KARINA AGUSTINI ZANIN

EFEITOS DA ADMINISTRAÇÃO REPETIDA DE ZOLPIDEM SOBRE AS FASES DA MEMÓRIA EM CAMUNDONGOS SUBMETIDOS À RESTRIÇÃO DE SONO

Tese Apresentada à Universidade Federal de São Paulo – Escola Paulista de Medicina para obtenção do Título de Doutor em Ciências

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UNIVERSIDADE FEDERAL DE SÃO PAULO

ESCOLA PAULISTA DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM PSICOBIOLOGIA

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(Clarice Lispector)

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Resumo & Abstract

RESUMO

Objetivos: O zolpidem (Zolp) é um fármaco seletivo para a subunidade α1 dos receptores $GABA_A$ com propriedades hipnóticas. Há relatos que sua administração pode induzir efeitos amnésticos. Até o presente momento nenhum estudo avaliou os efeitos cognitivos do Zolp em modelos experimentais de insônia. O objetivo desta Tese foi verificar os efeitos da administração repetida de Zolp (ou de sua retirada) sobre o processo de aprendizado/memória em camundongos submetidos à restrição de sono (RS). **Métodos:** Os camundongos foram submetidos à RS pelo método de *gentle handling*. Após a RS, os animais foram tratados com Zolp. Os paradigmas comportamentais utilizados foram a esquiva discriminativa em labirinto em cruz elevado (ED-LCE), a esquiva passiva (EP), reconhecimento de objetos (RO) e discriminação social (DS). O padrão de sono também foi analisado por meio do registro eletroencefalográfico. **Resultados:** A privação aguda de sono por 6 h promove amnésia, que é tolerada após a repetição do protocolo por 10 dias. Por outro lado, enquanto um curto período de privação de sono (3 h) não modificou a memória quando da sua ocorrência aguda, a repetição por 10 dias foi efetiva em prejudicar o desempenho na tarefa de ED-LCE. Ainda, todos os protocolos de privação de sono aumentaram a expressão de c-fos na amígdala basolateral e no giro denteado, estrutura que parece estar relacionada aos efeitos cognitivos da RS. Na tarefa de ED-LCE, a RS (3 h por 10 dias) promoveu déficits de memória quando realizada anteriormente ao treino ou ao teste. O prejuízo pré-treino foi revertido pela administração aguda de Zolp ou pela retirada do tratamento. O prejuízo de evocação foi revertido apenas após

o tratamento repetido com a droga. Ainda na ED-LCE, o antagonista β-cct reverteu o efeito agudo promnéstico do Zolp em animais submetidos à RS, mas não foi capaz de mimetizar os efeitos da retirada do tratamento. Na EP, uma tarefa de grande conteúdo emocional, a RS não foi capaz de promover déficits de retenção independente do momento em que ocorre. Já em paradigmas não-aversivos (RO e DS), a RS promoveu déficits de memória em ambos os modelos. O déficit de memória na tarefa de RO foi abolido pela administração de Zolp. Por outro lado, apenas a administração aguda dessa droga reverteu o prejuízo de memória induzido pela RS observado na tarefa de DS. Com relação ao padrão de sono, o protocolo de RS se mostrou efetivo em aumentar a vigília e, consequentemente, em reduzir significativamente o sono de ondas lentas (SOL), o sono paradoxal e o tempo total de sono. A administração de Zolp aumentou o SOL em ambas as fases do ciclo, mas diminuiu o sono paradoxal. Nos animais restritos de sono e tratados com Zolp, houve um aumento de SOL e de sono paradoxal e se manteve elevado no rebote. **Conclusão:** Os achados da presente Tese demonstram a importância da validação de modelos experimentais de insônia bem como a avaliação dos efeitos do tratamento com Zolp em condições de RS, uma vez que os efeitos dessa droga podem ser discrepantes dependendo das condições de sono e do método de avaliação empregado.

Palavras-chaves: zolpidem; memória; restrição de sono; registro de sono; camundongos.

ABSTRACT

Purpose: Zolpidem (Zolp) is an imidazopyridine agent, which selectively binds to the α_1 subunit into the $GABA_A$ receptors with mainly hypnotic properties. Zolp-induced amnestic effects have been reported. Cognitive effects of Zolp in animal models of insomnia remain poorly understood. Thus, the present work was tailored to examine the effects of the repeated administration of Zolp (or its withdrawal) on learning/memory phases in mice subjected to sleep restriction (SR). **Methods:** Mice were sleep restricted by gentle handling. After SR, animals were treated with Zolp. The behavioral paradigms employed were the plus-maze discriminative avoidance task (PM-DAT), the passive avoidance task (PAT), spontaneous object recognition (SOR) and social discrimination (SD). Sleep pattern was analyzed by electroencephalographic recording. **Results:** We observed that the deleterious effects of acute SR for 6 h may be tolerated when the protocol was repeated for 10 days. Oppositely, a shorter period of sleep deprivation (3 h) impaired memory in the PM-DAT when repeated during 10 days, but not when acutely employed. All of the SR protocols induced an increase in c-fos expression in the basolateral amygdala and dentate gyrus. Of note, c-fos expression in the dentate gyrus seems to be related to the cognitive effects of SR. In the PM-DAT, SR (3 h for 10 days) induced memory deficits when it occurred before training or testing. The pre-training impairment was abolished by the acute administration of Zolp or its withdrawal. Conversely, the retrieval deficit was abolished only after the repeated treatment with Zolp. The deleterious effects on memory were partially counteracted by the administration of the antagonist β-cct. In the PAT, a paradigm with a higher emotional

component, SR *per se* cannot promote retention deficits. In non-aversive paradigms (SOR and SD), SR promoted memory impairments in both tasks. The memory deficit in the SOR was abolished by Zolp administration. On the other hand, only the acute administration of this hypnotic counteracted the SR-induced memory impairment in the SD task. Regarding the sleep pattern, the SR protocol was effective in increasing wake time and consequently reducing slow-wave sleep (SWS), paradoxical sleep and total sleep time (TST). The acute administration of Zolp increased SWS in both cycle phases but decreased paradoxical sleep in control animals. An increase in SWS and paradoxical sleep were detected in sleep-restricted mice treated with Zolp, which persisted during rebound. **Conclusion:** The findings presented herein strengthen the importance to validate animal models of insomnia as well as the investigation of the effects of Zolp treatment under SR conditions since the effects of this drug may be dependable of the sleep conditions and the evaluation methods.

Keywords: zolpidem; memory; sleep restriction; sleep recording; mice.

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Introdução

1. INTRODUÇÃO

1.1 Sono

O sono é um processo fisiológico dividido em dois estados fundamentais, o sono NREM (do inglês, *non-rapid eye movements*) e o sono paradoxal ou REM (do inglês, *rapid eye movements*). É uma atividade que ocupa cerca de um terço de nossas vidas e é fundamental para uma boa saúde mental e emocional, além de ser essencial na manutenção de uma vida saudável (Everson et al., 1989; Rechtschaffen, 1998).

Fisiologicamente, é considerado como um estado funcional, cíclico e reversível que está presente em todas as faixas etárias e na maioria das espécies animais. Possui algumas características comportamentais, como uma imobilidade relativa e o aumento do limiar de resposta aos estímulos externos (Hoshino, 2008). O organismo tem grande necessidade de manifestar o sono e quando isso não se torna possível podem ocorrer alterações marcantes, incluindo a morte em caso de um período prolongado de privação de sono em animais (Rechtschaffen et al., 1998).

A compreensão moderna do sono originou-se dos primeiros registros da atividade elétrica cerebral em seres humanos. Esses registros, denominados de eletroencefalograma (EEG), permitiram a identificação de diferentes padrões durante o sono e ainda proporcionaram uma riqueza de informações sobre os potenciais corticais relacionados com mudanças fisiológicas distintas em cada fase do sono. Muitas alterações eletroencefalográficas ocorrem no decorrer de uma noite de sono, indicando uma sucessão bem ordenada e

cíclica de frequências de ondas nas fases de sono. Na primeira fase, ocorre o sono NREM, caracterizado pelo alentecimento progressivo da atividade cortical com três estágios: estágio S1 (sonolência); estágio S2 (sono “leve”) e estágio S3 (sono de ondas lentas propriamente dito). Esses estágios usualmente ocorrem em sequência, frequentemente com flutuações ao longo da noite. A seguir, ocorre o sono REM que tem como principais características o movimento rápido dos olhos e a atonia muscular, além de ser o período em que ocorrem os sonhos (Aserinsky e Kleitman, 2003; Dement e Kleitman, 1957). O sono REM é também denominado sono paradoxal, uma vez que o padrão eletroencefalográfico é semelhante ao da vigília, apesar de ser acompanhado pela atonia muscular que sugeriria um sono profundo (Jouvet et al., 1964). Em humanos, um ciclo de sono completo consiste de uma sequência de sono NREM e REM, e cada ciclo tem duração em média de 90 a 110 minutos. Em geral, são observados de quatro a seis ciclos durante uma noite de sono. Os episódios de sono REM aumentam do primeiro ao último ciclo de sono e podem durar até uma hora no fim da noite (Zisapel, 2007).

Na década de 70, foi realizada uma detalhada análise das várias fases do sono do rato. Esse estudo revelou a existência de semelhanças com o sono humano maiores do que se acreditava até então. Os episódios de sono em roedores frequentemente se concentram durante o período claro, enquanto que a vigília predomina no período escuro. Os roedores, em média, dormem cerca de 62% do período claro e 33% do período escuro, sendo que, durante a vigília, os animais realizam suas atividades vitais e sociais (alimentação, procriação, autolimpeza, interação social e exploração do ambiente). Somando-se os períodos de sono, verifica-se que os roedores dormem cerca de 50% das

24 horas, dividindo-se em sono de ondas lentas e sono paradoxal (Timo-Iaria et al., 1970).

1.2 Restrição de sono e insônia

Na sociedade moderna, as pessoas dormem menos do que o recomendado seja por estilo de vida, razões profissionais ou, ainda, em consequência de distúrbios do sono. De forma marcante, diversos estudos demonstram que o sono é essencial para garantir a sobrevivência. Consequentemente, a perda de sono promove alterações fisiológicas, como diminuição da tolerância à glicose (Spiegel et al., 1999), aumento da pressão arterial (Tochikubo et al., 1996), ativação do sistema nervoso simpático (Kato et al., 2000), diminuição nos níveis de leptina – hormônio da saciedade – (Spiegel et al., 2004) e aumento de marcadores inflamatórios (Meier-Ewert et al., 2004).

Do ponto de vista epidemiológico, um levantamento demonstrou que o número de americanos que relatavam dormir menos de 6 h durante a semana aumentou de 12% em 1998 para 15% em 2002 e, em 2009, alcançou 20%. Por outro lado, aqueles que relatavam dormir mais de 8 h por noite diminuiu de 35% em 1998 para 30% em 2002, chegando a 28% em 2009 (Tabela 1). Esses dados refletem a mudança no padrão de sono da sociedade moderna, revelando que os indivíduos estão cada vez mais submetidos a uma restrição crônica de sono.

A perda parcial de sono pode acontecer em decorrência da fragmentação do sono, da privação de estágios específicos do sono ou da restrição de sono. Assim, a fragmentação do sono é frequentemente observada

em indivíduos com distúrbios do sono como, por exemplo, a apneia obstrutiva do sono, nos quais a progressão normal dos estágios do sono é interrompida. A privação de estágios específicos parece ser menos comum e pode ocorrer quando a fragmentação do sono acontece exclusivamente durante um estágio específico (por exemplo, episódios de apneia principalmente durante o sono REM ou medicamentos que suprimem uma fase específica do sono). A restrição de sono, também chamada de débito de sono, é caracterizada por uma redução na duração do sono. Tal diminuição pode ocorrer em consequência a distúrbios do sono ou outras condições médicas, trabalhos em turno, responsabilidades sociais e domésticas e estilo de vida, sendo a mais comum na sociedade atual (Banks e Dinges, 2007).

Com relação à perda parcial de sono em decorrência de distúrbios do sono, um dos distúrbios mais frequentes na sociedade atual é a insônia. Esse distúrbio é caracterizado por dificuldade em adormecer, em manter o sono ou despertar precoce, associados à insatisfação com o padrão de sono ocorrendo no mínimo 3 noites por semana por pelo menos 3 meses, diante de oportunidade adequada para o sono (American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders, 2013). Este distúrbio resulta em prejuízo das funções diárias, tornando-se assim um importante objeto de saúde pública com graves implicações socioeconômicas (Billiard e Bentley, 2004; Revel et al., 2009; Roehrs e Roth, 2004; Roth e Drake, 2004).

Estudos demonstram que a insônia é o distúrbio do sono mais prevalente, afetando 10 a 25% da população adulta em diferentes países (Leger et al., 2008; Morin et al., 2006; Ohayon, 2002; Sivertsen et al., 2009; Soldatos et al., 2005). Ainda, episódios de insônia ocasional são relatados por

cerca de metade dos americanos adultos, enquanto que a insônia crônica afeta 10 a 14% dessa população nos Estados Unidos (Leger et al., 2008; Roehrs e Roth, 2004). No Brasil, um levantamento nacional baseado em questionário mostrou que 35% dos entrevistados reclamavam de algum sintoma de insônia com frequência acima de 3 vezes por semana (Bittencourt et al., 2009). Especificamente no estado de São Paulo, a prevalência de insônia objetiva, ou seja, demonstrada por avaliação polissonográfica, é de 32% (Castro et al., 2013). Nesse contexto, tem-se tornado importante a validação de modelos pré-clínicos para avaliação de potenciais tratamentos farmacológicos da insônia.

Tabela 1 – Relato do número de horas de sono nas últimas 2 semanas na população americana entre 1998 e 2009

	Dias úteis					Finais de semana				
	1998	2001	2002	2005	2009	1998	2001	2002	2005	2009
Menos de 6 h	12%	13%	15%	16%	20%	8%	7%	10%	10%	14%
Entre 6 e 7 h	23%	18%	24%	24%	23%	14%	10%	12%	15%	16%
Entre 7 e 8 h	28%	31%	29%	31%	27%	23%	21%	22%	24%	24%
Mais de 8 h	35%	38%	30%	26%	28%	53%	61%	52%	49%	44%
Média das horas de sono	-	7,0	6,9	6,8	6,7	-	7,8	7,5	7,4	7,1

Adaptado de National Sleep Foundation, 2009

Conforme revisado por Revel e colaboradores (2009), idealmente um modelo animal de insônia deve mimetizar as principais características da insônia em humanos, ou seja, deve exibir qualidade ou quantidade de sono diminuída no período em que o animal estaria fisiologicamente dormindo.

Nessa revisão, os autores avaliaram criticamente os potenciais modelos animais de insônia, tais como aqueles relacionados ao estresse, os farmacológicos, as perturbações do ritmo circadiano e modelos baseados em doenças, bem como situações experimentais capazes de interromper o sono (modelos relacionados à restrição de sono).

Entre os modelos relacionados à privação de sono, destaca-se a privação total de sono pelo método de *gentle handling* (Tobler et al., 1990). De fato, sugere-se que a privação total de sono causaria prejuízos maiores do que aqueles causados pela privação de um estágio específico do sono, como é o caso da privação de sono paradoxal. Em concordância, a privação de sono total por meio do método de *gentle handling* mostrou-se mais efetiva em promover amnésia do que a privação de sono paradoxal (Patti et al., 2010). Dessa maneira, a perturbação não seletiva de sono por algumas horas/dia durante períodos prolongados poderia ser um possível modelo de restrição de sono e, assim, de insônia experimental.

De importância para a presente Tese, no que diz respeito à cognição, o sono tem um papel crucial no aprendizado e formação de memórias. De fato, diversos estudos têm demonstrado que tanto a privação de sono paradoxal como a privação de sono total promovem prejuízos de memória em diversos modelos animais, como as tarefas de esquiva (Bueno et al., 1994), o labirinto aquático de Morris (Youngblood et al., 1997, 1999), o labirinto radial (Smith et al., 1998), reconhecimento de objetos (Palchykova et al., 2006) e a esquiva discriminativa em labirinto em cruz elevado (Alvarenga et al., 2008; Fernandes-Santos et al., 2012; Patti et al., 2010; Silva et al., 2004). Em protocolos de restrição de sono, os indivíduos são submetidos a curtos períodos de perda

total de sono (Banks e Dinges, 2007). Nesse aspecto, apesar de alguns modelos de restrição de sono em animais terem sido desenvolvidos recentemente (Everson e Szabo, 2009; Kim et al., 2007, 2012; Leemburg et al., 2010; McCoy et al., 2013; Yang et al., 2012; Zielinski et al., 2013), o emprego desse método ainda é menos frequente quando comparado ao emprego da privação de sono paradoxal ou total em trabalhos avaliando os possíveis efeitos neurocomportamentais da privação de sono. O pequeno número de estudos utilizando modelos animais de restrição de sono somado aos dados epidemiológicos – que refletem a mudança no padrão de sono da sociedade moderna – demonstram a importância do desenvolvimento de novos métodos de restrição de sono, um modelo com características translacionais.

Com relação a estudos em humanos, foi demonstrado que a restrição de sono prejudica o alerta, medido pelo teste de vigilância psicomotora, sendo que quanto menor o tempo permitido de sono maior a magnitude de redução no alerta. Além disso, os efeitos neurocomportamentais da restrição de sono parecem semelhantes àqueles da privação de sono total (Belenky et al., 2003; Van Dongen et al., 2003). No que diz respeito a modelos animais, a restrição de sono por meio do método da locomoção forçada promove déficits de memória espacial (McCoy et al., 2013; Zielinski et al., 2013). Contudo, os modelos de privação de sono por locomoção forçada induzem um estresse excessivo nos animais, o qual poderia influenciar os resultados comportamentais. De nosso conhecimento, apenas o estudo conduzido por Yang e colaboradores (2012) avaliou os efeitos da restrição de sono sobre a memória utilizando o *gentle handling*, um modelo de privação menos estressante (Palchykova et al., 2006). Nesse estudo, o protocolo de restrição

de sono impediu a consolidação de uma memória espacial em ratos adolescentes, mas não em adultos.

Os possíveis efeitos do sono sobre a memória de longo-prazo foram primeiramente considerados com base na hipótese restauradora do sono. Essa hipótese propõe que a síntese proteica é facilitada durante o sono (Idzikowski, 1984), o que poderia resultar numa potencialização dos mecanismos celulares que levam à consolidação da memória. Em animais, há indicações de que, após a aquisição de uma tarefa, ocorrem pequenas e vulneráveis "janelas de sono paradoxal", caracterizadas por um aumento na duração normal desse tipo de sono, proporcionais à quantidade de informações apresentadas durante a aquisição. Durante essas "janelas" ocorreria uma plasticidade neuronal acelerada, que seria sensível à interrupção do sono paradoxal (Smith, 1996). Contudo, embora a grande maioria dos estudos procure esclarecer o papel do sono paradoxal na formação da memória, um importante papel do sono de ondas lentas também tem sido relatado (Power, 2004; Gais e Born, 2004). Além disso, estudos sugerem que as fases de ondas lentas e paradoxal seriam complementares para os processos de memória que ocorreriam durante o sono (Diekelmann e Born, 2010; Ficca e Salzarulo, 2004).

A privação de sono pode promover alterações significativas em diversos sistemas de neurotransmissão central, dentre eles o dopaminérgico, o qual tem uma participação importante nos processos relacionados à formação da memória. Assim, a privação de sono parece estar relacionada com alterações na plasticidade da transmissão dopaminérgica. De fato, um aumento de diversos comportamentos relacionados à ação da dopamina, induzidos pela administração de agonistas diretos ou indiretos, tem sido observado após a

privação de sono (Arriaga et al., 1988; Ferguson e Dement, 1969; Frussa-Filho et al., 2004; Troncone et al., 1988; Tufik et al., 1978; Tufik, 1981a,b).

1.3 Zolpidem

Até a década de 1980, todos os hipnóticos não barbitúricos apresentavam estruturas químicas e farmacológicas semelhantes: os clássicos benzodiazepínicos (BZDs). Apesar da considerável eficácia, os BZDs causam efeitos adversos importantes como sedação persistente, ataxia, amnésia e tolerância, além de um considerável potencial de induzir dependência e abuso (Akhondzaded et al., 2002; Allison e Pratt, 2003). Nesse cenário, na tentativa de evitar tais efeitos, novos ligantes foram desenvolvidos. Assim, em 1988 foi lançado na França o zolpidem (Markowitz e Brewerton, 1996). Com a sua liberação pelo *US Food and Drug Administration* em 1992, o zolpidem se tornou o hipnótico mais vendido nos Estados Unidos e no continente europeu (Besnard et al., 1996).

O zolpidem é um derivado imidazopiridínico (Figura 1) e, assim como os BDZs, aumenta o efeito inibitório do ácido gama-aminobutírico (GABA) ao se ligar aos receptores $GABA_A$. Esses receptores são canais de íons cloreto com estrutura pentamérica formada pela combinação de pelo menos 19 subunidades diferentes pertencentes a 7 famílias principais: α, β, γ, δ, ε, π e θ (Fitzgerald et al., 2014; Tan et al., 2011). A função específica de cada subunidade do receptor $GABA_A$ tem sido estuda por métodos farmacológicos e comportamentais com a utilização de animais transgênicos, nos quais diferentes subunidades são excluídas ou modificadas (Tabela 2). Em sua

maioria, os receptores $GABA_A$ são compostos pelas subunidades 2α, 2β e 1γ (Nutt, 2006). Especificamente, os receptores que contêm as subunidades α1, α2, α3 ou α5 em combinação com quaisquer subunidades β e a subunidade γ2 são sensíveis aos BZDs clássicos como o diazepam (Hadingham et al. 1996; Wafford et al. 1996; Wisden et al. 1991). Por outro lado, o zolpidem apresenta maior afinidade pelos receptores $GABA_A$ que contem a subunidade α1. Já a afinidade pelas subunidades α2 e α3 é 10 a 20 vezes menor, enquanto que para a subunidade α5 é ainda menor (Fitzgerald et al., 2014). Essa seletividade confere ao zolpidem propriedades predominantemente hipnóticas e ausência relativa de efeitos anticonvulsivante e relaxante muscular que são associados aos BDZs (Depoortere et al., 1986; Lloyd e Zivkovic, 1988; Salvà e Costa, 1995; Zivkovic et al., 1988).

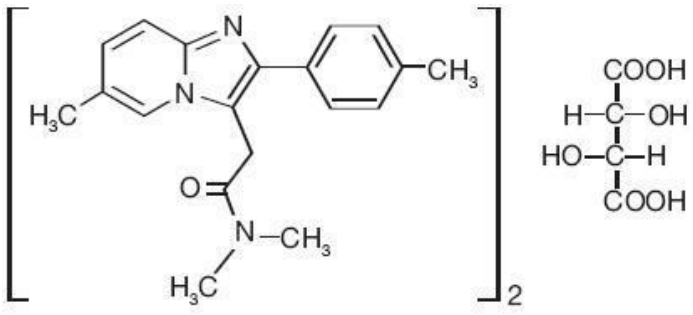

Figura 1 – Estrutura molecular do zolpidem.

Sob o aspecto farmacocinético, após a administração oral, o zolpidem é absorvido de maneira rápida, tendo uma biodisponibilidade de 70% (Langtry e Benfield, 1990). Sua meia-vida em humanos é de 1,5 a 2,5 horas (American Sleep Disorders Association, 1990), não possuindo nenhum metabólito ativo (Langtry e Benfield, 1990), características que evitam efeitos residuais pela manhã (American Sleep Disorders Association, 1990). A dose comumente

empregada na clínica é de 10 mg na hora de dormir. Essa dose parece promover problemas de memória, confusão, e sintomas psicóticos em 2% dos pacientes (American Sleep Disorders Association, 1990; Langtry e Benfield, 1990; Salvà e Costa, 1995). Com relação à arquitetura do sono, o zolpidem diminui a latência de sono e aumenta o tempo total de sono (American Sleep Disorders Association, 1990), podendo aumentar o sono de ondas lentas, geralmente reduzido em pacientes com insônia (Besset et al., 1995), sem, entretanto, alterar o sono REM (do inglês, *rapid eye movements*) ou estágio S2 do sono não-REM (Colle et al., 1991; Poyares et al., 2005).

Tabela 2 – Participação das diferentes subunidades do receptor $GABA_A$ nos efeitos comportamentais

	α1	α2	α3	α5	β2	β3	γ2	δ
Efeito dos benzodiazepínicos								
Sedação	+	-	-	-	+			
Ansiólise	-	+	-/+	-				
Amnésia	+			+				
Miorrelaxante	-		+					
Prejuízo motor	-	-	-					
Anticonvulsivante	+	-	-	-				
Potencialização do etanol				+				
Efeito anestésico	+				+	+		+
Ansiedade							+	
Aprendizado e memória				+				+

Adaptado de Nutt, 2006

Tendo em vista a semelhança entre o zolpidem e os clássicos BZDs, poder-se-ia argumentar que assim como ocorre com BZDs, o zolpidem poderia ter potencial de induzir abuso e dependência. Entretanto, devido a sua seletividade, sugeriu-se que esse risco seria diminuído (Evans et al., 1990; Perrault et al., 1990). Por outro lado, estudos e relatos recentes têm demonstrado que, opostamente ao esperado, o zolpidem apresenta potencial para abuso e dependência. Nesse sentido, Hajak e colaboradores (2003) realizaram um levantamento bibliográfico dos relatos de caso sobre abuso e dependência ao zolpidem entre 1966 e 2002. Nesse estudo, esses autores verificaram o total de 36 casos de dependência, com incidência semelhante entre homens e mulheres e com uma média de ingestão de 400 mg/dia. Entretanto, apesar desses relatos, os autores concluem que o zolpidem é uma droga relativamente segura, embora pacientes com histórico de dependência ou doenças psiquiátricas representem um grupo de risco. Em consequência, a prescrição de zolpidem deve ser cautelosa. Com efeito, Svitek e colaboradores (2008) relataram um caso de dependência em um homem jovem e insone sem histórico de abuso a outras substâncias, o qual consumia até 800 mg/dia de zolpidem e que desenvolveu síndrome de abstinência quando da retirada da droga. Com relação à tolerância, alguns estudos relatam o desenvolvimento de tolerância aos efeitos de ataxia e de indução do sono (Griffiths et al., 1992; Stoops e Rush, 2003; Ware et al., 1997).

Do ponto de vista neuroquímico, apesar de os BZDs e de o zolpidem atuarem no sistema GABAérgico, conhecidamente depressor do sistema nervoso central, sugere-se que haja um aumento compensatório nas sinapses excitatórias glutamatérgicas após a estimulação crônica de sinapses

GABAérgicas por meio do uso de BZDs (Stephens, 1995). Assim, o aumento da estimulação glutamatérgica poderia contribuir para tolerância e síndrome de abstinência relacionadas aos BZDs (Heikkinen et al., 2009). Nesse contexto, Heikkinen e colaboradores (2009) observaram que uma única administração de 5 mg/Kg de zolpidem é capaz de induzir um aumento na transmissão glutamatérgica dos neurônios dopaminérgicos da área tegmentar ventral (VTA), região de grande importância nos processos de reforço e recompensa associados às drogas de abuso (Di Chiara e Imperato, 1988; Schultz, 1998; Wise, 1996). Ainda, foi demonstrado que drogas moduladoras do sistema GABAérgico como os BZDs, podem inibir os interneurônios da VTA e da substância negra (Grace et al, 1980; Tan et al, 2010). Em consequência, os neurônios GABAérgicos deixam de inibir as células dopaminérgicas, aumentando a liberação de dopamina. Especificamente, foi demonstrando que esses interneurônios inibitórios expressam principalmente a subunidade α1, sítio de ação do zolpidem (Gao et al., 1993; Gao e Fritschy, 1994). Nada se sabe até o momento, entretanto, sobre o possível desenvolvimento de tolerância aos efeitos do zolpidem sobre o desempenho cognitivo.

1.4 Memória

A memória é geralmente definida como a capacidade de reter e manipular informações adquiridas anteriormente por meio da plasticidade neuronal. Ela pode ser classificada como implícita ou explícita, dependendo de como a informação é retida e evocada; ou memória de curto e longo prazo, dependendo de sua duração. A memória implícita ou não-declarativa inclui o

condicionamento clássico, habilidades e hábitos, e é em grande parte ou, com frequência, completamente inconsciente. A memória explícita ou declarativa, por outro lado, envolve a recuperação consciente de eventos ou fatos que tenham ocorrido. Esses tipos de memória são processados de formas diferentes e em partes diferentes do cérebro, embora estejam relacionados por meio de inúmeras conexões neurais (Thompson et al., 2002).

Assim, o hipocampo é considerado o responsável pela conversão de memória de curto prazo em memória de longo prazo e pela informação espacial, enquanto o neocórtex, pelo armazenamento da memória de longo-prazo declarativa (Jerusalinsky et al., 1997). Já o corpo estriado está envolvido com a memória de procedimento (Mishkin et al., 1984) e a amígdala parece ser especializada no processo de alerta e informação aversiva e participaria da memória para eventos com significado emocional (Cahill et al., 1990; Davis, 1992; Sarter et al., 1985). O septo medial também participa do processo de informação aversiva, por um mecanismo distinto daquele da amígdala (Izquierdo, 1991) e o córtex entorrinal, que apresenta conexões mono e polissinápticas com a amígdala, hipocampo e septo medial, processa informação espacial, aversiva, além de outras, estando particularmente envolvido com a memória associativa (Ferreira et al., 1992; Witter et al., 1989; Zola-Morgan et al., 1989).

Investigações farmacológicas também fornecem base para o possível papel exercido pela acetilcolina no processo de formação da memória, uma vez que um efeito amnéstico geralmente é verificado após a administração de antagonistas colinérgicos como a escopolamina (Givens e Olton, 1990; Glick e Zimmerberg, 1971; Weissman, 1967; Wiener e Messer, 1973). Além disso, o

tratamento com inibidores de colinesterase como a fisostigmina (Deutsch et al., 1979; Straton e Petrinovich, 1963) é sabiamente estimulador da memória, assim como o tratamento com agonistas colinérgicos como a nicotina (Battig, 1970; Erickson, 1971; Evangelista et al., 1970; Garg, 1969) ou a oxotremonina (Baratti et al., 1979). Apesar de a relação entre a acetilcolina e a função cognitiva, outros neurotransmissores como a serotonina (Steckler e Sahgal, 1995) e as catecolaminas (Dismukes e Rake, 1972; Randt et al., 1971) parecem também participar do processo de formação da memória.

Dentre os mecanismos celulares e moleculares envolvidos na memória e aprendizado, destacam-se a sensibilização e a potenciação de longa duração (LTP – *Long Term Potentiation*). A sensibilização consiste num processo de facilitação pré-sináptica da transmissão neuronal que ocorre nas memórias de curto e longo prazo. É um processo heterossináptico, no qual o aumento da força sináptica é induzido por interneurônios moduladores ativados pela estimulação. Estes liberam serotonina e outros neurotransmissores, que se acoplam a receptores transmembrânicos específicos ligados à proteína $G_{\alpha s}$, ativando a adenilil ciclase, que catalisa a conversão de ATP (adenosina trifosfato) em AMP_c (adenosina 3',5'-monofosfato cíclico), o qual ativa a proteína cinase A (PKA), causando, aliada à proteína cinase C (PKC), o aumento da liberação de neurotransmissores nos terminais sinápticos. A LTP é uma facilitação que consiste num aumento da amplitude dos potenciais pós-sinápticos excitatórios devido a um “trem” de estímulos em alta frequência. Ela possui uma fase precoce transitória (duração de aproximadamente 1 a 3 horas, não requer síntese de novas proteínas e é induzida por apenas uma série de estimulações) e uma fase tardia de consolidação (ocorre devido a 4 ou mais

séries de estimulações; com duração de no mínimo 24 horas e síntese de novas proteínas) (Kandel, 2003).

1.5 Zolpidem e memória

Conforme previamente comentado, o perfil farmacológico do zolpidem, embora qualitativamente diferente daquele apresentado pelos BZDs clássicos, possui intersecções farmacológicas com essas drogas (Evans et al., 1990). Nesse sentido, os efeitos amnésticos dos BZDs são amplamente conhecidos tanto em humanos (Lister, 1985) quanto em animais (Stackman e Walsh, 1992; 1995a,b). Ainda, apesar de aparentemente apresentar menos efeitos deletérios sobre os processos cognitivos em comparação aos BZDs, efeitos amnésticos do zolpidem não deixam de ser relatados tanto em investigações com modelos animais como em estudos clínicos.

No que concerne a animais de laboratório, a administração aguda de zolpidem atenuou a aquisição da tarefa de medo condicionado em camundongos (Sanger et al., 1986) e em ratos (Chodera et al., 1994). Ainda, a administração aguda de 1 – 10 mg/Kg dessa droga promoveu efeitos amnésticos em camundongos avaliados na esquiva passiva (Edgar et al., 1997; Tang et al., 1995) e prejudicou a memória de trabalho avaliada no modelo do labirinto radial (Herzog et al., 2000). Mais recentemente, verificamos que a administração aguda de 5 ou 10 mg/Kg de zolpidem (mas não de 2 mg/Kg) promove uma completa amnésia em camundongos avaliados na esquiva discriminativa em labirinto em cruz elevado. De fato, tal efeito amnéstico ocorreu somente quando o zolpidem foi administrado agudamente antes do

treino (efeitos sobre o aprendizado e a consolidação), mas não quando foi administrado imediatamente após o treino (efeito sobre a consolidação) ou antes do teste (efeito sobre a evocação). Conjuntamente, tais resultados sugerem que o zolpidem promove um déficit de memória anterógrada. Além disso, também foi verificado um atraso no aprendizado da tarefa após a administração aguda de 10 mg/Kg de zolpidem (Zanin et al., 2013). Ainda no que concerne aos efeitos cognitivos, a administração aguda dessa mesma dose também promove déficits de habituação em campo aberto, um modelo animal de memória implícita (Zanin et al., 2011). De relevância, a administração aguda desse hipnótico – em todas as doses (2, 5 ou 10 mg/Kg) – promoveu um marcante efeito hipolocomotor dose-dependente, refletindo o efeito sedativo dessa droga (Zanin et al., 2013).

Sob o aspecto clínico, estudos revelaram que a administração aguda de zolpidem produziu déficits de memória tais quais aqueles induzidos pela administração aguda de triazolam, um BZD clássico. Além disso, o zolpidem também promoveu uma distorção na avaliação subjetiva do desempenho em tarefas de aprendizado e memória, como tempo de reação e o reconhecimento de figuras (Evans et al., 1990). Cashman e colaboradores (1987) demonstraram que o zolpidem produziu amnésia anterógrada quando da administração aguda em mulheres, e que tal amnésia se mostrou dependente de dose. Por fim, a administração aguda de zolpidem, 2 ou 3 horas antes do horário de acordar, promoveu efeitos residuais no desempenho psicomotor e prejuízos tanto na memória de trabalho quanto na memória de curta duração (Danjou et al., 1999). Tomados em conjunto, os achados descritos acima

sugerem que a administração aguda de zolpidem promove um efeito inibitório sobre os processos de aprendizado e memória.

Apesar de haver poucos estudos que tenham avaliado os efeitos prejudiciais do tratamento repetido com zolpidem por um período clinicamente relevante sobre o desempenho cognitivo, Stoops e Rush (2003) demonstraram que houve prejuízo no desempenho de humanos tratados agudamente com zolpidem em 3 diferentes tarefas. Contudo, não houve diferença no desempenho quando comparado ao 1º dia após 4 dias de tratamento, sugerindo que não houve desenvolvimento de tolerância aos efeitos prejudiciais induzidos por essa droga. Ainda nesse sentido, Kleykamp e colaboradores (2012) também não observaram tolerância aos efeitos deletérios cognitivos do zolpidem mesmo após 22 a 30 dias de tratamento por todas as noites. Esses mesmos autores observaram que após a descontinuação do tratamento, apesar de haver uma tendência, não houve efeito significativo da abstinência sobre o desempenho cognitivo dos indivíduos. Entretanto, esses mesmos autores sugerem que a magnitude desses efeitos poderia aumentar com o uso de doses maiores da droga ou um prolongamento de seu uso por vários meses ou anos. Por outro lado, ao que sabemos, nenhum estudo avaliou os efeitos decorrentes da administração repetida dessa droga sobre o a aprendizado/memória em condições experimentais que mimetizem a insônia.

1.6 Genes de expressão imediata e família Fos

A estimulação neuronal ativa mecanismos distintos para processar e transmitir a informação. Essa ativação pode envolver alterações da atividade

eletrofisiológica neuronal ou na cascata de segundos mensageiros que evoca a produção de fatores que induzem ou inibem a transcrição de genes (Herdegen e Leah, 1998). Os genes ativados por fatores de transcrição podem produzir proteínas como as que constituem receptores, canais iônicos e as requeridas para síntese e regeneração de neurotransmissores. Entre os fatores de transcrição conhecidos, um dos mais estudados é o c-Fos, o qual é induzido rapidamente após a ativação sináptica, atingindo o pico entre 90 e 120 minutos após a estimulação (Nestler et al., 2001).

Mais especificamente, c-Fos é um proto-oncogene pertencente à família dos genes de expressão imediata, *immediate early genes* – IEGs. Esses genes são fatores de transcrição, ativados rápida e transientemente na cascata inicial da expressão gênica, responsável por iniciar o processo de mudanças adaptativas induzidas por estímulos extracelulares e atividade sináptica excitatória (Davis et al., 2003). A proteína c-Fos, produto do gene c-Fos, é uma proteína regulatória que, em conjunto com outros membros das famílias Fos e JUN, formam um complexo heterodimérico AP1, que por sua vez, se liga ao DNA para controlar a expressão gênica.

Na maioria das células, os níveis de expressão basal de c-Fos são relativamente baixos. Todavia, o seu RNAm e os níveis proteicos aumentam rapidamente após a aplicação de uma variedade de estímulos extracelulares, como por exemplo estresse (Del-Bel et al., 1993; Titze-de-Almeida et al., 1994), epilepsia (Herrera e Robertson, 1996), estímulos nociceptivos (Harris et al., 1996; Hunt et al., 1987) e drogas de abuso (Aston-Jones e Harris, 2004; Nestler, 2004). Essa ausência de expressão basal seguida de um aumento de expressão de c-Fos contribuem para a sensibilidade da técnica, onde a

presença da proteína c-Fos pode ser usada como um marcador de ativação celular gerada por estímulo. No sistema nervoso central, esse aumento pode ser detectado rapidamente (15-30 minutos para a ativação de RNAm e 30-60 minutos para a ativação da proteína c-Fos) pela técnica de imuno-histoquímica. A incubação do tecido em solução contendo o anticorpo anti-c-Fos, após a revelação, produz um precipitado escuro marcando o núcleo das células c-Fos positivas. A extensão e intensidade da expressão de c-Fos determinam as áreas cerebrais ativadas em uma determinada tarefa (Grzanna e Brown, 1993).

Os genes que codificam as proteínas da família Fos são classificados como IEGs devido ao seu rápido e transitório padrão de indução. Entretanto, uma subclasse de antígenos estáveis relacionados à proteína Fos (*Fos-related antigens* – FRAs) foram posteriormente identificados (Chen et al., 1995; Hope et al., 1994). Alguns desses antígenos crônicos são isoformas estabilizadas de delta-FosB (Hiroi et al., 1997, 1998). Resumidamente, como representado na figura 2, um estímulo agudo induz a síntese de c-Fos. O c-fos e a maioria dessas proteínas são instáveis e, portanto, dissipadas rapidamente após a estimulação. Após a repetição do estímulo, as isoformas de delta-FosB gradualmente acumulam em níveis elevados e fisiologicamente ativos, ao passo que a indução de c-Fos e outras proteínas da família Fos, é dessensibilizada após um estímulo repetido. Em consequência, as isoformas de delta-FosB tornam-se as proteínas da família Fos predominantes após um tratamento crônico e medeiam as modificações na expressão gênica.

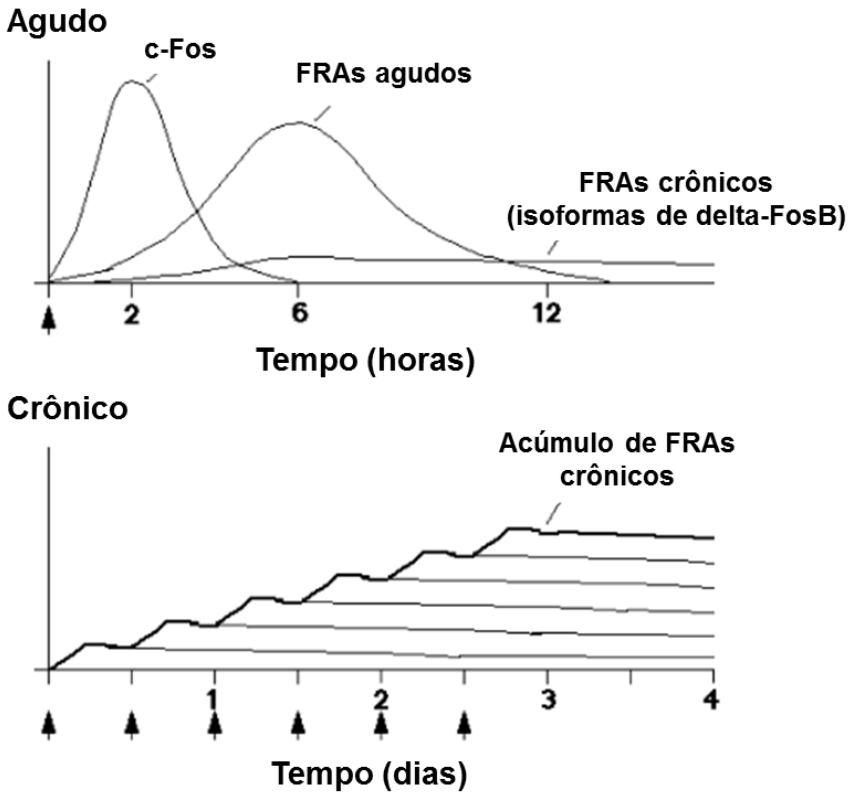

Figura 2 – padrão de expressão das proteínas Fos (adaptado de Nestler et al., 2001)

Com relação aos processos mnemônicos, a expressão pós-treino de RNA para c-Fos tem sido descrita em diversas tarefas, incluindo a esquiva ativa (Nikolaev et al., 1992), tarefas de discriminação de luminosidade (Grimm et al., 1997; Tischmeyer et al., 1990) e odor (Hess et al., 1995a,b), além da tarefa de alternância (Nagahara e Handa, 1995). De importância, diversos estudos têm demonstrado que a expressão da proteína c-Fos é aumentada no hipocampo após o aprendizado (Cammarota et al., 2000; Papa et al., 1995; Vann et al., 2000; Wan et al., 1999; Zhu et al., 1997). Com relação à evocação de tarefas, foi demonstrado que camundongos que aprenderam uma tarefa de discriminação espacial apresentaram uma maior expressão de proteínas c-Fos no hipocampo e no córtex entorrinal ao recordar uma memória recente, mas não uma memória de longa duração. Entretanto, diversas regiões corticais, como os córtices pré-frontal, temporal e cingulado anterior, foram mais ativadas

após a recordação de uma memória de longa duração (Bontempi et al, 1999; Maviel et al., 2004). Ainda, as regiões corticais que são recrutadas para dar suporte à memória de longa duração dependem da tarefa. Dessa forma, em uma tarefa olfativa, o teste da memória de longa duração foi associado com a atividade aumentada nos córtex piriforme, entorrinal e órbito-frontal, mas não o córtex cingulado anterior (Ross e Eichenbaum, 2006).

No que diz respeito ao sono, diversos estudos realizados em ratos (Cirelli et al., 1993, 1995; Garcia-Garcia et al., 1998; Grassi-Zucconi et al., 1993, 1994a,b; Novak e Nunez, 1998; O'Hara et al., 1993; Pompeiano et al., 1992, 1994, 1997; Sherin et al., 1996) e em camundongos (Basheer et al., 1997) demonstram que a expressão de c-Fos no cérebro é baixa, ou mesmo ausente, após algumas horas de sono, enquanto que essa atividade está aumentada em animais mantidos acordados por algumas horas. Nesse sentido, o padrão de expressão de c-Fos após algumas horas de privação de sono total é similar ao padrão observado após a vigília espontânea (Cirelli, 2002), sugerindo que a expressão de c-Fos está relacionada à vigília *per se*. Ainda, apesar de diferentes protocolos de privação de sono promoverem um aumento nos níveis de c-Fos, essa expressão não é necessariamente proporcional à "quantidade" de vigília. Nesse sentido, o aumento de c-Fos é mais acentuado após 3 horas de privação do que após 24 horas na maior parte das regiões cerebrais (Cirelli et al., 1995). Ainda, a expressão de c-Fos é induzida de maneira mais eficiente por estímulos novos do que por estímulos familiares (Anokhin et al., 1995; Hess et al., 1995b; Radulovic et al., 1998; Zhu et al., 1995), bem como por curtos períodos de privação ao invés de períodos prolongados (Cirelli et al., 1995, 2000).

Objetivos

2. OBJETIVOS

2.1 Objetivo geral

Considerando a crescente utilização do zolpidem no tratamento da insônia, as evidências prévias observadas por nosso grupo de pesquisa que sugerem alterações cognitivas induzidas pela administração aguda dessa droga, e a falta de estudos que abordem sistematicamente os efeitos do tratamento repetido com zolpidem sobre os processos mnemônicos, a presente Tese tem por objetivo analisar os efeitos do tratamento repetido com zolpidem ou de sua retirada abrupta sobre as diversas fases da formação da memória por meio dos paradigmas da esquiva discriminativa em labirinto em cruz elevado, esquiva passiva, reconhecimento de objetos e reconhecimento social. Ainda, visto que clinicamente o tratamento com zolpidem é iniciado após períodos variáveis de privação de sono, compararemos os resultados obtidos em camundongos mantidos em condições controle de sono ou submetidos à restrição de sono.

2.2 Objetivos específicos

Avaliar:

1. efeitos da privação de sono total por 3 ou 6 h agudamente ou durante 10 dias consecutivos no paradigma da esquiva discriminativa em labirinto em cruz elevado (Manuscrito 1);
2. efeitos da administração repetida de zolpidem (ou de sua retirada abrupta) sobre o aprendizado, consolidação e evocação no paradigma

da esquiva discriminativa em labirinto em cruz elevado em animais restritos de sono 3 h por dia durante 10 dias (Manuscrito 2);

3. efeitos da administração repetida de zolpidem (ou de sua retirada abrupta) sobre o aprendizado, consolidação e evocação no paradigma da esquiva passiva em animais restritos de sono 3 h por dia durante 10 dias (Manuscrito 3);
4. efeitos da administração repetida de zolpidem (ou de sua retirada abrupta) sobre o aprendizado/consolidação nos paradigmas de reconhecimento de objetos ou social em animais restritos de sono 3 h por dia durante 10 dias (Manuscrito 4);
5. efeitos do tratamento repetido com zolpidem no padrão de sono de camundongos restritos ou não de sono 3 h por dia durante 10 dias por meio de registro eletroencefalográfico (Manuscrito 5).

Manuscrito 1

TOLERANCE OR SENSITIZATION TO THE IMPAIRING EFFECTS OF TOTAL SLEEP DEPRIVATION IN MICE

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ABSTRACT

Purpose: It has been extensively demonstrated that paradoxical sleep deprivation impairs learning and memory. Although the total sleep deprivation (TSD) is more common in modern societies, its effects of on cognition remained overlooked. In agreement, we have already shown that acute TSD for 6 h induced memory impairments in mice subjected to the plus-maze discriminative avoidance task (PM-DAT). The present study was tailored to investigate whether 3 h or 6 h of acute or repeated TSD for 10 days could induce memory deficits in mice tested in the PM-DAT. Still, we examined the pattern of c-fos protein expression in the hippocampus and basolateral amygdala after TSD. **Design:** Male mice were kept in home cage (CTRL – control condition) or subjected to acute TSD by the gentle handling method for 3 h (TSD3h) or 6 h (TSD6h). The same condition of TSD was repeated for 10 consecutive days. At the ending of TSD period, animals were trained in the PM-DAT and 12 days after, animals were tested. **Results:** Acute TSD for 6h induced memory deficits, which were tolerated when the sleep deprivation was repeated for 10 consecutive days. Acute TSD for 3 h did affect memory. However, when the duration of the SR was prolonged, it induced amnesia. There was an increase in c-fos expression in the dentate gyrus, which was of higher magnitude after acute TSD for 3 h. All of SR groups also displayed increase in c-fos expression in the basolateral amygdala. **Conclusions:** TSD-induced memory deficits are critically influenced by duration and frequency of the sleep deprivation. Collectively, these data suggest that the amount of sleep loss is an important factor affecting performance.

Keywords: sleep restriction, memory, c-fos.

INTRODUCTION

It is well-established that sleep plays a critical role in learning and memory formation. Indeed, a myriad of studies have demonstrated that both paradoxical sleep deprivation and total sleep deprivation (TSD) methods leads to memory deficits in several animal models such as avoidance tasks (Bueno et al., 1994; Harris et al., 1982; Skinner et al., 1976), the Morris water maze (Youngblood et al., 1997, 1999), the radial maze (Smith et al., 1998), object recognition (Palchykova et al., 2006) and the plus-maze discriminative avoidance task (PM-DAT) (Alvarenga et al., 2008; Fernandes-Santos et al., 2012; Patti et al., 2010; Silva et al., 2004a).

Although the consequences of paradoxical sleep deprivation have been studied for many years, the effects of TSD on learning/memory as well as on anxiety and locomotor activity in animal models remained overlooked. Notwithstanding, Patti and colleagues (2010) have demonstrated that acute TSD for 6 h in adult mice promoted memory deficits in the PM-DAT when applied both before training and/or test and that these memory impairments are not related to state-dependency. Still, Fernandes-Santos and colleagues (2012) demonstrated that the TSD-induced retrieval deficits depends on the task involved and is influenced by sex. Indeed, Guan and colleagues (2004) reported that 6 h of TSD impaired spatial memory, but not spatial learning, and did not influence non-spatial learning or memory in rats evaluated in the Morris water maze. On the other hand, it was shown that 6 h of TSD did not affect spontaneous alternation behavior in the Y maze (Ramanathan et al., 2010). Similarly, Pierard and colleagues (2007) reported that TSD for 3 h had no

effects on the same model. Conversely, when the period of TSD was prolonged it impaired the ability of mice to run the alternation task. Still, mice subjected to 10 h of TSD decreased the spontaneous alternation behavior compared to non-deprived mice. Collectively, these data suggest that the amount of sleep loss is an important factor affecting performance.

It is known that neuronal stimulation activates several mechanisms to process information. This activation involves stimulation or inhibition of gene transcription (Herdegen and Leah, 1998). Among the transcriptional factors, one of the most studied is the c-fos, a proto-oncogene classified as an immediate early gene (IEGs). IEGs are responsible for initiating adaptive changes induced by extracellular stimuli or excitatory synapses (Davis et al., 2003). After activation, c-fos is rapidly induced reaching its peak after 90-120 min (Nestler et al., 2001).

The aim of the present study was to investigate whether different regimens of TSD (3 or 6 h acute or repeated during 10 consecutive days) would be able to induce memory impairments or other behavioral alterations in mice in the PM-DAT – an animal model which concomitantly evaluates learning/memory, anxiety-like behavior and locomotor activity. In addition, we analyzed the expression of c-fos in the dentate gyrus and basolateral amygdala.

MATERIAL AND METHODS

Subjects

Three-month-old Swiss male mice (outbred, raised, and maintained in the Centre for Development of Experimental Models in Medicine and Biology of Universidade Federal de São Paulo) were used. Animals weighing 35-40 g were housed under conditions of controlled temperature (22-23°C) and lighting (12h light, 12h dark; lights on at 6:45 a.m.). Food and water were available *ad libitum* throughout the experiments. Animals used in this study were maintained in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications Nº 8023, revised 2011) and with the Brazilian Law for Procedures for Animal Scientific Use (#11794/2008). The experimental procedures were approved by the Institutional Animal Care and Use Committee under the protocol #1741/10.

Total Sleep Deprivation (TSD)

Mice were submitted to TSD through the gentle handling method, described elsewhere (Tobler et al., 1990). This method consists of keeping the animal awake by tapping on the cage and, if necessary, by gently touching them with a soft brush if behavioral signs of sleep are observed. The animals were sleep deprived for 3 (10 AM to 1 PM) or 6 h (7 AM to 1 PM) during 1 or 10 consecutive days and immediately after the ending of sleep deprivation were submitted to behavioral session.

Plus-maze discriminative avoidance task (PM-DAT)

The apparatus employed was a modified elevated plus-maze, made of wood, containing two enclosed arms with sidewalls, and no top (28.5 x 7 x 18.5 cm), opposite to two open arms (28.5 x 7 cm). A non-illuminated, 100-W lamp and a hair dryer were placed over the exact center of one of the enclosed arms (aversive enclosed arm). In the training session, each mouse was placed at the center of the apparatus and, during a 10-min period, an aversive stimulus was administered every time the animal entered the enclosed arm containing the lamp and was continued until the animal left the arm. The aversive stimuli were the 100-watt light and an air blow produced by the hairdryer over the aversive enclosed arm. In the test session, mice were again placed in the center of the apparatus and were observed for 3 min; however, the mice did not receive an aversive stimulus when they entered the aversive enclosed arm (although the non-illuminated lamp and the hair dryer were still placed on the middle of this arm to help distinguish between the aversive and non-aversive arm). In all experiments, the animals were observed in a blind manner, and the apparatus was cleaned with a 5% alcohol solution after each behavioral session. Learning and memory were evaluated by the time spent in the aversive versus non-aversive enclosed arms in the training and testing session, respectively. Anxiety-like behavior was evaluated by the percent time spent in the open arms of the apparatus (time spent in open arms/time spent in both open and enclosed arms). The total number of entries into any of the arms was used to evaluate locomotion.

Perfusion procedure

The animals were deeply anesthetized with ketamine/xilazine. The chest cavity was opened, and the mice were perfused via the left ventricle with 100 ml phosphate-buffered saline (PBS, pH 7.2), followed by 100 ml of buffered 4% paraformaldehyde solution in PBS (pH 7.2), each one during approximately 10 min. The skulls were maintained in 4% paraformaldehyde solution for 24 h. After that, the fixed brains were removed from the skulls and kept in 4% paraformaldehyde solution at 4ºC until use. Then, the brains were transferred to 30% sucrose buffer solution and kept overnight at 4ºC. Brains were then frozen and coronal sections (50 µm thick) were cut in cryostat.

Immunohistochemistry

The tissue was rinsed in 0.1 M PBS (3 times for 5 min each) and incubated in 0.1 M PBS containing 5% normal goat serum and 0.2% Triton X-100 (Sigma®) for 30 min. Sections were then incubated overnight in 0.1 M PBS containing anti-C-Fos rabbit polyclonal antibody (1:3000) and 0.1% Triton X-100 at room temperature. After exposure to the primary antibody, the sections were then rinsed in 0.1 M PBS (5 times for 5 min each) and incubated in 0.1 M PBS containing biotinylated goat anti-rabbit IgG (1:200) and 0.1% Triton X-100 for 2 h. The sections were rinsed again using 0.1 M PBS (5 times for 5 min each) and incubated in avidin-biotin complex (Vectastain® ABC Kit) for 1h30min. The reaction was terminated by rinsing the tissue in 0.1 M PBS (5 times for 5 min each) and then in sodium acetate solution (5 times for 5 min each). Sections were mounted on gelatin-coated slides, dehydrated and coverslipped prior to viewing with a stereological microscope.

Stereology analysis

The stereological analysis was performed using the software Stereo Investigator (version 11.01, MicroBrightField, USA). An optical fractionator method was used to estimate the total number of nuclei positive c-fos of neurons in each region analyzed at high magnification with a 100x oil objective. The optical fractionator method (West et al., 1991) is a 3D probe placed through the reference space, which was used to estimate the number of nuclei of neurons in each region analyzed. After cutting the brain, the sampling of tissue sections followed a systematic, uniform, random sampling scheme to ensure that all parts of the structure had an equal probability of being sampled. Thus, the analysis of the sections started from a random position at the origin of the brain structures. Every 3 sections were included. A total of 11-14 (dentate gyrus) and 4-6 (basolateral amygdala) sections were obtained for stereological examination. The contour delineations (left and right) of the structures were made according to the atlas of Paxinos and Franklin (2001) and were drawn using a 4x objective.

Counts for the dentate gyrus were obtained from sections corresponding to planes ranging from -0.94 to -3.88 mm from bregma. For the basolateral amygdala, counts were obtained from sections corresponding to planes ranging from -0.58 to -2.06 mm from bregma. After the pilot stereological test, each counting frame, 75 x 60 µm for the dentate gyrus and 50 x 50 µm for the basolateral amygdala, was placed at an intersection of the lines forming a virtual grid (250 x 125 µm for the dentate gyrus and 200 x 200 µm for the basolateral amygdala) which was randomly generated and placed by the software within each structure of interest. The optical dissector height

(thickness) was 20 µm with a 2 µm top and bottom guard zones. The mean section thickness measured at every other counting frame site was used for final calculation of nuclei of neuron number for each region analyzed. The precision of the individual estimations is expressed by the coefficient of error (CE) and was calculated as described by Gundersen and colleagues (1999) and should not be over 0.10. Counting was made by a person blind to the condition using a 100x oil immersion objective. Still, it was previously defined that a cell should be counted when presenting a spherical or oval shape with color ranging from brown to black and well-defined margins.

Experimental design

Experiment I: Effects of acute total sleep deprivation for 3 or 6 h

Animals were assigned to one of the following groups: control (CTRL, n=12); total sleep deprived for 3 h (TSD 3 h, n=12) or total sleep deprived for 6 h (TSD 6 h, n=12). Mice allocated in CTRL groups were kept in their home cages throughout the experiment. On the other hand, mice allocated in the TSD groups were gently handled for 3 or 6 h before the training session. Immediately after the end of the TSD period animals were submitted to training in the PM-DAT. Twelve days after training, they were submitted to testing.

Experiment II: Effects of repeated total sleep deprivation for 3 or 6 h during 10 consecutive days

The same protocol of Experiment I was employed, but the sleep deprivation condition was repeated for 10 consecutive days.

Experiment III: Effects of different regimens of total sleep deprivation on c-Fos protein expression

Animals were assigned to one of the following groups: control (CTRL, n=5); total sleep deprived for 3 h (TSD 3 h/1 day, n=5); total sleep deprived for 6 h (TSD 6 h/1 day, n=5); total sleep deprived for 3 h during 10 days (TSD 3 h/10 days, n=5) or total sleep deprived for 6 h during 10 days (TSD 6 h/10 days, n=5). Mice allocated in CTRL groups were kept in their home cages throughout the experiment. On the other hand, mice allocated in the TSD groups were gently handled for 3 or 6 h daily for 1 or 10 days. On the 10^{th} day, mice allocated in the groups acutely sleep deprived were submitted to TSD on their respective period. Immediately after the end of the TSD period, animals were anesthetized and perfused, as described before.

Statistical analysis

Two-way ANOVA and Duncan's test were used to analyse time spent in the aversive versus non-aversive enclosed arms. Total number of entries in any of the arms, percent time spent in the open arms and the estimated total numbers of nuclei of neurons in the stereological counting method were compared using one-way ANOVA followed by Duncan's test. A probability of $p<0.05$ was considered significant for all comparisons made.

RESULTS

Experiment I: Effects of acute total sleep deprivation for 3 or 6 h

In the training session, analyzing time spent in the enclosed arms, two-way ANOVA showed significant effects of arm type (aversive vs. non-aversive) [$F(1,66)=241.77$; $p<0.001$], group (CTRL, TSD 3 h or TSD 6 h) [$F(2,66)=4.75$; $p=0.01$] and arm type x group interaction [$F(2,66)=4.50$; $p=0.01$]. Duncan's *post hoc* revealed that all groups discriminated the enclosed arms, spending significantly less time in the aversive enclosed arm then in the non-aversive one (Figure 1A). Regarding anxiety, ANOVA for the percent time spent in the open arms showed significant effects of group [$F(2,33)=9.34$; $p<0.001$]. The *post hoc* analyses showed that the sleep deprived for 3 h group (TSD 3 h) presented a higher exploration of the open arms compared to the other groups (Figure 1B). Analyzing motor activity, ANOVA for the total number of entries showed significant effects of group [$F(2,33)=5.82$; $p=0.007$]. In fact, mice in the TSD 3 h group presented an increased motor activity compared to the other groups (Figure 1C).

In the testing, two-way ANOVA for the time spent in the enclosed arms showed significant effects of arm type [$F(1,66)=71.72$; $p<0.001$] and arm type x group interaction [$F(2,66)=12.94$; $p<0.001$]. Duncan's *post hoc* revealed that only mice under control condition or sleep deprived for 3 h (CTRL and TSD 3 h groups) spent significantly less time in the aversive then in the non-aversive enclosed arm, remembering the task (Figure 1D). Still in this session, ANOVA did not show significant differences concerning the percent time in the open arms or the total number of entries (Figures 1E and 1F, respectively).

Experiment II: Effects of repeated total sleep deprivation for 3 or 6 h during 10 consecutive days

Two-way ANOVA for the time spent in the enclosed arms during training revealed significant effects only for arm type [$F(1,42)=316.19$; $p<0.001$]. Indeed, as demonstrated by Duncan's test, all of the groups spent less time in the aversive then in the non-aversive enclosed arm (Figure 2A). In relation to anxiety-like behavior, ANOVA for the percent time spent in the open arms did not show differences among groups (Figure 2B). In the same way, there were no differences in motor activity (Figure 2C).

During testing, two-way ANOVA demonstrated significant effects of arm type [$F(1,42)=14.93$; $p<0.001$] and arm type x group interaction [$F(2,42)=3.25$; $p<0.05$]. The *post hoc* analyses showed that only the group sleep deprived 3 h per day (TSD 3 h) did not discriminate the enclosed arm, while the CTRL and TSD 6 h groups spent significantly less time in the aversive enclosed arm then the non-aversive one (Figure 2D). Still in this session, ANOVA did not show differences neither for the exploration of the open arms nor for total number of entries (Figures 2E and 2F, respectively).

Experiment III: Effects of different regimens of total sleep deprivation on c-Fos protein expression

Analyzing the estimated number of c-fos positive cell in the dentate gyrus, one-way ANOVA showed significant effects of group $F(4,20)=9.33$; $p<0,001$]. Duncan's *post hoc* demonstrated that animals submitted to TSD presented an increase in expression of c-fos compared to CTRL group.

Additionally, those animals total sleep-deprived for 3 h during 1 day displayed an even greater expression of c-fos positive cells compared to the other groups (Figure 3A).

Regarding the expression of c-fos in the basolateral amygdala, one-way ANOVA showed significant effects of group [$F(4,20)=4.08$; $p<0,01$]. The *post hoc* analyzes by Duncan's test demonstrated that animals sleep-deprived presented an increase in the expression of c-fos compared to CTRL group (Figure 3B).

Figure 1

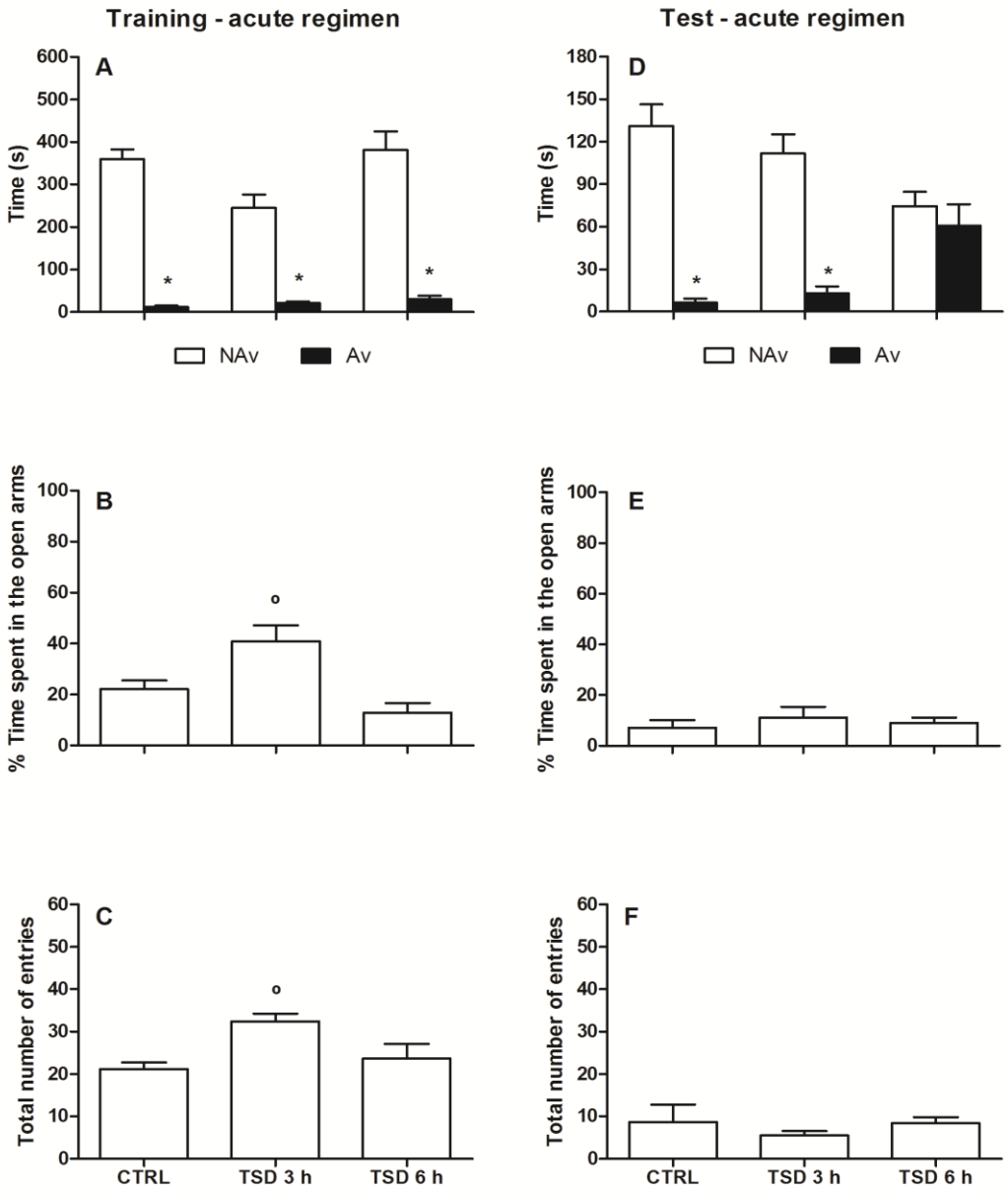

Figure 1: Effects of acute total sleep deprivation (TSD) on learning and consolidation of a discriminative task in mice. Animals were subjected to control condition (CTRL) or TSD for 3 or 6 h. After TSD period, mice were trained and 12 later tested. Results are presented as the mean ± S.E. of time spent in the non-aversive enclosed arm x time spent in the aversive enclosed arm in the training (**A**) and testing (**D**), percent time spent in the open arms in the training (**B**) and testing (**E**), and number of entries in the training (**C**) and testing (**F**). *p<0.05 compared to time spent in the non-aversive arm; ºp<0.05 compared to the other groups (one- or two-way ANOVA and Duncan's test).

Figure 2

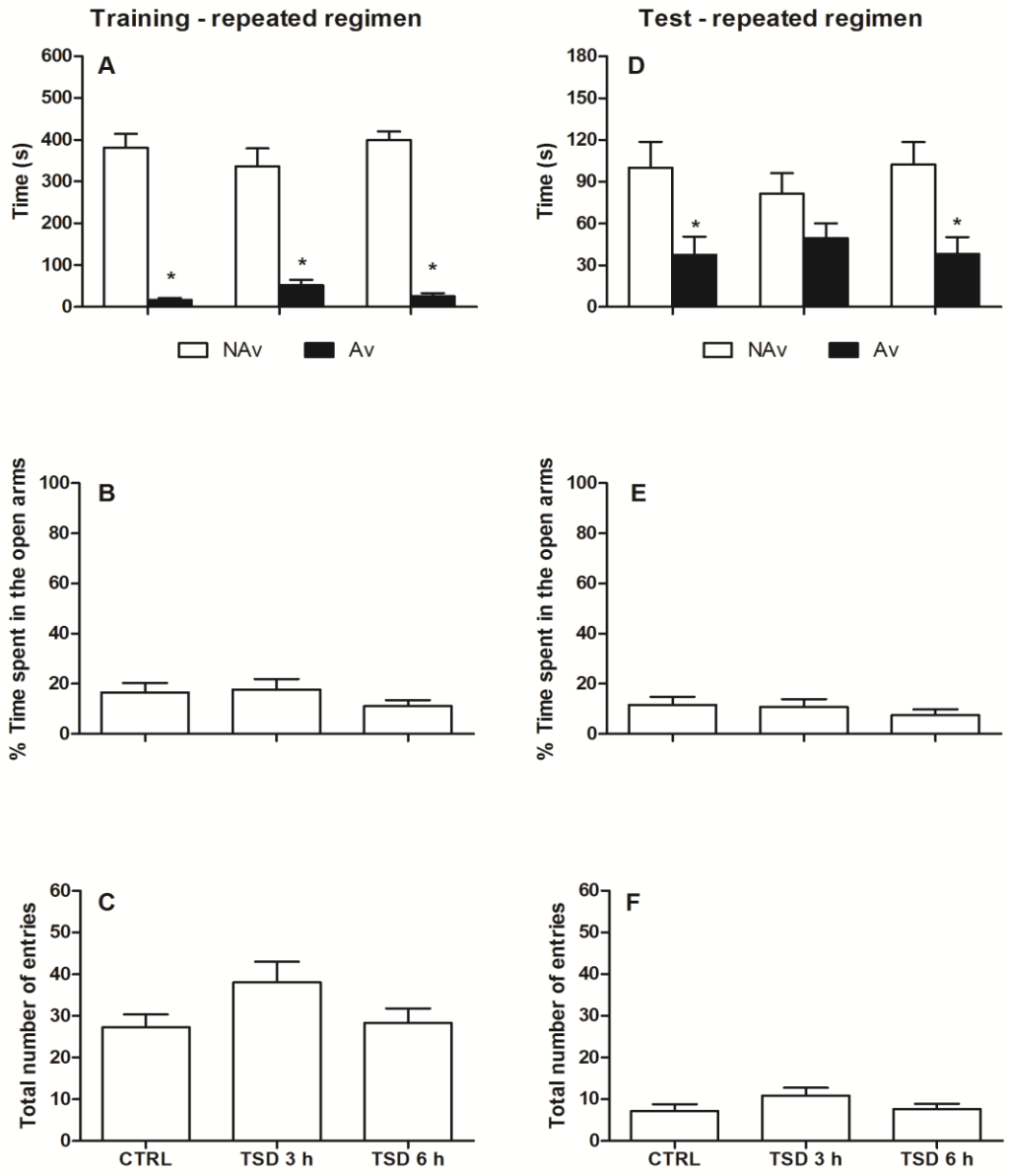

Figure 2: Effects of repeated total sleep deprivation (TSD) on learning and consolidation of a discriminative task in mice. Animals were subjected to control condition (CTRL) or TSD for 3 or 6 h during 10 consecutive days. In the 10th day, after TSD period, mice were trained and 12 later tested. Results are presented as the mean ± S.E. of time spent in the non-aversive enclosed arm x time spent in the aversive enclosed arm in the training (**A**) and testing (**D**), percent time spent in the open arms in the training (**B**) and testing (**E**), and number of entries in the training (**C**) and testing (**F**). *p<0.05 compared to time spent in the non-aversive arm (one- or two-way ANOVA and Duncan's test).

Figure 3

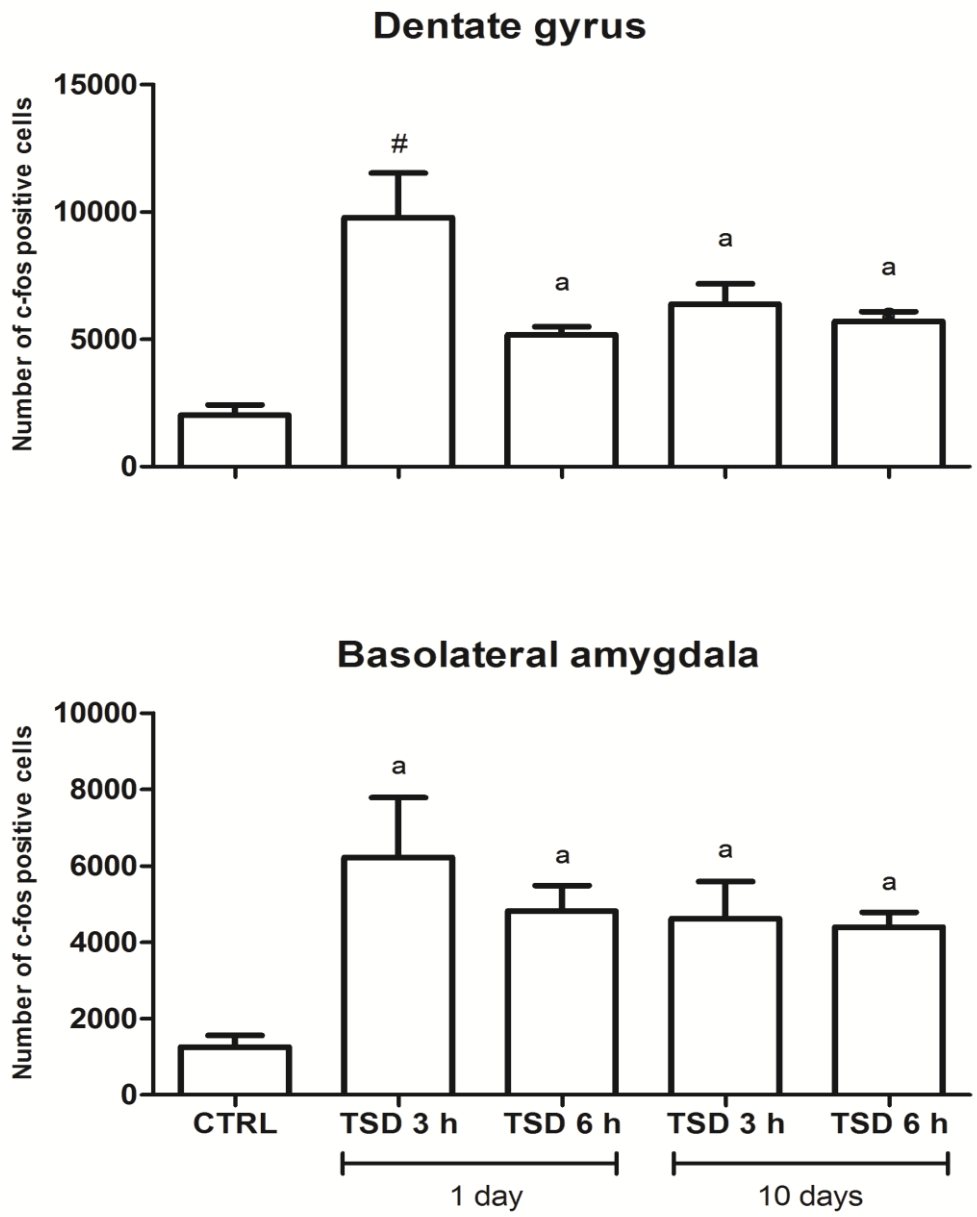

Figure 3: Effects of repeated total sleep deprivation (TSD) on c-fos protein expression. Animals were subjected to control condition (CTRL) or TSD for 3 or 6 h during 1 or 10 consecutive days. In the 10th day, after TSD period, mice were euthanized and the brains were collected. Results are presented as the mean ± S.E. of c-fos positive cells in the dentate gyrus (A) and basolateral amygdala (B). ª$p<0.05$ compared to CTRL group; #$p<0.005$ compared to the other groups (ANOVA and Duncan's test).

DISCUSSION

In the present study, our findings showed that the memory deficits induced by acute TSD for 6 h are tolerated when the sleep deprivation was repeated for 10 consecutive days. Conversely, while the acute TSD for only 3h did not affect memory, this procedure induced amnesia when an increase of TSD frequency occurred. Thus, TSD-induced memory deficits are critically influenced by duration and frequency of the sleep loss. The c-fos quantification suggests that the higher activation of the dentate gyrus after TSD may have prevented the cognitive impairment. In the basolateral amygdala, the stressing component of the TSD may have increased c-fos expression.

It is well-stablished that sleep plays a critical role on learning and memory processes. Thus, several studies have shown that paradoxical sleep deprivation promotes memory impairments in a variety of animal models (Alvarenga et al., 2008; Bueno et al., 1994; Patti et al., 2010; Silva et al., 2004a,b; Youngblood et al., 1999, 1997). In parallel, TSD models seem to mimic what occurs in humans, although its effects in animal models remains overlooked. In humans, sleep loss is frequently associated with TSD, chronic sleep restriction or sleep fragmentation. TSD involves the completely sleep loss over a period significantly prolonging wake time. Sleep restriction refers to a reduction of sleep time under the necessary to maintain optimal performance levels, being frequent in modern society (Banks and Dinges, 2007). On the other hand, sleep fragmentation is usually observed in patients with sleep disorders, such as sleep apnea (Reynolds and Banks, 2010).

In the experiment I, we evaluated the effects of acute TSD during 3 or 6 h prior to training. We observed that TSD for 3 h increased the exploration of the open arms, suggesting an anxiolytic effect. Still, this same group also presented an increased motor activity. On the other hand, extending the sleep deprivation period to 6 h, these modifications on emotionality or locomotion were no longer observed. In this concern, it has already been reported that acute TSD for 6 h did not modify anxiety-like behavior (Patti et al., 2010). This discrepancy could be due to the sleep deprivation period. In this vein, while 3 h of TSD led to alterations in the emotional levels (e.g., impulsivity and inquietude) of mice, the fatigue and exhaustion that could have been induced by being gentle handled for 6 h abolished emotional alterations. One could argue that the increased exploration of the open arms would be due to an artifactual effect of the hyperlocomotion presented by these animals. However, the percent time spent in the open arms of the apparatus during the training session has been validated as a measure of anxiety because classical anxiolytic agents (Calzavara et al., 2004; Gulick and Gould 2009a,b, 2011; Kameda et al., 2007) increase this parameter, and classical anxiogenic decrease it (Gulick and Gould 2009a; Silva and Frussa-Filho, 2000). In fact, the PM-DAT can dissociate the effects of pharmacological (such as ethanol administration) or procedure (such as sleep deprivation) manipulations on anxiety-like behavior and motor activity separately (Frussa-Filho et al., 2010).

When memory was analyzed, only acute TSD for 6 h promoted memory deficits in the test session. This result corroborates previous studies of our group showing that this sleep deprivation regimen induces amnesia when applied before training (Patti et al., 2010) or testing (Fernandes-Santos et al.,

2012) both in the PM-DAT and the passive avoidance task. Of note, other groups have also shown memory impairments induced by TSD when it occurred immediately (Palchykova et al., 2006) or 1 h (Prince et al., 2014) after training of object recognition tasks. These results seem to be in line with a molecular perspective, since Guan and colleagues (2004) reported that 6 h, but not 3 h of TSD, was able to induce alterations on the extracellular signal-regulated kinase 2 (ERK2) phosphorylation in the hippocampus, which is activated in response to many neurotransmitters and growth factors and by learning (Atkins et al., 1998; Cammarota et al., 2000; Swank and Sweatt, 2001) and long-term potentiation (LTP) (English and Sweatt, 1996; Wu et al., 1999). This protein kinase is involved in long-term synaptic plasticity (Angenstein et al., 1998; Impey et al., 1999; Mazzucchelli and Brambilla, 2000) and synaptic plasticity-related gene expression (Adams et al., 2000; Davis et al., 2000). Thus, these results could explain the memory impairments observed after 6 h of TSD but not after 3 h.

Insomnia is characterized by difficulties in falling asleep or maintaining sleep, low sleep quality or non-repairing sleep, leading to impairments on daily function and cognition (Billiard and Bentley, 2004). Thus, in the 2nd experiment we aimed to mimic the insomnia-induced cognitive deficits by a sleep restriction protocol. Thus, animals were subjected to TSD for 10 consecutive days. Our data showed that the repetition of TSD for 3 h abolished the observed anxiolytic and hiperlocomotor effects of the acute TSD for the same period, suggesting a tolerance development. In the same way, while the acute TSD for 6 h induced memory deficits, when it was repeated for 10 days, the impairment was no longer observed. Oppositely, the repeated TSD for 3 h promoted amnesia, which did not occur after the acute procedure. Our results suggest that

tolerance seems to occur for the behavioral modifications induced by the acute TSD for 3 h (anxiolytic and hiperlocomotor effects) as well as for the deleterious effects of acute TSD for 6 h on cognition.

Studies have shown that TSD in humans promote cognitive deficits especially with regard to vigilance (Doran et al., 2001; Van Dogen et al., 2004), constructive thinking (Killgore et al., 2008), spatial working memory (Heuer et al., 2005) and verbal memory (Harrison and Horne, 1998). Additionally, subjective measures such as fatigue, sleepiness and humor are strongly affected by acute periods of TSD (Pilcher and Huffcutt, 1996). Regarding sleep restriction, Van Dongen and colleagues (2003) demonstrated that restricting sleep to 2 or 4 h per night during 14 days induced cognitive deficits in the same magnitude of those promoted by TSD for 1 or 2 days, suggesting that even shorter periods of sleep restriction when lasting for days may impair behavioral function. These authors have also observed that subjective sleepiness was diminished in subjects chronically sleep-restricted compared to those totally sleep-deprived, suggesting tolerance to the subjective alterations induced by TSD.

As an attempt to find a possible mechanism for the behavioral findings of the different sleep deprivation protocol, we investigated the expression of c-fos protein in the dentate gyrus and in the basolateral amygdala. In the central nervous system, the c-fos protein expression may be induced by several stimuli, such as harmful, auditory, thermal, visual and somatosensory. In exception of GABA and glycine, the most important neurotransmitters such as dopamine are able to enhance c-fos expression (Cirelli and Tononi, 2000).

Our results demonstrated that TSD induced an increase in the number of c-fos positive cells in dentate gyrus. Of note, this proto-oncogene is sensitive to acute modifications, being rapidly induce and reaching its peak 90-120 min after stimulation. Corroborating our findings, several studies conducted in rats (Cirelli et al., 1993, 1995; Garcia-Garcia et al., 1998; Novak and Nunez, 1998; Sherin et al., 1996) and mice (Basheer et al., 1997) have shown that the cerebral expression of c-fos is usually low or even absent after normal sleep. Oppositely, such activity is enhanced in animals sleep-deprived for some hours. In this way, the pattern of c-fos expression after TSD is similar to that observed after spontaneous awakening (Cirelli et al., 1995), suggesting that c-fos expression is related to wake *per se*.

Acute TSD for 3 h induced a higher increment of c-fos expression than 6 h-TSD for 1 day or 3 h-TSD repeated for 10 days in the dentate gyrus. This pattern of activation seems to be in agreement with our behavioral findings. Specifically, animals subjected to acute 3 h-TSD retrieved the task. When the sleep condition was prolonged, animals displayed amnesia, which was accompanied by a reduction in the c-fos expression. Similarly, animals submitted to acute 6 h-TSD presented amnesia followed by a reduction in the c-fos. Interestingly, animals subjected to repeatedly 6 h-TSD displayed a trend towards increment of c-fos expression when TSD was applied for 10 days. Together, these findings, suggest that the better cognitive performance may be associated to the increased c-fos expression in the dentate gyrus, i.e. to a higher activation of this region. In fact, it was shown that the dentate gyrus granular neurons project to CA3 pyramidal neurons via mossy fiber projections, which seems to be essential during learning (Kesner et al., 2004).

Specifically concerning the c-fos expression pattern of animals subjected to TSD for 6 h during 10 days –which had memory preserved – higher levels of c-fos expression would be expected. This could be explained by the temporal pattern of c-fos expression since higher protein concentrations are found a few hours after the stimulus and then progressively decrease (Melia et al., 1994; Chauduri, 1997). Although several sleep deprivation protocols promote an increase of c-fos protein it does not seem to be related to the amount of time spent awake. In this regard, the enhancement in the number of c-fos positive cells is of higher magnitude after 3 h of sleep deprivation then after 24 h (Cirelli et al., 1995). Moreover, the c-fos expression is induced more efficiently by new stimuli than familiar one (Anokhin et al., 1995; Hess et al., 1995; Radulovic et al., 1998; Zhu et al., 1995), as well as shorter periods of sleep deprivation than prolonged periods (Cirelli et al., 1995, 2000).

It is well-known that the amygdaloid complex (mainly the basolateral portion) play a key role in the neurobiological mechanisms involved in aversive memory formation. In fact, it was shown that the inactivation of the basolateral amygdala impairs memory formation and consolidation of the PM-DAT in rats (Ribeiro et al., 2011). Herein, we observed an increase in c-fos expression in the basolateral amygdala, regardless of the sleep deprivation regimen employed. This result may be due to wake *per se*, as pointed before. Additionally, this finding could reflect the stressing component of the TSD procedure. Indeed, the activation of the hypothalamic-pituitary-adrenocortical axis has been reported after both acute (Campbell et al., 2002; Sgoifo et al., 2006) and repeated sleep restriction (Roman et al., 2006) by forced locomotion. Thus, once stress and glucocorticoids may influence memory formation (de

Kloet et al., 1999; McEwen and Sapolsky, 1995; McGaugh and Roozendaal, 2009), one could argue that the observed memory deficits could be a consequence of stress and not of TSD. However, this does not seem to be the case because although all groups presented an increase in c-fos expression, only those repeatedly sleep-deprived for 3 h or acutely deprived for 6 h showed amnesia. Notably, it was demonstrated a differentiation between the outcomes of stress and sleep loss on memory since metyrapone treatment did not prevent paradoxical sleep deprivation-induced memory impairment (Tiba et al., 2008).

Ideally, an insomnia animal model should exhibit decreased sleep quality or quantity during the period that the animal is supposed to be physiologically sleeping. Thus, there are potential models such as: electrical stimulation, drug-induced methods and those related to the interruption of sleep as water platform techniques and forced locomotion (Revel et al., 2009). However, these sleep deprivation methods may cause excessive stress or result in incomplete sleep loss, having unfavorable effects on experimental results. Oppositely, the gentle handling method seems to be less stressful. Thus, considering that the applied protocol of TSD for 3 h during 10 days animals were sleep-restricted during the light phase, as well as it promoted memory deficits, it brings the perspective of an efficient sleep restriction protocol to mimic insomnia characteristics.

Collectively, our results show that both the duration and frequency of TSD may critically influence the subsequent cognitive impairing-effect. Notably, not only the cognitive effects but also the behavioral alterations (anxiety and locomotion) may be tolerated or sensitized. These findings strengthen the importance of future studies evaluating the effects of short periods of sleep restriction.

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FIGURE LEGENDS

Figure 1: Effects of acute total sleep deprivation (TSD) on learning and consolidation of a discriminative task in mice. Animals were subjected to control condition (CTRL) or TSD for 3 or 6 h. After TSD period, mice were trained and 12 later tested. Results are presented as the mean ± S.E. of time spent in the non-aversive enclosed arm x time spent in the aversive enclosed arm in the training (**A**) and testing (**D**), percent time spent in the open arms in the training (**B**) and testing (**E**), and number of entries in the training (**C**) and testing (**F**). *p<0.05 compared to time spent in the non-aversive arm; ºp<0.05 compared to the other groups (two- or one-way ANOVA and Duncan's test).

Figure 2: Effects of repeated total sleep deprivation (TSD) on learning and consolidation of a discriminative task in mice. Animals were subjected to control condition (CTRL) or TSD for 3 or 6 h during 10 consecutive days. In the 10^{th} day, after TSD period, mice were trained and 12 later tested. Results are presented as the mean ± S.E. of time spent in the non-aversive enclosed arm x time spent in the aversive enclosed arm in the training (**A**) and testing (**D**), percent time spent in the open arms in the training (**B**) and testing (**E**), and number of entries in the training (**C**) and testing (**F**). *p<0.05 compared to time spent in the non-aversive arm (two- or one-way ANOVA and Duncan's test).

Figure 3: Effects of repeated total sleep deprivation (TSD) on c-fos protein expression. Animals were subjected to control condition (CTRL) or TSD for 3 or 6 h during 1 or 10 consecutive days. In the 10^{th} day, after TSD period, mice

were euthanized and the brains were collected. Results are presented as the mean ± S.E. of c-fos positive cells in the dentate gyrus (**A**) and basolateral amygdala (**B**). [a]$p<0.05$ compared to CTRL group; [#]$p<0.005$ compared to the other groups (ANOVA and Duncan's test).

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3.1.1 Conclusões parciais – manuscrito 1

No presente manuscrito buscamos caracterizar um período de privação de sono total que pudesse mimetizar os efeitos da insônia em humanos no que diz respeito aos aspectos cognitivos. Em outras palavras, um período de restrição de sono diário que fosse capaz de prejudicar a memória, assim como é observado na condição clínica. Em conjunto, os resultados apresentados no 1º manuscrito da presente Tese demonstraram que tanto a duração quanto a frequência da privação de sono parecem influenciar criticamente na indução de prejuízos cognitivos, levando à tolerância ou à sensibilização das alterações comportamentais e cognitivas. Especificamente, a privação de sono total aguda por 3 h induziu efeitos ansiolítico e hiperlocomotor sem prejudicar a memória. Por ouro lado, após a repetição durante 10 dias houve tolerância aos efeitos comportamentais e observamos um prejuízo cognitivo. A privação de sono total aguda por 6 h prejudicou o desempenho da tarefa. Contudo, após a sua repetição, tal prejuízo foi tolerado.

Para investigarmos um possível mecanismo para os achados comportamentais dos diferentes períodos de privação de sono, avaliamos a expressão da proteína c-fos no giro denteado e na amígdala basolateral. Todos os protocolos de privação de sono aumentaram a expressão de c-fos em ambas as regiões analisadas. Contudo, no giro denteado, a privação de sono aguda por 3 h promoveu um aumento de maior magnitude em relação aos demais grupos. Esses resultados sugerem que o melhor desempenho cognitivo estaria relacionado à maior ativação do giro denteado. Ainda, os resultados sugerem que os achados comportamentais estão relacionados à perda de sono

per se e não ao estresse inerente ao protocolo de privação, uma vez que a ativação da amígdala basolateral foi de mesma magnitude em todos os grupos. Coletivamente, esses dados sugerem que a privação de sono total desencadeia fenômenos plásticos de adaptação no sistema nervoso central.

Conforme revisado por Revel e colaboradores (2009), idealmente um modelo animal de insônia deve mimetizar as principais características da insônia em humanos, ou seja, deve exibir qualidade ou quantidade de sono diminuídas no período em que o animal estaria fisiologicamente dormindo. Nesse cenário, tendo em vista que a privação de sono total repetida por 3 h restringiu o sono dos animais no período diurno e, além disso, também promoveu déficits de memória no modelo da ED-LCE, escolhemos utilizar esse protocolo de insônia experimental nos experimentos subsequentes.

Considerando a crescente utilização do Zolp no tratamento da insônia e que clinicamente o tratamento com esse hipnótico se inicia após períodos variáveis de privação de sono, no 2º manuscrito avaliamos os efeitos do tratamento repetido com Zolp ou de sua retirada abrupta sobre o desempenho em camundongos mantidos sob o regime de restrição de sono avaliados na ED-LCE.

Manuscrito 2

INTERACTIONS BETWEEN ZOLPIDEM AND SLEEP RESTRICTION: BIDIRECTIONAL EFFECTS ON MEMORY IN MICE

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ABSTRACT

The cognitive effects of the repeated administration of Zolpidem (Zolp) in animal models of sleep restriction (SR) remain poorly understood. We evaluated the effects of the repeated administration of Zolp (or its withdrawal) on the memory phases of mice subjected to SR in the plus-maze discriminative avoidance task. Additionally, we investigated the possible reversion of effects by the antagonist β-cct. Mice were subjected to SR for 3 h per day during 10 days or left undisturbed and were then treated with saline (Sal) or Zolp. In the 10^{th} day, half of the animals treated with Sal received Zolp and the others received Sal. The same occurred for mice treated daily with Zolp. No modifications were found during the learning phase. Both the acute and repeated pre-training administration of Zolp promoted amnesia. SR induced memory deficits, which were counteracted by the acute administration of Zolp, or its withdrawal. No effects were found on memory consolidation after concomitant Zolp treatment and SR. When the drug was post-training administered in sleep-restricted mice, Zolp blocked the SR-induced memory impairment. For retrieval, only the repeated administration prevented amnesia. β-cct counteracted only the acute effects of Zolp. The interaction between SR and Zolp can positively or negatively modulate memory, depending on the phase. The α1-subunit activation explains the acute effects but not the long-term administration, which suggests the participation of other mechanisms. These data strengthen the importance of conducting future studies that evaluate the interaction between different SR periods and Zolp administration.

Keywords: hypnotics, zolpidem, sleep restriction, cognition, plus-maze discriminative avoidance task, rodents.

INTRODUCTION

It is well established that sleep plays a critical role in learning and memory formation. Indeed, many studies have demonstrated that both paradoxical sleep deprivation and total sleep deprivation for 6 h leads to memory deficits in several animal models (Fernandes-Santos et al., 2012; Palchykova et al., 2006). It has been suggested that total sleep deprivation could promote cognitive impairment of a higher magnitude than the impairments induced by the exclusive deprivation of paradoxical sleep (Patti et al., 2010). Experimentally, sleep restriction (SR) models, in which animals are repeatedly subjected to shorter periods of sleep, has been increasingly used as a strategy to mimic the sleep loss found in modern society. This represents an important translational model because SR is most prevalent because of medical conditions and lifestyle (Banks and Dinges, 2007).

In humans, studies have found that SR impairs behavioral alertness, with deterioration increasing in magnitude as the time allowed for sleep is reduced. Moreover, the neurobehavioral effects of SR appear to be similar to those of total sleep deprivation (Belenky et al., 2003; Van Dongen et al., 2003). In animal models, it has been demonstrated that SR using forced locomotion models impaired spatial memory (McCoy et al., 2013; Zielinski et al., 2013). Of note, Yang and colleagues (2012) evaluated the effects of SR on memory using the less stressing sleep deprivation method of gentle handling. In their study, the SR protocol hindered the consolidation of spatial memory in adolescent (but not in adult) rats.

Zolpidem (Zolp) is a hypnotic drug that is prescribed to treat insomnia. It is an imidazopyridine agent that selectively binds to the α1 subunit-containing $GABA_A$ receptor subtype (Sancar et al., 2007). Though Zolp appears to exhibit fewer deleterious psychomotor and cognitive effects, amnestic effects have been repeatedly reported both in humans (Fitzgerald et al., 2014) and in laboratory animals (Huang et al., 2010; Savić et al., 2005a,b). Specifically, we have recently demonstrated that the acute administration of 5 or 10 mg/kg Zolp (but not 2 mg/kg) induced amnesia in mice that were submitted to the plus-maze discriminative avoidance task (PM-DAT). Such impairment occurred when Zolp was administered either before or 2–3 h after training and was not related to state dependency, sedation or anxiolysis (Zanin et al., 2013). Additionally, the acute administration of 10 mg/kg Zolp promoted habituation deficits in the open field arena (Zanin et al., 2011).

From a clinical perspective, Zolp is typically prescribed as a repeated treatment for insomnia. To the best of our knowledge, no studies have characterized the effects of the repeated administration of Zolp on memory in animal models of SR. Thus, considering the increasing frequency of Zolp prescription, the previous evidence supporting the impairing effects induced by acute administration of this drug, and the absence of studies that systematically evaluated the effects of repeated treatment with Zolp in sleep-restricted animals, the aim of the present study was to evaluate the effects of repeated administration of Zolp (or its withdrawal) on the different memory phases (learning, consolidation and retrieval) of mice submitted to SR for 10 days and evaluated in the PM-DAT. Therefore, we performed the following order of experiments. First, we evaluated learning by a pre-training treatment and then

examined consolidation in 3 steps: 1- SR and treatment immediately after PM-DAT training; 2- Zolp administration at different time points after PM-DAT training; and 3- SR before training and Zolp injection 2 h after. Subsequently, we evaluated retrieval by repeating the protocol of experiment 1 before testing. Finally, to specifically evaluate a possible reversal of the amnestic effects of pre-training Zolp administration, we tested the antagonistic effects of β-carboline-3-carboxylate t-butyl-ester (β-cct).

MATERIAL AND METHODS

Subjects

Three-month-old Swiss male mice (outbred, raised, and maintained in the Centre for Development of Experimental Models in Medicine and Biology of Universidade Federal de São Paulo) were used. Animals weighing 35-40 g were housed under controlled temperature (22-23°C) and lighting (12 h light, 12 h dark; lights on at 6:45 a.m.) conditions. Food and water were available *ad libitum* throughout the experiments. The animals used in the present study were maintained in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications Nº 8023, revised 2011) and with the Brazilian Law for Procedures for Animal Scientific Use (#11794/2008). The experimental procedures were approved by the Institutional Animal Care and Use Committee under the protocol #1741/10.

Drugs

Zolp (Sanofi-Aventis®) was diluted in 0.9% saline solution (Sal), which was also the control solution, and was administered at a dose of 5 mg/kg. The dose of Zolp was chosen based on previous experiments conducted by our group that demonstrated memory deficits and relatively weak sedative effects in mice (Zanin et al., 2013).

To specifically evaluate a possible reversal of the effects, we used β-carboline-3-carboxylate t-butyl-ester (β-cct; Sigma®) in Experiment V. Although several β-carbolines appear to be inverse agonists, β-cct is an antagonist that does not potentiate pentylenetetrazol-induced convulsions or facilitate the

suppression of lever-pressing behavior by shock (Shannon et al., 1988). We chose the dose of 30 mg/kg, which was diluted with the aid of sonication in a solvent containing 85% distilled water, 14% propylene glycol, and 1% Tween-80, which was the vehicle control solution (Veh). The dose of β-cct was based on previous reports (Belzung et al., 2000; Savić et al., 2005a,b). All solutions were administered intraperitoneally in a volume of 10 ml/kg

Sleep Restriction (SR)

Mice were submitted to SR through the gentle handling method, as described previously (Tobler and Borbély, 1990). It consists of keeping the animal awake by tapping on the cage and, if necessary, gently touching them with a soft brush if behavioral signs of sleep are observed. The animals were sleep restricted for 3 h (from 10 AM to 1 PM) for 10 consecutive days. This time interval was chosen because this is when paradoxical sleep reaches its highest expression and slow wave sleep homeostatic pressure is generated (Franken et al., 1991).

Plus-maze discriminative avoidance task (PM-DAT)

The PM-DAT was used to concomitantly evaluate learning, memory, anxiety-like behavior, and motor activity, as described previously (Frussa-Filho et al., 2010). The aversive stimuli consisted of a 100-W light and an air blow produced by a 110-V hairdryer positioned over the aversive enclosed arm. The aversive stimuli were present during the 10-min training session. Tests sessions lasted 3 min, performed in the absence of the aversive stimuli. In all experiments, observations were recorded by an observer blinded to the

experimental conditions, and the apparatus was cleaned with a 5% alcohol solution after each behavioral session.

Specifically, learning was evaluated as a progressive decrease in percent time spent in the aversive enclosed arm (time spent in aversive enclosed arm/time spent in both enclosed arms) throughout the training. Learning and memory were evaluated by the time spent in the aversive versus the non-aversive enclosed arms in the testing. Anxiety-like behavior was evaluated by the percent time spent in the open arms of the apparatus (time spent in open arms/time spent in both open and enclosed arms). The total number of entries into any of the arms was used to evaluate locomotion.

Experimental design

Experiment I: effects of the repeated administration of Zolp or its withdrawal on learning and consolidation in sleep-restricted mice

Animals were kept in their home cages (control condition – CTRL) or subjected to SR by gentle handling for 3 h per day during 10 days. Every day, after the SR period, mice were treated with Sal (CTRL-Sal and SR-Sal) or Zolp (CTRL-Zolp and SR-Zolp). On the 10^{th} day, half of the animals treated with Sal received an acute Zolp injection, and the others received Sal. The same procedure was applied for mice that were treated daily with Zolp. The following groups were formed: CTRL-Sal/Sal (n=12), CTRL-Sal/Zolp (n=12), CTRL-Zolp/Sal (n=12), CTRL-Zolp/Zolp (n=12), SR-Sal/Sal (n=12), SR-Sal/Zolp (n=12), SR-Zolp/Sal (n=12) and SR-Zolp/Zolp (n=12). Thirty min after the last injection, mice were subjected to training in the PM-DAT. In this experiment, to

specifically evaluate learning, in addition to quantifying the total amount of time spent in the aversive enclosed arm in the training during the 10-min session, the progressive decrease in the exploration of the aversive enclosed arm was quantified min-by-min throughout the session, and the percent of time spent in the aversive enclosed arm in each min was calculated. Twelve days later, the animals were tested.

Experiment II: effects of the repeated administration of Zolp exclusively on memory consolidation in sleep-restricted mice

Initially, mice were trained in the PM-DAT. Immediately after training animals were subjected to CTRL or SR condition. Every day, after the SR period, mice were treated with Sal or Zolp, with the following groups formed: CTRL-Sal (n=10), CTRL-Zolp (n=10), SR-Sal (n=10), and SR-Zolp (n=10). Forty-eight hours after the last injection (12 days after training), mice were tested.

Experiment III: effects of the acute administration of Zolp immediately or 2 h after training on consolidation in sleep-restricted mice

Animals were maintained under CTRL or SR condition. On the 10^{th} day, after SR period, mice were trained. After training, mice received an acute injection of Sal or Zolp immediately after it or 2 h later. This time-point was chosen based on an additional experiment detailed in the Supplementary file. The Sal group received 2 Sal injections while the other groups received a single dose of Zolp and another Sal injection, forming the groups: CTRL-Sal (n=12);

CTRL-Zolp 0h (n=12); CTRL-Zolp 2h (n=12); SR-Sal (n=12); SR-Zolp 0h (n=12); SR-Zolp 2h (n=12). Twelve days after training, testing was performed.

Experiment IV: effects of the repeated administration of Zolp or its withdrawal on memory retrieval in sleep-restricted mice

Mice were trained in the PM-DAT. Forty-eight hours later, animals received the same treatment as described for the experiment I, forming the following groups: CTRL-Sal/Sal (n=12), CTRL-Sal/Zolp (n=12), CTRL-Zolp/Sal (n=12), CTRL-Zolp/Zolp (n=12), SR-Sal/Sal (n=12), SR-Sal/Zolp (n=12), SR-Zolp/Sal (n=12) and SR-Zolp/Zolp (n=12). Thirty min after the last injection, mice were subjected to testing.

Experiment V: effects of the acute administration of β-cct on memory in sleep-restricted mice treated with Zolp

Animals were kept under CTRL or SR condition. Every day, after the SR period, CTRL mice were treated with Sal (CTRL-Sal, n=10). Sleep-restricted mice received a Sal injection (SR-Sal, n=20) or Zolp (SR-Zolp, n=10). On the 10^{th} day, after the SR period, each animal received 2 injections at a 5-min interval. The 1^{st} injection was Veh or β-cct, and the 2^{nd} injection was Sal or Zolp, with the following groups formed: CTRL-Sal/Veh+Sal (n=10), SR-Sal/Veh+Sal (n=10), SR-Sal/β-cct+Zolp (n=10), and SR-Zolp/β-cct+Zolp (n=10). Thirty min after the 2^{nd} injection, mice were subjected to training and were tested 12 days later.

Statistical analysis

The decrease in the percent time spent in the aversive enclosed arm throughout the training was compared using ANOVA with repeated measures. MANOVA and Duncan's test were used to analyze the other parameters. A value of $p<0.05$ was considered significant for all comparisons.

RESULTS

Experiment I: effects of the repeated administration of Zolp or its withdrawal on learning and consolidation in sleep-restricted mice

During training, the percent time spent in the aversive enclosed arm min by min was analyzed using ANOVA with repeated measures, with time (min of observation) representing the within-subject variance and sleep condition (CTRL or SR), treatment (Sal or Zolp) and challenge injection (Sal or Zolp) all representing the between-subject variance. These analyses revealed significant effects of time [$F(9,792)=3.75$; $p<0.001$], treatment x challenge injection [$F(1,88)=7.22$; $p=0.009$] and sleep condition x treatment x challenge injection interactions [$F(1,88)=6.16$; $p<0.05$]. Thus, all animals progressively avoided the aversive arm (Figure S1). In accordance, MANOVA revealed significant effects of arm type (aversive x non-aversive) [$F(1,176)=750.38$; $p<0.001$], challenge injection [$F(1,176)=5.66$; $p<0.05$] and arm type x challenge injection interaction [$F(1,176)=11.08$; $p=0.001$]. Duncan's test revealed that all groups spent significantly less time in the aversive enclosed arm than in the non-aversive arm (Figure 1A).

Concerning anxiety-like behavior, MANOVA for the percent time spent in the open arms revealed no significant differences among groups (Figure 1B). Regarding locomotion, MANOVA for total number of entries revealed significant effects of sleep condition [$F(1,88)=5.79$; $p<0.05$] and challenge injection [$F(1,88)=60.08$; $p<0.001$]. Duncan's test showed that all of the animals that received Zolp before training displayed decreased motor activity compared to their respective control. Although sleep-restricted mice that were acutely treated

with Zolp (SR-Sal/Zolp) presented hypolocomotion, it was of a lesser magnitude compared to the respective control for the sleep condition (CTRL-Sal/Zolp) (Figure 1C).

In the testing, MANOVA revealed significant effects of arm type [$F(1,176)=10.39$; $p=0.002$], arm type x challenge injection [$F(1,176)=11.43$; $p=0.001$], arm type x sleep condition x challenge injection [$F(1,176)=8.65$; $p=0.004$] and arm type x sleep condition x treatment x challenge injection interactions [$F(1,176)=10.88$; $p=0.001$]. The *post hoc* test showed that for CTRL animals, only the group that was treated and challenged with Sal (CTRL-Sal/Sal) and the group that was subjected to Zolp withdrawal (CTRL-Zolp/Sal) discriminated the enclosed arms and spent less time in the aversive one. Regarding sleep-restricted mice, only the group that was acutely treated with Zolp (SR-Sal/Zolp) and the group that did not undergo treatment (SR-Zolp/Sal) discriminated the enclosed arms (Figure 1D). No differences were found either in the time spent in the open arms or in locomotion (Figures 1E and 1F, respectively).

Experiment II: effects of the repeated administration of Zolp exclusively on memory consolidation in sleep-restricted mice

During training, MANOVA revealed only significant arm type effects [$F(1,72)=387.84$; $p<0.001$]. As expected, because there was no pre-training manipulation, Duncan's *post hoc* test revealed that all of the groups spent significantly less time in the aversive enclosed arm than in the non-aversive arm. No differences were found concerning basal anxiety-like behavior or motor activity (Table S1).

During testing, MANOVA revealed significant effects of arm type [$F(1,72)=136.98$; $p<0.001$] and arm type x treatment interaction [$F(1,72)=4.75$; $p<0.05$]. Duncan's test revealed that all of the groups spent significantly less time in the aversive enclosed arm than in the non-aversive one (Figure 2A). MANOVA did not show significant effects of time spent in the open arms (Figure 2B). Regarding the total number of entries, there was a significant effect of sleep condition [$F(1,36)=6.95$; $p<0.05$]. The *post hoc* test demonstrated that both sleep-restricted groups presented increased motor activity compared to their respective controls (Figure 2C).

Experiment III: effects of the acute administration of Zolp immediately or 2 h after training on consolidation in sleep-restricted mice

MANOVA only revealed significant effects of arm type [$F(1,132)=532.95$; $p<0.001$] during the training. Duncan's *post hoc* test revealed that all of the groups spent significantly less time in the aversive enclosed arm than in the non-aversive arm. No differences were found regarding anxiety-like behavior or motor activity (Table S3).

In the testing, MANOVA followed by Duncan's test revealed significant effects of arm type [$F(1,132)=16.24$; $p<0.001$] and arm type x treatment interaction [$F(5,132)=4.72$; $p=0.001$]. Thus, for those animals in the CTRL condition, only those treated with Sal or Zolp immediately after training (CTRL-Sal and CTRL-Zolp 0h) retrieved the task. Those animals that were sleep-restricted and treated with Zolp immediately after training (SR-Zolp 0h) spent significantly less time in the aversive arm than in the non-aversive enclosed arm. The other groups did not discriminate between the enclosed arms (Figure

3A). One-way ANOVA did not reveal significant effects for the percent time spent in the open arms (Figure 3B) or for the total number of entries (Figure 3C).

Experiment IV: effects of the repeated administration of Zolp or its withdrawal on memory retrieval in sleep-restricted mice

During training, MANOVA revealed significant effects of arm type [$F(1,176)=1381.26$; $p<0.001$]. As expected, Duncan's test revealed that all of the groups spent significantly less time in the aversive enclosed arm than in the non-aversive one. No differences were found concerning basal emotionality or motor activity (Table S4).

MANOVA revealed significant effects of arm type [$F(1,176)=64.06$; $p<0.001$], arm type x sleep condition [$F(1,176)=6.85$; $p<0.05$], arm type x treatment [$F(1,176)=11.33$; $p=0.001$], arm type x challenge injection [$F(1,176)=9.20$; $p=0.003$] and arm type x treatment x challenge injection [$F(1,176)=6.93$; $p=0.009$] interactions in the testing. Duncan's *post hoc* test revealed that among the CTRL animals, only the mice subjected to Zolp withdrawal (CTRL-Zolp/Sal) did not discriminate the enclosed arms. Concerning the sleep-restricted animals, only mice that were repeatedly treated with Zolp (SR-Zolp/Zolp) spent significantly less time in the aversive than in the non-aversive enclosed arm (Figure 4A).

Further, MANOVA did not reveal significant effects for the percent time spent in the open arms (Figure 4B). For the total number of entries, MANOVA showed significant sleep condition [$F(1,88)=14.55$; $p<0.001$] and challenge injection [$F(1,88)=70.68$; $p<0.001$] effects. The *post hoc* analyses demonstrated

that the mice challenged with Zolp displayed decreased motor activity compared to their respective control groups, which were challenged with Sal. For sleep-restricted mice, those treated and challenged with Sal (SR-Sal/Sal) presented hyperlocomotion compared to their respective CTRL mice (CTRL-Sal/Sal). Similarly, those mice acutely treated with Zolp (SR-Sal/Zolp) also showed increased motor activity compared to those in the CTRL condition that were treated in the same manner (CTRL-Sal/Zolp) (Figure 4C).

Experiment V: effects of the acute administration β-cct on memory in sleep-restricted mice and treated with Zolp

During training, MANOVA showed a significant effect of arm type [$F(1,72)=261.06$; $p<0.001$]. Duncan's test demonstrated that all animals spent significantly less time in the aversive enclosed arm than in the non-aversive arm (Figure 5A). Regarding anxiety-like behavior, no significant effects were detected (Figure 5B). Similarly, no effects were found for the total number of entries (Figure 5C).

In the testing, MANOVA revealed significant effects of arm type x treatment interaction [$F(3,72)=11.54$; $p<0.001$]. Duncan's test showed that only mice in the CTRL group discriminated the enclosed arms (Figure 5D). When the percent time spent in the open arms was analyzed, no differences were observed (Figure 5E). Regarding the total number of entries, there were significant treatment effects [$F(3,36)=3.47$; $p<0.05$]. In fact, Duncan's test revealed that mice subjected to SR and treated with Sal (SR-Sal/Veh+Sal) or mice treated with Zolp and challenged with β-cct and Zolp (SR-Zolp/β-cct+Zolp) displayed higher motor activity compared to the control group (Figure 5F).

Figure 1

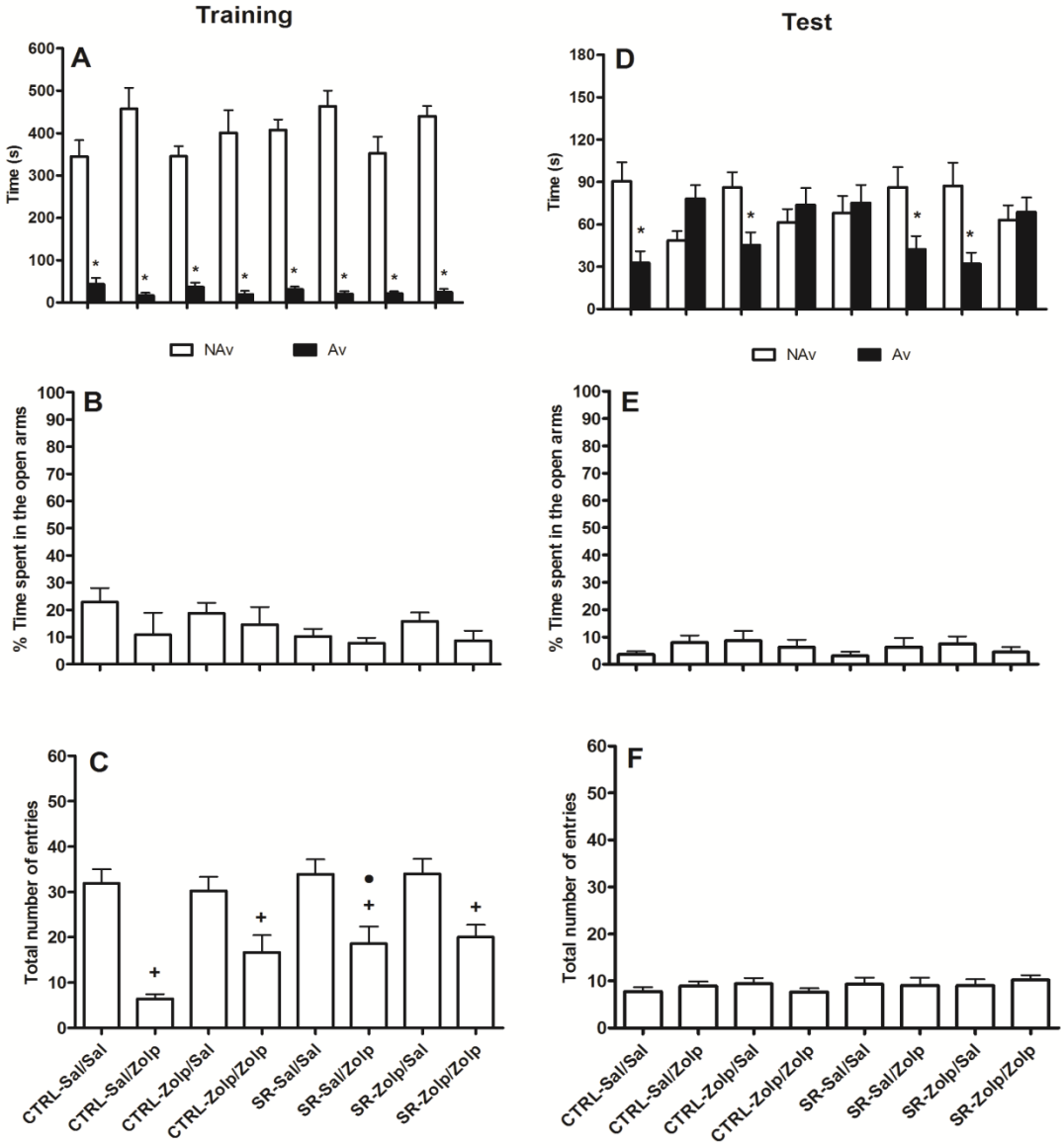

Figure 1: Effects of the repeated administration of zolpidem (Zolp), or its withdrawal, on learning and consolidation of memory for a discriminative task in mice submitted to sleep restriction (SR). Animals were subjected to a control condition (CTRL) or SR for 10 days and were treated daily with Sal or 5 mg/kg Zolp. On the 10th day, after the last injection, mice were trained and then tested 12 days later. The results are presented as the mean ± S.E. of time spent in the non-aversive enclosed arm x time spent in the aversive enclosed arm in the training (**A**) and testing (**D**), percent time spent in the open arms in the training (**B**) and testing (**E**), and number of entries in the training (**C**) and testing (**F**). *p<0.05 compared to the time spent in the non-aversive arm; +p<0.05 compared to the group of same sleep condition and treatment but different challenge injection; •p<0.05 compared to the group of same treatment and challenge injection but different sleep condition (MANOVA and Duncan's test).

Figure 2

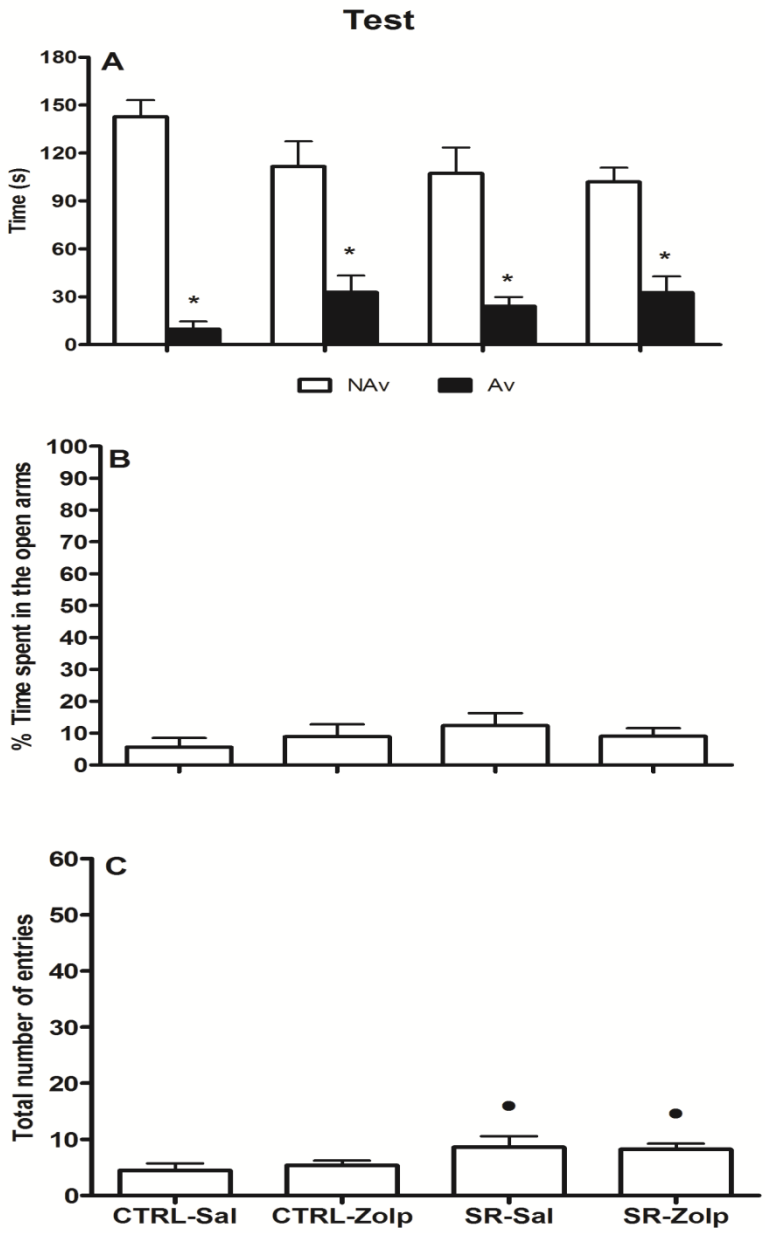

Figure 2: Effects of the repeated administration of zolpidem (Zolp) exclusively on memory consolidation of a discriminative task in mice submitted to sleep restriction (SR). First, mice were trained, followed by being subjected to control condition (CTRL) or SR for 10 days, and treated daily with Sal or 5 mg/kg Zolp. Forty-eight hours after the last injection, mice were tested. The results are presented as the mean ± S.E. of time spent in the non-aversive enclosed arm x time spent in the aversive enclosed arm (**A**), percent time spent in the open arms (**B**) and number of entries (**C**) during testing. *p<0.05 compared to the time spent in the non-aversive arm; •p<0.05 compared to the group of same treatment and challenge injection but different sleep condition (MANOVA and Duncan's test).

Figure 3

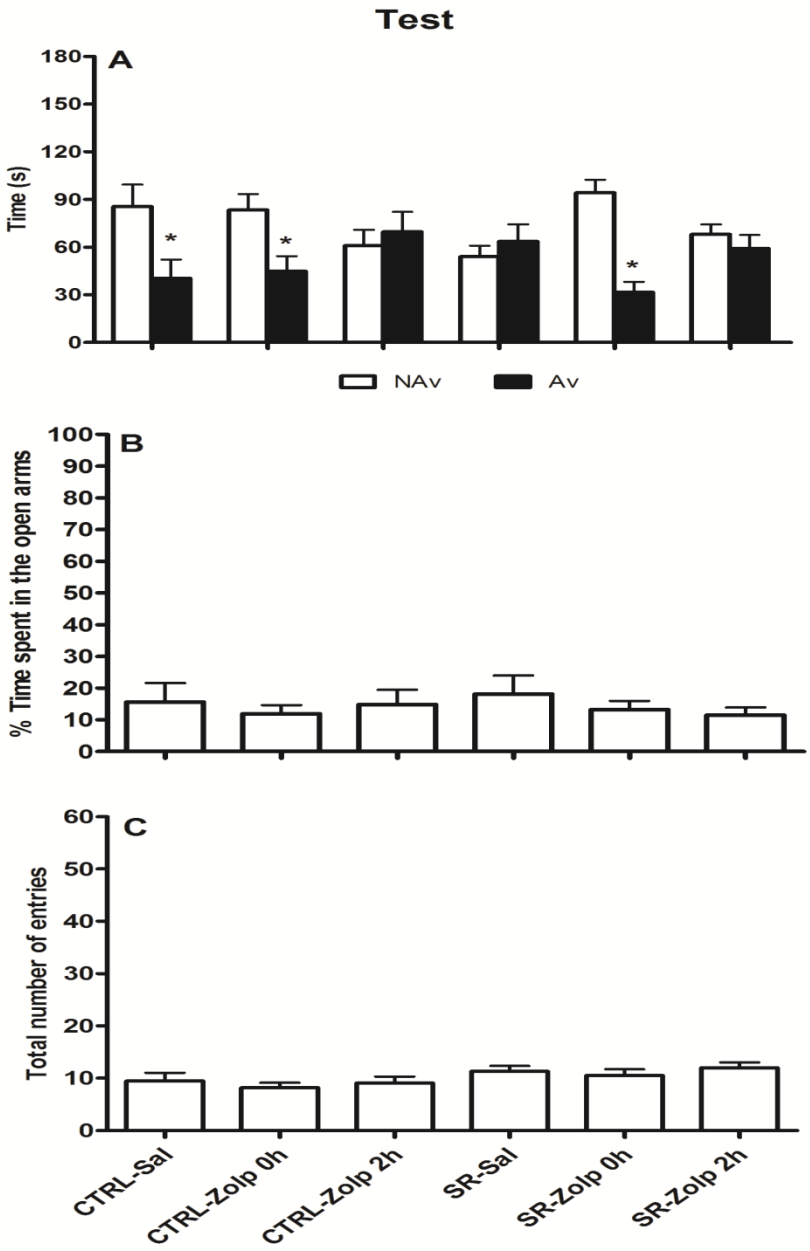

Figure 3: Effects of the acute administration of zolpidem (Zolp) immediately or 2 h after training on consolidation of a discriminative task in mice submitted to sleep restriction (SR). Animals were subjected to control condition (CTRL) or SR during 10 days. On the 10th day, after the SR period, mice were trained in the PM-DAT and were treated with Sal or 5 mg/kg Zolp immediately (0 h) or 2 h (2 h) after training. Testing was performed 12 days later. The results are presented as the mean ± S.E. of time spent in the non-aversive enclosed arm x time spent in the aversive enclosed arm (**A**), percent time spent in the open arms (**B**) and number of entries (**C**) during testing. *p<0.05 compared to the time spent in the non-aversive arm (MANOVA and Duncan's test).

Figure 4

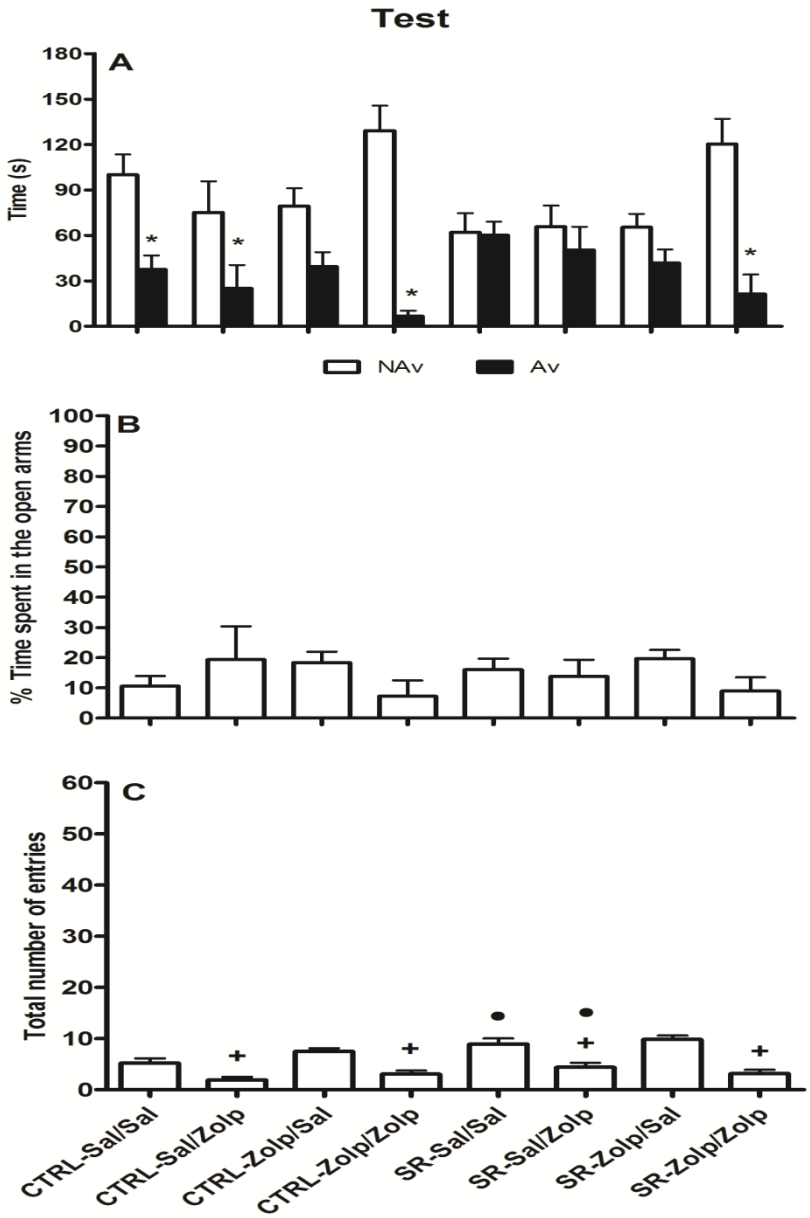

Figure 4: Effects of the repeated administration of zolpidem (Zolp) or its withdrawal on retrieval of a discriminative task in mice submitted to sleep restriction (SR). First, mice were trained. Forty-eight hours later, animals were subjected to control conditions (CTRL) or to SR for 10 days and were daily treated with Sal or 5 mg/kg Zolp. After the last injection, mice were tested. The results are presented as the mean ± S.E. of time spent in the non-aversive enclosed arm x time spent in the aversive enclosed arm (**A**), percent time spent in the open arms (**B**) and number of entries (**C**) during testing. *p<0.05 compared to the time spent in the non-aversive arm; +p<0.05 compared to the group of same sleep condition and treatment but different challenge injection; •p<0.05 compared to the group of same treatment and challenge injection but different sleep condition (MANOVA and Duncan's test).

Figure 5

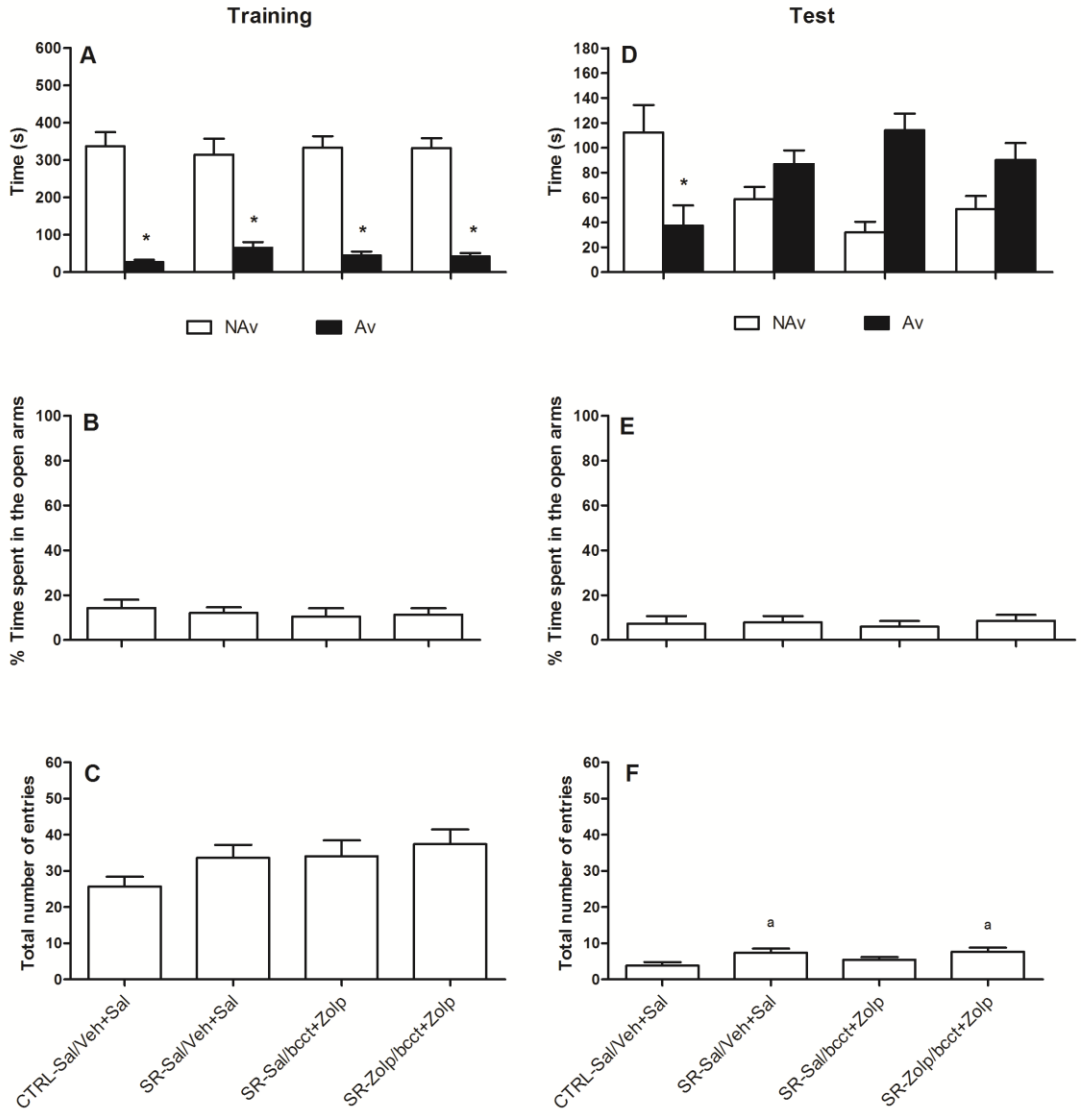

Figure 5: Effects of the acute administration of β-cct on discriminative memory in mice submitted to sleep restriction (SR) and treated with zolpidem (Zolp). Animals were subjected to control condition (CTRL) or SR for 10 days and were daily treated with Sal or 5 mg/kg Zolp. On the 10th day, after the SR period, each mouse received 2 injections at a 5-min interval. Thus, they received vehicle (Veh) followed by Sal or β-cct followed by Zolp injection. After the injection, mice were trained and then tested 12 days later. The results are presented as the mean ± S.E. of time spent in the non-aversive enclosed arm x time spent in the aversive enclosed arm in the training (**A**) and testing (**D**), percent time spent in the open arms in the training (**B**) and testing (**E**), and number of entries in the training (**C**) and testing (**F**). *$p<0.05$ compared to the time spent in the non-aversive arm; $^{a}p<0.05$ compared to the CTRL-Sal/Veh+Sal group (ANOVA and Duncan's test).

Supplementary information

Supplementary material and Methods

Additional experiment: effects of Zolp administered at specific intervals after training

Mice were trained in the PM-DAT and were then assigned to one of the following groups: CTRL (n=15), Sal (n=15), Zolp 1 h (n=15), Zolp 2 h (n=15), Zolp 3 h (n=15) or Zolp 6 h (n=15). Thus, mice received Zolp at different time points (1, 2, 3, or 6 h) after training. The CTRL group was handled at each time point, while the Sal group received Sal injections at all following time points. The Zolp groups received the drug at a single time point after training and received Sal injections at all of the remaining time points. The test session occurred 12 days later.

Supplementary results

Baseline performance of mice exposed to the training session in the PM-DAT in experiment II

Groups	TNAv	TAv	PTO	NE
CTRL-Sal	342.80 ± 37.30	30.10 ± 6.56 *	18.49 ± 4.00	30.90 ± 2.26
CTRL-Zolp	306.60 ± 28.15	34.00 ± 8.80 *	19.28 ± 2.90	36.90 ± 3.47
SR-Sal	319.70 ± 19.52	33.30 ± 10.06 *	22.60 ± 4.80	37.40 ± 3.78
SR-Zolp	324.10 ± 22.23	36.40 ± 5.27 *	20.63 ± 3.15	36.60 ± 3.44

Table S1 – Effects of the repeated administration of zolpidem (Zolp) exclusively on memory consolidation. The results are presented as the mean ± SE of time spent in the non-aversive enclosed arm (TNAv), time spent in the aversive enclosed arm (TAv), percent time spent in the open arms (PTO) and total number of entries (NE) during the training session in the plus-maze discriminative avoidance task (PM-DAT). *$p<0.05$ compared to the time spent in the non-aversive arm (MANOVA followed by Duncan's test).

Additional e*xperiment: effects of Zolp administered at specific intervals after training*

In the training, MANOVA only revealed significant effects of arm type [$F(1,168)=891.30$; $p<0.001$]. Duncan's test revealed that all of the groups spent significantly less time in the aversive enclosed arm than in the non-aversive one, as expected. There were no differences concerning anxiety-like behavior or motor activity (Table S2).

During testing, MANOVA followed by Duncan's test showed a significant effect only for arm type [$F(1,168)=71.42$; $p<0.001$]. Thus, the groups treated with Zolp 2 or 6 h after training did not discriminate the enclosed arms (Figure S2A). Concerning the exploration of open arms, there were no significant differences among groups (Figure S2B), and there were no effects on motor activity (Figure S2C).

Baseline performance of mice exposed to the training session in the PM-DAT in experiment SI

Groups	**TNAv**	**TAv**	**PTO**	**NE**
Ctrl	338.60 ± 18.80	23.67 ± 4.84 *	23.75 ± 6.13	35.80 ± 2.83
Sal	352.40 ± 89.23	28.8 ± 5.99 *	30.17 ± 7.75	34.33 ± 2.94
Zolp 1h	269.67 ± 23.49	27.47 ± 5.16 *	32.84 ± 5.91	43.40 ± 3.58
Zolp 2h	332.07 ± 28.55	18.20 ± 3.97 *	31.12 ± 6.77	32.33 ± 2.88
Zolp 3h	323.47 ± 18.18	26.20 ± 5.95 *	23.28 ± 5.75	36.73 ± 3.13
Zolp 6h	358.00 ± 31.06	24.40 ± 4.10 *	27.56 ± 6.95	40.27 ± 2.91

Table S2 – Effects of zolpidem (Zolp) administered in specific intervals after training in mice. The results are presented as the mean ± SE of time spent in the non-aversive enclosed arm (TNAv), time spent in the aversive enclosed arm (TAv), percent time spent in the open arms (PTO) and total number of entries (NE) during the training session in the plus-maze discriminative avoidance task (PM-DAT). *$p<0.05$ compared to the time spent in the non-aversive arm (MANOVA followed by Duncan's test).

Performance of mice that were sleep restricted and exposed to the training session in the PM-DAT in experiment III

Groups	**TNAv**	**TAv**	**PTO**	**NE**
CTRL-Sal	277.08 ± 37.30	32.50 ± 6.56 *	30.76 ± 7.19	43.75 ± 5.39
CTRL-Zolp 0h	287.75 ± 28.15	42.17 ± 8.80 *	21.66 ± 3.07	44.25 ± 3.84
CTRL-Zolp 2h	309.17 ± 19.52	41.75 ± 10.06 *	23.02 ± 3.26	42.75 ± 3.49
SR-Sal	328.50 ± 22.23	31.75 ± 5.27 *	18.95 ± 3.38	41.58 ± 3.10
SR-Zolp 0h	303.83 ± 25.37	37.08 ± 6.49 *	21.12 ± 3.95	45.33 ± 4.23
SR-Zolp 2h	304.00 ± 23.63	45.50 ± 11.37 *	20.52 ± 3.13	45.42 ± 3.39

Table S3 – Effects of the acute administration of zolpidem (Zolp) immediately or 2 h after training on consolidation in mice subjected to SR. The results are presented as the mean ± SE of time spent in the non-aversive enclosed arm (TNAv), time spent in the aversive enclosed arm (TAv), percent time spent in the open arms (PTO) and total number of entries (NE) during the training session in the plus-maze discriminative avoidance task (PM-DAT). *$p<0.05$ compared to the time spent in the non-aversive arm (MANOVA followed by Duncan’s test).

Baseline performance of mice exposed to the training session in the PM-DAT in experiment IV

Groups	**TNAv**	**TAv**	**PTO**	**NE**
CTRL-Sal/Sal	317.33 ± 19.51	14.92 ± 2.87 *	25.11 ± 2.96	30.67 ± 2.44
CTRL-Sal/Zolp	375.58 ± 23.61	12.33 ± 2.07 *	19.68 ± 3.58	28.83 ± 3.11
CTRL-Zolp/Sal	374.42 ± 28.22	8.58 ± 1.59 *	20.59 ± 4.45	29.25 ± 4.31
CTRL-Zolp/Zolp	329.83 ± 30.47	12.75 ± 2.39 *	26.16 ± 6.04	29.00 ± 2.27
SR-Sal/Sal	340.00 ± 27.91	11.83 ± 2.14 *	24.05 ± 4.62	26.25 ± 2.12
SR-Sal/Zolp	359.50 ± 29.65	13.83 ± 2.31 *	19.79 ± 4.19	27.83 ± 3.29
SR-Zolp/Sal	324.08 ± 21.20	22.08 ± 2.78 *	21.69 ± 3.13	34.33 ± 2.39
SR-Zolp/Zolp	370.33 ± 19.64	13.67 ± 3.09 *	17.71 ± 2.74	26.83 ± 2.81

Table S4 – Effects of the repeated administration of zolpidem (Zolp), or its withdrawal, on retrieval in mice subjected to SR. The results are presented as the mean ± SE of time spent in the non-aversive enclosed arm (TNAv), time spent in the aversive enclosed arm (TAv), percent time spent in the open arms (PTO) and total number of entries (NE) during training session in the plus-maze discriminative avoidance task (PM-DAT). *$p<0.05$ compared to the time spent in the non-aversive arm (MANOVA followed by Duncan's test).

Supplementary figures

Figure S1

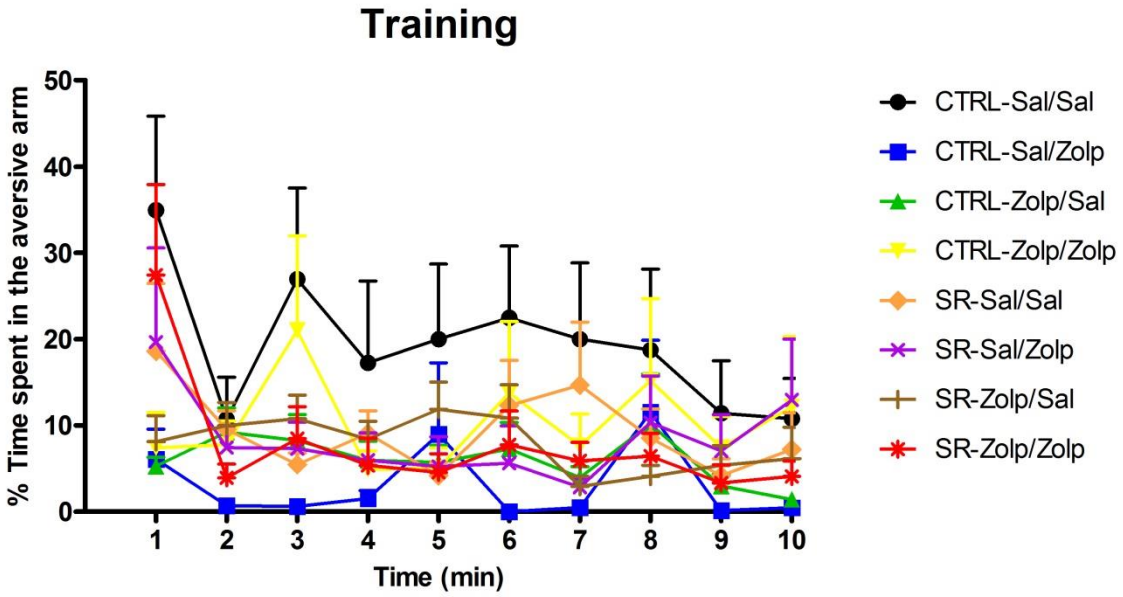

Figure S1: Effects of the repeated administration of zolpidem (Zolp), or its withdrawal, on learning of a discriminative task in mice subjected to SR. The results are presented as the mean ± SE of percent time spent in the aversive enclosed arm each min throughout the training in the plus-maze discriminative avoidance task (PM-DAT). ANOVA with repeated measures revealed significant effects of time factor and treatment x challenge injection and sleep condition x treatment x challenge injection interactions.

Figure S2

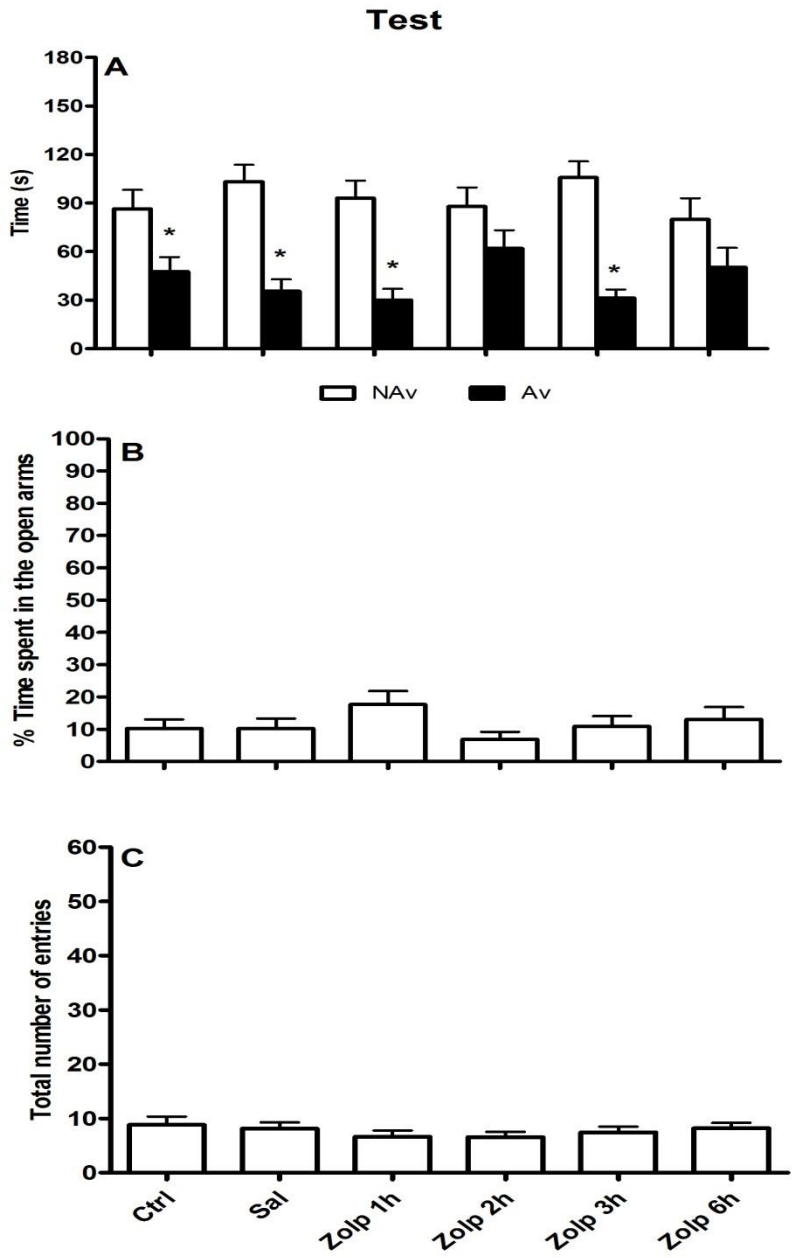

Figure S2: Effects of zolpidem (Zolp) administered in specific intervals after training in mice. The results are presented as the mean ± S.E. of time spent in the non-aversive enclosed arm x time spent in the aversive enclosed arm (**A**), percent time spent in the open arms (**B**) and the number of entries (**C**) during testing in the plus-maze discriminative avoidance task (PM-DAT). *p<0.05 compared to the time spent in the non-aversive arm (MANOVA and Duncan's test).

DISCUSSION

In the current study, we found that acute pre-training treatment with Zolp or its withdrawal were able to abolish the adverse cognitive effects induced by SR, despite the previously reported amnestic effects induced by the acute administration of this hypnotic (Zanin et al., 2013). Regarding consolidation, when Zolp was administered immediately after training (but not 2 h after it) in sleep-restricted mice, it counteracted the deleterious effects of SR. Interestingly, only the repeated treatment with Zolp was able to reverse the amnestic effects of SR on retrieval. In addition, the administration of the selective antagonist β-cct hindered the promnestic effects of the acute administration of Zolp in sleep-restricted mice, without mimicking the effects of the Zolp treatment withdrawal. These results suggest that the acute effects of Zolp are mediated, at least in part, by GABAergic receptors but that the effects of the repeated treatment are not exclusively related to this system.

To the best of our knowledge, this is the first study to systematically evaluate the effects of repeated treatment with Zolp, or its withdrawal, on memory phases in mice subjected to a repeated SR protocol. Thus, in experiment I, we assessed the effects of this treatment on learning of a discriminative task. Importantly, neither the repeated treatment with Zolp nor the SR protocol altered learning levels. These results corroborate our previous findings (Zanin et al., 2013) that the acute administration of Zolp in CTRL animals did not hamper learning in the same paradigm. Additionally, no effects on learning were detected after SR was combined or not with Zolp.

In the PM-DAT model, the percent time spent in the open arms of the apparatus during the training session has been validated as a measure of anxiety because classical anxiolytic agents increase this parameter (Gulick and Gould, 2011; Kameda et al., 2007), and classical anxiogenic drugs decrease it (Gulick and Gould 2009; Silva and Frussa-Filho 2000). We observed no effects of SR, Zolp treatment or its interactions on anxiety-like behavior. It has already been reported that acute total sleep deprivation does not modify anxiety-like behavior (Patti et al., 2010). Herein, we showed that a SR protocol is also not able to modify this behavior. Concerning Zolp treatment, we did not find any effects on anxiety-like behavior after the repeated administration of Zolp, as previously observed for acute administration (Zanin et al., 2013). Notably, the majority of studies reported a lack of effects of this drug on anxiety (Huang et al., 2010; Savić et al., 2004, 2005a,b).

Regarding motor activity, mice that were challenged with Zolp before training presented decreased locomotion compared to their respective controls, thereby corroborating the expected sedative effect of this drug (Zanin et al., 2011, 2013). In contrast, Zolp-induced hypolocomotion was of a lesser magnitude in sleep-restricted mice. Although it was not statistically significant, there was a trend towards tolerance to the Zolp-induced hypolocomotor effects in CTRL mice. Studies have reported the development of tolerance to the ataxic and sleep-inducing effects of Zolp in humans who were repeatedly treated with this drug (Stoops and Rush, 2003).

When memory was assessed, both the acute and repeated pre-training administration of Zolp induced memory impairment in CTRL mice. Regarding the acute amnestic effects of Zolp, this result is in line with our previous data

that showed that this hypnotic drug hindered memory in the discriminative avoidance task after acute administration (Zanin et al., 2013). Few studies have evaluated the effects of the repeated treatment with Zolp on cognition. Stoops and Rush (2003) demonstrated that the acute administration of Zolp promoted memory deficits in humans without the development of tolerance after repeated treatment. Similarly, Kleykamp and colleagues (2012) did not observe tolerance to the amnestic effect of Zolp. However, it could be suggested that the magnitude of these effects would increase if higher doses were administered or if treatment was prolonged. Indeed, the amnestic effects of Zolp occur after higher receptor occupation relative to its sedative effect (Savić et al., 2010). Concerning the effects of withdrawal, we did not find any effects on memory in mice kept under the CTRL condition. This finding is in agreement with a previous report that indicated no significant effects on cognitive performance of individuals after discontinuation of treatment (Kleykamp et al., 2012).

Repeated pre-training SR alone induced memory impairment. Similarly, sleep-restricted mice that were concomitantly treated with Zolp also displayed memory deficits. Although there are no reports evaluating the effects of Zolp in an animal model of SR, previous studies have reported that this drug may promote anterograde amnesia, i.e., forgetfulness of events that occurred shortly after drug intake (Tsai et al., 2007), thus corroborating the observed deficits in the present study. In contrast, the acute pre-training administration of Zolp, or treatment withdrawal, counteracted the SR-induced amnestic effects.

The interaction between Zolp and SR effects is intriguing. We speculate that a complex interaction among neurotransmission systems such as GABA and dopamine may play a role in the Zolp-induced reversal of the amnesia

promoted by SR. For example, it is well-known that paradoxical sleep deprivation increases several behaviors related to the dopaminergic system (Frussa-Filho et al., 2004; Troncone et al., 1988; Tufik, 1981), which is also important for memory persistence (Rossato et al., 2009). The occurrence of dopaminergic supersensitivity after paradoxical sleep deprivation is well documented. However, the employment of the multiple platform method for the deprivation of the paradoxical phase of sleep also markedly decreases the time spent in slow wave sleep (Silva et al., 2004). Thus, it could be hypothesized that total sleep deprivation could also induce dopaminergic supersensitivity, which could, in turn, lead to memory impairment (Xu et al., 2012). In parallel, it has already been demonstrated that GABA-modulating drugs such as benzodiazepines inhibits the GABAergic interneurons in relevant areas for dopaminergic transmission, such as the ventral tegmental area and substantia nigra (Tan et al., 2010; Grace et al., 1980). As a result, GABA neurons would no longer inhibit dopaminergic cells, thereby increasing dopamine release. Notably, it has been shown that these inhibitory interneurons primarily express the α1 subunit of the GABAergic receptor (Gao and Fritschy, 1994). In agreement with this assumption, the SR protocol employed herein may have induced dopaminergic supersensitivity, which is associated with the indirect Zolp-induced increase in dopaminergic activity leading to a lower hypolocomotor effect, as observed. Hence, it is tempting to speculate that Zolp administration increased dopamine release, which counteracted the SR-induced dopaminergic supersensitivity, thus blocking its deleterious effects on memory as occurred for motor behavior. This is an initial hypothesis that invites further investigations.

In experiment II, we evaluated the interactions between SR and Zolp exclusively on memory consolidation. Thus, neither the repeated post-training treatment with Zolp nor post-training SR hindered the consolidation of the task. Similarly, it has been reported that a similar protocol of post-training SR did not promote deficits in a spatial task in adult rats (Yang et al., 2012). Post-training SR increased motor activity, which could be due to the dopaminergic supersensitivity phenomenon. This hyperlocomotion effect strengthens the possible involvement of the dopaminergic system in the cognitive effects found in Experiment I. Notably, motor activity is closely related to dopamine neurotransmission (Kelly and Iversen, 1976), and sleep deprivation can potentiate spontaneous motor activity or amphetamine-induced hyperlocomotion in the open-field (Frussa-Filho et al., 2004). Notably, there was a trend towards SR-induced hyperlocomotion in the previous experiment. We acknowledge that the training environment may have impaired this effect because the behavioral sessions of the PM-DAT are qualitatively different: whereas the animal is exposed to a new and aversive environment during training, in the test session, the apparatus is no longer unknown and the aversive stimuli are absent, which makes the environment less anxiogenic. This difference between the sessions could have facilitated the expression of hyperlocomotion during testing in experiment II.

Previous studies have shown that consolidation processes have critical time points after training in which the memory trace can again become labile (Bekinschtein et al., 2010). We have demonstrated that memory persistence is impaired when acute 10 mg/kg Zolp is administered 2 or 3 h after (but not immediately, 1, or 6 h) the training (Zanin et al., 2013). Thus, to further

investigate the interactions between the post-training administration of Zolp and SR, we first determined an interval that was capable of promoting consolidation deficits after the administration of 5 mg/kg Zolp. Accordingly, mice treated with Zolp 2 or 6 h after training presented amnesia during testing, which suggests that the effects of this drug on memory consolidation are dose-dependent.

Considering the interactions between the time of drug administration and memory consolidation, in the experiment III, we investigated the effects of post-training administration of Zolp in mice submitted to SR for 10 days prior to training. In the present experiment, the drug administration occurred in distinct time points: immediately after training (ineffective in altering memory consolidation) or 2 h after it (an interval that is able to induce consolidation deficits). Importantly, SR-induced memory deficits were only abolished by Zolp when administered immediately after training. Specifically, this finding constitutes a retrograde facilitatory effect, i.e., an increased recall of information acquired before the drug administration, which has been previously demonstrated for Zolp (Fillmore et al., 2001; Kleykamp et al., 2012).

Comparing these findings with those obtained in experiment I, in which the acute pre-training administration of Zolp reversed SR-induced impairments, it could be suggested that such promnestic effects could be specifically related to consolidation processes. Importantly, even when it is administered before training, this hypnotic would still be available during the initial steps of consolidation because its half-life is approximately 0.3–1.5 h in rats (Garrigou-Gadenne et al., 1989). Conversely, when the administration occurred 2 h after training, the reversal effect was no longer observed, which suggests that the SR-induced cognitive impairments were established after this period. However,

the consolidation deficits promoted by the 2 h time point administration could have contributed to the deleterious effects of SR.

Though numerous studies have demonstrated that sleep loss impairs the acquisition and consolidation phases of memory (Stickgold et al., 2001), its effect on memory retrieval has been overlooked. Notably, some recent studies have reported an inhibitory effect of sleep deprivation on this stage of memory formation (Alvarenga et al., 2008; Fernandes-Santos et al., 2012; Patti et al., 2010; Takatsu-Coleman et al., 2013; Talhati et al., 2014). Thus, the SR protocol was applied immediately before testing in experiment IV. Neither Zolp treatment nor its withdrawal impaired memory retrieval. Again, these findings are in line with our previous results from the acute administration of the drug (Zanin et al., 2013). Regarding SR, it induced amnesia when applied immediately before testing. Indeed, previous studies from our group have shown that acute total sleep deprivation for 6 h promoted retrieval impairments in the PM-DAT both in male (Patti et al., 2010) and female (Fernandes-Santos et al., 2012) mice. Of note, the repeated pre-test Zolp treatment (but not its acute administration) abolished the retrieval deficits. These results suggest that this recovery phase of memory could be more sensitive to the deleterious effects of SR. In line with this assumption, the withdrawal of treatment specifically hindered memory retrieval.

To further investigate the involvement of the GABAergic receptors in the interaction between SR and Zolp, we designed experiment V using the α1-selective antagonist β-cct. This antagonist was able to reverse the Zolp-induced hypolocomotor effect. Previous studies have reported that β-cct counteracted the sedative effects of Zolp (Milić et al., 2012; Savić et al., 2005a). When

memory was evaluated, the administration of β-cct in mice that were subjected to SR and treated with Zolp promoted memory deficits. In this regard, we demonstrated that β-cct administration abolished the promnestic effect of the acute Zolp injection. Thus, mice challenged with β-cct and Zolp showed the same behavior as did those only subjected to SR: forgetting the task. Furthermore, the animals that were repeatedly treated with Zolp also displayed amnesia. However, in this case, the administration of β-cct did not counteract the cognitive effects of repeated Zolp treatment. A possible explanation that could be raised for the lack of effects of β-cct in mice repeatedly treated with Zolp is that the use of an antagonist (or the dose employed) was not sufficient to mimic the effects of a complete substance withdrawal. Thus, the deleterious effects of Zolp would remain. Finally, we observed increased locomotion in the group that was only sleep-restricted or subjected to SR and repeatedly treated with Zolp, which could reflect a habituation deficit.

These results suggest that the cognitive effects of the acute administration of Zolp are, at least in part, mediated by GABAergic receptors, whereas the effects promoted by the repeated treatment with this drug cannot be exclusively related to this mechanism. Studies have suggested that β-cct can reverse the sedative, anxiolytic and myorelaxant effects of classical benzodiazepines but that it is not able to abolish the amnestic effects of these drugs (Belzung et al., 2000; Milić et al., 2012; Savić et al., 2005a,b). Conversely, Makaron and colleagues (2013) reported that the cognitive impairments induced by both triazolam and Zolp were blocked by the administration of β-cct. These discrepant findings could be explained by the differences in the animal species (monkeys vs. mice) and the memory task employed. In that study, the memory

task used was associated with executive functions, which appears to be strongly influenced by α1 subunit-containing $GABA_A$ receptor subtypes (Jentsch et al., 1999).

Taken together, our results suggest that the interaction between SR and Zolp can positively or negatively modulate memory formation, depending on the phase analyzed. Specifically, the acute administration of Zolp prior to training or immediately after it (but not before testing) abolished the cognitive impairments induced by SR. Moreover, SR and the concomitant treatment with Zolp did not modify the consolidation of associative memory. Notably, the acute effects of Zolp administration (but not its withdrawal) can be explained by the GABAergic activation because β-cct counteracted the promnestic effect of acute Zolp administration. Therefore, the present findings strengthen the importance of conducting future studies to evaluate the interaction between sleep restriction periods and Zolp on memory, specifically addressing the consolidation phase.

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AUTHOR DISCLOSURES

Role of Funding Source

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Contributors

KAZ, CLP, DP and RF-F were responsible for the study concept and design. LBL-S, CSB, RS-B; AWH, LMBC, FT, ARB and SBG contributed to the acquisition of animal data. KAZ, CLP and DP assisted with data analysis and interpretation of findings. KAZ and CLP drafted the manuscript. DP and ST provided critical revision of the manuscript for important intellectual content. All authors critically reviewed content and approved final version for publication. The English language in this manuscript has been proofread and edited by

American Journal Experts (AJE), but we are entirely responsible for the scientific content of the paper.

Conflict of Interest

All authors disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the work that could inappropriately influence (bias) the present work.

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3.2.1 Conclusões parciais – manuscrito 2

Os resultados do presente manuscrito sugerem que a interação entre a restrição de sono e o tratamento com zolpidem afetam os processos de formação da memória de forma dependente da fase desse processo (aprendizado consolidação e evocação). Especificamente, há uma interação modulatória positiva entre a restrição de sono e o tratamento agudo com Zolp, uma vez que quando administrado prévia ou imediatamente após o treino, esse hipnótico foi capaz de reverter os déficits de memória induzidos pela RS. Por outro lado, quando esse hipnótico foi administrado imediatamente antes do teste, esse efeito não foi observado. No houve efeitos significativos sobre a fase de consolidação da memória associativa. De importância, não observamos tolerância após o tratamento repetido com a droga.

Para investigar a participação dos receptores GABAérgicos na interação entre a RS e o tratamento com Zolp, avaliamos os efeitos da administração prévia de β-cct, um antagonista GABAérgico seletivo para o sítio BZ_1 dos receptores $GABA_{A.}$ Os resultados sugerem que o efeito agudo do Zolp é mediado, ao menos em parte, por receptores GABAérgicos, uma vez que a administração de β-cct reverteu o efeito promnéstico da administração aguda de Zolp. Por outro lado, após o tratamento repetido, o efeito cognitivo não pode ser explicado exclusivamente por meio do sistema GABAérgico, sugerindo a interação com outros sistemas de neurotransmissão.

Nossos achados reforçam a importância de mais estudos que avaliem as interações entre períodos de RS e os efeitos do Zolp sobre a memória. Nesse

sentido, no 3º manuscrito investigamos os efeitos da interação entre a RS e o tratamento com Zolp em um modelo clássico de memória, a esquiva passiva.

Manuscrito 3

EFFECTS OF ZOLPIDEM ON MEMORY PHASES OF A PASSIVE AVOIDANCE TASK IN MICE SLEEP RESTRICTED

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ABSTRACT

Purpose: Zolpidem (Zolp), a hypnotic drug prescribed to treat insomnia, may have negative effects on memory. As far as we know, no studies have characterized the cognitive effects of Zolp in animal models of sleep restriction (SR). Unpublished data of our group found that the interaction between SR and Zolp can positively or negatively modulate memory of a discriminative avoidance task. Thus, the aim of this study was to evaluate the effects of the repeated administration of Zolp (or its withdrawal) on the memory phases of mice submitted to SR and tested in a classic memory task (passive avoidance task – PAT). **Design:** Mice were subjected to SR for 3 h per day during 10 days or left undisturbed (CTRL) and then were treated with saline (Sal) or Zolp. On the 10^{th} day, half of the animals treated with Sal received Zolp while the others received Sal. The same occurred for mice daily treated with Zolp. This protocol was applied before training, immediately after it or before testing. **Results:** In the PAT, the SR protocol did not induce memory deficits irrespective of the phase analyzed. The acute administration of Zolp induced amnesia both in CTRL and sleep-restricted mice while the repeated administration promoted deficits only in CTRL groups when administered before training. No effects were found on consolidation or retrieval. **Conclusions:** We demonstrated that only the acute (but not the repeated or drug withdrawal) Zolp-treatment modify memory retention irrespective of sleep condition.

Keywords: zolpidem, sleep restriction, memory, passive avoidance task.

INTRODUCTION

It is well established that sleep deprivation impairs cognitive function. Nonetheless, most studies have involved paradoxical or total sleep deprivation procedures. Indeed, it has been demonstrated that both paradoxical and total sleep deprivation methods leads to memory deficits in several animal models such as avoidance tasks (Bueno et al., 1994), the Morris water maze (Youngblood et al., 1997, 1999), the radial maze (Smith et al., 1998), object recognition (Palchykova et al., 2006) and in the plus-maze discriminative avoidance task (Alvarenga et al., 2008; Fernandes-Santos et al., 2012; Patti et al., 2010; Silva et al., 2004a). It remains unclear whether such effects would be observed after repeated sleep restriction (SR), which is more common in humans, thus representing a better translational model.

In SR models, the subjects are repeatedly submitted to shorter periods of sleep loss (Banks and Dinges, 2007). In humans, studies have found that SR impairs behavioral alertness, measured by psychomotor vigilance testing, with deterioration being of higher magnitude as time allowed for sleep was reduced. Moreover, the neurobehavioral effects of SR appear similar to those of total sleep deprivation (Belenky et al., 2003; Van Dongen et al., 2003). Regarding animal models, it was demonstrated that SR using forced locomotion models impaired spatial memory (McCoy et al., 2013; Zielinski et al., 2013). However, the forced locomotion models cause excessive stress in animals, which, in turn, can influence the behavioral results. From our knowledge, only Yang and colleagues (2012) evaluated the effects of SR on memory using a less stressing

sleep deprivation method, the gentle handling. In this study, the SR protocol hindered spatial memory in adolescent (but not in adult) rats.

In parallel, zolpidem (Zolp) is a sleep-enhancing drug prescribed to treat insomnia. This drug selectively binds to the α1 subunit-containing $GABA_A$ receptor subtype (Sancar et al., 2007). While Zolp seems to exhibit fewer deleterious psychomotor and cognitive effects, amnestic effects are still reported both in humans (Fitzgerald et al., 2014) and laboratory animals (Huang et al., 2010; Savić et al., 2005a,b; Zanin et al., 2011, 2013). Specifically, we have recently demonstrated that the Zolp-induced memory deficits are dose- and time-dependent in mice submitted to the plus-maze discriminative avoidance task. Notably, such impairments were not related to state dependency, sedation or anxiolysis (Zanin et al., 2013). Still, we have also demonstrated that the acute administration of 10 mg/kg Zolp promoted habituation deficits in the open filed arena (Zanin et al., 2011).

The one-trial step-through passive avoidance test is one of the most widely used memory tasks in rodents (Myhrer, 2003), and might be considered a measurement of explicit memory. In this paradigm, animals with cognitive deficits present decreased latency to step through in the test session. Such deficits could be induced by pharmacological manipulations (Isomae et al., 2003; Santucci and Shaw, 2003; Silva et al., 1999), consequent to neural lesions (Isomae et al., 2003), related to aging (Silva et al., 1996; Yasui et al., 2002) or due to sleep deprivation (Fernandes-Santos et al., 2012; Moreira et al., 2003, Patti et al., 2010; Silva et al., 2004a,b). However, as far as we know, there are no studies evaluating the effects of SR protocols in this memory task.

From a clinical perspective, Zolp is usually prescribed as a repeated treatment for insomnia. To date no studies have characterized the effects of Zolp on emotional memory as repeated administration in animal models of SR. Thus, considering the previous evidences supporting the impairing effects induced by the acute administration of this drug, as well as the absence of studies systematically evaluating the effects of Zolp in sleep restricted animals, the aim of the present study was to evaluate the effects of the repeated administration of Zolp (or its withdrawal) on the different memory phases (learning, consolidation and retrieval) of mice submitted to SR for 10 days and evaluated in the passive avoidance task (PAT).

MATERIAL AND METHODS

Subjects

Three-month-old Swiss male mice (outbred, raised, and maintained in the Centre for Development of Experimental Models in Medicine and Biology of Universidade Federal de São Paulo) were used. Animals weighing 35-40 g were housed under conditions of controlled temperature (22-23°C) and lighting (12h light, 12h dark; lights on at 6:45 a.m.). Food and water were available *ad libitum* throughout the experiments. Animals used in this study were maintained in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications Nº 8023, revised 2011) and with the Brazilian Law for Procedures for Animal Scientific Use (#11794/2008). The experimental procedures were approved by the Institutional Animal Care and Use Committee under the protocol #1741/10.

Drugs

Zolp (Sanofi-Aventis®), at the dose of 5 mg/kg, was diluted in saline 0.9% solution (Sal), which was also the control solution. The dose of Zolp was chosen based on previous experiments of our group, demonstrating both memory deficits and weaker sedative effects in mice (Zanin et al., 2013). All the solutions were administered intraperitoneally in a volume of 10 ml/kg

Sleep Restriction (SR)

Mice were submitted to SR through the gentle handling method as previously described (Tobler et al., 1990). This method consists of keeping the

animal awake by tapping on the cage and, if necessary, by gently touching them with a soft brush if behavioral signs of sleep are observed. The animals were sleep restricted for 3 h (from 10 AM to 1 PM) during 10 consecutive days. This time interval was chosen because this is when paradoxical sleep reaches its highest expression and slow wave sleep homeostatic pressure is generated (Franken et al., 1991).

Passive avoidance task (PAT)

PAT was conducted using methods described by Denti and Epstein (1972). The apparatus employed was a 2-way shuttle box with a guillotine door placed between the 2 modular testing chambers. One chamber was illuminated by a 40-W light, while the other remained dark. During the conditioning session, the animals were individually placed in the illuminated chamber facing away from the guillotine door. When the mouse entered the dark chamber, the door was quietly closed and a 0.4 mA foot shock was applied for 1 s through the grid floor. A test session was performed 12 days after conditioning using the same procedures, except for the foot shock. In both sessions (training and testing), the latency to enter the dark chamber was recorded, with a cut-off duration of 300 s.

Open field evaluation (OF)

The open-field apparatus used in the present study was a circular wooden arena (40 cm in diameter and 50 cm high) with an open top and floor divided into 19 squares. Hand-operated counters were used to score total locomotion (number of any floor unit entered) and stopwatches were used to

quantify duration of immobility (total of seconds of lack of movement). The observer was always unaware of the experimental design.

Experimental design

Experiment I: effects of the repeated administration of Zolp or its withdrawal on learning and consolidation in mice sleep-restricted

Animals were kept in their home cages (control condition – CTRL) or subjected to SR by gentle handling for 3 h per day during 10 days. Every day, after the SR period, mice were treated with Sal (CTRL-Sal and SR-Sal) or Zolp (CTRL-Zolp and SR-Zolp). On the 10^{th} day, half of animals treated with Sal received an acute Zolp injection while the others received Sal. The same procedure was applied for mice daily treated with Zolp. The following groups were formed: CTRL-Sal/Sal (n=12), CTRL-Sal/Zolp (n=12), CTRL-Zolp/Sal (n=12), CTRL-Zolp/Zolp (n=12), SR-Sal/Sal (n=12), SR-Sal/Zolp (n=12), SR-Zolp/Sal (n=12) and SR-Zolp/Zolp (n=12). Thirty min after the last injection, mice were subjected to training in the PAT. Considering that the PAT can be influenced by motor activity, mice were exposed to OF for 5 min immediately after training. Twelve days later animals were tested.

Experiment II: effects of the repeated administration of Zolp exclusively on memory consolidation in mice sleep-restricted

Initially, mice were trained in the PAT. Immediately after training, animals were kept in their home cages (CTRL) or were subjected to SR. Every day, after the SR period, mice were treated with Sal or Zolp and the following groups were

formed: CTRL-Sal (n=10), CTRL-Zolp (n=10), SR-Sal (n=10), and SR-Zolp (n=10). Forty-eight hours after the last injection (12 days after training) mice were tested.

Experiment III: effects of the repeated administration of Zolp or its withdrawal on memory retrieval in mice sleep-restricted

Mice were trained in the PAT. Forty-eight hours after it, animals received the same treatment as described for the experiment I, forming the following groups: CTRL-Sal/Sal (n=12), CTRL-Sal/Zolp (n=12), CTRL-Zolp/Sal (n=12), CTRL-Zolp/Zolp (n=12), SR-Sal/Sal (n=12), SR-Sal/Zolp (n=12), SR-Zolp/Sal (n=12) and SR-Zolp/Zolp (n=12). Thirty min after the last injection, mice were subjected to testing. Immediately after testing mice were exposed to OF for 5 min.

Statistical analysis

MANOVA and Duncan's test were used to analyze latency to enter the dark chamber in the PAT and the parameters in the OF. A probability of $p<0.05$ was considered significant for all comparisons made.

RESULTS

Experiment I: effects of the repeated administration of Zolp or its withdrawal on learning and consolidation in mice sleep-restricted

In the training session, analyzing the latency to enter the dark chamber, MANOVA showed significant effects of treatment (Sal or Zolp) [$F(1,88)=6.29$; $p<0.05$] and challenge injection (Sal or Zolp) [$F(1,88)=6.24$; $p<0.05$]. Duncan's *post hoc* revealed that in exception of mice sleep-restricted and daily treated with Zolp (SR-Zolp/Zolp), all the other groups challenged with Zolp showed an increased latency. Still, the animals under CTRL condition and abstinent from Zolp-treatment (CTRL-Zolp/Sal) as well as those sleep-restricted treated with Zolp (SR-Zolp/Sal and SR-Zolp/Zolp) showed a decreased latency compared to their respective control groups (Figure 1A).

Regarding exploration of the OF, MANOVA revealed significant effects of sleep condition (CTRL or SR) [$F(1,88)=4.73$; $p<0.05$] and challenge injection [$F(1,88)=38.01$; $p<0.001$]. Duncan's test demonstrated that only the group sleep-restricted acutely treated with Zolp (SR-Sal/Zolp) did not present a decreased motor activity. All the other groups challenged with Zolp (CTRL-Sal/Zolp, CTRL-Zolp/Zolp and SR-Zolp/Zolp) presented hypolocomotion compared to their respective control group challenged with Sal. However, in mice sleep-restricted the hypolocomotor effect was of less magnitude because there was an increase on motor activity when compared to CTRL groups (CTRL-Sal/Zolp x SR-Sal/Zolp and CTRL-Zolp/Zolp x SR-Zolp/Zolp). (Figure 2A). Analyzing immobility time, MANOVA showed significant effects only for challenge injection [$F(1,88)=57.50$; $p<0.001$]. The *post hoc* analysis revealed

that all animals challenged with Zolp displayed an increased immobility time, irrespective of sleep condition or treatment, when compared to their respective control groups (Figure 2B).

In the test session, MANOVA showed significant effects only for challenge injection [F(1,88)=10.92; p=0.001] in the latency to enter the dark chamber. Duncan's test demonstrated that all groups challenged with Zolp, except those under SR condition repeatedly treated with Zolp (SR-Zolp/Zolp), displayed a decrease in latency compared to their respective control groups (Figure 1B).

Experiment II: effects of the repeated administration of Zolp exclusively on memory consolidation in mice sleep-restricted

During training, analyzing the latency to enter the dark chamber, MANOVA did not show significant differences (Figure 3A). In the same way, no modifications were found in the test session (Figure 3B).

Experiment III: effects of the repeated administration of Zolp or its withdrawal on memory retrieval in mice sleep-restricted

In the training session, as expected, MANOVA did not show significant differences in the basal latency to enter the dark chamber (Figure 4A). In the test session, MANOVA showed significant effects only of challenge injection [F(1,88)=8.21; p=0.005]. Duncan's *post hoc* revealed that only mice under CTRL condition and acutely treated with Zolp (CTRL-Sal/Zolp) presented an increased latency compared to its respective control (Figure 4B).

Analyzing the OF parameters, MANOVA showed significant effects of challenge injection [$F(1,88)=23.41$; $p<0.001$] in total locomotion. Duncan's test revealed that mice challenge with Zolp (CTRL-Sal/Zolp, CTRL-Zolp/Zolp and SR-Zolp/Zolp), in exception of those sleep-restricted and acutely treated with Zolp (SR-Sal/Zolp), displayed a decreased locomotion compared to the respective control group challenged with Sal (Figure 5A). Regarding the immobility time, MANOVA demonstrated significant effects of challenge injection [$F(1,88)=24,41$; $p<0,001$]. The *post hoc* analysis showed that all animals challenged with Zolp, irrespective of sleep condition or treatment, presented an increased immobility time compared to their respective controls (Figure 5B).

Figure 1

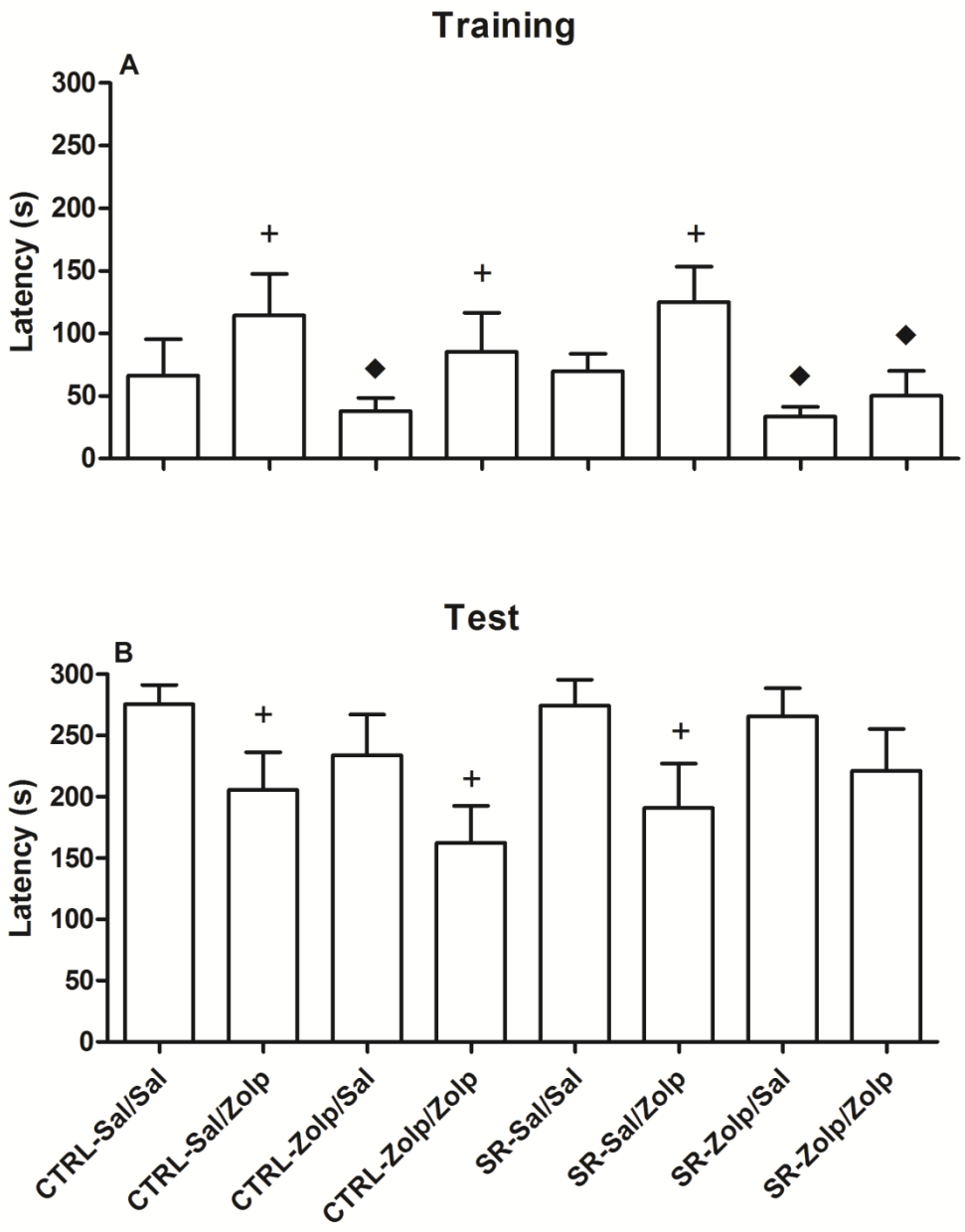

Figure 1: Effects of the repeated administration of Zolp or its withdrawal on learning and consolidation of a passive avoidance task in mice submitted to sleep restriction (SR). Animals were subjected to control condition (CTRL) or SR for 10 days and were daily treated with Sal or 5 mg/kg Zolp. On the 10th day, after the last injection, mice were trained and 12 later tested. Results are presented as the mean ± S.E. of latency to enter the dark chamber in the training (**A**) and testing (**B**). +$p<0.05$ compared to the group of same sleep condition and treatment but different challenge injection; ◆$p<0.05$ compared to the group of same sleep condition and challenge injection but different treatment (MANOVA and Duncan's test).

Figure 2

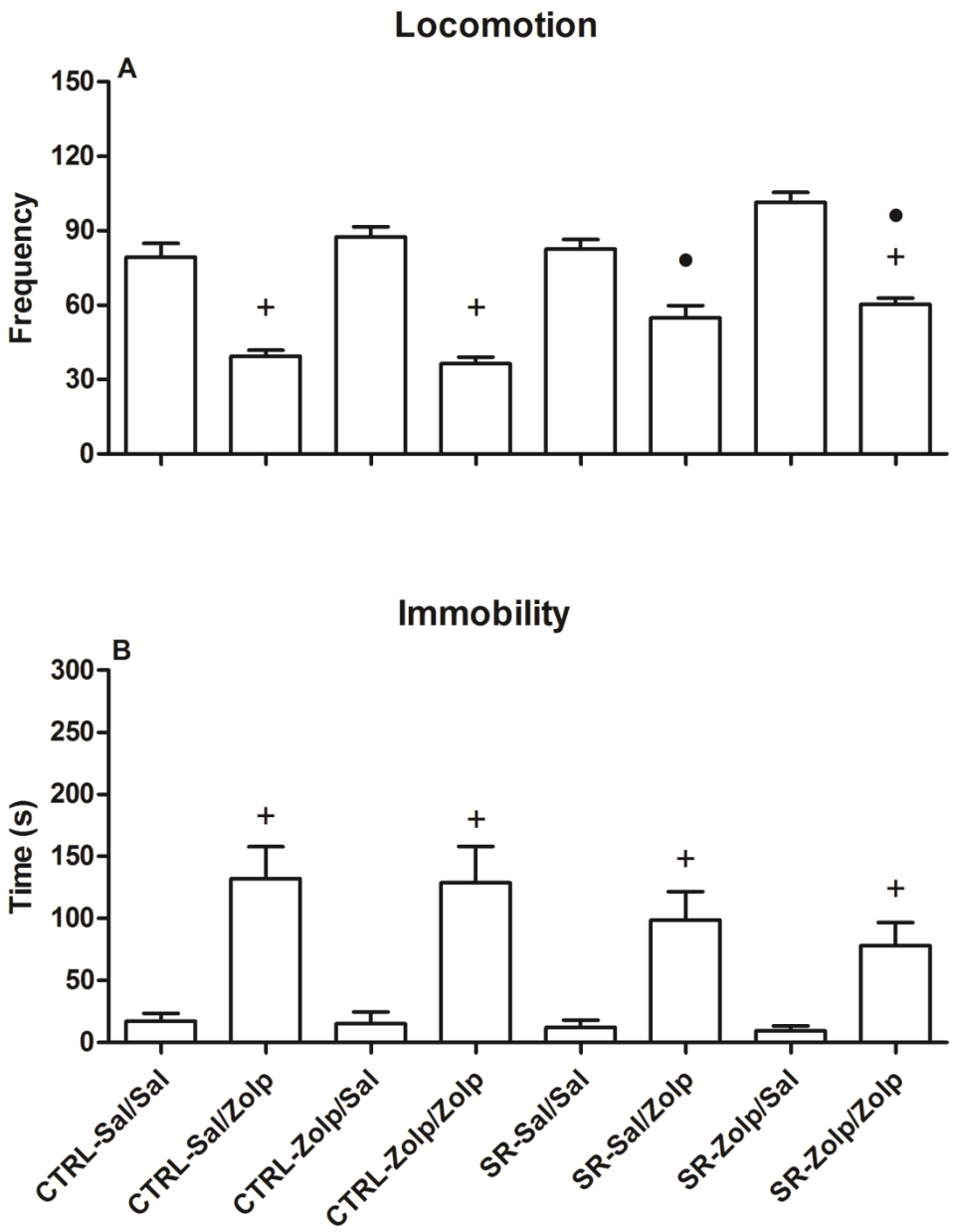

Figure 2: Effects of the repeated administration of Zolp or its withdrawal on open-field exploration in mice submitted to sleep restriction (SR). Animals were subjected to control condition (CTRL) or SR for 10 days and were daily treated with Sal or 5 mg/kg Zolp. On the 10th day, after the last injection, mice were trained and then exposed to the open-field. Results are presented as the mean ± S.E. of number of crossings through all quadrants (**A**) and immobility duration (**B**) immediately after training in the passive avoidance task. +$p<0.05$ compared to the group of same sleep condition and treatment but different challenge injection; •$p<0.05$ compared to the group of same treatment and challenge injection but different sleep condition (MANOVA and Duncan's test).

Figure 3

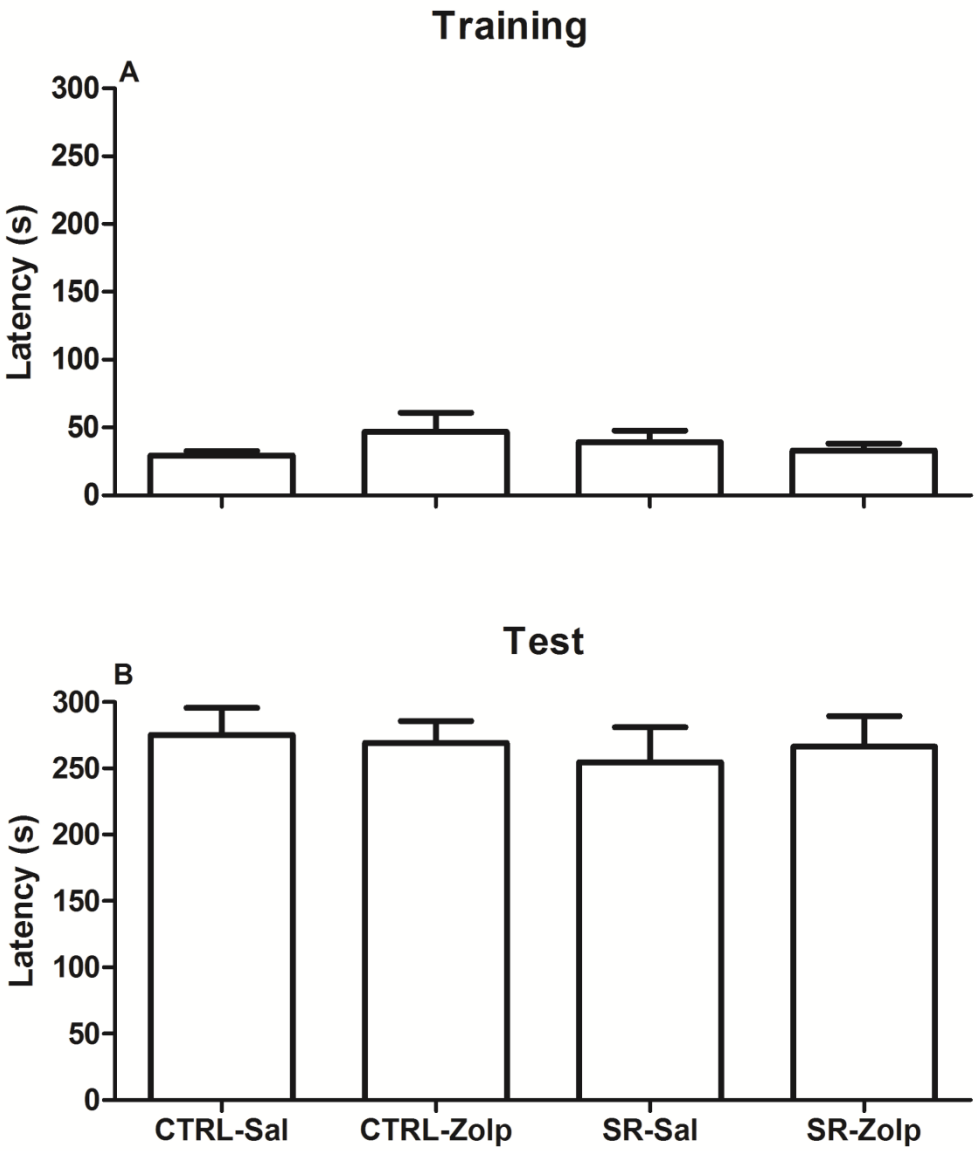

Figure 3: Effects of the repeated administration of Zolp exclusively on memory consolidation of a passive avoidance task in mice submitted to SR. Firstly, mice were trained. Immediately after, animals were subjected to control condition (CTRL) or SR for 10 days and were daily treated with Sal or 5 mg/kg Zolp. Forty-eight hours after the last injection, mice were tested. Results are presented as the mean ± S.E. of latency to enter the dark chamber in the training (**A**) and testing (**B**). There were no significant differences (MANOVA and Duncan's test).

Figure 4

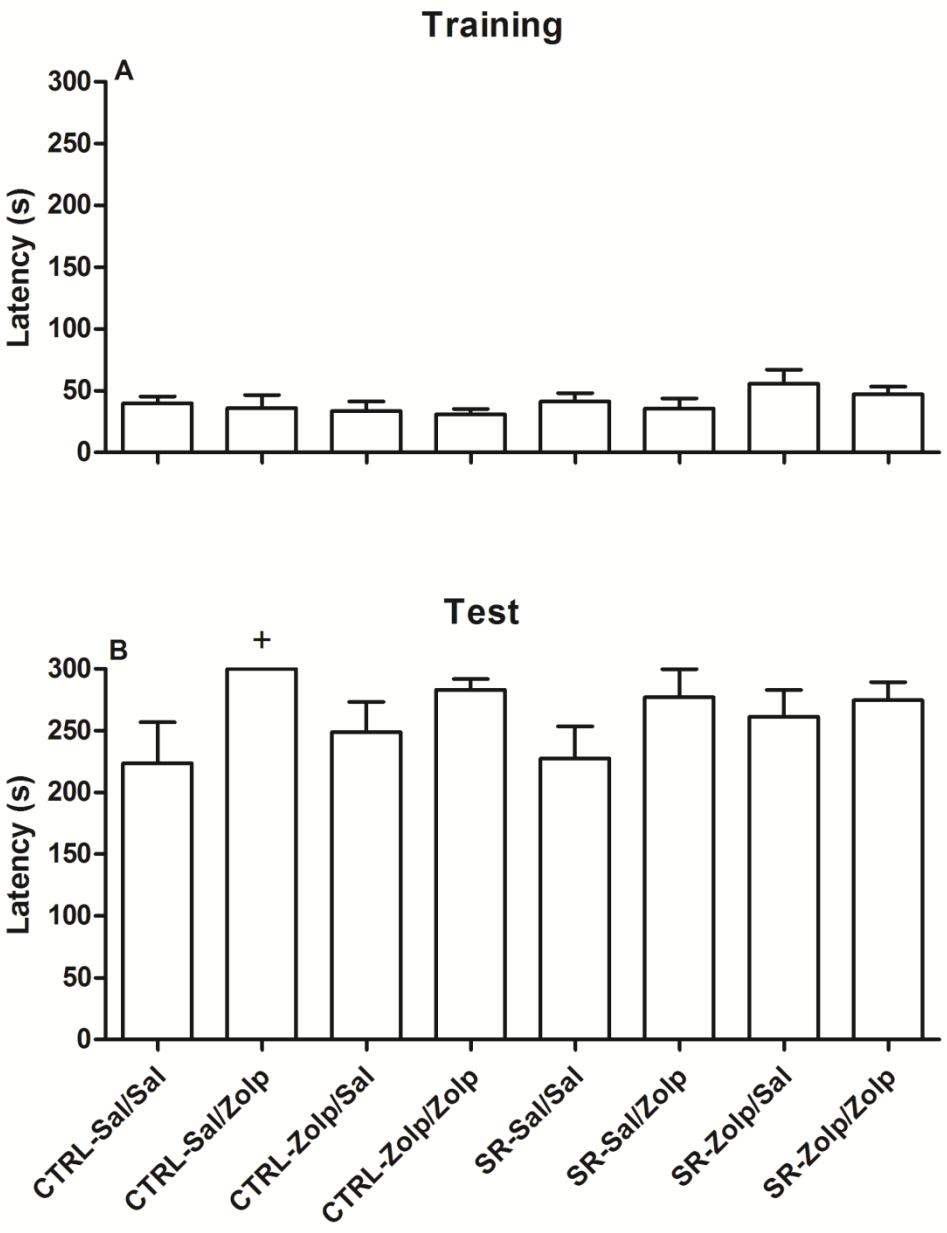

Figure 4: Effects of the repeated administration of Zolp or its withdrawal on retrieval of a passive avoidance task in mice submitted to sleep restriction (SR). Firstly, mice were trained. Forty-eight hours later, animals were subjected to control condition (CTRL) or SR for 10 days and were daily treated with Sal or 5 mg/kg Zolp. After the last injection, mice were tested. Results are presented as the mean ± S.E. of latency to enter the dark chamber in the training (**A**) and testing (**B**). +$p<0.05$ compared to the group of same sleep condition and treatment but different challenge injection (MANOVA and Duncan's test).

Figure 5

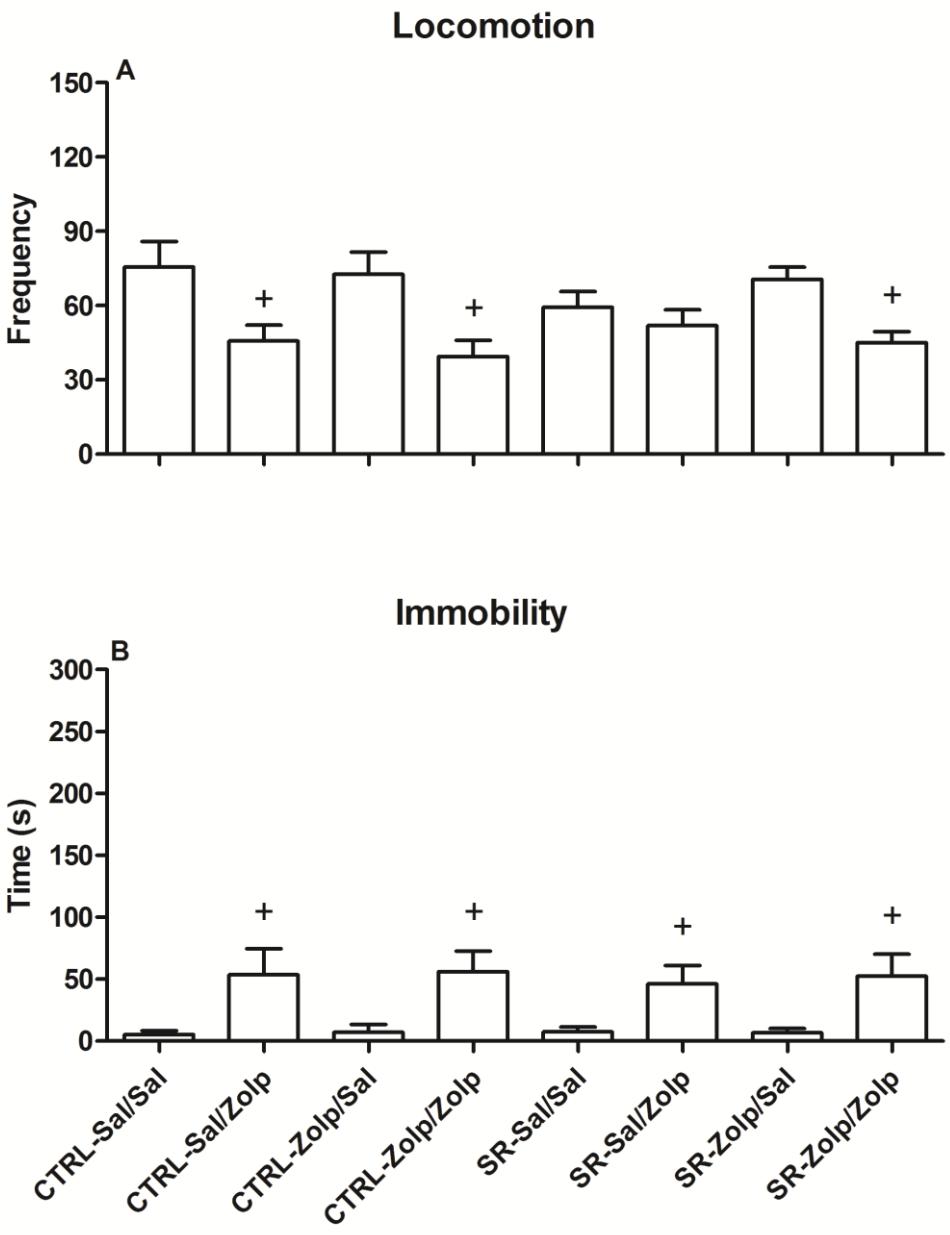

Figure 5: Effects of the repeated administration of Zolp or its withdrawal on open-field exploration in mice submitted to sleep restriction (SR). Firstly, mice were trained. Forty-eight hours later, animals were subjected to control condition (CTRL) or SR for 10 days and were daily treated with Sal or 5 mg/kg Zolp. After the last injection, mice were tested and then exposed to the open-field. Results are presented as the mean ± S.E. of number of crossings through all quadrants (**A**) and immobility duration (**B**) immediately after testing in the passive avoidance task. +$p<0.05$ compared to the group of same sleep condition and treatment but different challenge injection (MANOVA and Duncan's test).

DISCUSSION

In the present study we evaluated the effects of Zolp in mice sleep-restricted for 3 h during 10 consecutive days on memory phases (learning, consolidation and retrieval) in the PAT. Thus, we showed that SR *per se* did not impair emotional memory when it occurs before training or testing. Regarding the effects of Zolp, in control animals, the acute pre-training administration of Zolp hindered memory retention. The same was observed in sleep-restricted mice. Notably, neither SR nor Zolp or its interactions promoted consolidation deficits. When retrieval was analyzed, all of the animals, irrespective of sleep condition or treatment, retrieved the task. This lack of a possible negative effect could be due to (1) the stressing characteristic of the PAT and (2) the influence of the Zolp-induced hypolocomotion before training or testing.

In the 1st experiment we analyzed the effects of the repeated treatment with Zolp (or its withdrawn) on performance of mice subjected to SR in the PAT. Thus, in the training session we observed increase in latency to enter the dark chamber in mice acutely treated with Zolp. This result reflects the influence of motor activity in the PAT data. In fact, the acute treatment with Zolp promotes motor impairments, which in turn enhance the latency during training (Tang et al., 1995). On the other hand, sleep-restricted mice that were daily treated with Zolp did not differ from those abstinent of treatment and also presented a decreased latency compared to animals acutely treated. Although the PAT cannot detect alterations in locomotor activity, it could be suggested that in mice under SR there was a tolerance to the sedative effect of Zolp after repeated administration. Curiously, we observed that drug withdrawal decreased the

latency in both control and sleep-restricted groups. This finding could suggest a better performance in these groups. However, besides being strongly influenced by anxiety-like behaviors and motor activity, the PAT did not allow a specific evaluation of learning.

Considering the strong influence of locomotion on the latency to enter the dark chamber in the PAT, after training animals were exposed to the OF to evaluate motor activity. Thus, the pre-training administration of Zolp decreased locomotion in exception of the group sleep-restricted acutely treated with Zolp. Still, we found an increase in immobility time for all groups challenged with Zolp irrespective of sleep condition. These findings demonstrate that the acute effect of Zolp was of less magnitude in animals submitted to SR, suggesting an interaction between SR and Zolp treatment. Although we did not detect an increase in motor activity induced by SR, this decrease in the magnitude of the hypolocomotor effects of Zolp in mice sleep-restricted could reflect an enhanced locomotion promoted by SR. Specifically, sleep deprivation can potentiate both spontaneous and amphetamine-induced motor activity in the open-field (Frussa-Filho et al., 2004; Saito et al., 2014). We acknowledge that the foot shock applied during training of the PAT and the anxiogenic effect induced by the 1st exposure to a new environment (OF) may have impaired the expression of the SR-induced hyperlocomotion.

With regard to retention of the task, the animals acutely treated with Zolp (kept under CTRL condition) presented a decrease in latency suggesting an attenuation of memory processes. Indeed, several studies have reported deleterious effects of the acute Zolp administration in the PAT (Edgar et al. 1997; Fitzgerald et al., 2014; Foster et al. 2004; Sanger et al. 1986; Savić et al.,

2005b; Tang et al. 1995). Similarly, herein we found a negative effect of the repeated Zolp administration in the retention of the PAT (Mikolajczak et al., 1999). Regarding the sleep-restricted groups, only mice acutely treated with Zolp presented a decrease in latency to enter the dark chamber. It would be expected an impairing effect of SR *per se*. The absence of effect could be explained by the stressing component of the PAT since it involves painful and potentially life-threatening aversive stimuli, which could have strengthened the memory trace. Indeed, reinforcers can cause strong resistance of memory against forgetting (McGaugh, 2006).

As previously mentioned, the possible influence of Zolp-induced hypolocomotion in the performance of mice during the training cannot be excluded. Nevertheless, if this was the case, mice sleep-restricted and repeatedly treated with Zolp (SR-Zolp/Zolp group) should also present memory deficits since they presented decreased exploration in the OF, likewise all of the animals challenged with Zolp. This data suggests that, although being influenced by motor activity, our findings reflected the memory impairment induced by acute Zolp administration. Another possible explanation is the duration of SR. Thus, it could be suggested that longer periods of SR would precipitate memory deficits.

Fear-motivated memories involve both an explicit associative (the context) and an operant-like conditioning components (the shock) (Quillfeldt, 2010). Of note, acquisition and consolidation of avoidance tasks depends on the hippocampus (Izquierdo and Medina, 1997; Lorenzini et al., 1996; Rossato et al., 2006) and is also dependent on the basolateral amygdala, entorhinal and parietal cortex functioning (Izquierdo et al., 2006). It is known that neural

processing within the amygdala complex is important for assigning emotional value to events. In this concern, it was shown that Zolp enhanced the inhibitory postsynaptic paths in the basolateral amygdala (Kang-Park et al., 2004), which could explain the observed memory deficits. Specifically, after amygdala lesions, a cue that signs an aversive event fails to elicit fearful behavior and the association between cues and positive experiences is also deficient (Gallagher and Holland, 1994; LeDoux, 2000).

In the experiment II, we analyzed the interactions between SR and Zolp exclusively on memory consolidation. As expected, we did not find basal differences in the training session. Then, in the testing, neither the repeated treatment with Zolp nor the SR or their interactions modified the consolidation of the PAT. Concerning the SR lack of effects, it was reported that a similar protocol of post-training SR did not promote consolidation deficits in a spatial task in adult rats (Yang et al, 2012). Similarly, clinical studies demonstrated that the acute administration of Zolp did not promote consolidation deficits in a motor skill task (Morgan et al., 2010) or recall of word lists and digit symbol substitution test (Meléndez et al., 2005). In the present study, we also demonstrated that the repeated administration of Zolp did not impair consolidation of an emotional task. Notably, a meta-analysis by Myhrer (2003) reported that dopamine is the most influential neurotransmitter in PAT memory followed by glutamate. Conversely, drugs with effects on GABAergic or cholinergic activity seem to have a weaker participation on PAT memory formation.

While numerous studies have demonstrated that sleep plays a critical role in the acquisition and consolidation phases of memory formation (Smith et

al., 2009; Stickgold et al., 2001), its effect on memory retrieval remain overlooked, although some recent studies have reported an inhibitory effect on this stage (Alvarenga et al., 2008; Fernandes-Santos et al., 2012; Patti et al., 2010). In the present study, we demonstrated that the employed SR protocol did not impair emotional memory retrieval. Again, neither Zolp treatment nor its withdrawn altered memory recall. Curiously, mice under control condition and acutely treated with Zolp displayed increased latency which reflects the hypolocomotor effect of this drug that was administered prior to testing.

In agreement with this assumption, in the OF performance, mice treated with Zolp before testing showed increase in immobility time. Accordingly, there was a decrease in ambulation after Zolp administration in exception of the sleep-restricted group acutely treated with the drug. As mentioned before, it would be expected a hiperlocomotor effect of SR which was not detected. Again, this discrepancy could be due to the foot shock application and a possible anxiogenic effect induced by the novelty during the 1st exposure to the OF. Conversely, the attenuation of the Zolp-induced hypolocomotor effect in mice sleep-restricted would reflect an increased motor activity induced by SR modulating the acute effects of the drug.

Recently, studies in our laboratory found that the interaction between SR and Zolp can positively or negatively modulate memory of a discriminative avoidance task (Zanin et al., submitted data). Specifically, SR induced cognitive impairments were abolished by the acute administration of Zolp or its withdrawal. On the other hand, when administered to control animals, Zolp induced memory impairments. Such discrepant results could be due to the different memory tasks employed. Accordingly, Tipps and colleagues (2015)

have recently shown that ethanol withdrawn exerts a bidirectional effect on memory, in which context learning is impaired, but cued learning is improved. In line with our study, these results suggest that ethanol – a drug with a GABAergic component (Hevers and Lüddens, 1998) –.can also positively or negatively modulate memory depending on the task. In addition, compared to other animal memory tests that involve painful and potentially life-threatening aversive stimuli, the aversive stimuli used in the plus-maze discriminative avoidance task (light and sound) seem to be less stressful.

In summary, our results demonstrated that in the PAT, an animal model with a strong emotional component, SR during 10 days did not modify memory irrespective of the phase analyzed. Still, we corroborated previous findings showing the Zolp-induced anterograde amnesia. Finally, our findings showed that the PAT, although being a classical model to evaluate memory, might have been influenced by motor activity.

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3.3.1 Conclusões parciais – manuscrito 3

No paradigma da esquiva passiva, a RS repetida por 10 dias não foi capaz de prejudicar a memória quando realizada antes do treino ou teste na esquiva passiva. Ainda, ao contrário do observado anteriormente (manuscrito 2), a administração aguda pré-treino de Zolp prejudicou a retenção da tarefa nos animais restritos de sono. Por outro lado, assim como observado na ED-LCE, a RS, o zolpidem ou suas interações não foram capazes de modificar a consolidação da tarefa quando realizadas imediatamente após o treino.

Essas diferenças podem ter sido ocasionadas por: (1) diferenças qualitativas dos modelos, já que a EP poderia ser mais estressante por apresentar um estímulo doloroso, de modo que a RS não prejudicou a memória ou (2) influência da hipolocomoção induzida pelo zolpidem administrado anteriormente à exposição ao aparelho. Assim, considerando a influência do modelo de memória utilizado, no próximo manuscrito investigamos os efeitos da restrição de sono e o zolpidem em 2 tarefas não aversivas: reconhecimento de objetos e reconhecimento social.

Manuscrito 4

EFFECTS OF ZOLPIDEM ON MEMORY OF NON-AVERSIVE TASKS IN MICE SLEEP-RESTRICTED

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ABSTRACT

Purpose: Zolpidem (Zolp) and sleep restriction (SR) may have negative effects on memory. Preliminary data from our group found that the interaction between SR and Zolp can modulate the memory trace of a discriminative avoidance task. In the passive avoidance task, only the acute Zolp-treatment hindered memory regardless of SR. Herein we evaluated the effects of repeated administration of Zolp (or its withdrawal) in mice submitted to SR on spontaneous object recognition (SOR) and social discrimination (SD) tasks, which do not involve aversive components. **Design:** Mice were subjected to SR for 3 h per day during 10 days or left undisturbed (CTRL) and then were treated with saline (Sal) or Zolp. On the 10^{th} day, half of the animals treated with Sal received Zolp while the others received Sal. The same occurred for mice daily treated with Zolp. One hour after the last injection mice were submitted to behavioral sessions. **Results:** Zolp withdrawal impaired retention of SOR in CTRL mice. In the SD task, only the acute Zolp administration prevented memory deficits in CTRL animals. SR promoted deficits in the SOR and SD. The short-term memory impairment in the SOR task was abolished by Zolp regardless of the administration protocol, while only the acute administration of this drug counteracted the negative effects of SR in the SD task. **Conclusion:** The effects on memory of Zolp acute and long-term treatments and its withdrawal on sleep-restricted animals may vary depending on the task. Our data strengths the importance of future studies investigating different periods of SR as well as other memory tasks.

Keywords: zolpidem, sleep restriction, object recognition, social discrimination.

INTRODUCTION

There is a great amount of evidence showing that sleep is essential to ensure survival. Consequently, sleep loss has several adverse effects on endocrine functions, metabolic and inflammatory responses, and may cause a range of neurobehavioral deficits (Banks and Dinges, 2007). Partial sleep loss may be associated with to sleep fragmentation, specific sleep stages deprivation or sleep restriction (SR). Sleep fragmentation is generally present in sleep disorders (e.g., obstructive sleep apnea and insomnia), in which the normal progression and sequencing of sleep stages is disrupted. SR, which is also referred to as sleep debt, is characterized by reduced sleep duration. It may be experienced due to sleep disorders or other medical conditions, shift-work, social and domestic responsibilities and life-style (Banks and Dinges, 2007), thus representing an important translational model.

Specifically regarding cognition, it is well-established that sleep plays a critical role in learning and memory formation. Indeed, a myriad of studies have demonstrated that both paradoxical and total sleep deprivation leads to memory deficits in several animal models (Fernandes-Santos et al., 2012; Palchykova et al., 2006; Patti et al., 2010). Experimental protocols evaluating the animals neurobehavioral effects applying models of SR are less common than the considerable amount of work performed with total sleep deprivation or selective stage deprivation. In humans, SR impairs behavioral alertness and the neurobehavioral effects of SR appear similar to those of total sleep deprivation (Belenky et al., 2003; Van Dongen et al., 2003). Regarding animal models, it was demonstrated that SR by forced locomotion impaired spatial memory

(McCoy et al., 2013; Zielinski et al., 2013). In another study, SR by gentle handling hindered consolidation of a spatial memory in adolescent (but not in adult) rats (Yang et al., 2012).

In parallel, zolpidem (Zolp) is a hypnotic drug prescribed to treat insomnia. It is an imidazopyridine agent, which selectively binds to the α1 subunit-containing $GABA_A$ receptor subtype (Sancar et al. 2007). While Zolp seems to exhibit fewer deleterious effects, psychomotor and amnestic effects are still reported both in humans (Fitzgerald et al., 2014) and laboratory animals (Huang et al. 2010; Savić et al. 2005a,b; Zanin et al., 2011, 2013). From a clinical perspective, Zolp is prescribed as a long-term treatment for insomnia. Recently, studies in our laboratory found that the interaction between SR and Zolp can positively or negatively modulate memory of a discriminative avoidance task (Zanin et al., submitted data). Specifically, SR induced cognitive impairments, which were abolished by the acute administration of Zolp or its withdrawal. On the other hand, when administered to control animals, Zolp induced memory impairments. Conversely, when the same protocol was evaluated in the passive avoidance task, the employed SR protocol did not induce memory deficits and only the acute Zolp-treatment modified memory retention irrespective of sleep condition. Thus, the interaction between Zolp and SR may vary according to the task employed.

Spontaneous object recognition task assess the aspects of declarative memory in rodents (Dere et al., 2007; Ennaceur and Delacour, 1988). This task is based on the natural tendency of rodents to prefer novel stimuli in relation to familiar ones. Thus, animals are allowed to explore two objects. After a delay, the animal is again placed in the test arena with one object previous explored

(i.e., familiar) while the other object is novel. If memory is preserved, the animal spends more time exploring the novel object, signaling recognition memory (Winters et al., 2008). Similarly, in the social discrimination paradigm, the animal is initially exposed to an unfamiliar conspecific and then, both a familiar and a novel conspecific are simultaneously presented (van der Kooij and Sandi, 2012). The social recognition test measures short-term/working memory, i.e., the ability of retaining a small amount of information in a readily available state for a short period of time (Mathiasen and DiCamillo, 2010). Both tasks (object or social recognition) are simple in design and takes advantage of evaluating spontaneous behavior of animals with no artificial stimulus or positive/negative reinforcements.

Considering the increasing prescription of Zolp and the previous evidence supporting the influence of the memory task employed, as well as the absence of studies systematically evaluating the effects of the repeated treatment with Zolp in sleep-restricted animals, the aim of the present study was to evaluate the effects of the repeated administration of Zolp (or its withdrawal) on learning and memory of mice sleep-restricted in two different tasks without an aversive component, the spontaneous object recognition (SOR) and social discrimination (SD) tasks.

MATERIAL AND METHODS

Subjects

Three-month-old Swiss male mice (outbred, raised, and maintained in the Centre for Development of Experimental Models in Medicine and Biology of Universidade Federal de São Paulo) were used. Animals weighing 35-40 g were housed under conditions of controlled temperature (22-23°C) and lighting (12h light, 12h dark; lights on at 6:45 a.m.). Food and water were available *ad libitum* throughout the experiments. Animals used in this study were maintained in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications Nº 8023, revised 2011) and with the Brazilian Law for Procedures for Animal Scientific Use (#11794/2008). The experimental procedures were approved by the Institutional Animal Care and Use Committee under the protocol #1741/10.

Drugs

Zolp (Sanofi-Aventis®), at the dose of 5 mg/kg, was diluted in saline 0.9% solution (Sal), which was also the control solution. The dose of Zolp was chosen based on previous experiments of our group, demonstrating both memory deficits and weaker sedative effects in mice (Zanin et al., 2013). All the solutions were administered intraperitoneally in a volume of 10 ml/kg.

Sleep Restriction (SR)

Mice were submitted to SR through the gentle handling method, described elsewhere (Tobler et al., 1990). This method consists of keeping the

animal awake by tapping on the cage and, if necessary, by gently touching them with a soft brush if behavioral signs of sleep are observed. The animals were sleep restricted for 3 h (from 10 AM to 1 PM) during 10 consecutive days. This time interval was chosen because this is when paradoxical sleep reaches its highest expression and slow wave sleep homeostatic pressure is generated (Franken et al., 1991).

Spontaneous Object Recognition (SOR)

The object recognition test was carried out in an open-field arena, which consisted in a circular arena (96 cm diameter) enclosed by matte white walls (circumference 40 cm) with an open top and floor divided into 19 quadrants. In the familiarization session, the animals were placed in the arena containing two similar objects and left to explore them freely for 10 min but exploration of the objects was quantified in the first 5 min. The test session occurred 180 minutes later, in order to evaluate short-term memory. Twenty-four hours later, long-term memory was investigated. In the tests, one of the objects was substituted for a new object and the rat was introduced in the arena for more 5 min. In addition, herein we opted for not including a habituation session since it was demonstrated that the habituation phase does not influence performance and, thus, excluding this session reduces the total duration of the experiment (Leger et al., 2013).

The positions of the objects (familiar or novel) were randomly permuted for each experimental animal. Exploration was defined as sniffing or touching the object with the nose and/or forepaws. Sitting on or turning around the object was not considered exploratory behavior. Only in the familiarization session

immobility time was recorded in order to discard the possible influence of the sedative effects of Zolp. The time spent to explore each object was recorded by an observer blind to the treatment. The objects and the apparatus were cleaned with a 5% alcohol solution after each behavioral session.

The measure of discrimination considered was the discrimination ratio (DR), as described before (Ennaceur and Delacour, 1988). The DR is the difference between time spent exploring the two objects divided by the total time spent exploring both objects. DR index ranges from -1 to 1. Thus, a value higher than zero reveals an exploratory preference for the novel combination during test. Importantly, using the DR index individual differences in the level of exploration is taken into account (Akkerman et al., 2012).

Social discrimination task (SD)

The social recognition was performed in a 3-chambered sociability apparatus (Ugo Basile®, Varese, Italy). It is composed by a transparent acrylic box, divided into 3 compartments (20 x 40 x 22 cm) with a non-reflective, grey-colored floor and 2 round grid enclosures (7 x 15 cm high). The thin, widely spaced bars of the wire cage allowed nose contact between the bars, but prevented any social contact and limited the possibility of aggressive behavior. The 2 outer compartments (A and B) contained the grid enclosures and were separated by a central compartment with no cage inside and 2 sliding doors in order to allow mice moving freely between the compartments.

Firstly, the experimental animal was placed in the central compartment for habituation with the empty grid enclosures during 5 min. Then, an unfamiliar animal matched for body weight with the experimental animal was placed into

one of the wire cages. After 5 min, a 2nd unfamiliar mouse was inserted into the opposite compartment and exploration was allowed for more 5 min. The intruders were randomly selected and counterbalanced for each group. Time spent in target to the cage or sniffing the strange mouse was recorded by an observer blind to the treatment. The apparatus was cleaned with a 5% alcohol solution after each behavioral session.

The interaction with the 1st intruder confined to the small cage in one compartment is considered a measure of sociability while the social recognition memory is evaluated by the discrimination between the familiar intruder 1 and the novel intruder 2. In this condition, the possible preference for the new intruder is considered a measurement of retention (Nadler et al., 2004; Riedel et al., 2009).

Experimental design

Experiment I: effects of the repeated administration of Zolp or its withdrawal on SOR test in mice sleep-restricted

Animals were kept in their home cages (control condition – CTRL) or subjected to SR by gentle handling for 3 h per day during 10 days. Every day, after the SR period, mice were treated with Sal (CTRL-Sal and SR-Sal) or Zolp (CTRL-Zolp and SR-Zolp). On the 10^{th} day, half of animals treated with Sal received an acute Zolp challenge injection while the others received Sal. The same procedure was applied for mice daily treated with Zolp. The following groups were formed: CTRL-Sal/Sal (n=12), CTRL-Sal/Zolp (n=12), CTRL-Zolp/Sal (n=11), CTRL-Zolp/Zolp (n=12), SR-Sal/Sal (n=12), SR-Sal/Zolp

(n=12), SR-Zolp/Sal (n=12) and SR-Zolp/Zolp (n=12). Sixty min after the last injection, mice were subjected to training of SOR. This administration interval was chosen because animals present fewer hypolocomotor effects but the drug is still available, since its half-life is about 1.5 h in rodents (Garrigou-Gadenne et al., 1989). After training, animals were returned to their home-cage. Three hours later animals were submitted to test session 1 and after 24 h to test session 2.

Experiment II: effects of the repeated administration of Zolp or its withdrawal on SD test in mice sleep-restricted

Animals were treated as described previously, forming the following groups: CTRL-Sal/Sal (n=12), CTRL-Sal/Zolp (n=12), CTRL-Zolp/Sal (n=12), CTRL-Zolp/Zolp (n=12), SR-Sal/Sal (n=12), SR-Sal/Zolp (n=12), SR-Zolp/Sal (n=12) and SR-Zolp/Zolp (n=12). Sixty min after the challenge injection, mice were subjected habituation in the SR box. Five min after habituation, the 1st intruder was introduced into the box for 5 min (training session). Then, the 2nd intruder was placed in the other compartment during 5 min for testing.

Statistical analysis

The inter-group analyses were performed by MANOVA and Duncan's test and for intra-group analyses paired samples T-test was applied. A probability of $p<0.05$ was considered significant for all comparisons made.

RESULTS

Experiment I: effects of the repeated administration of Zolp or its withdrawal on SOR test in mice sleep-restricted

In the familiarization session, MANOVA for time spending exploring both objects revealed significant effects of sleep condition (CTRL vs. SR) [$F(1,87)=220.06$; $p<0.001$] and sleep condition x challenge injection (Sal vs. Zolp) interaction [$F(1,87)=4.46$; $p<0.05$]. Duncan's *post hoc* showed that mice only sleep-restricted (SR-Sal/Sal) and those sleep-restricted and abstinent of treatment (SR-Zolp/Sal) explored significantly more the objects compared to its respective control (CTRL-Sal/Sal and CTRL-Zolp/Sal, respectively). Still, the repeated treatment with Zolp (SR-Zolp/Zolp) decreased exploration compared to the group challenged with Sal (SR-Zolp/Sal). In addition, Zolp withdrawal in sleep-restricted mice induced an increase in exploration time compared to control group repeatedly treated with Zolp (CTRL-Zolp/Zolp) (Figure 1A). Regarding immobility time in this session, there were no significant differences among groups (Figure 1B).

In the 1st test session (3 h after the familiarization session), MANOVA for the DR showed significant effects of the interactions sleep condition x treatment [$F(1,87)=9.59$; $p<0.005$] and sleep condition x treatment x challenge injection [$F(1,87)=9.49$; $p<0.005$]. Duncan's test revealed that mice only sleep restricted (SR-Sal/Sal) displayed a lower DR compared to control (CTRL-Sal/Sal). Still, the control group abstinent of treatment (CTRL-Zolp/Sal) presented a decreased DR compared to all of the groups (with the exception of the SR-Sal/Sal group) (Figure 2).

In the 2nd test session (24 h after the familiarization session), MANOVA for the DR showed significant effects of treatment [$F(1,87)=6.38$; $p<0.05$] and sleep condition x challenge injection interaction [$F(1,87);=5.00$; $p<0.05$]. Thus, the *post hoc* analysis revealed that mice abstinent of treatment (CTRL-Zolp/Sal) presented a decreased performance compared to control group (CTRL-Sal/Sal) and the repeatedly Zolp-treated group (CTRL-Zolp/Zolp). Still, animals sleep-restricted and repeatedly treated with Zolp (SR-Zolp/Zolp) presented a worse performance compared to mice acutely treated with the drug and subjected to the same sleep condition (SR-Sal/Zolp) (Figure 2).

We performed a within-subjects analysis to compare performance between the test sessions. Thus, paired-samples T tests showed that only mice sleep-restricted and treated with Zolp (SR-Zolp/Zolp) presented a worse performance in the 2nd test compared to the 1st one [$T(11)=2.25$; $p<0.05$] (Figure 2).

Experiment II: effects of the repeated administration of Zolp or its withdrawal on SD test in mice sleep-restricted

In the habituation session, MANOVA did not show significant differences among groups for the time spent interacting with the empty cages (Figure 3A). In the training session, when the within-subject analysis was performed, all of the groups spent significantly more time exploring the 1st intruder than the empty cage [$T(11)=3.24$; $p<0.01$ for CTRL-Sal/Sal; 3.60; $p<0.005$ for CTRL-Sal/Zolp; 4.88; $p<0.001$ for CTRL-Zolp/Sal; 3.67; $p<0.005$ for CTRL-Zolp/Zolp; 3.75; $p<0.005$ for SR-Sal/Sal; 2.45; $p<0.05$ for SR-Sal/Zolp; 2.46; $p<0.05$ for

SR-Zolp/Sal; 2.43; p<0.05 for SR-Zolp/Zolp]. In addition, MANOVA followed by Duncan's test revealed significant effects of sleep condition [F(1,88);=4.38; p<0.05] and challenge injection [F(1,88);=5.60; p<0.05]. Indeed, sleep-restricted mice challenged with Zolp (SR-Sal/Zolp) explored less the empty cage compared to mice only sleep-restricted (SR-Sal/Sal). Still, mice subjected to SR and abstinent of treatment (SR-Zolp/Sal) presented a higher exploration of the empty cage compared to control (CTRL-Sal/Sal) and to the group in the same sleep condition continuously treated with Zolp (SR-Zolp/Zolp) (Figure 3B).

In the test session when the within-subject analysis was performed, paired-samples T test showed that among animals subjected to CTRL condition, only those treated with Sal (CTRL-Sal/Sal) or challenged with Zolp (CTRL-Sal/Zolp) showed an increased exploration of the 2nd intruder in relation to the 1st one [T(11)=2.29 and 2.87; p<0.05, respectively]. On the other hand, in the sleep-restricted mice, only those acutely treated with Zolp preferred to explore the 2nd intruder [T(11)=2.31; p<0.05]. Regarding the between-subject analysis, MANOVA did not show significant differences among groups (Figure 3C).

Figure 1

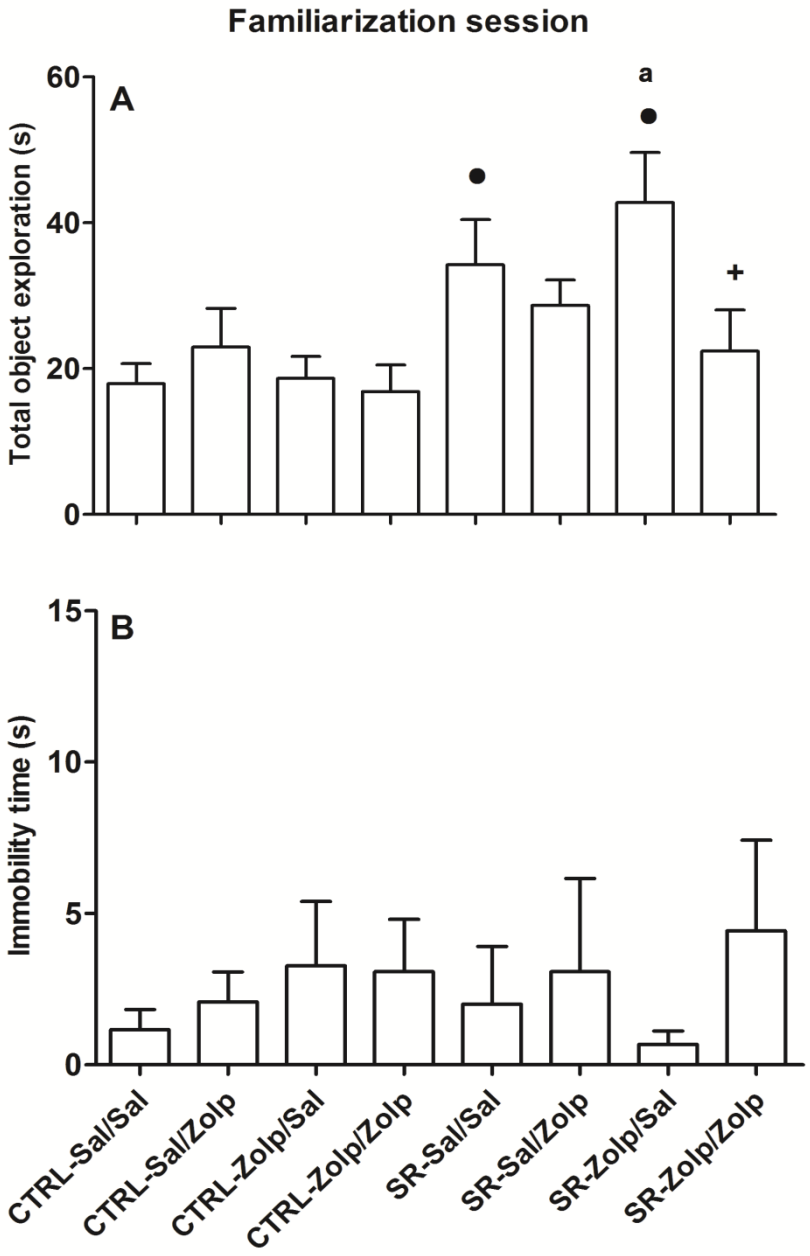

Figure 1: Effects of the repeated administration of Zolp or its withdrawal on object recognition task in mice submitted to sleep restriction (SR). Animals were subjected to control condition (CTRL) or SR for 10 days and were daily treated with Sal or 5 mg/kg Zolp. On the 10th day, after the last injection, mice were trained and tested 3 and 24 h later. Results are presented as the mean ± S.E. of total time spent in exploring the objects (**A**) and immobility time (**B**) in the familiarization session. •p<0.05 compared to the group of same treatment and challenge injection but different sleep condition; +p<0.05 compared to the group of same sleep condition and treatment but different challenge injection; ^{a}p<0.05 compared to the CTRL-Zolp/Zolp group (MANOVA and Duncan's test).

Figure 2

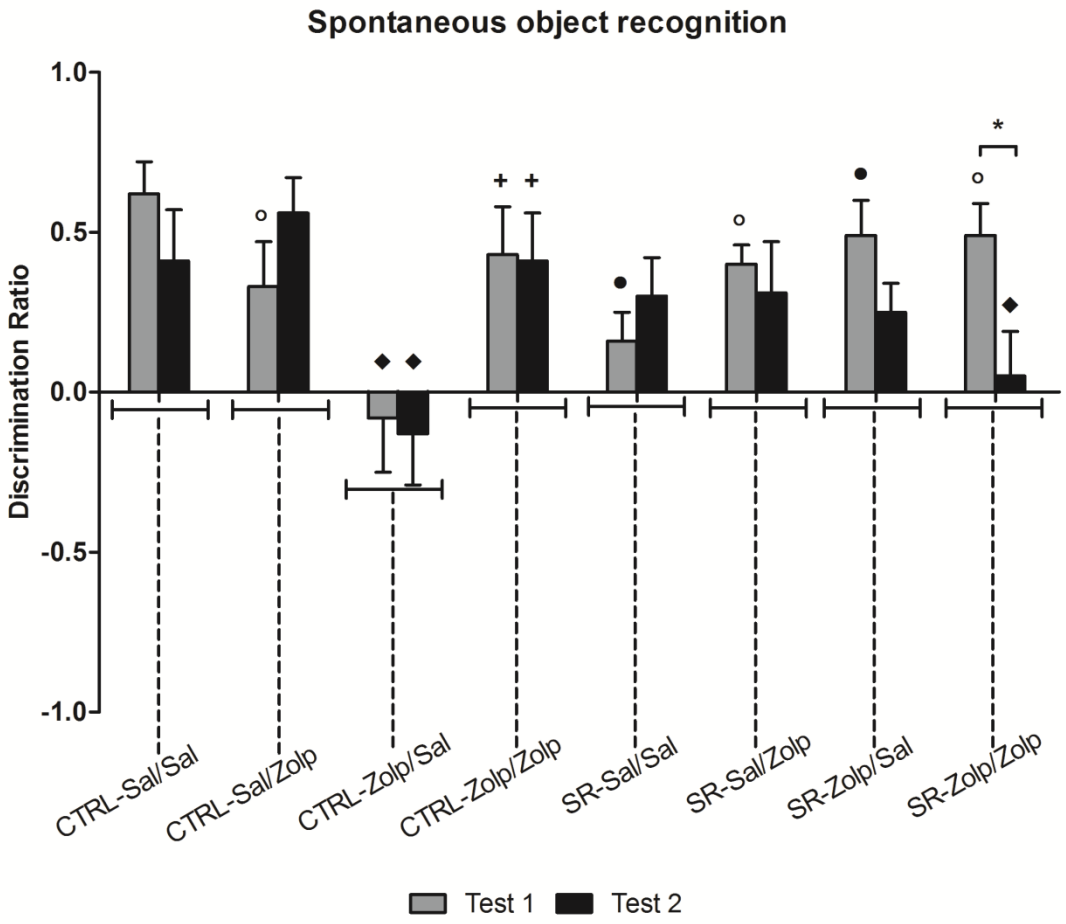

Figure 2: Effects of the repeated administration of Zolp or its withdrawal on object recognition task in mice submitted to sleep restriction (SR). Animals were subjected to control condition (CTRL) or SR for 10 days and were daily treated with Sal or 5 mg/kg Zolp. On the 10th day, after the last injection, mice were trained and tested 3 and 24 h later. Results are presented as the mean ± S.E. of discrimination ratio (DR) in the short- (test 1) and long-term (test 2) memory evaluation. □p<0.05 compared to the group of same treatment and challenge injection but different sleep condition; +p<0.05 compared to the group of same sleep condition and treatment but different challenge injection; ♦p<0.05 compared to the group of same sleep condition and challenge injection but different treatment; ºp<0.05 compared to the CTRL-Zolp/Sal group (MANOVA and Duncan's test); *p<0.05 compared to test 1 into the same group (paired-samples T test).

Figure 3

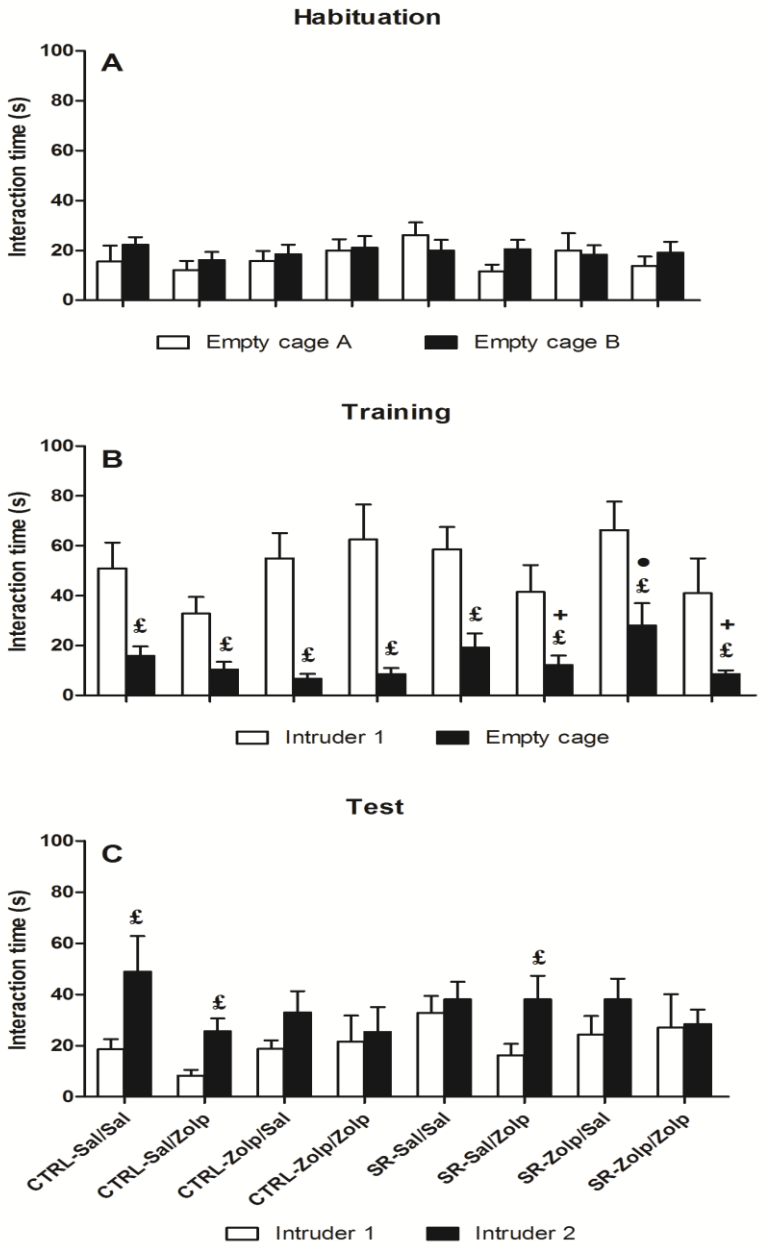

Figure 3: Effects of the repeated administration of Zolp or its withdrawal on social discrimination task in mice submitted to sleep restriction (SR). Animals were subjected to control condition (CTRL) or SR for 10 days and were daily treated with Sal or 5 mg/kg Zolp. On the 10th day, after the last injection, mice were habituated to the test box. Five min later were trained and 5 min after, tested. Results are presented as the mean ± S.E. of time spent interacting with the empty cages or the unfamiliar animal during habituation (**A**), training (**B**) and test (**C**) sessions. •p<0.05 compared to the group of same treatment and challenge injection but different sleep condition; +p<0.05 compared to the group of same sleep condition and treatment but different challenge injection; (MANOVA and Duncan's test); **£**p<0.05 compared to time exploring intruder 1 into the same group (paired-samples T test).

DISCUSSION

In the current study we showed that SR during 3 h for 10 consecutive days promoted short- and long-term memory deficits in 2 non-aversive tasks (SOR and SD). While the SR-induced short-term memory impairment in the SOR task was abolished by Zolp (regardless of the administration protocol), the administration of this hypnotic did not preserve long-term memory in SOR task. Moreover, Zolp withdrawal alone (but not the acute or repeated administration) was able to impair mice performance on SOR. In the SD task, SR promoted amnestic effects, which were counteracted by the acute Zolp administration. On the other hand, in the SD task, only the acute Zolp administration did not hinder memory in CTRL animals.

To the best of our knowledge, this is the first study that systematically evaluated the effects of the repeated treatment with Zolp or its withdrawal in non-aversive tasks in mice subjected to a repeated SR protocol. Thus, in experiment I we assessed the effects of this treatment on the SOR task. In the familiarization session, Zolp administration did not modify objects exploration. Conversely, SR induced an increase in exploration level, which was attenuated by the acute administration of Zolp and abolished by the repeated treatment with this drug. This Zolp-induced reduction in exploration could be due to the sedative effect of Zolp. Alternatively, a blockade of a possible SR-induced increase on impulsivity or motor activity may have occurred. Since there were no differences among groups on immobility time, it is unlike that the sedative effect of Zolp had influenced exploration. In addition, we have chosen the 1-h administration interval to minimize such hypolocomotor effect.

Distinct sleep deprivation protocols may increase impulsivity (Berro et al., 2014) as well as motor activity in laboratory animals. In fact, it was demonstrated that sleep deprivation potentiated both spontaneous (Frussa-Filho et al., 2004) and drug-induced (Arriaga et al., 1988; Ferguson and Dement, 1969; Frussa-Filho et al., 2004) motor activity in the open-field. Of note, motor activity is closely related to dopaminergic neurotransmission (Kelly et al., 1975; Kelly and Iversen, 1976). Similarly, preclinical and clinical studies have pointed that high impulsivity involves a dysregulation of the dopaminergic system (Dalley et al., 2011; Fineberg et al., 2010; Volkow et al., 2009). Although it is well-established that paradoxical sleep deprivation increase several behaviors related to the dopaminergic system (Frussa-Filho et al., 2004; Tufik et al., 1978; Tufik, 1981a,b; Troncone et al., 1988), it was shown that this procedure by the multiple platforms method also deprives time spent in slow wave sleep (Silva et al., 2004). Thus, it is tempting to speculate that SR could also have induced dopaminergic supersensitivity, which was suppressed by Zolp administration. Accordingly, it was demonstrated that GABA-modulating drugs such as benzodiazepines inhibits the GABAergic interneurons in important areas of dopaminergic transmission such as the ventral tegmental area and substantia nigra (Grace et al., 1980; Tan et al., 2010). As a result, GABA neurons no longer inhibit dopaminergic cells increasing dopamine release. Notably, it was shown that these inhibitory interneurons express mainly α1 subunit (Gao et al., 1993, 1994) – Zolp preferential site of action.

There is several discrimination measures used to evaluate memory in the SOR task. The most frequently used are the absolute difference in exploration (difference between time exploring the novel and the familiar objects) (King et

al., 2004; Messier et al., 1997) and the relative measure DR (Gaskin et al., 2003; Lieben et al., 2005). Comparing these measures, Akkerman and colleagues (2012) showed that the absolute difference in exploration was more vulnerable to differences in exploratory activity, while the DR, which corrects for exploratory differences, was more sensitive to subtle differences when exploration levels varied among groups. Considering that both the SR and Zolp may modify motor activity, we opted to apply the DR. Thus, in the short-term memory test (test 1), only Zolp withdrawal hampered retention in CTRL mice. Although mice subjected to SR presented a positive DR value, there was a significant decrease compared to animals in the CTRL-Sal/Sal group, suggesting that SR *per se* hindered short-term memory. Interestingly, irrespective from the administration regimen, Zolp counteracted the amnestic effect of SR.

As far as we know, no studies have addressed the effects of SR protocol or its interaction with Zolp on memory of SOR task. Notably, we have recently observed a similar behavioral pattern, i.e., Zolp-induced reversal of SR amnestic effects, when mice were exposed to an aversive task, the plus-maze discriminative avoidance (Zanin et al., submitted data). Although highly speculative, it could be suggested that, as happened for motor activity, Zolp administration increased dopamine release, which attenuated the SR-induced dopaminergic supersensitivity, blocking its deleterious effects on memory. In this regard, it has been suggested that hippocampal dopamine modulates memory encoding and consolidation during or early after training (Jay, 2003; O'Carroll et al., 2006; Talhati et al., 2014). This hypothesis deserves further investigations.

When long-term memory was evaluated (test 2), among animals which had their sleep undisturbed, in exception of the group abstinent of Zolp treatment, all of the groups presented high DR levels in the same magnitude of those observed during test 1, thus displaying a unmodified performance. Of note, Kleykamp and colleagues (2012) showed a trend towards a cognitive impairing effect induced by Zolp withdrawal in humans. Among sleep-restricted animals, although SR *per se* promoted short-term memory impairments, when long-term memory was evaluated this effect seems of less magnitude. This apparently improvement effect could be due to the 2nd exposition to the objects during the test 1. Thus, considering that mice were allowed 3-h sleep before being exposed to test 1, this session may have reinforced the memory trace for the familiar object or even worked like a new learning session. Still, in sleep-restricted mice repeatedly treated with Zolp, the long-term memory was attenuated although it did not modify short-term memory. In spite of a few studies evaluating the effects of the repeated treatment with Zolp on cognition, Stoops and Rush (2003) have demonstrated that the acute administration of Zolp promoted memory deficits in humans without toleration development after the repeated treatment.

No studies have evaluated the effects of SR protocols on long-term memory of a SOR task. Only few studies investigated the effects of acute total or paradoxical sleep deprivation protocols specifically on the consolidation phase using several variations of the SOR task however the results were controversial. When memory was evaluated in short periods after training, total sleep deprivation impaired object-place recognition (but not SOR), which is a hippocampal-dependent task (Binder et al., 2012; Inostroza et al., 2013). On the

other hand, when memory was evaluated 24 h after training, paradoxical sleep deprivation for 4 h impaired consolidation of object-place task (Ishikawa et al., 2014) and when extended for 6 h, it impaired the SOR (Chen et al., 2014). Conversely, when using the gentle handling method, sleep deprivation impaired consolidation immediately after training (Palchykova et al., 2006) as well as with a 1-h delay (Prince et al., 2014), depending on the duration of sleep deprivation. This discrepancy may be accounted for differences in the number of objects used or additional contextual cues and the duration of sleep deprivation. In addition, lesion studies demonstrated that the hippocampus is involved in object-place recognition, while SOR is dependent on the prefrontal and entorhinal cortex (Barker and Warburton, 2011; Mumby et al., 2002).

In the present study, we assessed social interaction in mice and determined sociability. Within this context, sociability is evaluated by social interaction with a 1st stranger confined to a small cage in one compartment, and after an interval, social recognition memory is evaluated by discrimination of a familiar stranger 1 from a novel stranger 2. This discrimination is typically observed in mice (Moy et al., 2004; Nadler et al., 2004; Riedel et al., 2009). We found that besides all of the groups preferred to explore the 1st intruder rather than the empty cage, in sleep-restricted mice, Zolp withdrawal increased the time exploring the empty cage. This increased exploratory behavior could be a result of a higher locomotor activity induced by SR, which was potentiated by Zolp withdrawal. Another explanation would be a reduction in sociability. This last explanation does not seem to be the case since the amount of time these animals spent exploring the 1st intruder was similar compared to the others. In addition, the increased cage exploration time was abolished by Zolp

administration in the repeatedly-treated group. This effect does not seem to be due to sedation. Specifically, Riedel and colleagues (2009) reported an enhanced sociability in diazepam-treated mice, which also expressed heightened activity, suggesting that locomotor activity may be independent of the amount of social activity.

During the testing, only the acute Zolp treatment did not impair memory in CTRL animals. No studies have investigated the effects of Zolp on SD, nevertheless it was shown that diazepam may induce SD deficits in mice (Riedel et al., 2009). Still, SR induced memory deficits, which were abolished by the acute administration of Zolp, but not its withdrawal or repeated treatment. Of note, previous studies have already reported that this drug promotes anterograde amnesia, i.e. forgetfulness of events that occurred shortly after drug intake (Danjou et al., 1999; Tsai et al., 2007). Additionally, these results gives further evidence that Zolp-induced negative or positive effects may vary depending on the emotional characteristic of the task (aversive vs. non-aversive) as well as the type of memory analyzed. In this respect, the SD test is designed to measure short-term/working memory (Mathiasen and DiCamillo, 2010), which refers to the capacity for holding a small amount of information in the mind in an active, readily available state for a short period of time.

Group or individual odors have great importance for rodents, since it is necessary for the determination of threat, communication of food sources, social hierarchy and for mate choice (Berry and Bronson, 1992; Jiming et al., 1994). Therefore, a memory for olfactory-based recognition represents a critical cognitive skill for these species. Herein we did not evaluate long-term memory for SD. However, as previously noted, rodents show poor memory in this

paradigm and generally remember a previous single encounter with companions for less than 1–2 h (Bluthé and Dantzer, 1993; Gheusi et al., 1994; Perio et al., 1989; Sekiguchi et al., 1991; Thor and Holloway,1982). Long-term memory of SD requires castration of males (Bluthé and Dantzer, 1990), a training to criterion procedure (Koogan et al., 2000) or stress application (Penka et al., 2004).

Collectively, our results suggest that the interaction between SR and Zolp is intriguing. Specifically, the short-term memory impairment in the SOR task was abolished by Zolp in all administration protocols, while only the acute administration of this drug counteracted the negative effects of SR in the SD task. In addition, albeit the repeated administration of Zolp reversed the effects of SR on short-term memory, it did not preserve long-term memory of SOR task. Thus, the positive or negative effects of Zolp are critically dependent on the task and the administration regimen. Therefore, the present findings point out to the need of future studies evaluating the interaction between Zolp and different SR periods, once that depending on the task, the effects of SR seems to be more deleterious than the effects of Zolp.

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3.4.1 Conclusões parciais – manuscrito 4

A RS promoveu déficits de memória de curta e longa durações em duas tarefas de conteúdo não-aversivo: o reconhecimento de objetos e o reconhecimento social. A administração de Zolp, independentemente do protocolo, reverteu o prejuízo da memória de curta-duração induzido pela RS no reconhecimento de objetos. Por outro lado, a administração repetida desse hipnótico não foi suficiente para preservar a memória de longa-duração. Ainda, a retirada do tratamento por si só prejudicou o desempenho.

No que diz respeito ao reconhecimento social, a RS promoveu amnésia, o que foi revertido apenas pela administração aguda de Zolp. Contudo, nesse paradigma, apenas a administração aguda da droga não prejudicou o desempenho na tarefa nos animais controle.

Em conjunto com os resultados obtidos nos manuscritos anteriores, os efeitos do Zolp sobre os processos de formação da memória são criticamente dependentes da tarefa e do regime de administração empregados. Dessa forma, nossos resultados reforçam a importância de estudos futuros avaliando a interação com Zolp e diferentes períodos de restrição de sono RS que mimetizem as situações clínicas.

Manuscrito 5

EFFECTS OF ZOLPIDEM ON SLEEP PATTERN IN AN ANIMAL MODEL OF SLEEP RESTRICTION

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ABSTRACT

Purpose: Zolpidem (Zolp) is a hypnotic drug prescribed to treat insomnia. Although polysomnographic findings after Zolp administration have already been described, they remain controversial. Additionally, no studies have characterized the effects of Zolp in animal models of sleep restriction (SR). Thus, the aim of this study was to investigate the effects of the repeated administration of Zolp on sleep parameters in mice submitted to SR. **Design:** Mice were subjected to control condition (CTRL) or to SR by gentle handling for 3 h per day during 10 days. The sleep pattern of mice was recorded before treatment to evaluate baseline parameters. After it, mice were subjected to CTRL or SR and after it were treated with saline (Sal) or Zolp during 10 days, forming the groups: CTRL-Zolp, SR-Sal and SR-Zolp. The parameters considered were: total sleep time (TST), slow-wave sleep (SWS), paradoxical sleep (PS) and wake (W). **Results:** The SR protocol enhanced W time and decreased SWS, PS and TST. Zolp administration increased SWS in both CTRL and SR groups. In the SR-Zolp group, the compensatory sleep enhancement was observed in the light phase. Still, only in the SR-Zolp group, the modifications persisted during rebound compared to baseline. **Conclusions:** Zolp increased SWS in both sleep conditions, but in CTRL mice it seemed to be tolerated. Still, in SR-Sal the SWS (but not PS) enhancement was reduced throughout days. Importantly, SR-Zolp group did not completely recover the sleep parameters to baseline levels during rebound.

Keywords: zolpidem, sleep restriction, sleep architecture

INTRODUCTION

Insomnia is the most prevalent sleep disturbance, affecting 10 to 25% of the general adult population in most countries (Léger et al., 2008; Morin et al., 2006; Ohayon, 2002; Sivertsen et al., 2009; Soldatos et al., 2005). Occasional episodes of insomnia symptoms are reported by one half of all American adults while chronic insomnia is estimated to affect 10–14% of this population in the US (Léger et al., 2008; Roehrs and Roth, 2004). In Brazil, a national survey based on screening questions revealed that 35% of the interviewed people complained of insomnia symptoms with frequencies greater than 3 times per week (Bittencourt et al., 2009). Specifically, a recent survey in São Paulo using polysomnography demonstrated that the prevalence of objective insomnia was 32% (Castro et al., 2013). This disorder negatively impacts physical and social performances becoming an important public health issue with remarkable socio-economic implications.

The current treatments for insomnia involve sleep hygiene measures, behavioral therapies and pharmacological treatments (Roth and Drake, 2004; Zammit, 2007). Concerning the pharmacological treatments, till the 80's the most employed drugs to treat insomnia were the benzodiazepines. Although its efficacy in inducing sleep, these drugs promote several adverse effects such as persistent sedation, ataxia, amnesia and tolerance as well as a high abuse potential (Akhondzaded et al., 2002; Allison and Pratt, 2003). In order to prevent such effects, new selective drugs were developed. Thus, in 1992 zolpidem (Zolp) was introduced into clinical practice in the USA becoming the most commonly prescribed hypnotic (Rush, 1998).

In this way, Zolp is an imidazopyridine agent which selectively binds to the α1 subunit-containing $GABA_A$ receptor subtypes (Lloyd and Zivkovic 1988; Puia et al. 1991; Sancar et al. 1997; Sanger et al. 1994). Due to this given selectivity, Zolp has mainly hypnotic properties with weaker anticonvulsant and myorelaxant effects (Depoortere et al., 1986; Lloyd and Zivkovic 1988; Zivkovic et al., 1988). While Zolp seems to exhibit fewer deleterious psychomotor and cognitive effects, amnestic effects are still reported (Danjou et al. 1999; Dingemanse et al. 2000; Huang et al. 2010; Salvà and Costa, 1995; Zanin et al., 2011, 2013). Of note, concerning sleep pattern, Zolp seems to decrease sleep latency and increase total sleep time (American Sleep Disorders Association, 1990). Still, it may enhance slow wave sleep which is usually reduced in insomniac patients (Besset et al., 1995). On the other hand, studies report no effects of Zolp on REM sleep or in S2 sleep stage (Colle et al., 1991; Poyares et al., 2005).

Although Zolp has proven to be safe and effective, some issues remain regarding its effects on sleep architecture, on tolerance development, and on possible rebound after the medication stops. From a clinical perspective, Zolp is usually prescribed as a repeated treatment for insomnia. However, as far as we know, no studies have systematically characterized the effects of Zolp on sleep pattern as repeated administration in animal models of sleep restriction (SR). Thus, the aim of this study was to evaluate the effects of the repeated administration of Zolp on sleep parameters of mice submitted to SR during 10 consecutive days.

MATERIAL AND METHODS

Subjects

Three-month-old Swiss male mice (outbred, raised, and maintained in the Centre for Development of Experimental Models in Medicine and Biology of Universidade Federal de Sao Paulo) were used. Animals weighing 35-40 g were housed under conditions of controlled temperature (22-23°C) and lighting (12h light, 12h dark; lights on at 7:00 a.m.). Food and water were available *ad libitum* throughout the experiments. Animals used in this study were maintained in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications Nº 8023, revised 2011) and with the Brazilian Law for Procedures for Animal Scientific Use (#11794/2008). The experimental procedures were approved by the Institutional Animal Care and Use Committee under the protocol #1741/10.

Drugs

Zolp (Sanofi-Aventis®), at the dose of 5 mg/kg, was diluted in saline 0.9% solution (Sal), which was also the control solution. The solutions were administered intraperitoneally in a volume of 10 ml/kg body weight. The dose of Zolp was chosen based on previous experiments of our group (Zanin et al., 2013).

Sleep Restriction (SR)

Mice were submitted to SR through the gentle handling method, described elsewhere (Tobler et al., 1990). This method consists of keeping the

animal awake by tapping on the cage and, if necessary, by gently touching them with a soft brush if behavioral signs of sleep are observed. The animals were sleep deprived for 3 h (from 10 AM to 01 PM) during 10 consecutive days. This time interval was chosen because this is when paradoxical sleep reaches its highest expression and slow wave sleep homeostatic pressure is generated (Franken et al., 1991).

Sleep pattern recording

Mice were surgically prepared with electrodes for electrocorticographic and electromyographic recordings in order to assess sleep-wake cycles. Anesthesia was induced by i.p. administration of 0.1ml/10g ketamine-xylazine solution. Two pairs of electrodes (steel screws) were implanted above the dura mater of the parietal cortex (above the hippocampus) in both hemispheres in the fronto-parietal medial derivation (1 pair on each side of the skull) for electrocorticographic recording. The following coordinates were used for each screw relative to the bregma (antero-posterior and medio-lateral coordinates, respectively): screw 1: 0.5, 0.8 mm; screw 2: -1.2, 1.8 mm; screw 3: -0.5, -0.5 mm; screw 4: -2.0, -2.0 mm. Bipolar derivations (screws 1 vs 2, and screws 3 vs 4; ipsilateral derivations) were used to access and classify brain activity in both hemispheres (Queiroz et al., 2013). One additional pair of nickel-chrome electrodes was implanted in the dorsal muscle of the neck for electromyographic recording. The electrodes were soldered to a connector, which was fixed to the cranium with acrylic dental cement. After surgery, mice were given antibiotic (veterinary pentabiotic® 0.5 ml/kg, intramuscularly) and anti-inflammatory (diclofenac, 25 mg/ml, i.p.) for 3 days, and were allowed to

recover from surgery for 2 weeks. After recovery, mice were habituated to the sleep recording system during 3 days.

After habituation, sleep recording was performed using the Somnologica software (EMBLA Medical digital polygraph, Reykjavik, Iceland). The sleep pattern was visually and manually scored in 10-second segments by a single blinded researcher to ensure consistency of the data. The following sleep parameters were considered: total sleep time in minutes (TST), percentage of slow-wave sleep time (time in slow wave sleep/TST – SWS), percentage of paradoxical sleep time (time in paradoxical sleep/TST – PS) and percentage of wake time (time awake/total recording time – W). SWS was classified as electrocortiographic voltage of 20–30 μV (usually 200–400 μV, peak to peak) and low frequency activity (delta waves, 1–4 Hz). Paradoxical sleep was classified as eletrocorticographic voltage below 20 μV with high and regular theta activity (6–10 Hz), especially in the fronto-parietal medial eletrocorticographic derivation, and muscle atonia in eletromiographic recording. The electrocorticographic and electromyographic signs were amplified and filtered by the system in 0,3-100 Hz and 30-300 Hz, respectively and the sampling frequency was fixed in 200 Hz.

Experimental design

Considering that the sleep record is performed in individual cages, 30 days before the beginning of treatment, all of the animals were placed individually in homecage to habituate to isolation. After this period, mice were subjected to surgery and then were habituated to the recording system, as described before.

All of the animals had their sleep recorded during 48 h before treatment for acquisition of basal sleep. On the 3rd day after the beginning of recording, mice were subjected to control condition (CTRL) or sleep restricted during 3 h for 10 consecutive days (SR). After the sleep deprivation period mice were treated with saline (Sal) or Zolp forming the following groups: CTRL-Zolp (n=4), SR-Sal (n=4) and SR-Zolp (n=4), which continued to be recorded till the beginning of sleep deprivation on the next day. On the 4th day the animals were disconnected of the system before the sleep deprivation period. On the 12th day (the 10th treatment-day) mice were connected to the recording system before the sleep deprivation protocol. Again, the animals had their sleep pattern recorded during the last treatment-day and then for 48 h after the end of treatment to evaluate rebound.

Statistical analysis

The sleep parameters throughout the treatment were compared using ANOVA with repeated measures. ANOVA and Duncan's test were used to analyze the parameters between groups and a paired-samples T test for intra-group analysis specifically during the sleep deprivation period. A probability of $p<0.05$ was considered significant for all comparisons made.

RESULTS

Considering that there were no significant differences among groups comparing the 1st and 2nd days of basal recording or rebound, we will show the results of the 24 h baseline record before the beginning of treatment and 24 h of rebound record after the end of treatment.

Analyzing the sleep parameters during the period of sleep deprivation (10 AM to 01 PM) in the 1st day, as expected, one-way ANOVA followed by Duncan's test revealed that mice subjected to SR presented a decrease in the percent time in SWS [$F(2,9)=345.37$; $p<0.001$] and in PS [$F(2,9)=24.09$; $p<0.001$] when compared to CTRL group (Figures 1A and 1B, respectively). On the other hand, analyzing the percent time awake, one-way ANOVA and Duncan's test showed an increase in this parameter [$F(2,9)=407.80$; $p<0.001$] in mice sleep-restricted compared to CTRL (Figure 1C). Still, analyzing TST animals subjected to SR presented a significant decrease compared to CTRL [$F(2,9)=406.67$; $p<0.001$] (Figure 1D).

Similarly, in the last day of SR (10th day) there was a decrease in the SWS [$F(2,9)=14.90$; $p=0.001$] and PS [$F(2,9)=7.58$; $p<0.05$] (Figures 1A and 1B, respectively). Still, there was an increase in W time [$F(2,9)=14.22$; $p=0.002$] in mice sleep deprived compared to CTRL group and consequently a decrease in TST [$F(2,9)=84.44$; $p<0.001$] (Figures 1C and 1D, respectively). Importantly, comparing the 1st to the 10th SR-day paired-samples T test did not show significant differences between days for all parameters analyzed (Figure 1), demonstrating no modifications in the magnitude of SR throughout time.

In order to evaluate the sleep pattern throughout the 10 SR-days in the light and dark phases we performed a repeated measures analysis from the beginning of baseline recording till the end of the 1st SR-day (1st period) and another analysis from the 10th SR-day till the end of rebound period (2nd period). The analysis was performed in an interval of every 3 h for all sleep parameters evaluated.

Regarding W time in the 1st period, ANOVA with repeated measures revealed significant effects of time [$F(15,135)=24.03$; $p<0.001$] and time x group interaction [$F(30,135)=7.17$; $p<0.001$]. In this way, in the 2nd period there were significant effects of time [$F(15,135)=17.95$; $p<0.001$] and time x group interaction [$F(30,135)= 2.95$; $p<0.001$]. Paired-samples T test demonstrated that in animals subjected to CTRL condition Zolp induced a decrease in W time during the dark phase (07 to 10 PM and 04 to 07 AM intervals) when compared to the same period during baseline record [$T(3)=3.25$; $p<0.05$ and 7.21; $p=0.005$, respectively]. Conversely, there was an increase in W during the rebound when compared to the 1st SR-day (07 to 10 PM interval) [$T(3)=5.57$; $p<0.05$] and to the 10th SR-day from 4 to 7 PM in the light phase [$T(3)=6.33$; $p=0.008$] and in the intervals from 07 to 10 PM [$T(3)=3.25$; $p<0.05$] and 10 PM to 01 AM [$T(3)=6.84$; $p=0.006$] in the dark phase, suggesting recovery to baseline levels (Figure 2).

Still analyzing the percent W, paired-samples T test showed that in mice only sleep-restricted (SR-Sal group), as expected there was an increase of this parameter in the interval from 10 AM to 01 PM in both the 1st and 10th days compared to baseline [$T(3)=44.96$; $p<0.001$ and 26.80; $p<0.001$, respectively], which decreased in relation to rebound [$T(3)=10.37$; $p<0.05$ and 8.87; $p=0.003$,

respectively]. Regarding de post-SR intervals, paired-samples T test revealed a reduction in W during the dark phase from 01 to 04 AM [T(3)=3.02; p<0.05] and from 04 to 07 AM [T(3)=3.81; p<0.05] in the 1st SR-day compared to same interval during baseline. Accordingly, there was also a decrease in this parameter from 10 PM to 01 AM in the 10th day compared to baseline. Comparing the SR days, there was an augment in W in the 10th day compared to the 1st day from 07 to 10 PM [T(3)=17.25; p<0.001]. In the rebound period, we observed an increase in W from 07 to 10 PM compared to the same interval in the 1st SR-day [T(3)=3,16; p<0,05] (Figure 2).

Concerning sleep restricted mice treated with Zolp (SR-Zolp group), there was an increase in W from 10 AM to 01 PM in the 1st and 10th days compared to the same period in baseline [T(3)=9.81; p=0.002 and 7.57; p=0.005, respectively], which decrease compared to rebound [T(3)=8.33; p=0.004 and 9.63; p=0.002, respectively]. Conversely, in the SR-Zolp group the decrease in W occurred already in the light phase from 04 to 07 PM [T(3)=3,71; p<0,05] in the 1st SR-day compared to baseline. In the same way, W was also reduced in the dark phase from 10 PM to 01 AM comparing the 1st day to baseline [T(3)=21.17; p<0.001]. Still, in the 10th day animals presented a decrease in W compared to baseline in the dark phase from 10 PM to 01 AM [T(3)=3.64; p<0.05] and from 01 AM to 04 AM [T(3)=3.62; p<0.05]. On the other hand, there was an augment in W during rebound compared to 10th day from 04 to 07 PM in the light phase [T(3)=5.07; p<0.05] and from 01 to 04 AM in the dark phase [T(3)=5.22; p<0.05]. However, unlike to what was observed for the other groups, during rebound a reduced W time was still observed from 10 PM to 01 AM compared to baseline [T(3)=3.42; p<0.05] (Figure 2).

Analyzing sleep, ANOVA with repeated measures revealed a significant time [$F(15,135)=24.48$; $p<0.001$] and time x group interaction [$F(30,135)=7.08$; $p<0.001$] for SWS in the 1st period. In the same way, in the 2nd period, there was a significant effect of time [$F(15,135)=19.44$; $p<0.001$] and time x group interaction [$F(30,135)=3.21$; $p<0.001$]. Regarding the CTRL-Zolp group, paired-samples T test showed that there was an increase in SWS in the end of the light phase (from 04 to 07 PM) during the 1st day and in the dark phase (from 07 to 10 PM and from 04 to 07 AM) during the 10th day compared to the same periods in baseline [$T(3)=3.97$; $p<0.05$; 3.27; $p<0.05$ and 3.96;$p<0.05$, respectively]. In the 10th day there was a decrease in SWS from 07 to 10 PM compared to the 1st day [$T(3)=3.27$; $p<0.05$], although being still higher than baseline. Still, there was a decrease in SWS during rebound compared to the 1st day from 04 to 07 PM [$T(3)=3.79$; $p<0.05$] and from 07 to 10 PM [$T(3)=6.45$; $p=0.008$] as well as compared to the 10th day from 04 to 07 PM [$T(3)=10.04$; $p=0.002$] and from 10 PM to 01 AM [$T(3)=3.91$; $p<0.05$], showing the recovery to baseline levels (Figure 3).

Regarding the SR-Sal group, paired-samples T test showed a significant decrease in SWS during the sleep deprivation periods (10 AM to 01 PM) in the 1st and 10th days compared to baseline [$T(3)=31.28$; $p<0.001$ and 21.99; $p<0.001$, respectively]. Consequently, there was an increase in SWS in those periods during rebound [$T(3)=8.86$; $p=0.003$ and 7.59; $p=0.005$, respectively]. Concerning the post-SR intervals, paired-samples T test demonstrated an increase in SWS during the dark phase from 10 PM to 01 AM in the 10th day compared to baseline [$T(3)=3.40$; $p<0.05$]. Still, in the 10th day there was a decrease in SWS from 07 to 10 PM compared to the 1st day [$T(3)=8.88$;

p=0.003]. During rebound, there was a decrease in this parameter from 04 to 07 PM compared to the same period in the 10th day [T(3)=4.10; p<0.05] (Figure 3).

Analyzing the group sleep restricted and treated with Zolp (SR-Zolp), paired-samples T test revealed a decrease in SWS during SR periods in the 1st and 10th days compared to the same period in baseline [T(3)=10.80; p=0.002 and 8.73; p=0.003, respectively], which in turn was enhanced during rebound [T(3)=7.78; p=0.004 and 8.90; p=0.003, respectively]. In the following periods, paired-samples T test showed an increase in SWS in the end of the light phase from 04 to 07 PM [T(3)=4.73; p<0.05] and from 10 PM to 01 AM [T(3)=15.29; p=0.001] during the 1st day compared to the same period in baseline. In the same way, there was an increase in this parameter in the dark phase from 01 to 04 AM [T(3)=3.15; p<0.05] in the 10th day compared to baseline. Still, the SWS was decreased during rebound compared to 10th day from 04 to 07 PM [T(3)=3.75; p<0.05] and from 07 to 10 PM [T(3)=3.11; p<0.05]. Conversely, during rebound, it was still observed an increased SWS from 10 PM to 01 AM compared to baseline [T(3)=3,53; p<0,05] (Figure 3).

Concerning PS during the 1st period, ANOVA with repeated measures showed a significant time [F(15,135)=7.44; p<0.001] and time x group interaction [F(30.135)=2,33; p<0.001]. Similarly, in the 2nd period there was a significant effect of time [F(15,135)=4.80; p<0.005] and time x group interaction [F(30,135)=1.83; p<0.05]. Analyzing the CTRL-Zolp group, paired-samples T test revealed a decrease in PS in the 1st and 10th days from 01 to 04 PM compared to the same period in baseline [T(3)=4.47; p<0.05 and 5.42; p<0.05, respectively]. Oppositely, in the end of the dark phase (from 04 to 07 AM) in the 1st and 10th days there was an increase in PS compared to baseline [T(3)=3.21;

p<0.05 and 4.47; p<0.05, respectively]. Still, during the 10th day, there was an increase in PS from 04 to 07 PM compared to the 1st day [T(3)=3.79; p<0.05]. Regarding the rebound period, there was an increase in PS from 01 to 04 PM compared to the 1st and 10th days [T(3)=7.62; p<0.005 and 4.29; p<0.05] (Figure 4).

In the SR-Sal group, paired-samples T test showed that, as expected, there was a decrease in PS during the SR periods (from 10 AM to 01 PM) in the 1st and 10th days compared to baseline [T(3)=8.38; p<0.005 and 8.37; p<0.005, respectively], which was enhanced in rebound [T(3)=9.97; p=0.002 and 9.96; p=0.002, respectively]. Regarding the post-SR periods, paired-samples T test revealed an increase in PS in the 1st day during the dark phase from 01 to 04 AM [T(3)=4.23; p<0.05] and in the 10th day from 10 PM to 01 AM [T(3)=3.30; p<0.05] and from 01 to 04 AM [T(3)=3.25; p<0.05] compared to the same intervals in baseline. Still, there was a decrease in this parameter during rebound compared to 10th day from 07 to 10 PM [T(3)=4.75; p<0.05], 10 PM to 01 AM [T(3)=4.06; p<0.05] and 01 to 04 AM [T(3)=4.45; p<0.05] (Figure 4).

Analyzing the SR-Zolp group, paired-samples T test showed, again, a decrease in PS from 10 AM to 01 PM during the SR period in the 1st and 10th days compared to baseline [T(3)=3.32; p<0.05 and 3.12; p<0.05, respectively] and a subsequent increase during rebound [T(3)=5.15; p<0.05 and 6.37; p=0.008, respectively]. During the following periods, paired-samples T test revealed and increase in PS from 10 PM to 01 AM [T(3)=6.28; p=0.008] in the 1st day compared to baseline. Still, there was an increase in PS in the 10th day from 01 to 04 AM [T(3)=5.36; p<0.05] compared to baseline. Concerning the rebound period, there was a decrease in PS from 04 to 07 PM [T(3)=3.06;

p<0.05] compared to the 1st day and from 01 to 04 AM [T(3)=4.54; p<0.05] and 04 to 07 AM [T(3)=7.37; p<0.005] compared to the 10th day. Conversely, during the rebound it was still observed an increase in PS from 07 to 10 AM compared to baseline [T(3)=3.31; p<0.05] (Figure 4).

Finally, we analyzed the TST. Thus, ANOVA with repeated measures revealed significant effects of time [F(15,135)=15.16; p<0.001] and time x group interaction [F(30,135)=4.74; p<0.001] in the 1st period. In the same way, in the 2nd period there was a significant effect of time [F(15,135)=16.71; p<0.001] and time x group interaction [F(30,135)=5.16; p<0.001]. Regarding the CTRL-Zolp group, paired-samples T test showed an increase in TST in the 10th day from 07 to 10 PM [T(3)=3.26; p<0.05] and 04 to 07 AM [T(3)=7.18; p=0.006] compared to baseline. On the other hand, in the 10th day there was a decrease in TST from 07 to 10 PM compared to the 1st day [T(3)=2.99; p<0.05]. During the rebound period, there was a decrease in TST from 07 to 10 PM compared to the 1st and 10th days [T(3)=16.95; p<0.001 and 3.25; p<0.05, respectively] (Figure 5).

In the SR-Sal group, paired-samples T test demonstrated that during the SR period there was a decrease in TST in the 1st and 10th days compared to baseline [T(3)=44.32; p<0.001 and 27.29; p<0.001, respectively] and that this parameter was increased in rebound [T(3)=10.42; p=0.002 and 9.10; p=0.003, respectively]. In the other intervals, paired-samples T test showed an increase in TST in the 1st day from 01 to 04 AM [T(3)=3.04; p<0.05] and in the 10th day from 10 PM to 01 AM [T(3)=3.57; p<0.05] compared to baseline. Nevertheless, in the 10th day there was a decrease in TST from 07 to 10 PM compared to the same period in the 1st day [T(3)=17.61; p<0.001]. Still, there was a decrease in

this parameter during rebound from 07 to 10 PM compared to the 1st day [T(3)=3.14; p<0.05] (Figure 5).

Finally, in the SR-Zolp group, paired-samples T test showed the expected decrease in TST from 10 AM to 1 PM (SR period) in the 1st and 10th days compared to baseline [T(3)=9.79; p=0.002 and 7.88; p=0.004, respectively] and, then, an increase compared to rebound [T(3)=8.31; p<0.05 and 9.88; p=0.002, respectively]. Analyzing the post-SR intervals, paired-samples T test revealed an increase in TST from 10 PM to 01 AM in the 1st day compared to baseline [T(3)=21.97; p<0.001]. Still, there was an increase in this parameter from 10 PM to 01 AM [T(3)= 3,63; p<0,05] and from 01 to 04 AM [T(3)=3,62; p<0,05] in the 10th day compared to baseline. Conversely, the TST in the 10th day was higher than in the 1st day from 04 to 07 PM [T(3)=3.61; p<0.05]. In the rebound period, there was a decrease in this parameter compared to the 10th day from 04 to 07 PM [T(3)=5.11; p<0.05] and from 01 to 04 AM [T(3)=5.21; p<0.05]. However, during the rebound, there was still an increase in TST from 10 PM to 01 AM compared to baseline [T(3)=3.43; p<0.05] (Figure 5).

Figure 1

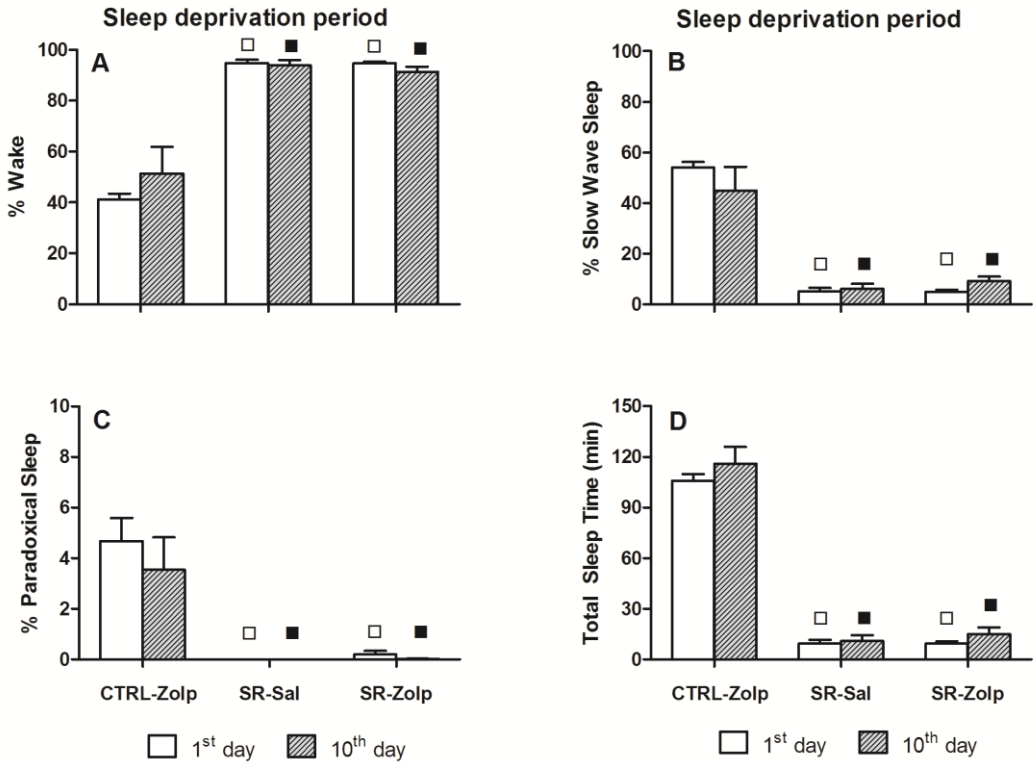

Figure 1: Electrophysiological recording of sleep parameters during the sleep restriction (SR) period in the 1st and 10th days. Results are presented as mean ± SE of percent time spent awake (**A**), percent time in slow wave sleep (**B**), percent time in paradoxical sleep (**C**) and total sleep time (**D**). At the end of sleep deprivation mice were treated with saline (Sal) or 5 mg/kg zolpidem (Zolp). □ $p<0.05$ compared to CTRL-Zolp group in the 1st day; ■ $p<0.05$ compared to CTRL-Zolp group in the 10th day (ANOVA and Duncan's test).

Figure 2

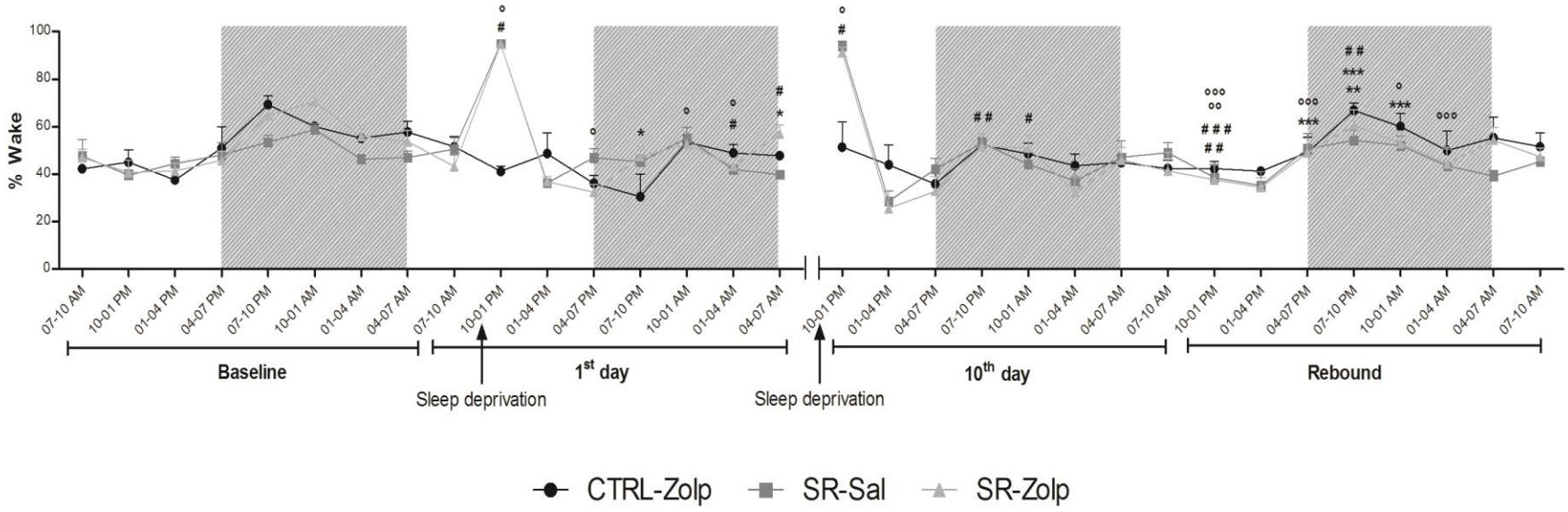

Figure 2: Electrophysiological recording of sleep parameters every 3 h throughout treatment. Results are presented as mean ± SE of percent time spent awake before the beginning (baseline), in the 1st and 10th sleep restriction (SR) days and the day after the end of treatment (rebound). At the end of sleep deprivation mice were treated with saline (Sal) or 5 mg/kg zolpidem (Zolp). ANOVA with repeated measures showed significant effects of time and time x group interaction. * p<0.05 compared to baseline, ** p<0.05 compared to 1st day or *** p<0.05 compared to 10th day for the CTRL-Zolp group; # p<0.05 compared to baseline, ## p<0.05 compared to 1st day or ### p<0.05 compared to 10th day for the SR-Sal group; ° p<0.05 compared to baseline, °° p<0.05 compared to 1st day or °°° p<0.05 compared to 10th day for the SR-Zolp group (paired-samples T test).

Figure 3

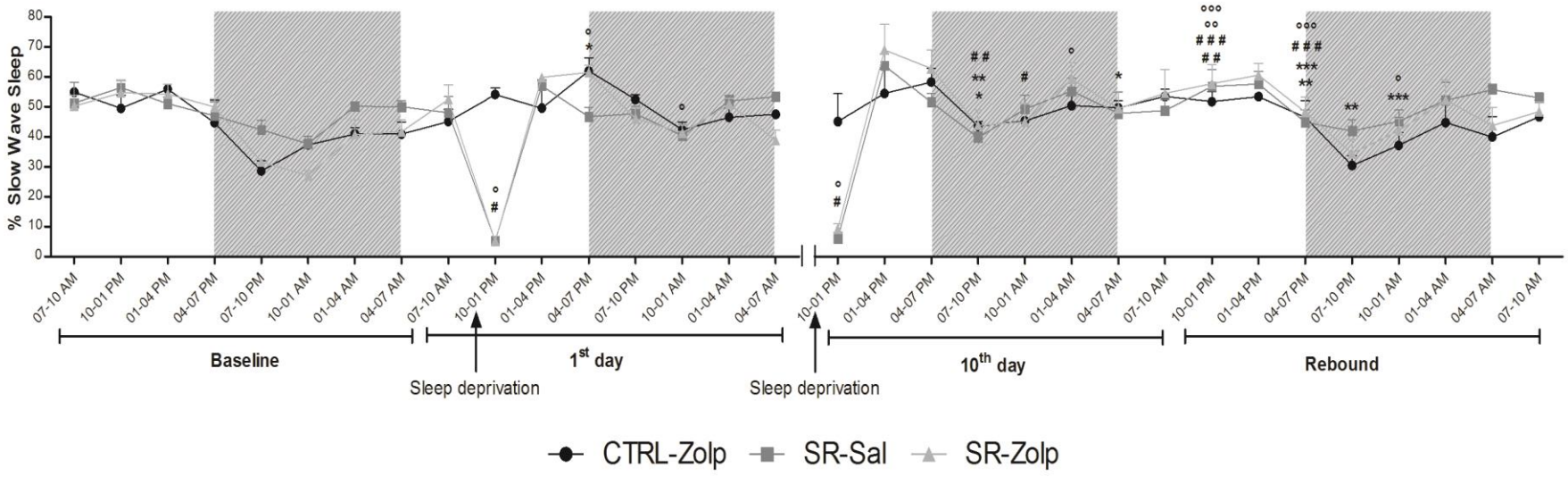

Figure 3: Electrophysiological recording of sleep parameters every 3 h throughout treatment. Results are presented as mean ± SE of percent time spent in slow-wave sleep before the beginning (baseline), in the 1st and 10th sleep restriction (SR) days and the day after the end of treatment (rebound). At the end of sleep deprivation mice were treated with saline (Sal) or 5 mg/kg zolpidem (Zolp). ANOVA with repeated measures showed significant effects of time and time x group interaction. * $p<0.05$ compared to baseline, ** $p<0.05$ compared to 1st day or *** $p<0.05$ compared to 10th day for the CTRL-Zolp group; # $p<0.05$ compared to baseline, ## $p<0.05$ compared to 1st day or ### $p<0.05$ compared to 10th day for the SR-Sal group; ° $p<0.05$ compared to baseline, °° $p<0.05$ compared to 1st day or °°° $p<0.05$ compared to 10th day for the SR-Zolp group (paired-samples T test).

Figure 4

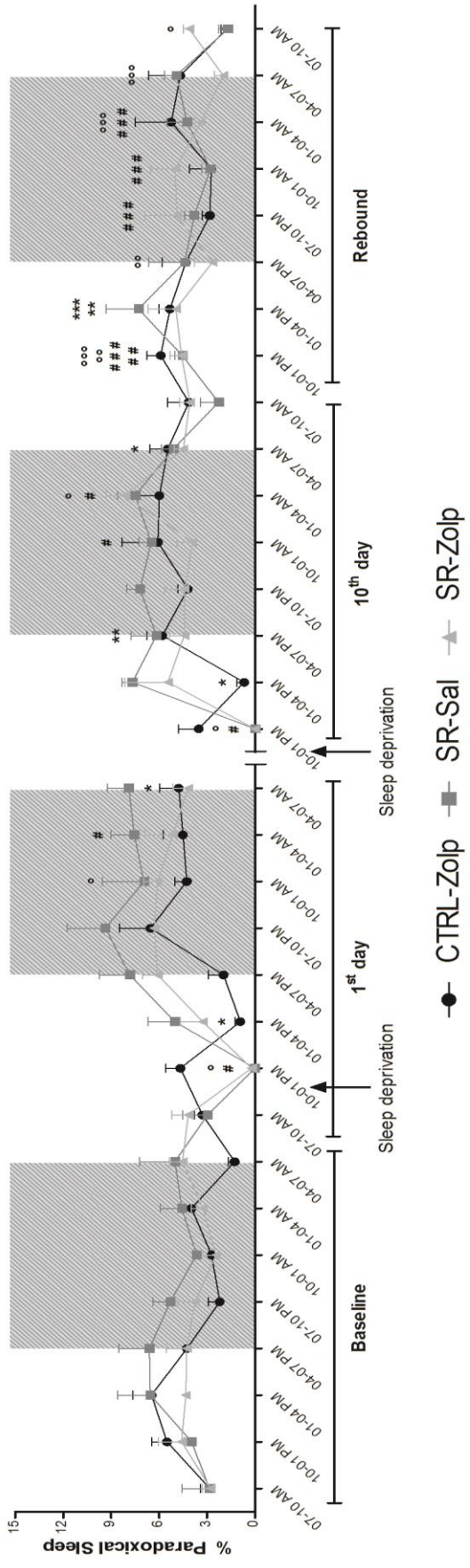

Figure 4: Electrophysiological recording of sleep parameters every 3 h throughout treatment. Results are presented as mean ± SE of percent time spent in paradoxical sleep before the beginning (baseline), in the 1st and 10th sleep restriction (SR) days and the day after the end of treatment (rebound). At the end of sleep deprivation mice were treated with saline (Sal) or 5 mg/kg zolpidem (Zolp). ANOVA with repeated measures showed significant effects of time and time x group interaction. * p<0.05 compared to baseline, ** p<0.05 compared to 1st day or *** p<0.05 compared to 10th day for the CTRL-Zolp group; # p<0.05 compared to baseline, ## p<0.05 compared to 1st day or ### p<0.05 compared to 10th day for the SR-Sal group; ° p<0.05 compared to baseline, °° p<0.05 compared to 1st day or °°° p<0.05 compared to 10th day for the SR-Zolp group (paired-samples T test).

Figure 5

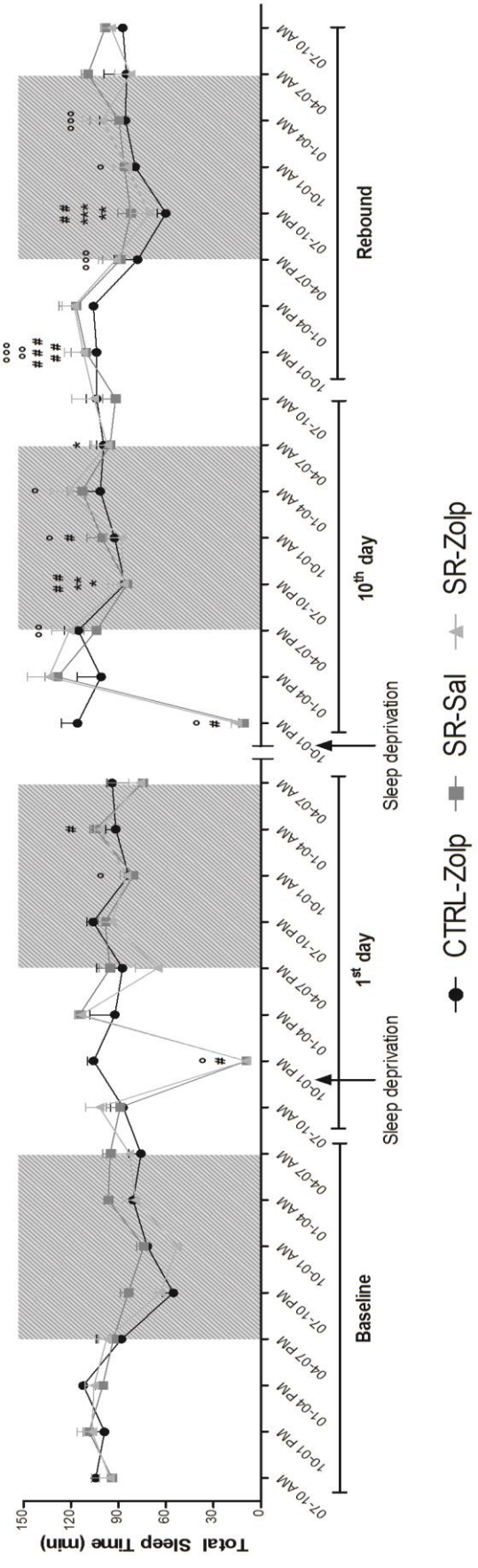

Figure 5: Electrophysiological recording of sleep parameters every 3 h throughout treatment. Results are presented as mean ± SE of total sleep time (min) before the beginning (baseline), in the 1st and 10th sleep restriction (SR) days and the day after the end of treatment (rebound). At the end of sleep deprivation mice were treated with saline (Sal) or 5 mg/kg zolpidem (Zolp). ANOVA with repeated measures showed significant effects of time and time x group interaction. * p<0.05 compared to baseline, ** p<0.05 compared to 1st day or *** p<0.05 compared to 10th day for the CTRL-Zolp group; # p<0.05 compared to baseline, ## p<0.05 compared to 1st day or ### p<0.05 compared to 10th day for the SR-Sal group; ° p<0.05 compared to baseline, °° p<0.05 compared to 1st day or °°° p<0.05 compared to 10th day for the SR-Zolp group (paired-samples T test).

DISCUSSION

In the present study, we showed that the chosen SR regimen was effective in enhancing wake time and as a consequence decreasing SWS, PS and TST. Of note, SR induced an increase in SWS, PS and TST in the dark phase, suggesting a compensatory effect. However, those alterations are tolerated throughout treatment, in exception of PS (still augmented in the last day). Regarding Zolp treatment, it induced an increase in SWS and reduced PS during the light phase in control animals. These modifications seem to be compensated in the dark phase as well as to be tolerated during treatment. Concerning the interaction between SR and Zolp, such compensatory increase in sleep parameters was already observed in the light phase and remained elevated during the role treatment. Curiously, only in the group SR-Zolp we still observed modifications on sleep pattern during rebound.

People in modern society sleep less than recommended for vocational or lifestyle reasons or sleep disturbances. Notably, significant impairments in cardiovascular, immune, endocrine, and cognitive functions have been reported in humans who sleep less than recommended (Banks and Dinges). Although animal models of SR have been recently developed (Everson and Szabo, 2009; Kim et al., 2007, 2012; Leemburg et al., 2010; McCoy et al., 2013; Zielinski et al., 2013; Yang et al., 2012), experimental research with models of SR are less used compared to the considerable amount of work evaluating the neurobehavioral effects of total sleep deprivation or selective PS deprivation. Thus, it remains unclear whether similar effects might be observed after SR, which is more common in humans representing a better translational model.

Herein, we specifically evaluated the effects of a SR protocol on sleep pattern. In addition, considering the increasing prescription of Zolp in the treatment of sleep disturbs (Rush, 1998), its interaction with SR was also analyzed.

Ideally, an insomnia animal model should exhibit decreased sleep quality or quantity during the period that the animal is supposed to be physiologically sleeping. Thus, there are potential models such as: electrical stimulation, drug-induced methods and those related to the interruption of sleep as the single or multiple water platform techniques and forced locomotion (Revel et al., 2009). However, these sleep deprivation methods may cause excessive stress or result in incomplete sleep loss, having unfavorable effects on experimental results. Considering this, we employed the gentle handling method, which seems to be less stressful, to keep mice awake for 3 h per day during 10 consecutive days. Through EEG monitoring, the animals had almost no SWS or PS and a consequent increase in wake time during the 3-h sleep deprivation period, showing the effectiveness of the method. Importantly, in the 10th day this increased wake time followed by a decreased sleep time was still observed.

Analyzing the intervals after the SR period, there was a decrease in wake time in the dark phase both in the 1st and 10th days. Furthermore, SWS was augmented in the last day. On the other hand, comparing the 1st and the 10th days, in the last day there was an increase in wake and a decrease in SWS, suggesting an adaptation to the effects of SR. The TST followed the same pattern of SWS. Concerning PS, we observed an increase in both the 1st and last days in the dark phase compared to baseline, characterizing the sleep rebound, a common outcome after sleep deprivation regulated by homeostatic process (Franken et al., 1991; Friedman et al., 1979; Machado et al., 2004,

2005). Oppositely to which was observed for SWS, the modification in PS was not compensated throughout days, which may explain the negative effects after SR. Regarding the acute effects of sleep deprivation (after the 1st day), there was an increase only in PS and a minor modification in SWS. This result is in line with previous work reporting a slight enhancement of sleep concomitant with a reduction in waking induced by 3-h sleep deprivation (Tobler et al., 1990).

Recently, Yang and colleagues (2012) evaluated the EEG of rats subjected to 4-h daily sleep deprivation during 7 consecutive days using the gentle handling method. The authors reported both SWS and PS increment even in the last days of SR while we observed a possible adaptation to the effects of SR instead. This discrepancy may be due to the different SR periods (7 vs. 10 days) and the animal model (rats vs. mice). Nevertheless, it was reported that acute sleep deprivation enhances SWS, although this enhancement is reduced after daily SR (Deurveilher et al., 2012; Kim et al., 2007, 2012; Zielinski et al., 2013). As suggested by other authors, we acknowledge that unlike the homeostatic response to acute sleep loss, SR induces an allostatic response (Kim et al., 2007, 2012). Specifically, after SR it may occur 2 physiological responses – homeostatic and allostatic. The 1st one maintains equilibrium by compensation to disrupt changes, while the allostatic response maintains stability through change (McEwen and Wingfield, 2003).

Concerning the effects of Zolp treatment, it induced a decrease in wake which was accompanied by an increase in all sleep parameters in the dark phase. Oppositely, although there was an increase in SWS in the light phase, we also observed a decrease in PS in the 1st day. Curiously, this increase in SWS remained till the last day followed by an increase in PS. These results

corroborate clinical findings in which Zolp was able to enhance SWS (Besset et al., 1995; Bettica et al., 2012). Probably, this increased time in SWS was responsible for the decrease in PS in the light phase. Likewise, the later increase in PS in the dark phase could be a compensation to its reduction during the light phase. Additionally, although still higher then baseline, in the 10th day there was a decrease in SWS in relation to the 1st day, suggesting a tolerance to Zolp effects on sleep architecture.

These results corroborate previous studies showing that Zolp has hypnotic effects increasing SWS sleep at the cost of waking and PS, but generally produces a milder suppressing effect on PS (Bettica et al., 2012; Chen et al., 2005; Depoortere et al., 1991; Fitzgerald et al., 2014; Mailliet et al., 2001; Renger et al., 2004). The effects of Zolp on PS are controversial. Some reports suggest that Zolp did not alter REM sleep in humans (Colle et al., 1991; Poyares et al., 2005) while others describe a decrease in PS but of less magnitude compared to benzodiazepines (Feinberg et al., 2000) or even a significant reduction in this sleep stage (Bettica et al., 2012; Brunner et al., 1991). Regarding laboratory animals, some authors reported that Zolp administration may decrease PS in rats (Fanselow and Bolles, 1979; Kopp et al., 2004; Renger et al., 2004) or even did not modify it in guinea pigs (Xi and Chase, 2008, 2009).

Few studies have examined the effects of Zolp on sleep architecture over extended administration schedules. Corroborating our data, Alexandre and colleagues (2008) showed that 5 mg/kg Zolp throughout 10 days increased SWS time and decreased PS time with no evidence of tolerance. Notably, this study reported that the values returned to baseline during withdrawal. Similarly,

Renger and colleagues (2004) demonstrated the same pattern of modifications after daily administration of at 10 mg/kg Zolp. In this case, the absence of a possible tolerating effect could be due to the duration of treatment (7 vs. 10 days). Indeed, when daily administered during 3 weeks Zolp increased SWS in the 1st week returning to baseline level in the following weeks (Ebert et al. 2008), suggesting that tolerance to the sleep-inducing effects of Zolp would develop with longer treatments. Of note, studies showed that tolerance to the sedative effects of Zolp may be developed after long periods in baboons (Griffiths et al., 1992) and humans (Ware et al., 1997). Importantly, all these alterations were completely abolished during recovery in the present study, while others (Renger et al., 2004) reported rebound effects after the repeated administration of Zolp. This discrepancy could be due to the employed doses (10 vs. 5 mg/kg).

Considering that Zolp is usually prescribed to insomniac patients, the evaluation of its effects after SR is important. Thus, oppositely to which was observed in the SR-Sal group, in the SR-Zolp group the decrease in wake occurred already in the light phase in the 1st day as well as in the dark phase both in the 1st and last treatment days. Concerning sleep, there was an increase in SWS in both cycle periods, similarly to the effects found in the CTRL-Zolp group. However, in the SR-Zolp group this effect persisted throughout treatment since there was no difference in the 10th day compared to the 1st. Analyzing PS, unlike the CTRL-Zolp group, Zolp administration after SR induced an increase in PS in the dark phase in both recording days. Thus, it could be suggested that such increase in PS would be due to sleep deprivation and not a Zolp effect *per se*. Regarding TST, as a consequence of the reduction in wake, there was an

increase of this parameter in the dark phase. However, differently from the control group, which tolerated Zolp effects, in the SR-Zolp group this enhancement was higher in the 10th day compared to the 1st one, suggesting that in sleep-restricted animals Zolp effects may be sensitized. To the best of our knowledge, this is the 1st study evaluating the effects of Zolp under conditions of SR. Thus, from a clinical perspective, our results suggest that Zolp may have interesting effects for insomniac patients once it sleep-inducing effects were not tolerated in sleep-restricted mice. However, under conditions of normal sleep, Zolp should be taken with cautions. Importantly, in exception of SR-Zolp group, after the end of treatment all of the animals recovered sleep to baseline levels. Although this group presented an increase in wake and a decrease in sleep parameters during rebound compared to the last treatment day, it was not sufficient to reach baseline.

Studies in our laboratory found that the interaction between SR and Zolp can positively or negatively modulate memory of a discriminative avoidance task (Zanin et al., submitted data). Specifically, SR induced cognitive impairments that were abolished by the acute administration of Zolp. On the other hand, when administered to control animals, Zolp induced memory impairments. In this respect, we could hypothesize that the unbalance between SWS and PS induced by Zolp in control mice observed herein may have impaired the task performance. In the same way, the modifications in PS found after SR, which were not tolerated over time, could explain the negative effects of SR on memory. Notably, several studies reported the important role of PS on mnemonic processes both in humans (Plihal and Born, 1999; Stickgold et al., 2000) and laboratory animals (Guan et al., 2004; Silvestri, 2005; Yang et al.,

2008). The curious positive effect of Zolp on memory of sleep-restricted mice could also be explained by the present findings. In this way, the SR-Zolp group displayed an anticipation of rebound after SR period, i.e., differently to which was observed in the SR-Sal group, the augment in SWS as well as in PS occurred still in the light phase and remained in the same magnitude over treatment, which could have contributed to a better sleep homeostasis.

The balance between SWS and PS seems to be crucial to memory processes. Although PS has been largely related to memory encoding, SWS sleep also plays a critical role in memory processing. Thus, the functional significance of SWS and the value of SWS-enhancing agents remain of interest (Dijik, 2009, 2010; Dijik et al., 2010; Maquet, 2010; Walsh, 2009). It was shown that the administration of gabaxadol, a $GABA_A$ receptor agonist, protected the body from the prejudicial effects of SR and that this protection would be related to changes in SWS (Walsh et al., 2008). Still, the enhancement of low-frequency EEG by electric stimulation increased memory consolidation (Marshall et al., 2006). Indeed, as reviewed by Walker (2009), SWS may be important for memory processing by preparing the brain for initial encoding before learning and facilitating the off-line memory consolidation after learning.

Collectively, the present study showed that the employed SR protocol adequately induced an increase in wake during sleep deprivation period, which was followed by a compensatory increase in sleep. Still, the enhancement in PS (but not in SWS) was not tolerated throughout days. In addition, although Zolp increased SWS in both conditions, in CTRL mice it seemed to be tolerated. Thus, we could hypothesize that the modifications in PS after SR contributed to its impairing effects, while the Zolp administration would facilitate the sleep

homeostasis mainly through its effects on SWS, counteracting the effects of SR. Finally, our results strengthened the importance of an adequate sleep balance as well as the need for further studies evaluating the effects of Zolp under conditions of reduced sleep.

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3.5.1 Conclusões parciais – manuscrito 5

No 5º manuscrito da presente Tese, demonstramos que o protocolo de RS empregado se mostrou efetivo em aumentar a vigília e, consequentemente, reduzir significativamente o sono de ondas lentas (SOL), o sono paradoxal e o tempo total de sono (TTS).

A administração de Zolp aumentou o SOL e diminuiu o sono paradoxal durante a fase clara nos animais controle, sendo que essa diminuição parece ser compensada durante a fase escura do ciclo e, ainda, todas as alterações tendem a ser toleradas ao longo do tratamento. Com relação aos efeitos observados após a restrição de sono por 3 h durante 10 dias, ocorreu um aumento compensatório no SOL, no sono paradoxal e no TTS na fase escura do ciclo. Ainda, ao longo do procedimento, exceto para o sono paradoxal que se manteve elevado até o 10º dia de privação de sono, parece haver uma adaptação do organismo às alterações induzidas pela RS.

Nos animais submetidos à RS e tratados com zolpidem o aumento compensatório em todos os parâmetros de sono ocorreu ainda na fase clara e se manteve elevado durante todo o experimento. De importância, ao contrário do observado nos demais grupos, os animais do grupo restrito de sono e tratado com Zolp ainda apresentavam alterações no padrão de sono durante o rebote quando comparado ao registro basal.

Em conjunto com os dados comportamentais, poderíamos sugerir que as modificações no sono paradoxal observadas após a restrição de sono teriam contribuído para os prejuízos induzidos pela diminuição do sono *per se*. Por outro lado, a antecipação do aumento compensatório de sono induzido pelo Zolp poderia explicar, ao menos em parte, a reversão dos déficits de memória

induzidos pela RS. Assim, esse hipnótico poderia ter facilitado a homeostase do sono, principalmente com relação ao SOL. Finalmente, nossos resultados demonstram a importância de um balanço adequado das fases do sono bem como sugerem que o Zolp pode apresentar efeitos positivos em condições semelhantes à insônia.

Conclusões

4. CONCLUSÕES

4.1 Conclusões específicas

Avaliar os efeitos da privação de sono total por 3 ou 6 h agudamente ou durante 10 dias consecutivos na tarefa de esquiva discriminativa em labirinto em cruz elevado e a posterior expressão de c-Fos

A privação de sono total aguda por 3 h promoveu uma diminuição nos níveis de ansiedade e um aumento da atividade locomotora. Esses efeitos foram abolidos quando a privação foi estendida para 6 h. Por outro lado, apenas a privação de sono total aguda por 6 h induziu déficits de memória. Já a privação de sono total repetida por 10 dias, independentemente de sua duração (3 ou 6 h), não promoveu modificações no aprendizado da tarefa discriminativa, nos níveis de ansiedade ou na atividade locomotora dos animais. Ainda, enquanto a privação aguda por 3 h promoveu efeito ansiolítico e hiperlocomotor, quando realizado repetidamente, esses efeitos não foram observados, sugerindo que houve tolerância comportamental. Ao contrário, após a repetição do protocolo, a privação por 3 h induziu déficits de memória, sugerindo uma sensibilização. Com relação ao período de 6 h, com a repetição da privação, o prejuízo cognitivo induzido pela privação aguda foi abolido. Os protocolos de privação de sono induziram um aumento da expressão de c-fos na amígdala basolateral e no giro denteado. Este último parece estar relacionado aos efeitos cognitivos da restrição de sono.

Em conjunto, nossos resultados sugerem que parece haver tolerância às alterações comportamentais promovidas pela privação de sono total aguda por

3 h (efeitos ansiolítico e hiperlocomotor) e ao efeito cognitivo deletério induzido pela privação de sono total aguda por 6 h (déficit de memória) e sensibilização do efeito cognitivo deletério quando a privação de sono total por 3 h é realizada de forma repetida. Ainda, esses dados sugerem que a privação de sono total desencadeia fenômenos plásticos de adaptação no sistema nervoso central.

Avaliar os efeitos da administração repetida de zolpidem (ou de sua retirada abrupta) sobre o aprendizado, consolidação e evocação da tarefa de esquiva discriminativa em labirinto em cruz elevado em animais restritos de sono 3 h por dia durante 10 dias

A administração aguda pré-treino de zolpidem, bem como a administração repetida, promoveu amnesia nos animais controle. Curiosamente, nos animais restritos de sono, tanto a administração aguda de zolpidem como a sua retirada abrupta foram capazes de reverter o prejuízo cognitivo induzido pelo protocolo de restrição de sono de 3 h por dia durante 10 dias. Com relação à consolidação, não houve efeitos do protocolo de restrição de sono ou de sua interação com o tratamento com zolpidem quando aplicados imediatamente após o treino. Contudo, quando o zolpidem foi administrado imediatamente (mas não 2 h) após o treino em animais que foram restritos de sono durante os 10 dias anteriores ao treino, o efeito deletério da restrição de sono foi abolido. Curiosamente, ao avaliar a evocação, apenas o tratamento repetido com zolpidem reverteu os prejuízos da restrição de sono. De importância, a administração do β-cct (antagonista seletivo α1) impediu o efeito benéfico da administração aguda de zolpidem em animais restritos de sono, mas não foi capaz de mimetizar o efeito da retirada abrupta. Esses resultados

sugerem que o efeito agudo do zolpidem pode ser mediado por receptores GABAérgicos, enquanto que os efeitos do tratamento repetido não parecem estar relacionados exclusivamente a esse sistema de neurotransmissão.

Dessa forma, nossos resultados sugerem que a interação entre a restrição de sono e o zolpidem pode modular a memória de maneira positiva ou negativa, dependendo da fase analisada. Esse efeito positivo do zolpidem poderia estar relacionado especificamente ao processo de consolidação, visto que mesmo quando administrado antes do treino a droga estaria presente no início da consolidação. Ainda, o efeito agudo dessa droga, mas não de sua retirada, pode ser explicado pela ativação GABAérgica, uma vez que o β-cct reverteu o efeito benéfico do zolpidem. Esses resultados fortalecem a importância de estudos futuros analisando a interação entre períodos de restrição de sono e o zolpidem sobre a memória, particularmente no que diz respeito à consolidação.

Avaliar os efeitos da administração repetida de zolpidem (ou de sua retirada abrupta) sobre o aprendizado, consolidação e evocação da tarefa de esquiva passiva em animais restritos de sono 3 h por dia durante 10 dias

Os resultados demonstram que, ao contrário do observado na esquiva discriminativa, no modelo da esquiva passiva, a restrição de sono *per se* não foi capaz de promover déficits de retenção quando realizada antes do treino ou do teste. Nos animais controle, assim como havíamos observado na esquiva discriminativa, a injeção desafio pré-treino de zolpidem promoveu déficits de retenção. Contudo, a injeção aguda de dessa droga também promoveu déficits de memória nos animais restritos de sono, diferentemente do observado para

esquiva discriminativa. Ainda, novamente nem a restrição de sono, o zolpidem ou suas interações foram capazes de promover déficits de consolidação quando o tratamento foi realizado imediatamente após o treino. Com relação ao tratamento pré-teste, não foram observadas modificações na evocação da tarefa. Essas diferenças observadas podem ter sido ocasionadas por: (1) diferenças qualitativas dos modelos, já que a esquiva passiva poderia ser mais estressante por utilizar um estímulo doloroso, de modo que a restrição de sono não prejudicou a retenção da tarefa ou (2) influência da hipolocomoção induzida pelo zolpidem administrado anteriormente ao treino ou ao teste.

Em conjunto com os experimentos anteriores, demonstramos que além da interação entre restrição de sono e o tratamento com zolpidem modular a memória de forma negativa ou positiva dependendo da fase da memória analisada, também pode apresentar diferenças dependendo do modelo de memória utilizado. Nesse sentido, no modelo da esquiva passiva as modificações cognitivas podem ser discrepantes se comparadas aos resultados obtidos na esquiva discriminativa. Nesse contexto, nossos resultados demonstram que a esquiva passiva, apesar de ser um modelo clássico para avaliação da memória, é fortemente influenciada pela atividade locomotora.

Avaliar os efeitos da administração repetida de zolpidem (ou de sua retirada abrupta) sobre o aprendizado/consolidação das tarefas de reconhecimento de objetos ou social em animais restritos de sono 3 h por dia durante 10 dias

A restrição de sono promoveu déficits de memória de curta e longa duração no modelo de reconhecimento de objetos, bem como no modelo de reconhecimento social. Curiosamente, independente do protocolo de

administração, o zolpidem reverteu o déficit de memória de curta duração na tarefa de reconhecimento de objetos, enquanto que apenas a administração aguda dessa droga aboliu o prejuízo observado na tarefa de reconhecimento social. Contudo, apesar de ter revertido o prejuízo na memória de curta duração, o tratamento repetido com zolpidem não foi capaz de preservar a memória de longa duração.

Avaliar os efeitos do tratamento repetido com zolpidem no padrão de sono de camundongos restritos ou não de sono 3 h por dia durante 10 dias por meio de registro eletroencefalográfico

O protocolo de restrição de sono se mostrou efetivo em aumentar a vigília e, consequentemente, reduzir significativamente o sono de ondas lentas (SOL), o sono paradoxal e o tempo total de sono (TTS). Ainda, a administração de zolpidem aumentou o SOL e diminuiu o sono paradoxal durante a fase clara nos animais controle, sendo que essa diminuição parece ser compensada durante a fase escura do ciclo. Ainda, essas alterações tendem a ser toleradas ao longo do tratamento. Com relação aos efeitos observados após a restrição de sono, ocorreu um aumento compensatório no SOL, no sono paradoxal e no TTS na fase escura do ciclo. Ao longo do procedimento, parece haver uma adaptação do organismo às alterações induzidas pela restrição de sono, exceto para o sono paradoxal que se manteve elevado durante todo tratamento. Nos animais restritos de sono e tratados com zolpidem, o aumento compensatório em todos os parâmetros de sono ocorreu ainda na fase clara e se manteve elevado durante todo o experimento. De importância, apenas esse grupo de

animais ainda apresentava alterações no padrão de sono durante o rebote quando comparado ao registro basal.

Em conjunto com os dados comportamentais, poderíamos sugerir que as modificações no sono paradoxal observadas após a restrição de sono teriam contribuído para os prejuízos induzidos pela diminuição do sono. Por outro lado, a administração de zolpidem pode ter facilitado a homeostase do sono, principalmente com relação ao SOL, impedindo os prejuízos da restrição de sono. Finalmente, nossos resultados demonstram a importância de um balanço adequado das fases do sono bem como sugerem que o zolpidem pode apresentar efeitos positivos em condições semelhantes à insônia.

4.2 Conclusão geral

Considerando a crescente utilização do zolpidem no tratamento da insônia, as evidências prévias observadas por nosso grupo de pesquisa que sugerem alterações cognitivas induzidas pela administração aguda dessa droga, e a falta de estudos que abordem sistematicamente os efeitos do tratamento repetido com zolpidem sobre os processos mnemônicos, a presente tese tem por objetivo analisar os efeitos do tratamento repetido com zolpidem ou de sua retirada abrupta sobre as diversas fases da formação da memória por meio dos modelos de esquiva discriminativa em labirinto em cruz elevado, esquiva inibitória, reconhecimento de objetos e reconhecimento social. Ainda, visto que clinicamente o tratamento com zolpidem é iniciado após períodos variáveis de privação de sono, compararemos os resultados obtidos em

camundongos mantidos em condições controle de sono ou submetidos à restrição de sono

O protocolo de restrição de sono de 3 h por dia durante 10 dias promoveu prejuízos de memória nas tarefas de esquiva discriminativa, reconhecimento de objetos e reconhecimento social. Por outro lado, em uma tarefa com grande conteúdo emocional aversivo (esquiva passiva), esses déficits não foram observados. Curiosamente, os resultados da presente Tese demonstram que a administração de zolpidem pode modular de maneira positiva a memória em animais restritos de sono, uma vez que reverteu a amnésia induzida pelo encurtamento do período de sono. Contudo, esse efeito benéfico é influenciado tanto pelo regime de administração da droga quanto pela tarefa de memória empregada. Ainda, a administração de zolpidem ou a restrição de sono separadamente alteraram a arquitetura do sono, mas esse efeito parece ser tolerado ao longo do tratamento. Por outro lado, quando somados, o zolpidem pode ter facilitado a homeostase do sono nos animais restritos, principalmente com relação ao sono de ondas lentas. Essa facilitação do processo de homeostase poderia justificar, ao menos parcialmente, a reversão dos efeitos negativos da restrição de sono após a administração de zolpidem.

Os achados da presente Tese demonstram a importância da validação de modelos experimentais de insônia bem como a avaliação dos efeitos do tratamento com zolpidem em condições de restrição sono, uma vez que os efeitos dessa droga podem ser discrepantes dependendo das condições de sono e do método de avaliação.

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Anexos

6.1 Anexo 1: Comitê de ética

UNIFESP
UNIVERSIDADE FEDERAL DE SÃO PAULO

Universidade Federal de São Paulo *Comitê de Ética em Pesquisa*
Escola Paulista de Medicina *Hospital São Paulo*

São Paulo, 26 de Novembro de 2010.
CEP 1741/10

Ilmo(a). Sr(a).
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PARECER DO COMITÊ DE ÉTICA INSTITUCIONAL

Ref: Projeto de pesquisa intitulado: **"Efeitos da administração repetida de zolpidem sobre as fases da memória em camundongos submetidos à insônia experimental".**

CARACTERÍSTICA PRINCIPAL DO ESTUDO: Estudo experimental crônico em camundongos.
RISCOS ADICIONAIS PARA O PACIENTE: Não se aplica.
OBJETIVOS: Analisar os efeitos do tratamento repetido com zolpidem sobre as diversas fases da formação da memória (aquisição, consolidação e evocação) de esquiva discriminativa, além dos efeitos induzidos pela retirada abrupta desse tratamento, tendo em vista seu potencial de abuso.
RESUMO: Serão utilizados camundongos Swiss machos, com 3 meses de idade.Será utilizado o zolpidem nas doses de 0,2, 0,5, 1,0, e 10mg/kg, diluído em solução salina 0,9%, sendo esta a solução controle. As soluções serão administradas intraperitonealmente num volume de 10 ml/kg. Como modelo comportamental para avaliação da memória será utilizada a esquiva discriminativa em labirinto em cruz elevado. Como modelos experimentais de insônia será utilizado o método de gentle handling, que consiste em manter os animais acordados por meio da manipulação com pincel de cerdas que apresentarem o comportamneto de dormir e, em um segundo momento, o modelo de perturbação de sono por estimulação sonora, que consiste em interromper de maneira não seletiva o sono dos animais por meio da apresentação de ruídos com frequência entre 125 e 2000Hz, e ruído contínuo com intensidade de 70dB. Ambos os modelos e insônia serão realizados por 10 dias durante 3 horas por dia. Para avaliação da imunohistoquímica, os animais serão anestesiados profundamente com ketamina e xilazina e será realizada a prefusão sistêmica , com a retirada dos cérebros para análise de c-Fos e Fos-B no hipocampo..
FUNDAMENTOS E RACIONAL: O estudo tem como proposiçãoprincipal verificar os efeitos da administração repetida de zolpidem sobre as diferentes fases do processo de aprendizado/memória em associação a dois modelos animais d einsônica, visto que clinicamente o tratamento é iniciado após períodos variáveis de fragmentação do sono..
MATERIAL E MÉTODO: Descritos os procedimentos experimentais, de modelos já padronizados pelos autores.
DETALHAMENTO FINANCEIRO: Sem financiamento externo.

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Tel.: (011) 5571-1062 - 5539.7162

CRONOGRAMA: R$ 1450,00.

OBJETIVO ACADÊMICO: Doutorado.

ENTREGA DE RELATÓRIOS PARCIAIS AO CEP PREVISTOS PARA: **21/11/2011** e **20/11/2012**.

O Comitê de Ética em Pesquisa da Universidade Federal de São Paulo/Hospital São Paulo **ANALISOU** e **APROVOU** o projeto de pesquisa referenciado.

1. Comunicar toda e qualquer alteração do projeto.
2. Comunicar imediatamente ao Comitê qualquer evento adverso ocorrido durante o desenvolvimento do estudo.
3. Os dados individuais de todas as etapas da pesquisa devem ser mantidos em local seguro por 5 anos para possível auditoria dos órgãos competentes.

Atenciosamente,

Prof. Dr. José Osmar Medina Pestana
Coordenador do Comitê de Ética em Pesquisa da
Universidade Federal de São Paulo/ Hospital São Paulo

1741/10

6.2 Anexo 2: Comprovante de submissão - Manuscrito 2

Gmail by Google

Camilla De Lima Patti Hissamura <clpatti@gmail.com>

Submission Confirmation

European Neuropsychopharmacology <ENP@elsevier.com> 27 de maio de 2015 20:21
Para: camila.patti@unifesp.br, clpatti@gmail.com

Interactions between zolpidem and sleep restriction: bidirectional effects on memory in mice
by Karina A Zanin; Camilla L Patti, Ph.D.; Leonardo B Lopes-Silva; Carolina S Bizerra; André W Hollais; Renan Santos-Baldaia; Liliane Ceccon; Fernanda Talhati; Aline R Borçoi; Stephanie B Grapiglia; Sergio Tufik; Roberto Frussa-Filho; Dalva Poyares
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