PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

FACULDADE DE BIOCIÊNCIAS

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

Vanessa Athaide Garcia

# Investigação do potencial uso do Modafinil como agente melhorador da memória

Orientadora: Dra. Nadja Schröder

Porto Alegre

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Tese apresentada como requisito para obtenção do grau de Doutor pelo Programa de Pós-graduação em Biologia Celular e Molecular da Faculdade de Biociências

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**RESUMO**

O Modafinil é um fármaco que promove o aumento do estado de alerta, e foi aprovado para o tratamento da sonolência diurna excessiva na narcolepsia e apnéia obstrutiva do sono. Alguns estudos indicam que o modafinil pode melhorar a aprendizagem, a memória e reverter déficits cognitivos induzidos pela privação de sono e estresse. No presente trabalho tivemos como objetivo caracterizar os efeitos do modafinil sobre a memória em ratos adultos saudáveis. Além disso, seus efeitos também foram estudados utilzando-se três modelos de declínio cognitivo para avaliar os efeitos do modafinil sobre a memória: o modelo da sobrecarga de ferro no período neonatal relacionado com as doenças neurodegenerativas, a separação materna no período neonatal que gera um prejuízo emocional e cognitivo na idade adulta e, por fim, a privação de sono paradoxal que interfere na consolidação da memória. No experimento I foram investigados os efeitos do fármaco sobre a consolidação e a evocação da memória utilizando-se distintos paradigmas de aprendizagem: reconhecimento de objetos e esquiva inibitória (tarefa de memória aversiva) em ratos adultos saudáveis. Ratos Wistar adultos receberam uma injeção aguda de modafinil (0,75; 7,5 ou 75 mg/kg) ou veículo imediatamente após o treino ou 1 hora antes do teste nas tarefas de reconhecimento de objetos ou de esquiva inibitória. A administração aguda de modafinil em ratos saudáveis não afetou a consolidação e nem a evocação da memória. O experimento II analisou os efeitos da administração aguda ou crônica de modafinil sobre os déficits de memória induzidos pela sobrecarga de ferro no período neonatal, um modelo de declínio cognitivo relacionado a distúrbios neurodegenerativos. Ratos tratados com ferro entre o 12º e 14º dia pós natal receberam, na idade adulta, uma injeção intraperitoneal aguda de modafinil (0,75; 7,5 e 75 mg/kg) ou veículo imediatamente após o treino da tarefa de reconhecimento de objeto. Uma injeção aguda de modafinil na dose mais elevada foi capaz de recuperar a memória de reconhecimento em ratos tratados com ferro. A fim de investigar os efeitos do tratamento crônico com modafinil, os ratos tratados com ferro receberam injeções diárias de modafinil nas mesmas doses por 17 dias. Vinte e quatro horas após a última injeção, os animais foram submetidos às tarefas de reconhecimento de objetos e esquiva inibitória. Os testes de retenção de memória foram realizados 24 horas após o treino. A administração crônica de modafinil recuperou completamente os déficits de memória induzidos pelo ferro, tanto na memória de reconhecimento quanto na memória emocional. O experimento III avaliou os efeitos da separação materna combinada com a privação de sono paradoxal (PSP) sobre a memória de reconhecimento. Ratos Wistar separados da mãe durante 14 dias, três horas por dia, foram submetidos a PSP por vinte e quatro horas na idade adulta. O modelo permitiu investigar a capacidade de reversão de tais déficits com a administração de modafinil que foi efetivo em recuperar o declínio cognitivo causado pela separação materna e pela PSP. A utilização e a comercialização do modafinil como estimulante são amplamente fundamentadas. Para dar suporte à sua aplicação na perda de memória associada às perturbações neurodegenerativas; ao estresse no início da vida e à PSP, pesquisas pré clínicas e clínicas adicionais são necessárias. Este estudo comprova por meio dos modelos de declínio cognitivo propostos, a efetividade do fármaco em reverter tais déficits de memória.

Palavras-chave: Modafinil – Memória de reconhecimento – Ferro – Esquiva Inibitória – Déficits de memória - Separação Materna - Privação de Sono.

## ABSTRACT

Modafinil is a drug that promotes increased alertness. It was approved for the treatment of excessive daytime sleepness in narcolepsy and obstructive sleep apnea. Some studies indicate that Modafinil can improve learning, memory and reverse cognitive loss induced by sleep deprivation and stress. This study was aimed to characterize the effects of modafinil on control adult rat's memory. Further, its effects were also studied using three models of cognitive decline to assess modafinil effects on memory. The iron overload model on neonatal period related to the degenerative diseases, maternal deprivation on neonatal period that generates emotional and cognitive impairment on adulthood, and, paradoxal sleep deprivation, which is known to interfere with on memory consolidation. The first experiment investigated the effects of the drug on memory consolidation and retrieval using distinct paradigms of learning; object recognition and inhibitory avoidance (aversive memory task) in healthy adult rats. Male Wistar rats received a single injection of acute modafinil (0.0; 0.75; 7.5 or 75 mg/kg) immediately after training or one hour before object recognition or inhibitory avoidance retention tests. Acute administration of modafinil to healthy rats did not affect memory consolidation or retrieval. The second experiment examined the acute or chronic administration of modafinil on the reversal of memory deficits induced by iron overload in the neonatal period, a model of cognitive decline related to neurodegenerative disorders. Rats treated with iron between 12 and 14 postnatal days received in adulthood, acute intraperitoneal injection of modafinil (0.0; 0.75; 7.5 and 75 mg/kg) immediately after training in the recognition task object. A single injection of modafinil at the highest dose was able to recover recognition memory in rats treated with iron. In order to investigate the effects of chronic treatment with modafinil, iron-treated rats received intraperitoneal injections of modafinil at the same doses for seventeen days. Twenty-four hours after the last injection, the animals were submitted to object recognition or inhibitory avoidance training. Memory retention tests were performed twenty four hours after training. Chronic administration of modafinil fully recovered memory deficits induced by iron both in recognition memory and emotional memory. Experiment III evaluated the effects of maternal deprivation combined with paradoxical sleep deprivation (PSP) on recognition memory. Wistar rats separated from their mothers for fourteen days, three hours per day, underwent PSP for twenty four hours in adulthood. The model allowed to investigate the ability to reverse such deficits with administration of modafinil that was effective in restoring cognitive decline caused by maternal deprivation and PSP. The use and commercialization of modafinil as a stimulant is widely proved. To support its application in the memory loss associated with neurodegenerative disorders, stress in early life and PSP, additional preclinical and clinical research are needed. This study shows, however, through the proposed models of cognitive decline, the drug effectiveness in reversing such memory deficits.



Key words: Modafinil – Recognition Memory – Iron – Inhibitory avoidance – Memory deficits, Maternal Separation, Sleep Deprivation.

# SUMÁRIO

# *Capítulo 1*

## *Introdução*

# 1 INTRODUÇÃO

## *1.1 Modafinil*

O Modafinil [2-(Difenilmetil-sufinil) acetamina] é um agente que promove o aumento do estado de alerta, primeiramente desenvolvido na França por volta de 1990. Tal fármaco tem se mostrado eficiente no tratamento da sonolência diurna excessiva relacionada à narcolepsia, no tratamento do distúrbio do sono relacionado à mudança de turno no trabalho e na síndrome da apneia e hipopneia obstrutiva do sono (Ferraro *et al*., 2001; Ballon & Feifel, 2006; Minzenberg & Carter, 2008). Estudos clínicos têm apontado efeitos positivos do modafinil quando utilizado para tratar outras patologias importantes como o distúrbio de hiperatividade e déficit de atenção, sonolência e fadiga relacionadas à doença de Parkinson, esclerose múltipla, depressão, sintomas negativos da esquizofrenia e nos déficits cognitivos causados pela doença de Alzheimer (Ferraro *et al.,* 1998; Taylor & Russo, 2000; Lang, 2002; Turner *et al.,* 2004; Kraft & Bowen, 2005; Morgan *et al.*, 2007). Ele tem sido popularmente categorizado como um psicoestimulante devido às suas propriedades promotoras da vigília (Minzenbergand, 2008). Embora tenha recebido essa classificação, evidências sugerem que o modafinil age em uma via neural diferente da anfetamina e da cocaína (Ballon & Feifel, 2006). Há várias hipóteses que tentam explicar o seu mecanismo de ação. Estudos com animais e humanos têm sugerido que ele ativa diretamente e indiretamente os sistemas dopaminérgico (De Saint Hilaire *et al*., 2001; Volkon *et al*., 2009), glutamatérgico (Ferraro *et al*., 1999), noradrenérgico (De Saint Hilaire *et al.,* 2001; Minzenberg *et al.,* 2008) e serotoninérgico (De Saint Hilaire *et al.,* 2001;

Minzenberg *et al*., 2008) em várias regiões do encéfalo incluindo o córtex pré-frontal, hipocampo, hipotálamo e estriado, considerando que ele inibe as vias GABAérgicas nas mesmas regiões (Figura 1) (Ferraro *et al*., 1999).

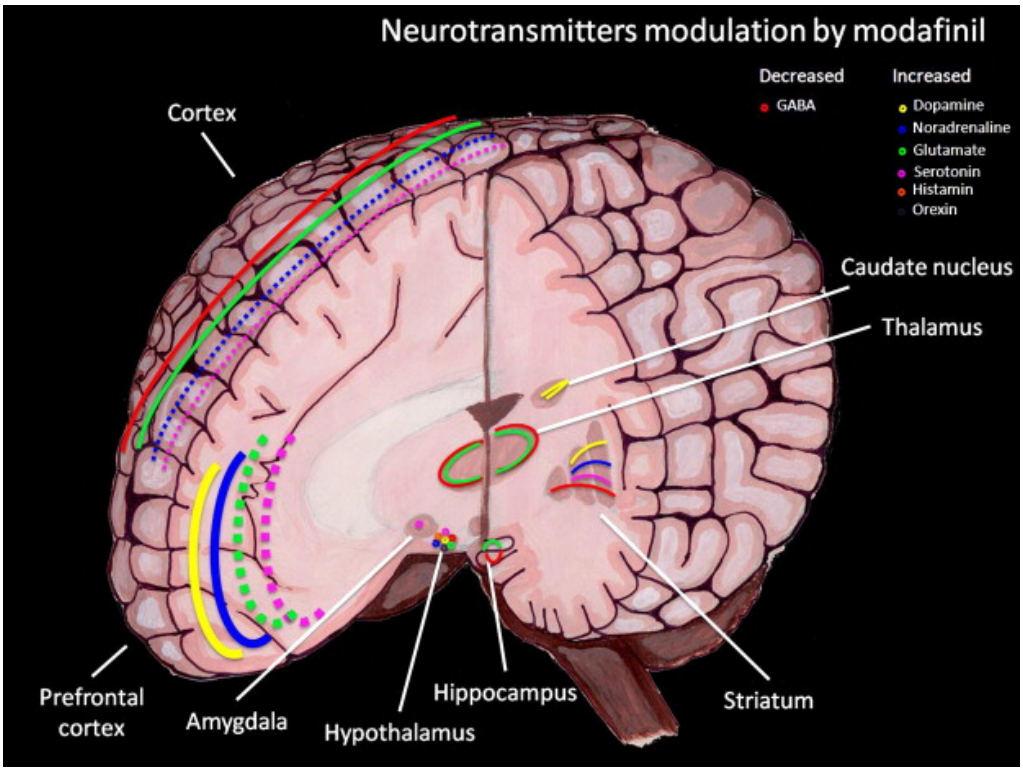


Figura 1- Modulação dos neurotransmissores pelo modafinil no encéfalo. Fonte: Scoriels et al., 2012.

Estudos tem demonstrado uma maior participação do sistema dopaminérgico nos efeitos do modafinil. Ferraro e colaboradores (1996) demonstraram que o modafinil aumenta, de forma dependente de dose, a liberação de dopamina no núcleo accumbens e que tal aumento é diminuido pelo antagonista do receptor GABAb baclofen, pelo antagonista GABAa muscimol e pelo inibidor da recaptação do neurotransmissor GABA SKF 89976A, demonstrando haver uma interação entre o sitema dopaminérgico e GABAérgico nos efeitos do modafinil. De fato, tais ações do modafinil no

sistema dopaminérgico podem ser secundárias à sua ação primária na diminuição da liberação do neurotransmissor GABA (Ferraro *et al*., 1997). Além disso, diversas características da hiperatividade dopaminérgica, como, por exemplo, o aumento da locomoção, são observados após o uso do modafinil. Dopheide e colaboradores (2007) demonstraram que o modafinil aumenta a liberação de dopamina em regiões estriatais e Zolkowska e colaboradores (2009) observaram haver uma correlação entre o aumento de dopamina no núcleo accumbens e um aumento de estereotipia em ratos sob o efeito do modafinil. De fato, o modafinil tem sido descrito até mesmo como agonista de receptores D2 (Korotkova *et al.,* 2007). Nesse sentido, o aumento da liberação de dopamina parece ser de extrema importância para os efeitos de aumento do estado de alerta causado pelo modafinil. Desse modo, níveis elevados de dopamina no núcleo accumbens e no córtex pré-frontal parece ser importante para o aumento do estado de alerta em experimentos realizados com ratos ( de Saint Hilaire et al., 2001; Murilo-Rodrigues et al., 2007).

Estudos realizados por Wisor e Eriksson (2005) e posteriormente por Morgan e colaboradores (2007), demonstraram que a interação entre o sistema adrenérgico e o sistema dopaminérgico é necessária para que os efeitos promotores da vigília ocorram além do aumento da atividade locomotora observada em animais.

O modafinil também parece modular os sistemas glutamatérgicos, GABAérgicos e serotoninérgicos, em regiões cerebrais que estão envolvidas no estado da vigília e nas funções cognitivas (Ward *et al*., 2004). A ação do modafinil inibindo a liberação de GABA no núcleo accumbens faz com que se tenha uma menor liberação de dopamina nessa região. Além disso, esse

mecanismo de ação indireto no sistema dopaminérgico pode explicar o baixo potencial de abuso do modafinil, já que drogas de abuso, como a anfetamina, agem diretamente aumentando a liberação de dopamina.

Estudos apontam que o modafinil modifica a atividade de áreas do encéfalo envolvidas com a memória, tais como o hipocampo e o cortex pré-frontal (Béracochéa *et al*., 2003). Dentro deste contexto, evidências indicam que o modafinil melhora as habilidades cognitivas em roedores após serem submetidos a diferentes paradigmas de aprendizagem. Ele melhora a discriminação visual e atenção em roedores (Morgan *et al*., 2007), facilita a aprendizagem e memória em tarefas espaciais e contextuais, em ratos adultos saudáveis (Burgos *et al.*, 2010; Tsanov *et al.,* 2010) e em camundongos (Beracochea *et al.,* 2008, Shuman *et al.,* 2009). Além disso, demonstrou-se que o modafinil recupera os déficitis de memória induzidos pela privação de sono (He *et al.,* 2011; Moreira *et al.,* 2010; Pierard *et al.,* 2007, 2011) ou pelo estresse crônico (Pierard *et al.,* 2006 ). No entando, dependendo do paradigma de aprendizagem utilizado, da dose e do intervá-lo de tempo da administração do fármaco, resultados contraditórios podem ser encontrados. Por exemplo, o modafinil piorou a memória emocional em camundongos (Burgos *et al.,* 2010; Fernandes *et al.,* 2013).Também foi demonstrado que o modafinil é efetivo na proteção contra danos neuronais incluindo danos na via nigro-estriatal (Fuxe *et al.,* 1992). Ele possui efeito protetor contra a toxicidade induzida por MPTP (1-metil-4-fenil-1,2,3,6-tetraidropiridina), modelo de doença de Parkinson, *in vivo* (Fuxe *et al.,* 1992). Ele reverteu de maneira dose-dependente a deficiência motora em macacos tratados com MPTP protegendo

a via nigro-estriatal da toxicidade (Jenner *et al.,* 2000). No entanto, não foram testados seus efeitos em modelos animais que envolvam prejuízo cognitivo.

No presente estudo foram propostos três modelos de declínio cognitivo. O modelo da sobrecarga de ferro no período neonatal relacionado com o envelhecimento e com as doenças neurodegenerativas. A separação materna no período neonatal que gera um prejuízo emocional e cognitivo observado na idade adulta. E por fim, o modelo de privação de sono paradoxal (PSP) que interfere na consolidação da memória.

## *1.2 Modelo de Declínio Cognitivo Induzido pela Administração de Ferro no Período Neonatal*

A deposição excessiva de ferro no Sistema Nervoso Central (SNC) tem sido relatada em um número de patologias neurodegenerativas como a doença de Alzheimer, doença de Parkinson, esclerose amiotrófica lateral e neuroferritinopatias (Rouault, 2001; Ong & Farooqui, 2005; Burn & Chinnery, 2006). O comprometimento do metabolismo do ferro é um dos fatores que pode desencadear o processo neurodegenerativo no encéfalo. No caso da doença de Parkinson e da doença de Alzheimer, o ferro tem desempenhado um importante papel na morte neuronal. A extensão e a intensidade do estresse oxidativo causado pelo aumento de ferro lábil, pode afetar a atividade de transcrição e as cascatas de sinalização que participam da sobrevivência ou da morte neuronal (Figura 2) (Salvador *et al.*, 2011). Além disso, a distribuição cerebral de ferro também 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.,* 2001, 2004; Martin *et al.,* 1998).

Apesar do excesso de ferro também ter sido observado em outras patologias como a esclerose múltipla, esclerose amiotrófica lateral e neuroferritinopatias, os eventos moleculares que conduzem à morte neuronal ainda não estão completamente compreendidos.

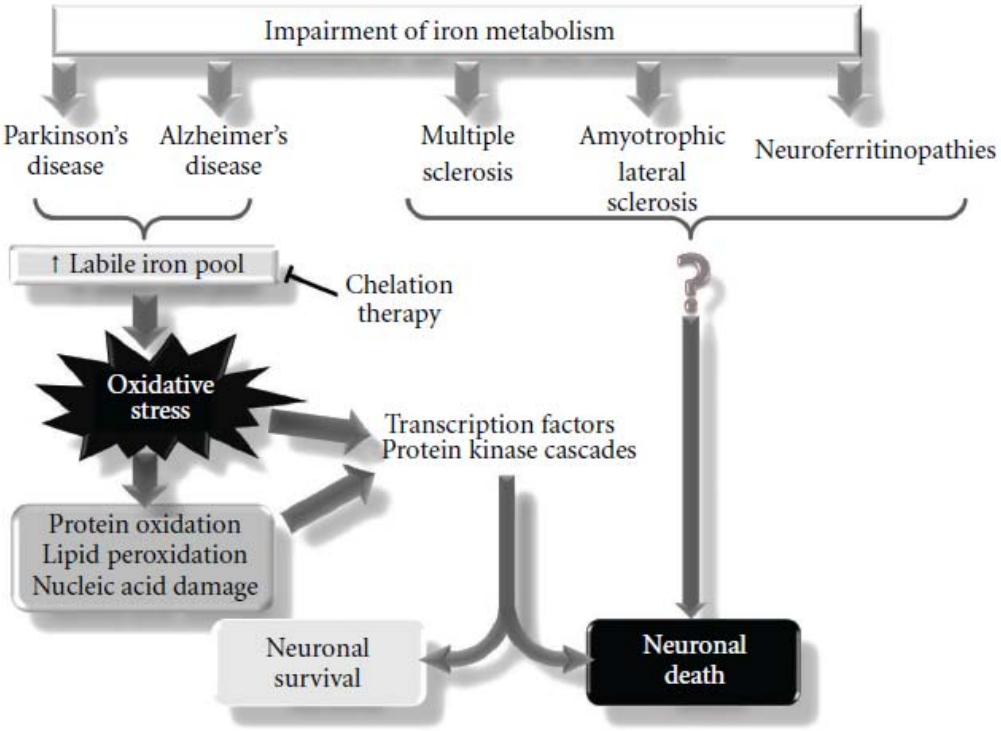


Fig 2 - Relação entre o comprometimento do metabolismo do ferro e doenças neurodegenerativas. Fonte: Salvador *et al*., 2011.

A suplementação excessiva de ferro no período neonatal induz a um acúmulo seletivo desse metal em algumas regiões cerebrais especificamente nos gânglios da base. 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 ao cérebro atinge seus níveis máximos durante o período pós-natal de rápido crescimento cerebral,

como pode ser observado na Figura 3 (Taylor & Morgan, 1990; Taylor *et al.,* 1991).

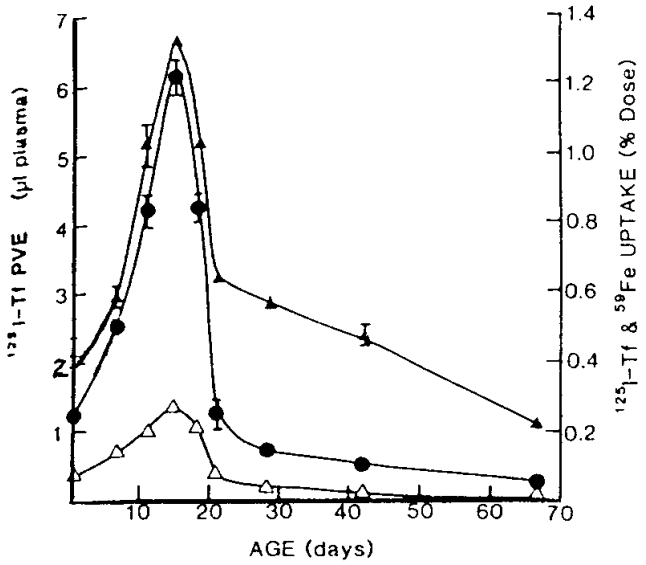


Fig 3 – Recaptação de ferro durante o período neonatal de rápido crescimento cerebral. Fonte: Taylor & Morgan, 1990.

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 dura, em humanos, desde o último trimestre da gravidez 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. Este

excesso foi associado com déficitis de memória de longo prazo em roedores (De Lima *et al.,* 2005a; Fagherazzi *et al,* 2012; Silva *et al,* 2012; Schröder *et al,* 2001, 2013). Foi observado também, que essa sobrecarga de ferro no período neonatal aumenta os parâmetros de estresse oxidativo em regiões cerebrais de ratos adultos (De Lima *et al.,* 2005b). Recentemente, verificamos que o tratamento neonatal com ferro produz um aumento na proteína glial acídica (GFAP), sendo esta um marcador astrocitário, sugerindo a presença de gliose reativa em camundongos adultos (Fernandez *et al.,* 2010) e em ratos (Fernandez *et al.,* 2011). Ainda, resultados recentes obtidos em nosso grupo de pesquisa demonstram que os marcadores apoptóticos, Par-4 e caspase-3 também se encontram aumentados em cérebros de animais adultos que foram tratados com ferro no período neonatal (Miwa *et al.,* 2011). Esses prejuízos causados podem permanecer na idade adulta (Kaur *et al.,* 2007). Sendo assim, eventos que ocorreram no início da vida, como por exemplo, o acúmulo de ferro no encéfalo, podem ter efeitos duradouros na função neuronal no adulto. Estes resultados sugerem que o prejuízo de memória associado a um tratamento com ferro, possa ser visto como um modelo de declínio cognitivo relacionado com distúrbios neurodegenerativos. Assim, ao longo dos últimos anos temos vindo a utilizar este modelo, a fim de investigar fármacos com potencial de uso como melhorador cognitivo para o tratamento de déficitis de memória associados ao envelhecimento e / ou doenças neurodegenerativas (De Lima *et al.,* 2005a; Fagherazzi *et al*, 2012; Schröder *et al*, 2001, 2013; Silva *et al*, 2012). Sendo assim, torna-se importante caracterizar o potencial neuroprotetor do modafinil em modelos animais que mimetizem prejuízos cognitivos associados a doenças neurodegenerativas. Ainda, não é conhecida

a possível interação entre os efeitos do modafinil e a sobrecarga de ferro cerebral, um possível agente etiológico de doenças neurodegenerativas.

## *1.3 Separação Materna*

Eventos estressantes no início da vida podem ter efeitos duradouros sobre os organismos vivos. Condições tais como a depressão pós-parto, negligência parental ou cuidados médicos especiais associados com o parto prematuro são estressantes para o recém-nascido porque interferem na interação da mãe com o bebê (Korosi *et al.,* 2012). Para roedores, primatas não-humanos, e seres humanos, a estimulação materna inadequada ou a privação materna no início da vida e na infância aumenta a resposta neuroendócrina ao estresse contribuindo com a etiologia de uma grande variedade de distúrbios neurocomportamentais e psiquiátricos (Chapman *et al*, 2004; Pietrek *et al*, 2012; van Winkel *et al,* 2013).

A vulnerabilidade a psicopatologias e a prejuízos cognitivos não é determinada somente pela interação entre gene e ambiente, mas sim por uma interação de quatro vias entre o gene, ambiente, desenvolvimento e sexo (Figura 4). Os genes são afetados pelo ambiente durante o período crítico de desenvolvimento seletivo de maneira sexo dependente. Eventos precoces adversos, no início da vida, podem modular a expressão de moléculas envolvidas na plasticidade celular no hipocampo contribuindo com alterações permanentes na estrutura e na função cerebral. Essas alterações podem levar a um aumento da vulnerabilidade de doenças psiquiátricas e da disfunção cognitiva (Ladd *et al*., 2000; Heim & Nemeroff, 2001).

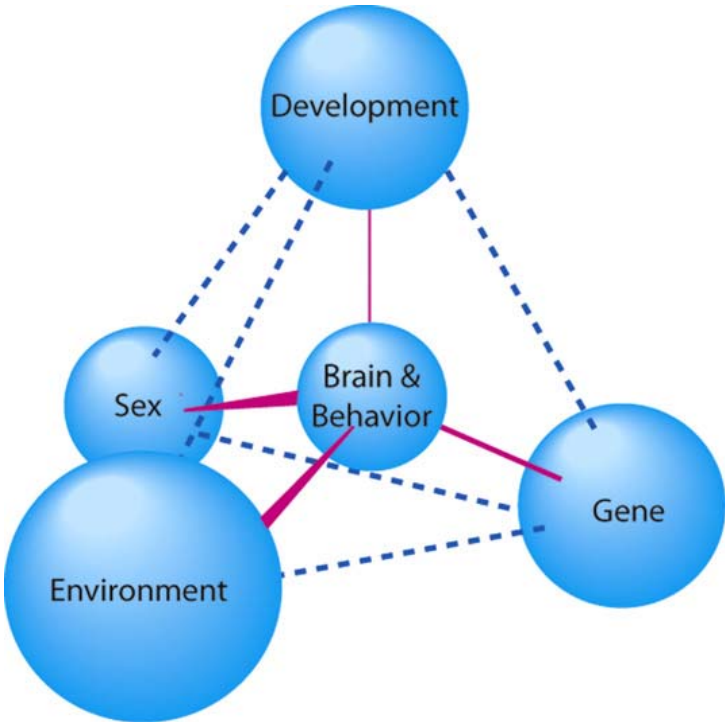


Fig 4 - Cérebro e comportamento em sua relação com desenvolvimento, gene ambiente e sexo. Fonte: Korosi *et al*., 2012.

Obesidade, tabagismo, abuso de drogas e aumento do uso de medicação psicotrópica, em adultos, também têm sido associados a experiências adversas na infância (Anda *et al*, 2007; Felitti, 2009). Em particular, a separação materna no período neonatal é conhecida como um dos mais fortes fatores de estresse para os animais recém-nascidos, com efeitos notáveis.

A privação diária de ratos recém-nascidos de suas mães provoca alterações citoquímicas, morfológicas e eletrofisiológicas permanentes no SNC (Ladd *et al*, 2000). Além disso, alguns estudos indicam que a separação materna durante o período crítico do desenvolvimento cerebral pode levar a deficiências na citoarquitetura em várias regiões do cérebro, tais como o hipocampo e o córtex, que são conhecidos por estarem

envolvidos com a aprendizagem e com a memória (McEwen, 2000; Oitzl *et al,* 2000 , Becker *et al*, 2007).

Estudos realizados em nosso grupo de pesquisa, e em outros, já demonstraram que a separação materna provoca déficits de memória persistentes em roedores (Aisa *et al*, 2007; 2009; Choy *et al,* 2008; de Lima *et al,* 2011a). Em outro estudo, nosso grupo também demonstrou que a exposição a um segundo agente estressor, como a exposição crônica ao psicoestimulante D-anfetamina, mais tarde na vida, potencializou os efeitos da separação materna sobre a cognição em ratos adultos (de Lima *et al,* 2011a; Pinheiro *et al* , 2012).

Sendo assim, torna-se relevante investigar se a privação de sono pode potencializar os efeitos cognitivos da separação materna na idade adulta. Animais expostos a um evento estressante precoce podem ser mais suscetíveis a uma segunda fonte de estresse mais tarde na vida, e esses estressores podem ter efeitos deletérios aditivos.

Feng e colaboradores (2007) demonstraram que ratos adultos submetidos à separação materna no período neonatal apresentavam perturbações de sono com características de insônia. Esses animais possuíam uma redução no tempo total de sono e um aumento no tempo total da vigília durante o período claro.

## *1.4 Sono*

Com o advento do registro eletroencefalográfico (EEG) por Hans Berger em 1929 e com a descrição de distintos padrões EEG presentes durante o sono por Davis e colaboradores (1937), tornou-se evidente a relação entre a fenomenologia do sono e os potenciais elétricos do sistema nervoso. Em 1953, Aserinsky e Kleitman descreveram a ocorrência cíclica de movimentos oculares rápidos durante o sono associada ao aumento da atividade cortical. No entanto, em 1957, Dement e Kleitman demonstraram que a atividade ocular ocorria simultaneamente à fase dessincronizada do sono e estava relacionada com o conteúdo dos sonhos, no ser humano, e, por isso, utilizaram a denominação de "sono de movimentos oculares rápidos" (REM, do inglês *rapid eye movement*). O sono REM, por seu padrão do EEG análogo ao da vigília, porém associado à acentuada diminuição do tônus muscular, foi denominado de sono paradoxal por Jouvet e Michel em 1959 (para revisão ver Datta e MacLean, 2007).

Em mamíferos, o sono e a vigília representam dois estados fisiológicos normalmente definidos por meio de métodos eletroencefalográficos. Ambos os processos, circadiano e homeostático são conhecidos por regular o ciclo entre esses dois estados (Borbély, 1982).

As investigações sobre o ciclo vigília-sono do rato demandam do registro de potenciais eletrofisiológicos corticais e subcorticais. O registro gráfico das oscilações dos potenciais elétricos do sistema nervoso denomina-se genericamente de oscilograma. Sua forma mais conhecida é o eletrencefalograma (EEG), obtido em animais, implantando-se elétrodos diretamente sobre a superfície cortical (Eletrocorticograma - ECoG) ou no interior do encéfalo.

## *1.5 Privação de Sono*

A necessidade diária de sono do homem moderno tem sido alvo de muitos estudos. Historicamente, o tempo de sono ocupou um terço de nossas vidas. No entanto, atualmente, o período de sono tem-se reduzido em função das atividades contemporâneas. Em conseqüência do cenário econômico, a população é induzida a prolongar a jornada de trabalho. Além disso, a exposição à luz artificial ampliou o período das atividades de lazer, prolongando a vigília. Essas questões socioeconômicas e culturais conduzem a uma redução significativa no tempo de sono (Tufik *et al*., 2009).

No modo de vida atual, a maior parte da privação do sono ocorre na fase REM, na segunda metade da noite. Assim, muitos investigadores desenvolveram e empregam diferentes metodologias para o estudo da privação de sono e enfocam na PSP (privação de sono paradoxal).

A privação de sono é uma preocupação social relevante devido às importantes implicações no desempenho individual e na saúde, como por exemplo, aumento das concentrações de cortisol e da atividade simpática, aumento da concentração de marcadores inflamatórios (Tasali *et al*., 2008).

Uma vez que cada sistema orgânico possui um papel distinto e crítico na adaptação de mudanças ambientais contínuas e desafiadoras, torna-se fundamental a condução de estudos visando investigar a maneira pela qual esses sistemas são afetados pela perda do sono. De fato, a privação de sono em homens e animais provoca diversas alterações, assim como aspectos negativos sobre o desempenho psicomotor e cognitivo, variações de humor, disfunções metabólicas, parâmetros comportamentais e avaliações cerebrais (Vogel, 1975; Suchecki *et al*., 2003; Silva *et al*., 2004; Frussa-Filho *et al*., 2004;

Hipólide *et al*., 2005; Antunes *et al.,* 2006; Allard *et al*., 2007; Guzman-Marin *et al*., 2007; Jhaveri *et al*., 2007; Perry *et al*., 2007; Beneditte *et al*., 2008; Martins *et al*., 2008; Tasali *et al.,* 2008; Alvarenga *et al*., 2008). Estilos de vida que envolve a privação de sono estão cada vez mais prevalentes. Evidências apontam que a privação de sono total e a restrição de sono (RS) podem afetar funções neurocomportamentais associadas com a excitação, a atenção, a memória e ao estado de estabilidade. Outros estudos indicam que os efeitos da privação de sono incluem uma ativação temporária dos principais sistemas neuroendócrinos de estresse, ou seja, do sistema simpático autonômico e do eixo hipotálamo-hipófise-adrenal (HPA) (Andersen *et al*, 2005; Meerlo *et al* , 2008). Fortes correlações entre a privação de sono paradoxal e a perda das funções cerebrais em animais e humanos têm sido observadas (Shaffery *et al*, 2002; Kim *et al*, 2005; Kopp *et al*, 2006; Vecsey *et al,* 2009). Consistentemente, os estudos têm mostrado que privação de sono prejudica significativamente o aprendizado e a memória (Alvarenga *et al*., 2008; Fernandes-Santos *et al*., 2012; Havekes *et al*., 2012; Joo *et al*., 2012).

O envolvimento do sono com aprendizado e com a memória tem sido extensivamente demonstrado em vários modelos animais incluindo os seres humanos (Wagner *et al.,* 2004; Walker *et al*., 2004; Rieth *et al.,* 2010; Diekelmann *et al*., 2010; 2011). Além disso, vários estudos eletrofisiológicos e de imagem fornecem um suporte abrangente para o entendimento de que o sono promove o processamento cognitivo (Hirase *et al*., 2001; Hoffman *et al*., 2002; Molle *et al.,* 2004; Ribeiro *et al.,* 2007).

Recentemente, foi demonstrado que o sono possui um papel fundamental na consolidação da memória (Mander *et al.,* 2011). O sono

adequado é necessário antes e depois de um evento, para que o aprendizado seja devidamente armazenado em longo prazo (Walker, 2009; Diekelmann & Born, 2010; Mander *et al.,* 2011). Ele aumenta consideravelmente a codificação e a consolidação da memória (Walker, 2006, 2009; Diekelmann & Born, 2010).

Conforme mostrado na Figura 5, durante o sono de ondas lentas (SOL) a memória novamente se codifica em uma armazenagem temporária (por exemplo: o hipocampo no sistema de memória declarativa) onde são reativadas para serem redistribuídas em uma armazenagem em longo prazo (por exemplo: o neocórtex). A consolidação do sistema durante o SOL se apóia em um diálogo entre o neocórtex e o hipocampo sobre o controle vertical pelas oscilações lentas neocorticais (vermelho). As fases de despolarização das oscilações lentas dirigem a reativação repetida das representações de memórias hipocampais junto com a formação das ondas agudas (azul) no eixo tálamo-cortical e no hipocampo (verde). Esse direcionamento sincrônico permite a formação do evento de um eixo de ondas onde a ondulação de ondas agudas e a informação da memória reativada associada tornam-se agregadas em eixos únicos (demonstrada em escala maior) (Born & Wilhelm, 2012).

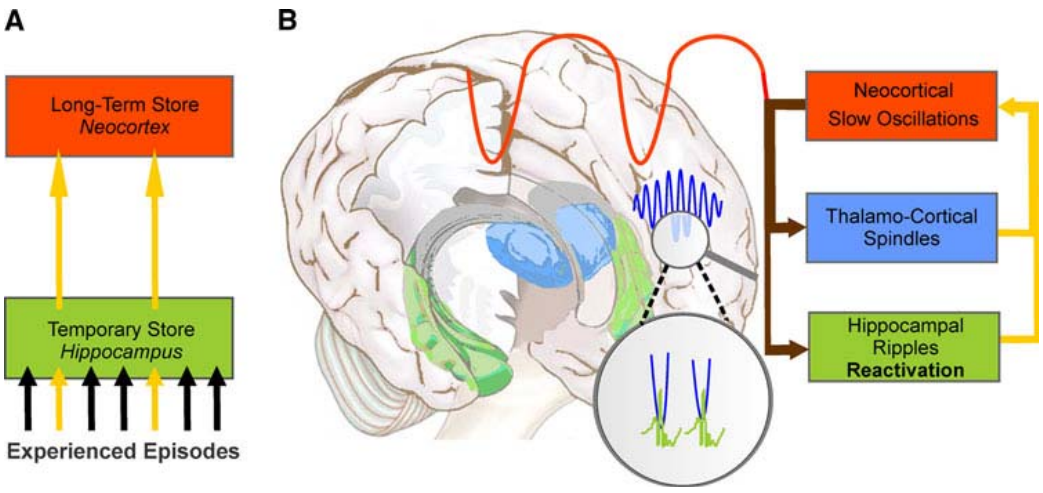


Fig 5 – Consolidação do sistema ativo durante o sono. Fonte: Born & Wilhelm, 2012.

Embora observações comportamentais demonstrassem claramente que dormir é importante para a consolidação da memória, os papéis das diferentes fases do sono ainda estão sendo decifrados. Devido a sua relação com os sonhos, o sono REM parece ser crítico para a formação da memória. Mas, a maioria dos estudos de EEG realizados até agora, tem relatado que o sono NREM especialmente SOL possui uma participação importante na retenção da memória. A privação de sono NREM ou de sono de SOL após a aprendizagem impede a consolidação e o aprimoramento posterior das memórias (Walker, 2006, 2009; Diekelmann & Born, 2010). O sono REM também tem sido associado com a memória emocional (Walker, 2009).

# 2. OBJETIVOS

## *2.1 Objetivo Geral*

Investigar o potencial terapêutico do modafinil, utilizando três modelos animais de prejuízo cognitivo induzido pelo tratamento neonatal com ferro, pela separação materna, pela privação de sono e também caracterizar os efeitos deste fármaco sobre a memória em ratos adultos saudáveis.

## *2.2Objetivos específicos*

2.2.1 Avaliar os efeitos do tratamento agudo com modafinil sobre a consolidação da memória de reconhecimento em ratos adultos saudáveis.

2.2.2 Avaliar os efeitos do tratamento agudo com modafinil sobre a evocação da memória de reconhecimento em ratos adultos saudáveis.

2.2.3 Avaliar os efeitos do tratamento agudo com modafinil sobre a consolidação da memória aversiva em ratos adultos saudáveis.

2.2.4 Avaliar os efeitos do tratamento agudo com modafinil sobre a evocação da memória aversiva em ratos adultos normais.

2.2.5 Avaliar o efeito do tratamento crônico com modafinil sobre a atividade em campo aberto (medida de atividade locomotora) em ratos tratados com ferro do 12° ao 14° dia de vida pós-natal.

2.2.6 Avaliar o efeito dos tratamentos agudo e crônico com modafinil sobre os prejuízos de memória de reconhecimento induzidos pelo tratamento com ferro do 12° ao 14° dia de vida pós-natal.

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

2.2.8 Avaliar o efeito da privação de sono paradoxal sobre a memória de reconhecimento, em ratos adultos normais, em três diferentes intervalos de tempo entre o treino e o teste na tarefa de reconhecimento do objeto novo.

2.2.9 Avaliar o efeito da separação materna associada com a privação de sono sobre a memória de reconhecimento em ratos adultos.

2.2.10 Avaliar o efeito do modafinil sobre os déficits de memória provocados pela separação materna e pela privação de sono paradoxal em ratos na idade adulta.

Os resultados relativos aos objetivos 2.2.1 até 2.2.7 estão apresentados no Artigo Científico I intitulado: "*Differential effects of modafinil on memory in naïve and memory- impaired rats"* aceito para publicação na revista "*Neuropharmacology".* E os resultados referentes aos objetivos 2.2.8 até 2.2.10 estão apresentados no Artigo Científico II intitulado: *"Modafinil ameliorates cognitive deficits induced by maternal separation and sleep deprivation"* aceito para publicação na revista "*Behavioural Brain Research".*

# *Capítulo 2*

***Artigo Científico I***

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# Differential effects of modafinil on memory in naïve and memory-impaired rats

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ABSTRACT

Modafinil is a wake-promoting drug and has been approved for the treatment of excessive daytime sleepiness in narcolepsy and obstructive sleep apnea. Modafinil was shown to improve learning and memory in rodents, and to reverse memory deficits induced by sleep deprivation or stress. However, depending on the memory paradigm used, modafinil might also impair memory. We aimed to investigate the effects of modafinil on memory consolidation and retrieval for object recognition and inhibitory avoidance in naïve adult rats. We also investigated whether acute or chronic administration of modafinil would reverse memory deficits induced by iron overload, a model of memory impairment related to neurodegenerative disorders. Adult naïve rats received modafinil (0.0, 0.75, 7.5 or 75 mg/kg) either immediately after training or 1 h prior to testing in object recognition or inhibitory avoidance. Iron-treated rats received modafinil immediately after training in object recognition. In order to investigate the effects of chronic modafinil, iron-treated rats received daily injections of modafinil for 17 days, and 24 h later they were trained in object recognition or inhibitory avoidance. Acute modafinil does not affect memory consolidation or retrieval in naive rats. A single injection of modafinil at the highest dose was able to recover recognition memory in iron-treated rats. Chronic modafinil completely recovered iron-induced recognition memory and emotional memory deficits. Additional preclinical and clinical studies are necessary in order to support the applicability of modafinil in recovering memory impairment associated with neurodegenerative disorders.



## 1. Introduction

Modafinil [(diphenyl–methyl) sulphinil-2-acetamide] is a psychostimulant that acts as a wake-promoting drug and has been approved for the treatment of excessive daytime sleepiness in narcolepsy and obstructive sleep apnea (Ballon and Feifel, 2006; Minzenberg and Carter, 2008). Though modafinil has been classified as a psychostimulant, evidence suggests that it acts on a neural pathway different from amphetamine and cocaine (Ballon and Feifel, 2006). While its precise mechanism of action is still not well identified, human and animal research suggest that it directly or indirectly activates the dopaminergic (de Saint Hilaire et al., 2001; Volkow et al., 2009), glutamatergic (Ferraro et al., 1999), noradrenergic (de Saint Hilaire et al., 2001; Minzenberg et al., 2008) and serotonergic (de Saint Hilaire et al., 2001) systems in several brain regions, including the prefrontal cortex, hippocampus, hypothalamus and striatum, whereas it inhibits GABAergic pathways in the same regions (Ferraro et al., 1999).

Evidence suggests that modafinil improves visual discrimination/attention in rodents (Morgan et al., 2007). Moreover, modafinil facilitates learning and memory in spatial and contextual tasks, in healthy adult rats (Burgos et al., 2010; Tsanov et al., 2010) and mice (Beracochea et al., 2008; Shuman et al., 2009). In addition, it was demonstrated that modafinil recovers memory deficits induced by sleep deprivation (He et al., 2011; Moreira et al., 2010; Piérard et al., 2007, 2011) or by chronic stress (Piérard et al., 2006). However, depending on the learning paradigm used, dose and time window of drug administration, contradictory results were found. For instance, modafinil was shown to impair emotional memory in rodents (Burgos et al., 2010; Fernandes et al., 2013).

Iron, the most abundant metal in the human body, is essential for many key biological processes related to the development of the

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nervous system, including myelination, neurotransmitter synthesis, and mitochondrial energy production (Connor et al., 1995). Over the last decades, many studies in humans as well as in animal models have shown that iron deficiency during neurological development leads to permanent cognitive deficits (Fretham et al., 2011; Lozoff, 2011). However, clinical and experimental evidence suggest a role of iron excess in neurodegenerative diseases. An abnormal iron homeostasis might cause severe cellular dysfunction or death, and it has been recognized as a triggering factor for different neurodegenerative disorders such as Parkinson's (PD), Alzheimer's (AD), and Huntington's (HD) diseases as well as amyotrophic lateral sclerosis (ALS) (Mills et al., 2010). Noteworthy, studies using brain imaging techniques show that iron concentrations, particularly in the parietal and temporal cortex, hippocampus and basal ganglia, positively correlate with poor performance in a variety of cognitive tests, both in healthy elderly individuals (Pujol et al., 1992; Sullivan et al., 2009; Bartzokis et al., 2011; Penke et al., 2012; Rodrigue et al., 2013) and in patients with dementia (Brass et al., 2006; House et al., 2006; Ding et al., 2009; Zhu et al., 2009).

In agreement with the observations that iron accumulation strongly correlates with poor cognitive performance in healthy and demented aged human subjects, we have demonstrated that brain iron accumulation induces persistent memory deficits in rodents (de Lima et al., 2005a; Fagherazzi et al., 2012; Schröder et al., 2001, 2013; Silva et al., 2012) and increases oxidative stress in brain regions related to memory formation in rats (de Lima et al., 2005a). Recently, we found that iron loading produces an increase in glial acidic protein (GFAP), which is an astrocyte marker, suggesting the presence of reactive gliosis in adult mice (Fernandez et al., 2010) and rats (Fernandez et al., 2011), and that the apoptotic markers, Par-4, and caspase-3 are also increased in the brains of adult animals treated with iron in the neonatal period (Miwa et al., 2011). Taken together, these results suggest that memory dysfunction associated with iron treatment may be viewed as a model of cognitive decline related to neurodegenerative disorders. Thus, over the last years we have been using this model in order to investigate drugs with potential use as cognitive enhancer for the treatment of memory deficits associated to aging and/or neurodegenerative disorders (de Lima et al., 2005b, 2007, 2008; Fagherazzi et al., 2012; Silva et al., 2012).

Thus, we aimed to evaluate whether the acute or chronic administration of modafinil would be able to reverse memory deficits induced by iron overload. Additionally, in order to better characterize the properties of modafinil on memory consolidation and retrieval, we tested healthy naïve adult rats using two different learning and memory paradigms, object recognition and the aversively motivated emotional memory task inhibitory avoidance.

## 2. Material and methods

### 2.1. Animals

For the experiments investigating the effects of acute modafinil on memory consolidation and retrieval in adult naïve control rats, male adult Wistar rats (200–250 g) were obtained from the State Health Science Research Foundation (FEPPS-RS, Porto Alegre, Brazil). For iron-induced memory impairment experiments, pregnant Wistar rats were obtained from FEPPS-RS. After birth each litter was adjusted within 48 h to eight rat pups 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 ±1 °C 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.

In order to reduce the possibility of litter effects, rat pups were randomly assigned to treatment groups and each treatment group (iron or vehicle, as described below) consisted of rats derived from 9 different litters. For postnatal treatments, animals were given standardized pellet food and tap water *ad libitum*. All behavioral experiments were performed at light phase between 9:00 and 16:30 h. All experimental procedures were performed in accordance to the principles of laboratory animal care and 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 for the Use of Animals (CEUA) of the Pontifical Catholic University. All efforts were made to minimize the number of animals used and their suffering.

### 2.2. Treatments

#### 2.2.1. Modafinil

For the experiments investigating the acute effects of modafinil on memory consolidation and retrieval in naïve animals, adult (3 months old at arrival) rats were trained and tested in the novel 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 (Tween 80–saline solution 1:16 v/v) or modafinil (Stavigile®, Libbs, USA) at the doses of 0.75, 7.5 and 75 mg/kg was administered intraperitoneally immediately after the training session for the investigation of its effects on memory consolidation and one hour before testing sessions for the investigation of effects on memory retrieval. The doses were selected based on pilot experiments performed in our laboratory and on a previous published study (Shuman et al., 2009).

For the investigation of the effects of modafinil on iron-induced memory impairments, adult (3 months old) rats treated neonatally with vehicle (Sorbitol 5%, Sorb) or iron (as described in detail below) received an acute intraperitoneal injection of vehicle or modafinil (at the doses of 0.75, 7.5 and 75 mg/kg) immediately after the training session of the object recognition task. For experiments investigating the chronic effects of modafinil (Mod) on iron-induced memory impairments, adult rats treated neonatally with vehicle (Sorb) or iron received a daily intraperitoneal injection of vehicle (Veh) or modafinil (Mod, at the doses of 0.75, 7.5, and 75 mg/kg) for 17 consecutive days. Training in the behavioral tasks of object recognition or inhibitory avoidance were performed 24 h after the last drug treatment. Drug solutions were freshly prepared immediately prior to administration.

#### 2.2.2. Iron neonatal treatment

The neonatal iron treatment has been described in detail elsewhere (Fagherazzi et al., 2012; Silva et al., 2012). Briefly, 12-day-old rat pups received orally a single daily dose (10 ml/kg solution volume) of vehicle (5% sorbitol in water, control group) or 30 mg/kg of body weight of $Fe^{2+}$ (iron carbonyl; Sigma–Aldrich, São Paulo, Brazil) via a metallic gastric tube, over 3 days (postnatal days 12–14).

### 2.3. Behavioral procedures

#### 2.3.1. Inhibitory avoidance task

The inhibitory avoidance (IA) behavioral training and retention test procedures were described in previous reports (Schröder et al., 2001; Silva et al., 2012). The IA apparatus was a 50 × 25 × 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 stepdown 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 (0.5 mA) and were removed from the apparatus immediately afterwards. A retention test trial was carried out 24 h after the training trial. The retention test trial was procedurally identical to training, except that no foot shock was presented. Stepdown latencies (in seconds) on the retention test trial (maximum 180 s) were used as a measure of IA retention.

#### 2.3.2. Object recognition task

The object recognition task was performed as previously described (de Lima et al., 2005a). Briefly, the object recognition task took place in an open-field apparatus (45 × 40 × 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). On the long-term memory (LTM) testing trial, performed 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% of trials in the right side and 50% of 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. 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. For the evaluation of the effects of modafinil on memory retrieval for the object recognition in naïve rats, animals were trained in this protocol, using two identical objects, and received Vehicle (Veh) or modafinil (Mod 0.75, 7.5, or 75 mg/kg) one hour before the test session. A recognition index calculated for each animal was expressed by the ratio TN/(TF + TN) [TF = time spent exploring the familiar object (A), TN = time spent exploring the novel object (B)].

*2.3.3. Modified protocol for the object recognition task*

Using two different objects during training might increase the difficulty of the task, thus preventing control animals from showing significant retention at a 24-h post-training delay (Tang et al., 1999), and we have previously used this protocol to investigate the effects of memory-enhancing drugs (Dornelles et al., 2007). Here we used this protocol in order to investigate the effects of modafinil on memory consolidation in adult naïve rats. Twenty four hours after habituation, training was conducted by placing individual rats for 5 min into the field, in which two distinct objects (A and B) were positioned in two adjacent corners, 10 cm from the walls. In a long-term retention test given 24 h after training, the rats explored the open field for 5 min in the presence of one familiar (either A or B) and one novel object (C). A recognition index calculated for each animal was expressed by the ratio TN/(TF + TN) [TF = time spent exploring the familiar object (A), TN = time spent exploring the novel object (C)].

*2.3.4. Open-field behavior*

In order to control for possible sensorimotor effects induced by chronic modafinil, behavior during habituation to the open field prior to object recognition training was evaluated after chronic modafinil administration, as previously described (de Lima et al., 2005b, 2008). The open-field floor was divided into 12 equal squares by black lines. Animals were placed in the rear left corner and left to explore the field freely for 5 min. Latency to start locomotion, line crossings, rearings, and the number of fecal pellets produced were counted. The number of crossings and rearings were used, respectively, as measures of locomotor activity and exploratory behavior, whereas the latency to start locomotion and the number of fecal pellets were used as measures of anxiety.

*2.4. Statistical analysis*

Data for latency to step-down and recognition indexes are expressed as mean ± S.E.M. Object recognition and inhibitory avoidance task data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* tests when necessary. Data from the experiment evaluating open field behavior were analyzed by ANOVA and are expressed as mean ± S.E.M. In all comparisons, $p$ values less than 0.05 were considered to indicate statistical significance.

## 3. Results

In order to characterize the effects of modafinil on memory consolidation of object recognition, we assessed the effects of post-training acute administration of modafinil on 24-h retention in rats exposed to two different objects during training. As previously described, this protocol does not produce significant recognition memory retention in control rats, when tested 24 h after training. Statistical comparison has revealed no significant differences in recognition indexes of training or testing sessions among the groups (Fig. 1A). Likewise, no significant differences were observed when time exploring objects during the training session was compared (Table 1). Next, we aimed to evaluate the effects of acute modafinil on memory retrieval. Thus, rats were trained using the classical object recognition protocol, with two identical objects, and received modafinil 1 h before the testing session. Again, statistical comparisons of recognition indexes in the training and testing sessions (Fig. 1B), and of the time exploring objects during training or testing sessions (Table 1) have revealed no significant differences among groups.

In the second experiment, we evaluated the effects of acute administration of modafinil on the consolidation and retrieval of memory for inhibitory avoidance. Fig. 2 shows the results of acute administration of modafinil in adult rats immediately after training (Fig. 2A) or 1 h before testing (Fig. 2B) in the inhibitory avoidance task. Modafinil, when administered immediately after training, at all doses, has not affected memory consolidation for inhibitory avoidance, as no significant differences in latencies to step-down among groups were found in the testing session (Fig. 2A). In the experiment evaluating the effects of modafinil 1 h before testing, statistical comparison of latencies to step-down has shown no significant differences among the groups, indicating that modafinil does not affect memory retrieval (Fig. 2B). Taken together, these results suggest that modafinil has no memory enhancing properties for neutral or aversively-motivated memory tasks in naïve rats.

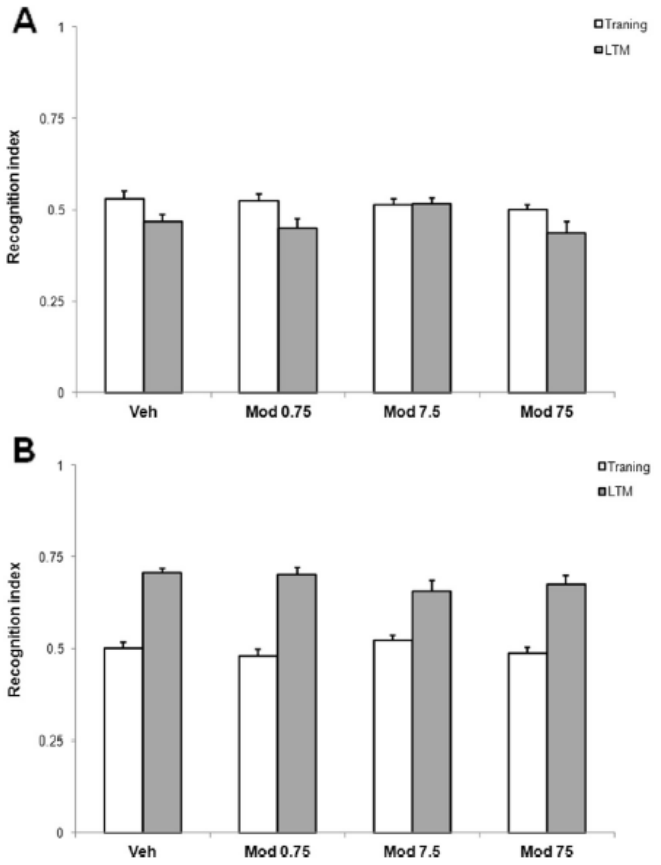


**Fig. 1.** Effects of acute modafinil (Mod) on memory consolidation (A) and retrieval (B) for the object recognition in naïve rats. (A) Post-training systemic administration of Mod on long-term memory retention of object recognition in naïve rats exposed to two different objects during training. Rats received a single intraperitoneal (i.p.) injection of vehicle (Veh) or Mod (0.75; 7.5 or 75 mg/kg) immediately after training and were tested for retention 24 h after training. (B) For the evaluation of the effects of modafinil on memory retrieval for object recognition, naïve rats were exposed to two identical objects in the training session and received Veh or Mod (0.75, 7.5, or 75 mg/kg) 1 h before testing. LTM retention test was performed 24 h after training. $N = 14–15$ per group. Data are expressed as mean ± SEM. No significant differences were found among groups.

Using a model of cognitive impairment induced by iron administration in the neonatal period, we aimed to investigate the effects of a single acute injection of modafinil immediately after training in ameliorating iron-induced memory impairment (Fig. 3A). One-way ANOVA revealed a significant difference among

**Table 1**
Total time (in seconds) exploring both objects in the training or testing trial of object recognition task in naïve rats submitted to acute modafinil administration either immediately after training (memory consolidation experiment) or one hour before the testing session (retrieval experiment).

| Group | Memory consolidation experiment | Memory retrieval experiment (training) | Memory retrieval experiment (testing) |
|---|---|---|---|
| Vehicle | 45.34 ± 3.08 ($n = 15$) | 32.57 ± 2.35 ($n = 14$) | 39.35 ± 3.69 ($n = 14$) |
| Mod 0.75 | 44.99 ± 1.94 ($n = 14$) | 32.48 ± 3.30 ($n = 15$) | 37.23 ± 3.74 ($n = 15$) |
| Mod 7.5 | 45.41 ± 3.37 ($n = 15$) | 35.03 ± 2.44 ($n = 15$) | 39.05 ± 3.47 ($n = 15$) |
| Mod 75 | 46.58 ± 3.57 ($n = 15$) | 38.60 ± 2.57 ($n = 15$) | 41.43 ± 4.31 ($n = 15$) |

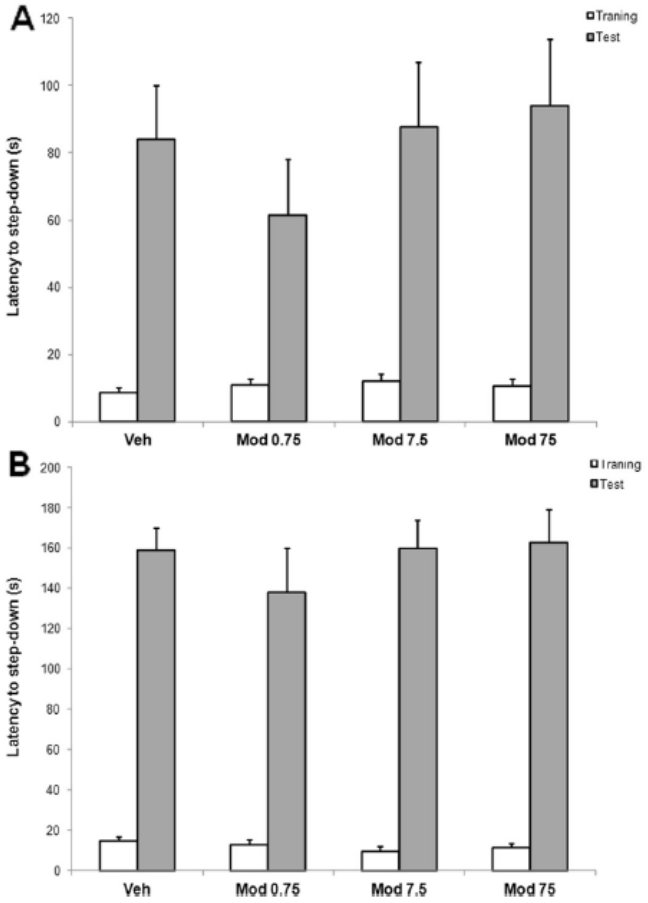


**Fig. 2.** Effects of acute modafinil (Mod) on memory consolidation (A) and memory retrieval (B) for the inhibitory avoidance task in naïve rats. Vehicle (Veh) or Mod (0.75, 7.5, or 75 mg/kg) were administered immediately after training or 1 h before testing. LTM retention test was performed 24 h after training. $N = 14–15$ per group in the memory consolidation experiment and 10–13 in the memory retrieval experiment. Data are expressed as mean ± SEM. No significant differences were found among groups.

the groups in recognition indexes in the retention test ($F_{(7,91)} = 33.76$, $p < 0.0001$), but not in training session, or in the total time exploring the objects during the training session (Table 2). Tukey's *post hoc* test showed that rats neonatally treated with iron that received vehicle (Iron-Veh) in the adulthood present significantly lower recognition indexes than the control group (Sorb-Veh, $p < 0.0001$) in the long term retention test, indicating that iron given in the neonatal period induces recognition memory impairment (Fig. 3A), as previously demonstrated. Acute administration of modafinil at the doses 0.75 and 7.5 mg/kg partially recovered iron-induced recognition memory deficits, as recognition indexes of these groups were significantly higher than the Iron-Veh group ($p < 0.05$ and $p < 0.01$, respectively), but were lower than controls (Sorb-Veh, both $p$'s $< 0.0001$) (Fig. 3A). Remarkably, iron-treated rats that received a single administration of modafinil at the dose of 75 mg/kg showed significantly higher recognition indexes than the Iron-Veh group's index ($p < 0.0001$), and no significant differences when compared to the control group, suggesting that, at this dose, acute modafinil completely reversed iron-induced deficits. The results also showed that modafinil by itself has no effect on recognition memory in adult control rats, revealed by comparisons between the control group (Sorb-Veh) and the groups treated neonatally with sorbitol and modafinil 0.75, 7.5 or 75 mg/kg immediately after training (Fig. 3A), confirming results obtained with naïve rats.

The effects of chronic administration of modafinil on iron-induced recognition memory deficits are shown in Fig. 3B. One-way ANOVA revealed a significant difference among the groups in the long-term retention test ($F_{(7, 91)} = 27.15$, $p < 0.0001$), but not in training session, or in the total time exploring objects during the training session (Table 2). Tukey's *post hoc* tests indicated that chronic administration of modafinil at all doses used completely recovered memory in animals that received iron in the neonatal period, as the recognition indexes of Iron–Mod groups were significantly higher than the Iron-Veh group (all $p$'s $< 0.0001$) and showed no significant differences when compared to the control group (Fig. 3B). In addition, results showed that chronic modafinil by itself has no effect on recognition memory in adult control rats, revealed by comparisons between the control group (Sorb-Veh) and groups treated neonatally with sorbitol and modafinil in the adulthood.

We also evaluated the effects of chronic administration of modafinil on iron-induced memory deficits using an aversively motivated learning paradigm (Fig. 4). Comparisons of latencies to step-down using ANOVA revealed a significant difference among the groups in the long-term retention test ($F_{(7, 80)} = 5.15$, $p < 0.0001$), but not in training trial. As previously described, iron administration in the neonatal period impairs inhibitory avoidance memory, as latencies to step-down in the testing session are significantly lower in the Iron-Veh group when compared to the control group (Sorb-Veh, $p < 0.0001$). Tukey's *post hoc* tests have also indicated that the groups treated with iron that received chronic modafinil at any dose do not statistically differ from the control group. Additionally, chronic administration of modafinil at the doses of 7.5 and 75 mg/kg to iron-treated rats produced a significant increase in retention test latencies in comparison to the Iron-Veh group ($p < 0.05$ and $p < 0.0001$, respectively). Chronic modafinil by itself had no effect on aversive memory in control rats, revealed by comparisons between the control group (Sorb-Veh) and groups treated with sorbitol in the neonatal period that received chronic modafinil at all doses used. Taken together, these results indicate that single acute injections or chronic administration of modafinil are able to ameliorate cognitive deficits related to iron accumulation.

In order to control for possible effects of iron administration or chronic adult modafinil treatment on general sensorimotor functions, we analyzed open filed behavior in iron-treated rats submitted to chronic modafinil (Table 3). One-way ANOVA showed no statistically significant differences among the groups in the number of crossings, latency to start locomotion, and defecation. Statistical comparison of the numbers of rearings has revealed a significant difference among the groups ($F_{(7, 91)} = 4.59$, $p < 0.05$). However, *post hoc* comparisons revealed no significant differences between the control group (Sorb-Veh) compared to all other groups. Additionally, direct comparisons of Iron-Veh group with all other groups have not shown any significant differences. These results suggest that neonatal iron treatment and chronic adult modafinil did not affect locomotion, exploration, or anxiety.

## 4. Discussion

The present findings show that modafinil, within a wide range of doses, when acutely administered immediately after training or prior to retention test does not affect consolidation or retrieval of two distinct types of memory, namely recognition memory and emotional memory in control rats. Recognition memory is based on a natural tendency of rodents in exploring novel objects; and the level of emotional arousal of this task is mainly related to the

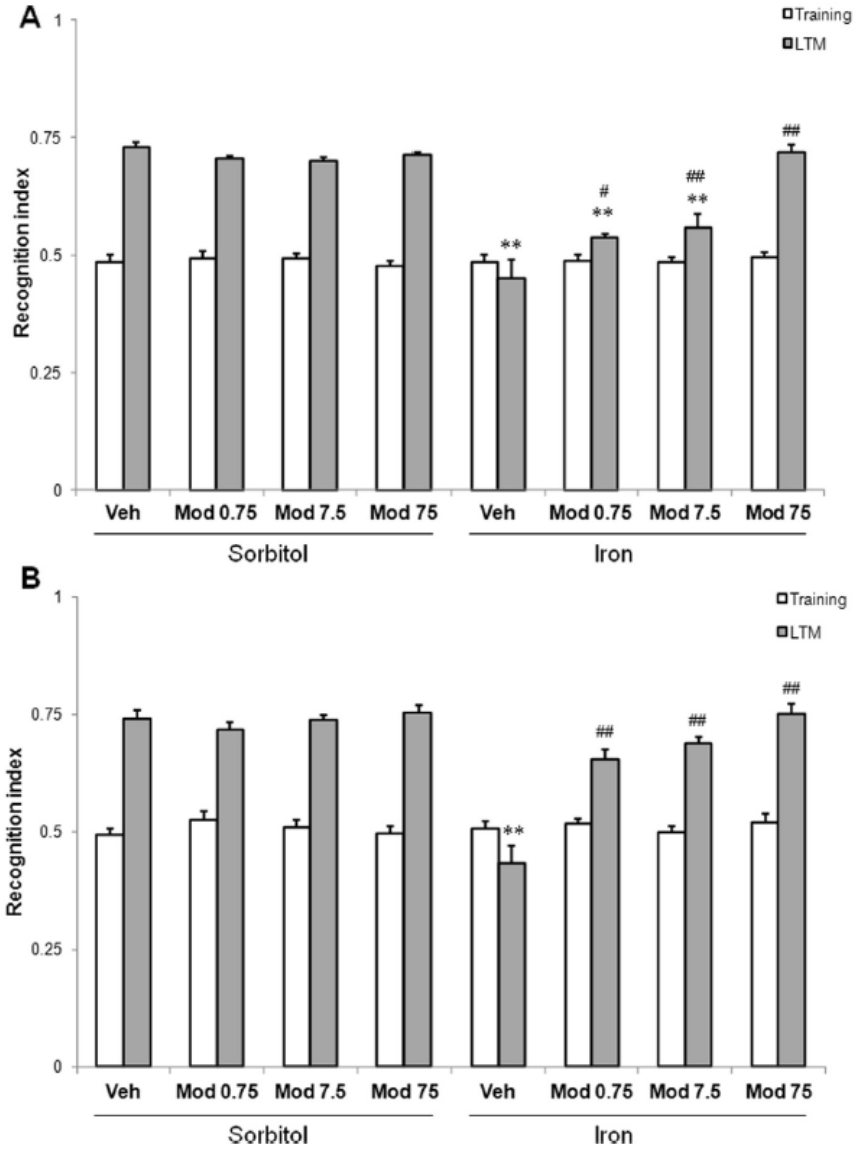


**Fig. 3.** (A) Effects of a single acute injection of modafinil (Mod) on iron-induced recognition memory deficits. Recognition indexes in training and retention test session are expressed as mean ± SEM. Vehicle (Veh) or Mod (0.75, 7.5 and 75 mg/kg) were administered immediately after training. $N = 12–13$ per group. Differences between sorbitol (Sorb)–Veh vs other groups are indicated as $^{**}p < 0.0001$; differences between Iron-Veh vs Iron-Mod are indicated as $\#\#p < 0.0001$. (B) Effects of chronic modafinil (Mod) on iron-induced recognition memory deficits. Recognition indexes in training and retention test sessions are expressed as mean ± SEM. A daily single injection of vehicle (Veh) or Mod (0.75, 7.5 and 75 mg/kg) were administered intraperitoneally for 17 consecutive days. Animals were trained in the novel object recognition task 24 h after the last injection. $N = 12–13$ per group. Differences between Sorb-Veh vs other groups are indicated as $^{**}p < 0.0001$; differences between Iron-Veh vs Iron-Mod are indicated as $\#p < 0.05$ and $\#\#p < 0.01$

exposure to a novel environment, which can be greatly reduced by habituating animals to the field prior training (Okuda et al., 2004). Conversely, inhibitory avoidance is known as a form of conditioning in which rodents learn to associate a neutral stimulus (context) to an aversive stimulus (electric shock), thus, activating neural circuits involved in emotional arousal, such as the noradrenergic system within the amygdala. It has been postulated that emotional memories tend to be more vivid and to persist longer than do memories of neutral or trivial events (McIntyre et al., 2003). The present data suggests that the level of emotional activation associated with the learning paradigm does not alter the response to modafinil in control, non memory-impaired, rats.

Experiments analyzing the effects of modafinil in memory-impaired rats, showed, for the first time, that both acute and chronic treatment ameliorate recognition and emotional memory, in a model of cognitive impairment relevant to aging and neurodegenerative disorders.

In order to control for effects on exploratory, sensorial and motor behavior or anxiety levels induced by modafinil that could interfere with memory acquisition or performance during the testing session, we analyzed the total time exploring objects during the training sessions or in the testing session of the object recognition task. Additionally, we analyzed open field behavior following the chronic treatment with modafinil. At the doses used in the present study, modafinil did not induce alterations in general exploratory behavior, as the time spent exploring objects and the number of rearings, crossings, latency to start locomotion, and number of fecal bolus produced were not different from the control groups.

Most of the previous studies investigating the effects of modafinil on memory in control animals were conducted using learning

**Table 2**
Total time (in seconds) exploring both objects in the training trial of object recognition task in iron-treated rats submitted to acute or chronic modafinil administration.

| Group | Acute modafinil treatment | Chronic modafinil treatment |
|---|---|---|
| Sorb-Veh | 40.14 ± 4.76 ($n = 12$) | 40.96 ± 4.88 ($n = 12$) |
| Sorb-Mod 0.75 | 34.33 ± 1.53 ($n = 13$) | 36.42 ± 3.05 ($n = 13$) |
| Sorb-Mod 7.5 | 38.88 ± 1.55 ($n = 13$) | 37.70 ± 3.61 ($n = 13$) |
| Sorb-Mod 75 | 38.31 ± 1.68 ($n = 13$) | 31.89 ± 2.72 ($n = 13$) |
| Iron-Veh | 35.46 ± 2.25 ($n = 12$) | 37.21 ± 2.61 ($n = 12$) |
| Iron-Mod 0.75 | 37.33 ± 1.92 ($n = 12$) | 33.55 ± 4.05 ($n = 12$) |
| Iron-Mod 7.5 | 36.99 ± 1.14 ($n = 12$) | 36.33 ± 2.81 ($n = 12$) |
| Iron-Mod 75 | 37.14 ± 2.23 ($n = 12$) | 32.96 ± 1.65 ($n = 12$) |

**Table 3**
Open-field behavior in iron-treated rats submitted to chronic modafinil administration.

| Group | Latency to start locomotion (s) (mean ± S.E.) | Number of crossings (mean ± S.E.) | Number of rearings (mean ± S.E.) | Number of fecal pellets (mean ± S.E.) |
|---|---|---|---|---|
| Sorb-Veh | 6.47 ± 0.64 | 65.00 ± 5.22 | 27.33 ± 2.06 | 2.58 ± 0.69 |
| Sorb-Mod 0.75 | 6.49 ± 0.88 | 57.84 ± 6.99 | 22.07 ± 2.47 | 2.46 ± 0.61 |
| Sorb-Mod 7.5 | 6.00 ± 1.09 | 71.84 ± 4.43 | 33.61 ± 4.00 | 3.92 ± 0.87 |
| Sorb-Mod 75 | 5.66 ± 0.46 | 78.69 ± 5.77 | 39.84 ± 3.41 | 2.61 ± 0.56 |
| Iron-Veh | 5.90 ± 0.55 | 73.91 ± 8.64 | 29.58 ± 4.98 | 2.16 ± 0.61 |
| Iron-Mod 0.75 | 5.74 ± 1.00 | 70.91 ± 5.53 | 25.00 ± 3.35 | 3.41 ± 0.43 |
| Iron-Mod 7.5 | 7.39 ± 0.67 | 81.66 ± 3.80 | 40.75 ± 2.34 | 1.66 ± 0.60 |
| Iron-Mod 75 | 6.44 ± 0.98 | 57.91 ± 7.71 | 23.41 ± 3.35 | 3.5 ± 0.67 |

Open-field behavior was analyzed during the habituation session for the object recognition task in iron-treated rats after chronic modafinil administration. Data are expressed as mean ± S.E.M.

and memory paradigms of spatial or contextual memory. However, the findings have been contradictory. For instance, chronic modafinil decreased the number of errors on the 8-arm radial maze, while the same treatment resulted in a decrease of successful responses in a complex operant conditioning learning (Burgos et al., 2010). Tsanov et al. (2010) reported that a chronic treatment with modafinil for a period of 16 days, starting seven days before the behavioral testing, improved acquisition of spatial memory in the Morris water maze in rats, without affecting swim speed, excluding possible pharmacological effects on motor skills. Similarly, using a dose of 75 mg/kg, administered daily pre training, Shuman et al. (2009) demonstrated that modafinil improved acquisition and memory in probe trials performed on the 7th and 8th day of the water maze testing, without affecting retrieval, as it had no effect when administered to trained mice only prior to the probe trial. In accordance with our findings, the authors also found that the same dose of modafinil, when administered acutely immediately after training, did not affect fear conditioning, a type of emotional memory. In contrast, it was recently demonstrated that post-training acute administration of high doses of modafinil (64 and 128 mg/kg) impaired emotional memory consolidation, tested on the plus-maze discriminative avoidance task, while a lower dose, when administered before the test in the same behavioral task, impaired retrieval (Fernandes et al., 2013). Additionally, in our study, control rats (sorbitol group) treated chronically with modafinil at all doses displayed lower latencies to step-down in the inhibitory avoidance test, although these effects were not statistically significant (Fig. 4). In contrast, in iron-treated rats, modafinil significantly improved step-down latencies, when compared to the iron-vehicle group. These differential effects may be related to the memory-impairing potential of modafinil in control animals, already described in a previous study (Fernandes et al., 2013). More studies are required in order to better understand the mechanisms involved in modafinil's memory-impairing effects in aversive memory tasks in control animals.

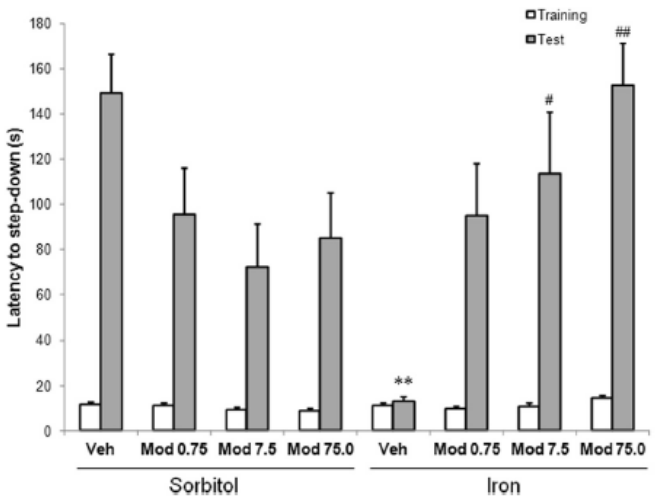


**Fig. 4.** Effects of chronic modafinil (Mod) on iron-induced memory deficits for the inhibitory avoidance task. Latencies to step-down in training and retention test session are expressed as mean ± SEM. A daily single injection of vehicle (Veh) or Mod (0.75, 7.5 and 75 mg/kg) was administered intraperitoneally for 17 consecutive days. Animals were trained in inhibitory avoidance task 24 h after the last injection. $N = 10–12$ per group. Differences between Sorb-Veh vs other groups are indicated as $^{**}p < 0.0001$; difference between Iron-Veh vs Iron-Mod is indicated as $\#\#p < 0.0001$.

To our knowledge, this is the first study investigating the effects of modafinil on memory consolidation and retrieval in the object recognition task in naïve animals. This task does not rely on aversive or appetitively-motivated stimulus, and, by using the present protocols, it does not use spatial information. Recognition memory tasks rely on the judgment of the prior occurrence of an object (Winters et al., 2008). Here we show that acute administration of modafinil, in a wide range of doses had no effect on recognition memory in control rats, but it was effective when acutely or chronically administered in reversing memory deficits induced by iron overload. One study showed that acute modafinil was able to ameliorate recognition memory in rats previously submitted to a subchronic treatment with phencyclidine (PCP), considered as an animal model of cognitive impairments associated with schizophrenia (Redrobe et al., 2010). As modafinil bears awakening properties and has been used to alleviate symptoms of excessive daytime sleepiness resulting from sleep disturbances, its potential in reversing memory deficits associated to sleep deprivation has been explored. Modafinil was shown to prevent spatial memory impairments induced by sleep deprivation in the water maze (He et al., 2011) and in a spontaneous alternation task in a T-maze (Piérard et al., 2007). Acute modafinil administered pretest also reversed contextual memory deficits in the 4-hole board (Pierard et al., 2011). Emotional memory deficits induced by sleep deprivation were also prevented by modafinil (Moreira et al., 2010).

Although the mechanisms underlying modafinil effects on memory are not completely understood, it was shown that chronic modafinil increases postsynaptic potentials, which are selectively correlated with theta rhythm augmentation in the dentate gyrus (Burgos et al., 2010), and upregulates synapsin I expression, and a synaptic plasticity-related gene, MMP-9, expression in the dorsal hippocampal CA3 region, suggesting that modafinil is able to enhance synaptic plasticity in the hippocampus (He et al., 2011). A short treatment of 4 days with modafinil increased glutamate receptor subunits GluR1, GluR2, NR1, and decreased levels of dopamine D2 receptors in the hippocampus of mice (Sase et al., 2012). In

contrast, a study using *in vivo* electrophysiology showed that modafinil blocked LTP (long-term potentiation, a form of synaptic plasticity related to learning and memory) induction in the prefrontal cortex of rats (Burgos et al., 2010).

Object recognition and inhibitory avoidance memory have been shown to critically depend on glutamatergic NMDA receptors (de Lima et al., 2005c; Roesler and Vianna, 1999) and to be modulated by dopaminergic (de Lima et al., 2011; Izquierdo et al., 1998) and adrenergic systems (Dornelles et al., 2007; McIntyre et al., 2002). Thus, it is possible that modafinil, by directly enhancing glutamatergic and catecholaminergic neural transmission, might modulate memory consolidation processes, leading to memory improvement in models of cognitive impairment. Another possibility, particularly related to the chronic effects of modafinil in reversing iron-induced effects might be related to modafinil neuroprotective properties. Studies suggested that modafinil may display neuroprotective properties in animal models of PD. Modafinil dose-dependently reversed the motor disability, the reduction of dopamine levels in the striatum, and the loss of tyrosine hydoxilase postitive neurons in the *substantia nigra pars compacta* in marmosets treated with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Jenner et al., 2000; van Vliet et al., 2006, 2008). Similar results had been previously found in mice treated with MPTP (Aguirre et al., 1999; Fuxe et al., 1992). Thus, we cannot rule out the possibility that chronic modafinil may have neuroprotective effects that would ultimately prevent memory loss in iron-treated rats. More studies are warranted in order to investigate the neurochemical mechanisms underlying modafinil effects.

In humans, a recent study has shown that acute modafinil improves spatial working memory, planning and decision making, as well as visual pattern recognition memory following delay (Müller et al., 2013). A seven-days treatment with modafinil (100 mg) enhanced performance in cognitive tasks dependent on the prefrontal cortex, a working memory task and a variable attentional control task in healthy volunteers (Rasetti et al., 2010). Studies also found that modafinil improved performance on a test of sustained attention (Dean et al., 2011; Randall et al., 2005) with no significant improvement on inhibitory control, and processing speed/attention (Dean et al., 2011), working memory (Dean et al., 2011; Randall et al., 2005; Turner et al., 2003), or short-term declarative memory (Randall et al., 2005). A single dose modafinil (300 mg) showed a tendency to improve performance in the One-Touch Stockings of Cambridge, a test of spatial planning and working memory, but failed to produce an enhancement on other cognitive tests of the Cambridge Neuropsychological Test Automated Battery (CANTAB), including digit spans and pattern recognition memory in healthy volunteers (Winder-Rhodes et al., 2010). Thus, in humans, using a variety of cognitive tests in healthy volunteers has also produced controversial results.

## 5. Conclusions

Additional preclinical and clinical studies are necessary in order to further support the applicability of modafinil in recovering memory impairment associated to neurodegenerative disorders. However, the question remains whether it can be considered as a memory enhancer for healthy individuals.

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# *Capítulo 3*

***Artigo Científico II***

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Research report

# Modafinil ameliorates cognitive deficits induced by maternal separation and sleep deprivation

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## HIGHLIGHTS

- Maternal separation induces memory impairment in adult rats.
- Sleep deprivation impairs recognition memory.
- Modafinil ameliorates deficits induced by maternal separation and sleep deprivation.

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## ABSTRACT

Animals exposed to an early adverse event may be more susceptible to a second source of stress later in life, and these stressors may have additive deleterious effects. Sleep deprivation is known to be a stressor, affecting multiple body functions such as the cognition. Modafinil enhances working memory and attention in healthy non-sleep deprived subjects and in animal models of sleep deprivation. The first aim of the present study was to investigate the effects of maternal separation (MS) combined with paradoxical sleep deprivation (PSD) in adulthood on recognition memory in rats. Second, we aimed to evaluate whether the administration of modafinil would be able to ameliorate memory deficits induced by MS and PSD. Wistar rat pups were initially distributed into MS and handling (H) groups, with their litters standardized in 4 females and 4 males. In adulthood, the male rats were submitted to PSD or control condition, being redistributed afterwards in modafinil- or vehicle-treatment immediately after the training session of object recognition task. PSD did not potentiate the cognitive deficit due to MS. However, modafinil was able to recover memory impairments associated to PSD and also to MS in the neonatal period. This study demonstrates for the first time that modafinil ameliorates cognitive deficits associated to MS and to PSD in adulthood, independent from MS in the neonatal period.



## 1. Introduction

Stressful events early in life can have lasting effects on living organisms. Adverse early life experiences might be implicated in an increased susceptibility to psychiatric diseases, such as depressive disorders and schizophrenia [1–3]. In addition, obesity, smoking, drug abuse, and increased use of psychotropic medication in adults have also been associated to adverse childhood experiences [4,5].

Neonatal maternal separation (MS), a widely used model of early life stress in rodents is known to cause permanent cytochemical, morphological and electrophysiological changes in the central nervous system (CNS) [6]. A study conducted by Feng and coworkers [7] has shown that adult rats submitted to MS present disturbed sleep with features of insomnia, including decreased total sleep and increased total wake time during the light period. Additionally, some studies indicate that MS during the critical period of brain development may lead to impairments of cytoarchitecture in various brain regions, such as hippocampus and cortex which are known to be involved in learning and memory [8–10].

We and others have already demonstrated that MS causes persistent memory deficits in rodents [11–14]. In addition, our group also showed that exposure to a second adverse event, such as

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chronic exposure to the psychostimulant D-amphetamine, potentiated the effects of MS on cognition in adult rats [14,15]. According to the "two-hit" hypothesis, animals exposed to an early adverse event may be more susceptible to a second source of stress later in life, and these stressors may have synergistic deleterious effects [16].

Evidence indicated that the effects of acute sleep deprivation (SD) include a temporary activation of the major neuroendocrine stress systems, i.e., the autonomic sympathetic system and the hypothalamic-pituitary-adrenal (HPA) axis [17,18]. Strong correlations between paradoxical sleep deprivation (PSD) and the loss of brain functions in animals and humans have been observed [19–22]. Consistently, studies have shown that SD significantly impairs learning and memory [23–26].

Currently, SD is very frequent among young people [27], who make use of stimulants to prolong wakefulness and to enhance cognitive, emotional and motivational functions [28]. Modafinil [(diphenyl-methyl) sulphinil-2-acetamide] is a medication that has been prescribed to improve excessive daytime sleepiness, alertness, attention, and memory in dementia [29,30]. Although its exact mechanism of action is not completely elucidated, modafinil has been shown to enhance dopaminergic and noradrenergic neurotransmission, increase glutamate release in the thalamus, hypothalamus, hippocampus and striatum, and to dose-dependently decrease GABA levels (for a review see [31]). Moreover, modafinil was shown to enhance spatial learning, working memory and attention in healthy non-sleep deprived animals [30,32] and humans [33,34].

Thus, the present study investigated the effects of MS-induced stress combined with PSD, as a second hit in the adulthood, on recognition memory in rats. We also aimed to evaluate whether the administration of modafinil would be able to recover memory deficits caused by MS and PSD.

## 2. Materials and methods

### 2.1. Animals

Pregnant Wistar rats were obtained from Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia (CEDEME) – Universidade Federal de São Paulo (UNIFESP), Brazil. After birth, each litter was adjusted within 48 h to contain 8 rat pups with the same proportion of male and female individuals. Pups were maintained together with their respective mother in individually ventilated cages with corn-cob bedding in a room at temperature of 22 ± 1 °C and a 12-h light/dark cycle. At the age of 4 weeks, the pups were weaned and the males were selected and raised in groups of 3 rats. The animals were supplied with standardized pellet food and tap water *ad libitum*. All behavioral experiments took place between 9:00 h and 16:00 h. Rats used in this study were maintained and treated in accordance with the guidelines established by the Ethical and Practical Principles of the Use of Laboratory Animals [35]. Since these experiments may result in a certain amount of stress-related behavior, the number of animals was kept at a minimum but sufficient to allow for statistical significances.

### 2.2. Maternal separation (MS)

MS was performed based on previous reports [14,15,36]. Rat pups were exposed to one of the following maternal rearing conditions from post-natal days 1–14 inclusive: (1) handling (H), animals were exposed to a daily 15-min period in which the dam was removed and the litter was weighed, (2) maternal separation (MS), animals were exposed to a daily 180-min period in which the dam was removed and the litter was weighed. During the separation period, rat pups of each litter were maintained together in a plastic cage with standard bedding material in an adjacent room to their dams on an incubator at the temperature of 35 °C to avoid hypothermia. After the separation period, pups were returned to the nest and rolled in home cage bedding material, and the dam was returned. In rats, the mother is routinely off the litter for periods of 20–25 min [37]. Thus, only the group exposed to an 180 min period of MS, but not the group exposed to a 15-min period of separation (handling), were exposed to a deprivation of maternal care.

### 2.3. Paradoxical sleep deprivation (PSD) procedure

The experimental groups were submitted to PSD for 24 (PSD-24 h), 48 (PSD-48 h) or 72 (PSD-72 h) h using the modified multiple platform method [38]. We used these periods because it has been reported that PSD impairs the acquisition of a variety of behavioral tasks. Modified multiple platform methods consist in placing the rats inside a tiled water tank (123 cm × 44 cm × 44 cm), containing 14 circular platforms, 6.5 cm in diameter, with water up to 1 cm of their upper surface. The rats could thus move around inside the tank by jumping from one platform to another. When they reached the paradoxical phase of sleep, muscle atonia set in and they fell into the water and woke. Throughout the study, the experimental room was maintained under controlled temperature (23 ± 1 °C) and a light–dark cycle (lights on at 07:00 h and off at 19:00 h). Food and water were provided *ad libitum* for all groups by placing chow pellets and water bottles on a grid located on top of the tank. Rats kept in their home cages were placed in the same room as the platform apparatus and were used as control in all the experiments. PSD method has proven to be effective in suppressing paradoxical sleep. Indeed, we have demonstrated that, in the same experimental conditions described here in rodents, the multiple platform technique resulted in significant decreases in slow wave sleep and completely abolished paradoxical sleep during the SD period.

### 2.4. Acute treatment with modafinil

For the experiments investigating the effects of modafinil on memory deficits induced by MS and PSD in adult animals (4 months), rats were trained and tested in the object recognition task. Vehicle (Tween 80-saline solution 1:16, v/v) or modafinil (Stavigile®, Libbs, USA) at the dose of 75 mg/kg ($n$ = 9–13 per group) was administered intraperitoneally immediately after the training session of the object recognition task. This dose was selected based on pilot experiments performed in our laboratory and previous published studies [30].

### 2.5. Object recognition task

The object recognition task was performed as previously described [14,15]. Briefly, the object recognition task took place in a Plexiglas cylinder (45 cm diameter) arena with corn-cob 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 a 5-min training trial in which they were exposed to two identical objects (A1 and A2). On the long-term memory (LTM) testing trial, performed 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 on the right side and 50% trials on 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 TN/(TF + TN) [TF = time spent exploring the familiar object (A); TN = time spent exploring the novel object (B)].

### 2.6. Experimental design

#### 2.6.1. Effect of PSD on recognition memory in three different time intervals between training and testing

Adult naïve Wistar rats not subjected to MS were trained in the object recognition task. After the training session, animals were submitted to PSD for 24 h (PSD-24 h group), 48 h (PSD-48 h group), or 72 h (PSD-72 group). For control groups, right after training, animals were returned to their home cages, and kept there for 24, 48 or 72 h (CTRL-24 h, CTRL-48 h, and CTRL-72 h groups). Immediately after the end of the PSD period, all animals, including the control groups were submitted to the test session in the object recognition task.

#### 2.6.2. Effects of MS, PSD and acute modafinil on object recognition memory retention

A separate set of animals, submitted to H or MS during the neonatal period, as described above, when adults, were trained in the object recognition task and subsequently distributed into the following groups: animals that received intraperitoneal (i.p.) injections of modafinil (M) 75 mg/kg, or vehicle (V) immediately after the training session. Those animals were further redistributed and either submitted to PSD for 24 h or returned to their home cages (control group, not submitted to paradoxical sleep deprivation, NPSD). After 24 h of PSD or NPSD, the animals were tested in the object recognition task. Experimental design for this experiment is shown in Fig. 1.

### 2.7. Statistical analysis

In the experiment designed to evaluate the effects of PSD on object recognition memory retention, data was analyzed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc tests.

The effects of early MS and subsequent administration of modafinil and PSD were analyzed using 3-way ANOVA. The model includes three fixed factors, each

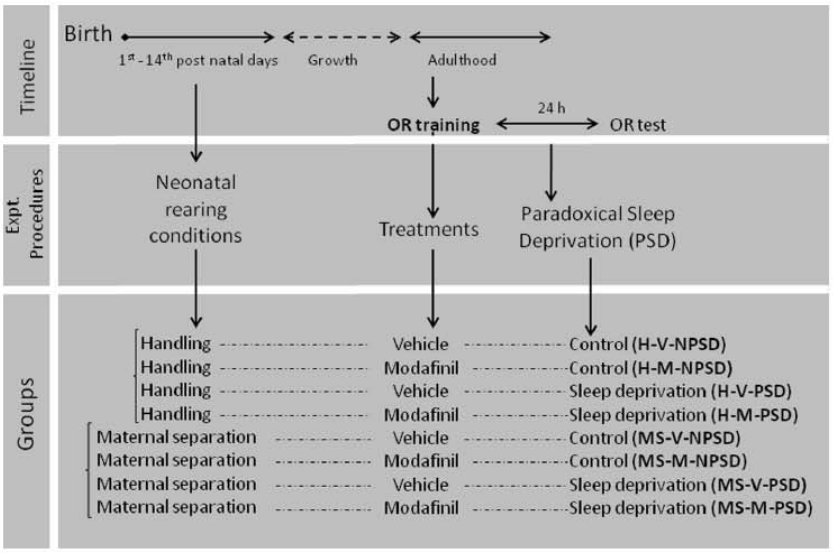


**Fig. 1.** Experimental design of the experiment that evaluated the effects of modafinil on memory deficits induced by MS associated with PSD. Male rat pups were exposed to one of the following rearing conditions from days 1 to 14: handling (H) or maternal separation (MS) from their mother daily for a period of 180 min. At the age of 3 months, both groups were trained in the object recognition task. Immediately after training, animals received i.p. injections of vehicle (V) or 75 mg/kg modafinil (M), and were subsequently submitted to paradoxical sleep deprivation (PSD) or returned to their home cages (not submitted to sleep deprivation, NPSD). Twenty-four hours after the training session they were tested in the object recognition task.

with two levels corresponding to MS (yes vs. no), modafinil administration (yes vs. no) and PSD (yes vs. no). To test the differences for interaction between the factors, ANOVA followed by Tukey's post hoc tests were used. The statistical significance was set at $p < 0.05$. All data are presented as mean ± SEM.

## 3. Results

### 3.1. Effect of PSD on recognition memory in three different time intervals between training and testing

Results for the experiment analyzing the effects of different time periods (24, 48, and 72 h) of PSD on recognition memory are shown in Fig. 2. One way ANOVA comparison of recognition indexes revealed a significant difference among the groups in LTM retention test ($F_{(5,59)} = 24.72$, $p = 0.0001$), but not in the training trial, or in the total time exploring both objects in the training session (data not shown). Post hoc analysis (Tukey's test) revealed that rats submitted to PSD for 24 or 48 h showed LTM impairments when compared to their respective control groups ($p = 0.028$ for PSD-24 h, and $p < 0.0001$ for PSD-48 h). PSD-72 h was not significantly different from its respective control group, CTRL-72 h. Consistent with previous results [39], groups tested 72 h after training session (both CTRL-72 h and PSD-72 h) showed no significant recognition memory retention, showing a statistically significant difference when compared to control groups tested 24 h and 48 h after training (CTRL-24 h and CTRL-48 h, both $p$'s $< 0.0001$), suggesting that object recognition memory does not persist for 72 h (Fig. 2).

### 3.2. Effects of MS, PSD and acute modafinil on object recognition memory retention

Based on the effects of PSD on recognition memory obtained in Experiment I, we chose the protocol of PSD-24 h in order to investigate a possible interaction with MS in the neonatal period. Results for recognition memory of rats that were submitted to MS and PSD and were treated with modafinil are shown in Fig. 3. Three-way ANOVA revealed a significant interaction between the effects of MS, PSD and modafinil administration on exploratory preferences in LTM retention ($F = 6.13$, df = 1, $p = 0.015$), but not in the training trial. Post hoc analysis (Tukey's test) revealed that animals that were separated from their mothers (MS) showed lower recognition indexes in LTM retention session when compared with the corresponding H group ($p = 0.000$), indicating that the MS *per se* impaired recognition memory. Both H and MS groups when subjected to PSD for 24 h and receiving vehicle (H-V-PSD and MS-V-PSD) showed similar LTM impairments (both $p$'s = 0.0001) when compared to the control group (H-V-NPSD). Results indicated that MS and PSD combined

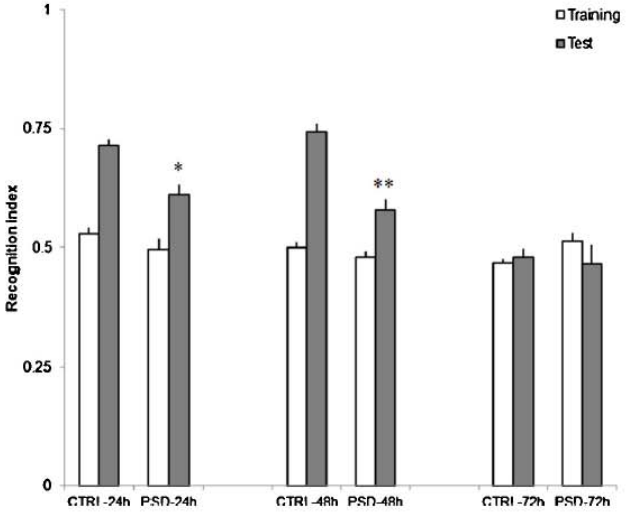


**Fig. 2.** Effects of three different time periods (24 h, 48 h, or 72 h) of PSD (PSD-24 h, PSD-48 h and PSD-72 h) on recognition memory. The proportion of the total exploration time that the animal spent investigating the novel object was the "Recognition Index" expressed by the ratio TN/(TF + TN), TF = time spent exploring the familiar object and TN = time spent exploring the novel object. Data expressed as mean ± SEM, $N = 10$–11 per group. Differences between PSD-24 h, and PSD-48 h and their respective control groups (CTRL-24 h and CTRL-48 h) are indicated as: *$p < 0.05$, **$p < 0.001$ (Tukey's post hoc test).

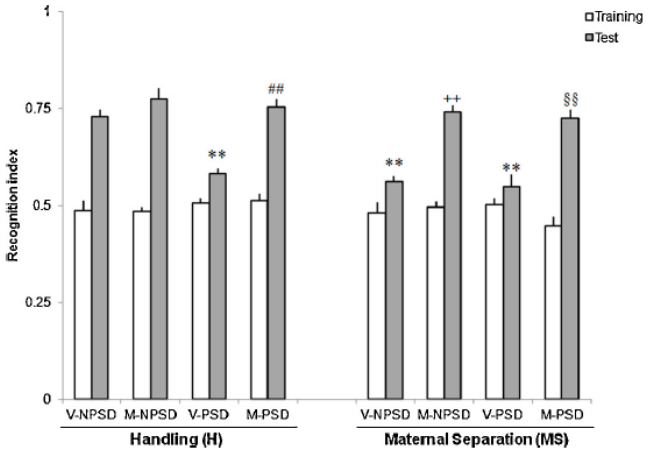


**Fig. 3.** Effects of modafinil (M) on recognition memory after exposure to maternal separation (MS) and PSD-24 h (PSD). Groups of animals submitted or not to maternal separation (MS and H, respectively) in the neonatal period were trained in the object recognition task, when adults. Immediately after the training session, animals were redistributed in groups that received modafinil (M) or vehicle (V) and were subsequently exposed to PSD for 24 h (PSD) or returned to their home cages (NPSD). Recognition memory retention was evaluated 24 h after the training session. 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. Data expressed as mean ± SEM, $N = 9–13$ per group. Differences between H-V-NPSD (control) group and all other groups are indicated as: $^{**}p < 0.001$; between H-V-PSD group from other groups as: $^{\#\#}p < 0.001$; between MS-V-NPSD group from other groups as: $^{++}p < 0.001$, between MS-V-PSD group from other groups as: $^{\S\S}p < 0.001$.

have not produced a synergistic deleterious effect on recognition memory, since recognition indexes of the group submitted to both MS and PSD (MS-V-PSD) were not significantly different from the group submitted to MS only (MS-V-NPSD, $p > 0.05$) or to PSD only (H-V-PSD, $p > 0.05$) (Fig. 3).

Modafinil was able to ameliorate memory deficits associated to MS, since recognition indexes of MS rats that received modafinil (MS-M-NPSD) were higher than those of MS rats that received vehicle (MS-V-NPSD, $p < 0.0001$). Moreover, MS rats that received modafinil did not differ statistically from the control group (H-V-NPSD, $p > 0.05$). Additionally, acute treatment with modafinil also prevented memory deficits induced by PSD, both in animals that were or were not separated from their mothers in the neonatal period, as their recognition indexes were higher than their respective controls (both $p$'s $< 0.0001$). Again, recognition indexes of PSD groups treated with modafinil did not differ from the control group ($p = 1.00$ for MS-M-PSD rats, and $p = 0.99$ for H-M-PSD group). Interestingly, modafinil itself did not affect memory in rats in the H-M-NPSD group, since this group was not statistically different from the control group (H-V-NPSD, $p = 0.82$).

## 4. Discussion

The present findings show that MS in the neonatal period produces significant cognitive impairments when adult animals were tested in the object recognition task, confirming previous reports by our research group [14,15]. Similarly, PSD in adulthood impairs recognition memory. Additionally, modafinil was able to ameliorate the cognitive deficits due to both MS and PSD and their combination, without affecting memory retention of control animals.

Accumulating evidence indicates that sleep plays an important role in neural plasticity [40] and in memory consolidation [41,42]. In the present study we found that 24 h of PSD, beginning immediately after learning, significantly disrupts recognition memory formation. In agreement with our findings, Palchykova and coworkers [43] have shown that 6 h of total SD, when starting immediately, but not after a delay of 6 h after training, disrupted memory consolidation for object recognition in mice. Recently, it was demonstrated that 6 h of total SD also impairs emotional memory consolidation, in a cued fear conditioning memory task in rats [44]. Male and female mice exposed to total SD, 6 h prior testing sessions, presented memory impairments in conditioned fear, in the passive avoidance task and in the plus-maze discriminative avoidance task [24].

We had hypothesized that PSD, acting as a second 'hit', would potentiate the cognitive deficits induced by MS. However, recognition memory impairment observed in the present study was similar in the group submitted to both MS and PSD in comparison to either MS or PSD alone. It is possible, however, that the experimental protocol we used constitutes a caveat for the interpretation of this hypothesis. In our experiment, either MS or PSD alone impaired retention to a level of exploratory preference comparable to training trial levels. Therefore, a "floor effect" might have prevented the observation of a synergistic effect between MS and PSD. Future experiments using reduced levels of severity of the MS or using a more sensitive memory test might unmask a possible "two-hit" interaction of MS and PSD and provide a more complete characterization of possible interactive effects of these two sources of stress.

Previous studies have already shown that modafinil restores memory impairments induced by SD in rodents. For instance, modafinil was able to improve contextual [45] and working memory [46] in mice submitted to total SD for 10 h. Modafinil was also shown to improve acquisition in naïve control rats and to reverse the long-term memory impairment induced by PSD, in the inhibitory avoidance task [47]. In humans, a double-blind placebo-controlled laboratory-based study demonstrated that modafinil attenuated the performance deficits induced by both prolonged wakefulness and circadian misalignment for several parameters including cognitive-psychomotor speed, visual attention and reaction times in healthy volunteers [48]. In another recent study, cognitive performance was assessed in resident doctors submitted to 24 h of supervised SD [49]. Modafinil was shown to improve performance on tests of higher cognitive function, such as solving working memory and planning problems, and decision making, and in the ability to flexibly redirect attention [49].

The present study revealed that modafinil was able to recover memory impairments not only associated to PSD, but also associated to MS in the neonatal period. To our knowledge, this is the first demonstration that modafinil ameliorates cognitive impairments associated both to PSD in the adulthood and to MS-related stress in the neonatal period. Although the mechanisms underlying modafinil effects on attenuating cognitive deficits are not completely understood, a study proposed that modafinil reverses the SD-induced failure in increasing *c-fos* expression following behavioral tasks, not only in areas involved in sleep–wake cycle regulation, but also in memory and emotions [46]. Modafinil treatment upregulated synapsin I expression, and a synaptic plasticity-related gene, MMP-9, expression in the dorsal hippocampal CA3 region, suggesting that modafinil was able to enhance synaptic plasticity [50]. Object recognition memory has been shown to depend on glutamatergic NMDA receptors [51] and to be modulated by adrenergic and dopaminergic systems [39,52]. Thus, it is possible that modafinil, by enhancing glutamatergic and catecholaminergic neural transmission, might be positively modulating memory consolidation processes, which could be responsible for modafinil memory-ameliorating effects. These mechanisms related to synaptic plasticity and modulation of memory consolidation may be effective in reversing MS-induced memory deficits.

In conclusion, the present findings provide evidence that modafinil is able to reverse memory deficits not only related

to sleep deprivation, but also to ameliorate memory deficits induced by neonatal stress, suggesting novel clinical applications for modafinil in treating cognitive deficits observed in neuropsychiatric conditions. Given the relevance of the present findings, subsequent studies investigating the cellular and molecular mechanisms that may underlie the effects of modafinil in neonatally stressed animals are warranted.

## Conflict of interest

The authors declare that they have no conflict of interest to declare.

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# *Capítulo 4*
## *Conclusões*

## 3. CONCLUSÕES

3.1 O modafinil administrado nas três doses (0.75; 7,5 e 75 mg/kg) imediatamente após o treino da tarefa de reconhecimento do objeto não apresentou nenhum efeito sobre a consolidação da memória em ratos adultos saudáveis.

3.2 O modafinil administrado nas três doses (0.75; 7,5 e 75 mg/kg) uma hora antes do teste da tarefa de reconhecimento do objeto não apresentou nenhum efeito sobre a evocação da memória em ratos adultos saudáveis.

3.3 O modafinil administrado nas três doses (0.75; 7,5 e 75 mg/kg) imediatamente após o treino da tarefa de esquiva inibitória não apresentou nenhum efeito sobre a consolidação da memória em ratos saudáveis.

3.4 O modafinil administrado nas três doses (0.75; 7,5 e 75 mg/kg) uma hora antes do teste da tarefa de esquiva inibitória não apresentou nenhum efeito sobre a evocação da memória em ratos adultos saudáveis.

3.5 A administração crônica de modafinil não alterou a atividade locomotora dos ratos tratados com ferro no período neonatal.

3.6 A adiministração aguda de modafinil nas doses de 0,75 e 7,5 mg/kg reverteu parcialmente o déficit cognitivo provocado pela sobrecarga de ferro no período neonatal. Quando administrado na dose de 75 mg/kg reverteu completamente o prejuízo de memória causado pelo tratamento com ferro no período neonatal. O tratamento crônico com modafinil nas três doses (0,75; 7,5 e 75 mg/kg) reverteu completamente o déficit de memória causado pela sobrecarga de ferro no período neonatal.

3.7 O tratamento crônico com modafinil nas doses de 7,5 e 75 mg/kg recuperou o prejuízo de memória aversiva causado pelo tratamento com ferro no período neonatal.

3.8 Os animais que foram submetidos a 24 e 48 horas de privação de sono paradoxal apresentaram um prejuízo de memória de longa duração na tarefa de reconhecimento do objeto.

3.9 A separação materna somada à privação de sono paradoxal, por 24 horas, na idade adulta não acentuou o déficit de memória causado pela separação materna no período neonatal.

3.10 A administração aguda de modafinil na dose de 75 mg/kg imediatamente após o treino da tarefa de reconhecimento de objeto recuperou os prejuízos de memória causados tanto pela separação materna no período neonatal quanto pela privação de sono paradoxal na idade adulta.

# *Capítulo V*
## *Considerações Finais*

## 4. CONSIDERAÇÕES FINAIS

O presente estudo avaliou, primeiramente, as propriedades farmacológicas do modafinil em ratos adultos saudáveis. Este é o primeiro estudo que investigou os efeitos do modafinil na consolidação e evocação da memória na tarefa de reconhecimento de objetos em ratos saudáveis. Esta tarefa não depende de estímulo aversivo e recompensa, e não usa informação espacial. Tarefas de memória de reconhecimento são baseadas na tendência que os roedores possuem em explorar o que é novo (Winters *et al*., 2008). Foi demonstrado que as tarefas de reconhecimento do objeto dependem do hipocampo, bem como, áreas corticais adjacentes ao hipocampo, tais como o córtex perirrinal (Dere *et al.,* 2007). Neste estudo, demonstramos que o modafinil administrado nas três doses propostas não apresentou efeito sobre a memória de reconhecimento, em animais saudáveis. Quando administrado nas três doses de forma aguda imediatamente após o treino, ou antes, do teste não afetou a consolidação e nem a evocação dos dois tipos distintos de memória, ou seja, a memória de reconhecimento e a memória emocional.

Um estudo demonstrou que a administração aguda de modafinil melhorou a memória de reconhecimento em ratos previamente submetidos a um tratamento subcrônico com fenciclidina (PCP), considerado como um modelo animal de prejuízos cognitivos associados com a ezquizofrenia (Redrobe *et al*., 2010).

Este estudo também investigou as propriedades farmacológicas do modafinil em três modelos de declínio cognitivo como a sobrecarga de ferro no período neonatal, a separação materna no período neonatal e a PSP.

Quando se avaliou a administração aguda e crônica do modafinil nos animais com prejuízo de memória causado pela sobrecarga de ferro, foi demonstrado, pela primeira vez, que ambos os tratamentos melhoraram as memórias de reconhecimento e emocional, em um modelo de disfunção cognitiva relevante para o envelhecimento e para doenças neurodegenerativas.

Embora os efeitos do modafinil sobre a memória não estejam completamente compreendidos, demonstrou-se que administração crônica de modafinil aumenta os potenciais pós-sinápticos, que são seletivamente correlacionados com o aumento do ritmo theta no giro denteado (Burgos *et al.* 2010), e regula positivamente a expressão da sinapsina I e de um gene relacionado com a plasticidade sináptica, o MMP-9, na região CA3 do hipocampo dorsal, sugerindo que o modafinil é capaz de aumentar a plasticidade sináptica no hipocampo (He *et al.,* 2011). Um tratamento de 4 dias com modafinil aumentou as subunidades GluR1, GluR2 e NR1 dos receptores de glutamato e diminuiu os níveis de receptores D2 de dopamina no hipocampo de camundongos (Sase *et al*., 2012).

Outra possibilidade, particularmente relacionada com os efeitos crônicos do modafinil em reverter os prejuízos de memória induzidos pela sobrecarga de ferro pode estar relacionada ao modafinil possuir propriedades neuroprotetoras. Estudos sugeriram que o modafinil pode exibir propriedades neuroprotetoras em modelos animais da doença de Parkinson. O modafinil de forma dependente da dose recuperou a incapacidade motora, a redução dos níveis de dopamina no estriado e a perda de neurônios positivos para tirosina hidroxilase na substância negra em saguis tratados com MPTP (1-metil-4-fenil-1,2,3,6-tetraidropiridina) (Jenner *et al,* 2000; van Vliet *et al,* 2006, 2008).

Resultados semelhantes foram previamente encontrados em camundongos tratados com MPTP (Fuxe *et al,* 1992; Aguirre *et al*, 1999) Assim, não podemos descartar a possibilidade de que o modafinil administrado cronicamente possa ter efeitos neuroprotetores que acabariam por impedir a perda de memória em ratos tratados com ferro.

Quando analisamos os efeitos da separação materna no período neonatal em ratos, nossos achados demonstraram que este modelo produziu prejuízos cognitivos na vida adulta desses animais confirmando resultados anteriores do nosso grupo de pesquisa (de Lima *et al,* 2011; Pinheiro *et al,* 2012). No presente estudo, nós submetemos os animais que foram separados da mãe no início da vida a um segundo agente estressor na idade adulta, a PSP de 24h.

Para isso, primeiramente nós analisamos o efeito da PSP sobre a memória de reconhecimento em três diferentes intervalos de tempo, 24h, 48h e 72h, entre o treino e o teste da tarefa. Nosso objetivo foi encontrar um intervalo de tempo ideal da PSP para a tarefa. Verificou-se então, que o intervalo de 24h de PSP, começando imediatamente após o aprendizado prejudicou significantemente a memória de reconhecimento. Baseado neste resultado, nós utilizamos o tempo de 24h de PSP como um segundo agente estressor.

Evidências indicam que o sono desempenha um papel importante na plasticidade neural (Wang *et al,* 2011) e na consolidação da memória (Wilhelm *et al*, 2012; Born & Wilhelm, 2012). Animais privados de sono não apresentam um bom desempenho em tarefas de memória (Alvarenga *et al.,* 2008; Patti *et al.*, 2010; Fernandez *et al.,* 2012). No entanto, quando submetemos os animais, que sofreram separação materna, a 24h de PSP na idade adulta, observamos

que a PSP não potencializou o deficit de memória causado pela separação materna. O índice de reconhecimento obtido na tarefa foi semelhante ao grupo que foi apenas submetido a separação materna ou exposto a 24h de PSP.

Com o objetivo de tentar reverter os déficitis de memória causados por esses dois modelos de prejuízos cognitivos, nós utilizamos o modafinil. Primeiro, por não haver na literatura nenhum trabalho mostrando a relação das propriedades farmacológicas do modafinil com estressores no início da vida. E segundo, porque ele promove o estado de alerta e tem sido usado para aliviar os sintomas da sonolência diurna excessiva resultante das perturbações de sono. O modafinil administrado imediatamente após o treino da tarefa de reconhecimento de objetos foi capaz de reverter os déficitis de memória causados tanto pela separação materna quanto pela PSP.

A memória de reconhecimento de objetos demonstra depender dos receptores glutamatérgicos NMDA (Roesler *et al,* 1999; Lima *et al,* 2005c) e de ser modulada pelos sistemas dopaminérgicos (Izquierdo *et al*., 1998; Lima *et al.*, 2011) e adrenérgicos (McIntyre *et al*, 2002; Dornelles et al, 2007). Sendo assim, é possível que o modafinil consiga aumentar diretamente a transmissão neural glutamatérgica e catecolaminérgica, podendo modular processos de consolidação da memória, levando à uma melhora dessa memória em modelos de disfunção cognitiva.

Estes mecanismos relacionados à plasticidade sináptica e modulação de consolidação da memória pode ser eficaz em reverter os déficitis de memória induzidos pela separação materna, sugerindo novas aplicações clínicas para o modafinil em tratar déficitis cognitivos observados em condições neuropsiquiátricas.

Foi demonstrado, recentemente, que a administração aguda de modafinil nas doses de 64 e 128 mg/kg prejudicou a consolidação da memória emocional em camundongos. A dose mais baixa, quando administrada antes do teste na mesma tarefa comportamental, dificultou a evocação da memória (Fernandes *et al.,* 2013). Em humanos, uma única dose de modafinil (300mg) melhorou o desempenho de voluntários saudáveis submetidos a um teste de memória de trabalho. Em contrapartida, não conseguiu produzir uma melhora em outros testes cognitivos (Winder-Rhodes *et al.,* 2010). Esses achados contraditórios encontrados tanto em animais quanto em humanos podem estar relacionados com o paradigma da tarefa cognitiva utilizada.

Prejuízos cognitivos, como os observados nas doenças neurodegenerativas vem crescendo em paralelo com a longevidade nos últimos tempos. Os poucos estudos já realizados demonstraram que a utilização do modafinil ocasiona melhoras cognitivas em ratos normais e possui efeitos neuroprotetores em modelos animais de neurodegeneração. No entanto, novas investigações são necessárias para confirmar estes achados iniciais, para identificar a especificidade destes efeitos no domínio da neuroquímica, neurodegeneração e cognição, e para avaliar outros fatores relevantes de uso clínico, como a relação de doses simples a regime de doses prolongadas, e a relação dos efeitos pró-cognitivos e neuroprotetores no desfecho clinico.

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