UNIVERSIDADE FEDERAL DO PAMPA

JEFFERSON ROSA DE MENEZES

ESTRATÉGIAS PARA PREVENÇÃO DE DÉFICITS COGNITIVOS ASSOCIADOS À DEPRIVAÇÃO MATERNAL

Uruguaiana

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PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

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Dissertação apresentada ao Programa de Pós-Graduação em Bioquímica da Universidade Federal do Pampa (UNIPAMPA, RS) como requisito para a obtenção de grau de Mestre em Bioquímica.

Orientador: Prof[a]. Dr[a]**.** Pâmela Billig Mello-Carpes

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Dissertação apresentada ao Programa de Pós-Graduação Stricto Sensu em Bioquímica da Universidade Federal do Pampa, como requisito para obtenção do da obtenção do Título de Mestre em Bioquímica.

Área de concentração: Bioquímica Farmacológica e Toxicológica

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URUGUAIANA

2017

Dedico à minha filha, meus pais, orientadora, família, professores, colegas e amigos pelo incentivo e compreensão durante todo o caminho percorrido.

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LISTA DE ABREVIATURAS

AMP: Proteínas dependentes do monofosfato adenosina

AMPA: Acido alfa-amino-3-hidroxi-5-metil-4isoxazol proiônico

AVE: Acidente vascular encefálico

BDNF: Fator neurotrófico derivado do cérebro

CamKII: Cálcio-calmodulina dependentes

CAT: Catalase

CREB: Proteína ligante ao elemento responsivo ao AMP cíclico

DM: Deprivação maternal

EA: Enriquecimento ambiental

ERK: Proteínas ativáveis extracelularmente

EROs: Espécies reativas de oxigênio

FRAP: Capacidade antioxidante total

GSH: Glutationa

MCD: Memória de curta duração

MLD: Memória de longa duração

MT: Memória de trabalho

NMDA: N-Metil-D-Aspartato

PKC: Proteínas cinases cálcio dependentes

SN: Sistema nervoso

SNC: Sistema nervoso central

SOD: Superoxido dismutase

TBARS: Espécies reativas ao ácido tiobarbitúrico

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RESUMO

Dissertação de Mestrado

Programa de Pós-Graduação em Bioquímica

Universidade Federal do Pampa, RS, Brasil

ESTRATÉGIAS PARA PREVENÇÃO DE DÉFICITS COGNITIVOS ASSOCIADOS À DEPRIVAÇÃO MATERNAL

Autor: Jefferson Rosa de Menezes

Orientador: Prof[a]. Dr[a]. Pâmela Billig Mello-Carpes

Data e local da defesa: Uruguaiana, 22 de fevereiro de 2017.

A deprivação maternal é um potente estressor na fase inicial da vida de mamíferos, ocasionando diversos déficits cognitivos que se mantêm na vida adulta. Dentre os mecanismos envolvidos nestes déficits estão o desiquilíbrio oxidativo e as alterações em determinadas proteínas, como o fator neurotrófico derivado do cérebro (do inglês *Brain-Derived Neurotrophic Factor*, BDNF). Estes eventos podem ocorrer em diferentes regiões do no cérebro, entre elas o hipocampo, principal região responsável pela formação e consolidação das memórias. Esta dissertação investigou os efeitos de diferentes estratégias neuroprotetoras (exercício físico, suplementação com chá verde, e, enriquecimento ambiental) nos déficits de memória advindos da deprivação maternal. Para avaliar a função mnemônica, foi utilizado um modelo animal de DM (em ratos Wistar) e os testes de reconhecimento de objetos, esquiva inibitória e labirinto aquático de Morris. Para avaliar o balanço redox hipocampal quantificamos espécies reativas de oxigênio (EROs), espécies reativas ao ácido tiobarbitúrico (TBARS), níveis de glutationa (GSH) e capacidade antioxidante total (FRAP), além disso, determinamos a atividade da enzima acetilcolinesterase e a quantificamos os níveis de BDNF. Nossos resultados demonstram que o exercício físico e o chá verde são estratégias antioxidantes eficazes de neuroproteção em um modelo de DM. Também demonstram que o enriquecimento ambiental é capaz de reverter os efeitos deletérios oriundos da DM por meio do incremento dos níveis de BDNF. Estes resultados revelam a possibilidade da utilização dessas intervenções como estratégia de neuroproteção.

Palavras chave: Memória. Exercício Físico. Chá verde. Enriquecimento ambiental. Estresse oxidativo. BDNF.

ABSTRACT

Master Thesis

Graduate Program in Biochemistry

Federal University of Pampa

STRATEGIES TO PREVENT COGNITIVE DEFICITS ASSOCIATED TO MATERNAL DEPRIVATION

Author: Jefferson Rosa de Menezes

Advisor: Pâmela Billig Mello-Carpes, PhD

Place and date: Uruguaiana, February 22th, 2017.

Maternal deprivation is a potent stressor in the early life of mammalian, leading to several cognitive deficits that remain until adulthood. Among the mechanisms involved in these deficits are the oxidative imbalance and the alterations in some proteins, such as Brain-Derived Neurotrophic Factor (BDNF), events that can occur in different brain regions, as the hippocampus, the main region responsible for the memory formation and consolidation. This master thesis investigated the effects of different neuroprotective strategies (physical exercise, green tea supplementation, and environmental enrichment) on memory deficits related to maternal deprivation (MD). To evaluate the mnemonic function was used an animal model of MD (using Wistar rats) and the object recognition, inhibitory avoidance and Morris water maze memory tests. To evaluate hippocampal redox balance we quantified the reactive oxygen species (ROS), the thiobarbituric acid reactive species (TBARS), the glutathione levels (GSH) and the total antioxidant capacity (FRAP), as well as determine the acetylcholinesterase activity and the BDNF levels. Our results show that physical exercise and green tea are effective neuroprotective antioxidant strategies in a MD model. They also demonstrate that environmental enrichment is capable to reverse the deleterious effects of MD by increasing BDNF levels. These results reveal the possibility of using these interventions as a neuroprotection strategy.

Key-words: Physical Exercise. Green tea. Environmental enrichment. Oxidative stress. BDNF.

APRESENTAÇÃO

Esta dissertação está dividida em três partes. A parte I é composta pela introdução, que apresenta uma breve revisão da literatura sobre o tema do trabalho, bem como sua importância, culminando na apresentação dos objetivos desta dissertação. Na parte II são apresentados os materiais e métodos, resultados e discussão, no formato de três artigos originais. O primeiro artigo já foi publicado no periódico *Physiology & Behavior*. O segundo foi submetido para apreciação no periódico *Physiology & Behavior* e encontra-se em revisão. O terceiro artigo será submetido ao periódico *International Journal of Developmental Neuroscience*. A parte III da dissertação é composta pelos itens discussão, conclusões e perspectivas, que englobam os comentários e as interpretações gerais desta dissertação, relacionados aos resultados dos três artigos. As referências bibliográficas finais contemplam apenas as citações da parte I e III, já que as citadas na parte II estão listadas ao final de cada artigo.

PARTE I

INTRODUÇÃO

1. Deprivação maternal

Vários fatores são determinantes para termos uma maturação adequada do Sistema Nervoso (SN). Podemos citar alguns destes como: o ambiente em que se vive, a qualidade da alimentação e também a presença de cuidado parental, seja maternal ou familiar (Benetti *et al.,* 2009). Na atualidade, cada vez têm-se achado mais normal o fato de uma criança ser afastada da presença materna logo nos primeiros meses de vida. Porém, há trabalhos que demonstram o quão benéfica é a presença da figura materna (biológica ou afetiva) no período inicial da vida, e mostram que quanto mais estreita essa relação for, melhor será o desenvolvimento desses indivíduos (Benneti *et al.,* 2009)

Diversos modelos animais são utilizados com a proposta de estudar os efeitos ocasionados pela privação da presença da mãe nos primeiros dias de vida. A deprivação maternal (DM) em ratos é um modelo de estresse realizado com fins experimentais. Nesta condição ocorre a interrupção temporária dos estímulos mãe-filhote, podendo a DM ser realizada através de diferentes protocolos. Dentre os protocolos utilizados em estudos com ratos de laboratório podemos citar aqueles que propõem uma privação única, como, por exemplo, privação maternal por 24h seguidas em um dos primeiros dias de vida (De La Fuente *et al.,* 2009), e alguns protocolos que fazem a retirada da mãe por um determinado período do dia (2-4h/dia) ao longo de vários dias consecutivos (8-15/dias) (Roceri *et al.,* 2004; Benetti *et al.,* 2009). Em nosso laboratório padronizamos a utilização de um protocolo que evita privações associadas (tais como, de alimentação, de calor, etc.), de forma que realizamos a retirada da mãe por 3h/dia durante 10 dias consecutivos (Mello *et al.,* 2009; Benetti *et al.,* 2009). Esse afastamento mãe-filhote causa diversos déficits cognitivos que persistem por um longo prazo nos animais que são submetidos a esse estresse logo no inicio da sua vida, fase esta, como mencionado previamente, importante para maturação do SN (Kuma *et al.,* 2004; Renard *et al.,* 2005).

Em roedores e outros mamíferos a relação mãe-filhote é de suma importância para o adequado desenvolvimento dos filhotes. Alterações nessa relação, como por exemplo, a privação por determinado tempo da presença da mãe no ambiente, podem ocasionar diversos prejuízos no comportamento desses animais (Benetti *et al.*, 2007; Benetti *et al.,* 2009).

Quando privados da presença da mãe, os animais apresentam déficits de memória (Renard *et al.,* 2005) que são acompanhados de modificações neuroquímicas e anatômicas, tais como a expressão reduzida do fator neurotrófico derivado do cérebro (BDNF) e do receptor N-Metil-D-Aspartato (NMDA). Além disso, diversos estudos demonstram uma correlação entre a DM e o aumento dos níveis de corticosterona, além de alterações no equilíbrio oxidativo, situações que influenciam as condições do tecido nervoso, afetando, também, suas funções (Roceri *et al.,* 2002; Ito *et al.,* 2006; Derin *et al.,* 2006).

Assim, considerando o impacto que a deprivação maternal pode ter na vida adulta, é fundamental o estudo e compreensão do efeito de intervenções promissoras neste modelo. Estudos prévios têm procurado investigar os efeitos adversos da DM e as possíveis vias que levam a estes efeitos. Aisa *et al.* (2009) demonstraram que a DM causa uma diminuição nos níveis de BDNF, bem como na proliferação celular na região do hipocampo, ocasionando um déficit de memória espacial. Outros estudos têm focado nos efeitos da DM no equilíbrio oxidativo. Schiavone *et al.* (2013) verificaram danos no DNA na região do hipocampo e déficits na memória espacial de ratos deprivados. A revisão de Schiavone *et al.* (2013) também mostrou que diversos trabalhos relacionam as alterações nos níveis corticosteroides na DM com a produção de espécies reativas de oxigênio. Ainda, de acordo com Schiavione *et al.* (2013) o estresse oxidativo causado pela exposição à DM tem inicio com a produção de radicais livres na mitocôndria, porém, outros mecanismos têm sido bastante discutidos, como o papel da enzima NADPH oxidase.

2. Memória

A memória é uma das principais funções cognitivas do SN. Considerando o tempo de duração, a memória pode ser classificada como memória de trabalho (MT), memória de curta duração (MCD) ou memória de longa duração (MLD). A MT é muito breve, durando no máximo alguns minutos (1-3), e serve basicamente para que possamos gerenciar as informações que estamos recebendo, sendo assim, ela determina por quanto tempo a informação vai nos ser útil e o passo posterior, podendo ser ignorada ou armazenada para formar uma memória propriamente dita. Assim, a MT, diferentemente das de curta e longa duração, não produz arquivos e é basicamente processada pelo córtex pré-frontal, dependendo da atividade elétrica dos neurônios dessa região do cérebro (Izquierdo, 2002).

A MCD já pode durar desde poucos minutos até algumas horas, dependendo de vários fatores, tais como a carga emocional despendida na aquisição da memória, o quão relevante

essa memória pode vir a ser para determinada pessoa, entre outros fatores. A MCD, assim como a MT não gera mudanças permanentes no nosso encéfalo, pois não requer síntese proteica e nem expressão gênica, mas é fundamental para que possamos administrar a informação enquanto a MLD está sendo formada. Esta última pode durar de algumas horas a vários anos, até uma vida inteira, e envolve mudanças estruturais necessárias para o armazenamento das informações em diversas estruturas cerebrais (Izquierdo *et al.*, 2004). Assim, embora partilhem de alguns mecanismos iniciais comuns, a MCD e a MLD têm peculiaridades individuais, especialmente nos processos neuroquímicos que envolvem a sua formação e consolidação, daí a importância do estudo de cada tipo de memória (Izquierdo & Medina, 1997; Izquierdo *et al*., 2004).

Na MCD, a circuitaria cerebral envolvida é abrangente, porém, as modificações permanentes são mínimas, não sendo necessária a síntese de novas proteínas nem expressão gênica, porque o papel da MCD é de reter a informação para que a mesma seja útil para processar o que está sendo feito com eficácia (Izquierdo *et al.*, 1998). Por outro lado, para que esteja disponível para evocação futura, a MLD envolve diversos processos após a aquisição. Dentre estes processos destacam-se a consolidação e a reconsolidação da memória.

No processo de consolidação da memória, diversas cascatas neuroquímicas se iniciam. Acredita-se que o passo inicial envolve a liberação de neurotransmissores, dentre eles o principal seria o glutamato, que, uma vez liberado, se une aos receptores AMPA (acido alfa-amino-3-hidroxi-5-metil-4isoxazol proiônico) na membrana pós-sináptica, permitindo a entrada de Na^{+} nas células, despolarizando-as (Izquierdo *et al.*, 2008). Após, ocorre a saída de Mg^{2+}, que é responsável por obstruir o receptor glutamatérgico NMDA, de forma que esse canal fica livre, permitindo a entrada de Ca^{2+} nas células (Izquierdo *et al.*, 2002). A entrada de Ca^{2+} é uma importante via de sinalização celular, e estimula direta e indiretamente as proteínas cinases cálcio dependentes (PKC), proteínas ativáveis extracelularmente (ERK), proteínas dependentes do monofosfato adenosina (AMP) cíclico (PKA), bem como as cálcio-calmodulina dependentes (CamKII), que irão ativar mecanismos intracelulares que culminam com síntese proteica, como por exemplo a fosforilação da proteína ligante ao elemento responsivo ao AMP cíclico (CREB), que, por sua vez, pode estar associada com a indução da síntese de outras proteínas, como o BDNF (Bernabeu *et al.*, 1997). O BDNF é um importante fator neurotrófico, relacionado com a plasticidade neuronal, maturação dos neurônios, aumento da arborização dendrítica, dentre diversas outras funções, as quais o fazem fundamental para o processamento mnemônico (Furini *et al.*, 2009).

Uma vez consolidadas, as MLD são passíveis de evocação (lembrança). No entanto, é importante ter claro que as memórias não são estáticas, rígidas. Pelo contrário, são lábeis (Izquierdo, 2008). Desta forma, toda vez que uma memória é evocada, ela se torna passível de modulação – processo denominado reconsolidação. Em termos moleculares, podemos dizer que a consolidação e a reconsolidação compartilham processos similares, porém não idênticos (Kelly *et al.,* 2003; Cammarota *et al.,* 2014). Utilizando a tarefa de reconhecimento de objetos, Rossato *et al.* (2007) demonstraram que a região hipocampal participa tanto na consolidação quanto na reconsolidação da memória. No entanto, Kelly *et al*. (2003) verificaram que há padrões diferentes nas proteínas cinases de sinalização extracelular (ERK) nos dois processos. Em relação à memória aversiva, Cammarota, *et al*. (2004) observaram que a administração no hipocampo de inibidores de síntese proteica em um tempo específico afeta a consolidação da memória, porém não afeta a reconsolidação. Ainda, Milekic *et al.* (2007) evidenciam a expressão de algumas proteínas, tais como o BDNF, na região do hipocampo, mostrando a importância da sua presença tanto para a consolidação quanto para a reconsolidação de memórias.

3. Estratégias de neuroproteção

Considerando que o estresse causado pela DM resulta em um considerável déficit cognitivo, em especial da memória, estudar maneiras de atenuar ou eliminar esses déficits é importante. O termo "neuroproteção" refere-se a alguma estratégia que tem como objetivo principal amenizar os danos sofridos por um determinado agente, especificamente na região do encéfalo, este, o principal componente do nosso sistema nervoso central (SNC) (Moretti *et al.,* 2015).

Considerando relatos prévios que relacionam os déficits cognitivos vinculados à DM ao desequilíbrio oxidativo, buscamos investigar estratégias que forneçam melhora da capacidade antioxidante (Stefania *et al.,* 2013; Rajendiran *et al.,* 2015). Para tal utilizamos duas estratégias diferentes: o exercício físico aeróbico e a suplementação com chá verde. Embora os efeitos do exercício físico aeróbico possam ir muito além da melhora da defesa antioxidante, sabe-se que o exercício é um importante estimulador das defesas antioxidantes endógenas (Ristow *et al.,* 2009). Adicionalmente, optamos por realizar um tratamento utilizando a suplementação com chá verde, um reconhecido antioxidante exógeno (Schimidt *et al.,* 2015).

Por fim, considerando que muitos trabalhos têm demonstrado a relação entre os déficits cognitivos da DM e uma redução nos níveis de BDNF (Roceri *et al*., 2004), optamos por avaliar o efeito de uma estratégia capaz de aumentar os níveis desta proteína neurotrófica, o enriquecimento ambiental (Vivinetto *et al.*, 2013).

3.1. Exercício físico como estratégia de neuroproteção

Diversas evidências mostram que exercício físico, independente da modalidade, traz inúmeros benefícios para a saúde do SN de humanos (Notarius *et al.*, 2015) e animais (Schimidt *et al.*, 2014; Sosa *et al.*, 2015; Cechetti *et al.*, 2012). Estudos comprovam melhoras no aprendizado, na memória e na plasticidade do sistema nervoso como resposta ao exercício (Flores *et al.*, 2014), sendo este capaz de aumentar a angiogênese cerebral (Allen *et al.*, 2009) e atenuar o declínio mental decorrente do envelhecimento (Flores *et al.*, 2014). Além disso, o exercício atenua as respostas neurais ao estresse, possivelmente contribuindo para a redução de patologias clínicas como hipertensão, insuficiência cardíaca, estresse oxidativo e imunodepressão (Arrick *et al.*, 2014). A falta do exercício físico, por sua vez, está relacionada a transtornos de humor, imunossupressão, piora do perfil lipídico, glicêmico e da qualidade do sono (Wilmore & Costil, 2001).

A adaptação que o exercício físico causa no SN tem implicações na prevenção e tratamento da obesidade, câncer, depressão (Dishman *et al.*, 2006), declínio cognitivo associado ao envelhecimento (Flores *et al.*, 2014) e distúrbios neurológicos, tais como doença de Parkinson, doença de Alzheimer (Li *et al.*, 2015), acidente vascular isquêmico (Sosa *et al.*, 2015; Schimidt *et al.*, 2014) e lesões medulares ou encefálicas (Dishman *et al.*, 2006). Embora tenhamos certa clareza sobre os benefícios do exercício físico à saúde cerebral como um todo, os mecanismos envolvidos na neuroproteção induzida pelo exercício parecem ser múltiplos e complexos, e não estão ainda completamente elucidados. Além disso, aspectos como intensidade, modalidade e duração parecem ser determinantes nos efeitos observados (Schimidt, *et al.*, 2016). Assim, para que seja possível associar o exercício físico à neuroproteção em diferentes tipos de doenças são necessárias avaliações criteriosas.

Em nosso trabalho optamos pela investigação dos efeitos do exercício físico aeróbico, já que diversas são as publicações que associam a prática deste tipo de exercício com uma melhora no equilíbrio oxidativo e, assim, melhora das funções cognitivas (Sosa *et al.*, 2015; Lin, *et al.*, 2015; Lai *et al.*, 2014). Alguns trabalhos demonstram que o exercício físico aeróbico é capaz de melhorar as funções do hipocampo (Van Praag, 2008) e que pode

prevenir danos oxidativos em diferentes fases da vida, inibindo a produção de EROs. Ji *et al.* (1992) destacaram a eficácia do exercício físico em promover a adaptação do corpo às necessidades requeridas pela prática de exercício, demonstrando que o exercício físico estimula a atividade de diversas enzimas antioxidantes, como a CAT, GPx e a SOD, além de induzir aumento na peroxidacão lipídica, que se inicia através da formação de radicais livres oriundos da prática de exercício. Outros trabalhos demonstram que o exercício físico aeróbico também tem efeitos sobre antioxidantes não enzimáticos. Tessier *et al.* (1995) observaram que a glutationa (GSH) pode ser reduzida durante a pratica de exercícios físicos; e, Hellsten *et al.* (1996), que a prática de exercício parece mobilizar antioxidantes não enzimáticos, como a vitamina E e a vitamina C, na tentativa de reduzir as EROs originados pela prática do exercício.

Obviamente, é preciso considerar que os efeitos do exercício sobre o SNC não são restritos à sua influência sobre o equilíbrio oxidativo. Diversos trabalhos investigando os mecanismos pelos quais o exercício atua no cérebro demonstram que ele é capaz de atuar direta e indiretamente sobre o SNC (Mablanda & Russel, 2010; Daniels *et al.*, 2012). Diretamente o exercício atua promovendo o aumento da circulação sanguínea cerebral, o incremento dos níveis de BDNF e de outros genes e proteínas relacionadas à plasticidade, a potenciação da atividade elétrica neuronal, e a modulação da liberação de alguns neurotransmissores, como a acetilcolina e a noradrenalina. Indiretamente, fatores e hormônios periféricos cuja produção é regulada pelo exercício, como os glicocorticoides, podem atravessar a barreira hemato-encefálica, atuando sobre o SNC. Em conjuntos, estas adaptações induzidas pelo exercício promovem mudanças estruturais (vasculares e neuronais) que se relacionam com a saúde cerebral (Cotman & Berchtold, 2002). Além disso, é importante considerar que o exercício físico é uma prática de baixo custo, com poucos efeitos adversos se realizada com orientação adequada.

3.2. Chá verde como estratégia de neuroproteção

Extratos vegetais e fitoquímicos purificados representam uma alternativa frequentemente testada para possíveis atividades farmacológicas que envolvem reações bioquímicas, tais como ensaios de oxidação (Wong *et al.*, 2015). A suplementação com

compostos que apresentam propriedades antioxidantes também tem sido estudada para prevenir déficits de memória associados ao desequilíbrio oxidativo (Schimidt *et al.,* 2015).

O chá verde tem um grande potencial antioxidante devido à presença de catequinas, como a epigalocatequina galato, a epicatequina, epicatequina galato e a epigalocatequina (Flores *et al.,* 2014). Além disso, é um produto de fácil acesso e sem efeitos colaterais significativos relatados. Vários estudos têm demonstrado o uso do chá verde como um potencial agente neuroprotetor em várias lesões cerebrais, como acidente vascular cerebral isquêmico e doença de Alzheimer (Schimidt *et al.,* 2015; Zang *et al.,* 2016.).

Sabemos que os eventos moleculares envolvidos na DM não são completamente compreendidos, mas há evidências de que eventos estressantes no início da vida podem promover numerosas cascatas moleculares, levando a maior permeabilidade da barreira hemato-encefálica, alterações da morfologia cerebral, estresse oxidativo, inflamação e consequente morte neuronal (Rajendiran *et al.,* 2015). O desequilíbrio oxidativo aumenta a susceptibilidade do tecido nervoso aos danos induzidos pela cascata molecular de reações bioquímicas (Stefania *et al.,* 2013). Por outro lado, conforme previamente mencionado, resultados de trabalhos prévios indicam que o consumo de chá verde promove neuroproteção, testada em modelos animais de diferentes injúrias ao SN, atenuando déficits de memória, diminuindo níveis de substâncias pró-oxidantes, e melhorando as defesas antioxidantes em diferentes regiões do cérebro (Flores *et al.*, 2014; Schimidt *et al.*, 2014).

Cabe ressaltar, também, o baixo custo envolvido na aquisição de chá e a ausência de efeitos colaterais, o que o torna uma alternativa eficiente para promoção de neuroproteção. Além disso, é importante destacar que, enquanto o exercício físico envolve considerável tempo para sua execução, além de deslocamento e outras adequações para sua prática, o consumo de chá não requer tais requisitos, o que pode aumentar a aderência ao tratamento.

3.3. Enriquecimento ambiental como estratégia de neuroproteção

O enriquecimento ambiental (EA) é uma ferramenta usada para gerar estímulos, criando um ambiente mais complexo e interativo e oferecendo estímulos diferentes dos que os sujeitos estão acostumados (Karyn *et al.,* 2003). Modelos para estudos envolvendo EA em roedores envolvem a manutenção dos animais em caixas moradia adaptadas, com objetos-estímulo de diferentes tamanhos e formatos (Vivinetto *et al.,* 2013).

Estudos prévios utilizando estes modelos demonstram que o EA é capaz de promover o aumento da espessura do córtex cerebral, bem como da arborização dentrítica e neurogênese (Pereira *et al.,* 2007). Adicionalmente, alguns autores demonstraram que o EA é capaz de reverter alguns dos déficits comportamentais e neuroquímicos relacionados à DM, tendo, também, efeitos em receptores glicocorticoides, aumentando a plasticidade cerebral e melhorando as funções cognitivas (Vivinetto *et al.,* 2013; Hutchinson *et al.,* 2012 , Francis *et al.,* 2002). O EA também parece agir sobre a cascata molecular que culmina no aumento do BDNF, que, conforme previamente mencionado, é um importante fator de crescimento neuronal, relacionado com a consolidação da memória e que está com seus níveis diminuídos após a DM (Leal-Galicia *et al.,* 2008; Benett *et al.,* 2006).

Estudos recentes como o de De Giorgio (2017) demonstram resultados promissores quando utilizando o enriquecimento ambiental para estimular pessoas com deficiência intelectual; embora seja difícil averiguar os mecanismos pelos quais isso ocorre, os resultados em testes de inteligência dessas pessoas mostram que o EA pode ser capaz de reparar danos neuronais e fazer com que esses indivíduos adquiram uma melhora motora considerável. Desta forma, o EA caracteriza-se como uma importante ferramenta, capaz de oferecer estímulos aos animais, de baixo custo e sem efeitos colaterais.

JUSTIFICATIVA

Estudos têm demostrado que a deprivação maternal (DM) é um dos mais potentes estressores naturais durante o período de desenvolvimento neonatal (Benetti *et al.,* 2009; Genest *et al.,* 2004). Em seres humanos, Voorhees *et al.* (2004) demonstraram que traumas fortes, abuso sexual e agressões físicas durante a infância são as principais causas de aumento do estresse e consequente surgimento de processos de desordem mental na vida adulta. Em modelos animais já tem sido demonstrado que a deprivação maternal (DM) é uma forma de mimetizar o estresse ocorrido em fase de desenvolvimento (Benetti *et al.,* 2009).

Considerando o já exposto, se faz necessário a investigação de estratégias que visem minimizar os danos causados pela DM. Portanto, a pesquisa do impacto de diferentes estratégias neuroprotetoras é importante. Para tal, intervenções que tenham custo reduzido e poucos efeitos adversos são bem-vindas, por isso, nessa dissertação propomos estudar os diferentes efeitos de estratégias como o exercício físico aeróbico, suplementação com chá verde e o EA e seus possíveis mecanismos frente ao déficit cognitivo induzido pela DM.

Diversos estudos têm utilizado o exercício físico como meio terapêutico para reverter déficits de memória e comportamento em ratos (Harrison *et al.,* 2013; Toy *et al.,* 2013; Ahlskog *et al.,* 2011; Shimidt *et al.,* 2014), inclusive em animais submetidos à DM (Marais *et al.,* 2011; Mello *et al.,* 2006). Paralelamente, alguns autores têm investigado o dano oxidativo consequente da DM (Uysal *et al.,* 2005), mas, embora se conheçam os efeitos antioxidantes do exercício físico (Schimidt *et al.,* 2014; Allen *et al.,* 2009; Sosa *et al.,* 2015) nenhum estudo antes do nosso, de acordo com nosso conhecimento, investigou o efeito do exercício no estresse oxidativo decorrente da DM. Da mesma forma, os efeitos do chá verde sobre os déficits de memória relacionados a diferentes injúrias do SNC já foram investigados, e o chá mostrou um considerável efeito neuroprotetor, melhorando a função mnemônica e o equilíbrio oxidativo (Zang *et al.,* 2016; Tamano *et al.,* 2013). No entanto, estes estudos com chá verde geralmente são realizados em modelos nos quais o momento da injúria é bem definido. Nós não encontramos trabalhos que avaliassem os efeitos do chá verde em um modelo de déficit de memória relacionado a um estresse no início da vida. Por fim, trabalhos prévios demonstram o potencial efeito benéfico do EA ao SNC. Leal-Galicia *et al.* (2008) demonstraram que a exposição a um ambiente enriquecido promove um impacto positivo na MCD e também causa uma manutenção da morfologia sináptica e um aumento na geração de novos neurônios na região do hipocampo. Vivinetto *et al.* (2013) verificaram que o EA é

capaz de causar uma aumento na expressão de receptores glicocorticoides, bem como uma melhora na memória aversiva, testada na esquiva inibitória.

OBJETIVOS

Objetivo geral

O objetivo geral deste trabalho é investigar os efeitos de diferentes estratégias de neuroproteção em um modelo de déficit cognitivo relacionado à deprivação maternal.

Objetivos específicos

Os objetivos específicos desta dissertação incluem:

- Verificar os efeitos da deprivação maternal (DM) sobre a função mnemônica de ratos Wistar, considerando seus efeitos sobre a Memória de Curta Duração (MCD), a Memória de Longa Duração (MLD), a consolidação e a reconsolidação da memória;

- Verificar os efeitos da DM sobre o equilíbrio oxidativo em tecido neural envolvido nas funções cognitivas (hipocampo) de ratos Wistar;

- Verificar os efeitos da DM sobre o Fator Neurotrófico Derivado do Cérebro (BDNF) em tecido neural envolvido nas funções cognitivas (hipocampo) de ratos Wistar;

- Verificar os efeitos neuroprotetores do exercício físico sobre a MCD, MLD e equilíbrio oxidativo hipocampal em um modelo de DM em ratos Wistar;

- Verificar os efeitos neuroprotetores da administração do chá verde sobre a MCD, MLD e equilíbrio oxidativo hipocampal em um modelo de DM em ratos Wistar;

- Verificar os efeitos neuroprotetores do enriquecimento ambiental sobre a MCD, consolidação e reconsolidação da MLD, e efeitos sobre os níveis de BDNF no hipocampo em um modelo de DM em ratos Wistar.

PARTE II

Os resultados desta dissertação estão organizados em três artigos científicos. O primeiro artigo, intitulado "*Physical exercise prevents short and long-term deficits on aversive and recognition memory and attenuates brain oxidative damage induced by maternal deprivation*", publicado na revista *Physiology & Behavior*, investiga a neuroproteção induzida pelo exercício físico aeróbico em um modelo DM, considerando seus efeitos comportamentais e bioquímicos.

O segundo artigo que compõe esta dissertação, intitulado "*Green tea protects against memory deficits related to maternal deprivation*", submetido à revista *Physiology & Behavior*, investiga a proposta da administração exógena de antioxidante, no caso pela suplementação com chá verde, em animais submetidos a DM no inicio da vida. São analisados os efeitos comportamentais e bioquímicos da DM e do chá verde.

O terceiro artigo que compõe esta dissertação, intitulado "*Maternal deprivation disrupts memory consolidation and reconsolidation. Environmental enrichment protects against these deficits*" é apresentado em modelo pronto para submissão, considerando as normas da revista *International Journal of Developmental Neuroscience.* Este trabalho investiga os efeitos da DM sobre a consolidação e reconsolidação da memória e níveis hipocampais de BDNF, bem como os efeitos do enriquecimento ambiental nestes parâmetros.

Artigo 1

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Physical exercise prevents short and long-term deficits on aversive and recognition memory and attenuates brain oxidative damage induced by maternal deprivation

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HIGHLIGHTS

- Maternal deprivation causes deficits in short- and long-term memory.
- Physical exercise avoids memory deficits related to maternal deprivation.
- Maternal deprivation promotes oxidative damage in hippocampus.
- Maternal deprivation promotes oxidative damage in prefrontal cortex.
- Physical exercise avoids brain oxidative damage caused by maternal deprivation.

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ABSTRACT

It is known from previous research that physical exercise prevents long-term memory deficits induced by maternal deprivation in rats. But we could not assume similar effects of physical exercise on short-term memory, as short- and long-term memories are known to result from some different memory consolidation processes. Here we demonstrated that, in addition to long-term memory deficit, the short-term memory deficit resultant from maternal deprivation in object recognition and aversive memory tasks is also prevented by physical exercise. Additionally, one of the mechanisms by which the physical exercise influences the memory processes involves its effects attenuating the oxidative damage in the maternal deprived rats' hippocampus and prefrontal cortex.

1. Introduction

Maternal deprivation (MD) is one of the most potent natural stressors during neonatal development and can results in permanent deficits during adulthood [1,2]. At the same time, in humans, a significant trauma experienced during childhood is the primary cause of increased stress and the subsequent emergence of mental disorders in adulthood [3]. Animal studies have demonstrated that MD results in behavioral changes that persist into adulthood [4–6], including increased anxiety [7], personality disorders [8], schizophrenia, depression [9], anhedonia [10], and memory deficits [6].

The impact of MD during the neonatal period is certainly related to the neural changes that occur during this period. For example, most granular neurons of the hippocampus develop and extend their axons between the 1st and 21st day of life [11]. During this period, the pups undergo an anatomical consolidation of their nervous system along with the continued proliferation and maturation of synapses [12]. The behavioral alterations observed in adult rats that were submitted to early-life stress could be related to alterations in gene expression [4,8,13], a reduction in brain-derived neurotrophic factor expression [14,15], an increase of corticosterone levels [16] and/or alterations in oxidative balance [17].

Considering the changes that have been observed in the adult brains of MD rats, several studies have attempted to identify strategies to avoid or decrease the behavioral deficits related to MD. Physical exercise can prevent the long-term memory deficits caused by MD, but little is known about the mechanisms involved in these this effect [18,19]. Because physical exercise has shown beneficial effects in improving oxidative balance [20], which is disrupted in the brains of MD rats [17], we investigated whether the effects of aerobic exercise on memory

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deficits are related to its effect on oxidative balance. Our results show that physical exercise prevents memory deficits on object recognition and inhibitory avoidance task, including short-term memory deficits for which the beneficial effects of physical exercise have not previously been studied. Additionally, physical exercise attenuates the oxidative damage induced by maternal deprivation in neural tissues.

2. Materials and methods

2.1. Animals

Pregnant female Wistar rats were obtained from Central Vivarium of Federal University of Santa Maria (RS/Brazil). All animals were maintained on a constant 12 h light/12 h dark cycle (lights on at 7:00 h) at controlled room temperature (23 ± 2 °C) and air humidity (60 ± 5%). Pregnant females were individually housed with sawdust bedding and with food and water available *ad libitum*. The day of delivery was considered to be day zero. At postnatal day 1 (PND-1), the MD protocol was initiated with half of the pups, which lasted until PND-10. Animals were weaned at 21 days of age (PND-21) and were housed 5 per cage in regular cages. Only the males were used in the following experiments. All experiments were conducted in accordance with the principles of laboratory animal care (NIH publication no. 80-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of the Local Institution (#001/2014).

The male rats were divided in four groups: (i) control, in which rats were not submitted to any intervention; (ii) deprived, which includes those rats submitted to MD as described below, without any additional intervention; (iii) physical exercise, which includes rats that were submitted to physical exercise as described below after PND-45; and (iv) deprived with physical exercise, which includes those rats submitted to MD from PND-1 until PND-10 and then submitted to physical exercise from PND-45 onward (Fig. 1). Rats in all four groups were submitted to the following behavioral tests starting at PND-100: open field; object recognition; inhibitory avoidance; tail flick; and elevated plus maze. After behavioral testing, the brains were isolated, and the hippocampus and prefrontal cortex dissected for use in the biochemical tests.

2.2. Maternal deprivation (MD) protocol

Female Wistar rats were maintained in individual boxes until their delivery day (considered to be day 0). Rats from groups (ii) and (iv) were submitted to maternal deprivation (MD) for 3 h per day during the light part of the cycle from PND-1 to PND-10. The MD protocol consisted of removing the mother from the residence box to other room. Pups were maintained in their home cage, and while the mothers were absent, the room temperature was increased to 32 °C to compensate for the absence of the mother's body heat [1]. At the conclusion of each daily deprivation session, the mothers were returned to their home boxes.

The rats in groups (i) and (iii) remained in their resident boxes together with their mothers during the first ten days of life. Only on PND-11 the boxes were cleaned normally again, according to the standard laboratory routine [21]. On PND-21, the animals were weaned, and the males were maintained in groups of 5 in plastic boxes with food and water available *ad libitum*, as with all the other animals in our animal housing facility.

2.3. Physical exercise protocol

Rats from groups (iii) and (iv) were submitted to chronic aerobic treadmill exercise during 8 weeks beginning on PND-45. One week prior to starting the training, all animals were placed in the treadmill for 10 min for habituation. On the first day of the second and fifth week, an indirect VO_2 maximum (peak oxygen uptake) test was conducted on a motorized rodent treadmill. The indirect VO_2 was used to determine and adjust the exercise intensity during the training period. An indirect measurement of VO_2 was determined as recommended by Brooks and White [22]: each rat ran on the treadmill at a low initial speed followed by speed increase of 5 m/min every 3 min, until they reached their exhaustion point. The intensity of physical exercise training (50 min/day; 5 day per week) was maintained between 50% and 70% of their respective VO_2 maximum for 8 weeks. Each training session started with a 10-min gradual acceleration followed by 30 min at the target intensity; the last 10 min of each session consisted of a gradual deceleration [23]. The treadmill used had individual 10 cm wide, 50 cm long lanes separated by plastic walls. No electric shock

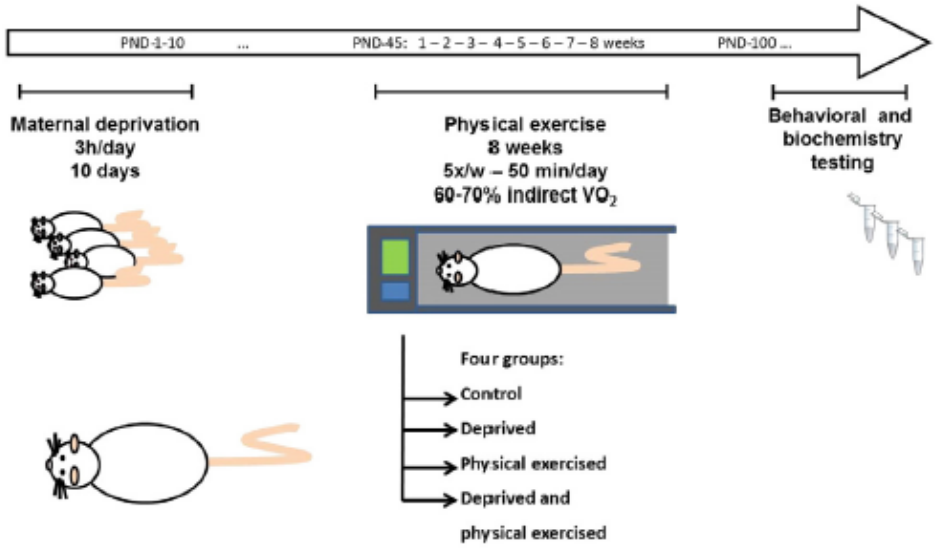

Fig. 1. Experimental design. Animals were submitted to maternal deprivation from PND-1 until PND-10 for 3 h per day (groups (ii) and (iv)). After, rats of the groups (iii) and (iv) were submitted to physical exercise lasting 8 weeks, from PND-45 on. In PND-100 the behavioral testing were started, followed by biochemical tests.

stimulation was used in this study to avoid possible stress effects. Control animals were transported to the experimental room and handled exactly as those in the physical exercise groups; however, they did not run on the treadmill.

2.4. Memory behavioral testing

2.4.1. Object recognition (OR) test

Recognition memory was evaluated through the object recognition (OR) test using a 40 × 50 × 50 cm open arena as described previously [24]. All animals were habituated to the experimental arena for 20 min/day for 4 days in the absence of any specific behavioral stimulus. The objects used in the training and testing sessions were made of metal or glass and were fixed to arena's floor. On the first day after habituation, the animals were placed in the arena with two different objects (named A and B) and were free to explore for 5 min (training session); the rats were tested 3 h later to evaluate short-term memory (STM) and 24 h later to evaluate long-term memory (LTM). In the tests, one of the objects was removed and replaced with a new object (C or D) and the rat was introduced in the arena for five minutes. The positions of the objects (familiar or novel) were randomly chosen for each experimental animal, and the arena was always cleaned between trials. Exploration was defined as sniffing or touching the objects with the nose and/or forepaws. The time spent exploring each object was recorded by an observer blind to the treatment and was expressed as a percentage of the total exploration time computed in seconds [25].

2.4.2. Inhibitory avoidance (IA) test

To evaluate aversive memory, rats were trained in a one-trial step-down inhibitory avoidance (IA) task using a 50 × 25 × 25 cm Plexiglas box with a 5 cm high, 8 cm wide, and 25 cm long platform placed on the left end of a series of conductive bronze bars that made up the floor of the box. For training, rats were gently placed on the platform facing the left rear corner of the training box. When they stepped down and placed their four paws on the grid, a 2 s, 0.5 mA scrambled foot shock was delivered. The tests were performed 3 h and 24 h after training, to evaluate the STM and the LTM, respectively. During the test, the rats were placed in the platform again, and the step-down latency was measured. A ceiling of 300 s for the step-down time was imposed [23].

2.5. Control behavioral tests

2.5.1. Open field (OF) test

The OF apparatus consisted of a 40 × 50 × 50 cm open arena painted white except for the frontal wall, which was made of glass. The floor was divided into 12 equal rectangles by black lines; the crossing of the lines was used to evaluate locomotion. The number of rearings performed by each animal was used to evaluate exploratory activity [1]. The rats were individually placed in the arena and observed for 5 min.

2.5.2. Elevated plus maze (EPM) test

To evaluate anxiety state, which could affect the results of memory tests, rats were exposed to an EPM. The apparatus was located 60 cm above the ground and presented two enclosed arms facing each other and two open arms, each measuring 50 × 10 cm. The closed arms had also side walls 20 cm high. The time spent and the total number of entries into the open and closed arms were recorded over a 5 min session [26].

2.5.3. Tail flick (TF) test

To ensure that the pain sensibility was not change during the training period, we performed the tail-flick test [27]. A metal rod that was progressively heated was applied to the tip of the tail to induce pain, and the reaction time (tail-flick latency) was measured as the interval between touching the metal to the tail on the metal and the voluntary withdrawal of the tail.

2.5.4. Rota-rod (RR) test

The RR test was used to ensure that the physical exercise practice did not result in impairments in the motor coordination, balance and muscle strength. The apparatus consisted of a cylinder with a diameter of 3 in.; suspended 20 cm from the device surface, driven by a gear that maintains a constant speed. The rat was placed on the cylinder and the time until the first fall, as well as the number of falls in a 300 s period, were measured. In the training session rotation speed was 16 rpm. In the testing, rotation speed was set at 26 rpm.

2.6. Biochemical testing

2.6.1. Tissue preparation

Rats from all groups were euthanized 24 h after the conclusion of the behavioral experiments. The brains were removed, and the bilateral hippocampus and prefrontal cortices were quickly dissected out and homogenized in 50 mM Tris HCl, pH 7.4, (1/5, *w*/*v*). Afterwards, samples were centrifuged at 2400 g for 10 min, and the supernatants (S1) were used for further analysis.

2.6.2. Glutathione (GSH)

GSH levels were fluorometrically determined [28]. An aliquot of homogenate was mixed (1:1) with perchloric acid ($HClO_4$) and centrifuged at 3000 g for 10 min. After centrifugation, the protein pellet was discarded and free-SH groups were identified in the clear supernatant. An aliquot of supernatant was incubated with ortho-phthalaldehyde, and fluorescence was measured at excitation of 350 nm and emission of 420 nm. The results were normalized to the mass of protein (in mg) and expressed as a percent of the control.

2.6.3. Reactive oxygen species (ROS)

ROS content was assessed by a spectrofluorometric method using 2,7-dichlorofluorescein diacetate (DCFH-DA) as a probe [29]. The sample (S1) was incubated in the dark with 5 μL DCFH-DA (1 mM). The oxidation of DCHF-DA to fluorescent dichlorofluorescein (DCF) was measured as a method of detecting intracellular ROS. The formation of the oxidized fluorescent derivative (DCF) was measured by DCF fluorescence intensity recorded at 520 nm (480 nm excitation) 30 min after the addition of DCFH-DA to the medium. The results were expressed as a percentage of control in arbitrary units (AU).

2.6.4. Thiobarbituric acid reactive substance (TBARS) levels

Lipoperoxidation was evaluated by the TBARS test [30]. One aliquot of S1 was incubated with a 0.8% thiobarbituric acid solution, acetic acid buffer (pH 3.2) and sodium dodecyl sulfate solution (8%) at 95 °C for 2 h, and the color reaction was measured at 532 nm. The results were expressed as nmol of malondialdehyde (MDA) per mg protein.

2.7. Statistical analysis

Data were checked for normality of distribution using the Shapiro–Wilk test. The OR test results were expressed as the percentage of total time spent exploring each object. The results were analyzed using a one-sample Student t-test, considering a theoretical mean of 50%. The IA results were expressed as the platform step-down latency in seconds and were analyzed using an intra-group Wilcoxon test to compare training *vs.* test. The OF, EPM, TF and RR tests results were analyzed using an ANOVA, and the biochemical results were compared using two-way ANOVA using Bonferroni correction for multiple comparisons. The differences were considered statistically significant when $P < 0.05$.

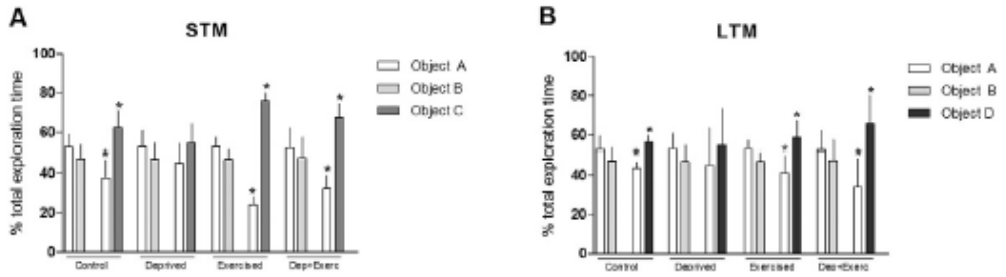

Fig. 2. Maternal deprivation causes short-term memory (STM) and long-term memory (LTM) deficits in object recognition (OR). Physical exercise avoids these deficits. A. The animals were trained on OR task and tested 3 h later. In the training session the animals were exposed to objects A and B and explored about 50% of the total exploration time each one. In the test session the rats were exposed to a familiar (A) and to a novel object (C). The MD group was not able to recognize the familiar and the new object in the test session, but the MD rats submitted to physical exercise were. B. The animals were trained on OR task and tested 24 h after training. In the training session the animals were exposed to objects A and B and explored about 50% of the total exploration time each one. In the test session the rats were exposed to a familiar (A) and to a novel object (D). The MD group could not differentiate the new and the familiar object, but the MD rats submitted physical exercise were. Data are expressed as mean ± SD of the percent of total exploration time; *P < 0.05 in one-sample t-test, considering a theoretical mean of 50%; n = 8–12 per group.

3. Results

3.1. Indirect maximal oxygen uptake

The treadmill running protocol enhanced physical aerobic capacity. Exercise training resulted in a significantly higher VO_2 maximum in the second measurement compared to start of the training ($P < 0.05$ in Student's t-test, data not shown).

3.2. Memory tests

3.2.1. Short- and long-term memory for object recognition

As expected, rats from all groups explored each of the objects (A and B) for a similar percentage of the time (about 50%) during the training session because both objects were novel (Fig. 2). Short-term memory (STM) was impaired in the deprived animals, and physical exercise ameliorated this memory deficits. In the STM test, the percent of time that control animals spent exploring the new object was significantly higher than 50% ($P = 0.01$), which indicates a preserved memory (Fig. 2A, control). Deprived rats spent similar times (approximately 50% of the total exploration time) exploring the familiar and the new objects ($P = 0.13$), which suggests a STM deficit (Fig. 2A, Deprived). Physical exercise improved STM of non-deprived rats ($P < 0.01$, Fig. 2A, Exercised) and was able to ameliorate the memory deficit induced by maternal deprivation, since deprived rats that were exposed to physical exercise spent more than 50% of the total exploration time exploring the new object (C) ($P < 0.01$, Fig. 2A, Dep + Exerc).

Similar results were observed for LTM test for OR: LTM was impaired in the deprived animals, and physical exercise ameliorated the memory deficits. MD causes memory deficits (the rats spent a similar percentage of time exploring the familiar and the new objects, A and D, respectively; $P = 0.47$, Fig. 2B, Deprived). Physical exercise was able to reverse the LTM deficit, as evidenced by the fact that the deprived and exercised rats spent more than 50% of the total exploration time exploring the new object (D) ($P < 0.01$, Fig. 2B, Dep + Exerc).

3.2.2. Short and long-term memory for aversive stimuli

Physical exercise reversed deficits in STM and LTM aversive memory. Our IA results demonstrate that deprived rats show deficits in STM and LTM towards aversive stimuli because there was no increase in the platform step-down latency of deprived rats when comparing training and test values ($P = 0.86$ for STM, Fig. 3A, Deprived; $P = 0.32$ for LTM, Fig. 3B, Deprived). Rats from the other groups, including the deprived and exercised group, increased their step-down latency, ($P < 0.001$ for STM, Fig. 3A, Dep + Exerc; $P < 0.002$ for LTM, Fig. 3B, Dep + Exerc).

3.3. Behavioral control tests

The protocols used (MD and physical exercise) did not affect locomotor and exploratory activities, anxiety, pain sensibility or motor coordination. There were no differences in the numbers of crossings ($P = 0.08$) and rearings ($P = 0.24$) in the OF test (Table 1, open field). The total number of entries and the time spent in the open arms in

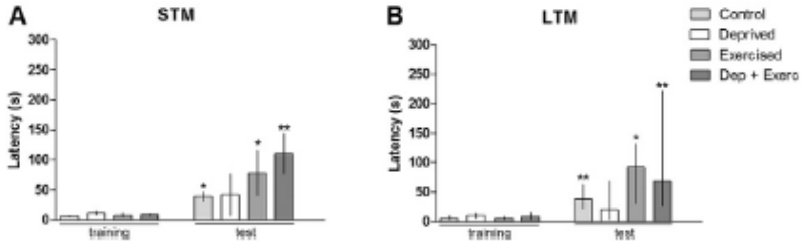

Fig. 3. Maternal deprivation (MD) causes deficits in short-term memory (STM) and long-term aversive memory (LTM) measured by inhibitory avoidance (IA) task; these deficits were avoided by physical exercise. A. Rats were trained in the IA task and STM was tested 3 h later. All animals showed a low step-down latency in the training. In the test, the MD group did not present increase in step-down latency, but MD rats submitted to physical exercise increased their latencies. B. Rats were trained in IA and LTM was tested 24 h later. All animals showed a low step-down latency in the training. In the test, the MD group did not present increase in step-down latency, but MD rats submitted to physical exercise presented. Data are expressed as median ± interquartile interval of step-down latency; *P < 0.05 and **P < 0.01 on training vs. test, Wilcoxon test; n = 8–12 per group.

Table 1
The maternal deprivation and the physical exercise do not alter locomotor and exploratory activities, anxiety, pain threshold, and motor coordination. Data are expressed as mean ± SD of the number of crossings and rearings (open field), the time spent and the number of entries in the open arms (plus maze), the latency time to tail withdrawal (tail flick), the latency to the first fall and the number of falls (Rota Rod). There were no differences between the groups (ANOVA; n = 8–10 per group for all tests).

Control behavioral tests		Control	Deprived	Exercised	Deprived and exercised
Open field	Crossings (n)	83.29 ± 5.99	61.00 ± 7.13	79.57 ± 4.75	62.57 ± 7.89
	Rearings (n)	37.63 ± 4.47	30.80 ± 2.90	35.43 ± 4.48	27.82 ± 3.27
Elevated plus maze	Time in open arms (s)	1.73 ± 1.58	2.14 ± 1.67	2.00 ± 0.88	1.00 ± 0.64
	Entrances in open arms (n)	2.62 ± 1.32	1.50 ± 0.65	1.28 ± 0.99	1.27 ± 0.77
Tail flick	Latency to tail withdrawn (s)	5.57 ± 0.57	5.48 ± 0.42	6.41 ± 0.59	6.06 ± 0.36
Rota-rod	Latency to the first fall (s)	25.00 ± 10.82	24.00 ± 17.06	34.57 ± 16.54	34.55 ± 10.69
	Falls (n)	0.25 ± 0.16	0.60 ± 0.22	0.28 ± 0.18	0.48 ± 0.35

the EPM were similar between all groups ($P = 0.51$ and $P = 0.12$, Table 1, elevated plus maze). The TF latency was also similar between the different groups ($P = 0.08$, Table 1, tail flick). In the RR test, there were no differences in the number of falls ($P = 0.06$) and in the latency for the first fall ($P = 0.11$) (Table 1, Rota-rod).

3.4. Biochemical tests

Exercise plays a neuroprotective role against hippocampal lipid peroxidation in deprived animals. There were no alterations in the antioxidant marker GSH in the hippocampus between all of the groups ($P = 0.11$ for MD effect; $P = 0.09$ for physical exercise effect; there are no interaction between effects, $P = 0.09$; Fig. 4A). Additionally, no differences were detected in hippocampal ROS ($P = 0.49$ for MD effect; $P = 0.31$ for physical exercise effect; there are no interaction between effects, $P = 0.36$; Fig. 4C). In our TBARS measurement, we found differences between groups ($P = 0.03$ for MD effect; $P = 0.23$ for physical exercise effect; there are no interaction between effects, $P = 0.17$; Fig. 4E). Therefore, in deprived rats compared to control rats, we detected an increase in hippocampal lipid peroxidation (TBARS) ($P = 0.03$).

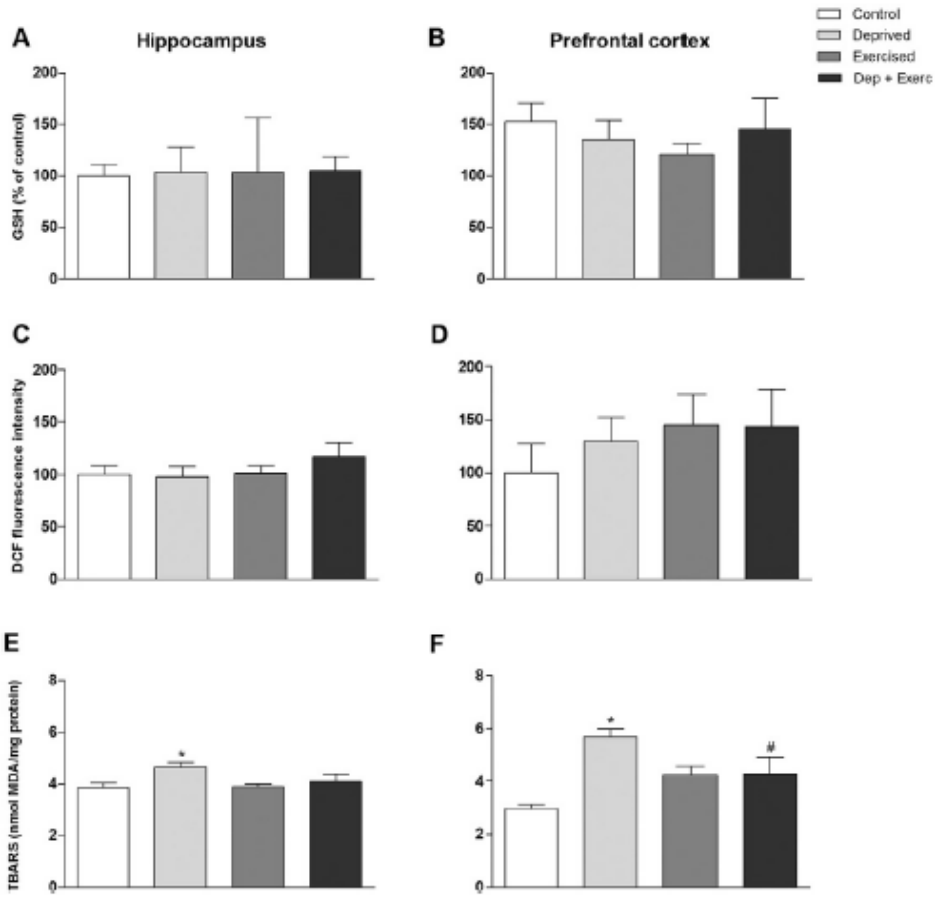

Fig. 4. Effects of maternal deprivation and physical exercise on GSH, ROS and lipid peroxidation in hippocampus and prefrontal cortex. A. Levels of GSH in the hippocampus, considering the percent of control. B. Levels of GSH in the prefrontal cortex, considering the percent of control C. Levels of ROS in the hippocampus measured by DCF fluorescent intensity. D. Levels of ROS in the prefrontal cortex measured by DCF fluorescent intensity. E. TBARS levels measured by MDA in hippocampus. F. TBARS levels measured by MDA in prefrontal cortex. Data are expressed as mean ± SD; *P < 0.05 compared to control group; #P < 0.05 compared to deprived group; two-way ANOVA with Bonferroni post-hoc; n = 8–12 per group.

However, lipid peroxidation did not increase in deprived and exercised rats ($P = 0.62$).

In prefrontal cortex biochemical analyses we observed similar results. There were no differences between the groups in GSH levels or ROS as measured by DCFH (GSH: $P = 0.92$ for MD effect; $P = 0.09$ for physical exercise effect; there are no interaction between effects, $P = 0.98$; Fig. 4B; ROS: $P = 0.62$ for MD effect; $P = 0.31$ for physical exercise effect; there are no interaction between effects, $P = 0.59$; Fig. 4D). In TBARS measurement, we found differences between groups ($P < 0.01$ for MD effect; $P = 0.93$ for physical exercise effect; there are interactions between the effects, $P < 0.01$; Fig. 4F). There was an increase on lipoperoxidation in the prefrontal cortex in MD rats compared to controls ($P < 0.01$, Fig. 4F), and the deprived and exercised rats showed decreased MDA levels compared to the deprived group ($P = 0.02$, Fig. 4F).

4. Discussion

Here, we demonstrate that physical exercise can ameliorate impairments in STM and LTM (in object recognition and IA task) caused by MD in rats. It is known that MD during early life is a potent stressor that leads to psychopathologies during adulthood due to deleterious effects on brain development [31–33]. Additionally, such alterations related to mother–pup interactions can cause social and cognitive problems in animals and humans [34–41].

MD is considered to be a useful model for studying of childhood neglect and abuse [42]. Additionally, MD experimental models permit the investigation of interventions with possible applications in humans with the goal of avoids behavioral and neuroendocrine abnormalities such as depression, anxiety disorders, and memory deficits. From these studies, we learned that pharmacological treatments [1,43,44], environmental enrichment [45], and physical exercise [1,18,19,46] could be possible strategies for reversing such deficits. Specifically regarding physical exercise, chronic aerobic exercise can prevent LTM deficits in different contexts [19]. However, our study is the first to address the effects of physical exercise on STM deficits caused by MD.

We show that physical exercise can prevent MD-related STM and LTM deficits. Considering that STM and LTM consolidation involve different neurobiological mechanisms [47] (for example, increased protein and gene expression is present only in LTM consolidation) [47], is important to know that physical exercise can have neuroprotective effects in STM and LTM consolidation in MD rats. However, the fact that these different types of memory involve some distinct neurobiological processes does not mean that the physiopathology of these deficits in MD rats does not involve a common cause, such as oxidative stress [48,49], which is one factor that is present in the brain of MD rats [42,50]. Additionally, it is likely that physical exercise affects memory through more than one mechanism. Here, we investigated the involvement of oxidative balance based on its role in the production of oxidative stress in the brain of MD rats [51–53]. Furthermore, it has been extensively shown that aerobic exercise improves brain oxidative status and promotes neuroprotection [54–56].

We demonstrated that physical exercise prevents oxidative damage (lipid peroxidation) in the hippocampus and prefrontal cortex. We specifically investigated the hippocampus and prefrontal cortex because previous studies have reported an oxidative misbalance in these regions in the brains of MD rats [42,50]. Farther, the hippocampus is one of the most important brain regions involved in memory processes [14,15], and it is highly susceptible to oxidative damage [57,58]. The prefrontal cortex, in turn, participates in numerous cognitive functions, including working memory [59], and is also related to the hippocampus during the processes of memory consolidation [60].

The oxidative damage observed in the brain of MD rats (increased lipid peroxidation) may be related to several disruptions. One hypothesis is related to dopaminergic system. It has been previously reported that MD increases dopamine turnover in the mesolimbic region of the brain [61] and induces dysfunction of the adult dopamine system [62] in laboratory animals. Dopamine is metabolized by monoamine oxidase, which produces hydrogen peroxide. Thus, the increased turnover of dopamine produces oxidative stress derived from the increased production of hydrogen peroxide. This generation of ROS could be a major component in decreased cell function and eventual cell death [63]. Previous studies have shown that increased ROS production can be induced by stress [64,65]. Unfortunately, the direct measurement of ROS production in living cells is difficult because they are highly reactive and have a short half-life [66]. Yet, the ROS can initiate radical chain reactions in some biological molecules, resulting in lipid peroxidation, which, in turn, can cause degeneration of membrane structure and loss of membrane protein function [66]. In the case of MD, the main event responsible for the oxidative imbalance occur in the firsts days of life, and, in these report, the biochemical measurements were made about 100 days after the end of the stressor event (MD). So, it is possible that ROS formed had already stabilized by reacting with lipids, which could explain the absence of increase in ROS, but the increase in oxidative damage (lipid peroxidation) found by us. [42,53], based on our findings of an increase in oxidative damage markers as demonstrated by the increase of lipid peroxidation (TBARS). We did not observe a change in the level of antioxidant enzyme, which is in agreement with a recent report [42].

Therefore, our results show that physical exercise ameliorates STM and LTM memory deficits through the avoidance or reduction of lipid peroxidation in the hippocampus and prefrontal cortex. However, we cannot discount the diverse effects of physical exercise on the central nervous system in this model of MD, including the suppression of apoptotic neuronal cell death, the enhancement of cellular proliferation in the hippocampus [18], the increase of synaptophysin and CaMKII in the ventral hippocampus [67], and the possible influence on neurohumoral or hormonal memory modulatory systems related to stress [19]. All of these mechanisms should be the subjects of future research.

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Artigo 2

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Keywords: Oxidative stress; Antioxidants; Early-life stress; Object recognition; Inhibitory avoidance.

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Abstract: Maternal deprivation in early life affects the development of the brain, causing cognitive losses in adulthood. Oxidative imbalance may be one of the factors that trigger these deficits. Therapies with antioxidant components, like green tea from Camellia Sinensis has been used to treat or prevent memory deficits in a variety of conditions related to oxidative stress. Here we demonstrate that short- and long-term memory deficits caused by maternal deprivation can be prevented by green tea antioxidant activity in hippocampus. Pregnant female rats were used. Her puppies were submitted to maternal deprivation and intake of green tea. Recognition memory and aversive memory were evaluated, as well as hippocampal oxidative status.

GREEN TEA PROTECTS AGAINST MEMORY DEFICITS RELATED TO MATERNAL DEPRIVATION

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Highlights

1. Maternal deprivation causes deficits in short- and long-term memory.
2. Green tea avoids memory deficits related to maternal deprivation.
3. Maternal deprivation promotes oxidative stress in hippocampus.
4. Green tea avoids hippocampal oxidative stress caused by maternal deprivation.

Abstract

Maternal deprivation in early life affects the development of the brain, causing cognitive losses in adulthood. Oxidative imbalance may be one of the factors that trigger these deficits. Therapies with antioxidant components, like green tea from *Camellia Sinensis* has been used to treat or prevent memory deficits in a variety of conditions related to oxidative stress. Here we demonstrate that short- and long-term memory deficits caused by maternal deprivation can be prevented by green tea antioxidant activity in hippocampus. Pregnant female rats were used. Her puppies were submitted to maternal deprivation and intake of green tea. Recognition memory and aversive memory were evaluated, as well as hippocampal oxidative status.

Keywords: Oxidative stress; Antioxidants; Early-life stress; Object recognition; Inhibitory avoidance.

1. Introduction

The first weeks of life are critical to neurodevelopment, thus adversities in early life can cause deficits in brain development [1]. Early-life maternal deprivation (MD) is considered a severe type of stress and promotes damage in different systems, which can remain until adulthood [1, 2]. There is evidence showing that MD is related to anxiety-like behavior [3], depressive-like behavior [3, 4], deficits in learning and memory [5], and neurodegenerative diseases, such as Alzheimer's disease [6] and Parkinson's disease [7].

The molecular events involved in MD are not completely understood, but there is evidence that stressful events in early life can promote numerous molecular cascades, leading to increased blood-brain barrier permeability, alterations in brain morphology, neuroinflammation and neuronal death [8]. An oxidative imbalance increases the susceptibility of brain tissue to damage induced by molecular cascades [9]. Thus, studies that aim to elucidate the biochemical cascades involved in MD and seek treatments that can minimize the brain damage and deficits in this condition are important.

One of the most important structures related to learning and memory capacity is the hippocampus [10]. Changes in antioxidant capacity, levels of free radicals and other parameters of oxidative status in the hippocampus have been found in conditions of MD stress exposure [11], indicating that this type of stress response leads to increased production of free

radicals in the hippocampus and oxidative stress [12]. In these conditions of hippocampal oxidative imbalance, learning and memory deficits are common [13].

Therefore, supplementation with antioxidant compounds has been studied to prevent memory deficits associated with oxidative imbalance [14, 15]. Green tea has great antioxidant potential due to the presence of catechins, such as epigallocatechin gallate, epicatechin gallate, epicatechin and epigallocatechin [12]. In addition, green tea is an easily accessible product with no reported side effects. Several studies have demonstrated the use of green tea as a potential neuroprotective agent in several brain injuries, such as ischemic stroke [14] and Alzheimer's disease [16]. However, we did not identify any previous research that investigated the use of green tea for the prevention of oxidative damage and memory deficits associated with MD.

Here, we demonstrate that maternal deprivation causes both short- and long-term memory deficits and hippocampal oxidative stress and that green tea supplementation can prevent the memory deficits and partially ameliorate the hippocampal oxidative imbalance observed in MD.

2. Material and methods

2.1 Animals and experimental design

Pregnant female Wistar rats were obtained from the Central Vivarium of the Federal University of Santa Maria (RS/Brazil). All animals were maintained on a constant 12-h light/12-h dark cycle (lights on at 7:00 a.m.) at a controlled room temperature (22 ± 2°C) and air humidity (60 ± 10%). Pregnant females were individually housed with sawdust bedding with food and water *ad libitum*. The day of delivery was considered day zero. At postnatal day 1 (PND-1), the MD protocol was initiated with half of the pups until PND-10. Animals were weaned at the age of 21 days (PND-21) and housed in regular cages with 4 animals per cage. Only the males were used for the following experiments. The females were assigned for use in other ongoing projects. All experiments were conducted in accordance with the principles of laboratory animal care (NIH publication nº 80-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of the Local Institution.

The male rats were divided into four groups: (i) control, in which rats were not submitted to any intervention; (ii) deprived, including those rats submitted to MD as described below, without any additional intervention; (iii) green tea supplementation, in

which rats received green tea mixed with drinking water from PND-21 to PND-80; and (iv) deprived + green tea supplementation, in which the rats were submitted to MD from PND-1 to PND-10 and received green tea mixed with drinking water from PND-21 to PND-80.

Rats from the four groups were submitted to behavioral tests starting on PND-81 as follow: open field, elevated plus maze, hot plate, object recognition, and inhibitory avoidance. After behavioral testing, the rat brains were isolated, and the brain tissues were dissected (bilateral hippocampus) for use in the biochemical tests. Figure 01 summarizes the experimental design.

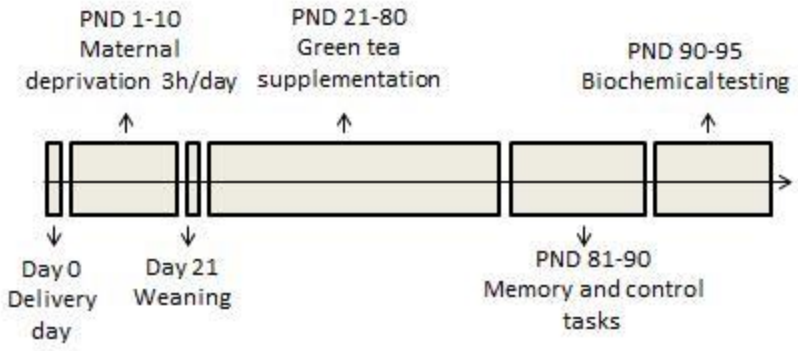

Fig 1. Experimental design. Day 0 was considered the day of the rats' born. On day 1 the MD protocol was initiated with half of the animals. Subsequently, each group was subdivided in two subgroups, with and without green tea supplementation. On day 81 the memory and control behavioral tests were started, following by the biochemical testing.

2.2. Maternal Deprivation

Female Wistar rats were maintained in individual boxes until the delivery day (considered day 0). Rats from groups (ii) and (iv) were subjected to maternal deprivation (MD) for 3 h per day from PND-1 to PND-10, always during the light portion of the cycle (09:00 a.m. to 15:00 p.m.). The MD protocol consisted of removing the mother from the residence box to another room. Pups were maintained in their home cage, and while the mothers were absent; the room temperature was raised to 32°C to compensate for the loss of the mother's body heat [17]. At the end of each daily deprivation session, the mothers were returned to their home boxes. Rats from groups (i) and (iii) remained in their resident boxes together with their mothers during the first ten days of life. The boxes were not regularly cleaned until PND-11, according to a standard laboratory routine [18]. On PND-21, the animals were weaned, and males were maintained in groups of 4 in plastic boxes with food and water *ad libitum*, similar to all the other animals in our animal housing facility.

2.3. Tea Supplementation

Rats from groups (iii) and (iv) received green tea mixed with drinking water (13.33 g/L), as previously described in the literature [19, 14]. The teas were purchased from a local supplier (Madrugada Alimentos LTDA, RS, Brazil) and were prepared daily with water boiled to 90°C, brewed for 3 min, filtered, cooled down and protected from light with aluminum foil, and were administered at an ambient temperature.

The intake volume for each day was monitored, and the mixture was analyzed by high-performance liquid chromatography (HPLC system YL9100, Young Lin, with diode array detector) to determine the presence of catechins (Table 1).

Table 1. Concentration (μg/mL) of catechins found in green tea. Data were obtained from HPLC analyses in comparison to the standard references solution.

Green tea catechins	**Concentration (μg/mL)**
(-)-Epigallocatechin (EGC)	213.68
(-)-Epicatechin (EC)	191.15
(-)-Epigallocatechin gallate (EGCG)	313.43
(-)-Epicatechin gallate (EG)	86.95

2.4. Control behavioral tests

2.4.1 Open field (OF) test

The OF apparatus consisted of a 40 x 50 x 50-cm open arena painted white, with the exception of the frontal wall which was made of glass. The floor was divided into 9 equal rectangles by black lines; line crossings were used to evaluate locomotion. The number of rearings performed by each animal was used to evaluate exploratory activity [20]. The rats were individually placed in the arena and observed during a 5-min session.

2.4.2 Elevated plus maze (EPM) test

To evaluate anxiety states, which could affect the results of the memory tests, rats were exposed to an EPM. The apparatus was located 60 cm above the ground and comprised two enclosed arms facing each other and two open arms, each measuring 50 x 10 cm. The closed arms had also 20-cm high sidewalls. The time spent and the total number of entries into the open and closed arms were recorded over a 5-min session [21].

2.4.3 Hot Plate (HP) test

To analyze the sensitivity/pain threshold of the rats, we used an HP test. This protocol consists of placing a rat on a heated metal plate and registering the time to withdrawal of the paws, and a ceiling of 20 s was imposed [22].

2.5 Memory tests

2.5.1 Object recognition short- and long-term memory

Recognition memory was evaluated through the object recognition (OR) test using a 40 x 50 x 50-cm open arena as described elsewhere [23]. All animals were habituated to the experimental arena in the absence of any specific behavioral stimulus for 20 min/day for 4 days. The objects used in the training and testing sessions consisted of metal or glass and were fixed to floor of the arena. On the first day after habituation, animals were placed in the arena containing two different objects (named A and B) and were free to explore for 5 min (training session). The rats were tested 3 h later to evaluate short-term memory (STM) and 24 h later to evaluate long-term memory (LTM). During the tests, one of the objects was removed, and a new object (C) took its place. The rat was then placed in the arena for five min. The positions of the objects (familiar or novel) were randomly permuted for each experimental animal, and the arena was always cleaned between trials. Exploration was defined as sniffing or touching the objects with the nose and/or forepaws. The time spent exploring each object was recorded by an observer masked to the treatment and expressed as a percentage of the total exploration time measured in seconds.

2.5.2 Aversive short- and long-term memory

To evaluate aversive memory, rats were trained in a single trial, step-down inhibitory avoidance (IA) task using a 50 x 25 x 25-cm plexiglass box with a 5-cm high, 8-cm wide, and 25-cm long platform on the left side of a series of electrifiable bronze bars, which made up the floor of the box. For training, rats were gently placed on the platform facing the left rear corner of the training box. When they stepped down and placed all four paws on the grid, a 2 s/0.6 mA scrambled foot shock was delivered. The tests were administered at 3 h and 24 h after training to evaluate the STM and the LTM, respectively. During the test, the rats were placed on the platform again, and the step-down latency was measured. A ceiling of 300 s was imposed on the step-down latency [24].

2.6 Biochemical testing

2.6.1 Tissue preparation

Rats from all groups were euthanized 24 h after the end of the behavioral experiments. The brain was removed, and the bilateral hippocampus was quickly dissected out and homogenized in 50 mM Tris HCl, pH 7.4 (1/5, w/v). Afterwards, samples were centrifuged at 2400 g for 10 min, and the supernatants (S1) were used for the assays.

2.6.2 Reactive oxygen species (ROS)

The ROS content was assessed with a spectrofluorimetric method using 2,7-dichlorofluorescein diacetate (DCFH-DA). The sample was incubated in darkness with 5 µl DCFH-DA (1 mM). The oxidation of DCHF-DA to fluorescent dichlorofluorescein (DCF) was measured for the detection of intracellular ROS. The formation of the oxidized fluorescent derivative (DCF), measured by DCF fluorescence intensity, was recorded at 520 nm (480 nm excitation) at 30 min after the addition of DCFH-DA to the medium. The results were expressed as the percentage of control in AU (arbitrary units) [25].

2.6.3 Lipid Peroxidation by TBARS

Lipid peroxidation was evaluated by the TBARS test [26]. An aliquot of S1 was incubated with a 0.8% thiobarbituric acid solution, acetic acid buffer (pH 3.2) and sodium dodecyl sulfate solution (8%) at 95° C for 2 h, and the color reaction was measured at 532 nm. The results were expressed as nmol of malondialdehyde (MDA) per mg protein.

2.6.4 Glutathione (GSH) levels

Glutathione (GSH) levels were determined fluorometrically. An aliquot of the homogenized sample was mixed (1:1) with perchloric acid (HClO4) and centrifuged at 3000 g for 10 min. After centrifugation, the protein pellet was discarded, and free GSH groups were determined in the clear supernatant. An aliquot of supernatant was incubated with ortho-phthalaldehyde, and fluorescence was measured at an excitation wavelength of 350 nm and an emission wavelength of 420 nm [27].

2.6.5 Ferric reducing/antioxidant power (FRAP) assay

Briefly, 50 µl S1 was added to 1.5 ml of freshly prepared FRAP reagent (300 mM acetate buffer (37°C) (pH 3.6), 10 mM HCl, 40 mM TPTZ and 20 mM FeCl3 ·6H2 at the ratio of 10: 1: 1) in a test tube and incubated for 10 min at 37°C. The absorbance of the blue

color complex was read against a reagent blank (1.5 ml of distilled water + FRAP reagent at 50°C) in 593 nm. The FRAP values were expressed as nmol of ferric ions reduced to a ferrous form/mg tissue [28].

2.6.6 Acetylcholinesterase (AchE) activity

The AChE activity was assessed by the Ellman method [29]. The reaction mixture was composed of 100 mM phosphate buffer pH (7.4) and 1 mM 5,5′-dithio-bis- 2-nitrobenzoic acid (DTNB). The method is based on the formation of a yellow anion, 4,4′-dithio-bis-acid nitrobenzoic, after adding 0.8 mM acetylthiocholine iodide. The change in absorbance was measured for 2 min at 30-s intervals at 412 nm (SpectraMax M5 Molecular Devices, CA, USA). The results were expressed as micromoles of acetylthiocholine iodide hydrolyzed/min/mg of protein. Proteins were measured according to Bradford [30] using bovine serum albumin as a standard.

2.7 Statistical analysis

Data were assessed for normality of distribution using the Shapiro-Wilk test. OR test results were expressed as the percent of total time exploration spent in each object; the results were analyzed using one-sample Student t-test, considering a theoretical mean of 50%. IA results, expressed as the platform step-down latency in seconds, were analyzed using intra-group Wilcoxon test to compare training *vs.* test. OF, EPM and HP tests results were analyzed using ANOVA. Biochemical results were compared using two-way ANOVA using Bonferroni correction for multiple comparisons. The differences were considered statistically significant at $P < 0.05$.

3. Results

3.1 Animal Weight and Fluid intake

The weight of the rats was measured each month, and we noted that weight gain was similar among all groups ($P > 0.05$; one-way ANOVA; data not shown). The fluid intake was also similar among all groups ($P > 0.05$; one-way ANOVA; data not shown).

3.2 Control behavioral tasks

We performed control behavioral tests to verify that maternal deprivation and green tea intake did not change important behavioral parameters that could affect the results of the

memory tasks. No changes in exploratory and locomotor activity, anxiety or pain sensitivity were observed (Table 2).

Table 2. MD and green tea supplementation did not affect the locomotor and exploratory activities (Open field), anxiety (Plus maze) and pain sensibility (Hot plate) (Two-way ANOVA; data expressed for mean ± SEM).

Behavioral task		Groups			
		Control	Green Tea	MD	MD + GT
Open Field	Crossings	62.2 ± 28.3	71.3 ± 38.7	72.0 ± 34.4	62.5 ± 48.6
	Rearings	20.0 ± 10.6	21.0 ± 15.6	19.0 ± 8.5	18.0 ± 12.1
Plus maze	Time in open arms (s)	58.2 ± 34.7	76.3 ± 20.9	68.8 ± 20.0	63.3 ± 37.8
	Entries in open arms	13.9 ± 6.7	17.0 ± 8.1	13.3 ± 6.7	15.6 ± 8.6
Hot plate	Time (s)	11.1 ± 2.5	10.6 ± 3.8	10.3 ± 1.9	8.7 ± 2.1

3.3 Memory evaluation

3.3.1 Object recognition memory

In the OR training, all rats explored each of the two new objects for a similar percentage of the total exploration time (approximately 50% for each object, Figure 2A and 2B, $P > 0.05$). At 3 h after training in the STM testing session, control rats explored the novel object (C) significantly more than 50% of the total exploration time ($P = 0.002$, Figure 2A/control). However, the MD rats spent approximately 50% of the total exploration time exploring each object ($P = 0.91$, Figure 2A/deprived). The rats supplemented with GT explored the novel object (C) significantly more than 50% of the total exploration time ($P = 0.03$, Figure 2A/green tea). GT did not prevent the recognition STM deficit associated with MD since the MD and supplemented rats spent approximately 50% of the total exploration time exploring each object ($P = 0.224$, Figure 2A/deprived + green tea).

In the LTM test at 24 h after training, control rats explored the novel object (D) significantly more than 50% of the total exploration time ($P = 0.002$, Figure 2B/control). However, the MD rats spent approximately 50% of the total exploration time exploring each object ($P = 0.90$, Figure 2B/deprived). The rats supplemented with GT explored the novel object (D) significantly more than 50% of the total exploration time ($P = 0.009$, Figure 2B/green tea). GT prevented the memory deficits associated with MD since the supplemented rats were able to distinguish between the familiar object and the new object ($P = 0.0009$, Figure 2B/deprived + green tea).

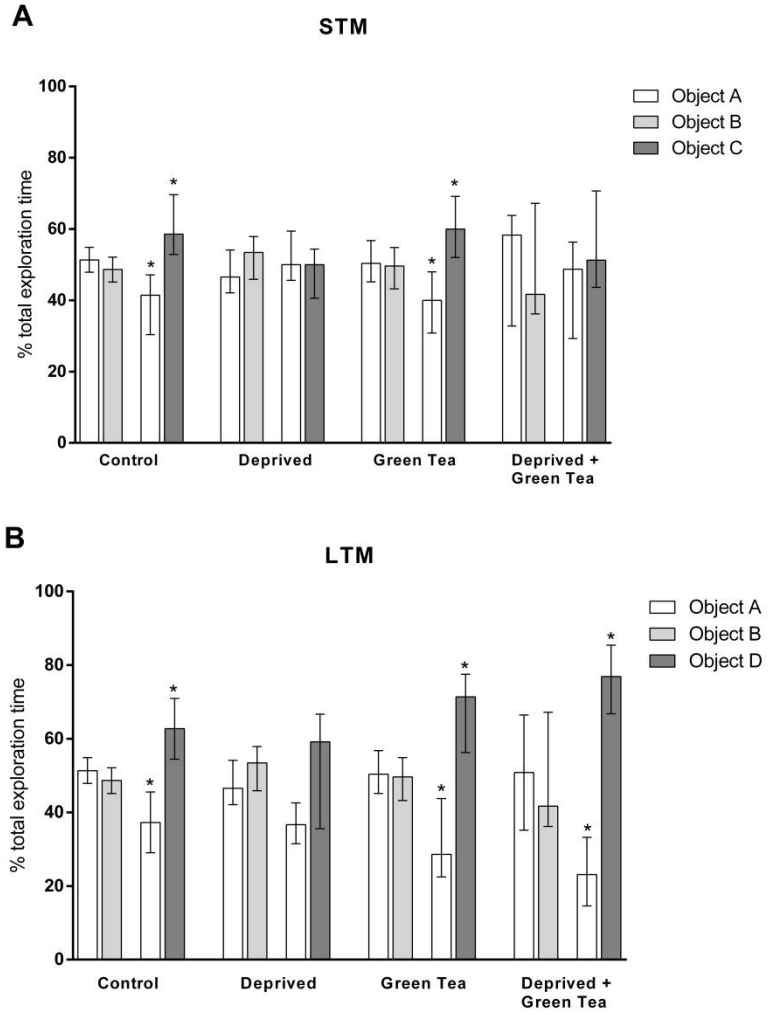

Fig 2. Effects of MD and GT supplementation on short and long-term memory evaluated on object recognition task. Maternal deprivation impairs short and long-term OR memory. GT prevents long-term OR memory deficit. Animals were exposed to two different objects (A and B) for 5 min in a training session. In the test session rats were exposed to a familiar object (A) and to a novel object (C) during 5 min. A. Short-term memory was measured 3h after the training. B. Long-term memory was measured 24h after the training. Data (mean ± SEM) are presented as the percentage of total exploration time. * $P \leq 0.05$, Student t-test, considering a theoretical mean of 50%.

3.3.2 Aversive memory

In the IA training, all rats had a low step-down latency time (Figure 3A and 3B). At 3 h after training in the STM testing session, control rats exhibited a significant increase in the latency time when compared to the test latency (P = 0.001, Figure 3A/control). However, the MD rats did not exhibit this increase (P = 0.80, Figure 3A/deprived). On the other hand, the rats supplemented with GT showed an increase in the latency time (P = 0.03, Figure 3A/green tea), and GT was able to prevent the short-term memory deficit caused by DM since the

deprived rats supplemented with GT exhibited an increase in the latency time (P = 0.02, Figure 3A/deprived + green tea).

In the LTM test performed at 24 h after the training, control rats showed a significant increase in the step-down latency time (P = 0.0001, Figure 3B/control). The MD rats did not exhibit this increase (P = 0.07, Figure 3B/deprived). However, the rats supplemented with GT showed an increase in the latency time (P = 0.01, Figure 3B/green tea), and GT was able to prevent the long-term memory deficit caused by MD since the deprived rats supplemented with GT exhibited an increase in the latency time (P = 0.0005, Figure 3B/ deprived + green tea).

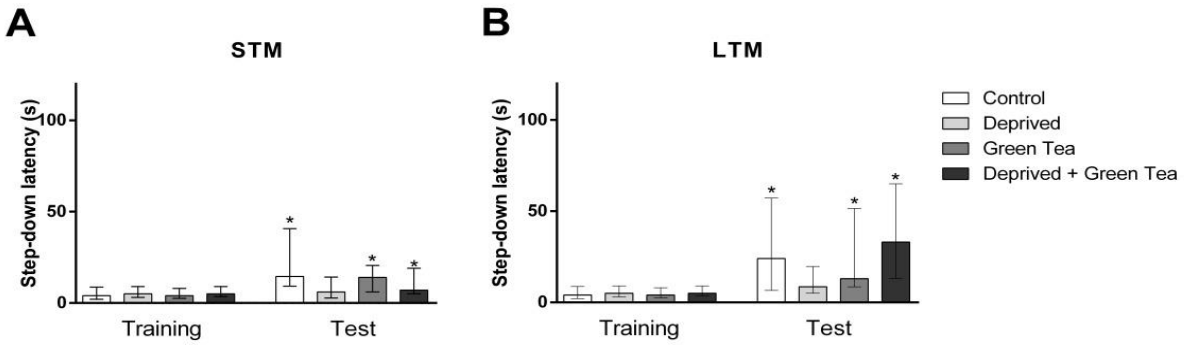

Fig 3. Effects of MD and GT supplementation on short and long-term aversive memory evaluated in IA task. Maternal deprivation impairs short and long-term aversive memory. Green tea administration avoids these deficits. Rats were trained in IA and 3h (A) or 24h (B) after were tested. Bars represent median ± interquartile range of step-down latencies. * $P \leq 0.05$ Wilcoxon test to compare training *vs.* test.

3.4 Biochemical testing

3.4.1 Reactive oxygen species (ROS) and lipid peroxidation

Maternal deprivation caused an increase in reactive oxygen species (Figure 4A/DCFH; P = 0.001), which was prevented by green tea supplementation. There were no differences in the levels of lipid peroxidation between the groups (Figure 4B/TBARS; P = 0.22).

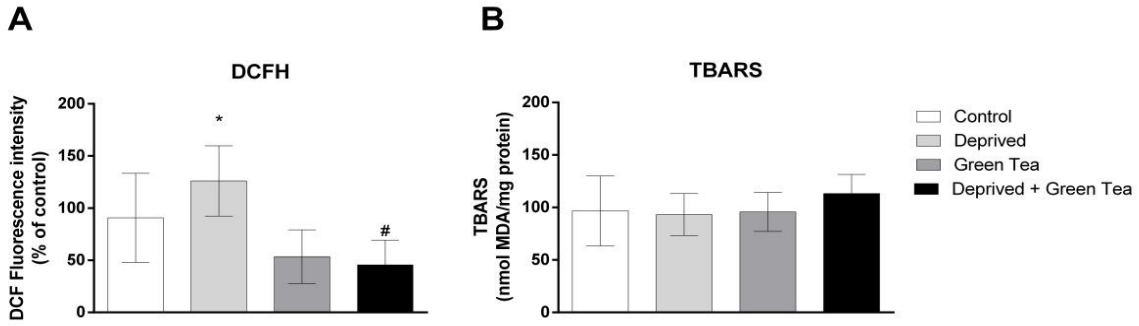

Fig 4. Effects of MD and GT supplementation on hippocampal pro-oxidant markers. MD promotes increase of reactive oxygen species and GT avoid this increase. The interventions had no effects on oxidative damage (lipid peroxidation). A. Reactive oxygen species (ROS) levels in hippocampus measured by DCFH. B. Lipid peroxidation in the hippocampus assessed by TBARS assay. * $P \leq 0.05$, Deprived vs. control group. $^{\#}$ $P \leq 0.05$, deprived vs. deprived + green tea group (Two-way ANOVA followed by Bonferroni post hoc test).

3.4.2 GSH and Ferric reducing/antioxidant power (FRAP) assay

Maternal deprivation and green tea supplementation promoted a decrease in GSH levels ($P < 0.05$; Figure 5A). Maternal deprivation did not alter the total antioxidant capacity ($P > 0.05$), but when associated with MD green tea increased the total antioxidant capacity ($P = 0.0043$; Figure 5B).

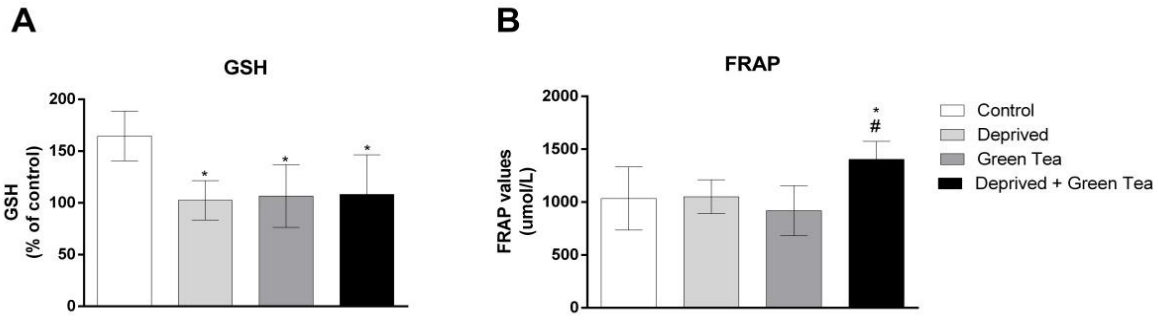

Fig 5. Effects of MD and GT supplementation on hippocampal antioxidant markers. Maternal deprivation and green tea promote decrease of GSH levels compared with control group. The association of MD and green tea supplementation promotes the increase of total antioxidant capacity (FRAP). A. GSH levels. B. Total antioxidant capacity measured by FRAP. * $P \leq 0.05$ (specific group in comparison to control). $^{\#}$ $P \leq 0.05$ (specific group in comparison to deprived group) (Two-way ANOVA followed by Bonferroni post hoc test).

3.4.3 Acetylcholinesterase

No changes in hippocampal AchE activity were detected (Figure 6; P = 0.94).

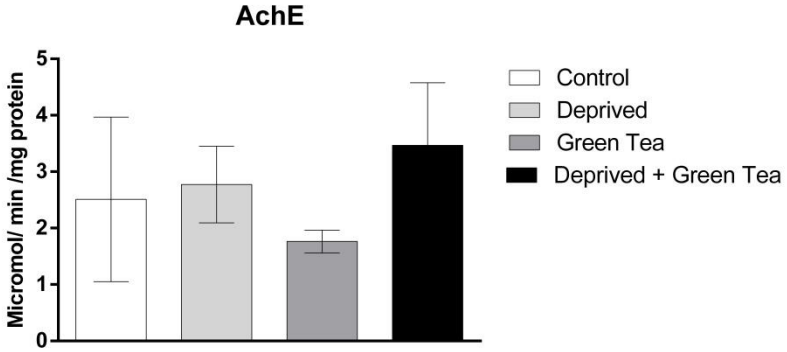

Fig 6. Effects of MD and GT supplementation in acetylcholinesterase (AchE) activity. Maternal deprivation and green tea supplementation had no effect in acetilcholinesterase activity. P = 0.94 in a two-way ANOVA.

4. Discussion

Our results demonstrate that green tea treatment is able to prevent recognition and aversive memory deficits related to maternal deprivation. In addition, GT circumvents increased ROS and improves, at least partially, the antioxidant defenses. According our best knowledge, this is the first investigation regarding the use of an exogenous antioxidant to prevent memory deficits related to MD.

In a previous study [31], our group demonstrated that MD causes deficits in both short- and long-term memory in different tasks, and that these deficits could be related to brain oxidative status since a disruption in the oxidative balance was detected, as confirmed here. Some previous studies have shown a relationship between memory deficits and oxidative stress [12, 14], indicating that the imbalance of antioxidant and pro-oxidant markers causes oxidative stress and damage in important regions of the central nervous system related to learning and memory, such as the hippocampus [10]. Similar work also demonstrated that certain types of stress in childhood can increase ROS in several tissues, such as the brain, and affect brain development, which makes the brain more susceptible to neurodegenerative diseases [32].

Considering the oxidative stress in the brain, several strategies have been investigated in different models of brain injury to find effective treatments that can block the consequences of neuronal oxidative stress. In a study performed by Zamini et al. [33], the authors showed that olive oil exerted protective effects in the CA1 region of the hippocampus by preventing

increased oxidative stress after an ischemia-reperfusion injury in the brain. In regard to green tea, Xu et al. [34] showed that the use of green tea improved not only the oxidative imbalance caused by cerebral ischemia but was also exerted beneficial effects through the modulation of anti-inflammatory levels. The use of antioxidant agents to prevent memory deficits related to oxidative stress is very common and is not restricted to natural compounds with antioxidant activities. In a previous work, our group demonstrated that aerobic exercise on a treadmill can reverse memory deficits related to MD and oxidative stress and associated damage in the hippocampus and prefrontal cortex [31]. Physical exercise is an interesting and well-known neuroprotective strategy, but it is important to consider that exercise requires time and public adhesion (i.e., the cooperation of patients, in the case of human), which suggest that physical exercise is not always the best strategy. On the other hand, green tea is cheap and easily accessible, and its inclusion in the diet does not demand very significant lifestyle changes.

It is important to consider that there are many mechanisms that can explain the deficits induced by maternal deprivation. Although some studies place a strong emphasis on the proliferation of cells in the hippocampus after oxidative stress [35], the increased production of ROS, altered antioxidant enzyme activities, and the increased DNA breaks index [36] after maternal deprivation are probably not the only mechanisms involved. Here, GT was able to partially prevent short-term memory deficits and completely block long-term memory deficits in recognition and aversive memory tasks. We only investigated the mechanisms related to oxidative stress since the antioxidant properties of GT exert the most pronounced effects. MD increased ROS levels, and GT prevented this increase. Additionally, MD decreased GSH, and although GT was not able to block the decrease in GSH in the MD rats, GT still increased the total antioxidant capacity. Thus, our results showed that the use of an exogenous antioxidant, GT, prevents oxidative damage resulting from maternal deprivation. Most likely, the antioxidant effects of GT are not the only mechanism by which this tea can prevent memory deficits since anti-inflammatory, anti-apoptotic, and anti-autophagy effects of GT were reported by others [37, 38] and could be involved in the effects observed here.

5. Conclusion

Green tea from *Camellia sinensis* protects against memory deficits related to maternal deprivation. The mechanisms involved in this effect seem to be related to the antioxidant properties of the tea since tea consumption prevented increased ROS and promoted enhancement of the total antioxidant capacity in the hippocampus of MD rats.

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Artigo 3

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MATERNAL DEPRIVATION DISRUPTS MEMORY CONSOLIDATION AND RECONSOLIDATION. ENVIRONMENTAL ENRICHMENT PROMOTES NEUROPROTECTION

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Abstract

Early-life stress causes a variability of deficits in central nervous system, because in this time the process of brain developmental is more intense. The maternal deprivation causes a deficit which will persist until adulthood life. Thereby, the environmental enrichment in rats is widely used to give stimuli and increases brain plasticity. Here we demonstrated that maternal deprivation causes memory deficits and that environmental enrichment (EE) acts as protector factor to these deficits. Pregnant female rats were used. Littermates are submitted to neonatal maternal deprivation from day 1 to 10 post-partum, and after weaning to EE. The maternal deprivation causes deficits on memory consolidation and reconsolidation and promotes the decrease of hippocampal BDNF levels. Additionally, the EE is able to protect against these deficits, reversing memory disrupting and normalizing BDNF levels in hippocampus of adult male rats.

Keywords: Recognition memory; Aversive memory; Spatial memory; BDNF.

1. Introduction

The development of mammals brain begins in utero and continues after being born, until adulthood. So, any kind of stress can affect directly the process of brain developmental and can cause various neurophysichiatric disorders, which will persist until adult life, such as, schizophrenia, depression and deficits in learning and memory (Aksu et al., 2012; Uysal et al., 2005).

Maternal deprivation is considered one of the most potent naturally occurring stressors that rats can go through during the neonatal period (Talge et al., 2007). Some authors, such as (Macri et al., 2011) have described that long periods of maternal deprivation in rodents are often used to mimic the stress caused in the infancy of humans, leading to similar impacts on neural, hormonal, and behavioral systems.

One of the main structures involved on learning and memory processes is the hippocampus, which shows a remarkable degree of structural and functional plasticity, and is particularly sensitive to stress. This structure continues to develop after birth and may be the brain region that is most vulnerable to the effects of chronic stress, that affect some process of memory, including short-term and long-term memory consolidation and, probably, reconsolidation (Benetti et al., 2009; Vivinetto et al., 2013).

Thereby, the environmental enrichment (EE) increases thickness of the cerebral cortex, as well of dentritic arborization, neurogenesis and long-term potentiation in rats (Francis et al., 2002). Some studies that investigate the effects of EE in rats that were exposed to MD demonstrated that the EE is able to reverse behavioral and neurochemical deficits,

promoting changes in glucocorticoids receptor cells (Hutchinson et al., 2012; Vivinetto et al., 2013). The EE also increases brain plasticity at the structural level, producing functional neurophysiological and memory enhancement (Leal-Galicia et al., 2008), probably by acting on a molecular cascade that culminates in the increase of brain derived neutrophic factor (BDNF) levels, an important neuronal growth factor, also important in the formation and consolidation memory processes (Bennett et al., 2006).

Here we demonstrate that maternal deprivation causes deficits on memory consolidation and reconsolidation and promotes the decrease of hippocampal BDNF levels. Additionally, the EE is able to protect against these deficits, reversing memory disrupting and normalizing BDNF levels in hippocampus.

2. Material and methods

2.1 Animals and experimental design

Pregnant female Wistar rats were obtained from Central Vivarium of Federal University of Santa Maria (RS/Brazil). All animals were maintained on a constant 12 h light/12 h dark cycle (lights on at 7:00 h) at controlled room temperature (22 ± 2 °C), and air humidity (at 60 ± 10%). Pregnant female were individually housed with sawdust bedding with food and water *ad libitum*. The day of delivery was considered as day zero. At postnatal day 1 (PND-1) MD protocol was initiated and was performed until PND-10 with male and female pups. Animals were weaned at the age of 21 days (PND-21), and housed in special large cages, 10 per cage. Only the males were considered for the follow experiments. Females were donned to use in others ongoing projects. All experiments were conducted in accordance with the principles of laboratory animal care (NIH publication nº 80-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of the Local Institution (protocol 14/2015).

The male rats were divided in four groups, with 8 to 12 rats each group: (i) control, which rats were not submitted to any intervention; (ii) deprived, including those rats submitted to MD, as will be described below, without any additional intervention; (iii) environmental enrichment group, which the rats were submitted to EE, as will be described below, from PND-21 to PND-80; and (iv) deprived more environmental enrichment, which the rats were submitted to MD and to EE.

All rats were submitted to behavioral tests starting on PND-81 as follow: Open Field; Elevated Plus Maze; Tail Flick; Object Recognition; Inhibitory Avoidance; and, Morris Water Maze. After behavioral testing, the rats' brains were isolated and brain tissues dissected

(hippocampus) to be used in the biochemical tests. The figure 01 summarizes the experimental design.

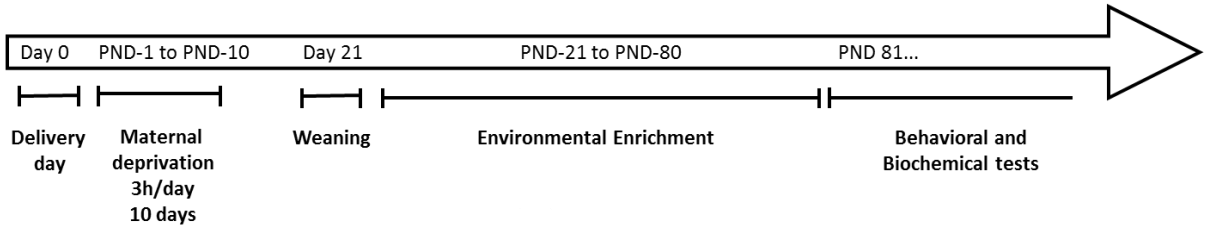

Fig. 1. Experimental design. Day 0 was considered the day of the rats' born. On PND-1 the MD protocol was initiated with half of the animals. Subsequently, each group was subdivided in two subgroups, with and without exposure to an EE. On PND-81 the memory and control behavioral tests were started, following by the biochemical testing.

2.2 Maternal Deprivation

Female Wistar rats were kept in individual boxes until the delivery day (considered as day 0). Rats from groups (ii) and (iv) were submitted to maternal deprivation (MD) for 3 hours per day from PND-1 to PND-10, every time during the light part of the cycle (09:00h a.m. to 15:00h p.m.). MD protocol consisted in removing the mother from the residence box to other room. Pups were kept in their home cage and while the mothers were absent; the room temperature was raised to 32°C to compensate the mother's body heat absence. At the end of each daily deprivation session, the mothers were returned to their home boxes (Benetti et al., 2009). Rats from groups (i) and (iii) remained in their resident boxes together with their mothers during the first ten days of life. Only on PND-11 the boxes were cleaned normally again, according to laboratory routine. On PND-21 the animals were weaned, and males were maintained in groups of 10 in large plastic boxes with food and water *ad libitum (Benetti et al., 2007).*

2.3 Environmental Enrichment

The environmental enrichment (EE) was conducted in an adapted cage, large than standard rats' housing boxes (25 x 60 x 35 cm). After the weaning day (PND-21) the rats are housed in these cages, with or without stimulating objects. In the groups submitted to EE, in which boxes were objects, the objects are frequently changed (every 15 days). Activities wheels were not included in the EE. Plastic tubes, little balls, sound objects and wooden houses were utilized (Sampedro-Piquero et al., 2013).

2.4 Control behavioral tests

2.4.1 Open field (OF)

The OF apparatus consisted of a 40 x 50 x 50 cm open arena painted white, except for the frontal wall which was made of glass. The floor was divided into 9 equal rectangles by black lines; crossings of the lines were used to evaluate locomotion. The number of rearing performed by each animal was used to evaluate the exploratory activity. The rats were individually placed in the arena and observed during 5 min (Bonini et al., 2006).

2.4.2 Elevated plus maze (EPM)

To evaluate anxiety state, which could affect the results of memory tests, rats were exposed to an EPM. The apparatus was located 60 cm above the ground and presented two enclosed arms face each other and two open arms, each measuring 50 x 10 cm. The closed arms had also side walls 20 cm high. The time spent and the total number of entries into the open and closed arms was recorded by infrared sensors over a 5 min session (Pellow et al., 1985).

2.4.3 Tail Flick (TF)

To analyze sensibility/pain threshold of the rats we used a TF test. This test consists in to put the rat in the apparatus with the tail on the metal rod and register the time spent for the movement of withdrawal of the tail of the metal rod. A ceiling of 12s was imposed (Tjolsen et al., 1989).

2.5 Short and long-term memory consolidation assessment

2.5.1 Object Recognition test

Recognition memory was evaluated through the object recognition (OR) test using a 40 x 50 x 50 cm open arena. All animals were habituated to the experimental arena in absence of any specific behavioral stimulus for 20 min/day during 4 days. The objects used in training and testing sessions were made of metal or glass, and were fixed to arena's floor. On the first day after habituation animals were placed in the arena containing two different objects (named A and B), free to explore during 5 min (training session). The rats were tested 3h later to evaluate short-term memory (STM) and 24h later to evaluate long-term memory (LTM). In the tests, one of the objects was removed and a new object (C) replaced it. The rat was then introduced in the arena for five minutes. Position of objects (familiar or novel) was randomly permuted for each experimental animal, and the arena was always cleaned between trials.

Exploration was defined as sniffing or touching the objects with the nose and/or forepaws. Time spent to explore each object was recorded by an observer blind to the treatment and expressed as a percentage of total exploration time computed in seconds (Mello-Carpes and Izquierdo, 2013)

2.5.2 Inhibitory Avoidance test

To evaluate the aversive memory rats were trained in a one-trial step-down inhibitory avoidance (IA) task using a 50 x 25 x 25 cm plexiglass box with a 5 cm-high, 8 cm-wide, and 25 cm-long platform on the left end of a series of electrifiable bronze bars, which made up the floor of the box. For training, rats were gently placed on the platform facing the left rear corner of the training box. When they stepped down and placed their four paws on the grid, a 2 s/0.6 mA scrambled foot shock was delivered. The tests were realized 3h and 24h after training, to evaluate the STM and the LTM, respectively. In the test rats were placed in the platform again and the step-down latency was measured. A ceiling of 300s to the step-down was imposed (Rossato et al., 2009).

2.6 Memory reconsolidation assessment

2.6.1 Morris Water Maze (MWM) test

To evaluate the memory reconsolidation we used the spatial memory paradigm MWM. The rats were trained in the water maze that is a black circular pool (200 cm in diameter) conceptually divided into four equal quadrants shown in the screen of computer for the purpose of recorder of data analysis by the software. A black circular platform (12 cm in diameter) was inserted two centimeters beneath the surface of the water and hidden from the rat's view in a specific quadrant. The water temperature was kept between 21 and 23°C. The water maze was located in a well-lit white room with several visual stimuli hanging on the walls to provide spatial cues and the lightning of the room was 60 lux projected in opposite the pool in the direction of spatial cues. The swimming path of the animals was recorded using a video camera mounted above the center of the pool and analyzed using a video tracking and analysis system. Training using the spaced training protocol was carried out during 5 successive days. On each day, rats received eight consecutive training trials, during which the hidden platform was kept in a constant location. A different starting location was used on each trial, which consisted of a swim followed by a 30-s platform sit. Any rat that did not find the platform within 60 s was guided to it by the experimenter (Da Silva et al., 2013). Rats were trained in the spatial version of the MWM during 5 d as stated above, and 24 h after

the last training session were submitted to eight 60-sec reversal trials in which the platform was placed in the opposite quadrant of the pool. Memory retention was evaluated in a probe test carried out 24h after the last reversal trial (Rossato et al., 2006)

2.7 Immunoblot essays to access BDNF protein expression

Animals were killed by decapitation and the hippocampus was rapidly dissected out and homogenized in ice-chilled buffer (20 mM Tris-HCl, pH 7.4, 0.32 M sucrose, 1 mM EDTA, 1mM EGTA, 1mM PMSF, 10 µg/ml aprotinin, 15 µg/ml leupeptin, 10 µg/ml bacitracin, 10 µg/ml pepstatin, 15 µg/ml trypsin inhibitor, 50 mM NaF, and 1 mM sodium orthovanadate). Protein concentration was determined using the BCA protein assay (Pierce, Rockford, IL), and equal amounts of protein were fractionated by SDS-PAGE before electrotransferred to polyvinylidene difluoride membranes (PVDF; Immobilon-P, Millipore, MS). After verification of protein loading by Ponceou S staining, the blots were blocked in Tween-Tris buffer saline (TTBS; 100 mm Tris-HCl, pH 7.5, containing 0.9 % NaCl and 0.1 % Tween 20) and incubated overnight with anti-BDNF antibody (N20, 1:5,000, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-β-actine antibody (1:1,000, Sigma-Aldrich, St Louis, MO; control). The membranes were washed in TTBS and incubated with HRP-coupled anti-IgG antibody, washed again, and the immunoreactivity detected using a West-Pico enhanced chemiluminescence kit (Pierce, IL). Densitometric analysis was carried out in an ImageQuant RT-ECL system (GE, Piscataway, NJ).

2.8 Statistical analysis

Data were checked for normality of distribution using Shapiro-Wilk test. OR test results were expressed as the percent of total time exploration spent in each object; the results were analyzed using one-sample Student t-test, considering a theoretical mean of 50%. IA results, expressed as the platform step-down latency in seconds, were analyzed using an intra-group Wilcoxon test to compare training *vs.* test results. OF, EPM and TF tests results were analyzed using ANOVA. Biochemical results were compared using two-way ANOVA using Bonferroni correction for multiple comparisons. The differences were considered statistically significant when $P < 0.05$.

3. Results

3.1 Animals' weight

The rats' weight was measured each month and we note that the weight gain was similar in all groups ($P > 0.05$; one way ANOVA; data not shown).

3.2 Control behavioral tests

We performed behavioral control tests to verify if the maternal deprivation or the exposure to environmental enrichment does not change some important behavioral parameter that could affect memory tasks results. No alteration on exploratory and locomotor activity, anxiety behavior or pain sensitivity was observed (table 1).

Table 1. Control behavioral tasks. MD and EE exposition did not affect the locomotor and exploratory activities (Open field), anxiety behavior (Elevated Plus maze) and pain sensibility (Tail Flick) (Two-way ANOVA; data expressed for mean ± SEM).

Behavioral task		**Groups**			
		Control	**MD**	**EE**	**MD + EE**
Open Field	Crossings (n)	35.2 ± 12.4	32.1 ± 14.2	28.7 ± 15.4	34.5 ± 16.3
	Rearings (n)	29.0 ± 11.6	26.6 ± 12.4	22.9 ± 14.2	31.2 ± 13.7
Elevated Plus Maze	Time in open arms (s)	54.1 ± 3.9	39.4 ± 9.2	41.2 ± 9.6	40.1 ± 6.9
	Entries in open arms (n)	13.3 ± 4.9	11.9 ± 6.4	19.1 ± 5.3	21.3 ± 4.1
Tail Flick	Time (s)	3.1 ± 2.3	2.2 ± 1.4	2.8 ± 1.3	2.6 ± 1.1

3.3 Short and long-term memory consolidation

In the OR training, all the rats explore for a similar percent of total exploration time the two new objects (about 50% each, figure 2A and 2B, $P > 0.05$). 3 h after training, in the STM (figure 2A) testing session, control rats explored the novel object (C) significantly more than 50% of total exploration time ($P = 0.0016$, figure 2A/control). However, the MD rats spent about 50% of the total exploration time exploring each object ($P = 0.06$, figure 2A/deprived). The rats that exposure to EE explored the novel object (C) significantly more than 50% of total exploration time ($P = 0.0004$, figure 2A/EE). The EE avoided recognition STM deficit related to MD, since the MD rats submitted to EE spent more than 50% of the total exploration time exploring the new object ($P = 0.0001$, figure 2A/deprived + EE).

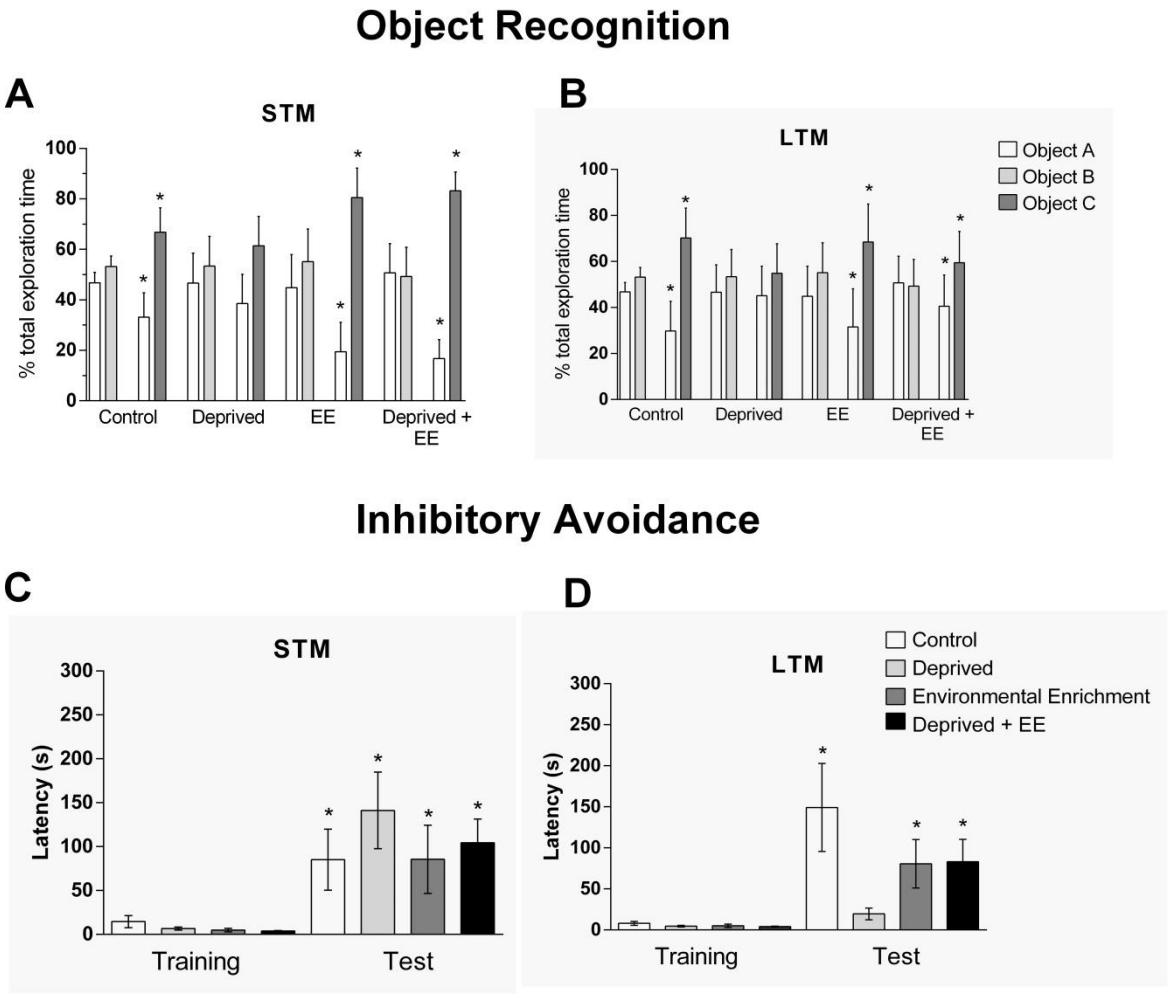

Fig. 2. Effects of MD and EE on short and long-term memory consolidation evaluated on object recognition (A and B) and inhibitory avoidance test (C and D). Maternal deprivation impairs short and long-term OR memory and long-term IA memory. EE prevents OR and IA memory deficits. Data from OR (mean ± SEM) are presented as the percentage of total exploration time. * $P \leq 0.05$, Student t-test, considering a theoretical mean of 50%. On IA bars represent median ± interquartile range of step-down latencies. * $P \leq 0.05$ Wilcoxon test to compare training *vs.* test to IA task.

In the test of LTM, 24h after training, control rats explored the novel object (C) significantly more than 50% of total exploration time (P = 0.0031, figure 2B/control). However, the MD rats spent about 50% of the total exploration time exploring each object (P = 0.31, figure 2B/deprived). The rats that were exposed to EE explored the novel object (C) significantly more than 50% of total exploration time (P = 0.002, figure 2B/EE). The EE avoided memory deficits related to MD, since the rats submitted to EE were able to distinguish the familiar object from the new object (P = 0.034, figure 2B/deprived + EE).

In the IA training, all the rats present a low step-down latency time (figure 2C and 2D). 3h after training, in the STM testing session, rats from all groups significantly increased

their latency time when compared to training ($P < 0.05$, figure 2C). In the LTM test, performed 24h after the training, control rats had a significantly increase in the step-down latency time ($P = 0.03$, figure 2D/control). The MD rats did not present this increase ($P = 0.06$, figure 2D/deprived). However, the rats submitted to EE presented an increase in the latency time ($P = 0.031$, figure 2D/EE) and the EE was able to prevent the long-term memory deficit caused by MD, since the deprived rats submitted to EE after the MD presented an increase in the latency time ($P = 0.001$, figure 2D/MD + EE).

3.4 Memory reconsolidation

During the spatial memory consolidation all the animals were able to learn (observe the latencies to find the platform on MWM over the 5 training days on Fig 3A). After, in the reversal training, the animals were trained to find the platform in a different position (memory reconsolidation). In the reconsolidation test different parameters of learning were analyzed.

The time spent to find the place where the platform was allocated during reversal training was higher for the deprived animals (aim is considered the first place of the platform, used during the 5 days of consolidation training; reversed is considered the novel place of the platform, used during the reversal/reconsolidation training), but MD animals exposed to EE shown similar latencies to controls ($P < 0.01$, fig 3B). The deprived rats spent a similar time in the pool quadrant where the platform was during the consolidation training days (aim) and in the pool quadrant where the platform was during the reconsolidation training days (reversed); however, the MD animals exposed to EE spent more time in the reversed quadrant, as the controls ($P < 0.01$, fig 3C). Regarding the number of crossings in the place where the platform was during consolidation (aim) and reconsolidation (reversed) training, the deprived rats crossed a similar number of times in each one; MD rats exposed to EE, as the control groups, crossed more times the place where the platform was in the reversal training ($P < 0.01$, fig 3D). The distance swim in each quadrant (aim and reversed) was also similar in the deprived rats; MD rats submitted to EE, on the other hand, swim a higher distance in the reversed quadrant, as the control groups ($P < 0.01$, fig 3E). All these differences are should be related to memory reconsolidation, as the swimming velocity was not different between all the groups ($P > 0.01$, fig 3F).

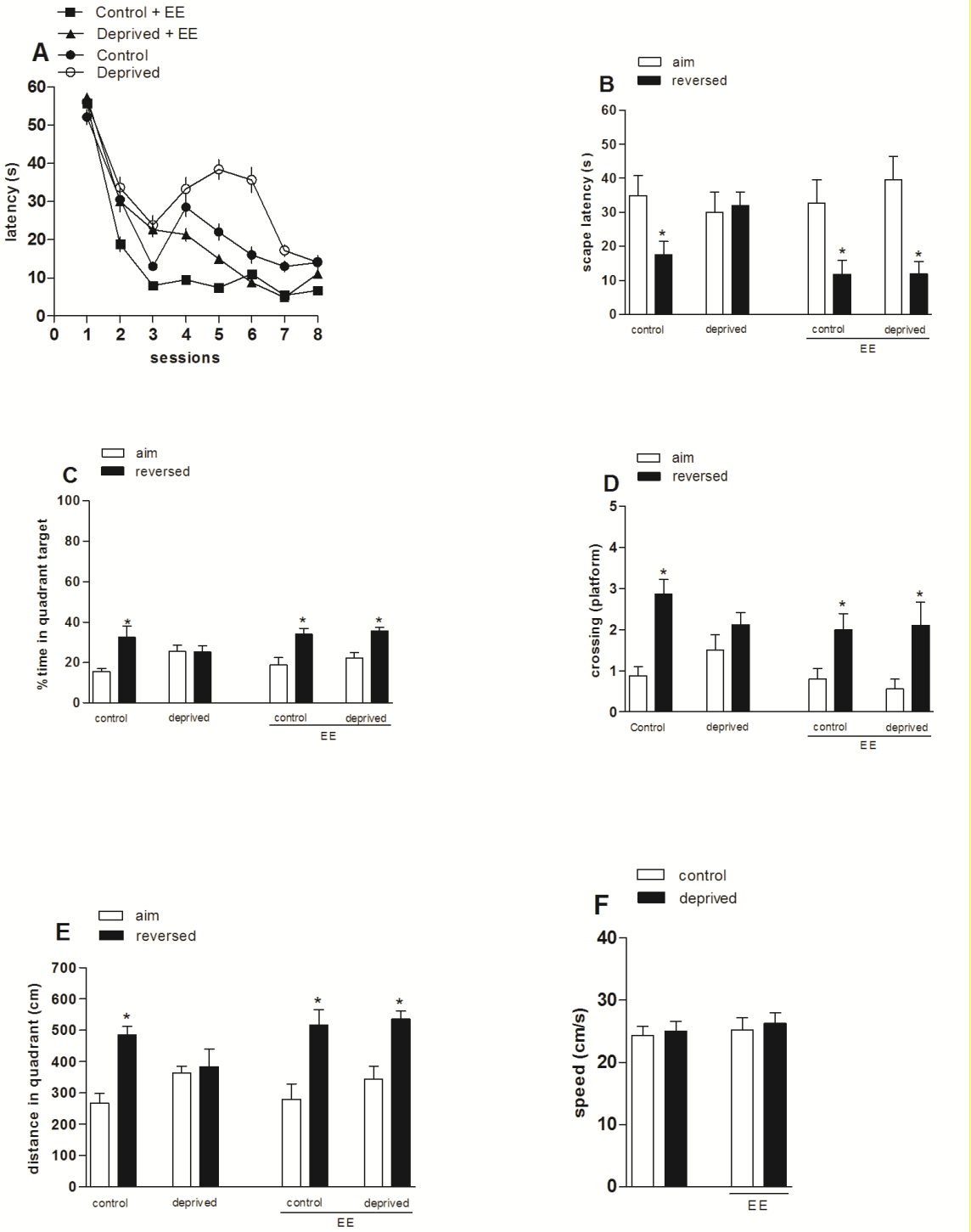

Fig 3. Effects of MD and EE on the spatial memory reconsolidation. Animal were trained an evaluated on Morris Water Maze. MD impairs memory. During the consolidation phase (first five days) male rats were trained to swim by different position of the pool to find the platform (aim). On the day sixth, animal were trained to find the platform moved to opposite position of the pool (reversed). Deprived animals shown memory impairment. Spatial memory deficit was prevented when deprived animals were exposed to EE immediately after weaning until adulthood. Data from MWM are presented as mean ± SEM. * $P \leq 0.05$, two-way ANOVA followed by Dunn's pot hoc.

3.5 BDNF expression

To determine the involvement of the BDNF in the deficits caused by MD and protection caused by EE we performed a western blot analyzes. As control, we measured β-actin levels. BDNF protein expression was significant less in the deprived rats when compared to controls (P = 0.041, figure 4B). The EE maintained the BDNF levels similar to control group, even in the group previously submitted to MD (P > 0.05). β-actin protein expression was similar in all groups (P > 0.05 for each group compared to the control group).

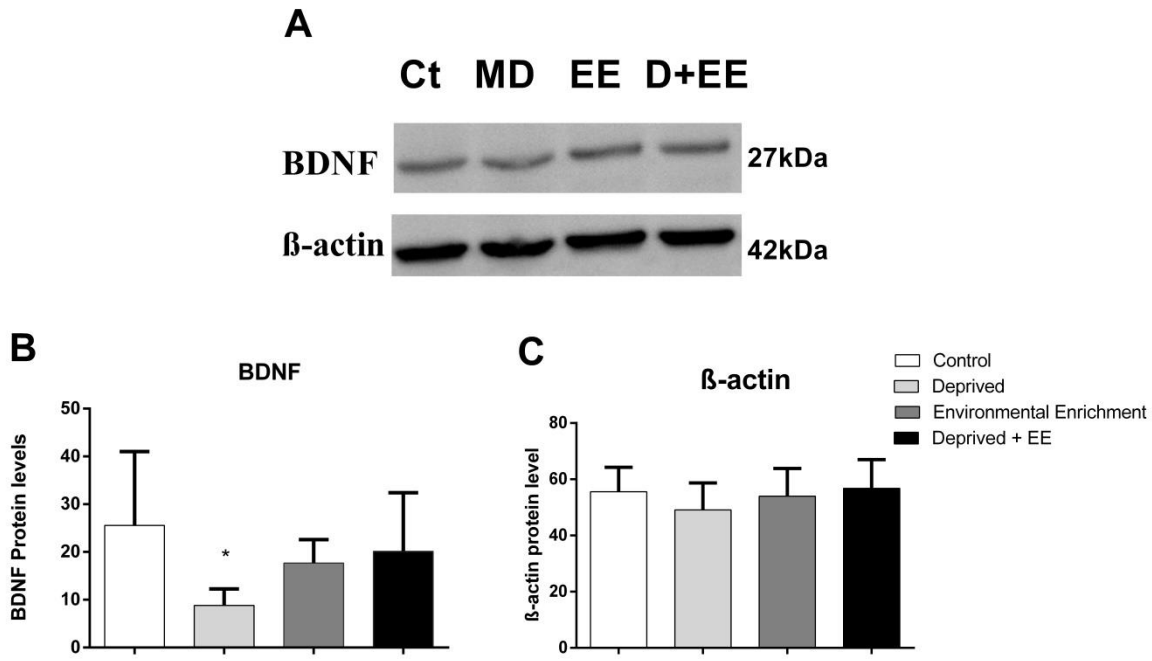

Fig. 4. Effects of MD and EE on hippocampus expression of BDNF. The MD decreases the BDNF protein levels and the EE prevent this. BDNF levels are measured by western blot analyzes. A. Illustrative image of membranes. Ct = Control; MD = Maternal Deprivation; EE = environmental enrichment and D+EE = Maternal Deprivation + environmental enrichment B. Representative graphs for BNDF. C. Representative graphs for β-actin. (* $P \leq 0.05$ Two-way ANOVA followed by Bonferroni post hoc test).

4. Discussion

Our results show that MD causes deficits on memory consolidation and reconsolidation, promoting a decline in hippocampal BDNF protein levels. Additionally, the EE is able to prevent memory deficits originating by MD, reversing the BDNF decrease.

The memory consolidation deficits caused by MD are already well described in the literature by us and others (Bian et al., 2015). These deficits are related to alterations

promoted by MD in specific neurotransmitters systems (Benetti et al., 2015), oxidative status (Neves et al., 2015), and expression of plasticity related proteins, as BDNF (Reus et al., 2013) and CREB (Shu et al., 2015). However, at least according our best knowledge, this is the first work that relates the effects of MD on memory reconsolidation. Reconsolidation is a protein synthesis-dependent process whereby memories become again vulnerable to changes by pharmacological or environmental agents (Bonini et al., 2007), being essential to guarantee memory persistence. Reconsolidation seems also to depend on different proteins, including BDNF (Radiske et al., 2015).

Shu et al. demonstrated that childhood neglect, as infancy maternal separation, may decrease the neurobehavioral plasticity, thereby blunting the responses to adulthood stress and increasing the susceptibility to depression (Shu et al., 2015). Reus GZ et al. (2013) show that the BDNF levels are altered in various regions of brain, such as, amygdala, prefrontal cortex and hippocampus, when the rats are exposed to a model of depression. In humans, parental maltreatment or neglect or familiar strife can also compromise cognitive development (Van Voorhees and Scarpa, 2004).

On the other hand, our results highlight the potential of EE to prevent or avoid cognitive deficits, in this case, related to MD. Some authors show the EE is able to prevent age-related recognition memory deficits and increase neurogenesis and synaptic markers in the hippocampus (Leal-Galicia et al., 2008). EE also causes sprouting and synaptogenesis and are considerable a key in a role to recovery of learning and memory deficits after ischemic damage (Briones et al., 2006).

Previous works investigated the effects of EE on MD models. Koe et al. (2016) demonstrated that a single short episode of EE in adulthood can reduce anxiety-like behavior in MD rats, what is accompanied by on basolateral amygdala neurons in adulthood, long after initial stress treatment. Hui et al. (2011) shown that Sprague-Dawley rats exposed to a MD for 180 min (PND 2-14) had decreased sucrose preference and impaired long-term memory in the MWM in adulthood and the EE exposure was able to reverse these deficits; additionally, the authors also identified that the EE cause the significantly increased N-acetylaspartate, an important for measure of neuronal integrity. Vivinetto et al. (2013) shown that EE post MD (4.5h in the first 3 weeks of life), can reverse cognitive deficits in IA latency. In this work, the authors also found that the EE increased a c-Fos expression on a CA1 hippocampal neurons. Our work add information to the previous knowledge, showing that, in addition to memory consolidation, MD disrupts memory reconsolidation and EE was able to prevent these

memory deficits. We also demonstrate that MD promotes BDNF decreases, and, EE was able to avoid this decrease.

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PARTE III

DISCUSSÃO

Os resultados desta dissertação, apresentados no formato de três artigos científicos, demonstram que a DM causa prejuízos cognitivos, isto é, déficits de diferentes tipos de memória em um modelo animal. Os mecanismos envolvidos nestes déficits podem ser variados, e os resultados aqui apresentados comprovam o envolvimento de ao menos dois mecanismos distintos: o desequilíbrio oxidativo e a diminuição dos níveis de BDNF. Os dois processos são sabidamente importantes para a formação, consolidação e reconsolidação da memória. Ainda, nós demonstramos que diferentes estratégias podem ser utilizadas para evitar ou amenizar os prejuízos mnemônicos causados pela DM.

O primeiro estudo apresentado teve como objetivo investigar o papel neuroprotetor do exercício físico em ratos submetidos à DM. Para isso, submetemos os animais à oito semanas de exercício aeróbico em esteira rolante a partir do seu quadragésimo quinto dia de vida. A função mnemônica dos animais, assim como o equilíbrio oxidativo hipocampal, foram avaliados e revelaram o potencial neuroprotetor do exercício. Já se sabe que o exercício físico apresenta inúmeros benefícios para o SN, incluindo melhora na sua plasticidade (Lambert & Evans, 2005) e diminuição da formação de espécies reativas de oxigênio (Schimidt *et al.*, 2014). Também, há estudos que demonstram que a realização de exercício físico aeróbico traz outros benefícios, como a redução da destruição da dopamina na substancia cinzenta do cérebro, em animais que foram submetidos a uma lesão em um dos hemisférios cerebrais (Mabandla & Russel, 2010).

De acordo com Castillha-Ortega *et al.* (2013), o exercício físico demonstrou ter um potencial antioxidante, fazendo com que houvesse um aumento na neurogênese no hipocampo e uma melhora em funções cognitivas de ratos. Hamakawa *et al.* (2013) também demonstraram resultados positivos no dano oxidativo cerebral causado por um acidente vascular transitório; o exercício reverteu o dano oxidativo e os prejuízos motores causados pelo acidente vascular. Ainda, corroborando com nossos resultados, Schimidt *et al.* (2014) em seus experimentos demonstraram que a isquemia-reperfusão cerebral causa uma diminuição nas defesas antioxidantes, diminuindo os níveis de glutationa e catalase, o que foi prevenido através de uma intervenção com exercícios físicos aeróbicos.

Assim, os resultados do primeiro artigo corroboraram com os achados prévios sobre o potencial antioxidante do exercício e permitiram demonstrar a capacidade do exercício físico

em proteger contra o déficit mnemônico causado pela DM, modulando marcadores pró-oxidante (diminuição das espécies reativas de oxigênio), o que teve um papel benéfico para as funções cognitivas estudadas nesse trabalho.

Em vista dos resultados do primeiro artigo (envolvimento do estresse oxidativo hipocampal no déficit de memória dos ratos deprivados) resolvemos investigar se um reconhecido antioxidante exógeno também poderia oferecer alguma forma de neuroproteção aos animais submetidos à DM. Com base em outros estudos realizados pelo nosso e por outros grupos (Schimidt *et al.*, 2014; Flores *et al.*, 2014; Li *et al.*, 2014) podemos afirmar que o chá verde é capaz de prevenir déficits cognitivos advindos de diferentes injúrias ao SN, tais como, doença de Alzheimer e diferentes modelos de acidente vascular encefálico (AVE), devido, ao menos em parte, à sua ação antioxidante.

Considerando os danos oxidativos advindos da DM relatados na literatura, tais como o aumento da produção de radicais livres e consequente dano no DNA de células nervosas e também o aumento da atividade inflamatória (Schiavone *et al.*, 2013), e levando em consideração a incapacidade de se realizar exercícios que alguns indivíduos têm por motivos variados, é de suma importância que haja uma intervenção diferenciada, que não exija grande esforço físico. Estudos em animais e em seres humanos revelaram que o consumo de chá verde pode elevar a capacidade antioxidante no plasma (Leenen *et al.*, 2000). Nesse sentido a utilização da suplementação com chá verde pode ser considerada uma estratégia relevante.

Em um trabalho realizado por Li *et al.* (2007) os autores conseguiram verificar o aumento da expressão das enzimas superóxido dismutase (SOD) e catalase (CAT), que representam uma importante defesa antioxidante, fundamental para manter o equilíbrio oxidativo. De acordo com Van Acker (1996) o chá verde tem sua ação antioxidante potencializada pela presença de hidroxilas nos anéis A, B e D, possibilitando as catequinas capturarem as espécies reativas de oxigênio, como o radical superóxido, radical hidroxila e o peroxido de hidrogênio, todos altamente danosos aos lipídios, proteínas e ao DNA celular; além disso, também apresenta uma ação inibidora de lipoperoxidação (Nanjo *et al.*, 1999; Bixby *et al.*, 2005).

Em conjunto, estes resultados sugerem que a atividade antioxidante do chá verde, além de ocorrer diretamente, por meio da neutralização de espécies reativas, pode se processar através de mecanismos indiretos, como, por exemplo, a preservação e modulação de enzimas antioxidantes (Skrzydlewska *et al.*, 2002). Estes resultados corroboraram com os resultados do nosso primeiro trabalho, demonstrando que os danos de memória oriundos da DM estão

intimamente associados ao desiquilíbrio oxidativo provocado pela DM e que a utilização de um antioxidante, seja ele endógeno ou exógeno, é capaz de reverter esse déficit.

No terceiro artigo que compõe esta dissertação, visando outra abordagem para prevenção dos efeitos deletérios oriundos da DM, buscamos realizar uma análise dos níveis de um fator neurotrófico, o BDNF. O BDNF é importante para o crescimento e maturação neuronal, tendo amplo envolvimento nos processos de aquisição, consolidação e reconsolidação da memória (Roceri *et al.,* 2004). Outros trabalhos previamente realizados demonstraram os efeitos que um estresse provocado logo no início da vida pode causar a nível molecular, tais como a diminuição nos níveis de BDNF, o aumento dos níveis de corticosterona e uma possível demência na vida adulta, cujos mecanismos ainda não estão completamente compreendidos (Lippmann *et al.,* 2007; Ranaa *et al.,* 2015).

Considerando que trabalhos prévios demonstram que o EA faz com que haja aumento dos marcadores de plasticidade sináptica, diminuição dos níveis de ansiedade e também aumenta a neurogênese (Fan *et al.,* 2007; Monteiro *et al.,* 2014) melhorando os níveis de BDNF, e promovendo melhora consequente da memória espacial, aversiva e de reconhecimento (Hu *et al.,* 2013; Ji *et al.*, 2016), demonstramos que a exposição ao EA é uma estratégia adequada para prevenir déficits de memória e diminuição dos níveis de BDNF em ratos deprivados.

Tomados em conjunto, os resultados dessa dissertação são capazes de elucidar um pouco mais os mecanismos envolvidos nos déficits mnemônicos causados pela DM, demonstrando que este estresse no início da vida causa prejuízos à MCD e MLD na vida adulta, além de prejudicar a consolidação e reconsolidação da memória. Adicionalmente elucidam ao menos dois mecanismos envolvidos nestes prejuízos de memória: o desequilíbrio oxidativo e a diminuição dos níveis de BDNF. Também, demonstramos que três diferentes tipos de abordagem são capazes de impedir que os déficits se evidenciem na vida adulta dos animais: o exercício físico aeróbico, a suplementação com chá verde, e a exposição ao enriquecimento ambiental. Cabe ressaltar a capacidade de cada uma das intervenções propostas em atingir seus objetivos, cada uma com um diferente mecanismo de ação, apostando em agentes antioxidantes endógenos (exercício aeróbico), antioxidantes exógenos (consumo de chá verde) e intervenção comportamental (enriquecimento ambiental).

CONCLUSÕES

Os resultados desta dissertação permitem concluir que a deprivação maternal causa prejuízos na função mnemônica de ratos Wistar, considerando seus efeitos sobre MCD e a MLD (consolidação e reconsolidação). Ainda, verificamos que a DM traz prejuízos ao equilíbrio oxidativo e níveis de BDNF no hipocampo.

Verificamos também a eficácia de algumas estratégias neuroprotetoras. Demonstramos os efeitos benéficos do exercício físico aeróbico e do chá verde sobre a MCD e a MLD, bem como seus efeitos sobre o equilíbrio oxidativo hipocampal após a exposição à DM. Ainda, demonstramos os efeitos do enriquecimento ambiental sobre os déficits da MCD e a consolidação e reconsolidação da MLD em ratos deprivados, através da prevenção da diminuição dos níveis de BDNF.

PERSPECTIVAS

Seguindo a linha de pesquisa direcionada ao entendimento do declínio cognitivo associado à DM, temos a perspectiva de iniciar outras pesquisas, buscando analisar outros mecanismos que possam estar envolvidos nos efeitos adversos desta intervenção, pois acreditamos que eles vão além do estresse oxidativo e alterações nos níveis de BDNF, embora possam estar relacionados a estes. Dentre os mecanismos adicionais que pretendemos investigar, incluem-se as alterações nos níveis de alguns neurotransmissores, tais como a dopamina.

Ainda, para dar sequência às nossas pesquisas envolvendo neuroproteção e DM, pretendemos investigar os efeitos de tratamentos de curto prazo, visto que os estudos que realizamos até então se dedicaram à verificação dos efeitos benéficos de tratamentos de duração mais longa. Embora as alternativas investigadas sejam todas de baixo custo, o tempo de tratamento influencia na aderência do paciente ao mesmo, sendo assim, verificar se as alternativas propostas também têm efeitos quando realizadas por períodos curtos pode abrir a possibilidades e ampliar seu uso.

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ANEXOS

Anexo I

Certificado de aprovação do projeto pelo CEUA-Unipampa – Projeto estresse oxidativo

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DO PAMPA
(Lei nº 11.640, de 11 de janeiro de 2008)

Pró-Reitoria de Pesquisa

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Fone: (55) 3413 4321, E-mail: ceua@unipampa.edu.br

CERTIFICADO DE APROVAÇÃO DE PROTOCOLO PARA USO DE ANIMAIS EM PESQUISA

Número de protocolo da CEUA: **001/2014**

Título: **EFEITOS DO EXERCÍCIO FÍSICO NO DÉFICIT DE MEMÓRIA E ESTRESSE OXIDATIVO DE TECIDOS NEURAIS CAUSADOS PELA DEPRIVAÇÃO MATERNAL**

Data da aprovação: **23/04/2014**

Período de vigência do projeto: De: **04/2014** Até: **04/2017**

Pesquisador: **PÂMELA BILLIG MELLO CARPES**

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Alessandra S. K. Tamajusuku Neis
Professor Adjunto
Coordenadora da CEUA/UNIPAMPA

Anexo II

Certificado de aprovação do projeto pelo CEUA-Unipampa – Projeto enriquecimento ambiental

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DO PAMPA
(Lei nº 11.640, de 11 de janeiro de 2008)

Pró-Reitoria de Pesquisa

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Fone: (55) 3413 4321, E-mail: ceua@unipampa.edu.br

CERTIFICADO DE APROVAÇÃO DE PROTOCOLO PARA USO DE ANIMAIS EM PESQUISA

Número de protocolo da CEUA: **014/2015**

Título: **O enriquecimento ambiental como fator de prevenção de déficits cognitivos associados à deprivação maternal**

Data da aprovação: **12/06/2015**

Período de vigência do projeto: De: **06/2015** Até: **06/2018**

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Anexo III

Comprovante de submissão de manuscrito à revista *Physiology & Behavior*

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