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Efeito do Butirato de Sódio sobre o Déficit de Memória Induzido pela Sobrecarga Neonatal com Ferro

Porto Alegre

2011

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Efeito do Butirato de Sódio sobre o Déficit de Memória Induzido pela Sobrecarga Neonatal com Ferro

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RESUMO

O excesso de ferro no encéfalo tem sido relacionado com a patogênese de diversas doenças neurodegenerativas, como por exemplo, as doenças de Alzheimer e de Parkinson. Reconhece-se o período neonatal como crítico para o estabelecimento do conteúdo normal de ferro no cérebro adulto, podendo, de forma determinante alterar a distribuição cerebral deste metal de acordo com o grau de absorção apresentado. Estudos realizados anteriormente em nosso laboratório indicaram que a administração de ferro no período neonatal prejudica severamente a memória em ratos adultos.

Muitos estudos demonstram que a transcrição gênica é um processo necessário para a plasticidade e a consolidação da memória de longa duração. A acetilação das histonas ocorre nos resíduos lisina, neutralizando sua carga positiva, diminuindo a afinidade entre a proteína e o DNA, levando o relaxamento da estrutura da cromatina, permitindo o recrutamento da maquinaria transcricional. A deacetilação das histonas é um processo reversível, que ocorre pela ação das histonas deacetilases (HDACs), as quais representam a principal via de manipulação farmacológica do epigenoma, com promissores valores terapêuticos. Nos últimos anos ocorreu um aumento no número de estudos sugerindo que o Butirato de sódio (NaBut), um inibidor específico de HDACs, apresenta um possível potencial como agente terapêutico no tratamento de doenças neurodegenerativas. No entanto, a caracterização de seus efeitos é limitada pela falta de estudos utilizando modelos animais adequados que reproduzam aspectos de doenças neurodegenerativas. Portanto, o objetivo deste estudo foi determinar se os déficits de memória induzidos pelo tratamento neonatal com ferro podem ser revertidos pela administração de NaBut. Para tanto, ratos machos receberam veículo (5% de sorbitol em água) ou ferro (10,0 mg/kg) via oral do 12° ao 14° dia pós-natal. Ao atingirem a idade adulta, ambos os grupos foram divididos em grupos experimentais que receberam injeções agudas de NaBut (1,2g/Kg) ou veículo por via intraperitoneal, imediatamente após a sessão de treino nas tarefas de esquiva inibitória e reconhecimento do objeto novo.

Os resultados mostraram que uma injeção aguda imediatamente após a sessão de treino não afetou a memória em ratos controles, mas foi capaz de reverter os déficits de memória induzidos pelo tratamento neonatal com ferro, tanto na tarefa de reconhecimento de objeto quanto na tarefa de esquiva inibitória.

A partir dos resultados obtidos nos testes de memória de reconhecimento de objeto, e no teste relacionado à memória aversiva, a esquiva inibitória, podemos sugerir que o NaBut apresenta resposta satisfatória como modulador da memória, permitindo-nos inferir sua potencialidade como agente terapêutico para o tratamento dos prejuízos de memória que acompanham muitas doenças neurodegenerativas.

Palavras-chave: ferro – neurodegeneração – butirato de sódio - memória.

ABSTRACT

Iron accumulation in the brain has been associated to the pathogenesis of neurodegenerative disorders. We have previously demonstrated that iron overload in the neonatal period results in severe and persistent memory deficits in adult rats. Here, using the animal model of cognitive impairment induced by iron overload we tested the effects of NaBut in ameliorating memory. Rats received vehicle or 10.0 mg/kg Fe^{+2} orally at postnatal days 12-14. When animals reached the age of 2 months, they received an acute intraperitoneal injection of vehicle or NaBut (1.2 g/kg) immediately after the training session in either novel object recognition or inhibitory avoidance tasks. Retention test sessions were performed 24 hours after training. Animals that received iron in the neonatal period showed severe memory deficits. A single acute injection of NaBut was able to recover memory deficits in iron-treated rats. The results provide evidence that NaBut may be considered for the treatment of cognitive decline associated with neurodegenerative disorders.

Keywords: sodium butyrate; object recognition; inhibitory avoidance; neurodegeneration; iron.

LISTA DE ABREVIATURAS

DNA – Ácido desoxirribonucléico

DP – Doença de Parkinson

DA – Doença de Alzheimer

NaBut – Butirato de Sódio

HDAC – Histona Deacetilase

HDACi – Inibidor de Histona Deacetilase

SAMP-8 – SENESCENCE ACCELERATED PRONE MOUSE - modelo de envelhecimento acelerado estabelecido por seleção fenotípica

MAO-B – Monoamino oxidase B

AChE – Acetilcolinesterase

PDE4 – Fosfodiesterase tipo 4

H4K12 – Histona 4, acetilada na lisina 12 -

H1 – Histona 1

H2 – Histona 2

H3 – Histona 3

H4 – Histona 4

LTM – Memória de longa duração

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1. REFERENCIAL TEÓRICO

Como resultado das mudanças nas taxas de mortalidade e de fertilidade nas últimas décadas no Brasil, estima-se que a população acima de 65 anos irá crescer de 2,7% em 1960 para aproximadamente 14% antes de 2050, um aumento três vezes mais rápido do que o observado nos países desenvolvidos. Devido ao envelhecimento populacional, a prevalência e a incidência de doenças neurodegenerativas, comuns em idosos, também têm aumentado no Brasil. De acordo com Chaimowicz *et al.*, 2006, a demência é uma condição comum entre idosos, aumentando significativamente com a idade, duplicando sua incidência a cada 5 anos, a partir dos 60 anos; utilizando-se a doença de Parkinson como exemplo, observa-se que são 10 % dos novos casos de demência, sendo a incidência anual da Doença de Parkinson (DP) de aproximadamente 107 casos para cada 1000 pessoas, representando 80% dos casos de parkinsonismo, com prevalência de 550 casos para cada 1000 idosos acima dos 70 anos.

A manifestação dessas desordens vem crescendo significativamente em nível mundial: no Reino Unido cerca de 5% da população acima de 65 anos apresenta algum tipo de demência, sendo que a prevalência é crescente a medida que a idade aumenta, chegando a 20% nos idosos acima de 80 anos (Edwardson & Kirkwood, 2002); nos Estados Unidos, estima-se que 4,5 milhões de habitantes sofram da Doença de Alzheimer (DA) (Fillit, 2002); de acordo com estudos na população suíça, aproximadamente 10% dos idosos entre 85 e 88 anos que não apresentam um quadro característico de demência

passam a desenvolver a doença a cada ano e no Japão, onde a expectativa de vida é maior que a de qualquer outro país (75,6 anos para os homens e 81,4 anos para as mulheres), a incidência da DA é proporcionalmente a mais alta do mundo (Post, 1999).

O estudo dos mecanismos envolvidos no desenvolvimento das patologias neurodegenerativas, assim como de medidas preventivas e terapêuticas, torna-se muito importante, pois, esses ainda não foram completamente elucidados. Além disso, esse tipo de doença gera uma profunda sobrecarga emocional, social e econômica, o que prejudica o estabelecimento de um envelhecimento bem sucedido entre a população de idosos.

Um crescente corpo de evidências clínicas e experimentais sugere a participação do ferro em doenças neurodegenerativas, particularmente no mecanismo de morte celular na DP, pois a maioria das reações de formação de radicais hidroxil, induzidas pelo metabolismo da dopamina, envolve a presença de ferro. Além disso, evidências sugerem que o estresse oxidativo participe no mecanismo de morte neuronal, devido à formação excessiva de peróxido de hidrogênio e radicais livres derivados de oxigênio (Figura 1), que podem causar danos à célula através de reações de peroxidação lipídica e alterações na fluidez da membrana.

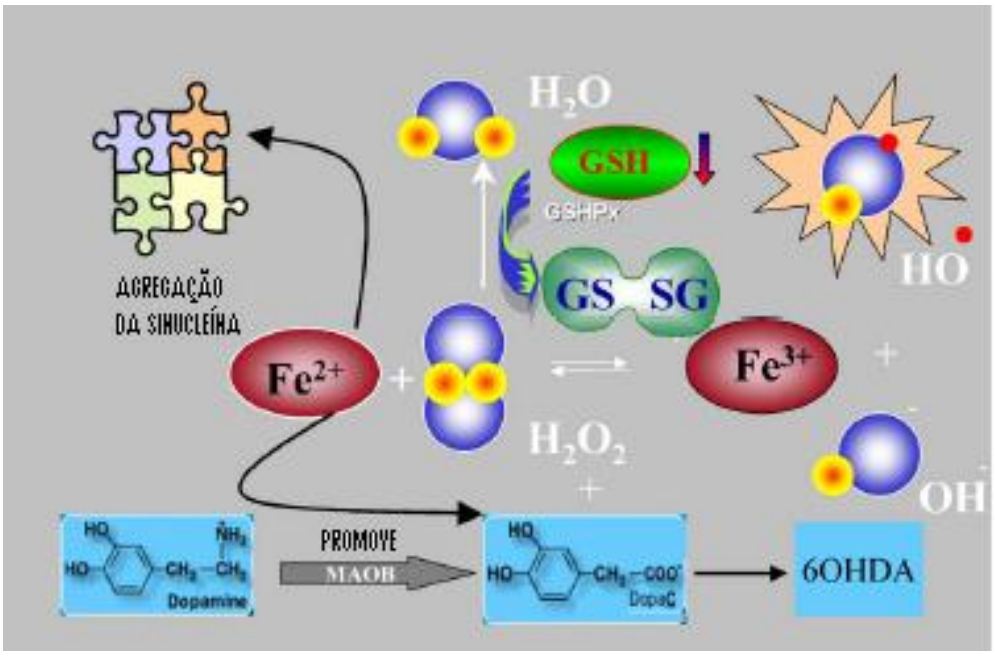

Figura 1 – Envolvimento do ferro em processos oxidativos, demonstrando a formação de radicais livres a partir do peróxido de hidrogênio. (Pollla *et al.*, 2003.)

O período neonatal é crítico para o estabelecimento do conteúdo de ferro no cérebro adulto. Investigações a respeito da captação de ferro pelo cérebro indicaram que o transporte de ferro para o cérebro atinge seus níveis máximos durante o período pós-natal de rápido crescimento cerebral, como indicado na Figura 2 (Taylor & Morgan, 1990; Taylor *et al*., 1991). Além disso, a distribuição cerebral de ferro altera-se com o envelhecimento, podendo ter alguma relação com disfunções nas vias de manutenção da homeostasia desse metal e, conseqüentemente, promovendo os depósitos nas regiões onde seu metabolismo é mais alto, podendo, desse modo, participar de eventos neurodegenerativos (Zecca *et al*., 2004; 2001; Martin *et al.*, 1998).

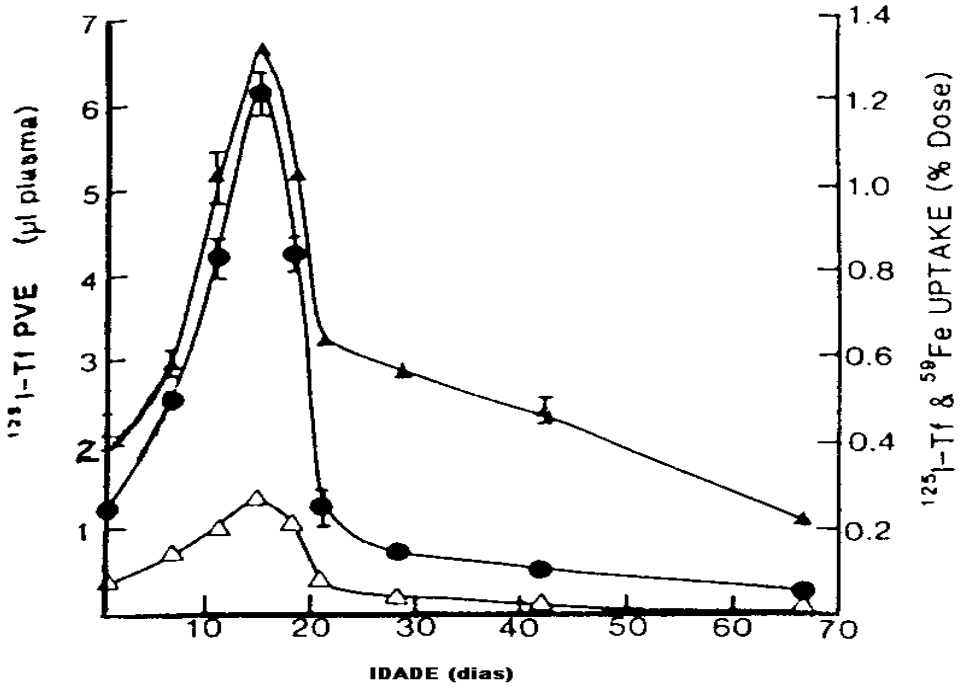

Figura 2 – Transporte de Ferro e transferrina na barreira hemato-encefálica ao longo da vida em ratos. (Taylor & Morgan, 1990)

Enquanto no passado a ênfase havia sido dada ao combate à deficiência de ferro (anemia), a aplicação indiscriminada de suplementação de ferro a crianças durante seu primeiro ano de vida tornou importante estudar os mecanismos através dos quais o organismo pode se proteger contra o excesso desse metal (Bothwell, 1995).

De fato, Fredriksson e colaboradores (Fredriksson *et al*., 1999), utilizando camundongos, descreveram pela primeira vez que o tratamento sistêmico com ferro durante o período de rápido desenvolvimento cerebral (período que vai, em humanos, desde o último trimestre da gestação até um ano de vida) produz acúmulo seletivo de ferro nos gânglios da base, além de causar disfunções neurocomportamentais. Os resultados mostraram, ainda,

que camundongos (Fredrikson *et al*., 2000) e ratos (Schröder *et al*., 2001) tratados com ferro do 10° ao 12° dia de vida pós-natal apresentam hipoatividade motora, bem como déficits no aprendizado e memória em duas diferentes tarefas comportamentais, o labirinto radial de oito braços e a esquiva inibitória.

Recentemente, foi verificado que ratos tratados com ferro do 12° ao 14° dia de vida pós-natal apresentam déficits de memória de reconhecimento quando adultos (De Lima *et al*., 2005a). Foi observado também que a administração de ferro no período neonatal induz um aumento significativo na peroxidação lipídica na substância nigra, no córtex e no hipocampo, bem como um aumento de danos oxidativos a proteínas nestas mesmas regiões cerebrais de ratos adultos. Adicionalmente, a análise revelou que ocorre uma diminuição da atividade da superóxido dismutase (enzima anti-oxidante) na substância nigra, no córtex e no hipocampo (De Lima *et al.*, 2005a). Esses resultados sugerem que o ferro possa estar exercendo seus efeitos deletérios sobre a cognição através do aumento dos níveis de estresse oxidativo cerebral.

Além disso, ratos submetidos à administração de ferro do 12° ao 14° dia de vida pós-natal, tratados com selegilina (um fármaco utilizado no tratamento da DP) simultaneamente ao tratamento com ferro ou tardiamente (na fase adulta), foi constatado que esse fármaco é capaz de proteger e reverter os déficits cognitivos induzidos pela exposição ao ferro na tarefa de reconhecimento do objeto novo (De Lima *et al*., 2005b).

Mais recentemente outros estudos foram realizados em nosso laboratório utilizando o modelo animal no sentido de investigar compostos com propriedades neuroprotetoras e melhoradoras da memória no modelo de sobrecarga neonatal com ferro (De Lima *et al*., 2007; 2008a).

1.1. INFLUÊNCIA DAS HISTONAS NO PROCESSO DE TRANSCRIÇÃO

As histonas são os principais constituintes protéicos da cromatina, sua massa é aproximadamente igual a do DNA total da célula. Essas proteínas são relativamente pequenas e possuem um caráter básico acentuado, contendo grandes proporções de aminoácidos carregados positivamente (LISINA e ARGININA) em sua constituição, o que favorece a interação destas proteínas com a fita dupla de DNA, carregada negativamente. A acetilação das histonas ocorre nos resíduos lisina, especificamente no grupo amino da cadeia lateral, efetivamente neutralizando sua carga positiva, diminuindo a afinidade entre a proteína e o DNA, levando ao relaxamento da estrutura da cromatina, permitindo o recrutamento da maquinaria transcricional (Roth & Sweat, 2009).

As histonas estão divididas em cinco classes, caracterizadas de acordo com a proporção relativa de cada um dos aminoácidos básicos, podendo ser reconhecidas em todos os eucariotos (Zaha et al, 1996).

As histonas **H3** e **H4** estão entre as proteínas conhecidas mais conservadas ao longo do processo evolutivo, possuindo seqüências idênticas mesmo em organismos pouco relacionados, sugerindo uma função

possivelmente idêntica em todos os eucariotos. As classes de histonas **H2A** e **H2B** são também identificadas em todos os grupos eucarióticos, mas com variação espécie-específica considerável em suas seqüências de aminoácidos. A classe de histonas **H1** compreende uma série de proteínas relacionadas, com homologia parcial nas seqüências de aminoácidos e apresentam tanto variação entre espécies, quanto entre tecidos diferentes de uma mesma espécie.

A acetilação de resíduos de lisina das histonas centrais (H2A, H2B, H3 e H4) *in vivo* é fortemente relacionada com o aumento da atividade transcricional. Os grupos acetil são incorporados às histonas na fase S (de replicação do DNA) do ciclo celular, sendo removidos antes do início da mitose. A acetilação de histonas, aparentemente, não afeta nem a estrutura nem a estabilidade das partículas centrais, mas altera a conformação da ligação ao DNA. Sendo assim a acetilação diminui a estabilidade da fibra de 30nm, evitando uma maior compactação da cromatina e, por conseguinte, favorecendo a transcrição. (Zaha *et al.*, 1996) As modificações epigenéticas da cromatina promovem uma importante modulação da transcrição gênica, tendo envolvimento significativo na regulação da plasticidade sináptica e da memória (Roth & Sweat, 2009). Entre as modificações epigenéticas está a desacetilação das histonas que, normalmente, reprime a expressão gênica (Barrett & Wood, 2008; Kouzarides, 2007).

Sendo assim, já é reconhecido o envolvimento das histonas em muitos processos neurais superiores, como plasticidade sináptica e memória (Stefanko *et al.*, 2009), além de suas funções normais no processo transcricional, bem

como ação de uma família de enzimas conhecidas como histona-deacetilases (HDACs) no processo de regulação gênica, que tem a função de manter a cromatina condensada, inibindo o processo de transcrição (Lozano *et al.*, 2008).

1.2. ACETILAÇÃO E DESACETILAÇÃO DAS HISTONAS E SUA RELAÇÃO COM A MEMÓRIA

A deacetilação das histonas é um processo reversível, que ocorre pela ação das HDACs, as quais estão num total de onze diferentes isoformas, que representam a principal via de manipulação farmacológica do epigenoma, com promissores valores terapêuticos (Roth & Sweat, 2009). Entre outros, o Butirato de Sódio (NaBut) é um inibidor reversível não competitivo das HDACs.

Estudos demonstraram que o bloqueio da atividade das HDACs através da administração sistêmica ou intra-hipocampal do NaBut, melhora a memória para condicionamento ao medo (Vecsey *et al*., 2007) e memória de reconhecimento (Stefanko *et al*., 2009).

O tratamento de animais "*in vivo*" com inibidores das HDACs reforça a formação da memória de longa duração, indicando que alterações na acetilação das histonas modulam a estrutura da cromatina, a formação da memória de longa duração e a aprendizagem (Levenson *et al*, 2004). Resultados deste mesmo estudo demonstram que a acetilação dos diferentes tipos de histonas é dependente do tipo de memória formado. Esta

especificidade sugere o envolvimento de alterações epigenéticas no processo de formação da memória e na aprendizagem, não por causa das alterações de transcrição gênica, mas envolvendo alterações específicas no maquinário epigenético que parecem causar impacto nos diferentes tipos de formação da memória (Franklin & Mansuy, 2010).

Entende-se que o código epigenético pode estar associado à memória, como um modificador da cromatina que influencia a expressão de genes necessários a cognição, e também nos processos de formação de memória de longa duração e extinção de medo contextual (Roth & Sweat, 2009).

Fontán-Lozano e colaboradores (2008) demonstraram que a aquisição da memória de reconhecimento envolve a acetilação da histona H3 (Ac-H3), estando a forma acetilada aumentada no hipocampo, uma área cerebral crucial para os processos cognitivos. Além disso, os autores demonstraram que a administração de inibidores de HDACs melhoram os processos cognitivos em animais com déficit cognitivo induzido por Kainato/Acido Kainico, e também pelo envelhecimento em animais mutantes SAMP-8.

Outro estudo demonstrou que o aumento da acetilação de histonas pelo uso de inibidores das HDACs induz o brotamento de dendritos e um aumento no número de sinapses (Fischer *et al*., 2007). Os autores concluem o estudo sugerindo que a inibição das HDACs pode representar uma estratégia terapêutica para doenças neurodegenerativas em que há perda/prejuízo de

memória, e propõem a necessidade de estudos adicionais em modelos animais adequados.

De acordo com o exposto acima, conclui-se que as histonas desempenham um importante papel no equilíbrio dinâmico da cromatina, sendo, as HDACs, enzimas que atuam nas histonas promovendo desacetilação da cauda amino-terminal. A inibição do processo de acetilação pelo NaBut, um representante da classe de fármacos inibidores de HDACs, tem demonstrado uma atividade moduladora da memória em alguns processos neurodegenerativos, de acordo com o que é observado nos estudos mais recentes com o NaBut.

No presente trabalho, investigamos os efeitos da administração sistêmica de NaBut sobre a memória aversiva e de reconhecimento de longa duração em modelo animal de ratos Wistar, de disfunção cognitiva induzida por sobrecarga de ferro no período neonatal associada ao desenvolvimento de doenças neurodegenerativas, causadas pelo acúmulo de ferro no tecido cerebral.

2. OBJETIVOS

Este trabalho teve como principal objetivo investigar o potencial terapêutico do uso de Butirato de Sódio (NaBut), através da utilização de um modelo animal, avaliando os efeitos do tratamento agudo com NaBut sobre prejuízos de memória.

2.1. Objetivos Específicos

- Avaliar o efeito do tratamento agudo com NaBut sobre os prejuízos de memória de reconhecimento de objetos novos induzidos pelo tratamento com ferro do 12° ao 14° dia de vida pós-natal.

- Avaliar o efeito do tratamento agudo com NaBut sobre os prejuízos de memória aversiva (esquiva inibitória) induzidos pelo tratamento com ferro do 12° ao 14° dia de vida pós-natal.

3. ARTIGO

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Assunto: Submission Confirmation

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Corresponding Author: Dr. Nadja Schroder

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Research paper

Memory impairment induced by brain iron overload is accompanied by reduced H3K9 acetylation and ameliorated by sodium butyrate

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Abstract

Iron accumulation in the brain has been associated to the pathogenesis of neurodegenerative disorders. We have previously demonstrated that iron overload in the neonatal period results in severe and persistent memory deficits in adult rats. Alterations in histone acetylation have been associated with memory deficits in models of neurological disorders. Here we examine histone acetylation in the brain and the effects of the histone deacetylase inhibitor (HDACi) sodium butyrate (NaB) on memory in the neonatal iron overload model in rats. Rats received vehicle or 30.0 mg/kg Fe^{+2} orally at postnatal days 12-14. When animals reached adulthood, they were given training in either novel object recognition or inhibitory avoidance. Histone acetylation in the dorsal hippocampus and the effects of NaB were examined in separate sets of rats. Iron overload led to a reduction in H3 lysine 9 acetylation in the hippocampus, without affecting the acetylation of other H3 and H4 lysine residues. A single systemic injection of NaB (1.2 g/kg) immediately after training ameliorated iron-induced memory impairments. The results suggest that a reduction in H3K9 acetylation might play a role in iron-induced memory impairment, and support the view that HDACis can rescue memory dysfunction in models of brain disorders.

Keywords: sodium butyrate; object recognition; inhibitory avoidance; neurodegeneration; iron; histone acetylation.

Iron is the most abundant metal in the brain, where it participates as a cofactor in key enzymatic reactions, which are relevant to neurotransmitter synthesis and degradation, dendritic arborization, and myelinization (Gerlach et al., 1994; Beard, 2008; Todorich et al., 2009). In addition, iron is part of metalloproteins involved in oxygen transport and energetic metabolism. However, it is widely recognized that iron, in the ferrous form, can react with hydrogen peroxide via the Fenton reaction producing the very reactive hydroxyl radical which can damage cellular components including membrane lipids, proteins and DNA (Halliwell and Gutteridge, 1992; Smith et al., 1997).

Some studies have indicated that iron concentration in the brain progressively increases during the aging process in normal individuals (Connor et al., 1990; Bartzokis et al., 1994), and it is currently known that iron selectively accumulates in the brains of patients suffering from neurodegenerative disorders (for a review Zecca et al., 2004). Iron-induced oxidative stress has been implicated in the pathogenesis of Alzheimer's, Parkinson's and Huntington's diseases, among others (Kell, 2010). Remarkably, recent studies have shown that iron content in brain regions has been positively correlated with poorer performance in cognitive testing in Alzheimer's patients (Ding et al., 2009). In addition, redox-active iron levels in the cerebrospinal fluid increased with the degree of cognitive impairment from normal to MCI subjects (Lavados et al., 2008).

We have previously demonstrated that iron, when administered to rodents in the neonatal period induces persistent memory deficits that are relevant to neurodegenerative disorders. Iron treatment disrupts spatial memory, assessed by the eight-arm radial maze and aversive memory

assessed by the inhibitory avoidance task (Schröder et al., 2001). Additionally, iron neonatal treatment has also proven to impair recognition memory (de Lima et al., 2005a; 2007; 2008a), which was associated to increased oxidative stress markers in brain regions relevant to memory formation (de Lima et al., 2005a). The precise mechanism underlying the disruptive effect of iron accumulation on memory is poorly understood.

Recently, memory decline related to aging has been associated with changes in epigenetic modification of chromatin, including altered histone acetylation (Peleg et al., 2010; Penner et al., 2010; 2011; Sweatt, 2010). Accumulating evidence indicates that chromatin remodeling through histone acetylation is a key epigenetic mechanism regulating gene transcription during memory formation. Histone deacetylases (HDACs) induce chromatin condensation and repress gene transcription. Memory formation has been associated with histone modifications including increased acetylation of H3 and H4 lysine residues (for recent reviews, see Barrett and Wood, 2008; Mikaelsson and Miller, 2011), whereas reduced H4K12 acetylation has been associated with memory impairment in aged mice (Peleg et al., 2010). Sodium butyrate (NaB) is an HDAC inhibitor (HDACi) that potently inhibits Class I HDACs, comprised of HDACs 1, 2, 3, and 8 (Kilgore et al., 2010). Systemic administration of NaB or other Class I HDACis enhances memory formation in rats and mice (Levenson et al., 2004), ameliorates ageing-related memory impairments in rats and mice (Peleg et al., 2010; Reolon et al., 2011), and improves memory deficits in models of neurodegenerative disorders (Alarcón et al., 2004; Kilgore et al., 2010).

In the present study, we verified whether neonatal iron overload in rats produces changes in the acetylation of H3K9, H3K14, H4K5, and H4K12 in the dorsal hippocampus. In addition, we examined the effects of posttraining systemic administration of NaB on the formation of novel object recognition memory and inhibitory avoidance in adult rats given neonatal iron.

2. Experimental procedures

2.1 Animals

Pregnant Wistar rats were obtained from the State Health Science Research Foundation (FEPPS-RS). After birth each litter was adjusted within 48 h to eight rat pups, and to contain offspring of both genders in about equal proportions. Each pup was kept together with its mother in a plastic cage with sawdust bedding in a room temperature of 21± 1ºC and a 12/12 h light/dark cycle. At the age of 3 weeks, pups were weaned and the males were selected and raised maintained in groups of three to five in individually ventilated cages with sawdust bedding. For postnatal treatments, animals were given standardized pellet food and tap water *ad libitum*.

All behavioral experiments were performed at light phase between 09:00 h and 16:30 h. All experimental procedures were performed in accordance with the NIH Guide for Care and Use of Laboratory Animals (NIH publication No. 80-23 revised 1996) and approved by the Institutional Ethics Committee of the Pontifical Catholic University (CEUA 10/00143). All efforts were made to minimize the number of animals and their suffering.

2.2 Treatments

2.2.1 Iron neonatal treatment

The neonatal iron treatment has been described in detail elsewhere (Perez et al., 2010; Rech et al., 2010; de Lima et al., 2008a). Briefly, 12-day-old rat pups received orally a single daily dose (10 ml/kg solution volume) of vehicle (5% sorbitol in water) (control group, n = 26) or 30 mg/Kg of body weight of Fe^{2+} (iron carbonyl, Sigma-Aldrich, São Paulo, Brazil) (n = 29) via a metallic gastric tube, over 3 days (postnatal days 12-14). In this model, iron is given orally during the period of maximal iron uptake by the brain, so that the model correlates with dietary iron supplementation to infants. We previously characterized that this treatment protocol induces a selective accumulation of iron in the rat basal ganglia (Schröder et al., 2001).

2.2.2 Sodium butyrate

For the investigation of the effects of NaB on iron-induced memory impairments, adult rats treated neonatally with vehicle or iron (as described above) received an acute intraperitoneal injection of vehicle (saline solution, NaCl 0.9 g%) or NaB (1.2 g/kg, Sigma-Aldrich, São Paulo, Brazil) immediately after the training session of the object recognition task. Ten days later, groups were semi-randomized, in order to guarantee that a rat would not receive the same previous treatment, and were trained and tested in the inhibitory avoidance task. Vehicle or NaB were administered intraperitoneally immediately after the training session of either inhibitory avoidance or novel object recognition task. The combination of neonatal iron treatment and adult acute

NaB treatment resulted in four experimental groups: vehicle in the neonatal period treated with saline (Veh-Sal), vehicle in the neonatal period treated with NaB (Veh-NaB), iron in the neonatal period treated with saline (Iron-Sal), iron in the neonatal period treated with NaB (Iron-NaB). Drug solutions were freshly prepared immediately prior to administration.

2.3 Behavioral procedures

2.3.1 Inhibitory avoidance

We used the single-trial step-down IA conditioning as an established model of fear-motivated, hippocampus-dependent memory (Izquierdo and Medina, 1997). In IA training, animals learn to associate a location in the training apparatus with an aversive stimulus (footshock). The IA behavioral training and retention test procedures were described in previous reports (Schröder et al., 2001; Quevedo et al., 2004). The IA apparatus was a 50 x 25 x 25-cm acrylic box (Albarsch, Porto Alegre, Brazil) whose floor consisted of parallel caliber stainless steel bars (1 mm diameter) spaced 1 cm apart. A 7-cm wide, 2.5-cm high platform was placed on the floor of the box against the left wall. On the training trial, rats were placed on the platform and their latency to step down on the grid with all four paws was measured with an automatic device. Immediately after stepping down on the grid, rats received a mild foot shock (0.4 mA) and were removed from the apparatus immediately afterwards. A retention test trial was carried out 24 after the training trial. The retention test trial was procedurally identical to training, except that no footshock was

presented. Step-down latencies (s) on the retention test trial (maximum 180 s) were used as a measure of IA retention (Schröder et al., 2001; Quevedo et al., 2004).

2.3.2 Object recognition

The OR task was performed as previously described (de Lima et al., 2007; 2008a). Briefly, the OR task took place in an open field apparatus (45 x 40 x 60 cm) with sawdust covering its floor. On the first day, rats underwent a habituation session during which they were placed in the empty open field for 5 min. On the following day, rats were given one 5-min training trial in which they were exposed to two identical objects (A1 and A2). The objects were positioned in two adjacent corners, 9 cm from the walls. On the long-term memory (LTM) testing trial (24 h after the training session), rats were allowed to explore the open field for 5 min in the presence of two objects: the familiar object A and a novel object B. These were placed in the same locations as in the training session. In long-term retention test trial, the novel object was placed in 50% trials in the right side and 50% trials in the left side of the open field. All objects were made of plastic Duplo Lego Toys and had a height of about 10 cm. Objects presented similar textures, colors, and sizes, but distinctive shapes. Between trials the objects were washed with 10% ethanol solution. Object exploration was measured by an experimenter blind to group treatment assignments; using two stopwatches to record the time spent exploring the objects during the experimental sessions. Exploration was defined as follows: sniffing or touching the object with the nose. Sitting on the object was not

considered as exploration. A recognition index calculated for each animal was expressed by the ratio $T_N/(T_F+T_N)$ [T_F= time spent exploring the familiar object (A); T_N= time spent exploring the novel object (B)].

2.4 Histone extraction

Adult rats treated with vehicle or iron in the neonatal period were either habituated to the open-field or trained in the object recognition task for five minutes, as described above. One hour after the behavioral procedure, animals were decapitated and their dorsal hippocampi were quickly removed and snap-frozen on nitrogen. The tissue was homogenized (n = 3-4 per group), placed in a cooled protease inhibitor solution (Complete Mini, Roche Applied Science, São Paulo, Brazil) and stored at -80ºC for subsequent analysis. Histones were extracted in RIPA (Sigma-Aldrich, São Paulo, Brazil) homogenization buffer 1x containing 1mM sodium orthovanadate. After 20 minutes in ice, samples were centrifuged at 12000 rpm for 1-min. The supernatant was collected, and the same volume of 0.2N HCl was added and acid extraction of histones was carried out over night at 4°C. In the next day, samples were centrifuged at 6500 g for 10 minutes at 4°C. The supernatants were saved and the protein content was determined using Bradford assay (Bradford, 1976). Aliquots were stored at -20°C.

2.5 Western blot analysis

Twenty-five μg total protein was separated on a 7.5% SDS-polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane. Membranes were blocked with 5% non fat dry milk in TBS containing 0.05% Tween-20 and were incubated overnight with the following antibodies: anti-ß-actin (ab34731, Abcam) at 1:3000, anti-histone H3 (ab1791, Abcam) at 1:3000, anti-acetyl histone H3 (Lys-14, K14, ab52946, Abcam) at 1:1000; anti-acetyl histone H3 (Lys-9, ab10812, Abcam) at 1:500, anti-histone H4 (ab10158, Abcam) at 1:200, anti-acetyl histone H4 (Lys-5, K5, ab51997, Abcam) at 1:3000, and anti-acetyl histone H4 (Lys-12, K12, ab61238, Abcam) at 1:700. Goat anti-rabbit (ab6721, HRP) radish-conjugated secondary antibodies were used and detected using Western Lighting Western Blot Chemiluminescence (NEL 104001EA, Perkin Elmer). Pre-stained molecular weight protein markers (Benchmark marker, Invitrogen) were used to determine the detected bands' molecular weight and confirm antibodies target specificity. The densitometry quantification was performed using ImageJ software (http://rsb.info.nih.gov/ij/). Total blotting protein levels of were normalized according to each sample's ß-actin protein levels. Results were expressed as a ratio of acetylated H3 and H4 residues to total histone.

2.6 Statistical analysis

Behavioral data were analyzed as previously described (de Lima et al., 2005a,b; 2007; 2008a,b). Data for latency to step-down and recognition indexes are expressed as mean (S.E.M.). Comparisons among experimental groups were performed using a Kruskal-Wallis analysis of variance followed by Mann-

Whitney *U*-tests, two-tailed when necessary. Western blotting data was analyzed using an ANOVA followed by a Tukey's multiple comparison test. In all comparisons, *P* values less than 0.05 were considered to indicate statistical significance.

3. Results

Rats given neonatal iron overload showed a reduction of H3K9 acetylation, irrespective of the animals being trained or not (*P*s < 0.001 and 0.01 for trained and non-trained rats, respectively, in comparison to controls given Veh and habituation). OR training did not affect H3K9 acetylation in rats not giving iron (*P* = 0.91 compared to controls) (Figure 1A). There was no difference among groups in the acetylation of H3K14. Although there was an apparent iron-induced increase in H4K5 and H4K12 acetylation in both trained and habituated rats, these comparisons did not reach statistical significance (H4K5, *P* = 0.10, Fe-Hab *versus* Veh-Hab; *P* = 0.11, Fe-Trai *versus* Veh-Hab; H4K12, *P* = 0.29, Fe-Hab *versus* Veh-Hab; *P* = 0.10, Fe-Trai *versus* Veh-Hab; Figure 1B).

We also used the model of cognitive impairment induced by neonatal iron administration to investigate the possible memory-ameliorating effect of a single posttraining injection of NaB. Results are shown in Figure 2. Statistical comparison of recognition indexes using Kruskal-Wallis analyses of variance showed significant difference among groups in long-term retention test ($H_{(3)}$ = 31.17, *P* < 0.0001), but not in training session ($H_{(3)}$ = 3.64, p = 0.30). Comparison of the total time exploring both objects during the training session

showed no statistically significant differences among groups ($H_{(3)}$ = 4.32, *P* = 0.23, data not shown). Further analyses with Mann-Whitney U tests showed that rats neonatally treated with iron that received saline in adulthood present significantly lower recognition indexes than the control group veh-sal (*P* < 0.0001) in long-term retention test, indicating that iron given in the neonatal period induces severe recognition memory impairment.

Iron-treated rats that received a single acute administration of NaB showed a significantly higher recognition index than the iron-sal group's index (*P* < 0.0001). Additionally, this group presented no statistically significant difference when compared to the control group (*P* = 0.51), suggesting that NaB was able to completely reverse iron-induced recognition memory deficits (Figure 2). NaB had no effect by itself on recognition memory in control rats, since we found no significant difference between the control group (veh-sal) and veh-NaB group (*P* = 0.67).

Similar results were found when animals were tested in the IA task (Figure 3). Statistical comparison of latencies to step-down using Kruskal-Wallis analyses of variance showed a significant difference among groups in long-term retention test ($H_{(3)}$ = 17.35, *P* = 0.001), but not in training session ($H_{(3)}$ = 2.17, *P* = 0.54). Further analyses with Mann-Whitney U tests showed that rats neonatally treated with iron that received saline in adulthood present significantly lower latency in the long-term retention test than the control group veh-sal (*P* = 0.001), indicating that iron given in the neonatal period induces IA memory impairment. NaB improved IA memory in animals that received iron in the neonatal period, as the step-down latency of the iron-NaB group was significantly higher than the iron-sal group (*P* = 0.002). NaB was able to

completely reverse iron-induced memory deficits as the latency to step-down of this group presented no statistically significant differences when compared to the control group (P = 0.82). Again, results showed that NaB has no effect by itself on IA memory in adult control rats, revealed by comparisons between the control group (veh-sal) and the group that was given vehicle in the neonatal period and NaB (P = 0.55).

Discussion

In the present study, we examined the acetylation of H3 and H4 lysine residues in the hippocampus in the neonatal iron model of memory impairment in rats, and the effects of systemic administration of NaB on the consolidation of recognition and fear memory in the neonatal iron-induced model of memory dysfunction. We found a reduction in H3K9 acetylation, without significant changes in H3K14, H4K5, and H4K12 acetylation, in the dorsal hippocampus of rats given neonatal iron, providing the first evidence that brain iron overload might be related to alterations in chromatin plasticity. Peleg and coworkers (2010) reported that age-related memory impairment was associated with a reduction in acetylation of H4K12, but not H3K9, and most previous evidence indicates that memory formation is associated with acetylation of other H3 and H4 residues, such as H3K14 and H4K5/8/12/16 (reviewed in Barrett and Wood, 2008). H3K9, among other H3 and H4 residues, is likely to be a target for HDAC3, which might play a critical role as a negative regulator of memory for OR and other types of long-term memory and is inhibited by NaB (McQuown and Wood, 2011; McQuown et al., 2011).

In contrast to previous studies describing that behavioral training produces increases in the acetylation of H3 and H4 lysine residues (reviewed in Barrett and Wood, 2008), in our experiments OR training did not affect histone acetylation in rats given iron or vehicle neonatally in comparison to their respective control groups given simple habituation to the training box. It is possible that exposure to a novel environment and habituation by itself produced enhancements in histone acetylation similar to those induced by OR training. Future experiments comparing histone acetylation in habituated rats with naïve, non-habituated control rats, could clarify this issue. It is also possible that OR training produces alterations in H3 and H4 acetylation in brain areas other than the dorsal hippocampus. The hippocampus was selected as the target brain area for the present study because of its involvement in memory for both OR and IA, as well as because of previous evidence of a role for alterations in histone acetylation in the hippocampus in memory impairment (Peleg et al., 2010).

A single posttraining injection of NaB did not affect memory in rats not given iron showing normal levels of memory retention, but ameliorated iron-induced deficits in memory for both NOR and IA. Because there was no difference among groups in the total time exploration time during NOR training or in IA training latencies, and NaB injections were given after training, the effects induced by iron and NaB are unlikely to be related to alterations in locomotion, anxiety, motivation, or sensorial function. Using the same model, we have previously shown that memory deficits induced by neonatal iron overload parallel those related to aging (de Lima et al., 2005b; 2008a; Rech et al., 2010) and are associated with pathological and behavioral features of

neurodegenerative disorders such as Parkinson's and Alzheimer's disease. For example, neonatal iron leads to an increase in oxidative stress and apoptotic markers in the hippocampus and cortex as well as astrogliosis and dysfunction of cholinergic pathways in the adulthood (de Lima et al., 2005a; Fernandez et al, 2011; Miwa et al., 2011; Perez et al., 2010). In the AbetaPP/PS1 transgenic mouse model of Alzheimer's disease, neonatal iron results in increase astrocytosis and changes in brain fatty acid composition (Fernandez et al., 2010). In rats, both neonatal iron overload and aging are associated with alterations in the expression of mRNA for proteins involved in iron homeostasis in the brain (Dornelles et al., 2010).

We have also found that treatment with iron chelators can rescue memory impairments associated with both neonatal iron and aging, indicating that brain iron may play a role in aging-related memory dysfunction (de Lima et al., 2007; 2008b). In addition, several pharmacological treatments targeting brain signaling that enhance memory and synaptic plasticity in animal models or patients with neurodegenerative disorders can rescue iron-induced impairments in recognition memory in rats. These drugs include the acetylcholinesterase (AChE) inhibitor galantamine, the muscarinic receptor agonist oxotremorine (Perez et al., 2010), and the phosphodiesterase type 4 (PDE4) inhibitor rolipram (de Lima et al., 2008b).

Treatment with HDACis has been recently shown to ameliorate memory deficits in both aged mice and mouse models of neurodegeneration (Fontán-Lozano et al., 2008; Francis et al., 2009; Kilgore et al., 2010; Mikaelsson and Miller, 2011; Peleg et al., 2010). The present results are consistent with those previous studies as well as with our recent observation that NaB rescues

deficits in OR memory in aged rats (Reolon et al., 2011), and indicate that HDACis can also ameliorate memory impairment associated with brain iron accumulation. Molecular mechanisms possibly involved in mediating the beneficial effects of HDACis on models of memory impairment might include activation of genes regulated by the CREB:CREB-binding protein (CBP) transcriptional complex (Vecsey et al., 2007; Haettig et al., 2011; McQuown et al., 2011). Aging-related memory impairment in mice has been associated with alterations in histone H4 lysine 12 (H4K12) acetylation, leading to altered expression of a range of genes involved in memory consolidation (Peleg et al., 2010). Further studies examining alterations in specific histone subtypes after brain iron accumulation, aging and other models of memory dysfunction, will allow the identification of the most promising HDACis to treat cognitive impairments associated with neurodegeneration and aging.

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Figure captions

Fig. 1. Histone H3 (A) or H4 (B) acetylation in the hippocampus of rats treated neonatally with vehicle (Veh) or iron (Fe) submitted to a training trial in the object recognition task or habituation to the open field. Data are shown as means ± S.E.M ratio of acetylated H3 and H4 residues to total histone; n= 3–4 animals per group; ** $P < 0.01$, differences between the control group (given Veh in the neonatal period and habituation in adultohood, Veh-Hab) *versus* other groups.

Fig. 2. Effects of a single acute injection of NaB on iron-induced recognition memory deficits. Saline or NaB (1.2 mg/kg) were administered immediately after the training session. Long-term memory (LTM) retention test was performed 24 h after training. Behavioral testing was carried out when animals were 2 months old. The proportion of the total exploration time that the animal spent investigating the novel object was the "Recognition Index" expressed by the ratio TN/(TF+TN), TF = time spent exploring the familiar object and TN = time spent exploring the novel object. n = 12 – 15 per group. Data expressed as mean ± S.E.M. recognition index. Differences between veh-sal vs other groups are indicated as: ** P <0.001; difference between iron-sal vs iron-NaB is indicated as: ## P <0.001.

Fig. 3. Effects of a single acute injection of NaB on iron-induced IA memory deficits. Saline or NaB (1.2 mg/kg) were administered immediately after the training session. Long-term memory (LTM) retention test was performed 24 h

after training. Behavioral testing was carried out when animals were 2 months old. n = 13 – 15 per group. Data expressed as mean ± S.E.M. retention test step-down latencies (s). Differences between veh-sal vs other groups are indicated as: ** *P* <0.001; difference between iron-sal vs iron-NaB is indicated as: ## *P* <0.001.

Figure 1

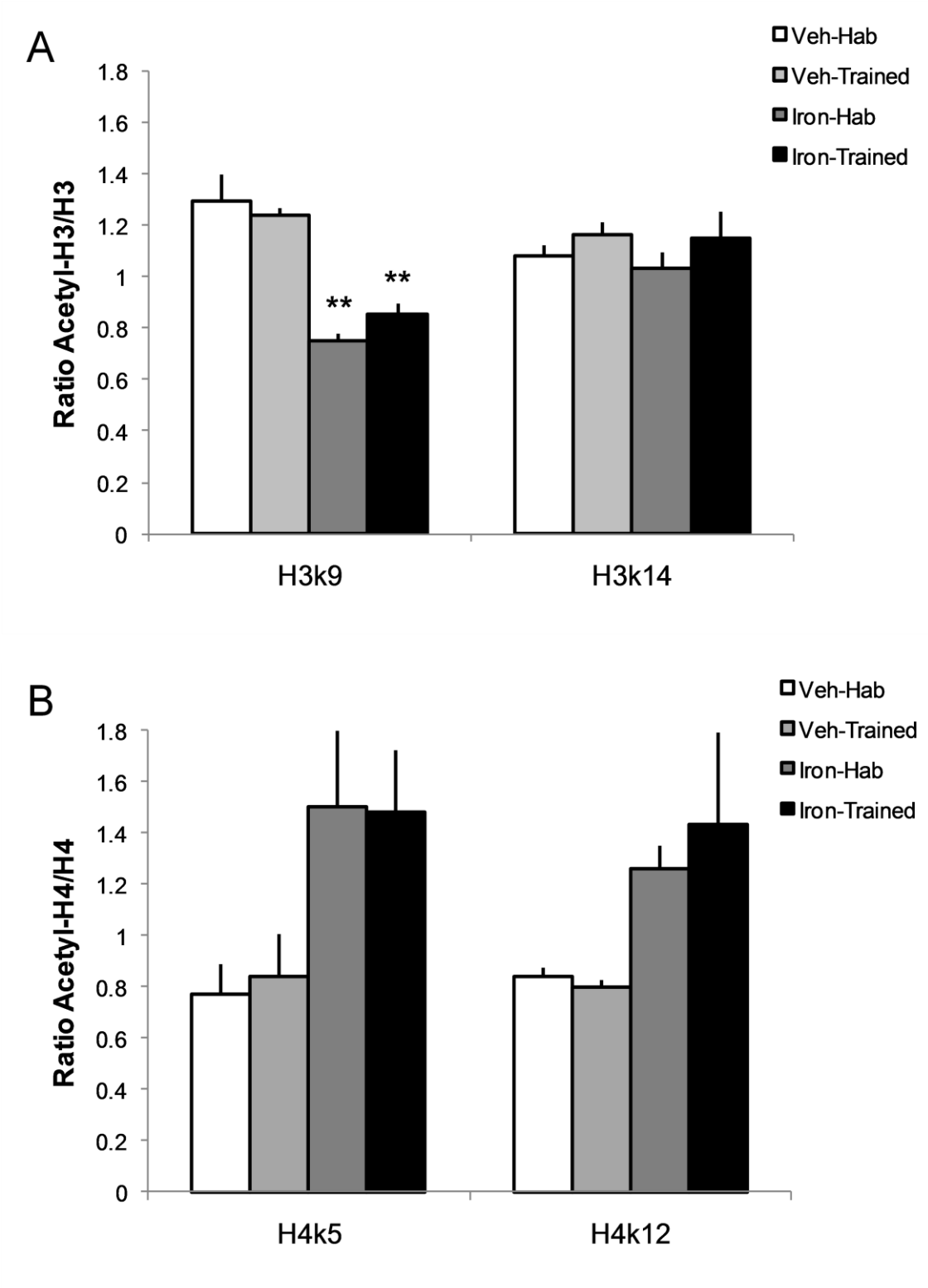

A
Veh-Hab
Veh-Trained
Iron-Hab
Iron-Trained
Ratio Acetyl-H3/H3
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
H3k9
H3k14
B
Veh-Hab
Veh-Trained
Iron-Hab
Iron-Trained
Ratio Acetyl-H4/H4
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
H4k5
H4k12

Figure 2

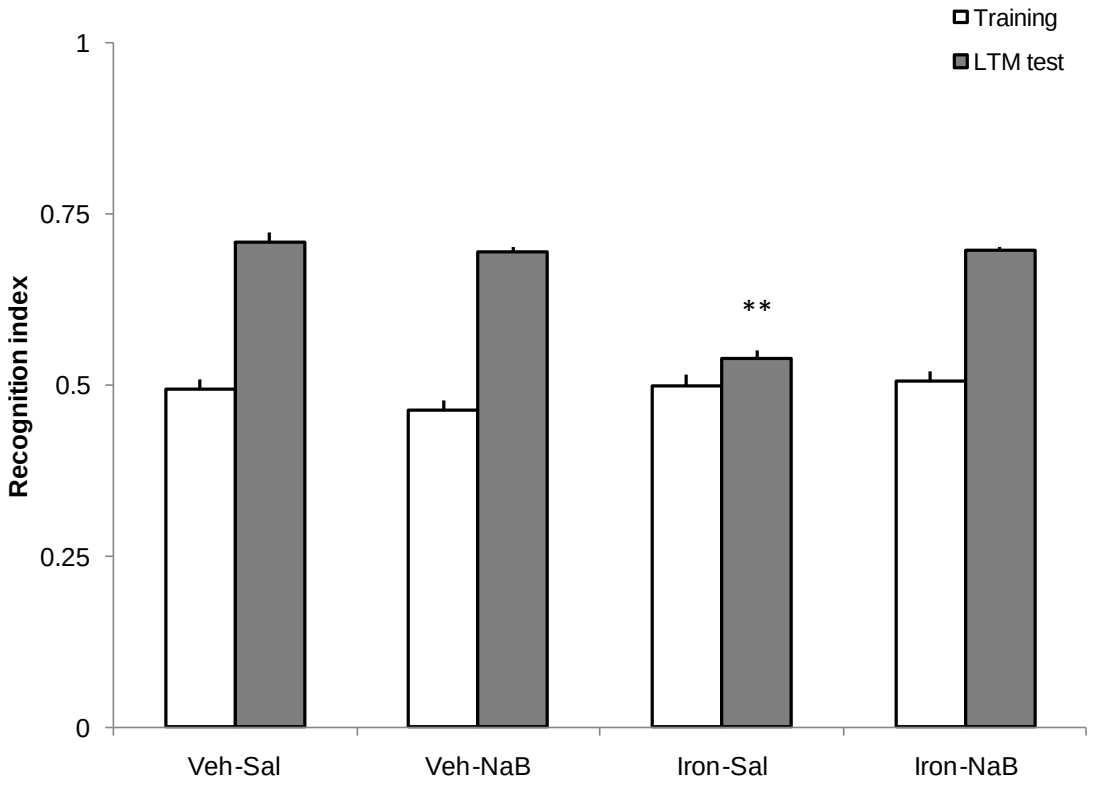
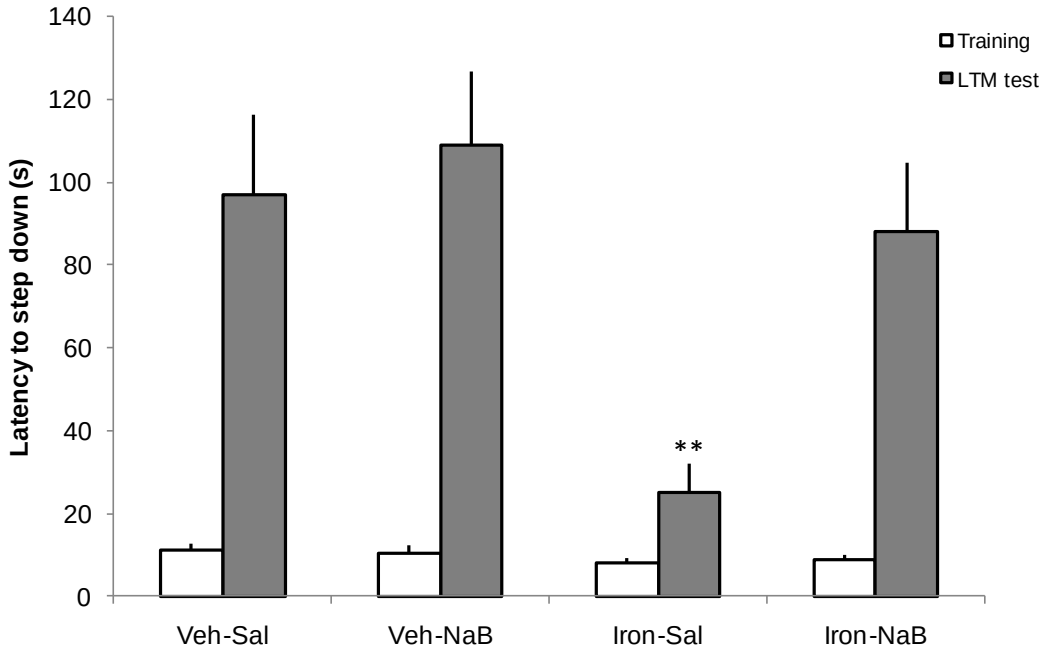


Figure 3



4. CONSIDERAÇÕES FINAIS

O ferro é o metal mais abundante no tecido cerebral, participando de inúmeros e importantes processos fisiológicos, como por exemplo, co-fator de reações enzimáticas, na síntese e degradação de neurotransmissores, mielinização, formação de dendritos (Beard, 2088; Gerlach *et al*., 1994; Todorich *et al*., 2009), além de estar envolvido no transporte de oxigênio e no metabolismo energético.

O íon ferro, na forma ferrosa pode reagir com o peróxido de hidrogênio, via reação de Fenton, produzindo radicais hidroxil reativos, causando estresse oxidativo e por consenguinte dano celular (Halliwell and Gutteridge, 1992; Smith *et al*., 1997) no tecido cerebral, sendo, por este motivo, associado a doenças neurodegenerativas, (review Zecca *et al*., 2004). O estress oxidativo induzido pelo ferro está relacionado às doenças de Alzheimer's, Parkinson's e Huntington's, entre outras (Kell, 2010), mais prevalentes no envelhecimento, visto que a concentração de ferro aumenta progressivamente em indivíduos adultos normais durante o processo de envelhecimento (Connor *et al*., 1990; Bartzokis *et al*., 1994).

A sobrecarga de ferro no período neonatal leva a um aumento do estresse oxidativo e de marcadores de apoptose (De Lima *et al.,* 2005a, 2005b, 2005c, Miwa *et al.*, 2010), podendo estar associado ao déficit de memória. Neste estudo buscamos compreender os efeitos da administração sistêmica de NaBut sobre a consolidação da memória de reconhecimento e de medo, em modelo animal com déficit cognitivo induzido por tratamento neonatal com ferro.

Utilizamos o tratamento agudo, com uma única injeção, pós-treino, de NaBut, que demonstrou não afetar a consolidação da memória no grupo de animais tratado com veículo, no período pós-natal, apresentando níveis normais de consolidação da memória. Entretanto os animais tratados com ferro no período pós-natal, com déficit de memória induzido pela sobrecarga de ferro, apresentaram melhora significativa, de acordo com os resultados apresentados durante o experimento, sem alterações sensoriais, de motivação, locomoção ou aumento da ansiedade.

Inferimos, então, que o NaBut tem a capacidade de resgatar déficits de memória de reconhecimento de objeto e de memória aversiva em ratos com prejuízos de memória, causados por acúmulo de ferro por sobrecarga na ingesta desse metal no período neonatal.

Estes resultados são consistentes com os vários estudos, recentes, que demonstram que o déficit de memória associado ao envelhecimento está relacionado a modificações epigenéticas da cromatina, incluindo alterações na acetilação das histonas (Peleg *et al*., 2010; Penner *et al*., 2010a; 2010b; Sweatt, 2010).

Portanto, o NaBut, pertencente a uma classe de inibidores das HDACs, atua sobre mecanismos moleculares envolvidos nos processos de formação e de consolidação da memória, demonstrando ser um promissor inibidor de HDACs em tratamento de problemas cognitivos associados a neurodegeneração.

4.1. Perspectivas

A partir dos resultados encontrados no presente trabalho, surgiu o interesse em dar continuidade com estudos que examinam as alterações em subtipos específicos de histonas e nas suas formas acetiladas após o tratamento neonatal com ferro. Sendo assim, estes estudos permitirão melhor caracterização dos mecanismos envolvidos no prejuízo de memória causado pelo ferro e sua relação com a maquinaria epigenéticas, bem como o uso de inibidores de HDACs no tratamento de problemas cognitivos associados a neurodegeneração.

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