MARIA NOÊMIA MARTINS DE LIMA

Déficits de Memória Induzidos pelo Tratamento Neonatal com Ferro e pelo Envelhecimento: Estratégias de Neuroproteção

Tese submetida ao Programa de Pós-graduação em Gerontologia Biomédica da Pontifícia Universidade Católica do Rio Grande do Sul como parte dos requisitos necessários à obtenção do Grau de Doutor em Gerontologia Biomédica.

Orientadora: Dra. Nadja Schröder

Porto Alegre

2007

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Para meu avô, José Martins de Lima

(*in memoriam*)

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(Tutu D)

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(Lispector C)

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"Os homens deveriam saber que do cérebro, e somente do cérebro, provêm nossos prazeres, alegrias, risadas e brincadeiras, bem como nossas tristezas, dores, desgostos e medos. Através dele, em particular, nós pensamos, vemos, ouvimos e distinguimos o feio do bonito, o ruim do bom, o agradável do desagradável... É a mesma coisa que nos faz loucos ou delirantes, nos excita com espanto e medo, seja de noite ou de dia, traz insônia, erros inoportunos, ansiedade sem sentido, a distração e atos que são contrários aos hábitos..."

(Hipócrates)

RESUMO

O excesso de ferro no encéfalo tem sido relacionado com a patogênese de diversas doenças neurodegenerativas, por exemplo, as doenças de Alzheimer e de Parkinson. Tem sido demonstrado que o período neonatal é crítico para o estabelecimento do conteúdo normal de ferro no cérebro adulto e também se sabe que o envelhecimento altera a distribuição cerebral deste metal. Nós descrevemos anteriormente que a administração de ferro no período neonatal prejudica severamente a memória de reconhecimento em ratos adultos e que o envelhecimento também induz prejuízos significativos na memória de reconhecimento. O objetivo deste estudo foi determinar se os déficits de memória induzidos pelo tratamento neonatal com ferro e pelo envelhecimento poderiam ser revertidos através de três diferentes estratégias farmacológicas. No Experimento I, ratos machos receberam veículo (5% de sorbitol em água destilada) ou ferro (10,0 mg/kg via oral) do 12° ao 14° dia pós-natal. Ao ati ngirem a idade adulta, os grupos foram divididos em três outros grupos experimentais que receberam 6 injeções de salina ou desferroxamina (DFO, um quelante de ferro). Os animais foram submetidos à tarefa de reconhecimento do objeto novo (RON) 24 h após a última injeção. Os resultados indicaram que o tratamento com DFO na idade adulta foi capaz de reverter o prejuízo de memória de reconhecimento induzido pelo tratamento neonatal com ferro. No Experimento II, ratos machos (23 meses de idade) receberam 6 injeções de salina ou DFO (300,0 mg/Kg ip). Os animais foram submetidos ao RON 24 h após a última injeção. Os ratos tratados com DFO apresentaram índices de reconhecimento normais, enquanto que os ratos do grupo salina apresentaram déficits de memória de reconhecimento. Também foi demonstrado que o DFO reduziu os danos oxidativos a proteínas no córtex e no hipocampo desses animais. No Experimento III, os ratos foram submetidos ao mesmo tratamento neonatal com ferro realizado no Experimento I. Ao atingirem a idade adulta, os grupos foram divididos em 3 outros grupos experimentais que receberam veículo (1% de DMSO em salina) ou SKF 38393 [um agonista de receptores dopaminérgicos do tipo D_1] (5,0 mg/Kg ip) ou GBR 12935 [um inibidor da recaptação de dopamina] (5,0 ou 10,0 mg/Kg ip) imediatamente após o treino do RON. Tanto a administração de SKF 38393 quanto de GBR 12935 foi capaz de

reverter o prejuízo de memória induzido pelo tratamento neonatal com ferro. No Experimento IV, os ratos foram submetidos ao tratamento neonatal com ferro como descrito nos Experimentos I e III. Ao atingirem a idade adulta, os grupos foram divididos em quatro outros grupos experimentais que receberam veículo ou rolipram [um inibidor da fosfodiesterase] (0,01; 0,03 ou 0,1 mg/kg ip) imediatamente após o treino do RON. Os ratos tratados com ferro, que receberam rolipram (0,03 e 0,1 mg/Kg), apresentaram memória de reconhecimento normal. No Experimento V, ratos machos (24 meses de idade) receberam veículo ou rolipram (0,1 mg/kg ip) imediatamente após o treino do RON. O tratamento com rolipram reverteu o prejuízo de memória de reconhecimento induzido pelo envelhecimento. Os resultados, em conjunto, mostram que a terapia com quelante de ferro e o aumento dos níveis do AMPc foram capazes de reverter os déficits de memória de reconhecimento induzidos pelo tratamento neonatal com ferro e pelo envelhecimento.

Palavras-chave: desferroxamina, envelhecimento, ferro, GBR 12935, memória de reconhecimento, ratos, rolipram, SKF 38393.

ABSTRACT

Excess of iron in the brain has been implicated in the pathogenesis of several human neurodegenerative diseases, for example Alzheimer's and Parkinson's diseases. It has been shown that the neonatal period is critical for the establishment of normal iron content in the adult brain and it is also known that aging alters the cerebral distribution of this metal. We have previously described that neonatal administration of iron severely impairs recognition memory in adult rats. In addition, we also described that old rats present recognition memory deficits. The aim of the present study was to determine if iron- and aging-induced recognition memory deficits could be reverted by three different pharmacological strategies. In Experiment I, male rats received vehicle (5% sorbitol in water) or iron (10.0 mg/kg orally) at postnatal days 12 to 14. When they reached the age of 3 months both groups were divided in three experimental groups receiving 6 injections of saline or desferroxamine (DFO, an iron chelator agent) (30.0 or 300.0 mg/kg ip). The animals were submitted to a novel object recognition task (NOR) 24 h after the last injection. Iron-treated rats showed long-term recognition memory impairment. Iron-treated rats, that received DFO (300.0 mg/Kg), showed long-term recognition memory indexes similar to those seen in vehicle group. In Experiment II, male Wistar rats (23 months old) received 6 injections of saline or DFO (300.0 mg/kg ip). The animals were submitted to NOR 24 h after the last injection. DFO-treated rats showed normal recognition memory while the saline group showed long-term recognition memory deficits. It was also demonstrated that DFO reduced the oxidative damage to proteins in cortex and hippocampus. In Experiment III, rats were submitted to the same neonatal treatment with iron performed in Experiment I. When they reached adulthood both groups were divided in three experimental groups receiving vehicle (1% DMSO in saline solution) or SKF 38393 [a dopamine D_1 receptor agonist] (5.0 mg/kg ip) or GBR 12935 [a dopamine reuptake inhibitor] (5.0 or 10.0 mg/Kg ip) immediately after NOR training. Iron-treated rats that received SKF 38393 and GBR 12935 (10.0 mg/Kg) showed normal recognition memory. In Experiment IV, rats were submitted to the same neonatal treatment with iron performed in Experiments I and III. When they reached adulthood both groups were divided in four experimental groups receiving vehicle or rolipram, a phosphodiestase inhibitor, (0.01, 0.03 or 0.1

mg/kg ip) immediately after NOR training. Iron-treated rats, that received rolipram (0.03 and 0.1mg/Kg), showed normal recognition memory. In Experiment V, male rats (24 months old) received vehicle or rolipram (0.1 mg/kg ip) immediately after NOR training. Rolipram-treated rats showed normal recognition memory while the vehicle group presented recognition memory deficits. Taken together, the results show that the iron chelation therapy and the cAMP pathway stimulation were able to revert the iron- and aging-induced recognition memory deficits.

Key-words: aging, desferroxamine, GBR 12935, iron, rats, recognition memory, rolipram, SKF 38393.

SUMÁRIO

LISTA DE ABREVIATURAS

- **AADC**, descarboxilase de aminoácido aromático (do Inglês: *aromatic amino acid decarboxylase*)
- **AMPc**, adenosina monofosfato cíclico (do Inglês: *cyclic adenosine monophosphate*)
- **ATP**, trifosfato de adenosina (do Inglês: *adenosine triphosphate*)
- **CREB**, proteína ligante de elemento responsivo ao AMPc (do Inglês: *cAMP response element binding*)
- **DA**, doença de Alzheimer
- **DAT**, transportador de dopamina
- **DFO**, deferoxamina
- **DH**, doença de Huntington
- **DNA**, ácido desoxirribonucleico (do Inglês: *desoxyribonucleic acid*)
- **DMSO**, dimetilsulfoxido
- **DP**, doença de Parkinson
- **EUA**, Estados Unidos da América
- **GABA**, ácido gama aminobutírico (do Inglês: *gamma aminobutyric acid*)
- **GMPc**, guanosina monofosfato cíclico (do Inglês: *cyclic guanosine monophosphate*)
- **ip**, intraperitoneal
- **LTM**, memória de longa duração (do Inglês: *long-term memory*)
- **LTP**, memória de curta duração (do Inglês: *long-term potentiation*)
- **MAO**, monoamino oxidase
- **PDE4**, fosfodiesterase do tipo 4 (do Inglês: *phosphodiesterase type 4*)
- **PKA**, proteína cinase dependente de AMPc (do Inglês: *cAMP-dependent protein kinase*)
- **SN**, substância negra
- **SNC**, sistema nervoso central
- **TH**, tirosina hidroxilase

1 INTRODUÇÃO

O aumento na expectativa de vida é um fenômeno que vem se manifestando de forma crescente em escala mundial. Essa mudança no padrão de distribuição etária da população ocasionou uma maior prevalência de patologias relacionadas ao envelhecimento, tais como as doenças neurodegenerativas [doenças de Alzheimer (DA) e de Parkinson (DP), por exemplo][1,2].

Como resultado das mudanças nas taxas de mortalidade e de fertilidade nas últimas décadas no Brasil, estima-se que a população acima de 65 anos irá crescer de 2,7% em 1960 para aproximadamente 14% antes de 2050, um aumento três vezes mais rápido do que o observado nos países desenvolvidos[3]. Em decorrência desse fenômeno, tem sido observado um aumento significativo da prevalência e da incidência de doenças neurodegenerativas tanto no Brasil[4], quanto nos países desenvolvidos: no Reino Unido cerca de 5% da população acima de 65 anos apresenta algum tipo de demência, sendo que a prevalência é crescente à medida que a idade aumenta, chegando a 20% nos idosos acima de 80 anos[5]; nos Estados Unidos da América (EUA), estima-se que 4,5 milhões de habitantes sofram da DA[6]; de acordo com estudos na população suíça, aproximadamente 10% dos idosos entre 85 e 88 anos que não apresentam um quadro característico de demência passam a desenvolver a doença a cada ano e no Japão, onde a expectativa de vida é maior que a de qualquer outro país (75,6 anos para os homens e 81,4 anos para as mulheres), a incidência da DA é proporcionalmente a mais alta do mundo[7].

As doenças neurodegenerativas são desordens progressivas que afetam determinadas populações neuronais do sistema nervoso central (SNC), levando à morte neuronal e à ruptura de circuitos neurais.

1.1 Ferro, Envelhecimento e Doenças Neurodegenerativas

Evidências clínicas e experimentais indicam a participação do ferro nos mecanismos que levam à morte celular nas patologias neurodegenerativas em que é observado o acúmulo desse metal. De fato, estudos demonstram a elevação da concentração de ferro na substância negra (SN) de portadores da DP[8,9,10,11,12]. Adicionalmente, depósitos de ferro também têm sido encontrados no SNC de

pacientes com as doenças de Alzheimer[13,14], de Huntington[15], de Hallervorden-Spatz[16], Ataxia de Friedreich[17], Esclerose Amiotrópica Lateral[18], bem como em idosos que apresentam características de envelhecimento benigno[19,20,21].

O ferro é um dos metais mais abundantes no corpo humano e o cérebro contém uma concentração substancialmente maior deste metal do que qualquer outro órgão. Entre as funções do ferro, destaca-se a participação na constituição estrutural de proteínas transportadoras de oxigênio, o envolvimento no processo de fosforilação oxidativa em nível mitocondrial e a regulação gênica. No tecido nervoso o ferro catalisa reações envolvidas no metabolismo energético, sendo essencial para processos relacionados à síntese, degradação e mecanismos de ação de vários neurotransmissores e neuromoduladores, entre os quais o ácido gama-aminobutírico (GABA), o glutamato, a dopamina, a noriepinefrina e as endorfinas[22].

O período neonatal é crítico para o estabelecimento do conteúdo de ferro no cérebro adulto. Investigações a respeito da captação de ferro pelo cérebro, indicam que o transporte de ferro ao cérebro atinge seus níveis máximos durante o período pós-natal de rápido crescimento cerebral (período durante o qual ocorrem processos cruciais para o estabelecimento das estruturas e funções encefálicas e aquisição de aspectos sensório-motores essenciais)[23,24]. Além disso, a distribuição cerebral de ferro altera-se durante os processos de desenvolvimento e envelhecimento[25]. Esse fato pode ter alguma relação com disfunções nas vias que asseguram a homeostasia desse metal o que, conseqüentemente, poderia estar promovendo a deposição de ferro nas regiões cerebrais onde seu metabolismo é mais alto. Desse modo, o ferro poderia estar participando dos eventos que levam à neurodegeneração[26,27,28,29,30]. Como o ferro catalisa a formação de radicais hidroxil, que são extremamente tóxicos, acredita-se que o estresse oxidativo esteja envolvido no processo de morte neuronal[31,32]. Também é importante considerarmos que durante o processo de envelhecimento não patológico, ocorre naturalmente um aumento nos níveis de ferro em regiões específicas do SNC[26,33].

Camundongos[34,35] e ratos[36] submetidos à sobrecarga de ferro durante o período neonatal apresentam, quando adultos, alterações no conteúdo encefálico deste metal, no comportamento motor, na memória espacial e de esquiva inibitória. Estes resultados tornam-se bastante importantes se considerarmos que no ser humano esta fase de rápido crescimento cerebral inicia-se no último trimestre de gravidez e estende-se ao longo do primeiro ano de vida[37], justamente quando as

crianças são expostas à aplicação indiscriminada de fórmulas lácteas suplementadas com ferro, as quais podem apresentar conteúdo 10 (Europa) a 100 (EUA) vezes maior deste metal do que o leite materno[38]. Portanto, enquanto no passado a ênfase havia sido dada ao combate à deficiência de ferro (anemia), a aplicação indiscriminada de suplementação de ferro a crianças durante seu primeiro ano de vida tornou importante estudar os mecanismos através dos quais o organismo pode se proteger contra o excesso desse metal[39].

Recentemente, foi verificado que ratos submetidos à sobrecarga de ferro do 12° ao 14° dia de vida pós-natal (período no qual o cérebro dos roedores atinge o maior nível de susceptibilidade à absorção de ferro) apresentam déficits de memória de reconhecimento quando adultos[40]. O mesmo estudo revelou, ainda, que a exposição ao ferro no período neonatal induz um aumento significativo na peroxidação lipídica e nos danos a proteínas (2 parâmetros indicativos de estresse oxidativo) no córtex cerebral, no hipocampo e na SN de ratos adultos. Adicionalmente, o estudo demonstrou que ocorre uma diminuição da atividade da superóxido dismutase (enzima anti-oxidante) no córtex cerebral, no hipocampo e na SN de ratos adultos. Esses resultados sugerem que o ferro possa estar exercendo seus efeitos deletérios sobre a cognição através do aumento dos níveis de estresse oxidativo cerebral em regiões que participam do processamento da memória.

Além disso, o mesmo grupo de pesquisadores constatou que a selegilina (um inibidor da MAO amplamente utilizado no tratamento da DP) é capaz de proteger contra (quando é administrada simultaneamente com o ferro) e reverter (quando é administrada somente na idade adulta) os déficits de memória de reconhecimento induzidos pela exposição à sobrecarga de ferro do 12° ao 14° dia de vida pós-natal[41].

Ainda, esses pesquisadores verificaram, em um outro estudo, que ratos velhos (21 a 23 meses de idade), quando comparados com ratos jovens (3 meses de idade), apresentam déficits de memória de reconhecimento. Quando os ratos velhos são submetidos a um tratamento com selegilina (o mesmo protocolo aplicado aos ratos que foram expostos à sobrecarga de ferro no período neonatal), eles apresentam uma reversão desses déficits de memória induzidos pelo envelhecimento[42].

Portanto, já foi bem estabelecido que o modelo animal, no qual os animais são expostos a uma sobrecarga de ferro no período de desenvolvimento no qual a

absorção de ferro atinge os níveis mais altos (dose compatível com a de fórmulas lácteas utilizadas na alimentação de bebês humanos), é um instrumento importante para o teste de drogas com possível ação neuroprotetora, uma vez que o modelo é capaz de mimetizar as perdas cognitivas observadas em ratos velhos, bem como é passível de ter essas perdas revertidas através dos mesmos tratamentos farmacológicos que as revertem em ratos velhos.

1.2 Memória

A memória, uma das mais importantes funções cognitivas do ser humano, pode ser entendida como a incrível habilidade que possuímos de armazenar informações e conhecimentos sobre nós mesmos e o mundo que nos cerca. Ela é a base para o desenvolvimento da linguagem, do reconhecimento das pessoas e dos objetos que encontramos todos os dias, para sabermos quem somos e para termos a consciência da continuidade de nossas vidas. Sem a memória, a cada dia, ou a cada momento, estaríamos começando uma nova vida, sem podermos nos valer do que aprendemos anteriormente[43].

Em relação ao conteúdo, as memórias podem ser classificadas como *declarativas* ou *procedurais*. As memórias *procedurais* são aquelas relacionadas às capacidades/habilidades motoras, ou sensoriais. As memórias que registram fatos, eventos, ou conhecimento são chamadas *declarativas*, porque nós, seres humanos, podemos relatar como as adquirimos. Entre elas - as referentes a eventos aos quais presenciamos, ou dos quais participamos - são denominadas *episódicas* e, ainda, as de conhecimentos gerais são denominadas *semânticas*[44].

Um dos exemplos mais profundamente estudados da memória declarativa é a memória de reconhecimento, que é a capacidade de julgar um item recentemente encontrado como familiar. A memória de reconhecimento em seres humanos consiste de dois componentes: um episódico, que diz respeito à habilidade de recordar do episódio (situação) no qual um objeto foi introduzido (objeto novo), e um componente familiar, que se relaciona com a habilidade de reconhecer um objeto como já conhecido (ou familiar), mas sem a necessidade da lembrança do próprio episódio[45].

A memória de reconhecimento pode ser testada em roedores usando tarefas de reconhecimento de objetos que são baseadas na tendência espontânea que os

roedores apresentam de explorar objetos novos quando os animais se lembram dos objetos aos quais eles foram previamente expostos. As vantagens desse tipo de teste incluem o fato de que eles não são baseados em reforços positivos (como a utilização de alimentos) ou negativos (como a utilização de choques elétricos)[46,47,48,49]. Além disso, esse tipo de tarefa revelou ser dependente tanto do hipocampo (uma região cerebral importante para o processamento da memória), quanto da via nigro-estriatal (uma região do SNC rica em ferro)[50,51,52,53]. Ainda, tem sido proposto que essa tarefa apresenta analogia com testes de memória de reconhecimento que são amplamente utilizados em seres humanos para caracterizar síndromes amnésicas, pois fornecem um índice acurado do grau de severidade geral de prejuízos da memória declarativa[54,55].

Muitas das patologias neurodegenerativas que se manifestam em idades avançadas envolvem déficits de memória. O estudo dos mecanismos envolvidos no processo de perda cognitiva, assim como de medidas preventivas e terapêuticas, torna-se muito importante, visto que, esses ainda não foram totalmente estabelecidos. Além disso, esse tipo de patologia gera uma profunda sobrecarga emocional, social e econômica, o que prejudica o estabelecimento de um envelhecimento bem sucedido entre a população de idosos.

1.3 Estratégias Terapêuticas para o Tratamento dos Déficits Cognitivos Associados ao Envelhecimento e às Doenças Neurodegenerativas

Atualmente, três classes de fármacos têm se destacado dentre as investigações clínicas e experimentais que buscam novas terapias para o tratamento dos déficits cognitivos associados ao envelhecimento e das doenças neurodegenerativas:

1. os quelantes de ferro;
2. fármacos que modulam a neurotransmissão dopaminérgica;
3. fármacos que modulam a atividade da via da adenosina monofosfato cíclico (AMPc).

A deferroxamina (DFO) é um quelante de metais que vem sendo utilizado no tratamento de patologias que envolvem o acúmulo de ferro no organismo, como a talassemia maior (doença hereditária que afeta a capacidade do organismo de produzir hemoglobina) e a aceruloplasminemia (doença congênita onde a proteína

ceruloplasmina, que normalmente liga-se ao ferro, está ausente no sangue)[56]. Após ter sido constatado que diversas doenças neurodegenerativas envolvem o acúmulo de ferro no SNC, sua utilização passou a ser investigada também como um possível agente terapêutico para esta classe de patologias, uma vez que ele seria capaz de diminuir a formação de radicais livres que, por sua vez, têm sido apontados como os principais responsáveis pela morte neuronal nessas patologias[57,58,59]. Entretanto, existem poucos estudos na literatura a respeito dos efeitos neurocomportamentais decorrentes do uso do DFO[60,61,62,63].

Já foi demonstrado que o sistema dopaminérgico, além de estar envolvido na modulação da atividade motora, também está envolvido no processamento de diferentes tipos de memória[64]. Tem sido sugerido que a dopamina exerceria seus efeitos sobre a memória através do aumento da manutenção da potenciação de longa duração (LTP), que é um dos principais mecanismos de plasticidade neural proposto para explicar como a memória é armazenada[65]. A dopamina possui uma família de diferentes tipos de receptores que, ao serem estimulados, podem induzir diferentes efeitos sobre a memória. Os receptores dopaminérgicos da família D_1, especialmente, estão expressos em regiões cerebrais envolvidas com o processamento da memória de reconhecimento[66,67,68,69]. De fato, a estimulação da neurotransmissão dopaminérgica através do SKF 38393 (que é um agonista seletivo de receptores dopaminérgicos da família D_1 demonstrou melhorar a memória em reconhecimento social em roedores[70]. Além disso, tem sido proposto que esse fármaco modula a memória de reconhecimento de objetos tanto para o caráter familiaridade quanto para o espacial[67].

Uma outra forma de se modular a neurotransmissão dopaminérgica é através da manipulação da expressão dos transportadores de dopamina (DATs). Os DATs são os responsáveis pela recaptação desse neurotransmissor na fenda sináptica, o que faz com que o estímulo desencadeado pela dopamina seja encerrado. A expressão dos DATs também revelou ser um fator importante na modulação da memória[71]. O aumento da estimulação dopaminérgica através do uso do GBR 12935 (que é um inibidor dos DATs) recentemente começou a ser investigado em estudos comportamentais em roedores[72,73,74].

Os neurônios dopaminérgicos da via nigro-estriatal são os mais susceptíveis ao declínio funcional e à morte neuronal que ocorrem durante os processos de envelhecimento patológico e não-patológico no cérebro humano. Tem sido proposto

que essa maior susceptibilidade estaria relacionada ao metabolismo da dopamina, o qual promove a formação de radicais livres citotóxicos. Adicionalmente, tem sido sugerido que os déficits cognitivos associados ao envelhecimento não patológico estejam relacionados, ao menos em parte, com a diminuição da neurotransmissão dopaminérgica. De fato, o conteúdo de dopamina estriatal (região cerebral que recebe aferências dos neurônios dopaminérgicos da SN) no cérebro humano decai rapidamente em uma taxa de aproximadamente 15% por década após os 45 anos. Na população saudável, acredita-se que a perda da dopamina estriatal seja de aproximadamente 40% em indivíduos com 75 anos[75]. No caso da DP (doença neurodegenerativa caracterizada principalmente pela morte de neurônios dopaminérgicos da SN *pars compacta*), a perda de neurônios ocorre de forma muito mais intensa. A manifestação dos sintomas motores da DP (tais como tremor de repouso e dificuldade em iniciar movimentos) só ocorre quando mais de 60% dos neurônios da SN já foram perdidos[76]. Apesar da DP ser considerada primariamente como uma desordem motora, a alta prevalência de complicações neuropsiquiátricas nos pacientes acometidos por essa doença sugere que os déficits de dopamina possam estar afetando outros processos neurais importantes para a modulação do aprendizado e da cognição[77]. A Figura 1 resume os principais eventos envolvidos na neurotransmissão dopaminérgica.

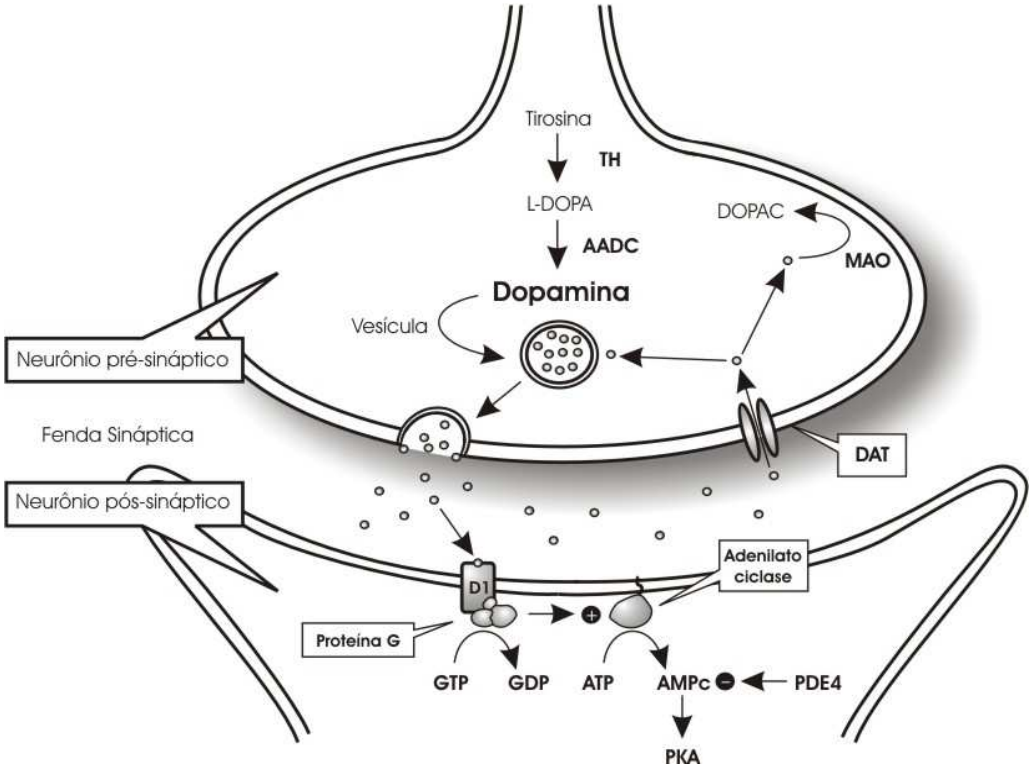

Figura 1. Neurotransmissão dopaminérgica. A dopamina é sintetizada a partir do aminoácido tirosina. A enzima tirosina hidroxilase (TH) converte a tirosina em dopa que é convertido em dopamina pela enzima descarboxilase de aminoácido aromático (AADC). A dopamina é armazenada em vesículas até que o neurônio pré-sináptico seja estimulado. Após terem sido liberadas na fenda sináptica, as moléculas de dopamina irão se ligar aos seus receptores na membrana do neurônio pós-sináptico. A ligação da dopamina com o seu receptor dopaminérgico do tipo D_1 ativa a proteína G, que, por sua vez, irá estimular a adenilato ciclase a converter ATP em AMPc. O AMPc ativa a proteína cinase dependente de AMPc (PKA). A enzima fosfodiesterase do tipo 4 (PDE4) inibe o AMPc descontinuando a cascata bioquímica. Os transportadores de dopamina (DAT) são responsáveis pela recaptação da dopamina pelo neurônio pré-sináptico. Uma vez que tenha retornado ao neurônio, a dopamina pode ser reutilizada, ou degradada pela enzima monoamino oxidase (MAO).

Cabe ressaltar que a ativação dos receptores dopaminérgicos das famílias D_1/D_5, além de modular diretamente a formação da memória (conforme foi descrito anteriormente), também demonstrou estimular a via do AMPc que, por sua vez, já revelou estar envolvida na consolidação da memória[78,79]. Em concordância, Bach e colaboradores (1999)[80] realizaram um estudo onde foi verificado que o aumento dos níveis de AMPc através do uso de agonistas de receptores dopaminérgicos das famílias D_1/D_5 e de inibidores da fosfodiesterase (discutido abaixo), foi capaz de reverter os déficits de memória espacial induzidos pelo envelhecimento em

camundongos da linhagem C57BL/B6 e que essa reversão está relacionada a um reforço na fase tardia da LTP[80].

Portanto, fármacos que aumentam a neurotransmissão dopaminérgica podem ser considerados uma alternativa, não só para o tratamento de patologias que envolvem prejuízos motores, como também para o tratamento do esquecimento senil benigno, bem como de patologias neurodegenerativas e psiquiátricas que envolvem prejuízos cognitivos.

O rolipram é um inibidor da fosfodiesterase do tipo 4 (PDE4) que é uma enzima que hidroliza os nucleotídeoas cíclicos do AMPc e da guanosina monofosfato cíclico (GMPc). A inibição da enzima PDE4 aumenta a disponibilidade intracelular do AMPc cerebral. Um dos efeitos do AMPc é estimular uma outra enzima chamada de proteína cinase dependente de AMPc (PKA). As proteínas cinases catalizam reações químicas de fosforilação, ou seja, a transferência de grupamentos fosfato (PO_3^{-2}) do trifosfato de adenosina (ATP) para determinados sítios em proteínas. A fosfolrilação, neste caso, modifica a conformação da proteína, consequentemente modificando a sua atividade. A proteína ligante de elemento responsivo ao AMPc (CREB) torna-se ativa quando é fosforilada pela PKA. Uma vez ativada, a CREB irá se ligar a regiões específicas do DNA, regulando, dessa forma, a transcrição gênica. Portanto, a ativação gênica induzida pela via do AMPc→PKA→CREB pode ser considerada como um fator chave para a consolidação da memória[78,79,81]. Um número substancial de estudos experimentais tem mostrado que a inibição da enzima PDE4, através do uso do rolipram, produz um efeito positivo sobre a memória espacial[82,83,84], sobre a memória de esquiva inibitória[85,86,87,88,89], sobre condicionamento contextual ao medo[82,84,90] e sobre a memória de reconhecimento[81,90,91,92,93]. Esses achados dão suporte à idéia de que o aumento dos níveis de AMPc (através da utilização de inibidores da PDE4) possa ser um possível alvo terapêutico para patologias neurodegenerativas e psiquiátricas que envolvem prejuízos cognitivos.

No presente estudo foi analisado o efeito neuroprotetor de três diferentes classes de fármacos: um quelante de ferro (desferroxamina), dois fármacos que modulam a neurotransmissão dopaminérgica (SKF 38393 e GBR 12935) e um fármaco que modula a atividade da via do AMPc (rolipram), sobre os déficits de memória de reconhecimento induzidos pelo tratamento neonatal com ferro e pelo envelhecimento (ver Quadro I).

2 OBJETIVOS

2.1 Objetivo Geral

Avaliar o efeito do tratamento com de três diferentes classes de fármacos: um quelante de ferro (DFO), fármacos que modulam a neurotransmissão dopaminérgica (SKF 38393 e GBR 12935) e que modulam a atividade da via do AMPc (rolipram) sobre os déficits de memória induzidos pelo tratamento neonatal com ferro e pelo envelhecimento.

2.2 Objetivos Específicos

Avaliar o efeito do tratamento crônico com DFO (30,0 e 300,0 mg/Kg ip) em ratos na fase adulta sobre os déficits de memória de reconhecimento induzidos pelo tratamento com ferro (10,0 mg/Kg via oral) do 12° ao 14° dia de vida pós-natal.

Avaliar o efeito do tratamento crônico com DFO (300,0 mg/Kg ip) em ratos com 24 meses de idade (fase de envelhecimento) sobre os déficits de memória de reconhecimento induzidos pelo envelhecimento, bem como sobre a medida de danos oxidativos a proteínas em estruturas cerebrais envolvidas no processamento da memória (córtex, hipocampo e estriado).

Avaliar o efeito do tratamento agudo com SKF 38393 (5,0 mg/Kg ip) e com GBR 12935 (5,0 e 10,0 mg/Kg ip) em ratos na fase adulta sobre os déficits de memória de reconhecimento induzidos pelo tratamento com ferro (10,0 mg/Kg via oral) do 12° ao 14° dia de vida pós-natal.

Avaliar o efeito do tratamento agudo com rolipram (0,01; 0,03 e 0,1 mg/Kg ip), em ratos na fase adulta sobre os déficits de memória de reconhecimento induzidos pelo tratamento com ferro (10,0 mg/Kg via oral) do 12° ao 14° dia de vida pós-natal.

Avaliar o efeito do tratamento agudo com rolipram (0,1 mg/Kg ip) em ratos com 24 meses de idade (fase de envelhecimento) sobre os déficits de memória de reconhecimento induzidos pelo envelhecimento.

Quadro I. Distribuição dos Experimentos em Relação aos Artigos que Compõem a Tese

Experimentos	**Procedimentos**	**Artigo**
I	Administração de ferro (10,0 mg/Kg via oral) no do 12° ao 14° dia de vida pós-natal e administração de 6 injeções de desferal (30,0 ou 300,0 mg/Kg ip) a ratos Wistar machos com 3 meses de idade (fase adulta). Realização da tarefa de reconhecimento do objeto novo 24 h após a administração da última injeção.	De Lima MN, Presti-Torres J, Caldana F, Grazziotin MM, Scalco FS, Guimarães MR, Bromberg E, Franke SI, Henriques JAP, Schröder N. Desferoxamine reverses neonatal iron-induced recognition memory impairment in rats. Eur J Pharmacol. 2007. (no prelo)
II	Administração de 6 injeções de desferal (300,0 mg/Kg ip) a ratos com 23 meses de idade (fase de envelhecimento). Realização da tarefa de reconhecimento do objeto novo 24 h após a administração da última injeção. Medida de danos oxidativos a proteínas em estruturas cerebrais envolvidas no processamento da memória (córtex, hipocampo e estriado).	De Lima MN, Dias CP, Presti-Torres J, Dornelles A, Garcia VA, Scalco FS, Guimarães MR, Petry RC, Bromberg E, Constantino L, Budni P, Dal-Pizzol F, Schröder N. Reversion of age-related recognition memory impairment by iron chelation in rats. Neurobiol Aging. 2007. (no prelo)
III	Administração de ferro (10,0 mg/Kg via oral) no do 12° ao 14° dia de vida pós-natal e administração de SKF 38393 (5,0 mg/Kg ip) ou GBR 12935 (5,0 ou 10,0 mg/Kg ip) a ratos Wistar machos com 6 meses de idade (fase adulta) imediatamente após a sessão de treino da tarefa de reconhecimento do objeto novo.	De Lima MN, Presti-Torres J, Garcia VA, Guimarães MR, Schröder N. Modulatory effects of dopaminergic stimulation on iron-induced recognition memory deficits. (a ser submetido)
IV	Administração de ferro (10,0 mg/Kg via oral) no do 12° ao 14° dia de vida pós-natal e administração de rolipram (0,01; 0,03 ou 0,1 mg/Kg ip) a ratos Wistar machos com 6 meses de idade (fase adulta) imediatamente após a sessão de treino da tarefa de reconhecimento do objeto novo.	De Lima MN, Presti-Torres J, Garcia VA, Guimarães MR, Roesler R, Schröder N. Amelioration of recognition memory impairment associated with iron loading and aging by the type 4-specific phosphodiesterase inhibitor rolipram. Neuroscience. 2007. (submetido)
V	Administração de rolipram (0,1 mg/Kg ip) a ratos Wistar machos com 24 meses de idade (fase de envelhecimento) imediatamente após a sessão de treino da tarefa de reconhecimento do objeto novo.	

ARTIGO I

DESFEROXAMINE REVERSES NEONATAL IRON-INDUCED RECOGNITION MEMORY IMPAIRMENT IN RATS

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Desferoxamine reverses neonatal iron-induced recognition memory impairment in rats.

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Abstract

We have previously demonstrated that rats given iron neonatally presented memory deficits. The aim of the present study was to evaluate the effect of desferoxamine, a metal chelating agent, on memory deficits in an iron overload model in rats. Male rats received vehicle or iron orally at postnatal days 12-14 and desferoxamine (30 or 300 mg/kg) in the adulthood. After desferoxamine treatment, they were trained in a novel-object recognition task. Iron-treated rats showed recognition memory impairments when compared to controls. Iron-treated rats that received desferoxamine 300 mg/kg, showed normal recognition memory, suggesting that desferoxamine can reverse recognition memory deficits associated with iron accumulation. Further research is required to examine whether the findings from animal models of iron overload have implications for humans.

Key-words: iron, recognition memory, desferoxamine.

1 Introduction

Increasing evidence has indicated that excessive iron in selective regions of the brain may generate cytotoxic free radical formation, thereby possessing implications for the etiology of neurodegenerative disorders (Zecca et al., 2004; Thomas and Jankovic, 2004). Increased levels of iron have been reported in normal brain aging of rats (Benkovic and Connor, 1993; Focht et al., 1997) and human subjects (Bartzokis et al., 2007), as well as in several neurodegenerative disorders, such as Parkinson's (Dexter et al., 1994; Griffiths et al., 1999), Alzheimer's (Ong and Farooqui, 2005; Quintana et al., 2006) and Huntington's (Bartzokis et al., 1999; Bartzokis and Tishler, 2000) diseases. Despite years of investigation, it is still not known why iron levels are abnormally high in some regions of the brain in neurodegenerative disorders. Also, it is not clear whether iron accumulation in the brain is an initial event that causes neuronal death or is a consequence of the disease process.

The use of animal models have greatly increased our understanding of the iron regulatory mechanisms and the pathogenesis of neurodegenerative disorders related to iron deposition in the brain (Anderson and Powell, 2000; Grabill et al., 2003; Zhang et al., 2005). In previous reports we have demonstrated that iron supplementation in the neonatal period induces selective iron accumulation in brain regions, specifically in the basal ganglia, which was associated with long-term memory deficits in adult mice (Fredriksson et al., 1999, 2000) and rats (Schröder et al., 2001; de Lima, 2005a; 2005b).

Therefore, it would be of great interest the assessment of iron chelation in later stages of life as a possible therapeutic strategy on functional deficits induced by elevated neonatal dietary iron feeding. The present study was performed in order to evaluate the effect of the iron-chelator desferoxamine on iron-induced recognition memory deficits.

2 Materials and methods

Pregnant Wistar rats were obtained from Fundação Estadual de Pesquisa e Produção em Saúde, Porto Alegre, RS, Brazil. After birth, each litter was adjusted within 48h to contain eight rat pups. Each pup was maintained together with its

respective mother in a plastic cage in a room at 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 to 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 Care and Use of Laboratory Animals (NIH publication No. 80-23 revised 1996) and approved by the Ethics Committee of the Pontifical Catholic University (CEP-996/04). The neonatal iron treatment has been described in detail elsewhere (Schröder et al., 2001; de Lima et al., 2005a; 2005b). Briefly, 12-day-old rat pups received orally a single daily dose (10 ml/kg solution volume) of vehicle (5% sorbitol in water) (control group) or 10 mg/kg of body weight of Fe^{2+} (Ferromyn®, AB Hässle, Göteborg, Sweden) via a metallic gastric tube, over 3 days (post-natal days 12-14). In this model, iron is given orally during the period of maximal iron uptake by the brain, so that the model correlates with dietary iron supplementation to infants. Both groups were further divided into three experimental groups receiving intraperitoneal (i.p.) injections of saline (NaCl 0.9%) or desferoxamine mesylate (Desferal, Novartis, SP, Brazil) at the doses of 30 or 300 mg/kg in a 1.0 ml/kg injection volume dissolved in saline. Desferoxamine injections were given three times per week for 2 weeks starting when the animals reached the age of 2 months. The dose of desferoxamine was chosen on the basis of previous studies (Lan and Jiang, 1997; Freret et al, 2006) and pilot experiments performed in our laboratory.

Animals were trained in a novel object recognition task 24 h after the last administration of desferoxamine. On the first day, rats were submitted to a habituation session to the training arena (an open field (45 x 40 x 60 cm) made of plywood with a frontal glass wall), during which they were placed in the empty arena for 5 min. On the following day, rats were given one 5-min training trial in which they were exposed to two identical objects (A1 and A2). The objects were positioned in two adjacent corners, 9 cm from the walls. On the short-term memory testing trial (90 min after the training session), rats were allowed to explore the open field for 5 minutes in the presence of two objects: the familiar object A and a novel object B. These were placed in the same locations as in the training session. On the long-term memory testing trial (24 h after the training session), the same groups of rats were allowed to explore the open field for 5 minutes in the presence of two objects: the familiar object A and a third novel object C. All objects presented similar textures,

colors, and sizes, but distinctive shapes. Object exploration was measured using two stopwatches to record the time spent exploring the objects during the experimental sessions. Exploration was defined as follows: sniffing or touching the object with the nose. A recognition index calculated for each animal was expressed by the ratio $T_B/(T_A+T_B)$ [T_A= time spent exploring the object A; T_B= time spent exploring the object B], as previously described (Schröder et al., 2003; de Lima et al., 2005a; 2005b; de Lima et al., 2006). Comparisons among groups were performed with a Kruskal-Wallis analysis of variance followed by Mann-Whitney U tests. P values of less than 0.05 were considered to indicate statistical significance. Statistical comparison of total time exploring both objects during training and testing trials was made using one way analysis of variance (ANOVA) (Schröder et al., 2003; de Lima et al., 2005a; 2005b; de Lima et al., 2006).

3 Results

Statistical comparison of recognition indexes showed that there were no significant differences among groups in the training trial or in the short-term memory retention trial, 90 min after training session (Fig. 1).

In the long-term memory retention trial, performed 24 h after training session, statistical comparison of recognition indexes showed that groups treated with iron neonatally and that received saline or desferoxamine in the dose of 30 mg/kg in the adulthood showed severe impairments in recognition memory (Fig. 1), as their recognition indexes were significantly lower than the control group.

Iron-treated rats that received desferoxamine at the dose of 300 mg/kg showed normal recognition memory, as their recognition indexes did not differ significantly from the control group, and were significantly different from the iron plus saline group, indicating that iron chelation in the adulthood was able to reverse neonatal iron-induced recognition memory deficits (Fig. 1).

Desferoxamine treatment by itself did not affect the performance of animals in the novel object recognition task, since recognition indexes of the groups treated with vehicle plus desferoxamine at both doses did not differ statistically of those treated with vehicle plus saline.

Table 1 shows that there were no significant differences in the total time exploring both objects between experimental groups compared to the control group

(vehicle plus saline) during training and retention test trials, thus indicating that iron and/or desferoxamine effects on memory are not related to general sensorimotor parameters such as locomotion, motivation, and exploratory activity.

4 Discussion

The present results show that iron chelation therapy in the adulthood was able to reverse the cognitive impairment induced by neonatal iron loading in rats. Although the molecular mechanisms involved in the deleterious effects of iron on cognition still need further investigation, a recent study performed in our laboratory have indicated that desferoxamine was able to reverse age-induced recognition memory impairments and to reduce protein oxidative damage in aged rats (de Lima et al., 2007). A possible relation between iron effects and oxidative stress has been previously proposed, since our studies have also demonstrated that our iron-neonatal treatment induces lipid peroxidation and protein carbonylation in hippocampus, cortex and substantia nigra (Dal-Pizzol et al., 2001). Recently, it was shown that iron load in the early stages of life induces recognition memory impairment possibly by inducing oxidative damage in the brain (de Lima et al., 2005b). It has been proposed that iron accumulation in the brain mediates oxidative damage, and neuronal death associated with neurodegenerative disorders (Jenner, 2003; Ke and Qian, 2003; Barnham et al., 2004; Zecca et al., 2004). It was found that in neurological diseases such as Alzheimer's disease, Parkinson's disease, dementia with Lewy bodies, and Huntington's disease iron accumulation occurs in brain regions more susceptible to neuronal degeneration. The reason why iron accumulates in the brain is still a matter of controversy. Some authors have raised the hypothesis that both genetic and non-genetic factors may be involved (Quintana et al., 2006; Bartzokis et al., 2007). Although it is known that iron uptake by the brain is higher during the development of the nervous system, there is a continuous iron uptake resulting in iron accumulation during the aging process (Connor et al., 1990; Connor et al., 1995). Thus, it is possible that dietary iron may represent a modifiable risk factor for age-associated neurodegenerative disorders.

As a consequence of the understanding that iron accumulation may be a common feature of age-associated neurodegenerative disorders, the therapeutic role for chelating agents is promising. Desferoxamine is a metal chelator agent with

antioxidants properties. Previous studies have shown that in rats that were submitted to a controlled traumatic brain injury, desferoxamine pretreatment improved spatial memory (Long et al., 1996). It was also demonstrated that intracerebroventricular pretreatment with desferoxamine prevented the fall in striatal and frontal cortex dopamine, dihydroxyphenylacetic acid, and homovalinic acid, as well as striatal tyrosine hydroxylase activity and dopamine turnover resulting from 6-hydroxydopamine (6-OHDA) lesion of dopaminergic neurons (Ben-Shachar et al., 1991). Desferoxamine also protected against 6-OHDA-induced deficit in locomotor activity, and exploratory behavior (Youdim et al., 2004). A few human studies have indicated that iron chelators may slow the clinical progression of the dementia associated with Alzheimer's disease (Crapper et al., 1991; Regland et al., 2001). However, it is important to note that brain iron requirements might differ in rodents and humans due to their differential time courses of maturation and myelination, which require iron (Roskams and Connor, 1994; Bartzokis et al., 2001). Also, iron deficiency in both rats and humans has been associated with cognitive and neurophysiological deficits (reviewed in Lozoff and Georgieff, 2006; McCann and Ames, 2007). Thus, further research is required to examine whether the findings from animal studies suggesting that iron chelation produces beneficial effects on cognitive function can be extrapolated to humans.

In conclusion, the present results extend our previous findings which implicate brain iron accumulation in cognitive decline observed in normal aging and possibly in neurodegenerative disorders. Moreover, it supports the view that iron chelation therapy could be considered as a target for the development of new strategies of treatment of functional deficits associated with neurodegenerative disorders that involve iron accumulation.

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Table 1. Total amount of time spent exploring both objects during object recognition training and retention test trials in rats treated with iron in the neonatal period and desferoxamine in the adulthood.

Group	N	training trial	90-min test trial	24-h test trial
Veh-Sal	10	30,12 ± 3,39	46,86 ± 9,09	38,15 ± 4,13
Veh-desferoxamine 30	9	20,79 ± 3,01	33,94 ± 6,84	36,44 ± 2,66
Veh-desferoxamine 300	9	35,52 ± 5,56	30,42 ± 3,49	22,28 ± 4,11
Fe-Sal	12	31,87 ± 3,69	38,60 ± 3,81	35,64 ± 5,13
Fe-desferoxamine 30	9	28,41 ± 4,14	32,13 ± 4,20	27,79 ± 4,25
Fe-desferoxamine 300	9	38,91 ± 4,53	30,57 ± 4,28	26,24 ± 3,09
Overall	58	30,97 ± 1,76	35,78 ± 2,34	31,45 ± 1,80

Data expressed as mean ± S.E.M.

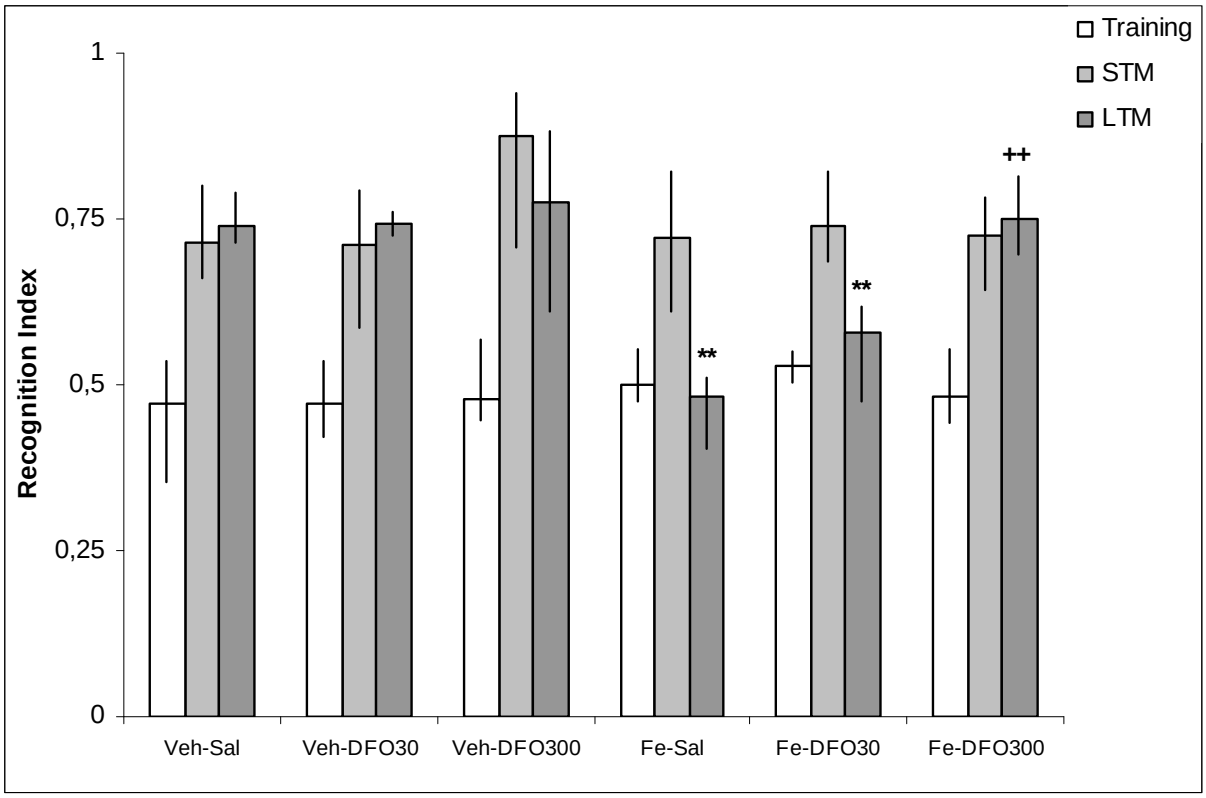

Figure 1. Effect of desferoxamine (DFO) on iron-induced recognition memory deficits. Behavioral procedure was carried out when animals were 3 months old. They were trained in a novel object recognition task. Testing trials were conducted 90 min (short-term memory, STM) or 24 h (long-term memory, LTM) after the training session. Data are expressed as median [interquartile ranges] "Recognition Index" which is defined by the ratio $T_B/(T_A+T_B)$, T_A = time spent exploring the familiar object and T_B = time spent exploring the novel object. *N* = 9-12 per group. Statistical comparisons between vehicle-saline and other experimental groups are indicated (** $P < 0.01$). Statistical comparisons between iron-saline and other experimental groups are indicated ($^{++}$ $P < 0.01$).

ARTIGO II

REVERSION OF AGE-RELATED RECOGNITION MEMORY IMPAIRMENT BY IRON CHELATION IN RATS

Original Article

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Reversion of age-related recognition memory impairment by iron chelation in rats.

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Abstract

It is now generally accepted that iron accumulates in the brain during the ageing process. Increasing evidence demonstrate that iron accumulation in selective regions of the brain may generate free radicals, thereby possessing implications for the etiology of neurodegenerative disorders. In a previous study we have reported that aged rats present recognition memory deficits. The aim of the present study was to evaluate the effect of desferoxamine (DFO), an iron chelator agent, on age-induced memory impairment. Aged Wistar rats received intraperitoneal injections of saline or DFO (300 mg/kg) for two weeks. The animals were submitted to a novel object recognition task 24 h after the last injection. DFO-treated rats showed normal recognition memory while the saline group showed long-term recognition memory deficits. The results show that DFO is able to reverse age-induced recognition memory deficits. We also demonstrated that DFO reduced the oxidative damage to proteins in cortex and hippocampus. Thus, the present findings provide the first evidence that iron chelators might prevent age-related memory dysfunction.

Key-words: aging, recognition memory, desferoxamine, neuroprotection, iron, oxidative stress, protein carbonyl, rat.

1 Introduction

It is now generally accepted that iron accumulates in the brain during the ageing process [50,59,71]. In humans, it is known that concentrations of non-haem iron increase in the putamen, motor cortex, prefrontal cortex, sensory cortex and thalamus during the first 30-35 years of life [29,42]. Recent studies have shown that levels of ferritin, the major iron storage protein, in older individuals were higher than in younger controls in the frontal cortex, caudate nucleus, putamen substantia nigra and globus pallidus [10,70]. A study comparing cellular and regional distribution of ferritin and iron between young and aged rats has indicated that in the normal aging brain there is an intracellular accumulation of iron in neurons [5].

Excessive iron content in selective regions of the brain may generate cytotoxic free radical formation, thereby possessing implications for the etiology of neurodegenerative disorders [52,64]. Increased levels of iron have been reported in several neurodegenerative disorders, such as Parkinson's (PD) [17,18,20,28,32,57], Alzheimer's (AD) [8,39,48,51,53] and Huntington's (HD) [3,4] diseases. Despite years of investigation, it is still not known why iron levels are abnormally high in some regions of the brain in neurodegenerative disorders. Also, it is not clear whether iron accumulation in the brain is an initial event that causes neuronal death or is a consequence of the disease process.

A recent study involving human subjects was the first to correlate iron content, as measured by quantitative magnetic resonance (MR) imaging, and cognitive impairments in elderly participants. Accordingly, R2 an MR imaging parameter affected by changes in brain iron concentration and water content, was different in elderly participants with mild to severe levels of cognitive impairment compared with healthy controls [30], suggesting that iron misregulation might play a role in the decline in cognitive function observed in aged individuals.

The use of animal models has greatly increased our understanding of the iron regulatory mechanisms and the pathogenesis of neurodegenerative disorders related to iron deposition in the brain [2,27,72]. In previous reports we have demonstrated that iron supplementation in the neonatal period induces a selective iron accumulation in brain regions, especially in the basal ganglia, which was associated with memory impairments in adult mice [23,24] and rats [60]. In addition, iron supplementation in this period induces lipid peroxidation and protein carbonylation in

substantia nigra [12]. Moreover, it was shown that iron load in the early stages of life induces recognition memory impairment possibly by inducing oxidative damage in the brain [14].

Desferoxamine (DFO) is a metal chelator agent with antioxidants properties. Recently, with the observation that several neurodegenerative diseases involve iron accumulation in the central nervous system, DFO and other metal chelating agents became also investigated as a possible therapeutic agent for this class of pathologies [11,22].

However, there is little information in the literature about the possible cognitive effects of iron chelation therapy in normal aged subjects or in patients with age-related neurodegenerative disorders. Thus, the purpose of the present study was to evaluate the effect of DFO on age-related recognition memory deficits. In order to do that, we submitted aged male Wistar rats (24 months old) treated subchronically with DFO to a novel object recognition task. Additionally, parameters of oxidative stress in cerebral regions related to memory formation were evaluated.

Recognition memory can be tested in rodents using object recognition tasks that are based on spontaneous activity and the natural preference that rats display to explore a novel object more than a familiar one when the animal remembers previous exposure to familiar object. Advantages associated with this class of measure include the fact that performance does not depend on the retention of a rule, and is not based on usual positive or negative reinforcers, such as food deprivation or application of an electric shock [7,19,21,46]. Moreover, these tasks might depend both on the hippocampus and the nigrostriatal dopaminergic pathway [13,44,45,61,66], brain regions that are severely affected in neurodegenerative disorders in which iron is overloaded.

2 Methods

2.1 Animals

Male Wistar rats were obtained from the State Foundation for Health Science Research (FEPPS-RS, Porto Alegre, Brazil). Animals were kept 3 to a cage on a 12-h light/dark cycle with food and water available *ad libitum*. All behavioral experiments took place between 9:00 and 17:00. 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 Behavior (SBNeC) recommendations for animal care and were approved by the Ethics Committee of the Pontifical Catholic University (CEP-996/04).

2.2 Drugs and pharmacological procedures

Aged animals (23 months-old) received intraperitoneal (ip) injections of saline (NaCl 0.9%) or desferoxamine mesylate (Desferal, Novartis, SP, Brazil), 300 mg/kg in a 1.0 ml/kg injection volume dissolved in saline three times per week for 2 weeks. The dose of DFO was chosen on the basis of previous studies [25,34] and pilot experiments performed in our laboratory. Dinitrophenylhydrazine and trichloroacetic acid were purchased from Sigma, St. Louis, MO, USA.

2.3 Novel object recognition memory

Twenty-four hours after open field exploration (see below), animals were trained and tested in a novel object recognition task as previously described [13-16,61]. Training in the object recognition task took place in the same arena used for the open field, except that the arena floor was covered with sawdust during the recognition memory task training and test trials. The open field exploration was thus used as a context habituation trial for the recognition memory task. The object recognition test required that the rats recalled which of two plastic objects they had been previously familiarized with. Twenty-four hours after arena exploration, training was conducted by placing individual rats into the field, in which two identical objects (objects A_1 and A_2; Duplo Lego toys) were positioned in two adjacent corners, 9 cm from the walls. Animals were left to explore the objects until they had accumulated 30 s of total object exploration time or for a maximum of 20 min. One rat in each group reached the 20-min ceiling and was excluded from the experiment. In a short-term memory (STM) test given 1.5 h after training, the rats explored the open field for 5 minutes in the presence of one familiar (A) and one novel (B) object. All objects presented similar textures, colors, and sizes, but distinctive shapes. A recognition index calculated for each animal was expressed by the ratio $T_B/(T_A+T_B)$. [T_A = time spent exploring the familiar object A; T_B = time spent exploring the novel object B].

Between trials the objects were washed with 10% ethanol solution. In a long-term memory (LTM) test given 24 h after training, the same rats explored the field for 5 minutes in the presence of familiar object A and a novel object C. Recognition memory was evaluated as for the short-term memory test. Exploration was defined as sniffing or touching the object with the nose and/or forepaws. Sitting on the object was not considered exploration.

2.4 Open field behavior

In order to control for possible sensorimotor effects induced by DFO, behavior during exploration of an open field was evaluated 24 h after the last injection. The open field was a 40 X 45 cm arena surrounded by 50 cm high walls, made of plywood with a frontal glass wall. The floor of the arena was divided into 12 equal squares by black lines. Animals were placed in the rear left corner and left to explore the field freely for 5 min. Latency to start locomotion, line crossings, rearings and the number of fecal pellets produced were counted [16].

2.5 Oxidative stress analysis

After completion of behavioral procedures animals were killed by decapitation and the brain regions (cortex, hippocampus and striatum) from seven rats randomly selected from each group were isolated and stored at -80^{0}C for posterior analyses. All the results were normalized by the protein content [38]. The oxidative damage to proteins was assessed by the determination of carbonyl groups based on the reaction with dinitrophenylhydrazine (DNPH) as previously described [35]. Briefly, proteins were precipitated by the addition of 20% trichloracetic acid and redissolved in DNPH and the absorbance read at 370 nm.

2.6 Statistical analysis

Behavioral data were analyzed as previously described [13-16,61]. Data for recognition indexes are expressed as median (interquartile ranges). Comparisons between groups were performed using Mann-Whitney U tests. Comparison of time (in seconds) spent exploring the familiar and novel objects in retention test trials within

each individual group was performed by Wilcoxon test. Data from the experiment evaluating open field behavior were analyzed by independent samples t-test. Biochemical data are expressed as mean ± S.D. Data were analyzed by independent samples t-test. In all comparisons, *P* values less than 0.05 were considered to indicate statistical significance.

3 Results

3.1 Effects of DFO on age-related impairment of object recognition memory

Fig. 1 shows the effect of DFO on object recognition memory in aged rats. There was no significant difference between groups in the training trial (median [interquartile ranges] percentages of time exploring object A2 were 53.72 [44.96/58.83] in the saline-treated group and 47.09 [39.68/52.03] in the group treated with DFO, $P = 0.102$). DFO-treated animals showed a significantly higher STM retention than animals given saline ($P < 0.05$), as their recognition index was higher than the saline group. Saline-treated aged rats showed no preference towards the novel object in the LTM retention test, as Wilcoxon test has indicated no significant difference between the time spent exploring the familiar and the novel object (median [interquartile ranges] time in seconds exploring object A was 5.01[3.16/11.34] and object C was 7.60 [4.24/12.65] during LTM test, $P = 0{,}441$). However, rats treated with DFO showed a significant preference in exploring the novel object during the LTM retention trial (median [interquartile ranges] time in seconds exploring object A was 5.51[3.14/8.35] and object C was 13.37 [12.34/18.32] during LTM test, $P = 0{,}008$). There was no significant difference between groups in the total time exploring both objects during LTM retention test (Mann-Whitney U test, $P = 0.222$). Moreover, as shown in Fig. 1, statistical comparison of LTM recognition indexes has indicated a significant difference ($P < 0.05$) between the groups. These results indicate that iron chelation reversed the age-related impairment in object recognition memory.

3.2 Open field behavior in aged rats treated with DFO

Results for open field behavior in aged rats treated with saline or DFO are shown in Fig. 2. DFO did not affect the number of crossings ($P = 0.818$) or rearings

(P = 0.879) performed, latency to start locomotion (P = 0.224), or defecation (P = 0.365). These results indicate that the DFO-induced improvement of performance in the recognition memory task could not be attributed to alterations in sensorimotor functions such as locomotion, exploratory behavior, motivation, or anxiety.

3.3 Oxidative stress analyses in aged rats treated with DFO

Figure 4 shows the effect of DFO on the level of protein carbolylation, which was used as an index of protein damage. DFO significantly reduced protein carbonyl content in the hippocampus (P = 0.009) and cortex (P = 0.004) of aged rats (Fig. 3 A and B) without affecting striatal levels of protein carbonylation (P = 0.538; Fig. 3C).

4 Discussion

The present study has investigated the possibility that iron chelation therapy could attenuate age-related memory deficits. In a previous report we had demonstrated that ageing induces long-term object recognition memory impairments in rats [16]. The data shown here demonstrate for the first time that iron chelation was able to reverse age-induced memory deficits. In addition, DFO treatment induced an enhancement of short-term memory retention and decreased oxidative damage to proteins in specific brain areas that are involved in memory formation, such as the cortex and the hippocampus. Thus, the present findings give support to the hypothesis that, at least in part, age-associated cognitive deficits might be related to oxidative damage promoted by misregulation in iron metabolism.

It is known that DFO is a potent iron chelator, and several studies were performed in order to elucidate the mechanisms by which DFO can exert its potential beneficial effects. It was demonstrated that intracerebraventricular (icv) pretreatment with DFO prevented the reduction in striatal and frontal cortex dopamine (DA), dihydroxyphenylacetic acid (DOPAC), and homovalinic acid (HVA), as well as the striatal tyrosine hydroxylase (TH) activity and DA turnover resulting from 6-hydroxydopamine (6-OHDA) lesion of dopaminergic neurons [6]. DFO also protected against 6-OHDA-induced deficit in locomotor activity, rearing, and exploratory behavior in a novel environment [69]. It was also demonstrated that iron plays a key role in the nigral damage induced by the inhibition of proteasome and that reducing

iron reactivity by DFO may prevent dopaminergic degeneration and reduce abnormal protein aggregation [73]. DFO ability to revert cognitive impairments induced by traumatic brain injury was studied in rats. It was shown that DFO improved spatial memory performance in the Morris water maze [37].

Despite the low permeability of DFO into the blood-brain barrier (BBB), a study has shown that intraperitoneal injections of DFO for two weeks was able to decrease iron content in brain regions and influence dopamine metabolism in ferrrocene loaded rats [68]. More recent studies in which this drug was administrated peripherally have demonstrated that DFO is able to reduce free iron levels in cerebrospinal fluid after intracerebral hemorrhage [67], to decrease striatal free radical formation induced by methamphetamine [49], and to decrease the size of brain damage and to improve behavioral recovery after ischemia [25]. Although in the present study we have not quantified iron content in rats' brains, the use of similar dose and regimen of administration together with the fact that protein carbonyl levels in brain regions have been reduced in DFO treated rats, strongly suggests that the drug was able to cross the BBB and reach brain regions where iron is inducing oxidative damage to proteins. DFO penetration into the brain in our study maybe due either to the ability of DFO to cross BBB itself or to age-associated changes in the BBB [43,63]. In the search for compounds with neuroprotective properties, novel chelators with greater central nervous system availability have been studied in animal models of neurodegenerative disorders [31,56,62,69] and also in AD patients [55,58].

In the present study DFO-treated rats were submitted to the novel object recognition task. This task has been increasingly used in recent years as a model for the investigation of the neurobiological mechanisms of learning and memory. Whereas most studies investigating learning and memory in rodents use spatial and/or emotionally motivated behavioral tasks, the object recognition task provides a tool for assessing non-spatial, non-aversive memory sensitive to genetic and pharmacological manipulations as well as aging process [7,16,54,61].

Analysis of open field behavior following DFO treatment has demonstrated that the dose of DFO used (300 mg/kg) in the present study has not affected general activity, thus not affecting rats' ability to explore objects. In another study using the same dose of DFO, Freret et al. [25] found no evidence of toxicity in the chronically DFO-treated rats, such as a decrease in weight, or any neurological or visual

impairment. In agreement with that, we found no significant difference in body weights between the groups at the last day of DFO treatment (data not shown).

The molecular mechanisms underlying the reversion of recognition memory deficits by iron chelation are unknown. However, the present results suggest that, at least in part, the mechanism might be related to the inhibition of iron-induced oxidative damage in the brain. In the present study we found that DFO was able to reduce protein carbonylation in the cerebral cortex and in the hippocampus without affecting striatal levels of protein carbonyls. Conversely, we have previously shown that, in rats given iron overload, the content of protein carbonyls was not altered in the striatum, whereas it was increased in other brain regions [12]. Thus, the present result is consistent with previously observed differential effects of iron on oxidative stress in the striatum, hippocampus and cortex.

The imbalance between pro-oxidants and antioxidants resulting in oxidative stress associated with ageing has been extensively demonstrated [for a review see 59]. Over the years a number of studies have consistently reported the oxidative protein damage in brain regions, especially the hippocampus of aged rats [1,9,47]. Evidence indicates that agents that reduce oxidative stress might also enhance memory performance in aged rats [65]. It has been proposed that oxidized protein accumulate, contributing to the aging process [26]. Increasing evidence has suggested that oxidative stress is implicated in age-related cognitive decline, and antioxidants have been used to assess the role of oxidative damage in memory senescence [41]. Accordingly, a recent report has indicated that protein carbonyl levels are increased in aged Wistar rats and that antioxidant treatment attenuates cognitive deficits in senescent-accelerated OXYS rats [33]. Supplementation with N-acetylcysteine has also proved to delay age-associated memory impairment in mice [40]. Mice receiving chronic systemic administration of two synthetic catalytic scavengers of reactive oxygen species, from 8 to 11 months presented an almost complete reversion of age-associated cognitive deficits and increase in oxidative stress in the brain [36].

The present study supports the view that cognitive deficits associated with ageing might be related to iron accumulation in the brain, and provides the first evidence that iron chelators might prevent age-related memory dysfunction.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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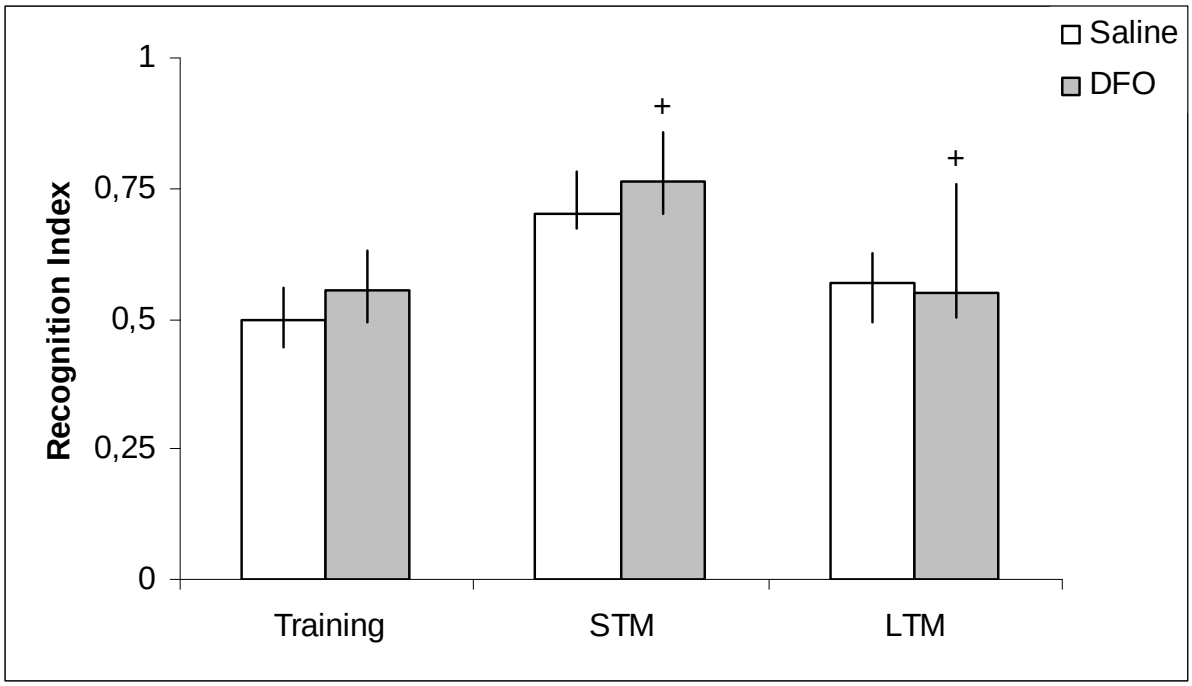

Figure 1. Effect of DFO on age-induced recognition memory deficits. Short-term retention (STM) was tested 1.5 h after training and long-term retention (LTM) 24 h after training. Behavioral testing was carried out when animals were 24 months old. The proportion of the total exploration time that the animal spent investigating the novel object was the "Recognition Index" expressed by the ratio $T_B/(T_A+T_B)$, T_A = time spent exploring the familiar object and T_B = time spent exploring the novel object. Data expressed as median [interquartile ranges], *N* = 9 animals per group. Differences between vehicle- and DFO-treated groups are indicated: + $P < 0.05$.

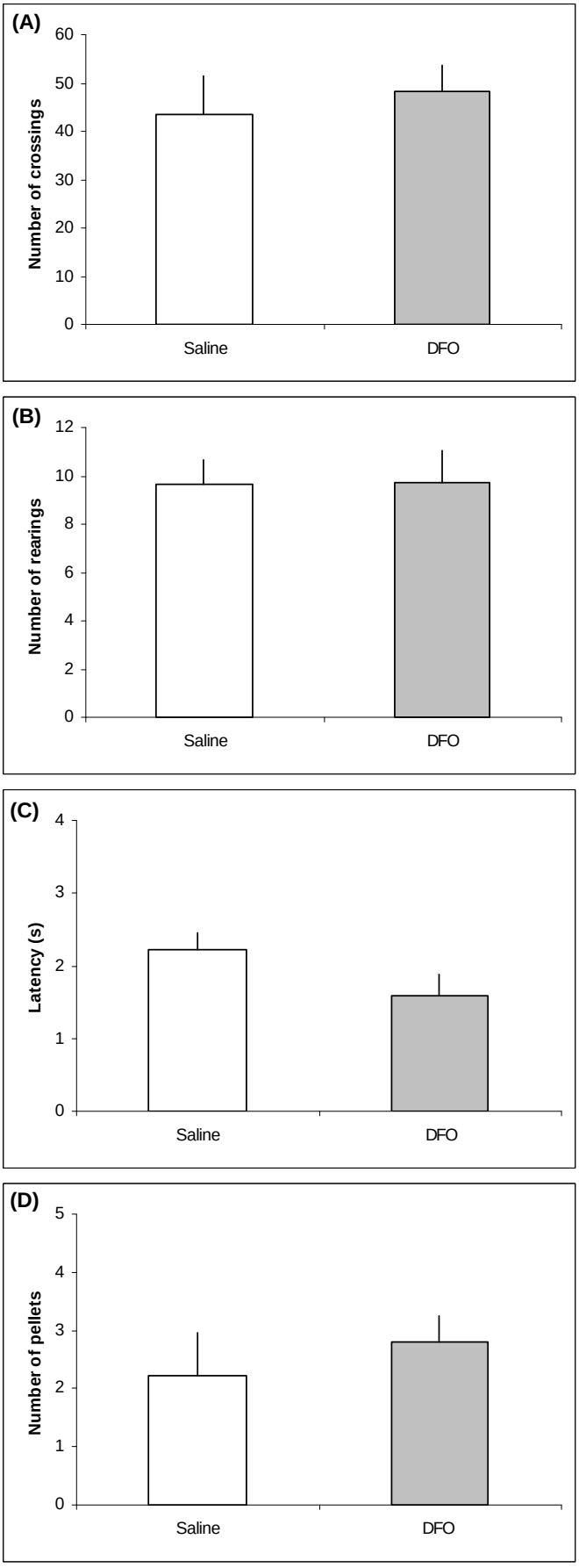
(A)
Number of crossings
60
50
40
30
20
10
0
Saline
DFO
(B)
Number of rearings
12
10
8
6
4
2
0
Saline
DFO
(C)
Latency (s)
4
3
2
1
0
Saline
DFO
(D)
Number of pellets
5
4
3
2
1
0
Saline
DFO

Figure 2. Open field behavior in aged rats treated with systemic injections of saline (NaCl 0.9%) or DFO (300 mg/kg) for 2 weeks. Animals were left to explore the arena for 5 min 24 h after the last injection. Data are mean ± S.E. number of crossings **(A)**, number of rearings **(B)**, latency to start locomotion (s) **(C)** and number of fecal pellets **(D)**. *N* = 9 animals per group. There were no significant differences between groups.

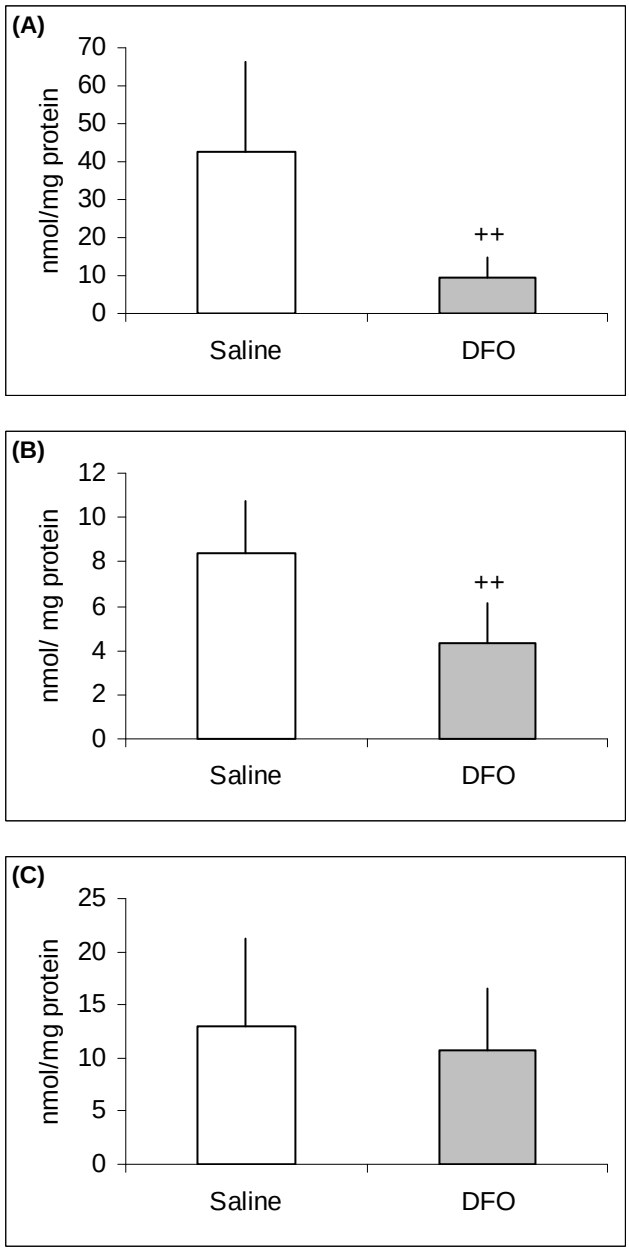

Figure 3. Protein carbonyl content in brain regions of aged rats following DFO treatment. Protein carbonyl content was measured in the hippocampus **(A)**, cortex **(B)**, and striatum **(C)** of 7 rats from each group, as described in Material and Methods section. Values are expressed as means ± S.D. Differences between vehicle- and DFO-treated groups are indicated: ++ $P < 0.01$.

ARTIGO III

MODULATORY EFFECTS OF DOPAMINERGIC STIMULATION ON IRON-INDUCED RECOGNITION MEMORY DEFICITS

Original Article

Modulatory effects of dopaminergic stimulation on iron-induced recognition memory deficits.

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Abstract

Excess of iron in the brain has been implicated in the pathogenesis of several human neurodegenerative diseases, for example Alzheimer's (AD) and Parkinson's (PD) disease. It has been shown that the neonatal period is critical for the establishment of normal iron content in the adult brain and it is also known that aging alters the cerebral distribution of this metal. We have previously described that neonatal administration of iron severely impaired recognition memory in adult rats. Thus, the aim of the present study was to determine if dopaminergic stimulation by SKF 38393 (a dopamine D_1 receptor agonist) and GBR 12935 (a dopamine reuptake inhibitor) could revert iron-induced recognition memory deficits. In order to do that, male Wistar rats received vehicle (5% sorbitol in water) or iron (10.0 mg/Kg) orally from postnatal days 12 to 14. These animals were submitted to a novel object recognition memory task when they reached the age of 6 months. Vehicle- and iron-treated animals received an intraperitoneal injection of vehicle (1% DMSO in saline solution 0.9% NaCl) or SKF 38393 (5.0 mg/Kg) or GBR 12935 (5.0 or 10.0 mg/Kg) immediately after training in the novel object recognition task. Object recognition task consisted of a 5-min training trial, when they explored two copies of the same object. In retention test trials, one of the objects was replaced by a novel object. Results have indicated that SKF 38393 (5.0 mg/Kg) and GBR 12935 (10.0 mg/Kg) attenuated iron-induced recognition memory deficits.

Key-words: iron, neonatal, SKF 38393, GBR 12935, recognition memory, rats, dopamine.

1 Introduction

The involvement of iron in several brain metabolic processes and normal development of neurological systems during a critical perinatal period, wherein deficiencies in this metal are associated with disruptions in behavioral performance, has been indicated (Youdim and Yehuda, 2000; Youdim et al., 1991; Ben-Shachar et al., 1986). However, there is also accumulating evidence that excessive iron in selective regions of the brain may generate cytotoxic free radical formation and cause alterations in iron metabolism, thereby possessing implications for the etiology of neurologic disorders (Zecca et al., 2004; Thomas and Jankovic, 2004; Kaur and Andersen 2004; Sengstock at al., 1993). Increased levels of iron in selective brain regions have been reported in several neurodegenerative disorders, such as Parkinson's (PD), Huntington's (HD), Hallervorden-Spatz and Alzheimer's (AD) diseases, amyotrophic lateral sclerosis (ALS) as well as in normal brain aging (Jellinger, 1999). In some experimental models of PD where degeneration of nigrostriatal dopaminergic neurons has been observed, there is evidence for iron-induced oxidative stress as a pathogenic factor (Berg and Youdim, 2006; Youdim et al., 2004; Leret et al., 2002). However, these models of PD are generally focused on the motor alterations associated with this disorder.

The effects of iron administration during the neonatal period on cognition have been well documented. Adult mice (Fredriksson et al., 1999; 2000) and rats (Schröder et al., 2001) that received iron during a critical period of development, which corresponds to the period of maximal uptake of iron by the brain, showed spatial memory deficits when tested in the radial arm maze. In addition, this treatment has proven to disrupt performance in the inhibitory avoidance task, a type of aversively motivated conditioning in rats (Schröder et al., 2001). We have found that iron neonatal treatment impairs long-term recognition memory in adult rats and induces oxidative damage in brain regions implicated in memory formation, thus raising the possibility that iron-induced cognitive deficits are at least partially mediated by oxidative stress (De Lima et al., 2005a).

Additionally, we have demonstrated that selegiline, a monoaminoxidase B (MAO-B) inhibitor, that have also been proposed to enhance the release of dopamine, and to block dopamine uptake (Ebadi et al., 2006), protected against iron-induced recognition memory deficits (De Lima et al., 2005b).

Dopamine (DA) is a key neurotransmitter that plays an important role modulating not only motor activity but also normal cognitive process and neuropsychiatric pathologies such as schizophrenia. Many aspects of learning function such as reward, attention and fear have been shown to be influenced by the dopaminergic system (for a review see Seamans and Yang, 2004). More recently, it has become evident that distinct DA receptors in different brain areas are involved on recognition memory processing (Hotte et al., 2005; Belcher et al., 2005; Ventura et al., 2004; Wooley et al., 2003; Besheer et al., 1999). The selective and high efficacy D_1 receptor agonist, SKF 82958, facilitated social recognition in a model of social recognition memory (recognition of a juvenile by an adult rat) (Di Cara et al., 2006). In addition, it has been proposed that the DA D_1 receptor agonist SKF 81297 modulates recognition memory for familiarity and place of objects as well as temporal memory for objects (Hotte et al., 2005). The dopamine transporter (DAT) reuptake inhibitor, GBR 12935, also revealed to modulate object recognition memory in an animal model of schizophrenia (Castagne et al., 2004).

Thus, the purpose of the present study was to investigate if dopaminergic stimulation could revert iron-induced recognition memory deficits. In order to do that, rats were treated with iron or vehicle in the neonatal period, and at the age of 6 months they where divided in groups that received SKF 38393 (a DA D_1 receptor agonist) or GBR 12935 (a DAT inhibitor) immediately after object recognition training.

2 Material and methods

2.1 Animals

Pregnant Wistar rats were obtained from Fundação Estadual de Pesquisa e Produção em Saúde (FEEPS-RS), Porto Alegre, RS, Brazil. After birth, each litter was adjusted within 48 h to eight rat pups and to contain offspring of both genders in about equal proportions. Each pup was kept together with its mother in a plastic cage with sawdust bedding in a room temperature of 22 ± 1°C and a 12:00/12:00h light/dark cycle. At the age of 4 weeks, pups were weaned and males were selected and raised in groups of three to five rats. For postnatal treatments, animals were supplied with standardized pellet food and tap water *ad libitum*. Behavioral testing started when animals reached the age of 6 months. 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 Behavior (SBNeC) recommendations for animal care. The protocol for this research was approved by the Institutional Ethics Committee of the Pontifical Catholic University (CEP-996/04).

2.2 Treatments

The neonatal iron treatment has been described in detail elsewhere (Schröder et al., 2001; De Lima et al., 2005a; 2005b). Briefly, 12-day-old rat pups received orally a single daily dose of vehicle (5% sorbitol in water) (control group) or 10.0 mg/Kg of body weight of Fe^{2+} (Ferromyn®, AB Hässle, Göteborg, Sweeden; iron concentration in the solution was 1.0 mg/ml) via a metallic gastric tube, over 3 days (postnatal days 12-14). In this model, iron is given orally during the period of maximal iron uptake by the brain, so that the model correlates with dietary iron supplementation to infants. We previously characterized that this treatment protocol induces a selective accumulation of iron in the rat basal ganglia (Schröder et al., 2001). SKF 38393 (5.0 mg/kg) and 1-[2-(Diphenylmethoxy)ethyl]-4-(3-phenylpropyl)-piperazine dihydrochloride (GBR 12935) (5.0 or 10.0 mg/Kg) were administered intraperitonealy immediately after the training trial in the object recognition task. SKF 38393 and GBR 12935 were dissolved in 1% dimethyl sulfoxide (DMSO) in saline solution (0.9% NaCl) in a 1.0 ml/Kg injection volume and were purchased from Sigma-Aldrich (Saint Louis, MO, USA). The doses of SKF 38393 and GBR 12935, as well as the method of injection, were chosen on the basis of previous studies (Van Galeen et al., 2006; Gagnaire and Micillino, 2006; Castagne et al., 2004; SKF 38393: Dubrovina, 2006; Armentero et al., 2002; Cestari and Castellano, 1997).

2.3 Object recognition

An open field apparatus (45 x 40 x 60 cm) with sawdust covering its floor was used in the novel object recognition task. On the first day, rats were submitted to a habituation session during which they were placed in the empty open field for 5 min. On the following day, rats were given one 5-min training trial in which they were exposed to two identical objects (A1 and A2). All objects were made of plastic Duplo

Lego Toys and had a height of about 10 cm. Objects presented similar textures, colors and sizes, but distinctive shapes. The objects were positioned in two adjacent corners, 9 cm from the walls. Between trials, the objects were washed with a 10% ethanol solution. On the long-term memory (LTM) testing trial (24 hours after the training session), rats were allowed to explore the open field for 5 minutes in the presence of two objects: the familiar object A and a novel object B. These were placed in the same locations as in the training session. In retention test trials, the novel object was placed in 50% trials in the right side and 50% in the left side of the open field. Object exploration was measured by one experimenter blind to group treatment assignments, using two stopwatches to record the time spent exploring the objects during the experimental sessions. Exploration was defined as follows: sniffing or touching the object with the nose. Sitting on the object was not considered as exploration. A recognition index calculated for each animal was expressed by the ratio $T_B/(T_A+T_B)$ [T_A= time spent exploring the familiar object; T_B= time spent exploring the novel object], as previously described (De Lima et al., 2005a; 2005b; Schröder et al., 2003).

2.4 Statistical analysis

Comparisons among groups were performed with a Kruskal-Wallis analysis of variance followed by Mann-Whitney U tests when necessary. Comparisons between sessions within the same group were performed with a Wilcoxon test. *P* values of less than 0.05 were considered to indicate statistical significance.

3 Results

Statistical comparison of recognition indexes showed that groups treated neonatally with iron and receiving vehicle in adulthood showed impaired long-term retention of recognition memory, revealed by comparisons between groups treated with iron followed by vehicle and the respective control group given vehicle followed by vehicle. Comparisons in recognition indexes between training and the long-term memory (LTM) trial within each group indicated that animals given iron showed no significant difference between training and retention test performances, suggesting

that these animals had a complete memory blockade reveled by the lack of preference towards the novel object in the long-term retention test trial (Figs 1 and 2).

Figure 1 shows that SKF 38393 was able to improve recognition memory in control rats and partially revert iron-induced memory impairment. Figure 2 shows that iron-treated rats that received GBR 12935 (10.0 mg/Kg) showed normal long-term recognition memory. These findings indicate that dopaminergic stimulation could revert iron-induced recognition memory deficits. There were no significant differences between vehicle-vehicle and iron-vehicle groups in the total time exploring both objects during acquisition (training session), indicating that the treatment with iron did not affect sensorimotor parameters such as locomotion, motivation and anxiety (overall mean ± S.E. time exploring both objects during the training trial was 37,064 ± 1,2491).

4 Discussion

In the present study, iron-treated rats presented long-term recognition memory deficits consistent with those seen in previous studies performed in our laboratory (De Lima et al., 2005a; 2005b). Since we have already described that selegiline (a MAO-B inhibitor) was able to revert iron-induced recognition memory deficits (De Lima et al., 2005b), in this study we investigated if dopaminergic stimulation via direct DA receptor agonism and DAT inhibition could also revert these deficits. We found that iron-treated rats given SKF 38393 and GBR 12935 showed no deficits in recognition memory. These results are in agreement with the view that stimulation of the dopaminergic system is able to reverse iron-induced recognition memory deficits.

Previous reports have described an important role for DA receptor activation in synaptic plasticity and memory processing, and the effects of DA receptor agonists and antagonists in different rodent models of learning and memory have been extensively characterized (Ponnusami et al., 2005; Fujishiro et al., 2005; Seamans and Yang, 2004; Sajikumar and Frey, 2004; Wall et al., 2003; Passetti et al., 2003; Umegaki et al., 2001; Chen et al., 1995; Huang et al., 1995). The DAT is also a target for the development of pharmacotherapies for a number of central disorders including AD, PD, schizophrenia, Tourette's syndrome, Lesch-Nyhan disease, attention deficit hyperactivity disorder, obesity, depression, and stimulant abuse as well as normal

aging (Kliethermes and Crabbe, 2006; Van Gaalen et al., 2006; Swant and Wagner, 2006; Runyon and Carroll, 2006; Desai et al., 2005; Erixon-Lindroth et al., 2005; Castagne et al., 2004).

Previous reports have indicated that injection of the selective DA uptake blocker GBR 12935 (15 mg/kg i.p.) increases DA, as well as NE and, to a lesser extent, 5-HT in the ventral tegmental area and nucleus accumbens (Reith et al, 1997), and produces a significant enhancement in LTP of Schaffer collateral synapses in the CA1 region of rat hippocampus (Swant and Wagner, 2006). GBR 12935 elevated locomotion in C57BL/6J mice at the maximally active dose of 10 mg/kg (Tolliver and Carney, 1994) and rats (Zhu et al., 2004).

The precise mechanisms underlying the effects of iron on cognition remains to be clarified. It is well known that iron content in the brain overlaps with the distribution of dopaminergic neurons (Roskams and Connor, 1994). Accordingly, we have previously reported that our iron neonatal treatment induces a selective increase in iron levels in the *substantia nigra* and the basal ganglia in rats (Schröder et al., 2001) and mice (Fredriksson et al., 1999). It has been proposed that iron accumulation in these areas can induce damage by interacting with hydrogen peroxide originated from dopamine metabolism. Moreover, it has been hypothesized that this mechanism could be involved in cell death during normal aging as well as in neurodegenerative disorders such as PD (Youdim et al., 2005; Floyd and Hensley, 2002).

It is generally accepted that iron accumulates in the brain as a function of age (Martin et al., 1998; Zecca et al., 2004). Besides, it has been found that neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA), used to investigate the mechanism of dopaminergic neurodegeneration, induce an increase in iron concentration in the *substantia nigra* of rats, mice and monkeys (Oestreicher et al., 1994; Temlett et al., 1994), thus implicating iron accumulation in the degenerative process.

The object recognition task has been increasingly used in recent years as a model for the investigation of the neurobiological mechanisms of learning and memory. Whereas most studies investigating learning and memory in rodents use spatial and/or emotionally motivated behavioral tasks, the object recognition task provides a tool for assessing non-spatial, non-aversive memory sensive to genetic and pharmacological manipulations as well as aging process (Rampon et al., 2000; Schröder et al., 2003; De Lima et al., 2005b; 2005c; 2007).

In summary, young adult rats treated with iron for 3 days during the neonatal period show consistent and reproducible neurofunctional deficits. The reversion of iron-induced memory deficits can provide insights on the elucidation of the mechanisms underlying the effects of iron. Most importantly, the neonatal iron administration model can be considered an important tool in identifying drugs with neuroprotective or cognitive enhancing properties since it can be related to cognitive decline associated with either normal aging or neurodegenerative disorders involving brain iron accumulation.

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Abbreviations

AD, Alzheimer´s disease; ALS, amyotrophic lateral sclerosis; DA, dopamine; DAT, dopamine transporter; DMSO, dimethyl sulfoxide; FEPPS-RS, State Foundation for Health Science Research; HD, Huntington’s disease; ip, intraperitoneal; LTM, long-term memory; MAO, monoaminoxidase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 6-OHDA, 6-hydroxydopamine; PD, Parkinson´s disease; SBNeC, Brazilian Society for Neuroscience and Behavior.

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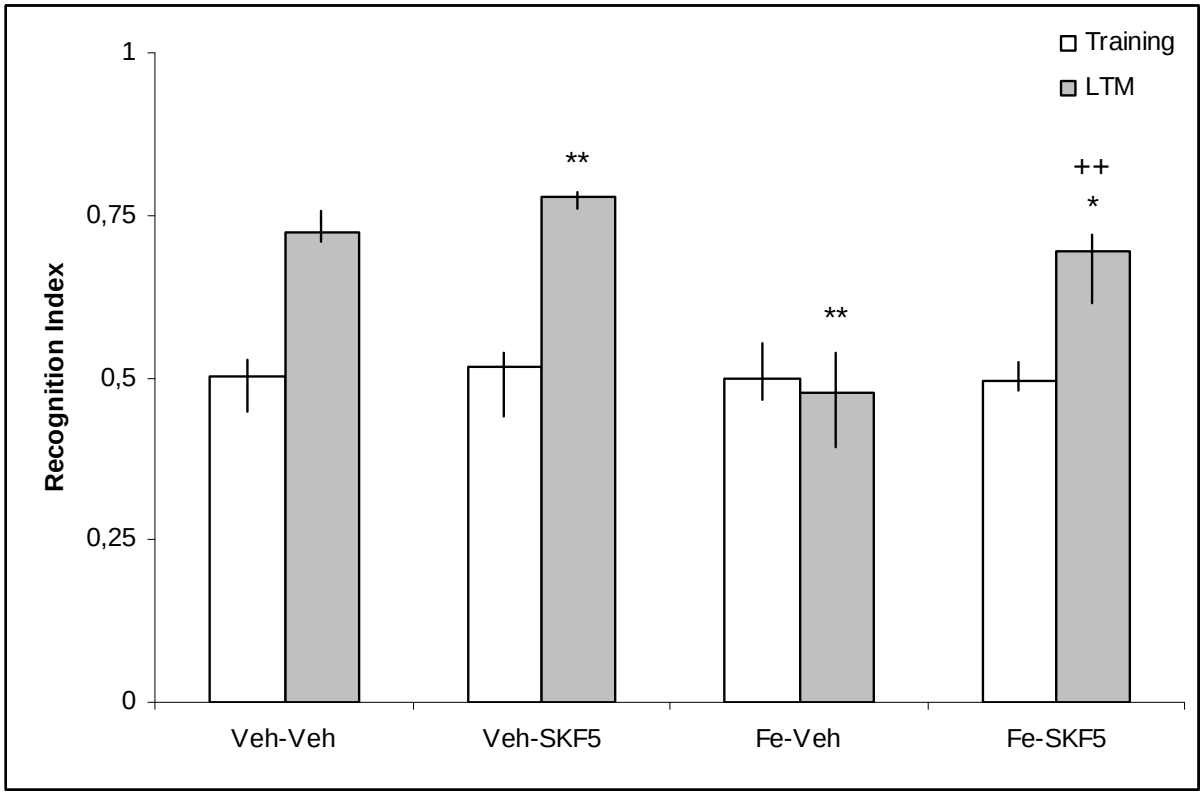

Figure 1. Effects of dopaminergic activation via SKF 38393 (a dopamine D_1 receptor agonist) on iron-induced recognition memory deficits. The long-term (LTM) retention test was performed 24 h after training. Behavioral testing was carried out when animals were 6 months old. The proportion of the total exploration time that the animal spent investigating the novel object was the "Recognition Index" expressed by the ratio $T_B/(T_A+T_B)$ [T_A = time spent exploring the familiar object; T_B = time spent exploring the novel object]. Data expressed as median [interquartile ranges], *N* = 9-11 per group. Differences between vehicle-vehicle *vs* other groups are indicated as: * $P < 0.05$ and ** $P < 0.01$ (Mann-Whitney U test); and between iron-vehicle *vs* other groups are indicated as: $^{++}P < 0.01$ (Mann-Whitney U test).

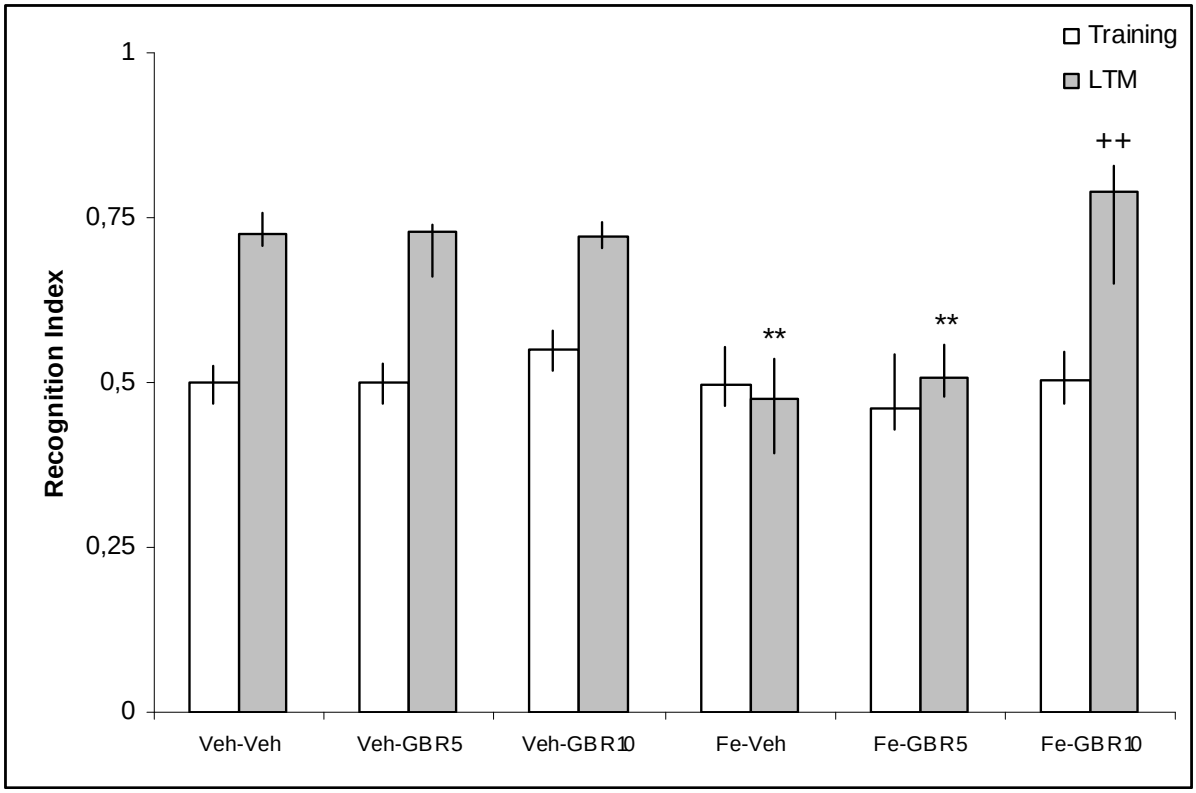

Figure 2. Effects of dopaminergic activation via GBR 12935 (a dopamine reuptake inhibitor) on iron-induced recognition memory deficits. The long-term (LTM) retention test was performed 24 h after training. Behavioral testing was carried out when animals were 6 months old. The proportion of the total exploration time that the animal spent investigating the novel object was the "Recognition Index" expressed by the ratio $T_N/(T_F+T_N)$ [T_F = time spent exploring the familiar object; T_N = time spent exploring the novel object]. Data expressed as median [interquartile ranges], *N* = 9-11 per group. Differences between vehicle-vehicle *vs* other groups are indicated as: ** $P < 0.01$ (Mann-Whitney U test); and between iron-vehicle *vs* other groups are indicated as: $^{++}P < 0.01$ (Mann-Whitney U test).

ARTIGO IV

AMELIORATION OF RECOGNITION MEMORY IMPAIRMENT ASSOCIATED WITH IRON LOADING AND AGING BY THE TYPE 4-SPECIFIC PHOSPHODIESTERASE INHIBITOR ROLIPRAM

Rapid Report

(Neuropharmacology Section)

Neuroscience

Amelioration of recognition memory impairment associated with iron loading or aging by the type 4-specific phosphodiesterase inhibitor rolipram.

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Abstract

Increasing evidence indicates that iron deposition in the brain might play a role in cognitive dysfunction associated with neurodegenerative disorders and aging. Previous studies have not examined whether iron-induced memory deficits can be attenuated by acute treatments with memory-enhancing agents. Phosphodiesterase type 4 (PDE4) inhibitors such as rolipram (ROL) ameliorate memory impairments in several rodent models of amnesia and have been proposed as candidate cognitive-enhancing drugs. Here we show that a single posttraining systemic injection of ROL dose-dependently attenuates the impairment of memory for novel object recognition (NOR) in rats given neonatal iron loading, a model of brain iron accumulation. Posttraining administration of ROL also enhanced NOR retention in aged rats. These findings provide the first evidence that stimulation of an intracellular second messenger signaling pathway can attenuate iron-induced memory impairment, and support the view that PDE4 inhibitors might ameliorate cognitive dysfunction associated with aging and neurodegenerative disorders.

Key-words: iron, rolipram, phosphodiesterase type 4 inhibitors, aging, memory dysfunction, memory consolidation.

A growing body of evidence indicates that iron deposition in the brain might play a critical role in the pathogenesis of neurodegenerative disorders such as Alzheimer's disease (AD), as well as in cognitive deficits associated with normal aging (for recent reviews, see Sipe et al., 2002; Casadesus et al., 2004; Sadrzadeh and Saffari, 2004; Berg and Youdim, 2006). Our group was the first to develop an animal model that enabled us to demonstrate that deposition of iron in the brain produces cognitive dysfunction (Fredriksson et al., 1999; 2000; Schröder et al., 2001; de Lima et al., 2005b; 2005c). The increasingly accepted view that iron might mediate deficits in brain function associated with neurodegenerative disorders, together with our previous findings that iron loading can induce memory impairments, suggest that the search for therapies capable of reversing iron-induced memory deficits is warranted. We have recently shown that iron-induced memory deficits in rats can be reversed by administration of the monoamine oxidase (MAO) inhibitor selegiline (de Lima et al., 2005b). However, previous studies have not examined whether memory dysfunction selectively associated with brain iron accumulation could be reversed by a single administration of agents targeted to signaling mechanisms underlying synaptic plasticity and memory formation.

The cAMP/protein kinase A/cAMP regulatory element-binding protein (cAMP/PKA/CREB) signaling pathway is crucially involved in synaptic plasticity and memory consolidation (Abel et al., 1997; Bevilaqua et al., 1997; Bach et al., 1999; Schafe et al., 1999; Quevedo et al., 2004), and drugs that increase cAMP levels are proposed as potential cognitive enhancers for the treatment of patients with memory dysfunction (for a recent review, see Arnsten et al., 2005). Agents that enhance cAMP signaling include inhibitors of the phosphodiesterase type 4 (PDE4) isoform, an enzyme that catalyzes hydrolysis of cAMP. Rolipram (ROL), a specific PDE4 inhibitor, has been shown to enhance both hippocampal long-term potentiation (LTP) and memory in mice (Barad et al., 1998). In addition, ROL reverses the inhibition of the cAMP/PKA/CREB pathway and LTP induced by β-amyloid peptide in rat hippocampal slices (Vitolo et al., 2002) and ameliorates deficits in LTP and memory in aged mice as wel as in several pharmacological and genetic rodent models of amnesia (Imanishi et al., 1997; Bach et al., 1999; Zhang et al., 2000; Alarcon et al., 2004; Gong et al., 2004; Zhang et al., 2004; Rutten et al., 2006).

Based on these findings, we asked whether administration of ROL could ameliorate memory deficits in rats given neonatal iron loading, a model of cognitive

dysfunction associated with brain iron accumulation developed by our group (Schröder et al., 2001; de Lima et al., 2005b; 2005c). Because we have observed that some of the cognitive and neurochemical alterations induced by iron parallel those associated with aging (de Lima et al., 2005a; 2007; Dias et al., 2007), and an iron chelating agent reverses memory deficits in aged rats (de Lima et al., 2007), we also examined the effects of ROL on memory retention in aged rats.

EXPERIMENTAL PROCEDURES

Subjects

Subjects were male Wistar rats (State Foundation for Health Science Research-FEPPS-RS, Porto Alegre, RS, Brazil). For **Experiment 1**, rats were given a neonatal iron treatment as described in previous reports (Schröder et al., 2001; de Lima et al., 2005b; 2005c). Thus, pregnant Wistar rats were obtained from FEEPS-RS and after birth, each litter was adjusted within 48 h to eight rat pups and to contain offspring of both genders in about equal proportions. Each pup was kept together with its mother in a plastic cage with sawdust bedding in a room temperature of 22 ± 1°C and a 12:00/12:00h light/dark cycle. At the age of 4 weeks, pups were weaned and males were selected and raised in groups of three to five rats. Behavioral testing started when animals reached the age of 6 months.

For **Experiment 2**, aged (23 months-old) weighing 480-600 g were used (de Lima et al., 2005a; 2007). All animals were maintained in groups of three to five in a plastic cage with sawdust bedding in a room at temperature of 22 ± 1°C and a 12h light/dark cycle and were supplied with standardized pellet food and tap water *ad libitum*. All behavioral experiments took place between 9:00 and 17:00. All experimental procedures were performed in accordance with the NIH Guide for Care and Use of Laboratory Animals (NIH publication No. 80-23 revised 1996) and approved by the Ethics Committee of the Pontifical Catholic University (CEP-996/04).

Neonatal iron treatment

In **Experiment 1**, rats were given a neonatal iron load as described in previous reports (Schröder et al., 2001; de Lima et al., 2005b; 2005c). Briefly, 12-day-old rat

pups received orally a single daily dose (10.0 mg/kg solution volume) of vehicle (VEH, 5% sorbitol in water) or 10.0 mg/kg of Fe^{2+} (Ferromyn®, AB Hässle, Göteborg, Sweeden; iron concentration in the solution was 1.0 mg/ml) via a metallic gastric tube, over 3 days [postnatal (PN) days 12-14]. In this model, iron is given orally during the period of maximal iron uptake by the brain, so that the model correlates with dietary iron supplementation to infants. We have previously demonstrated that this treatment protocol induces a selective accumulation of iron in the rat basal ganglia (Schröder et al., 2001).

Rolipram treatment

Immediately after behavioral training, adult rats treated orally with iron or its VEH in the neonatal period (**Experiment 1**) were given a single intraperitoneal (i.p.) injection of vehicle [VEH, 1% dimethyl sulfoxide (DMSO) in saline (0.9% NaCl)] or ROL (0.01, 0.03 or 0.1 mg/kg) dissolved in VEH. In **Experiment 2**, aged rats were given a posttraining i.p. injection of VEH or ROL at 0.1 mg/kg. The doses of ROL were chosen on the basis of previous studies (Imanishi et al., 1997; Zhang et al., 2000; 2004). Drug solutions were prepared freshly before each experiment.

Novel object recognition

The novel objet recognition (NOR) procedure uses the natural preference for novel objects displayed by rats and mice to assess cognitive alterations associated with aging, genetic manipulations, or drug treatments. We have previously shown that both neonatal iron load and aging alter NOR memory in rats (de Lima et al., 2005a; 2005b; 2005c; 2007). The NOR task was carried out in an open field apparatus (45 x 40 x 60 cm) made of plywood with sawdust covering its floor. On the first day, all animals were submitted to a habituation session during which they were placed in the empty open field and left to freely explore the arena for 5 min. In **Experiment 1**, NOR training was conducted by giving the rats a 5-min training trial in which they were exposed to two identical objects (A1 and A2). All objects were made of plastic Duplo Lego Toys and had a height of about 10 cm. Objects presented similar textures, colors and sizes, but distinctive shapes. The objects were positioned in two adjacent corners, 9 cm from the walls. Between trials, the objects were

washed with a 10% ethanol solution. On a memory retention testing trial carried out 24 hours after training, rats were allowed to explore the open field for 5 minutes in the presence of two objects: the familiar object A and a novel object B. These were placed in the same locations as in the training trial. In retention test trials, the novel object was placed in 50% trials in the right side and 50% in the left side of the open field. Object exploration was measured by one experimenter blind to group treatment assignments, using two stopwatches to record the time spent exploring the objects during training and test trials. These training and test procedures have been previously shown as adequate to examine NOR deficits induced by neonatal iron administration (de Lima et al., 2005b; 2005c).

Because aged rats might show alterations in locomotion that could affect object exploration during NOR training, in **Experiment 2** a different training procedure was used in which animals were left to explore the objects until they had accumulated 30 s of total object exploration time or for a maximum of 20 min as described in previous studies (de Lima et al., 2005a; 2007; Dias et al., 2007). Retention testing was carried out 24 h after training as in **Experiment 1**. For both **Experiments 1** and **2**, exploration was defined as sniffing or touching the object with the nose. Sitting on the object was not considered as exploration. A recognition index calculated for each animal was expressed by the ratio $T_B/(T_A+T_B)$ [T_A= time spent exploring the familiar object; T_B= time spent exploring the novel object], as previously described (de Lima et al., 2005a; 2005b; 2005c, 2006; 2007; Dias et al., 2007).

Statistical analysis

Data for NOR retention are expressed as median (interquartile ranges). Comparisons between groups were performed using a Kruskal-Wallis analysis of variance followed by Mann-Whitney U tests, two-tailed when necessary (de Lima et al., 2005a; 2005b; 2005c, 2006; 2007; Dias et al., 2007).

RESULTS

Experiment 1

All groups showed comparable levels of exploration during the training trial. Overall median (interquartile ranges) total time exploring both objects during the training trial was 34.1 (28.9/44.6). Results for exploratory preferences are shown in Fig. 1. Kruskal-Wallis tests showed a significant difference among groups in exploratory preferences in the 24-h retention test trial (df = 7, H = 38.03, $P < 0.001$), but not in the training trial (df = 7, H = 4.37, $P = 0.74$). Further analyses with Mann-Whitney U tests showed that neonatal iron administration induced a significant deficit in NOR retention ($P < 0.01$, comparison between the group treated with VEH plus VEH and the group treated with iron plus VEH). Posttraining systemic administration of ROL alone did not affect retention, but attenuated the iron-induced retention deficit in a dose-dependent manner. Iron-treated rats given ROL at 0.03 or 0.1 mg/kg showed enhanced NOR retention compared to rats given iron and VEH (Mann-Whitney U tests, $P < 0.05$ and $P < 0.01$ respectively), whereas ROL at 0.01 mg/kg did not rescue the iron-induced retention impairment ($P = 0.85$, comparison between the group given iron and VEH and the group given iron and ROL at 0.01 mg/kg). The results indicate that posttraining systemic administration of ROL dose-dependently attenuated the NOR retention impairment associated with neonatal iron loading.

Experiment 2

Results for exploratory preferences in aged rats treated with VEH or ROL at 0.1 mg/kg are shown in Fig. 2. Mann-Whitney U tests showed a significant difference between groups in exploratory preferences in the 24-h retention test trial ($P < 0.01$), but not in the training trial ($P = 0.44$). The results indicate that posttraining systemic administration of ROL enhanced consolidation of NOR memory in aged rats.

DISCUSSION

Consistent with previous studies (de Lima et al., 2005b; 2005c), we have demonstrated that one daily oral administration of iron during PN days 12-14

impaired 24-h retention of NOR tested in the adulthood in rats. We have previously described the impairing effects of neonatal iron treatment in memory for other tasks in both rats and mice, and demonstrated that the iron-induced memory impairments are associated with an accumulation with iron in brain areas including the basal ganglia (Fredriksson et al., 1999; 2000; Schröder et al., 2001). Moreover, we have previously shown that the effects of neonatal iron on performance in NOR and other memory tasks in rats could not be attributed to sensorimotor impairments (Schröder et al., 2001; de Lima et al., 2005b; 2005c). The present results also showed that a single, posttraining injection of the PDE4 inhibitor ROL by itself did not affect NOR memory in adult rats, but attenuated the iron-induced memory impairment. In addition, ROL administration enhanced NOR memory in aged rats. The use of posttraining injections of ROL indicates that its effects were selectively related to modulation of the consolidation phase of NOR memory formation, and rules out the possibility that the ROL effects were due to drug-induced alterations in attentional, motivational, motor, or sensory-perceptual mechanisms at training.

The view that brain iron accumulation might play a role in neurodegenerative disorders is now well established (Sipe et al., 2002; Casadesus et al., 2004; Sadrzadeh and Saffari, 2004; Berg and Youdim, 2006). However, only more recently human studies have indicated that an increase in brain iron concentration might mediate memory loss in normal aging (House et al., 2006). Consistent with this view, previous reports from our laboratory have indicated that NOR memory deficits induced by neonatal iron administration in rats paralell those observed in aged animals (de Lima et al., 2005a; 2005b; 2005c; 2007), and we have recently shown that deficits in NOR memory in aged rats were reversed by systemic administration of deferoxamine, an iron chelating agent (de Lima et al., 2007). Thus, the use of animal models of brain iron accumulation associated with memory impairment, such as the neonatal iron treatment used in the present study, might contribute to the identification of candidate cognitive enhancers for the treatment of memory loss associated with aging or brain disease.

Previous studies have not examined whether acute administration of drugs with memory-enhancing properties could ameliorate memory deficits in iron-treated rats. ROL has been previously shown to enhance synaptic plasticity and memory memory in aged mice (Bach et al., 1999) as well as in several other rodent models of memory and amnesia (Imanishi et al., 1997; Barad et al., 1998; Zhang et al., 2000; Vitolo et

al., 2002; Alarcon et al., 2004; Gong et al., 2004; Zhang et al., 2004; Rutten et al., 2006). Thus, ROL and other PDE4 inhibitors have been proposed as cognitive enhancers with potential clinical usefulness (Arnsten et al., 2005). The rationale for the investigation of PDE4 inhibitors as cognitive enhancers is based on the well established critical role of the cAMP/PKA/CREB signaling pathway in underlying synaptic plasticity and memory consolidation (Abel et al., 1997; Bevilaqua et al., 1997; Bach et al., 1999; Schafe et al., 1999; Quevedo et al., 2004). Although the molecular mechanisms mediating the memory-impairing effects of brain iron remain to be elucidated, it is possible that iron interferes with protein kinase intracellular signaling pathways crucially involved in synaptic plasticity. For instance, recent evidence indicates that iron-dependent generation of hydroxyl radicals modulate the activation of the mitogen-activated kinase (MAPK)/extracellular-regulated kinase (ERK) pathway in PC12 neuroblastoma cells (Munoz et al., 2006). Our finding that a posttraining systemic administration of ROL ameliorates NOR memory deficits in rats given neonatal iron suggests that alterations in cAMP signaling might be involved in iron-induced cognitive impairment, and extends to iron-induced amnesia previous studies indicating that ROL might act as an effective cognitive enhancer in a range of experimental models of amnesia. Furthermore, the finding that ROL administration enhanced NOR retention in aged rats supports previous studies (Bach et al., 1999) indicating that PDE4 inhibitors could ameliorate age-related memory dysfunction. Because posttraining injections of ROL were used in the present study, our findings also provide strong evidence suggesting that the cognitive-enhancing properties of PDE4 inhibitors are specifically related to a facilitation of the consolidation phase of memory.

CONCLUSION

In summary, the present study shows that a single systemic administration of the PDE4 inhibitor ROL after training attenuates NOR memory deficits associated with brain iron accumulation or aging in rats. These findings provide the first evidence that iron-induced cognitive dysfunction might be ameliorated by stimulation of an intracellular signaling pathway. Together, our findings support the view that PDE4 inhibitors could be developed as cognitive-enhancing agents for the treatment of memory loss associated with aging and neurodegenerative disorders.

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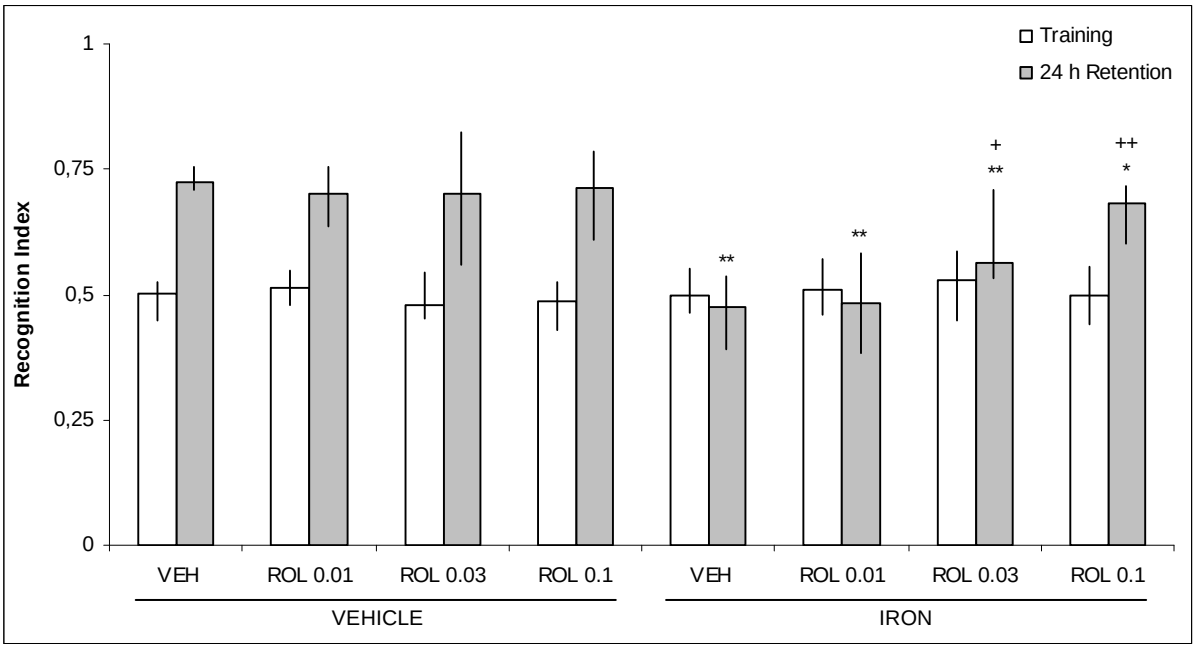

Figure 1. Posttraining systemic administration of rolipram (ROL) attenuates novel object recognition (NOR) memory impairment induced by neonatal iron loading. Male rats were given a single daily dose of vehicle (VEH) or iron (Fe^{2+}, 10.0 mg/kg) from postnatal days (PN) 12-14. At 6 months of age, animals were given an intraperitoneal (i.p.) injection of VEH or ROL (0.01, 0.03 or 0.1 mg/kg) immediately after NOR training. Memory retention was tested 24 h after training. Data are median (interquartile ranges) exploratory preference during the training and 24-h retention test trials. N = 10 animals per group, * $P < 0.05$ and ** $P < 0.01$ compared to rats treated with VEH (neonatal period) and VEH (adulthood); $^{+}$ $P < 0.05$ and $^{++}$ $P < 0.01$ compared to rats treated with iron (neonatal period) and VEH (adulthood).

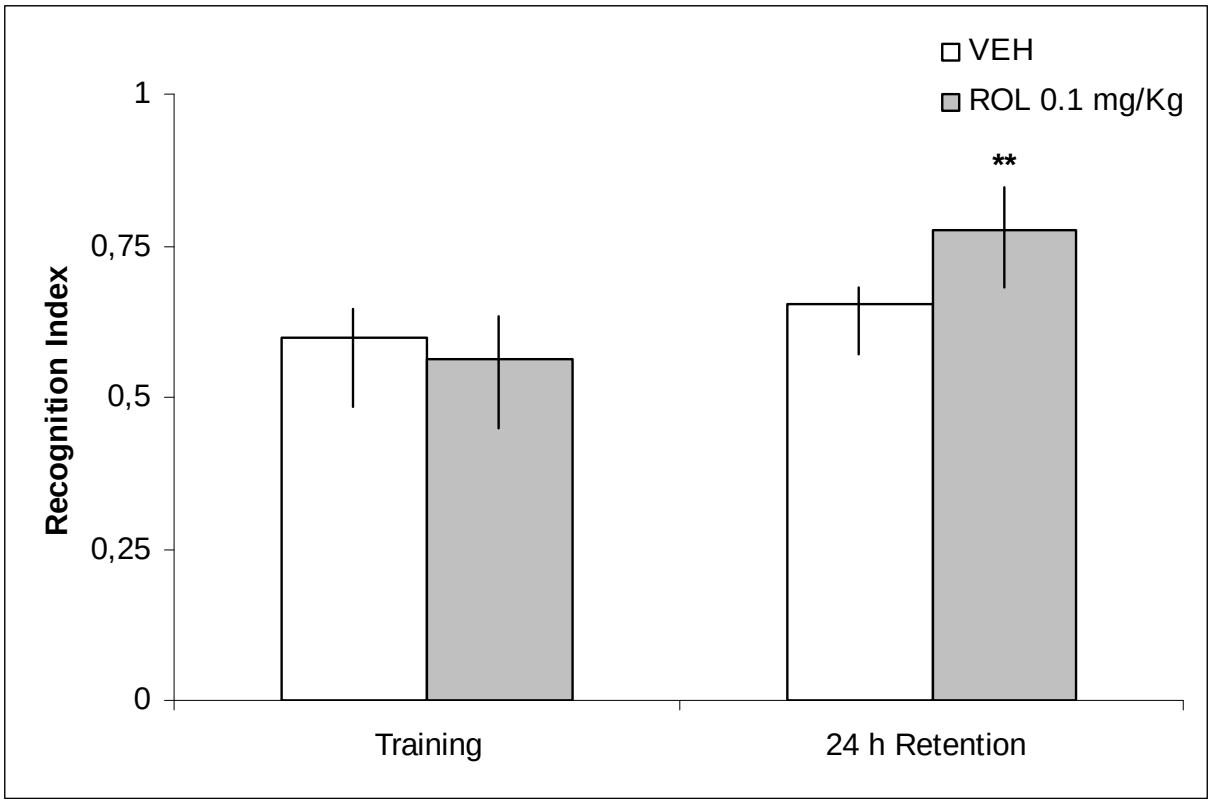

Figure 2. Posttraining systemic administration of rolipram (ROL) enhances novel object recognition (NOR) memory in aged rats. Twenty-three month-old male rats were given an intraperitoneal (i.p.) injection of VEH or ROL (0.01, 0.03 or 0.1 mg/kg) immediately after NOR training. Memory retention was tested 24 h after training. Data are median (interquartile ranges) exploratory preference during the training and 24-h retention test trials. N = 9 animals per group, * P < 0.05 compared to VEH-treated rats.

7 CONSIDERAÇÕES FINAIS

O excesso de ferro no encéfalo tem sido relacionado com a patogênese de diversas doenças neurodegenerativas que envolvem prejuízo cognitivo, as quais são mais prevalentes em populações de idosos. Tem sido demonstrado que o período neonatal é crítico para o estabelecimento do conteúdo normal de ferro no cérebro adulto[34,35,36] e também se sabe que o envelhecimento altera a distribuição cerebral deste metal. Foi descrito anteriormente que a administração de ferro no período neonatal prejudica severamente a memória de reconhecimento em ratos adultos e que o estresse oxidativo poderia estar relacionado com a indução desses déficits[40]. Adicionalmente, também foi demonstrado que ratos velhos apresentam déficits de memória de reconhecimento[41] similares aos observados nos animais que são submetidos à sobrecarga de ferro no período neonatal. Ainda, foi verificado que tanto os déficits induzidos pela administração neonatal de ferro quanto os déficits induzidos pelo envelhecimento podem ser revertidos pela selegilina (um inibidor da MAO)[41,42].

Modelos animais de envelhecimento já demonstraram ser instrumentos importantes não somente para o entendimento do processo de envelhecimento em si, como também para o entendimento do processamento da memória nesta fase da vida e o desenvolvimento de novos tratamentos que possam auxiliar na reversão dos déficits de memória associados às patologias neurodegenerativas que se manifestam nesse período. Como já foi estabelecido que o modelo animal em que é feita a administração de ferro no período neonatal é capaz de mimetizar os déficits de memória associados ao envelhecimento e que esses déficits podem ser revertidos através do mesmo tipo de terapia farmacológica aplicada a ratos velhos, torna-se possível investigarmos outras estratégias farmacológicas que possam reverter os déficits de memória através desse modelo.

O objetivo deste estudo foi determinar se os déficits de memória induzidos pelo tratamento neonatal com ferro e pelo envelhecimento poderiam ser revertidos através de três diferentes estratégias farmacológicas. Para tanto, foram realizados 5 experimentos nos quais foram testadas três classes de fármacos com possível ação terapêutica: **1)** um quelante de ferro [DFO]; **2)** dois fármacos que modulam a

neurotransmissão dopaminérgica [SKF 38393 (um agonista de receptores dopaminérgicos do tipo D_1) e GBR 12935 (um inibidor da recaptação de dopamina)] e **3)** um fármaco que modula a atividade da via do AMPc [rolipram].

Nos Experimentos I e II, foram obtidos efeitos similares em relação à indução dos déficits de memória nos ratos tratados com ferro no período neonatal e nos ratos velhos. Ao se administrar um quelante de ferro, que sabidamente interfere na formação de radicais livres, aos ratos tratados com ferro no período neonatal e aos ratos velhos, onde os níveis cerebrais de ferro estão naturalmente aumentados, foi verificada a mesma reversão dos déficits de memória de reconhecimento em ambos os grupos. Adicionalmente, o DFO reduziu os níveis de danos oxidativos a proteínas em regiões cerebrais importantes para o processamento da memória (córtex e hipocampo) nos ratos velhos. A obtenção desses resultados dá suporte à visão de que um dos mecanismos de ação pelo qual o ferro exerce seus efeitos deletérios sobre a memória esteja relacionado com a formação de radicais livres, tanto durante o envelhecimento normal quanto em doenças neurodegenerativas.

No Experimento III, foi verificado que a estimulação da neurotransmissão dopaminérgica também é capaz de reverter os déficits de memória induzidos pela administração neonatal de ferro, o que corrobora a idéia de que o sistema dopaminérgico é especialmente afetado pela sobrecarga de ferro e que, o mesmo, estaria envolvido na consolidação da memória de reconhecimento. Como já foi descrito que os receptores dopaminérgicos do tipo D_1 estão expressos em regiões cerebrais envolvidas na formação da memória de reconhecimento[66,67,68,69], acreditamos que os efeitos observados em nosso experimento estejam relacionados à ativação desses receptores promovida pela administração do SKF 38393 na fase de consolidação da memória. Também acreditamos que os efeitos induzidos pelo GBR 12935 estejam relacionados ao aumento da disponibilidade da dopamina induzido pela inibição dos DATs, uma vez que já foi demonstrado que o GBR 12935 liga-se aos DATs em regiões cerebrais importantes para a formação da memória[94,95].

Nos Experimentos IV e V, mais uma vez foram obtidos efeitos similares em relação à indução dos déficits de memória nos ratos tratados com ferro no período neonatal e nos ratos velhos. Os resultados demonstram que o aumento nos níveis de AMPc na fase de consolidação da memória de reconhecimento é capaz reverter os déficits de memória em ambos os grupos. Apesar dos níveis de AMPc não terem sido medidos nesses animais, acreditamos que os resultados obtidos estejam

relacionados ao aumento dos níveis de AMPc causado pela administração de rolipram, uma vez que tem sido proposto que seja essa a propriedade através da qual o rolipram estaria promovendo a melhora da memória[81,82,83,84,85,86,87,88,89,90,91,92,93].

Ao se analisar os resultados dos Experimentos III, IV e V em conjunto, pode-se inferir que o mecanismo através do qual foi produzida a melhora na memória de reconhecimento foi a estimulação da via do AMPc, uma vez que, tanto o uso de um agonista de receptores dopaminérgicos da família D_1 quanto o uso de um inibidor da fosfodiesterase produziram efeitos similares sobre a performance dos animais.

A relevância desse trabalho está no fato de ter sido demonstrado que a administração de baixas quantidades de ferro por um período curto em uma fase suscetível da vida é capaz de produzir efeitos comportamentais que podem estar relacionados com processos neurodegenerativos manifestados na fase adulta, os quais apresentam semelhanças com os efeitos observados naturalmente na fase de envelhecimento. Adicionalmente, foi demonstrado que esses efeitos da sobrecarga de ferro podem ser revertidos através da utilização de diferentes estratégias farmacológicas. Estudos posteriores serão necessários para esclarecer os mecanismos celulares e moleculares através dos quais esse metal, amplamente utilizado na dieta infantil, acumula-se no cérebro e de que maneira sua homeostasia é rompida desencadeando o processo neurodegenerativo.

A tarefa de reconhecimento do objeto novo demonstrou ser uma ferramenta importante para o estudo das alterações cognitivas relacionadas ao envelhecimento e a modelos de doenças neurodegenerativas, bem como para o teste de fármacos com possível ação neuroprotetora.

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ANEXOS

Cartas de Aceite e de Submissão dos Artigos que Compõem a Tese

Artigo: De Lima MN, Dias CP, Presti-Torres J, Dornelles A, Garcia VA, Scalco FS, Guimarães MR, Petry RC, Bromberg E, Constantino L, Budni P, Dal-Pizzol F, Schröder N. Reversion of age-related recognition memory impairment by iron chelation in rats. *Neurobiol Aging*. 2007. (no prelo)

Neurobiology of Aging
Ms. No.: NBA-06-500R1
Title: Reversion of age-related recognition memory impairment by iron chelation in rats.
Corresponding Author: Dr. Nadja Schroder
Authors: Maria NM de Lima; Caroline P Dias; Juliana Presti-Torres; Arethuza Dornelles; Vanessa A Garcia; Felipe S Scalco; Marcelo R Guimarães; Roberta C Petry; Elke Bromberg; Larissa Constantino; Patricia Budni; Felipe Dal-Pizzol; Schroder N.

Dear Dr. Schroder,

I am pleased to inform you that your manuscript referenced above has been accepted for publication in Neurobiology of Aging.

We will complete final editorial processing and forward your paper to our typesetter. As soon as it is assigned an issue, the publisher will be in touch with you. Minor changes, abbreviations, etc., will be made by our copy editor, and you will be able to check them when you receive page proofs. In approximately 45 days your manuscript will be published on line and appear at PubMed, ahead of print publication.

We appreciate your interest and hope to receive more manuscripts from you and your colleagues for consideration for publication in Neurobiology of Aging.

Sincerely,

Paul D. Coleman, Ph.D.
Editor-in-Chief
Neurobiology of Aging

Shanti Sharma, Ph.D.
Managing Editor
Neurobiology of Aging
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Artigo: De Lima MN, Presti-Torres J, Caldana F, Grazziotin MM, Scalco FS, Guimarães MR, Bromberg E, Franke SI, Henriques JAP, Schröder N. Desferoxamine reverses neonatal iron-induced recognition memory impairment in rats. *Eur J Pharmacol*. 2007. (no prelo)

Ref.: Ms. No. EJP-27052R2
European Journal of Pharmacology

Dear Dr Schroder,

Thank you for your manuscript no EJP-27052R2 entitled Desferoxamine reverses neonatal iron-induced recognition memory impairment in rats which can be accepted for publication in the EUROPEAN JOURNAL OF PHARMACOLOGY as it stands.

Your manuscript will appear under the heading: Section: Behavioral pharmacology

I have forwarded the manuscript to the publisher, who will contact you directly with regard to galley proofs and other business matters.

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I remain,

Yours sincerely,

W.H. Gispen, Ph.D.

****
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Artigo: De Lima MN, Presti-Torres J, Garcia VA, Guimarães MR, Roesler R, Schröder N. Amelioration of recognition memory impairment associated with iron loading and aging by the type 4-specific phosphodiesterase inhibitor rolipram. *Neuroscience*. 2007. (submetido)

Dear Dr. Schroder,

Your submission entitled "AMELIORATION OF RECOGNITION MEMORY IMPAIRMENT ASSOCIATED WITH IRON LOADING OR AGING BY THE TYPE 4-SPECIFIC PHOSPHODIESTERASE INHIBITOR ROLIPRAM" has been received for consideration in Neuroscience.

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Lista de Artigos Publicados durante o Período de Realização do Curso de Doutorado (outubro de 2004 - abril de 2007)

1 Budni P, De Lima MN, Polydoro M, Moreira JC, Schröder N, Dal-Pizzol F. Antioxidant effects of selegiline in oxidative stress induced by iron neonatal treatment in rats. *Neurochem Res*. 2007;32(6):965-72. DOI: http://dx.doi.org/10.1007/s11064-006-9249-x

2 Dias CP, De Lima MN, Presti-Torres J, Dornelles A, Garcia VA, Scalco FS, Guimrães MR, Constantino L, Budni P, Dal-Pizzol F, Schröder N. Memantine reduces oxidative damage and enhances long-term recognition memory in aged rats. *Neuroscience*. 2007;146(4):1719-25. DOI: http://dx.doi.org/10.1016/j.neuroscience.2007.03.018

3 Dornelles A, De Lima MN, Grazziotin M, Presti-Torres J, Garcia VA, Scalco FS, Roesler R, Schröder N. Adrenergic enhancement of consolidation of object recognition memory. *Neurobiol Learn Mem*. 2007;88(1):137-42. DOI: http://dx.doi.org/10.1016/j.nlm.2007.01.005

4 Presti-Torres J, De Lima MN, Scalco FS, Caldana F, Garcia VA, Guimaraes MR, Schwartsmann G, Roesler R, Schröder N. Impairments of social behavior and memory after neonatal gastrin-releasing peptide receptor blockade in rats: Implications for an animal model of neurodevelopmental disorders. *Neuropharmacology*. 2007;52(3):724-32. DOI: http://dx.doi.org/10.1016/j.neuropharm.2006.09.020

5 De Lima MN, Presti-Torres J, Dornelles A, Bromberg E, Schröder N. Differential effects of low and high doses of topiramate on consolidation and retrieval of novel object recognition memory in rats. *Epilepsy Behav*. 2007;10(1):32-7. DOI: http://dx.doi.org/10.1016/j.yebeh.2006.09.007

6 De Lima MN, Luft T, Roesler R, Schröder N. Temporary inactivation reveals an essential role of the dorsal hippocampus in consolidation of object recognition memory. *Neurosci Lett*. 2006;405(1-2):142-6. DOI: http://dx.doi.org/10.1016/j.neulet.2006.06.044

7 De Lima MN, Laranja DC, Caldana F, Grazziotin MM, Garcia VA, Dal-Pizzol F, Bromberg E, Schröder N. Selegiline protects against recognition memory impairment induced by neonatal iron treatment. *Exp Neurol*. 2005;196(1):177-83. DOI: http://dx.doi.org/10.1016/j.expneurol.2005.07.017

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9 De Lima MN, Polydoro M, Laranja DC, Bonatto F, Bromberg E, Moreira JC, Dal-Pizzol F, Schröder N. Recognition memory impairment and brain oxidative stress induced by postnatal iron administration. *Eur J Neurosci*. 2005;21(9):2521-8. DOI: http://dx.doi.org/10.1111/j.1460-9568.2005.04083.x

10 Schröder N, De Lima MN, Quevedo J, Dal-Pizzol F, Roesler R. Impairing effects of chronic haloperidol and clozapine treatment on recognition memory: possible relation to oxidative stress. *Schizophr Res*. 2005;73(2-3):377-8. DOI: http://dx.doi.org/10.1016/j.schres.2004.06.015

11 De Lima MN, Laranja DC, Bromberg E, Roesler R, Schröder N. Pre- or post-training administration of the NMDA receptor blocker MK-801 impairs object recognition memory in rats. *Behav Brain Res*. 2005;156(1):139-43. DOI: http://dx.doi.org/10.1016/j.bbr.2004.05.016