological disorders. Instead, combination interventions targeting multiple mechanisms and thereby mimicking endogenous programs of neuroprotection and neural repair offer greater potential benefit [35]. Recently, we used this approach to show that ER combined with Erythropoietin (EPO) and epidermal growth factor (EGF) was more effective than the growth factors or ER alone in promoting behavioural recovery following forelimb motor cortex stroke in rats [36]. In the same vein, the present study was undertaken to examine effects of a combinational therapy consisting of CsA and ER in attempts to promote cognitive and motor recovery in a rat model of perinatal hypoxic-ischemia injury.

2. Materials and methods

2.1. Animals

Seven pregnant Sprague-Dawley rats were acquired from Charles River Laboratories (Montreal, Quebec, Canada) and housed on a reverse 12 h light/dark cycle until parturition with food and water freely available. At post-natal day 7 (PND) pups from the 7 dams were randomly divided into two experimental groups: HI (n = 17 females; n = 14 males) and sham (n = 9 females; n = 9 males). Subsequent behavioural testing was done during the dark phase. All procedures were in accordance with guidelines set by the Canadian Council on Animal Care and the University of Ottawa Animal Care Committee.

2.2. Surgical procedures

At PND 7, rat pups were anesthetized with isoflurane, had their left common carotid artery exposed, isolated from the nerve and vein and ligated using 4-0 surgical silk. After a 2.5 h delay, pups were placed in a hypoxic chamber for 90 min with O_2 levels and temperature maintained at 8% and 37 °C respectively [5,6]. Upon conclusion of this hypoxic episode, pups were returned to their home cage. Sham-operated animals were submitted to manipulation, anesthesia and neck incision, but did not receive arterial occlusion or exposure to the hypoxic environment.

2.3. Cyclosporine A (CsA) administration

Our initial plan was to begin CsA administration (15.0 mg/kg, i.p.) in the first 5–14 days after HI as we have done previously with ER and drug therapies in adult animals [25,36]. Unfortunately, we encountered an extremely high mortality rate (~50%) in neonatal rats which was due to toxicity of the CsA vehicle Cremophor EL [37] since equal numbers of vehicle and CsA treated pups died. Consequently, we delayed CsA until weaning (PND 21), when all HI pups were implanted subcutaneously on the flank with osmotic minipumps (Alzet, Cupertino, USA) delivering CsA (420 mg/mL; BioShop, Burlington, Canada) or vehicle (Cremophor EL; ethanol:cremophor—65:35). Rats were anesthetized during osmotic minipump implantation with 1.5%-2% isoflurane. Pumps had a total fill volume of 100 μl and pumped at a rate of 0.14 μl/h [2]. The vehicle and/or CsA solutions at this later developmental time point resulted in no deaths or detectable morbidity. Minipumps were kept in place until the end of experiments.

2.4. Enriched rehabilitation

At PND 21, after osmotic pump implantation, pups were weaned from their mothers and separated by sex (Fig. 1A). Animals in groups receiving ER were housed in large enrichment cages (groups of four to five, Fig. 1B) while those in non-rehabilitation groups were pair housed in standard cages. Experimental groups were defined with respect to ER condition and drug delivery. Rats were randomly divided into four experimental groups: (CsA or vehicle): CsA + Rehabilitation (n = 4 females; n = 4 males); CsA + NoRehabilitation (n = 5 females; n = 2 males); Vehicle + Rehabilitation (n = 4 females; n = 5 males); Vehicle + NoRehabilitation (n = 4 females; n = 3 males).

Enrichment cages contained objects of varied shapes and texture for exploration (i.e., shelves, plastic tubing, ladders, ramp) that were changed on a weekly basis. In addition to being housed in enriched environments, enriched groups were exposed to rehabilitative reach training 4 h/day, 6 days/week for 4 weeks (PND 21 until PND 48). Rehabilitation consisted of reaching for a food reward in a Plexiglas© chamber using only the impaired limb (right). The reaching apparatus was filled with 8 g of sugar pellets (45 mg; Research Diets, New Brunswick, NJ) and was used to encourage (rather than force) coordinated use of affected forelimb. Procedures for rehabilitative reach training were modified from those described previously [25]. Animals were trained to reach through a 1.1-cm-wide vertical slot to obtain food pellets situated in a well

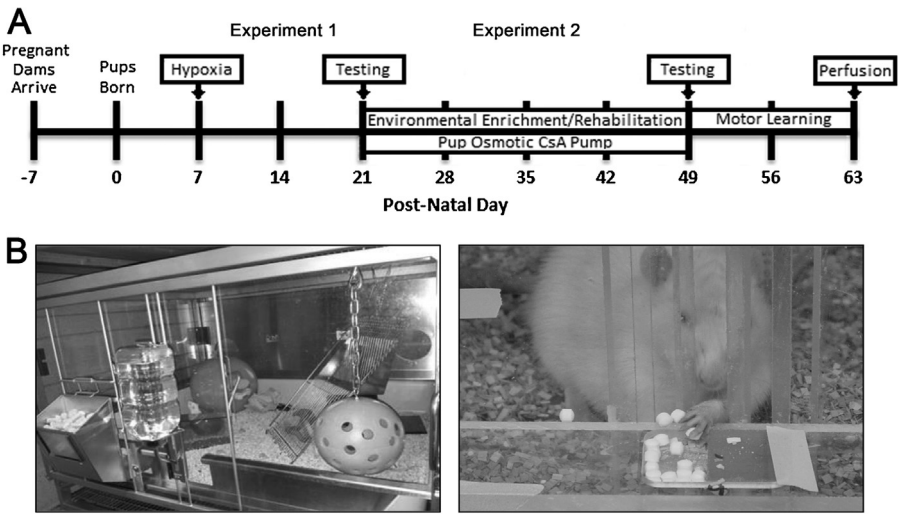

Fig. 1. (A) Time course of interventions. Experiment 1: motor function and cognitive tests at PND 17. Experiment 2: combinational therapy of cyclosporine A and enriched rehabilitation from PND 21 to 49 followed by motor learning performance (staircase test). (B) Enrichment cage shown on left, daily reach training shown on right.

2 cm from the front of a Plexiglas box on a shelf 3.5 cm high. The slot was positioned 1.4 cm from the right wall of the reaching box to discourage use of the left (unaffected) paw (Fig. 1B). At the end of each session remaining sugar pellets were weighed. The non-rehabilitation group did not receive rehabilitative therapy but to control for any possible effects of sugar on recovery, standard-treated animals were fed the average daily amount of sugar pellets eaten by an enriched animal (i.e., ~8 g/d). Animals were kept in enrichment cages until the end of experiments.

2.5. Behavioural testing

2.5.1. Cylinder test

To examine HI effects on spontaneous forelimb use during upright postural support movements [38,39], animals were placed into a clear Plexiglas© cylinder (20 cm in diameter) situated on a glass tabletop and videotaped from below. Each session consisted of 5 min in the cylinder or a minimum of 20 upright wall contacts. Forelimb wall contacts number (single-limb contacts and bilateral contacts) used for postural support was counted. Contralateral forelimb usage was calculated using the following equation [40]:

$$\text{Forelimb asymmetry}\,(\%) = \left[\frac{\left(\#\text{ofcontralateralcontact} + 1/2\#\text{ofbilateral}\right)}{(\#\text{ofipsilateral} + \text{contralateral} + \text{bilateralcontacts})}\right] * 100$$

2.5.2. Ladder rung test

Rats were trained (4 trials, twice in each direction) to cross a horizontal ladder situated 30 cm above a Table with variably spaced rungs [41]. Animals were videotaped crossing the apparatus in 4 trials using an irregular spaced rung pattern that was varied between each test session. Forelimb and hindlimb errors resulting in a limb falling through the plane of the bars (misses, deep slips, or slight slips) were counted. Steps precluding and following a stop were not counted. No significant differences in fore- and hindlimb errors were observed; therefore these errors were combined and expressed as total number of errors for each side (unaffected vs affected).

2.5.3. Open field

The rectangular open-field arena (98 cm × 98 cm × 30 cm) was divided in 16 zones (each zone was 24.5 × 24.5 cm). Animals were placed individually in the center, always facing the same direction, and were video-recorded for 5 min [10]. The distance traveled, number of crossings, instances of rearing and grooming, and latency to leave center of open field were measured automatically using EthoVision tracking software (Noldus Information Technology, Inc.).

2.5.4. Novel object recognition

This test is based on the natural behaviour of animals to spend more time exploring a new, rather than a formerly encountered object [42]. The novel object recognition test took place one day after open field testing. On the test day, a session consisting of two trials was given. The inter-trial interval was 5 min. In the first trial, two different objects were placed equidistant from the sidewalls. Rats were placed into the center of the arena and allowed to explore the two objects (A and B) for 5 min. Time spent exploring each object was recorded. During the second trial, one of the objects presented in the first trial was replaced by a novel object (B was changed to C) and rats were left in the box for 5 min. The time spent exploring both the familiar and novel object was recorded using EthoVision tracking software (Noldus Information Technology, Inc.). Exploration was defined by sniffing the object within a distance of 2 cm and/or touching it with the nose [42]. Object position was randomly assigned to avoid confounds of object and place preference. After each exposure, apparatus and objects were cleaned carefully with 70% alcohol to remove olfactory stimuli. Object preference index was calculated as follows: difference in exploration time in each object divided by the total time spent exploring the two objects.

2.5.5. Montoya staircase task

This test provides a sensitive measurement of independent forelimb skilled reaching and motor learning ability [43,44]. Staircase acquisition began after exposure to ER (6 weeks after HI). During the staircase training period all animals were mildly food restricted (~12 g of standard laboratory chow to maintain approximately 85–95% of their free-feeding body weight). Acquisition occurred over a 10-day period with animals receiving two 15-min trials/day beginning at PND 49.

2.6. Experiment 1: effects of neonatal hypoxia-ischemia on early motor and cognitive function

Ten days after hypoxic-ischemic surgery (PND 17), rat pups (sham (n = 18) and HI rats (n = 31)) were evaluated for deficits in motor and cognitive function using cylinder, ladder rung, open field and novel object recognition tests.

2.7. Experiment 2: combinational therapy of CsA and ER to promote motor recovery in rats exposed to neonatal HI

The same HI rats as in experiment 1 were divided into 4 experimental groups with respect to both an enriched rehabilitation (ER) condition and drug intervention: CsA + Rehabilitation (n = 8); CsA + No Rehabilitation (n = 7); Vehicle + Rehabilitation (n = 9); Vehicle + No Rehabilitation (n = 7). From PND 21 until PND 49 rats were exposed to the combinational therapy and then evaluated on the same motor and cognitive tasks (cylinder, ladder rung, open field, novel object recognition, and staircase task). Staircase acquisition occurred after exposure to the combinational therapy (i.e. PND 49-63).

2.8. Histological assessment

Following behavioural testing, animals were deeply anesthetized (5% isoflurane) and transcardially perfused with ice-cold 0.9% heparinized saline, followed by 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Brains were removed and post-fixed in 4% PFA overnight at 4 °C, then transferred into 20% sucrose-PBS until saturated. The brains were then frozen in isopentane on dry ice, sectioned on a cryostat at 20 μm and stained with cresyl violet to assess brain damage. Every 10th section of tissue was saved, which produced on average 50 sections per brain. Ten different coordinate planes for both the left and right hemispheres relative to Bregma (1.20, 0.70, 0.20, −0.30, −0.80, −1.30, −1.80, −2.30, −2.80, −3.30 mm) were measured using StereoInvestigator (MicroBrightfield Bioscience, Williston, Vermont, USA) software. The hippocampal area of both left and right hemispheres was also measured at −3.30 mm relative to Bregma. The surface area of each section was multiplied by both the section thickness (20 μm) and the interval between sections. This calculation results in an estimated volume of tissue in the space between each measured section. The sum of volumes for all measured sections was calculated to provide hemisphere and cortex volume for each animal.

2.9. Statistical analysis

Pretreatment data were analyzed using unpaired *T*-test and post-treatment data were analyzed by one-way or repeated-measures (staircase) analysis of variance (ANOVA). All analyses were followed by *post-hoc* tests for multiple comparisons, when appropriate. Brain damage (area and volume) was analyzed using Mann-Whitney U non-parametric tests. Experimenters performing volume and area analyses were blind to the experimental groups. Values are mean ± SEM. Statistical significance was set at $p < 0.05$. Statistics were performed using SPSS (SPSS, IBM, Armonk, New York).

3. Results

Since both male and female rats were included in this experiment, sex was initially included as an independent variable in all analyses. However, as no significant effects of sex were observed in any analyses this variable is not included in further discussion for simplicity of interpretation.

3.1. Infarct area and volume assessment

Hypoxia-ischemia resulted in injury primarily localized to the ipsilateral side (left). Animals were analyzed for hemisphere, cortex and hippocampal injury. Since there was no difference between HI groups, we collapsed animals by HI surgery and used the non-parametric Mann-Whitney *U* test to compare HI and sham animals. Mann-Whitney *U* tests showed that HI resulted in decreased volume of left hemisphere and cortex and also decreased area of left hippocampus while the right side was unaffected ($p < 0.001$); Fig. 2A–I.

3.2. Experiment 1: effects of neonatal HI on early motor and cognitive function

3.2.1. Cylinder test

In the cylinder test unpaired Student's *t*-test showed no difference in the proportion of touches between HI and sham rats at PND 17 ($p > 0.05$). Proportion of touches in HI rats using the unaffected (left) and affected paws 57% and 42% respectively in comparison to Sham rats that performed at 52% and 47% (data not shown).

3.2.2. Open field

Unpaired Student's *t*-test showed HI rats exhibited fewer zone crosses in the first minute of open-field exploration ($p < 0.05$) compared to Shams. There were no differences between groups when considering total number of crossings, distance and latency to leave arena center for the full 5-min trial (Table 1).

3.2.3. Ladder rung test

Horizontal ladder data analysis revealed a significantly greater number of foot slip errors per step for both right (affected; $p < 0.001$) and left (unaffected; $p < 0.05$) paws in HI animals (Fig. 3A). There were no significant differences in the number of steps required to cross the ladder (Sham: 59.33 ± 1.26; HI: 61.76 ± 1.84).

3.2.4. Novel object recognition

Novel object recognition, a test of declarative memory, wasn't affected by HI ($p > 0.05$). HI rats spent ~67% of time with the novel object while shams spent ~73% (Table 2).

3.3. Experiment 2: combinational therapy of CsA and ER to promote motor recovery in rats exposed to neonatal HI

3.3.1. Cylinder test

One-way ANOVA indicated no significant effects for *drug* ($F(1,30) = 1.2419$, $p > 0.05$) and *rehabilitation* ($F(1,30) = 0.0007$, $p > 0.05$) either for the affected (right) or unaffected (left) forelimb between groups ($p > 0.05$; data not shown).

3.3.2. Open field

We did not detect a significant *rehabilitation x drug* interaction ($F(3,27) = 0.003$, $p > 0.05$, therefore *rehabilitation* and *drug* main effects were assessed. One-way ANOVA indicated a significant *rehabilitation* effect ($F(1,30) = 6.5242$, $p < 0.01$) for crossing number during the first minute of open-field exploration. Once there was a significant main effect for *rehabilitation*, we collapsed groups across rehabilitated and non-rehabilitated animals. Unpaired Student's *t*-test showed ER rats had a greater number of zone crosses in the first minute of open-field exploration compared to non-rehabilitation animals ($p < 0.05$). Considering total number of crossings, distance

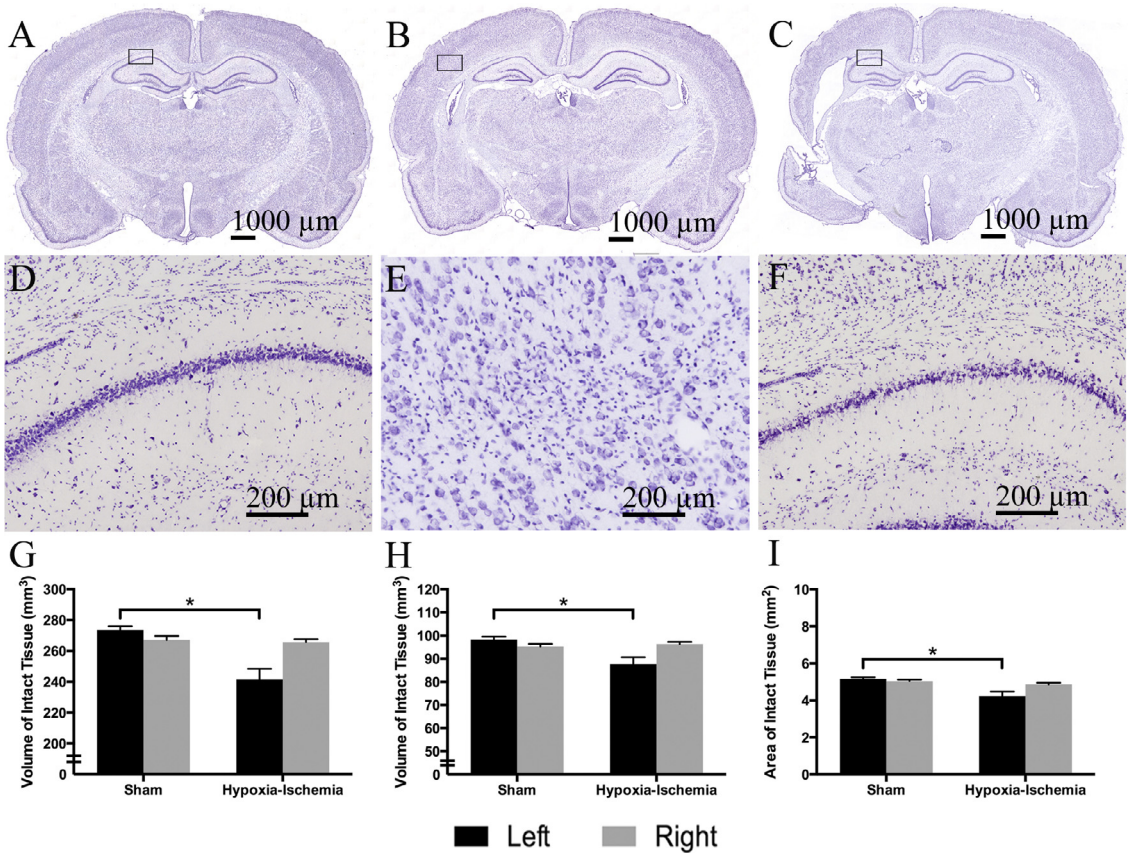

Fig. 2. Area and volume assessment of hemispheric, cortical and hippocampal tissue. Cresyl violet stained representative coronal sections of brain from (A) sham and (B, C) hypoxia-ischemia groups. Figures (D–F) show the inset regions from figures (A–C) respectively. (D) The CA1 region of the hippocampus in sham rats appeared to have a higher density of neurons than rats undergoing (F) hypoxia-ischemia. (E) Throughout the neocortex of HI rats, scattered microinfarcts were present. (G) Decreased total left (ligated) hemispheric volume of HI group compared to sham. (H) Decreased volume of left cortex in HI group compared to sham. (I) Decreased area of left hippocampus in HI group compared to sham. All data represent mean ± SEM using non-parametric Mann-Whitney *U* test (*$p < 0.001$). All structures in right side were unaffected relative to sham.

Table 1
Performance in the open-field task both prior to treatment (Experiment 1) and following rehabilitation and CsA treatment (Experiment 2A). In experiment 1, rats that received hypoxia-ischemia crossed between zones of the open field significantly less than those receiving a sham surgery. In Experiment 2, all animals received hypoxia-ischemia. No drug by group interaction was observed, but a significant main effect of rehabilitation showed that rats receiving rehab crossed between open field zones significantly more than those that did not receive rehab (2B). Unpaired student's *t*-test; *$p < 0.05$.

Experiment	Group	Crossings 1st minute	Total crossings	Distance (cm)	Latency (s)
1	Sham	*18.67 ± 8.1*	49.89 ± 8.2	1253.28 ± 133.5	24.19 ± 7.4
	Hypoxia-Ischemia	*11.23 ± 15.1**	39.26 ± 5.1	1253.39 ± 113.7	41.18 ± 10.2
2A	Vehicle + No Rehab	24.71 ± 1.8	130.57 ± 4.7	3354.65 ± 78.0	3.29 ± 0.7
	Vehicle + Rehab	32.78 ± 2.6	148.22 ± 13.1	3512.17 ± 185.6	4.39 ± 0.9
	CsA + No Rehab	28.14 ± 4.9	134.57 ± 17.1	3460.56 ± 334.4	2.93 ± 0.2
	CsA + Rehab	35.875 ± 2.4	149.38 ± 8.2	3554.65 ± 197.5	5.19 ± 2.5
2B	No Rehab	*26.43 ± 3.4*	132.57 ± 8.5	3407.60 ± 165.6	3.11 ± 0.3
	Rehab	*34.33 ± 1.5**	148.76 ± 7.9	3532.16 ± 134.6	4.77 ± 1.2

Unpaired Student's *t*-test.
* $p < 0.05$.

Table 2
Performance in the novel-object preference index prior to treatment (Experiment 1) and following rehabilitation and CsA treatment (Experiment 2). Rats receiving hypoxia-ischemia were not significantly impaired, relative to shams, on this task, and no effects of treatment on novel-object preference were observed.

Experiment	Groups	Proportion of Time with Novel (%)
1	Sham	73.13 ± 4.9
	Hypoxia-Ischemia	67.48 ± 3.8
2	Vehicle + No Rehab	57.33 ± 8.5
	Vehicle + Rehab	55.06 ± 7.6
	CsA + No Rehab	61.45 ± 6.1
	CsA + Rehab	67.31 ± 5.7

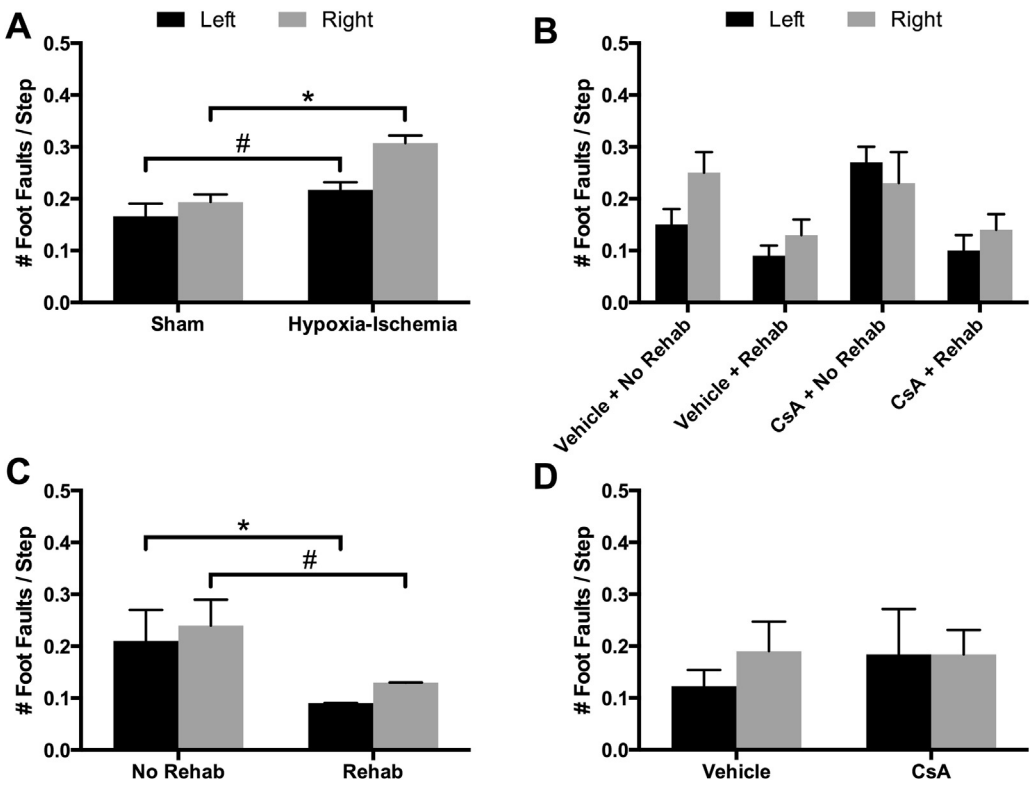

Fig. 3. Foot faults per step in ladder walking task, mean ± SEM. (A) In Experiment 1, animals in the HI group made significantly more errors with both the right and left paws in the ladder walking task. *p < 0.001; #p < 0.05. (B) Experiment 2, performance of all experimental conditions presented as number of errors per total of steps. All groups received HI injury. No significant time by group interaction was observed. (C) Post hoc analysis collapsing rehab and no rehab animals. Rehab condition demonstrated significant improvements over animals in the standard conditions both the affected and unaffected paws (*p < 0.001; #p < 0.05). (D) Post-hoc analysis collapsing CsA and vehicle animals for the left and right paw (p > 0.05). No significant differences between groups were observed.

and latency to leave arena center there is no difference between groups (Table 1).

3.3.3. *Ladder rung test*

One-way ANOVA indicated a *rehabilitation* effect ($F(1,30) = 16.0242$, $p < 0.001$) and a *drug* effect ($F(1,30) = 4.2336$, $p < 0.05$) for left (unaffected) paws in the horizontal ladder-rung test. There was also a significant main effect of *rehabilitation* ($F(1,30) = 6.756$, $p < 0.001$) for the right paws (affected, Fig. 3B). Collapsing rehab and no rehab animals, *post hoc* analysis showed that the rehab group makes significantly fewer errors than the no rehab group with right ($p < 0.01$) and left paws ($p < 0.001$, Fig. 3C). However, unpaired Student's *t*-test showed no significant difference between CsA and vehicle drug treatment for the left paws ($p > 0.05$; Fig. 3D). Additionally, there were no between-group differences in the number of steps required to cross the ladder (CsA + Rehab: 45.37 ± 1.26; CsA + NoRehab: 45.0 ± 1.58; Vehicle + Rehab: 47.87 ± 1.72; Vehicle + NoRehab: 46.85 ± 1.89).

3.3.4. *Novel object recognition*

Novel-object preference index wasn't affected by *rehabilitation* ($F(1,30) = 0.0310$, $p > 0.05$) or *drug* ($F(1,30) = 2.4356$, $p > 0.05$). Therefore, declarative memory was not influenced by combinational therapy (Table 2).

3.3.5. *Montoya staircase task*

Number of pellets retrieved for left paw (unaffected) increased within days; repeated measures ANOVA revealed significant *days* effect ($F(9,21) = 64.5623$, $p < 0.001$) representing a motor learning effect without differences between groups across time (Fig. 4A). Repeated measures ANOVA revealed a significant *rehabilitation* ($F(1,30) = 7.1776$, $p < 0.01$) and *days* effect ($F(9,21) = 26.5755$, $p < 0.001$) for affected forelimb (right, Fig. 4B). Collapsing rehab and no rehab animals, *post hoc* analysis showed that the no rehab group retrieved significantly fewer pellets than the rehab group across time ($p < 0.001$; Fig. 4C). The same difference is observed when comparing average group performances ($p < 0.001$; Fig. 4D).

4. Discussion

The Rice-Vannucci rat model of neonatal HI produces unilateral brain injury, causing damage mainly in the artery-occluded hemisphere [13]. In this model, most animals do not display any obvious locomotor or postural abnormalities so it is important to choose tests that are sufficiently sensitive to reveal sensorimotor and cognitive deficits.

In the first experiment, we investigated effects of neonatal HI event on early motor and cognitive function using a battery of tests. In a second experiment, we investigated the efficacy of a combined therapy of CsA and motor rehabilitation (i.e. enriched rehabilitation) on recovery of motor and cognitive function using the behavioural tests employed in experiment 1 and examined the acquisition of a novel skilled reaching task, the Montoya staircase.

In experiment 1 we found abnormalities in the open field and ladder tests but not in cylinder or novel object recognition tests. Open field evaluation conducted 17 days after injury showed that HI animals made fewer crossings than shams in the first minute of the test period, suggesting decreased exploratory activity or possibly increased anxiety [10,45]. Earlier studies have reported both hyper- and hypoactivity following neonatal hypoxia–ischemia [10,18,46] which may be due to differences in testing such as pre-exposing animals to the testing environment which results in habituation or averaging activity over long test sessions.

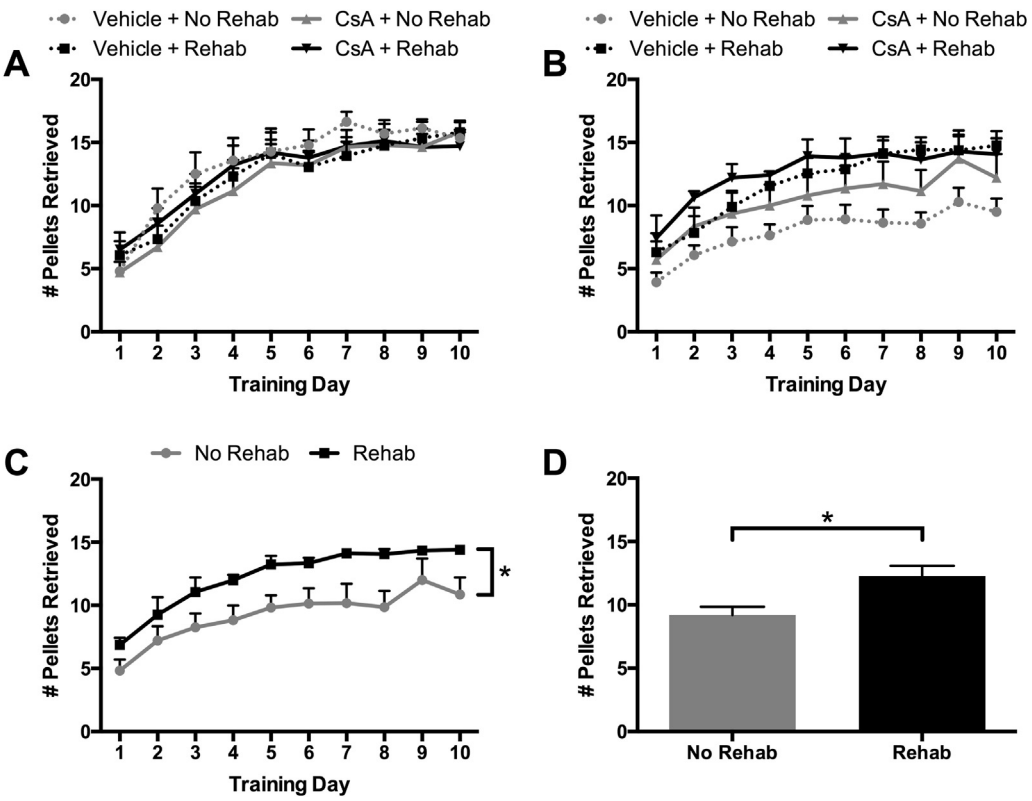

Fig. 4. Number of pellets retrieved in staircase reaching task averaged by training day, mean ± SEM. (D) All groups performed similarly with the left (unimpaired) paw in the staircase reaching task. (A) There was no significant time by group interaction for performance in the staircase reaching task with the right (impaired) paw. (B) Performance of rehab vs. non-rehab groups collapsed across time for the right paw. Animals in the rehab condition demonstrated superior performance over animals in the standard conditions at all time points (*$p < 0.001$). (C) Group performance for the right paw averaged across time. Overall, animals in rehab groups retrieved significantly more pellets than animals in groups that did not receive rehab (*$p < 0.001$).

Similarly, in the ladder rung test HI animals made significantly more foot faults than shams. Ladder rung walking has been reported to be sensitive in detecting long-term impairment in placement, stepping, and coordinated limb use during locomotion following different types of brain injury [25,41] in adult animals and the present findings show this also to be the case with HI injury (Fig. 3A).

The behavioural data in experiment 2 showed that ER produces significantly greater motor recovery than CsA therapy. Enriched rehabilitation increased number of zone crosses in open-field exploration and enhanced coordinated limb use on the ladder task and accelerated and improved new motor learning (i.e. acquisition of staircase skilled-reaching). Open field-testing conducted at PND 49 showed that ER animals exhibited a greater number of zone crossings in the first minute of open-field exploration when compared to non-rehabilitated animals. Ladder test outcomes suggest that the rehabilitation condition improved use of both affected and unaffected paws when compared to the non-rehabilitated group. In the staircase test, we found preserved motor learning capacity for all groups across time and greater functional improvement for ER groups independent of drug intervention. Contralateral (right) paw-reach performance was more impaired in non-rehabilitated rats indicating that this test is useful in detecting treatment effects. Enriched rehabilitation had no effect in the novel object recognition test.

In the present study CsA had no effect on behavioural outcomes relative to ER. A previous study reported that early CsA administration promoted endogenous neural precursor cell activation in mice [47]. However, other studies describing CsA effects on immature brain injury such as infarct volume [2,32,48,49] have produced inconsistent results. Based on the time window of other therapeutic interventions [50] it is possible that CsA administered 2 weeks after the HI procedure was too late to be efficacious since HI causes progressive damage and apoptosis in cerebral cortex, striatum and hippocampus [51,52] over the first several days after HI. Notably, there are reports that CsA administered immediately after or several hours after ischemic injury may attenuate cell death and protect the immature rat brain [2,32]. Unfortunately, Cremophor EL, the vehicle used to dissolve CsA proved so highly toxic in young rat pups that it precluded early CsA treatment. Our results suggest that very late CsA intervention has no effect on functional recovery after neonatal HI.

Assessment of HI injury shows clear hippocampal, cortical and hemispheric atrophy ipsilateral to arterial occlusion that was not affected by the combined therapy of CsA and ER. These results are in agreement with other findings [14,15,18,45,53] including a recent clinical trial in which CsA had no effect on infarct size [54].

In summary, the present study demonstrated that ER enhanced recovery of motor function after neonatal HI and improved learning of new motor skills. The present findings in an HI model are congruent with previous work reporting functional benefits of ER in models of focal ischemia [24–26] in adult animals. Interestingly, these studies found that there is an early “critical or sensitive” time window, lasting approximately 30 days after stroke in the adult rat, when ER is most effective. In the present study, the ER was initiated 14 days after HI injury and was very effective in enhancing motor learning (i.e. staircase acquisition), attenuating sensory-motor deficits (e.g. ladder test) and blunting the anxiety response in a novel environment (i.e. open-field). It remains to be determined if the “critical period” in the young brain remains open for a longer period of time than that of the adult brain.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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5. CAPÍTULO 3

Artigo: Role of stimulation for children at social-environmental risk in Southern Brazil

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Role of stimulation for children at social-environmental risk in Southern Brazil

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In the past few decades, studies about infant development have trying to answer pending questions regarding interdependence of the environment in which children are inserted, health and the developmental process. Especially, the literature has shown that some important factors as socioeconomic status, family support and environment are crucial for proper neurobehavioral development.[1,2] Additionally, children who receive inadequate or disruptive stimulation have a tendency of developing cognitive, social or emotional problems in later stages of life.[3] Given that young child's brain develops through stimulation of the sensing pathways (e.g. seeing, hearing, touching, smelling, tasting) from early experiences, is easy to understand the emergence of early child development programs.

Early stimulation programs have been developed to improve capabilities and to minimize disabilities in children with or at risk for neurodevelopmental disorders considering two main risk factors: *social-environmental risk* in which encompasses children that live in a low socioeconomic status and limited stimulation at home and *biological/medical risk* that involves children with disorders known to induce developmental delay (e.g. cerebral palsy) and/or as a result of preterm birth or low birth weight.[2,4,5] These early programs have goals that are accomplished through developmental, educational, and therapeutic services for children with support for their families.[1,2,4] Many studies address the role of early intervention in infants biologically at risk of developmental disorders, those with prenatal, perinatal, and neonatal complications.[5-9] Nevertheless, studies of early intervention in infants in social-environmental risk are scant.[10-12] In contrast with this finding, it is well established that socio-economic disadvantaged families generally offer less

diversity and quality of cognitive, social and motor experiences provided in the environment.[5,13] Recent literature has stated that the quality and quantity of environmental stimuli in the family context was essential for the children development.[5,12]

In basic neuroscience research field, the experimental studies have helped us to understand how the stimuli from a child's experiences influence neural network of the brain. In rats, it was well established that the immature brain has great plasticity and is highly susceptible to environmental stimuli.[2,14] Therefore, several studies in rodents have demonstrated that exposition to a variety of stimuli, in early stages of life, have influence on central nervous system development causing persistent changes in physiological and behavioral processes in adulthood.[15-18] We have investigated the benefits of environmental stimulation as neuroprotective strategy for brain damage caused by an experimental model of neonatal hypoxia-ischemia.[19-22] This brain lesion cause cognitive impairments and sensory deficits, affecting rodents' neurobehavioral development as hypoxic-ischemic encephalopathy in humans.[23,24] Our outcomes showed that both late and early environmental stimulation was able to recover functional deficits and to prevent hippocampal dendritic spine density loss after neonatal HI injury.[19-22] Notably, our previous experimental data support the potential of environmental stimulation after brain damage in rodents. Thus, we decided to study, on a translational view, the possible benefits of an early stimulation program may offer to infants in a socio-environmental risk.

Considering that (a) social-environmental condition is a risk factor for infant neurobehavioral development delay; (b) the brain development is influenced by

environmental conditions, familiar care and offered stimulation; and (c) early stimulation programs are designed to involve parents and/or other caregivers to a full infant neurodevelopment and improve outcomes for children's behavior, learning and health; this work aimed to assess motor development using Alberta Infant Motor Scale (AIMS; 0-18-month-olds); establish relationship between social risk factors and environmental affordances for motor development using Affordances in the Home Environment for Motor Development (AHEMD) and evaluate possible delays or impairments in functional independence using Pediatric Evaluation of Disability Inventory (PEDI) in children from 0 to 2 year old inserted into an early stimulation program in south of Brazil.

Methods

Participants

Six families of children at social-environmental risk were recruited from Better Early Childhood Program (BECP) which integrates Health Department of Rio Grande do Sul, in Porto Alegre Southern Brazil. Recruitment procedures, informed consent, and data collection for the current study were approved by Human Subjects Committee of Universidade Federal do Rio Grande do Sul (n. 2252). The infant aged range was from 2 to 24 months old (mean ±standard deviation: 13±9.2 months). Eligibility to participate was determined by confirming that family was registered in BECP and child didn't attend daycare center or school. Eight children (6 boys, 2 girls) met the selection criteria to participate in the study.

Better Early Childhood Program (BECP)

BECP has socio-educational activities geared to families with children from zero to six years and pregnant women, which are in a social vulnerability situation. The stimulation program purpose is to promote full development based on home visiting programs that focus on enhancing parent-child interactions and overall parenting practices to facilitate child development. Once a week BECP visitor agents guide the parents or caregivers how to stimulate infants accordingly with developmental stage. In each session BECP visitor agent offers an educational toy while interact with child. The activities should cover physical, psychological, intellectual and social aspects. Stimulation is a procedure based on the experience of environment, rich in quality that may contribute to the child development. This moment is important to evaluate aforementioned developmental aspects. At the end of each month, visitors should report children development evolution with positive and negative aspects.

Procedure

The coordinators responsible for BECP were informed about the study by a letter and asked to disclose the research among families in attendance by visitor agents. Parents could send back a reply form if they wanted to participate. Parents were called and given more information about the study and then, occurred the first home visit. Information related to health, daily life, household income, parent education and children development was collected by questionnaire. Home visits were scheduled once a month and consisted of a standardized observation and a free-play session with main caregiver and BECP visitor agent. During the standardized observation, researcher assessed

motor development using AIMS. Additionally home opportunities for infant motor development (AHEMD) and child functional independence (PEDI) were evaluated. All home visits were photographed and scored afterwards. The neurobehavioral development data was collected during 4 months (1 visit/month, 45 minutes each).

Instruments

General information

General information was assessed through census applied by BECP visitor agent to parents with questions regarding pre- and postnatal medical conditions (birth date, gestational age and need for pediatric intensive care unit), household income, parent's education, number of adults and children living in the house, etc.

Alberta Infant Motor Scale (AIMS)

Alberta Infant Motor Scale is largely used for evaluation and diagnosis of motor development in the first 18 months of life.[25] Accordingly with the instrument, the AIMS allow analyzing free movements, without manipulation or reflex assessment. Evaluation is based on natural movement patterns. The AIMS consists of 58 motor items arranged in four subscales: prone (21), supine (9), sitting (12), and standing (16). Total score is determined by the sum of the subscale scores (58). Percentile ranks and categorizations of motor development (delay ≤5%, at risk of delay 5-25%, typical development ≥25%) are provided.[25] Recently AIMS has been validated for use with Brazilian samples.[26]

Affordances in the Home Environment for Motor Development (AHEMD)

The identification of social/environmental risk factors was estimated through AHEMD. This tool evaluates home opportunities for infant motor development from 3 to 42 months, as well as reflects children physical activity. The instrument consists in an introductory questionnaire and five subscales: outside space, inside space, variety of stimulation, fine-motor toys and gross-motor toys.[27] A translated version to Portuguese was used.

Pediatric Evaluation of Disability Inventory (PEDI)

PEDI is a pediatric tool used to evaluate functional performance in children aged between 6 months to $7^{1/2}$ years.[28] Changes in performance in functional skills and independence in daily activities, amount of caregiver assistance, and modification and adaptive equipment can be monitored using PEDI. There is a fully translated Portuguese version available. PEDI was used to measure functioning and performance in self-care, social function and mobility using two dimensions of functional skills and caregiver assistance. Functional skills scales are designed to identify child capability or performance limitation in specific skill. Functional skills items are scored as unable or limited capability (0) or capable (1) of performing. Caregiver assistance scale measures the amount of assistance given in daily activities in self-care, mobility and social function areas. A six-point ordinal scale is used, from independent to total assistance, according to definitions given in the manual. The items of each separate domain are added to raw scores and converted into scaled scores (normative or continuous) using the tables provided in the manual.[29] Normative score provide an estimate of child's functional performance along the continuum of a specified scale independently of age. To each age stage there is a

normality range between 30 and 70, where scores below 30 represents development delay and scores above 70 means enhanced development performance. Continuous score allows comparison between function fields.

Statistical analysis

Descriptive statistics were used to summarize data. Nonparametric statistics was used to compare AIMS raw score over 4 months using Friedman test for repeated measures analysis and Wilcoxon post hoc test for multiple comparisons. While, parametric repeated-measures analysis of variance (ANOVA) was performed to compare PEDI continuous score between 3 domains of functional skills and caregiver assistance followed by Bonferroni's test for multiple comparisons. Relationships between motor development and environment (home) factors were evaluated by calculation of Spearman's correlation coefficient (r_s). Correlation coefficients <0.30 were considered weak, those between 0.30 and 0.70 were considered moderate and coefficients >0.70 were considered strong.[30] A value of $p<.05$ was considered statistically significant. All statistical analysis was performed using Statistical Package for the Social Sciences version 17.0 (StatSoft USA).

Results

Alberta Infant Motor Scale (AIMS)

The results of AIMS are presented in Table 1. Means, standard deviations (SD), raw scores, percentiles over the four evaluations are reported. Children over 18 months of age were removed from AIMS analysis, and then we split children over 12 months and under 12 months of age to facilitate

descriptive analysis. Infants under 12 months of age scored lower in AIMS analysis; consequently they were classified with risk of delay in motor development. While infants over 12 months of age scored within the normal range, so they were classified as typical motor development. Although there is no statistical difference, the pattern of progress over the four months in infants under 12 months of age suggests that those children will also reach the typical motor development.

Table 1

Affordances in the Home Environment for Motor Development (AHEMD)

The descriptive outcomes showed that 37.5% of families live with 2 wages, also 50% of fathers and 25% of mothers did not complete the elementary school. Most of parents (75%) reported that they live in a very small home and in a crowded house, where in 37.5% of cases, there are 5 children living in the same house. To investigate the relation between environment factors and motor development, Spearman's correlation were used. For the total sample we found positive, significant and strong associations for household income and number of fine-motor toys ($r_s(3) = 0.88$, $p=0.044$). There is also a trend to strong association for household income and number of gross-motor toys ($r_s(3) = 0.86$, $p=0.058$). Additionally, we correlated stimulation time by BECP and motor development by AIMS. There was a strong, positive correlation between stimulation time and raw AIMS score, which was statistically significant ($r_s(6) = 0.91$, $p =0.001$). There is no association between environment factors and PEDI normative score ($p>0.05$). Notably, lower socio-economic families offer less diversity of cognitive and motor experiences in the

home environment nevertheless the early stimulation program is able to improve motor capabilities in those children.

Pediatric Evaluation of Disability Inventory (PEDI)

The results of PEDI are presented in Table 2. Maximum, minimum, mean and standard error for the mean (SEM) are reported. Children under 6 months of age were removed from analysis. Outcomes related to normative score showed that infants were classified in typical development range (between 30 and 70) in both functional skill and caregiver assistance domain. Parametric repeated-measures ANOVA were performed to compare PEDI continuous score between 3 domains of functional skills and caregiver assistance followed by Bonferroni test for multiple comparisons. Figure 1A shows a significantly highest continuous score in functional skill performance ($F(2,4)= 6.40$, $p=0.022$) on social function when compared to self-care. And significantly lowest continuous score in caregiver assistance performance ($F(2,4)= 7.15$, $p=0.017$) on social function when compared to self-care (Figure 1B). Then, we can infer that infants performed better on social activities than self-care skills as dressing, eating and toileting tasks in functional skill. On the other hand, excessive caregiver assistance resulted in greater dependence on social function skill.

Table 2

Figure 1: PEDI continuous score. (A) Functional skill performance. (B) Caregiver assistance performance. ANOVA followed by Bonferroni's test *Difference of social function from self-care ($p<0.05$).

Discussion

In the present study we aimed to assess the association among home environment factors, impairments in functional independence and motor development in infants aged 2 to 24 months inserted into Better Early Childhood Program in Southern Brazil. The results of this study indicate that: (1) infants had typical motor development and probably the younger children will reach the same pattern of motor progress; (2) the low socioeconomic status and limited home affordances represent risk conditions for neurobehavioral development delay; (3) infants presented a better performance on social activity in functional skill. We may affirm that early stimulation program contributed, to some extent, to neurobehavioral outcomes once there was no strong evidence of motor delays.

Outcomes related to AIMS showed that 60% of the sample exhibited below average behavior, in other words, the evaluated children presented a risk of delay in motor development. This finding in general, supports previous reports on Brazilian children in the first 2 years old.[5,31] But this outcome may be discussed carefully because we should take into account that children under 12 months of age will still achieve the upper development stages. Interestingly, we showed an association between greater time of stimulation and better motor development performance on AIMS. Experimental studies also support the concept that exposure to an enriched environment could enhance the animal's sensorimotor and cognitive functions.[32,33] And these functional changes have been associated with morphological changes such as increase of dendritic spine density or improvement of cortical synaptic plasticity.[21,34]

Environmental factors confirmed the social-environmental vulnerability situation of those evaluated families. The socioeconomic status of the families

studied was extremely unfavorable where 37.5% live with 2 wages and 50% of parents have had only elementary school education. Both factors were previously reported as predictive of child motor development.[5,31,35] Overall, a higher level of parents' education is frequently associated with more appropriate child care and lower family income is associated with high risk for motor deficits. In the present study some home affordances were associated with infant motor development; we highlight that for lower income families there was less access to toys. In addition, lack of experience associated with limited space inside the home could negatively affect the child's acquisition of new skills. It has been demonstrated the importance of age-appropriate toys to motor development.[5,13] Considering that lower socio-economic families offer less diversity of cognitive and motor experiences in the home environment, the stimulation programs have a key role of orienting and giving support to the families. Comparing with animal studies adopting environmental enrichment, it is recognized that is important to use objects with different shapes and textures, which are changed at least once a week.[21] This procedure guarantee the novelty factor which is crucial for exploration and, consequently, for neurodevelopment stimulation.

Regarding to PEDI assessment, child performance in functional skills at home environment and the amount of caregiver assistance were examined. All child samples had a typical development accordingly with normative score that provide an estimate of child's functional performance along the continuum of a specified scale independently of age. Our findings indicate greater dependence in social function when compared to self-care in caregiver assistance domain. The excessive care or negligence can represent a risk for typical development in this case. Thus, the caregivers should encourage the independence of daily

living activities. These findings adds to the existing literature the importance of early stimulation programs at social-environmental risk communities that not only encourage parents to stimulate their child but also instructs them about the main neurobehavioral development milestones.

Low socioeconomic status is characteristic of suburban communities of large cities in developmental countries and tends to generate poor stimuli environments frequently unfavorable to typical neurobehavioral development (social-environmental risk). Together biological (e.g. child-centered) and social factors (e.g. environment- centered) may offer positive and/or negative consequences for child development.[10,36] Low family income and low parental education are two aspects related to inappropriate environment for child development. Then, early stimulation programs are targeted to act into the context that child lives, detect the risk factors that they may be exposed and prevent diseases and delays. For this purpose many programs use home visiting to enhance parent-child interactions to facilitate child neurodevelopment. This guidance is to empower caregivers how to improve home opportunities to stimulate their child through variables such as home organization, availability of toys, encourage language development in daily activities. Stimulation is nothing more to offer the child the opportunity to develop their skills, helping to achieve full neurobehavioral development. In this study, despite of social vulnerability of children evaluated, we may affirm that BECP assist in preventing neurobehavioral development delay. Similarly, previous experimental data support the potential of environmental stimulation preventing motor and cognitive deficits in both healthy or diseased cases.[21,22,37,38] Environmental enrichment paradigm, involves an experimental setting in which groups of

animals are kept in large cages containing tunnels, platforms, toys, running wheels and that potentiates social interactions, learning, memory and sensory as well as motor stimulation.[34] In the same way appropriate home environment stimulation can potentiate child development. Notably the brain plasticity plays an important role on development outcome. Therefore the early detection of infants at risk for developmental disorders offers the opportunity for intervention at young age, i.e. during a phase in which the central nervous system is characterized by considerable plasticity.

The limitations of this study was firstly the small sample size nevertheless there was a great homogeneity of infants because they were selected from the same community thus sharing the same social-environmental influences. Secondly, there were a low number of families assisted by BECP due to lack of human resources (visitor agent) during the research period.

Conclusion

Social-environmental conditions have influence on cognitive and motor experiences in the home environment and may affect the neurobehavioral development outcomes of infants. However, appropriate stimuli offered during early stages of life, as provided by Better Early Childhood Program, play an important role in preventing neurobehavioral development delays.

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AUTHORS CONTRIBUTION

C.P.S. and L.O.P. designed the study; C.P.S. performed experiments; collected and analyzed data; C.P.S. and L.O.P. wrote the manuscript. All authors read and approved the final manuscript.

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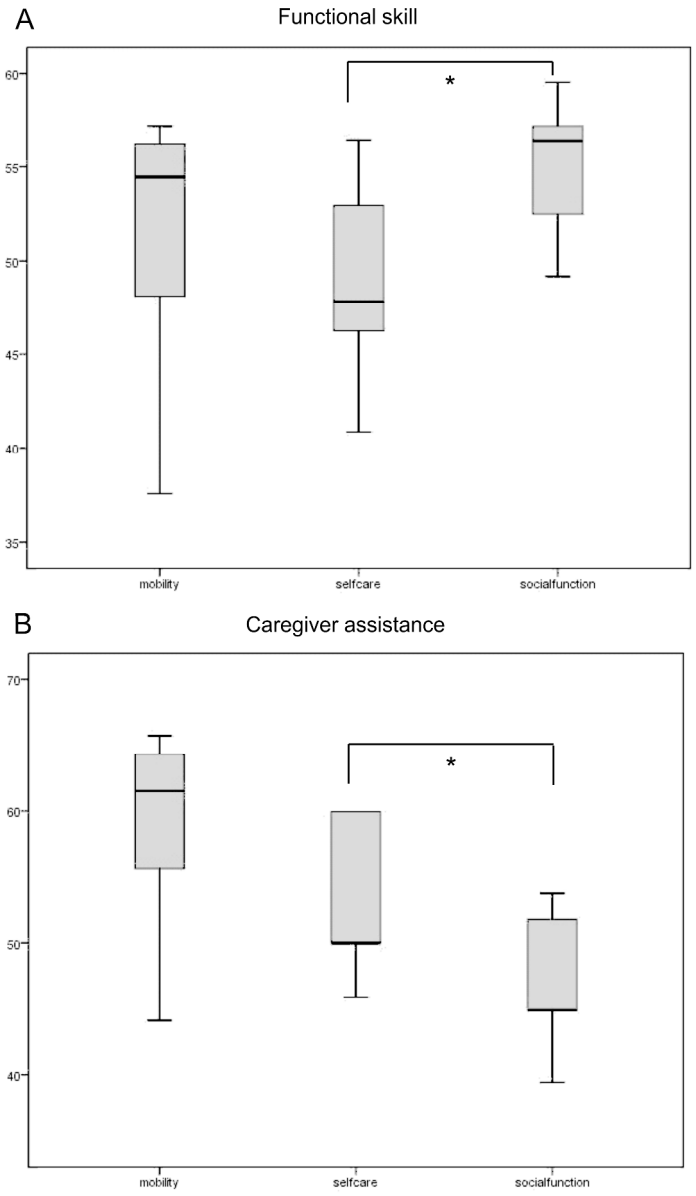
Table 1. AIMS raw scores, percentiles and categorization during 4 months of evaluation (n = 5).

	1st evaluation				2nd evaluation				3rd evaluation				4th evaluation				
	Raw scores		**Percentile**		**Raw scores**		**Percentile**		**Raw scores**		**Percentile**		**Raw scores**		**Percentile**		Motor development
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	categorization
Semesters (months)																	
< 12 months (n=3)	5.33	2.31	20.00	25.98	9.67	2.08	21.67	24.66	12.33	1.15	21.67	24.66	15.67	0.58	21.67	24.66	risk of delay
> 12 months (n=2)	51.50	7.78	47.50	60.10	52.50	7.78	47.50	60.10	53.00	7.07	47.50	60.10	54.00	5.66	47.50	60.10	typical development

Table 2. Normative score outcomes assessed by PEDI (n = 5).

Variable	Maximum	Minimum	Mean	S.E.M.
Functional skill: self care	64.8	38.5	52.08	3.16
Functional skill: mobility	53.3	16.1	44.02	3.66
Functional skill: social function	82.5	53.1	65.42	3.66
Caregiver assistance: self care	71.7	49.5	58.58	3.7
Caregiver assistance: mobility	50.7	28	42.54	4.56
Caregiver assistance: social function	49	37.2	43.4	4.4



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6. DISCUSSÃO

Esta tese teve 3 objetivos principais: a) estudar os efeitos terapêuticos do enriquecimento ambiental precoce sobre os aspectos do desenvolvimento de ratos submetidos à HI neonatal; b) avaliar o efeito combinado do tratamento farmacológico utilizando ciclosporina A e reabilitação na recuperação funcional de ratos submetidos à HI neonatal e; c) descrever o desenvolvimento motor de crianças de 0 a 2 anos de idade junto ao programa Primeira Infância Melhor (PIM) em Porto Alegre-RS.

O experimento apresentado no Capítulo 1 teve como principal enfoque o efeito do enriquecimento ambiental precoce sobre o desenvolvimento dos reflexos neurológicos, uma vez que estes têm uma importante função preditiva sobre a maturação encefálica (Lubics et al., 2005). Ten e colaboradores (2003) já mostraram que a avaliação dos reflexos neurológicos pós HI neonatal fornece uma medida objetiva e não invasiva capaz de predizer déficits neurofuncionais a longo prazo. Neste primeiro trabalho evidenciamos que, independente da lesão, a exposição ao EA precoce afetou alguns aspectos do desenvolvimento dos reflexos e a maturação de algumas características físicas como a antecipação da abertura dos olhos, desdobramento das orelhas, erupção do incisivo, reflexos de contração das pálpebras e das orelhas. Além disso, na avaliação diária dos reflexos, os animais estimulados apresentaram melhor resposta nos reflexos do endireitamento, geotaxia negativa e aversão à queda. De maneira geral, observamos que ao longo do tempo o desempenho dos animais HI se igualou ao desempenho dos animais controles, corroborando com os dados da literatura (Lubics et al., 2005; Brockmann et al., 2013). Sabe-se que o sistema sensório-motor é crucial para o desenvolvimento e adaptação

ao ambiente e por isso alguns autores já reportaram que roedores mesmo quando submetidos à HI neonatal conseguem completar o desenvolvimento dos reflexos (veja Golan e Huleihel, 2006 para revisão). Esse achado representa a grande plasticidade encefálica dos roedores neonatos (Balduini et al., 2003) além da importância ontogênica dos reflexos para a sobrevivência da espécie (Fox, 1985). Portanto, demonstramos um benefício importante da estimulação através do ambiente enriquecido precoce sobre o desenvolvimento de habilidades necessárias para expressar os sinais e reflexos neurológicos.

Em relação aos achados morfológicos, a HI provocou a perda tecidual nas três estruturas avaliadas: estriado, corpo caloso e neocórtex. Demonstramos que o EA precoce foi capaz de prevenir a atrofia no corpo caloso ipsilateral à lesão e, interessantemente, no neocórtex contralateral à lesão. Esse achado sugere uma reorganização e adaptação do hemisfério não lesionado, como anteriormente demonstrado por Jansen e Low (1996b). Rodrigues e colaboradores (2004) aplicaram em roedores com HI neonatal um protocolo de estimulação tátil do 8º ao 21º dia pós-natal, e encontraram recuperação no hipocampo após o período de estimulação. Alguns resultados recentes do nosso grupo de pesquisa mostraram que a exposição ao ambiente enriquecido, iniciada 2 semanas após a HI e mantida por 9 semanas, resultou na prevenção da perda de densidade de espinhos dendríticos no hipocampo (Rojas et al., 2013). Portanto os danos morfológicos podem ser minimizados desde cedo se houver a disponibilidade de estímulos apropriados.

Conforme descrito no Capítulo 2, o segundo artigo desta tese teve como objetivo avaliar, pré e pós o tratamento combinado de CsA e reabilitação, a recuperação funcional de ratos submetidos à HI neonatal. Na primeira etapa do

experimento, foram avaliados os déficits cognitivos e motores dez dias após a indução do modelo de HI neonatal (no 17º dia pós-natal). De maneira geral, os estudos com HI neonatal avaliam o comportamento cognitivo na vida adulta e são bem conclusivos: há déficit de aprendizado e memória (Ikeda et al., 2001; Chou et al., 2001; Arteni et al., 2003; Pereira et al., 2007, 2008; Carletti et al., 2012; Rojas et al., 2013). Contudo, o mesmo não pode ser afirmado em relação ao comprometimento motor, ou seja, há uma inconsistência na literatura sobre os déficits motores em decorrência da HI. Já foram relatados hipoatividade (Charriaut-Marlangue et al., 2014) e hiperatividade (Lubics et al., 2005; Sanches et al., 2012) no campo aberto, ausência de déficit locomotor no teste *rotarod* (Balduini et al., 2003; Lubics et al., 2005; Rojas et al., 2013) e no índice de assimetria do uso dos membros anteriores no teste do cilindro (de Paula et al., 2009, Im et al., 2010), entre outros achados. Os resultados apresentados na primeira parte do experimento mostraram o efeito da HI neonatal apenas no teste do campo aberto e no teste da escada horizontal, onde os animais realizaram uma menor atividade exploratória no primeiro minuto de avaliação do campo aberto e maior número de erros durante a locomoção na escada horizontal. Logo, esses testes foram sensíveis na detecção do déficit motor dos animais submetidos à HI neonatal. As inconsistências apresentadas na literatura em relação à detecção dos déficits motores no modelo de HI neonatal podem estar relacionados com a diversidade dos protocolos utilizados, tempo pós-lesão que ocorre a avaliação motora e a pouca sensibilidade dos testes motores.

Na segunda etapa do experimento, os mesmos animais foram tratados com CsA e com a reabilitação motora, que consistiu em 4 semanas de exposição ao

EA associado ao treinamento da tarefa de alcance com a pata afetada (4h/dia, 6 dias/semana). Os resultados mostraram apenas o efeito da reabilitação motora, que por sua vez levou ao aumento da atividade exploratória no campo aberto e diminuição do número de erros na escada horizontal. Efeitos positivos da reabilitação motora em modelo de isquemia focal foram demonstrados previamente nos estudos de Biernaskie e Corbett, 2001 e Biernaskie et al., 2004, onde os autores sugerem que o enriquecimento combinado com tarefas específicas é capaz de aumentar a plasticidade neuronal através das áreas não lesionadas, bem como melhorar a função, se iniciado precocemente. Enquanto os testes do cilindro e escada horizontal avaliam a motricidade ampla, o teste do staircase tem a finalidade de avaliar a motricidade fina dos membros anteriores e o aprendizado motor (Montoya et al., 1991). O staircase foi aplicado após 4 semanas de tratamento combinado de CsA e reabilitação motora e foi altamente sensível para avaliar o efeito do tratamento. Nossos achados mostraram que os animais reabilitados apresentaram melhora da função motora da pata anterior acometida pela HI. Por outro lado, a administração de CsA não apresentou nenhum efeito sobre a recuperação dos animais submetidos à HI neonatal. Esse fato pode ser explicado pela administração tardia da CsA (2 semanas após HI) através da implantação de bomba osmótica subcutânea. No presente estudo, o veículo utilizado para dissolver a CsA causou alta mortalidade dos animais (~50%) o que justifica a administração tardia da CsA a partir do 21° dia pós natal. Corroborando com nossos achados Puka-Sundvall et al. (2001) não obtiveram efeitos neuroprotetores com administração de CsA, assim como Leger et al. (2010) não demonstraram efeitos benéficos da CsA na recuperação de isquemia

neonatal severa. Apesar da HI gerar um dano progressivo e desencadear eventos bioquímicos que podem durar vários dias e até semanas, sugerimos que a intervenção com CsA deve ser iniciada mais cedo para causar efeitos benéficos como aqueles evidenciados em modelos de isquemia focal em roedores adultos (Nakajima et al, 2000;. Hossain 2008; Erlandsson et al., 2011; Dibajnia e Morshead, 2013). Em relação aos achados morfológicos, houve atrofia significativa do córtex, hemisfério cerebral e hipocampo ipsilateral à oclusão arterial nos animais submetidos à HI e a terapia combinada não teve efeito sobre essas medidas morfológicas. Esses resultados estão de acordo com outros achados da literatura sobre a ausência de efeito do EA e da CsA sobre o volume da lesão tecidual (Pereira 2007, 2008; Nighoghossian et al., 2015).

No estudo do Capítulo 3 avaliamos o desenvolvimento neuropsicomotor de crianças de 0 a 2 anos de idade junto ao programa Primeira Infância Melhor (PIM) em Porto Alegre-RS. Os resultados apresentados contribuem para uma melhor compreensão do desenvolvimento das crianças inseridas em programas de ação preventiva na saúde pública, como o PIM. Ressalta-se aqui que o programa é voltado para crianças que se encontram em condições socioeconômicas desfavoráveis, não apresentam alterações neurológicas e não frequentam escolas de educação infantil; tais fatos reforçam o papel do PIM em viabilizar que aspectos do desenvolvimento sejam potencializados com a orientação adequada de estímulos no ambiente domiciliar. Algumas limitações foram encontradas na realização deste estudo. Primeiramente em relação ao reduzido tamanho da amostra estudada (n=8) enquanto o cálculo

amostral indicou um n=30 para resultados satisfatórios de representatividade da população. E segundo a ausência de um grupo controle.

Através da análise do processo de aquisições das habilidades motoras da criança nos primeiros anos de vida, é possível identificar o quanto essa etapa é crítica para o desenvolvimento, visto que grande número de alterações acontece em um curto período de tempo. No período pós-natal constata-se a crescente presença do ambiente como potencializador dos comportamentos motores, cognitivos e de aprendizado (Almeida et al., 2006; Saccani et al., 2013). Deste modo, para que ocorra o processo de desenvolvimento adequado, é fundamental considerar as condições de estimulação e do contexto proporcionado à criança. Estudos sobre desenvolvimento infantil indicam que os fatores de risco biológico (baixo peso ao nascimento, prematuridade e tempo de internação hospitalar) e os fatores de risco sociais/ambientais podem ser determinantes no atraso neuropsicomotor e se manifestam de diferentes formas e intensidades, variando nas diferentes fases do desenvolvimento (Mancini et al., 2004). Nesse sentido, a introdução de ações preventivas ou corretivas sobre os desvios do desenvolvimento, como a estimulação precoce, auxilia no alcance de um desenvolvimento neuropsicomotor pleno e satisfatório uma vez que esta faz uso tanto de recursos humanos quanto de recursos do ambiente (Guimarães et al., 2015; Santos et al., 2009).

Nossos achados junto às crianças atendidas pelo programa PIM em Porto Alegre-RS mostraram que 60% apresentaram comportamento motor abaixo da média esperada indicando risco de atraso no desenvolvimento motor. A baixa condição socioeconômica das famílias estudadas está diretamente relacionada

com o maior risco de atraso. Esses resultados corroboram com os estudos de Halpern et al., 2000 e Lima et al., 2004 que afirmaram que as crianças de famílias com rendimentos mais baixos têm uma maior probabilidade (50%) de apresentar atraso no desenvolvimento motor. Além disso, é importante ressaltar que para as famílias de baixa renda, o acesso a brinquedos não é uma prioridade e um grande número de moradores na casa muitas vezes prejudica a aquisição de novas habilidades pela criança. Ademais, as defasagens encontradas podem estar relacionadas à falta de experimentação e exploração dos movimentos e dos ambientes em virtude, muitas vezes, do pouco espaço físico nos domicílios que estas crianças vivem (Guimarães et al., 2015).

Em relação à avaliação pediátrica de incapacidade (PEDI), observamos que as crianças inseridas no PIM estão com suas habilidades de interação social e comunicação íntegra no domínio da habilidade funcional. Entretanto, quando se analisou a assistência do cuidador nessa mesma função, as crianças se mostraram mais dependentes, indicando que, embora apresentassem as habilidades necessárias, continuavam a receber ajuda de seus cuidadores. Nesse caso, o auxílio excessivo do cuidador pode se tornar um risco para o desenvolvimento. Pensando nisto, os pais e cuidadores devem ser incentivados a estimular a independência funcional da criança nas atividades de vida diária.

Em suma, o modelo experimental de HI neonatal não afetou o desenvolvimento dos reflexos e a maturação das características físicas (Capítulo 1), contudo, causou prejuízo motor e funcional nos roedores (Capítulo 2). Por sua vez, a estimulação através do enriquecimento ambiental teve efeito

sobre o desenvolvimento de habilidades necessárias para expressar os reflexos neurológicos e foi capaz de reverter os déficits motores e funcionais quando associado à tarefa de alcance. Além disso, o EA precoce foi capaz de minimizar a atrofia em estruturas como corpo caloso e neocórtex. Em relação às crianças de 0 a 2 anos avaliadas junto ao PIM detectamos um risco ao atraso no desenvolvimento infantil diretamente relacionado com o baixo nível socioeconômico das famílias avaliadas e consequentemente à menor quantidade de estímulos ofertados. Conjuntamente esses resultados ampliam os conhecimentos acerca da estimulação ambiental como uma estratégia de reabilitação neurofuncional em roedores e como potencializadora do desenvolvimento infantil.

A pesquisa experimental no campo das neurociências nos ajuda a entender como os estímulos do ambiente podem influenciar os aspectos da plasticidade do encéfalo em desenvolvimento. Um ambiente com estímulos adequados favorecerá o desenvolvimento integral, uma vez que possibilita a aquisição de habilidades motoras, cognitivas e sociais. O paradigma do enriquecimento ambiental e o Programa Primeira Infância Melhor apresentam uma relação estreita entre si, pois, ambos utilizam a estimulação ambiental como recurso para minimizar desordens e potencializar o desenvolvimento cerebral e global. Como vimos nesta tese, para os roedores submetidos à HI neonatal, o EA tem papel de neuroproteção e reabilitação em alguns aspectos funcionais. E para as crianças em risco socioambiental, o PIM tem o papel de orientar as famílias, a partir de suas culturas e experiências, para que promovam o desenvolvimento integral de seus filhos no ambiente que estão inseridos. Sendo assim, é seguro afirmar que a estimulação ambiental resulta em efeito

benéfico tanto após o dano cerebral decorrente da HI neonatal quanto para crianças em situação de vulnerabilidade social. Em ambos os casos o ambiente teve uma forte influência sobre os aspectos do desenvolvimento neuropsicomotor. Porém, mais pesquisas são necessárias para ampliar o entendimento desta relação translacional entre os mecanismos relacionados com os efeitos do ambiente sobre o encéfalo imaturo e o desenvolvimento de programas de estimulação para crianças em risco biológico e/ou risco socioambiental.

7. CONCLUSÕES

Os dados obtidos por meio dos trabalhos desenvolvidos permitem concluir que:

- Independentemente da lesão hipóxico-isquêmica, a exposição ao enriquecimento ambiental precoce (8º ao 20º dia pós-natal) estimulou a maturação precoce de algumas características físicas e de alguns reflexos. Ademais, o EA foi capaz de prevenir atrofia do corpo caloso ipsilateral à lesão e do neocortex contralateral à lesão.

- A avaliação do efeito da HI neonatal pré-tratamento combinado mostrou prejuízo em algumas tarefas motoras (campo aberto e escada horizontal), porém não houve comprometimento da atividade cognitiva (reconhecimento de objetos).

- Após o tratamento combinado, apenas o componente de reabilitação motora (enriquecimento ambiental associado à tarefa de alcance) teve efeito na recuperação funcional dos ratos submetidos à HI neonatal. O componente farmacológico, CsA, não causou nenhum efeito sobre a recuperação dos roedores. Ademais, a terapia combinada não recuperou a atrofia do hipocampo, córtex e hemisfério cerebral ipisilateral à lesão.

- As crianças atendidas pelo programa Primeira Infância Melhor em Porto Alegre-RS apresentaram um desenvolvimento motor normal, porém houve indicativo de risco de atraso; tal risco apresenta relação com as famílias avaliadas, nas quais há um baixo nível socioeconômico possibilitando poucas oportunidades de estimulação no ambiente. E por fim, quanto à Avaliação Pediátrica de Incapacidade, no domínio da assistência ao cuidador as crianças mostraram dependência nas habilidades de interação social e comunicação.

8. PERSPECTIVAS

- Investigar outras variáveis morfológicas que possam ser influenciadas pelo enriquecimento ambiental precoce, tais como, arborização dendrítica e densidade de espinhos dentríticos.

- Avaliar as possíveis adaptações morfológicas na estrutura muscular (comprimento do sarcômero, área de secção transversa e número total de fibras) após enriquecimento ambiental em ratos submetidos à HI neonatal.

- Avaliar fator neurotrófico derivado da glia (GDNF) e fator neurotrófico derivado do cérebro (BDNF) que podem ter relação entre o enriquecimento ambiental e a melhora dos parâmetros funcionais da HI neonatal.

- Avaliar a neurogênese no estriado, hipocampo e neocórtex de ratos submetidos à HI neonatal através do marcador de proliferação celular bromodesoxiuridina (BrdU).

- Orientação junto ao Programa Primeira Infância Melhor para implementação de avaliações que auxiliem na detecção e acompanhamento de crianças em risco e/ou com atraso do desenvolvimento.

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ANEXOS

ANEXO 1

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 23260
Título: Avaliação da estimulação precoce através do ambiente enriquecido em lesão encefálica do tipo hipóxia-isquemia: modelo experimental

Pesquisadores:

Equipe UFRGS:

LENIR ORLANDI PEREIRA SILVA - coordenador desde 01/07/2012
Clarissa Cristini Pedrini Schuch - pesquisador desde 01/07/2012
Bruna Ferrary Deniz - pesquisador desde 01/07/2012
Joseane Jiménez Rojas - pesquisador desde 01/07/2012
Ramiro Diaz - pesquisador desde 01/07/2012
IOHANNA DECKMANN - pesquisador desde 01/07/2012
SILVIA BARBOSA - Técnico de Laboratório desde 01/07/2012

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 25/06/2012 - Sala de Reuniões do 2º andar da Reitoria, Campus Central, em seus aspectos éticos e metodológicos, para a utilização de 120 ratos Wistar, machos ou fêmeas, de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008 que disciplina a criação e utilização de animais em atividades de ensino e pesquisa.

Porto Alegre, Quarta-Feira, 11 de Junho de 2014

BRUNO CASSEL NETO
Vice Pró-Reitor de Pesquisa

ANEXO 2

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA

Comitê De Ética Em Pesquisa Da Ufrgs

PROPESQ

CARTA DE APROVAÇÃO

Comitê De Ética Em Pesquisa Da Ufrgs analisou o projeto:

Número: 22522
Título: AVALIACAO DA ESTIMULACAO PRECOCE ATRAVES DO AMBIENTE ENRIQUECIDO EM LESAO ENCEFALICA DO TIPO HIPOXIA-ISQUEMIA: MODELO EXPERIMENTAL A CLINICA

Pesquisadores:

Equipe UFRGS:

LENIR ORLANDI PEREIRA SILVA - coordenador desde 20/03/2012
LUÍSA HELENA MACHADO MARTINATO - pesquisador desde 20/03/2012
Clarissa Cristini Pedrini Schuch - pesquisador desde 20/03/2012
Bruna Ferrary Deniz - pesquisador desde 20/03/2012
Joseane Jiménez Rojas - pesquisador desde 20/03/2012
Ramiro Diaz - pesquisador desde 20/03/2012

Comitê De Ética Em Pesquisa Da Ufrgs aprovou o mesmo , em reunião realizada em 28/06/2012 - Sala de Reuniões do 2º Andar do Prédio da Reitoria, por estar adequado ética e metodologicamente e de acordo com a Resolução 196/96 e complementares do Conselho Nacional de Saúde.

Porto Alegre, Quarta-Feira, 11 de Julho de 2012

JOSÉ ARTUR BOGO CHIES
Coordenador da comissão de ética

ANEXO 3

Primeira Infância Melhor

Programa PIM - Primeira Infância Melhor
Av. Borges de Medeiros, 1501 - 6º andar - Ala Norte
CAFF - Centro Administrativo Fernando Ferrari
Bairro Praia de Belas - CEP: 90119-900
Porto Alegre - RS - Brasil

TERMO DE CONCORDÂNCIA

Eu, Cândida Kirst Bergmann responsável pelo departamento de pesquisa do Programa Primeira Infância Melhor do município de Porto Alegre, declaro estar informado (a) da metodologia que será desenvolvida na pesquisa: **A estimulação do desenvolvimento neuropsicomotor através do programa Primeira Infância Melhor: uma aproximação da neurociência com o desenvolvimento *integral* da criança** coordenada por Lenir Orlandi Pereira.

Ciente de que sua metodologia será desenvolvida conforme a resolução CNS 196/6 e das demais resoluções complementares autorizo a realização da pesquisa nesta instituição.

Porto Alegre, 5 de maio de 2012

CK Bergmann ID 3529010

Assinatura e carimbo do responsável

SECRETARIA ESTADUAL DA SAÚDE
PRIMEIRA INFÂNCIA MELHOR
Av. Borges de Medeiros, 1501 - 6º A - Ala Norte
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