PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

FACULDADE DE BIOCIENCIAS

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

Kelem Vedovelli

Efeitos Do Ambiente Enriquecido Nos Níveis Centrais E Periférico De Bdnf E Sua Relação Com O Desempenho Na Tarefa De Reconhecimento De Objetos Em Ratos

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Efeitos do Ambiente Enriquecido nos níveis centrais e periférico de BDNF e sua Relação com o Desempenho na Tarefa de Reconhecimento de Objetos em Ratos

Dissertação apresentada como requisito para obtenção do grau de Mestre pelo Programa de Pós-graduação em Biologia Celular e Molecular da Faculdade de Biociências

Orientadora: Dra Elke Bromberg

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RESUMO

Estudos com modelos de organismos demonstraram que o ambiente enriquecido induz mudanças celulares, estruturais e comportamentais ,e aumento nos níveis do fator neurotrófico derivado do cérebro (BDNF) no sistema nervoso central. Essas evidências sugerem que BDNF possa ser um interessante marcador biológico dos efeitos de estilo de vida na cognição e outros parâmetros comportamentais em humanos. Para testar esta hipótese, analisamos os efeitos do ambiente enriquecido na memória de longa duração, através do reconhecimento do objeto , e os níveis de BDNF no hipocampo, córtex frontal e soro de ratos expostos a um protocolo experimental que pode ser mais facilmente relacionado com estudos de interação com humanos. Os animais foram mantidos durante dez semanas em condição social (condições padronizadas de laboratório) ou enriquecido (aumento da oportunidade para exercícios físicos e experiências de aprendizado). Na sétima semana os animais foram submetidos a testes comportamentais (campo aberto e teste do reconhecimento do objeto) e no final da décima semana foram eutanisados para análise dos níveis de BDNF. Os animais mantidos na condição enriquecida mostraram melhora na performance no teste de memória, mas em contrapartida não apresentaram alteração significativa nos níveis centrais e periféricos de BDNF. Os resultados deste estudo são importantes para destacar a necessidade de desenvolver protocolos experimentais usando o modelos animais que se assemelham mais próximo às características dos estudos com seres humanos e motivar mais investigações para determinar as circunstâncias sob que BDNF poderia ser um marcador biológico dos efeitos do enriquecimento do ambiente.

Palavras-Chaves: BDNF,hipocampo, córtex frontal, ambiente enriquecido, reconhecimento do objeto.

Abreviaturas: BDNF (fator neurotrófico derivado do cérebro).

ABSTRACT

Studies with organisms models demonstrated that cellular, structural and behavioral changes induced by environmental enrichment are related to increased levels of BDNF in the brain. These evidences suggest that BDNF could be an interesting biomarker of the effects of lifestyle on cognition and other behavioral parameters in humans. To test this hypothesis, we analyzed the effects of environmental enrichment on long term memory for object recognition and on BDNF levels of hippocampus, frontal cortex and serum of rats exposed to an experimental protocol that could be more easily translated to human intervention studies. Animals were maintained for 10 weeks in a social (standard laboratory conditions) or enriched (increased opportunity for physical exercise an learning experiences) condition. In the 7^{th} week they were submitted to behavioral testing (open field and novel object memory task) and at the end of the 10^{th} week they were killed and BDNF levels were analyzed. Animals maintained in the enriched condition showed enhanced performance on the memory task in the absence of any significant alteration in central or peripheral BDNF levels. The results of this study are important to highlight the need to develop experimental protocols using animal models that more closely resemble the characteristics of studies with humans and motivate more investigations to determine the conditions under which BDNF could be a biomarker of the effects of environment enrichment.

KEY WORDS : BDNF, hippocampus, frontal cortex , environmental enrichment, novel object recognition

ABREVIATIONS:

BDNF- brain-derived neurotrophic factor

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CAPÍTULO 1

1.1 INTRODUÇÃO

1.1.1 FATOR NEUROTRÓFICO DERIVADO DO CÉREBRO (BDNF)

O fator neurotrófico derivado do cérebro (BDNF) é uma pequena proteína e membro da família de neurotrofinas, encontrado no sistema nervoso, onde desempenha papel importante no desenvolvimento e manutenção do sistema nervoso periférico (SNP) e central (SNC), na plasticidade, sobrevivência e proliferação neuronal (Yulug et al., 2009). No encéfalo é abundante especialmente no hipocampo, córtex cerebral, estriado e amígdala, estruturas que estão envolvidas com memória e aprendizagem (Vaynman et al., 2003). Devido às evidências de seu envolvimento na neurogênese hipocampal, suas funções e mecanismos de ação têm sido estudados principalmente na fase pós natal, em doenças neurodegenerativas e patologias associadas ao declínio cognitivo (Scheneider et al. 2000; Karege et al. 2002).

O BDNF exerce seus efeitos por meio de receptores tirosina quinase (TrkB), aos quais se liga com grande afinidade, resultando no recrutamento de proteínas que ativam duas vias de sinalização intracelular. Uma destas vias ativa a proteína quinase-1 dependente de fosfatidilinositol-3 (PI-3K), e a proteína quinase B (AKT), que fosforilam e desativam agentes pró-apoptóticos (Halbook et al.,2006; Kermani et al.,2007; Santos et al.,2010). O outro caminho envolve a TrkB, que induz a ativação de proteínas intracelulares como, Ras, Raf, MEKs e sinais extracelulares regulados pelas quinases (ERKs) os quais ativam um ou mais fatores de transcrição que irão induzir a transcrição de BDNF, além de regular a expressão de genes envolvidos na formação da LTP (potenciação de longa duração), na modulação da secreção de neurotransmissores (como a serotonina), na plasticidade sináptica, na resistência ao estresse e na sobrevivência celular . Essa atividade é dependente da ativação dos canais de cálcio voltagem-dependentes voltagem dependente de cálcio (Mattson et al., 2004; Sossin et al., 2007; Santos et al.,2100).

Assim a ativação destas cascatas modulam a proliferação, sobrevivência e manutenção do sistema neuronal, sugerindo que a deficiência de BDNF leva a déficits funcionais e alterações no desenvolvimento pós-natal e maturação de neurônios do encéfalo (Mattson et al. 2004).

Grande parte dos estudos a respeito das funções do BDNF é realizado em roedores, onde normalmente seus níveis são quantificados em estruturas cerebrais (hipocampo e córtex frontal). Já em estudos em modelos humanos, o BDNF é analisado somente perifericamente não sendo observado em estruturas encefálicas pela inviabilidade. Contudo poucos estudos reportam a concentração desta neurotrofina no plasma, local esse, que seria de fundamental importância de se quantificar, visto que já foi observado seu aumento, em um estudo com indivíduos com doenças de retardo mental , que recebiam tratamento com estímulo cognitivo (Mattson et al., 2004).

Um grande número de estudos de transtornos psiquiátricos e doenças neurodegenerativas mostraram alterações nos níveis de BDNF nestas circunstâncias patológicas, indicando que BDNF poderia ser um marcador biológico interessante nestas situações (Cunha et al., 2006; Kim et al., 2007; Pillai et al.,2010). A primeira evidência da presença de BDNF no plasma e soro de humanos foi em 1995, em um estudo de Rosenfeld e colaboradores. Há estudos experimentais que relatam que o BDNF pode cruzar a barreira hematoencefálica, sugerindo com isso, que as concentrações no sistema nervoso central podem ser paralelas com as concentrações de BDNF, nos níveis plasmáticos (Pan et al., 1998; Klein et al., 2010)

Outro estudo mostrou também que BDNF foi capaz de cruzar a barreira hematoencefálica em roedores. Os resultados indicaram que a passagem do fator neurotrófico do cérebro para o plasma foi associado com a reabsorção do fluido cérebro-espinhal por diferença de concentração do fluxo, (Karege et al*.,* 2002; Lang et al., 2007).

Porém, as relações entre os níveis centrais e periféricos de BDNF ainda não estão bem estabelecidas, uma vez que os mecanismos de troca de BDNF através da barreira hematoencefálica ainda não estão descritos (embora existam várias evidências de sua ocorrência) (Pan et al.,1998). É importante considerar o fato de que as plaquetas são importantes locais de armazenamento e liberação de BDNF (Karege et al., 2002). Estudo recente mostrou que mais de 99% das proteínas são estocadas nas plaquetas e podem ser liberadas no soro (Karege et al*.,* 2002). Uma vez que os estudos utilizando modelos animais indicam a participação do BDNF em importantes aspectos funcionais do SNC, o interesse na elucidação do papel desta neurotrofina em diferentes aspectos fisiológicos (como no envelhecimento) e

patológicos (como em doenças neurodegenerativas) tem crescido (Murer et al.,2000).

Os fatores neurotróficos são responsáveis pelo crescimento neuronal e o estabelecimento das conexões sinápticas entre os neurônios (Kim et al.,2007). Tais fatores exercem um papel chave sobre a organização de redes neurais durante os estágios iniciais do desenvolvimento pós-natal, bem como no cérebro adulto. Evidências clínicas (Sklar et al., 2002; Cunha et al., 2006; Rosa et al., 2006) e experimentais (Itoh et al., 2004) revelaram que os níveis de fatores neurotróficos estão alterados em pacientes com transtornos do humor, bem como em modelos animais desses transtornos (Hashimoto et al., 2004). Vários estudos já demonstraram alterações nos níveis plasmáticos de BDNF em doenças neurodegenerativas, especialmente naquelas relacionadas ao envelhecimento e ao declínio cognitivo (Halbach et al., 2010; Erickson et al., 2010) e em transtornos psiquiátricos, como a depressão e o transtorno de humor bipolar (Yan et al., 2010; Scalzo et al., 2010). De uma forma geral, estes estudos indicam um declínio do BDNF plasmático, estando de acordo com as hipóteses dos mecanismos patofisiológicos das doenças em questão, e reforçando a idéia de que o BDNF periférico é um bom indicador de seus níveis centrais. Entretanto, é importante considerar que, em se tratando de patologias, é esperada uma alteração de vários parâmetros físico-químicos, de forma que seria mais fácil detectar alterações periféricas, por se tratarem de situações patológicas, elas são mais pronunciadas do que níveis centrais de BDNF (Sklar et al., 2002; Karege et al., 2002).

Além do interesse na participação do BDNF em alterações patológicas, os estudos com animais têm sugerido que fatores como atividade física e enriquecimento ambiental seriam capazes de aumentar os níveis de BDNF no sistema nervoso central (Bayne et al. 2005; Segovia et al. 2009). Entretanto, há relativamente poucos estudos de alterações nos níveis de BDNF induzidas por diferentes ambientes nos seres humanos, muitos se restringem ao efeito do exercício e a na maioria deles, são relativos aos efeitos agudos da atividade física em BDNF.

Porém, neste caso, é mais difícil de estabelecer um paralelo com seres humanos, pois é possível que, apesar de ocorrer elevação dos níveis de BDNF em diferentes estruturas cerebrais, talvez a variação do mesmo não seja suficiente para

ser detectada perifericamente. Portanto, o ponto de partida para a investigação em humanos é justamente a verificação da possibilidade de se utilizar o BDNF periférico como indicador de suas alterações centrais.

A falta de estudos com seres humanos reflete a necessidade de esclarecer a importância que cada componente que compõe o ambiente enriquecido (físico, social e cognitivo) exerce no sistema nervoso central, bem como o efeito da idade , a época de exposição ao ambiente enriquecido, que influenciam na relação entre níveis centrais e periféricos de BDNF. Esses fatores tornam-se importantes, já que os estudos em animais medem níveis centrais de BDNF e os estudos humanos dependem da avaliação de concentrações periféricas (soro ou plasma). Considerando estudos humanos, e o enriquecimento ambiental como uma intervenção, o social versus a condição enriquecida é o mais conivente como metodologia para ser avaliado em um modelo experimental com animal e comparado com humanos. Embora nós saibamos que BDNF pode cruzar a barreira hematoencefálica (Karege et al., 2002; Lang et al., 2007) e já se obteve correlações entre níveis central e periférico de BDNF em alguns animais, como ratos e porcos (Klein et al. 2010), nós ainda não sabemos a quantidade de alterações centrais de BDNF necessárias para induzir a modificação periférica mensurável de BDNF. O único estudo que analisou se as alterações induzidas da correlação central de BDNF com sua medida periférica foram realizadas com os ratos expostos ao tratamento eletro choque (Sartorius et al. 2009), uma situação muito diferente das circunstâncias fisiológicas e que provavelmente pode induzir modificações mais pronunciadas de BDNF.

1.1.2 ENRIQUECIMENTO DO AMBIENTE E BDNF

O cérebro pode ser modificado como conseqüência da interação entre o ambiente e a constituição genética (Murer et al. 2000; Van Praaga et al*.,* 2000; Pham et al. 2002).

Um dos modelos experimentais utilizados para investigar os efeitos da estimulação ambiental é a manutenção de animais em um ambiente enriquecido.

Nesse ambiente os animais são abrigados em condições que potenciam a estimulação motora, sensorial e interação social, condições que conduzem a melhorias no aprendizado e na memória. Donald Hebb (1949) foi o pioneiro demonstrando resultados da estimulação ambiental desde cedo no desenvolvimento sistema nervoso central e na sinaptogênese. Ele sugeriu que esse estímulo seja importante no desenvolvimento neurofisiológico e comportamental (Cooper et al. 2005).

Em geral os roedores são abrigados em caixas mais espaçosas e com outros animais com oportunidade de promover interação social. O ambiente é composto por uma variedade de objetos dentre eles: túneis, bolas, brinquedos que são trocados frequentemente. Estes objetos constituem-se em fatores estimuladores do sistema sensorial e motor (Van Praaga et al*.,* 2000).

O enriquecimento ambiental causa mudanças neuroquímicas, neuroanatômicas e comportamentais em muitas espécies de animais (Van Praaga et al., 2000; Cooper et al., 2005; Zhu et al., 2006). Apresenta um impacto nas condições neuroanatômicas, como, mudanças na anatomia neural, modificação da expressão genética, no número de espinhas dendríticas (Mora et al. 2007). Além disso, induz mudanças neuroquímicas, como a melhora na sinaptogênese para neurogênese e aumento nos níveis de neurotrofinas em diferentes regiões do cérebro como no hipocampo, córtex pré-frontal e amígdala (Falkenberg et al.,1992; Pietropaolo et al., 2004; Gelfo et al., 2010) . O hipocampo é a estrutura cerebral mais analisada em estudos, por estar envolvido no aprendizado e na memória. Estudos mostram essa estrutura influenciada pelo enriquecimento ambiental, apresentando níveis elevados de neurotrofinas, sugerindo que estejam envolvidos na plasticidade sináptica, influenciando no processo de aprendizado e memória (Rossi et al.,2006).

Por sua vez as alterações comportamentais desencadeadas pelo ambiente enriquecido, auxiliam na regulação do estresse (Segovia et al., 2009) ajudando na manutenção da memória pelo aumento da expressão de BDNF (Ickes et al., 2000), melhorando o aprendizado em animais velhos (Undine et al., 2007; Gobbo et al., 2004), sugerindo um efeito neuroprotetor em doenças neurodegenerativas. Assim, supõe-se que esses fatores ambientais influenciem de forma benéfica no desenvolvimento do sistema nervoso central e no processo de envelhecimento (Pham et al., 2002). Estudos mostram que na fase jovem ocorre continua

modificação das conexões neuronais e que este remodelamento também ocorre na idade adulta no cérebro, mostrando que o sistema nervoso é capaz de responder a mudanças na plasticidade durante toda a vida. Essa plasticidade pode ser definida como mudanças observadas no sistema nervoso ou no comportamento. Então plasticidade comportamental refere-se a alguma mudança significativa observada no comportamento enquanto que plasticidade neural pode se referir a mudanças na função e estrutura do sistema nervoso a níveis moleculares que influenciam na plasticidade comportamental (Ickes et al*.* 2000).

1.1.3 MEMÓRIA E BDNF

A memória, umas das mais importantes funções cognitivas do ser humano, pode ser entendida como a habilidade que possuímos de armazenar informações e conhecimentos sobre nós mesmos e o mundo que nos cerca. É a base para o desenvolvimento da linguagem, do reconhecimento das pessoas e dos objetos que encontramos todos os dias, para sabermos quem é e para termos a consciência da continuidade de nossas vidas. Sem a memória, a cada dia, ou a cada momento, estaríamos começando uma nova vida, sem podermos nos valer do que aprendemos anteriormente (Yassuda et al*.*, 2002).

Em relação ao conteúdo, a memória pode ser classificada como *declarativa* ou *procedural.* As memórias *procedurais* são aquelas relacionadas às capacidades, habilidades motoras ou sensoriais. As memórias que registram fatos, eventos, ou conhecimentos são chamadas de *declarativas,* porque nós seres humanos, podemos relatar seu conteúdo. Entre elas, as referentes a eventos aos quais participamos, são denominadas *episódicas* e, ainda, as de conhecimentos gerais são denominadas *semânticas* (Milner et al*.,* 1998).

Um dos exemplos mais profundamente estudados da memória declarativa é a memória de reconhecimento, que é a capacidade de julgar um item recentemente encontrado como familiar. A memória de reconhecimento em seres humanos consiste de dois componentes: um episódico que diz respeito à habilidade de lembrar-se do episódio (situação) no qual um item (que pode ser um objeto, uma face, uma música) foi introduzido (item novo), e um componente familiar, que se

relaciona com a habilidade de reconhecer um item como já conhecido (ou familiar), mas sem a necessidade da lembrança do próprio episódio (Manns et al., 2003).

A memória de reconhecimento pode ser testada em roedores usando tarefas de reconhecimento de objetos que são baseadas na tendência espontânea que os roedores apresentam de explorar objetos novos quando os animais se lembram dos objetos aos quais eles foram previamente expostos. As vantagens desse tipo de teste incluem o fato de que eles não são baseados em reforços positivos (como a utilização de alimentos) ou negativos (como a utilização de choques elétricos) (Mumby et al., 2001; Clark et al*.,* 2005).

Além disso, esse tipo de tarefa revelou ser dependente tanto do hipocampo (uma região cerebral importante para o processamento da memória), quanto da via nigro-estriatal. Estudos recentes têm focado a expressão abundante de BDNF no hipocampo, córtex e estriado. Evidências já indicaram que BDNF está envolvido na manutenção da (LTP), aprendizado espacial e reconhecimento. A LTP é induzida pela alta freqüência de sinapses excita tórias, resultando do modelo de aprendizado dependente da plasticidade do cérebro (Ernfors et al., 2003).

Com base no exposto até o momento, investigamos os efeitos do enriquecimento ambiental nos níveis centrais e periféricos de BDNF e a possível relação dos mesmos com o desempenho em testes de memória de reconhecimento de ratos. Embora seja esperado um aumento dos níveis de BDNF central (Schneider et al., 2000; Ickes et al., 2000) e uma correlação dos mesmos com o desempenho cognitivo dos animais, somente a confirmação de uma correspondência com alterações nos níveis periféricos de BDNF permitirá inferências mais concretas a respeito do papel do BDNF em aspectos cognitivos de seres humanos e a análise dos mecanismos através dos quais o enriquecimento ambiental exerce seus efeitos plásticos e neuroprotetores no SNC.

A fim de contribuir com a elucidação de algumas destas situações, mantivemos ratos por 10 semanas em uma condição social ou enriquecida e investigamos se estas condições de moradia teriam efeitos na memória de longa duração do reconhecimento do objeto (uma tarefa que fornece uma analogia próxima os testes do reconhecimento que são amplamente utilizados nos seres humanos), e em níveis centrais e periféricos de BDNF. As condições experimentais e a escolha dos parâmetros a serem analisados foram escolhidas porque poderiam

mais facilmente ser correlacionadas com situações experimentais de estudos humanos para investigar o efeito da intervenção ambiental na cognição e no BDNF.

1.2 OBJETIVOS

1.2.1 OBJETIVO GERAL

Avaliar os efeitos do ambiente enriquecido (maior oportunidade de experiências de aprendizado e atividade física) nos níveis centrais e periféricos de BDNF e sua relação com o desempenho na tarefa de reconhecimento de objetos em ratos.

1.2.2 OBJETIVOS ESPECÍFICOS

- Avaliar os efeitos do enriquecimento ambiental (maior oportunidade de experiências de aprendizado e atividade física) nos níveis centrais (hipocampo e córtex frontal) e periféricos (soro) de BDNF através do kit ELISA em ratos adultos.

- Verificar se existe relação entre níveis de BDNF em estruturas cerebrais (hipocampo, córtex frontal) e no soro .

CAPÍTULO 2

Artigo científico que será submetido à revista *Neuroscience*

Effects of increased opportunity for physical exercise and learning experiences on recognition memory and BDNF levels in brain and serum of rats

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ABSTRACT

Studies with animal models showed that cellular, structural and behavioral changes induced by environmental enrichment are related to increased levels of BDNF in the brain. These evidences suggest that BDNF could be an interesting biomarker of the effects of lifestyle on cognition and other behavioral parameters in humans. To test this hypothesis, we analyzed the effects of environmental enrichment on long term memory for object recognition and on BDNF levels of hippocampus, frontal cortex and serum of rats exposed to an experimental protocol that could be more easily translated to human intervention studies. Animals were maintained for 10 weeks in a social (standard laboratory conditions) or enriched (increased opportunity for physical exercise a learning experiences) condition. In the 7^{th} week they were submitted to behavioral testing (open field and novel object memory task) and at the end of the 10^{th} week they were killed and BDNF levels were analyzed. Animals maintained in the enriched condition showed enhanced performance on the memory task in the absence of any significant alteration in central or peripheral BDNF levels. The results of this study are important to highlight the need to develop experimental protocols using animal models that more closely resemble the characteristics of studies with humans and motivate more investigations to determine the conditions under which BDNF could be a biomarker of the effects of environment enrichment.

KEY WORDS: BDNF, hippocampus, frontal cortex, environmental, novel object recognition

ABREVIATIONS : BDNF- brain-derived neurotrophic factor

1. Introduction

An increasingly body of evidences from animal models has shown that enriched environment, namely rearing conditions that provide an increased opportunity for physical exercise, learning experiences and social interaction, can improve development and function of brain over the lifetime (Van Praaga et al., 2000 ; Cooper .,2005; Zhu et al.,2006).

Among the behavioral changes induced by environmental enrichment are improvement in motor function (Johansson ., 1996; Risedal et al., 2002), learning and memory (Duffy et al., 2001; Gomez-Pinilla., 2005; Rossi *et al*., 2006), as well as emotionally-related behaviors (Zhu et al., 2006 ; Koh et al., 2007). Cellular alterations, such as increases in neurogenesis, synaptic density and neuronal plasticity (Mora et al.,2007) has also been observed in animals housed in complex environments and seem to be related to improvement in behavioral parameters (Pham et al.,2002 ; Gobbo., 2004). These changes in neural circuits have been associated with alterations in neurotrophic factors, especially brain-derived neurotrophic factor (BDNF). It is well documented that the expression and levels of BDNF are prone to environmentally induced changes (Falkenberg et al., 1992; Pietropaolo et al., 2004; Gelfo et al., 2010) and that increases in BDNF induced by enriched environment are associated with better performance in different behavioral tasks (Schneider et al., 2000; Ickes et al., 2000; Gomez-Pinilla., 2005; Hopkins et al., 2010). The binding of BDNF to TrKB and p75 receptors (Kermani et al, 2007; Halbook et al, 2006) activates biochemical cascades that can lead to cell proliferation, survival and plasticity (Mattson et al., 2004; Sossin et al., 2007) and, at least in part, explain the effects of enriched housing conditions on brain and behavior.

All these evidences suggest that BDNF would be an interesting biomarker of the effects of environmental enrichment on cognition and other behavioral parameters in humans.

A great numbers of studies on psychiatric (Sklar *et al*., 2002; Itoh *et al*., 2004 Cunha *et al*., 2006; Rosa *et al*., 2006; Kim *et al*., 2007) and neurodegenerative disorders (Mattson et al., 2004; Yan et al., 2010; Scalzo et al., 2010; Pillai et al., 2010) have shown consistent alterations of BDNF in these pathological conditions.

However, there are relatively few studies of environmentally induced alterations of BDNF in humans, all of them restricted to the effect of exercise and most of them related to the acute effects of physical activity on BDNF (Winter et al., 2007; Nofuji et al., 2008; Zoladz et al., 2008; Rasmussen et al., 2009). At least in part, this lack of studies with humans reflects the need to clarify some issues, as the relative importance off each component of the enriched environment (physical, social and cognitive) on the central nervous system, the effects of age and time of exposure to the enriched environment and the relation between central and peripheral levels of BDNF, since animal studies measure central BDNF levels and human studies depend on the evaluation of peripheral (serum or plasma) concentrations.

Studies that compared healthy animals maintained in enriched and impoverished conditions (isolation, no stimulation for physical or learning experiences) showed higher brain levels of BDNF in animals of the enriched condition (Pham et al., 1999; Pham et al., 2002; Rossi et al., 2006; Branchi et al., 2006; Zhu et al., 2006). However, results from studies that compared animals in a social (2 to 5 animals in and standard cage) and in an enriched condition (more than 5 animals in a greater cage with toys) were not so consistent, ranging from data showing important to mild (or no significant) effects of enrichment on BDNF levels (Rossi et al., 2006; Zhu et al., 2006; Parks at al., 2008; Bakos et al., 2009). Considering human studies, and environmental enrichment as an intervention, the social versus enriched condition would be the most common situation. Could mild alterations of BDNF in central nervous system lead to detectable peripheral alterations of this neurotrophin? Although we know that BDNF can cross the blood brain barrier (Pan et al., 1998; Karege et al., 2002; Lang

et al., 2007) and that there are correlations between central and peripheral BDNF in some animals (Klein et al., 2010), and biomarkers of cortical integrity and peripheral BDNF in humans (Lang et al., 2007), we still don't know the amount off central BDNF alterations necessary to induce measurable peripheral modification of this neurotrophin. The only study that analyzed if an induced alteration of central BDNF correlate with its peripheral measure was done with rats exposed to electroconvulsive treatment (Sartorius et al., 2009); a situation very different from physiological conditions and that probably can induce more pronounced modifications of BDNF.

In order to contribute to the elucidation of some of these issues, we maintained rats for 10 weeks in a social or enriched condition and investigated if these housing conditions had effects on long term memory of object recognition (a task that provides a close analogy with recognition tests that are widely used in humans) and on central (hippocampus and frontal cortex) and peripheral (serum) BDNF levels. The experimental conditions and the choice of the parameters to be analyzed were selected because they could be more easily translated to human studies to investigate the effect of environmental intervention on cognition and BDNF.

1.Experimental Procedures

2.1 Animals

Male Wistar rats (30 days of age at the time of arrival) were obtained from the State Health Science Research Foundation (FEPPS-RS, Porto Alegre, Brazil). Animals were kept (4-5 to a cage) on a room temperature of 21 ± 1°C and a 12/12 h light/dark cycle with food and water *ad libitum*. All behavioral experiments were performed at light phase between 09:00 h and 16:30 h. The experimental procedures were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals (NIH publication No. 80-23 revised 1996). All experimental protocols were approved by the institutional animal care committee. All efforts were made to minimize the number of animals and their suffering.

1.2Housing conditions

The animals used in this study were randomly assigned to one of two housing environments, characterizing two experimental conditions: a) Social condition (control group, n=15), in which rats remained in the standard methacrylate cages (31x42x17cm), with no access to any stimulus objects and handled only during routine cage changing; b) Enriched condition (Enriched group, n=10), in which animals were maintained in a large cage (34x52x59cm) with two floors, ramps and several stimulus objects or “toys” selected from a pool of 40 objects, such ladders, boxes, balls, tunnels, brushes, baby music toys, and platforms. The objects were changed three times a week in random combination. Although these rats could observe ongoing activity in the room, they received only minimal contact with animal care workers during routine cage changing.

The animals were kept in these conditions for a period of 2 months, (beginning at the 40nd postnatal day) before behavioral testing. After behavioral testing they were maintained for another two weeks in the different housing conditions before blood sampling for BDNF evaluation. Weight gain was measured at the beginning and end of the experiment and no significant differences between groups were observed (data not shown).

2.3 Open Field behavior

Habituation and reactions to spatial changes were evaluated by on open field. The open field was a 40cm x 45cm arena surrounded by 50cm high walls, made of plywood with a frontal glass wall. The floor of the arena was divided into 12 equal squares by black lines. On the first day, animals were handled for 1 min. On second day, all animals were placed in the rear left corner and left to explore the field freely for 5 min. Latency to start locomotion, line crossings, rearing and the number of fecal pellets produced were counted (De Lima et al., 2005c).

2.4 Novel Object recognition memory (NOR)

The NOR task uses the natural preference for novel objects displayed by rats and was used to assess cognitive alterations associated with environmental enrichment. Twenty-four hours after open field exploration, animals were trained and tested in a novel object recognition task as previously described (Schroder et al., 2003; De Lima et al., 2005a, b, c; 2006; Schroder et al., 2003).Training in the object recognition task took place in the same arena used for the open field, except that arena floor was covered with sawdust during the recognition memory task training and test trials. The open field exploration was thus used as a context habituation

trial for the recognition memory task. The object recognition test required that the rats recalled which of two plastic objects they had been previously familiarized with. Twenty-four hours after arena exploration, training was conducted by placing individual rats into the field, in which two identical objects (Duplo Lego toys) were positioned in two adjacent corners, 9 cm from the walls. In a long-term memory (LTM) test given 24h after training, the rats explored the open field for 5 min in the presence of familiar object (F) and a novel object (N). All objects presented similar textures, colors, and sizes, but distinctive shapes. Object exploration was measured using two stopwatches to record the time spent exploring the objects during training and test trials. A recognition index calculated for each animal was expressed by the ratio $T_N / (T_F + T_N)$ [T_F= time spent exploring the familiar object F; T_N= time spent exploring the novel object N] (De Lima et al., 2005, 2006, 2008a, b; Dias et al., 2007). Between trials the objects were washed with 10% ethanol solution. Exploration was defined as sniffing or touching the object with the nose and/or forepaws. Sitting on the object was not considered exploration.

2.5 BDNF ELISA procedure

All rats were euthanized by decapitation two weeks after LTM to avoid any interference of the performed behavioral tests on BDNF levels. Brains were immediately removed; frontal cortex and hippocampus were rapidly dissected and frozen in liquid nitrogen. Blood for serum BDNF determination was collected in anticoagulant-free tubes and kept at room temperature for one hour and then at $4C^0$ for a second hour before centrifugation at 3000 rpm during 10 min. Serum was then stored at $-20C^o$ until use.

BDNF levels in serum and in brain tissues [homogenized in phosphate buffer solution (PBS) with protease inhibitor cocktail(Sigma)] were determined by sandwich-ELISA using

monoclonal antibodies specific for BDNF (R&D Systems, Minneapolis, Minnesota). Briefly, microtiter plates (96-well flat-bottom) were coated overnight at room temperature with the monoclonal anti-BDNF antibody at 4 ug /ml in PBS. Then, plates were washed three times with wash buffer (PBS, pH 7.4, with 0.05% Tween 20) and were blocked for 1 hour at room temperature with PBS containing 5% nonfat milk powder. After another washing, plates were coated for 2 hours at room temperature with the samples diluted 1:2 (tissues) and 1:10 (serum) in sample diluents (PBS with 1% BSA) and standard curve ranged from 7.8 to 500 pg/mL of BDNF. Plates were washed again and was added a biotinylated anti-BDNF antibody at 0.2 ug/mL in PBS, which was incubated for 2 hours at room temperature. After washing, the incubation with streptavidin-peroxidase conjugate (diluted 1:200 in sample diluents) for 20 min at room temperature was performed. Plates were then washed and incubated with the substrate for 20 minutes at room temperature. Finally, the stop solution (H_2SO_4 1M) was added and the amount of BDNF was determined by absorbance at 450 nm with correction at 540 nm. The standard curve demonstrated a direct relationship between optical density (OD) and BDNF concentration. Total protein was measured by Lowry's method using bovine serum albumin as a standard.

2.6 Statisticals Analiysis

Comparisons of behavioral data from social and enriched housing conditions were performed using Mann-Whitney U-testes, except for the results of crossings and rearing, which were analyzed by independent samples *t-test*. Within group comparisons of recognition indexes were analyzed with Wilcoxon test. Between groups comparisons of BDNF levels in serum and brain tissue samples were performed using independent-samples t tests. Parametric data were expressed as mean $\pm$ S.E.M and nonparametric data as median (interquartile ranges). P< 0, 05 was considered to indicate statistical significance.

3. Results

3.1 Open Field behavior

Behavior during exploration of an open field was evaluated in order to control for sensorimotor effects that could be induced by different housing conditions. Results showed no significant differences in latency to start locomotion (p=0,815), number of crossings (p=0,083) and rearing (p=0,107) between experimental groups. Number of fecal pellets was slightly, but significantly (p=0,024), greater for animals of the social condition [2(0/4)] than for rats maintained in the enriched condition [0(0/0)].

3.2 Effects of enriched environment on the Object Recognition Task

Statistical comparison of recognition indexes showed that there was no significant difference (p=0,81) between animals of the social (0,51 [0,46 /0,56] and the enriched conditions (0,53[0,43/0,63]) in the training trial. Within group analyses indicated that animals of the social group showed significant preference (p=0,036) in exploring the novel object during the LTM retention trial (time expended exploring object F was [23(17, 89/34, 71)] and object N was [33, 19(22, 44/36, 9)]. Animals of the enriched condition group also preferred the novel object in the LTM retention test, as Wilcoxon test has indicated a significant difference (p=0,04) between the time spent exploring the familiar and the novel object (time expended exploring object F was [16,78(13,43/36,83)] and object N was [50,6(33,20/77,13)]. However, recognition indexes in the LTM retention trial were significantly (p=0,014) higher for rats housed in the enriched environment (0, 65[0, 62/0, 73]) than for rats maintained in the social condition (0, 55[0, 48/0, 64]) (Fig. 1).

3.3 Measurement of BDNF in the two brain regions of the rats

The BDNF levels in hippocampus (110,45 ± 12,35) and frontal cortex (17,91± 3,92) of rats living in the enriched environment were not significantly different (p>0,2) from those of the hippocampus (161,98 ± 38,19) and frontal cortex (23,06 ± 3,07) of rats maintained in the social condition (Fig. 2A and F. 2B).

3.4 Measurement of BDNF in the serum of the rats

Serum levels of BDNF were also not significantly different (p=0,60), in animals maintained in the enriched condition (978.65 ± 45.95) in relation to animals of the social condition (1009.24± 35.73) (Fig. 2C).

4.Discussion

The present study has investigated the effect of environment enrichment on long term memory for object recognition and BDNF levels in brain (hippocampus and frontal cortex) and serum in rats. The data shown here demonstrate that animals maintained in the enriched condition had enhanced performance on the memory task in relation to the social condition. This enhanced performance can not be attributed to any unspecific sensorimotor effect that could be induced by the different housing conditions, since no significant between groups differences were seen in the open field parameters. Thus, these animals clearly showed an improved retrieval in the memory task. These results are in line with other studies of the effects of environment enrichment (Ickes et al., 2000; Gobbo., 2003; Shun-Wei et al., 2006; Rossi et al; 2006) and, as expected, indicate that increased opportunity for learning experiences and physical activity can improve cognitive performance, regardless of the effects of social interaction. However, the observed effects of the enriched environment on memory

occurred in the absence of any significant alteration in central or peripheral BDNF levels. Although unexpected, the results obtained in this study for BDNF in hippocampus and frontal cortex are not unique (Parks et al, 2008 and Bakos et al, 2009).

While this is not the first study to compare social and enriched conditions, it was the only one that maintained the same number of rats in the two conditions. In other studies (Rossi et al, 2006; Parks et al., 2008 and Bakos et al., 2009) the enriched housing, besides offering opportunity for learning experiences and physical activity, also encouraged a greater social interaction, since 2 to 3 times more animals were maintained in the enriched cages than in the social condition. Thus, our experimental design certainly diminished the difference in social interaction between experimental groups. Looking to our results for brain BDNF from this perspective, it could be possible that only between groups differences in sensory-motor stimulation are not enough to produce significant differences in BDNF levels. In favor to this hypothesis are the studies of Parks et al. (2008) and Bakos et al. (2009), which even maintaining more animals in the social than in the enriched condition, also found no significant differences in hippocampal or frontal levels of BDNF in healthy male rats. Moreover, the great majority of studies that showed increased levels of BDNF in brain, especially in the hippocampus, compared enriched and isolated animals (Pham et al., 1999; Ickes et al., 2000). In this conditions the BDNF differences between groups are improved, since isolation rearing of animals is considered to be a model of social stress (Arakawa et al., 2003; Bianchi et al., 2006) and could depress BDNF levels (Jacobsen et al., 2006; Yulug et al., 2009; Weintraubb et al., 2010), in addition to induce behavioral and physiological changes (Jones et al., 2010). Although this type of experimental design facilitates the investigation of the cellular and behavioral effects of BDNF, it could be hardly translated to the investigations of environment enrichment and BDNF in humans, since isolation is not a common situation for healthy humans.

Besides the reduction of social interaction differences between our experimental groups, there are some other experimental aspects that should be considered in the interpretation of the BDNF results. First we must analyze the possible effects of age and time period during which the animals were maintained in the enriched environment.

Our animals were young and their exposure to the enriched condition occurred when BDNF levels tend to triplicate in frontal cortex and almost double in the hippocampus and serum as a result of postnatal development of the nervous system (Karege et al., 2002). Thus, these developmental alterations of BDNF could have obscured the effects of environmental enrichment if they were mild. It would be interesting to investigate this same experimental protocol in older animals, in which the BDNF reached stable values or is already declining because of aging (Halbach .,2010; Erickson et al.,2010) . In relation to the time period during which the animals were maintained in the enriched condition, it could be argued that 10 weeks may have been insufficient to induce changes in the levels of BDNF. However, previous studies have shown that even smaller periods of time, such as 6 to 8 weeks, are capable to alter BDNF expression (Schneider et al., 2001) and levels in hippocampus (Rossi et al, 2006).

Second, it is important to note that some studies show important regional differences in the hippocampal BDNF levels in response to environment enrichment (Schneider et al., 2001; Ickes et al., 2000; Bayne., 2005; Segovia et al., 2009). Zhu et al (2006), for example, showed that although dorsal hippocampus has higher BDNF concentrations, only ventral hippocampus showed increased (nearly 17%) BDNF levels in mice that were maintained in the enriched tested condition. In this condition, animals were maintained in an enriched environment, tested in different tasks for behavior analysis (such as elevated plus-maze, open-field, novel-objects exploration and food neophobia) and killed 8 days later for neurothrophin analysis. In our study we used a similar protocol, namely exposure to an enriched

environment and behavioral testing (open-field and novel-objects exploration) before killing animals for neurothrophin analysis. However, we used total hippocampus. Thus, it could be that significant between group differences in hippocampus were missed because they where slight and occurred in only a sub region of this structure, and whole hippocampus analysis could have "diluted" these results. Moreover Zhu and colleagues (2006) showed that brain BDNF levels appear to be sensitive to behavioral testing stimuli, reducing the effects of environmental enrichment on neurothrophin concentrations. The authors speculated that behavioral testing could involve some degree of stress or cognitive demand that would likely impinge on BDNF, an aspect that could not be ruled out in our study. It is important to highlight that although the testing protocol used in this study could interfere with the BDNF modulation by environmental enrichment, it is closer to the situations that are found in studies with humans, in which it is virtually impossible to prevent the subjects to do activities other than those proposed in the experimental enrichment protocol and control for everyday stress situations.

Data on serum levels of BDNF obtained in this study were within the values previously described in the literature (Klein et al., 2010), and no significant differences between experimental groups were seen. These results were not surprising, given the absence of significant alterations of BDNF in brain. If there was any BDNF alteration in other brain structures that we have not investigated, it was too small to be reflected in serum levels.

In the absence of significant alterations of BDNF, other factors should be considered to explain the improved performance on LTM for object recognition seen in rats exposed to an increased opportunity for learning experiences and physical exercise. An increasingly body of evidences shows that other neurotrophins, as NGF and NT-3, can be modulated by housing conditions (Ickes et al.,2000; Pham et al.,2002; Shun-Wei et al.,2006) and are related to cellular and behavioral changes resultant from the effects of enriched environment (Gobbo et

al.,2003; Rossi et al.,2006). Besides alterations in neurotrophins, enriched environment could also act on other brain aspects, like neurotransmitter system (Chan et al., 2008; Hoffman et al., 2009; He S et al.,2010) known to modulate learning and memory mechanisms In conclusion, our results indicated that BDNF was not an adequate biomarker of the long term effects of increased physical and learning opportunities on cognition, since young animals exposed to the enriched environment showed increased long-term memory for object recognition without any alterations in central (hippocampus and frontal cortex) or peripheral BDNF levels. However, this study did not aim to draw a final conclusion about the potential of BDNF as a biomarker in intervention studies about the effects of environmental enrichment in healthy humans. We believe that the results of this study are important to highlight the need to develop experimental protocols using animal models that more closely resemble the characteristics of studies with humans and motivate more investigations to determine the conditions under which BDNF could be a good biomarker of the effects of environment enrichment. As discussed above, it is possible that age has an important role in the determination of the potential role of BDNF as a biomarker. Future studies should address this issue, since there are a great number of clinical evidences showing that an enriched life style can slow the rate of cognitive decline in late life (Valenzuela et al, 2007).

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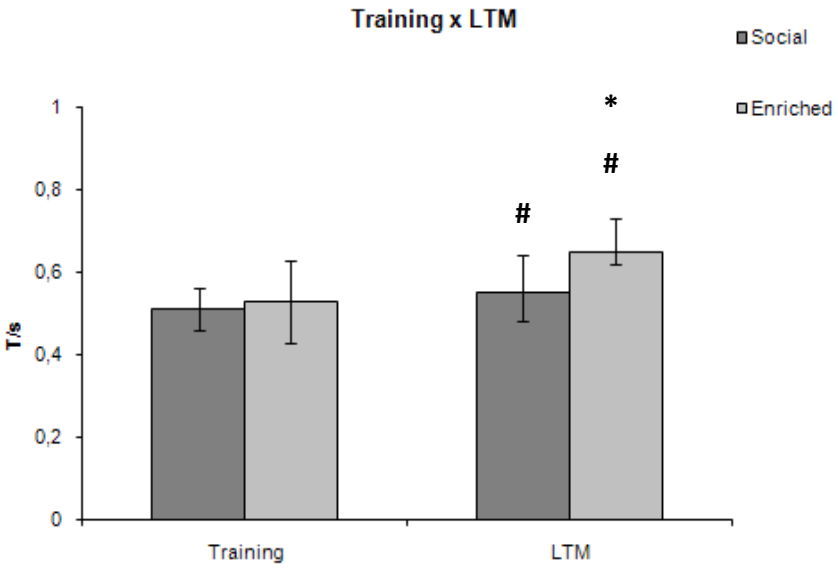
6.Figure Legends

6.1 Fig. 1. Effect of environmental enrichment on recognition memory. Animals were maintained for two months in social or enriched housing conditions, trained on the object recognition task and tested 24h later for long-term retention. Recognition index is expressed by the ratio T_N / (T_F+T_N), T_F=time spent exploring the familiar object and T_N=time spent exploring the novel object. Data expressed as median and interquartile ranges. *P<0,05 in relation to training trail of rats maintained in the enriched condition and in relation to LTM test of control animals.

6.2 Fig.2. Brain- derived neurotrophic factor (BDNF) levels in (A) frontal cortex , (B) frontal cortex and (C) serum of animals maintained in the social (control) and enriched conditions. Values are expressed as mean ± S.E.M. Differences between social condition and enriched condition are indicated: *$p<0,05$.

Fig 1.


Training x LTM
Social
Enriched
1
0,8
0,6
0,4
0,2
0
T/s
*
#
#
Training
LTM

.

Fig. 2A.

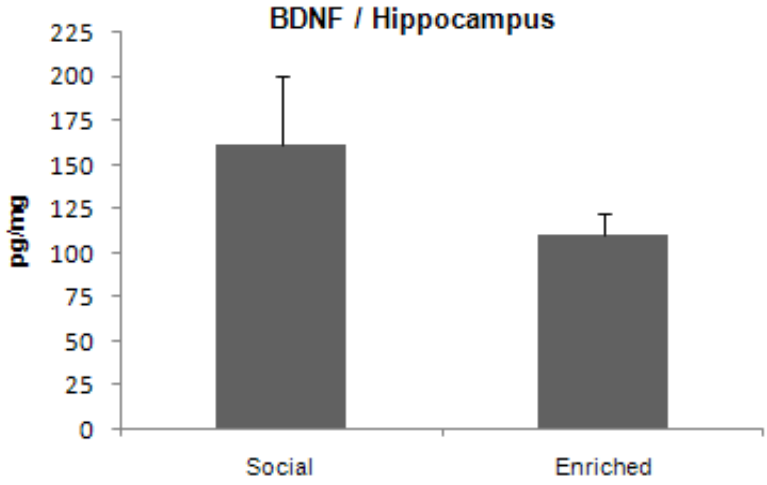

BDNF / Hippocampus
225
200
175
150
125
100
75
50
25
0
pg/mg
Social
Enriched

Fig. 2B.

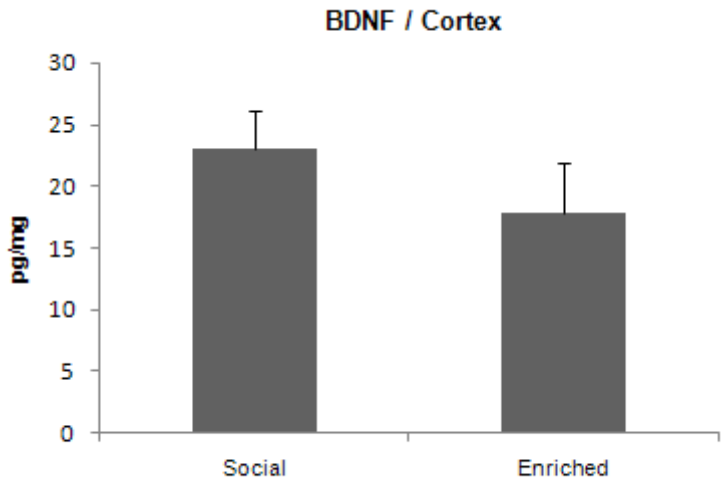
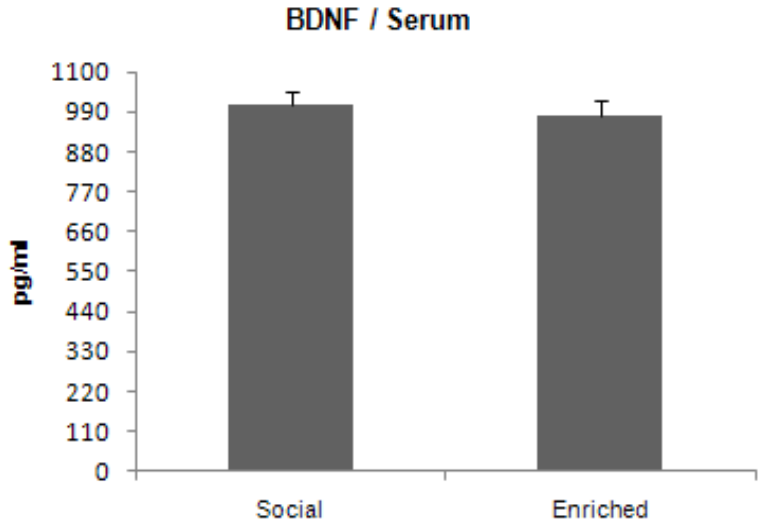

BDNF / Cortex
30
25
20
15
10
5
0
pg/mg
Social
Enriched

Fig. 2C.


BDNF / Serum
1100
990
880
770
660
550
440
330
220
110
0
pg/ml
Social
Enriched

CAPÍTULO 3

Considerações finais

3.1 CONSIDERAÇÕES FINAIS

A maioria dos estudos sobre os efeitos do ambiente enriquecido mantêm os animais do grupo controle em uma situação de isolamento, enquanto os animais do ambiente enriquecido são mantidos em grupos (Schneider et al., 2001; Pitropaolo et al., 2004; Shun-wei et al; 2006). Este tipo de intervenção não nos permite separar a contribuição relativa da interação social em relação a estimulação sensório-motora e atividade física espontânea e, possivelmente, acentua as diferenças nas tarefas comportamentais e nos níveis de neurotrofinas.

Animais mantidos sozinhos são considerados um modelo de estresse social (Arakawa., 2003). Nessa condição, demonstram uma variedade de mudanças comportamentais e psicológicas, como déficit no aprendizado e na discriminação no reconhecimento do objeto (Jones et al.,2010).

Em função destas alterações, este estudo abordou os efeitos do enriquecimento sobre a memória e o BDNF, através de uma série de aspectos metodológicos, pois o objetivo foi traçar um paralelo o mais próximo possível com o ser humano para avaliar a importância da estimulação sensório-motora e atividade física espontânea sobre parâmetros que refletem aspectos funcionais do SNC. Por isso usamos como grupo controle (social) um conjunto de animais que não foi mantido em isolamento, uma tarefa de memória neutra e avaliamos o BDNF no plasma. Esse trabalho foi o primeiro a manter que manteve o mesmo número de ratos nas duas situações de ambiente (ambiente enriquecido / social), o desenho do nosso modelo experimental diminuiu as diferenças da interação social entre os grupos. Além disso, esse modelo experimental facilita a investigação dos efeitos celulares e comportamentais induzidos pelo ambiente enriquecido e pode ser traduzido com estudos de ambiente enriquecido e BDNF com humanos, já que o isolamento não é uma situação comum de pessoas saudáveis.

Outro aspecto importante que deve ser considerado, além da diferença da interação social, é a idade dos animais e o tempo de moradia no ambiente enriquecido.

Observamos que não houve aumento significativo de BDNF no hipocampo, córtex e consequentemente no plasma ao longo do tempo de experimento. A ausência de alteração pode ter sido mascarada pela fase de desenvolvimento pós-

natal destes animais, que por si só já tende a duplicar os níveis dessa neurotrofina no SNC (Karege et al.,2002). Talvez em animais mais velhos se teria uma alteração nos níveis de BDNF no grupo do ambiente enriquecido, já que ocorre um declíneo desta neurotrofina com o envelhecimento.

Outro fator relevante foi o tempo que os animais foram mantidos, pode ter sido insuficiente para mudanças nos níveis de BDNF. Pietropaolo (2004) , relatou que o tempo de quatro dias já seria o suficiente pra produzir mudanças nos níveis de BDNF em ratos jovens, porém não em ratos adultos . Outro estudo observou o aumento de BDNF em ratos expostos durante o período de um ano no ambiente enriquecido (Ickes et al., 2000).

Contudo os animais abrigados nessas condições demonstraram uma melhora na memória , quando submetidos ao teste de reconhecimento do objeto, mostrando que o ambiente enriquecido apresenta uma influência positiva nesse aspecto, pela oportunidade que o enriquecimento ambiental proporciona com experiências de atividade física e aprendizado que podem melhorar o desempenho cognitivo.

Estudos futuros serão importantes, já que um grande número de evidências clínicas mostram que um estilo de vida “enriquecido” pode diminuir um declíneo cognitivo na vida mais tarde (Valenzuela et al., 2007).

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Anexo-

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