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FACULDADE DE BIOCÊNCIAS  
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

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**Efeito do tratamento neonatal com ferro e do envelhecimento sobre  
as proteínas Par-4 e Caspase-3 em estruturas cerebrais de ratos**

Porto Alegre  
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Dissertação apresentada como requisito para obtenção do grau de Mestre pelo Programa de Pós-Graduação em Biologia Celular e Molecular da Faculdade de Biociências da Pontifícia Universidade Católica do Rio Grande do Sul.

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Porto Alegre  
2010

## AGRADECIMENTOS

À Profª Mônica Vianna, por sua orientação, ensinamentos, dedicação e incentivo. Muito obrigada.

À Profª Nadja Schröder, por sua co-orientação e incentivo, possibilitando o primeiro trabalho em conjunto com a Drª Mônica.

Ao meu pai, Roberto, por ter proporcionado meus estudos.

À minha mãe, Wanda, e ao meu irmão, Fábio, por sempre terem acreditado em mim, mesmo quando eu não acreditava mais.

Ao Diego, por ser uma pessoa paciente, me dando todo apoio nos momentos difíceis. Muito obrigada.

À colega de Pós-Graduação Liana Fernandez.

Às colegas e amigas de jornada, Gabriela Carvalho e Marialice Soares, pelas horas de estudo e aprendizado, pela descontração, apoio e carinho. Obrigada meninas!

À Raquel Mattos, pelo seu profissionalismo excelente, pela amizade e por sempre ajudar quando achamos que tudo está perdido.

Às técnicas Terezinha Stein e Rosalva Meuer pelos seus ensinamentos.

## RESUMO

O processo de envelhecimento é caracterizado por alterações morfológicas e fisiológicas ao longo do tempo. No cérebro, este processo é marcado por alterações estruturais e funcionais levando a disfunção e ao declínio cognitivo. Nos mamíferos o ferro é encontrado em diversas áreas do cérebro, sendo essencial em muitos processos bioquímicos que são importantes no desenvolvimento e manutenção das funções neurobiológicas normais. Estudos revelam que há aumento do conteúdo de ferro tanto em cérebros de ratos velhos como em humanos idosos quando comparados com indivíduos jovens, indicando que durante o processo de envelhecimento existe um desequilíbrio no metabolismo desse metal. Com o avanço da idade, há aumento da morte celular programada que está relacionada a desordens neurológicas. A apoptose envolve cascatas de sinalização que são ativadas em diversas situações. Dentre as proteases envolvidas, a Par-4 e caspase-3 apresentam importante papel na sinalização apoptótica. Par-4 foi primeiramente identificada entre genes pró-apoptóticos ativados em resposta a insultos às células cancerosas na próstata. A caspase executora caspase-3, pertence a família de enzimas proteolíticas com resíduo cisteína no seu sítio catalítico que cliva proteínas em resíduos aspartato. Esta protease é importante no controle da apoptose no tecido nervoso responsável pela transdução e execução dos sinais de morte. Devido à carência de estudos que relacionem as proteínas pró-apoptóticas Par-4 e caspase-3 em modelos de neurodegeneração incluindo a ausência de trabalhos que relacionem estas proteases com estudos sobre os efeitos do ferro no sistema nervoso, este trabalho teve como objetivo avaliar alterações destas proteínas nas regiões do hipocampo, estriado e córtex de ratos adultos (3 meses) e idosos (24 meses) controles ou tratados com ferro durante o período neonatal através de imunohistoquímica. Para tanto, os animais controles foram tratados com dose oral diária de 5% sorbitol em água e os animais experimentais com 10 mg  $Fe^{2+}/kg$  de peso corporal do 12º ao 14º dia de vida pós-natal. Os resultados obtidos mostram aumento da imunorreatividade de ambas proteínas nas regiões CA1, CA3 e córtex ao longo do envelhecimento normal. Imunorreatividade de Par-4 mostrou significativo aumento nas regiões CA1, CA3, DG e córtex nos animais adultos tratados com ferro e significativa diminuição nos animais velhos tratados com ferro em relação aos seus respectivos controles. Imunorreatividade de caspase-3 mostrou significativo aumento nas regiões CA1, CA3 e córtex nos animais adultos tratados com ferro e significativa diminuição nos animais velhos tratados com ferro. Não houve diferença significativa no estriado para ambas proteínas. Os resultados indicam que a sobrecarga de ferro no período neonatal ocasiona maior apoptose na fase adulta, aumentando as chances de desenvolvimento de desordens neurológicas.

Palavras-chaves: ferro – apoptose - Par-4 - caspase-3 - envelhecimento

## ABSTRACT

The aging process is characterized by morphological and physiological changes over time. In the brain, this process is marked by structural and functional alterations leading to dysfunction and cognitive decline. In mammals, iron is found in several areas of the brain, is essential in many biochemical processes that are important in the development and maintenance of normal neurobiological functions. Studies show that there is an increase of iron existing in both the brains of aged rats and elderly humans compared with young subjects, indicating that during the aging process there is an imbalance in the metabolism of this metal. With advancing age, there is an increase of programmed cell death that is related to neurological disorders. Apoptosis involves signaling cascades that are activated in different situations. Among the proteases involved, the Par-4 and caspase-3 play an important role in apoptotic signaling. Par-4 was the first identified between pro-apoptotic genes activated in response to insults to cancer cells in the prostate. The executioner caspase-3, belongs to the family of proteolytic enzymes with cysteine residue in its catalytic site that cleaves proteins at aspartate residues. This protease is important about control of apoptosis in neural tissue responsible for the transduction and execution of the signs of death. Due to lack of studies that relate the protein Par-4 and caspase-3 in neurodegeneration's models including a shortage of studies that relate these proteases with studies on the effects of iron in the nervous system, the goal of this study was evaluate changes of these proteins in regions of the hippocampus, striatum and cortex of adult rats (3 months) and aged rats (24 months) controls or treated with iron during the neonatal period by immunohistochemistry. Therefore, the control animals were treated with orally daily dose of 5% sorbitol in water and the experimental group with 10 mg Fe<sup>2+</sup> / kg body weight of 12 to 14 days of postnatal life. The results show increased immunoreactivity of both proteins in CA1, CA3 and cortex regions during normal aging. Par-4 immunoreactivity showed a significant increase in the CA1, CA3, DG and cortex regions in adult iron-treated animals and a significant decrease in aged iron-treated animals in relation to their respective controls. Caspase-3 immunoreactivity showed a significant increase in the CA1, CA3 and cortex regions in adult iron-treated animals and a significant decrease in aged iron-treated animals. There was no significant difference in the striatum for both proteins. Our results indicate that iron overload in the neonatal period leads to increased apoptosis in adulthood, increasing the chances of developing neurological disorders.

Keywords: iron – apoptosis - Par-4 - caspase-3 - aging

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# **CAPÍTULO 1**

## **INTRODUÇÃO E OBJETIVOS**

# 1 INTRODUÇÃO

## 1.1 – Processo de envelhecimento

O processo biológico pelo qual ocorrem as alterações das características morfológicas e fisiológicas no organismo vivo ao longo do tempo é conhecido como envelhecimento. As características desse processo incluem a perda gradativa das funções biológicas, aumento da probabilidade de morte e/ou estão associadas com patologias. Estudos *in vitro* e *in vivo* têm mostrado que após a idade reprodutiva, a perda da função celular e a quantidade de células de um organismo são progressivamente diminuídas com o passar dos anos (Harper, 1982). No caso dos humanos, essas alterações trazem consigo reflexos no comportamento, habilidade intelectual e capacidade física no cumprimento das atividades diárias (Kluger *et al.*, 1997). Entre as variáveis externas que afetam o envelhecimento, talvez a mais importante seja a nutrição (Harper, 1982; Joseph *et al.*, 1983; Bartus, 1990; Kluger *et al.*, 1997; Shukitt-Hale *et al.*, 1998; Joseph *et al.*, 2000). Numerosos estudos mostram que em diferentes modelos animais, o consumo de suplementos antioxidantes e a restrição de dieta retardam déficits comportamentais observados durante o envelhecimento (Dubey *et al.*, 1996; Eastwood, 1999; Greenwood & Winocur, 1999; Masoro, 2000; Casadesus *et al.*, 2002). Porém, há controvérsias sobre o uso de suplementos ricos em ferro durante o período neonatal que podem acarretar no surgimento de desordens neurológicas na fase adulta (Fomon *et al.*, 2000; Rao & Georgieff, 2007).

O aumento na expectativa de vida é um fenômeno que vem se manifestando de forma crescente em escala mundial (Ricklefs & Finch, 1995; Arking, 1998). Essa mudança no padrão de distribuição etária da população ocasionou maior prevalência de doenças associadas ao envelhecimento. A Figura 1 ilustra em nível mundial, a evolução da expectativa de vida ao nascer da população.



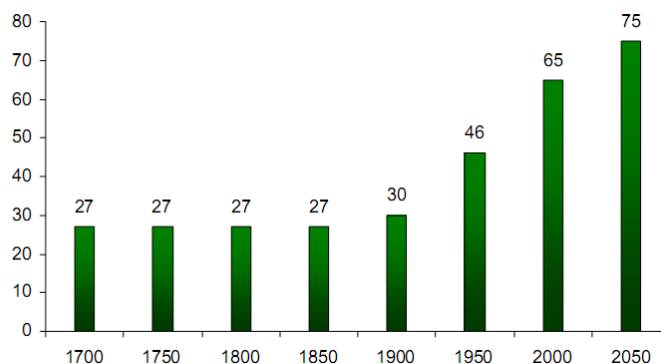


Figura 1 - Evolução da expectativa de vida mundial (1700 – 2050).  
Fonte: Lee, RD (2003, p. 167).

O envelhecimento do cérebro é um processo caracterizado por alterações estruturais e funcionais levando a disfunção e ao declínio cognitivo. Durante a fase de envelhecimento o surgimento de patologias como doença de Parkinson (DP) e doença de Alzheimer (DA) (Ricklefs & Finch, 1995; Arking, 1998; Maccione *et al.*, 2001) é frequente. Em pacientes com DP é observada profunda disfunção motora devido à degeneração de neurônios dopaminérgicos na substância negra. A causa da DP ainda é desconhecida, porém, o aumento do estresse oxidativo e a disfunção mitocondrial em neurônios de dopamina são características principais da doença (Jenner & Olanow, 1998). Já a DA é caracterizada pelo progressivo declínio cognitivo e distúrbios emocionais que são fortemente relacionados com a degeneração sináptica e morte dos neurônios nas estruturas límbicas (hipocampo e amígdala) e regiões associadas ao córtex cerebral (Cummings *et al.*, 1998). Portanto, as doenças neurodegenerativas são desordens progressivas típicas do envelhecimento que afetam determinadas populações neuronais do sistema nervoso central, levando à morte neuronal e à ruptura de circuitos neurais.

## 1.2 – Ferro no sistema nervoso central (SNC)

Nos mamíferos o ferro é encontrado em diversas áreas do cérebro (Hill & Switzer, 1984), sendo essencial em muitos processos bioquímicos que são importantes no desenvolvimento e manutenção das funções neurobiológicas normais (Hill & Switzer, 1984; Andrews, 2002).

Atualmente, tem-se sugerido a relação da disfunção nas vias de homeostase do ferro e a patogênese de doenças neurodegenerativas (Martin *et al.*, 1998) caracterizado pelo aumento do estresse oxidativo que medeia a morte celular e o prejuízo cognitivo associado ao envelhecimento (Dexter *et al.*, 1989; Butterfield *et al.*, 2002; Liu *et al.*, 2003; Mattson, 2004; Mecocci *et al.*, 2004).

O acúmulo de ferro no cérebro tem sido associado à doença de Parkinson (DP) e doença de Alzheimer (DA) (Dexter *et al.*, 1991; Kienzl *et al.*, 1995). A substância negra é a região com maior depósito desse metal em portadores de DP, acredita-se que essa é a região cerebral mais afetada pela perda neuronal (Dexter *et al.*, 1991; Hirsch *et al.*, 1992; Jellinger *et al.*, 1993; Kienzl *et al.*, 1995; Ebaldi *et al.*, 1996; Griffiths *et al.*, 1999). Depósitos de ferro também têm sido encontrados no núcleo caudado de indivíduos com a doença de Huntington (Bartzokis *et al.*, 1999) e ao redor das placas senis de pacientes com DA (Lynch *et al.*, 2000; Falangola *et al.*, 2005).

Estudos revelam que há aumento do conteúdo de ferro tanto em cérebros de ratos velhos como em humanos idosos quando comparados com indivíduos jovens, indicando que durante o processo de envelhecimento existe um desequilíbrio no metabolismo desse metal (Zecca *et al.*, 2001).

Fredriksson e colaboradores demonstraram pela primeira vez em estudos utilizando camundongos e ratos, que o tratamento sistêmico com ferro durante o período de rápido desenvolvimento cerebral (período que vai, em humanos, desde o último trimestre de gravidez até um ano de vida) produz acúmulo de ferro nos gânglios da base, além de causar disfunções neurocomportamentais (Fredriksson *et al.*, 1999). Os ratos tratados com ferro do 10º ao 12º 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 *et al.*, 1999; Schröder *et al.*, 2001). Em estudo recente, verificou-se que a memória de reconhecimento também está prejudicada em ratos tratados no período neonatal com ferro (de Lima *et al.*, 2005).

Evidências do envolvimento do metabolismo anormal do ferro em diversas patologias relacionadas ao SNC têm promovido um grande esforço dos pesquisadores na tentativa de entender os mecanismos que participam da distribuição desse elemento no encéfalo (Taylor & Morgan, 1990). À medida que esses estudos avançam, é marcante o impacto do conteúdo de ferro

da dieta alimentar sobre o metabolismo desse metal no SNC. O período neonatal é crítico para o estabelecimento do conteúdo de ferro cerebral nos adultos, tornando-se importante estudar os possíveis efeitos tóxicos da sobrecarga desse metal nessa fase (Taylor & Morgan, 1990; Taylor *et al.*, 1991; Benkovic & Connor, 1993).

### 1.3– Apoptose

Apoptose é um exemplo de morte celular programada que envolve a regulação por cascatas de sinalização que são ativadas em diversas situações. A indução e execução da apoptose requer cooperação de várias moléculas, incluindo moléculas de sinalização, receptores, enzimas e proteínas reguladoras de genes (Launay *et al.*, 2005). O princípio do processo de apoptose pode acontecer por uma via extrínseca, onde é necessário um sinal externo que se ligará ao receptor localizado na superfície celular, ou intrínseca, que requer fatores endógenos conhecidos como “sinais de estresse” (danos ao DNA) provenientes do interior da própria célula. A Figura 2 mostra esquematicamente a cascata intracelular quando há um sinal que levará a célula a morte.

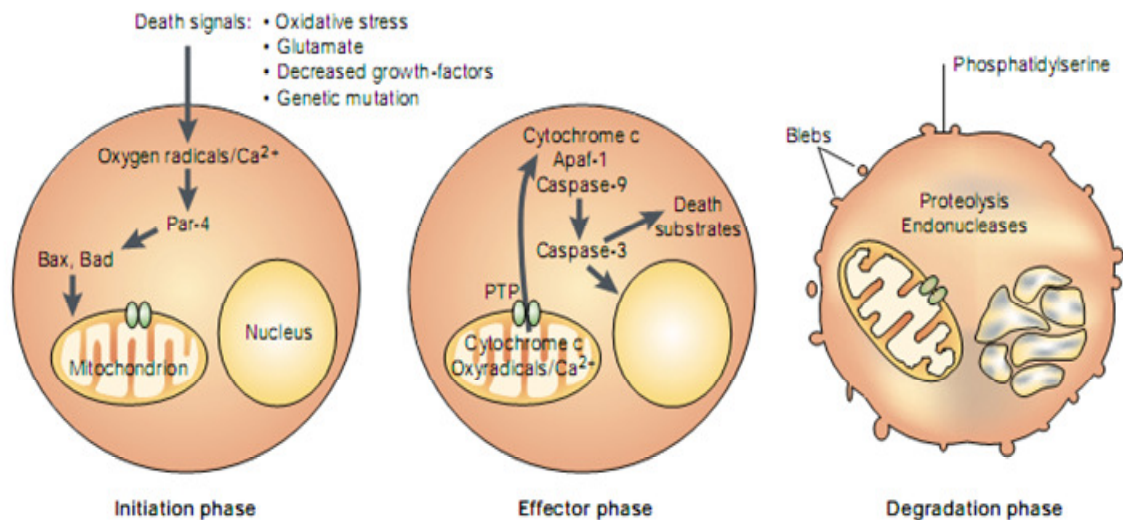


Figura 2 – Características morfológicas e bioquímicas da apoptose.  
Fonte: Mattson, MP (2000, p. 120)

O processo de apoptose pode ser modulado em várias situações e dificuldades na regulação dos sinais relacionados com a indução da morte celular e estão associados a uma série de doenças. A resistência das células a morte é associada a muitos tipos de câncer e, a identificação de alterações moleculares responsáveis pela imortalização dessas células é importante para pesquisas nesta área (Wyllie, 1997).

Apoptose também está relacionada a desordens neurológicas que tem como característica central a morte neuronal (Stennicke & Salvesen, 1998; Mattson, 2000). Em contraste ao rápido *turnover* de células em tecidos proliferativos, os neurônios comumente sobrevivem durante o tempo de vida do organismo. Esta característica é necessária para a manutenção da função dessas células dentro do circuito neuronal. Durante o desenvolvimento do sistema nervoso central e periférico, muitos neurônios sofrem apoptose durante a fase de sinaptogênese (Oppenheim, 1991). As razões para esta perda de células nervosas incluem desde competição por espaço ou fatores tróficos até a eliminação de vias redundantes (Pettmann & Henderson, 1998).

Durante o processo de envelhecimento é observada maior perda de neurônios, o que explica o declínio funcional do cérebro (Haug, 1986; Wickelgren, 1996a; Wickelgren, 1996b). Com o avanço da idade, há aumento da apoptose no córtex e na região CA1 hipocampal (zonas cruciais para função da memória) (West & Gundersen, 1990; West *et al.*, 1994). Sugere-se que o aumento do estresse oxidativo (Shukitt-Hale, 1999) e da inflamação (Hauss-Wegrzyniak *et al.*, 2000) podem estar envolvidos no déficit comportamental durante o envelhecimento.

Nos mamíferos os diversos tipos celulares (principalmente os neurônios) co-expressam proteases conhecidas como Par-4 (*prostate apoptosis response 4*) (Sells *et al.*, 1994) e múltiplas caspases que podem agir em diferentes estágios na cascata enzimática envolvida na morte celular programada (Chan & Mattson, 1999).

### **1.3.1 – Par-4**

A proteína Par-4 foi primeiramente identificada entre genes pró-apoptóticos ativados em resposta a insultos às células cancerosas na próstata (Sells *et al.*, 1994).

Par-4 apresenta domínio zíper de leucina na sua região C terminal e duas seqüências sinal de localização nuclear (SNL) na região N terminal. Possui 100% de homologia em humanos, ratos e camundongos, sugerindo que sua função e regulação é similar entre os mamíferos (El-Guendy & Rangnekar, 2003). O domínio zíper de leucina é essencial para a ativação da morte celular programada devido a sua função de interação proteína-proteína (Diaz-Mecco *et al.*, 1996; Sells *et al.*, 1997).

Estudos revelam a presença de Par-4 em diversos tecidos de origem endodérmica, mesodérmica e ectodérmica de ratos com variações de expressão (Boghaert *et al.*, 1997). Análises do RNAm e expressão da proteína Par-4 em vários tecidos demonstram níveis baixos em diferentes tipos celulares, incluindo neurônios (Boghaert *et al.*, 1997). Os níveis desta proteína encontram-se elevados no cérebro, pulmão e testículo, porém encontram-se baixos no coração e fígado. Através de imunohistoquímica, notou-se baixo nível da proteína em muitos neurônios em condições basais. Expressão de Par-4 mostra-se elevada em certas populações neuronais, tais como, células piramidais do hipocampo e neurônios das camadas intermediárias do córtex cerebral. (Boghaert *et al.*, 1997).

Consistente com sua função apoptótica, seus níveis estão elevados em células que estão morrendo, por exemplo, em células do ducto da próstata em ratos castrados e neurônios em processo de degeneração (El-Guendy & Rangnekar, 2003). Esta proteína medeia à interação com proteínas que modulam a apoptose, incluindo proteína kinase C  $\zeta$  (PKC  $\zeta$ ), Bcl-2 e componentes da sinalização de NF- $\kappa$ B (Diaz-Meco *et al.*, 1996; Frutos *et al.*, 1999; Camandola & Mattson, 2000).

A indução da Par-4 parece ser a ligação entre a cadeia de eventos que levam a disfunção mitocondrial, ativação de caspase e morte celular seguida da exposição de neurônios a diferentes estímulos apoptóticos como o peptídeo  $\beta$  amilóide (Guo *et al.*, 1998), agentes geradores de oxiradicais (Duan *et al.*, 1999a,b) e retirada do fator trófico (Camandola & Mattson, 2000).

Camandola e colaboradores demonstraram que Par-4 promove apoptose suprimindo a sinalização envolvendo ativação de NF- $\kappa$ B e expressão de Bcl-2 (Camandola & Mattson, 2000). Este mecanismo é consistente com habilidade da ativação de NF- $\kappa$ B (Barger *et al.*, 1995; Mattson *et al.*, 1997; Tagliatela *et al.*, 1997) e super expressão de Bcl-2 (Greenlund *et al.*, 1995; Kruman *et al.*, 1997; Guo *et al.*, 1997) de prevenir apoptose em células expostas aos estímulos apoptóticos. A Figura 3 mostra esquematicamente o mecanismo de ação da Par-4.

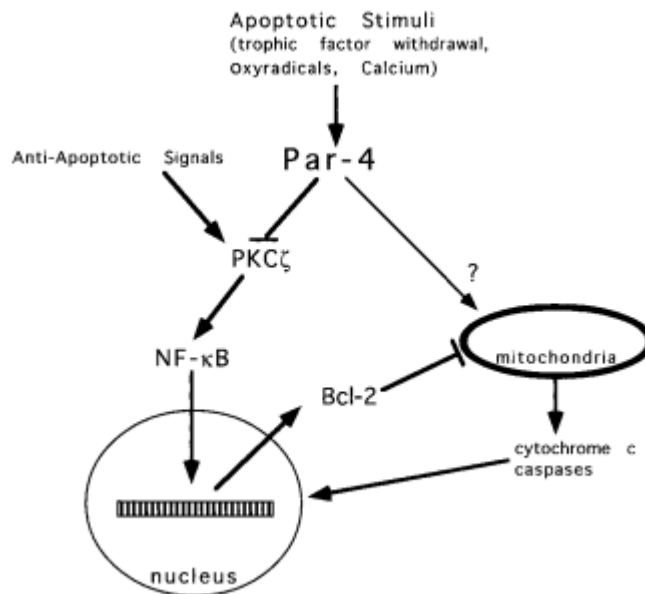


Figura 3: Mecanismo proposto de ação de Par-4 na apoptose neuronal.  
 Fonte: Camandola & Mattson (2000, p.138)

Pesquisas apontam o seu papel na apoptose neuronal induzido em modelos experimentais, tais como, retirada do fator trófico e exposição a insultos oxidativos e metabólicos (Mattson *et al.*, 1999). Estudos com tecidos pós-morte de pacientes e modelos animais de desordens neurodegenerativas (doença de Alzheimer, Parkinson e Huntington e esclerose lateral amiotrófica) mostraram aumento nos níveis de Par-4 em neurônios vulneráveis (Guo *et al.*, 1998; Duan *et al.*, 1999a; Kruman *et al.*, 1999; Pedersen *et al.*, 2000). Tabela 1 mostra as desordens neurodegenerativas em que a Par-4 pode ter função.

Tabela 1 - Desordens neurodegenerativas em que a Par-4 pode estar envolvida	
Desordens	Evidências
Doença de Alzheimer	Aumento do RNAm e da proteína Par-4 em neurônios afetados no tecido humano; peptídeo $\beta$ -amilóide induz Par-4 em cultura de neurônios do hipocampo; RNA antisense previne apoptose induzida pelo peptídeo $\beta$ -amilóide
Doença de Parkinson	Aumento dos níveis da proteína Par-4 em neurônios dopaminérgicos vulneráveis na substância negra de macacos e ratos com administração de MPTP; RNA antisense protege os neurônios dopaminérgicos dos insultos relevantes a doença de Parkinson
Doença de Huntington	Aumento de Par-4 nos neurônios estriais seguido da administração de 3NP; RNA antisense protege cultura de neurônios estriais contra a indução de morte celular
Esclerose Lateral Amiotrófica	Aumento dos níveis da proteína Par-4 em neurônios motores no tecido humano e camundongos com ALS; insultos oxidativos induz Par-4 em cultura de neurônios motores; RNA antisense previne apoptose em cultura de neurônios motores
Derrame Cerebral	Aumento dos níveis da proteína Par-4 seguida da oclusão arterial cerebral em ratos; privação glicose/oxigênio induz Par-4 em cultura de neurônio hipocampal; RNA antisense previne apoptose induzida pela excitotoxicidade e insultos metabólicos

Adaptado de Mattson MP, Duan W, Chan SL, Camandola S. Par-4: An Emerging Pivotal Player in Neuronal Apoptosis and Neurodegenerative Disorder. *J Mol Neurosci.* 1999; 13: 17-30.

A Par-4 é rapidamente induzida em neurônios que irão sofrer apoptose e exerce ação pró-apoptótica nos estágios iniciais da morte celular, antes da ativação de caspases e alterações na mitocôndria (Chan *et al.*, 1999).

### 1.3.2 – Caspases / Caspase-3

Caspases são enzimas proteolíticas com resíduo cisteína no seu sítio catalítico que cliva proteínas em resíduos aspartato (Earnshaw *et al.*, 1999; Yuan & Yankner, 2000; Troy & Salvesen, 2002) e são consideradas indispensáveis para promover a cascata de sinalização intracelular que leva à morte celular programada (Stennicke & Salvesen, 1998). Quatorze caspases já foram identificadas, possuindo distintas funções no processo inflamatório e na apoptose (Wolf & Green, 1999). Baseado na homologia da seqüência de aminoácidos, caspases podem ser divididas em três subfamílias:

Tabela 2- Subfamílias das caspases e seus membros

Subfamília	Função	Membro
I	Ativador Apoptótico	Caspase-2
		Caspase-8
		Caspase-9
		Caspase-10
II	Executor Apoptótico	Caspase-3
		Caspase-6
		Caspase-7
III	Mediador Inflamatório	Caspase-1
		Caspase-4
		Caspase-5
		Caspase-11
		Caspase-12
		Caspase-13
		Caspase-14

Adaptado de Fan TJ, Han LH, Cong RS, Liang J. Caspase family and apoptosis. *Acta Biochim Biophys Sin.* 2005; 37: 719-727.

Sugere-se que a existência de múltiplas caspases deva-se ao fato de algumas serem envolvidas na amplificação da resposta através da clivagem de pró-formas de outras caspases (Chan & Mattson, 1999).

A atividade das caspases é regulada por outras proteínas, como as pertencentes à família Bcl-2 (*B cell leukemia- 2*) com membros anti e pró-apoptóticos e proteína inibidora de apoptose (*inhibitor of apoptosis proteins*, conhecida como IAP). Bcl-2 e Bcl-x<sub>L</sub> são considerados reguladores negativos da apoptose neuronal (Merry & Korsmeyer, 1997). A super expressão de Bcl-2 protege contra estímulos de indução apoptótica, tais como, retirada do fator trófico, neurotransmissor excitatório glutamato, danos oxidativos, glucocorticóides e agentes danosos ao DNA (Sentman *et al.*, 1991; Guo *et al.*, 1997; Kruman *et al.*, 1997). Estudos sugerem que Bcl-2 age antes da ativação da caspase prevenindo a morte celular. Enquanto a Bcl-2 inibe ativação da caspase, seus membros pró-apoptóticos (Bax e Bad) são substratos da caspase-3 sendo responsáveis pela ativação da cascata de morte (Cheng *et al.*, 1997; Clem *et al.*, 1998).

As IAPs em mamíferos são pertencentes à família de inibidores de caspases identificados a partir da homologia ao baculovírus IAP. Algumas IAPs possuem a característica de suprimir apoptose pela inativação da pró-caspase 9 (Deveraux *et al.*, 1998), caspases 3 e 7 (Roy *et al.*, 1997). Especula-se que a ativação das caspases possua relação com o aumento da apoptose em diversas patologias. A Figura 4 resume os principais eventos envolvidos na morte celular programada quando ativada através das vias extrínseca e intrínseca.



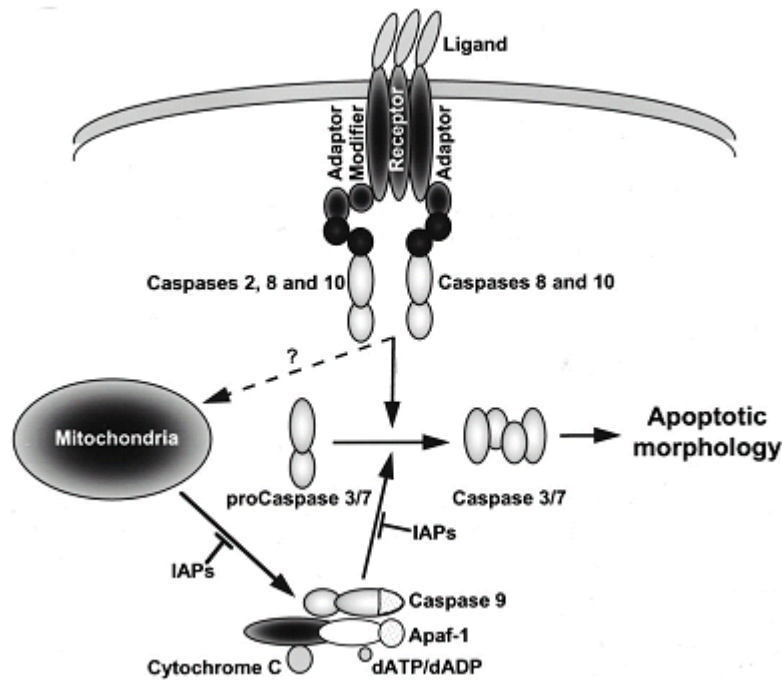


Figura 4 – Na via extrínseca a ativação apoptótica ocorre através da ligação de um sinal extracelular ao receptor na superfície celular, a cascata apoptótica é acionada mediante recrutamento de caspases que levarão a célula à morte. Na via intrínseca há fatores endógenos conhecidos como “sinais de estresse” (danos ao DNA) provenientes do interior da própria célula. Estes sinais acabam sendo detectados pelas mitocôndrias que sofrem desacoplamento da cadeia respiratória e liberam para o citosol o citocromo c. O citocromo c citoplasmático se liga a APAF-1 (*apoptotic protease activation factor-1*) e este complexo, na presença de ATP, ativa a caspase-9 que, por sua vez, ativará a caspase 3 e 7 e levará a célula a morte (Fan et al., 2001). Adaptado de Stennicke HR, Salvesen GS (1998, p. 17).

A caspase executora Caspase-3 é importante no controle da apoptose no tecido nervoso (Salvesen, 2002; Troy & Salvesen, 2002). Ela é responsável pela transdução e execução dos sinais de morte. Estudos evidenciam que o nível de estresse oxidativo pode levar a célula à morte, possivelmente pela modulação diferenciada da atividade das caspases (Hortelano *et al.*, 1997; Leist *et al.*, 1997). Contudo, se torna claro que certas proteases não são meramente enzimas degradativas, mas são moléculas de sinalização altamente reguladas que controlam processos biológicos críticos via proteólise específica (Los *et al.*, 2001).

Recentemente, pesquisas apontam o papel das caspases em vários aspectos não apoptóticos na fisiologia celular. Caspases estão envolvidas na atividade de citocinas durante a inflamação, progressão do ciclo celular, diferenciação de progenitores celulares durante a eritropoiese e células musculares e proliferação de linfócitos T (Fadell *et al.*, 2000; Los *et al.*,

2001; Fernando *et al.*, 2002; Robertson & Zhivotovsky, 2002). No tecido nervoso há crescente evidência sobre os mecanismos apoptóticos não estarem somente envolvidos na morte celular programada, mas podem regular a plasticidade sináptica e motilidade do cone de crescimento (Gilman & Mattson, 2002; Perfettini & Kroemer, 2003).

O sistema nervoso possui uma complexidade estrutural e funcional devido à característica de que suas células, altamente diferenciadas, não se dividem (Mattson, 2000). Durante a evolução, o sistema nervoso desenvolveu mecanismos que protegem as células da morte neuronal. Os sintomas das desordens neurodegenerativas enfatizam a importância de meios que promovam a sobrevivência e a plasticidade das células nervosas. Existem diversas vias de sinalização anti-apoptótico (Mattson, 2000). Fatores neurotróficos podem proteger os neurônios contra apoptose através de ativação de receptores associados a cascatas de sinalização que envolvem quinases e levam à produção de proteínas que promovem a sobrevivência celular. A Figura 5 ilustra alterações na sinalização sináptica em desordens neurodegenerativas.

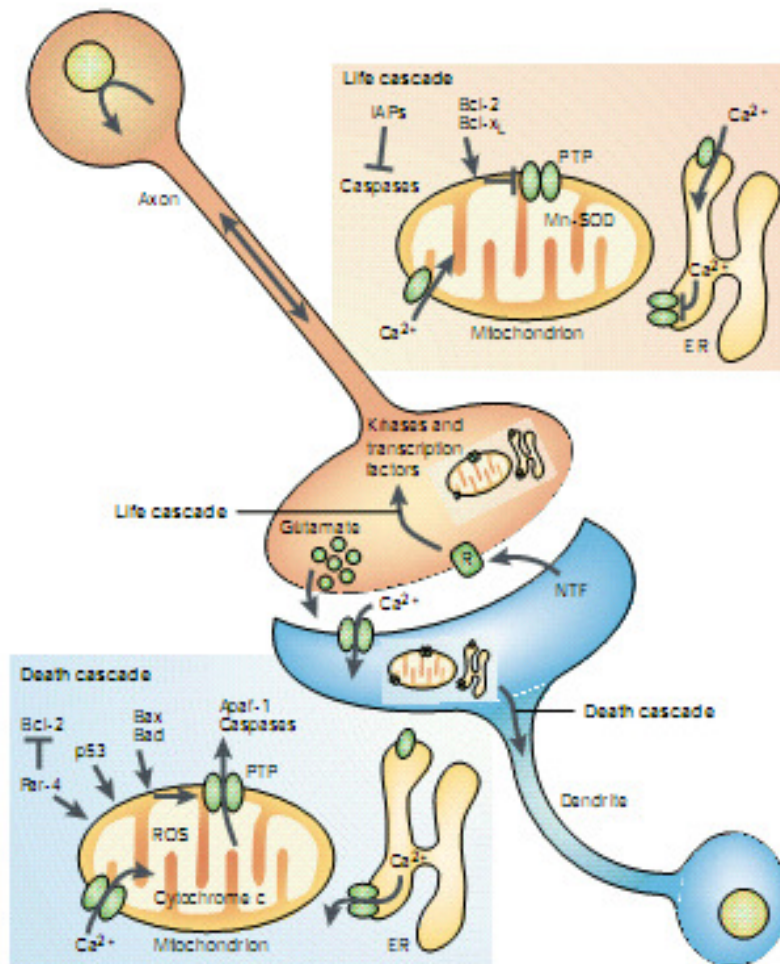


Figura 5 – Alteração na sinalização sináptica em desordens neurodegenerativas. Estressores (idade e doença) promovem ativação apoptótica através de cascatas bioquímicas nos terminais sinápticos e nos neuritos. Por exemplo, a superativação dos receptores de glutamato reduz a disponibilidade energética ou aumenta o estresse oxidativo (por meio de espécies reativas ao oxigênio – ROS – *reactive oxygen species*), levando ao influxo de Ca<sup>2+</sup> nos terminais pós-sinápticos dos dendritos, acionando fatores que ativarão caspases induzindo à célula a morte. Já na sinalização anti-apoptótica, pode ocorrer ativação de receptores (R) pelo fator neurotrófico (NTF) nos terminais do axônio que estimulará cascata de quinases e fatores de transcrição e aumentará a produção de proteínas que promovam a sobrevivência (Bcl-2, Bcl-x<sub>L</sub>) e proteínas inibidoras de apoptose (IAPs).  
Fonte: Mattson, MP (2000, p. 120)

A expressão de proteínas pertencentes à família das caspases em cérebros de ratos durante o desenvolvimento e envelhecimento bem como sua localização subcelular em cérebros de ratos adultos, sugere que as caspases podem contribuir para a regulação da plasticidade sináptica (Shimohama *et al.*, 2001a,b). Yan *et al.* (2001) estudou a expressão da atividade da caspase-3 em células mitóticas e pós-mitóticas na região anterior do cérebro de ratos e demonstrou a atividade da caspase-3 em células em divisão nas zonas proliferativas e migrando para o bulbo olfatório ao se diferenciarem em neurônios. Portanto, a caspase-3 ativa pode ter uma função no processo

celular normal, como a diferenciação neuronal, a migração e a plasticidade (Kudryashov *et al.*, 2001, 2002).

A potenciação de longa duração, conhecida como LTP (*long-term potentiation*), é um fenômeno eletrofisiológico caracterizado pela facilitação duradoura da resposta sináptica. Este fenômeno, descrito primeiramente por Bliss e Lomo (1973), é considerado, desde então, um modelo dos eventos celulares envolvidos em aprendizado e memória. A forma mais tradicional de LTP, dependente do receptor ionotrópico glutamatérgico NMDA (N-methyl-D-aspartate) na região CA1 do hipocampo, é provavelmente a forma mais estudada de plasticidade sináptica. Algumas enzimas proteolíticas tem se mostrado essenciais para a LTP (Gulyaeva *et al.*, 2003). Tomimatsu e colaboradores tem mostrado em estudos recentes, o importante papel do sistema proteolítico na LTP (Tomimatsu *et al.*, 2002).

Atualmente, tem-se sugerido o envolvimento da caspase-3 no fenômeno da LTP. Gulyaeva *et al.* (2003) demonstrou prejuízo na LTP pelo uso de inibidor específico de caspase-3, sugerindo que substratos ou produtos da caspase-3 estão envolvidos no processo. Assim sendo, há evidências de que a caspase-3 é essencial para plasticidade sináptica de longa duração.

Dentre as caspases, este estudo dará enfoque à caspase-3 executora, vista sua importância central na apoptose somada as evidências de suas funções não apoptóticas (Fadeel *et al.*, 2000; Los *et al.*, 2001; Fernando *et al.*, 2002; Gilman & Mattson, 2002; Perfettini & Kroemer, 2003). Devido à carência de estudos que relacionem as proteínas pró-apoptóticas Par-4 e caspase-3 em modelos de neurodegeneração e a ausência de trabalhos que relacionem estas proteases com estudos sobre os efeitos do ferro no sistema nervoso, torna-se importante avaliar possíveis alterações nestas proteínas que podem estar ocorrendo em decorrência do tratamento neonatal com ferro, já que este modelo experimental já está bem estabelecido mostrando diversas alterações na fase de envelhecimento (Sobotka *et al.*, 1996; Fredriksson *et al.*, 1999; Schröder *et al.*, 2001; de Lima *et al.*, 2005; Fredriksson & Archer, 2007).

## **2 OBJETIVOS**

### **2.1– Objetivo Geral**

Avaliar eventuais alterações nas proteínas Par-4 e caspase-3 em diferentes regiões do cérebro de ratos adultos (3 meses) e idosos (24 meses) controles e tratados com ferro durante o período neonatal através de imunohistoquímica.

### **2.2– Objetivos Específicos**

Avaliar a presença de Par-4 e caspase-3 na região do hipocampo, estriado e córtex em ratos adultos (3 meses) normais (não tratados com ferro no período neonatal) através de imunohistoquímica.

Avaliar a presença de Par-4 e caspase-3 na região do hipocampo, estriado e córtex em ratos velhos (24 meses) normais (não tratados com ferro no período neonatal) através de imunohistoquímica.

Avaliar a presença de Par-4 e caspase-3 na região do hipocampo, estriado e córtex em ratos adultos (3 meses) previamente tratados com ferro durante o período neonatal através de imunohistoquímica.

Avaliar a presença de Par-4 e caspase-3 na região do hipocampo, estriado e córtex em ratos velhos (24 meses) previamente tratados com ferro durante o período neonatal através de imunohistoquímica.

Comparar possíveis alterações das proteínas Par-4 e caspase-3 entre os grupos controle e experimental

**CAPÍTULO 2**

**ARTIGO CIENTÍFICO**

**SUBMETIDO AO PERIÓDICO NEUROTOXICITY RESEARCH EM 19/01/2010 E**

**ACEITO EM 23/03/2010**

7

## 3 Neonatal Iron Treatment Increases Apoptotic Markers 4 in Hippocampal and Cortical Areas of Adult Rats

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6 Gustavo Vedana · Raquel Mattos · Liana Lisboa Fernandez · Arlete Hilbig ·  
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8 Received: 19 January 2010/Revised: 13 March 2010/Accepted: 23 March 2010  
9 © Springer Science+Business Media, LLC 2010

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10 **Abstract** Oxidative stress, cellular damage, and neuronal  
11 apoptosis are believed to underlie the progressive cognitive  
12 decline that accompanies natural aging and to be exacer-  
13 bated in neurodegenerative diseases. Over the years, we  
14 have consistently demonstrated that iron neonatal treatment  
15 induces oxidative stress and memory deficits in adult rats,  
16 but the mechanisms underlying these effects remained  
17 undefined. The purpose of this study was to examine  
18 whether neonatal iron overload was associated with apop-  
19 totic cell death in adult and old rats. We analyzed Par-4 and  
20 caspase-3 immunoreactivity in specific brain areas includ-  
21 ing the hippocampus CA1, CA3 and dentate gyrus (DG),  
22 the adjacent cortex and the striatum in adult (3 months-old)  
23 and aged (24 months-old) rats from control (vehicle-treat-  
24 ed) and neonatally iron-treated groups. Neonatal iron  
25 treatment consisted of a daily oral administration of 10 mg/  
26 kg of Fe<sup>2+</sup>, for three consecutive days, from post-natal  
27 12–14. Control aged animals showed increased levels of  
28 both markers when compared to untreated adult animals.  
29 When adults were compared, iron-treated animals presented  
30 significantly higher Par-4 and caspase-3 immunoreactivities

in CA1, CA3 and cortex. In the DG, this effect was statisti- 31  
cally significant only for Par-4. Interestingly, when control 32  
and iron-treated aged animals were compared, a significant 33  
decrease in both apoptotic markers was observed in the 34  
later groups in the same areas. These results may be inter- 35  
preted as an acceleration of aging progressive damages 36  
caused by iron overload and may contribute to a better 37  
understanding of the damaging potential of iron accumula- 38  
tion to brain function and the resulting increased suscepti- 39  
bility to neurodegeneration. 40

**Keywords** Iron · Neurodegeneration · Aging · 42  
Apoptosis · Rat 43

### 44 Introduction

45 Increasing evidence suggest that iron and its major storage 46  
protein ferritin accumulate in specific brain regions during 47  
aging under physiological conditions (Connor et al. 1995; 48  
Zecca et al. 2001; Polla et al. 2003; Schipper 2004; Zecca 49  
et al. 2004). The deleterious potential of excessive iron 50  
accumulation and its altered metabolism on brain cells is 51  
corroborated by evidences indicating that under pathologi- 52  
cal conditions, there is an exacerbation of the aging 53  
associated changes in iron content in specific brain areas 54  
(Dexter et al. 1991; Kienzl et al. 1995). Iron overload has 55  
been implicated in the pathogenesis of several human 56  
neurodegenerative disorders, i.e., Parkinson's disease (PD) 57  
and Alzheimer's disease (AD), Huntington's (HD), and 58  
neurodegeneration with brain iron accumulation (NBIA), 59  
involving aberrant brain iron metabolism and resulting cell 60  
damages (Dexter et al. 1991; Kienzl et al. 1995; Reich- 61  
mann et al. 1995; Swaiman 1991; Youdim et al. 1993; Lee 62  
et al. 2006).

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Springer

	Journal : Large 12640	Dispatch : 30-3-2010	Pages : 9
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- 63 The immediate postnatal period is critical for the  
64 establishment of iron content in the adult brain and its  
65 regional distribution profile (Connor et al. 1995). It has  
66 been shown that both iron transport and transferrin trans-  
67 porter binding sites are maximal during the postnatal peri-  
68 od of rapid brain growth, reaching highest values around  
69 the 15th day after birth in rats (Taylor and Morgan 1990).  
70 Dwork and colleagues (Dwork et al. 1990) demonstrated  
71 that once iron is absorbed during this critical period in  
72 development, it is retained in the brain. Earlier studies from  
73 our group have shown that iron supplementation in the  
74 neonatal period induces a selective iron accumulation in  
75 specific brain regions, especially in the basal ganglia,  
76 which was correlated to behavioral changes in adult mice  
77 (Fredriksson et al. 1999, 2000) and rats (Schröder et al.  
78 2001) including memory deficits (de Lima et al. 2005a)  
79 that could be ameliorated selegiline (de Lima et al. 2005b)  
80 or iron chelation (de Lima et al. 2008). The cognitive  
81 deficits resulting from iron supplementation were accom-  
82 panied by an increased oxidative damage in brain regions  
83 (de Lima et al. 2005a). We have also demonstrated that  
84 neonatal iron supplementation in mice can lead to  
85 increased GFAP immunoreactivity in adulthood, suggest-  
86 ing a reactive gliosis response that could be following  
87 oxidative damage-induced neuronal loss (Fernandez et al.  
88 2009).
- 89 Neuronal death mediated by apoptosis is believed to  
90 underlie cognitive and behavioral symptoms of aging and  
91 neurodegenerative diseases in humans and animal models  
92 (Morrison and Hof 1997; Mattson 2000). Many signals can  
93 mediate neuronal apoptosis initiation, including neurotro-  
94 phic factor deprivation (McKay et al. 1999; Mattson and  
95 Kroemer 2003; Stefanis 2005) and iron-induced oxidative  
96 stress (Polla et al. 2003).
- 97 The protease prostate apoptosis response-4 (Par-4), ini-  
98 tially identified as an up regulated molecule in prostate  
99 tumor cells undergoing apoptosis (Sells et al. 1994), and  
100 now known to be essential in developmental and pathol-  
101 ogy neuronal death, regulates the early stages of apop-  
102 tosis (Guo et al. 1998; Mattson et al. 1999). Levels of Par-4  
103 increase rapidly in response to various apoptotic stimuli  
104 through enhanced translation of Par-4 messenger RNA  
105 (Mattson et al. 1999; El-Guendy and Rangnekar 2003).  
106 Par-4 induction seems to link the chain of events leading to  
107 mitochondrial dysfunction, caspase activation and cell  
108 death (Guo et al. 1998; Chan et al. 1999; Duan et al. 1999;  
109 Guo et al. 2001). The caspase members of proteolytic  
110 enzymes comprise a large family involved in activation,  
111 execution and mediation of apoptosis (Earnshaw et al.  
112 1999; Yuan and Yankner 2000; Troy and Salvesen 2002).  
113 Among the executioner caspases, caspase-3 plays a critical  
114 role in neuronal apoptosis in the nervous system under  
115 physiological and pathological conditions (Troy and  
116 Salvesen 2002; Salvesen 2002; Shimohama et al. 1999;  
117 Lynch and Lynch 2001; Rohn 2008) playing important  
118 roles in neurodegeneration (Friedlander 2003). Oxidative  
119 damage mediated by reactive oxygen species (ROS) have  
120 been shown to induce caspase activation and result  
121 in neuronal apoptosis associated to Parkinson's disease  
122 (Tatton et al. 2003).
- 123 Oxidative stress, cellular damage, and neuronal apop-  
124 tosis are believed to underlie the progressive cognitive  
125 decline that accompanies natural aging. Although over the  
126 years, we have consistently demonstrated that iron neonatal  
127 treatment induces cognitive changes in adult rats, the  
128 mechanisms involved remain to be clarified. Thus, the  
129 purpose of this study was to examine whether neonatal iron  
130 overload is associated with apoptotic cell death in adult and  
131 old rats. We analyzed Par-4 and caspase-3 immunoreac-  
132 tivity in specific brain areas including the hippocampal  
133 formation subareas CA1, CA3 and DG, the adjacent cortex  
134 and the striatum in adult and aged rats from control and  
135 neonatally iron-treated groups.
- Methods** 136
- Animals** 137
- 138 Pregnant Wistar rats were obtained from Fundação Esta-  
139 dade de Pesquisa e Produção em Saúde (FEEPS), Porto  
140 Alegre, Brazil. After birth, each litter was adjusted within  
141 48 h to eight rat pups. Each pup was maintained together  
142 with its respective mother in a plastic cage with sawdust  
143 bedding in a room at a temperature of  $22 \pm 1^\circ\text{C}$  and a 12 h  
144 light: dark cycle. At the age of 4 weeks the pups were  
145 weaned and the males were selected and raised in groups of  
146 three to five rats. At postnatal treatment, the animals were  
147 supplied with standardized pellet food and tap water  
148 ad libitum. All experimental procedures were performed in  
149 accordance with the NIH Guide for the Care and Use of  
150 Laboratory Animals, and the Brazilian Society for Neuro-  
151 science and Behaviour (SBNeC) recommendations for  
152 animal care. The protocol for this research was approved  
153 by the Institutional Ethics Committee of the Pontifícia  
154 Universidade Católica do Rio Grande do Sul.
- Animal Treatment** 155
- 156 The neonatal iron treatment was performed as previously  
157 described (Schröder et al. 2001; de Lima et al. 2005a, b,  
158 2007, 2008). Ten 12-day-old rat pups received orally  
159 a single daily dose of 10.0 mg  $\text{Fe}^{2+}$ /kg body weight  
160 in solution volume (Ferromyn<sup>®</sup>, AB Hässle, Göteborg,  
161 Sweden) and nine ones received vehicle (5% sorbitol in  
162 water, control group) via a metallic gastric tube, over

163 3 days (post natal days 12–14). The period of treatment  
 164 was chosen based on studies that show that iron uptake by  
 165 the brain increases rapidly in the first 15 days of life,  
 166 decreasing after the 15th day of life in rats (Taylor and  
 167 Morgan 1990; Taylor et al. 1991).

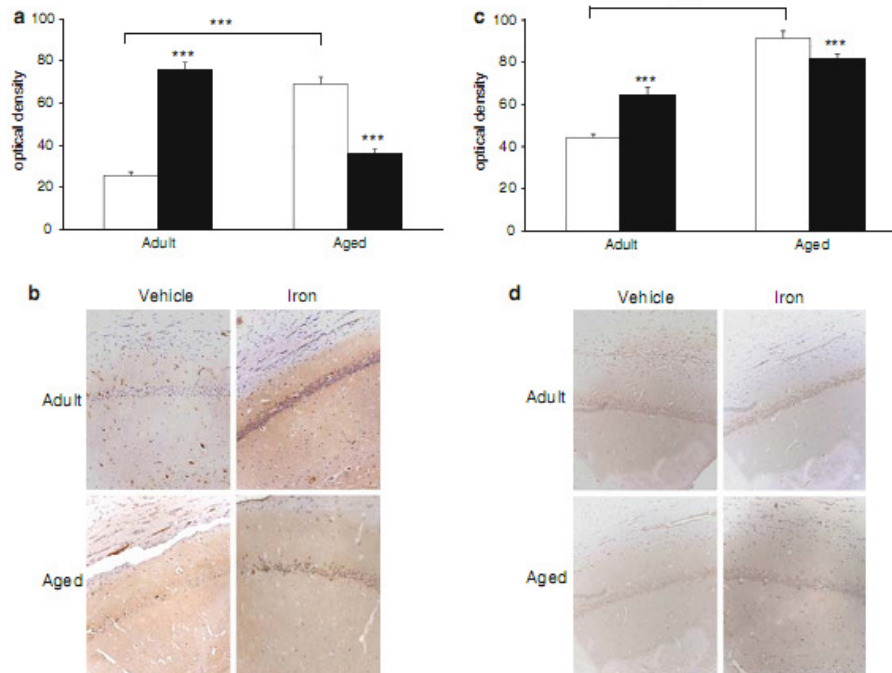
#### 168 Immunohistochemical Analyses (IHC)

169 Five iron-treated and 3 sorbitol-treated 3-month-old rats,  
 170 and five iron-treated and six sorbitol-treated 24-month-old  
 171 rats were perfused through the left cardiac ventricle for  
 172 20 min with 4% paraformaldehyde in phosphate buffer.  
 173 The brains were rapidly removed, and immersed in the  
 174 same fixative solution at room temperature. Immediately  
 175 afterwards, the brains were cut coronally and embedded in  
 176 paraffin. Serial, 5- $\mu$ m-thick coronal sections were obtained  
 177 with a microtome, and used for immunohistochemistry.  
 178 De-waxed sections corresponding to Paxinos and Watson  
 179 (Paxinos and Watson 2005) striatum (Figs. 25–33), hip-  
 180 pocampus (Figs. 33–37) and cortex (Figs. 33–37) were  
 181 immersed in 3% hydrogen peroxide in 100% methanol for

182 15 min to inhibit endogenous peroxidase activity. Then,  
 183 sections were boiled in 10 mM citrate buffer, pH 6.0, for  
 30 min or treated with formic acid for 3 min, for antigen  
 184 retrieval. After rinsing in phosphate-buffered saline (PBS),  
 185 the sections were incubated with normal horse serum for  
 186 2 h and then with the primary antibody overnight at 4°C in  
 187 humid chambers. The following primary antibodies were  
 188 used following the manufacturer instructions: anti-Par-4  
 189 rabbit polyclonal (AB75686, Abcam Inc. Cambridge, UK),  
 190 dilution 1:800, anti-Caspase-3 rabbit polyclonal (AB59388,  
 191 Abcam Inc. Cambridge, UK), dilution 1:100. The sections  
 192 were washed three times in PBS and immunostained  
 193 by a MAX Polymer Detection Kit (secondary biotinylated  
 194 universal, HRP polymer conjugate, and DAB chromogen,  
 195 ZIMED) for optical detection. 196

#### Densitometry and Statistical Processing of Data 197

The immunohistochemical images were captured using 198  
 Olympus BX 50 microscope, DC300F camera and capture 199  
 system Leica IM 50. The selection was done manually 200



**Fig. 1** Densitometric analysis of Par-4 (a) and caspase-3 (c) immunoreactivities in CA1 area. Data were expressed as mean  $\pm$  standard error of the mean (SEM). Comparisons among groups were performed using ANOVA followed by Tukey post hoc test. \*\*\* Indicates

$P < 0.001$ . Representative histological sections of Par-4 (b) and caspase-3 (d) immunohistochemistry of CA1 of adult and aged rats treated with vehicle (open bars) or iron (filled bars) in the neonatal period. Magnification: 10 $\times$

201 including striatum, hippocampus or cortex. To analyze  
 202 the images, Image J software was used (<http://www.uhn>  
 203 [research.ca/facilities/wcif/download.html](http://www.uhnresearch.ca/facilities/wcif/download.html)). For each image,  
 204 color functions and color de-convolution, H DAB vectors:  
 205 color 2 R: 0.26814753, G: 0.57031375, B: 0.77642715 were  
 206 applied. The color 2 image (brown) was inverted and the  
 207 measurement was obtained as a percentage of the DAB  
 208 chromogen. Data were expressed as mean  $\pm$  standard error  
 209 of the mean (SEM). Comparisons among groups were per-  
 210 formed using ANOVA followed by Tukey post hoc test.  
 211  $P$  values of less than 0.05 were considered statistical signif-  
 212 icant and are indicated in the figure as \*  $P < 0.05$ ,  
 213 \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$ .

Author Proof

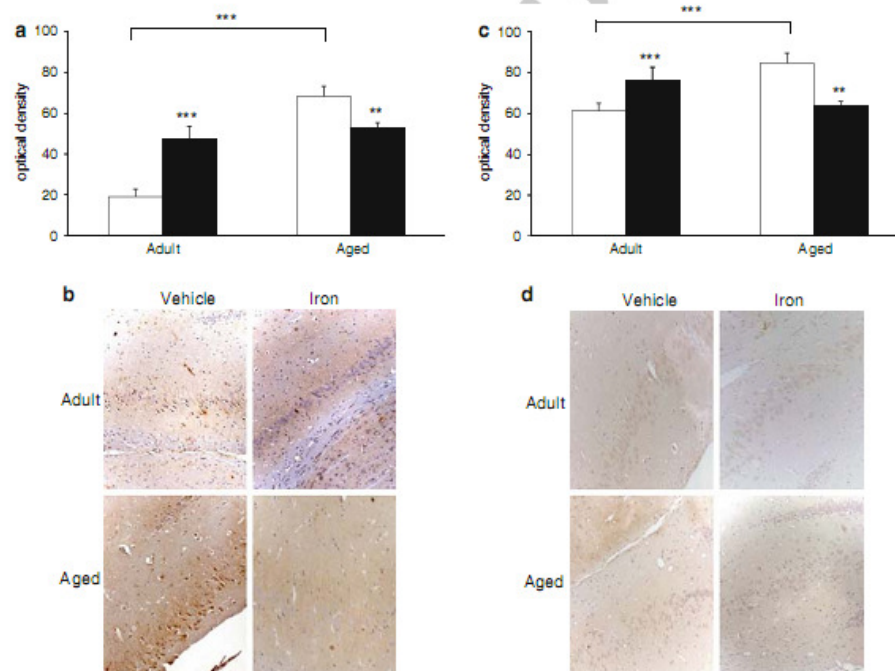
## 214 Results

215 The studied hippocampal regions showed a similar profile in  
 216 terms of immunoreactivity to both apoptotic molecules  
 217 studied. When adult animals were compared, iron-treated  
 218 animals presented significantly higher Par-4 and caspase-3

219 immunoreactivities in the CA1 (Fig. 1a–d) and CA3  
 220 (Fig. 2a–d) areas in comparison to their respective control  
 221 groups ( $P < 0.001$  for all comparisons). Interestingly,  
 222 when control and iron-treated aged animals were com-  
 223 pared, a significant decrease in both apoptotic markers was  
 224 observed in the later groups in those areas ( $P < 0.001$  in  
 225 CA1, and  $P < 0.01$  in CA3).

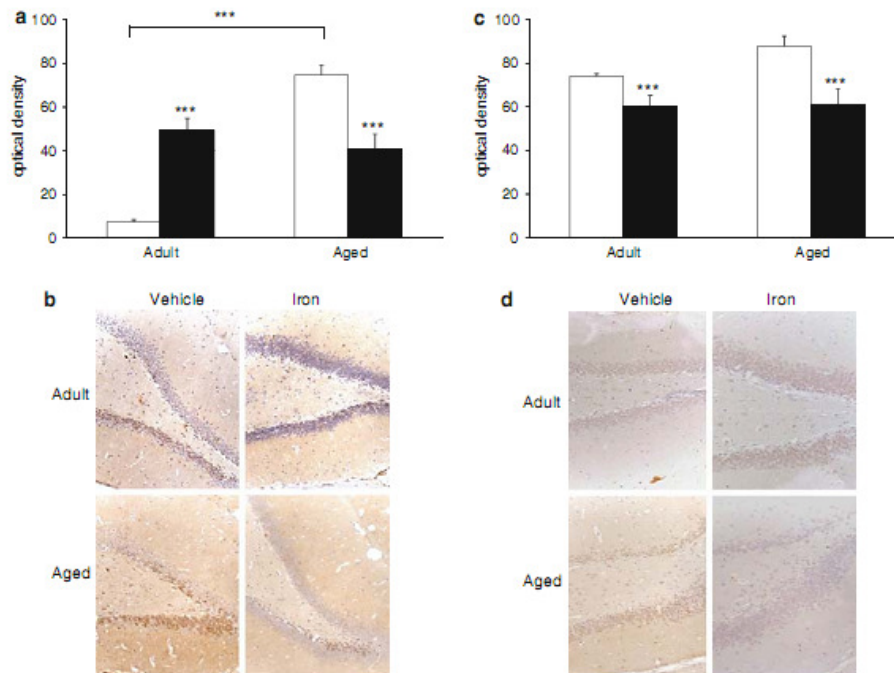
226 At the DG formation Par-4 showed an equivalent profile to  
 227 that above described, being increased in adult treated  
 228 animals when compared to their controls and decreased  
 229 when the equivalent aged groups were compared ( $P <$   
 230 0.001 to both comparisons, Fig. 3a–b). Caspase-3 immu-  
 231 noreactivity at this area, however, had lower levels in iron-  
 232 treated animals at both ages when they were compared to  
 233 their age-matching controls ( $P < 0.001$ , Fig. 3c, d).

234 As shown in Fig. 4, the analyzed cortical area showed a  
 235 profile comparable to that observed in CA1 and CA3. Iron-  
 236 treated animals showed significantly higher levels of Par-4  
 237 and caspase-3 at the adult age ( $P < 0.05$ ), while that effect  
 238 was inverted at a later period in life, when aged treated  
 239 animals showed significant less reactivity to both markers  
 240 than their respective control group ( $P < 0.001$ ).



**Fig. 2** Densitometric analysis of Par-4 (a) and caspase-3 (c) immu-  
 noreactivities in CA3 area. Data were expressed as mean  $\pm$  standard  
 error of the mean (SEM). Comparisons among groups were performed  
 using ANOVA followed by Tukey post hoc test. \*\* Indicates

$P < 0.01$  and \*\*\* indicates  $P < 0.001$ . Representative histological  
 sections of Par-4 (b) and caspase-3 (d) immunohistochemistry of CA3  
 of adult and aged rats treated with vehicle (open bars) or iron (filled  
 bars) in the neonatal period. Magnification: 10 $\times$



**Fig. 3** Densitometric analysis of Par-4 (a) and caspase-3 (c) immunoreactivities in DG area. Data were expressed as mean  $\pm$  standard error of the mean (SEM). Comparisons among groups were performed using ANOVA followed by Tukey post hoc test. \*\*\* Indicates

$P < 0.001$ . Representative histological sections of Par-4 (b) and caspase-3 (d) immunohistochemistry of DG of adult and aged rats treated with vehicle (open bars) or iron (filled bars) in the neonatal period. Magnification: 10 $\times$

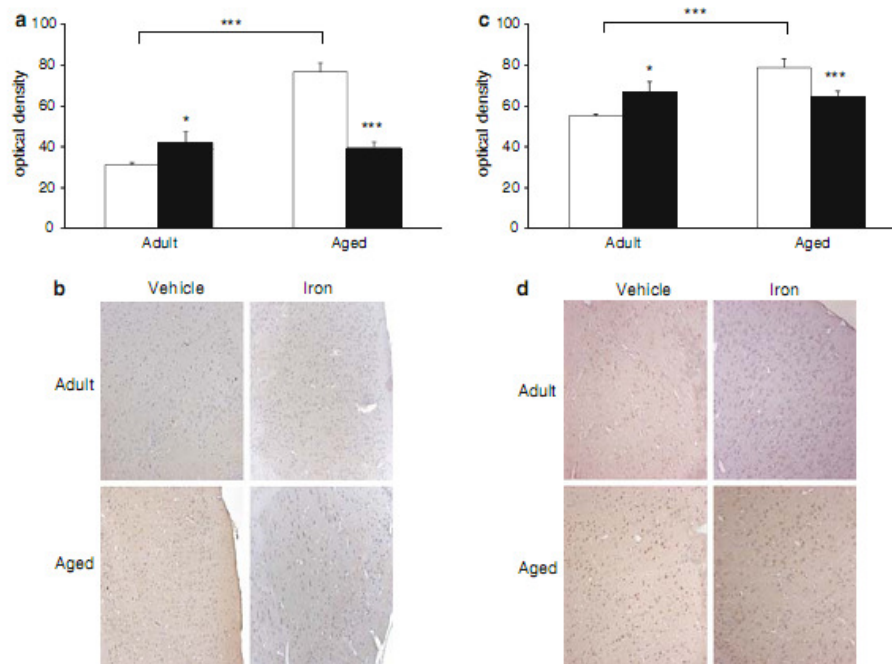
241 Finally, when Par-4 and caspase-3 were studied in the  
242 striatum of adult and aged animals, no significant effect of  
243 the neonatal iron treatment was observed (Fig. 5).

244 Our data also demonstrates a generalized increase in  
245 Par-4 and caspase-3 immunoreactivity in hippocampal  
246 CA1 (Fig. 1) and CA3 (Fig. 2) areas and cortex (Fig. 4)  
247 associated to normal aging in untreated animals when adult  
248 and aged control animals are compared. Par-4 immunore-  
249 activity was significantly increased in DG, and, despite a  
250 clear tendency, this effect was not statistically significant  
251 for caspase-3 (Fig. 3) ( $P = 0.072$ ). This profile was also  
252 observed in the striatum, where no significant changes were  
253 found in Par-4 ( $P = 0.079$ ) and caspase-3 ( $P = 0.053$ )  
254 immunoreactivity when adult and aged controls were  
255 compared (Fig. 5).

## 256 Discussion

257 The present findings show that iron, given to rats in the  
258 neonatal period, induces increased levels of apoptotic  
259 markers, Par-4 and caspase-3 in hippocampal subregions

and cortex of adult rats. The increased Par-4 and caspase-3  
260 levels observed in adult iron-treated animals were compa-  
261 rable to the levels of both markers in aged control indi-  
262 viduals, suggesting that iron overload accelerates the  
263 apoptotic cell death rate that normally increases with  
264 age (Lynch and Lynch 2001). Further studies evaluating  
265 apoptotic DNA fragmentation and quantification of apop-  
266 totic-related protein levels could be performed in order  
267 to better characterize iron-induced apoptotic mechanisms  
268 and refine the present findings. The premature apoptosis  
269 observed in iron-treated adults might be related to previous  
270 findings from our group showing cognitive deficits in adult  
271 animals that had received iron supplementation in the  
272 neonatal period at levels comparable to those typically  
273 observed in aged individuals (de Lima et al. 2005a, b, c;  
274 2008). The hippocampal formation is known to be critically  
275 involved in cognition, participating in memory acquisition  
276 and consolidation for a myriad of tasks (Eichenbaum 2004)  
277 and our findings of iron-induced increase in Par-4 and  
278 caspase-3 levels in hippocampal and cortical areas could  
279 explain these animals' memory deficits (de Lima et al.  
280 2005a, b, 2007, 2008).  
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**Fig. 4** Densitometric analysis of Par-4 (a) and caspase-3 (c) immunoreactivities in cortex area. Data were expressed as mean  $\pm$  standard error of the mean (SEM). Comparisons among groups were performed using ANOVA followed by Tukey post hoc test.

\* Indicates  $P < 0.05$  and \*\*\* indicates  $P < 0.001$ . Representative histological sections of Par-4 (b) and caspase-3 (d) immunohistochemistry of cortex of adult and aged rats treated with vehicle (open bars) or iron (filled bars) in the neonatal period. Magnification:  $10\times$

Author Proof

282 Interestingly, no significant differences were found in  
 283 Par-4 and caspase-3 levels in the striatum of iron-treated  
 284 rats compared to their age-matching controls. We have  
 285 previously described that iron supplementation in the  
 286 neonatal period does not alter the level of protein carbonylation, an index of oxidative protein damage, and superoxide dismutase (SOD) activity in striatum of adult rats (de Lima et al. 2005a; Dal-Pizzol et al. 2001), in contrast to iron-induced alterations found in the *substantia nigra*, hippocampus, and cortex in those studies. Taking together these findings suggest that the striatum might be more resistant to oxidative stress and apoptotic activation than hippocampal and cortical areas.

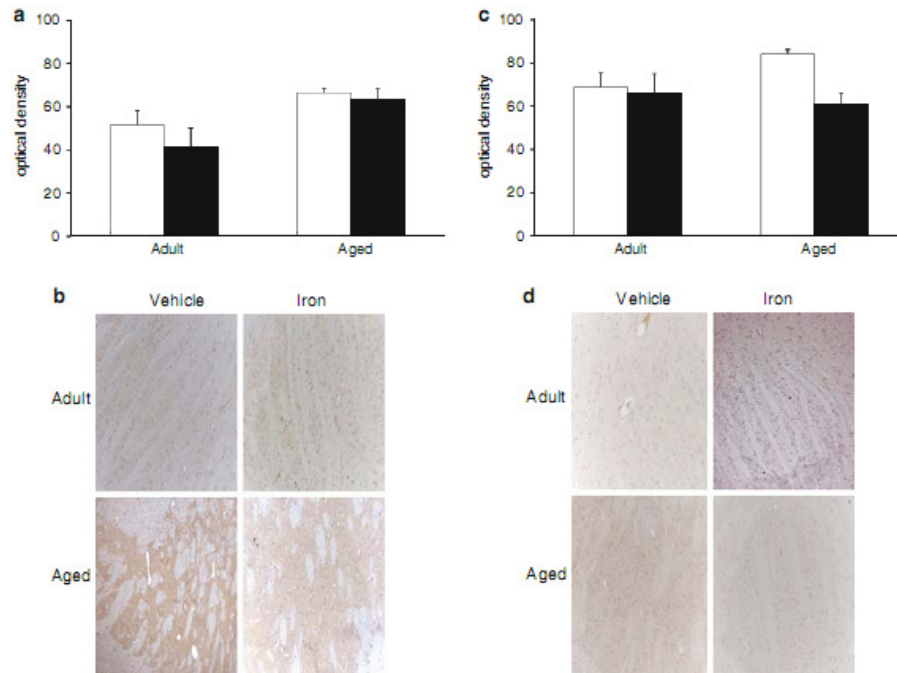
295 Neuronal apoptosis can be initiated by signals including  
 296 growth-factor deprivation, excessive excitatory glutamatergic neurotransmission and oxidative stress that activate intracellular cascades of events that can include production of Par-4 and procaspase-3 cleavage and caspase-3 activation [reviewed in Mattson 2000]. Our study quantified protein levels of Par-4 and caspase-3 using immunohistochemistry as it reflects the active form of both apoptotic markers as protein levels have been previously shown to

rise due to increased messenger RNA translation in response to various apoptotic stimuli (Mattson et al. 1999; El-Guendy and Rangnekar 2003).

307 Aging occurs naturally throughout life and is associated with a significant intensification of apoptosis under physiological conditions and/or susceptibility to cell death after challenges in different tissues (Lacelle et al. 2002; Barja 2004). Our findings substantiate this concept, showing increased levels of proteins involved in apoptosis activation and execution in control aged animals when compared to adults, in accordance to previous reports (Adams et al. 1996; Tagliatela et al. 1996; Anglade et al. 1997; Dorszewska et al. 2004; Mansour et al. 2008).

317 Apoptosis has also been extensively related to neurodegenerative disorders (reviewed in 22]. Iron accumulation, that naturally occurs during aging (Zecca et al. 2001; Polla et al. 2003; Schipper 2004; Zecca et al. 2004), shown to be exacerbated in patients of PD, AD, HD, and NBIA (Dexter et al. 1991; Kienzl et al. 1995). Iron-induced apoptosis has been previously demonstrated using in vitro cellular models of neurotoxic injury (Kooncumchoo et al. 2006; Zhang et al. 2009). To our knowledge, this is the first

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**Fig. 5** Densitometric analysis of Par-4 (a) and caspase-3 (c) immunoreactivities in striatum area. Data were expressed as mean  $\pm$  standard error of the mean (SEM). Comparisons among groups were performed using ANOVA followed by Tukey post hoc test. No

significant changes in striatum. Representative histological sections of Par-4 (b) and caspase-3 (d) immunohistochemistry of striatum of adult and aged rats treated with vehicle (open bars) or iron (filled bars) in the neonatal period. Magnification: 10 $\times$

326 demonstration that iron supplementation in vivo induces  
327 apoptosis in cerebral tissue in adult rats.

328 Iron overload is believed to contribute to the develop-  
329 ment of neurodegeneration and exacerbation of normal  
330 apoptotic rates largely due to its participation in the Fenton  
331 reaction and production of reactive oxygen species (ROS)  
332 which result in cell damage (Dexter et al. 1991; Kienzl  
333 et al. 1995; Reichmann et al. 1995; Swaiman 1991; You-  
334 dim et al. 1993; Lee et al. 2006). Accordingly, we have  
335 previously shown that adult iron-treated rats show  
336 increased oxidative damage in the hippocampus and cortex  
337 (de Lima et al. 2005a; Dal-Pizzol et al. 2001), areas in  
338 which we now observe increased apoptosis at the same life  
339 period. We have also shown in prior studies that at this  
340 period iron-treated animals have increased GFAP-immu-  
341 noreactivity in equivalent brain areas (Fernandez et al.  
342 2009), suggesting an active gliosis that we may speculate  
343 are taking over spaces left by neuronal death.

344 Taken together our findings showing higher Par-4 and  
345 caspase-3 levels in control aged animals when compared to  
346 iron-treated groups of the same age may be explained by

the precocious damage caused by iron overload and inte-  
grated to previous findings may contribute to a better  
understanding of the damaging potential of iron accumu-  
lation to brain function and the resulting increased sus-  
ceptibility to neurodegeneration. Iron supplementation at  
very early stages of development results in brain accumu-  
lation and subsequent toxicity, increasing oxidative damage  
up to a level that natural defenses fail and neuronal apop-  
totic rates are exacerbated during adulthood, resulting in  
memory deficits. The mitotic responsiveness and more  
resistant nature of astrocytes may allow their proliferation  
in areas previously occupied by neurons at this early stage,  
resulting in lower levels of apoptotic proteins in iron-treated  
aged individuals. These findings may have implications for  
the understanding of iron's role in the pathogenesis of  
neurodegenerative disorders.

**Acknowledgments** This research was partially funded by CNPq  
grant 476316/2006-5 and FAPERGS PROADE3 0521807 to Monica  
Vianna. Monica Vianna is supported by CNPq 312137/2006-0 fel-  
lowship, Nadja Schröder is supported by 301368/2006-6 fellowship,  
and Clivia Miwa is supported by CAPES-MEC fellowship.

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553



## **CAPÍTULO 3**

### **CONSIDERAÇÕES FINAIS**

O envelhecimento é um processo que ocorre naturalmente por toda a vida e está associado com significativo aumento da apoptose em condições fisiológicas e/ou suscetibilidade a morte celular após injúrias (Barja, 2004). A perda de neurônios resulta em prejuízos funcionais tais como demência e deficiência neuro-motora (Higami & Shimokawa, 2000).

O ferro é um metal essencial para o SNC (Zhang *et al.*, 2005). Muitos processos no desenvolvimento normal do SNC, incluindo respiração oxidativa, mielinização dos axônios e síntese de neurotransmissores necessitam de ferro (van Gelder *et al.*, 1998; Hentze *et al.*, 2004). Atualmente, tem sido sugerida relação entre disfunções nas vias de homeostase do ferro e a patogênese de doenças neurodegenerativas (Martin *et al.*, 1998).

O modelo apresentado de sobrecarga de ferro no período neonatal é bem conhecido por induzir acúmulo de ferro em regiões do cérebro, especificamente nos gânglios da base, região associada com déficits na memória de longa duração em camundongos adultos (Fredriksson *et al.*, 1999; Fredriksson *et al.*, 2000) e ratos (Schröder *et al.*, 2001; de Lima *et al.*, 2005a; de Lima *et al.*, 2005b; de Lima *et al.*, 2007; de Lima *et al.*, 2008) e pelo aumento do estresse oxidativo que medeia a morte celular e o prejuízo cognitivo associado ao envelhecimento (Dexter *et al.*, 1989; Butterfield *et al.*, 2002; Liu *et al.*, 2003; Mattson, 2004; Mecocci *et al.*, 2004; de Lima *et al.*, 2005).

O trabalho desenvolvido e cuja síntese está apresentada no Capítulo 2 teve como objetivo avaliar alterações nas proteínas pró-apoptóticas Par-4 e caspase-3 em diferentes regiões do cérebro de ratos adultos e idosos que receberam sobrecarga de ferro no período neonatal e seus respectivos controles. Os resultados demonstram um aumento da imunorreatividade de ambas proteínas nas regiões CA1, CA3 e córtex ao longo do envelhecimento normal. O significativo aumento de Par-4 e caspase-3 nos animais idosos não tratados sugere aumento proeminente e consistente na taxa de apoptose associada ao envelhecimento.

As regiões estudadas do hipocampo indicaram similares perfis de imunorreatividade para ambas proteínas analisadas. CA1 e CA3 mostraram significativo aumento de imunorreatividade de Par-4 e caspase-3 nos animais adultos tratados com ferro quando comparados com seus respectivos controles. Quando os animais idosos dos grupos controle e experimental foram comparados, foi observado significativa diminuição de ambas proteínas nas mesmas áreas. O giro dentado não apresentou diferença significativa nos níveis de caspase-3 e teve aumento de Par-4 nos animais adultos experimentais quando comparados com seus controles e apresentou-se

diminuído quando grupos equivalentes de idosos foram comparados. O estriado não apresentou mudanças significativas para as proteínas estudadas.

Durante o processo de envelhecimento, há aumento do conteúdo normal de ferro nas diferentes regiões do cérebro. O acúmulo deste metal é encontrado em pacientes com DP, DA e DH sendo marcadas pelo aumento da apoptose. (Mattson, 2000).

Estudos *in vitro* (Kooncumchoo *et al.*, 2006; Kobayashi *et al.*, 2008; Hasegawa *et al.*, 2009; Zhang *et al.*, 2009) tem demonstrado a morte neuronal devido a toxicidade do ferro.

O presente trabalho é a primeira demonstração *in vivo* de que a sobrecarga de ferro induz apoptose no tecido cerebral de ratos adultos.

Os resultados sugerem que há aumento prematuro da apoptose pela suplementação de ferro no período neonatal, já que os níveis de Par-4 e caspase-3 nos animais adultos tratados assemelham-se aos observados nos animais idosos do grupo controle. Estas observações reforçam a idéia de que o acúmulo de ferro acelera a taxa de morte celular, que normalmente aumenta com a idade, e é apoiada por descobertas de aumento de gliose nos animais tratados com ferro já na idade adulta (Fernandez *et al.*, 2009). A suplementação de ferro nos estágios iniciais do desenvolvimento resulta no seu acúmulo no cérebro e toxicidade, aumentando o dano oxidativo. As elevadas taxas de apoptose neuronal na idade adulta resultam nos déficits de memória já descritos nesta fase (Schröder *et al.*, 2001; de Lima *et al.*, 2005a; 2005b). Estes resultados fornecem melhor entendimento do papel do ferro na patogênese de desordens neurodegenerativas e auxiliam para estudos complementares que avaliem os mecanismos apoptóticos envolvidos nestas desordens que são cruciais para estabelecer a real contribuição deste metal e seus mecanismos de ação.

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