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EFEITOS NEUROCOMPORTAMENTAIS DO TRATAMENTO NEONATAL COM FERRO: *Possível modelo para o estudo de doenças neurodegenerativas*

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Resumo

O excesso de ferro no cérebro tem sido relacionado com a patogênese de várias doenças neurodegenerativas humanas. O período neonatal é crítico para o estabelecimento da concentração de ferro no cérebro adulto. Nesta tese são avaliados os efeitos neurocomportamentais do tratamento neonatal com ferro. No primeiro estudo (capítulo II) camundongos NMRI foram tratados oralmente com 3.7 ou 37.0 mg Fe^{+2}/kg diariamente do 10° ao 12° dias de vida pós-natal. Os animais tratados com Fe^{+2} 37.0 mg/kg apresentaram marcada hipocinesia para os três parâmetros da atividade motora: locomoção, respostas de orientação e atividade total nos primeiros vinte minutos, no entanto foram mais ativos nos vinte minutos finais da sessão de uma hora. Os camundongos tratados com a dose mais baixa (Fe^{+2} 3.7 mg/kg/dia) apresentaram diminuição apenas no número de respostas de orientação no primeiros vinte minutos e um aumento da locomoção e das respostas de orientação nos vinte minutos finais da sessão. Na tarefa do labirinto radial, os camundongos tratados com Fe^{+2} 37.0 mg/kg apresentaram um maior número de erros, bem como uma latência maior em obter os 8 pellets no 3° dia testado, quando comparados com os controles. A análise do conteúdo total de ferro (μg/g) mostrou que a dose de 37.0 mg/kg induziu um aumento nos níveis de ferro nos gânglios da base, mas não no córtex frontal.

No segundo grupo de experimentos (capítulo III) os camundongos foram tratados com ferro (7.5 mg/kg) em três diferentes fases do período neonatal: do 3° ao 5°, do 10° ao 12° e do 19° ao 21° dia de vida. Confirmando os resultados obtidos no primeiro estudo, o tratamento com ferro do 10° ao 12° dia causou hipocinesia para os três parâmetros da atividade motora nos primeiros vinte minutos, sendo que a respostas de orientação neste caso, continuaram diminuídas no 2° período de 20 minutos. Novamente, encontrou-se um aumento dos três parâmetros nos vinte minutos finais da sessão. O tratamento com ferro do 3° ao 5° dia, produz efeitos sobre a atividade motora, mas em uma menor extensão que aqueles produzidos pelo tratamento do 10° ao 12° dia. Na tarefa do labirinto radial, os camundongos tratados do 10° ao 12° dia apresentaram maior número de erros e maior latência na segunda e terceira (última) sessão, enquanto que nos tratados do 3° ao

5° dia o mesmo ocorreu somente na última. A análise do conteúdo total de ferro (μg/g) mostrou que quando o ferro foi administrado do 3° ao 5° e do 10° ao 12° encontrou-se uma elevação nos níveis de ferro nos gânglios da base, mas não no córtex frontal. O tratamento neonatal com ferro do 19° ao 21° dia de vida não produziu efeitos em nenhum dos parâmetros das tarefas comportamentais estudadas, além de não alterar o conteúdos de ferro nos gânglios da base.

Com o objetivo de investigar a interação dos efeitos do ferro administrado no período neonatal com tratamento com MPTP (capítulo IV), grupos de camundongos C57 Bl/6 receberam Fe^{2+} (7.5 mg/kg), ou veículo do 10° ao 12° dia de vida, seguido, aos 3 meses de idade pela administração de MPTP (2 x 20 ou 2 x 40 mg/kg, s.c.) ou salina. A combinação do tratamento neonatal com ferro com a exposição dos camundongos adultos ao MPTP produziu déficits severos na atividade motora, acentuando os efeitos do MPTP. A análise neuroquímica mostrou que a combinação do ferro com MPTP produz uma depleção mais acentuada do conteúdo de DA estriatal, do que aquela encontrada nos animais tratados apenas com MPTP.

No capítulo V ratos foram tratados com ferro em quatro diferentes doses do 10° ao 12° dia de vida. Os ratos que receberam Fe^{+2} 30.0 mg/kg (dose mais alta) apresentaram diminuição no número de cruzamentos nos 5 minutos finais da sessão na tarefa do campo aberto. Na tarefa do labirinto radial todas as doses utilizadas (de 2.5 a 30.0 mg/kg/dia) causaram aumento na latência em visitar os oito braços e menor número de escolhas corretas nas 8 primeiras entradas no 5° (último) dia testado.

O conhecimento dos efeitos funcionais do excesso de ferro adquirido pelo cérebro durante o período neonatal ainda é limitado, no entanto os resultados obtidos apontam para o envolvimento do ferro em processos neurodegenerativos.

Palavras-chave: Ferro - administração – pós-natal – comportamento motor – labirinto radial - gânglios da base – dopamina – MPTP - parkinsonismo.

Abstract

Excess iron in the brain has been implicated in the pathogenesis of several human neurodegenerative disorders. The neonatal period is critical for the establishment of normal iron content in the adult brain. In the first study (Chapter II), the long-term neurobehavioural effects of iron exposure during this period were assessed by treating NMRI mice orally with 0.0, 3.7 or 37.0 mg Fe^{2+}/kg b. w. on postnatal days 10 - 12. Spontaneous motor behaviour and radial arm maze learning were tested at the age of 3 months. It was found that the mice treated with the higher dose of Fe^{2+} 37.0 mg/kg b. w., were hypoactive during the first 20 min of testing but hyperactive during the final 20 min, showing an almost complete lack of habituation of spontaneous activity in the test chambers. These changes were also seen in animals treated with the lower dose of Fe^{2+}, 3.7 mg/kg b. w., but the effects were lesser pronounced indicating dose-response relationship. In the radial arm maze, the Fe^{2+} 37.0 mg/kg group evidenced significantly both more errors in arm choices and longer latencies to acquire all eighth pellets. Analysis of brain iron content indicated significantly more total iron (µg/g) in the basal ganglia, but not frontal cortex, of the higher, 37 mg/kg, dose group. For the second study (Chapter III), newborn mice were administered Fe^{2+} (7.5 mg/kg, b.w.) on either Days 3-5, 10-12 or 19-21, or vehicle (saline) at the same times, postnatally.

It was found that mice treated with Fe^{2+} during postnatal Days 10-12 were marked hypokinesic during the 1st 20-min test period and hyperkinesic during the 3rd and final 20-min test period. These mice showed an almost complete lack of habituation of spontaneous motor activity parameters to the test chambers. In the radial arm maze the Days 10-12 treatment group evidenced significantly both more errors in arm choices and longer latencies to acquire all eight pellets; these mice showed also a severe trial-to-trial retention deficit as indexed by retention quotients. These behavioural deficits were observed also in animals treated with Fe^{2+} during postnatal Days 3-5, but the effects were less pronounced indicating the higher susceptibility of the brain for Fe^{2+}-induced damage, during Days 10-12 post partum. Treatment with Fe^{2+} on Days 19-21 did not induce behavioural alterations in comparison with its respective control (vehicle) group. Analysis of total brain iron content

indicated significantly more iron (μg/g) in the basal ganglia, but not frontal cortex, of mice from days 3-5 and 10-12 Fe^{2+} treatment groups.

In order to study the interactive effects of postnatal iron and adult MPTP treatments (Chapter IV), groups of C57 Bl/6 mice were administered iron (Fe^{2+}) 7.5 mg/kg, b. wt., p.o. or vehicle (saline) on Days 10-12 post partum followed, at 3 months of age, by administration of either MPTP (2 x 20 or 2 x 40 mg/kg, s.c.) or saline. Behavioural testing was started three weeks later. MPTP treatment of adult mice caused a dose-related hypokinesia tOhroughout the 3 x 20-min test periods; in the mice that received both neonatal iron and MPTP severe deficits of motor activity (akinesia) were obtained. Neurochemical analyses of striatal DA levels demonstrated that the depletions were most severe under conditions of combined neonatal iron and adult MPTP treatment; postnatal iron enhanced DA loss after the 2 x 20 mg/kg dose of MPTP. Furthermore, these depletions were associated with an almost total akinesia by these groups when locomotion and rearing counts were expressed as a percentage of the Veh-sal group. The analysis of total iron content (μg/g) in brain regions indicated notably elevated levels in the basal ganglia, but not in the frontal cortex, of mice administered Fe^{2+} on Days 10-12 after birth.

In the Chapter V Wistar rats were treated neonatally with 0.0; 2.5; 7.5; 15.0 or 30.0 mg Fe+2/kg b.w. on days 10-12 after birth. Results show that the highest iron dose (30.0 mg/kg) caused a decrease in the number of crossings in the final 5-minute period of the session. In the radial arm maze task all the iron doses used in this study produced a higher latency in visiting the 8 arms and a decrased number of correct choices in the first 8 entries in the last trial.

The knowledge of the long-term effects of iron entering the brain during this critical period of rapid brain growth are limited. Increased amounts of iron in the brain, especially in the basal ganglia may contribute to neurodegenerative processes.

Keywords: Iron administration - postnatal - motor behaviour - radial maze learning - basal ganglia – dopamine – MPTP - parkinsonism.

Lista de Abreviaturas e Símbolos Utilizados

6-OHDA – 6-Hidróxi dopamina

ANOVA – análise de variância

DA – dopamina

DNA – ácido desóxi ribonucléico

DOPAC – ácido di-hidróxi fenilacético

DP- Doença de Parkinson

EUA – Estados Unidos da América

H_2O_2 – peróxido de hidrogênio

HVA – ácido homovanílico

MAO – monoamino oxidase

MPTP – 1-metil-4-fenil-1,2,3,6-tetrahidropiridina

OH^- - íon hidroxil

• OH – radical hidroxil

RNA – ácido ribonucléico

ROS – espécies reativas de oxigênio (do inglês Reactive Oxygen Species)

Índice

Capítulo I

Introdução

I.1. O papel do ferro no cérebro e sua distribuição

O ferro é um dos metais mais abundantes no corpo humano e o cérebro contém uma concentração substancialmente maior deste metal do que de qualquer outro (Janetzky et al, 1997).

O ferro é essencial para as células cerebrais uma vez que desempenha funções importantes em muitos processos metabólicos, incluindo a síntese de DNA, RNA e proteínas, como co-fator de enzimas heme e não-heme, na formação da mielina e no desenvolvimento da árvore dendrítica (Youdim et al, 1991). Além disso, é um componente chave dos grupos heme nos citocromos e em proteínas ferro-sulfúricas na mitocôndria, permitindo a transferência de elétrons na respiração celular. Nos neurônios esta função é particularmente importante pois estes são dependentes do metabolismo aeróbico. Ainda, muitas enzimas que existem exclusivamente no cérebro, tais como a tirosina hidroxilase e a monoamino oxidase utilizam ferro como cofator (Janetzky et al, 1997).

O ferro é, portanto, essencial para o desenvolvimento neurológico normal (Connor et al, 1995). Estudos relatam (Yehuda et al, 1986) que a deficiência de ferro em ratos nos estágios iniciais do desenvolvimento do cérebro tem sido relacionada com alterações comportamentais incluindo déficits no aprendizado e na memória.

Uma vez que o ferro participa de tantos eventos importantes no cérebro, é necessário que ele esteja numa forma facilmente disponível nas células. Entretanto, o cérebro também necessita de mecanismos que o protejam do estresse oxidativo induzido pelo ferro (ver item I.3). A proteína que torna o ferro disponível quando necessário e também protege adequadamente a célula dos excessos deste metal é a proteína

intracelular ferritina (Cheepsunthorn et al, 1998). Aproximadamente 1/3 do conteúdo cerebral de ferro encontra-se ligado à ferritina. A síntese desta proteína é induzida sempre que os níveis celulares de ferro estão acima dos níveis requeridos para o metabolismo normal da célula (Casey et al, 1988).

O perfil de distribuição do ferro no cérebro sugere que sua presença está relacionada a vias específicas (Hill & Switzer, 1984). Os níveis mais altos deste metal encontram-se (em ordem decrescente): no globo pálido e na substância negra; no núcleo rubro, no putâmen, no tálamo e no núcleo caudato; no cerebelo e no córtex (Hill & Switzer, 1984; Benkovic & Connor, 1993).

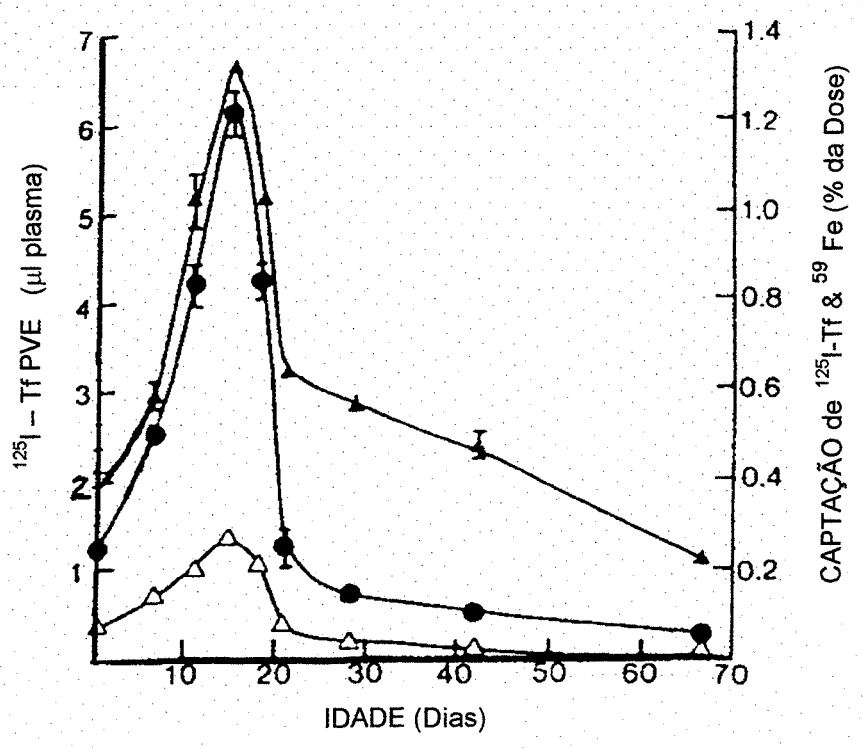
I.2. Transporte do ferro através da barreira hemato-encefálica

O ferro é transportado no plasma e nos fluidos intersticiais por uma glicoproteína chamada transferrina. As células endoteliais dos capilares cerebrais possuem receptores para a transferrina e são capazes de captá-la por endocitose. Com base nestas observações, é proposto que o ferro é transportado do plasma ao cérebro por transcitose mediada por receptores do complexo ferro-transferrina (Taylor & Morgan, 1990).

Estudos em ratos (Taylor & Morgan, 1990; Taylor et al, 1991) demonstraram que o período de rápido crescimento cerebral (primeiras duas semanas de vida) é acompanhado por um aumento no transporte específico de transferrina e de ferro para o cérebro. A figura 1 mostra a curva do transporte de ferro no cérebro de ratos, o qual aumenta rapidamente nos primeiros quinze dias após o nascimento, declinando logo

após até o vigésimo primeiro dia e caindo praticamente a zero no rato adulto. Pode ser visto ainda, que o transporte de transferrina apresenta o mesmo padrão, atingindo os níveis máximos de captação pelo cérebro no décimo quinto dia de vida pós-natal. Este perfil de captação parece ser específico para o cérebro, uma vez que foi diferente do perfil encontrado tanto para o fêmur quanto para o fígado. Em um outro estudo (Dwork et al, 1990), no qual foi utilizado ferro radioativo, foi demonstrado que o metal absorvido pelo cérebro em ratos de 15 dias permanece no cérebro, não retornando ao plasma. O ferro adquirido pelo cérebro nesta idade é redistribuído dentro do cérebro, assumindo um perfil similar ao do ferro total na idade adulta (Dwork et al, 1990).

Figura 1 (adaptada de Taylor & Morgan, 1990): Efeito da idade na captação de transferrina (Δ, ▲) e ^{59}Fe (●) pelo cérebro no rato.



I.3. O ferro e o estresse oxidativo

A hipótese do "estresse oxidativo" infere um desequilíbrio entre a formação de oxidantes celulares e processos antioxidativos. Devido à formação excessiva de peróxido de hidrogênio (H_2O_2) e radicais livres derivados de oxigênio, o estresse oxidativo pode causar danos à célula através de reações de peroxidação lipídica e alterações na fluidez da membrana (Gerlach et al, 1994).

O peróxido de hidrogênio é produzido em tecidos humanos por uma série de enzimas (Halliwell, 1992). Em neurônios dopaminérgicos o H_2O_2 é gerado principalmente pela reação de desaminação oxidativa da dopamina pela monoamino oxidase (MAO), bem como pela sua auto-oxidação não-enzimática (a qual é estimulada por ferro e outros metais de transição) (figura 2). O peróxido de hidrogênio é relativamente inerte e atóxico para as células. No entanto, em presença de ferro sua molécula é reduzida formando radicais hidroxil (•OH) através da Reação de Fenton (Janetzky et al, 1997).

Os radicais hidroxil são os mais reativos das espécies reativas de oxigênio (ROS), podendo difundir-se e reagir rapidamente com muitas das moléculas encontradas nas células vivas. Estas reações incluem quebra do DNA, alterações químicas da deóxi-ribose, de membranas lipídicas e carbohidratos, levando a uma cascata de eventos que resulta em danos ao sistema mitocondrial de transporte de elétrons, descompartimentalização da homeostase do cálcio intracelular, indução de proteases, aumento na peroxidação lipídica da membrana, e, finalmente à morte celular (Lan & Jiang, 1997b; Halliwell, 1992; Youdim et al, 1993).

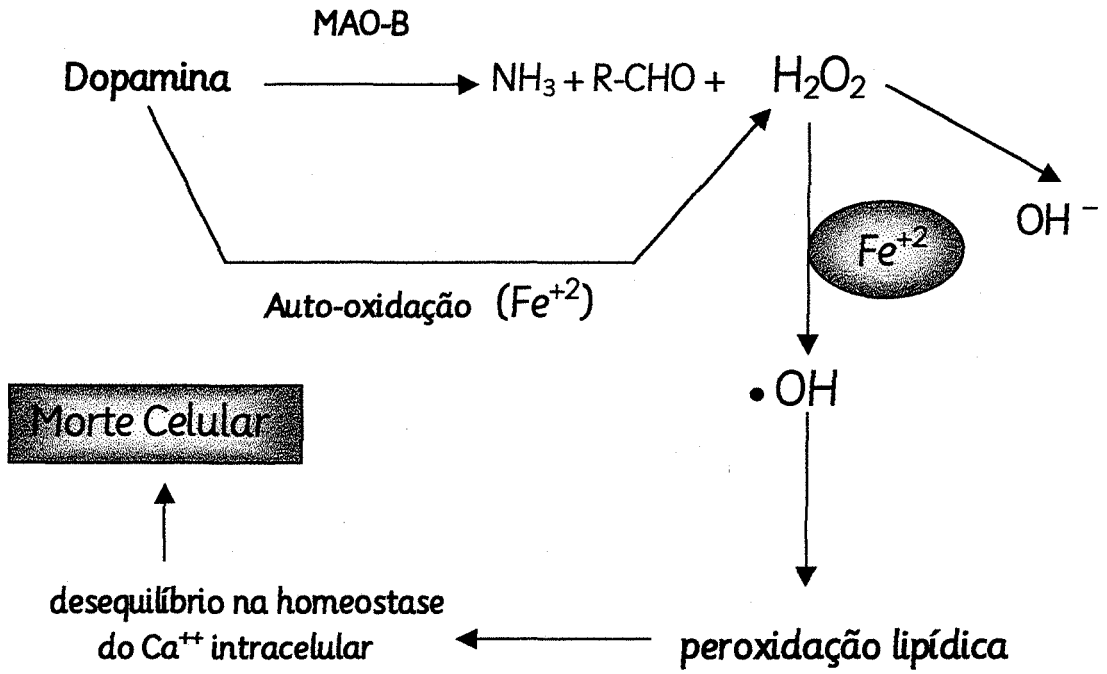


Figura 2 (adaptada de Janetzky et al, 1997): Importância do Ferro (Fe^{+2}) nas reações de formação dos radicais •OH em neurônios dopaminérgicos.

I.4. O ferro e a Doença de Parkinson

A Doença de Parkinson (DP) é uma doença neurodegenerativa progressiva caracterizada pela degeneração dos neurônios dopaminérgicos negro-estriatais incluindo a perda de corpos celulares na *pars compacta* da substância negra (Götz et al, 1990).

A etiologia da neurodegeneração, no entanto, permanece obscura. Recentemente, um crescente corpo de evidências clínicas e experimentais sugere a participação do ferro no mecanismo de morte celular na DP, pois: (1) o ferro catalisa a formação de radicais hidroxil que são extremamente tóxicos (2) evidências sugerem que o estresse oxidativo participe no mecanismo de morte neuronal (3) estudos demonstram a elevação da

concentração de ferro na substância negra de portadores de PD (Hirsch & Faucheux, 1998).

Ao longo da última década vários estudos, utilizando diferentes métodos (detecção de metais por espectroscopia e por microanálise por raio-x, além de técnicas histoquímicas), mostram a presença de concentrações anormais de ferro na substância negra de portadores da Doença de Parkinson (Griffiths et al, 1999; Dexter et al, 1991; Ebadi et al, 1996; Faucheux et al, 1993; Jellinger et al, 1993; Kienzl et al, 1995).

I.5. MPTP: Modelo animal da Doença de Parkinson

Na última década o composto 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP) foi estabelecido como uma neurotoxina seletivamente dopaminérgica o qual causa uma síndrome parkinsoniana em humanos (Davis et al, 1979) e primatas (Langston et al, 1983). As principais características desta síndrome consistem em um padrão de distúrbios motores envolvendo tremores, rigidez e hipocinesia. Estes distúrbios do movimento têm sido associados a um quadro neuropatológico de degeneração neuronal na *pars compacta* da substância negra destes pacientes.

A utilização do MPTP em camundongos e em primatas não-humanos oferece um dos mais úteis modelos animais para o estudo da Doença de Parkinson, pois mostrou-se ser o modelo que mais acuradamente reproduz todos os aspectos da doença que acomete seres humanos (Gerlach & Riederer, 1996).

Em geral, os roedores parecem ser menos sensíveis que humanos e primatas aos efeitos neurotóxicos do MPTP, no entanto foi verificado que camundongos de diferentes

cepas podem desenvolver uma pronunciada degeneração do sistema negro-estriatal após injeções sistêmicas da neurotoxina. Os camundongos da cepa C-57 BL6, que são pigmentados, quando tratados repetidamente com MPTP invariavelmente demonstram um conjunto de sintomas típicos, entre os quais está a diminuição da atividade locomotora (Sundström et al, 1990). Tais efeitos comportamentais são acompanhadas de alterações neuroquímicas que incluem severa depleção dos níveis de dopamina (DA) (-80%) e seus metabólitos no estriado, e poucas alterações em outros sistemas neurotransmissores (Sundström et al, 1987).

I.6. Consumo de ferro

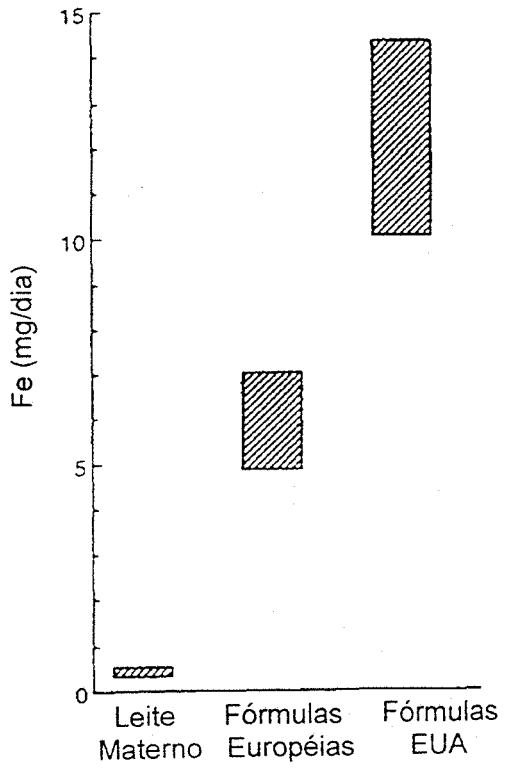
A concentração de ferro no leite materno (níveis normais em torno de 0.2 a 0.4 mg/l) é considerada baixa, embora recém-nascidos alimentados somente com leite materno raramente desenvolvam anemia ferropriva. A concentração de ferro no leite materno não parece ser afetada pelo estado materno, uma vez que estudos demonstram que mulheres anêmicas e mulheres recebendo suplementação de ferro apresentam níveis de ferro no leite semelhantes aos de mulheres não anêmicas (Zavaleta et al, 1995). A figura 3 mostra que crianças de até um ano de idade cuja alimentação é baseada em fórmulas lácteas fortificadas recebem diariamente um aporte de ferro que corresponde a 10 (níveis europeus) e 100 (níveis nos EUA) vezes os níveis do leite materno (Lönnerdal, 1997).

Enquanto no passado a ênfase havia sido dada ao combate à deficiência de ferro, a aplicação indiscriminada de suplementação de ferro tornou importante estudar os

mecanismos através dos quais o organismo pode se proteger contra o excesso deste metal (Bothwell, 1995).

Uma vez que a questão da suplementação com ferro em crianças saudáveis no seu primeiro ano de vida é controversa e a maioria das fórmulas alimentares infantis contém concentrações de ferro muito mais elevadas que as do leite materno, é importante determinar se a exposição a este metal no período neonatal pode produzir algum efeito neurocomportamental.

Figura 3 (adaptada de Lönnerdal, 1997): Consumo diário de ferro (em mg) por crianças alimentadas com diferentes dietas.



I.7. Objetivos

I.7.1. Objetivo geral

O objetivo geral desta tese é avaliar os efeitos comportamentais e neuroquímicos da exposição neonatal de duas espécies de roedores (camundongos e ratos) ao ferro.

Nos dias de hoje está bem estabelecido o envolvimento do ferro em doenças neurodegenerativas , especialmente na DP, o que torna fundamental a investigação dos possíveis mecanismos que poderiam estar levando ao acúmulo deste metal no cérebro e sua possível contribuição para o processo neurodegenerativo. Uma vez que o período neonatal é de extrema importância para o estabelecimento dos níveis normais de ferro no cérebro adulto, há a necessidade de estabelecer as possíveis conseqüências decorrentes da exposição a este metal neste período.

I.7.2. Objetivos Específicos

1. Avaliar os efeitos do tratamento com duas diferentes doses de ferro (3.7 e 37.0 mg/kg) durante três dias do período neonatal (do 10° ao 12° dia) sobre o comportamento motor em camundongos adultos.
2. Avaliar os efeitos do tratamento com duas diferentes doses de ferro (3.7 e 37.0 mg/kg) durante três dias do período neonatal (do 10° ao 12° dia) sobre o aprendizado da tarefa do labirinto radial em camundongos adultos.

3. *Verificar se o tratamento com ferro (3.7 e 37.0 mg/kg) do 10° ao 12° dia de vida pós-natal causaria alterações na concentração de ferro nos gânglios basais e no córtex frontal dos camundongos adultos.*
4. *Avaliar os efeitos do tratamento com ferro (dose de 7.5 mg/kg) em três diferentes fases do período neonatal (do 3° ao 5°, do 10° ao 12° ou do 19° ao 21° dia) sobre o comportamento motor em camundongos adultos.*
5. *Avaliar os efeitos do tratamento com ferro (dose de 7.5 mg/kg) em três diferentes fases do período neonatal (do 3° ao 5°, do 10° ao 12° ou do 19° ao 21° dia) sobre o aprendizado da tarefa do labirinto radial em camundongos adultos.*
6. *Verificar se o tratamento com ferro (dose de 7.5 mg/kg) em três diferentes fases do período neonatal (do 3° ao 5°, do 10° ao 12° ou do 19° ao 21° dia) causaria alterações na concentração de ferro nos gânglios basais e no córtex frontal dos camundongos adultos.*
7. *Investigar uma possível interação entre os efeitos produzidos pelo tratamento neonatal com ferro (7.5 mg/kg, administrado do 10° ao 12° dia) e a exposição ao MPTP sobre o comportamento motor em camundongos adultos*
8. *Investigar uma possível interação entre os efeitos produzidos pelo tratamento neonatal com ferro (7.5 mg/kg, administrado do 10° ao 12° dia) e a exposição ao MPTP sobre os níveis estriatais de Dopamina em camundongos adultos.*

9. Avaliar o efeito do tratamento com quatro diferentes doses de ferro (2.5, 7.5, 15.0 e 30.0 mg/kg) durante três dias do período neonatal (do 10° ao 12° dia) sobre o comportamento motor em ratos adultos.
10. Avaliar o efeito do tratamento com quatro diferentes doses de ferro (2.5, 7.5, 15.0 e 30.0 mg/kg) durante três dias do período neonatal (do 10° ao 12° dia) sobre o aprendizado da tarefa do labirinto radial em ratos adultos.

I.8. Organização dos trabalhos que compõem esta tese

No **capítulo II** estão descritos os experimentos iniciais, os quais encontram-se publicados no periódico *Toxicology and Applied Pharmacology.* Foram utilizadas duas doses (3.7 e 37.0 mg/kg/dia) de Fe^{+2} que foram administradas a camundongos NMRI por três dias (do décimo ao décimo segundo dia de vida). Ao atingirem a idade adulta os animais foram testados para a atividade locomotora e para o aprendizado na tarefa do labirinto radial de 8 braços. Após os testes comportamentais, o conteúdo de ferro no cérebro deste animais foi dosado por espectroscopia de absorção atômica. Os resultados mostraram que a exposição neonatal produziu acumulação de ferro nos gânglios da base, além de produzir hipoatividade no comportamento motor e déficits no aprendizado do labirinto radial.

No **capítulo III**, apresentado sob forma de um manuscrito que está submetido à revista *Developmental Brain Research,* encontra-se um segundo grupo de experimentos, no qual os tratamentos com ferro (7.5 mg/kg/dia) foram realizados em três diferentes fases do período neonatal (do 3° ao 5°; ou do 10° ao 12° ou do 19° ao 21° dias de vida)

visando investigar se os déficits comportamentais observados nos experimentos iniciais seriam específicos à fase do 10° ao 12° dia de vida (fase que corresponde à máxima captação de ferro pelo cérebro) ou se estenderiam durante todo o período neonatal. Os resultados mostraram que os efeitos são máximos quando o ferro é administrado do 10° ao 12° dia, sendo bem menos severos quando administrado do 3° ao 5° dia de vida. Já a administração de ferro do 19° ao 21° dia não produz déficits comportamentais.

No **capítulo IV** (manuscrito em fase de preparação para ser submetido), estudou-se o efeito do tratamento neonatal com ferro combinado com a exposição ao MPTP. Para isso, camundongos C57 BL6 foram tratados do 10° ao 12° dia de vida com ferro (7.5 mg/kg/dia) e quando adultos receberam duas doses diferentes de MPTP. Após o tratamento com MPTP o comportamento motor destes animais foi testado. Posteriormente, foi analisado o conteúdo de ferro nos gânglios da base e o conteúdo de DA no estriado destes animais. Os resultados mostram que o ferro acentua os efeitos deletérios do MPTP, pois os animais que receberam MPTP ambos os tratamentos apresentaram acentuada diminuição na atividade locomotora e nos níveis de DA estriatal.

No sentido de verificar se os efeitos da exposição neonatal ao ferro se estenderiam a outra espécie de roedores, ratos Wistar foram tratados oralmente com 2.5; 7.5; 15.0 ou 30.0 mg Fe^{+2}/kg de peso corporal do 10 ° ao 12° dia de vida pós natal. Na idade adulta a atividade locomotora destes animais foi testada na tarefa do campo aberto e o aprendizado na tarefa do labirinto radial de 8 braços. No **capítulo V** estão descritos os resultados deste grupo de experimentos mostrando que a dose mais alta de

ferro (30.0 mg/kg) produziu diminuição no número de cruzamentos no campo aberto. Na tarefa do labirinto radial, todas as doses de ferro induziram déficits no aprendizado.

Capítulo II

Neonatal iron exposure induces neurobehavioural dysfunction in adult mice

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Neonatal Iron Exposure Induces Neurobehavioural Dysfunctions in Adult Mice

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Abstract: Neonatal Iron Exposure Induces Neurobehavioural Dysfunctions in Adult Mice. Fredriksson, A., Schröder, N., Eriksson, P., Izquierdo I. and Archer T. (1999). *Toxicol. Appl. Pharmacol.* **xxx**, xxx-xxx.

Excess iron in the brain has been implicated in the pathogenesis of several human neurodegenerative disorders, i.e. Parkinson's and Alzheimer's disease. The neonatal period is critical for the establishment of normal iron content in the adult brain. In the present study, the long-term neurobehavioural effects of iron exposure during this period were assessed by treating NMRI mice orally with 0.0, 3.7 or 37.0 mg Fe^{2+}/kg b. w. on postnatal days 10 - 12. Spontaneous motor behaviour and radial arm maze learning were tested at the age of 3 months. It was found that the mice treated with the higher dose of Fe^{2+} 37.0 mg/kg b. w., were hypoactive during the first 20 min of testing but hyperactive during the final 20 min, showing an almost complete lack of habituation of spontaneous activity in the test chambers. These changes were also seen in animals treated with the lower dose of Fe^{2+}, 3.7 mg/kg b. w., but the effects were lesser pronounced indicating dose-response relationship. In the radial arm maze, the Fe^{2+} 37.0 mg/kg group evidenced significantly both more errors in arm choices and longer latencies to acquire all eighth pellets. Both dose groups showed attenuated performance increments on successive trials. Analysis of brain iron content indicated significantly more total iron (μg/g) in the basal ganglia, but not frontal cortex, of the higher, 37 mg/kg, dose group. The knowledge of the long-term effects of iron entering the brain during this critical period of rapid brain growth are limited. Increased amounts of iron in the brain, especially in the basal ganglia may contribute to neurodegenerative processes.

Key words: Iron supplementation - neonatal - Days 10 - 12 - NMRI mice - Fe^{+2} - high/low doses - locomotion - rearing - habituation - radial maze – errors – latency – deficits - total iron content - basal ganglia - parkinsonism.

Introduction

Iron, the most abundant metal ion in the brain (Connor et al. 1995), is involved in numerous metabolic processes including oxidative metabolism, DNA, RNA and protein synthesis, as a cofactor for numerous enzymes such as monoamine synthetic and degradative enzymes, and myelin synthesis (Janetzky et al. 1997; Youdim et al. 1991). It is widely accepted that iron is essential in the neonatal brain for normal neurological development (Connor et al. 1995). Iron deficiency at a tender age in humans, during the period of early development of the brain, has been related to behavioural alterations including deficits in learning and memory (Youdim et al. 1991). However, due to its capacity of catalysing oxidation reactions which may result in cytotoxic free radical formation (Sengstock et al. 1993), iron accumulation in the brain has been implicated in the pathogenesis of several human neurodegenerative disorders, for instance Parkinson's disease (PD) and Alzheimer's disease (Dexter et al. 1991; Kienzl et al. 1995) as well as the aberrant iron metabolism in the brain as typified by the Hallervorden-Spatz syndrome (Swaiman 1991).

Recent findings implicate iron overload in clinical disorders afflicting the brain and central nervous system (Fellman et al. 1998; Rustin et al. 1998; Walshe and Cox 1998). Animal studies have demonstrated the potential neurotoxicity of this metal. Intranigral iron infusions in adult rats produced a dose dependent neuronal death (Sengstock et al. 1993), while, young rats exposed to excess dietary iron evidenced neurobehavioural deficits (Sobotka et al. 1996). Studies concerning iron uptake by the brain have shown that iron transport as well as transferrin (the iron mobilization protein) binding sites are maximal during the period of rapid brain growth (second week of post natal life in mice and rats) (Taylor and Morgan 1990) and the maximum uptake by the brain occurs in 15-days old rats (Taylor et al. 1991). Dwork

and co-workers (Dwork et al. 1990) have demonstrated that the iron acquired by the brain during this period remains in the brain, without returning to plasma sites. Therefore, there appears to be a particular phase within the neonatal period that may be critical for the establishment of normal iron content in the adult brain.

In mammals, the fetus can be indirectly exposed during gestation via maternal intake of toxic agents. During the neonatal period, offspring may be affected by toxic agents by ingesting mother's milk, or be directly exposed to xenobiotics. In many mammalian species a rapid growth of the brain occurs during perinatal development, the so-called "brain growth spurt" (Davison and Dobbing 1968). In the human, this period begins during the third trimester of pregnancy and continues throughout the first year of life. In mouse and rat the corresponding period is neonatal, spanning the first 3-4 weeks of life. This is the critical period during which the brain undergoes several fundamental developmental phases, viz. maturation of axonal and dendritic outgrowth, establishment of neural connections, synaptogenesis, multiplication of glia cells with accompanying myelinization, and cell, axonal and dendritic death (Kolb and Whishaw 1989). This is also the period when animals acquire many new motor and sensory faculties (Bolles and Woods 1964), including advances in spontaneous motor behaviour (Campbell et al. 1969).

During the major part of the infancy, the breast-fed infant will have an iron intake of 0.14-0.32 mg/day (Dewey et al. 1984; Dewey and Lonnerdal 1983). Maternal iron status does not appear to affect the concentration in breast milk (Lonnerdal 1986). It is likely that milk iron concentration is under homeostatic control due to the fact that both anemic women (Celada et al. 1982) and women consuming iron supplements (Zavaleta et al. 1995) have levels of iron in their breast milk comparable with those of nonanemic women. Infants fed iron-fortified formula will have an iron intake of 4.8-7.0 mg/day (Europe) or 10-14 mg/day

(United States) (Lonnerdal 1997).

Taking into account the situation that iron supplementation to healthy infants during their first year of life has been controversial (Schulz-Lell et al. 1987) and that most infant formulae contain a much higher content of iron than breast milk (Lonnerdal 1997) it is relevant to determine whether or not iron exposure (Fe^{+2}) during a selected neonatal period may produce long term deficits in neurobehavioural parameters and/or to alter iron distribution in the brain. Thus spontaneous motor behaviour and radial arm maze acquisition performance were chosen to indicate possible functional deficits. Some expected relationship between iron accumulation in the basal ganglia and these behavioural measures from the viewpoint of the putative role of iron in PD was considered whereas our previous research from lesioning catecholamine systems implicated the possible relevance of the frontal cortex.

Material and Methods

Animals

Pregnant NMRI mice were purchased from B&K, Sollentuna, Sweden. Each litter adjusted within 48 h to 8-10 mice and to contain offspring of either sex in about equal number, was kept together with its respective mother in a plastic cage in a room at temperature of 22 ± 1° C and a 12/12 hours constant light/dark cycle (light on between 06.00 and 18.00 hrs). The male offspring only were used in this study. At the age of 4 weeks the mice were weaned and the males were placed and raised in groups of 4 to 6 animals in a room maintained for male mice only. The animals were supplied with standardized pellet food and tap water ad libitum.

Drugs

Ferromyn® (Iron succinate: 3.7 mg Fe^{++}/ml, AB Hässle, Göteborg, Sweden). Dosages, expressed as mg Fe^{2+}/kg b. w., were administered orally via a metallic gastric tube in a volume of 10 ml/kg b. w.. Saline was used as vehicle and to prepare the different doses of Fe^{2+}. Ferromyn S is applied to the treatment of anemia and as a prophylactic measure for blood donors and pregnant women.

Treatment

Groups of male mice, derived from three to four different litters, were treated with vehicle, 3.7 or 37 mg Fe^{2+} / kg b. w. days 10-12 post partum. For behavioural testing each treatment group consisted of 8 mice, each litter contributing 2 or 3 pups to each treatment group.

Iron content analysis

Mice were killed by cervical dislocation within two weeks after completion of behavioural testing. Brain regions from 4 mice in each group were dissected (Glowinski and Iversen 1966) and stored in minus 70° C until analysed. Determination of total iron was performed by MeAna - Konsult, Uppsala, Sweden, an accredited lab. fulfilling the specifications of SS-EN 45 001 (an accredited laboratory, appointed by the Swedish Board for technical accreditation (SWEDAC) meeting the requirements set up in the quality standards SS-EN 45 001). The tissues were dissolved in analytical bombs under the influence of 3 ml supra pure HNO_3 and heat (150° C for 2 hrs). Analysis was made using atomic absorption spectroscopy (instrument Perkin-Elmer 4100 ZL equipped with a graphite furnace).

Behavioural Measurements and Apparatus

Activity test chambers: An automated device, consisting of macrolon rodent test cages (40 x 25 x 15 cm) each placed within two series of infra-red beams (at two different heights, one low and one high, 2 and 8 cm, respectively, above the surface of the sawdust, 1 cm deep), was used to measure spontaneous motor activity (RAT-O-MATIC, ADEA Elektronic AB, Uppsala, Sweden). The distance between the infra-red beams was as follows: the low levels beams were 73 mm apart lengthwise and 58 mm apart breadthwise in relation to the test chamber; the high level beams, placed only along each longside of the test chamber were 28 mm apart. According to the procedures described previously (Archer et al. 1986), the following parameters were measured: LOCOMOTION was measured by the low grid of infra-red beams. Counts were registered only when the mouse in the horizontal plane, ambulating around the test-cage. REARING was registered throughout the time when at least one high

level beam was interrupted, i.e. the number of counts registered was proportional to the amount of time spent rearing. TOTAL ACTIVITY was measured by a sensor (a pick-up similar to a gramophone needle, mounted on a lever with a counterweight) with which the test cage was constantly in contact. The sensor registered all types of vibration received from the test cage, such as those produced both by locomotion and rearing as well as shaking, tremors, scratching and grooming. All three behavioural parameters were measured over three consecutive 20-min. periods. The motor activity test room, in which all 12 ADEA activity test chambers, each identical to the home cage, were placed, was well-secluded and used only for this purpose. Each test chamber (i.e. activity cage) was placed in a sound-proofed wooden box with 12 cm thick walls and front panels, and day-lighting. Motor activity parameters were tested on one occasion only at the age of 3 months.

Radial arm maze: The radial eight-arm maze, a procedure sensitive to deficits in spatial learning performance (Olton et al. 1978; Olton and Werz 1978), was modified and adapted to evaluate maze learning performance in mice (Fredriksson et al. 1996). Radial arm maze testing was performed when the animals were three months of age. The 8 arms (each 36.0 cm long, 7.1 cm wide and 2.0 cm high enclosing walls) of the maze extended radially from a central hub (16 x 16 cm). The maze was placed on a table in the centre of the test room. At testing, all the mice were placed on a total food deprivation for 24 hours. For the learning trials, a food pellet (10 mg) was placed at the extremity of each arm. At the start of each test trial, the mouse was placed in the central hub and then monitored for its instrumental learning performance, i.e., the latency until all 8 pellets were collected and the number of arms visited in collecting all 8 pellets subtracted by 8 provided the number of errors per animals. Errors were defined as re-entries to arms already visited. Within each arm,

photocell beams were located at distances of 6.0, 18.0, 30.0 cm from the central hub. The photocells of each arm registered every time a mouse approached to within 3.0 cm of the food cup, thus monitoring every arm entrance from 6 to 30 cm depth. An upper time limit of 10 min was applied throughout. The maze-learning test room was secluded, without any explicitly arranged extra-maze cues. Each mouse was tested for one trial only, on each of three consecutive days. Each animal was observed carefully to ensure it consumed each pellet.

Statistical analysis

The locomotion, rearing and total activity data over three consecutive 20-min. periods in the activity test chambers as well as the latency until all 8 pellets were collected and the number of errors, over three consecutive testing days in the radial maze were submitted to a split-plot ANOVA design (Kirk 1995). Brain levels of iron were submitted to an one-way ANOVA design (Kirk 1995). Pairwise testing between the different treatment groups was performed with the Tukey HSD test (Kirk 1995).

Results

Weight-gain

No significant differences between the groups regarding measures of weight-gain ($F(2, 21) = 0.16$, $p=0.85$) were observed after one week as a consequence of the different treatments employed. Neither were there any significant differences between the group mean weights ($F(2, 21) = 0.08$, $p=0.92$) when the behavioural testing started. Mean body weight values were as follows: Saline: 33.8±1.7, Fe^{2+} 3.7 mg/kg: 34.1±1.6 and Fe^{2+} 37.0 mg/kg: 34.5±1.5.

Spontaneous motor activity

There were significant interaction effects (Groups X Time periods) for all the three parameters of spontaneous behaviour (Locomotion: $F(4, 42) = 21.55$, $p<0.0001$; Rearing: $F(4, 42) = 27.84$, $p<0.0001$; Total activity: $F(4, 42) = 17.09$, $p<0.0001$) measured over three consecutive 20 min periods. Comparison of group means indicated that the highest dose group, Fe^{2+} 37.0 mg/kg, showed fewer locomotion counts during the 1st 20-min period but more counts than the vehicle-treated mice during the final 20 min: the low dose group, Fe^{2+} 3.7 mg/kg, also showed more locomotor activity during the 3rd period. Significant dose differences were seen during the 1st and final periods. Rearing behaviour was affected in both dose groups: during the 1st 20-min period (also the 2nd 20-min period for the Fe^{2+} 37.0 mg/kg group) counts were fewer than the vehicle-treated mice but more during the final 20-min period. Dose differences were seen during the 1st period (see Figure 1). For total activity, fewer counts were obtained for the Fe^{2+} 37.0 mg/kg group during the 1st period but more counts during

the third period. Compared to the Fe^{2+} 3.7 mg/kg group, the Fe^{2+} 37.0 mg/kg group obtained fewer counts during the 1st two periods. In order to assess the extent of habituation to the test cages over each 20-min interval an habituation quotient was derived by dividing numbers of counts during the first 20-min by that obtained during the second 20-min period, and counts during the second 20-min period by those obtained during the third 20-min period. In each case the result of each division was multiplied by 100 to give a quotient representing the reduction of counts from the first to the second to the third period for each mouse (cf.). Thus the obtained quotients were subjected to split-plot ANOVA that indicated a significant Groups x Quotients interaction: $F(3, 20) = 189.21$, $p<0.0001$. Mean quotient values per group were as follows:-

		1st quotient[1]	2nd quotient[2]
Saline	Locom.	215±18	1642±15
	Rear.	242±15	1982±9
3.7	Locom.	181±18*	147±11*
	Rear.	208±9*	143±6*
37.0	Locom.	171±12*	95±6*
	Rear.	106±11*	83±7*

Values represent mean habituation quotients ± SD

*p<0.01, Tukey HSD tests versus appropriate saline comparison

[1]first 20-min / second 20-min x 100

[2]second 20-min / third 20-min x 100

The habituation quotient analysis indicates that the reductions in activity counts from the first to the second to the third 20-min intervals were significantly greater in the saline group than in the iron dose groups.

Insert Figure 1 here

Radial arm maze learning

There were significant interaction effects (Groups X Days) for both the parameters of radial arm maze learning (Number of errors: $F(4, 42) = 6.59$, $p<0.0003$; Latency to the last pellet: $F(4, 42) = 3.17$, $p<0.0229$) measured over three consecutive testing days. Comparison of group means indicated that the highest dose group, Fe^{2+} 37.0 mg/kg, compared with both the vehicle and the Fe^{2+} 37.0 mg/kg dose group, showed a greater number of errors as well as longer latencies to acquire all eight pellets during the final of three consecutive test days in the maze. Note that the Fe^{2+} 3.7 mg/kg dose group although not significantly different from controls was intermediate and also made less errors compared with the Fe^{2+} 37.0 mg/kg dose group on Day 2.

There were significant main effects (Days) for both the parameters of radial arm maze learning (Number of errors: $F(2, 42) = 25.44$; Latency to the last pellet: $F(2, 42) = 33.39$) between the treatments. Tukey HSD tests indicate that while the vehicle group improved learning from Days 1 to 2 to 3 the Fe^{2+} 37.0 mg/kg dose group, and to a lesser extent the Fe^{2+} 3.7 mg/kg dose group, failed to decrease numbers of errors or demonstrate shorter latencies

to the last pellet.

Insert Figure 2 here

Analysis of brain iron content

There was a significant treatment effect for the total iron content in the basal ganglia ($F(2, 11)= 9.76$) in contrast to the frontal cortex ($F(2, 11)=0.49$). Tukey HSD test indicated that both groups that had received iron, i.e. Fe^{2+} 3.7 mg/kg and Fe^{2+} 37.0 mg/kg, neonatally, showed increased concentrations of total iron (µg/g) in the basal ganglia. This effect was statistically significant only in the case of the higher dose, Fe^{2+} 37.0 mg/kg.

Insert Table 1 here

Discussion

The results presented above may be summarised as follows: (1) The neonatal treatment with Fe^{2+} (3.7 or 37.0 mg/kg, days 10-12) did not influence body weight gain. (2) Mice that had received the higher dose Fe^{2+} 37.0 mg/kg showed marked hypokinesia for locomotion, rearing and total activity during the first two 20-min periods but were more active during the final 20 min; the lower dose Fe^{2+} 3.7 mg/kg group was hypokinesic for rearing during the first 20 min and hyperkinesic during the final 20 min for locomotion and rearing parameters. (3) In the radial arm maze learning, the Fe^{2+} 37.0 mg/kg group made more errors and showed longer latencies on the final third day of testing. (4) In the activity test chambers and radial arm maze, respectively, deficits in habituation to the test chamber and deficits in performance increments over successive test trials were evidenced for both Fe^{2+} dose groups. (5) Analysis of total iron content (μg/g) in brain regions indicated notably elevated levels in the basal ganglia, but not frontal cortex, of the Fe^{2+} 37 mg/kg group.

During early life, infants usually consume a diet that is heavily dominated by milk. It is generally believed that breast-fed infants absorb adequate quantities of minerals and trace elements, whereas there is some concern about how well infants can utilize these nutrients from cow's milk formulae and other infant diets. Therefore, most infant formulae contain much higher concentrations of minerals and trace elements than those of breast milk. Present understanding of how infants may utilize these nutrients from different diets is very limited (Lonnerdal 1997). Iron balance studies performed in full term male infants from their 3rd until their 17th week of life showed that the group receiving a formula (iron content 10.35 mg/l) retained up to 40 times more iron than the breast- fed babies (Schulz-Lell et al. 1987). From comparison milk-based, iron-fortified formulae containing 7.4 and 12.7 mg/L iron and breast-feeding during the first year of life, conclusions may be drawn that both support

normal growth and iron status of healthy, term, normally fed infants during the first year and both are well tolerated and accepted (Bradley et al. 1993). Regional differences in water content of iron may add a significant amount to the fortified formulae.

Several studies have demonstrated that a selective and significant elevation of iron is found in the substantia nigra of patients with Parkinson's disease (Dexter et al. 1987; Drayer et al. 1986; Riederer et al. 1989). In laboratory studies, Sengstock et al. (Sengstock et al. 1993) demonstrated neurodegenerative effects of infused iron (ferric chloride, 1.25 – 6.3 nmol unilaterally) in the rat substantia nigra. They obtained a very high correlation between amount of iron infused intranigrally and magnitude of striatal DA, DOPAC and HVA within the ipsilateral striatum. Similarly, Youdim et al. (Youdim et al. 1991) obtained substantial reductions in striatal dopaminergic markers following infusions of 5 µl of 179 mM into rat substantia nigra. Further, Sengstock et al. (Sengstock et al. 1994) described evidence that excess Fe infusion into the rat substantia nigra may be capable of inducing persistent or progressive neurodegeneration changes relevant to PD. Thus, unilateral Fe infusions (1.25 or 2.10 nmol into the substantia nigra) caused neuronal losses in the substantia nigra pars compacta, a dose-dependent and progressive nigral atrophy, progressive reductions of DA and HVA following infusion of 1.25 nmol Fe, and progressive increases in apomorphine-induced rotational behaviour (an established marker for parkinsonism in the rat laboratory). The authors postulated the pattern of changes establish intranigral Fe infusion as an animal model of PD (Sengstock et al. 1994). Besides these findings, MPTP-lesioned hemiparkinsonian monkeys and 6-OHDA lesioned rats exhibit iron accumulation in substantia nigra (Oestreicher et al. 1994; Temlett et al. 1994).

The effects of iron 'overload' within the substantia nigra and upon striatal DA neurons have been described above (Sengstock et al. 1994; Sengstock et al. 1993), but the functional

consequences of this overload are of necessary comparison to the present issue. Sobotka and co-workers (Sobotka et al. 1996) fed weaning rats diets consisting of Fe at 4 (Fe-deficient), 35 (control), 350, 3500 or 20000 ppm for 12 weeks, testing for behavioural and b. w. changes at different intervals (food-intake or calculated dose not shown). Body weight was reduced in the Fe-deficient rats and those that received 20000 ppm, and to a lesser extent those that received 3500 ppm. Similarly, total activity was decreased in the Fe-deficient rats and 20000 ppm group, and Slope, a measure of habituation, was deficient in the 3500 ppm Fe group also. Avoidance learning and prepulse inhibition were both defective in the Fe-deficient rats and 20000 ppm group, while by diet week 10 prepulse inhibition was defective even in the 3500 ppm group. Brain concentrations of total nonheme iron (μg/g) indicated a significant reduction of Fe in Fe-deficient (4 ppm) group and a significant excess in the 20000 ppm group that should be compared directly with the behavioural deficits described (Sobotka et al. 1996). These behavioural deficits appear to conform with the present hypokinesia on locomotion, rearing and total activity, the deficits in radial arm performance, the attenuated habituation in the activity test chambers, consecutive daily performance increments in the maze and the significantly elevated total iron concentrations in the basal ganglia of the mouse species tested in view of the discussed role of these functions in disorders such as PD (Schmidt and Kretschmer 1997).

Our knowledge of the long-term effects of iron entering the brain during this critical period of rapid brain growth are limited. However, both the clinical evidence demonstrating consequences of iron overload (Rustin et al. 1998) and the preclinical results appear to underline the putatively deleterious effects of excesses of the metal. Increased amounts of iron in the brain, especially in the basal ganglia may contribute to neurodegenerative processes.

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Table 1. Total brain iron in frontal cortex and basal ganglia (µg/g wet weight) in four months old NMRI male mice following an oral exposure to iron. Groups of male mice, derived from three to four different litters, were treated with vehicle, 3.7 or 37 mg Fe^{2+} / kg b. w. days 10-12 post partum. Statistical analysis of data was performed using One-way ANOVA. Pairwise testing between each of the treatment groups was performed with the Tukey HSD test.

	Frontal Cortex	Basal Ganglia
Control	26.3±3.5	38.5±3.4
Low dose (3.7 mg/kg)	27.2±4.7	46.8±8.0
High dose (37.0 mg/kg)	29.5±5.5	61.1±9.2**

Data are presented as mean ± SD, n=4 in each treatment group, ** $p \leq 0.01$ compared with Control.

Figure 1. *Spontaneous behaviour in three months old NMRI male mice following an oral exposure to iron. Groups of male mice (n = 8), derived from three to four different litters, were treated with vehicle, 3.7 or 37 mg Fe^{2+} / kg b. w. days 10-12 post partum. For measurement of motor activity, see Materials and Methods. Statistical analysis of behavioural data was performed using ANOVA with a Split-plot design. There were significant Group x Period interactions (F(4,42) = 21.55, F(4,42) = 27.84 and F(4,42) = 17.09) for the variables Locomotion, Rearing and Total activity, respectively. Pairwise testing between each of the treatment groups was performed with the Tukey HSD test. Bars represent mean + SD. Letters (upper case .01 and lower case .05) indicate significant differences where A represent comparison between vehicle and iron treated groups and B between the iron treated groups.*

Figure 2. *Radial arm maze learning in three months old NMRI male mice following an oral exposure to iron. Groups of male mice (n = 8), derived from three to four different litters, were treated with vehicle, 3.7 or 37 mg Fe^{2+} / kg b. w. days 10-12 post partum. Statistical analysis of behavioural data was performed using ANOVA with a Split-plot design. There were significant Group x Days interactions for both the parameters of radial arm maze learning (Number of errors: F(4,42) = 6.59; Latency to the last pellet: F (4,42) = 1.80) measured over three consecutive testing days. Pairwise testing between each of the treatment groups was performed with the Tukey HSD test. Bars represent mean + SD. Letters (upper case .01 and lower case .05) indicate significant differences where A represent comparison between vehicle and iron treated groups and B between the iron treated groups.*

Figure 1

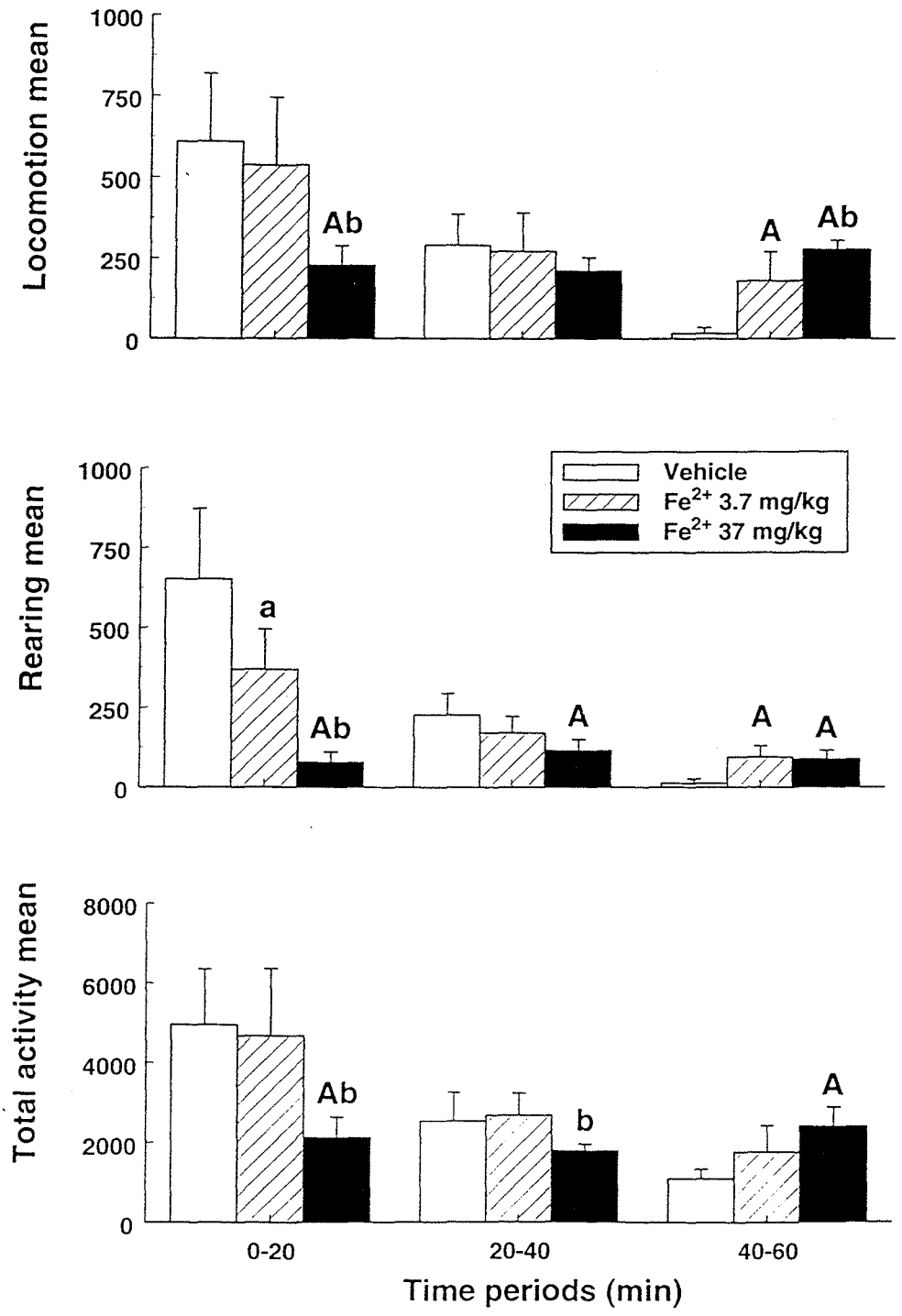
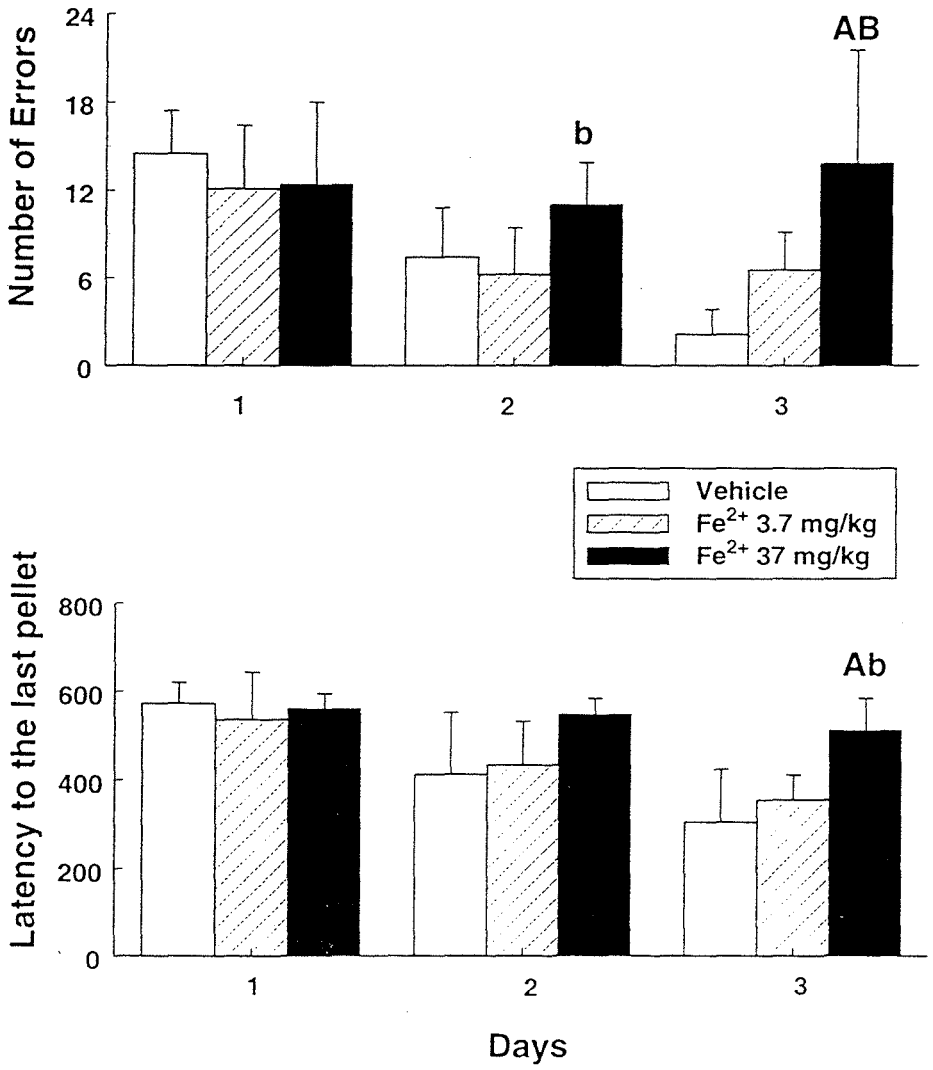


Figure 2


Number of Errors
24
18
12
6
0
1
2
3
b
AB
Vehicle
Fe2+ 3.7 mg/kg
Fe2+ 37 mg/kg
Latency to the last pellet
800
600
400
200
0
1
2
3
Ab
Days

Capítulo III

Maze learning and motor activity deficits in adult mice induced by iron exposure during a critical postnatal period

Developmental Brain Research, submetido

Maze Learning and Motor Activity Deficits in Adult Mice Induced by Iron Exposure During a Critical Postnatal Period

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Abstract

Newborn mice were administered Fe^{2+} (iron succinate: 7.5 mg/kg, b.w.) on either Days 3-5, 10-12 or 19-21, or vehicle (saline) at the same times, postnatally.

Spontaneous motor behaviour and radial arm maze learning were tested at the age of three months. It was found that mice treated with Fe^{2+} during postnatal Days 10-12 were marked hypokinesic during the 1st 20-min test period and hyperkinesic during the 3rd and final 20-min test period. These mice showed an almost complete lack of habituation of spontaneous motor activity parameters to the test chambers. In the radial arm maze the Days 10-12 treatment group evidenced significantly both more errors in arm choices and longer latencies to acquire all eight pellets; these mice showed also a severe trial-to-trial retention deficit as indexed by retention quotients. These behavioural deficits were observed also in animals treated with Fe^{2+} during postnatal Days 3-5, but the effects were less pronounced indicating the higher susceptibility of the brain for Fe^{2+}-induced damage, during Days 10-12 post partum. Treatment with Fe^{2+} on Days 19-21 did not induce behavioural alterations in comparison with its respective control (vehicle) group. Analysis of total brain iron content indicated significantly more iron (μg/g) in the basal ganglia, but not frontal cortex, of mice from days 3-5 and 10-12 Fe^{2+} treatment groups. The contribution of iron-overload during the immediate postnatal to later functional deficits seems implicated and the kinetics of iron uptake to the brain and its regional distribution at this critical period of development await elucidation.

Theme: Disorders of the nervous system Topic: Neurotoxicity

Keywords: Iron administration - postnatal - motor behaviour - habituation - radial maze learning - basal ganglia - NMRI mice.

1. Introduction

During perinatal development of the brain, there are periods of neuronal tissue development that be critical for a normal maturation [36]. Iron, the most abundant metal in the human body and brain [7], is involved in several metabolic processes and is essential for normal neurological development (cf. [26,51], and iron deficiency during critical periods of development is associated with disruptions of behavioural performance, e.g. in learning and memory tasks [52]. On the other hand, there is accumulating evidence that excessive iron deposits in the brain, which may generate cytotoxic free radical formation [43], and alterations in iron metabolism play an important role in many neurologic diseases [18,33,45]. Iron-overload is implicated in the pathogenesis of several human neurodegenerative disorders, i.e. Parkinson's disease (PD) and Alzheimer's disease (AD) [12,27]) and in the Hallervorden-Spatz syndrome, involving aberrant brain iron metabolism [46]. Several studies have demonstrated that a selective and significant increases of iron are found in the substantia nigra of patients afflicted with Parkinson's disease [13,14,37].

The immediate postnatal period is critical for establishment of normal iron content in the adult brain and its regional distribution. Investigations of cerebral iron uptake indicate that both iron transport and transferrin, the iron mobilization protein, binding sites are maximal during the postnatal period of rapid brain growth, essentially during the second week post partum in rats and mice [48], and maximal brain iron uptake occurs in 15-day-old rats [47]. Dwork et al. [15] showed that iron acquired by the brain during this period of development is retained in the brain without being returned to plasma sites. In many mammalian species, a period of rapid brain growth occurs during perinatal development, termed "brain growth spurt" [9]. In humans this period begins during the third trimester of pregnancy and is maintained throughout the first year of life whereas in rats and mice the corresponding period occurs during the first three-to-four weeks of postnatal life. During this critical period of neuronal development the essential processes of brain structure and function are established [38] and fundamental sensory-motor faculties are acquired [3,5]. Earlier studies have shown that low dose exposure to xenobiotics during a critical phase of the neonatal development of the mouse brain can lead to disruption of brain function in the adult [1,16,17]. Thus, it appears that there may exist a critical neonatal period of brain development associated with the establishment of normal iron content in the adult brain.

Recent findings implicate cerebral iron overload in neurologic disorders afflicting the brain and CNS [19,38,51]. Laboratory studies have indicated that intranigral iron infusions to adult rats produced a dose-dependent neuronal death [43], and further that young rats exposed to excess dietary iron evidenced neurobehavioural deficits [44]. In the present study the long-term neurobehavioural effects of iron exposure during this period were assesed by treating NMRI mice orally with Ferromyn S (7.5 mg Fe^{2+} /kg b.w.) either days 3-5, 10-12 or 19-21 post partum. Spontaneous motor behaviour and radial arm maze learning were tested at the age of three months.

2. Material and methods

Animals

Pregnant NMRI mice were purchased from B&K, Sollentuna, Sweden. Each litter adjusted within 48 h to 8-10 mice and to contain offspring of either sex in about equal number, was kept together with its respective mother in a plastic cage in a room at temperature of 22 ± 1° C and a 12/12 hours constant light/dark cycle (lights on between 06.00 and 18.00 hrs). The male offspring only were used in this study. At the age of 4 weeks the mice were weaned and the males were placed and raised in groups of 4 to 6 animals in a room maintained for male mice only. The animals were supplied with standardized pellet food and tap water ad libitum. Experiments were carried out in accordance with the European Communites Council Directive of 24 November 1986 (86/609/EEC) after approval from the local ethical committee.

Drugs

Ferromyn® (Iron succinate: 3.7 mg Fe^{++}/ml, AB Hässle, Göteborg, Sweden). Dosages, expressed as mg Fe^{2+}/kg b. w., was administered orally via a metallic gastric tube in a volume of 10 ml/kg body weight. Saline was used as vehicle and to prepare the dose of Fe^{2+}. Ferromyn S is applied to the treatment of anemia and as a prophylactic measure for blood donors and pregnant women.

Treatment

Groups of male mice, derived from three to four different litters, were treated with vehicle or 7.5 mg Fe^{2+} / kg b. w. either days 3-5, 10-12 or 19-21 post partum. For behavioural testing each treatment group consisted of 10 mice, each litter contributing not more than 1 or 2 pups to each treatment group.

Iron content analysis

Mice were killed by cervical dislocation within two weeks after completion of behavioural testing. Brain regions from 4 mice in each group were dissected [25] and stored in minus 70° C until analysed. Determination of total iron was performed by MeAna - Konsult, Uppsala, Sweden, an accredited laboratory, appointed by the Swedish Board for technical accreditation (SWEDAC) meeting the requirements set up in the quality standards SS-EN 45

001. The tissues were dissolved in analytical bombs under the influence of 3 ml supra pure HNO_3 and heat (150° C for 2 hrs). Analysis was made using atomic absorption spectroscopy (instrument Perkin-Elmer 4100 ZL equipped with a graphite furnace).

Behavioural Measurements and Apparatus

Activity test chambers: An automated device, consisting of macrolon rodent test cages (40 x 25 x 15 cm) each placed within two series of infra-red beams (at two different heights, one low and one high, 2 and 8 cm, respectively, above the surface of the sawdust, 1 cm deep), was used to measure spontaneous motor activity (RAT-O-MATIC, ADEA Elektronic AB, Uppsala, Sweden). The distance between the infra-red beams was as follows: the low levels beams were 73 mm apart lengthwise and 58 mm apart breadthwise in relation to the test chamber; the high level beams, placed only along each longside of the test chamber were 28 mm apart. According to the procedures described previously [2], the following parameters were measured: LOCOMOTION was measured by the low grid of infra-red beams. Counts were registered only when the mouse in the horizontal plane, ambulating around the test-cage. REARING was registered throughout the time when at least one high level beam was interrupted, i.e. the number of counts registered was proportional to the amount of time spent rearing. TOTAL ACTIVITY was measured by a sensor (a pick-up similar to a gramo-phone needle, mounted on a lever with a counterweight) with which the test cage was constantly in contact. The sensor registered all types of vibration received from the test cage, such as those produced both by locomotion and rearing as well as shaking, tremors, scratching and grooming. All three behavioural parameters were measured over three consecutive 20-min. periods. The motor activity test room, in which all 12 ADEA activity test chambers, each identical to the home cage, were placed, was well-secluded and used only for this purpose. Each test chamber (i.e. activity cage) was placed in a sound-proofed wooden box with 12 cm thick walls and front panels, and day-lighting. Motor activity parameters were tested on one occasion only, over three consecutive 20-min periods, at the age of 3 months.

Radial arm maze: The radial eight-arm maze, a procedure sensitive to deficits in spatial learning performance [34,35], was modified and adapted to evaluate maze learning performance in mice [22]. Radial arm maze testing was performed when the animals were

three months of age. The 8 arms (each 36.0 cm long, 7.1 cm wide and 2.0 cm high enclosing walls) of the maze extended radially from a central hub (16 x 16 cm). The maze was placed on a table in the centre of the test room. At testing, all the mice were placed on a total food deprivation for 24 hours. For the learning trials, a food pellet (10 mg) was placed at the extremity of each arm. At the start of each test trial, the mouse was placed in the central hub and then monitored for its instrumental learning performance, i.e., the latency until all 8 pellets were collected and the number of arms visited in collecting all 8 pellets subtracted by 8 provided the number of errors per animals. Errors were defined as re-entries to arms already visited. Within each arm, photocell beams were located at distances of 6.0, 18.0, 30.0 cm from the central hub. The photocells of each arm registered every time a mouse approached to within 3.0 cm of the food cup, thus monitoring every arm entrance from 6 to 30 cm depth. An upper time limit of 10 min was applied throughout. The maze-learning test room was secluded, without any explicitly arranged extra-maze cues. Each mouse was tested for one trial only, on each of three consecutive days. Each animal was observed carefully to ensure it consumed each pellet. Fe 2+ and vehicled mice were tested 2 days after the test of spontaneous motor behaviour.

Statistical analysis

The locomotion, rearing and total activity data over three consecutive 20-min. periods in the activity test chambers as well as the latency until all 8 pellets were collected and the number of errors, over three consecutive testing days in the radial maze were submitted to a split-plot ANOVA design [28]. Brain levels of iron were submitted to an one-way ANOVA design [28]. Pairwise testing between the different treatment groups was performed with the Tukey HSD test [28].

3. Results

Weight-gain

No significant differences between the groups regarding measures of weight-gain ($F(5, 54) = 0.03$) were observed after one week as a consequence of the different treatments employed. Neither were there any significant differences between the group mean weights ($F(5, 54) = 0.11$) when the behavioural testing started.

Spontaneous motor activity

Postnatal administration of Fe^{2+} on Days 10-12, and to a lesser extent on Days 3-5, disrupted sponataneous motor behaviour. There were significant interaction effects (Groups X Time periods) for all the three parameters of spontaneous behaviour (Locomotion: $F(10, 108) = 10.05$; Rearing: $F(10, 108) = 11.96$; Total activity: $F(10, 108) = 6.40$) measured over three consecutive 20 min periods. Comparison of group means indicated that the Fe^{2+} dose group treated on Days 10-12, showed fewer locomotion, rearing and total activity counts compared to vehicle animals during the 1st 20-min period, and for rearing the 2nd 20-min period as well as, but more counts than the vehicle-treated mice during the final (3rd) 20 min (see Figure 1). The Fe^{2+} dose

Insert Fig. 1 here

group treated on Days 3-5, showed fewer rearing during the 1st 20-min period but more locomotion, rearing and total activity counts than the vehicle-treated mice during the final 20 min. Significant differences between Fe^{2+} groups and treatment days were seen during the 1st and final periods. Locomotion, rearing and total activity behaviours were affected more in the Days 10-12 treatment group during the 1st 20-min period with fewer counts than both the mice treated days 3-5 or 19-21, together with more locomotion during the final 20-min period. Fewer counts were seen for the rearing variable the second (compared to Days 19-21 Fe^{2+} treated mice) and the final 20-min period (compared to Days 3-5 Fe^{2+} treated mice). Compared to mice treated on Days 19-21, both the Days 3-5 and 10-12 Fe^{2+} treated mice showed significantly more counts for all three variables.

Habituation to Activity test chambers

Habituation is a relatively simple, nonassociative form of learning in situations where repeated measures of behaviour are monitored. In order to access the extent of habituation to the activity test chambers over each successive 20-min interval, an habituation quotient for each mouse was derived by dividing the numbers during the 1st 20-min by that obtained during the 2nd 20-min period, and counts during the 2nd 20-min period by those obtained during the 3rd 20-min period. In each case the result of each division was multiplied by 100 to provide a quotient representing the reduction of activity counts from the first to the second to the third period for each mouse (cf. [8,21]. Thus, the obtained quotients were subjected to split-plot ANOVA that indicated significant Groups x Quotients interactions, as follows: Locomotion: $F(5, 108) = 95.67$, Rearing: $F(5, 108) = 43.13$; and Total activity: $F(5, 108) = 68.58$. The mean quotient values per group (i.e. Days 3-5, 10-12, and 19-21 treatment) are presented in Table 1. The habituation quotient analysis reveals that the reductions in motor

Insert Table 1 here

Activity from the 1st to the 2nd to the 3rd 20-min intervals of testing were significantly greater in the vehicle group than in the Days 3-5 and Days 10-12 Fe^{2+} (7.5 mg/kg) treatment groups. Habituation quotients were significantly higher in the vehicle group than in the Days 3-5 and Days 10-12 treatment groups, respectively.

Radial arm maze learning

Radial arm maze learning performance was disrupted by the neonatal administration of Fe^{+2} on Days 3-5 and 10-12, with a more pronounced detrimental effect on Days 10-12. Thus, there were significant interaction effects (Groups X Days) for both the applied parameters of radial arm maze learning (Number of errors: $F(10, 108) = 4.67$; Latency to the last pellet: $F(10, 108) = 4.30$) measured over three consecutive testing days. Figure 2 presents the mean

Insert Fig. 2 here

Number of errors and the Latency (secs) to acquire all eight pellets. Comparison of group means (Tukey HSD tests) indicated that the Fe^{2+} group treated on Days 10-12, showed a

greater number of errors as well as longer latencies to acquire all eight pellets during the final (i.e. 3rd trial) of three consecutive test days in the maze compared with all the other groups (see figure 2). Note that the Fe^{2+} group treated days 3-5 was intermediate for both parameters day 3 and made significantly more errors compared with the Fe^{2+} group treated days 19-21. There were significant main effects (days) for both the parameters of radial arm maze learning (Number of errors: $F(2, 108) = 168.23$; Latency to the last pellet: $F(2, 108) = 87.21$) between the treatments. Tukey HSD tests indicate indirectly that while the vehicle groups and the Fe^{2+} group treated on Days 19-21 improved learning from Days 1 to 2 to 3, whereas the Fe^{2+} group treated on Days 10-12, and to a lesser extent the Fe^{2+} group treated on Days 3-5, failed to decrease numbers of errors or demonstrate shorter latencies to the last pellet. In order to demonstrate directly the differences between groups in showing learning improvements from the 1st to 2nd to 3rd trials, retention quotients were tabulated.

Learning retention over consecutive trials

Retention quotients pertaining to number of errors and latency to last pellet acquisition from the 1st, 2nd and 3rd trials (on three consecutive days) in the radial arm maze, derived by dividing numbers of errors/secs during the 1st trial by that obtained during the 2nd trial, and the errors/secs during the 2nd trial by that obtained during the 3rd trial. In each case the result was multiplied by 100 to provide a quotient representing the retention of radial maze learning over test trials with a 24-hr interval. Thus, the obtained retention quotients were subjects to split-plot ANOVA that indicated significant Groups x Quotients interactions:
Number of Errors: $F(5, 108) = 34.21$; Latency to last pellet: $F(5, 108) = 41.77$. Table 2 presents the retention quotients of mice treated with vehicle or Fe 2+ 7.5 mg/kg on Days 3-5, 10-12 and 19-21 after birth. Tukey HSD testing indicated

Insert Table 2 here

Significantly lower retention quotients by the Days 10-12 Fe 2+ group (ranging from 96 to 126) than by the respective vehicle group (ranging from 131 to 615), and to a lesser degree by the Days 3-5 Fe 2+ group (ranging from 180 to 107) compared to the respective vehicle group

(ranging from 334 to 134). A retention quotient of 100 or less indicates a complete failure of retention from one trial to the next.

Analysis of brain iron content

There was a significant treatment effect for the total iron content in the basal ganglia ($F(5, 18) = 24.33$) in contrast to the frontal cortex ($F(5, 18) = 0.55$, ns). Tukey HSD test indicated that the Fe^{2+} groups treated on Days 3-5 and 10-12, showed increased concentrations of total iron (µg/g) in the basal ganglia compared with all vehicle-treated groups and also the Fe^{2+} groups treated on Days 19-21 (see Table 3).

Insert Table 3 here

4. Discussion

The results of neonatal administration of Fe^{2+} during different periods postpartum, i.e. Days 3-5, 10-12 or 19-21 may be summarised as follows:-

(1) Neonatal treatment of mice with Fe^{2+} 7.5 mg/kg on Days 10-12, and to a lesser extent on Days 3-5 (rearing only), demonstrated a marked hypokinesia for locomotor, rearing and total activity during the 1st 20-min test period that was maintained during the 2nd 20-min period for rearing only (but not for the Days 3-5 group. During the 3rd 20-min test period both the Days 10-12 and Days 3-5 Fe^{2+} groups were hyperkinesic over all three parameters of motor activity.

(2) Neonatal treatment with Fe^{2+} on Days 10-12, and to a lesser extent on Days 3-5, marked impaired acquisition performance in the radial arm maze as indexed by both more errors and longer latenies to acquire all eight pellets on the 3rd test trial (third and final day of testing). Mice treated on Days 10-12 showed also longer latencies on the 2nd test trial compared with their respective vehicle group.

(3) In the motor activity test chambers, deficits in habituation to the test chambers as indexed by habituation quotients point to interference of a simple, nonassociative type of learning by the Days 10-12 and Days 3-5 Fe^{2+} groups.

(4) In the radial arm maze, deficits in retention of the maze-configuration stimuli as indexed by retention quotients suggest some interference of memory-for-maze-cues by the groups administered Fe^{2+} on Days 10-12 and 3-5 postnatally. (5) The analysis of total iron content (µg/g) in brain regions indicated notably elevated levels in the basal ganglia, but not frontal cortex, of mice administered Fe 2+ on Days 3-5 and 10-12, but not on Days 19-21. This effect was most pronounced in mice treated on Days 10-12. (6) Neonatal treatment with Fe 2+ 7.5 mg/kg on either Days 3-5, 10-12 or 19-21 did not influence body weight gain. Ancilliary to the above main findings it was shown that the vehicle treatment on Days 3-5, postnatally, elevated the habituation quotient, whereas vehicle treatment on Days 10-12 increased the retention quotient, compared to the others vehicle groups, respectively. These results support our earlier findings that neonatal exposure to iron can cause behavioural disorder in adults [23] and also that the induction of these disturbances in the adult animal occurs during a defined critical stage of the the neonatal development of the mouse brain [1,16,17].

Factors affecting iron intake during infancy would appear to be of some relevance on the basis of the present results. Breast-fed infants have a relatively constant iron intake of 0.14 - 0.32 mg/day during the major portion of infancy [10,11], and maternal iron status appears not to affect breast milk concentrations [29]. It is probable that the iron concentrations of maternal milk are under homeostatic control due to the finding that both anemic women [6] and women consuming iron supplements [53] have breast milk levels of iron comparable with those of nonanemic women. Infants fed on an iron-fortified diet formula will consume an iron intake of 4.8 - 7.0 mg/day (Europé) or 10 - 14 mg/day (United States) (cf. [30]. Iron balance studies performed in full term male infants from their third until their 17t^h week of life indicated that the group receiving an iron-fortified diet formula (iron content 10.35 mg/l) retained upto 40 times more iron than the breast-fed infants [41]. From comparisons of milk-based, iron-fortified formulae containing 7.4 and 12.7 mg/L iron and breast-feeding infants during the first year of life, it seems evident that both normal growth and iron status of infants are supported [4]. Regional differences in water iron content may add significant amounts to the fortified diet formulae.

In laboratory investigations potentially neurodenerative effects of iron administered to experimental animals have been observed. For example, Sengstock et al. [43] demonstrated neurochemical alterations following the infusion of iron (ferric chloride, 1.25 - 6.3 nmol unilaterally) into the substantia nigra of rats. They obtained a very high correlation between the amount of iron infused intranigrally and the concentrations of dopamine (DA), dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) within the ipsilateral striatum of each rat. Further, Sengstock et al. [42] indicated that excess iron infusion into the substantia nigra of rats induced persistent or progressive neurodegenerative changes relevant to PD. They infused iron into the substantia nigra (1.25 or 2.10 nmol) causing neuronal loss in the substantia nigra pars compacta, a dose-dependent and progressive nigral atrophy, progressive reductions of DA and HVA following infusion of 1.25 nmol, and progressive increases in apomorphine-induced rotational behaviour (an established marker for parkinsonism in the rat laboratory). This pattern of neuropharmacological alterations by unilateral intranigral iron infusion was postulated to offer an animal model of PD [42], bearing in mind the unilateral intranigral 6-hydroxydopamine administration technique

(cf. [50]. Similarly, Youdim et al. [52] obtained substantial reductions in striatal dopaminergic markers following infusions 5 μl of 179 mM ferric chloride into the substantia nigra of rats. Finally, it has been shown that MPTP-lesioned hemiparkinsonian monkeys and 6-OHDA lesioned rats exhibit iron accumulation in the substantia nigra [32,49].

The functional consequences of iron overload to the developing brain are relevant to the present findings. Sobotka et al. [44] fed weanling rats diets consisting of iron either at 4 (iron-deficient), 35 (control), 350, 3500 or 20000 ppm for 12 weeks, testing for behavioural and body weight changes at different intervals (food-intake or calculated dosage not shown). Body weight reductions were found in the iron-deficient (4 ppm) rats and those that received 20000 ppm, and to a lesser extent those that had been administered 3500 ppm. Similarly, total activity was decreased in the iron-deficient rats and the 20000 ppm group, whereas Slope, a measure of habituation to the test chamber, was deficient in the 3500 ppm group as well. Avoidance learning and prepulse inhibition were both impaired in the iron-deficient rats and the 20000 ppm group, whereas by Week 10 of the iron-modulated diet prepulse inhibition, a sensory-gating assessment technique [24], was defective even in the 3500 ppm group. Brain concentrations of total nonheme iron (μg/g) indicated a significant reduction of iron in the iron-deficient (4 ppm) group and significant excess in the 20000 ppm group that were directly comparable to the behavioural deficits obtained [44]. These functional deficits are confirmed and extended by the present results that include initial hypokinesic effects upon locomotion, rearing and total activity of Fe^{2+} administration (7.5 mg/kg) during postnatal Days 10-12, hyperkinesia during the 3rd and final 20-min test period by the group administered Fe^{2+} during postnatal Days 3-5 as well, and deficits in radial arm maze performance by both the Days 10-12 and 3-5 postnatal administration groups. Furthermore, both these groups showed an impaired habituation to the activity test cages, retention deficits from the 1st to the 2nd to the 3rd trials in the radial arm maze and significantly elevated total iron concentrations (μg/g) in the basal ganglia, but not frontal cortex, of the mice brains. The pattern of behavioural deficits observed has been discussed in the functional context of disorders such as PD or AD [20,40].

Both clinical [39] and preclinical [23] results demonstrated the deleterious consequences of postnatal iron overload for later functional development and behavioural performance. The

present findings of a relatively sensitive and narrow critical period for this disruptive influence indicate the importance of understanding the kinetics and distribution of iron uptake into the brain and into specific and selectively-sensitive regions [31].

Acknowledgements

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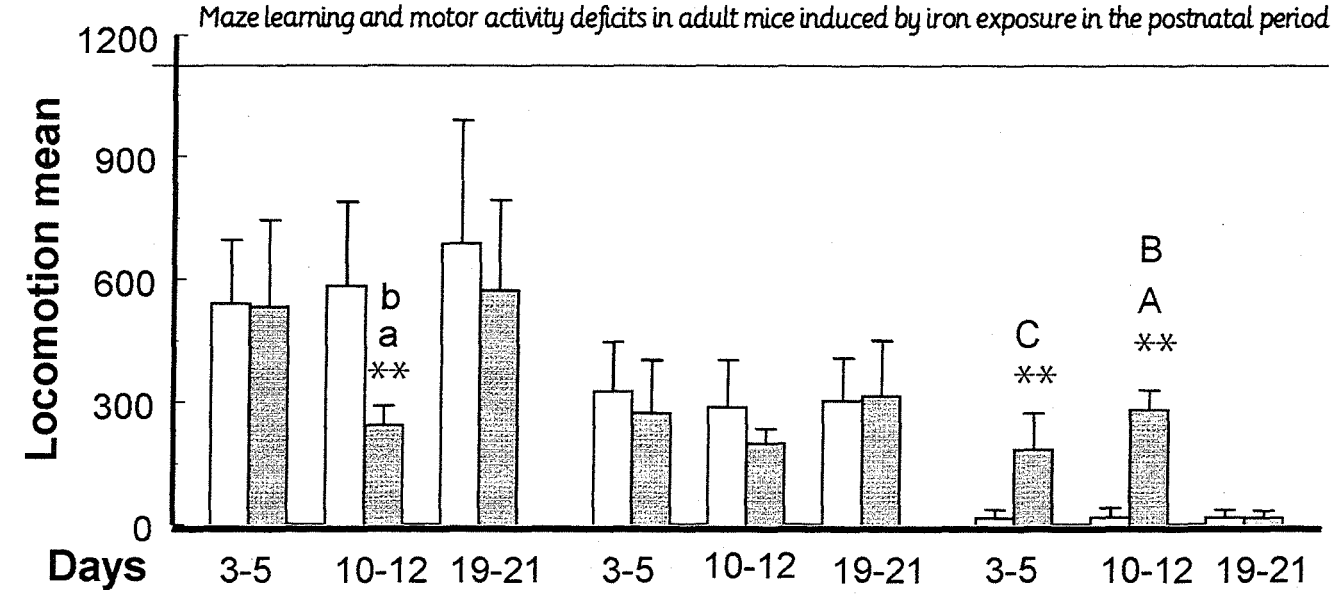
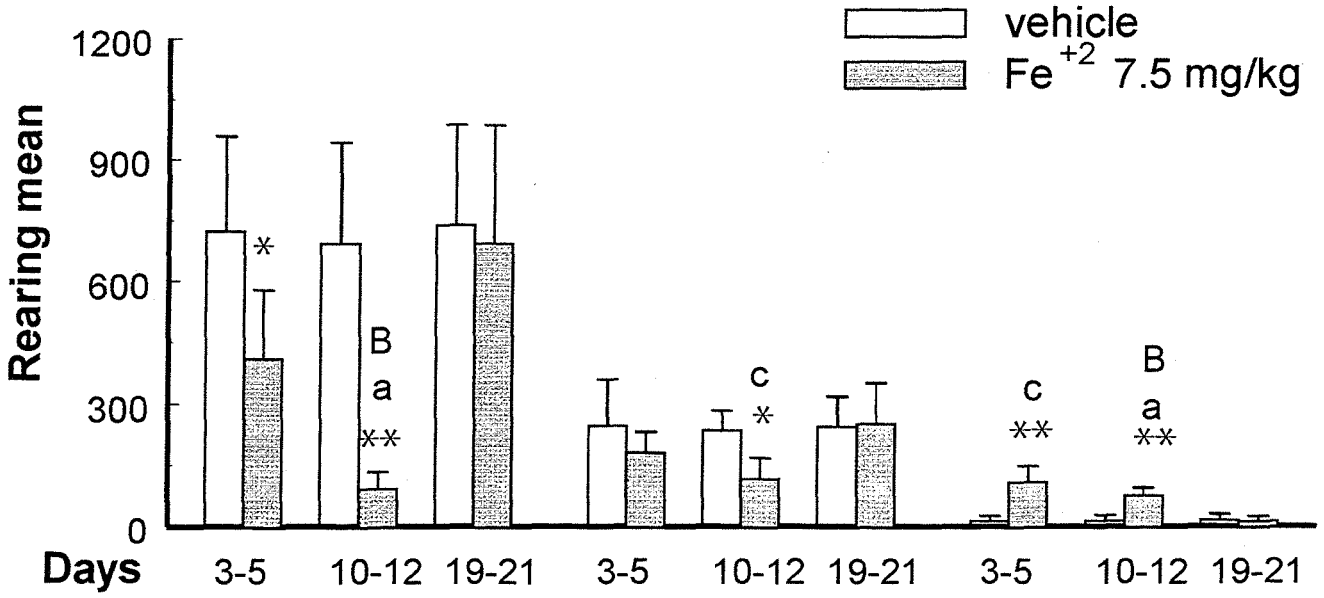
Figure 1. *Spontaneous behaviour in three months old NMRI male mice following an oral exposure to iron. Groups of male mice (n = 10), derived from five to six different litters, were treated with vehicle, or 7.5 mg Fe^{2+} / kg b. w. either days 3–5, 10-12 or 19-21 post partum. For measurement of motor activity, see Materials and Methods. Statistical analysis of behavioural data was performed using ANOVA with a Split-plot design. There were significant interaction effects (Groups X Time periods) for all the three parameters of spontaneous behaviour (Locomotion: $F(10, 108) = 10.05$; Rearing: $F(10, 108) = 11.96$; Total activity: $F(10, 108) = 6.40$) measured over three consecutive 20 min periods. Pairwise testing between each of the treatment groups was performed with the Tukey HSD test. Bars represent mean + SD. ** $p \leq 0.01$ and * $p \leq 0.05$ compared with the Control group exposed the same days. Letter in* **upper case** $p \leq 0.01$ *and* **lower case** $p \leq 0.05$. *where* ^{A}*represents comparision between treatment days 3-5 versus 10-12,* ^{B}10-12 versus 19-21 and ^{C}3-5 versus 19-21.


Locomotion mean
1200
900
600
300
0
Days
3-5
10-12
19-21
3-5
10-12
19-21
3-5
10-12
19-21
b
a
**
C
**
B
A
**


vehicle
Fe+2 7.5 mg/kg
Rearing mean
1200
900
600
300
0
Days
3-5
10-12
19-21
3-5
10-12
19-21
3-5
10-12
19-21
*
B
a
**
c
*
c
**
B
a
**

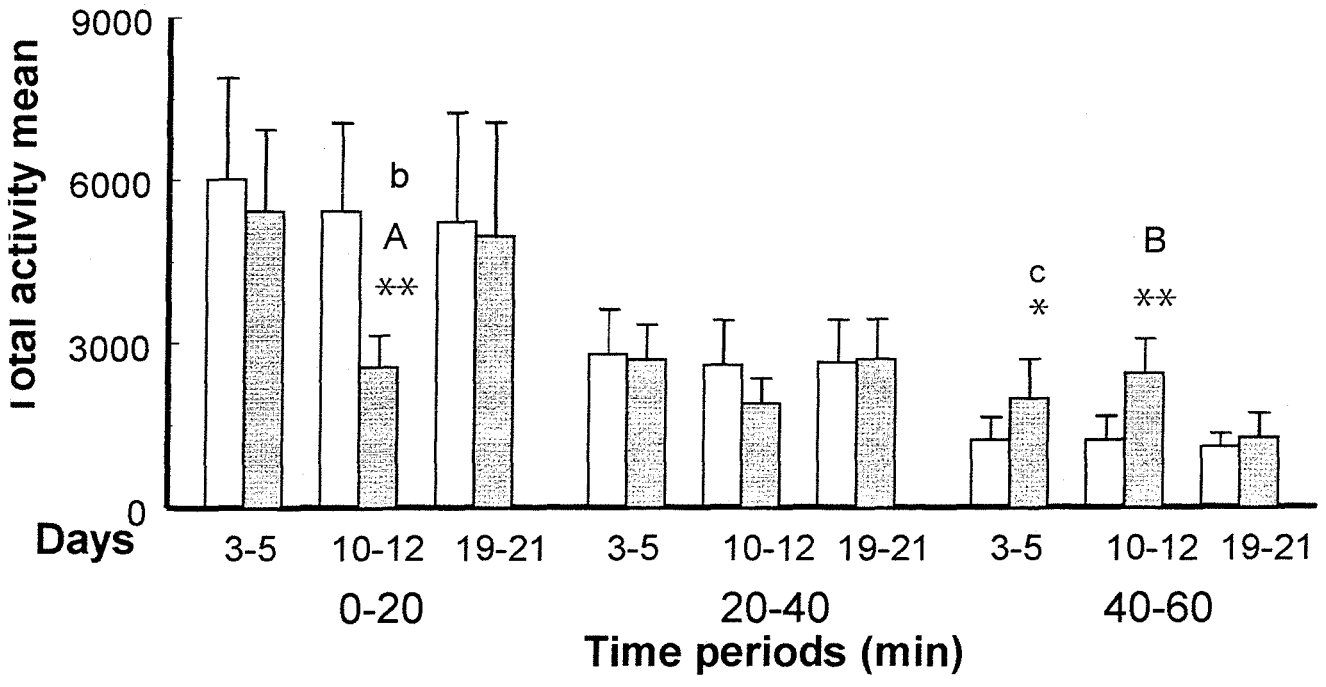

Total activity mean
9000
6000
3000
0
Days
3-5
10-12
19-21
3-5
10-12
19-21
3-5
10-12
19-21
b
A
**
c
*
B
**
0-20
20-40
40-60
Time periods (min)

Figure 2. *Radial arm maze learning in three months old NMRI male mice following an oral exposure to iron. Groups of male mice (n = 10), derived from five to six different litters, were treated with vehicle, or 7.5 mg Fe^{2+} / kg b. w. either days 3–5, 10-12 or 19-21 post partum. Statistical analysis of behavioural data was performed using ANOVA with a Split-plot design. There were significant Group x Days interactions for both the parameters of radial arm maze learning (Number of errors: F(10, 108) = 4.67; Latency to the last pellet: F(10, 108) = 4.30) measured over three consecutive testing days. Pairwise testing between each of the treatment groups was performed with the Tukey HSD test. Bars represent mean + SD. ** $p \leq 0.01$ and * $p \leq 0.05$ compared with the Control group exposed the same days. Letter in* **upper case** *$p \leq 0.01$ and* **lower case** *$p \leq 0.05$. where ^{A}represents comparision between treatment days 3-5 versus 10-12, ^{B}10-12 versus 19-21 and ^{C}3-5 versus 19-21.*

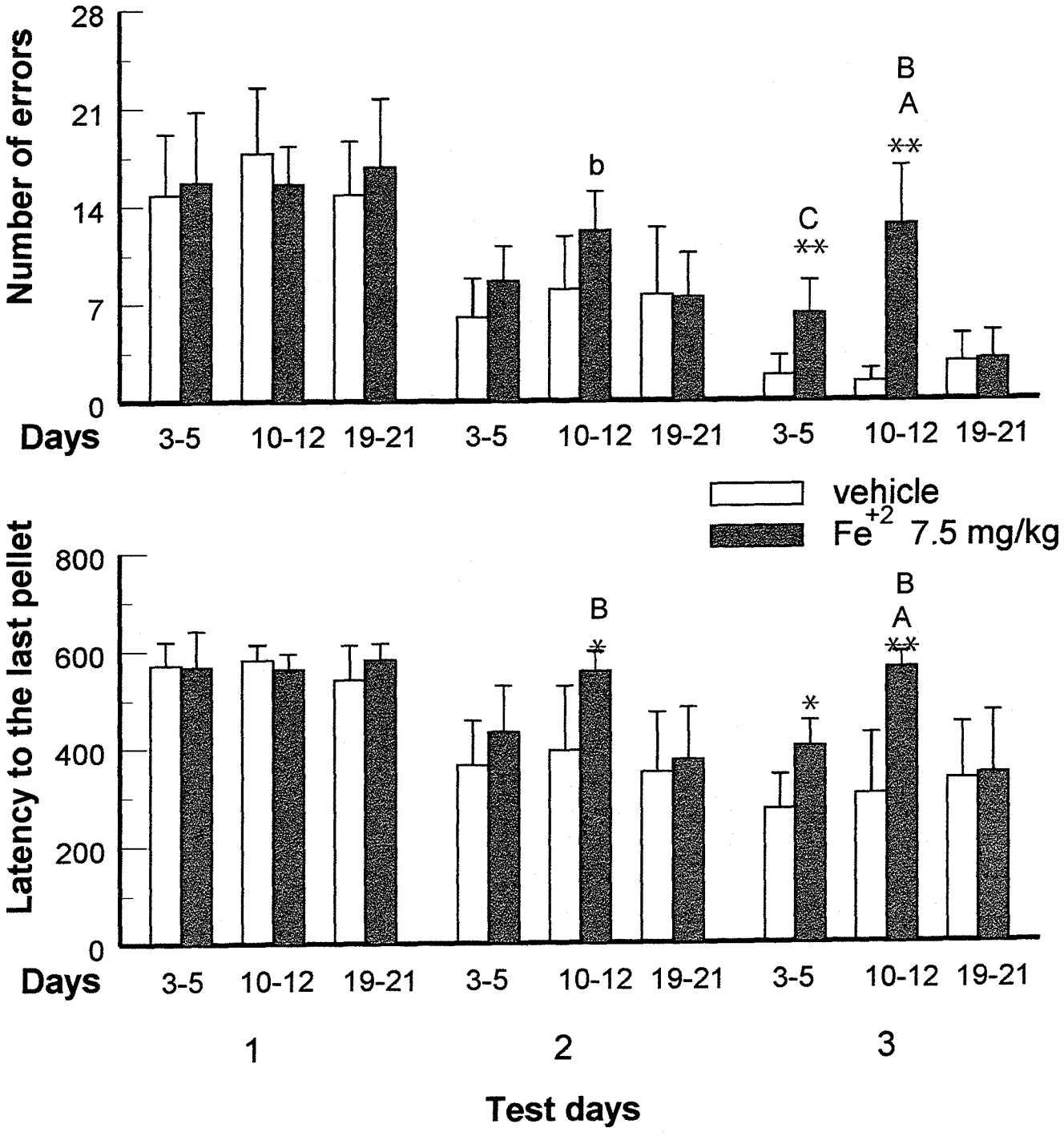

Number of errors
28
21
14
7
0
b
C
**
B
A
**
Days
3-5
10-12
19-21
3-5
10-12
19-21
3-5
10-12
19-21
vehicle
Fe+2 7.5 mg/kg
Latency to the last pellet
800
600
400
200
0
B
*
*
B
A
**
Days
3-5
10-12
19-21
3-5
10-12
19-21
3-5
10-12
19-21
1
2
3
Test days

Table 1. Spontaneous motor activity habituation quotients.

Treatment	Locomotion Quotients		Rearing Quotients		Total activity Quotients	
	1^{st1}	2^{nd2}	1^{st1}	2^{nd2}	1^{st1}	2^{nd2}
Days 3-5						
Vehicle	166±32	2038±175	293±29	2050±161	216±27	228±21
7.5 mg Fe^{2+}	194±36	147±18^{A}	226±13^{a}	167±19^{A}	201±24	136±14^{A}
Days 10-12						
Vehicle	204±27	1694±151*	294±18	1815±144	209±39	216±24
7.5 mg Fe^{2+}	122±33^{A}	70±13^{A}	78±31^{A}	151±38^{A}	136±11^{A}	76±34^{A}
Days 19-21						
Vehicle	228±29	1684±132*	301±28	1758±151*	198±21	241±19
7.5 mg Fe^{2+}	181±34	1852±172	275±34	2083±159	184±22	212±23

*Habituation quotients pertaining to locomotion, rearing and total activity counts from the 1st, 2nd and 3rd 20-min test periods in the activity test chambers, derived by dividing the respective number of counts per mouse during the 1st 20-min by that obtained during the 2nd 20-min period, and counts during the 2nd 20-min period by that obtained during the 3rd 20-min period. In each case the result was multiplied by 100. Motor activity was measured in four-month-old NMRI male mice, derived from five to six different litters, that were treated with either vehicle, or 7.5 mg Fe^{2+} / kg b. w. on either Days 3–5, 10-12 or 19-21 post partum. Values represent habituation quotients ± SD. $^{A}p<0.01$ versus respective vehicle (Days 3-5, 10-12 or 19-21), $^{a}p<0.05$, *p<0.01 versus Days 3-5 vehicle, ^{1} 1st 20-min / 2nd 20-min x 100 and ^{2} 2nd 20-min / 3rd 20-min x 100.*

Table 2. Radial arm maze retention quotients.

Treatment	Errors Quotients		Secs Quotients	
	1^{st1}	2^{nd2}	1^{st1}	2^{nd2}
Days 3-5				
Vehicle	247 ± 36	334 ± 30*	157 ± 14	134 ± 16
7.5 mg Fe^{2+}	180 ± 38[a]	138 ± 24[A]	130 ± 16[a]	107 ± 8[A]
Days 10-12				
Vehicle	224 ± 42	615 ± 43	148 ± 16	131 ± 13
7.5 mg Fe^{2+}	126 ± 27[A]	96 ± 36[A]	101 ± 7[A]	98 ± 7[A]
Days 19-21				
Vehicle	195 ± 43	282 ± 34*	155 ± 19	119 ± 31
7.5 mg Fe^{2+}	227 ± 41	255 ± 26	155 ± 14	108 ± 12

Retention quotients pertaining to number of errors and latency to last pellet acquisition from the 1st, 2nd and 3rd trials (on three consecutive days) in the radial arm maze, derived by dividing numbers of errors/secs during the 1st trial by that obtained during the 2nd trial, and the errors/secs during the 2nd trial by that obtained during the 3rd trial. In each case the result was multiplied by 100. Radial arm maze learning was measured in four-month-old NMRI male mice, derived from five to six different litters, that were treated with either vehicle, or 7.5 mg Fe^{2+} / kg b. w. on either Days 3–5, 10-12 or 19-21 post partum. Values represent mean retention quotients ± SD. [A]$p<0.01$ versus each respective vehicle (Days 3-5, 10-12 and 19-21), [a]$p<0.05$, *$p<0.01$ versus Days 10-12 vehicle, [1]1st trial / 2nd trial x 100 and [2]2nd trial / 3rd trial x 100.

Table 3. Total brain iron.

Treatment	Frontal Cortex	Basal Ganglia
Days 3-5		
Vehicle	25.3±1.5	35.2±3.1
7.5mg Fe^{2+}/kg	24.6±2.5	44.5±2.5*^{C}
Days 10-12		
Vehicle	24.8±3.4	35.9±2.6
7.5mg Fe^{2+}/kg	25.9±1.8	53.1±3.9**^{AB}
Days 19-21		
Vehicle	23.3±2.3	34.2±2.1
7.5mg Fe^{2+}/kg	25.4±2.4	37.7±3.1

Total brain iron in frontal cortex and basal ganglia (µg/g wet weight) in four months old NMRI male mice following an oral exposure to iron. Six groups of male mice, derived from five to six different litters, were treated with vehicle, or 7.5 mg Fe^{2+} / kg b. w. either days 3–5, 10-12 or 19-21 post partum. Statistical analysis of data was performed using One-way ANOVA. Pairwise testing between each of the treatment groups (Fe^{2+} versus vehicle) was performed with the Tukey HSD test. Data are presented as mean ± SD, n=4 in each treatment group, ** $p \leq 0.01$ and * $p \leq 0.05$ compared with respective Vehicle group. Letter in **upper case** $p \leq 0.01$ and **lower case** $p \leq 0.05$. ^{A}represents the comparision between Fe^{2+} on treatment Days 3-5 versus 10-12, ^{B}10-12 versus 19-21 and ^{C}3-5 versus 19-21.

Capitulo IV

Postnatal iron and adult MPTP induced neurodegenerative and functional deficits in mice

Manuscrito em preparação

Postnatal Iron and Adult MPTP Induced Neurodegenerative and Functional Deficits in mice

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Abstract

To study the interactive effects of postnatal iron and adult MPTP treatments, groups of C57 Bl/6 mice were administered iron (Fe^{2+}) 7.5 mg/kg, b. wt., p.o. or vehicle (saline) on Days 10-12 post partum followed, at 3 months of age, by administration of either MPTP (2 x 20 or 2 x 40 mg/kg, s.c.) or saline. Behavioural testing was started three weeks later. Postnatal iron administration to mice induced hypoactivity during the 1st 20-min period of testing and hyperactivity during the 3rd and final 20-min period for all three parameters of motor activity. MPTP treatment of adult mice caused a dose-related hypokinesia throughout the 3 x 20-min test periods; in the mice that received both neonatal iron and MPTP severe deficits of motor activity (akinesia) were obtained. Habituation quotient analysis indicated marked disruptions of habituation to the novel test chambers by the mice administered iron both from the 1st to the 2nd test period and from the 2nd to the 3rd, although in each case the disruption was greatest from the 1st to the 2nd period. Combined neonatal iron and MPTP treatment drastically worsened this disruption of normal activity during the 1st to 2nd periods. MPTP treatment caused habituation deficits during the 1st to 2nd periods but enhanced habituation during the 2nd to 3rd periods. Neurochemical analyses of striatal DA levels demonstrated that the depletions were most severe under conditions of combined neonatal iron and adult MPTP treatment; postnatal iron enhanced DA loss after the 2 x 20 mg/kg dose of MPTP. Furthermore, these depletions were associated with an almost total akinesia by these groups when locomotion and rearing counts were expressed as a percentage of the Veh-sal group. The analysis of total iron content (μg/g) in brain regions indicated notably elevated levels in the basal ganglia, but not in the frontal cortex, of mice administered Fe^{2+} on Days 10-12 after birth. The role of iron-overload in parkinsonism with associated functional deficits incorporates important interactive propensities with MPTP that may further elucidate focal aspects of the neurodegenerative disease profile.

Keywords: Iron-overload; postnatal; MPTP; three months; spontaneous motor behaviour; habituation; dopamine; total iron; basal ganglia; C57 Bl/6 mice.

1. Introduction

During perinatal development of the brain, there are periods of neuronal tissue development that are critical for a normal maturation [40]. Iron, the most abundant metal in the human body and brain [5], is involved in several metabolic processes and is essential for normal neurological development [cf. 30, 59], and iron deficiency during critical periods of development is associated with disruptions of behavioural performance, e.g. in learning and memory tasks [59]. On the other hand, there is accumulating evidence that excessive iron deposits in the brain, which may generate cytotoxic free radical formation [46], and alterations in iron metabolism play an important role in many neurologic diseases [13, 15, 38, 49]. Iron-overload is implicated in the pathogenesis of several human neurodegenerative disorders, i.e. Parkinson's disease (PD) and Alzheimer's disease (AD) [8, 32] and in the Hallervorden-Spatz syndrome, involving aberrant brain iron metabolism [51]. Several studies have demonstrated that a selective and significant increases of iron are found in the substantia nigra of patients afflicted with Parkinson's disease [9, 11, 41].

The immediate postnatal period is critical for establishment of normal iron content in the adult brain and its regional distribution. Investigations of cerebral iron uptake indicate that both iron transport and transferrin, the iron mobilization protein, binding sites are maximal during the postnatal period of rapid brain growth, essentially during the second week post partum in rats and mice [53], and maximal brain iron uptake occurs in 15-day-old rats [52]. Dwork et al. [12] showed that iron acquired by the brain during this period of development is retained in the brain without being returned to plasma sites. In many mammalian species, a period of rapid brain growth occurs during perinatal development, termed "brain growth spurt" [7]. In humans this period begins during the third trimester of pregnancy and is maintained throughout the first year of life whereas in rats and mice the corresponding period occurs during the first three-to-four weeks of postnatal life. During this critical period of neuronal development the essential processes of brain structure and function are established [42] and fundamental sensory-motor faculties are acquired [3, 4]. Thus, it appears that there may exist a critical neonatal period of brain development associated with the establishment of normal iron content in the adult brain.

1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) induces parkinsonism in human and nonhuman primates [36], inducing the loss of substantia nigra cells in the pars compacta of adult animals. It was previously shown that injections of MPTP (2 x 40 mg/kg, s.c.) in C57 BL/6 mice induced L-Dopa reversible hypoactivity [24, 50]; a less rigorous dose treatment, e.g. 2 x 20, 25 or 30 mg/kg of MPTP has been found not to reduce motility in the C57 black mice although dopamine (DA) concentrations may indicate upto 50-80% reduction [29, 48, 57]. The parameters of MPTP treatment in this mouse strain are long-lasting (upto and above 52 weeks after treatment) with a good correlation between the functional defect, hypokinesia, the neurochemical concomitant, a severe depletion of DA, and a dose- and time-dependent recovery of several parameters of motor behaviour after treatment with L-Dopa [cf. 17, 18, 21, 22, 23, 24].

The purpose of the present study was to examine the functional and neurochemical consequences of administering a 7.5 mg/kg dose of Fe^{2+} to mouse pups on Days 10-12 after birth and then treating these animals with either low (2 x 20 mg/kg) or high (2 x 40 mg/kg) doses of MPTP or saline in order access the effects of an iron-overload upon the induction of parkinsonism and/or AD in the laboratory [cf. 26, 44].

2. Methods and Materials

2.1. Animals

Pregnant C57 BL/6 mice were purchased from B&K, Sollentuna, Sweden. Each litter was adjusted within 48 h to 8-10 mice and to contain offspring of either sex in about equal numbers, was kept together with its respective mother in a plastic cage in a room at temperature of 22 ± 1°C and a 12/12 hours constant light/dark cycle (lights on between 06.00 and 18.00 hrs). The male offspring only were used in this study. At the age of 4 weeks the mice were weaned and the males were placed and raised in groups of 4 to 6 animals in a room maintained for male mice only. The animals were supplied with standardized pellet food and tap water ad libitum. In the experiment described, 10-12-day old male C57 BL/6

mice (ALAB, Sollentuna, Sweden), weighing 22-25 g were administered Fe^{2+} (see below) or saline. At three months of age these mice were administered either MPTP or saline. Free access to food and water was maintained throughout. They were housed in groups of 6 animals and tested only during the hours of light (08.00-15.00 hrs). Behavioural testing was initiated three weeks following treatment with MPTP. All testing was performed in a normally lighted room. This test room, in which all 12 ADEA activity test chambers, each identical to the home cage, were placed, was well-secluded and used only for this purpose. Each test chamber (i.e. motor activity test cage) was placed in a sound-proofed wooden box with 12 cm thick walls and front panels and a small double-glass window to allow observation; each box had a dimmed lighting.

2.2. Behavioural measurements and apparatus

An automated device, consisting of macrolon rodent test cages (40 x 25 x 15 cm) each placed within two series of infra-red beams (at two different heights, one low and one high, 2 and 8 cm, respectively, above the surface of the sawdust, 1 cm deep), was used to measure spontaneous and/or drug-induced motor activity of MPTP and control mice (RAT-O-MATIC, ADEA Elektronic AB, Uppsala, Sweden). The distance between the infra-red beams was as follows: the low levels beams were 73 mm apart lengthwise and 58 mm apart breadthwise in relation to the test chamber; the high level beams, placed only along each longside of the test chamber were 28 mm apart. According to the procedures described previously [1], the following parameters were measured: LOCOMOTION was measured by the low grid of infra-red beams. Counts were registered only when the mouse in the horizontal plane, ambulating around the test-cage. REARING was registered throughout the time when at least one high level beam was interrupted, i.e. the number of counts registered was proportional to the amount of time spent rearing. TOTAL ACTIVITY was measured by a sensor (a pick-up similar to a gramophoneneedle, mounted on a lever with a counterweight) with which the test cage was constantly in contact. The sensor registered all types of vibration received from the test cage, such as those produced both by locomotion and rearing as well as shaking, tremors, scratching and grooming. All three behavioural parameters were measured over consecutive 30-min. periods but .

2.3. Treatment and chemicals

Ferromyn® (Iron succinate: 7.5 mg Fe^{++}/ml, AB Hässle, Göteborg, Sweden). Dosages, expressed as mg Fe^{2+}/kg b. w., was administered orally via a metallic gastric tube in a volume of 10 ml/kg body weight. Saline was used as vehicle and to prepare the dose of Fe^{2+}. Ferromyn S is applied to the treatment of anemia and as a prophylactic measure for blood donors and pregnant women. MPTP (Research Biochemical Inc., Natick, MA. USA, either 2 x 20 or 2 x 40 mg/kg, s.c., 24 hr interval, administered four to six weeks before behavioural testing). Dosages are expressed as the free base. The dose of MPTP applied has not been found to affect food/water intake excessively. However, in the experiments special precautions are taken to facilitate each animal's ability to acquire food/water by placing each appropriately on the floor of the cage for the first two days following the MPTP treatment.

2.4. Neurochemical analysis.

Mice were killed by cervical dislocation within two weeks of completion of behavioural testing and striatal regions dissected out. Determination of DA was performed using an high-performance liquid chromatograph with electrochemical detection (HPLC-EC), according to [2], as modified by [58]. Striatal regions were rapidly dissected out and stored at -80°C until neurochemical analysis. DA concentrations was measured as follows: The frozen tissue samples were weighed and homogenized in 1 ml of 0.1 M perchloric acid, and alpha-methyl-5-hydroxytryptophan was added as an internal standard. After centrifugation (12 000 rpm, i.e. 18600 g, 4oC, 10 min) and filtration, 20 µl of the supernatant was injected into the HPLC-EC to assay DA. The HPLC system consisted of a PM-48 pump (Bioanalytical Systems, BAS) with a CMA/240 autoinjector (injection volume: 20 µl), a precolumn (15 x 3.2 mm, RP-18 Newguard, 7 µm), a column (100 x 4.6 mm, SPHERI-5, RP-18, 5 µm), and an amperometric detector (LC-4B, BAS, equipped with an Ag/AgCl reference electrode and a MF-2000 cell) operating at a potential of +0.85V. The mobile phase, ph 2.69, consisted of K_2HPO_4 and citric acid buffer (pH 2.5), 10% methanol, sodium octyl sulphate, 40 mg/l, and EDTA. The flow rate was 1 ml/min, and the temperature of the mobile phase was 35°C.

2.5. Iron content analysis

Mice were killed by cervical dislocation within two weeks after completion of behavioural testing. Brain regions from 4 mice in each group were dissected [28] and stored

in minus 70° C until analysed. Determination of total iron was performed by MeAna - Konsult, Uppsala, Sweden, an accredited lab. fulfilling the specifications of SS-EN 45 001 (an accredited laboratory, appointed by the Swedish Board for technical accreditation (SWEDAC) meeting the requirements set up in the quality standards SS-EN 45 001). The tissues were dissolved in analytical bombs under the influence of 3 ml supra pure HNO_3 and heat (150° C for 2 hrs). Analysis was made using atomic absorption spectroscopy (Instrument Perkin-Elmer 4100 ZL equipped with a graphite furnace).

2.6. Experimental Design

C57 BL/6 10-12 days-old mouse pups were administered Fe^{2+} (Iron succinate: 7.5 mg Fe^{++}/kg, p.o., b.w.) or saline (Vehicle), and returned to the respective mothers. At the age of three months, Fe^{2+}-treated and saline mice were administered either MPTP (2 x 20 or 2 x 40 mg/kg, s.c., on two occasions separated by a 24-hr interval) or saline to provide the following experimental groups: Veh-Sal, Veh-MPP20, Veh-MPP40, Fe-Sal, Fe-MPP20, and Fe-MPP40.

2.7. Statistical analysis

The locomotion, rearing and total activity data over three consecutive 20-min. periods in the activity test chambers, as well as the habituation quotients were submitted to a split-plot ANOVA design [33]. Brain (frontal cortex and basal ganglia) regional levels of iron, locomotion, rearing and total activity over the full 60-min period, and striatal levels of DA each were submitted to an one-way ANOVA completely randomised design [33]. Pairwise testing between the different treatment groups was performed with the Tukey HSD test. The 1% level of significance was maintained throughout unless where otherwise stated.

3. Results

3.1. Weight gain

No significant differences between the groups regarding measures of weight gain were observed one week after treatment with Fe^{2+}/saline: [$F(5, 54) = 0.07$]. Neither were there any significant differences between the group mean weights: [$F(5, 54) = 0.16$]

as a consequence of the different treatments employed when the behavioural testing started.

3.2. Spontaneous motor activity

Postnatal administration of Fe^{2+} on Days 10-12 disrupted motor behaviour; treatment with MPTP three months after birth caused a dose-related hypokinesic effect upon locomotor, rearing and total activity. The postnatal treatment of mice with Fe^{2+} followed by MPTP (at 3 months age) drastically potentiated the akinesia observed in these animals. Thus, there were significant Groups x Time periods interaction effects for all the three parameters of motor behaviour [Locomotion: $F(10, 108) = 48.74$; Rearing: $F(10, 108) = 34.16$; and

Total Activity: $F(10, 108) = 37.91$] measured over three consecutive 20-min periods.

Figure 1 presents the locomotion, rearing and total activity counts of mice treated postnatally with either Fe^{2+} or saline, and at 3 months age with either MPTP

Insert Figure 1 here

(20 or 40 mg/kg) or saline. Comparisons of the group means with Tukey HSD tests indicated that the following differences were obtained:

Fe-sal < Veh-sal on the 1st 20-min, and Fe-sal > Veh-sal on the 3rd 20-min period.

Veh-sal > Veh-MPP20 > Veh-MPP40 for each parameter at the 1st and 2nd 20-min periods, but not 3rd 20-min period.

Fe-sal > Fe-MPP20 > Fe-MPP40 for each parameter at the 1st and 2nd 20-min periods but not the 3rd 20-min period.

Veh-MPP20 > FeMPP20 and Veh-MPP40 > FeMPP40 for each parameter at the 1st and 2nd, but not 3rd, period.

Comparisons of each parameter of motor activity over the total 60-min test period (see Table 3) indicate the dose-related deficits induced by MPTP and the exacerbative effects of postnatal Fe^{2+} administration. Thus, one-way ANOVA indicated significant Between-groups effects for Locomotion: $F(5, 54) = 113.37$;

Rearing: $F(5, 54) = 88.67$; and, Total activity: $F(5, 54) = 49.85$. Tukey HSD tests indicated significant effects due to each dose of MPTP, neonatal iron and the combination of these

treatments (Table 3, below). Each parameter of activity for each group is tabulated as a percentage of the Veh-sal group in order to allow a direct comparison with the percent DA analysed for these groups. It will be noted from the percent of Veh-sal group values that the Fe^{2+}-treated mice administered MPTP (20 or 40 mg/kg) were almost wholly akinesic.

3.3. Habituation to Activity test chambers

Habituation is a relatively simple, nonassociative form of learning in situations where repeated measures of behaviour are monitored. In order to access the extent of habituation to the activity test chambers over each successive 20-min interval, an habituation quotient for each mouse was derived by dividing the number of counts during the 1st 20-min period by that obtained during the 2nd 20-min period, and counts during the 2nd 20-min period by those obtained during the 3rd 20-min period. In each case the result of each division was multiplied by 100 to provide a quotient representing the reduction of activity counts from the first to the second to the third period for each mouse [cf. 6, 19, 20]. Thus, the obtained quotients were subjected to split-plot ANOVA that indicated significant Groups x Quotients interaction effects, as follows: Locomotion: $F(10, 108) = 28.81$; Rearing: $F(10, 108) = 16.70$; Total activity: $F(10, 108) = 25.43$. The mean quotient values per treatment group and period (i.e. 1st to 2nd and 2nd to 3rd) are presented in Table 1.

Insert Table 1 here

Habituation quotient analysis reveals that the reductions in motor activity from the 1st to the 2nd period were significantly greater in the Veh-sal group than in the other groups. However, for locomotion and rearing activity habituation quotients from the 2nd to the 3rd periods for the Veh-MPP20 and Veh-MPP40 groups were dose-dependently larger than the Veh-sal group; these groups demonstrated severely reduced activity for these parameters so the result is something of a 'floor-effect'. For total activity, the quotient of the Veh-sal group was higher than that of all the other groups. Postnatal treatment with Fe^{2+} markedly reduced the quotients (i.e. close to or less than 100) but again the later treatment with MPTP, resulting in extremely low levels of activity, raised the locomotion and rearing quotients in comparison with the Fe-sal group.

3.4. Analysis of brain iron content

Postnatal administration of Fe^{2+} 7.5 mg/kg induced higher levels of iron in the basal ganglia, but not frontal cortex, four months after birth. Thus, there was a significant Between-groups treatment effect in the basal ganglia [F(5, 18) = 37.40] in contrast to the frontal cortex [F(5, 18) = 1.11, ns]. Tukey HSD tests indicated

Insert Table 2 here

Significantly higher levels of total iron (μg/g) in the basal ganglia of mice that had been treated with Fe^{2+} on Days 10-12 after birth, i.e. groups Fe-sal, Fe-MPP20 and Fe-MPP40. No significant differences were obtained in the frontal cortex.

3.5. Neurochemical Analysis.

Fe^{2+}+MPTP, Fe^{2+}-treated, MPTP-treated mice tested in the motor activity test chambers displayed severe depletions of DA in the striatum in comparison with the saline-treated mice at 4 months age (see Table 3). One-way ANOVA indicated a significant Between-groups effect F(5, 35) = 116.76. Pairwise testing indicated that MPTP treatment at 3 months induced a dose-dependent depletion of DA in the striatum. Comparison of the Veh-MPP20 group with the Fe-MMP20 indicated a significant greater loss of DA by the latter suggesting a potentiation of the neurotoxic effects of MPTP in mice neonatally administered Fe^{2+}.

Insert Table 3 here

4. Discussion

The results of combining postnatal administration of iron (Days 10-12 after birth) with MPTP (20 or 40 mg/kg) at three months age may be summarised as follows:- (1) Postnatal iron administration to mice induced an hypokinesia during the 1st 20-min period of testing and an hyperkinesia during the 3rd and final 20-min period for all three parameters of motor activity. (2) MPTP treatment of adult mice caused a dose-related hypokinesia throughout the 3 x 20-min test periods; in the mice that received both neonatal iron and MPTP severe deficits of

motor activity were obtained. (3) Habituation quotient analysis indicated marked disruptions (as evidenced by quotients of around 100 or less) in habituation to the novel test chambers by the mice administered iron both from the 1st to the 2nd test period and from the 2nd to the 3rd, although in each case the disruption was greatest from the 1st to the 2nd period. Combined neonatal iron and MPTP treatment drastically deteriorated this disruption during the 1st to 2nd periods but raised the quotients during the 2nd to 3rd periods even though these remained less than those of the Veh-sal group. (4) MPTP treatment caused habituation deficits during the 1st to 2nd periods but enhanced habituation during the 2nd to 3rd periods, possibly reflecting a peculiar 'floor-effect'. (5) Neurochemical analyses of striatal DA demonstrated that depletions were most severe under conditions of combined neonatal iron and adult MPTP. Furthermore, these depletions were associated with an almost total akinesia by these groups when locomotion and rearing counts were expressed as a percentage of the Veh-sal group. (6) The analysis of total iron content (μg/g) in brain regions indicated notably elevated levels in the basal ganglia, but not in the frontal cortex, of mice administered Fe^{2+} on Days 10-12 after birth.

The functional consequences of iron overload to the developing brain are the subject of some recent attention but the profound effects of a combined iron overload and MPTP administration of the adult animal have never previously been observed. Sobotka et al. [47] fed weanling rats diets consisting of iron either at 4 (iron-deficient), 35 (control), 350, 3500 or 20000 ppm for 12 weeks, testing for behavioural and body weight changes at different intervals (food-intake or calculated dosage not shown). Body weight reductions were found in the iron-deficient (4 ppm) rats and those that received 20000 ppm, as well as the 3500 ppm group but to a lesser extent. Total activity was reduced for iron-deficient and 20000 ppm groups, whereas Slope, a measure of habituation to the test chamber, was disrupted in the 3500 ppm group too. Avoidance learning and prepulse inhibition, a sensory-gating assessment technique [27], were both deficient in these two same groups. It should be noted that by Week 10 even the 3500 ppm group were defective on the prepulse inhibition test. These latter data [sobotka] bear consideration in the light of the drastic alterations to sensory attentional mechanisms induced, above, by the postnatal iron or adult MPTP administrations but most particularly by the combination treatment. Brain concentrations of total nonheme iron (μg/g) indicated a significant reduction of iron in the iron-deficient group

and a significant excess of the metal in the 20000 ppm group that were directly comparable to the behavioural deficits observed [47]. These functional deficits were confirmed and extended by the recent findings of Fredriksson et al. [25] indicating deficits of spontaneous motor behaviour, radial arm maze performance and habituation to test chamber quotients by groups of NMRI mice administered Fe^{2+} at the 3.7 and 37.0 mg/kg dosages, postnatally on Days 10-12. Here too, the functional disruptions were associated with significant increases in total iron in the basal ganglia but not frontal cortex. These results have been reinforced by the observation (Fredriksson et al. submitted manuscript) that the critical period for the disruptive effects of a 7.5 mg/kg dose of Fe^{2+} was Days 10-12 postnatally, wherein in addition to motor activity, habituation and radial arm maze deficits marked intertrial retention deficits were obtained in the iron-overload groups; once again all these deficits were associated with increased total iron in the basal ganglia, but not in the frontal cortex.

The potentially neurodegenerative effects of iron administration to experimental animals in the laboratory have been studied. Sengstock et al. [46] found alterations of brain neurochemistry following the infusion of iron (ferric chloride, 1.25 - 6.3 nmol unilaterally) into the substantia nigra of rats. They obtained a very high correlation between the amount of iron infused intranigrally and the concentrations of DA, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) within the ipsilateral striatum of each rat. Sengstock et al. [45] further demonstrated that excess iron infusion into the substantia nigra (1.25 or 2.10 nmol) induced neuronal losses in the pars compacta, a dose-dependent and progressive nigral atrophy, progressive decreases of DA and HVA following infusions of 1.25 nmol, and progressive increases in apomorphine-induced rotational behaviour (an established functional marker for parkinsonism in the laboratory[55]). In the present study, the association between postnatal iron administration, DA concentrations and motor activity (the functional marker of parkinsonism in the present case) was neither simple nor straightforward. Thus, the Fe-sal group, demonstrating a marked elevation of basal ganglia total iron, was found to have only a non-significant 9% loss of DA but significant 24%, 67% and 28% losses of locomotion, rearing and total activity (see Table 3), respectively. On the other hand, the functional deficits of the Fe-MPP20 group were potentiated markedly in comparison with the Veh-MPP20 group (cf. 92% versus 64%, 91% versus 48%, and 67% versus 52%, for locomotion, rearing and total activity, respectively) and DA loss by the former (72%) was significantly more than that

of the latter (46%). This evidence then indicates that the combination both neonatal iron overload with an adult low dose of MPTP exacerbates both the functional and neurochemical deficits, resulting in a more directly neurodegenerative involvement of iron. Since the MPTP dosages were administered three months after the iron treatment, which by itself did not affect DA concentrations, it seems plausible that postnatal iron renders DA neurons, most probably in the substantia nigra, particularly vulnerable to the neurotoxic action of MPTP. Since it has been shown that MPTP-lesioned hemiparkinsonian monkeys and 6-OHDA lesioned rats exhibit iron accumulation in the substantia nigra [37, 54], it seems likely that the basal ganglia iron accumulation of the neonate conveyed a DA neuron vulnerability to the adult animals that later MPTP precipitated the destruction of those neurons. Note that Youdim et al. [59] found substantial reductions in striatal dopaminergic markers following infusions of 5 μl of ferric chloride into the substantia nigra of rats.

Recent findings by Lan and Jiang [34] offer further evidence for the role of iron overload or accumulation in the brain as one critical neurodegenerative agent in PD. They established a chronic cerebral iron-loaded model by feeding mice a high iron diet that caused significant increases in brain iron concentrations as well as increases in oxidised glutathione (GSSG), decrease in total glutathione (oxidised plus reduced glutathione, GSSG + GSH) and thereby an increase in the GSSG/(GSSH+GSH) ratios. Hydroxyl radical (.OH) levels in the striatum and brainstem were increased significantly also. This excess-iron diet did not, by itself, alter either DA or lipid peroxidation (LPO) concentrations in the striatum but a single 30 mg/kg i.p. dose of MPTP to these animals induced a great enhancement in these neurochemical abnormalities. It was concluded that whereas iron does induce oxidative stress it was not responsible for neuronal destruction per se. In agreement with present conclusions it was suggested that the brain iron-overload is a setting for DA neuronal damage, promoted by triggering or precipitating factors, such as MPTP. Further analysis of the neurodegenerative actions of iron and MPTP [35] in demonstrating the protective roles of desferrioxamine and vitamin E suggested that while iron-overload may contribute to the neuronal loss in PD, iron chelators and antioxidants retard the neurodegenerative progression. Finally, Jellinger [31] has outlined a PD pathology implicating major factors including a misregulation of iron metabolism, iron-induced oxidative metabolism and free radical formation. The main lines of

evidence involve: 1) an increase in brain iron, 2) the decreased availability of glutathione and other antioxidants, 3) an increase in LPO products and reactive oxygen species, and 4) an impairment of mitochondrial electron transport mechanisms.

The neurodegenerative implications of iron-overload for PD are demonstrated in both preclinical [25] and clinical [43] studies. The present results provide certain robust functional concomitants to studies that have provided analyses of several aspects of iron involvement in PD and AD pathology [10, 39, 56]. It ought to bear borne in mind, however, that study of control subjects, PD patients and MPTP-treated monkeys [14], indicating transferrin receptor involvement in iron of PD patients' straitum, provides a note of caution in underlining the discrepancies between 'acute' nigrostriatal destruction and the chronic, idiopathic, implacable nature of the human PD condition.

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Table 1. Spontaneous motor activity habituation quotients for locomotion, rearing and total activity by postnatal Fe^{2+} -treated, MPTP-treated or saline-treated mice.

Treatment:	Veh-sal	Veh-MPP20	Veh-MPP40	Fe-sal	Fe-MPP20	Fe-MPP40
Activity						
Locomotion:						
1st quotient	269±101	87±36^{A}	72±29^{A}	112±53*	60±13^{X}	18±3^{Y,*}
2nd quotient	1812±72	5733±41^{A}	7112±34^{B}	86±32*	2410±24^{X,*}	1100±26^{Y,*}
Rearing:						
1st quotient	279±43	212±52^{a}	187±49^{b}	68±47*	54±19	28±3^{X,*}
2nd quotient	2234±53	3091±24^{A}	3286±31^{A}	100±51*	1334±35^{X,*}	1300±34^{X,*}
Total activity:						
1st quotient	176±95	100±86^{a}	99±72^{b}	112±97	103±88	85±54
2nd quotient	304±91	231±76	189±56^{b}	87±122*	135±88^{A}	116±66^{B}

Habituation quotients pertaining to locomotion, rearing and total activity counts from the 1st, 2nd and 3rd 20-min test periods in the motor activity test chambers, derived by dividing the respective number of counts per mouse during the 1st 20-min by that obtained during the 2nd 20-min period, and counts during the 2nd 20-min period by that obtained during the 3rd 20-min period. In each case the result was multiplied by 100. Motor activity was measured in three-and-a-half-month-old C57 BL/6 male mice, derived from five to six different litters, that were treated either with Fe^{2+} 7.5 mg/kg b.w. on Days 10-12 post partum or vehicle followed at three months age by treatment with either MPTP (20 or 40 mg/kg) or saline.

Values represent habituation quotients ± SD.

$^{A}p < 0.01$, $^{a}p < 0.05$, Tukey HSD tests, versus Veh-sal, $^{B}p < 0.01$, $^{b}p < 0.05$, versus Veh-MPP20; $^{X}p < 0.01$, versus Fe-sal, $^{Y}p < 0.01$, versus Fe-sal and Fe-MPP20

$^{*}p < 0.01$, versus respective vehicle group.

[1] 1st 20-min period / 2nd 20-min period x 100 and [2] 2nd 20-min period / 3rd 20-min period x 100.

Table 2. Total iron concentrations in brain regions of postnatal Fe^{2+} -treated, MPTP-treated or vehicle/saline-treated mice.

Treatment	Frontal Cortex (%)	Basal Ganglia (%)
Veh-sal	27.78±5.50	38.48±4.11
Veh-MPP20	29.73±4.52 (107)	42.87±1.61 (111)
Veh-MPP40	24.80±5.02 (89)	38.82±4.35 (101)
Fe-sal	28.36±5.34 (102)	66.28±3.99* (172)
Fe-MPP20	30.86±4.08 (112)	64.03±6.23* (167)
Fe-MPP40	32.12±4.75 (116)	68.03±6.33* (177)

Total iron concentration in the frontal cortex and basal ganglia brain regions (μg/g wet weight) in four-month-old C57 BL/6 male mice. Six groups of male mice, derived from five to six different litters, were treated with 7.5 mg Fe^{2+} /kg b.w. on Days 10-12 post partum. Statistical analysis of data was performed using one-way ANOVA. Pairwise testing was performed with Tukey HSD tests.

Values represent means ± SD

*$p < 0.01$, versus Veh-sal. (%) = percent of Veh-sal for each respective region.

Table 3. Striatal dopamine concentrations and motor activity counts over the total 60-min test period of mice postnatally treated with iron or vehicle followed by MPTP or saline at three months age.

Treatment:	Veh-sal	Veh-MPP20	Veh-MPP40	Fe-sal	Fe-MPP20	Fe-MPP40
DA ()	14.28±1.27	7.81±1.66^{A}	2.15±0.68^{B}	13.04±1.72	4.00±0.89^{C}	1.56±0.88^{D}
(%)		(54)	(15)	(91)	(28)	(11)
Locom.	892±81	323±30^{A}	122±17^{B}	681±57*	77±7^{C}	12±2^{D}
(%)	---	(36)	(14)	(76)	(8)	(1)
Rearing	1027±118	535±56^{A}	335±33^{B}	347±49*	97±14^{C}	37±7^{D}
(%)	---	(52)	(32)	(33)	(9)	(3)
Total Act.	10012±737	4815±272^{A}	3854±216^{B}	7230±570*	3319±121^{C}	2519±91^{D}
(%)	---	(48)	(38)	(72)	(33)	(25)

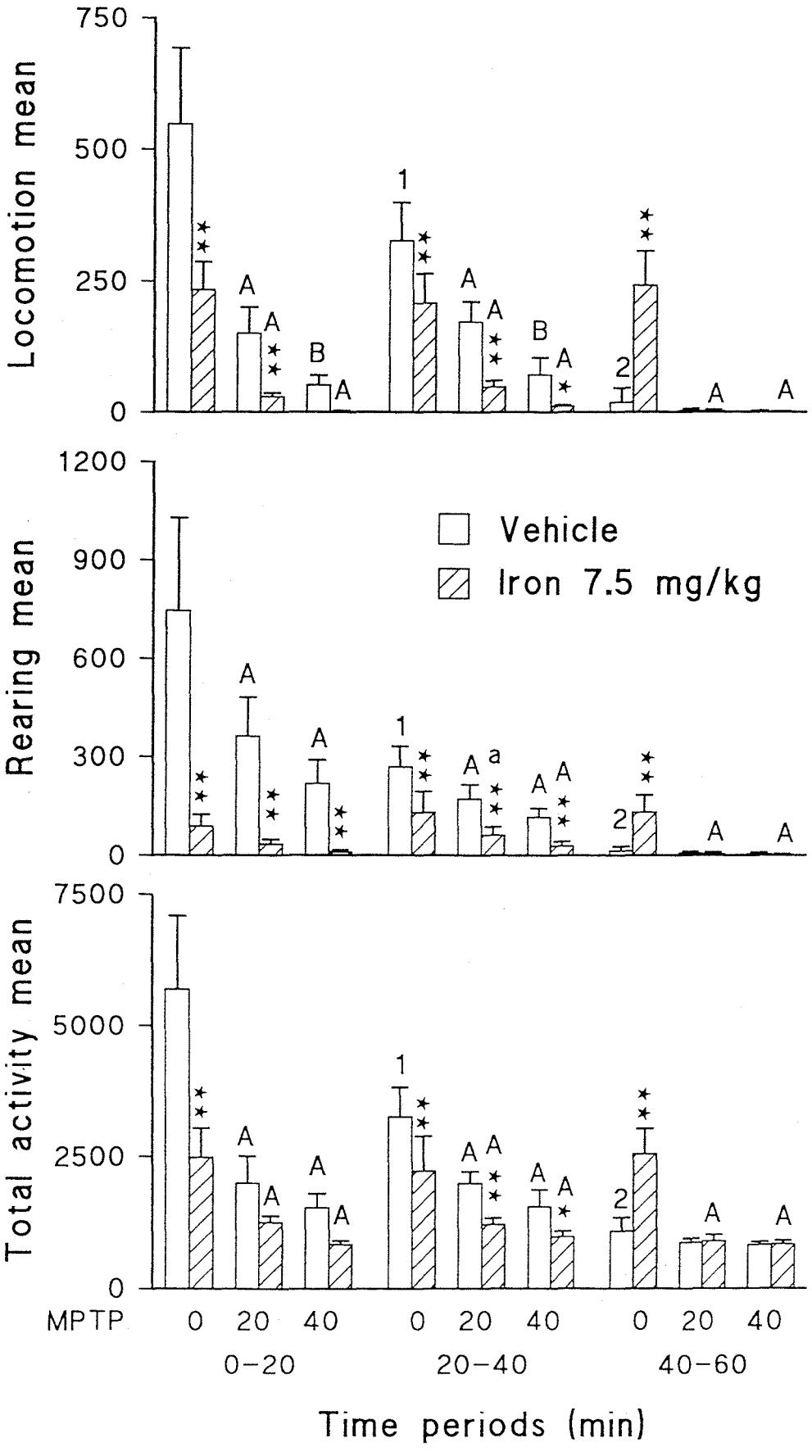
Values represent means ± SD of 10 mice. (%) = percent of Veh-sal group.

Motor activity was measured in three-and-a-half-month-old C57 BL/6 male mice, derived from five to six different litters, that were treated either with Fe^{2+} 7.5 mg/kg b.w. on Days 10-12 post partum or vehicle followed at three months age by treatment with either MPTP (20 or 40 mg/kg) or saline.

$^{A}p < 0.01$, Tukey HSD tests, versus Veh-sal; ^{B}versus Veh-MMP20; ^{C}versus Fe-sal; ^{D}versus Fe-MMP20; *versus respective Veh-sal.

Figure Captions

Figure 1. Spontaneous motor behaviour by three and a half month old C57 Bl/6 male mice postnatally administered iron (Fe^{2+}) orally and then administered MPTP as adults. Locomotion, rearing and total activity counts by groups of mice (n = 10), derived from five to six different litters, and treated with either 7.5 mg Fe^{2+}/kg b. wt., or vehicle (saline) on Days 10-12 post partum. MPTP (2 x 20 or 2 x 40 mg/kg, s.c.) or saline were administered at three months of age. Split-plot ANOVA was used to analyse each behavioural parameter over consecutive 20-min periods. Significant Groups x Time periods interaction effects were obtained: [Locomotion: $F(10, 108) = 48.74$; Rearing: $F(10, 108) = 34.16$; and Total activity: $F(10, 108) = 37.91$]. Pairwise testing between iron-treated and vehicle groups, as well as within groups between different Time periods, was performed with the Tukey HSD test.
Values represent means ± SD.
** $p < 0.01$, * $p < 0.05$, versus respective vehicle group, Tukey HSD test.
^{A} $p < 0.01$, ^{a} $p < 0.05$, versus saline; ^{B} $p < 0.01$, versus saline and –MPP20.
^{1} $p < 0.01$, versus Veh-sal 0-20 period; ^{2} $p < 0.01$, versus Veh-sal 20-40 period.


Locomotion mean
750
500
250
0
Rearing mean
1200
900
600
300
0
Total activity mean
7500
5000
2500
0
Vehicle
Iron 7.5 mg/kg
MPTP
0 20 40 0 20 40 0 20 40
0–20
20–40
40–60
Time periods (min)

Capítulo V

Efeito do tratamento neonatal com ferro em ratos adultos

V.1. Introdução

Os resultados apresentados nos capítulos anteriores demonstraram que o tratamento neonatal de camundongos com ferro constitui uma importante ferramenta no estudo de doenças neurodegenerativas das vias negro-estriatais, uma vez que ficou determinado que este metal acumula-se seletivamente na substância negra, produzindo efeitos de ordem motora e cognitiva quando estes animais atingem a idade adulta.

Tornou-se importante, a partir daí, determinar se estes efeitos seriam verificados também em outras espécies, já que o modelo animal clássico para o estudo da DP, MPTP aplica-se somente a camundongos da cepa C57 BL6 e estudos demonstram que ratos tratados mesmo com doses elevadas desta droga desenvolvem apenas leves sintomas neurológicos (Gerlach e Riederer, 1996). Portanto, a obtenção de efeitos do tratamento neonatal com ferro em ratos, ampliaria grandemente a utilização desse modelo no estudo de doenças neurodegenerativas, além de possibilitar uma melhor caracterização e entendimento dos efeitos encontrados.

Para tanto, foi escolhida a tarefa do campo aberto como medida da atividade motora e o labirinto radial de oito braços para avaliação do aprendizado e memória nestes animais. Foram utilizadas quatro diferentes doses na faixa de 2.5 a 30.0 mg Fe^{+2}/kg, com o objetivo de determinar a dose mais adequada para a realização de estudos posteriores.

V.2. Material e Métodos

V.2.1. Animais e Tratamento

Ratas Wistar prenhes foram obtidas do Biotério do Depto. de Bioquímica do Instituto de Ciências Básicas da Saúde da UFRGS, Porto Alegre, Brasil. Cada ninhada foi ajustada em 8 filhotes de ambos os sexos e foi mantida com sua respectiva mãe em sua caixa moradia em ambiente climatizado com ciclos claro/escuro de 12 horas, recebendo ração comercial e água "ad libitum". A partir do décimo dia de idade os ratos machos receberam uma dose oral diária (via tubo intra-gástrico) de 0,0; 2,5; 7,5; 15,0 ou 30, mg/kg de peso corporal de succinato de ferro (Ferromyn®, AB Hässle, Göteborg, Suécia) por três dias. Na idade de 3-4 semanas os animais foram desmamados e os machos foram colocados em grupos de 3 a 8 ratos por caixa. Cada grupo de tratamento consistiu de ratos de, pelo menos, quatro ninhadas diferentes.

V.2.2. Tarefas Comportamentais

V.2.2.1. Campo aberto

Esta tarefa foi realizada quando os animais tinham 90 dias de idade. Os animais eram colocados no canto esquerdo de uma caixa de madeira (40 x 40 x 50 cm) cujo assoalho é dividido em 12 quadrados iguais por linhas pretas. As sessões tinham duração de 15 minutos (divididos em três períodos consecutivos de 5 min. cada), durante os quais os animais exploraram a caixa livremente e foi registrado o número de cruzamentos e o número de respostas de orientação (rearings).

V.2.2.2. Labirinto radial de oito braços

A tarefa do labirinto radial de 8 braços foi realizada quando os animais tinham de 120 a 150 dias de idade. Os 8 braços (78 cm de comprimento, 8 cm de largura e 1 cm a altura das paredes) do labirinto estendem-se radialmente de uma plataforma central circular (28 cm de diâmetro). Antes de iniciar o treinamento na tarefa, os animais foram submetidos a um regime de deprivação alimentar parcial até que seus pesos corporais fossem reduzidos a 80% do peso inicial. Os animais foram submetidos a uma sessão diária durante 5 dias consecutivos. Em cada sessão um pellet de ração (100 mg) era colocado na extremidade de cada um dos 8 braços, e o rato era colocado no centro do labirinto. Registrou-se a latência para que todos os braços fossem visitados e o número de erros que foi definido como o número de reentradas nos braços já visitados. Outro parâmetro registrado foi o número total de acertos nas 8 primeiras entradas. Os animais podiam escolher os braços livremente até que atingissem os 8 acertos ou até que 10 minutos houvessem passado.

V.2.3. Análise Estatística

Para a tarefa do campo aberto os dados relativos ao número de cruzamentos e o número de respostas de orientação foram analisados utilizando ANOVA de uma via, seguido por teste de Duncan quando necessário.

Na tarefa do labirinto radial a latência, bem como o número de respostas corretas foram analisados através da ANOVA de medidas repetidas seguida pelo teste de Fisher para verificação das diferenças entre os grupos.

V.3. Resultados e Discussão

V.3.1. Campo Aberto

A figura 1 A mostra o número de cruzamentos no campo aberto obtidos em uma sessão de quinze minutos, a qual foi dividida em três períodos consecutivos de 5 min. cada. Nos últimos 5 minutos os animais tratados com a dose mais alta de ferro (30.0 mg/kg) apresentaram menor número de cruzamentos do que os ratos do grupo controle. O número de respostas de orientação não foi alterado por nenhuma das doses utilizadas neste estudo, na sessão de quinze minutos do campo aberto (figura 1B).

A diminuição no número de cruzamentos nos 5 minutos finais da sessão na tarefa do campo aberto obtida para a dose mais alta (Fe^{+2} 30.0 mg/kg) indica que o tratamento neonatal com ferro em ratos, produz alterações no comportamento motor confirmando o que foi encontrado anteriormente em camundongos. Provavelmente em ratos este efeito manifeste-se em menor extensão, uma vez que, em camundongos, foram obtidos efeitos motores a partir de doses mais baixas, o que não ocorreu em ratos. Além disso o número de respostas de orientação não foi alterado em ratos. No entanto, parte das diferenças

encontradas pode ser atribuída às diferenças entre as duas diferentes metodologias aplicadas para a medida do comportamento motor em ratos e camundongos.

V.3.2. Labirinto Radial

Houve uma interação significativa (grupos x sessões) para ambos os parâmetros do labirinto radial (número de acertos nas 8 primeiras visitas: $F(4,52) = 3.38$, $p < 0,02$; Latência: $F(4,52) = 3.46$, $p < 0.02$) medidas nos 5 dias consecutivos. A comparação entre os grupos indicou que na primeira sessão não havia diferença entre os grupos para o parâmetro latência (Figura 2 A). No entanto, no segundo e no quarto dia os grupos tratados com 2.5; 15.0 e 30.0 mg Fe^{+2}/kg apresentaram uma latência significativamente maior do que a do grupo controle para completar a tarefa. No terceiro e no último (5°) dia todos os grupos tratados apresentaram uma latência significativamente maior do que a do grupo controle, sendo que, no quinto dia, houve diferença significativa entre a latência do grupo tratado com dose mais alta (30.0 mg/kg) e o grupo tratado com a dose mais baixa (2.5 mg/kg).

No parâmetro do número de escolhas corretas nas 8 primeiras entradas (Figura 2 B), tanto no primeiro como no segundo dia, os grupos tratados com 7.5 e 30.0 mg/kg apresentaram um número menor de escolhas corretas quando comparados com o grupo controle. No terceiro dia os grupos tratados com 7.5 e 15 mg/kg obtiveram um menor número de escolhas corretas, enquanto que no quarto dia os animais tratados com 15.0 e 30.0 mg/kg apresentaram menos escolhas corretas que o grupo controle. No último dia,

todos os grupos apresentaram número significativamente menor de escolhas corretas do que o grupo controle.

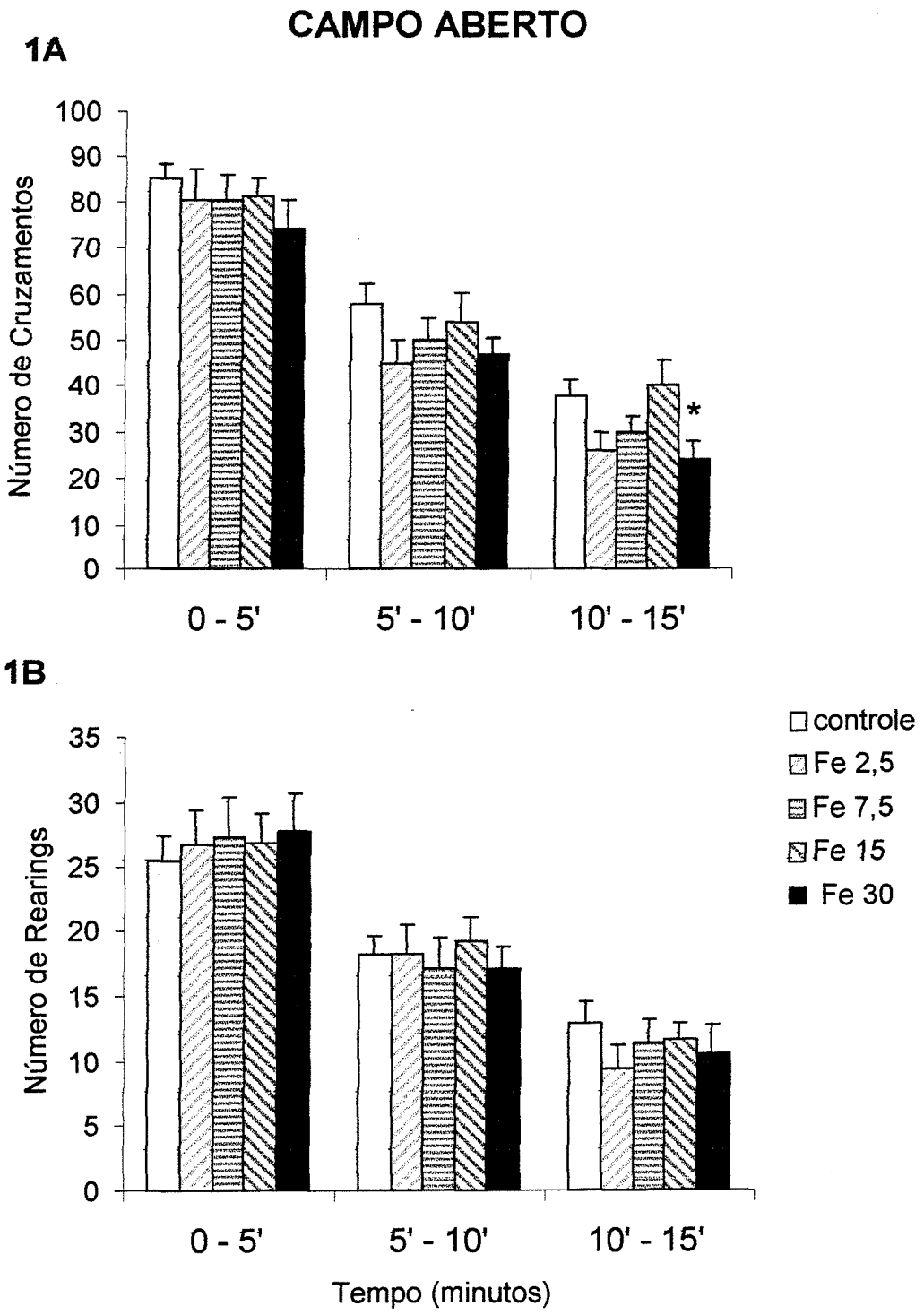
Pode-se verificar, através dos resultados obtidos no labirinto radial, que o tratamento neonatal com ferro em ratos produziu déficits na capacidade de aprendizado para esta tarefa semelhantes àqueles encontrados em camundongos. Para os parâmetros avaliados obteve-se déficits em todas as doses utilizadas, confirmando o que havia sido encontrado anteriormente com camundongos.

Em conjunto, estes resultados indicam que o tratamento neonatal com ferro é capaz de produzir alterações neurocomportamentais em ratos, da mesma forma que foi demonstrado em camundongos. Com isso, ampliam-se a utilidade e a validade deste modelo, uma vez que os resultados anteriores foram confirmados e reproduzidos e o tratamento mostrou-se aplicável a diferentes espécies.

LEGENDAS PARA AS FIGURAS:

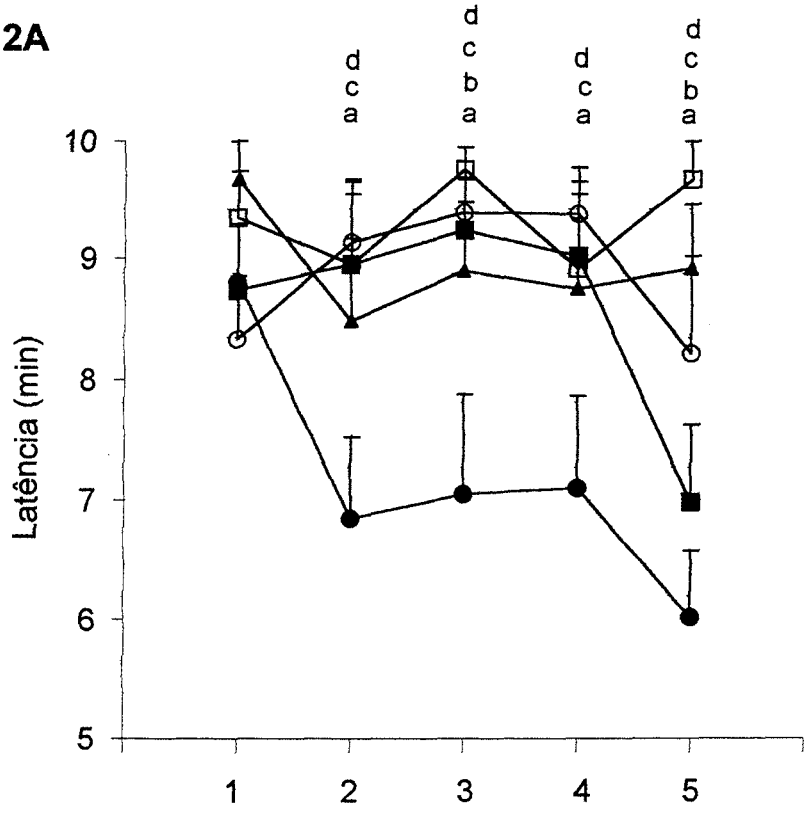
Figura 1: *Tarefa do campo aberto realizada em ratos de 90 dias de idade os quais receberam tratamento neonatal com ferro. Grupos de ratos Wistar (n = 12-16) receberam 0.0; 2.5; 7.5; 15.0 ou 30.0 mg Fe^{+2}/kg de peso corporal do 10° ao 12° dia pós-natal. Análise estatística dos dados referentes ao número de cruzamentos* **(1A)** *e número de respostas de orientação* **(1B)** *foi realizada utilizando-se ANOVA de uma via, seguida do teste de Duncan. O critério para significância foi p< 0.05 (representado na figura por *).*

Figura 2: *Tarefa do labirinto radial de 8 braços realizada em ratos de 120 a 150 dias de idade os quais receberam tratamento neonatal com ferro. Grupos de ratos Wistar (n = 11-14) receberam 0.0; 2.5; 7.5; 15.0 ou 30.0 mg Fe^{+2}/kg de peso corporal do 10° ao 12° dia pós-natal. Análise estatística foi realizada através da utilização da ANOVA de medidas repetidas. Houve uma interação significativa (grupos x sessões) para ambos os parâmetros do labirinto radial (Latência* **(2A)**: *F(4,52) = 3.46, p < 0.02; número de escolhas corretas nas 8 primeiras visitas* **(2B)**: *F(4,52) = 3.38, p < 0,02) medidas nos 5 dias consecutivos. Teste de Fisher foi utilizado para avaliar as diferenças entre os grupos. As letras representam diferenças significativas entre os grupos (a = diferença entre o grupo de 2.5 mg/kg X controle; b = grupo 7.5 mg/kg X controle; c = grupo de 15.0 mg/kg X controle; d = grupo de 30 mg/kg X controle).*

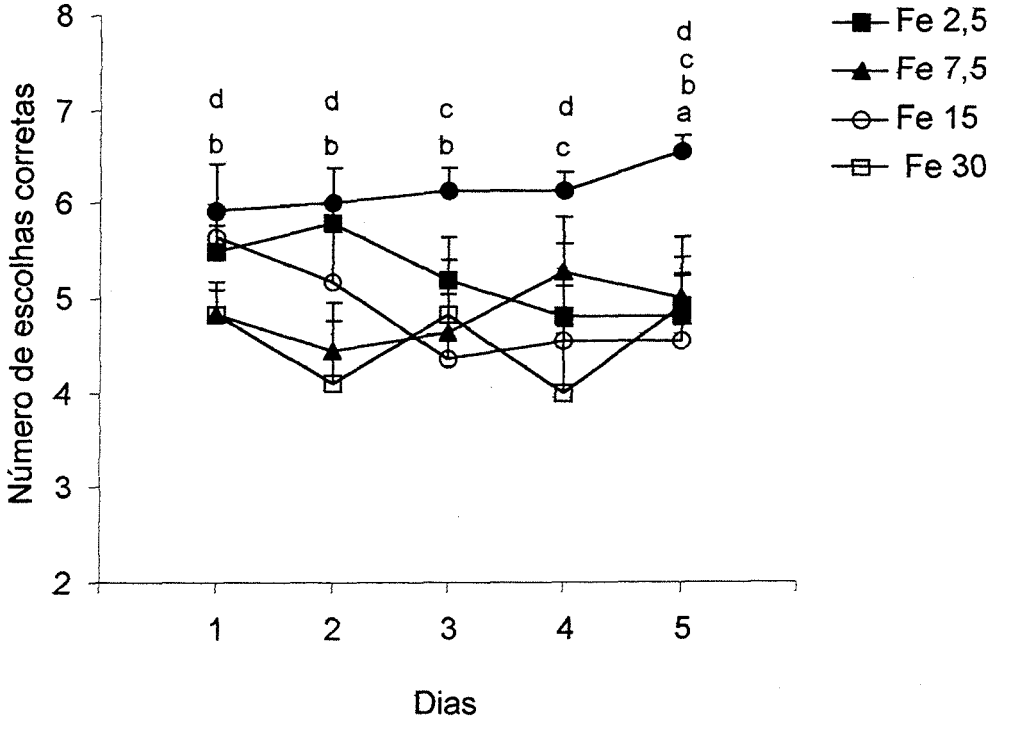

CAMPO ABERTO
1A
Número de Cruzamentos
100
90
80
70
60
50
40
30
20
10
0
*
0 - 5'
5' - 10'
10' - 15'
1B
Número de Rearings
35
30
25
20
15
10
5
0
0 - 5'
5' - 10'
10' - 15'
Tempo (minutos)
controle
Fe 2,5
Fe 7,5
Fe 15
Fe 30

LABIRINTO RADIAL

2A



2B



Capítulo VI

Discussão Geral

Os resultados apresentados nesta tese podem ser resumidos como se segue:

No primeiro estudo (capítulo II) verificou-se que os animais tratados com Fe^{+2} 37.0 mg/kg apresentaram marcada hipocinesia para os três parâmetros da atividade motora: locomoção, respostas de orientação (rearings) e atividade total nos primeiros vinte minutos, no entanto foram mais ativos nos vinte minutos finais da sessão de uma hora. Os camundongos tratados com a dose mais baixa (Fe^{+2} 3.7 mg/kg/dia) apresentaram diminuição apenas no número de respostas de orientação no primeiros vinte minutos e um aumento da locomoção e das respostas de orientação nos vinte minutos finais da sessão.

Na tarefa do labirinto radial, os camundongos tratados com Fe^{+2} 37.0 mg/kg apresentaram um maior número de erros, bem como uma latência maior em obter os 8 pellets no 3° (último) dia testado, quando comparados com os controles. Ambas as doses de ferro utilizadas induziram déficits na habituação dos camundongos à caixa utilizada para testar a atividade motora. A análise do conteúdo total de ferro (µg/g) mostrou que a dose de 37.0 mg/kg induziu um aumento nos níveis de ferro nos gânglios da base, mas não no córtex frontal.

No segundo grupo de experimentos (capítulo III) os camundongos foram tratados com ferro (7.5 mg/kg) em três diferentes fases do período neonatal: do 3° ao 5°, do 10° ao 12° (mesma fase estudada no primeiro grupo de experimentos) e do 19° ao 21° dia de vida. Confirmando os resultados obtidos no primeiro estudo, o tratamento com ferro do 10° ao 12° dia causou hipocinesia para os três parâmetros da atividade motora: locomoção, respostas de orientação, e atividade total nos primeiros vinte minutos, sendo que a respostas de orientação neste caso, continuaram diminuídas no 2° período de 20

minutos. Novamente, encontrou-se um aumento dos três parâmetros nos vinte minutos finais da sessão. O tratamento com ferro do 3° ao 5° dia, produz efeitos sobre a atividade motora, mas em uma menor extensão que aqueles produzidos pelo tratamento do 10° ao 12° dia. Nos primeiros 20 minutos da sessão somente o número de respostas de orientação foram diminuídas, enquanto que os outros parâmetros permaneceram inalterados neste período. Nos 20 minutos finais da sessão observou-se aumento nos três parâmetros, à semelhança do tratamento do 10° ao 12° dia. Ainda, foi possível observar que o tratamento nas duas fases produziu déficits na habituação à caixa utilizada para testar a atividade motora.

Na tarefa do labirinto radial, os camundongos tratados do 10° ao 12° dia apresentaram maior número de erros e maior latência na segunda e terceira (última) sessão, enquanto que nos tratados do 3° ao 5° dia o mesmo ocorreu somente na última. A análise do conteúdo total de ferro (μg/g) mostrou que quando o ferro foi administrado do 3° ao 5° e do 10° ao 12° encontrou-se uma elevação nos níveis de ferro nos gânglios da base, mas não no córtex frontal.

O tratamento neonatal com ferro do 19° ao 21° dia de vida não produziu efeitos em nenhum dos parâmetros das tarefas comportamentais estudadas, além de não alterar o conteúdo de ferro nos gânglios da base. Isto pode ser entendido facilmente considerando-se que nesta fase do período neonatal os níveis de transporte de transferrina e ferro ao cérebro são aproximadamente sete vezes menores do que os níveis observados do 10° ao 15° dia pós-natal (Taylor & Morgan, 1990). Da mesma forma, isso também

explica a obtenção de efeitos máximos quando o tratamento foi realizado do 10° ao 12° dia.

O terceiro estudo (capítulo IV) reproduz os efeitos do tratamento neonatal com ferro em uma cepa diferente de camundongos. Enquanto os dois estudos anteriores foram realizados com camundongos NMRI , para o terceiro estudo utilizou-se camundongos C57/ Bl6 (cepa sensível ao MPTP). O ferro produziu novamente hipocinesia nos primeiros vinte minutos e hipercinesia nos vinte minutos finais para todos os parâmetros medidos da atividade motora. Conforme já foi demonstrado anteriormente (Fredriksson et al, 1990; Sundström et al, 1990) o MPTP por si só produziu hipocinesia dependente da dose ao longo dos três períodos consecutivos de 20 minutos para os três parâmetros da atividade motora. A combinação do tratamento neonatal com ferro com a exposição dos camundongos adultos ao MPTP produziu déficits severos na atividade motora, acentuando os efeitos do MPTP. A análise neuroquímica mostrou que a combinação do ferro com MPTP produz uma depleção mais acentuada do conteúdo de DA estriatal, do que aquela encontrada nos animais tratados apenas com MPTP.

No capítulo V encontra-se o último grupo de experimentos apresentados nesta tese, os quais relacionam-se com a aplicabilidade dos estudos anteriores em ratos. Neste caso, foram empregadas quatro diferentes doses de ferro as quais foram administradas do 10° ao 12° dia pós-natal. Os ratos tratados com Fe^{+2} 30.0 mg/kg apresentaram diminuição no número de cruzamentos nos 5 minutos finais da sessão na tarefa do campo aberto. Na tarefa do labirinto radial todas as doses utilizadas (de 2.5 a 30.0 mg/kg/dia) causaram

aumento na latência em visitar os oito braços e menor número de escolhas corretas nas 8 primeiras entradas no 5° (último) dia testado.

Com base nos resultados apresentados nesta tese, os quais, em conjunto demonstram que tratamento neonatal com ferro é capaz de produzir efeitos motores e cognitivos tanto em camundongos quanto em ratos, parece ser de grande relevância considerar os fatores que afetam o consumo de ferro durante a infância. Crianças que se alimentam de leite materno têm um consumo relativamente constante de ferro que varia de 0.14 a 0.32 mg/dia durante a maior parte da infância (Dewey et al, 1984; Dewey & Lönnerdal, 1983). É provável que o conteúdo de ferro no leite esteja sob um controle homeostático, uma vez que estudos demonstram que mulheres anêmicas e mulheres recebendo suplementação de ferro apresentam níveis de ferro no leite semelhantes aos de mulheres não anêmicas (Zavaleta et al, 1995). Crianças alimentadas com fórmulas lácteas consomem diariamente de 4.8 a 7.0 mg (níveis de fórmulas européias) ou de 10.0 a 14.0 mg (níveis de fórmulas nos E.U.A.) de ferro (Lönnerdal, 1997), os quais correspondem à cerca de 10 e 100 vezes (respectivamente) aos níveis diários de crianças alimentadas com leite materno.

Há alguns anos vem sendo estudado o potencial neurotóxico do ferro através de estudos que se utilizam da infusão intracerebral na substância negra de sais deste metal. Ben-Shachar & Youdim (1991) encontraram redução no conteúdo de DA e HVA (ácido homovanílico) estriatais após infusões de ferro na sustância negra de ratos. Similarmente, Sengstock e colaboradores (1993) demonstraram alterações neuroquímicas decorrentes da infusão intranigral de cloreto de ferro em ratos. Neste estudo os autores

encontraram uma elevada correlação entre a quantidade de ferro infundida e a concentração de DA, DOPAC (ácido di-hidróxi fenil acético) e HVA no estriado *ipsi lateral* à infusão em ratos. Em outro estudo, Sengstock et al (1994) demonstraram que a infusão de ferro na substância negra de ratos causou morte neuronal, além de produzir uma progressiva atrofia na substância negra e reduções progressivas no comportamento rotacional induzido por apomorfina (marcador bem estabelecido para parkinsonismo em ratos). Estes autores postularam, então, que a infusão intranigral de ferro poderia constituir-se em um novo modelo animal para D. P. Ainda, há trabalhos que demonstram que macacos tratados com MPTP (Temlett et al, 1994), bem como ratos lesionados com 6-OHDA (6-Hidróxi Dopamina) (Oestreicher et al, 1994; He et al, 1996) apresentaram acumulação de ferro na substância negra.

O crescente interesse na utilização do ferro para estudo de doenças neurodegenerativas no sistema negro-estriatal é provavelmente decorrente da existência de inúmeros trabalhos que demonstram a presença de concentrações elevadas de ferro na substância negra de pacientes da Doença de Parkinson (Griffiths et al, 1999; Dexter et al, 1991; Ebadi et al, 1996; Faucheux et al, 1993; Jellinger et al, 1993; Kienzl et al, 1995).

Existem poucos estudos, no entanto, que investigam as conseqüências funcionais da acumulação de ferro. Sobotka et al (1996) alimentaram ratos com dietas com diferentes concentrações de ferro: 4 (grupo deficiente em ferro), 35 (grupo controle), 350, 3.500 ou 20.000 ppm por doze semanas, durante as quais os animais foram pesados e submetidos a testes comportamentais. A ração era fornecida *ad libitum* e o estudo não indica o consumo de comida diário e nem a dosagem de ferro recebida diariamente.

Reduções de peso foram encontradas nos ratos que receberam a ração deficiente em ferro e nos ratos que receberam a ração contendo 3.500 e 20.000 ppm de ferro. Este estudo relata que os grupos que receberam as rações contendo 3.500 e 20.000 ppm apresentaram déficits comportamentais incluindo diminuição na atividade locomotora e na habituação à caixa. Estes déficits funcionais foram confirmados e avaliados mais profundamente através dos trabalhos desenvolvidos nesta tese, nos quais obtivemos déficits na atividade locomotora e no aprendizado da tarefa do labirinto radial decorrentes do tratamento com uma quantidade definida de ferro por um período determinado. Os animais tratados com ferro não apresentaram alterações no seu peso corporal, o que permite uma melhor interpretação dos resultados obtidos.

O estudo no qual combinamos o tratamento neonatal com ferro com a exposição ao MPTP mostra que o ferro é capaz de potenciar os déficits neuroquímicos e funcionais induzidos pelo MPTP, indicando mais diretamente a participação do ferro com os processos envolvidos na neurodegeneração. Uma vez que o MPTP é uma toxina que age seletivamente nos neurônios dopaminérgicos da substância negra e o ferro administrado no período neonatal potenciou os seus efeitos, parece plausível que o ferro acumulou-se nesta estrutura. É provável que o ferro acumulado nesta estrutura torne os neurônios dopaminérgicos mais vulneráveis à ação do MPTP, o qual desencadearia a destruição dos mesmos.

Um estudo recente (Lan & Jiang, 1998) oferece evidências sobre o papel da acumulação do ferro como um agente crítico no processo degenerativo. Estes autores administraram ferro de maneira crônica na ração de camundongos, o que resultou num

aumento significativo da concentração de ferro no cérebro destes animais. O excesso de ferro não aumentou, por si só, os níveis de DA nem a peroxidação lipídica no estriado, no entanto, uma simples dose de MPTP a estes animais induziu uma grande alteração nestes parâmetros neuroquímicos. Estes resultados parecem estar de acordo com os resultados descritos no capítulo IV desta tese, os quais sugerem que o excesso de ferro contribui grandemente para os efeitos danosos de agentes desencadeadores do processo neurodegenerativo, como por exemplo o MPTP. Finalmente, Jellinger, 1999 apontou a desregulação do metabolismo do ferro e no metabolismo oxidativo induzido pelo ferro e a formação de radicais livres como fatores importantes implicados na patologia da Doença de Parkinson.

Os resultados aqui apresentados, juntamente com estudos clínicos (Rustin et al, 1998) demonstram o envolvimento do excesso de ferro em doenças neurodegenerativas, tais como DP. Estudos posteriores serão necessários para esclarecer os mecanismos através dos quais este metal, amplamente utilizado na dieta infantil, acumula-se em regiões específicas do cérebro, e de que maneira a homeostasia é rompida desencadeando o processo degenerativo.

Entretanto, a relevância deste trabalho está no fato de que demonstramos pela primeira vez a susceptibilidade de um período definido da vida em que a administração de baixas quantidades de ferro por um tempo curto é capaz de produzir efeitos comportamentais e neuroquímicos que podem estar relacionados com processos neurodegenerativos. Efeitos semelhantes foram obtidos anteriormente apenas através de exposições por períodos prolongados a quantidades muito elevadas deste metal.

Conclusões

1. Ambas as doses do tratamento neonatal com ferro (3.7 e 37.0 mg/kg) causaram hipocinesia em todos os parâmetros da atividade motora, embora os efeitos tenham sido menos pronunciados em camundongos tratados com a dose mais baixa.

2. O tratamento com a dose mais alta de ferro (37.0 mg/kg) produziu déficits no aprendizado da tarefa do labirinto radial.

3. O tratamento neonatal com 37 mg/kg de ferro causou elevação da concentração de ferro nos gânglios basais, mas não no córtex frontal dos camundongos.

4. O tratamento neonatal com ferro (7.5 mg/kg) do 10° ao 12°, e em menor extensão do 3° ao 5° dia de vida, causou hipocinesia.

5. O tratamento neonatal com ferro do 10° ao 12°, e em menor extensão do 3° ao 5° dia de vida, produziu déficit no aprendizado da tarefa do labirinto radial.

6. Camundongos tratados com ferro do do 3° ao 5° ou do 10° ao 12° dia de vida apresentaram aumento na concentração de ferro nos gânglios basais mas não no córtex frontal.

7. O ferro acentuou os efeitos deletérios do MPTP, pois a combinaçào dos dois tratamentos produziu severos déficits motores em camundongos adultos.

8. A análise neuroquímica demonstrou que camundongos tratados com ferro e MPTP apresentaram uma maior depleção nos níveis de DA estriatal do que camundongos que receberam somente o MPTP.

9. O tratamento neonatal com a dose mais alta de ferro (30.0 mg/kg) em ratos causou diminuição no número de cruzamentos no campo aberto.

10. Os ratos tratados com todas as doses de ferro utilizadas (de 2.5 a 30.0 mg/kg) apresentaram déficits no aprendizado da tarefa do labirinto radial.

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