UNIVERSIDADE FEDERAL DO PAMPA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

UM ESTUDO COMPORTAMENTAL E BIOQUÍMICO DE ESTRATÉGIAS PARA PROMOÇÃO DA PERSISTÊNCIA DAS MEMÓRIAS DE LONGA DURAÇÃO

TESE DE DOUTORADO

Liane da Silva de Vargas

Uruguaiana, RS, Brasil
2016

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Por

Liane da Silva de Vargas

Tese apresentada ao Programa de Pós-Graduação em Bioquímica da Universidade Federal do Pampa (UNIPAMPA, RS), como requisito parcial para a obtenção do grau de **Doutora em Bioquímica**

Orientadora: Prof[a]. Dr[a]. Pâmela Billig Mello Carpes

Uruguaiana, RS, Brasil

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Universidade federal do pampa
Programa de pós-graduação em bioquímica

A Comissão Examinadora, abaixo assinada, aprova a
Tese de Doutorado

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Elaborada por

Liane da Silva de Vargas

Como requisito parcial para a obtenção do grau de
Doutora em Bioquímica

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Uruguaiana, RS, Brasil
2016

“*Somos aquilo que nos lembramos.”*

Norberto Bobbio

“*As conquistas não definem a nossa força, mas sim o amor pelo que lutamos.”*

Massivi Suburbano

DEDICO

À minha família, meus pais Celi e Vanderlei, meu irmão Rafael e minha irmã Liziane, por me mostrar o sentimento mais puro e sincero: o amor incondicional.

DEDICO

À minha mestre, Pâmela, por ser fonte de exemplo e admiração, tanto na ciência quanto na vida.

AGRADECIMENTOS

Acredito que as coisas não acontecem ao acaso, da mesma maneira, as pessoas não entram em nossa vida sem um motivo. Por isso, gostaria de agradecer primeiramente a **Deus**, por ter permitido que eu encontrasse tantas pessoas especiais, as quais sem dúvida alguma contribuíram para que eu conseguisse realizar mais este sonho.

À minha **família** quero dedicar a minha mais profunda gratidão. Meus pais **Vanderlei** e **Celi**, meus irmãos, **Liziane** e **Rafael**, minha madrinha **Carmen**, obrigada por estarem sempre ao meu lado, incentivando-me, apoiando minhas decisões, além de serem meus exemplos e o motivo pelo qual busco sempre melhorar.

Com todo carinho, respeito e admiração, direciono agradecimentos especiais a minha querida mestre e orientadora **Pâmela**. Obrigada por ter me proporcionado grandes oportunidades, pelos seus ensinamentos, os quais eu levarei além da ciência, para vida. Obrigada também pela sua compreensão, apoio e confiança, não tenho palavras para descrever a admiração que tenho por você.

Agradeço as minhas **amigas Assisenses**, irmãs do coração, em especial a **Alia, Inha, Lauren, Milene** e **Taline**, pelo carinho, por compreenderem a minha ausência durante esses anos (nem sempre, mas na maioria das vezes), por estarem comigo sempre em pensamento, pelo carinho e por esses 20 e poucos anos de amizade. Vocês moram no meu coração!

Ás minhas amigas do “3B”, **Maíra, Teka, Bibiana** e **Tais**, que mesmo de longe, se fazem presentes e alegram os meus dias.

À minha amiga **Tânia Regina**, que se tornou minha segunda mãe em Uruguaiana, sempre me incentivando, apoiando e me dando muito carinho. Obrigada por tudo.

À minha amiga Carmem, mais conhecida como **Neiva**, pela amizade e companheirismo, nos momentos bons e ruins, durante esses últimos anos.

À equipe do **Grupo de Pesquisa em Fisiologia** (GPFis), os quais de alguma forma estiveram sempre ao meu lado, seja ajudando nos experimentos (**Ben-Hur, Karine** e **Rithiele**), como também nas parcerias de “quartas”, “Recis” e afins, (**Mauren**, **Jefferson** e **Rithiele)**. Sou muito grata por tê-los comigo no dia a dia do lab.

Ao professor **Chárist**on e demais pesquisadores do **Neurolab** da PUC-RS por me auxiliarem na condução dos experimentos de LTP e influxo de cálcio.

Ao Professor **Rafael** e ao **Mateus** por conduzirem as análises de HPLC.

À **Universidade Federal do Pampa** por tornar possível o sonho da graduação e pós-graduação em uma universidade pública e de qualidade.

Ao **PPG Bioquímica**, pela vaga no programa e por toda assistência dada pela coordenação, professores e funcionários.

À **CAPES** pela bolsa de estudos concedida.

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Bioquímica
Universidade Federal do Pampa

UM ESTUDO COMPORTAMENTAL E BIOQUÍMICO DE ESTRATÉGIAS PARA PROMOÇÃO DA PERSISTÊNCIA DAS MEMÓRIAS DE LONGA DURAÇÃO

Autora: Liane da Silva de Vargas
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Local e data da defesa: Uruguaiana, 03 de novembro de 2016

A persistência é a principal característica da memória de longa duração (MLD). Uma vez consolidada, a MLD pode persistir por horas, dias ou anos, sendo que a sua persistência irá depender de diferentes fatores. Considerando a importância da memória no cotidiano de cada indivíduo, sendo ela responsável pela construção da personalidade e também pela manutenção das nossas ações, torna-se necessário e indispensável que haja a persistência de algumas memórias. Nesse sentido, é importante investigar os mecanismos envolvidos nesse processo, visando não só o entendimento das suas bases neurobiológicas, as quais ainda não são totalmente claras, mas também, buscar por estratégias que mantenham ou melhorem a memória ao longo do tempo. Diante disso, este trabalho teve como objetivo investigar diferentes estratégias para promoção da persistência das MLD. A tese é composta de dois estudos principais que buscaram investigar: (i) o efeito de uma sessão única de exercício físico, uma estratégia não farmacológica, na persistência da memória de reconhecimento de objetos (RO) em roedores; e, (ii) o efeito do tratamento com a Metilprednisolona (MP), um fármaco glicocorticoide, na persistência da memória aversiva em roedores. Na primeira etapa, demonstramos que a ativação noradrenérgica é necessária para que haja a persistência da memória de RO e que uma sessão única de exercício físico após a aprendizagem é capaz de promover a persistência da memória de RO por meio da ativação do sistema noradrenérgico hipocampal. Na segunda etapa, demonstramos que o tratamento crônico por 10 dias com baixa dose de MP promove a persistência da memória aversiva, além de promover o aumento

do influxo de Ca^{2+} em cultura de células de hipocampo e facilitar a indução da LTP (Potenciação de longa duração) nessa mesma estrutura. Com base nos resultados obtidos, podemos concluir que o exercício físico pode ser adotado como estratégia comportamental para a promoção da persistência das MLD. Além disso, o uso de glicocorticoides também tem potencial para ser utilizado como estratégia farmacológica que melhora a memória, entretanto seu efeito depende da dose, e estudos futuros são necessários para melhor elucidar os mecanismos de ação envolvidos, bem como seus efeitos colaterais.

Palavras chave: Persistência da memória; noradrenalina; exercício físico; glicocorticoides; LTP (potenciação de longa duração); hipocampo.

ABSTRACT

Doctoral Thesis
Graduation Program in Biochemistry
Federal University of Pampa

A BEHAVIORAL AND BIOCHEMISTRY STUDY OF STRATEGIES TO PROMOTE HEE LONG-TERM MEMORY PERSISTENCE

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Advisor: Dr. Pâmela Billig Mello Carpes
Site and date: Uruguaiana, November 3, 2016

Persistence is the main characteristic of long-term memory (LTM). When consolidated the LTM may persist for hours, days or years, and the persistence will depend of different factors. Considering the importance of memory in individual's daily life, being responsible for personality construction and also for the maintenance of our actions, it is necessary and essential that some memories persist along the time. Therefore, it is important to investigate the mechanisms involved in this process, not only to understanding of its neurobiology, which is not entirely clear, but also to find strategies to maintain or improve memory over time. Thus, this study aimed to investigate different strategies for promoting LTM persistence. This thesis is composed of two main studies that pursued to investigate: (i) the effect of one-single physical exercise session, a non-pharmacological strategy, in the persistence of object recognition memory (OR) in rodents; and (ii) the effect of treatment with methylprednisolone (MP), a glucocorticoid drug, on persistence of aversive memory in rodents. In the first stage, we show that noradrenergic activation is required to the persistence of OR memory and that one-single exercise session after learning promotes OR memory persistence through noradrenergic hippocampal system activation. In the second stage, we demonstrated that a chronic treatment for 10 days with low MP dose promotes aversive memory persistence, promotes increased Ca^{2+} influx in hippocampal cell culture and facilitates LTP induction in the same structure. Based on the results obtained, we can conclude that physical exercise can be adopted as a behavioral strategy for promoting the persistence of LTM. In addition, the use of glucocorticoids

also has potential to be used as a pharmacologic strategy that improves memory. However its effect depends on the dose, and future studies are needed to better elucidate the mechanisms involved, as well as its side effects.

Key-words: Memory persistence; norepinephrine; physical exercise; glucocorticoids; LTP (long-term potentiation); hippocampus.

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AMPA - ácido α-amino-3-hidroxi-5-metil-4-isoxazol propiônico

AMPc – Adenosina monofosfato cíclica

BDNF - Fator neurotrófico derivado do cérebro, do ingês *brain-derived neurotrophic fator*

Ca^{2+} - Íon cálcio

CA1 – Região CA1 do hipocampo dorsal, do latim Cornu ammonis (Corno de Ammon)

CaMKII - Proteína cinase dependente de cálcio/calmodulina do tipo II

CREB – Proteína ligante do elemento responsivo ao AMPc

D1 – Receptor de dopamina tipo D1

ERKs – Proteínas cinase ativáveis extracelularmente

GABA - ácido δ-aminobutírico

GMPc – Monofosfato cíclico de guanosina, do inglês *guanosine monophosphate cyclique*

HIPP – Hiocampo

HPLC - Cromatografia líquida de alta eficiência, do inglês *High performance liquid chromatography*

LC – Locus coeruleus

LTP – Potenciação de longa duração, do inglês Long-term potentiation

MCD – Memória de curta duração

Mg^{2+} - Íon magnésio

mGlu-R - receptores glutamatérgicos metabotrópicos

Min - Minutos

MLD – Memória de longa duração

MP – Metilprednisolona

MT – Memória de trabalho

mRNA – Ácido ribonucléico mensageiro (do inglês messenger ribonucleic acid)

MUS - Muscimol

NA - Noradrenalina

Na^{2+} - Íon Sódio

NMDA - N-metil-D-aspartato

NTS – Núcleo do trato solitário

PKA - proteínas cinases dependentes do AMPc

PKC - proteínas cinases cálcio dependentes

PKG - proteínas cinases dependentes do GMPc

PGi – Nucleo paragigantocelular

SNC – Sistema nervoso central

TIM - timolol

VTA – Área tegumentar ventral, do inglês *ventral tegmental área*

SUMÁRIO

APRESENTAÇÃO

A presente tese é composta por dois estudos principais, os quais buscaram, respectivamente, investigar os efeitos de uma estratégia não farmacológica e de uma farmacológica na promoção da persistência de diferentes tipos de memória.

A tese é organizada em três partes principais. Na primeira parte (introdução) estão descritos os temas que fundamentam essa tese, bem como os objetivos do estudo. A segunda parte da tese é composta pelos métodos, resultados e referências de cada estudo, apresentados na forma de artigos publicados ou submetidos a periódicos internacionais com qualis CAPES. Os resultados do primeiro estudo da tese são apresentados na forma de 1 artigo publicado no periódico "*Neurobiology of Learning and Memory*" e 1 manuscrito submetido ao mesmo periódico. Os resultados do segundo estudo da tese estão apresentados na forma de 1 manuscrito submetido ao periódico "*Behavioural Brain Research*". Por fim, a terceira parte da tese é composta pelas sessões de discussão e conclusão, nas quais apresentamos interpretações gerais sobre os manuscritos. Ao final desta tese, encontram-se as referências bibliográficas utilizadas na primeira e na terceira parte da tese.

PARTE I

INTRODUÇÃO

1 Memória

Define-se por memória a capacidade de adquirir, formar, conservar e evocar informações (IZQUIERDO, 2011). A memória é considerada como um processo de armazenamento de informações, cujos diferentes sistemas anatômicos e funcionais do Sistema Nervoso Central (SNC) são responsáveis por mediar; sistemas estes que funcionam independentemente, mas de forma cooperativa (SQUIRE, 1992; IZQUIERDO, 2011).

Considerando que as memórias são provenientes de experiências, elas podem, portanto, ser relembradas a qualquer instante (SQUIRE, 1992; IZQUIERDO, 2011). No entanto, sabemos que nem sempre lembramos tudo que vivenciamos, muitas vezes não sentimos falta, ou nem mesmo notamos a perda de algumas lembranças, mas é muito comum precisarmos de uma determinada informação e não a recordamos mais.

O processo de formação da memória envolve inicialmente três fases: a aquisição, a consolidação e a evocação. A aquisição, também chamada de aprendizagem, corresponde ao período no qual a informação é adquirida, ou seja, é neste momento em que há o primeiro contato com a informação. A consolidação corresponde à fase de armazenamento, e é neste período que se desencadeiam processos bioquímicos complexos que são necessários para que a informação seja armazenada. A evocação corresponde ao momento em que a informação é relembrada, ou seja, é o momento em que recordamos de algo que foi previamente aprendido (SQUIRE, 2004; IZQUIERDO, 2011). Ainda, existe outro processo, considerado por alguns autores como uma nova fase da memória, chamado de persistência da memória, ou consolidação tardia, que acontece 12 horas após a aquisição, por meio da modulação de mecanismos que regulam a duração das memórias por 7 dias ou mais (BEKINSCHTEIN et al., 2007). Estes processos são sequenciais e, cada um, dependente do processo prévio.

Temos ainda outros caminhos que a memória pode seguir após o seu armazenamento, por exemplo, uma memória previamente consolidada pode passar por um processo de esquecimento natural, diante desse processo, a informação original não estará mais disponível para a recuperação. Por outro lado, uma memória pode sofrer um processo de extinção, como resultado de um novo aprendizado. A extinção de uma memória não significa o seu esquecimento, mas sim, a inibição da memória original (THOMPSON, 1976; BOUTON, 1993). As memórias podem também sofrer modificações, por um processo chamado de reconsolidação, no qual uma memória previamente consolidada sofre uma desestabilização durante a sua evocação seguida de posterior estabilização (DUDAI & EISENBERG, 2004). Em humanos a reconsolidação permite a incorporação de novas informações à memória original que está sendo evocada (FORCATO et al., 2010). Sabe-se que os processos de formação das memórias, bem como outros processos, estão sob modulação de diversos sistemas de neurotransmissores (IZQUIERDO, 2011). Além disso, nos primeiros minutos ou horas após a aquisição, as memórias estão sujeitas à interferência de alguns fatores que podem influenciar sua consolidação, alguns exemplos são as experiências prévias, drogas e outros tratamentos, emoções, etc., os quais poderão também influenciar a persistência da informação adquirida (CAHILL & MCGAUGH, 1998; IZQUIERDO & MCGAUGH, 2000; IZQUIERDO, 2011; VARGAS et al., 2014). A figura 1 ilustra as fases do processo de formação, evocação e persistência da memória e sua relação com os tipos de memória de acordo com o tempo de duração, bem como os momentos nos quais a memória pode sofrer interferências.

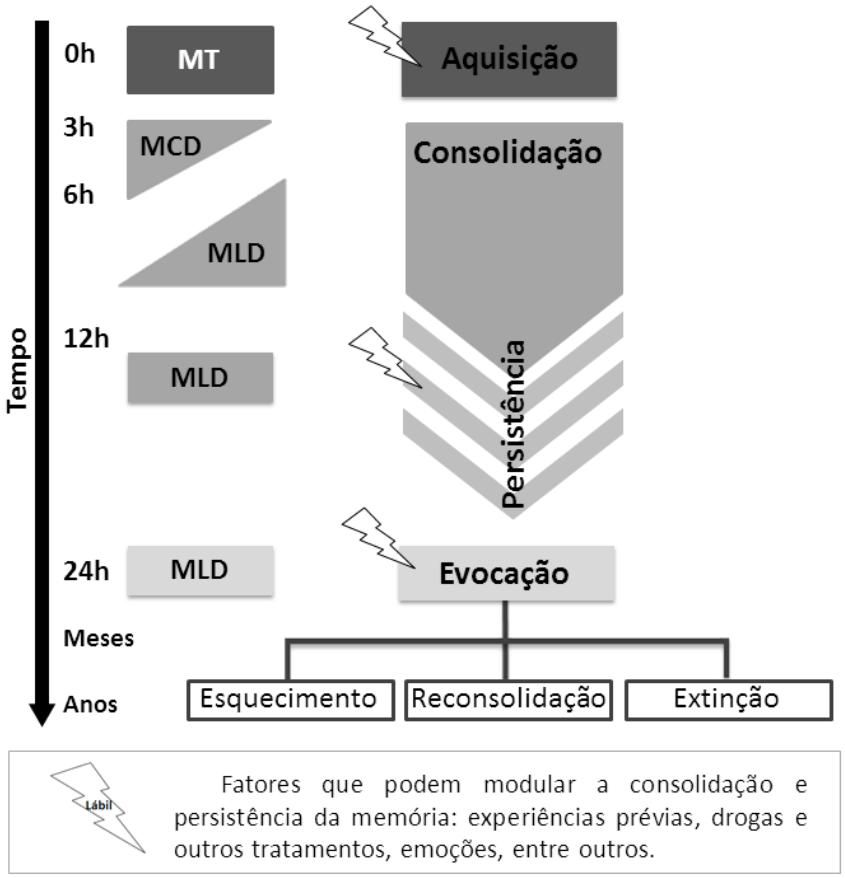

Figura 1. Fases da memória e sua relação com os tipos de memória de acordo com o tempo de duração. Nos primeiros minutos ou horas após a aquisição, as memórias estão sujeitas à interferência de alguns fatores que podem influenciar sua consolidação e persistência. MT = Memória de Trabalho. MCD = Memória de Curta Duração. MLD = Memória de Longa Duração. h = horas. Fonte: adaptada de (MELLO-CARPES, 2010).

1.1 Tipos de memória

Existem diferentes tipos de memórias, sendo elas classificadas basicamente de acordo com a sua natureza ou tempo de duração. De acordo com a natureza as memórias podem ser classificadas em memórias explícitas (ou declarativas), e memórias implícitas (ou de procedimentos). As memórias declarativas referem-se à fatos, eventos ou conhecimentos, podendo ser facilmente relatadas, sendo classificadas de duas formas: episódicas ou semânticas. As memórias episódicas referem-se a eventos específicos dos quais participamos ou assistimos, enquanto que as memórias semânticas

remetem a conhecimentos gerais (SQUIRE & KANDEL, 2003; IZQUIERDO, 2011). As memórias implícitas, por sua vez, correspondem a um conjunto de habilidades e hábitos que não podem ser facilmente descritos por meio de palavras e são adquiridas por meio da prática e repetições (por exemplo, tocar violão, andar de bicicleta, dirigir).

Considerando o tempo que as memórias duram, podemos dizer que existem memórias que duram apenas alguns segundos, enquanto que outras duram horas, dias, meses ou até mesmo a vida inteira. Assim, de acordo com o tempo, podemos classificar as memórias como: memórias de trabalho (MT), de curta duração (MCD) ou de longa duração (MLD; IZQUIERDO, 2011; ver figura 1).

A memória de trabalho, também chamada de memória imediata, é aquela que tem duração de segundos, no máximo poucos minutos (1-3 min). É uma memória muito breve que tem basicamente o papel de “gerenciamento”, ou seja, ela serve para selecionar e manter uma determinada informação por um tempo suficiente para que ela se torne ou não uma memória propriamente dita. A MT, diferentemente das demais, não produz arquivos, sendo processada fundamentalmente pelo córtex pré-frontal, dependendo simplesmente da atividade elétrica dos neurônios dessa região (DASH et al, 2007; IZQUIERDO, 2011).

A memória de curta duração, por sua vez, pode durar minutos ou poucas horas, enquanto que a memória de longa duração permanece durante horas, dias, meses ou muitas décadas, sendo, neste último caso, também chamada de memória remota. A MCD mantém a informação disponível enquanto a MLD está sendo formada (UNSWORTH & ENGLE, 2007), ambas são consolidadas por células especializadas do hipocampo e de áreas do córtex com as quais ele se conecta. A MCD, assim como a MT, não causa mudanças permanentes, já que não requerem mudanças na expressão gênica ou síntese proteica, alterações estas que acontecem durante a formação da MLD a fim de conservar estruturalmente a informação referente a elas em sinapses

modificadas em diversas regiões cerebrais (IZQUIERDO & MEDINA, 1997; IZQUIERDO & MCGAUGH 2000; IZQUIERDO, 2011).

1.2 Mecanismos da consolidação das memórias de longa duração

A formação de uma MLD requer uma série de processos metabólicos que compreendem diversas fases e que necessitam um período entre três e oito horas (IZQUIERDO & MEDINA, 1997; IZQUIERDO, 2011). Enquanto esses processos não estiverem concluídos, as MLD são lábeis. O conjunto de todos esses processos, juntamente com o seu resultado, denomina-se consolidação (IZQUIERDO, 2011).

Os mecanismos envolvidos na consolidação da memória começaram a ser desvendados após uma sequência de estudos, os quais culminaram com a descoberta de um processo eletrofisiológico, em 1973, chamado potenciação de longa duração (LTP, do inglês *long-term potentiation*) (BLISS & LOMO, 1973). A LTP consiste em um persistente aumento da resposta de neurônios a uma estimulação breve e repetitiva de um, ou de um conjunto de axônios, que fazem sinapses com elas (IZQUIERDO, 2006; 2011). Este foi o primeiro processo eletrofisiológico observado cuja duração podia ser medida não em segundos, mas em horas, semanas ou até mesmo meses. Diante dessa longa duração, semelhante à das MLD, foi proposto que a LTP poderia ser a base para formação das memórias de longa duração (IZQUIERDO et al., 2006; 2008). No entanto, mesmo havendo semelhanças entre os mecanismos moleculares da formação da memória com a LTP, sabe-se também que existem algumas diferenças (IZQUIERDO et al., 1992; IZQUIERDO & MEDINA, 1995; IZQUIERDO, 2011).

Acredita-se que a formação da memória se inicia por meio do aumento na liberação de neurotransmissores, principalmente o glutamato (IZQUIERDO & MCGAUGH, 2000; MCGAUGH & IZQUIERDO, 2000). Uma vez liberado, o glutamato se une em receptores específicos na membrana pós-sináptica, denominados receptores AMPA (ácido α-amino-3-hidroxi-5-metil-4-isoxazol propiônico), o que permite a entrada de íons Na^{2+} na célula, produzindo

despolarização. Em consequência da despolarização, a célula expulsa o íon Mg^{2+}, que normalmente obstrui o receptor glutamatérgico do tipo NMDA, este canal, então "desobstruído", passa a ser funcional, respondendo ao glutamato e permitindo a entrada de íons Ca^{2+} na célula. Os receptores glutamatérgicos metabotrópicos (mGlu-R) também podem ser ativados, induzindo a liberação de Ca^{2+} das reservas intracelulares (IZQUIERDO & MEDINA, 1995; IZQUIERDO, 2011).

O aumento de Ca^{2+} intracelular é uma importante via de sinalização, e estimula direta ou indiretamente uma série de enzimas chamadas proteínas cinases, como as proteínas cinases cálcio dependentes (PKC), a proteína cinase cálcio/calmodulina dependentes (CaMKII), as dependentes do GMPc (PKG), as dependentes do AMPc (PKA) e as proteínas ativáveis extracelularmente (ERKs), que, por sua vez, ativam mecanismos intracelulares que culminam com a síntese proteica. Um exemplo, é a fosforilação de fatores de transcrição no núcleo, em especial, a proteína ligante do elemento responsivo ao AMPc (CREB), feita pelas proteínas cinases PKA, PKC e CaMKII, a qual induz a síntese de RNA mensageiros (mRNA), levando à síntese de várias proteínas, tais como: o fator neurotrófico derivado do cérebro (BDNF do inglês *brain-derived neurotrophic factor*) (MCGAUGH, 2000; IZQUIERDO & MCGAUGH 2000; IZQUIERDO, 2011). Cabe ressaltar que todos esses processos estão sujeitos à modulação, inclusive por outros neurotransmissores diferentes do glutamato, como por exemplo, a dopamina, a noradrenalina, a serotonina, a acetilcolina, o ácido ð-aminobutírico (GABA) e poliaminas, modulação esta que irá influenciar inclusive a persistência da memória adquirida (CAHILL & MCGAUGH, 1998; MCGAUGH, 2000; 2002; IZQUIERDO, 2000). A figura 2 ilustra a sequência de eventos moleculares que acontecem durante a formação da memória.

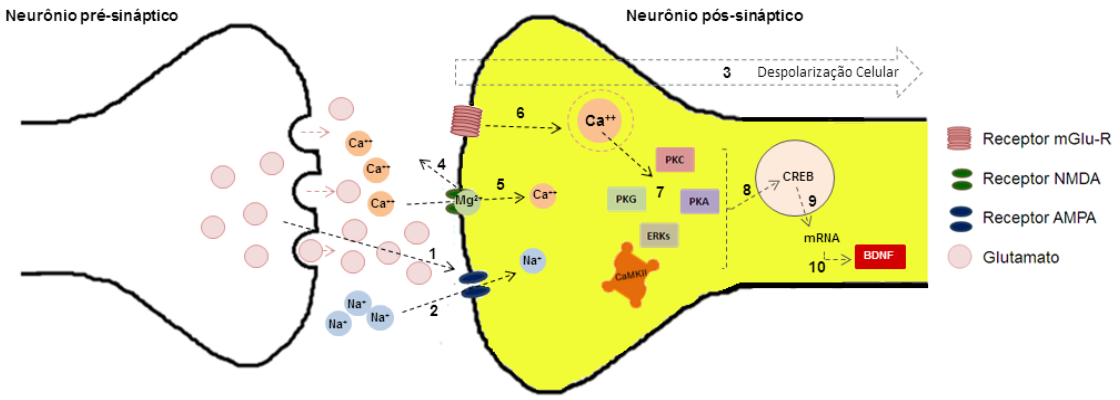

Figura 2. Sequência dos mecanismos moleculares envolvidos na formação da memória. Aumento da liberação de glutamato na fenda sináptica, o qual se liga a receptores AMPA (1) permitindo a entrada de Na^{2+} na célula (2) produzindo a despolarização (3). A despolarização expulsa o Mg^{2+} que estava ligado ao receptor NMDA (4), tornando-o funcional, permitindo a entrada de Ca^{2+} na célula (5). Os receptores mGlu-R também são ativados aumentando a concentração de Ca^{2+} na célula (6), condição esta que estimula uma série de proteínas cinases (7), levando a fosforilação de fatores de transcrição no núcleo (8), culminando com a síntese proteica (9,10). Fonte: produzida pelo próprio autor.

1.3 Persistência da memória

A principal característica da MLD é a persistência (MCGAUGH, 2000; IZQUIERDO et al., 2006). Como foi mencionada previamente, uma vez consolidada, a MLD pode persistir por horas, dias ou anos (MCGAUGH, 2000), sendo que a sua persistência, em condições fisiológicas, irá depender de diferentes fatores, tais como a idade, o nível do estímulo emocional no momento da consolidação, o estado de alerta, entre outros (CAHILL & MCGAUGH, 1998; MEDINA et al., 2008). É importante destacar que existem algumas doenças nas quais a persistência da memória muitas vezes se encontra prejudicada, como no caso da doença de Alzheimer e também da doença de Parkinson. Em ambas ocorrem alterações em sistemas de neurotransmissores e, em detrimento disso, a persistência de memórias fica comprometida (XU et al., 2012).

Já se sabe que acontecimentos que envolvem um forte grau de alerta emocional são recordados por mais tempo e com maiores detalhes do que acontecimentos neutros (RUBIN & FRIENDLY, 1986; BRADLEY et al., 1992; PALOMBA et al., 1997; OCHSNER, 2000), e que memórias enriquecidas emocionalmente envolvendo estruturas do sistema límbico são as mais afetadas pela adrenalina e hormônios glicocorticoides (DE QUERVAIN et al., 2009). Evidências demonstram que o estresse agudo e exposições breves a glicocorticoides podem facilitar a aprendizagem e a memória (JOELS et al., 2012; SARABDJITSINGH et al., 2016). Por outro lado, déficits cognitivos devido a níveis excessivos de corticosteroides têm sido observados em doenças crônicas ou durante situações de estresse que levam a hipersecreção das glândulas suprarrenais (BELANOFF et al, 2001a; 2001b; LOSEL & WEHLING, 2003; STAHN & BUTTGEREIT, 2008). Embora haja um constante progresso buscando elucidar a influência da emoção na cognição, ainda não se sabe exatamente quando e porque as emoções prejudicam ou facilitam a cognição.

É comum nos lembrarmos, também, por anos alguns fatos ou eventos que não envolveram nenhum grau de alerta emocional, como as leis da física aprendidas no colégio ou as letras de músicas das quais não gostamos muito (IZQUIERDO, 2011). Essas observações influenciaram pesquisas visando investigar se existem mecanismos posteriores à consolidação celular que expliquem a persistência da MLD por poucos ou muitos dias.

Pesquisas prévias definiram alguns processos que determinam a persistência da memória, tais processos ocorrem no hipocampo, estrutura que desempenha um papel crucial para a consolidação e evocação da memória (SQUIRE & KANDEL, 2003). Estes processos iniciam 12 horas após a aquisição, sendo que um deles envolve a ativação de neurônios dopaminérgicos da área tegumentar ventral (VTA, do inglês *ventral tegmental area*), cujos axônios inervam a região CA1 do hipocampo, e nele estimulam receptores D1 de dopamina, levando a uma rápida síntese e liberação imediata de BDNF no hipocampo. A ativação desse sistema resulta no fortalecimento de sinapses hipocampais, as quais participam na consolidação e persistência da

memória por pelo menos duas ou três semanas a mais (MEDINA et al., 2008; ROSSATO et al., 2009).

Ainda, processos bioquímicos tardios vinculados à liberação de BDNF e à persistência da memória foram descritos, incluindo um pico tardio de c-Fos e outras proteínas, que, quando inibidos, diminuem ou então anulam a persistência (BEKINSCHTEIN et al., 2007; 2008; IZQUIERDO, 2011). Eckel-Mahan e colaboradores (2008) também descrevem a relação da ativação de um sistema cíclico e circadiano, o qual consiste em um aumento, a cada 12 horas, da atividade da CAMKII e das ERKs no hipocampo a partir do momento em que a memória é adquirida, ativação esta importante para a manutenção da memória (ECKEL-MAHAN et al. 2008).

Diversos estudos corroboram o envolvimento da síntese proteica no processo de persistência da MLD (BEKINSCHTEIN et al., 2007; 2008; 2014) , a citar o experimento realizado por Bekinschtein e colaboradores (2007), no qual demonstraram que a infusão intra-hipocampal de anisomicina, um inibidor de síntese proteica, 12 horas após o aprendizado, não altera a retenção da memória de dois dias, entretanto causa amnésia quando os animais são testado 7 dias após o treino, tanto na tarefa de esquiva inibitória, quanto na tarefa de medo condicionado ao contexto (BEKINSCHTEIN et al., 2007). Sabe-se também que a reativação da via de sinalização intracelular envolvendo AMPc/MAPK/CREB no hipocampo parece ser necessária para que haja a persistência da MLD (ECKEL-MAHAN et al. 2008).

Existem várias possibilidades de mecanismos modulando a persistência da memória. O sistema noradrenérgico, o qual tem sido apontado como mediador na melhora da memória em uma variedade de paradigmas de aprendizagem, tais como o medo condicionado ao contexto, reconhecimento de objetos e extinção do medo condicionado ao contexto (LALUMIERE et al., 2003; ROOZENDAAL et al., 2006; BERLAU & MCGAUGH, 2006; MELLO-CARPES & IZQUIERDO, 2013), também tem demonstrado influência sob a persistência da memória. Parfitt e colaboradores (2012) investigaram o efeito da interação entre o estresse por imobilização e a persistência da memória,

bem como um possível efeito do estresse mediado pela ação β-adrenérgica na persistência da memória, e mostraram que houve uma melhora na persistência causada pelo estresse promovido 12 horas após o treino na esquiva inibitória por meio da imobilização, efeito este que foi revertido com a administração de um antagonista β-adrenérgico previamente ao estresse, indicando que os receptores β-adrenérgicos estão envolvidos no efeito da persistência da MLD promovida pelo estresse (PARFITT et al., 2012). Chai et al. (2014) mostraram que a infusão de noradrenalina (NA) na região CA1 do hipocampo 12 horas após a extinção (fase da consolidação tardia) promove a persistência da extinção até 14 dias; efeito este que foi bloqueado pelo propranolol (antagonista β-adrenérgico), pelo Rp-cAMPS (inibidor da PKA) e pela anisomicina e emetina (inibidores de síntese proteica), mostrando que o aumento da atividade noradrenérgica hipocampal durante a fase tardia da extinção promove a persistência da extinção da MLD (CHAI et al., 2014).

É importante destacar, no entanto, que estes processos bioquímicos relacionados à persistência da MLD que ocorrem no hipocampo 12 horas após o aprendizado dependem da prévia consolidação da memória. McGaugh (2000) destaca que a melhora na persistência da memória geralmente é resultado de mecanismos que melhoram a consolidação desta. Portanto, não somente intervenções 12h após a aprendizagem, como também intervenções na janela temporal de consolidação, podem interferir na persistência das MLD.

1.4 O hipocampo

O processamento da consolidação e evocação das memórias declarativas envolve a participação das seguintes áreas cerebrais: formação hipocampal (hipocampo, giro denteado, subículo e pré-subículo), córtex parahipocampal e áreas conectadas ao hipocampo como a amígdala, córtex entorrinal, córtex perirrinal, giro do cíngulo, área pré-frontal e córtex de associação parietal (SQUIRE & KANDEL, 2003).

Experimentos utilizando manipulações farmacológicas e bioquímicas no hipocampo e suas conexões evidenciam a importante participação dessas estruturas para o processamento da memória (MORRIS, 1989; CHOU & LEE, 1995; IZQUIERDO & MEDINA, 1995; BERNABEU et al., 1996; RUBIN et al., 2000). Mas, mesmo havendo a participação das áreas previamente citadas, o hipocampo é considerado a estrutura central para a formação de memórias declarativas (MCGAUGH 2000; IZQUIERDO et al., 2006; ROOZENDAL et al., 2008; IZQUIERDO, 2011), o qual apresenta conexões sinápticas diretas ou indiretas com as demais áreas envolvidas.

O nome “hipocampo” vem do seu formato curvado apresentado em secções coronais do cérebro humano, o qual se assemelha a um cavalo-marinho (Grego: *hippos* = cavalo, *campi* = curva). Ele se localiza bilateralmente no lobo temporal e é um importante componente do sistema límbico (Figura 3).

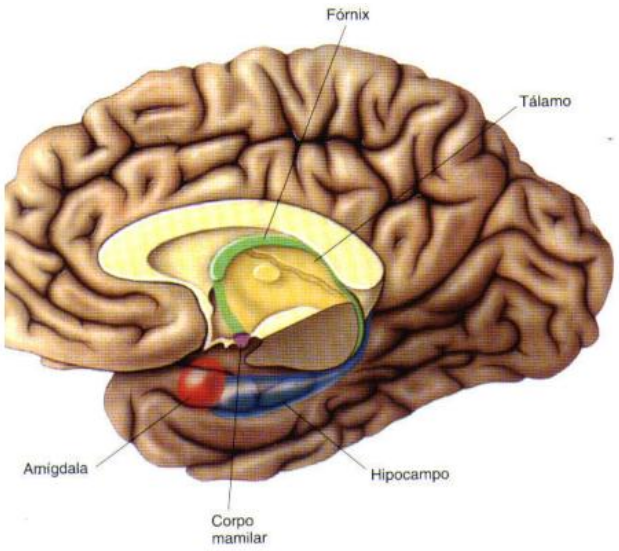

Figura 3. Localização do hipocampo no cérebro humano. Fonte: Bear et al, 2002.

O hipocampo é subdividido em diversas regiões, as quais compõe a circuitaria interna hipocampal: CA1 → Subículo → Córtex entorrinal → Giro denteado → CA3 → CA1 (RAMÓN Y CAJAL, 1893). Este circuito é ativo e participa da formação das memórias (IZQUEIRDO e MEDINA, 1997). Embora

várias outras estruturas internas do hipocampo sejam capazes de evidenciar a plasticidade, a região CA1 tem a função relacionada ao aprendizado e à memória melhor documentada.

Alguns estudos demonstram que a ativação de diferentes áreas e suas vias pode ter repercussões no hipocampo e influenciar os processos mnemônicos. Como citamos anteriormente, a própria ativação da VTA é um exemplo disso, sua ativação culmina na rápida síntese de BDNF e sua imediata liberação no hipocampo, promovendo a persistência da memória (IZQUIERDO, 2011). Outro exemplo é a via NTS-PGi-LC-HIP (Núcleo do Trato Solitário - Núcelo Paragigantocelular- *Locus Coeruleus* - Hipocampo), cuja ativação é necessária para a consolidação da memória de reconhecimento de objetos, de forma que a inibição das regiões do NTS, PGi ou LC através da infusão de muscimol (agonista dos receptores gabaérgicos) até 3 horas após a aprendizagem prejudica a consolidação da memória. Da mesma forma, a infusão intra-hipocampal de timolol (antagonista β-adrenérgico) prejudica a consolidação e persistência da memória, sugerindo que esta via contribui para a consolidação da memória por meio de mecanismos noradrenérgicos hipocampais (MELLO-CARPES & IZQUIERDO, 2013).

1.5 Intervenções para melhora da memória

Considerando a importância da memória no cotidiano de cada indivíduo, sendo ela responsável pela construção da personalidade e também da manutenção das nossas ações, torna-se necessário e indispensável que haja a persistência de algumas memórias. A capacidade de discriminar entre pessoas ou objetos familiares e novos, entre um ambiente neutro e um que oferece perigo, por exemplo, são habilidades fundamentais para o comportamento em mamíferos (RIESENHUBER & POGGIO, 2002; SQUIRE et al., 2007), uma vez que proporcionam a vantagem adaptativa da experiência prévia para a solução de questões inerentes à sobrevivência. Diante desse contexto, muitas pesquisas vêm sendo desenvolvidas não só com o objetivo de entender os

mecanismos subjacentes aos processos de formação da memória, mas também visando estratégias que possam servir como tratamento de doenças que apresentam como sintoma o déficit cognitivo, como por exemplo, as Doenças de Alzheimer e de Parkinson, ou que sirvam como estratégias auxiliadoras, facilitando o processo de aprendizagem.

A exposição ao estresse é uma condição que pode levar a prejuízos cognitivos, provocando efeitos deletérios tanto na estrutura como na função cerebral (MCEWEN, 1999a; 1999b). Já está bem estabelecido na literatura que hormônios relacionados ao estresse, por exemplo, os glicocorticoides, podem influenciar distintos processos cognitivos, podendo atuar de maneira negativa, como também positiva (MCGAUGH & ROOZENDAAL, 2002; RASHIDY-POUR et al., 2009), prejudicando ou melhorando a memória. Embora haja uma vasta gama de investigações a respeito desse tema, os resultados ainda são contraditórios. Por exemplo, estados de estresse crônico normalmente estão associados ao prejuízo da memória, enquanto que o estresse agudo promove benefícios na aquisição da memória (DE KLOET et al., 1999; JOELS et al., 2011; SANDI, 2011). Além disso, o efeito dos hormônios e fármacos glicocorticoides parece ser dose dependente. Em geral, doses moderadas promovem uma melhora da memória, enquanto as doses mais elevadas são normalmente menos eficazes ou podem mesmo prejudicar a consolidação da memória (ROOZENDAAL et al., 1999).

Os glicocorticoides são amplamente utilizados na terapia de alergias, inflamações, desordens autoimunes e no tratamento de traumatismos crânio-encefálicos, para o controle do edema (KAJIYAMA et al., 2010). Nesse sentido, considerando o uso frequente e o fácil acesso a fármacos dessa família, torna-se importante esclarecer quais são seus efeitos nos processos cognitivos, pois isto irá alertar quanto ao seu uso, o qual pode de fato prejudicar a memória, proporcionando a busca por alternativas medicamentosas com o mesmo fim terapêutico, mas também poderá colaborar com o tratamento de distúrbios cognitivos, considerando que existem evidências que apontam para seus efeitos benéficos em relação à memória.

Trabalhos envolvendo a manipulação dos sistemas de neurotransmissores mostram a importância dos mesmos para os processos de formação e consolidação da memória. O sistema colinérgico, por exemplo, é um importante modulador para o desempenho da memória (MUNERA ET AL., 2000; LOPES et al., 2008). Izquierdo e colaboradores, (1991; 1992), ao comparar o efeito de antagonistas e agonistas colinérgicos sobre a consolidação da memória em ratos, mostraram que a inibição dos receptores colinérgicos hipocampais impede a consolidação da memória aversiva, em contrapartida, os agonistas facilitam este processo (IZQUIERDO, et al., 1991; 1992). Recentemente, Partiff e colaboradores (2012) demonstraram que os receptores colinérgicos muscarínicos e nicotínicos da região CA1 do hipocampo estão envolvidos também com a persistência da memória (PARTIFF et al., 2012).

Outro importante modulador da atividade neuronal relacionada com as diferentes formas de aprendizagem e memória é o sistema dopaminérgico (JAY, 2003). Memo e colaboradores (1988) demonstraram que a administração de antagonistas dopaminérgicos, como a escopolamina, promove amnésia após a tarefa na esquiva inibitória em ratos. Também a modulação da evocação da memória envolve, entre outras, as fibras dopaminérgicas, sendo a evocação melhorada pela ativação dos receptores D1 (IZQUIERDO, 2011). Recentemente, a dopamina teve também destacado seu importante papel na persistência da memória. Rossato e colaboradores (2009) verificaram que a memória aversiva de longa duração desapareceu rapidamente quando o antagonista dos receptores D1, SCH 23390, foi injetado no hipocampo dorsal de ratos horas depois da experiência com medo. Por outro lado, a aplicação intrahipocampal do agonista D1, SK38393, ao mesmo tempo pós-treinamento tornou a memória persistente (ROSSATO et al., 2009).

Adicionalmente, pesquisas têm sido voltadas à investigação de estratégias comportamentais que possam influenciar a consolidação e a persistência da memória, já que, em muitos casos, o uso de fármacos pode causar efeitos colaterais, fornecendo riscos à saúde, sendo, nestes casos, terapias comportamentais boas alternativas. De Carvalho e colaboradores

(2013) demonstraram que uma breve exposição a um ambiente novo melhora a extinção do medo condicionado em ratos (DE CARVALHO et al., 2013). Recentemente, Menezes et al. (2015) demonstraram que a exposição a um ambiente novo facilita o processo extinção e que este efeito se dá pela ativação de receptores de dopamina D1 na região do hipocampo (MEZENES et al., 2015). Moncada e colaboradores (2011) destacaram que, além do sistema dopaminérgico, a ativação noradrenérgica parece ser fundamental para o efeito da novidade sobre a MLD (MONCADA et al., 2011). Ainda, um estudo realizado com escolares mostrou que uma experiência nova (novidade) melhora a memória relacionada à atividades literárias ou gráficas quando a novidade é aplicada próxima às sessões de aprendizagem, de forma que os alunos que haviam experimentado uma nova aula de ciências 1 hora antes ou depois da leitura de uma história apresentaram melhor desempenho do que os grupos controles ao recordar eventos da história (BALLARINI et al., 2013). Tal efeito de promoção da memória de longo prazo também foi reproduzido com outro tipo de novidade (uma aula de música) e também após outro tipo de tarefa de aprendizagem (uma memória visual) (BALLARINI et al., 2013).

Assim como a novidade, o exercício físico regular também tem seus efeitos positivos na função mnemônica, principalmente relacionados com a melhora da função cognitiva (MELLO et al., 2008; NEVES et al., 2015). Os efeitos da prática regular de exercício físico sobre o sistema nervoso incluem o aumento da neurogênese hipocampal (FABEL et al., 2003; VAN PRAAG et al., 2005; DURING & CAO, 2006) a redução de variáveis relacionadas ao estresse oxidativo (OGONOVSKY et al., 2005; RADAK et al., 2006), o aumento dos níveis do BDNF (BERCHTOLD et al., 2005; VAYNMAN et al., 2006), o incremento da vascularização cerebral (ISAACS et al., 1992), e uma variedade de mudanças morfológicas (ARIDA et al., 2004). Além disso, estudos têm demonstrado que o exercício físico é capaz de promover o aumento dos níveis de NA e outros neurotransmissores no organismo (MEEUSEN & DEMEIRLEIR 1995; PAGLIARI & PEYRIN, 1995; SEGAL et al., 2012). Entretanto, são poucos os estudos voltados à investigação dos efeitos do exercício agudo sobre a cognição.

Segal e colaboradores (2012) demonstraram que a prática de exercício físico agudo após a aprendizagem melhora a consolidação da memória tanto em idosos saudáveis como em idosos com comprometimento cognitivo leve, sugerindo que com o exercício há uma ativação do sistema noradrenérgico, o qual foi mensurado indiretamente por meio da alfa amilase salivar (SEGAL et al., 2012). Ainda, existem alguns estudos voltados à utilização de compostos alternativos que poderiam contribuir para a melhora cognitiva induzida pelo exercício. Schimidt et al. (2014) demonstraram que tanto o exercício aeróbico como a suplementação com chá verde traz benefícios relacionados a neuroproteção em um modelo animal de isquemia reperfusão cerebral, atenuando os déficits cognitivos provenientes dessa condição (SCHIMIDT et al., 2014).

Diante do exposto, nesta tese de doutorado propomos investigar diferentes estratégias que possam qualificar a persistência da MLD, incluindo os efeitos de uma estratégia não farmacológica (exercício físico) e de outra farmacológica (uso de fármacos corticoides) na persistência de diferentes tipos de memória.

2 Justificativa

Considerando a importância da memória no cotidiano de cada indivíduo, sendo ela responsável pela construção da personalidade e também da manutenção das nossas ações, se torna necessário e indispensável que haja a persistência de algumas memórias. Nesse sentido, é importante investigar os mecanismos envolvidos nesse processo, visando não só o entendimento das bases neurobiológicas da persistência da MLD, as quais ainda não são totalmente claras, mas também, a busca por estratégias que mantenham ou melhorem a persistência da memória ao longo do tempo (BEKINSCHTEIN et al., 2007; SCHIMIDT et al., 2014; VARGAS et al., 2014; NEVES et al., 2015). Segundo McGaugh (2000), a melhora na persistência da memória pode se dar como resultado da melhora na consolidação desta. Assim, intervenções no período de consolidação da memória poderiam promover sua persistência.

Estudos prévios têm procurado investigar se estratégias farmacológicas e não farmacológicas podem contribuir para a persistência da memória (MELLO-CARPES et al., 2008; ANDRADE-TALAVERA et al., 2015; MELLO-CARPES et al., 2013). Já se sabe que o exercício físico é uma prática comportamental não invasiva que favorece a liberação de noradrenalina (MEEUSEN & DEMEIRLEIR, 1995), um importante neurotransmissor envolvido no processo de consolidação e persistência da memória, em especial da memória de reconhecimento de objetos (MELLO-CARPES & IZQUIERDO, 2013), entretanto os resultados relacionados ao exercício referem-se, em sua maioria, aos efeitos advindos da sua prática regular e por um longo tempo, havendo poucos estudos voltados aos efeitos do exercício agudo. No entanto, nem sempre é simples promover o engajamento das pessoas na prática regular de exercício físico, sendo assim, é importante pesquisar os efeitos do exercício agudo na memória.

Além disso, estudos anteriores demonstram que existe uma modulação induzida por hormônios relacionados ao estresse ou pelo tratamento com glicocorticoides na memória, e que esta pode ser tanto positiva como negativa,

prejudicando ou melhorando o desempenho mnemônico (MCGAUGH & ROOZENDAAL, 2002; DE QUERVAIN et al., 2009; RASHIDY-POUR et al., 2009). Ainda, o efeito dessa modulação parece ser dependente da dose utilizada (ROOZENDAAL et al., 1999), demonstrando a necessidade de estudos nesse tema, já que o uso de tratamentos medicamentosos com glicocorticoides está amplamente presente em meio a população, e ainda não estão claros os seus efeitos nos processos cognitivos.

Assim, nesta tese de doutorado procuramos investigar estas diferentes estratégias e seu uso na promoção da persistência da memória de longa duração. O trabalho é dividido em dois estudos principais, que buscam, respectivamente, investigar os efeitos de uma estratégia não farmacológica (exercício físico) e de outra farmacológica (uso de um fármaco glicocorticoide) na persistência de diferentes tipos de memória.

No primeiro estudo apresentado na tese investigamos se uma sessão de exercício físico após um aprendizado de reconhecimento pode ter um efeito positivo na persistência desta memória, cujo período de duração normalmente é limitado há alguns poucos dias em roedores. A memória de reconhecimento é representada pela capacidade de discriminar entre pessoas ou objetos familiares e novos. Este tipo de memória exige que as características específicas de um determinado evento sejam identificadas, discriminadas e comparadas com as características de memórias previamente adquiridas (STECKLER et al., 1998). Em roedores este tipo de memória é avaliada por meio da tarefa de reconhecimento de objetos (ENNAUCER & DELACOUR, 1988). Nossa hipótese é de que o exercício agudo, assim como o exercício crônico, também pode ter efeitos positivos sobre a aprendizagem, uma vez que o exercício físico é uma prática comportamental não invasiva que favorece a liberação de noradrenalina (MEEUSEN & DEMEIRLEIR, 1995). Estudos prévios do nosso grupo demonstraram que a ativação do sistema noradrenérgico hipocampal é fundamental para a consolidação da memória de reconhecimento de objetos (RO) (MELLO-CARPES & IZQUIERDO, 2013), assim, no primeiro estudo desta tese: (i) investigamos o papel do sistema noradrenérgico na promoção da persistência da memória de RO; e, (ii)

propomos o uso do exercício físico dentro da janela de consolidação da memória, de forma que este processo possa sofrer influência dos mecanismos que são ativados durante o exercício, especialmente a ativação do sistema noradrenérgico.

O segundo estudo da tese envolve a investigação do efeito do tratamento crônico com diferentes doses de Metilprednisolona (MP), um fármaco glicocorticoide, na persistência da memória aversiva em roedores. A memória aversiva corresponde à capacidade de discriminar entre um ambiente neutro e um ambiente perigoso, evitando-o, e é mensurada a partir do teste de esquiva inibitória em roedores (IZQUIERDO et al., 1997). Considerando que existe uma modulação induzida por hormônios relacionados ao estresse ou pelo tratamento com glicocorticoides na memória e que esta pode ser tanto positiva como negativa, além de depender da dose utilizada, nossa hipótese é de que a MP possa influenciar na consolidação e persistência da memória aversiva. Além disso, tanto a memória de reconhecimento como a aversiva são dependentes do hipocampo (IZQUIERDO et al., 1997), e podem sofrer alterações por diversos sistemas de neurotransmissores (MONLEÓN et al., 2009), podendo, portanto, sofrer influência do exercício, bem como dos glicocorticoides.

3 OBJETIVOS

3.1 Objetivo Geral

Investigar diferentes estratégias com potencial de qualificar a persistência das memórias de longa duração.

3.2 Objetivos específicos

- Investigar a participação do sistema noradrenégico na promoção da persistência da memória de reconhecimento de objetos;
- Verificar se uma única sessão de exercício físico é capaz de promover a persistência da memória de reconhecimento de objetos;
- Verificar se o efeito de uma sessão de exercício físico sobre a memória está relacionado com a ativação de mecanismos noradrenérgicos no hipocampo;
- Avaliar o efeito de diferentes doses de Metilprednisolona (fármaco corticoide) na persistência da memória aversiva;
- Investigar o efeito do tratamento com a Metilprednisolona no influxo celular de cálcio e indução da LTP hipocampal.

PARTE II

Neurobiology of Learning and Memory 127 (2016) 84–92

Contents lists available at ScienceDirect

Neurobiology of Learning and Memory

journal homepage: www.elsevier.com/locate/ynlme

Hippocampal noradrenergic activation is necessary for object recognition memory consolidation and can promote BDNF increase and memory persistence

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ARTICLE INFO

Article history:
Received 11 September 2015
Revised 23 November 2015
Accepted 27 November 2015
Available online 10 December 2015

Keywords:
Norepinephrine
Nucleus of the Solitary Tract
BDNF
Memory persistence

ABSTRACT

Previously we showed that activation of the Nucleus of the Solitary Tract (NTS)–Nucleus Paragigantocellularis (PGi)–Locus coeruleus (LC) pathway, which theoretically culminates with norepinephrine (NE) release in dorsal hippocampus (CA1 region) and basolateral amygdala (BLA) is necessary for the consolidation of object recognition (OR) memory. Here we show that, while the microinjection of the beta-noradrenergic receptor blocker timolol into CA1 impairs OR memory consolidation, the microinjection of norepinephrine (NE) promotes the persistence of this type of memory. Further, we show that OR consolidation is attended by an increase of norepinephrine (NE) levels and of the expression of brain derived neurotrophic factor (BDNF) in hippocampus, which are impaired by inactivation of the NTS–PGi–LC pathway by the infusion of muscimol into the NTS.

1. Introduction

It is known that emotionally arousal-induced memory consolidation requires noradrenergic activation of the basolateral amygdala (BLA) (Beldjoud, Barsegyan, & Roozendaal, 2015; McGaugh, 2000) as studied in inhibitory avoidance (IA) and other tasks (Beldjoud et al., 2015; McGaugh, 2015). Previous research strongly suggests that the NE release in the BLA during the consolidation of emotional memories depends on arousal induced by activation of the NTS (Nucleus of the Solitary Tract)–PGi (Paragigantocellularis nucleus)–LC (Locus Coeruleus)–BLA pathway: Garcia-Medina and Miranda (2013) found that stimulation of the NTS promotes the release of NE in lateral and basolateral amygdala, and Roozendaal, Williams, and McGaugh (1999) showed that the activation of glucocorticoid receptors in NTS facilitates memory consolidation of inhibitory avoidance learning, among other researches. This pathway (NTS–PGi–LC) was proposed to play a major role in the modulation of the consolidation of aversive behaviors by Cedric Williams, James McGaugh and their associates over 20 years ago (Clayton and Williams, 2000a,b; King & Williams, 2009; Miyashita & Williams, 2004; Williams & McGaugh, 1993) and was suggested to play a similar role in that of OR by two of the present authors two years ago (Mello-Carpes & Izquierdo, 2013).

Previously, our group demonstrated that basolateral and central amygdala noradrenergic activation is not necessary to promote object recognition task (OR) consolidation (Mello-Carpes & Izquierdo, 2013), although others had shown that the noradrenergic activation of basolateral amygdala can modulate the consolidation of this memory (Roozendaal, Castello, Vedana, Barsegyan, & McGaugh, 2008). OR memory is viewed as a relatively non-emotional declarative memory; however, considering that there is a lot of variability across OR procedures among different studies it is not easy to draw a meaningful conclusion about this. However, all OR task protocols do involve the presentation of novelty to the animal, and the detection of and reaction to novelty are major functions of the hippocampus (Acquas, Wilson, & Fibiger, 1996; Menezes et al., 2015; Netto et al., 1985), we hypothesized that this type of memory may also require the participation of this pathway, but using taking as the last stage hippocampal noradrenergic activation, instead of that of the amygdala. In a previous paper (Mello-Carpes & Izquierdo, 2013) we showed that, although the

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http://dx.doi.org/10.1016/j.nlm.2015.11.014
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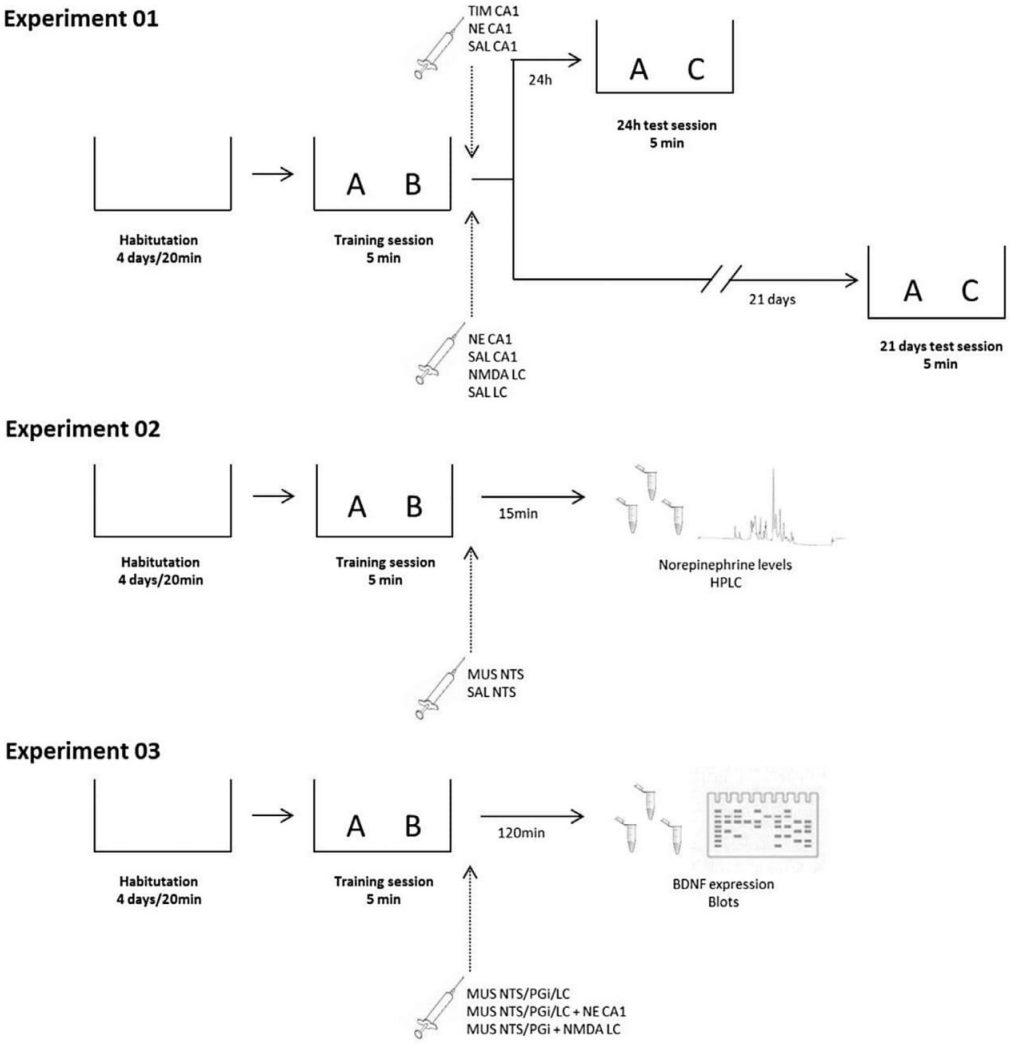

Fig. 1. Experimental design. The experiments were organized in three stages. Experiment 1: The rats were habituated to the object recognition arena without any object for 4 days (20 min/day). In the training session rats were exposed to two different objects (A and B) for 5 min; immediately after that the received bilateral hippocampal infusions (1 μl/side in CA1) of vehicle (VEH; saline), timolol (TIM; 1 μg/μl for CA1) or norepinephrine (NE; 1 μg/μl) and, on the test session, realized 24 h after, animals were exposed to a familiar (A) and a novel object (C) for five minutes to evaluate long-term memory retention. Other group of animals received bilateral LC (Locus Coeruleus) infusions (0.25 μl/side) of vehicle (VEH; saline) or NMDA (NMDA; 0.1 μg/μl) or bilateral hippocampal infusions (1 μl/side in CA1) of vehicle (VEH; saline) or norepinephrine (NE; 1 μg/μl). On the test session, realized 21 days after, all animals were exposed to a familiar (A) and a novel object (C) for five minutes to evaluate long-term memory persistence. Experiment 2: The rats were habituated to object recognition arena without any object for 4 days (20 min/day). On training session rats were exposed to two different objects (A and B) for 5 min and immediately after that received bilateral infusions (0.5 μl/side in NTS) of vehicle (VEH; saline) or muscimol (MUS 0.01 μg/μl); 15 min later the hippocampus were removed and prepared for HPLC determination of norepinephrine levels. Experiment 3: The rats were habituated to object recognition arena without any object for 4 days (20 min/day). On training session rats were exposed to two different objects (A and B) for 5 min and immediately after that received bilateral infusions of different drugs or combinations of drugs and/or vehicle in (MUS in NTS/PGi/LC; or MUS in NTS/PGi/LC + NE CA1; or MUS NTS/PGi + NMDA LC); 120 min later the hippocampus were removed and prepared for immunoblot determination of BDNF protein expression.

connection of the medullary nuclei to the amygdala is not involved in OR consolidation, the connection of NTS–PGi–LC pathway to hippocampus is. Indeed, other evidences from previous works indicates an important role of the hippocampus in the consolidation of this task (Balderas, Rodriguez-Ortiz, & Bermudez-Rattoni, 2015; Clarke, Cammarota, Gruart, Izquierdo, & Delgado-García, 2010; Cohen & Stackman, 2015; Furini et al., 2010; Myskiw et al., 2008).

In addition to our previous data, here we show that, while the hippocampal CA1 injection of β-adrenergic blocker timolol impairs OR memory consolidation, the injection of NE promotes the persistence of this memory. The LC stimulation, which culminates with the increase of NE release in hippocampus, promotes memory persistence too. Also, we demonstrate that OR learning promotes the hippocampal increase of NE levels, which are disrupted by inactivation of NTS–PGi–LC pathway after OR training. These results confirm the hippocampal noradrenergic modulation of OR memory.

Still, considering that recent findings suggest that OR consolidation requires an increase of BDNF expression in CA1 region of

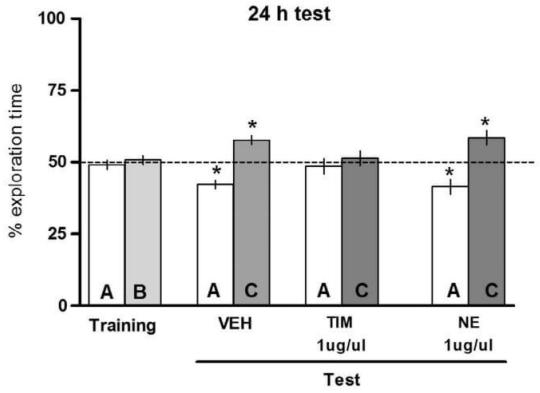

Fig. 2. Norepinephrine is necessary to OR memory consolidation. On training session (day 1) rats were exposed to two different objects (A and B) for 5 min and immediately after that received bilateral infusions (1 μl/side in CA1) of vehicle (VEH; saline), timolol (TIM; 1 μg/μl for CA1) or norepinephrine (NE; 1 μg/μl). On test session (day 2), animals were exposed to a familiar (A) and a novel object (C) for five minutes to evaluate long-term memory retention. The infusion of β-adrenergic antagonist timolol in CA1 region of dorsal hippocampus immediately after training impairs retention of object recognition long-term memory. The infusion of NE in CA1 region of dorsal hippocampus immediately after training does not affect retention of object recognition long-term memory. Data (mean ± SD) are presented as percentage of total exploration time. $^{*}P \leqslant 0.01$ in one-sample Student's *t*-test with theoretical mean = 50; $n = 9$–12 per group.

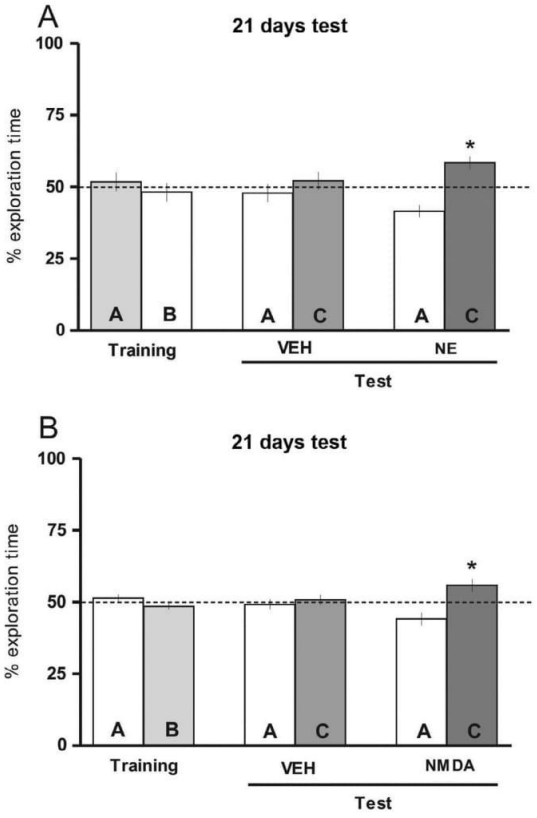

Fig. 3. Norepinephrine promotes OR memory persistence. On training session (day 1) rats were exposed to two different objects (A and B) for 5 min. In the first experiment (3A), immediately after training the rats received bilateral infusions (1 μl/side in CA1) of vehicle (VEH; saline) or norepinephrine (NE; 1 μg/μl). In the second experiment (3B), immediately after training the rats received bilateral infusions (0.25 μl/side in LC) of vehicle (VEH; saline) or NMDA (NMDA; 0.1 μg/μl). On test session (21 days later), animals were exposed to a familiar (A) and a novel object (C) for five minutes to evaluate long-term memory retention. Figure (A) shows that the infusion of NE in CA1 region of dorsal hippocampus immediately after training promotes memory persistence of object recognition long-term memory. Fig. 2B shows that the induction of the increase of NE release on hippocampus through LC stimulation with NMDA promotes persistence of object recognition long-term memory. Data (mean ± SD) are presented as percentage of total exploration time. $^{*}P \leqslant 0.01$ in one-sample Student's *t*-test with theoretical mean = 50; $n = 9$–12 per group.

dorsal hippocampus 120 min after OR training (Furini et al., 2010) and that BDNF has an essential role in memory formation and persistence (Bekinschtein et al., 2008; Romero-Granados, Fontan-Lozano, et al., 2010) we decided investigate the BDNF protein expression in hippocampus and demonstrated that it is disrupted when NTS–PGi–LC pathway is inactivated after OR training. The activation of a downstream point of the pathway after the inactivation, or the exogenous infusion of noradrenaline in hippocampus, or the stimulation of LC, however, permits the BDNF increase expect and necessary to OR learning.

2. Material and methods

Male Wistar rats (3-month-old, 350–380 g) purchased from FEPPS (Fundação Estadual de Produção e Pesquisa em Saúde do Rio Grande do Sul, Porto Alegre, Brazil) or from Central Vivarium of Federal University of Pelotas (RS/Brazil) were used. The animals were housed 5 to a cage and maintained with free access to food and water under a 12 h light–dark cycle, with lights on at 8:00 AM. The temperature of the animal room was kept at 22–24 °C. All experiments were conducted in accordance with the "Principles of laboratory animal care" (NIH publication n° 80–23, revised 1996). Fig. 1 summarizes the experiments conducted.

2.1. Surgery and drug infusion procedures

In order to implant the rats with indwelling cannulas, they were deeply anesthetized with thiopental (i.p., 30–50 mg/kg) and 27-gauge cannulas were placed, stereotaxically aimed at the NTS (A – 13.3, L ± 1.0, V – 7.9 mm), PGi (A – 12.8, L ± 1.6, V – 12 mm), LC (A – 9.7, L ± 1.3, V – 7.1 mm) and/or CA1 region of the dorsal hippocampus (A – 4.2, L ± 3.0, V – 2.0 mm) (coordinates according to Paxinos and Watson (1986). The cannulae were affixed with dental cement. Animals were allowed to recover from surgery for 4 days before submitting them to any other procedure. Some of the animals received bilateral cannulae implants into the NTS, PGi or LC and the CA1 region of the dorsal hippocampus; that is, they carried 4 brain cannulae each.

At the time of drug delivery, 30-gauge infusion cannulas were tightly fitted into the guides. Infusions (0.25 μl/side in LC, 0.5 μl/side in NTS and PGi, and 1.0 μl/side in CA1 region of hippocampus) were carried out over 60 s with an infusion pump, and the cannulas were left in place for 60 additional seconds to minimize backflow. The doses and volume used were based on pilot experiments and on previous studies showing the effect of each compound on learning and behavioral performance (Clayton & Williams, 2000a,b; Furini et al., 2010; Lemon, Aydin-Abidin, Funke, & Manahan-Vaughan, 2009; Mello-Carpes & Izquierdo, 2013). The placement of cannulas was verified postmortem: 2–4 h after the last behavioral test, a 4% methylene-blue solution was infused in the same

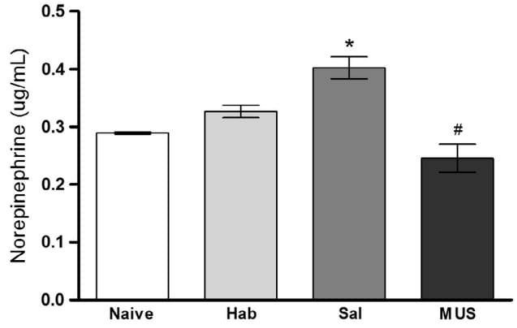

Fig. 4. Consolidation of object recognition memory promotes a norepinephrine increase 15 min after training; NTS–PGi–LC pathway inhibition prevents this increase. Cannulated animals were habituated for 4 days (Hab) and some of them were trained in the OR task. Some animals were not exposed to OR apparatus (Naïve controls). Rats trained in the OR received an intra-NTS infusion of saline (Sal) or Muscimol (MUS) to promote the inhibition of NTS–PGi–LC–CA1 pathway and 15 min later were euthanized, and the bilateral hippocampus was removed for HPLC determination of NE levels. The tissue content of NE in the hippocampus homogenate (μg/ml) was higher in the trained rats infused by saline when compared to naïve or habituated ones. The tissue content of NE in the hippocampus homogenate (μg/ml) was lower in the trained rats infused by muscimol when compared to the ones that received saline. $P < 0.01$ (One-way ANOVA). $^{*}P < 0.01$ for saline vs. naïve and saline vs. habituated. $^{\#}P < 0.01$ for saline vs. muscimol (Tukey's Multiple Comparison Test). $n = 4$ per group analyzed in triplicate.

volume used in each of the mentioned places as described earlier, and the extension of the dye 30 min thereafter was taken as an indication of the presumable diffusion of the vehicle or drug previously given to each animal. Only data from animals with correct implants and no significant adjacent structure tissue spread were analyzed.

2.2. Drugs, antibodies and reagents

Muscimol (MUS), timolol (TIM), NMDA and NE were purchased from Sigma–Aldrich (St. Louis, MO). The drugs were dissolved in saline and stored at −20 °C, protected from light until use, at which time an aliquot was thawed and diluted to working concentration in saline 0.9% (pH 7.2). The doses and volume used were based on a previous study of our laboratory and others, as cited before (Mello-Carpes & Izquierdo, 2013). Anti-BDNF and anti-tubulin antibody were from Santa Cruz Biotechnology (Santa Cruz, CA) and/or Sigma–Aldrich (St Louis, MO). Other reagents used in the experiments were of analytical grade and obtained from standard commercial suppliers.

2.3. Object recognition task

Training and testing in the object recognition task (OR) were carried out in an open-field arena (50 × 50 × 50 cm) built of polyvinyl chloride plastic, plywood and transparent acrylic as described previously (Ennaceur & Delacour, 1988; Mello-Carpes & Izquierdo, 2013). The first procedure consisted in the habituation of the animals to the training box. Each animal was placed in the apparatus for 20 min of free exploration per day during 4 consecutive days before the training. On the training day, two different objects (A and B) were placed in the apparatus; animals were allowed to explore them freely for 5 min. The objects were made of metal, glass, or glazed ceramic. Exploration was defined as sniffing or touching the objects with the nose and/or forepaws. Sitting on or turning around the objects was not considered exploratory behavior. A video camera was positioned over the arena, and the rats' behavior was recorded using a video tracking and analysis system for later evaluation. The experiments were performed by an observer blind to the treatment condition of the animals.

In the experiments that involved a test session, this was carried out 24 h or 21 days later. One of the objects was randomly exchanged for a novel object (C) and rats were reintroduced into the apparatus for an additional 5 min period. To avoid confounds by lingering olfactory stimuli and preferences, the object and the arena were cleaned after testing each animal trial with 70% ethanol.

2.4. Norepinephrine levels

The determination of hippocampal NE levels was made by HPLC (High Performance Liquid Chromatography). Levels of NE in homogenates prepared from the hippocampus were determined using a reverse-phase HPLC system (YL9100, Young Lin). Rats' brains were removed and bilateral hippocampus were quickly dissected out in an iced surface and homogenized in 50 mM Tris HCl, pH 7.4, (1/10, w/v). Afterwards, samples were centrifuged at 2400*g* for 20 min, and supernatants were filtered and then stored at −80 °C until use (Menezes et al., 2015; Nirogi et al., 2012). The HPLC system consisted of a Vacuum Degasser (YL9101) and quaternary pump (YL9110) connected to a reversed phase column (SYNERGI 4μ FUSION-RP 80 Å 250 × 4.60 mm; Phenomenex) on a Column Compartment (YL9131) coupled to a Diode Array Detector (YL9160). The mobile phase consisted of methanol and water (12/88, v/v) adjusted to pH 3 with phosphoric acid. To separate NE, we used the programming isocratic with a flow rate of 0.8 mL/min. The sample was filtered through 0.22 μm syringe filters. We injected 20 μL samples into the HPLC system by an auto sampler device (YL9150). The detection was at 198 nm by DAD. Chromatograms were recorded and integrated by PC integration software (YL-Clarity). All analyses were run in triplicate. The analytical parameters were as follows: linear range, 0.1–10.0 μg/ml; determination coefficient, 0.999; and calibration equation, $y = 628.12x - 34.342$. Norepinephrine for HPLC was supplied by Sigma–Aldrich Brazil. Other reagents used in this experiment were of analytical grades and obtained from standard commercial suppliers.

2.5. Immunoblot essays to measure BDNF protein expression

Animals were killed by decapitation and the CA1 region of the dorsal hippocampus rapidly dissected out and homogenized in ice-chilled buffer (20 mM Tris–HCl, pH 7.4, 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μg/ml aprotinin, 15 μg/ml leupeptin, 10 μg/ml bacitracin, 10 μg/ml pepstatin, 15 μg/ml trypsin inhibitor, 50 mM NaF, and 1 mM sodium orthovanadate). Protein concentration was determined using the BCA protein assay (Pierce, Rockford, IL), and equal amounts of protein were fractionated by SDS–PAGE before electrotransferred to polyvinylidene difluoride membranes (PVDF; Immobilon-P, Millipore, MS). After verification of protein loading by Ponceou S staining, the blots were blocked in Tween-Tris buffer saline (TTBS; 100 mm Tris–HCl, pH 7.5, containing 0.9% NaCl and 0.1% Tween 20) and incubated overnight with anti-BDNF antibody (N20, 1:5000, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-tubulin antibody (1:1000, Sigma–Aldrich, St Louis, MO). The membranes were washed in TTBS and incubated with HRP-coupled anti-IgG antibody, washed again, and the immunoreactivity detected using a West-Pico enhanced chemiluminescence kit (Pierce, IL). Densitometric analysis was carried out in an ImageQuant RT-ECL system (GE, Piscataway, NJ).

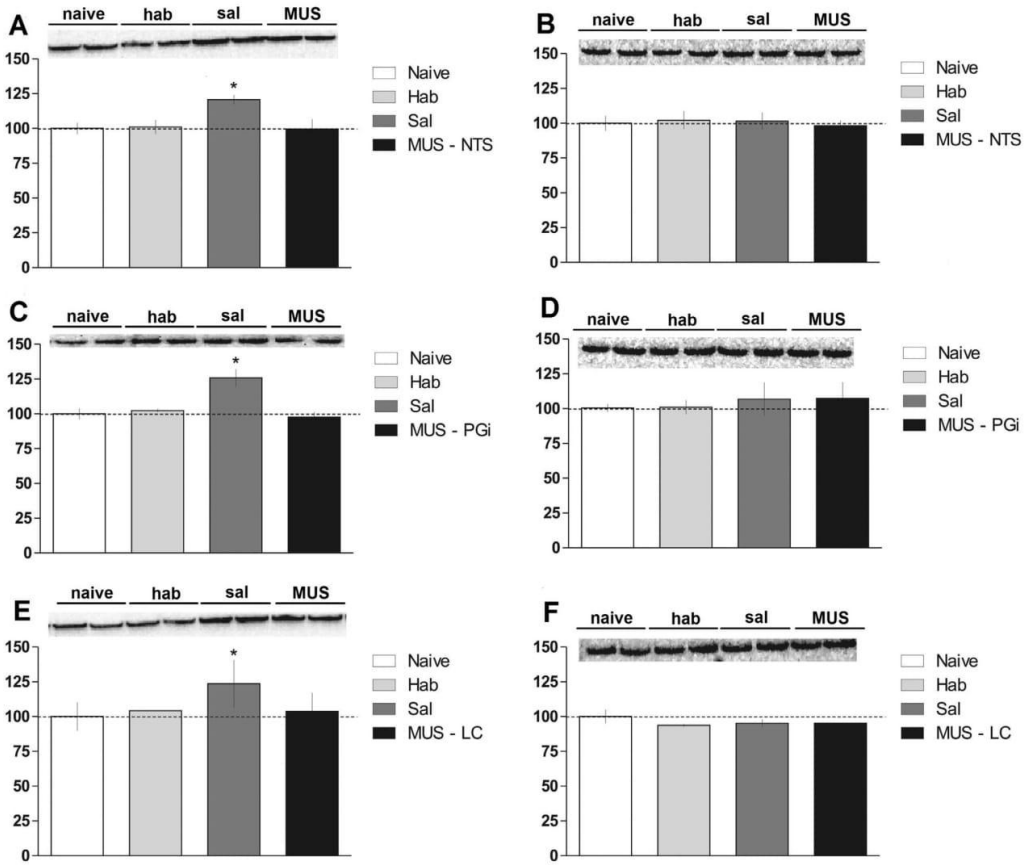

Fig. 5. Consolidation of object recognition memory promotes a BNDF increase 120 min after training; this increase is blocked by infusion of muscimol in NTS (A), PGi (C) and LC (E). Consolidation of object recognition memory did not alter tubulin protein levels 120 min after training. Additionally, any alteration was seemed after infusion of muscimol in NTS (B), PGi (D) and LC (F). Cannulated animals were habituated for 4 days (Hab) and some of them were trained in the OR task. Some animals were not exposed to OR apparatus (Naïve controls). Trained animals rats were exposed to two different objects for 5 min and immediately after that received bilateral infusions of saline (Sal) or muscimol (MUS) in NTS, PGi (0.01 μg/μl; 0.5 μl/side) or LC (0.02 μg/μl; 0.25 μl/side). 120 min after that, the animals are killed by decapitation, the dorsal CA1 region of hippocampus dissected out and total homogenates submitted to SDS–PAGE followed by immunoblot analysis with antibodies against BDNF (A, C and E) or tubulin (B, D and F). Bars show the percentage levels respect to naive animals. Data are expressed as mean ± SD. $^{*}P \leqslant 0.05$ in Dunnett's comparison test after ANOVA; $n = 5$ per group.

2.6. Statistical analyses

Object exploration time in OR task was converted to percent of total exploration time and so a one-sample *t*-test was used to compare the percent of total time exploration spent in each object with a theoretical mean (50%). In HPLC results, the data of the groups were compared using an One-Way ANOVA followed by Tukey's Multiple Comparison Test. The immunoblots' data were analyzed using ANOVA followed by Dunnett's post hoc test. All data were expressed as mean ± SD. The sample size (*n*, number of animals in each group) for each experiment is stated in the figure legends. The differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Hippocampal norepinephrine is necessary for OR memory consolidation and can promotes memory persistence

To measure the effect of NE infusion into CA1, rats were trained in the OR learning task and immediately after training received bilateral intra-CA1 infusion of VEH, TIM or NE (1 μg/μl; 1 μl/side). LTM was evaluated 24 h later. In the LTM retention test session, rats that received TIM were not able to remember the familiar object, and spent about the same time exploring each object (Fig. 2, $P = 0.622$), while the VEH rats spent significantly more than 50% of total exploration time exploring the novel object (Fig. 2, $P < 0.0001$). Rats that received NE after training explored the novel object significantly longer than the familiar one (Fig. 2, $P = 0.006$), similar to control (VEH) rats.

Considering these results we went on to investigate the effects of NE on OR memory persistence. Rats were trained in the OR task and tested them 21 days later. We did this in two conditions: (i) after a NE infusion directly into the CA1 region of dorsal hippocampus (Fig. 3A), and (ii) after LC activation to promote increment of NE release in CA1 (Fig. 3B). In both conditions we observed that the rats had their memory preserved 21 days after training, and spent more time exploring the novel object ($P < 0.01$), while the VEH rats were not able to recognize the new object, spending around 50% of total exploration time in each one.

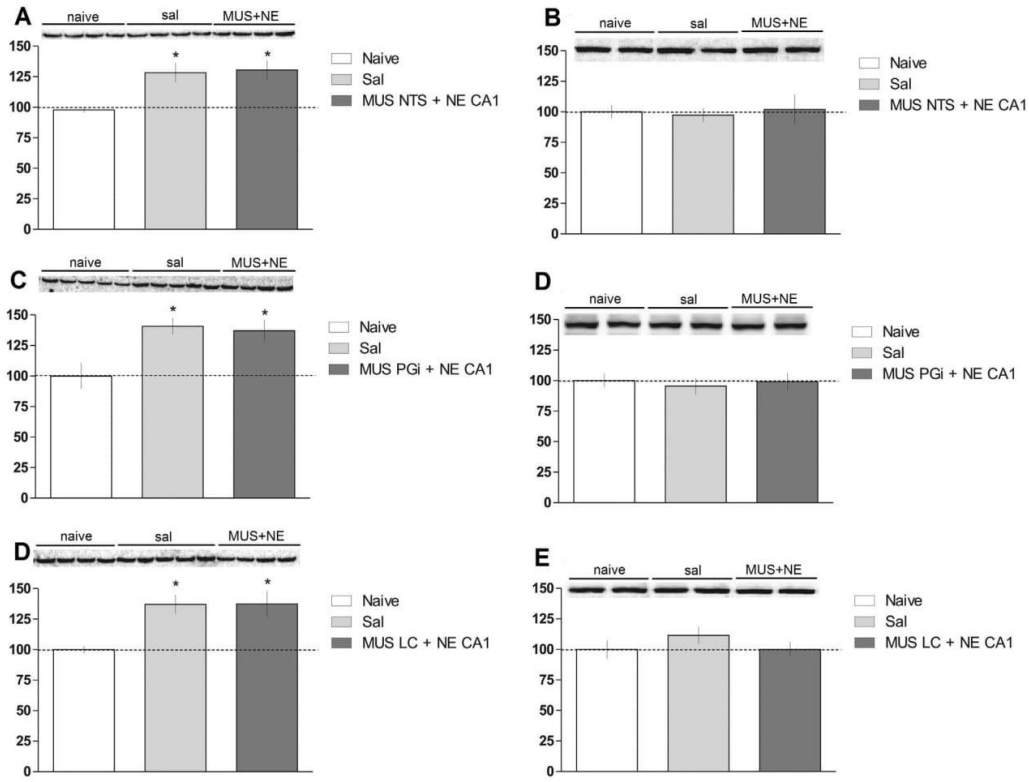

Fig. 6. Block of the increase of BDNF by infusion of muscimol in NTS (A), PGi (C) and LC (E) are reversed by infusion of NE in CA1 region of dorsal hippocampus. The infusion of muscimol in NTS (B), PGi (D) and LC (F) followed by infusion of NE in CA1 region of dorsal hippocampus did not alter tubulin protein levels 120 min after training. After the cannula implantation procedure, animals were divided into naïve controls and animals trained in the OR task. Trained rats were exposed to two different objects for 5 min and immediately after that received bilateral infusions of saline (Sal) or muscimol (MUS) into NTS, PGi (0.01 μg/μl; 0.5 μl/side) or LC (0.02 μg/μl; 0.25 μl/side) + norepinephrine (NE) in CA1 (1 μg/μl; 1 μl/side). One-hundred-and-twenty min after that, they were killed by decapitation, the dorsal CA1 region of hippocampus dissected out and total homogenates were submitted to SDS–PAGE followed by immunoblot analysis with antibodies against BDNF (A, C and E) or tubulin (B, D and F). Bars show the percentage levels respect to naive animals. Data are expressed as mean ± SD. $^{*}P \leqslant 0.05$ in Dunnett's comparison test after ANOVA; $n = 5$ per group.

3.2. OR training is accompanied by an increase of norepinephrine levels on hippocampus, which is impaired by NTS–PGi–LC pathway inactivation

Sixteen animals were divided in four groups: (i) Naïve ($n = 4$); (ii) Habituated, which rats were just habituated to OR apparatus (Hab) ($n = 4$); (iii) Saline (Sal), which rats were trained in OR task and received a saline infusion in NTS ($n = 4$); and (iv) muscimol (MUS), which rats were trained in OR task and received a MUS infusion in NTS ($n = 4$). Rats from group (ii), (iii) and (iv) were euthanized for hippocampus dissection 15 min later the last behavioral procedure. The CA1 regions of both dorsal hippocampi were homogenized and processed for HPLC determination of NE levels. The rats that received Sal presented an increase of hippocampal NE levels when compared to Naïve and Saline groups ($P < 0.01$). Inactivation of the NTS–PGi–LC pathway by MUS infusion into NTS blocked this increase (Fig. 4, $P < 0.01$).

3.3. OR training culminates in an increase of BDNF expression on hippocampus which is impaired by inactivation of the NTS–PGi–LC pathway and reversed by norepinephrine injection on hippocampus and by LC stimulation with NMDA

We verified that inactivation of NTS (Fig. 5A), PGi (Fig. 5C) or LC (Fig. 5E) with MUS blocked the BDNF increase that occurred 120 min post training in the OR task ($P = 0.02$ for NTS, PGi and LC). We also measured the tubulin levels as a control procedure and did not find any differences between groups (Fig. 5B, D and F) ($P > 0.05$ in all analyses).

Importantly, the infusion of NE in CA1 region immediately after infusion of MUS in NTS (Fig. 6A), PGi (Fig. 6C) or LC (Fig. 6E) reverts the effect previously described, and permits the increase of BDNF expression 120 min post training in OR ($P = 0.01$ to NTS and PGi; $P = 0.02$ to LC). There are no differences between groups in tubulin levels (Fig. 6B, D and F) ($P > 0.05$ in all analyses).

Also, stimulation of LC with NMDA after MUS injection in NTS (Fig. 7A) or PGi (Fig. 7C) reverts the block of the increase of BDNF expression 120 min after training in OR ($P = 0.02$ to NTS and PGi). There are no differences between groups in tubulin levels (Fig. 7B and D) ($P > 0.05$ in all analyses).

4. Discussion

This study provides important complementary evidence about the role of hippocampal NE in OR consolidation (Mello-Carpes & Izquierdo, 2013). In the first set of experiments we show that hippocampal NE acting on β-adrenergic receptors is necessary to OR memory consolidation, since the injection of timolol (a β-blocker) after OR training impairs memory consolidation. Otherwise, the injection of NE just after OR learning, although it did not affect

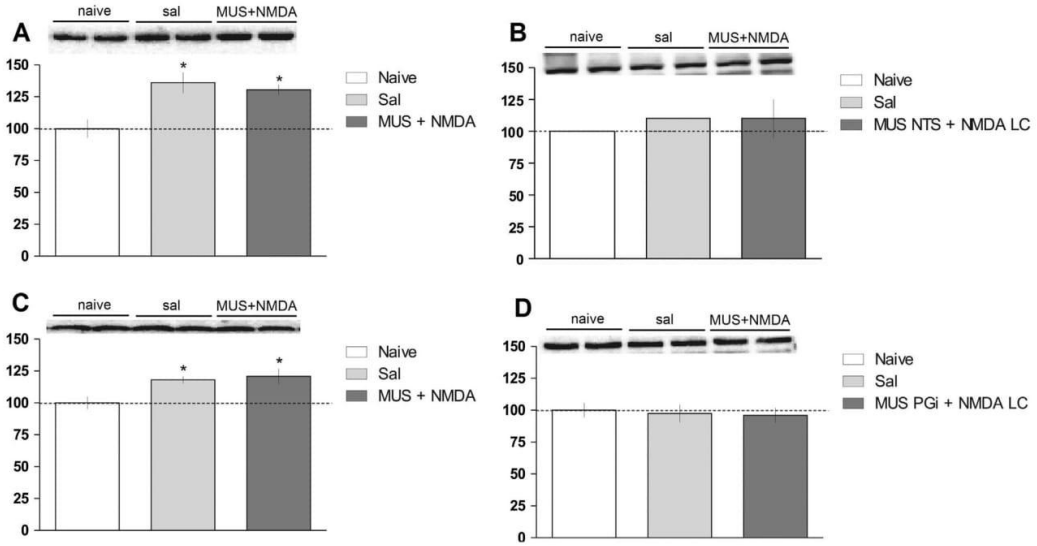

Fig. 7. Blockade of the BDNF increase in CA1 by infusion of muscimol in NTS (A) and PGi (C) are reversed by infusion of NMDA in LC. The infusion of muscimol in NTS (B) and PGi (D) followed by infusion of NMDA in LC did not alter tubulin protein levels 120 min after training. Cannulae implanted animals were either trained in the OR task or left untrained (naïve). Trained animals rats were exposed to two different objects for 5 min and immediately after that received bilateral infusions of saline (Sal) or muscimol (MUS) in NTS or PGi (0.01 μg/μl; 0.5 μl/side) + NMDA in LC (0.01 μg/μl; 0.25 μl/side). 120 min after that, the animals are killed by decapitation, the dorsal CA1 region of hippocampus dissected out and total homogenates submitted to SDS–PAGE followed by immunoblot analysis with antibodies against BDNF (A and C) or tubulin (B and D). Bars show the percentage levels respect to naive animals. Data are expressed as mean ± SD. $^*P \leqslant 0.05$ in Dunnett's comparison tests after ANOVA; $n = 5$ per group.

memory consolidation by itself, promoted memory persistence, since the rats that received NE could remember the original memory for at least 21 days, while the controls did not. Other authors also demonstrated that noradrenergic activation influences the consolidation of OR memory tasks. (Dornelles et al., 2007) used systemic injections of epinephrine which promoted memory improvement, an effect that could be blocked by prior propranolol administration. Roozendaal et al. (2008) provided evidences that the post training BLA norepinephrine administration promotes a dose dependent enhancement of OR memory, while propranolol administration promotes a dose dependent OR memory impairment. The authors concluded that a post training noradrenergic BLA activation can enhance a low-arousing experience, inducing LTM consolidation.

Taken together with those previous data, our results suggest that hippocampal NE is essential for OR memory consolidation and its release depends on activation of the NTS–PGi–LC pathway. Activation of this pathway is presumably related to the presentation of a novelty to the animal (the objects during the OR task training), which would promote an alert state (King & Williams, 2009) and stimulate peripheral arousal. The modulatory action of various peripheral hormones and drugs in memory consolidation is well known (Izquierdo et al., 2006; Izquierdo & Medina, 1997; McGaugh, 2000). The brainstem NTS receives peripheral information and various stimuli from the periphery, and thus, plays a vital role in transmitting these information to the limbic structures that participate in mnemonic processes (Clayton and Williams 2000a,b).

Miyashita and Williams (2004) observed an increase of NE in rat hippocampus after a peripheral injection of epinephrine; this increase did not occur when NTS was inhibited, what suggests that the inhibition of the NTS activity interrupts the transmission of information from the periphery to the hippocampus (Miyashita & Williams, 2004). The NTS projects to the PGi (Babstock & Harley, 1992) and LC (Takigawa & Mogenson, 1977), the largest noradrenergic input to hippocampus (Haring & Davis, 1985; Loughlin, Foote, & Grzanna, 1986; Loy, Koziell, & Moore, 1980). McGaugh and colleagues had studied the role of the NTS–PGi–LC pathway in the consolidation of emotional memories (McGaugh, 2000; Williams & McGaugh, 1993) and demonstrated that peripheral injection of epinephrine improves the retention of aversive memory. Here, the infusion of NE directly into the CA1 region of hippocampus immediately after training did not affect the consolidation of recognition memory when compared to control animals. It is difficult to observe memory enhancement in the OR task protocol used here, since memory is usually at the measurable maximum. Moreover, even if the exogenous injection of NE in the hippocampus after learning has not improved OR memory, tests up to 21 days after the training showed that the animals that received NE, unlike the controls, remain with the ability to discriminate the familiar and the new object, which is viewed as an increase of persistence. Since, as discussed by McGaugh (2000, 2015), enhanced persistence is usually the result of enhanced consolidation, it is probably legitimate to infer that when the former is seen the latter had taken place (McGaugh, 2000, 2015).

It is important to consider that different protocols to evaluate OR memory are currently used. The nature of the protocol certainly could influence the results observed and this could make it difficult to draw conclusions on the role of each structure and neurotransmitter system in OR. Whether or not OR memory consolidation requires the hippocampus, for example seems to depend on the version of the task used (see reviews: Ameen-Ali, Easton, and Eacott, 2015 and, Cohen and Stackman Jr., 2015). An interesting work form Balderas et al. (2015) differentiates the role of hippocampus in object and object-in-context memory tasks. Ameen-Ali et al. (2015) conclude that the contribution of hippocampus in OR memory is not so clear, but evidence suggests that the hippocampus is involved in integrating object information and spatial

and contextual information, which permits the formation of episodic memories.

Additionally, some details of task procedures that vary between different labs can influence results. The use of habituation, the use of identical or different objects on OR training session (Dornelles et al., 2007) and the duration of training session (Roozendaal et al., 2008) are factors that could influence the results obtained. In our case, using the standard 4 days of habituation and a 5 min training session with two different objects, we verify that OR memory consolidation requires hippocampal noradrenaline, and OR learning promotes increase of NE levels, which is blocked by NTS–PGi–LC pathway inactivation through muscimol injection on NTS. This results agree with the hypothesis that OR task promotes NTS–PGi–LC–CA1 activation, what culminates with NE release in hippocampus, essential to this memory consolidation.

There is evidence about the involvement of BDNF on memory consolidation and persistence (Bekinschtein et al., 2007; Bekinschtein et al., 2008; Romero-Granados et al., 2010; Slipczuk et al., 2009). In our third set of experiments we investigated the BDNF protein expression on hippocampus after OR learning and our results agree with Furini et al. (2010), demonstrating that 120 min after training in the task of OR there is an increase in BDNF expression in the CA1 region of the dorsal hippocampus. As previously demonstrated, the inactivation of NTS, PGi or LC blockers the learning in OR task (Mello-Carpes & Izquierdo, 2013); also, here we verified that the inactivation of any of this brainstem nucleus also avoids BNDF increase in hippocampus. The infusion of exogenous noradrenaline in hippocampus (CA1) after NTS, PGi or LC inhibition allowed the BDNF increase (and the OR learning, as showed by Mello-Carpes & Izquierdo, 2013). Additionally, proving the pathway organization, the NTS or the PGi inactivation combined with the subsequently activation of a downstream point of the pathway (by NMDA injection on LC) also allowed the BDNF increase.

Both β-noradrenergic mechanisms, probably hippocampal (Parfitt, Barbosa, Campos, Koth, & Barros, 2012), and BDNF-mediated hippocampal mechanisms (Bekinschtein et al., 2008) have been attributed a role in the persistence of fear-motivated learning in rats. The present data endorse both postulations, and extend them to OR, a non-aversive, more "cognitive" behavior. Further, they suggest that the effects on persistence are initiated at the time of consolidation, as most data on the role of the latter on persistence show (Furini, Myskiw, & Izquierdo, 2014; McGaugh, 2000, 2015). In addition, they point to an involvement of the NTS–PGi–LC–CA1 pathway proposed by McGaugh, Williams and their coworkers in aversive tasks (Clayton and Williams, 2000a,b; Miyashita & Williams, 2004; Williams & McGaugh, 1993) and more recently also by us in OR (Mello-Carpes & Izquierdo, 2013) to regulate memory consolidation by noradrenergic endings in the hippocampus and amygdala.

Acknowledgments

This work was supported by research grants and fellowship from CAPES/Brazil (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq/Brazil (Conselho Nacional de Pesquisa) and Federal University of Pampa/Brazil.

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Manuscript Details

Manuscript number	YNLME_2016_29
Title	ONE-SINGLE PHYSICAL EXERCISE SESSION AFTER OBJECT RECOGNITION LEARNING PROMOTES MEMORY PERSISTENCE THROUGH HIPPOCAMPAL NORADRENERGIC MECHANISMS
Article type	Research Paper

Abstract

Previously we showed the involvement of the hippocampal noradrenergic system in consolidation and persistence of object recognition (OR) memory. Here we show that one-single physical exercise session immediately after learning promotes OR memory persistence for at least 21 days and that in parallel it increases norepinephrine levels in hippocampus. Additionally, the effects of exercise on memory are avoided by an hippocampal beta-adrenergic antagonist infusion, what suggest that the exercise effect on memory could be related to noradrenergic mechanisms. Together, these results suggest that acute physical exercise can be adopted as a non-pharmacological intervention that assists in memory consolidation and persistence, with little or no side effects.

Keywords	Acute physical exercise; running; norepinephrine; hippocampus; memory persistence.
Taxonomy	Neurological Mechanisms of Learning, Hippocampus, Neurological Mechanisms of Memory
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Suggested reviewers	James McGaugh, Juliana Bonini, Benno Roozendaal

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Highlights:

Acute physical exercise promotes memory persistence.

Physical exercise effects on memory are avoided by timolol.

Physical exercise effects on memory are similar to norepinephrine effects.

Acute physical exercise increases norepinephrine levels in hippocampus.

ONE-SINGLE PHYSICAL EXERCISE SESSION AFTER OBJECT RECOGNITION LEARNING PROMOTES MEMORY PERSISTENCE THROUGH HIPPOCAMPAL NORADRENERGIC MECHANISMS

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Abstract: Previously we showed the involvement of the hippocampal noradrenergic system in consolidation and persistence of object recognition (OR) memory. Here we show that one-single physical exercise session immediately after learning promotes OR memory persistence for at least 21 days and that in parallel it increases norepinephrine levels in hippocampus. Additionally, the effects of exercise on memory are avoided by an hippocampal beta-adrenergic antagonist infusion, what suggest that the exercise effect on memory could be related to noradrenergic mechanisms. Together, these results suggest that acute physical exercise can be adopted as a non-pharmacological intervention that assists in memory consolidation and persistence, with little or no side effects.

Key-words: Acute physical exercise; running; norepinephrine; hippocampus; memory persistence.

1 Introduction

Recognition memory refers to the ability to discriminate between a familiar and a new characteristic (Squire, Wixted, and Clark, 2007) This type of memory requires that specific characteristics of a particular event are identified, discriminated and compared with the memory of characteristics previously experienced (Steckler, Drinkenburg, Sahgal, and Aggleton, 1998), providing adaptive advantage of previous experience in solving issues related to the survival.

The object recognition task (OR) has been widely used to evaluate the mechanisms involved in the formation of declarative memories (Moses, Cole, Driscoll, and Ryan, 2005; Reed, Squire, Patalano, Smith, and Jonides, 1999). Previously we showed that activation of the pathway Nucleus of the Solitary Tract – Nucleus Paragigantocellularis – *Locus coeruleus* (NTS–PGi–LC) is necessary for the consolidation of OR memory and its activation culminates with norepinephrine (NE) release in CA1 hippocampus region (Mello-Carpes and Izquierdo, 2013). Several studies have indicating an important role of the hippocampus in OR memory consolidation (Balderas, Rodriguez-Ortiz, and Bermudez-Rattoni, 2015; Clarke, Cammarota, Gruart, Izquierdo, and DelgadoGarcia, 2010; Cohen and Stackman, 2015; Furini, Rossato, Bitencourt, Medina, Izquierdo, and Cammarota, 2010; Myskiw, Rossato, Bevilaqua, Medina, Izquierdo, and Cammarota, 2008). Recently we demonstrated that the hippocampal CA1 injection of beta-adrenergic blocker timolol impairs OR memory consolidation, while the injection of NE promotes the persistence of this memory (Mello-Carpes, da Silva de Vargas, Gayer, Roehrs, and Izquierdo, 2016). In addition we also show that OR learning promotes the hippocampal increase of NE levels, indicating that hippocampal noradrenergic activation is necessary for OR memory consolidation and persistence (Mello-Carpes et al., 2016).

It is well established in the literature that the noradrenergic system plays a crucial role in modulating the ability of concentration and mnemonic performance in rats (Izquierdo and Medina, 1997; McGaugh, 1989; 2000; MelloCarpes et al., 2016). This was demonstrated in a variety of learning

paradigms, such as contextual fear conditioning, OR, and extinction of contextual fear conditioning (Berlau and McGaugh, 2006; LaLumiere, Buen, and McGaugh, 2003; Mello-Carpes et al., 2016; Roozendaal, Okuda, Van der Zee, and McGaugh, 2006). Evidence has shown that there is a strong relationship between training-induced endogenous noradrenergic activation and enhanced memory (McIntyre, Hatfield, and McGaugh, 2002; Segal and Cahill, 2009).

In parallel, studies have shown the effects of physical exercise on memory (Hotting and Roder, 2013; Mello, Benetti, Cammarota, and Izquierdo, 2008; Neves, Menezes, Souza, and Mello-Carpes, 2015). Although the effects of long-term aerobic exercise on memory and its benefits to the central nervous system (CNS), including neuroprotection, has been extensively described (Ahlskog, Geda, Graff-Radford, and Petersen, 2011; Baker, Frank, Foster-Schubert, Green, Wilkinson, McTiernan, Plymate, Fishel, Watson, Cholerton, Duncan, Mehta, and Craft, 2010; Neves et al., 2015; Schimidt, Vieira, Altermann, Martins, Sosa, Santos, Mello-Carpes, Izquierdo, and Carpes, 2014), there are fewer studies investigating the acute effects of physical exercise and their results are inconsistent so far (Chang, Labban, Gapin, and Etnier, 2012; Hotting, Schickert, Kaiser, Roder, and Schmidt-Kassow, 2016). Physical exercise is able to promote increase on NE levels (Chatterton, Vogelsong, Lu, Ellman, and Hudgens, 1996; Pagliari and Peyrin, 1995; Segal, Cotman, and Cahill, 2012), and even one-single physical exercise session could have this effect (Segal et al., 2012).

Thus, considering that the NE is an important neurotransmitter involved in the consolidation and persistence of recognition memory, as shown by findings recently published by our group (Mello-Carpes et al., 2016; Mello-Carpes and Izquierdo, 2013), and that physical exercise is a non-invasive behavioral practice that favors the release of NE (Meeusen and De Meirleir, 1995), we investigated here the effects of one-single physical exercise session after OR learning and demonstrated that physical exercise can promote OR memory persistence through hippocampal noradrenergic mechanisms.

2 Material and methods

2.1 Rats and groups

Adult male Wistar rats (3 months old) purchased from the vivarium of the Federal University of Santa Maria (RS/Brazil) were used. They were housed four per cage and maintained under controlled light and environmental conditions (12 h light/12 h dark cycle at a temperature of 23 ± 2 °C and humidity of 50 ± 10%) with free access to food and water. All experiments were conducted in accordance with the “Principles of laboratory animal care” (NIH publication n 80-23, revised 1996).

The rats were divided in six groups: (i) naïve (just for NE levels measurements); (ii) control, in which rats were just trained in the OR task; (iii) treadmill habituation, which rats were habituated to treadmill and trained in the OR task; (iv) physical exercise, in which rats were habituated to treadmill, trained in OR task and submitted to one-single physical exercise session in the treadmill for 30 minutes immediately after OR training; (v) physical exercise + timolol (TIM), which rats were habituated to treadmill, trained in OR task, submitted to one-single physical exercise session on treadmill and received a TIM intra-hippocampal infusion immediately after the physical exercise session; and (vi) Norepinephrine (NE), in which rats were trained in the OR task and received NE intra-hippocampal infusion immediately after. Rats from group (i) and some rats from groups (ii), (iii) and (iv) were euthanized for hippocampus dissection for posterior biochemical analyses. Figure 1 summarizes the experiments conducted.

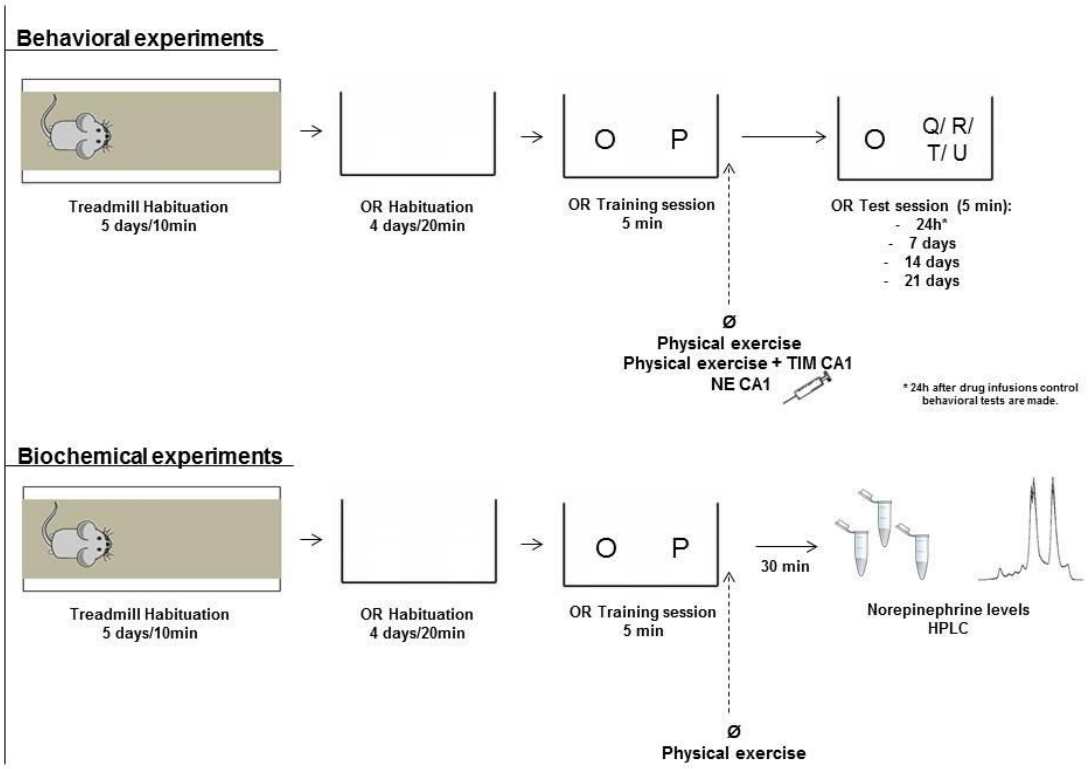

Fig. 1. Experimental design. Behavioral and biochemical experiments were conducted. **For behavioral experiments** rats were divided in five groups: control, treadmill habituation, physical exercise, physical exercise + timolol and norepinephrine. All animals were habituated and trained in OR test, and, according groups, they were submitted or not to: treadmill habituation; one-single physical exercise session, followed or not by intra-hippocampal timolol infusion; intra-hippocampal norepinephrine infusion, or nothing. OR test sessions were made 24h, 7, 14 and 21 days after training. **For biochemical experiments** rats were divided in four groups: naïve, control, treadmill habituation and physical exercise. These groups were submitted or not to: treadmill habituation; OR habituation and training; one-single physical exercise session after OR training. 30 minutes after OR training the rats were euthanized and their hippocampus were dissected and quickly prepared to norepinephrine levels measurement by HPLC.

2.2 Surgery and drug infusion procedures

In order to implant brain cannulas, the rats that were used in the behavioral experiments were deeply anesthetized with ketamine and xylazine (i.p., 75 mg/kg and 10 mg/kg, respectively) and 27-gauge cannulas were placed, stereotaxically aimed at CA1 region of the dorsal hippocampus (A – 4.2, L 3.0, V – 2.0 mm) (coordinates according to (Paxinos and Watson, 1986). The cannulae were affixed with dental cement. Animals were allowed to recover from surgery for 4 days before submitting them to any other procedure.

At the time of drug delivery, 30-gauge infusion cannulas were tightly fitted into the guides. Infusions (1 µl/side in CA1 region of hippocampus; 1µg/µl of TIM or NE dissolved in saline for groups (v) and (vi), respectively; groups (ii), (iii) and (iv) received just saline infusion) were carried out over 60 s with an infusion pump, and the cannulas were left in place for 60 additional seconds to minimize backflow. The doses and volume used were based on pilot experiments and on previous studies showing the effect of each compound on learning and behavioral performance (Mello-Carpes et al., 2016; Mello-Carpes and Izquierdo, 2013). Cannula placements were verified postmortem: 2–4 h after the last behavioral test: A 4% methylene-blue solution was infused at the same volume used in the experiments as described earlier, and the extension of the dye 30 min thereafter was taken as an indication of the presumable diffusion of the vehicle or drug previously given to each animal (Mello-Carpes and Izquierdo, 2013).

2.3 Reagents and drugs

Timolol (TIM) and NE were purchased from Sigma–Aldrich (St. Louis, MO). The drugs were dissolved in saline and stored at 20°C, protected from light until use, at which time an aliquot was thawed and diluted to working concentration in saline 0.9% (pH 7.2). Other reagents used in the experiments were of analytical grade and obtained from standard commercial suppliers.

2.4 Physical exercise protocol

One-single physical exercise session was performed immediately after OR training in a motorized treadmill built for rodents (Insight Ltda, Sao Paulo, Brazil). For this, the rats was previously adapted to the treadmill in order to avoid novelty and/or stress effects. The protocol used was an adaptation made from the protocol proposed by (Malek, Huttemann, Lee, and Coburn, 2013), in which the running exercise was performed at an intensity of 60–70% maximal oxygen uptake (VO_2) (treadmill belt velocity between 9 m/min and 13 m/min) in an one-single physical exercise session with 30 min of duration, realized

immediately after OR learning. For adaptation to the treadmill, one week before the exercise session the rats were habituated to it for two days (2 a 5 m/min for 10 minutes) and then were subjected to the “good runner protocol”, which consists in placing the animals on the treadmill (8 m/min for 10 min) without tilting and there evaluate the level of trainability in a range from 1 to 5 points for three consecutive days; at the end the animals that maintained an average of three or more points were included in the exercise group (Arida, Scorza, Gomes da Silva, Cysneiros, and Cavalheiro, 2011). In the next and last day, an indirect VO_2 running test was performed to determine the individual intensity of exercise (starting with low velocity and increasing it in 5 m/min every 3 min until the rat was unable to keep running). Time to fatigue (min) and the work volume (m/min) were considered as an indirect measure of VO_2 maximum (Brooks and White, 1978; Cechetti, Worm, Elsner, Bertoldi, Sanches, Ben, Siqueira, and Netto, 2012). Here, we denominated the two days of habituation in the treadmill plus the three days of the good runner protocol plus the VO_2 test day as "treadmill habituation", and one group (iii) was submitted to these habituation period without the physical exercise session to isolate the possible effects of these habituation *per se* (see fig. 1).

2.5 Behavioral experiments

2.5.1 Object Recognition task

Training and testing in the object recognition (OR) task were carried out in an arena (50 x 50 x 50 cm) built of polyvinyl chloride plastic, plywood and transparent acrylic as described by (Ennaceur and Delacour, 1988). First, the animals were habituated to the OR apparatus by placing them in it for 20 min per day to freely explore it during 4 consecutive days before the training. On training day two different objects (O and P) were placed in the apparatus and the animals were allowed to explore them freely for 5 min. The objects were made of metal, glass, or glazed ceramic. Exploration was defined as sniffing or touching the objects with the nose and/or forepaws. Sitting on or turning around the objects was not considered exploratory behavior. 24 h, 7, 14 or 21 days

later, on test phase, one of the objects was randomly exchanged for a novel object (named Q, R, S and T, respectively) and rats were reintroduced into the apparatus for an additional 5 min period of free exploration (see figure 1 – behavioral experiments). To avoid confounds by lingering olfactory stimuli and preferences, the object and the arena were cleaned after testing each animal with 70% ethanol.

The effect of the intra-hippocampal infusions and/or of the exercise were studied by posttraining administration, following the classic studies of (McGaugh, 2000) showing that treatment given at that time are more likely to affect consolidation and, possibly, persistence (see references in (Izquierdo, Furini, and Myskiw, 2016).

2.5.2 Open field and plus maze

To analyze exploratory and locomotor activities and to ensure that the drugs infusion or others procedures proposed in the study design did not impair such behaviors, altering the results of the memory tests, 24 h after saline, TIM or NE infusion, rats were placed on the left quadrant of a 50 x 50 x 39 cm open field (OF) made with wood painted white, with a frontal glass wall. Black lines were drawn on the floor to divide it into 12 equal quadrants. Crossing and rearing, as measures for locomotor and exploratory activities respectively, were measured over 5 min (Bonini, Bevilaqua, Zinn, Kerr, Medina, Izquierdo, and Cammarota, 2006). To evaluate the animals' anxiety state, 24 h after infusions rats were exposed to an elevated plus maze (PM) as described by (Pellow, Chopin, File, and Briley, 1985).

The total number of entries into the four arms, the number of entries and the time spent into the open arms were recorded over a 5 min session.

2.6 Norepinephrine levels measurement

The determination of hippocampal NE levels by HPLC (High Performance Liquid Chromatography) was measured NE in homogenates prepared from the hippocampi using a reverse-phase HPLC system (YL9100, Young Lin). Rats' brains were removed and bilateral hippocampus were quickly dissected out in an iced surface and homogenized in 50 mM Tris HCl, pH 7.4 (1/10, w/v). Afterwards, samples were centrifuged at 2400g for 20 min, and supernatants were filtered and then stored at 80°C until use (Menezes, Alves, Borges, Roehrs, de Carvalho Myskiw, Furini, Izquierdo, and Mello-Carpes, 2015; Nirogi, Abraham, Jayarajan, Medapati, Shanmuganathan, Kandikere, Irappanavar, Saralaya, Benade, Bhyrapuneni, and Muddana, 2012). The HPLC system consisted of a Vacuum Degasser (YL9101) and quaternary pump (YL9110) connected to a reversed phase column (SYNERGI 4l FUSION-RP 80 Å 250 x 4.60 mm; Phenomenex) on a Column Compartment (YL9131) coupled to a Diode Array Detector (YL9160). The mobile phase consisted of methanol and water (12/88, v/v) adjusted to pH 3 with phosphoric acid. To separate NE, we used the programming isocratic with a flow rate of 0.8 mL/min. The sample was filtered through 0.22 µm syringe filters. We injected 20 µL samples into the HPLC system by an auto sampler device (YL9150). The detection was at 198 nm by DAD. Chromatograms were recorded and integrated by PC integration software (YLClarity). All analyses were run in triplicate. The analytical parameters were as follows: linear range, 0.1–10.0 µg/ml; determination coefficient, 0.999; and calibration equation, y = 628.12x - 34.342. Norepinephrine for HPLC was supplied by Sigma-Aldrich Brazil. Other reagents used in this experiment were of analytical grades and obtained from standard commercial suppliers.

2.7 Statistical Analyzes

Object exploration time in the OR task was converted to percent of total exploration time and so a one-sample t-test was used to compare the percent of total time exploration spent in each object with a theoretical mean (50%).

Additionally, the discrimination index (DI) on 24h and 21 days tests were calculated taking into account the difference of time spent exploring the new (T novel) and the familiar (T familiar) objects: DI = ([(T novel – T familiar)/(T novel + T familiar)] x 100 (%)), and was used as a memory parameter (Flores, Martins, Schimidt, Santos, Izquierdo, Mello-Carpes, and Carpes, 2014); the DI data were analyzed using one-way ANOVA followed by t-tests. The OF and PM results were analyzed using one-way ANOVA. The HPLC results were compared using one-way ANOVA followed by Tukey's multiple comparison test. All data were expressed as mean ± SD. The sample size (n, number of animals in each group) for each experiment is stated in the figure legends. The differences were considered statistically significant at $P \leq 0.05$.

3 Results

3.1 Object recognition memory task

We evaluated the persistence of memory in the OR task testing rats from different groups 24h, 7, 14 or 21 days after training (fig. 2A). In the OR training, the rats from all groups explored the two new objects for a similar percent of total exploration time (O and P; Fig. 2B-F, training, $P > 0.05$). The animals from the control group, which were not exposed to any additional procedure besides the OR task, explored significantly more than 50% of total exploration time the new object (Q) in the 24h test ($P = 0.003$), but not in subsequent testing days (Fig. 2B, $P > 0.05$).

The rats who were habituated to the treadmill were able to remember the familiar object up to 14 days after training and spent significantly more than 50% of total exploration time exploring the novel object until this day (Fig. 2C, $P < 0.05$), but were unable to recognize the new object on the 21 day test (Fig. 2C, $P > 0.05$); so, they did show good consolidation but a limited persistence. On the other hand, the rats that were exposed to one-single physical exercise session immediately after OR training were able to remember the familiar object up to 21 days after training, spent significantly more than 50% of total exploration time exploring the novel object in the different days of tests (Fig. 2D,

P ≤ 0.05), and so they showed both good consolidation and persistence of object recognition memory. However, the animals that received intra-hippocampal timolol (TIM) infusion after physical exercise were not able to remember the familiar object, and spent about the same time exploring each object in all tests and sessions (Fig. 2E, P > 0.05). The norepinephrine (NE) infusion, in turn, promoted an effect similar to that of one-single physical exercise session, i.e., it increased memory persistence (Fig. 2F, P < 0.05).

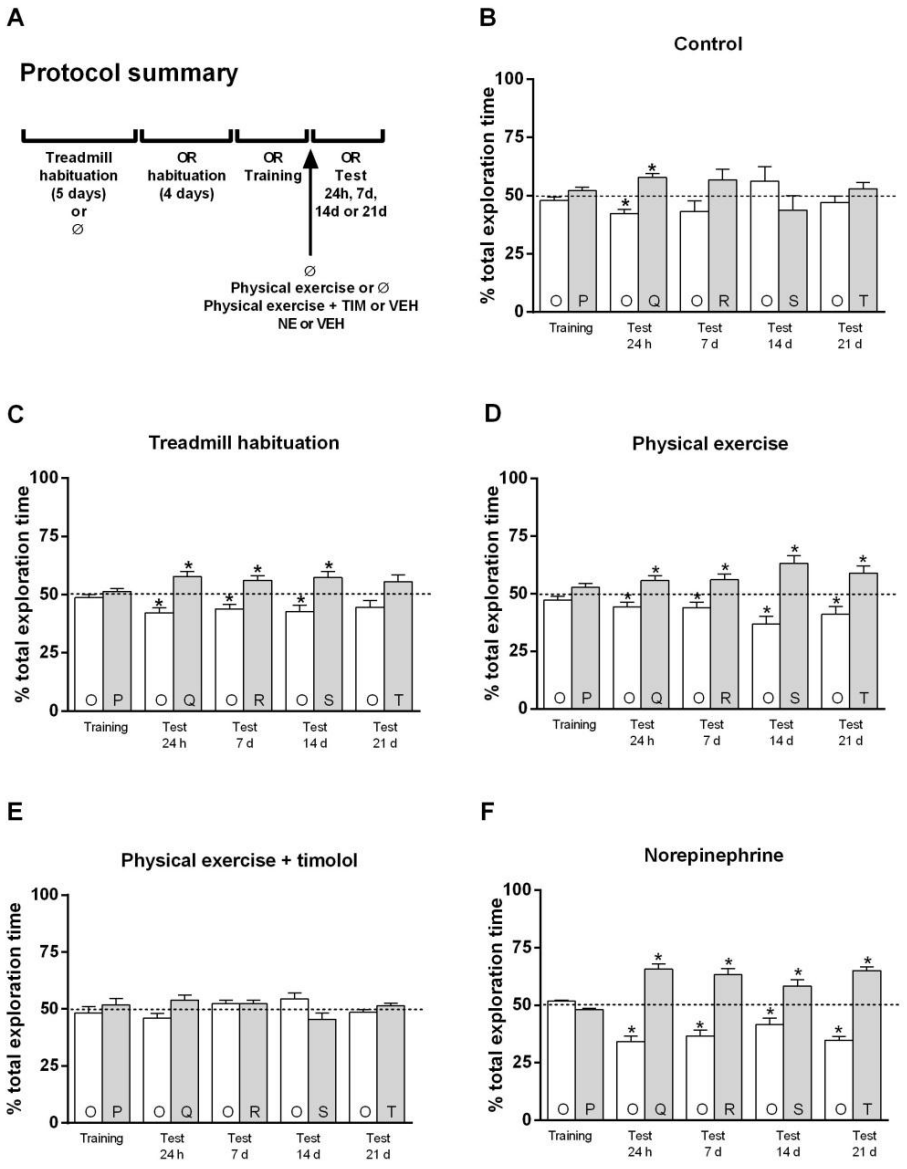

Fig. 2. One-single physical exercise session after learning promotes OR memory persistence; TIM infusion blocks this effect and NE infusion has an effect similar to that of exercise. A. Summary of the experiments conducted. Some rats were habituated to treadmill before the OR task. On OR training session all rats were exposed to two different

objects (O and P) for 5 min. According the group, immediately after training the rats were submitted to one-single physical exercise session on treadmill for 30 minutes (D) or were submitted to one-single physical exercise session on treadmill for 30 minutes followed by bilateral intra-hippocampal infusions (1µl/side in CA1) of timolol (1µg/µl) (E) or immediately after training and received just an intra-hippocampal infusion (1µl/side in CA1) of norepinephrine (NE; 1µg/µl). 24 h, 7, 14 or 21 days later, on test phase, one of the objects was randomly exchanged for a novel object (named Q, R, S and T, respectively) and rats were reintroduced into the apparatus for an additional 5 min period of free exploration. Control animals are able to recognize the objects until 24h after training (B). Animals habituated to treadmill present memory until 14 days test (C). The physical exercise immediately after training promotes persistence of OR memory for at least l 21 days (D). The infusion of TIM in CA1 region of dorsal hippocampus immediately after physical exercise avoids this effect (E). The infusion of NE in CA1 region of dorsal hippocampus immediately after training promotes persistence of OR memory until 21 days (F). Data (mean ± SD) are presented as percentage of total exploration time; *P ≤ 0.05 in one-sample Student's t-test with theoretical mean = 50%; n = 10 per group/test.

Considering the 24h OR discrimination index (DI), it is possible to verify that all groups, except that one that received TIM, were able to consolidate the OR memory (Fig 3A, $P < 0.05$), and that the group that received NE had a higher DI than the others (Fig 3A, $P < 0.05$). On day 21, the group that received TIM has a lower DI index, which is not different from that of the control group (the memory trace naturally decays). On the other hand, one-single session of physical exercise and NE improve DI in comparison to the control group (Fig 3B, $P < 0.01$).

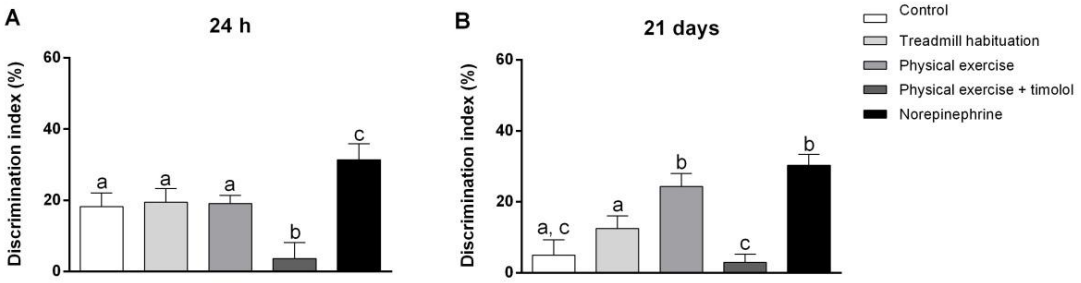

Fig. 3. Timolol (TIM) intra-hippocampal infusion after OR learning disrupts memory consolidation. One-single physical exercise session and intra-hippocampal norepinephrine (NE) infusion after OR learning promotes memory persistence. Discrimination index for novel object in the 24h (A) and 21 days (B) tests. Groups (bars) with different letters were significantly different ($P < 0.01$ on ANOVA followed by t-tests). Data is presented as mean ± SD; n = 10 per group/test.

It is important highlight that in all days of OR task there were no significant differences between groups in the total exploration time (table 1).

3.2 Control experiments

Rats were exposed to OF and PM tests 24h after the drug infusions to verify exploratory and locomotor activity and anxiety, respectively. The intra-hippocampal infusion of TIM or NE (1.0 µl/side; 1 µg/µl) did not affect the number of crossings and rearing during the 5 min long free exploration session at the open field (Table 1 – Open Field). Similarly, no effects in the total number of entries or in time spent at open arms during the plus maze session were found (Table 1 – Plus maze).

Table 1. Infusion of timolol, norepinephrine or saline and the others procedures proposed in the study design have no effect on total exploration time in training and tests on OR task, on anxiety in a plus maze, and on locomotor and exploratory activities in an open field ($P > 0.05$; one-way ANOVA). Data are expressed as mean ± SD of the total exploration time, in seconds, on OR training and tests (OR; n = 10 per group/day), the total time spent in the open arms (plus maze; n = 10 per group), and the number of crossings and rearings (open field; n = 10 per group).

	Control	Treadmill habituation	Physical exercise	Physical exercise + timolol	Norepinephrine
OR total exploration time (s)					
Training	73.3 ± 23.16	51.1 ± 19.33	54.6 ± 17.19	73.3 ± 23.16	68.8 ± 19.69
Test (24h)	46.4 ± 9.52	71.4 ± 20.58	55.7 ± 21.54	51.6 ± 9.22	48.0 ± 20.43
Test (7d)	41.3 ± 12.45	39.2 ± 16.82	43.8 ± 17.28	41.1 ± 9.48	41.7 ± 12.93
Test (14d)	48.4 ± 20.47	44.9 ± 24.61	46.9 ± 22.61	37.6 ± 10.74	34.6 ± 12.20
Test (21d)	70.8 ± 21.59	58.3 ± 16.33	62 ± 24.33	54.3 ± 12.53	49.3 ± 15.81
Plus maze					
Time in open arms (s)	86.3 ± 38.7	86.1 ± 7.8	112.3 ± 24.1	100.4 ± 16,1	103.7 ± 15.7
Open field					
Crossings	59.3 ± 21.0	73.6 ± 24.6	85.2 ± 18.5	75.4 ± 15.8	88.0 ± 16.5
Rearings	23.4 ± 10.4	28.6 ± 6.8	38.2 ± 13.6	31.8 ± 12.7	40.1 ± 17.1

3.3 Norepinephrine levels

To test our hypothesis that a single physical exercise session could be promoting the persistence of memory through the increased release of NE on hippocampus, sixteen animals were divided in four groups: (i) naïve (n = 4); (ii) control, which rats were just trained in OR task (n = 4); (iii) treadmill habituation, which rats were habituated to treadmill and trained in OR task (n = 4); and, (iv) physical exercise, which rats were habituated to treadmill, trained in OR task and submitted to one-single physical exercise session on treadmill for 30 minutes immediately after OR training (n = 4). Rats from group (ii), (iii) and (iv) were euthanized for hippocampus dissection 30 min after OR training. The CA1 regions of both dorsal hippocampi were homogenized and processed for HPLC determination of NE levels. All the rats trained on OR presented a higher hippocampal level of NE than naïve ones (Fig. 4, $P < 0.01$). Additionally, the rats submitted to the one-single physical exercise session immediately after OR training presented an increase of hippocampal NE levels when compared to other groups (Fig. 4, $P < 0.05$).

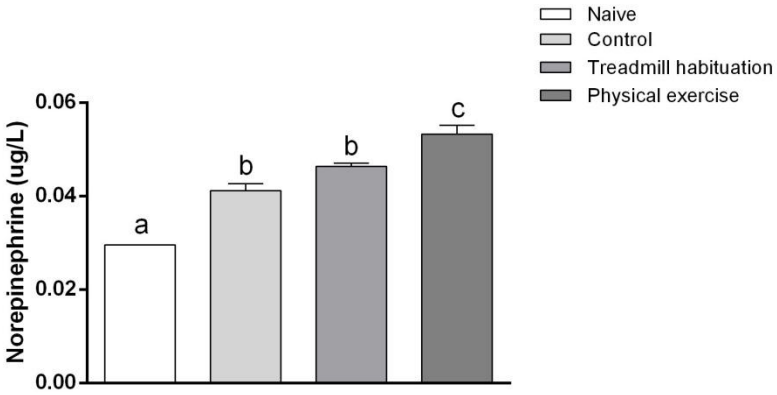

Fig. 4. Object recognition learning increases norepinephrine (NE) levels in the hippocampus. Physical exercise promotes an additional increase of NE levels. Groups (bars) with different letters were significantly different in their tissue content of NE in the hippocampus homogenate (µg/L) ($P < 0.01$ on ANOVA followed by Tukey's multiple comparison test). Data presented as mean ± SD; n = 4 per group/test.

4 Discussion

Our results show that a single session of physical exercise after learning promotes OR memory persistence through hippocampal noradrenergic mechanisms. We have previously demonstrated that activation of the NTS–PGi–LC pathway is necessary for the consolidation of OR memory and this activation culminates by a norepinephrine (NE) release in the hippocampus (Mello-Carpes and Izquierdo, 2013). While intrahippocampal infusion of the β-blocker timolol) after OR training impairs memory consolidation, that of NE just after OR learning promoted memory persistence for at least 21 days (MelloCarpes et al., 2016). These results indicate that there is an involvement of hippocampal noradrenergic system in consolidation of OR memory and suggest that NE could promote OR memory persistence.

Physical exercise, in turn, is known to increase brain NE levels (Hamilton, Fogle, and Meston, 2008; Kitaoka, Fujikawa, Miyaki, Matsumura, Fushiki, and Inoue, 2010; Meeusen and De Meirleir, 1995; Pagliari and Peyrin, 1995). Experiments made with microdialysis show that treadmill running promotes increase in NE release in the rodents' brain, and this effect is sustained through the duration of running (Pagliari and Peyrin, 1995). Additionally, (Kitaoka et al., 2010) show that rats that were subjected to running at 15 m/min (incline 3º) for 60 min increased significantly the noradrenergic and dopaminergic activities in the ventromedial hypothalamus. In human studies, physical exercise significantly enhances salivary alpha-amylase (sAA) levels, a biomarker for NE (Chatterton et al., 1996; Hamilton et al., 2008).

Considering that the pharmacological manipulation of the noradrenergic system could be potentially dangerous in the population, specially the elderly, because of the drugs' side effects, the use of physical exercise could be a good alternative to mobilize noradrenergic system. Reports about chronic physical exercise effects on learning and memory can be easily found in the literature (Hotting and Roder, 2013; Neves et al., 2015), but it is not always simple to promote people's engagement in the regular practice of physical exercise. In this sense, the effects of a single session of physical exercise on memory is

important, might represent an alternative. There are few studies on the acute effect of exercise effects on memory. (Segal et al., 2012) shows that postlearning acute aerobic exercise (6 minutes at 70% VO2 max on a stationary bicycle) enhanced memory both in patients with mild cognitive impairment (aMCI) and in controls, with a concomitant elevation of endogenous NE in both groups (indirectly measured via a biomarker, salivary alpha-amylase). Our present findings support those data, with direct brain measurements.

We show that a single session of treadmill running in rats immediately after OR training enhances the persistence of this memory for at least 21 days. The involvement of the hippocampal NE system in that effect is clear, since the exercise's effects disappeared when hippocampal beta-adrenergic receptors were blocked by timolol infusion. Additionally, direct measurements demonstrated that hippocampal NE levels increase after a physical exercise session. Although the OR learning promotes NE increase *per se*, this increase is higher if the rat is submitted to a single physical exercise session. It is important to highlight that we have controlled for some intervenient factors, as the fact that stress caused by treadmill running and/or its novelty by the simple expedient of habituating the rats to the treadmill; although this control procedure did by itself cause a small memory enhancement.

The results of this study provide new evidence that complement our previous findings about the role of hippocampal NE in OR memory consolidation and persistence (Mello-Carpes et al., 2016; Mello-Carpes and Izquierdo, 2013), and show the acute exercise, when performed immediately after memory acquisition, contributes to activation of NE system, increasing NE levels in the hippocampus, and promoting memory persistence. Therefore, we suggest that single sessions of physical exercise can be adopted as a non-pharmacological intervention that assists in memory consolidation and persistence with little or no side effects.

5 Acknowledge

This work was supported by research grants and fellowship from CAPES/Brazil (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) grated to II and PBMC, CNPq/Brazil (Conselho Nacional de Pesquisa) granted to PBMC, and Federal University of Pampa/Brazil granted to PBMC. LSV was supported by CAPES/Brazil. BSN was supported by FAPERGS. II was supported by CNPq.

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FIGURES

Figure 1

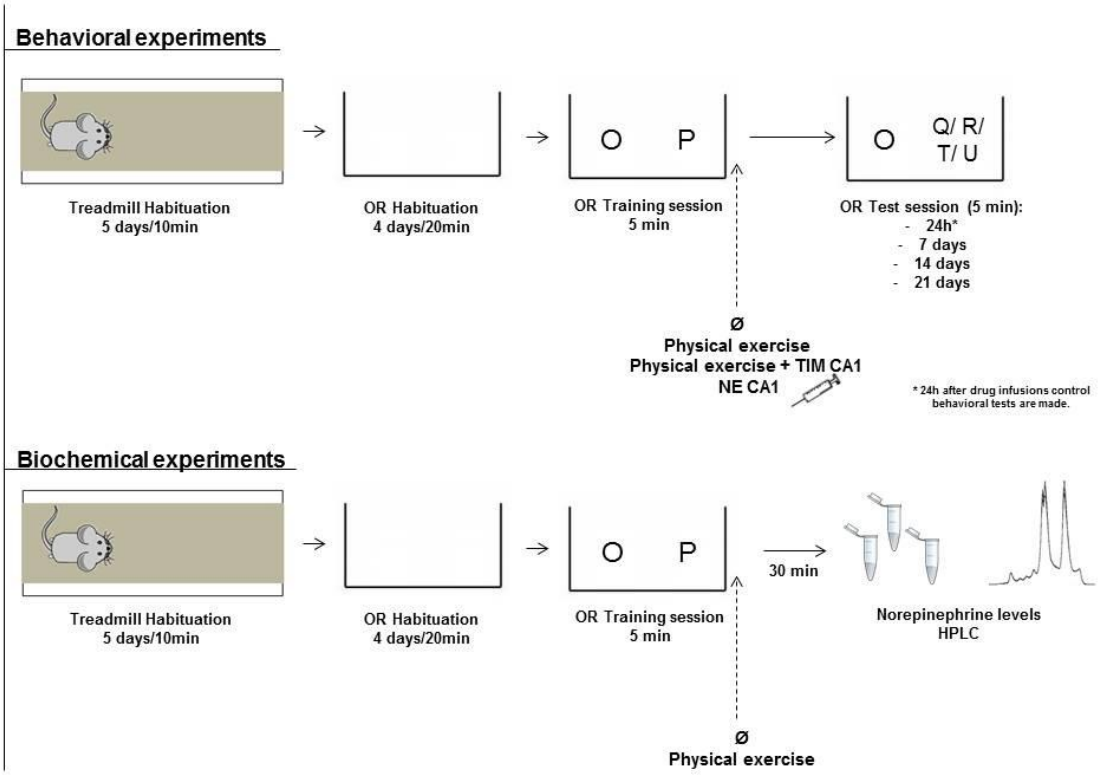

Figure 2

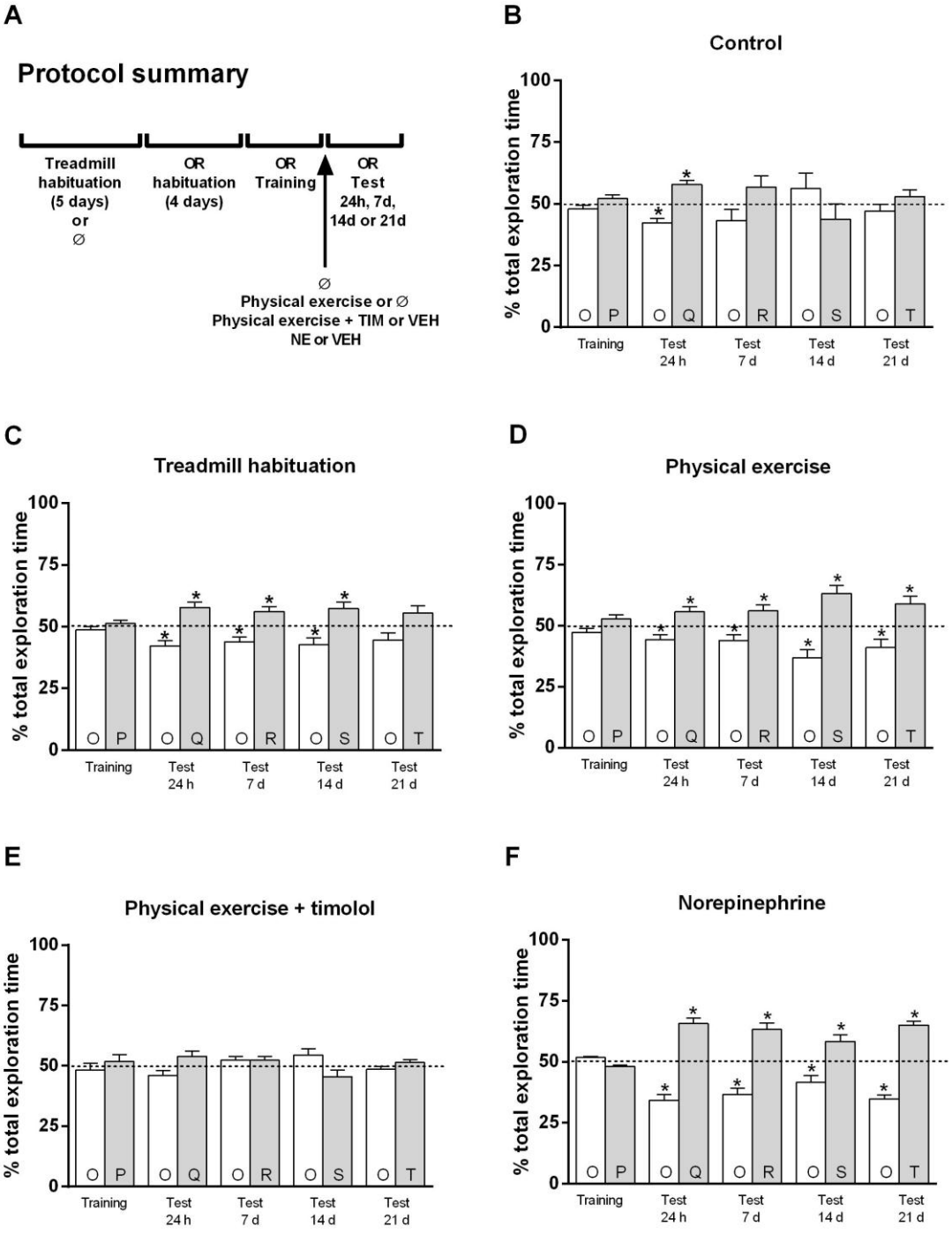
A
Protocol summary
Treadmill habituation (5 days) or ∅
OR habituation (4 days)
OR Training
OR Test 24h, 7d, 14d or 21d
∅
Physical exercise or ∅
Physical exercise + TIM or VEH
NE or VEH
B
Control
C
Treadmill habituation
D
Physical exercise
E
Physical exercise + timolol
F
Norepinephrine
% total exploration time
0
25
50
75
100
O P
O Q
O R
O S
O T
Training
Test 24 h
Test 7 d
Test 14 d
Test 21 d

Figure 3

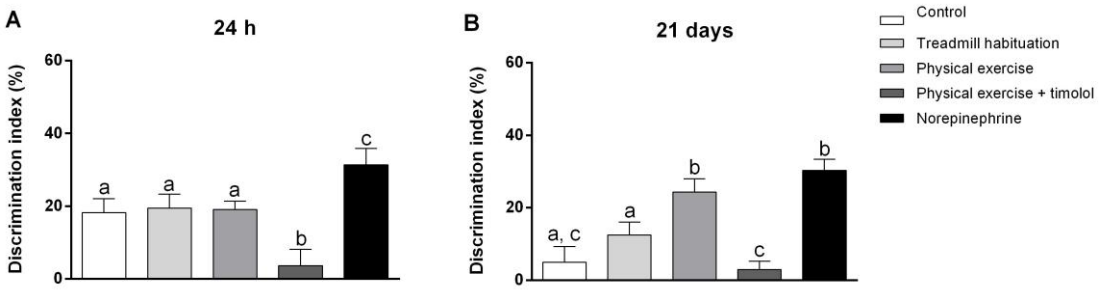
A
24 h
Discrimination index (%)
60
40
20
0
a
a
a
b
c
B
21 days
Discrimination index (%)
60
40
20
0
a, c
a
b
c
b
Control
Treadmill habituation
Physical exercise
Physical exercise + timolol
Norepinephrine

Figure 4

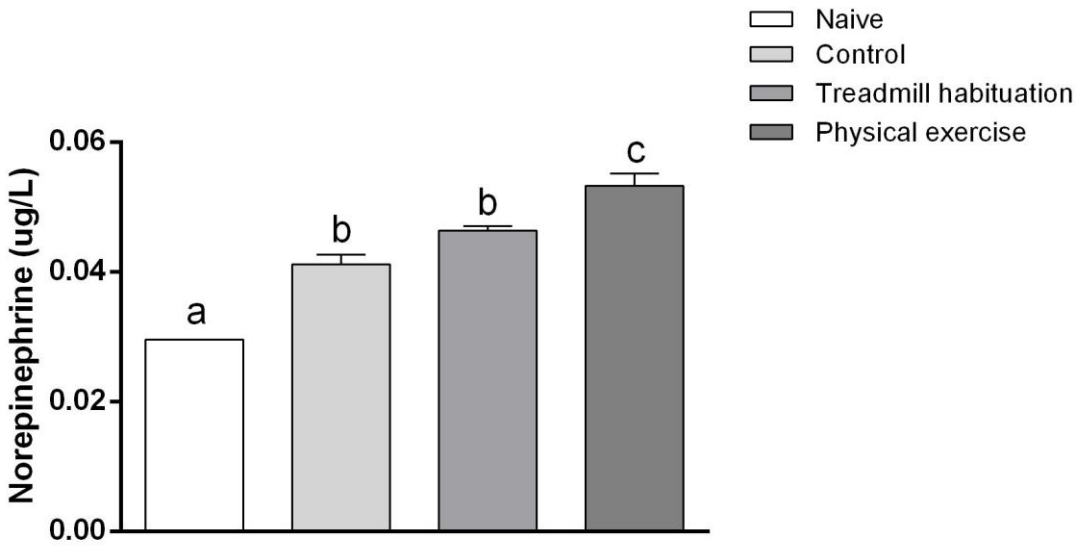
Naive
Control
Treadmill habituation
Physical exercise
Norepinephrine (ug/L)
0.06
0.04
0.02
0.00
a
b
b
c

FIGURE LEGENDS

Fig. 1. Experimental design. Behavioral and biochemical experiments were conducted. **For behavioral experiments** rats were divided in five groups: control, treadmill habituation, physical exercise, physical exercise + timolol and norepinephrine. All animals were habituated and trained in OR test, and, according groups, they were submitted or not to: treadmill habituation; one-single physical exercise session, followed or not by intra-hippocampal timolol infusion; intra-hippocampal norepinephrine infusion, or nothing. OR test sessions were made 24h, 7, 14 and 21 days after training. **For biochemical experiments** rats were divided in four groups: naïve, control, treadmill habituation and physical exercise. These groups were submitted or not to: treadmill habituation; OR habituation and training; one-single physical exercise session after OR training. 30 minutes after OR training the rats were euthanized and their hippocampus were dissected and quickly prepared to norepinephrine levels measurement by HPLC.

Fig. 2. One-single physical exercise session after learning promotes OR memory persistence; TIM infusion blocks this effect and NE infusion has an effect similar to that of exercise. A. Summary of the experiments conducted. Some rats were habituated to treadmill before the OR task. On OR training session all rats were exposed to two different objects (O and P) for 5 min. According the group, immediately after training the rats were submitted to one-single physical exercise session on treadmill for 30 minutes (D) or were submitted to one-single physical exercise session on treadmill for 30 minutes followed by bilateral intra-hippocampal infusions (1µl/side in CA1) of timolol (1µg/µl) (E) or immediately after training and received just an intra-hippocampal infusion (1µl/side in CA1) of norepinephrine (NE; 1µg/µl). 24 h, 7, 14 or 21 days later, on test phase, one of the objects was randomly exchanged for a novel object (named Q, R, S and T, respectively) and rats were reintroduced into the apparatus for an additional 5 min period of free exploration. Control animals are

able to recognize the objects until 24h after training (B). Animals habituated to treadmill present memory until 14 days test (C). The physical exercise immediately after training promotes persistence of OR memory for at least l 21 days (D). The infusion of TIM in CA1 region of dorsal hippocampus immediately after physical exercise avoids this effect (E). The infusion of NE in CA1 region of dorsal hippocampus immediately after training promotes persistence of OR memory until 21 days (F). Data (mean ± SD) are presented as percentage of total exploration time; *P ≤ 0.05 in one-sample Student's t-test with theoretical mean = 50%; n = 10 per group/test.

Fig. 3. Timolol (TIM) intra-hippocampal infusion after OR learning disrupts memory consolidation. One-single physical exercise session and intra-hippocampal norepinephrine (NE) infusion after OR learning promotes memory persistence. Discrimination index for novel object in the 24h (A) and 21 days (B) tests. Groups (bars) with different letters were significantly different (P < 0.01 on ANOVA followed by t-tests). Data is presented as mean ± SD; n = 10 per group/test.

Fig. 4. Object recognition learning increases norepinephrine (NE) levels in the hippocampus. Physical exercise promotes an additional increase of NE levels. Groups (bars) with different letters were significantly different in their tissue content of NE in the hippocampus homogenate (µg/L) (P < 0.01 on ANOVA followed by Tukey's multiple comparison test). Data presented as mean ± SD; n = 4 per group/test.

TABLES

	Control	Treadmill habituation	Physical exercise	Physical exercise + timolol	Norepinephrine
Plus maze					
Time in open arms (s)	86.3 ± 38.7	86.1 ± 7.8	112.3 ± 24.1	100.4 ± 16,1	103.7 ± 15.7
Open field					
Crossings	59.3 ± 21.0	73.6 ± 24.6	85.2 ± 18.5	75.4 ± 15.8	88.0 ± 16.5
Rearings	23.4 ± 10.4	28.6 ± 6.8	38.2 ± 13.6	31.8 ± 12.7	40.1 ± 17.1
Total exploration time (s)					
Training	73.3 ± 23.16	51.1 ± 19.33	54.6 ± 17.19	73.3 ± 23.16	68.8 ± 19.69
Test (24h)	46.4 ± 9.52	71.4 ± 20.58	55.7 ± 21.54	51.6 ± 9.22	48 ± 20.43
Test (7d)	41.3 ± 12.45	39,2 ± 16.82	43.8 ± 17.28	41.1 ± 9.48	41.7 ± 12.93
Test (14d)	48.4 ± 20.47	54.9 ± 24.61	46.9 ± 22.61	37.6 ± 10.74	34.6 ± 12.20
Test (21d)	70.8 ± 21.59	58.3 ± 16.33	62 ± 24.33	54.3 ± 12.53	39.3 ± 15.81

Table 1. Infusion of timolol, norepinephrine or saline and the others procedures proposed in the study design, have no effect on anxiety in a plus maze, on locomotor and exploratory activities in an open field ($P > 0.05$; one-way ANOVA) and does not affect exploration in the OR task.. Data are expressed as mean ±SD of the total number of entries and the time spent in the open arms (plus maze; n = 10 per group), the number of crossings and rearings (open field; n = 10 per group).

Manuscript Details

Manuscript number	BBR_2016_74
Title	THE EMERGING ROLE OF METHYLPREDNISOLONE AS CONGNITIVE ENHANCER IN RATS: INVOLVEMENT OF CALCIUM SIGNALING
Article type	Research Paper

Abstract

Acute and chronic stress influences cognitive performance in opposite directions through the release of glucocorticoids. Corticosteroids bind, with different affinities, to mineralocorticoid and glucocorticoid receptors expressed throughout the brain, namely in the hippocampus, amygdala and prefrontal cortex. Understanding the mechanisms underlying glucocorticoid effects in memory is paramount to design new strategies for the treatment of cognitive impairment induced by stress. Here, we demonstrate that a low daily dose of methylprednisolone (MP, 5 mg /kg, i.p.) for 10-days favors aversive memory persistence in adult rats, without any effect on the exploring behavior, locomotor activity, anxiety levels and pain perception. Enhanced performance on the inhibitory avoidance task was correlated with long-term potentiation (LTP), a phenomenon that was significantly strengthen in hippocampal slices of rats injected with MP (5 mg/kg) during 10 days. Additionally, in vitro incubation with MP (30-300 μM) concentration-dependently increased intracellular [Ca2+]i in cultured hippocampal neurons depolarized by KCl (35 mM). In conclusion, a low daily dose of MP for 10 days promotes aversive memory persistence in rats. The mechanism(s) underlying MP-induced enhancement of hippocampal performance may involve calcium signals and LTP induction in the hippocampus of adult rats.

Keywords	Inhibitory avoidance; Aversive memory; Glucocorticoids; Long-term potentiation.

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Legends - figures.docx [Figure]

Table and legends.docx [Table]

Highlights.docx [Highlights]

Highlights

- Treatment with low dose of methylprednisolone promotes memory persistence;
- A low daily dose of MP for 10 days increases LTP in the hippocampus of adult rats;
- The methylprednisolone actions should involve calcium signaling.

THE EMERGING ROLE OF METHYLPREDNISOLONE AS CONGNITIVE ENHANCER IN RATS: INVOLVEMENT OF CALCIUM SIGNALING

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Abstract

Acute and chronic stress influences cognitive performance in opposite directions through the release of glucocorticoids. Corticosteroids bind, with different affinities, to mineralocorticoid and glucocorticoid receptors expressed throughout the brain, namely in the hippocampus, amygdala and prefrontal cortex. Understanding the mechanisms underlying glucocorticoid effects in memory is paramount to design new strategies for the treatment of cognitive impairment induced by stress. Here, we demonstrate that a low daily dose of methylprednisolone (MP, 5 mg/kg, i.p.) for 10-days favors aversive memory persistence in adult rats, without any effect on the exploring behavior, locomotor activity, anxiety levels and pain perception. Enhanced performance on the inhibitory avoidance task was correlated with long-term potentiation (LTP), a phenomenon that was significantly strengthen in hippocampal slices of rats injected with MP (5 mg/kg) during 10 days. Additionally, *in vitro* incubation with MP (30-300 µM) concentration-dependently increased intracellular $[Ca^{2+}]_i$ in cultured hippocampal neurons depolarized by KCl (35 mM). In conclusion, a low daily dose of MP for 10 days promotes aversive memory persistence in rats. The mechanism(s) underlying MP-induced enhancement of hippocampal performance may involve calcium signals and LTP induction in the hippocampus of adult rats.

Key-words: Inhibitory avoidance; Aversive memory; Glucocorticoids; Long-term potentiation.

1 Introduction

Exposure to stress has deleterious effects on brain structure and function, which can be manifested either immediately after stress [1] or as a long-term vulnerability, leading to cognitive deficits [2]. It is well recognized that stress-related hormones (e.g. epinephrine, glucocorticoid) can influence both negatively or positively several distinct cognitive processes [3, 4]. Emotionally-enriched memories involving limbic structures are the most affected by epinephrine and glucocorticoid hormones [5]. Mounting evidences show that acute stress and pulsatile exposure to glucocorticoids can transiently facilitate learning and memory [6, 7]. On the other hand, cognitive deficits due to excessive levels of corticosteroids have been observed in chronic disease or during stressful situations leading to hypersecretion of adrenal glands [8-11].

Several brain regions involved in memory consolidation, such as amygdala, hippocampus and prefrontal cortex, are highly enriched in high-affinity mineralocorticoid and low-affinity glucocorticoid receptors and both receptors can be activated during stressful conditions [12, 13], thereby affecting memory performance [5]. These receptors regulate gene transcription, but can also act non-genomically [10, 11]. Glucocorticoids acting via genomic mechanisms are generally excitatory in limbic structures, thus providing the persistence of emotional aspects related to a stressful event. Mineralcorticoid receptors rapidly enhance and glucocorticoid receptors suppress neuronal activity via non genomic pathways [6].

The hippocampus is one of the brain regions mostly affected by stressful situations as in this region memory consolidation is complex, mainly because ontogenetic and connectivity differences [14]. Memory formation in the hippocampus may involve a variety of physiological synaptic plasticity phenomena, which can be electrophysiologically perceived as long-term potential (LTP) [15]. Strong evidence now exists that calcium elevation initiates LTP through the activation of a major synaptic protein, calcium–calmodulin dependent protein kinase II (CaMKII) [16, 17]. Data from several groups support the idea that calcium entry through NMDA receptors activates calmodulin both

in the cytoplasm and in the channel nanodomain [16, 18]. The complex Ca2+ calmodulin-CaMKII is then transferred from bulk cytoplasm of dendritic spines to the postsynaptic density (PSD) where it enhances AMPA-mediated transmission in two ways. One of these mechanisms implicates phosphorylation of the receptor itself, thus increasing the average conductance of the channel. Alternatively, phosphorylation of extrasynaptic stargazin leads to its binding to PSD, thereby increasing the number of AMPA receptors anchored to the synapse. Memory consolidation seems to be dependent on CaMKII activation also by stimulating fusion of vesicles containing AMPA receptors with the plasma membrane, thus increasing the extrasynaptic channel concentration [16, 18]. Interestingly, glucocorticoid receptors activation in pyramidal cells of the hippocampus and prefrontal cortex increase the expression of AMPA receptors and strengthen glutamatergic synaptic transmission through pathways partially overlapping with those involved in LTP. There is, however, a gap in our knowledge regarding the mechanisms that regulate glucocorticoid-induced enhancement of glutamatergic synaptic and LTP, and whether calcium signaling is involved.

Corticosteroids are used for decades as first line medications for the treatment of inflammatory and autoimmune diseases. Despite the high number of studies aiming at explaining glucocorticoid-induced mechanisms involved in memory reinforcement, this is not yet completely understood. In the present study, we investigated the effects of methylprednisolone on aversive memory persistence in rodents, as well as the molecular mechanisms underlying the corticoid effect on calcium signaling and LTP induction in the rat hippocampus.

2 Materials and methods

2.1 Experimental Animals

Adult male Wistar rats (three months old, 250-280g) were purchased from the Central vivarium of Federal University of Santa Maria (RS/Brazil) and housed four per cage under controlled light and environmental conditions (12h light/12h dark cycle at 23 ± 2°C and 50 ± 10% humidity) with food and water *ad libitum*.

The study was performed according to and approved by the Ethics Committee for Animal Use from Federal University of Pampa, under the protocol number 021/2013.

Two-day-old Wistar rats of both sexes were used for primary cell cultures. To this end, pregnant female rats were purchased from the Centre of Biological Experimental Models from the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS). The animals were kept under an alternate 12 hours cycle of light/dark, at a constant 24°C temperature, with access to water and food *ad libitum*. The newborn rats were kept with the dams until the moment of sacrifice. A total of 12 newborn rats were used to perform cell cultures. The study was performed according to and approved by the Ethics and Research Committee and Ethics Commission in Animal Use from PUCRS, under the protocol number 13/00340. No more than one offspring from each litter and dam were used in these experiments.

All experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH, 1996).

2.2 Drugs and reagents

Sodium Thiopental (Thiopentax®) was purchased from Cristalia Produtos Químicos Farmacêuticos LTDA (Itapira, SP, Brazil). All other chemicals and reagents employed in the experiments were of the highest purity and were obtained from Sigma, Aldrich, Merck, Invitrogen, and BioRad. Methylprednisolone sodium succinate (MP, Solu-Medrol®) was from Laboratórios Pfizer (São Paulo, SP, Brazil).

2.3 Experimental protocols

2.3.1 Behavioral assays

To perform behavioral experiments, 30 animals were equally divided into three groups: control, experimental (i) and experimental (ii). The last two groups were treated daily with MP dissolved in saline and administered intraperitoneally (i.p.) at low (5 mg/kg) and high (30 mg/kg) doses, respectively; the treatment was prolonged for 10 consecutive days and was performed every day at 05:00 PM

[19]. The rats from the control group received saline instead of MP. At the end of treatment, animals from the different groups were submitted to tests of aversive memory (figure 1A/Behavioral experiments).

2.3.1.1 Handling of animals

Two days before starting behavioral tests, the animals were subjected to two rounds of handling. During each session the animals were transported from the *vivarium* to the room where the experiments were to be conducted, removed from the cage and handled by the experimenter for 5 minutes.

2.3.1.2 Inhibitory avoidance task

Inhibitory avoidance (IA) is a commonly used behavioral task to investigate learning and memory processes in rodents [20, 21]. The device used in this study for the inhibitory avoidance task (IA) consisted of a 50.0 x 25.0 x 50.0 cm metal box, with an acrylic made front. The floor of the apparatus consisted of an array of parallel electrified bars with a platform box of 5.0 cm height by 7.0 cm wide placed in the left side of the box. During IA training, rats were carefully placed on the elevated platform and they receive a single aversive footshock (0.5 mA for 2 s) when stepping down from the platform and all four legs contacted with the electrified bars [22]. After this event, the animals were immediately placed into their housing-boxes. Even though IA training consists of a single trial, the brain processes underlying task acquisition are complex. Rats must encode different pieces of information in order to acquire a correct association between a particular location within the apparatus and the aversive stimulus of footshock, which involve the hippocampus, the amygdala and the prefrontal cortex. Retention of the training was tested at 24 hours and then every week for the next 2 weeks after training by measuring rats' latency to step down from the platform. Longer retention test latencies (compared to training ones) are interpreted as indicating better memory/persistence of memory.

2.3.1.3 Control behavioral tasks

After to finish IA protocol, all animals were subjected to behavioral control experiments to avoid biases of the effect of MP injection on other behavioral parameters, influencing the IA (memory) learning. To analyze exploratory and locomotor behavior, each rat was submitted to an Open Field (OF) test, in which crossing and rearing were monitored over 5 min [23]. To evaluate anxiety, rats were exposed to an Elevated Plus Maze (EPM). The time spent and the total number of entries into the open and closed arms were recorded over a 5 min session [24]. Finally, to ensure that pain sensitivity did not change upon MP administration, we used the Tail Flick (TF) test [25].

2.3.2 Electrophysiology

2.3.2.1 Preparation of brain slices for electrophysiology

In this set of experiments, rats (n = 5 per group) were injected either with saline (control) or with the lowest dose (5 mg/Kg) of MP according to the protocol described for the behavioral experiments. At eleven's day after starting saline or MP injections, the animals were deeply anesthetized with 40 mg/Kg thiopental and decapitated (figure 1B/Electrophysiology). Brains were removed en bloc and stored briefly in ice-cold, coronal hippocampal slices (400 µm thick) were cut with a vibro slicer (MA752, Campden Instruments, USA) and kept for at least 1 h in oxygenated (95% O_2 plus 5% CO_2) artificial cerebrospinal fluid (ACSF) containing (in mM): 130 NaCl, 3.5 KCl, 1.3 NaH_2PO_4, 2 $MgCl_2$, 2 $CaCl_2$, 10 d-glucose, and 24 $NaHCO_3$, pH 7.4, at 23–25°C.

2.3.2.2. Electrophysiology recordings of field excitatory postsynaptic potentials (fEPSPs)

For extracellular electrophysiology recordings, individual hippocampal slices from control and low dose methylprednisolone-injected rats (n=5 per group) were transferred to a submersion-type recording chamber, which was continuously superfused with oxygenated (95% O_2 plus 5% CO_2) ACSF at a flow rate of 3.0 ml/min. Field excitatory postsynaptic potentials (fEPSP) were triggered by electrical stimulation of the Schaffer collaterals with constant-current pulses of 0.2 ms duration delivered every 20s (0.05 Hz) using a

differential alternating current stimulator (Isoflex M.P.I., Israel); the stimulation electrode consisted of a twisted bipolar pair of 75 μm platinum–iridium wires (A-M Systems, Carlsborg, WA, USA). Field EPSPs were recorded extracellularly on the pyramidal layer of the CA1 hippocampal region using an Axoclamp 2-B amplifier (Axon Instruments, Foster City, CA, USA). Field EPSPs were amplified and low-pass filtered at 600 Hz (Cyber Amp 320, Axon Instruments), digitized (Digidata 1322A, Axon Instruments) and recorded on a computer using the Axoscope 9.2 software (Axon Instruments). The amplitude of fEPSPs was measured using the Clampfit 9.2 software (Axon Instruments). Recorded values were normalized on a per recording basis and then plotted as the mean of 2-min time periods (6 fEPSPs) ± S.E.M. corresponding to one to three slices per experimental animal.

At the beginning of each recording, an input–output (I/O) curve for the fEPSP amplitude relative to the stimulus intensity was recorded using 50 μA stepwise increases (ranging from 50 to 250 μA) until saturation of the fEPSP amplitude. Current intensity was then adjusted to evoke baseline fEPSP amplitude ranging from 50–60% of the maximal fEPSP amplitude obtained by the I/O curve. Baseline responses obtained to 0.05 Hz paired-pulse stimuli (0.2 ms) were recorded for 20 min before the induction of long-term potentiation (LTP). After reaching stable baseline fEPSP recordings, LTP was induced using a high-frequency stimulation (HFS) protocol consisting of four trains of 1 s duration delivered at 100 Hz frequency with an inter-train interval of 20 s. Field EPSPs were monitored for at least 60 min after tetanic stimuli.

2.3.3 Calcium images

2.3.3.1. Primary hippocampal cell cultures

Primary hippocampal cell cultures were prepared as described previously (Gan et al., 2011). Briefly, 1- to 2-day-old Wistar rats were sacrificed by cervical dislocation followed by decapitation. Hippocampi were removed, triturated, and the resulting cell homogenates were plated onto 13-mm coverslips coated with poly-L-lysine at a density of 3×10^5 cells.ml^{-1}. Cultures were, then, incubated with culture media consisting of Neurobasal-A Medium (Invitrogen,UK)

supplemented with 2% (v/v) B-27 (Invitrogen, UK) and 2 mM L-glutamine; hippocampal cell cultures were maintained at 37°C, in a humidified atmosphere with 5% CO_2 for 13-16 days *in vitro* (DIV). After 5 DIV, cytosine-D-arabinofuranoside (Ara-C, 10 mM) was added to inhibit glial cell proliferation. Single-cell Ca^{2+} imaging experiments were performed on cells taken from at least three separate cultures obtained from different rats.

2.3.3.2. Single-cell $[Ca^{2+}]_i$ imaging

All imaging experiments were performed on a digital epifluorescence imaging system (WinFluor, J. Dempster, University of Strathclyde) mounted on a Nikon Eclipse 2000 microscope using a 20x objective. Hippocampal cultures (DIV 10–14) were loaded with Fluo-4 AM (5 µM, 45–60 min, room temperature, Invitrogen, UK) prior to experiments. Experiments were performed on cultures continually perfused (1–2 $mL.min^{-1}$) with HEPES-buffered saline (HBS) containing (in mM): NaCl 140, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2, HEPES 10, D-glucose 10, pH was adjusted to 7.4 and osmolarity adjusted to 310 mOsm with sucrose if required. All drugs were added via the perfusate. Cells were identified as astrocytes based on their morphological characteristics and their lack of response to high extracellular potassium (35 mM). Data were calculated as changes in fluorescence ratio and expressed as $\Delta F/F_0$.

2.4. Statistical analysis

For statistical analysis of the inhibitory avoidance task results the Wilcoxon test was used to compare training and testing step-down latencies; to compare step-down latencies in each test day between different groups a Kruskall-Wallis test followed by Dunn's *post hoc* was used. For control behavioral tasks, one way ANOVA test was used. All the other data were compared by one way ANOVA followed by Tukey´s test as *post hoc*. Differences were considered significant when $P < 0.05$.

3 Results

3.1 Behavioral tests

3.1.1 Low dose of MP enhances aversive memory persistence

Twenty four hours after inhibitory avoidance (IA) training, all groups of animals had higher latencies to step-down from the platform compared with the training period (consolidation phase; Figure 2/test 24h; $P < 0.01$). Aversive memory was maintained in all groups of animals 7 days after the IA training (Figure 2/test 7d; $P < 0.01$), but from this time onwards retention of the training was only observed in rats treated with the lower dose (5 mg/kg) of MP (Figure 2/test 14d; $P = 0.007$ for 5 mg/kg MP dose; $P > 0.05$ for other groups). Additionally, in the comparisons between groups' step-down latencies in each test day, only in day 14 differences were found, being the group (ii) step-down latency higher than the others (Figure 2/test 14d; $P = 0.04$ for saline *vs.* 5 mg/kg; $P = 0.66$ for saline *vs.* 30 mg/kg; $P = 0.02$ for 5 mg/kg *vs.* 30 mg/kg). While aversive memory performance returned to control levels in the group of animals treated with 30 mg/Kg MP, rats injected with the lower dose (5 mg/kg) of MP maintained higher latency times in the IA test than control animals until day 14 after training (Figure 2).

3.1.2 Treatment with MP did not alter locomotor and exploratory activities, anxiety test performance and pain sensibility

To avoid biases concerning the effect of MP on behavioral parameters that might influence IA (memory) performance, we subjected all animals to open field (OF), elevated plus maze (EPM), and tail flick (TF) tests. Our intention was to evaluate the effect of low and high doses of MP injection on exploratory/locomotor behavior (OF), anxiety levels (EPM), and pain perception (TF) of experimental rats. Table 1 show that treatment with MP did not affect any of the parameters under evaluation ($P > 0.05$).

3.2 Treatment of rats with a low dose of MP during ten consecutive days increases long-term potentiation (LTP) in isolated hippocampal slices

Synaptic transmission recordings from CA1 pyramidal neurons were elicited by electrical stimulation in the Schaffer collaterals. Since *in vivo* treatment of rats with a low dose of MP prolongs aversive memory performance, we asked ourselves whether this could translate the plasticity phenomena operated in hippocampal slices *in vitro* as changes in LTP amplitude. Figure 3A and 3B shows that the LTP amplitude was significantly higher in hippocampal slices isolated from rats that have been treated with a low daily dose of MP (5 mg/kg), for 10 days as compared to control littermates, which received saline instead of MP (control group, 185.7 ± 0.24 mV, n=6 *vs.* MP group, 309.3 ± 0.23 mV, n=5, $p<0.0001$).

3.3 MP concentration-dependently increases $[Ca^{+2}]_i$ levels in cultured neurons of the rat hippocampus

Epifluorescence microscopy in the time-lapse mode was used to monitor intracellular calcium ($[Ca^{2+}]_i$) dynamics in cells superfused with MP (0.3-300µM). MP concentration-dependently increased $[Ca^{2+}]_i$ in cultured hippocampal neurons under resting conditions (Figure 4). The effect of MP (0.3-300µM) was significantly higher after neuronal depolarization with KCl (35 mM). In fact, intracellular $[Ca^{2+}]_i$ after MP (300 µM) application increased by 1262±30% above the baseline, but it was further enhanced ($p<0.05$) to 2194±10% following KCl (35 mM) application (n=35 cells).

4 Discussion

Glucocorticoids are well recognized to be crucially involved in the regulation of memory consolidation [5]. Previous studies have shown that their action can be both beneficial or detrimental to mnemonic processes, depending on very specific conditions: for example, chronic stress states are normally associated with memory impairment, while acute stress is associated with benefits in memory acquisition [26-28]. Furthermore, the effects of glucocorticoids also depend on factors as: (i) the rhythm of application and dosage, since their action in memory consolidation considers an inverted U-shape dose-response relationship; and, (ii) the time of application, considering the memory stage,

since the effects of it can differ between encoding, consolidation, retrieval and reconsolidation [29]. In general, moderate doses enhance memory, while higher doses are normally less effective or may even impair memory consolidation [30].

Our results show that daily i.p. treatment with a low dose of MP during 10 days is amenable to induce persistence of aversive memory considering that the animals treated with MP (5 mg/Kg) kept higher latencies to step-down from the platform in the IA test during 14 days, while control animals only maintained aversive memory for 7 days. Data show that aversive memory consolidation achieved 24 hours after training did not differ among the three groups of tested animals, control (injected with saline) and treated with a low (5 mg/Kg) or high (30 mg/Kg) dose of MP. Unlike previous studies we focused our attention to make clear the differences on aversive memory persistence after a short period (10 days) of treatment with a low dose of MP. As discussed by McGaugh [31, 32], enhanced memory persistence is usually the result of mechanisms leading to promotion of consolidation. Considering this, it is legitimate to infer that treatment with a low dose of MP strengthens memory consolidation leading to prolonged IA memory, although no differences between groups were perceived immediately (24 hours) after training.

Glucocorticoids, via genomic and non-genomic mechanisms, may influence a wide variety of cellular functions, including cell signaling, ion channel properties, as well as cell structure, which may be related to synaptic plasticity and memory consolidation [33-35]. Long-term potentiation (LTP) is considered the electrophysiological translation of synaptic plasticity phenomena in several brain regions, including the hippocampus [17]. It consists of an increase in the postsynaptic response observed for hours, days or weeks after a short repetitive high frequency stimulation of presynaptic afferents, and has been considered to be the basis of long term memory (LTM) consolidation [36]. Data from our study show that following a 10-day treatment with a low dose of MP injected i.p. to living rats favored LTP in slices of the hippocampus.

Whether facilitation of LTP induction is directly related to aversive memory persistence in MP treated animals requires further investigation. Recent findings from another group have shown that animals submitted to acute stress had higher LTP than unstressed control animals [37]. Additionally, induction of LTP was facilitated when corticosterone (200 nM) or the synthetic glucocorticoid receptor agonist dexamethasone (200 nM) were applied for 30 min before the tetanus [37]. Differences in the chemical nature and timing of application of the corticosteroid may justify results disparity among our and Whitehead's works. Despite these differences, data from both studies agree that facilitation of LTP caused by corticosteroids takes more than 10 min to occur and, therefore, it is not a rapid process probably by implicating an underlying genomic mechanism in detriment of a faster non-genomic pathway. Although it has been shown that acute stress or exposure to glucocorticoids can cause a rapid increase in glutamate release (see e.g. [38, 39] and these changes in excitatory synaptic transmission have been suggested to regulate synaptic plasticity, no underlying rapid non-genomic mechanism has been revealed [37].

It has been demonstrated that consolidation of aversive memory requires plasticity phenomena, such as LTP, in the CA1 region of the dorsal hippocampus, and activation of AMPA, NMDA and metabotropic glutamate receptors [40, 41]. Acute stress and glucorticoids may trigger rapid synaptic insertion of Ca2+-permeable AMPA receptors to facilitate LTP in the hippocampus during high frequency stimulation [37], but the molecular mechanism(s) underlying glucocorticoid-induced enrichment of AMPA receptors at the synapse is unknown. Stimulation of postsynaptic AMPA and NMDA glutamate receptors in the hippocampus may synergize to increase intracellular [Ca2+]I levels, which may lead to activation of CaMKII [40, 42]. CaMKII plays pivotal roles in LTP by phosphorylating substrates through elaborate regulatory mechanism, and is known to be both necessary and sufficient for LTP [43-46]. CaMKII has been hypothesized to act as "a frequency detector" which integrates multiple Ca2+ pulses; and, as a "memory molecule" which remembers the past history of synaptic activation (reviewed by [17, 47]. These

two components operate within a defined time window during the LTP induction, being the calcium required only during the first second after LTP induction [43]

Corticosteroids can affect calcium homeostasis both in physiological, as well as in pathological conditions, using distinct mechanisms [48, 49]. For instance, dexamethasone (1 μM) decreases intracellular [Ca2+]i in Ca2+-free cultured hypothalamic neurons by activating plasma membrane calcium pumps (PMCA) [48]. Conversely, activation of glucocorticoid receptors in CA1 pyramidal neurons of the mouse hippocampus favor [Ca2+]i influx through voltage-sensitive L type channels [50]. Here, we show that MP concentration-dependently increased resting [Ca2+]i levels above baseline in cultured hippocampal neurons. Interestingly, MP-induced [Ca2+]i rises were significantly potentiated upon depolarizing hippocampal neurons with KCl (35 mM). This phenomenon was verified even after washing out the corticoid from the incubation medium, meaning that the corticoid may act under these circumstances via an intracellular pathway in order to prime calcium responses to subsequent stimuli. We are aware that these experiments were performed with cultured hippocampal neurons isolated from newborn rats and, therefore, these are hardly comparable to LTP experiments made in hippocampal slices from control adult rats. Notwithstanding this, contrasting results obtained under these two experimental conditions may have different interpretations besides the age of the animals, duration of exposure and stimulation conditions, *i.e.* tetanus vs. high KCl when triggering LTP and depolarization of hippocampal neurons, respectively.

As previously mentioned, intracellular [Ca2+]i rises is a crucial determinant to LTP induction, a phenomena observed in the hippocampus of rats treated during 10 days to low daily doses of MP, who also exhibiting aversive memory persistence. Thus, our results demonstrate that exposure to low doses of MP increases aversive memory persistence and suggest, for the first time, that the molecular mechanism(s) underlying cognitive enhancement caused by MP might involve Ca2+ signals that culminates in LTP induction.

5 Acknowledgements

This work was supported by research grants from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/Brazil), and from the Federal University of Pampa granted to PBMC, II, CDB and LV. LSV was supported by CAPES/Brazil. RG was supported by FAPERGS. II was supported by CNpq. PCS was supported by University of Porto/Banco Santander Totta.

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FIGURES

Figure 1

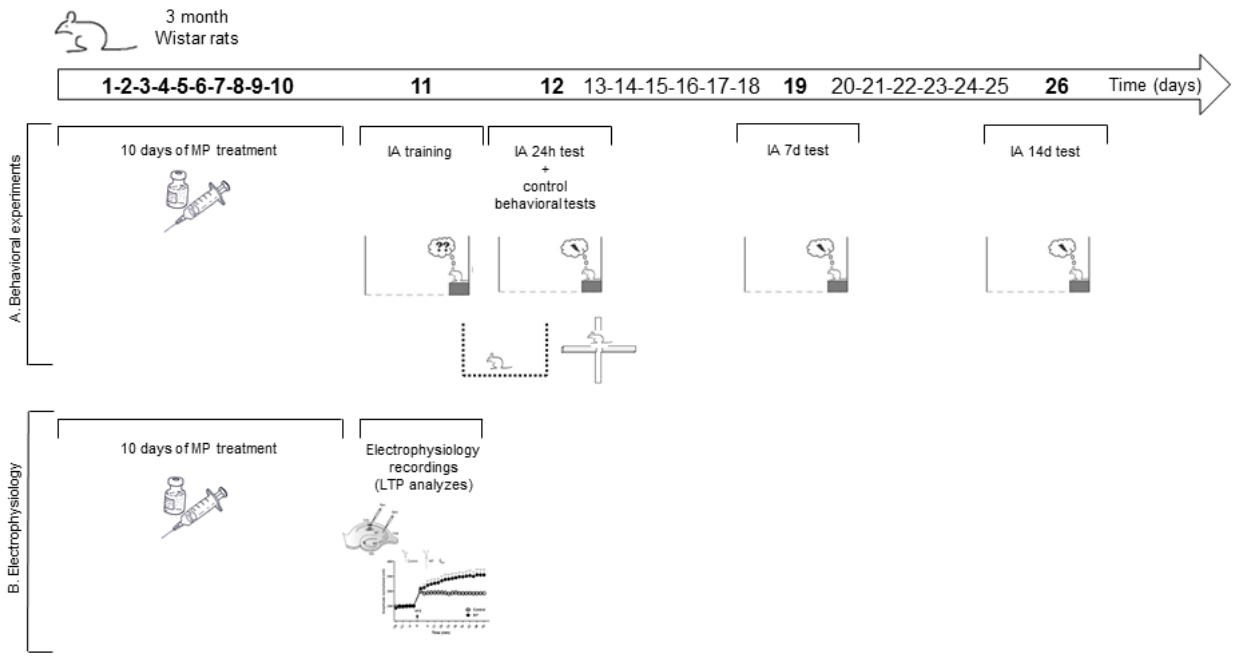

Figure 2

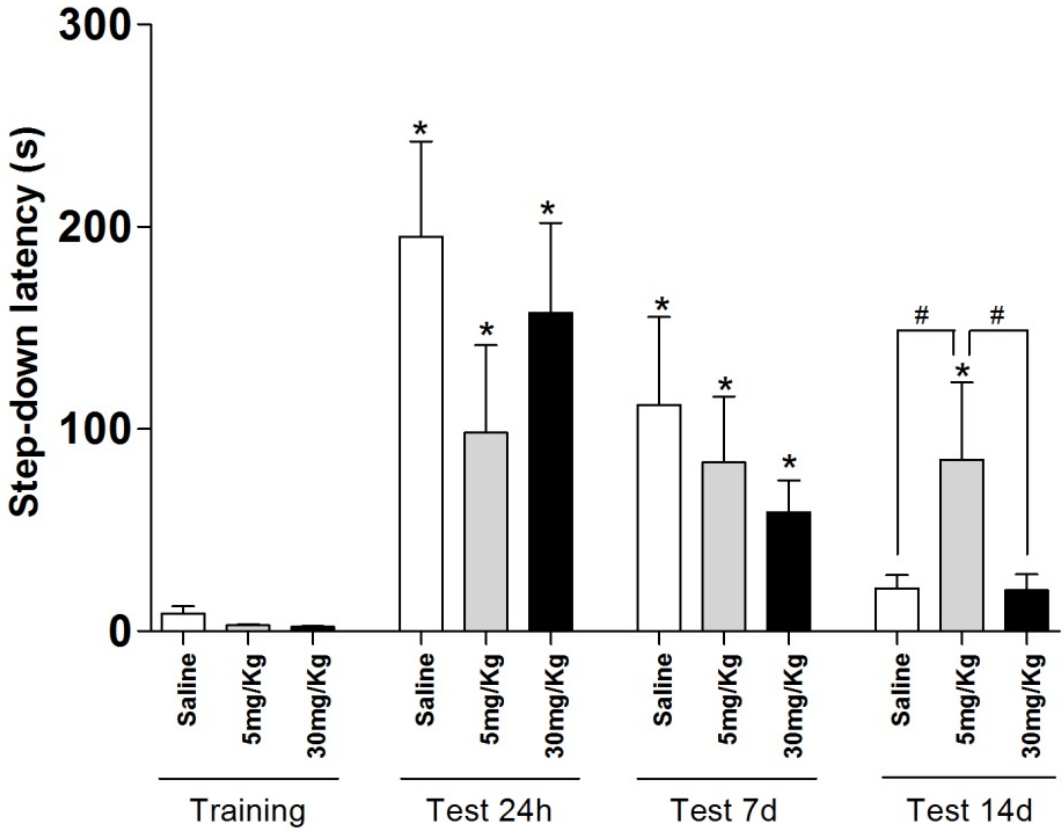
Step-down latency (s)
300
200
100
0
Saline
5mg/Kg
30mg/Kg
Training
Test 24h
Test 7d
Test 14d
*
#

Figure 3

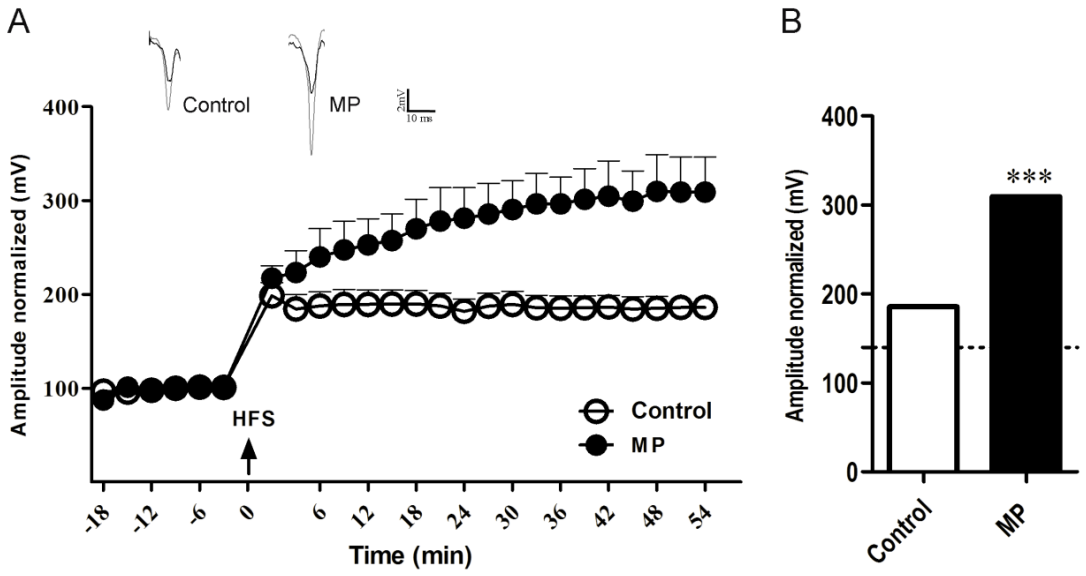
A
Control
MP
2mV
10 ms
Amplitude normalized (mV)
400
300
200
100
HFS
Control
MP
-18
-12
-6
0
6
12
18
24
30
36
42
48
54
Time (min)
B
Amplitude normalized (mV)
400
300
200
100
0
***
Control
MP

Figure 4

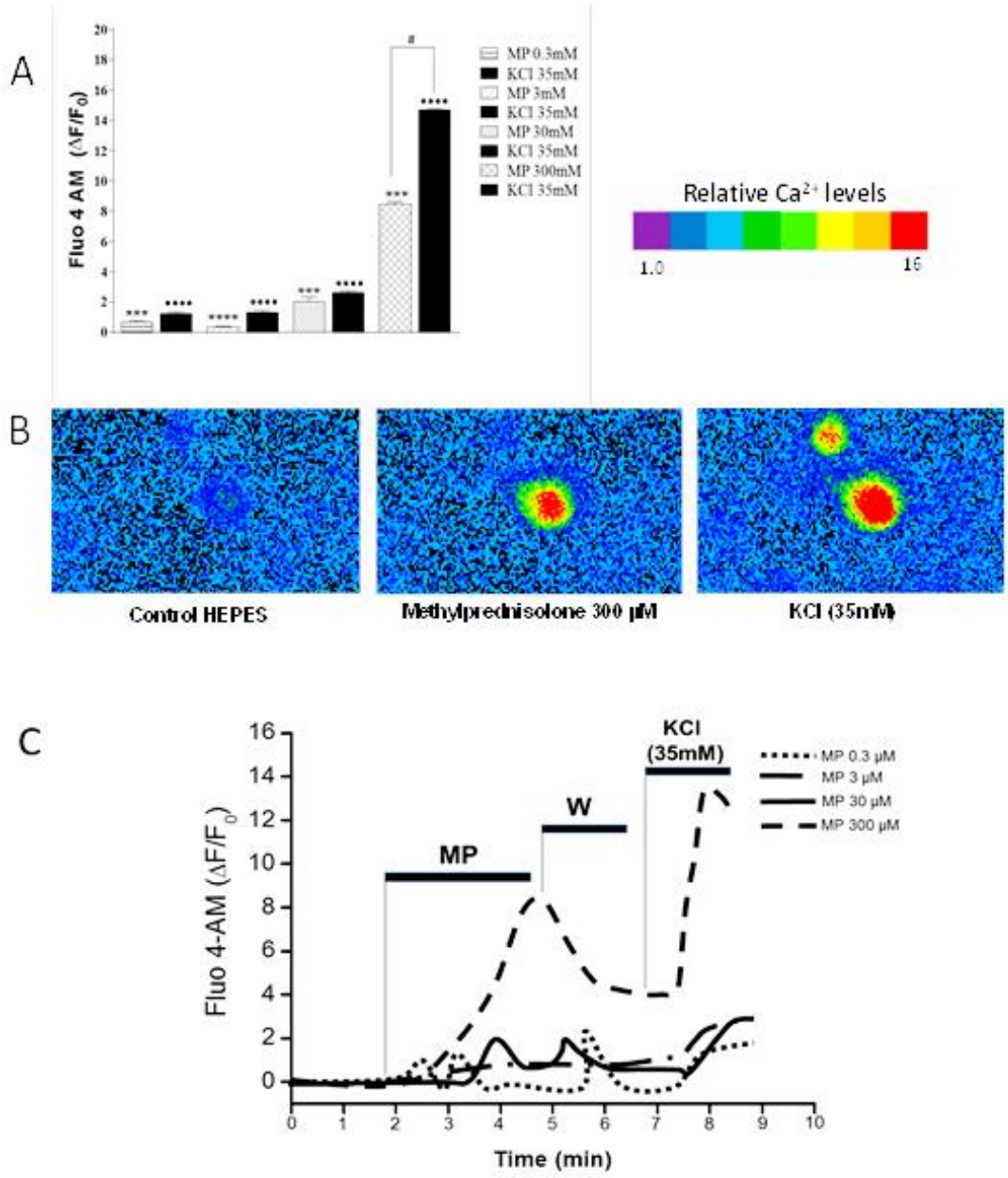
A
Fluo 4 AM (ΔF/F0)
MP 0.3mM
KCl 35mM
MP 3mM
KCl 35mM
MP 30mM
KCl 35mM
MP 300mM
KCl 35mM
Relative Ca2+ levels
1.0
16
B
Control HEPES
Methylprednisolone 300 µM
KCl (35mM)
C
Fluo 4-AM (ΔF/F0)
MP
W
KCl (35mM)
MP 0.3 µM
MP 3 µM
MP 30 µM
MP 300 µM
Time (min)

FIGURE LEGENDS

Figure 1. Summary of experimental design. Male Wistar rats (3-month) were treated with methylprednisolone (MP) for 10 days. **A.** The behavioral experiments were initiated one day after the end of the treatment. On day 11 after starting saline or MP injections, the animals were submitted to inhibitory avoidance (IA) training and to behavioral control tests (open field, elevated plus maze and tail flick). On day 12 the animals were submitted to IA test to evaluate the consolidation of aversive memory (24h after training), and on days 19 and to evaluate the persistence of aversive memory (7 and 14 days after training). **B.** The electrophysiological experiments were conducted one day after the end of the treatment. On day 11 after starting saline or MP injections, the animals were deeply anesthetized and decapitated. Brains were removed and individual hippocampal slices were prepared for extracellular electrophysiology recordings.

Figure 2. Effects of 10-day treatment with two different doses of MP in the consolidation and persistence of aversive memory. All animals showed a low step-down latency during IA training. Aversive memory consolidation was observed in all groups of animals 24 hours after IA training. Aversive memory persisted in all groups of animals until 7 days after training, but on day 14 aversive memory only persisted in the group treated with the lowest MP dose. *$P < 0.01$ (Wilcoxon test; training *vs.* test). $^{\#}P < 0.05$ (Krukall-Wallis followed by Dunn's post hoc). n = 10/group.

Figure 3 Ten-day treatment with a low dose of MP increases LTP the rat hippocampus. Ten-day treatment with a low dose of MP increases LTP the rat hippocampus. **A.** Time-course of fEPSP recordings normalized to the pre-tetanus (HFS) amplitude in rat hippocampal slices from control animals (open circles) and MP-treated rats with a 5 mg/Kg dose applied i.p. during 10 days (black circles). Abscissa show the time (in minutes) at which the fEPSP was

recorded; zero time corresponds to tetanus (HFS) application. fEPSP recordings started 20 min before HSF and were prolonged for 54 min after induction of LTP. Corresponding representative traces of slices 10 min before and 54 min after LTP induction. Calibration bar for all analog sweeps: 2 mV/10 ms. Histograms represent normalized amplitudes of fEPSP recordings obtained between 42 and 54 min after HFS application in control animals (open bar) and MP-treated animals (black bar). Asterisks indicate significant differences between groups ***P < 0.0001 when compared to the control group. n = 6-10/group.

Figure 4. MP concentration-dependently increase $[Ca^{+2}]_i$ levels in cell cultures of the rat hippocampus. A. Each bar represents the mean ± S.E.M of the fluorescence after treatment with methylprednisolone (0.3, 3, 30 and 300µM) followed by KCl (35mM). **B.** Representative pseudocolor images of calculated calcium influx of control HEPES and during the onset of methylprednisolone (300µM) and high K^+. Note that methylprednisolone induces a significant increase in calcium influx in higher concentrations. *P < 0.05 compared to resting conditions; **P < 0.01 compared to resting conditions. **C.** Frequency and amplitude of calcium oscillations in developing hippocampal neurons at 10-14 days. The cells were loaded with FLUO-4 AM as a calcium fluorescence probe. The graph represents the mean responses (n = 50 cells each) of the calcium oscillations during the pre-intervention phase, the post treatment phase with different concentrations of methylprednisolone, washout followed by high potassium application. The application of methylprednisolone significantly increased the amplitude of calcium oscillations. The results were expressed as mean ± SEM. Statistical significance was determined by One-way Anova followed by Tukey test as post hoc to compare cells infused with MP (**P < 0.01) with those infused with KCl (****P < 0.0001); $^{\#}P \leq 0.0001$ with Student t-test. MP: methylprednisolone application; W: washout.

TABLE

		Control	Low Dose MP	High Dose MP
Open Field (OF)	Crossings (n)	57.00 ± 9.55	73.60 ± 7.76	74.82 ± 7.97
	Rearings (n)	22.40 ± 3.66	26.56 ± 2.15	26.44 ± 4.73
Elevate Plus Maze (EPM)	Total entries (n)	7.83 ± 1.65	6.08 ± 1.21	8.85 ± 1.99
	Time in open arms (s)	11.67 ± 1.65	13.67 ± 4.29	15.79 ± 6.54
Tail Flick (TF)	Latency (s)	5.49 ± 0.38	5.28 ± 0.40	5.02 ± 0.31

Table 1. Treatment with MP had no effect on locomotor and exploratory activities, anxiety tests and pain perception. Data are expressed as mean ± SE of the total time of exploration activities in the open field test (OF), i.e. the number of crossings and rearings, the time spent and the number of entries in the open arms of the elevated plus maze test (EPM), and the time latency for pain perception in the tail flick test (TF). There were no statistical significant differences among the 3 groups (ANOVA; n = 8 per group for all tests).

Parte III

DISCUSSÃO

Esta tese buscou investigar diferentes estratégias que pudessem qualificar a persistência da MLD. Assim, o trabalho foi dividido em dois estudos principais, que buscaram, respectivamente, investigar os efeitos de uma estratégia não farmacológica (exercício físico) envolvendo mecanismos noradrenérgicos, bem como investigar os efeitos de uma estratégia farmacológica (uso de fármaco glicocorticoide) na promoção da persistência da memória.

Os resultados do primeiro estudo ratificaram o papel do sistema noradrenérgico hipocampal na consolidação e persistência da memória de RO, mostrando que estas dependem do aumento dos níveis de NA no hipocampo e aumento da expressão de BDNF nessa mesma estrutura. Adicionalmente, demonstramos que a persistência da memória de RO depende da ativação do sistema noradrenérgico a partir da via NTS-PGi-LC-Hipocampo. Após a identificação do papel da NA na persistência da memória de RO, demonstramos que uma única sessão de exercício físico realizada imediatamente após a aprendizagem aumenta os níveis de NA no hipocampo, sendo capaz de promover a persistência da memória de RO.

Recentemente, Mello-Carpes e Izquierdo (2013) demonstraram que a ativação da via NTS-PGi-LC-Hipocampo é necessária para a consolidação da memória de RO, a qual provavelmente culmina com a liberação de NA no hipocampo. Dornelles et al. (2007) mostraram que a injeção sistêmica de adrenalina melhora a memória e que este efeito pode ser bloqueado pela administração de propranolol, antagonista dos receptores beta-adrenérgicos, antes do aprendizado. Ainda, Roozendal e colaboradores (2008) evidenciaram que a administração de NA na amigdala basolateral promove uma melhora da memória, enquanto que a administração de propranolol prejudica a mesma, ambos efeitos dependentes da dose utilizada. Nossos resultados corroboram com estes dados prévios e deixam claro o importante papel do sistema

noradrenérgico na consolidação da memória de RO, além de evidenciarem que a ativação deste sistema contribui para a persistência da memória.

Já está bem estabelecido na literatura que o sistema noradrenérgico é fundamental para concentração e desempenho cognitivo de roedores, modulando estes processos (MCGAUGH, 1989; IZQUIERDO & MEDINA, 1997; MELLO et al., 2013; 2016). Isto já foi demonstrado em uma variedade de paradigmas comportamentais, tais como o medo condicionado ao contexto, a tarefa de RO, e a extinção do medo condicionado ao contexto (MCINTYRE et al., 2002; SEGAL & CAHILL, 2009). Cientes dessa contribuição, mas considerando que a manipulação farmacológica do sistema noradrenérgico pode oferecer riscos à saúde, especialmente na população idosa, buscamos por uma alternativa comportamental que pudesse ativar este sistema, reproduzindo seus efeitos na memória. A estratégia proposta aqui foi o uso do exercício físico.

Embora esteja bem documentado na literatura os benefícios provenientes do exercício físico regular para o sistema nervoso (ISAACS et al., 1992; FABEL et al., 2003; ARIDA et al., 2004; VAN PRAAG et al., 2005; DURING & CAO, 2006; BERCHTOLD et al., 2005; VAYNMAN et al., 2006), especialmente no que se refere a fatores relacionados aos processos cognitivos, como a neuroplasticidade (HÖTTING & RÖDER, 2013), o aumento do volume hipocampal (SMITH et al., 2010), melhora das funções executivas (HÖTTING et al., 2012), entre outros aspectos citados anteriormente na introdução desta tese, a prática de exercício regular nem sempre é possível, e o número de estudos sobre os efeitos de uma única sessão de exercício sobre a memória é bastante limitado e os seus resultados são inconsistentes até agora. Alguns estudos mostram efeitos benéficos, nenhum efeito, ou até mesmo efeitos prejudiciais (LABBAN & ETNIER, 2011; SEGAL et al., 2012; MCNERNEY & RADVANSKY 2015).

Os modelos de consolidação da memória enfatizam a natureza dinâmica das representações da memória, propondo duas principais fases: uma em que a memória é considerada lábil, sendo, portanto suscetível a aprimoramentos ou

melhorias, e outra em que a memória é estável, sendo bastante insensível a qualquer tipo de tratamento ou intervenção (MCGUGH 1966; 2000; NADER & HARDT, 2009; NADEL et al., 2012). Mesmo que hoje se saiba que existem processos neuroquímicos mais tardios (cerca de 12h após o aprendizado) que estão relacionados à persistência da memória (MEDINA et al., 2008; ECKEL-MAHAN et al. 2008; ROSSATO et al., 2009), esta fase também depende da consolidação inicial. Segundo McGaugh (2000), a melhora na persistência da memória geralmente é resultado de mecanismos que melhoram a consolidação desta.

Assim, considerando que uma fase lábil da memória é vista logo após a aprendizagem, ou então após a reativação de uma memória previamente adquirida, fica claro que se o exercício for realizado durante as fases iniciais da consolidação da memória, esta poderá sofrer a influência do mesmo. No entanto, a maioria dos estudos que relatam efeitos benéficos do exercício físico agudo na MLD, os participantes praticam o exercício antes ou durante a aprendizagem, não deixando claro se o exercício estaria atuando na aquisição, na consolidação da memória, ou em ambas (WINTER et al., 2007; SCHMIDT-KASSOW et al., 2010; 2013; 2014; LABBAN & ETNIER, 2011).

Os resultados de estudos com humanos em que os participantes realizam exercícios após aprendizagem são contraditórios. Por exemplo, Labban e Etnier (2011) não encontraram melhora significativa da memória em participantes que se exercitaram após a aprendizagem em comparação com participantes que ficaram em uma condição de repouso, enquanto a memória melhorou nos participantes que tinham se exercitado antes de aprendizagem. Este achado contrasta com os resultados de Segal et al. (2012) e McNerney e Radvansky (2015) que mostraram que o exercício aeróbico imediatamente após a aquisição melhora a memória.

Segal e cols. (2012) mostraram que o exercício agudo feito após a aprendizagem, durante 6 minutos a um intensidade de 70% do VO_2 máximo em uma bicicleta estacionária, melhora a memória, tanto em pacientes com comprometimento cognitivo leve, como em indivíduos saudáveis, promovendo

uma elevação concomitante de NA endógena em ambos grupos (medida indiretamente através da AAs). Nossos achados sustentam estes dados prévios e adicionam medidas de NA realizadas diretamente no hipocampo, além da uma avaliação da persistência da memória ao longo do tempo.

Assim, na segunda parte do primeiro estudo desta tese mostramos que uma sessão única de exercício físico é capaz de promover a persistência da memória de RO por meio da ativação noradrenérgica hipocampal. O envolvimento do sistema NA hipocampal nesse efeito foi claro, uma vez que o efeito do exercício desapareceu quando os receptores β-adrenérgicos do hipocampo foram bloqueados pela infusão de timolol. Além disso, medições diretas demonstraram que houve um aumento nos níveis de NA no hipocampo após a sessão de exercício físico. Embora a aprendizagem na tarefa de RO promova o aumento de NA *per se*, este aumento foi maior quando os animais foram submetidos à sessão de exercício.

A NA é capaz de modular a expressão de proteínas chave para memória (KOBAYASHI & YASOSHIMA, 2001). Nossos achados mostraram que o aumento dos níveis hipocampais de NA promovido pela aprendizagem no RO aumenta a expressão de BDNF, o que provavelmente se deu em virtude da ligação da NA aos beta-receptores da membrana celular do hipocampo, o que ativou o CREB (SARA, 2009), levando ao aumento da expressão de BDNF. O BDNF é necessário em diferentes tipos de aprendizados, relacionando-se com a plasticidade sináptica e processos mnemônicos (BEKINSCHTEIN ET AL., 2007, 2008). Assim como o nosso resultado, Furini e colaboradores (2010) também demonstraram que a expressão proteica de BDNF aumenta 120 minutos após o aprendizado na tarefa de RO, e que a aprendizagem depende deste processo.

Resumidamente, os resultados do primeiro estudo desta tese fornecem novas evidências que complementam descobertas anteriores do nosso grupo sobre o papel da NA hipocampal na consolidação e persistência da memória de RO (Mello-Carpes e Izquierdo, 2013), sugerindo, adicionalmente, que o exercício agudo pode ser adotado como uma intervenção não farmacológica

que auxilia na consolidação e persistência da memória, com pouco ou nenhum efeito colateral.

No segundo estudo desta tese mostramos que o tratamento com baixas doses de MP (um fármaco glicocorticoide) é capaz de promover a persistência da memória aversiva em roedores, e que este efeito está provavelmente relacionado à sinalização intracelular de Ca^{2+} e indução de LTP no hipocampo. Neste estudo tratamos os animais durante 10 dias com diferentes doses de MP (uma dose alta e uma dose baixa), e verificamos que no grupo tratado com a baixa dose a memória persistiu por 14 dias, diferente do grupo tratado com uma alta dose e do grupo controle, nos quais a memória persistiu por 7 dias apenas. Estes resultados sugerem que o uso crônico de glicocorticoide não prejudica a memória, e, além disso, que o uso de uma baixa dose pode ter efeitos positivos sobre a memória, promovendo sua persistência.

Sabe-se que a exposição a hormônios relacionados ao estresse poderia provocar efeitos deletérios na estrutura e na função cerebral, influenciando os processos cognitivos (MCEWEN, 1999a; 1999b). Estudos prévios revelam que os glicocorticoides são capazes de modular tanto positiva como negativamente os processos mnemônicos, levando a melhora ou prejuízo da memória (MCGAUGH & ROOZENDAAL, 2002; RASHIDY-POUR et al., 2009). Assim, percebe-se que os resultados de investigações a respeito desse tema ainda são contraditórios. Estudos defendem que os efeitos dos hormônios e fármacos glicocorticoides são dependentes da dose, sendo que doses moderadas têm mostrado efeitos positivos sobre a memória, enquanto que doses mais elevadas podem interferir negativamente na memória, podendo prejudicar a sua consolidação, e, portanto, a sua persistência (ROOZENDAAL et al., 1999). Por outro lado, existem estudos que apontam que o tempo de exposição a estes hormônios é o determinante para seus efeitos benéficos, uma vez que estados de estresse crônico são comumente associados ao prejuízo da memória, enquanto que o estresse agudo é responsável por promover benefícios na aquisição da memória (DE KLOET et al., 1999; JOELS et al., 2011; SANDI, 2011).

Interessantemente, nossos resultados mostraram que o efeito da MP foi dose dependente, entretanto, não houve prejuízo da memória no tratamento com uma alta dose de MP, conforme os estudos anteriores relatam, mas sim a melhora da memória, evidenciada pela persistência desta, nos animais tratados com uma baixa dose de MP. Como discutido anteriormente e fundamentado por McGaugh (2000; 2015), a melhora da persistência da memória é geralmente o resultado de mecanismos que conduzem à promoção da consolidação. Considerando isso, podemos inferir que o tratamento com uma dose baixa de MP fortalece a consolidação da memória, levando à sua persistência.

Os glicocorticoides podem influenciar uma vasta variedade de funções celulares, incluindo a sinalização celular, as propriedades de canais iônicos, bem como a estrutura celular, que podem ser relacionadas com a plasticidade sináptica e a consolidação da memória (REVEST et al., 2005; BISAZ et al, 2009). A potenciação de longa duração (LTP) é considerada a tradução eletrofisiológica de fenômenos de plasticidade sináptica, em várias regiões do cérebro, incluindo o hipocampo (HIDALGO & ARIAS-CAVIERES, 2016), sendo considerada como a base da consolidação das MLD (IZQUIERDO et al., 2008). Aqui, mostramos que o tratamento com a MP em baixa dose favorece a indução de LTP. A consolidação da memória aversiva requer fenômenos de plasticidade, tais como a LTP, na região CA1 do hipocampo, além da ativação de receptores AMPA, NMDA e dos receptores metabotrópicos de glutamato (RIEDEL et al., 2003; AHMED & FREY, 2005). Sabe-se que o estresse agudo e os glicocorticóides podem facilitar a LTP hipocampal durante uma estimulação de alta frequência por meio da inserção sináptica rápida dos receptores AMPA permeáveis ao Ca^{2+} (WHITEHEAD et al., 2013), mas o mecanismo molecular subjacente a potencialização dos receptores AMPA induzida pelos glicocorticoides na sinapse ainda é desconhecido.

Além disso, os glicocorticoides podem afetar a homeostase do Ca^{2+}, tanto em condições fisiológicas, como patológicas, utilizando mecanismos distintos (CHEN et al., 2011; SUWANJANG et al., 2013). Esses mecanismos envolvem tanto a ativação de bombas de Ca^{2+} na membrana plasmática

(SUWANJANG et al., 2013), como também a ação de receptores de Ca^{2+} do tipo L sensíveis a voltagem (CHAMEAU et al., 2007). Nossos resultados corroboram com estes dados, demonstrando que a MP aumentou de maneira dose-dependente o influxo Ca^{2+} em cultura de células de hipocampo. Sabe-se que o aumento da concentração de Ca^{2+} é um determinante importante para a indução da LTP, fenômeno este que foi observado no hipocampo de ratos tratados durante 10 dias com baixas doses de MP, os quais também apresentaram a persistência da memória aversiva. Assim, nossos resultados sugerem que o mecanismo molecular subjacente à melhora cognitiva pela MP pode envolver a sinalização de Ca^{2+}, que culmina na indução da LTP.

Tomados em conjunto, os resultados dos dois estudos desta tese fornecem evidências de que é possível utilizar estratégias não-farmacológicas e farmacológicas para melhora da memória e promoção da sua persistência. Embora os estudos apresentados tenham utilizado diferentes paradigmas de avaliação da memória (o primeiro utilizou a tarefa de reconhecimento de objetivos, enquanto o segundo utilizou a esquiva inibitória), ambos os mecanismos envolvidos foram investigados no hipocampo, estrutura cujo papel fundamental na consolidação das memórias foi discutido na primeira parte desta tese.

CONCLUSÕES

Com base nos nossos resultados podemos concluir que:

1. A ativação do sistema noradrenérgico hipocampal é necessária para consolidação e persistência da memória de RO;

2. Uma sessão única de exercício físico após a aprendizagem promove a persistência da memória de RO;

3. O exercício físico promove a persistência da memória de RO por meio da ativação do sistema noradrenérgico hipocampal;

4. O tratamento crônico por 10 dias com baixa dose de MP promove a persistência da memória aversiva;

5. Baixas doses de MP promovem o aumento do influxo de Ca^{2+} em cultura de células de hipocampo;

6. O tratamento crônico com baixa dose de MP facilita a indução da LTP no hipocampo.

Por fim, podemos concluir que o exercício agudo pode ser adotado como estratégia comportamental para a promoção da persistência das MLD. Além disso, o uso de glicocorticoides também tem potencial para ser utilizado como estratégia farmacológica que melhora a memória, entretanto seu efeito depende da dose, e estudos futuros são necessários para melhor elucidar os mecanismos de ação envolvidos, bem como seus efeitos colaterais.

PERSPECTIVAS

Os resultados dessa tese permitiram confirmar algumas hipóteses previamente levantadas pela literatura. Entretanto, a partir da demonstração de que uma única sessão de exercício físico após a aprendizagem, bem como o uso de baixas doses de MP são capaz de promover a persistência da memória de reconhecimento e da memória aversiva, respectivamente, novas perguntas surgiram com o intuito de melhor compreender os mecanismos envolvidos no efeito dessas duas estratégias na persistência da memória.

Assim, minhas perspectivas futuras envolvem a continuidade de trabalhos de pesquisa em colaboração com o Grupo de Pesquisa em Fisiologia da Unipampa para que possam ser investigadas questões como:

1. Verificar se o efeito do exercício físico agudo (uma única sessão) sobre a persistência se reproduz mesmo quando realizado com menor duração;
2. Verificar se o exercício físico agudo promove a persistência de outros tipos de aprendizado como, por exemplo, a persistência da extinção da memória aversiva;
3. Realizar uma pesquisa translacional, avaliando o efeito de uma única sessão de exercício físico após a aprendizagem em escolares;
4. Aprofundar a investigação dos mecanismos envolvidos no efeito da MP sobre a persistência da memória;
5. Verificar se o tratamento com a MP pode influenciar outros mecanismos cognitivos, como a extinção e persistência da extinção da memória aversiva.

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ANEXOS

Anexo I – Certificado de aprovação do projeto pelo CEUA-UNIPAMPA

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DO PAMPA
(Lei nº 11.640, de 11 de janeiro de 2008)

Pró-Reitoria de Pesquisa

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Fone: (55) 3413 4321, E-mail: ceua@unipampa.edu.br

CERTIFICADO DE APROVAÇÃO DE PROTOCOLO PARA USO DE ANIMAIS EM PESQUISA

Número de protocolo da CEUA: **021/2013**

Título: **UM ESTUDO DOS EFEITOS DA METIL PREDNISOLONA NA CONSOLIDAÇÃO E PERSISTÊNCIA DA MEMÓRIA EM RATOS**

Data da aprovação: **15/10/2013**

Período de vigência do projeto: De: **10/2013** Até: **10/2016**

Pesquisador: **PÂMELA BILLIG MELO CARPES**

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Alessandra S. K. Tamajusuku Neis
Professor Adjunto
Coordenadora da CEUA/UNIPAMPA

Anexo II – Comprovante de submissão de manuscrito à Revista Neurobiology of Learning and Memory

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