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Liana Lisboa Fernandez

Alterações neuropatológicas induzidas
pelo tratamento neonatal com ferro e
pelo envelhecimento em ratos e em
camundongos transgênicos e suas
implicações em processos
neurodegenerativos

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Alterações neuropatológicas induzidas pelo tratamento neonatal com ferro e pelo envelhecimento em ratos e em camundongos transgênicos e suas implicações em processos neurodegenerativos

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RESUMO

O presente estudo foi planejado para investigar alterações neuropatológicas em ratos adultos e velhos, e em camundongos transgênicos APP/PS1 submetidos à administração de ferro suplementar num período crítico pós-natal, com o objetivo de estudar a contribuição de fatores de risco ambientais e genéticos na patogênese de doenças neurodegenerativas. Nenhuma diferença significativa foi vista na abundância das proteínas β -amilóide, tau fosforilada e na α -sinucleína, analisados por IHC no encéfalo, quando ratos tratados com ferro e sem ferro são comparados. Aumento de astrocitose, detectada por densitometria de astrócitos imunoreativos marcados por GFAP, foi encontrado em ratos velhos (24 meses) tratados com ferro na substância negra e estriado e no hipocampo de ratos adultos (3 meses) tratados com ferro quando comparados com controles pareados por idade. Nenhuma modificação nas placas de β -amilóide foram vistas em camundongos transgênicos APP/PS1 tratados e não tratados. Nenhuma diferença na reação microglial foi observada quando comparados os 4 grupos: transgênicos com ferro (TgFe), transgênicos com sorbitol (TgSb), wild type com ferro (WtFe), wild type com sorbitol (WtSb). Ainda, aumento em astrocitose, revelado por densitometria de astrócitos reativos marcados por GFAP, e aumento de níveis de expressão de GFAP, revelados por western blotting, foram encontrados em camundongos tratados com ferro (tanto Tg como Wt) quando comparados com TgSb e WtSb. Este aumento foi acompanhado por alterações significativas na composição de ácidos graxos no cérebro de camundongos APP/PS1 que levaram à diminuição do índice de capacidade peroxidativa de membrana e redução do dano oxidativo protéico. Os presentes achados claramente documentam que o excesso de ferro durante o período neonatal impacta na composição celular e molecular de cérebros de ratos adultos e velhos e de camundongos transgênicos APP/PS1. Estas observações podem encorajar mais estudos focados nos efeitos de suplementações na dieta de crianças.

ABSTRACT

The present study was aimed to investigate neuropathological changes in adult and old rats, and in APP/PS1 transgenic mice subjected to supplementary iron administration in a critical postnatal period in order to study the contribution of environmental and genetic risk factors to the pathogenesis of neurodegenerative disorders. No significant differences in abundance of proteins β -amyloid, phosphorylated tau and α -synuclein analysed by IHC in brain were seen, when treated and non-treated rats were compared. Increased astrocytosis, revealed by densitometry of immunoreactive astrocytes marked with GFAP, was found in old (24 months) treated rats in substantia nigra and striatum and in the hippocampus of adult (3 months) treated rats when compared to age-matching controls. No modifications in β -amyloid burden were seen in treated and non-treated APP/PS1 mice. No differences in microglial reactions were observed when comparing the four groups of mice: transgenic with iron (TgFe), transgenic with sorbitol (TgSb), wild type with iron (WtFe) and wild type with sorbitol (WtSb). Yet increased astrocytosis, as revealed by densitometry of immunoreactive astrocytes marked with GFAP, and increased expression levels of GFAP, as revealed by western blotting, were found in iron-treated mice (both Tg and Wt) when compared with TgSb and WtSb. This was accompanied by significant changes in brain fatty acid composition in APP/PS1 mice that lead to a lower membrane peroxidizability index and to reduced protein oxidative damage. The present findings clearly document that excess of iron during the neonatal period impacts in the cellular and molecular composition of the adult and old brain rats and APP/PS1 transgenic mice. These observations may encourage further studies focused on the effects of dietary supplementations in children.

LISTAS DE ABREVIATURAS

AASA: Ácido aminoadípico

ACL: Comprimento de cadeia média

APOE: Apolipoproteína E

APP/PS1: Proteína Precursora do Amilóide/ Presenilina 1

APP: Proteína Precursora do Amilóide

A β : Peptídeo beta-amilóide

BHE: Barreira Hemato-Encefálica

BHL: Barreira Hemato-Liquórica

CEL: Carboxietilisina

CL: Corpos de Lewy

CML: Carboximetilisina

DA ou AD: Doença de Alzheimer

DAB: Tetrahydroclorito diaminobenzidina

DBI: Índice de ligação dupla

DMT-1: Transportador Divalente de Metal 1

DP ou PD: Doença de Parkinson

ENF: Emaranhados Neurofibrilares

Fe: Ferro

FEEPS: Fundação Estadual de Pesquisa e Produção em Saúde

Fr: Ferritina

GC/MC: Cromatografia a gás/ espectroscopia de massas

GFAP: Proteína Ácida fibrilar da Glia

GSA : Glutathiona sulfonamida- ácido glutâmico

Hfe: Proteína de Hemocromatose Hereditária

Hs: Hemossiderina

IHC: Imunohistoquímica

IL1, IL6, IL8: Interleucinas 1,6 e 8

IREs: Elementos Reguladores do Ferro

IRPs: Proteínas Reguladoras do Ferro

MDAL: Malondialdehidolisina

MUFA: Ácidos Graxos Monoinsaturados

PBS: Solução tampão fosfato

PI: Índice de capacidade peroxidativa

PS: Placas senis

PUFA: Ácidos Graxos Polinsaturados

RL: Radicais Livres

ROS: Espécies Reativas de Oxigênio

Sb: Sorbitol

SBNeC: Sociedade Brasileira de Neurociências e Comportamento

SFA: Ácidos Graxos Saturados

SN: Substância Negra

SNC: Sistema Nervoso Central

Tf: Transferrina

TfR: Receptor de Transferrina

Tg: Transgênico

UFA: Ácidos Graxos Insaturados

Wt: Wild type, selvagem

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CAPÍTULO 1:

1. INTRODUÇÃO

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2.1 OBJETIVOS GERAIS

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1. INTRODUÇÃO

Com o aumento da expectativa de vida, têm crescido o número de indivíduos idosos e conseqüentemente a prevalência de doenças associadas ao envelhecimento, como as doenças neurodegenerativas (ARKING, 1998).

Uma tendência atual é relacionar as enfermidades neurodegenerativas com alterações em proteínas específicas, as quais se acumulam em regiões específicas no SNC, conforme mostrado na Figura 1, porque são sintetizadas de forma anômala, encontram-se em excesso, mudam sua conformação ou têm dificuldades para serem eliminadas (PRUSINER, 2001).

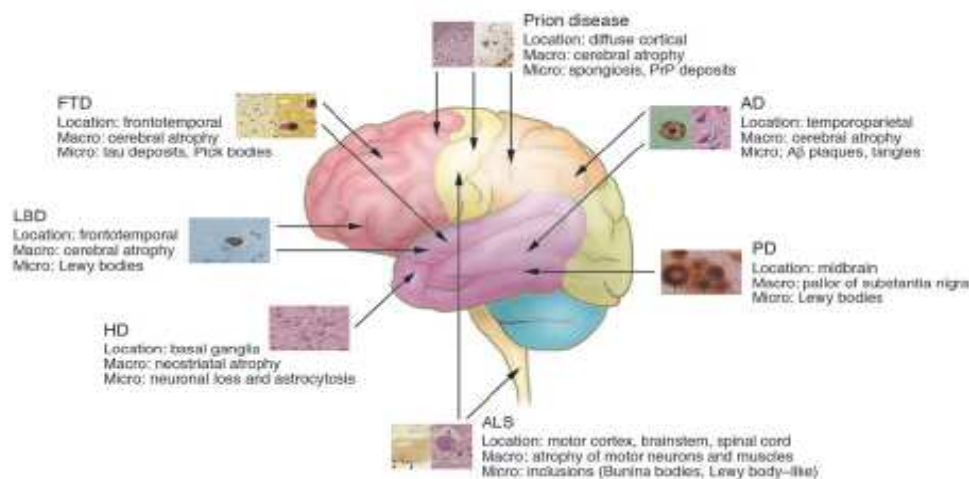


FIG.1 Localização anatômica macroscópica e microscópica das alterações características das doenças neurodegenerativas (BERTRAM, 2005).

Estes agregados protéicos estão presentes também em cérebros de idosos normais, porém em menor quantidade do que em indivíduos com doenças neurodegenerativas (KELLER, 2004). O entendimento da fisiologia destas proteínas, e dos mecanismos que as levam a se acumular formando agregados, poderão contribuir para o entendimento da fisiologia do envelhecimento e a fisiopatologia das doenças neurodegenerativas.

Uma forma especializada de agregado protéico intracelular frequentemente observada durante o envelhecimento normal é conhecida como lipofuscina. A análise bioquímica revela que a lipofuscina é um complexo dinâmico de agregação protéica intracelular composta primariamente de proteína e lipídio com vestígios de carboidratos e metais (KELLER, 2004). Com o tempo, este grânulo se acumula em células de vida longa sendo conhecido como um marcador do envelhecimento celular. Sabe-se que células pós-mitóticas como os neurônios exibem acúmulo de lipofuscina relacionado à idade (KELLER, 2004). O grau de acúmulo de lipofuscina nas camadas e áreas corticais representa o curso oposto da mielinização cortical: neurônios de projeção muito mielinizados e camadas densamente mielinizadas possuem muito poucos grânulos de lipofuscina enquanto àqueles mielinizados por último no mesocórtex temporal são os mais ricamente pigmentados do córtex humano (KELLER, 2004).

As alterações neuropatológicas características da Demência de Alzheimer (DA) são: placas de amilóide extracelulares ou placas senis (PS) (Figura 2) e os emaranhados neurofibrilares (ENF) de proteína tau hiperfosforilada. A PS contém um pequeno e tóxico produto A β 40 e/ou o A β 42 resultado da clivagem da proteína precursora do amilóide (APP) pela rota da β -secretase (BOSSY-WETZEL, 2004; SELKOE, 2004).

A DA é a principal causa de demência e é uma patologia multifatorial resultado da interação de fatores genéticos e ambientais (BOSSY-WETZEL, 2004; SELKOE, 2004; PRINCE, 1998; BLENNOW, 2006).

A DA familiar é uma doença muito rara, de início precoce causada por mutações na Proteína Precursora do Amilóide (APP) ou de genes da Presenilina (PS1, PS2) ambas ligadas ao metabolismo do A β . Por outro lado a DA esporádica é muito comum e sua causa desconhecida, provavelmente porque é uma doença heterogênea causada pelo envelhecimento associado a interações genéticas (polimorfismo gene APOE) e fatores de risco ambientais (BERTRAM, 2005; FERNANDEZ, 2005; BOLCHELT, 1996).

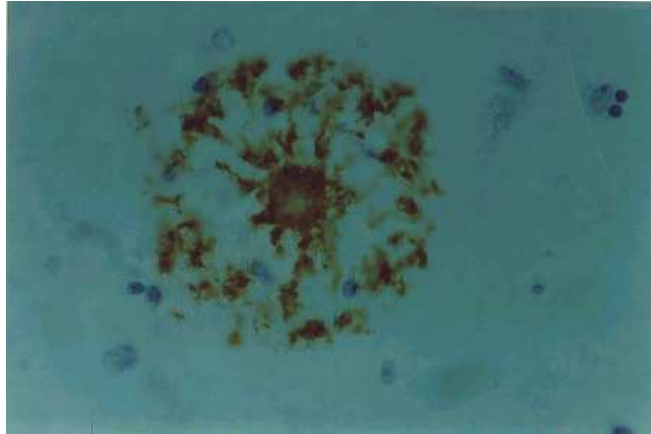


FIG.2 Placa Senil: imunohistoquímica (IHC) para β -amilóide (FERNANDEZ, 1997).

Na Doença de Parkinson (DP) observam-se níveis elevados de agregados de α -sinucleína, uma proteína encontrada na membrana das vesículas do terminal pré-sináptico, com função desconhecida, formando os Corpos de Lewy (CL) quando acumula-se na forma de inclusões citoplasmáticas, por provável sobrecarga do sistema ubiquitina-proteossoma (MOORE, 2005).

É interessante observar que os neurônios mais finos, longos, e pouco mielinizados são os mais suscetíveis à DA e DP. Estes neurônios vulneráveis apresentam agregados de proteína tau ou de α -sinucleína na proximidade de grânulos de lipofuscina (KELLER, 2004; BRAAK 2006; SCHULTZ, 2004).

O estresse oxidativo tem sido considerado um dos principais mediadores do declínio progressivo da função celular observada no envelhecimento normal. O aumento dos níveis de ácidos nucléicos, lipídios e proteínas oxidadas encontrados no envelhecimento causam a inibição ou dano enzimático múltiplo, levando a alterações na síntese de proteínas, produção de energia, dinâmica do citoesqueleto e transdução de sinal, funções fundamentais das células (KELLER, 2004).

A oxidação protéica também aumenta a agregabilidade de moléculas, alterando seu formato e seu índice de degradação, diminuindo o *turnover* de proteínas específicas, determinando interações entre proteínas, formando os agregados protéicos. Estes agregados alteram o tráfego intracelular e sobrecarregam as vias proteolíticas proteossomal e lisossomal (KELLER, 2004).

Acredita-se que o aumento dos níveis de oxidação protéica tem importante papel na agregação da proteína tau e da α -sinucleína tanto pela ação direta das espécies reativas de oxigênio (ROS) como indireta pela inibição da atividade proteossômica induzida por ROS (KELLER, 2004).

O ferro é o metal mais abundante do cérebro. Ele participa dos principais processos neuronais, incluindo síntese de neurotransmissores e mielinização dos axônios (QUINTANA, 2006).

O ferro em sua forma ferrosa é quimicamente uma fonte irrefutável de estresse oxidativo porque ele é capaz de catalizar a formação de radicais livres via reação de Fenton (QUINTANA, 2006; ZECCA, 2004).

Concentrações de ferro cerebral não são estáticas; elas aumentam com a idade e em várias doenças e diminuem quando o ferro é deficiente na dieta (PINERO, 2000). A concentração de ferro no cérebro é muito baixa no primeiro ano de vida e grande quantidade deste metal é necessária durante o desenvolvimento cerebral especialmente durante a mielinogênese onde é encontrado em altos níveis nos oligodendrócitos (FALANGOLA, 2005; ZHANG, 2005). Os oligodendrócitos são as células cerebrais que mais contêm ferro (BARTZOKIS, 2007). A concentração de ferro adquirida até os 20 anos é mantida para o resto da vida do indivíduo (ZECCA, 2004).

O aumento do ferro cerebral com a idade ocorre em concentrações diferentes, em diferentes regiões do Sistema Nervoso Central (SNC). As maiores concentrações de ferro no encéfalo humano adulto são encontradas nos núcleos da base especificamente no globo pálido, no núcleo rubro e na substância negra (SN) (QUINTANA, 2006; FALANGOLA, 2005; GAASCH, 2007).

Em cérebros normais, o ferro não é tóxico apesar de seus altos níveis, provavelmente devido a mecanismos homeostáticos eficientes (FALANGOLA, 2005; CASTELLANI, 2007).

A ferritina é a principal molécula responsável pelo estoque de ferro não tóxico no cérebro. Observa-se um aumento da H-ferritina até os 20 anos e da L-ferritina durante o envelhecimento normal o que pode indicar um mecanismo citoprotetor, seqüestrando o ferro livre e gerando complexos estáveis de ferro

solúvel (Fe^{+3}), protegendo as células do dano oxidativo causado pelo ferro livre (Fe^{+2}) (ZECCA, 2004; QUINTANA, 2006; GAASCH, 2007). A Hemossiderina armazena ferro na sua forma insolúvel (PRINCE, 1998). Um excesso de ferro intracelular pode ser encontrado no reservatório de ferro lábil, forma muito mais transitória (GAASCH, 2007). A ferritina é encontrada no SNC no citoplasma e núcleos dos oligodendrócitos. Portanto a ferritina é particularmente abundante em axônios mielinizados, aqueles pouco comprometidos pela DA. Disfunção na ferritina (com eventual degradação à hemossiderina) poderia resultar num aumento de íons ferrosos tóxicos, levando a uma produção de radicais livres que induziriam tanto ao estresse oxidativo quanto o dano de mielina relacionado à idade e ao declínio cognitivo na DA (QUINTANA, 2006).

A neuromelanina é o principal local de estoque de ferro em neurônios da SN em indivíduos normais e é responsável por 10-20% do conteúdo total de ferro do sistema nervoso. É um pigmento completamente ausente no primeiro ano de vida aumentando continuamente até o fim da vida. (ZECCA, 2004). Tem uma forte afinidade pelo ferro e outros metais apresentando provavelmente um papel protetor contra o acúmulo de ferro intraneuronal. A síntese da neuromelanina depende aparentemente da concentração citosólica da dopamina, porque um produto da oxidação da dopamina é seu precursor. (ZECCA, 2004). Portanto, a síntese da neuromelanina protege contra a toxicidade da dopamina, prevenindo seu acúmulo citoplasmático (ZECCA, 2004; RIEDERER, 2004).

A existência de uma relação tem sido sugerida entre disfunções nas vias de manutenção da homeostasia do ferro, principalmente nas regiões onde seu metabolismo é mais alto, e a patogênese de doenças neurodegenerativas (BENKOVIC, 1993; MARTIN, 1998; QUIAN, 2001). Estudos demonstram a elevação da concentração de ferro na SN de portadores de DP (DEXTER, 1991; JELLINGER, 1993; FAUCHEUX, 1993; KIENZI, 1995; EBALDI, 1996; GRFFITHS, 1999) e ao redor das PS de pacientes com DA (LYNCH, 2000; RIEDERER, 2004).

Sabe-se que a perda da neuromelanina da SN é um marcador neuropatológico da DP (FASANO, 2003). Evidências histológicas demonstram que os neurônios mais pigmentados são os primeiros a degenerarem (FASANO, 2006).

O papel do ferro, da neuromelanina e da ferritina na patogênese da DP não são claros, porém um déficit no acúmulo de ferro pela neuromelanina e da capacidade de estocá-lo pela ferritina pode indicar uma migração progressiva do ferro de seus estoques para o citosol, contribuindo para neurodegeneração (ZECCA, 2004; FASANO, 2006).

A barreira hemato-encefálica e hemato-liquórica controlam a captação de ferro para o cérebro, através da regulação da expressão de proteínas receptoras de transporte, como a expressão de receptor da transferrina no endotélio e células do plexo coróide (RIEDERER, 2004). O aumento significativo do ferro no sistema nervoso de pacientes com DP pode estar relacionado também com uma alteração na barreira hematoencefálica (RIEDERER, 2004).

Também foi descrita que a neurodegeneração do sistema nervoso induzida por inibidores de proteossoma pode ser prevenida por quelantes de ferro (ZHANG, 2005).

Sabe-se que o A β liga-se com Cu⁺², Fe⁺² e Zn⁺² e esta ligação com metais induz a uma alteração da conformação do A β tipo *β -sheet*, resultando num aumento da agregabilidade (BOSSY-WETZEL, 2004).

Acredita-se que o A β pode mediar efeitos danosos às células ligando-se a esses metais que geram reações de redução e oxidação, liberando radicais livres (BOSSY-WETZEL, 2004). A análise dos dados disponíveis indica que o A β possui propriedades de uma apolipoproteína ligante de metais que influencia o transporte e metabolismo lipídico (KONTUSH, 2004). Como o fragmento A β tem uma alta afinidade por ligar-se a metais iônicos, ferro e cobre livre ligar-se-iam rapidamente no A β solúvel e o complexo A β -metal formaria agregados que estimulariam uma resposta inflamatória da microglia levando a fagocitose destes complexos. Portanto, as ligações dos metais com A β pode ser um mecanismo de remoção dos íons metálicos do espaço extracelular e de eliminação dos

mesmos do cérebro. Isto sugere que o A β pode ajudar a limitar a toxicidade de metais, auxiliando mecanismos antioxidantes cerebrais. Caso este complexo não seja eliminado, então formaria o centro da placa, agregando com o tempo mais A β (BISHOP, 2004).

O envolvimento direto do ferro na formação da placa senil também induz, além da ativação da micróglia, a ativação de astrócitos reativos, levando à síntese de várias citocinas (IL-1, IL-6, IL-8) que ativam os macrófagos a produzir grande quantidade de ROS. A ativação da micróglia também libera ferro da ferritina de uma maneira dependente de superóxido, resultando em oxidação lipídica. (CASTELLANI, 2007).

Os emaranhados neurofibrilares são outro sítio de acúmulo de ferro (CASTELLANI, 2007). O Fe⁺³ é essencial para induzir agregação da proteína tau hiperfosforilada, levando à formação de emaranhados neurofibrilares (YAMAMOTO, 2002). Além disso, o A β induz a desregulação de cinases/fosfatases associadas com a hiperfosforilação da tau, constituindo uma via crítica para neurodegeneração (EGAÑA, 2003). Por isso diversas pesquisas com quelantes de metais têm sido realizadas em busca de novas terapias para DA (FINEFROCK, 2003; WHITE, 2006; HUANG, 2004).

Fredriksson (1999), e Schroder (2001), utilizando camundongos e ratos, descreveram pela primeira vez que o tratamento sistêmico com ferro durante o período de rápido desenvolvimento cerebral (nos humanos este período equivale ao último trimestre da gestação até o primeiro ano de vida) produz acúmulo seletivo de ferro nos gânglios da base, além de causar disfunções neurocomportamentais. Ratos e camundongos tratados com ferro do 10^o ao 12^o dia de vida pós-natal apresentam hipoatividade motora, bem como déficits no aprendizado e memória em duas tarefas comportamentais, o labirinto radial de oito braços e a esQUIVA inibitória (FREDRIKSSON, 1999; FREDRIKSSON, 2000; SCHRODER, 2001). Subsequentemente, diversos estudos realizados em nosso laboratório demonstraram que o tratamento com ferro no período neonatal também produz prejuízos em memória de reconhecimento (DE LIMA, 2004, 2005a).

Este modelo animal foi útil na demonstração do efeito neuroprotetor da selegilina (inibidor da monoaminoxidase B), rolipram (inibidor fosfodiesterase) e desferoxamina (quelante de ferro) com reversão dos déficits cognitivos (DE LIMA, 2004; 2007, 2008, 2005b).

O camundongo transgênico PS1/APP é um modelo de patologia da DA, superexpressando ambos genes mutantes APP e PS1, demonstrando precocemente (aos 6 meses) extensa deposição de amilóide (BOLCHELT, 1996; SCHEUNER, 1996; MC GOWAN, 1999). Este animal transgênico é usado para compreender o desenvolvimento das alterações neuropatológicas da DA.

É evidente o impacto do conteúdo de ferro na dieta alimentar sobre o metabolismo desse metal no sistema nervoso central. Como o período neonatal é crítico para o estabelecimento do conteúdo de ferro cerebral nos adultos, torna-se importante estudar os possíveis efeitos tóxicos da sobrecarga desse metal nessa fase, suas conseqüências no envelhecimento e possíveis associações com a fisiopatologia de doenças neurodegenerativas.

Por isso este trabalho se propõe a contribuir na elucidação do papel do ferro como agente indutor da agregação protéica encontrada nas diversas desordens neurodegenerativas e no próprio envelhecimento normal.

2. OBJETIVOS

2.1 Objetivo Geral

Avaliar as alterações neuropatológicas induzidas pelo tratamento neonatal com ferro e pelo envelhecimento em ratos e em camundongos transgênicos.

2.2 Objetivos Específicos

- Avaliar a presença de proteína tau hiperfosforilada em ratos tratados com ferro neonatal comparados com ratos controles na fase adulta (3 meses) e no envelhecimento (24 meses) através de IHC;
- Avaliar a presença de beta-amilóide em ratos tratados com ferro neonatal comparados com ratos controles na fase adulta (3 meses) e no envelhecimento (24 meses) através de IHC;
- Avaliar a presença de alfa-sinucleína em ratos tratados com ferro neonatal comparados com ratos controles na fase adulta (3 meses) e no envelhecimento (24 meses) através IHC;
- Avaliar a presença de GFAP em ratos tratados com ferro neonatal comparados com ratos controles na fase adulta (3 meses) e no envelhecimento (24 meses) através IHC;
- Avaliar a presença de β -amilóide e microglia em camundongos transgênicos e selvagens tratados com ferro neonatal comparados com camundongos transgênicos APP/PS1 e selvagens controles na fase adulta (6 meses) através IHC;
- Avaliar a presença de GFAP em camundongos transgênicos APP/PS1 e selvagens tratados com ferro neonatal comparados com camundongos transgênicos e selvagens controles na fase adulta (6 meses) através de IHC e WB;

- Avaliar a distribuição de lipídios e concentração de marcadores de estresse oxidativo nos camundongos transgênicos APP/PS1 tratados com ferro comparados com os tratados com sorbitol, através de espectroscopia de massas.

CAPÍTULO 2

ARTIGO

TÍTULO: Ferro e neurodegeneração / *Iron and neurodegeneration*

REVISTA: *SCIENTIA MEDICA*,2007;17(4): 218-224.

Ferro e Neurodegeneração
Iron and neurodegeneration

Liana L. Fernandez¹, Luis Henrique T. Fornari², Malu Viter², Nadja Schroder³

¹Doutoranda Biologia Celular e Molecular PUCRS, Prof. Assistente FFFCMPA, Coordenadora do Ambulatório de Demências da Santa Casa de Misericórdia de porto Alegre.

²Acadêmicos Medicina FFFCMPA

³Doutora, Prof. Pós-graduação Biologia Celular e Molecular PUCRS

Liana Lisboa Fernandez, Cel. Bordini, 675/204
Fone/Fax:33325983, lfernandez@plugin.com.br

RESUMO:

O ferro tem tido um papel importante na participação dos mecanismos da neurodegeneração. Esta revisão tem por objetivo abordar aspectos do seu metabolismo: absorção, transporte e estoque; sua participação no estresse oxidativo; e, por fim, hipóteses de sua participação nas doenças neurodegenerativas mais freqüentes: Demência de Alzheimer (DA) e Doença de Parkinson (DP). Foram utilizados artigos de revisão e originais acessados pelo Pubmed e Scopus, dos últimos sete anos, assim como livros textos. Foram excluídos artigos que o principal enfoque era o uso de drogas e mutações genéticas relacionadas com metais. Concluiu-se que o desequilíbrio na homeostase do ferro, aumentando o estresse oxidativo é uma via importante em relação à patogênese da neurodegeneração. Torna-se fundamental o esclarecimento se estas alterações são causa ou consequência do processo neurodegenerativo.

DESCRITORES: ferro, neurodegeneração, estresse oxidativo, Demência de Alzheimer, Doença de Parkinson

ABSTRACT:

Aims: Increasing evidence has indicated that iron plays an important role in the pathogenesis of neurodegenerative disorders. The aim of this article is to review aspects related to iron absorption, transport and storage in the human body. Additionally, the role of iron in oxidative stress in the central nervous system, and its implications to prevalent neurodegenerative disorders, with special reference to Alzheimer's dementia and Parkinson's disease are discussed. Data source: A systematic review of all published

English literature was conducted on Medline, Ovid, and Scopus, from January 2000 through September 2007. Textbooks were used as well. Studies focusing mainly on pharmacological therapies and metal-related genetic mutations were not included. Summary of the findings: This article review the iron metabolism like absorption, transport and storage, and its

influence in oxidative stress and in the most important neurodegenerative disorders, Alzheimer's dementia and Parkinson disease. Conclusions: The reviewed literature strongly suggests that iron-induced oxidative stress is a central pathway in the pathogenesis of neurodegenerative disorders. These data warrant further investigation in order to clarify whether disruption in iron homeostasis plays a causative role or is a consequence of the neurodegenerative process.

KEYWORDS: iron/metabolism; nerve degeneration; oxidative stress; Alzheimer disease; Parkinson disease.

Scientia Medica, Porto Alegre, v. 17, n. 4, p. 2

INTRODUÇÃO:

O ferro tem sido descrito como um elemento importante na participação dos mecanismos da neurodegeneração. O entendimento do seu metabolismo e das disfunções relacionadas ao estresse oxidativo é fundamental para desvendar a patofisiologia de doenças neurodegenerativas, como DP e DA, cada vez mais prevalentes no nosso meio devido ao aumento da expectativa de vida.

MATERIAL e MÉTODOS:

A revisão foi feita utilizando-se livros textos, artigos de revisão e originais retirados do Pubmed e Scopus sobre o assunto: *iron, neurodegeneration process, Alzheimer, Parkinson*, nos últimos sete anos. Excluíram-se artigos que enfocavam principalmente drogas, como quelantes de metais, e mutações genéticas relacionadas com acúmulo de ferro. Utilizou-se livros textos com enfoque no metabolismo do ferro e estresse oxidativo. A revisão concentrou-se na fisiologia do metabolismo do ferro, estresse oxidativo e possíveis alterações relacionadas às patologias neurodegenerativas: DP e DA.

RESULTADOS E DISCUSSÃO:

ABSORÇÃO, TRANSPORTE, ESTOQUE DO FERRO E ACESSO AO SISTEMA NERVOSO CENTRAL (SNC)

São três as proteínas que medeiam a distribuição e o transporte de ferro: Transferrina (Tf), Receptor de Transferrina (TfR) e Ferritina (Fr). A Tf conduz ferro para os tecidos que possuem TfR. A maior parte dos íons férricos ligados à Tf provém da degradação da hemoglobina de eritrócitos velhos; este processo é realizado pelos macrófagos do sistema reticuloendotelial (baço, fígado, medula óssea) e proporciona uma reciclagem de ferro. Pouco ferro ligado à Tf é proveniente da alimentação (1%). Fr e hemossiderina (Hs) são exemplos de proteínas com função de armazenamento de ferro intracelular. A Fr é composta por uma concha protéica externa (apoferritina - a qual contém de 4000 a 5000

íons férricos) e por um núcleo hidroxifosfato de ferro; a Hs provém da digestão lisossomal de agregados de moléculas de Fr¹.

As proteínas reguladoras do ferro (IRPs) percebem as concentrações de ferro intracelular e participam da manutenção da sua homeostase. Estas IRPs (IRP1 e IRP2) ligam-se a elementos reguladores do ferro (IREs) presentes nos mRNAs que codificam proteínas envolvidas com o metabolismo do ferro controlando suas traduções. O TfR e a Fr são dois alvos importantes das IRPs e são responsáveis pela captação e estoque do ferro celular respectivamente².
Fig.1.

A absorção do ferro ocorre no duodeno, preferencialmente a partir do ferro reduzido. A quantidade de ferro absorvida é regulada conforme as necessidades do organismo através de mudanças na expressão de DMT-1 (transportador divalente de metal 1) envolvido na captação de ferro da luz intestinal através das microvilosidades e, de ferroportina (transportador de ferro que controla a saída de ferro da célula para o plasma portal), conforme a concentração de ferro nos enterócitos vilosos das criptas intestinais. Tais enterócitos possuem TfRs associados à proteína Hfe (proteína da hemocromatose hereditária) na sua superfície basal¹. A Hfe normal interage com TfR, atenuando sua capacidade de mediar a entrega do ferro para o interior da célula².

A expressão do DMT-1 é controlada da mesma forma que o TfR. Fig1 Na deficiência de ferro, a Tr (baixo índice de saturação) fornece pouco ferro aos enterócitos vilosos das criptas intestinais. Aumenta a síntese de DMT-1 e a sua conseqüente expressão nas microvilosidades intestinais. Embora não haja evidências experimentais de que os níveis de ferroportina aumentem durante a deficiência de ferro, supõe-se que um mecanismo análogo envolvendo IRP/IRE ocorra e culmine no aumento da expressão de ferroportina. Os aumentos nas concentrações de DMT-1 e ferroportina resultam num aumento da transferência de ferro do enterócito para o sangue portal¹. Fig.2

A principal causa da deficiência de ferro, caracterizada pela perda progressiva dos estoques de Fr e Hs, é a perda crônica de sangue. O baixo padrão de

saturação da Tr está relacionado à deficiência na síntese de hemoglobina e conseqüente anemia ferropênica (hipocrômica e microcítica)¹. A deficiência de ferro também tem sido descrita como tendo papel na patofisiologia da síndrome das pernas inquietas³.

Uma das principais causas da sobrecarga de ferro é o aumento da absorção intestinal, o que predispõe a uma deposição nociva de ferro tissular. Na Hemocromatose Hereditária, há absorção exacerbada de ferro, em função da mutação na proteína Hfe. As concentrações intracelulares de ferro nos enterócitos permanecem baixas, o que determina a absorção contínua de ferro¹. Em relação aos tecidos (especialmente fígado, coração, glândula pituitária e cérebro) ocorre um excesso de seqüestro de ferro. A Hemocromatose tem sido descrita como fator de risco para DP e DA².

Concentrações de ferro cerebral não são estáticas; elas aumentam com a idade e em várias doenças e diminuem quando o ferro é deficiente na dieta⁴. Pinero. O aumento do ferro cerebral com a idade ocorre em concentrações diferentes, em diferentes regiões do SNC. As maiores concentrações de ferro no encéfalo humano adulto são encontradas nos núcleos da base especificamente no globo pálido, no núcleo rubro e na substância negra^{5,6,7}. O ferro também está presente na substância branca, especialmente durante o desenvolvimento cerebral, onde é encontrado em altos níveis nos oligodendrócitos para formação de mielina^{5,8}. A barreira hemato-encefálica (BHE) e a barreira hemato-liquórica (BHL) controlam a captação do ferro para dentro de cérebro pela regulação da expressão de receptores de proteínas de transporte, tais como TfR no endotélio e em células do plexo coróide^{5,9,10,11}. Para manter a homeostase do ferro no SNC, o sistema IRP/IRE regula a expressão de Fr e TfR^{2,9}. O ferro ligado a Tf da circulação sistêmica é endocitado pelas células endoteliais cerebrais na sua forma férrica (Fe+3)¹⁰. No endossoma é convertido a sua forma ferrosa (Fe+2) na presença do pH ácido. É transportado ao citossol através da DMT-1 aumentando sua concentração no reservatório de Fe+2 lábil^{2,7}. Daí o ferro pode ativar o sistema IRP/IRE; ser sequestrado por chaperonas ou proteínas de estoque (Fr, neuromelanina, Hs, metalotioninas, frataxina); ser reoxidado a Fe+3 e ser

exportado da célula via ceruloplasmina/IRG ou ferroportina; participar de reações catalizadas por ferro gerando espécies reativas de oxigênio (ROS)^{2,7,9,10}. Ferro não ligado a Tf é encontrado dentro do cérebro, no fluido extracelular, sugerindo que neurônios e outras células do cérebro podem potencialmente capturar ferro de uma forma livre de Tf, como por exemplo DMT1, receptor de lactoferrina (neurônios) e ceruloplasmina^{7,11}.

Em cérebros normais, o ferro não é tóxico apesar de seus altos níveis, provavelmente devido a mecanismos homeostáticos eficientes^{5,9}.

Um terço a três-quartos de todo ferro cerebral está estocado dentro das células gliais ligado a Fr (Fe^{+3}) na sua forma solúvel, protegendo as células do dano oxidativo causado pelo ferro livre (Fe^{+2})^{6,7}. A Hs armazena ferro na sua forma insolúvel⁷. Um excesso de ferro intracelular pode ser encontrado no reservatório de ferro lábil, forma muito mais transitória⁷. Os oligodendrócitos são as células cerebrais que mais contêm ferro¹². Na pars compacta da substância negra (SN) a neuromelanina é conhecida como principal armazenador de ferro. Ela não é detectável durante o primeiro ano de vida e aumenta a partir da segunda década, continuamente, até os 80 anos^{9,13}. Já a Fr na SN é muito baixa no primeiro ano de vida, aumenta até os 20 anos e permanece constante até os 90 anos¹³. Os níveis de ferro aumentam até a quarta década de vida e permanecem constantes até os 90 anos na SN de indivíduos normais¹³. No caso de uma BHE alterada o ferro passa a ser continuamente capturado pelo cérebro levando a um acúmulo excessivo deste metal¹⁴.

FERRO E ESTRESSE OXIDATIVO

O oxigênio apresenta um papel essencial em nosso organismo, porém, também, um papel tóxico¹⁵. Durante a respiração mitocondrial, o oxigênio molecular é reduzido em água pelas células, para a formação de energia, produzindo concomitantemente pequenas quantidades de radicais livres¹⁶. Através de processos enzimáticos e não-enzimáticos que ocorrem normalmente na célula, o oxigênio aceita elétrons livres e se transforma em radicais de oxigênio altamente reativos (ROS: H_2O_2 , O^{2-}). A geração de radicais livres (RL:OH) atua fazendo

parte do mecanismo de defesa antimicrobiana humana que se destina a destruir microorganismos invasores, células tumorais e outras células alvos de remoção. Por outro lado, podem ser extremamente tóxicos danificando lipídios, proteínas, DNA e RNA celulares, levando a várias formas de lesão celular. Porém, as células possuem sistemas de defesa (proteínas quelantes de metais, enzimas de defesa, antioxidantes) para prevenir lesões causadas por ROS. Um desequilíbrio entre a taxa de geração e a capacidade de remoção celular de radicais livres causa um estresse oxidativo, que pode ser causa direta de uma patologia ou, estar associado a uma forma de perpetuar o dano celular causado por outro processo patológico¹⁵.

Metais de transição, como o ferro (ou o cobre) doa ou aceita elétrons livres durante reações intracelulares e catalisa a formação de radicais livres, como na reação de Fenton: H_2O_2 (ROS) + Fe^{2+} = Fe^{3+} + OH (RL) + OH^- . Visto que a maior parte do ferro intracelular está na forma férrica (Fe^{3+}) ele primeiro precisa ser reduzido para a forma ferrosa (Fe^{2+}) para participar da reação de Fenton¹⁶.

No estado metabólico normal, o superóxido favorece a oxidação de Fe^{2+} a Fe^{3+} . No entanto, se a concentração intracelular de superóxido é elevada, a reação favorece a redução de Fe^{3+} a Fe^{2+} perpetuando a reação de Fenton formando mais radicais hidroxila⁹. FIG3

Os níveis das ROS, no entanto, são minimizados pela ligação dos íons a proteínas de armazenamento e de transporte (p.ex., Tr, Fr, lactoferrina e ceruloplasmina), que agem como quelantes e assim, minimizam a formação de OH^- ¹⁶.

De todos os órgãos, o cérebro deve ser considerado o mais sensível ao estresse oxidativo devido as seguintes características:

- Alto consumo de oxigênio (20% de todo organismo);
- Altos níveis de ferro e ascorbato (cruciais para peroxidação lipídica da membrana, através da Reação de Fenton);
- Relativamente baixos níveis de agentes protetores antioxidantes;
- Tendência a acumular metais;

- Microglia (macrófagos do SNC) produz ROS sob ativação e é capaz de secretar citocinas inflamatórias;
- Contém altas concentrações de neurotransmissores auto-oxidáveis (dopamina, noradrenalina) que reagem com O₂ produzindo ROS;
- Contém aminoácidos excitotóxicos (glutamato);
- Contém enzimas (monoamino oxidase, tirosina, etc) que produzem H₂O₂ como produtos finais de suas atividades;
- E tem alto tráfego de Ca²⁺ através da membrana neuronal seguindo interferência com transporte de íons (pela ruptura de metabolismo energético)^{17,18,19}.

Ocorrendo um excesso ou desregulação na homeostase do ferro em áreas cerebrais relevantes, aumenta o dano oxidativo induzido por ferro, levando a processos neurodegenerativos com conseqüente morte neuronal^{5,20,21}.

NEURODEGENERAÇÃO:

Uma classificação clara de neurodegeneração pode basear-se nas principais alterações neuropatológicas: presença de componentes protéicos anormais que se acumulam no cérebro, levando à perda neuronal, dependentes da idade. Acúmulos de β -amilóide (A β) nas placas senis (PS) e de proteína tau hiperfosforilada nos emaranhados neurofibrilares (ENF) da DA; da α -sinucleína nos corpos de Lewy (CL) na DP; agregados da proteína huntingtina na Doença de Huntington; corpos de Pick na demência de Pick são alguns exemplos. Um mecanismo comum no desenvolvimento de processos neurodegenerativos é a presença de alteração na conformação de proteínas (por oxidação protéica ou dano oxidativo de RNA), gerando estruturas intermediárias, que formam oligômeros solúveis (considerados os mais tóxicos) que posteriormente se agregam formando protofibrilas e por fim fibrilas que são consideradas marcadores de neurodegeneração. Tanto a agregação protéica como o estresse oxidativo, presentes nestas patologias, estão associados com o envolvimento de metais^{9,22,23,24}.

Há uma tentativa durante o envelhecimento normal, de manter o turnover destas proteínas anômalas, via sistemas de degradação proteolítica proteossomal e lisossomal, levando a formação e acúmulo do pigmento lipofuscina dentro das células de vida longa como os neurônios. A lipofuscina é um agregado protéico, composto por proteína, lipídio, traços de carboidratos e também metais, oriunda da degradação lisossomal. Ainda não se sabe o papel da lipofuscina no envelhecimento ou nas doenças relacionadas à idade, porém parece que ela pode induzir neurotoxicidade pela geração de ROS. Além disso, a degradação lisossomal de mitocôndrias possivelmente é uma das fontes metabólicas de ferro dentro de uma célula danificada levando ao aumento do estresse oxidativo. O aumento da quantidade de proteínas oxidadas ou a diminuição de sua degradação leva a formação dos agregados relacionados com a neurodegeneração²⁵.

PAPEL DE FERRO NA DA:

Existe duas vezes mais ferro na neuropila de pacientes com DA que em pacientes não demenciados⁵. A Fr aumenta com a idade e seu acúmulo é considerado fator de risco para DA⁶. Sabe-se que a proteína A β tem sítios de ligação com íons metais e que a interação entre A β e ferro é um dos fatores responsáveis por agregação e depósito do A β ^{5,26}. Existem evidências que se ligando com o ferro o A β está agindo como quelante para diminuir o dano oxidativo de altas concentrações de ferro livre, porém o acúmulo deste complexo parece contribuir para aumentar o estresse oxidativo apresentando paradoxalmente um efeito neuroprotetor e neurotóxico^{5,9,22,27,28}. Acredita-se que oligômeros de A β solúveis sejam tóxicos e seus níveis correspondam ao grau de neurodegeneração na DA^{18,19}.

O envolvimento do ferro diretamente na formação da PS também induz a ativação da microglia e astrócitos reativos levando a síntese de várias citocinas (IL1, IL6, IL8) que ativam macrófagos a produzirem grande quantidade de ROS. A ativação da microglia também libera ferro da ferritina de uma maneira dependente de superóxido resultando em oxidação lipídica⁹.

Os ENF são outros sítios de acúmulo de ferro⁹. O Fe+3 é essencial para induzir agregação da proteína tau hiperfosforilada levando a formação de ENF²⁹. Além disso, o A β induz a desregulação de kinases/fosfatases associadas com a hiperfosforilação da tau constituindo uma via crítica para neurodegeneração³⁰. Em relação a modelos animais, Schroder e cols demonstraram em ratos adultos, que receberam excesso de Fe+2, 10-12 dias após o nascimento, déficits de memória nos testes de campo aberto e labirinto radial, comprovando um efeito danoso tardio do ferro em comportamentos aprendidos³¹.

PAPEL DO FERRO NA DP:

A neuromelanina da SN armazena grande quantidade de ferro que pode migrar progressivamente para o citosol, durante a patogênese da DP, aumentando ROS, fazendo com que os neurônios dopaminérgicos nigrais sejam peculiarmente susceptíveis ao estresse oxidativo. Evidências histológicas têm demonstrado que neurônios mais pigmentados são os primeiros a degenerarem na DP³². Qualquer evento que concorra para aumentar o potencial oxidativo destes neurônios (mobilização de Fe, aumento concentração de dopamina intracelular, estabilização de protofibrilas protéicas: α -sinucleína) poderiam constituir o evento inicial desencadeante da cascata de degradação oxidativa³³. Além de contribuir para o estresse oxidativo o ferro induz a agregação de α -sinucleína contribuindo para formação de CL^{34,35}.

Zhang e cols demonstraram papel neuroprotetor de um quelante de ferro num modelo de degeneração nigral induzido por inibidor de proteossomo, diminuindo a perda de neurônios dopaminérgicos e presença α -sinucleína de CL o que confirma o papel importante do ferro como causa das alterações neuropatológicas da DP³⁵.

CONCLUSÃO:

O ferro é um metal fundamental para homeostase do organismo. Porém quando em excesso, não conseguindo ser estocado pela Fr e neuromelanina, passa a desencadear reações oxidativas aumentando o estresse oxidativo. Um

desequilíbrio entre a formação de radicais livres e das enzimas de defesa contra seus danos, leva a oxidação de elementos fundamentais celulares para um funcionamento normal, trazendo alteração na conformação de proteínas e aumento de sua agregabilidade formando fibrilas e por fim, neurodegeneração.

Um modelo animal que comprove este processo é fundamental para o entendimento da fisiopatologia das doenças neurodegenerativas (DP e DA as mais freqüentes) e para o desenvolvimento de novas drogas.

ANEXOS

Fig 1

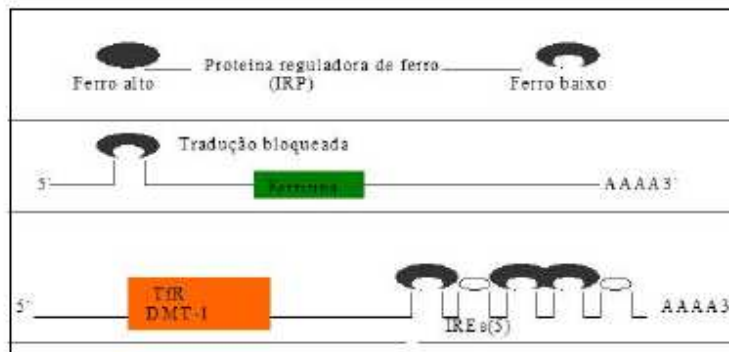


Figura 1 - Quando o ferro celular está deficiente, a IRP(1) liga-se a estruturas em alças IREs localizadas nos mRNAs da Fr e de TfRs. A ligação da IRP ao IRE localizado a montante (dentro da região 5' não traduzida) no mRNA da Fr provoca um bloqueio de sua tradução, reduzindo a concentração de Fr intracelular. A ligação da IRP ao IRE localizado a jusante (dentro da região 3' não-traduzida) no mRNA do TfR provoca a sua estabilização e, portanto, aumento de tradução, elevando a expressão de TfR. Contrariamente, quando há sobrecarga de ferro intracelular, a IRP diminui a sua capacidade de se ligar aos IREs. Conseqüentemente, há um aumento na expressão de Fr e uma diminuição na expressão de TfR1. Figura baseada em Hoffbrand.¹ O excesso de ferro direciona a IRP2 ao proteossoma para ser degradada.

Fig 2

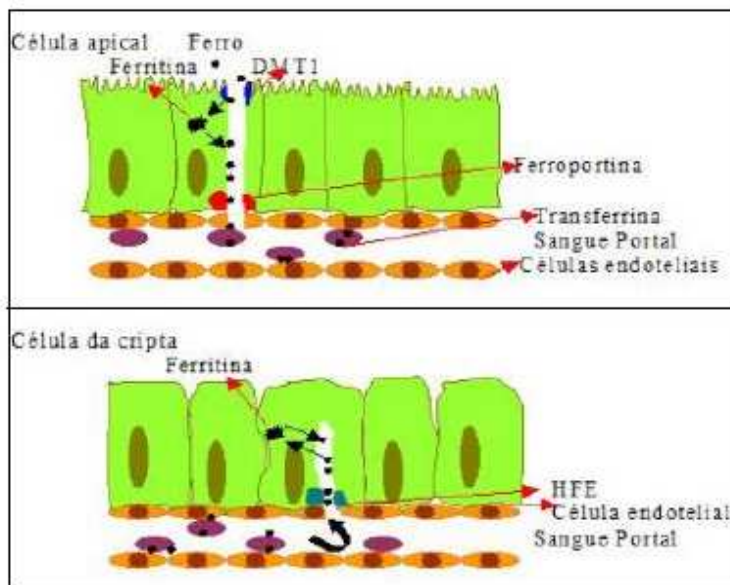


Figura 2 - Controle da absorção do ferro pelos enterócitos. Baseada em Hoffbrand.¹

Fig.3

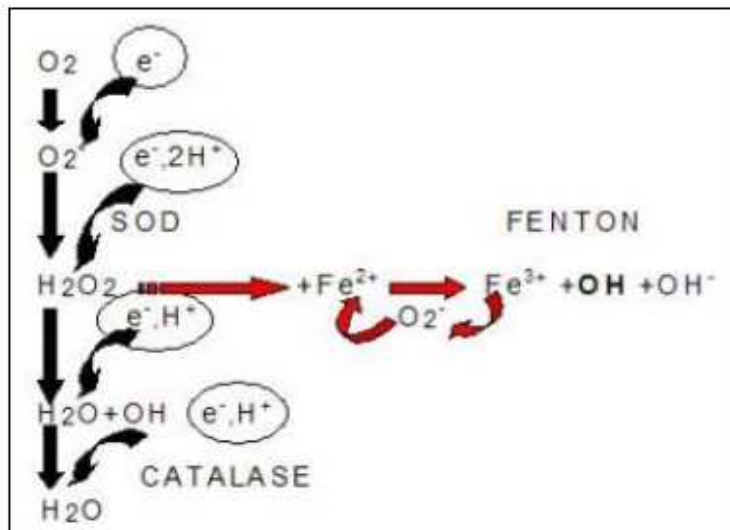


Figura 3 - Perpetuação da formação de radicais hidroxila pela reação de Fenton na presença de excesso de superóxido. Baseada em Robbins¹⁵ e Marks.¹⁶

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CAPÍTULO 3

ARTIGO CIENTÍFICO

TÍTULO: Neuropathological changes following iron administration in adult and old rats

REVISTA: manuscrito em preparação para submissão

Research paper

Section Editor:

Neuropathological changes in adult and old rats following early post-natal iron administration

Liana Lisboa Fernandez^{1,2}, Maria Noêmia Martins de Lima¹, Felipe Scalco¹, Gustavo Vedana¹, Clívia Miwa¹ Arlete Hilbig², Nadja Schröder¹

¹Neurobiology and Developmental Biology Laboratory, Faculty of Biosciences, Pontifical Catholic University, 90619-900 Porto Alegre, RS, Brazil

²Health Basic Science Department, Federal University of Medical Science, Porto Alegre, RS, Brazil

*Correspondence to: Liana Lisboa Fernandez, Rua Coronel Bordini, 675, sala 204, Porto Alegre, CEP 90440-001, RS, Brasil. Tel.fax:+55 51 33325983
E-mail address: llfernandez@plugin.com.br

Abstract

The present study was aimed to investigate neuropathological changes in adult and aged rats subjected to supplementary iron administration in a critical postnatal period in order to study the contribution of environmental risk factors to the pathogenesis of neurodegenerative disorders. Ten rats received iron between 12th and 14th post-natal days; 9 rats received vehicle (sorbitol 5% in water) in the same period. Five iron-treated and 3 sorbitol-treated rats were killed at the age of 3 months (adults) while 5 iron-treated and 6 sorbitol-treated rats were killed at age of 24 months (aged) and their brains processed for immunohistochemistry. No significant differences in β -amyloid, phosphorylated tau and α -synuclein were seen when iron-treated and non-treated rats were compared. Increased astrocytosis, revealed by densitometry of GFAP-immunoreactive astrocytes, was found in old (24 months) iron-treated rats in substantia nigra and striatum and in the hippocampus of adult (3 months) iron-treated rats when compared to age-matching controls. These findings suggest that transient dietary iron supplementation during the neonatal period is associated to cellular imprinting in the brain later in life.

Key words: neurodegeneration; Alzheimer's disease; Parkinson disease; iron; GFAP; astrocytosis.

Introduction

Iron is the most abundant transition metal in the body and in the brain. It has versatile functions as a cofactor and biocatalyst in many vital as well as potentially damaging reactions in the cell. Iron is an essential cofactor in cytochrome oxidase, glucose-6-phosphate dehydrogenase, NADH dehydrogenase, aldehyde dehydrogenase, succinate dehydrogenase, and aconitase enzymes among others, all of which are enzymes involved in production of ATP. Iron plays a crucial role in function of hemoglobin and the cytochromes a, b, and c, and in the biosynthesis of cholesterol and lipids, which are necessary precursors in the formation of neuronal membranes and myelin^(1,2,3). Because of the high oxygen utilization in the brain and high content of myelin, and the crucial role of iron for the production of several neurotransmitters such dopamine, norepinephrine, serotonin and GABA, there is an especially important link between iron and brain function^(1,2,3). Excess of iron have been implicated in free radical formation via the Fenton reaction⁽⁴⁾. Free radicals may damage proteins, nucleic acids, and lipids, causing irreversible damage to cells and inducing cell death by apoptosis or necrosis. It has been suggested that disrupted iron homeostasis leading to iron accumulation in one or more brain regions is associated with numerous conditions such as Alzheimer's disease (AD) and Parkinson's disease (PD)^(1,2,3). It has been proposed that an imbalance in the synthesis of iron-storage proteins, ferritin, hemosiderin, neuromelanin, and the amount of free iron would increase the risk of cellular damage by oxidative stress. In fact, these dysfunctions of iron-storing proteins would be a normal aging event that would explain the predisposition of neurodegenerative disorders in elderly^(5,6,7,8). Regions undergoing degeneration display an excess of iron relative to ferritin levels which may indicate that iron accumulates at a faster rate than ferritin is produced⁽⁴⁾. AD is characterized by the presence of two neuropathological markers: senile plaques enriched with A β -peptide deposition (extracellular), and neurofibrillary tangles formed by hyperphosphorylated tau protein (intracellular)^(9,10,11,12). Studies have shown that iron co-localizes and influences the aggregation of both AD markers^(13,14,15,16,17,18). Moreover, iron-

induced A β and plaque formation induces activation of microglia and reactive astrocytes⁽⁴⁾. Interestingly, Bishop and co-workers⁽¹⁹⁾ observed that when iron was combined with A β , the neurotoxicity of this metal was substantially reduced, suggesting that this complex may be a mechanism to remove iron from the extracellular space and to facilitate its clearance from the brain, reducing oxidative stress^(19,20).

α -synuclein is the main component of the abnormal protein depositions constituting the Lewy bodies, intracytoplasmic inclusions in the cell body of neurons, recognized as the hallmark of PD^(16,21). The Lewy bodies are located mainly in dopaminergic neurons in the substantia nigra and noradrenergic neurons in the locus coeruleus⁽²²⁾. Elevated levels of iron in microglia, astrocytes, oligodendrocytes and dopaminergic neurons is observed in substantia nigra *pars compacta*^(16,21). Fasano et al⁽²³⁾ emphasized that nigral neuromelanin stores large amounts of iron that can be released during PD pathogenesis. The high content of dopamine, neuromelanin, and high levels of iron make dopaminergic nigral neurons peculiarly susceptible to oxidative stress^(23,24).

Iron has also been implicated in promotion of α -synuclein aggregation either directly or by increasing levels of oxidative stress suggesting an important role for it in Lewy body formation^(16,21,25). Zhang and coworkers⁽²⁶⁾ found that co-injection of the iron chelator desferroxamine not only attenuates the lactacystin-induced dopamine neuron loss, but also reduces the presence of ubiquitin-positive intracellular inclusions in the substantia nigra.

In 1999, Fredriksson and collaborators observed neurobehavioral dysfunctions and increased content of total iron in the basal ganglia in adult mice exposed to a neonatal iron administration^(27,28). Subsequently, Schröder and coworkers⁽²⁹⁾ observed memory impairments in the radial arm maze and in the inhibitory avoidance task in adult rats following postnatal iron administration. Studies by De lima et al⁽³⁰⁾ demonstrated that recognition memory was also impaired by iron neonatal treatment. These effects were reversed by selegiline⁽³¹⁾ and the iron chelator desferroxamine⁽³²⁾. It was reported that iron

supplementation in the neonatal period induces oxidative stress in substantia nigra, but not in cerebellum and striatum of adult rats ⁽³³⁾. In another study, oxidative stress parameters were investigated in other brain regions, demonstrating that iron administration as early as postnatal days 5-7 and as late as days 19-21 induces oxidative stress at least in the hippocampus, cortex and substantia nigra ⁽³⁰⁾.

Based on these findings, the present study was designed to investigate histopathological modifications in adult (3 months) and old (24 months) rats subjected to transient iron administration at a critical postnatal period and to correlate the findings with neurogeneration.

Material and methods

Animals

Pregnant Wistar rats were obtained from Fundação Estadual de Pesquisa e Produção em Saúde (FEEPS), Porto Alegre, Brazil. After birth, each litter was adjusted within 48 h to eight rat pups. Each pup was maintained together with its respective mother in a plastic cage with sawdust bedding in a room at a temperature of 22 ± 1 °C and a 12 h light: dark cycle. At the age of 4 weeks the pups were weaned and the males were selected and raised in groups of three–five rats. At postnatal treatment, the animals were supplied with standardized pellet food and tap water ad libitum. All experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and the Brazilian Society for Neuroscience and Behaviour (SBNeC) recommendations for animal care. The protocol for this research was approved by the Institutional Ethics Committee of the Pontificia Universidade Católica do Rio Grande do Sul.

Animal treatment

The neonatal iron treatment was performed as previously described^(29,30,31,32,34). Ten twelve-day-old rat pups received orally a single daily dose of 10.0 mg Fe²⁺/kg body weight in solution volume (Ferromyn®, AB Hässle, Göteborg, Sweden) and 9 ones received vehicle (5% sorbitol in water, control group) via a metallic gastric tube, over 3 days (post natal days 12–14). The period of treatment was chosen based on studies that show that iron uptake by the brain increases rapidly in the first 15 days of life, decreasing after the 15th day of life in rats^(35,36).

Immunohistochemical analyses (IHC)

Five iron-treated, 3 sorbitol-treated 3-month-old rats, 5 iron-treated and 6 sorbitol-treated 24-month-old rats were perfused through the left cardiac ventricle for 20 min with 4% paraformaldehyde in phosphate buffer. The brains were rapidly removed, and immersed in the same fixative solution at room temperature. Immediately afterwards, the brains were cut coronally and

embedded in paraffin. Serial, 5- μ m-thick coronal sections were obtained with a microtome, and used for immunohistochemistry. De-waxed sections corresponding to striatum (sections of figure 27 to 38 from Paxinos and Watson 2005)⁽³⁷⁾, hippocampus (sections of figure 54 to 66 from Paxinos and Watson 2005)⁽³⁷⁾ and substantia nigra (sections of figure 71 to 80 from Paxinos and Watson 2005)⁽³⁷⁾ were immersed in 3% hydrogen peroxide in 100% methanol for 15 min to inhibit endogenous peroxidase activity. Then, sections were boiled in 10 mM citrate buffer, pH 6.0, for 30 min or treated with formic acid for 3 min, for antigen retrieval. After rinsing in phosphate-buffered saline (PBS), the sections were incubated with normal horse serum for 2 h and then with the primary antibody overnight at 4°C in humid chambers. The following primary antibodies were used: anti-A β (mouse, monoclonal anti-human beta-amyloid, Dako), dilution 1:60, pretreated for 3 min with formic acid and citrate; anti-tau (pT²⁰⁵) (rabbit, polyclonal, anti-tau INVITROGEN) 1:250, pretreated with citrate; anti- α -synuclein (rabbit, ZYMED) 1:200, pretreated with formic acid and citrate; anti-GFAP (mouse, monoclonal anti-human glial fibrillary acidic protein, DAKOCYTOMATION), dilution 1:400, pretreated for 20 min with boiling citrate. The sections were washed three times in PBS and immunostained by a MAX Polymer Detection Kit (secondary biotinylated universal, HRP polymer conjugate, and DAB chromogen, ZYMED) for optical detection.

Densitometry and statistical processing of data

The immunohistochemical images were captured using AxisVision AC Real 4.5 software through inverted microscope with a 10x objective coupled to an AxioCam IC Zeiss camera. The selection section was done manually including striatum, hippocampus or substantia nigra. To analyze the images, Image J software was used (<http://www.uhnresearch.ca/facilities/wcif/download.html>). For each image, color functions and color de-convolution, H DAB vectors: color 2 R: 0.26814753, G: 0.57031375, B: 0.77642715 were applied. The color 2 image (brown) was inverted and the measurement was obtained as a percentage of the DAB chromogen. Data were expressed as mean \pm standard error of the mean

(SEM). Comparisons between groups were performed using the independent samples T-TEST. p values of less than 0.05 were considered statistical significant and are indicated in the figure as * $p < 0.05$ and ** $p < 0.01$.

Results

No significant changes in β -amyloid, phosphorylated tau and α -synuclein were seen in iron- and vehicle-treated rats in striatum, hippocampus and substantia nigra of adult and aged rats. Representative pictures with respective positive controls (human brain) are shown in Figure 1.

INSERT Fig 1 HERE

Increased astrogliosis, revealed by densitometry of GFAP-immunoreactive astrocytes, was found in hippocampus of 3-month old iron-treated rats ($p < 0,05$) when compared to vehicle- treated rats, as shown in Figure 2.

INSERT Fig 2 HERE

Statistical analysis of densitometric immunohistochemistry for GFAP showed an increase in GFAP levels in the substantia nigra and striatum (both p 's < 0.05), but not in hippocampus, in aged rats that received iron in comparison to the group treated with vehicle in the neonatal period (Figure 3).

INSERT Fig 3 HERE

Discussion

The present findings show that transient iron intake during neonatal period induces a region-specific astrocytic gliosis in adult and old rats, and that this

change is not accompanied by apparent differences in A β -amyloid, phosphorylated tau nor in α -synuclein. Iron administration in rats' early life has no role in A β metabolism, in contrast to the well-known interactions of A β and iron in older individuals^(19,20). Similarly, lack of differences regarding α -synuclein and phosphorylated tau do not give support to the concept that iron accelerates α -synuclein aggregation and Lewy body formation^(16,21,25) nor that it modifies tau phosphorylation patterns⁽¹⁸⁾.

Kimoto et al⁽³⁸⁾, studying glial cells in mouse hippocampus during postnatal development, concluded that glial cells may play a role in the maintenance and neuronal function. Their findings suggest that glial neurotrophic factors may be important for neuronal growth and maturation of oligodendrocyte during postnatal development. They emphasize that astrocytes perform a wide range of adaptative functions in the mammalian nervous system, including neurotransmitter uptake, synthesis and secretion of trophic factors, aid in the repair and regeneration of wounds, and regulation of synaptic density⁽³⁸⁾. Astrocytes are the most numerous cells in the brain, dwell in both grey and white matter and act as the main element of the homeostatic system of the brain. They shape the microarchitecture of the brain, form neuronal-glia-vascular units, regulate the blood-brain barrier, control microenvironment of the central nervous system and defend nervous system against a multitude of insults⁽³⁹⁾.

Acute and chronic brain insults trigger a specific glial reaction, generally known as reactive astrogliosis, represented by a complex morphofunctional remodeling of astrocytes⁽³⁹⁾. Reactive astrogliosis is a defensive brain reaction which is aimed at isolation of the damage area from the rest of the central nervous system (CNS) tissue, reconstruction of the blood-brain barrier and facilitation of the remodeling of brain circuits in areas surrounding the lesioned region⁽³⁹⁾. There are two types of astrogliosis: astrocytes surrounding the lesion undergo a robust hypertrophy and proliferation, which ultimately ends up in complete substitution of previously existing tissue architecture with a permanent glial scar; and damage distant astrogliosis do not distort the normal architecture of CNS tissue but astroglial cells modify their appearance and undergo multiple

biochemical and immunological changes although permit growth of neuritis and synaptogenesis, thus facilitating remodeling of neuronal networks. In particular, dystrophic astrocytes may have reduced ability for glutamate uptake, thus increasing the overall brain vulnerability to glutamate excitotoxicity. In contrast, the reactive astroglial cells may serve another function- the function of neuronal killers ⁽³⁹⁾.

GFAP, an intermediate filament protein, is increasingly expressed in response to astrocytic activation⁽⁴⁰⁾. It has been shown that GFAP expression also increases with ageing in the human brain and that astrocytes may respond to preclinical AD molecular pathology ⁽⁴¹⁾. Increased astroglial responses precede the oxidative impairments of critical cellular components in an AD animal model ⁽⁴⁰⁾. Recent studies have shown that the number of astrocytes is significantly increased in the hippocampus in APP/PS1 mice compared with age- and gender-matched wild-type littermates ⁽⁴²⁾. We also found in another study, that astrocytic gliosis is an early event in the course of APP/PS1-related pathology and that iron administration during the neonatal period and its late effects are independent of the genetic substrate of the individuals, as similar astrocytic responses was observed in APP/PS1 and wild-type littermates as well ⁽⁴³⁾. In the present study we also observed an increased astrogliosis in hippocampus of adult rats that received iron overload early in life, which might be related to iron-induced hippocampal based memory impairments, previously described by our research group. Moreover, in old iron-treated rats we found the same astrocytic responses in striatum and substantia nigra.

At certain iron concentrations, the defenses of brain cells may fail, and homeostatic mechanisms are overwhelmed. Under such conditions, differential cellular responses in brain-associated cell types are recruited to manage cell survival ^(4,15). It has been demonstrated that astrocytes are more resistant to free iron and oxidative stress toxicity than neurons and brain vascular endothelial cells, and may even provide transient protection to other cells within the brain ^(15,44). Initially astrocytes play a protective role vis a vis in thiamine deficiency-induced neurodegeneration, but in later stages an increase in GFAP-positive

astrocytes is observed, an event that is indicative of astrocyte activation along with increased neurodegeneration. It is not known whether astrocytes fail to produce protective factors in the later stages or if neuronal cells are too impaired to respond to these factors ⁽⁴⁾.

The present study shows that increased iron availability to the CNS tissue at early stages of development in rats results in significant increased GFAP-astrocyte expression levels that are maintained later in life in different regions. The early hippocampal response suggests increased cell vulnerability compared with substantia nigra and striatum, perhaps due to the presence of higher levels of iron-storage proteins in these regions (neuromelanin and ferritin). Although these results suggest a permanent cellular defense to transient iron overload during development, it may be questioned whether these changes are protective or facilitators of later brain damage. Absence of increased β -amyloid, phosphorylated tau and α -synuclein in iron-treated rats are indicators of a scenario consistent with a successful adaptative response. On the other hand increased astrocytic gliosis usually reflects impaired neuronal/glial homeostasis ^(39,41,45). It is possible based in Castellani et al hypothesis that brain protein deposit pathology is therefore a host response rather than a manifestation of cytotoxic protein injury. The pathology would be a manifestation of cellular adaptation, as a defense against oxidative injury ⁽⁴⁶⁾ in our case caused by iron overload early in life.

The present findings clearly document that excess of iron during the neonatal period also impacts in the cellular composition of the adult and old brain. These observations may encourage further studies focused on the effects of dietary supplementations in children.

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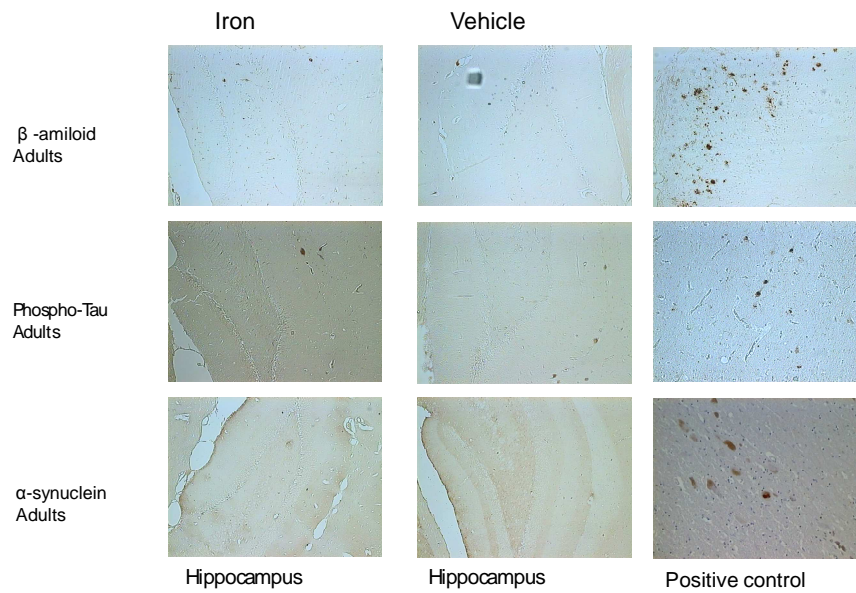
Figure legends

Figure 1. (A) Representative histological sections of β -amyloid, phosphorylated tau and α -synuclein immunohistochemistry of hippocampus of adult rats treated with vehicle or iron in the neonatal period. Magnification: 10x.

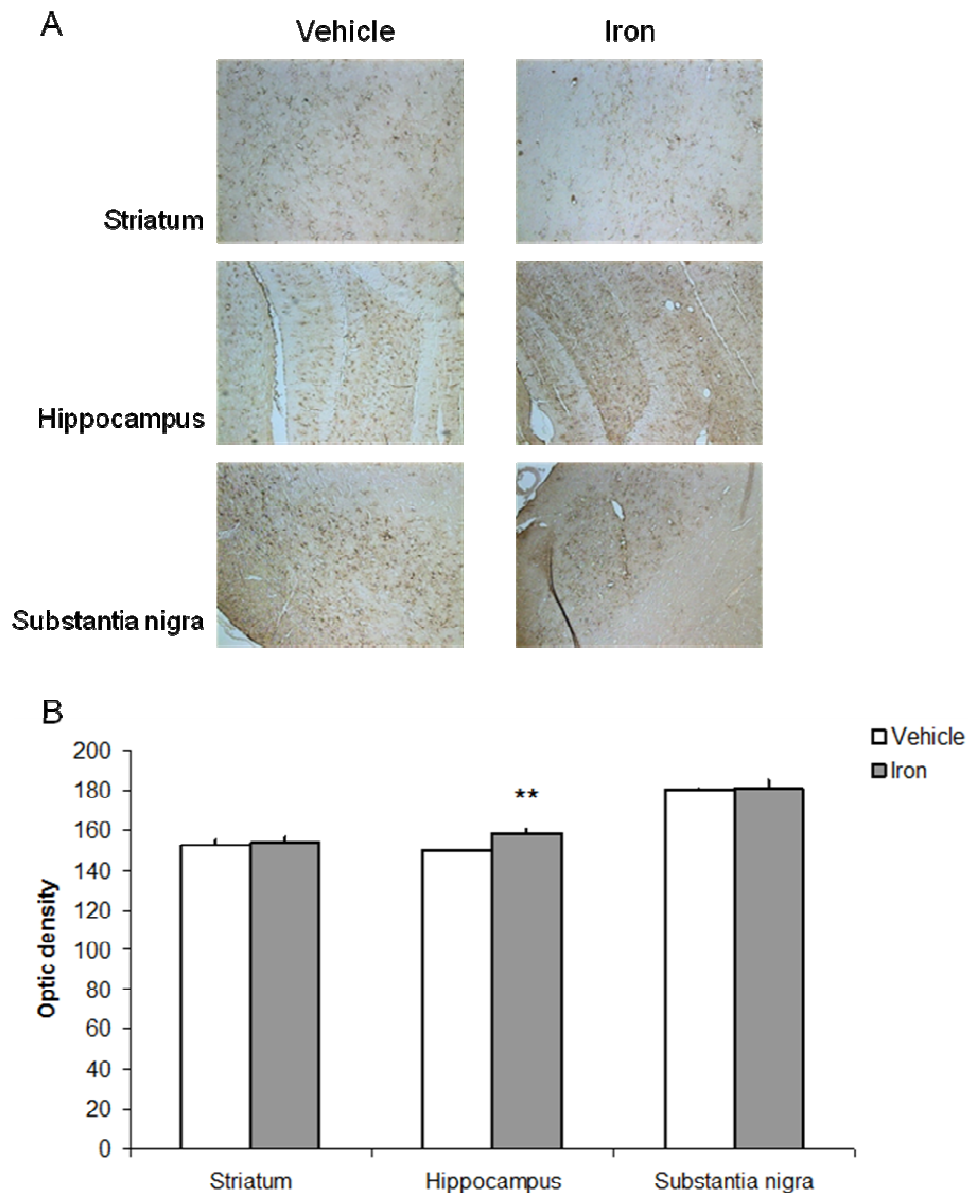


Figure 2. (A) Representative histological sections of GFAP immunohistochemistry of striatum, hippocampus and substantia nigra of adult rats treated with vehicle or iron in the neonatal period. Magnification: 10x. (B) Densitometric analysis of GFAP immunohistochemistry. Data were expressed as mean \pm standard error of the mean (SEM). Comparisons between groups were performed using the independent samples T-TEST. ** indicates $p < 0.01$.

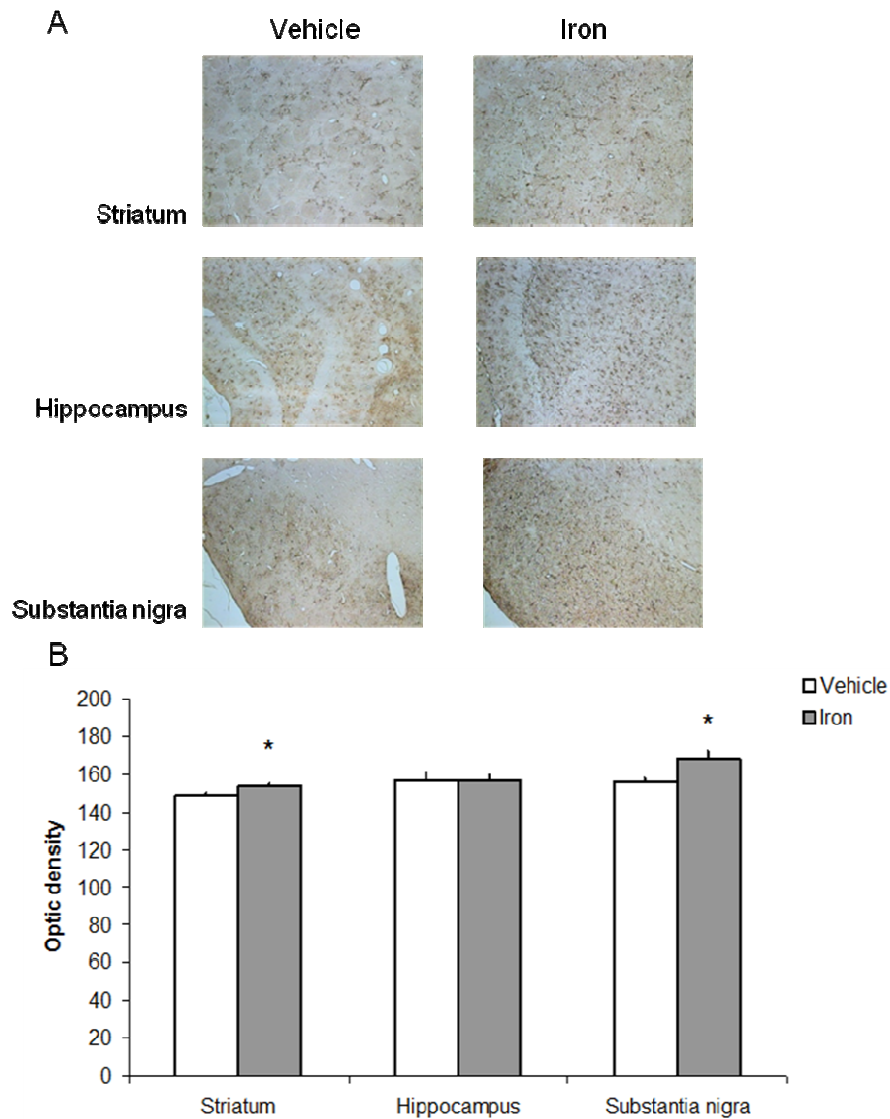


Figure 3. (A) Representative histological sections of GFAP immunohistochemistry of striatum, hippocampus and substantia nigra of old rats treated with vehicle or iron in the neonatal period. Magnification: 10x. (B) Densitometric analysis of GFAP immunohistochemistry. Data were expressed as mean \pm standard error of the mean (SEM). Comparisons between groups were performed using the independent samples T-TEST. * indicates $p < 0.05$.

CAPÍTULO 4

ARTIGO CIENTÍFICO

TÍTULO: Effects of increased iron intake during the neonatal period on the brain of adult APP/PS1 transgenic mice

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Effects of increased iron intake during the neonatal period on the brain of adult APP/PS1 transgenic mice

Liana Lisboa Fernandez^{1,2,3}, Marga Carmona³, Manuel Portero-Otin⁴, Alba Naudi⁴, Reinald Pamplona⁴, Nadja Schröder¹, Isidro Ferrer³

¹*Neurobiology and Developmental Biology Laboratory, Faculty of Biosciences, Pontifical Catholic University, 90619-900 Porto Alegre, RS, Brazil*

²*Health Basic Science Department, Federal University of Medical Science, Porto Alegre, RS, Brazil*

³*Institut de Neuropatologia, Servei Anatomia Patològica, IDIBELL-Hospital Universitari de Bellvitge, Universitat de Barcelona, Carrer Feixa LLarga sn, Hospitalet de Llobregat, CIBERNED, Spain.*

⁴*Department of Experimental Medicine, University of Lleida-IRBLLEIDA, 25008 Lleida, Spain.*

*Correspondence to: Liana Lisboa Fernandez, Rua Coronel Bordini, 675, sala 204, Porto Alegre, CEP 90440-001, RS, Brasil. Tel., fax: +55 51 33325983

E-mail address: llfernandez@plugin.com.br

Short title: Late effects of iron intake in APP/PS1 mice

Abstract

The present study was aimed to investigate neuropathological changes in APP/PS1 transgenic mice (Tg), as a model of Alzheimer's disease, subjected to supplementary iron administration in a critical postnatal period, in order to reveal the interaction of genetic and environmental risk factors in the pathogenesis of the disease. Twelve Tg and 10 wild-type (Wt) littermates were administered iron between the 12th and 14th post-natal days (TgFe, WtFe); 11 Tg and 15 Wt received vehicle (sorbitol 5%) alone in the same period (TgSb, WtSb). Mice were killed at the age of six months and processed for morphological and biochemical studies. No modifications in β -amyloid burden were seen in iron-treated and non-iron-treated APP/PS1 mice. No differences in microglial reactions were observed when comparing the four groups of mice. Yet increased astrogliosis, as revealed by densitometry of GFAP-immunoreactive astrocytes, and increased expression levels of GFAP, as revealed by gel electrophoresis and western blotting, were found in iron-treated mice (both Tg and Wt) when compared with TgSb and WtSb. This was accompanied by significant changes in brain fatty acid composition in APP/PS1 mice that lead to a lower membrane peroxidizability index and to reduced protein oxidative damage, as revealed by reduced percentages of the oxidative stress markers: glutamic semialdehyde, aminoadipic semialdehyde, N^ε-carboxymethyl-lysine, N^ε-carboxyethyl-lysine, and N^ε-malondialdehyde-lysine. These findings demonstrate that transient dietary iron supplementation during the neonatal period is associated with cellular and metabolic imprinting in the brain in adult life, but it does not interfere with the appearance of amyloid plaques in APP/PS1 transgenic mice.

Key words: neurodegeneration; Alzheimer's disease; APP/PS1 transgenic mice; iron; GFAP; docosahexaenoic acid, docosapentaenoic acid, peroxidizability index, protein oxidation

Introduction

The number of old people has risen with the increase of life expectancy and, as a consequence, the prevalence of age-related diseases including neurodegenerative disorders has increased as well [1]). Neuropathological changes in many degenerative diseases of the nervous system are characterized by the presence of abnormal proteins which accumulate in the brain, leading to a selective loss of neurons in an age-dependent manner [1-3]. Alzheimer's disease (AD) is the leading cause of dementia in the elderly [1, 2, 4]. AD is a multisystemic disease involving the neuronal circuits in the entorhinal and perirhinal cortex, hippocampal complex, amygdala and neocortex, together with the basal forebrain cholinergic system, striatum, thalamus and several nuclei of the brain stem. The neuropathological hallmarks of AD are extracellular deposits of β -amyloid peptides ($A\beta$) consisting of diffuse or neuritic plaques, and amyloid angiopathy; and intraneuronal deposits of hyperphosphorylated tau manifested as neurofibrillary pre-tangles and tangles, dystrophic neurites of neuritic plaques, and neuropil threads [1-4]. Sporadic AD is a common multifactor complex disorder resulting from the interaction of both genetic (e.g. *APOE*) and environmental factors in aged individuals [4-6]. Familial AD is a very rare autosomal dominant disease, often with early onset, caused by mutations in the amyloid precursor protein and presenilin genes (*APP*, *PSEN1*, *PSEN2*) both linked to $A\beta$ metabolism [3-5].

Several transgenic animal models have been produced to recapitulate particular aspects of AD. APP/PS1 transgenic mice over-express both mutant human APP and PS1, and show early and extensive $A\beta$ deposition in the form of plaques and amyloid angiopathy [7-9]. APP/PS1 transgenic mice show progressive amyloid plaque formation, dendritic spine loss, astrogliosis and behavioral deficiencies [10-13].

Increasing evidence shows that, in spite of vital physiological functions, iron may play a role in metal-ion-mediated oxidative damage under certain conditions, most clearly pronounced with aging [14-20]. Accumulation of proteins related with

iron storage as ferritin and hemosiderin occurs in the hippocampus, and probably other regions, in AD [21]. However, regions undergoing degeneration display an excess of iron relative to ferritin levels which may indicate that iron accumulates at a faster rate than ferritin is produced [22]. Moreover, iron-activated A β and plaque formation induces activation of microglia and reactive astrocytes [22]. Furthermore, iron co-localizes with A β in amyloid plaques in APP/PS1 transgenic mice [23].

Several studies have shown that neonatal iron exposure may induce neurobehavioral and cognitive dysfunctions in adult mice [24, 25]. Similar deficits are observed in adult rats after postnatal iron administration [26-30]. These effects are reversed by selegiline [30] and the iron chelator desferoxamine [28]. In these models, iron is given orally during the period of maximal iron uptake by the brain so that the rodent models mimic dietary iron supplementation to human infants [28].

The present study was designed to investigate histological and biochemical modifications in adult APP/PS1 transgenic mice subjected to transient iron administration at a critical postnatal period. The main objective was to evaluate the interaction of genetic and environmental factors in the pathology of the disease.

Material and methods

Animal treatment

Twenty three 6-month-old APP/PS1 transgenic mice (Tg) from the Jackson Laboratory (Bar Harbor, Maine, USA) and 25 age-matched littermate control mice (Wt) were used. The mouse line used in this study expressed a Mo/Hu APP695swe construct in conjunction with the exon-9-deleted variant of human presenilin 1 (PS1-dE9) [8]. Details of neuropathological and behavioural aspects of these mice are reported elsewhere [11, 13].

Twelve Tg and 10 Wt were subjected to iron administration between the 12th and the 14th postnatal days (TgFe, WtFe); 11 Tg and 15 Wt received vehicle alone

during the same period (TgSb, WtSb). The neonatal iron treatment was performed as previously described [24-30]. Briefly, 12-day-old mouse pups received a single oral daily dose (10.0 ml/kg solution volume) of vehicle (5% sorbitol in water) (control group) or 10.0 mg/kg of body weight of iron carbonyl (Sigma; iron concentration in the solution was 1.0 mg/ml) via a metallic gastric tube for 3 days. All the process was conducted with the approval of the Committee for Animal Care and Research of the Autonomous Government of Catalunya.

Immunohistochemical analyses (IHC)

Three TgFe, 3 TgSb, 4 WtFe and 4 WtSb 6-month old mice were perfused through the left cardiac ventricle for 20 min with 4% paraformaldehyde in phosphate buffer. The brains were rapidly removed and immersed in the same fixative solution at room temperature. Immediately afterwards, the brains were cut sagittally and embedded in paraffin. Serial, 5- μ m-thick coronal sections were obtained with a microtome, and used for immunohistochemistry. De-waxed sections were immersed in 3% hydrogen peroxide in 100% methanol for 15 min to inhibit endogenous peroxidase activity. Then the sections were boiled in 10 mM citrate buffer, pH 6.0, for 30 min or treated with formic acid for 3 min, for antigen retrieval. After rinsing in phosphate-buffered saline (PBS), the sections were incubated with normal horse serum for 2 h and then with the primary antibody overnight at 4°C in humid chambers. The following primary antibodies were used: anti-A β (monoclonal mouse anti-human beta-amyloid, Dako, Barcelona, Spain), dilution 1:25, pretreated for 3 min with formic acid; anti-GFAP (monoclonal mouse anti-human glial fibrillary acidic protein, DAKOCYTOMATION), dilution 1:250, pretreated for 20 min with boiling citrate; and anti-lectin of *Lycopersicon esculentum* (L-0651, Sigma, Madrid, Spain), dilution 1:100, with no pre-treatment.

The sections were washed three times in PBS and immunostained by a standard avidin–biotin complex method (secondary biotinylated universal Dako and Streptavidin HRP, Dako), washed and finally treated for 3 min with 0.01% H₂O₂

and 0.05% diaminobenzidine tetrahydrochloride (DAB, Sigma) for optical detection, except for lectin, in which the sections were treated only with streptavidin and DAB.

Western blot (WB) analysis

Nine TgFe, 8 TgSb, 6 WtFe and 11 WtSb 6-month-old mice were killed by rapid decapitation and all brains were separated in regions and frozen in -80°C . Samples of the posterior hemisphere (including the hippocampus) of all animals were homogenized in a glass homogenizer in 600 μl of RIPA buffer (25mM Tris, HCl pH 7.6, 150mM NaCl, 1%NP-40, 1% sodium deoxyenolate, 0.1% SDS) with phosphatase inhibitors (complex mini-protease inhibitors 1:10ml, NaV 200mM, NaF 1M, PMSF 100mM) and centrifuged at 10,000 rpm for 5 min at 4°C . Pellet fractions were discarded and protein concentrations of the supernatants were determined with the BCA method with bovine serum albumin as a standard. Samples containing 20 μg of protein were loaded onto 10% acrylamide gels. Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred to nitrocellulose membranes (400 mA for 90 min). Then the membranes were incubated with 5% skimmed milk in TBS-T buffer (100 mM Tris-buffered saline, 140 mM NaCl and 0.1% Tween 20, pH 7.4) for 1 h at room temperature, and incubated in primary antibody anti-GFAP (monoclonal mouse anti-human glial fibrillary acidic protein) diluted 1:3,000 in TBS-T containing 3% BSA (Sigma) overnight at 4°C . Subsequently, the membranes were incubated with the secondary antibody (anti-rabbit) labeled with horseradish peroxidase (Dako) in a dilution of 1:1,000. Finally, membranes were developed with the chemiluminescence ECL Western blotting system (Amersham/Pharmacia, Barcelona, Spain) followed by apposition of the membranes to autoradiographic films (Hyperfilm ECL, Amersham). The monoclonal antibody to β -actin (Sigma) was used at a dilution of 1:5,000 as a control of protein loading.

Densitometry and statistical processing of data

The immunohistochemical images were captured using AxisVision AC Real 4.5 program, an inverted microscope with a 20x objective, and an AxioCam IC Zeiss camera. The selection was made manually including hippocampus or adjacent temporal cortex. To analyze the image the Image J software was used (<http://www.uhnresearch.ca/facilities/wcif/fdownload.html>). For each image, color functions and color de-convolution, H DAB vectors: color 2 R: 0.26814753, G: 0.57031375, B: 0.77642715 were applied. The color 2 image (brown) was inverted and the measurement was obtained as a percentage of the DAB chromogen. Data were expressed as mean \pm standard error of the mean (SEM). Comparisons between groups were performed using the independent samples T-TEST. p values of less than 0.05 were considered statistically significant and these are indicated in the figure as * $p<0.05$ and ** $p<0.01$.

Western blotting analysis was performed by measuring protein levels using densitometry of all bands, measured with Total Lab v2.01 software. The results were normalized with β -actin. Data were expressed as mean \pm SEM. Comparisons among groups were performed with a one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. p values of less than 0.05 were considered statistically significant and these are indicated in the figure as * $p<0.05$ and ** $p<0.01$.

Mass spectrometry analysis of protein oxidative damage markers

The same animals used for gel electrophoresis and western blotting were utilized for the study of brain lipid composition and oxidative stress markers (4 TgFe, 4 TgSb). The frontal and part of the parietal cortex were used for study.

The markers of protein oxidation--the protein carbonyls glutamic (GSA) and amino adipic (AASA) semialdehydes, glycooxidation (carboxyethyl-lysine CEL, and carboxymethyl-lysine CML), and lipoxidation (malondialdehydelysine MDAL, and CML)--were determined by gas chromatography/mass spectrometry (GC/MS). The trifluoroacetic acid methyl ester derivatives of these five markers were measured in acid hydrolyzed de-lipidated and reduced protein samples using an

isotope dilution method as previously described [31], with an HP6890 Series II gas chromatograph (Agilent, Barcelona, Spain), an MSD5973A Series and a 7683 Series automatic injector, an HP-5MS column (30-m x 0.25-mm x 0.25- μ m), and the described temperature program [31]. Quantification was performed by external standardization using standard curves constructed from mixtures of deuterated and non-deuterated standards. Analyses were carried out by selected ion-monitoring GC/MS (SIM-GC/MS). The ions used were lysine and [$^2\text{H}_8$]lysine, m/z 180 and 187, respectively; 5-hydroxy-2-aminovaleric acid and [$^2\text{H}_5$]5-hydroxy-2-aminovaleric acid (stable derivatives of GSA), m/z 280 and 285, respectively; 6-hydroxy-2-aminocaproic acid and [$^2\text{H}_4$]6-hydroxy-2-aminocaproic acid (stable derivatives of AASA), m/z 294 and 298, respectively; CML and [$^2\text{H}_4$]CML, m/z 392 and 396, respectively; CEL and [$^2\text{H}_4$]CEL, m/z 379 and 383, respectively; and MDAL and [$^2\text{H}_8$]MDAL, m/z 474 and 482, respectively. The amounts of product were expressed as the μ molar ratio of GSA, AASA, CML, CEL or MDAL/mol lysine.

Fatty acid analysis

Fatty acyl groups of brain lipids were analyzed as methyl ester derivatives by GC/MS as previously described [31]. Separation was performed in an SP2330 capillary column (30 m x 0.25 mm x 0.20 μ m) in a GC Hewlett Packard 6890 Series II gas chromatograph (Agilent). A Hewlett Packard 5973A mass spectrometer was used as detector in the electron-impact mode. Identification of fatty acyl methyl esters was made by comparison with authentic standards and on the basis of mass spectra. Results are expressed as mol%. The following fatty acyl indexes were also calculated: saturated fatty acids (SFA); unsaturated fatty acids (UFA); monounsaturated fatty acids (MUFA); polyunsaturated fatty acids from n-3 and n-6 series (PUFAn-3 and PUFAn-6); average chain length (ACL) = $[(\Sigma\% \text{Total}14 \times 14) + (\Sigma\% \text{Total}16 \times 16) + (\Sigma\% \text{Total}18 \times 18) + (\Sigma\% \text{Total}20 \times 20) + (\Sigma\% \text{Total}22 \times 22)]/100$; double bond index (DBI) = $[(1 \times \Sigma \text{mol}\% \text{ monoenoic}) + (2 \times \Sigma \text{mol}\% \text{ dienoic}) + (3 \times \Sigma \text{mol}\% \text{ trienoic}) + (4 \times \Sigma \text{mol}\% \text{ tetraenoic}) + (5 \times \Sigma \text{mol}\% \text{ pentaenoic}) + (6 \times \Sigma \text{mol}\% \text{ hexaenoic})]$, and peroxidizability index (PI) = $[(0.025 \times$

$\Sigma\text{mol\% monoenoic}) + (1 \times \Sigma\text{mol\% dienoic}) + (2 \times \Sigma\text{mol\% trienoic}) + (4 \times \Sigma\text{mol\% tetraenoic}) + (6 \times \Sigma\text{mol\% pentaenoic}) + (8 \times \Sigma\text{mol\% hexaenoic})]$.

Statistical analyses

All values were expressed as means \pm SEM. Comparisons between experimental groups were statistically analyzed with Student's t tests. The minimum level of statistical significance was set at $p < 0.05$ in all the mass spectrometry analyses.

Results

Immunohistochemistry

APP/PS1 mice treated neonatally either with iron or vehicle and killed at six months presented A β plaques in hippocampus (Fig. 1 a1, b1) and temporal cortex (Figure1 a2, b2). A β plaques were not observed in the hippocampus (Fig.1 c1, d1) and temporal cortex (Fig.1 c2, d2) in Wt mice irrespectively of neonatal treatments. No significant differences in the number of plaques were seen in Tg mice treated with iron when compared with Tg mice treated with vehicle alone (data not shown).

In order to investigate the possibility of an inflammatory reaction in the tissue, labeling of microglia cells using lectin showed no differences between both Wt and Tg mice groups at six months of age treated with iron or sorbitol in the neonatal period.

INSERT Fig 1 HERE

In contrast, statistical analysis of densitometric immunohistochemistry for GFAP showed an increase in GFAP levels in the hippocampus ($p < 0.05$), but not in temporal cortex, in Wt mice that received iron in comparison to the group treated with vehicle alone in the neonatal period (Fig 2). Moreover, GFAP levels were

also significantly increased in the temporal cortex of APP/PS1 mice treated with iron in comparison to the Tg group treated with vehicle alone ($p < 0.05$) (Fig. 3).

INSERT Fig 2 and Fig 3 HERE

Western blotting

In order to confirm immunohistochemical findings, gel electrophoresis and western blotting of total homogenates including the parietal cortex and hippocampus was carried in Tg and Wt mice. Statistical comparison of GFAP band optic density normalized with β -actin showed significant differences among the different groups. Tukey post hoc test revealed that iron treatment in the neonatal period produced an increase in GFAP expression levels in Wt mice when compared with Wt mice treated with sorbitol alone ($p < 0.01$). Increased GFAP expression was seen in Tg mice when compared with Wt independently of neonatal iron exposure ($p < 0.01$) (Fig 4). Expression levels of GFAP in Tg mice at six months treated with iron were higher, although not significantly so, when compared with mice treated with sorbitol alone.

INSERT Fig 4 HERE

Fatty acid composition

Table I shows the fatty acid composition of brain lipids from both untreated and treated APP/PS1 transgenic mice. Iron administration altered the fatty acid composition of total brain lipids, so that the total number of double bonds (DBI) and peroxidizability index (PI) was significantly decreased ($p < 0.020$ and $p < 0.025$, respectively). The fatty acids mainly responsible for the decrease in DBI and PI were basically from the PUFA n-3 series. Thus, iron transiently administered at neonatal period significantly increased, at the age of six months,

the levels of saturated fatty acids such as 16:0 ($p < 0.027$) and 18:0 ($p = 0.05$), whilst it decreased the highly unsaturated eicosapentaenoic acid (20:5n-3) and docosapentaenoic acids (22:5n-3). The content of the highly unsaturated arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3) maintained stable. No changes were detected for monounsaturated fatty acids and PUFA n-6 contents. The membrane acyl composition indicated that the membranes maintained a similar, even though slightly lower, fatty acid average chain length (around 18 carbon atoms) in the Tg-Fe group when compared with the group treated with sorbitol alone.

Oxidative stress markers

Concerning the steady-state level of the protein oxidative damage markers, the concentrations of all the five different oxidative (the specific protein carbonyls GSA and AASA), glycoxidative (CEL and CML) and lipoxidative (CML and MDAL) markers analyzed were significantly decreased by iron treatment in the Tg animal group (Table II). The mean magnitude of all these decreases taken together was 23.0% in the brain of the Tg treated with iron when compared with the group treated with sorbitol alone.

Discussion

The present findings have shown that transient iron intake during neonatal period induces astrocytic gliosis in APP/PS1 transgenic mice and in age-matched littermates at the age of six months, and that this change is not accompanied by apparent differences in microglial reactions and, more particularly, in A β burden, as revealed by the amount of β -amyloid plaques, in APP/PS1 transgenic mice at least, within the range of the period studied. With minor differences, GFAP changes are consistent with densitometry in GFAP immunostained sections and gel electrophoresis and western blotting. Administration of iron at very early stages of development resulting in very similar effects in APP/PS1 and age-matched littermates points to the fact that A β metabolism has no role in iron removal and later astrocytic responses in the neonatal period, in contrast to the

well-known interactions of A β and iron in older individuals [32]. Similarly, the lack of differences regarding microglia does not give support to the concept that soluble A β -metal complex may stimulate inflammatory responses and thereby promote rapid phagocytosis of the peptide [33].

Acute and chronic brain insults trigger a specific glial reaction, generally known as reactive astrogliosis, represented by a complex morphofunctional remodeling of astrocytes [12]. GFAP expression also increases with ageing in the human brain; astrocytes may respond to preclinical AD molecular pathology [34]. In line with these observations in AD, recent studies have shown that the number of astrocytes is significantly increased in the hippocampus in APP/PS1 mice compared with age- and gender-matched wild-type littermates [10]. Exactly the same results have been reproduced in the present study, thus indicating that astrocytic gliosis is an early event in the course of APP/PS1-related pathology. Additionally, and critically, iron administration during the neonatal period and later effects are independent of the genetic substrate of the individual, as similar astrocytic responses occur in APP/PS1 and wild-type littermates.

The brain is unique in its homeostatic management of iron in that saturation of iron-handling proteins can occur more rapidly in the brain than in the peripheral organs [20]. Iron accumulation and overload have been identified as a severe toxic event affecting brain cells. At certain iron concentrations, the defenses of brain cells may fail, and homeostatic mechanisms are overwhelmed. Under such conditions, differential cellular responses in brain-associated cell types are recruited to manage cell survival [20]. For instance, it has been demonstrated that astrocytes are more resistant to free iron toxicity than neurons and brain vascular endothelial cells, and may even provide transient protection to other cells within the brain [20]. A similar response may also occur at less toxic doses of iron, thus explaining astrocytic reactions in adult animals following neonatal administration of 10.0 mg/Kg of body weight of iron carbonyl in postnatal days 12, 13 and 14.

Mechanisms leading to accompanying metabolic changes in the brain of adult mice treated with iron in the neonatal period are difficult to follow as a single point at the age of six months is available for study in APP/PS1 mice. Yet it can be assumed, as a working hypothesis, that iron treatment during the neonatal period may enhance Fenton-type reactions, leading to oxidative stress in this period. These reactions may be followed by net flux of free radical generation leading to protein modifications and membrane susceptibility to lipid peroxidation, as well as qualitative and quantitative changes in cell types. Concerning protein modifications, the percentage of change in the five measured markers of protein oxidative damage, resulting in reduced glutamic and aminoadipic semialdehydes, N^ε-carboxymethyl-lysine, N^ε-carboxyethyl-lysine and N^ε-malondialdehyde-lysine, is compatible with a homeostatic response to one-time increased oxidative damage [35]. Similarly, the changes in fatty acid composition are clearly compatible with an adaptive response of brain to iron intake. Thus, the depletion of 20 and 22 carbon n-3 precursors would reflect an attempt to spare the levels of docosahexaenoic acid. The conserved docosahexaenoic levels, allowing for the essential functions of this fatty acid in neurons, seem to confirm a successful response. These changes in lipid composition lead to a lower lipid peroxidizability, a fact that would also contribute to reduced protein oxidative damage [36]. The results of fatty acid analyses and levels of protein oxidative damage are complementary, and, taken together, reflect an adaptation to increased oxidative damage geared to curve protein oxidative damage and lipid peroxidizability. Whether these changes are specific to APP/PS1 transgenic mice is not known, but in all likelihood they are not. Studies in a limited number of wild-type littermates treated with iron or with sorbitol alone (n=2) per group) showed a similar pattern as that observed in APP/PS1 mice (unpublished observations). In summary, the present study shows that increased iron availability to the nervous tissue at early stages of development in APP/PS1 mice results in significant cellular (increased astrocytes and increased GFAP expression levels) and metabolic (brain lipid composition, and reduced index of peroxidizability and oxidative protein damage) responses that are maintained later in life. Although

these results suggest a permanent cellular and molecular defense to transient iron overload during development, it may be questioned whether these changes are protective or facilitators of later brain damage. The absence of increased microglial responses, the lack of increased amyloid burden, together with reduced index of lipid peroxidizability and oxidative protein damage in iron-treated mice, are indicators of a scenario consistent with reduced vulnerability to oxidative stress. Yet increased astrocytic gliosis usually reflects impaired neuronal/glial homeostasis [12, 34, 37]. It is well documented that perinatal iron deficiency is causative of brain damage persisting into adult life [38-42]. The present findings clearly document that excess of iron during the neonatal period also impacts in the cellular and molecular composition of the adult brain. Furthermore, early Fe administration during development does not interfere with the appearance of amyloid plaques in APP/PS1 transgenic mice.

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Table I. Fatty acyl composition (%) of total lipids in the frontal cortex of APP/PS1 transgenic mice treated with sorbitol (Tg sorbitol) or with iron (TgFe) during the neonatal period and killed at six months of age. Values are means \pm SEM from n=4 samples. ACL: average chain length; SFA: saturated fatty acids; UFA: unsaturated fatty acids; MUFA: monounsaturated fatty acids; PUFA_{n=3}: polyunsaturated n-3 fatty acids; PUFA_{n=6}: polyunsaturated n-6 fatty acids; PI: peroxidizability index .

	Tg sorbitol	Tg Fe	p values
14:0	0.68 \pm 0.10	0.60 \pm 0.05	0.569
16:0	21.47 \pm 0.56	25.38 \pm 1.22	0.027
16:1n-7	0.52 \pm 0.07	0.80 \pm 0.15	0.143
18:0	19.20 \pm 0.26	21.04 \pm 0.72	0.054
18:1n-9	18.23 \pm 0.42	18.55 \pm 0.50	0.633
18:2n-6	4.31 \pm 0.26	2.97 \pm 0.23	0.008
18:3n-3	0.13 \pm 0.02	0.91 \pm 0.47	0.152
18:4n-6	0.16 \pm 0.02	0.17 \pm 0.03	0.628
20:0	0.27 \pm 0.02	0.32 \pm 0.09	0.618
20:1n-9	1.01 \pm 0.05	1.09 \pm 0.07	0.409
20:2n-6	0.33 \pm 0.02	0.26 \pm 0.08	0.438
20:3n-6	0.32 \pm 0.04	0.33 \pm 0.03	0.742
20:4n-6	7.26 \pm 0.50	7.62 \pm 0.29	0.560
20:5n-3	8.23 \pm 1.26	4.82 \pm 1.27	0.078
22:0	1.16 \pm 0.08	0.90 \pm 0.15	0.163
22:4n-6	2.43 \pm 0.45	2.04 \pm 0.04	0.423
22:5n-6	0.46 \pm 0.11	0.41 \pm 0.04	0.678
22:5n-3	2.34 \pm 0.59	0.71 \pm 0.09	0.034
24:0	0.74 \pm 0.09	0.43 \pm 0.09	0.050
22:6n-3	9.70 \pm 1.46	9.94 \pm 0.34	0.878
24:5n-3	0.49 \pm 0.08	0.30 \pm 0.05	0.103
24:6n-3	0.56 \pm 0.09	0.40 \pm 0.02	0.129
ACL	18.63 \pm 0.04	18.36 \pm 0.06	0.014
SFA	43.52 \pm 0.83	48.67 \pm 1.29	0.015
UFA	56.48 \pm 0.83	51.33 \pm 1.29	0.015
MUFA	19.76 \pm 0.31	20.45 \pm 0.56	0.330
PUFA	36.71 \pm 0.69	30.89 \pm 1.55	0.014
PUFA_{n=3}	21.45 \pm 0.60	17.07 \pm 1.13	0.014
PUFA_{n=6}	15.26 \pm 0.66	13.81 \pm 0.52	0.136
DBI	188.96 \pm 4.39	163.20 \pm 6.86	0.020
PI	196.65 \pm 5.86	165.71 \pm 8.68	0.025

Table II. Markers of oxidative damage in frontal cortex in APP/PS1 mice treated with iron (TgFe) in vehicle or with sorbitol alone (Tg) in neonatal period and killed at the age of six months. Values are means \pm SEM from n=4 samples. GSA: Glutamic semialdehyde; AASA: aminoadipic semialdehydes; CML: N^ε-carboxymethyl-lysine; CEL: N^ε-carboxyethyl-lysine; MDAL: N^ε-malondialdehyde-lysine. Units: μ mol/mol lysine.

	Tg Sorbitol	Tg Fe	p (Tg vs TgFe)
GSA	6885.50 \pm 314.48	5987.27 \pm 155.05	0.034
AASA	86.88 \pm 3.63	64.59 \pm 2.92	0.001
CEL	270.17 \pm 10.40	212.93 \pm 6.80	0.002
CML	352.37 \pm 25.20	233.11 \pm 14.38	0.003
MDAL	1263.65 \pm 96.18	938.03 \pm 29.30	0.012

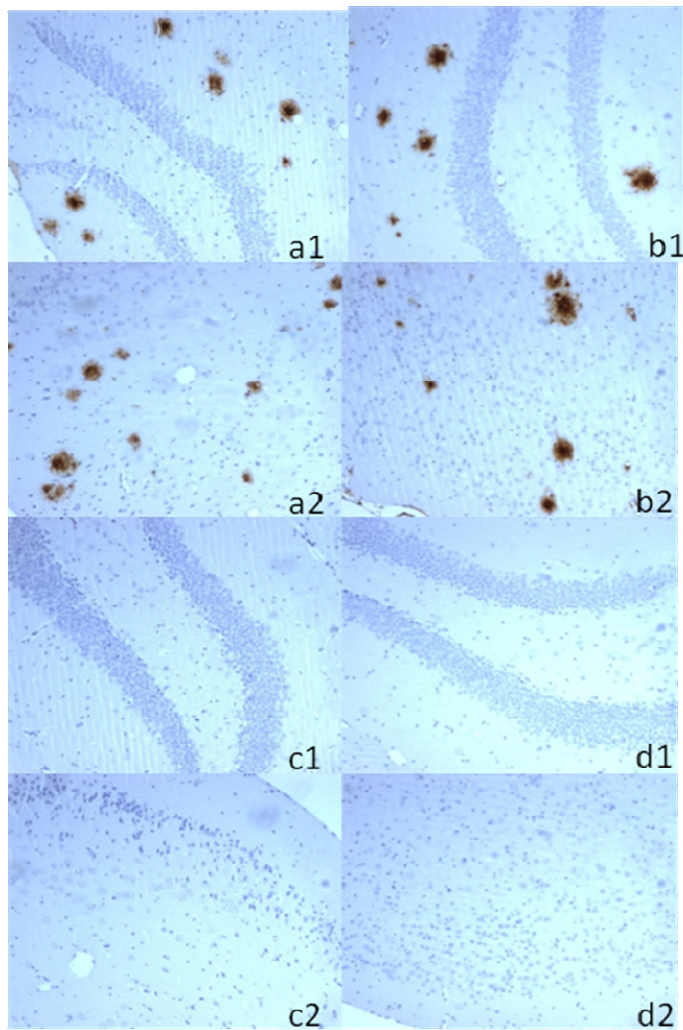
Figures

Fig 1. Representative histological sections of A β immunohistochemistry of hippocampus (1) and temporal cortex (2) of (a) TgFe group; (b) TgSb group; (c) WtFe group; (d) WtSb group show amyloid plaques. Magnification: 20x. No differences among groups are observed.

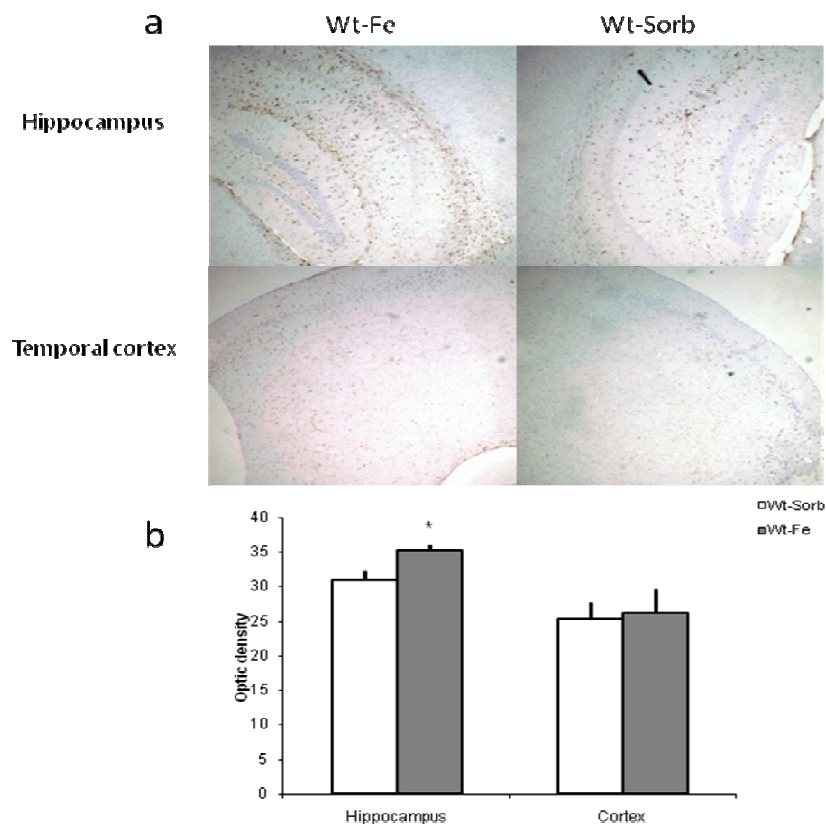


Fig 2. Representative histological sections of GFAP immunohistochemistry of the hippocampus and temporal cortex of WtFe and WtSb groups show immunostained astrocytes. Magnification: 20x (a). Statistical analysis reveals a significant increase in GFAP in the hippocampus of Wt animals that received iron (* $p < 0.05$, independent samples t-test) (b).

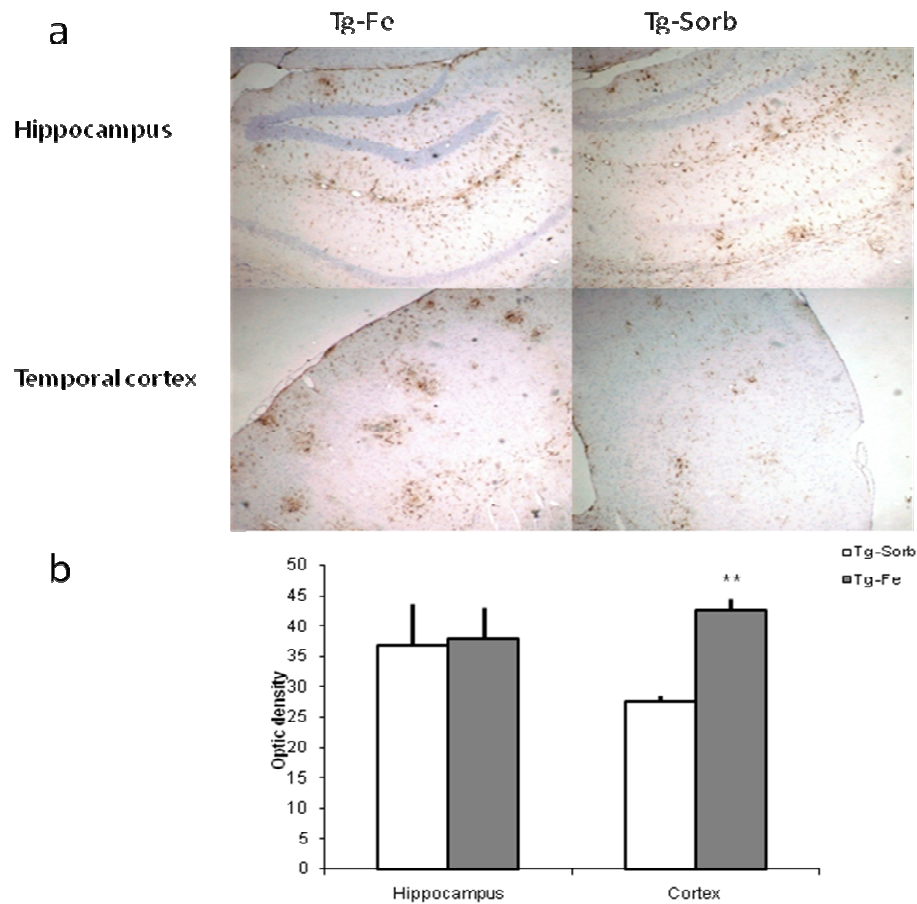


Fig 3. Representative histological sections of GFAP immunohistochemistry of the hippocampus and temporal cortex of TgFe and TgSb groups show immunostained astrocytes. Magnification: 20x (a). A significant increase in GFAP is observed in the temporal cortex of transgenic animals that received iron (** $p < 0.01$, independent samples t-test) (b).

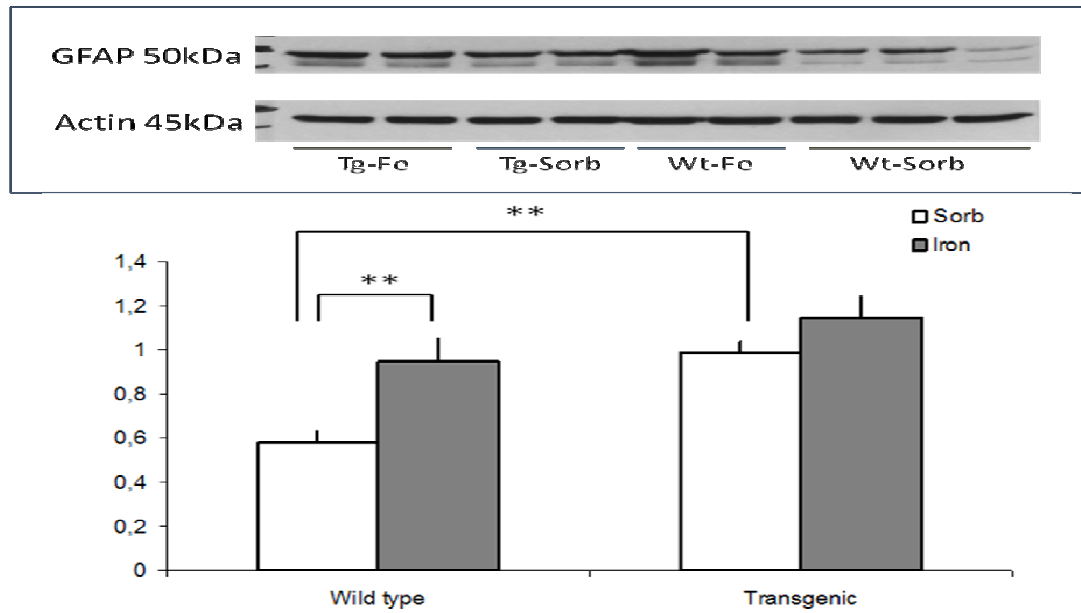


Fig 4. Western blot analysis of GFAP in 1: WtSb (n=11); 2: WtFe (n=6); 3: TgSb (n=8); and 4: TgFe (n=9). Increased expression levels of GFAP are seen in WtFe group versus WtSb, and in TgSb group versus WtSb; ** $p < 0.001$.

CAPÍTULO 5

1. CONSIDERAÇÕES FINAIS

1. CONSIDERAÇÕES FINAIS

O ferro tem sido descrito como um elemento importante na participação dos mecanismos da neurodegeneração (BOSSY-WETZEL, 2004).

Nossos achados demonstraram que administração transitória de ferro durante o período neonatal induz a uma gliose astrocítica em camundongos transgênicos APP/PS1 e em camundongos selvagens na idade de seis meses, e esta alteração não é acompanhada de diferenças aparentes em reações microgliais e especialmente na formação de placas reveladas pela quantidade de β -Amilóide, nos camundongos transgênicos APP/PS1 ao menos no período estudado. Alterações na GFAP foram consistentes na densitometria de GFAP em cortes imunocorados e em gel de eletroforese por western blotting.

Encontramos também que a ingesta transitória de ferro durante o período neonatal induz gliose astrocítica em ratos adultos (3 meses) e velhos (24 meses), em diferentes regiões. Nos ratos adultos encontramos aumento da GFAP no hipocampo e em ratos velhos, no estriado e substância negra. Esta alteração não foi acompanhada de diferenças aparentes no β -amilóide, tau fosforilada e nem em α -sinucleína.

Portanto, a administração de ferro em estágios precoces do desenvolvimento parece não interferir no metabolismo do β -amilóide tanto em ratos como em camundongos transgênicos APP-PS1 nem em reação microglial (camundongos transgênicos) em contraste com interações bem-estabelecidas do β -A e ferro em indivíduos idosos (Kontush, 2004; BISHOP, 2004). Da mesma forma, a falta de efeito em relação a α -sinucleína e tau fosforilada em ratos não dão suporte à hipótese que o ferro acelera a agregação de α -sinucleína na formação de corpos de Lewy (MOLIN-HOLGADO, 2007; GAETA, 2005; SAYRE, 2005) nem na modificação dos padrões de fosforilação da tau (EGAÑA, 2003).

Mecanismos que levam a alterações metabólicas no cérebro de camundongos adultos tratados com ferro no período neonatal são difíceis de caracterizar somente avaliando camundongos APP-PS1 com seis meses de idade. Porém, podemos assumir como hipótese que o ferro durante o período

neonatal pode ter aumentado reações do tipo Fenton levando a um estresse oxidativo neste período. Estas reações podem ter sido seguidas por um fluxo de geração de radicais livres que levaram a modificações de proteínas e membrana suscetíveis a peroxidação lipídica, tanto quanto a alterações qualitativas e quantitativas em tipos celulares. Apesar de modificações proteicas, a percentagem de alterações em cinco marcadores de dano oxidativo proteico, resultando em diminuição semialdeídos glutâmico e aminodípico, N^ε-carboximetil-lisina, N^ε-carboxietil-lisina and N^ε-malondialdeído-lisina, é compatível com resposta homeostática a aumento do dano oxidativo prévio (BREUSING, 2008).

Semelhantemente, alterações na composição de ácidos graxos são claramente compatíveis com uma resposta adaptativa do cérebro a ingestão de ferro. Então, a depleção de precursores 22 e 24 carbonos poderia refletir uma tentativa de poupar níveis de ácido docosahexaenóico. A conservação de níveis de docosahexaenóico, permitindo funções essenciais de ácidos graxos nos neurônios, parece confirmar uma resposta bem sucedida. Estas alterações na composição lipídica levam a menor capacidade peroxidativa, fato que poderia contribuir para um dano oxidativo proteico reduzido (PAMPLONA, 2008). Os resultados das análises de ácidos graxos e dano oxidativo proteico são complementares e juntos refletem uma adaptação ao aumento de dano oxidativo gerado para reverter o rumo do dano oxidativo proteico e da capacidade peroxidativa lipídica.

Em resumo, o presente estudo demonstra que uma disponibilidade aumentada de ferro para o tecido nervoso em estágios precoces do desenvolvimento de camundongos APP-PS1 e ratos resulta em resposta celular significativa (aumento de astrócitos e aumento de níveis de expressão de GFAP) assim como resposta metabólica (composição lipídica cerebral, e redução de índice da capacidade peroxidativa e dano oxidativo proteico em camundongos APP-PS1) mantidos em fases mais tardias da vida.

Apesar destes resultados sugerirem defesa celular e molecular permanente à sobrecarga de ferro durante o desenvolvimento, pode-se

questionar se estas alterações são protetoras ou facilitadoras de dano cerebral tardio. A ausência de um aumento de resposta microglial, ausência de aumento β -A, α -sinucleína, tau fosforilada, junto com redução do índice de capacidade peroxidativa lipídica e dano oxidativo protéico em camundongos e ratos, são indicadores de um cenário consistente com redução de vulnerabilidade a estresse oxidativo. Mas o aumento de gliose astrocítica geralmente reflete dano na homeostase neurônio/glial (RODRIGUEZ, 2009; WHARTON, 2009; SALMIRA, 2009). Está bem documentado que uma deficiência de ferro neonatal é causa de dano cerebral persistente no adulto (WU, 2008, GEORGIEFF, 2008; TRAN, 2009; CARLSON, 2009; COLLARD, 2009). Os resultados deste trabalho claramente demonstram que um excesso do ferro durante o período neonatal também impacta na composição celular e molecular do cérebro adulto.

CAPÍTULO 6

1. CONCLUSÕES

1. CONCLUSÕES

- Nenhuma modificação no β -A, tau fosforilada e α -sinucleína foi detectada em ratos adultos e velhos tratados e não tratados com ferro no estriado, hipocampo e substância negra.
- Aumento de astrocitose, revelada pela densitometria de GFAP- astrócitos imunoreativos, foi encontrado no hipocampo de ratos adultos tratados com ferro e no estriado e substância negra de ratos velhos.
- Nenhuma diferença significativa no número de placas foi encontrada nos camundongos transgênicos APP-PS1 tratados com ferro ou somente veículo.
- Aumento de astrocitose, revelado pela densitometria de GFAP- astrócitos reativos e por gel de eletroforese em western blotting, em camundongos transgênicos APP/PS1 e em camundongos selvagens com seis meses de idade foi encontrado no hipocampo e córtex temporal dos animais tratados com ferro neonatal.
- Nenhuma diferença na reação microglial foi encontrada em camundongos transgênicos APP-PS1 e selvagens tratados e não tratados com ferro neonatal.
- Gliose astrocítica é um evento precoce no curso da patologia relacionada com APP/PS1.
- A administração do ferro durante o período neonatal e seus efeitos tardios independe do substrato genético do indivíduo, já que a resposta astrocítica ocorre em animais transgênicos e em selvagens.
- Os resultados das análises dos ácidos graxos e dos níveis de dano oxidativo proteico são complementares, e, juntos, refletem uma adaptação a um aumento de dano oxidativo gerado em algum momento.

- Em resumo, o presente estudo demonstra que um aumento na disponibilidade de ferro para o tecido nervoso em estágios precoces do desenvolvimento de ratos e camundongos transgênicos APP/PS1 resulta em resposta significativa celular (aumento de astrócitos e aumento dos níveis de expressão da GFAP) e metabólica (composição lipídica, e índice reduzido de capacidade peroxidativa e dano oxidativo protéico em camundongos) que são mantidos em fases tardias da vida.
- Os achados presentes claramente documentam que o excesso de ferro durante o período neonatal impacta na composição celular e molecular do cérebro adulto.
- Estas observações podem encorajar futuros estudos focados nos efeitos da suplementação de ferro na dieta de crianças.

CAPÍTULO 7

1. REFERÊNCIAS BIBLIOGRÁFICAS

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ANEXO I

1. Comprovante de Submissão do Artigo Científico do Capítulo 4

ANEXO 1

Journal of Alzheimer's Disease

History of Manuscript 09-901			
"Effects of increased iron intake during the neonatal period on the brain of adult APP/PS1 transgenic mice"			
		Date	Action
		2009-08-10	Out For Review
		2009-08-10	Assigned to Editor (Smith)
		2009-08-09	Record Created/Needs Editor Assigned
		2009-08-09	Acknowledgment Sent to Author
		2009-08-09	Manuscript Accessioned
		2009-08-07	Manuscript Submitted