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EFEITO DA MORFINA SOBRE A PERSISTÊNCIA DA MEMÓRIA DE MEDO CONDICIONADO CONTEXTUAL EM RATOS

TESE DE DOUTORADO

Gerusa Paz Porto

Santa Maria, RS, Brasil

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EFEITO DA MORFINA SOBRE A PERSISTÊNCIA DA MEMÓRIA DE MEDO CONDICIONADO CONTEXTUAL EM RATOS

Gerusa Paz Porto

Tese de Doutorado, apresentada ao Programa de Pós-Graduação em Farmacologia, Área de Concentração em Farmacologia, da Universidade Federal de Santa Maria (UFSM), como requisito parcial para obtenção do grau de
Doutor em Farmacologia

Orientador: Prof. Dr. Carlos Fernando de Mello
Co-orientadora: Prof.ª Maribel Antonello Rubin

Santa Maria, RS, Brasil
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aprova a Tese de Doutorado

EFEITO DA MORFINA SOBRE A PERSISTÊNCIA DA MEMÓRIA DE MEDO CONDICIONADO CONTEXTUAL EM RATOS

elaborada por
Gerusa Paz Porto

como requisito parcial para obtenção do grau de
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COMISSÃO EXAMINADORA:

Carlos Fernando de Mello, Dr.
(Presidente/Orientador)

Bernardo Baldisserotto, Dr. (UFSM)

Clóvis Milton Duval Wannmacher, Dr. (UFRGS)

Luis Fernando Freire Royes, Dr. (UFSM)

Rafael Roesler, Dr. (UFRGS)

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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Farmacologia
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EFEITO DA MORFINA SOBRE A PERSISTÊNCIA DA MEMÓRIA DE MEDO CONDICIONADO CONTEXTUAL EM RATOS

AUTORA: GERUSA PAZ PORTO
ORIENTADOR: CARLOS FERNANDO DE MELO
CO-ORIENTADORA: MARIBEL ANTONELLO RUBIN
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Está bem estabelecido que os receptores opióides modulam redes cerebrais envolvidas no aprendizado e memória. Estudos utilizando várias tarefas de aprendizado têm mostrado que a morfina prejudica muitas formas de memória. Uma característica comum entre estes estudos é a administração de morfina pré ou pós-treinamento, a fim de verificar se este opióide altera o processo de consolidação da memória que ocorre até 6 horas após o aprendizado. Estudos recentes em animais têm descrito que manipulações farmacológicas realizadas 12 horas após o treino alteram a persistência da memória. Contudo, pouco se sabe sobre o papel da morfina nesta fase mnemônica. Portanto, o objetivo deste estudo foi avaliar o efeito de morfina sobre a persistência da memória de medo e investigar o seu possível mecanismo de ação. Ratos Wistar machos adultos foram submetidos à tarefa de medo condicionado ao contexto, sendo a memória desta tarefa medida como percentual de observações de imobilidade ("congelamento") 2, 7 ou 14 dias após o treinamento. A administração de morfina (10 mg/kg, i.p.) 12 horas após o treino não alterou as respostas de congelamento ao contexto de ratos testados 2 dias após o treino, mas reduziu o congelamento ao contexto nas sessões de teste realizadas 7 e 14 dias após o treino. O decréscimo do congelamento ao contexto induzido pela injeção de morfina 12 horas após o treino não foi revertido pela injeção de morfina (10 mg/kg, i.p.) antes da sessão de teste, indicando que os efeitos deste opióide não foram decorrentes de dependência de estado. A administração do antagonista de receptores opióides naloxona (1 mg/kg, i.p.) preveniu o efeito da morfina. A injeção intrahipocampal dos ativadores das enzimas adenilil ciclase e proteína quinase dependente de AMPc (PKA), forcolina (0,13 µg/sítio) e 8-Br-AMPc (7,5 µg/sítio), respectivamente, também preveniu o efeito de morfina. Os resultados sugerem que a morfina, administrada 12 horas após o treinamento do medo condicionado ao contexto não altera a formação da memória, mas prejudica a persistência do traço de memória por meio de mecanismos mediados pelos receptores opióides e a via de sinalização AMPc/PKA. Estes achados indicam que os opióides apresentam um potencial terapêutico na prevenção de doenças psiquiátricas relacionadas a memórias de medo.

Palavras-chave: Opióide. Manutenção da memória. PKA.

ABSTRACT

Ph. D. Thesis
Graduation Program in Pharmacology
Federal University of Santa Maria, RS, Brazil

EFFECT OF MORPHINE ON THE PERSISTENCE OF CONTEXTUAL FEAR CONDITIONING MEMORY IN RATS
AUTHOR: GERUSA PAZ PORTO
ADVISOR: CARLOS FERNANDO DE MELO
CO-ADVISOR: MARIBEL ANTONELLO RUBIN
Defense Place and Date: Santa Maria, January 16th, 2015.

It is established that opioids modulate brain networks involved in learning and memory. Studies using various learning tasks have shown that morphine impairs many forms of memory. A common feature of these studies is that morphine was administered pre- or post-training, in order to determine whether this opioid alters memory consolidation that occurs up to 6 hours after learning. Recent studies in animals have shown that pharmacological manipulations performed 12 hours after training alter memory persistence. However, little is known about the role of morphine in this mnemonic phase. Therefore, the aim of this study was to evaluate the effect of morphine on the persistence of fear memory and investigate its putative mechanism of action. Adult male rats were submitted to contextual fear conditioning task and the memory of this task was measured as percentage of observations of immobility ("freezing") 2, 7 or 14 days after training. Morphine administration (10 mg/kg, i.p.), 12 hours post-training, did not alter freezing to context of rats tested 2 days after training but reduced, at the dose of 10 mg/kg, contextual freezing in the testing session carried seven and fourteen days after training. The decrease of contextual freezing induced by injection of morphine 12 hours post-training was not reverted by the injection of morphine (10 mg/kg, i.p.) before the testing session, indicating that its effect was not due to state dependence. The administration of opioid receptor antagonist, naloxone (1 mg/kg, i.p.) inhibited the decrease of contextual freezing induced by morphine. The intrahippocampal injection of activators of enzymes adenylyl cyclase and cAMP-dependent protein kinase (PKA), forskolin (0.13 μg/site) and 8-Br-cAMP (7.5 μg /site), respectively, also prevented the effect of morphine. The findings suggest that morphine administered 12 hours after training of contextual fear conditioning does not alter the formation of memory, but impairs the persistence of the memory trace by mechanisms mediated by opioid receptors and cAMP/PKA signaling. These findings indicate that opioids have therapeutic potential in preventing psychiatric disorders related to fear memories.

Key-words: Opioid. Maintenance of memory. PKA

LISTA DE FIGURAS

Figura 1 - Estrutura da morfina... 12
Figura 2 - Principais metabólitos da morfina... 13
Figura 3 - Mecanismo de ação da morfina.. 19
Figura 4 - Efeito da administração de 8-Br AMPc sobre o efeito da morfina 12 horas após o treino em imagens representativas de *western blot* e análises densitométricas da pPKA, PKA total e razão pPKA/PKAtotal na fração citosol 1 hora após a injeção de morfina.................................. 88
Figura 5 - Efeito da administração de 8-Br AMPc sobre o efeito da morfina 12 horas após o treino em imagens representativas de *western blot* e análises densitométricas da pCREB, CREB total e razão pCREB/CREB total na fração nuclear 1 hora após a injeção de morfina........................ 89

ARTIGO 1

Figure 1 - Effect of morphine (3 and 10 mg/kg, i.p.) injection 12 h post-training on freezing to context.. 34
Figure 2 - Effect of morphine (10 mg/kg, i.p.) injection 12 h post-training on freezing to context in shocked and nonshocked animals………………. 35
Figure 3 - Lack of effect of morphine (10 mg/kg, i.p.) injection 6, 9, or 24 h post-training on freezing to context……………………………………………... 36
Figure 4 - Effect of morphine (10mg/kg, i.p.) injection 30 min bEfeito da administração de 8-Br AMPc sobre o efeito da morfina 12 horas após o treino em imagens representativas de *western blot* e análises densitométricas da pCREB, CREB total e razão pCREB/CREB total na fração nuclear 1 hora após a injeção de morfinaefore test on freezing to context of animals injected with morphine (10 mg/kg, i.p.)12 h post-Training... 37
Figure 5 - Effect of naloxone (1 mg/kg, i.p., 30 min before morphine) on morphine (10 mg/kg, i.p., 12 h post-training)-induced decrease of freezing score... 38

ARTIGO 2

Figure 1 - Effect of forskolin (0.13μg/μl, intrahippocampal) injected 30 min before morphine (10 mg/kg, i.p.) 12 hours post-training on freezing to context of animals.. 60
Figure 2 - Effect of 8-Br-cAMP (7.5μg/μl, intrahippocampal) injected 30 min before morphine (10 mg/kg, i.p.) 12 hours post-training on freezing to context of animals……………………………………………………………………….. 60

LISTA DE ABREVIATURAS E SIGLAS

AMPc	Adenosina monofosfato cíclica
AP5	Antagonista dos receptores NMDA
BDNF	Fator neurotrófico derivado do cérebro
8 Br-AMPc	Ativador da proteína quinase A
CaMKII	Proteína quinase II dependente de cálcio/calmodulina
CREB	Proteína ligante ao elemento de resposta ao AMPc
ERK	Proteína quinase regulada por sinais extracelulares
GDP	Guanosina difosfato
GTP	Guanosina trifosfato
LY294002	Inibidor da PI3K
M3G	Morfina-3-glicuronídeo
M6G	Morfina-6-glicuronídeo
M3S	Morfina-3-sulfato
NMDA	N-metil-D-aspartato
PBS	Tampão fosfato salino
PI3K	Fosfatidilinositol-3-quinase
PKA	Proteína quinase A
PKI	Inibidor da proteína quinase A
SKF38393	Antagonista dos receptores D1
TLR4	Receptores tipo *Toll 4*
U0126	Inibidor da proteína quinase ativada por mitógeno
U-73122	Inibidor da fosfolipase C

SUMÁRIO

INTRODUÇÃO.. 9

ARTIGO 1- EFFECT OF MORPHINE ON THE PERSISTENCE OF LONG TERM MEMORY IN RATS... 25

Abstract.. 27

Introduction.. 28

Methods.. 29

Results.. 33

Discussion.. 39

References... 42

ARTIGO 2 - MORPHINE IMPAIRS THE PERSISTENCE OF MEMORY VIA A cAMP/PKA-DEPENDENT PATHWAY.. 46

Abstract.. 48

Introduction.. 49

Material and methods... 50

Results.. 52

Discussion.. 53

Conclusion.. 55

References... 56

DISCUSSÃO.. 61

CONCLUSÕES.. 65

REFERÊNCIAS... 66

APÊNDICE... 85

1 INTRODUÇÃO

Memória é o processo de retenção e reconstrução de conhecimentos aprendidos (KANDEL et al., 2014). Conforme a emoção presente no momento do aprendizado, pode apresentar diversos níveis de significância e até durar por um longo tempo (FEDERMAN et al., 2013; ROOZENDAAL e McGAUGH, 2011). De acordo com o tempo que perdura, a memória pode ser classificada como de imediata, de curta ou de longa duração (CAMMAROTA et al., 2004).

As memórias de longa duração são aquelas que possuem como característica a persistência ao longo do tempo (BEKINSCHTEIN et al., 2007; 2010). Para que sejam armazenadas por um longo prazo, é preciso que ocorra uma cascata de eventos que promova mudanças estruturais nos neurônios, decorrente da transcrição gênica que origina proteínas para o crescimento de novas sinapses e transcritos de RNA reguladores da expressão gênica (ALBERINI e KANDEL, 2014).

Estudos realizados com roedores têm mostrado que a manipulação farmacológica, no período próximo às 12 horas seguintes ao aprendizado, pode interferir na persistência da memória de longa duração (BEKINSCHTEIN et al., 2007; BEKINSCHTEIN et al., 2010; ROSSATO et al., 2009). Estes estudos têm implicado os sistemas serotoninérgico, noradrenérgico e colinérgico nesta fase tardia de modulação da memória, uma vez que a administração da venlafaxina, um inibidor da recaptação de serotonina e noradrenalina, e a administração do antagonista de receptor muscarínico escopolamina, tornam as memórias adquiridas menos persistentes (PARFITT et al., 2012a; SLIPCZUK et al., 2013). Ao mesmo tempo, tais estudos têm verificado que a estimulação da via de sinalização da proteína quinase dependente de adenosina monofosfato cíclica (PKA) (ROSSATO et al., 2009) está envolvida nos mecanismos da persistência da memória, a despeito de outras vias de sinalização como a da fosfatidilinositol-3-quinase e da proteína alvo da rapamicina em mamiferos (PI3K/mTOR), da CamKII e da fosfolipase C (BEKINSCHTEIN et al., 2008; 2010).

Um número significativo de estudos tem mostrado que a administração de morfina, um dos principais alcalóides do ópio, prejudica a memória de

longa duração de diferentes tarefas em roedores (ARDJMAND et al., 2011; FARAHMANDFAR et al., 2010; IZQUIERDO, 1979; WESTBROOK e GOOD, 1997). Nestes estudos, morfina foi administrada antes ou logo após o treino, e os animais foram testados aproximadamente 24 horas após. Tais experimentos buscavam determinar se a morfina interfere nos processos de consolidação que ocorrem até aproximadamente 6 horas após o aprendizado (CAMMAROTA et al., 2004) A despeito destes estudos que revelaram um efeito redutor da injeção de morfina até 6 horas pós-treino sobre a memória, o efeito da administração tardia de opióides, dentro da janela temporal associada à modulação da persistência da memória, permanece não determinado.

A descoberta de fármacos que possam prejudicar a persistência da memória poderia ser uma estratégia para evitar que memórias de medo consolidadas sejam responsáveis pela ocorrência de transtornos de ansiedade que tragam consequências sociais e ocupacionais desfavoráveis aos indivíduos acometidos. A administração de um fármaco após um evento traumático poderia por exemplo, ser uma estratégia útil, para prevenir o desenvolvimento do transtorno do estresse pós traumático.

No presente estudo foi investigado se a morfina prejudica a persistência da memória de medo condicionado ao contexto. Além disto, investigou-se o papel da adenilil ciclase e da via de sinalização da PKA neste efeito.

1.2 Ópio

O ópio é o látex extraído das cápsulas da *Papaver sominiferum*, uma planta da família das Papaveráceas, conhecida popularmente como papoula e que é caracterizada por apresentar folhas solitárias e frutos encapsulados. O ópio e seus derivados vêm sendo utilizados com finalidade terapêutica, desde os primórdios da humanidade (DUARTE, 2005).

Em 4000 A.C., a papoula era referida nos ideogramas dos Sumérios como “planta da alegria” devido às suas propriedades euforizantes. Papiros médicos egípicios indicam que muitas fórmulas terapêuticas continham ópio. No Papiro de Ebers (1552 a.C.), o ópio era recomendado para promover a sedação em crianças. Na Grécia antiga, Hipócrates, considerado o pai da medicina, prescrevia um suco de papoula, o mecônio, como narcótico e para tratar a leucorréia (DUARTE, 2005).

Os árabes dominaram o comércio no Oceano Índico e introduziram o ópio na Índia e posteriormente na China. Entre os séculos X e XIII, o ópio se espalhou da Ásia para todas as partes da Europa (BROWNSTEIN, 1993). Em 1680, o médico Inglês Thomas Sydenham criou um láudano, uma preparação líquida de ópio, vinho de cereja, açafrão, cravo e canela (BARAKA, 2000). No início do século XVI, manuscritos já surgiram relatando abuso e tolerância ao ópio na Turquia, Egito, Alemanha e Inglaterra (BROWNSTEIN, 1993). Em 1721, foi publicada na Farmacopeia de Londres, uma fórmula denominada de Elixir Paregórico, contendo ópio, mel, cânfora, anis e vinho (DUARTE, 2005).

Em 1806, o farmacêutico alemão Freidrich Wilhelm Adam Sertürner isolou, pela primeira vez, o alcaloide do ópio morfina, pela dissolução do ópio em ácido, seguida da neutralização com amônia (BARAKA, 1982). Sertürner inicialmente denominou o alcaloide de *principium somniferum* em homenagem a Morfeu, o Deus grego dos sonhos (HAMILTON e BASKETT, 2000).

Com a invenção da seringa hipodérmica em meados de 1850, a morfina começou a ser usada para pequenos procedimentos cirúrgicos, para a dor pós-operatória e a dor crônica e como um adjuvante de anestésicos gerais (BROWNSTEIN, 1993; DORRINGTON e POOLE, 2013). Em meados de 1860 a morfina foi usada em hospitais psiquiátricos para controlar rapidamente a agitação e agressividade (BAN, 2001).

Após a descoberta da morfina, outros alcaloides do ópio foram isolados, assim como foram desenvolvidos uma ampla gama de agonistas e antagonistas opióides sintéticos (BROWNSTEIN, 1993). A relação entre a estrutura química e a atividade farmacológica destes compostos levou à proposta de sítios de reconhecimento específicos, que resultou na descoberta dos receptores opióides (PASTERNAK, 2014).

1.3 Morfina

A morfina é um alcaloide benzilisoquinolínico que atua diretamente sobre o sistema nervoso central para aliviar a dor. Ela representa o mais prevalente e principal agente ativo do ópio e constitui 10 a 20% do peso total das sementes secas da papoula, juntamente com os compostos fenantrênicos codeína (0,5%), tebaína (0,2%), papaverina (1,0%), e noscapina (6,0%) (ANTON et al., 2009).

Em relação à estrutura, a morfina é constituída por um anel benzênico com um grupo hidroxila fenólico na posição 3 e um grupo hidroxila alcoólico na posição 6, além de um átomo de nitrogênio terciário, o qual é crucial para as suas propriedades analgésicas, pois é responsável pela sua passagem pela barreira hematoencefálica (TRESCOT et al., 2008) (Figura 1).

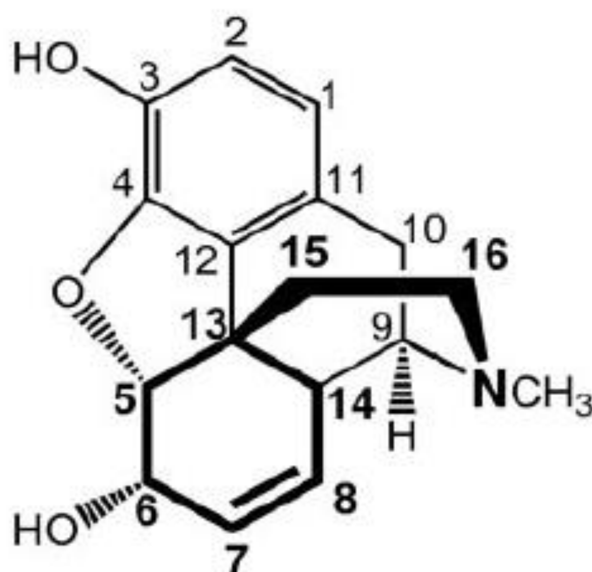

Figura 1 – Estrutura da morfina. Fonte: BOETTCHER et al., 2005.

A metabolização da morfina ocorre no fígado. Neste órgão, por ação de várias isoformas de enzimas UDP—glucoronosiltransferases, mas principalmente da isoforma UGT2B7, cerca de 90% da morfina é convertida no metabólito morfina-3-glicuronídeo (M3G), e 10% em morfina-6-glicuronídeo (M6G) (DE GREGORI et al., 2012) (Figura 2).

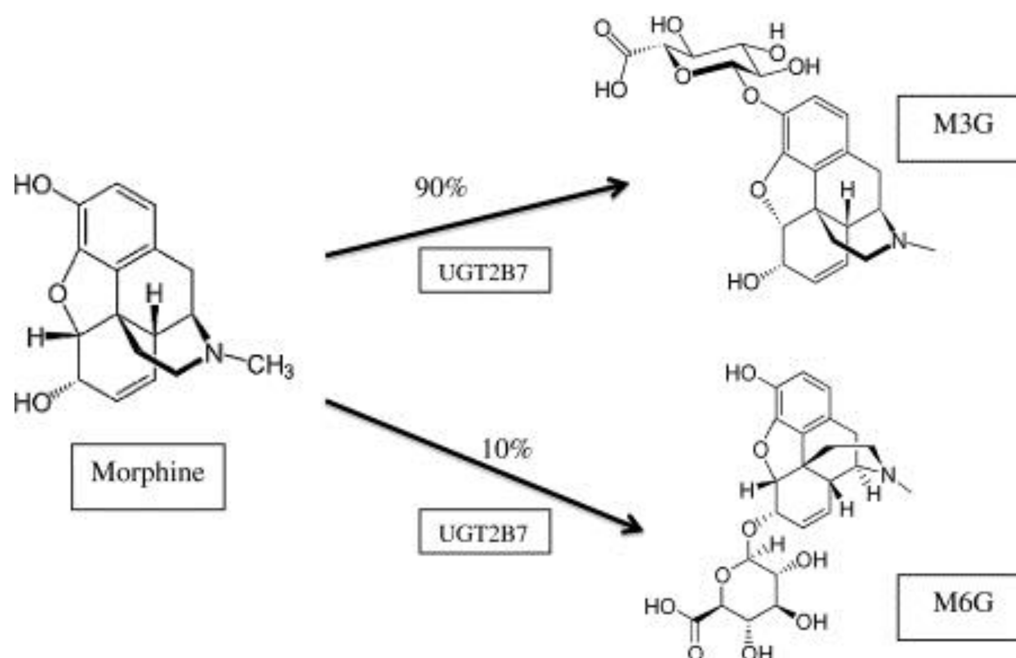

Figura 2 – Principais metabólitos da morfina: morfina-3-glicuronídeo e morfina-6-glicuronídeo. Fonte: DE GREGORI et al., 2012.

O metabólito M6G é um agonista de receptores opióides (FRANCES et al., 1990; PASTERNAK et al., 1987) que apresenta atividade analgésica influenciada pela idade, sexo e pelas variantes do gene que codifica estes receptores (WITTWER e KERN, 2006). Estudos realizados com animais demonstraram que a M6G possui maior potência analgésica que a morfina (COLLER et al., 2009; PAUL et al., 1989). Já o metabólito M3G não possui atividade analgésica (HEWETT et al., 1993; PENSON et al., 2000) e não se liga em receptores opióides (BARTLETT e SMITH, 1995; LOSER et al., 1996; ULENS et al., 2001). Em ratos, ao contrário do que ocorre em humanos (PENSON et al., 2000), o M3G antagoniza a analgesia induzida pela morfina (GONG et al., 1992; SMITH et al., 1990) e induz alodínia após a

administração intratecal ao se ligar a receptores "*toll–like*" (TLR4) (LEWIS et al., 2010) e à proteína 2 acessória mieloide (MD2) (KOMATSU et al., 2009). Além disto, o M3G apresenta efeito neuroexcitatório, provavelmente mediado pela via de sinalização TLR4 (DUE et al., 2012), sendo responsável pela ocorrência de mioclonias (BARTLETT et al., 1994; HALLIDAY *et al.*, 1999; SMITH, 2000).

A morfina pode ser também metabolizada a morfina-3-sulfato (M3S) (YEH *et al.*, 1977), morfina-6-sulfato (FROLICH et al., 2011) e hidromorfona (CONE *et al.*, 2008; HUGHES *et al.*, 2012; WASAN et al., 2008), a qual exibe alta atividade analgésica (KUMAR et al., 2008). Além disto, este alcalóide pode ser desmetilado pelas enzimas CYP3A4 e CYP2C8 à normorfina que igualmente possui atividade analgésica (FROLICH et al., 2011; PROJEAN et al., 2003; SULLIVAN et al., 1989).

Evidências indicam também que o metabolismo deste opióide possa ocorrer no cérebro. Estudos com homogeneizados de cérebro humano têm mostrado que estes produzem os metabólitos M3G e M6G a partir de concentrações nanomolares de morfina (YAMADA et al., 2003). Adicionalmente, as isoformas UGT1A6 e 2B7 das UDP-glucoronosiltransferases, responsáveis pelo metabolismo no fígado também são encontradas em neurônios e astrócitos (BUCKLEY e KLAASSEN, 2007; KING et al., 1999; NAGANO et al., 2000; SULEMAN et al., 1998).

1.4 Receptores opióides

A morfina se liga aos receptores opióides, os quais denominam-se μ (mu) (PASTERNAK e PAN, 2011), κ (kappa) e δ (delta) (DHAWAN et al., 1996; GRANIER, 2012; MARTIN et al., 1976). Estes receptores são

estruturalmente compostos por 7 domínios transmembrana, com três alças extracelulares e três intracelulares. A porção C-terminal se localiza no meio intracelular e a porção N-terminal no extracelular (DAVIS et al., 2005; PASTERNAK, 2010; TRESCOT et al., 2008). Os receptores opióides apresentam uma homologia de 65 a 70% e divergem na segunda e terceira alças extracelulares e nos grupamentos N e C terminal (JORDAN e DEVI, 1998). Estudos de cristalografia revelaram que tais receptores apresentam hélices transmembrana alinhadas de uma forma similar ao redor do sitio de ligação, e que possuem um resíduo de aspartato na terceira hélice, que interage com o ligante por meio de ligações iônicas (COX, 2013). Os três subtipos de receptores são acoplados a uma proteína Gi/o, sensível à toxina pertussis (FILIZOLA e DEVI, 2013), como veremos em mais detalhe, a seguir.

Os receptores mu são o principal alvo da morfina e dos peptídeos opióides endógenos denominados beta-endorfinas (DHAWAN et al., 1996; KIEFFER, 1999; VALLEJO et al., 2011). Embora estudos de atividade com radioligantes, tenham classificado tais receptores em subtipos µ1 e µ2, de acordo com diferenças na região C terminal (PASTERNAK e PAN, 2011; WOLOZIN e PASTERNAK, 1981), abordagens na área de biologia molecular já identificaram mais de 30 variantes em camundongos, 16 em ratos e 19 em humanos (PASTERNAK, 2014). No sistema nervoso, estes receptores estão localizados no núcleo caudado, putâmen, neocortex, tálamo, núcleo acumbens, amígdala, hipocampo, no corno dorsal da medula espinhal e nos nervos periféricos (DHAWAN et al., 1996; DILTS e KALIVAS, 1989; LE MERRER et al., 2009; PASTERNAK, 2010). Quando estimulados, os receptores µ inibem a nocicepção, o ciclo respiratório e a motilidade intestinal; causam euforia, dependência física, tolerância, imunossupressão e êmese (ALFARAS-MELAINIS et al., 2009; AL-HASANI e BRUCHAS, 2011; CONTET et al., 2004; KIEFFER, 1999; MATTHES et al., 1996; PASTERNAK e PAN, 2011; TRESCOT et al., 2008; VALLEJO et al., 2011).

Os receptores kappa são o sítio alvo dos peptídeos opióides endógenos dinorfinas A *(*COX*,* 2013; DHAWAN et al., 1996; WUSTER et al., 1981). Estes receptores apresentam 3 subtipos: K1a, K1b e K2 *(*ZUKIN et al., 1988) e, quando ativados, aumentam a diurese, reduzem a temperatura,

elevam os níveis de corticosteroides, provocam analgesia, sedação e efeitos disfóricos (CRAFT et al., 2000; DHAWAN et al., 1996; PASTERNAK e PAN, 2011; VALLEJO et al., 2011; VONVOIGTLANDER et al., 1983; WANG et al., 2010). Tais receptores estão localizados principalmente no hipotálamo, pituitária, amígdala, área tegmental ventral, núcleo caudado, putâmen e núcleo acumbens (DHAWAN et al., 1996; LE MERRER et al., 2009; MENG et al., 1993).

Os receptores delta, alvo dos peptídeos endógenos encefalinas (PASTERNAK e PAN, 2011; WUSTER et al., 1981), foram clonados em 1992, (EVANS et al., 1992; KIEFFER et al., 1992) seguidos dos mu (CHEN et al., 1993; THOMPSON et al., 1993; WANG *et al.*, 1993) e kappa (CHEN *et al.*, 1994) em 1993 e 1994, respectivamente. Estes receptores existem na forma de 2 subtipos: δ1 e δ2 (JIANG et al., 1991) e estão localizados em regiões corticais, núcleo acumbens, amígdala e corpo estriado (BAKOTA et al., 1998; BLACKBURN et al., 1988; DHAWAN et al., 1996; GOUARDERES et al., 1993; LE MERRER et al., 2009; MCLEAN et al., 1986). Quando estimulados, os receptores delta provocam aumento da atividade motora, analgesia, tolerância e redução da motilidade gastrointestinal (DHAWAN et al., 1996; KEST et al., 1996; PASTERNAK e PAN, 2011; VALLEJO et al., 2011; ZHU et al., 1999).

1.5 Mecanismo de ação da morfina

A ligação da morfina aos receptores opióides induz uma mudança conformacional no receptor que é seguida pelo seu acoplamento à proteína Gi/o, que se encontra na forma inativa (BURFORD et al., 1998; BURFORD et al., 2000; GALEOTTI et al., 2001; MASSOTTE et al., 2002; RAFFA et al.,

1994; SANCHEZ-BLAZQUEZ et al., 1995; SANCHEZ-BLAZQUEZ et al., 2001). Nesta condição, a proteína Gi/o é constituída por um complexo heterotrimérico composto pela união das subunidades α, associada com guanosina difosfato (GDP), β e γ. Após o acoplamento com o receptor, ocorre a dissociação de GDP da subunidade α e a sua troca por guanosina trifosfato (GTP), seguida da dissociação do complexo α-GTP do complexo βγ (AL-HASANI e BRUCHAS, 2011; HALES, 2011; STANDIFER e PASTERNAK, 1997). O complexo α-GTP promove inibição da enzima adenilil ciclase e, consequentemente, redução dos níveis intracelulares de adenosina monofosfato cíclico (AMPc) (FELDBERG e SILVA, 1977; LAW et al., 2000; PIROS et al., 1995; SHARMA et al., 1975; SHARMA et al., 1977; TRABER et al., 1975), levando a uma redução da atividade da PKA (BACHRACH et al., 1979; VETTER et al., 2006) e da ativação da proteína ligante do elemento responsivo ao AMPc (CREB) (GUITART et al., 1992). O complexo formado pelas subunidades βγ promove a abertura dos canais de potássio, levando ao efluxo destes íons e à hiperpolarização do neurônio, com consequente redução da excitabilidade neuronal (DASCAL, 2001; IPPOLITO et al., 2002; TORRECILLA et al., 2008; YANG et al., 1998). Adicionalmente, o complexo βγ pode promover o fechamento dos canais de cálcio dependentes de voltagem dos tipos N, P, Q, L, bem como do tipo T, localizados em terminais pré-sinápticos e relacionados à liberação de neurotransmissores (PIROS et al., 1995; SCHROEDER et al., 1991; TSUNOO et al., 1986; WU et al., 2004; ZOLLNER e STEIN, 2007). A reativação da enzima adenilil ciclase, o fechamento dos canais de potássio e a abertura dos canais cálcio ocorre quando há hidrólise do GTP para GDP, e novamente a união da subunidade α-GDP com o complexo βγ (AL-HASANI e BRUCHAS, 2011) (Figura 3).

A estimulação de receptores opióides pela morfina pode também ativar a proteína quinase II dependente de cálcio/calmodulina (CaMKII) (LOU et al., 1999) e, também por meio de interações do complexo βγ, estimular a via de sinalização da fosfolipase C (NARITA et al., 2001). Adicionalmente, embora sem evidências diretas de que seja por meio de receptores opióides, estudos têm sugerido que a morfina ativa as vias de sinalização do

fosfatidilinositol-3-quinase (NARITA, 2003; NARITA et al., 2004) e da ERK (ASENSIO et al., 2006; SEO et al., 2008).

Um aspecto importante a ressaltar é que os mecanismos citados envolvendo a via de sinalização da enzima adenilil ciclase estão relacionados à ação aguda da morfina. A administração crônica de morfina está associada a uma ativação da enzima adenilil ciclase (CHAKRABARTI et al., 1998; DUMAN et al., 1988; JOLAS et al., 2000; TERWILLIGER et al., 1991), aumento dos níveis de AMPc (KURIYAMA et al., 1978), ativação da PKA (NESTLER e TALLMAN, 1988; TERWILLIGER et al., 1991) e da CREB (CAO et al., 2010; REN et al., 2013).

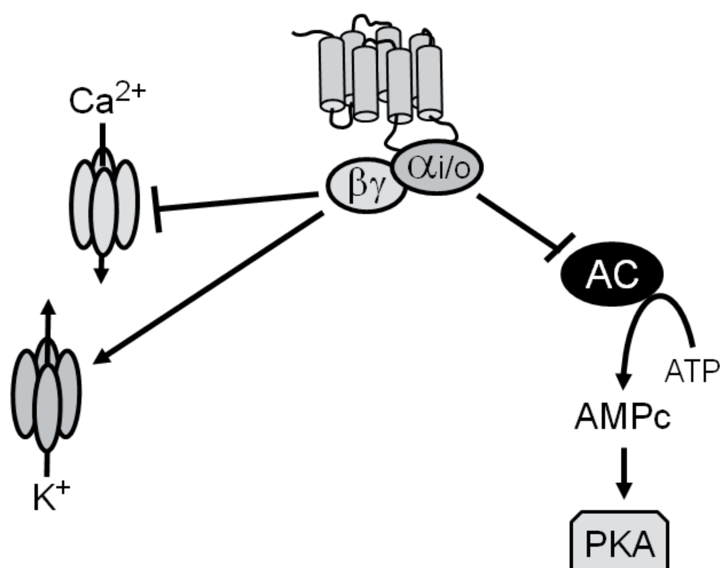

Figura 3 – Mecanismo de ação da morfina. ↓, ativação; ⊥, inibição; AC: adenilil ciclase; AMPc: adenosina monofosfato cíclica. Fonte: adaptado de Aquino-Miranda e Arias-Montano, 2012.

1.6 Efeitos farmacológicos da morfina

Devido às suas propriedades analgésicas, a morfina permanece como um dos fármacos mais frequentemente prescritos para dores moderadas e intensas, mesmo 200 anos após a sua descoberta (VALLEJO et al., 2011). Embora promova outros efeitos desejáveis, como a sensação de bem estar, sedação (BION et al., 1986) e efeito antitussígeno (ADCOCK et al., 1988; KAMEI et al., 1989; KARLSSON et al., 1990; YANAURA et al., 1975), a morfina pode promover vários efeitos adversos agudos e crônicos. Os efeitos agudos incluem náuseas, vômitos (CHINACHOTI et al., 2013; GUPTA et al., 1989; WIRZ et al., 2008; YOSHIKAWA e YOSHIDA, 2002), depressão respiratória (DAHAN et al., 2013; KIMURA et al., 2013; SAM et al., 2011), constipação (HOLZER, 2009; NORTH e TONINI, 1977) e déficit de memória (KAMBOJ et al., 2005; NAGHIBI et al., 2012; ZARRINDAST et al., 2013). Os efeitos decorrentes do uso crônico compreendem tolerância (SHEN et al.,

2012), dependência (BACK et al., 2011; GHAZAL et al., 2013), depressão do sistema imunológico (BROWN et al., 2012; NUGENT et al., 2011), alterações na neurogênese (ZHENG et al., 2013) e efeitos neuroinflamatórios (TUMATI et al., 2012).

1.6.1 Efeitos da morfina sobre a memória

Embora algumas investigações tenham constatado ausência de efeitos sobre a memória (BEATTY, 1983; KAHVECI et al., 2006) e outras terem verificado efeitos modulatórios positivos (MONDADORI e WASER, 1979; NAKAMA-KITAMURA e DOE, 2003; SHIIGI e KANETO, 1990; SHIIGI et al., 1990), a maioria dos estudos sugere que este opióide promove amnésia (CASTELLANO, 1975; CASTELLANO et al., 1984; FARAHMANDFAR et al., 2010; NASEHI et al., 2013; WESTBROOK et al., 1997; ZARRINDAST et al., 2013).

Vários estudos têm revelado que a administração sistêmica de morfina, em doses que variam de 1 a 15 mg/kg, antes das sessões de treinamento, prejudica a memória nas tarefas de esquiva inibitória (BABAEI et al., 2012; LU et al., 2010; NAGHIBI et al., 2012; NASEHI et al., 2013; REZAYOF et al., 2006; ZARRINDAST et al., 2006; ZARRINDAST et al., 2013) labirinto aquático de Morris (FARAHMANDFAR et al., 2010; ZHU et al., 2011) e labirinto em Y (BABAEI et al., 2012; CASTELLANO, 1975; MA et al., 2007). Adicionalmente, a administração direta no núcleo acumbens, septo medial e na amígdala de ratos, prejudica a memória nas tarefas de medo condicionado ao contexto (WESTBROOK et al., 1997), alternância espontânea (RAGOZZINO et al., 1992) e esquiva inibitória (RAGOZZINO e GOLD, 1994). Evidências têm mostrado que alguns destes efeitos estão relacionados à interação com receptores opióides (CASTELLANO, 1975; ZHU et al., 2011) e com o sistema dopaminérgico (REZAYOF et al., 2006), uma vez que são revertidos por naloxona e prevenidos pela administração do agonista dopaminérgico apomorfina, respectivamente.

Ainda sob a ótica de um efeito deletério sobre processos mnemônicos, vários investigadores têm mostrado que a morfina, quando administrada após o treino, causa amnésia em animais submetidos às tarefas de esquiva inibitória (BARATTI et al., 1984; BIANCHI et al., 2010; CASTELLANO et al., 1984; CASTELLANO et al., 1994; CESTARI e CASTELLANO, 1997; COSTANZI et al., 2004; GALEOTTI et al., 2001; INTROINI et al., 1985; ZARRINDAST et al., 2002; ZARRINDAST et al., 2005; ZARRINDAST et al., 2011; ZARRINDAST et al., 2013), esquiva ativa (IZQUIERDO, 1979) e medo condicionado (RUDY et al., 1999).

Os efeitos prejudiciais da administração pós-treino de morfina na esquiva inibitória parecem ser devidos, pelo menos em parte, a uma ação na área tegmental ventral (ZARRINDAST et al., 2005). A estimulação de receptores opióides (CASTELLANO et al., 1984), a ativação de proteínas Gi1 e Gi3 (GALEOTTI et al., 2001) e a ativação da fosfolipase C β3 (BIANCHI et al., 2010) têm sido implicadas neste efeito da morfina. Da mesma forma, um papel para os sistemas glutamatérgico (CESTARI e CASTELLANO, 1997) dopaminérgico (CASTELLANO et al., 1994) e canabinóide (COSTANZI et al., 2004) também tem sido proposto, pois a administração dos antagonistas dos receptores glutamatérgico MK801 e dopaminérgico SCH 2339 e do agonista canabinóide anandamida, em doses que não têm efeito per se, potencializam o prejuízo de memória induzido por morfina.

Todos os estudos mencionados acima investigaram o efeito da administração de morfina nas fases iniciais da consolidação da memória, que classicamente se estende até seis horas após a aquisição da tarefa (CAMMAROTA et al., 2004). Contudo, estudos recentes sugerem a existência de uma segunda janela temporal, em torno de 12 horas pós-treino, cuja manipulação farmacológica está associada ao prejuízo ou melhora na persistência da memória de longo prazo. Neste contexto, evidências têm indicado que a administração na região CA1 do hipocampo do inibidor de síntese proteica anisomicina (BEKINSCHTEIN et al., 2007; 2008) e de antagonistas de receptores NMDA, como o AP5, (ROSSATO et al., 2009), beta adrenérgicos como o propranolol (PARFITT et al., 2012a) e nicotínicos como a mecamilamina (PARFITT et al., 2012b), são capazes de prejudicar a memória. O estudo de Rossato e colaboradores (2009) sugeriu a participação

dos receptores dopaminérgicos na persistência da memória, pois a administração via intrahipocampal do agonista de receptores D1, SKF38393, no período de 12 horas após o treinamento, favorece a manutenção do traço mnemônico. Este estudo também sugeriu a ativação da via de sinalização da PKA neste efeito, pois a administração do ativador da enzima PKA, 8 Br-AMPc, e do inibidor, PKI, no hipocampo, aumenta e reduz, respectivamente, a persistência da memória.

Enquanto que estudos de Bekinschtein e colaboradores, (2007) e Rossato e colaboradores, (2009) têm sugerido a participação do fator neurotrófico derivado do encéfalo (BDNF) na persistência da memória, Bekinschtein et al. (2008), têm sugerido a participação da via de sinalização da ERK, e descartado a participação das vias de sinalização da enzima PI3K/mTOR e da fosfolipase C. Evidências obtidas de tais estudos, têm revelado que a administração intrahipocampal de anticorpo anti-BDNF (BEKINSCHTEIN et al., 2007; ROSSATO et al., 2009) e do inibidor da proteína quinase ativada por mitógeno, U0126 12 horas após o treinamento, prejudica a persistência da memória, enquanto que a administração do inibidor da PI3K, LY294002 e do inibidor da fosfolipase C, U-73122, não altera a retenção da memória 7 dias após o treinamento (BEKINSCHTEIN et al., 2008).

Dentre as possibilidades de atuação da morfina anteriormente citadas estão: a inibição da enzima adenilil ciclase com consequentemente redução da ativação da PKA, a ativação das vias de sinalização da fosfolipase C, fosfatidilinositol-3-quinase e ERK, bem como a ativação da CaMKII. Considerando que há estudos que descartam o envolvimento da proteína CamKII e das vias da fosfatidilinositol-3-quinase e da fosfolipase C nos eventos hipocampais que ocorrem 12 horas após o treino envolvidos na persistência da memória e que poucos estudos tem implicado a via da ERK nos efeitos agudos da morfina, é plausível que a administração tardia de morfina (12 horas pós-treino) prejudique a persistência da memória, por meio da diminuição da atividade da enzima adenilil ciclase e redução da ativação da via de sinalização da PKA no hipocampo**.** Como até o presente momento nenhum estudo investigou tal efeito da morfina, tampouco os mecanismos pós-transducionais nele envolvidos, neste estudo avaliou-se o efeito da

administração tardia de morfina sobre a persistência da memória e o envolvimento da adenilil ciclase e da PKA neste efeito.

2 OBJETIVOS

2.1 Objetivo Geral

Investigar o efeito da morfina sobre a persistência da memória de medo contextual de ratos e o seu respectivo mecanismo de ação.

2.2 Objetivos Específicos

- Verificar o efeito da morfina sobre a persistência da memória de ratos com a utilização da tarefa de condicionamento de medo ao contexto;

- Determinar se o efeito da morfina sobre a persistência da memória contextual é mediado pela ativação de receptores opióides;

- Avaliar se o possível efeito deletério da morfina sobre a persistência da memória contextual é decorrente do fenômeno de dependência de estado;

- Avaliar o envolvimento da via de sinalização AMPc/PKA/CREB no possível efeito deletério da morfina sobre a persistência da memória contextual;

ARTIGO 1

Original paper

Effect of morphine on the persistence of long term memory in rats

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EFFECT OF MORPHINE ON THE PERSISTENCE OF LONG-TERM MEMORY IN RATS

Gerusa Paz Porto[a,1], Laura Hautrive Milanesi[a,1],
Maribel Antonello Rubin[a,b], Carlos Fernando Mello[a,*]

[a]Graduation Program in Pharmacology, Department of Physiology and Pharmacology, Center of Health Sciences, Universidade Federal de Santa Maria, Santa Maria, RS, 97105-900, Brazil.
[b] Department of Chemistry, Center of Exact and Natural Sciences, Universidade Federal de Santa Maria, Santa Maria, RS, 97105-900, Brazil.

*Corresponding author. Fax: + 55 55 3220 8870.
E-mail adress: cf.mello@smail.ufsm.br (C.F. Mello).
[1] These authors contributed equally to this work.

Abstract

Rationale Current evidence suggests that pharmacological manipulation around

12 hours after training alters the persistence of long-term memory. However, no
study has addressed whether opioids modulate the persistence of fear. The
current study examined whether morphine alters the persistence of the memory
of contextual fear conditioning

Methods Male adult Wistar rats were injected with saline (NaCl 0.9%, intraperitoneally (i.p.)) or morphine (3 and/or 10 mg/kg, i.p.) 6, 9, 12 or 24 h post-training and tested 2 or 7 days after training, when freezing responses were assessed. The involvement of state dependence and opioid receptors in the effect of morphine was investigated by respectively injecting naloxone (1 mg/kg, i.p.) 30 min before morphine, and morphine (10 mg/kg, i.p.) 30 min before testing.

Results Morphine (10 mg/kg, i.p., 12 h post-training) did not alter freezing to context in animals tested 2 days after training, but impaired freezing to context when testing was carried out 7 or 14 days after training. Morphine (10 mg/kg, i.p.) administration 6, 9 or 24 h post-training did not alter freezing measured 2 or 7 days after training. Pre-test morphine improved recall, but did not alter the deleterious effect of 12 h post-training morphine. The deleterious effect of morphine was prevented by naloxone, indicating that opioid receptors are involved in this effect.

Conclusions Our findings indicate an inhibitory role for opioid receptors in memory persistence. This is relevant from both the experimental and clinical point of views, since it may have implications for the prevention post-traumatic stress disorder (PTSD).

Keywords: Morphine, Long-term memory, Persistence, Fear conditioning, Rat.

Introduction

Long term memories are those that last several hours, days, weeks or even longer periods (Davis and Squire 1984). The formation of these memories requires an early consolidation process which extends up to 6 h post-training and includes activation of gene expression, protein synthesis and resulting synaptic plasticity (Izquierdo et al. 2004; Kandel 2001; Schafe et al. 2001). A significant body of evidence has indicated that during this period memories can be negatively modulated by opioids (Bodnar 2011; Castellano et al. 1988; Galizio et al. 2013; Goeldner et al. 2009; Tramullas et al. 2008; Ukai et al. 2001). In line with this view, morphine impairs not only the consolidation but also the acquisition of long term memory in several tasks, such as radial, Y, and Morris water maze (Castellano 1975; McNamara and Skelton 1992; Miladi-Gorji et al. 2011; Spain and Newsom 1991) active and passive avoidance (Aguilar et al. 1998; Castellano et al. 1994; Introini et al. 1985; Izquierdo 1979; Ragozzino and Gold 1994) and contextual fear conditioning (McNally and Westbrook 2003). Notwithstanding, a few studies have reported that morphine may have no effect on memory acquisition (Saha et al. 1990) or improve memory consolidation (Castellano and Pavone 1984; Mondadori and Waser 1979).

Accumulating evidence suggests that memory persistence, one exclusive feature of long-term memories (Bekinschtein et al. 2010), depends on protein synthesis that occurs around 12 h after acquisition. During this period, there is activation of NMDA receptors in the ventral tegmental area (VTA) followed by activation of dopaminergic receptors in the dorsal hippocampus (Rossato et al. 2009), culminating with an increase of brain-derived neurotrophic factor (BDNF) expression in the CA1 region (Bekinschtein et al. 2008). Accordingly, learning-induced changes in hippocampal c-Fos, Homer 1a, Akt, CamKIIa, and ERK2 levels around 18–24 h after inhibitory avoidance training have been also described in the hippocampus (Bekinschtein et al. 2010). Moreover, other neurotransmitters and neuromodulators seem to play a role in this late consolidation process (Medina et al. 2008). For instance, the activation

of muscarinic and nicotinic receptors at the CA1 region 12 h post-training improves memory persistence (Parfitt et al. 2012). On the other hand, the systemic administration of corticosterone or stress exposure during this time window impairs the persistence of long-term memory (Yang et al. 2013).

Although opioid receptors have been implicated in the acquisition, early consolidation and memory retrieval, no study has addressed whether delayed administration of opioids alters the persistence of long-term fear-related memories. Therefore, in the current study, we investigated whether the delayed (12 h post-training) administration of morphine alters the persistence of the contextual fear memory.

Methods

Subjects and housing conditions

Adult male Wistar rats weighing between 200 and 250 g were used. The animals were housed to a cage (five animals per cage) and maintained on a 12-h light/12 dark cycle at a temperature of 22°C with water and standard lab chow (Guabi, Santa Maria, Rio Grande do Sul) ad libitum. All experimental procedures were carried out in accordance with institutional guidelines and were approved by the local Animal Care and Use Committee (015/2013), according to the Brazilian legislation.

Drugs

Morphine sulfate and naloxone chlorhydrate were purchased from Cristália (São Paulo, Brazil). All drugs were dissolved in sterile 0.9 % NaCl just before each experiment and administered intraperitoneally (i.p.). Doses were selected based on previous studies (Mariani et al. 2011) and delivered in a volume of 0.1 ml/100 g body weight.

Apparatus

Fear conditioning was conducted in a conditioning chamber (30 x 25 x 25 cm) located in a well-lit room. The front and ceiling walls of the chamber were made of clear acrylic plastic, whereas the lateral and rear walls were made of opaque plastic. The floor of the chamber consisted of 32 stainless steel rods spaced 1 cm apart and connected to a shock generator. The chamber was cleaned and dried before each rat occupied it with a 30% ethanol solution to minimize olfactory cues.

Procedure

In the fear conditioning training session the animals were placed in the conditioning chamber and, after a 3 min adaptation period, received three 1 s-0.4-mA footshocks. The shocks were separated 40 s apart. After the last shock, they were left in the chamber for 60 s before returning to their home cages. Memory retention was evaluated in a test session carried out at 2, 7 or 14 days after training. Animals were tested only once. In the test, trained animals were placed again in the chamber, but did not receive shocks. Freezing, defined as the absence of all movement except that required for respiration (Fanselow 1980), was measured for 8 min by instantaneous observations every 4 s. The percentage of observations scored as freezing was then calculated and taken as a contextual fear conditioning measure. Observations were rated by the same observer who was blind to the test condition.

Experimental design

Experiment 1: effect of morphine on the persistence of contextual fear conditioning

Animals were injected with saline (0.9% NaCl, i.p.) or morphine (3 and 10 mg/kg, i.p.) 12 h post-training. Testing was performed at 2, 7 or 14 days after training. During the test, the number of observations scored as freezing was recorded as described above, as well as the percentage of freezing.

Experiment 2: determination of whether morphine-induced effects depend on
shock delivery (conditioning)

Since the injection of morphine 12 h post-training reduced memory persistence in experiment 1, we tested whether this effect was specific to animals that learned the task. Shocked and nonshocked animals were injected with saline (0.9% NaCl, i.p.) or morphine (10 mg/kg, i.p.) 12 h post training. Testing was performed at 7 days after training. During the test, the number of observations scored as freezing was recorded as described above, as well as the percentage of freezing.

Experiment 3: determination of the time window of morphine effect on the
persistence of contextual fear conditioning

Since the injection of morphine 12 h post-training reduced memory persistence in experiment 1, we tested whether this effect could occur if morphine was injected at other time intervals after training. Animals were injected with saline (0.9% NaCl, i.p.) or morphine (10 mg/kg, i.p.) 6, 9 and 24 h post-training. Testing was performed at 2 or 7 days after training. During the test, the number of observations scored as freezing was recorded as described above, as well as the percentage of freezing.

Experiment 4: effect of pre-test morphine on the impairment of contextual fear conditioning persistence induced by morphine

In order to investigate whether the deleterious effect of morphine was due to state dependence, animals were injected with saline (0.9% NaCl, i.p.) or morphine (10 mg/kg, i.p.) 12 h post-training and with saline or morphine (10 mg/kg, i.p.) 30 min before testing. The testing session was performed 7 days after training. During the test, the number of observations scored as freezing was recorded as described above, and the percentage of freezing calculated.

Experiment 5: effect of naloxone on morphine-induced impairment of contextual fear conditioning

In order to investigate whether the deleterious effect of morphine was mediated by opioid receptors, animals were injected with saline (0.9% NaCl, i.p.) or naloxone (1 mg/kg, i.p.) 11.5 h post-training and saline or morphine (10 mg/kg, i.p.), 12 h post-training. The testing session was performed 7 days after training. During the test, the number of observations scored as freezing was recorded as described above, as well as the percentage of freezing.

Data analysis

Percentages of freezing are expressed as mean and standard error of the mean (SEM). Data were subjected to arcsin transformation before analysis in order to meet the assumptions for parametric tests and analyzed by *t* test for independent samples and one-way or two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test, when appropriate. A $p<0.05$ was considered significant. *F* or *t* values are shown only if $p<0.05$.

Results

Experiment 1

Figure 1a, b, c shows the effect of morphine (3 and/or 10 mg/kg, i.p.) 12 h post-training on freezing to context when testing session was performed 2, 7 or 14 days after training, respectively. Since 3 mg/kg morphine did not alter freezing responses at 2 and 7 days (Fig. 1a, b, respectively), only the dose of 10 mg/kg was tested at 14 days. Statistical analysis (one-way ANOVA) revealed that morphine did not alter freezing to context of animals tested 2 days after training (Fig. 1a). On the other hand, morphine, at the dose of 10 mg/kg, reduced the freezing scores of the animals tested 7 days ($F(2,33)=4.26$, $p=0.023$, $\eta 2=0.205$, one-way ANOVA followed by Bonferroni's test, Fig. 1b) and 14 days after training ($t(13.47)=2.23$, $p<0.05$, *t* test, Fig. 1c], when compared with saline controls. Partitioning of the total sum of squares into trend components revealed a highly significant linear component: $F(1,33)=7.91$, $p=0.008$, indicating that morphine decreased freezing scores linearly with the dose.

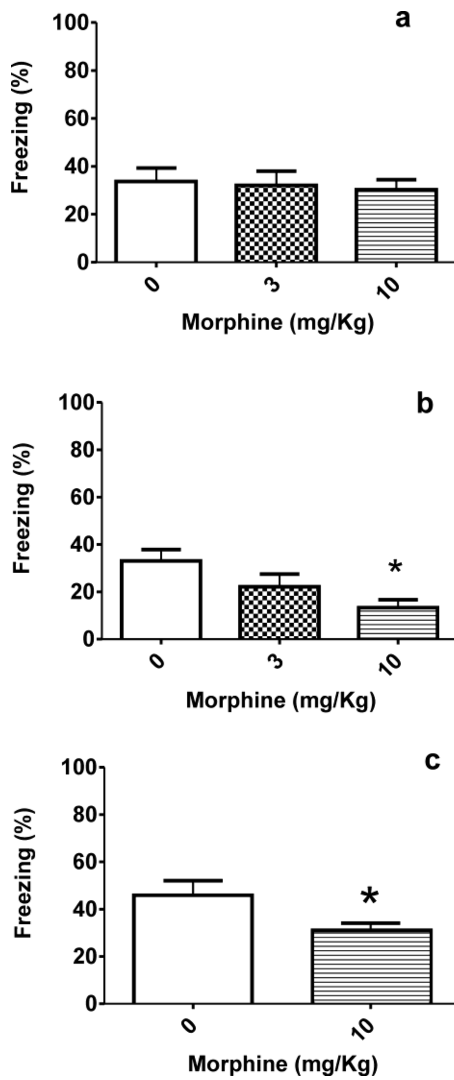

Fig. 1 Effect of morphine (3 and 10 mg/kg, i.p.) injection 12 h post-training on freezing to context. Testing session was performed 2 (**a**), 7 (**b**) or 14 (**c**) days after training. *p<0.05 compared with saline (0.9% NaCl, i.p.) control by the Bonferroni's *t* test. Data are means ± SEM (n=10-13 in each group)

Experiment 2

Figure 2 shows the effect of morphine (10 mg/kg, i.p.) 12 h post-training on freezing to context to shocked and nonshocked animals, measured 7 days after

training. Statistical analysis (two-way ANOVA) revealed a significant treatment by shock interaction: F(1,37)=4.92, *p*=0.033, η2=0.12, indicating that morphine decreased scores only in those animals that received footshock.

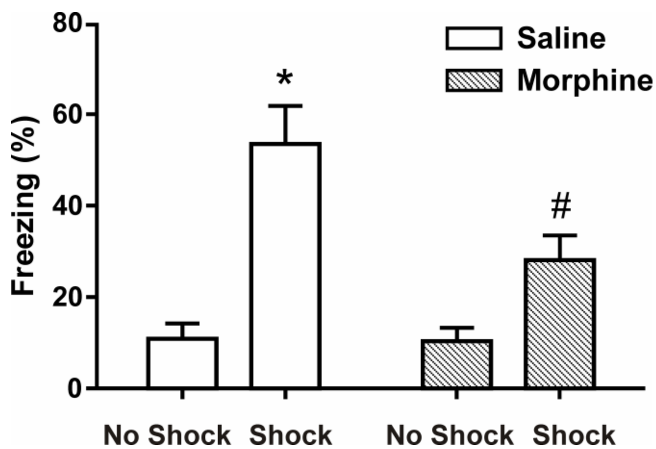

Fig. 2 Effect of morphine (10 mg/kg, i.p.) injection 12 h post-training on freezing to context in shocked and nonshocked animals. Testing session was performed 7 days after training. *p<0.05 compared with nonshocked saline (0.9% NaCl, i.p.) #p<0.05 compared with shocked saline (0.9% NaCl, i.p.) by the Bonferroni's t test. Data are means ± SEM (n=9-11 in each group)

Experiment 3

Figures 3a-f shows that morphine administration at 6, 9 and 24 h post-training did not alter contextual freezing of animals tested either 2 or 7 days after training, respectively.

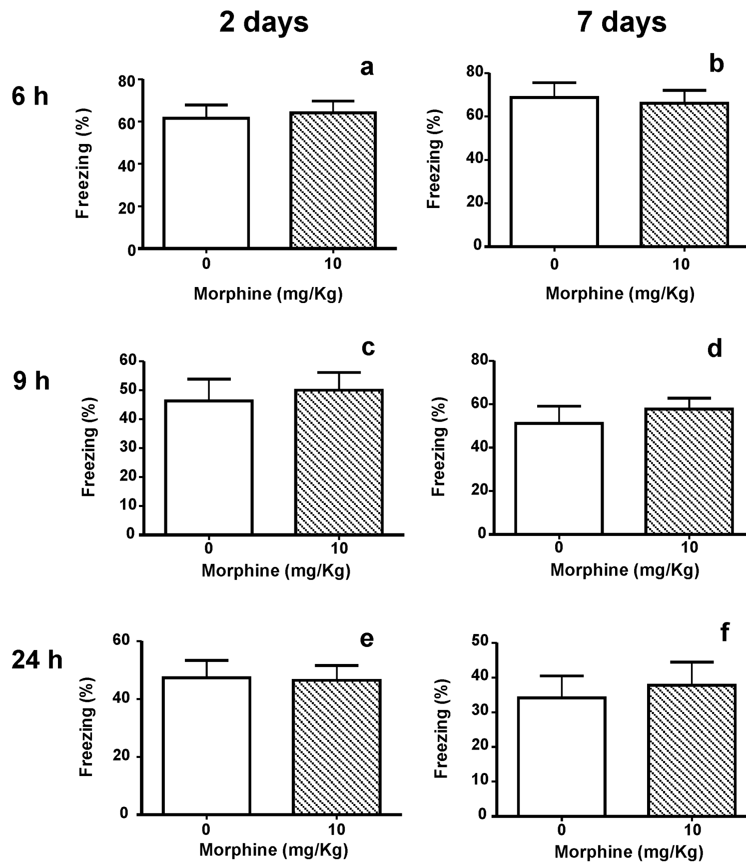

Fig. 3 Lack of effect of morphine (10 mg/kg, i.p.) injection 6, 9 or 24 h post-training on freezing to context. Testing session was performed 2 (a,c,e) and 7 (b,d,f) days after training. Data are means ± SEM (n=9-12 in each group)

Experiment 4

Figure 4 shows the effect of morphine (10 mg/kg, i.p.) 12 h post-training and 30 min before test on freezing to context, measured 7

days after training. Statistical analysis (two-way ANOVA) revealed a significant effect of 12 h post-training treatment, indicating that late morphine (10 mg/kg, i.p.) administration impaired freezing at testing (F(1,36)=9.73, *p*=0.004). Statistical analysis also revealed a significant effect of pre-test treatment (F(1,36)=4.45, *p*=0.042), because pre-test morphine increased contextual freezing scores at testing, regardless of previous post-training treatment. No significant interaction was found, i.e., pre-test morphine did not alter the effect of post-training morphine, ruling out state dependence as a possible explanation for the currently reported morphine-induced impairment of memory persistence.

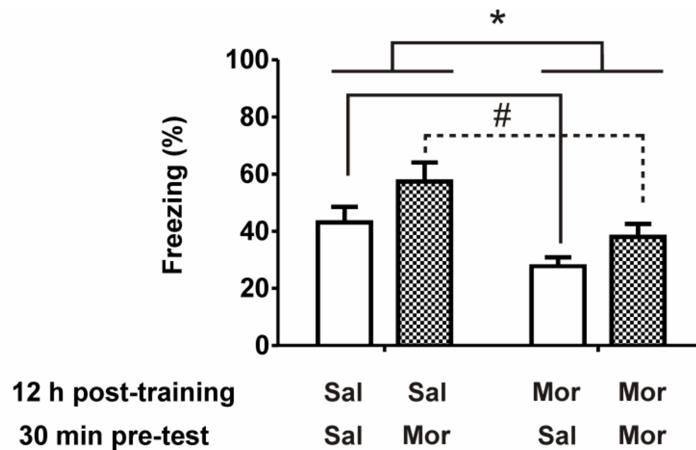

Fig. 4 Effect of morphine (10 mg/kg, i.p.) injection 30 min before test on freezing to context of animals injected with morphine (10 mg/kg, i.p.) 12 h post-training. Testing session was performed 7 days after training. *p<0.05 between pooled post-training saline (*Sal-Sal* and *Sal-Mor* groups) and pooled post-training morphine (*Mor-Sal* and *Mor-Mor* groups) by the Bonferroni's *t* test. # p<0.05 between pooled pre-test saline (*Sal-Sal* and *Mor-Sal* groups) and pooled pre-test morphine (*Sal-Mor* and *Mor-Mor* groups). Lines indicate pooled groups. Data are means ± SEM (n=10 in each group)

Experiment 5

Figure 5 shows the effect of naloxone (1 mg/kg, i.p., 30 min before 12 h post-training morphine) on freezing to context, measured 7 days after training. Statistical analysis revealed a significant pre-treatment (saline or

naloxone) by treatment (saline or morphine) interaction ($F(1,76)=5.10$, $p=0.027$), indicating that naloxone prevented morphine-induced decrease of contextual freezing.

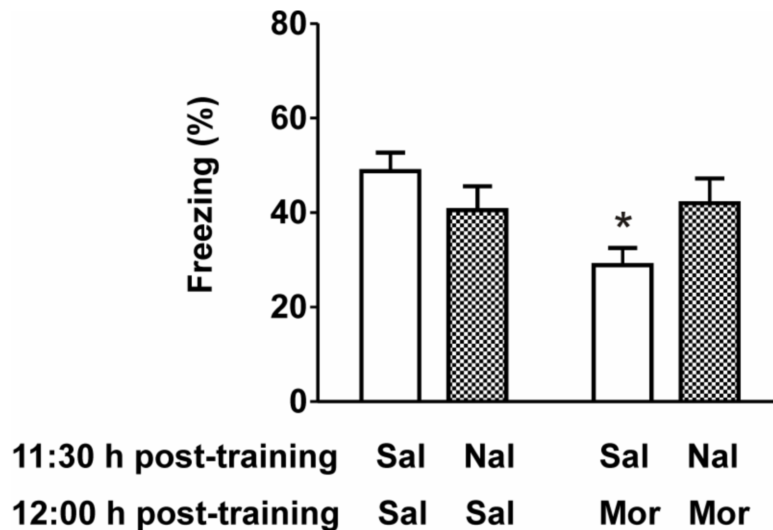

Fig. 5 Effect of naloxone (1 mg/kg, i.p., 30 min before morphine) on morphine (10 mg/kg, i.p., 12 h post-training)-induced decrease of freezing scores. Testing session was performed 7 days after training. *$p<0.05$ compared with saline-saline (0.9% NaCl, i.p.) and naloxone-morphine groups by the Bonferroni's *t* test. Data are means ± SEM (n=20 in each group)

Discussion

In the current study, we showed that the injection of morphine (10 mg/kg, i.p.) 12 h post-training reduces freezing to context in animals tested 7 and 14 days after training but does not alter memory retention at 2 days (Fig. 1a-c). Morphine decreased freezing scores only in shocked animals, indicating that this is a specific effect (Fig. 2). We also verified that morphine (10 mg/kg, i.p.) injection 6, 9 and 24 h after training does not alter

memory retention at 2 and 7 days (Fig. 3a-f). The time window for morphine-induced decrease of freezing scores (12 h post-training) coincides with that observed by Bekinschtein et al. (2007) who have shown that anisomycin injection into the CA1 region 12 h after training decreases freezing scores measured 7 days, but not 2 days, after training session. Those authors have concluded that the 12 h post-training injection of anisomycin does not prevent memory formation but impairs the persistence of long term memory. In line with this view, our results suggest that morphine alters the persistence of the memory of fear.

It has been reported that post-training injection of morphine induces state dependence in rats (Izquierdo 1979; Mariani et al. 2011; Zarrindast et al. 2012). According to this view, animals that have received morphine post-training would properly recall the information if they were tested in the presence of the opioid. Therefore, if state dependence ensued, the pre-test administration of morphine should restore the memory of animals previously injected with morphine (Izquierdo 1979; Mariani et al. 2011; Zarrindast et al. 2012). The second experiment showed that the pre-test administration of morphine did not reverse the decrease of freezing scores induced by 12 h post-training morphine, indicating that its effects on contextual fear memory are not related to state dependence.

Interestingly, the injection of morphine 30 min before testing (7 days post-training) increased freezing to context (Fig. 4), indicating that morphine facilitated late retrieval, regardless whether the animals received morphine 12 h post-training, or not. The effects of morphine on memory retrieval are controversial. While most of the previous studies suggest that morphine impairs the retrieval of many tasks (Bruins Slot and Colpaert 1999; Farahmandfar et al. 2012; Saha et al. 1991; Zarrindast et al. 2006, 2013), Shiigi et al. (1990) have shown that morphine may facilitate retrieval. Moreover, in that study, a role for mu-opioid receptors in the facilitatory effect of pre-test morphine on memory retrieval was proposed, because the nonspecific opioid antagonist naloxone, unlike the selective kappa-opioid antagonist nor–BNI, completely antagonized this effect of morphine. They have also shown that DTLET, a selective delta opioid agonist, does

not alter retrieval indicating that delta receptors are not involved in memory retrieval. However, as discussed by Roesler and McGaugh (2010), when an animal is given a treatment before memory retention testing, any resulting alteration in behavioral performance might be due to the treatment's effects on sensory and motor functioning in place of or in addition to effects on retrieval. Such treatments thus confound the interpretation of the findings, making it difficult to assess the treatment-induced influence on mechanisms mediating memory expression.

The last experiment showed that naloxone prevents morphine-induced long-lasting reduction of freezing scores. It has long been suggested that the antagonism of morphine mnemonic effects by naloxone indicates the involvement of opioid receptors (Beatty 1983; Bruins Slot and Colpaert 1999; Mariani et al. 2011; Shiigi et al. 1990; Zarrindast and Rezayof 2004). As a logical extension of this idea, our findings suggest the involvement of opioid receptors in the current effect of morphine on memory persistence.

Context fear conditioning experiments carried out by Bekinschtein et al. (2007) show that protein synthesis that occurs around 12 h post-training is critical for the persistence of long-term memory. Moreover, the systemic administration of corticosterone or stress exposure during this time window impairs the persistence of long term memory of fear (Yang et al. 2013). Bekinschtein et al. (2008) have also reported that BDNF facilitates memory persistence by itself, transforming a nonlasting long-term memory trace of inhibitory avoidance into a persistent one, in an ERK-dependent manner. Moreover, Rossato et al. (2009) have shown that the persistence of inhibitory avoidance memory depends on the activation of D1 receptors and, consequently, on the increase of cAMP/PKA/CREB signaling pathway and hippocampal BDNF expression, which occurs 12 h after acquisition. Interestingly, the 11 h post-training systemic injection of the antidepressant venlafaxine selectively impairs the persistence of the inhibitory avoidance (Slipczuk et al. 2013), and the activation of muscarinic and nicotinic receptors at the CA1 region 12 h post-training improves (Parfitt et al. 2012) the persistence of the inhibitory avoidance memory. Moreover, the delayed (12 h post-training) activation of N-methyl-D-

aspartate receptors in the VTA has been implicated in the persistence of inhibitory avoidance memory (Rossato et al. 2009). It is also worth pointing out that the administration of methylphenidate 12 h after acquisition enhances human memory persistence (Izquierdo et al., 2008). Therefore, accumulating evidence suggests that late neurochemical processes, which occur around 12 h after acquisition, selectively modulate memory persistence in both rats and humans. In this regard, our study suggests a role for opioid receptors in the persistence of fear memory that temporally coincides with these studies, and that opioids may pharmacologically modulate the maintenance of the memory of fear. From the clinical point of view, one might argue that opioids could be used as possible therapeutics aiming to the prevention of disorders related to fear memory persistence, such as the PTSD. Interestingly, the use of morphine during trauma care is associated with a reduced risk of PTSD development after serious injury in veterans (Holbrook et al. 2010) further supporting our view. At last, in studies that have investigated the involvement of particular brain regions in memory for aversively motivated tasks, the effects of amygdala or insular cortex post-training lesions on retention depended on the level of the unconditioned stimulus applied, including the number of training trials and shock intensity (Bermudez-Rattoni et al. 1997; Parent et al. 1995a,b). Therefore, one must consider the currently reported effects of morphine observing the shock intensity used, and the possibility that the effect of morphine may change with shock increase.

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ARTIGO 2

Original paper

Morphine impairs the persistence of memory via a cAMP/PKA-dependent pathway

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MORPHINE IMPAIRS THE PERSISTENCE OF MEMORY VIA A cAMP/PKA-DEPENDENT PATHWAY

Gerusa Paz Porto[a,1], Cristiane Signor[b,1], Laura Hautrive Milanesi[a,1], Vinícius Rafael Funck[a], Maribel Antonello Rubin[a,b], Carlos Fernando Mello[a,*]

[a]Graduation Program in Pharmacology, Department of Physiology and Pharmacology, Center of Health Sciences, Universidade Federal de Santa Maria, Santa Maria, RS, 97105-900, Brazil.

[b] Department of Chemistry, Center of Exact and Natural Sciences, Universidade Federal de Santa Maria, Santa Maria, RS, 97105-900, Brazil.

*Corresponding author. Fax: + 55 55 3220 8870.

E-mail adress: cf.mello@smail.ufsm.br (C.F. Mello).

[1] These authors contributed equally to this work.

Abstract

Aims:

Morphine is an opioid that impairs the persistence of memory. Although PKA signaling has been implicated in memory persistence, no study has investigated whether morphine alters persistence of long-term memory by reducing the cAMP/PKA signaling. Therefore, in the present study we investigated whether drugs that alter cAMP/PKA signaling modify the effect morphine on maintenance of memory.

Main methods:

Adult rats were training in contextual fear conditioning task and 11.5 hours post-training were injected with the adenylyl cyclase activator forskolin (0.13 μg/μl) or vehicle (phosphate buffer saline/DMSO) or with activator PKA, 8-Br-cAMP (7.5μg/μl) or vehicle (phosphate buffer saline), via intrahippocampal, followed by morphine (10 mg/kg, i.p.) 30 min later. The testing session was performed 7 days after training and during the test, the number of observations scored as freezing was recorded.

Key findings:

The intrahippocampal administration of Forskolin (0.13 μg/μl), or 8-Br-cAMP (7.5 μg/μl), did not alter freezing to context in animals tested seven days after training but prevented morphine-induced decrease of contextual freezing.

Significance:
Our findings support a role for cAMP/PKA signaling in deleterious effect of morphine on memory persistence.

Key words: opioid, persistence of long-term memory, cyclic AMP, protein kinase A

Introduction

Current evidence suggests that late consolidation processes that occur around 12 hours post-training in the hippocampus, are required to the persistence of fear memories [1,2]. These processes depend on the activation of NMDA receptors in the ventral tegmental area (VTA), activation of D1 dopaminergic receptors in the dorsal hippocampus [3] and protein synthesis [4]. Accordingly, increased brain-derived neurotrophic factor (BDNF) expression in the CA1 region [5] and changes in hippocampal c-Fos, Homer 1a, Akt, CamKIIa, and ERK2 levels around 18–24 h after training have been associated with memory persistence [6].

It has been suggested that ERK and cAMP/PKA pathways, but not PI3K/mTOR cascade, are activated in the hippocampus around 12 hours post-training [3,5]. In this time window, the intrahippocampal injection of PI3K inhibitor LY294002 and mTOR inhibitor rapamycin have no effect on memory of rats tested 2 and 7 days after inhibitory avoidance training. In contrast, blocking ERK activation in the hippocampus by injecting the MEK inhibitor U0126 impairs memory persistence and blocks the positive modulatory effects of BDNF [5]. Furthermore, Rossato and colleagues [3] have shown that while the intra-CA1 infusion of PKA inhibitor PKI hampers memory persistence, the infusion of the PKA activator 8-Br-cAMP enhances it, evidencing a role for PKA in the maintenance of long term memory.

We have recently shown that morphine injection 12 hours after training impairs freezing behavior of contextual fear conditioning when animals are tested 7 days after training, suggesting that opioid receptors may modulate the persistence of fear memories [7]. Although morphine binds to mu, delta and kappa opioid receptors which are coupled to G_q and G_i proteins [8-13], it is still to be determined whether adenylyl cyclase or phospholipase C-related pathways are involved in the deleterious effects of morphine on fear memory persistence. Considering that cAMP-dependent mechanisms are involved in memory persistence, and that the modulation of hippocampal events 12 hours after learning could be useful to prevent the persistence of fear memories related to Posttraumatic Stress Disorder (PTSD) this study investigated whether the cAMP/PKA signaling pathway is involved in the effect of morphine on the persistence of fear memory in rats.

Material and methods

Animals

All animal experiments reported in this study were conducted in accordance with Brazilian law No. 11.794/2008, were approved by the local Animal Care and Use Committee (process 015/2013) and are in agreement with institutional guidelines. Male Wistar rats (200-250 g) were bred in the Animal House of the Federal University of Santa Maria, housed 4 to a cage, and maintained on a 12 h light/12 dark cycle at a temperature of 22 °C with access to water and rodent laboratory chow (Guabi, Santa Maria, RS, Brazil) *ad libitum.* Behavioral tests were conducted during the light phase of the cycle (between 9:00 AM and 5:00 PM).

Surgery

Rats were anaesthetized with ketamine/xylazine (80 and 10 mg/kg, respectively, i.p.), and implanted with two 27-gauge guide cannulas placed 1 mm above the dorsal hippocampus at the following coordinates: A, 4 mm; L, 3.0 mm; and V, 2.0 mm [14].

Drugs:

Morphine sulfate was purchased from Cristália (São Paulo, Brazil) and injected intraperitoneally in a volume of 0.1 ml/100 g body weight. Sterile 0.9 % NaCl (saline) was used as control. Forskolin and 8-Br-cAMP were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and dissolved in 200 mM phosphate buffer saline (PBS) in 50% DMSO and 100 mM phosphate buffer saline (PBS), respectively.

Behavioural and infusion procedures

One week after surgery, the animals were subjected to a single training session in a conditioning chamber, consisting of a 30 x 25 x 25-cm box with a grid floor containing 32 stainless steel rods spaced 1 cm apart and connected to a shock generator. In the fear-conditioning training session the animals were placed in the conditioning chamber and, after a 3 min adaptation period, received three 1 s-0.4 mA footshocks. The shocks were separated 40 s apart. After the last shock, they were left in the chamber for 60 s before returning to their home cages. Freezing behavior was defined as the absence of all movement except the required for respiration [15]. Memory retention was evaluated in a test session carried out at 7 days after training.

In the test, trained animals were placed again in the chamber, but did not receive shocks, and freezing was measured for 8 min by instantaneous observations every 4 s. The percentage of observations scored as freezing was then calculated and taken as a contextual fear conditioning measure. Forskolin and 8-Br-cAMP were injected bilaterally into the hippocampus (1 μl/brain hemisphere over 3 min) via an infusion pump 11.5 hours after training session, using a 30-gauge needle fitted into the guide cannula. The tip of the infusion needle protruded 1.0 mm beyond that of the guide cannula into the dorsal hippocampus. The injection needles were left in place for additional 60 s to minimize backflow.

Experimental design

Experiment 1

Animals were injected with PBS in 50% DMSO or forskolin (0.13 μg/μl, intrahippocampal) 11.5 hours post-training and with saline or morphine (10 mg/kg, i.p.) 30 min later. Forskolin is an adenylyl cyclase activator [16,17]. The testing session was performed 7 days after training. During the test, the number of observations scored as freezing was recorded as described above, as well as the percentage of freezing.

Experiment 2

Animals were injected with PBS (i.p.) or 8-Br-cAMP (7.5 μg/μl, intrahippocampus) 11.5 hours post-training and with saline or morphine (10 mg/kg, i.p.) 30 min later. 8-Br-cAMP is cAMP analog that activates PKA [18]. The testing session was performed 7 days after training. During the test, the number of observations scored as freezing was recorded as described above, as well as the percentage of freezing.

Statistical analysis

Data are presented as means ± S.E.M. Two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test, was used to determine statistical significance, Differences were considered to be significant at $p \leq 0.05$.

Results

Experiment 1:

Effect of forskolin on the deleterious effect of morphine on the memory persistence.

Fig. 1 shows the effect of morphine alone or in combination with forskolin on memory retention. Statistical analysis (two-way ANOVA) showed a significant interaction of 11.5 hours post-training treatment (200 mM phosphate buffer saline in 50% DMSO or forskolin, 0.13 μg/μl, intrahippocampus) with 12 hours post-training treatment (saline or morphine, 10 mg/kg, i.p.) [$F(1,47)=6.6$, $p=0.01$], indicating that forskolin prevented morphine-induced decrease of contextual freezing.

Insert Fig. 1 here

Experiment 2:

Effect of 8-Br-cAMP on the deleterious effect of morphine on the memory persistence.

Fig. 2 shows the effect of morphine alone or in combination with 8-Br-cAMP. Statistical analysis (two-way ANOVA) showed a significant interaction of 11.5 hours post-training treatment (saline or morphine, 10 mg/kg, i.p.) with 12 hours post-training treatment (saline or morphine, 10 mg/kg, i.p.) [$F(1,20)=7.9$, $p=0.01$], indicating that 8-Br-cAMP prevented morphine-induced decrease of contextual freezing.

Insert Fig. 2 here

Discussion

This study shows that the intrahippocampal administration of forskolin and 8-Br-cAMP prevent the impairing effect of morphine on memory persistence, suggesting the involvement of the cAMP/PKA pathway in the memory persistence deficit induced by morphine.

Previous studies have shown that morphine, when injected 12 hours post-training, impairs the persistence of fear conditioning memory, since it decreases freezing scores measured 7 days, but not 2 days after training session [7]. This effect seems to be mediated by the activation of opioid receptors, since it is prevented by naloxone [7].

A substantial number of studies have associated the hippocampus with maintenance of contextual memories [4,5,19,20]. Zelikowsky and colleagues [21] have shown that rats with dorsal hippocampal lesions were able to form long-term contextual fear memories, but were unable to maintain a memory 30 days after training. In addition, it has been proposed that hippocampal dopaminergic activation ensures the temporal persistence of hippocampal memory traces, since intrahippocampal infusion of the D1/D5 dopaminergic antagonist SCH23390 impairs the persistence of new memories over time [22]. Rossato and colleagues [3] have shown that memory persistence depends on activation of D1 receptors and, consequently, on an increase of cAMP/PKA/CREB signaling pathway activity and brain-derived neurotrophic factor (BDNF) hippocampal expression, which occur 12 hours after acquisition. D1 receptors activate cAMP/PKA/CREB signaling pathway [23,24]. On the other hand, opioid receptors are coupled to Gi protein that inhibits adenylyl cyclase and decreases cAMP intracellular levels [9,25].

The first experiment showed that the administration of forskolin, an adenylyl cyclase activator that increases intracellular cAMP levels [17] prevented morphine-induced impairment of the persistence of conditioned fear assessed 7 days after training. Importantly, the dose of forskolin that prevented the effect of morphine did not alter performance *per se*. Thus, it is unlike that the currently reported reversal of the morphine effect by forskolin is due to physiological antagonism. Forskolin has been used to prevent or restore the harmful effects of treatments on memory and to suggest that these effects may involve a decrease of adenylate cyclase activity [26-28]. It is well known that morphine by stimulating Gi/o protein-coupled opioid receptors [25] inhibits adenylate cyclase, reducing intracellular cAMP levels [29,30]. By these means morphine hampers the forskolin-induced intracellular production of cAMP [31]. Considering that the hippocampus plays an important role in the memory of contextual fear conditioning [32,33] and that it expresses μ-opioid receptors [34-36], it is tempting to propose that morphine impaired memory persistence by stimulating opioid hippocampal receptors and ultimately decreasing cAMP

production. In addition, our results also agree with Galeotti and colleagues, who have also implicated the Gi protein in the memory impairment induced by morphine, since the amnesic effect of morphine is prevented by pertussis toxin, a bacterial toxin that inactivates the alfa subunit of Gi proteins [13].

The results of the second experiment showed that intrahippocampal infusion of 8-Br-cAMP, a cAMP membrane-permeant analog [18], 11.5 hours after training did not alter the persistence of memory, but prevented the impairment of memory persistence induced by morphine. Considering that the pretreatment with 8-Br-cAMP did not modify the performance of the animals in the fear conditioning task, we can rule out that the prevention of morphine amnesia is due to a precognitive effect of 8-Br-cAMP and can suggest that the ability of this activator of PKA to rescues deficits in memory produced by morphine indicates that effect of morphine is related to disruption of PKA signaling.

The activation of PKA signaling promotes the phosphorylation of a variety of substrates, including the cAMP responsive element binding protein (CREB) transcription factor [37,38]. Suzuki and colleagues [39] have shown that CREB plays an important role in memory persistence. Those authors have shown that transgenic mice that have upregulation of CREB activity, unlike wild-type, maintained conditioned freezing levels greater in the training context than in the novel environment when retention test was carried at 1 month after training. Phosphorylated CREB transcription factor can increase BDNF expression, a factor that is sufficient to induce persistence of long-term memory storage [5] and promotes neuroplasticity, differentiation and neuronal survival [40,41].

In our study morphine was injected by the intraperitoneal route. Although this opioid can act on any brain structure, our experiments suggest an important role for the hippocampus in the systemic effect of morphine because the restoration of the cAMP/PKA pathway in this cerebral structure was enough to reverse the observed impairment of memory persistence. Moreover, the present findings confirm a role for the hippocampus in persistence of memory around 12 h after training, in accordance with other investigations [20,42,43]. They agree with findings Rossato and colleagues [3] indicating that PKA signaling pathways in the CA1 region is involved in long-term memory persistence of fear memories.

Conclusion

Our findings suggest the involvement of hippocampal cAMP and PKA pathways in the impairment of persistence of contextual fear conditioning induced by morphine.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Legends

Fig. 1 Effect of forskolin (0.13 μg/μl, intrahippocampal) injected 30 min before morphine (10 mg/kg, i.p.) 12 hours post-training on freezing to context of animals. Testing session was performed 7 days after training. *$p<0.05$ compared with PBS/DMSO/Saline and Forskolin/Morphine groups by two-way anova followed by the Bonferroni's *t* test. Data are means +/- SEM (n=12-13 in each group). Forsk: forskolin; PBS/DMSO: phosphate buffer saline (PBS) in 50% DMSO (vehicle of forskolin); Sal: saline; Mor: morphine.

Fig. 2 Effect of 8-Br-cAMP (7.5 μg/μl, intrahippocampal) injected 30 min before morphine (10 mg/kg, i.p.) 12 hours post-training on freezing to context of animals. Testing session was performed 7 days after training. *$p <0.05$ compared with PBS/Saline and 8-Br-cAMP/Morphine groups by two-way anova followed by the Bonferroni's *t* test.

Data are means +/- SEM (n=6-7 in each group). 8-Br: 8-Br-cAMP; PBS: phosphate buffer saline (vehicle of 8-Br-cAMP); Sal: saline; Mor: morphine.

Fig. 1

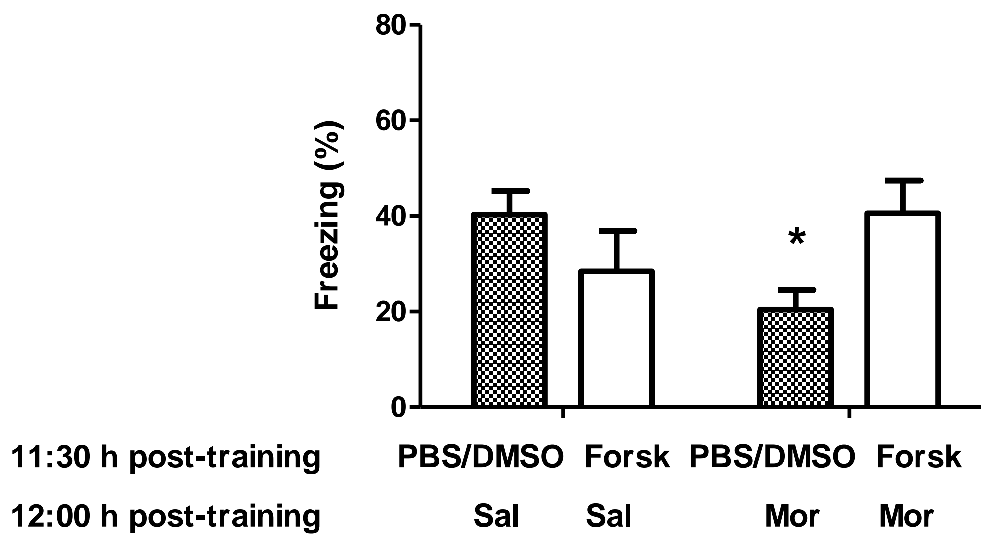
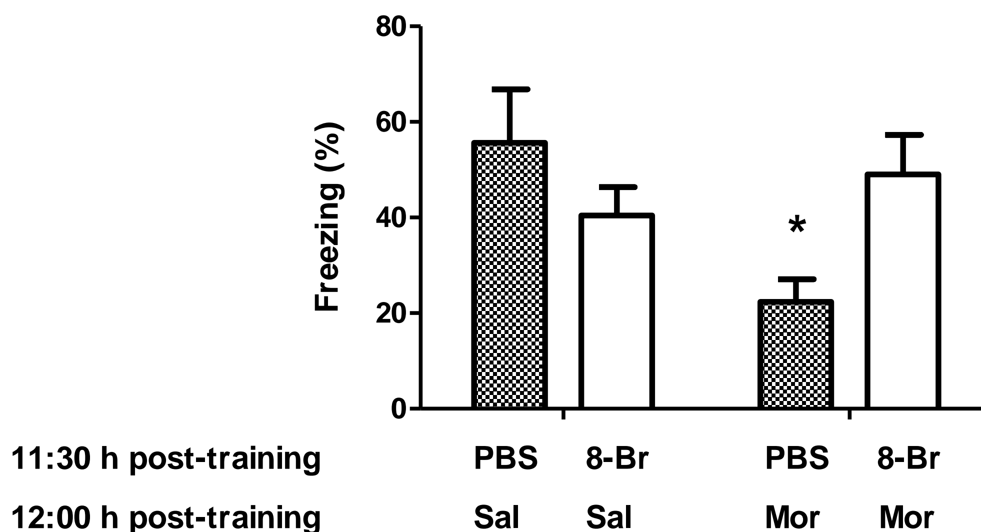


Fig. 2



DISCUSSÃO

A persistência de memórias decorrentes de experiências emocionalmente significantes é um fenômeno adaptativo de extrema relevância para a sobrevivência, pois nos auxilia a lembrar tanto de situações favoráveis como responder a situações potencialmente ameaçadoras (MARTIJENA e MOLINA, 2012). Entretanto, sob certas condições, memórias traumáticas ou de medo persistentes podem levar a transtornos de ansiedade que são responsáveis pela perda da qualidade de vida, impedimento do sucesso e da produtividade de indivíduos acometidos (MAHAN e RESSLER, 2012).

Muitos trabalhos têm examinado como memórias consolidadas são mantidas ao longo do tempo. Evidências sugerem que alterações moleculares e mudanças estruturais sinápticas têm um importante papel neste processo (JOHANSEN et al., 2011). Estudos prévios sugerem que muitos mecanismos moleculares da fase de consolidação incluindo ativação de quinases, formação de fatores de transcrição e produção de proteínas também estão envolvidos na persistência da memória (BEKINSCHTEIN et

al., 2010; KATCHE et al., 2012). Além disso, estudos recentes têm mostrado que a persistência da memória pode ser modulada pelos sistemas glutamatérgico (ROSSATO et al., 2009), noradrenérgico (PARFITT et al., 2012a), colinérgico (PARFITT et al., 2012b) e serotoninérgico (SLIPCZUK et al., 2013) 12 horas após o aprendizado.

Um aspecto relevante é que pouco é sabido sobre o envolvimento do sistema opióide na persistência da memória de longo prazo. Dentro do contexto de transtornos de ansiedade relacionados a memórias aversivas, a descoberta de um fármaco que prejudica a persistência de uma memória de medo poderia ser uma ferramenta útil na terapêutica. Neste sentido, a primeira parte deste trabalho propos-se a investigar o efeito do opióide morfina sobre a persistência da memória de medo contextual em ratos.

Neste estudo, nós demonstramos que a administração de morfina 12 horas após o treino da tarefa de condicionamento de medo ao contexto, prejudicou a memória de ratos submetidos a sessões de teste 7 e 14 dias após o treino, mas não interferiu na memória quando a sessão de teste foi realizada 2 dias após o treino. Em consonância com interpretações dos experimentos de Bekinschtein e colaboradores (2007) concluímos que este opióide prejudicou a persistência, e não a formação da memória de medo.

Uma vez que um efeito deletério da morfina sobre a persistência da memória foi observado, decidiu-se investigar o mecanismo de ação de tal efeito. Considerando que morfina é capaz de promover o fenômeno dependência de estado quando administrada próximo ao treino (IZQUIERDO, 1979; MARIANI et al. 2011; ZARRINDAST et al. 2012), investigamos se este fenômeno poderia ter influenciado no efeito deste opióide. Para isto, animais que foram tratados com morfina 12 horas após o treino também foram tratados com morfina no periodo anterior ao teste. Constatamos que não houve reversão do efeito da morfina sobre a persistência da memória. Interessantemente, ao avaliar resultados de outros grupos controles deste experimento, constatamos também que a injeção de morfina 30 minutos antes do teste, por si só, melhorou a performance no teste executado 7 dias após o treino. Tal efeito foi por nós interpretado de forma cautelosa, pois conforme Roesler e McGaugh (2010), este efeito poderia ser atribuído

também ou exclusivamente a efeitos do tratamento sobre o sistema locomotor ou sensorial.

Vários autores têm sugerido o envolvimento de receptores opióides no efeito da morfina sobre a memória, pois observaram que a injeção do antagonista de receptores opióides naloxona é capaz de prevenir estes efeitos (BRUINS SLOT e COLPAERT, 1999; MARIANI et al. 2011; SHIIGI et al. 1990; ZARRINDAST e REZAYOF, 2004). Com o intuito de verificar envolvimento dos receptores opióides no efeito de morfina sobre a persistência da memória, realizamos o pré-tratamento dos animais com a naloxona e verificamos que esta foi capaz de prevenir o efeito da morfina, evidenciando o papel destes receptores no efeito.

Estudo de Rossato e colaboradores (2009) têm indicado que a persistência da memória depende da ativação de receptores D1 hipocampais e consequentemente de um aumento da ativação da via de sinalização AMPc/PKA, a qual ocorre 12 horas após o aprendizado. Neste período, a ativação da via AMPc/PKA inicia com a ativação da enzima adenilil ciclase pela proteína Gs, seguida de um aumento nos níveis de AMPc, o qual ativa a PKA (proteína quinase dependente de AMPc). Esta proteína é composta por 4 subunidades: 2 regulatórias e duas catalíticas. Quando o nível de AMPc aumenta nas células, o AMPc se liga as subunidades regulatórias da PKA, causando dissociação das subunidades regulatórias das catalíticas, as quais se movem para o núcleo onde podem fosforilar uma variedade de substratos como o fator de transcrição CREB (elemento responsivo de ligação ao AMPc) (KANDEL, 2012; WANG e ZHANG, 2012). O CREB pode aumentar a transcrição do BDNF, um fator neurotrófico que induz persistência da memória de longo prazo (BEKINSCHTEIN et al., 2008). Considerando que a morfina pode exercer seus efeitos farmacológicos por meio de várias vias de sinalização (LOU et al., 1999; NARITA et al., 2004; SEO et al., 2008) incluindo a via da AMPc/PKA (MILANÉS et al., 1999, RATKA et al., 1991) optou-se por investigar o papel desta via no efeito da morfina sobre a persistência da memória. Para isto, animais que receberam morfina 12 horas após o treinamento foram pré-tratados via intrahipocampal com o ativador da enzima adenilil ciclase forscolina (UBOLDI e SAVAGE, 2002) ou com o

análogo do AMPc, ativador da enzima PKA, 8-Br-AMPc (BRANSKI et al., 2008).

Conforme esperado, a administração de forscolina e de 8-Br-AMPc preveniu o efeito deletério da morfina sobre a persistência da memória, sugerindo que este efeito seja mediado por uma redução dos níveis de AMPc e por uma redução da ativação da proteína quinase A. É importante destacar que as doses de forscolina e de 8-Br-AMPc não tinham efeito *per se*, o que indica que este efeito não foi decorrente de antagonismo fisiológico. O uso de forscolina para prevenir ou restaurar os efeitos prejudiciais de tratamentos sobre a memória tem sido empregado por vários autores, sugerindo que estes podem envolver inibição da enzima adenilil ciclase (ANDO et al., 1987; KINNEY et al., 2003; SATO et al., 2004). Considerando que o hipocampo é uma estrutura envolvida no medo condicionado contextual (DAUMAS et al., 2005; XU et al., 2014), e que expressa receptores opióides mu em abundância, com um relevante papel nos mecanismos de memória (GIANNOPOULOS e PAPATHEODOROPOULOS, 2013; JAFARI-SABET e JANNAT-DASTJERDI, 2009; MEILANDT et al., 2004), é possível que a morfina tenha prejudicado a persistência da memória por estimular receptores opióides hipocampais e promover a inibição da enzima adenilil ciclase e da proteína quinase A nesta estrutura.

Na maioria dos estudos sobre memória publicados na literatura, a morfina é administrada de forma sistêmica (BABAEI et al., 2012; BIANCHI et al., 2010; ESMAEILI et al., 2009; SIERRA et al., 2013; ZHANG et al., 2008; ZHU et al., 2011). Deste modo, este opióide pode atuar sobre várias estruturas do sistema nervoso central. Entretanto, parece que seu efeito sobre a persistência é mais importante a nível de hipocampo, pois a elevação da atividade da via de sinalização AMPc/PKA somente nesta estrutura foi suficiente para reverter o prejuízo da memória.

Nos últimos anos, um número significante de estudos clínicos observacionais têm revelado que o uso de morfina após diversos tipos de traumas como acidentes de trânsito, assaltos, quedas e queimaduras, pode exercer efeito protetor contra o desenvolvimento do transtorno do estresse pós-traumático (BRYANT el., 2009; SAXE et al., 2001; STODDARD et al 2009, NIXON et al. 2010, HOLBROOK et al 2010). Este efeito tem sido

hipoteticamente atribuído pelos autores aos seus efeitos deletérios sobre a memória. Recentemente, foi mostrado que a administração de tratamentos farmacológicos em humanos 12 horas após o aprendizado é capaz de influenciar a persistência da memória (IZQUIERDO et al., 2008). Sob esta ótica, tais achados associados aos resultados deste trabalho indicam que a morfina e possivelmente demais opióides podem futuramente apresentar um efeito promissor no tratamento de transtornos desta natureza.

CONCLUSÕES

Neste estudo a administração tardia de morfina (12 horas pós-treino) reduziu o congelamento contextual de ratos em teste realizado sete dias após o treinamento, mas não dois dias após o treino. Tais achados sugerem que a administração de morfina neste periodo não interfere na formação da memória, mas prejudica a persistência da memória de medo contextual. Este efeito não é decorrente do fenômeno dependência de estado, visto que não foi revertido pela administração deste opióide antes do teste de evocação. É um efeito mediado por receptores opióides, pois foi prevenido pela administração do antagonista opióide naloxona.

Os resultados comportamentais também sugerem que o mecanismo pelo qual morfina prejudica a persistência da memória envolve uma redução da atividade da enzima adenilil ciclase hipocampal, pois a forscolina, um ativador desta enzima, em dose sem efeito *per se* preveniu o efeito deste opióide. É possível também que este efeito seja decorrente de uma redução da ativação da enzima PKA, pois também foi prevenido pela administração do ativador desta enzima, o 8-Br-AMPc, em doses que não têm efeito *per se.* .A confirmação desta última hipótese depende da conclusão dos resultados de western blot previstos conforme apêndice A, apresentado no final desta tese.

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APÊNDICE – Experimentos de western blot

Os resultados até agora obtidos mostram um importante efeito redutor da morfina sobre a persistência da memória de medo em ratos mediado pelos receptores opióides. As demais abordagens comportamentais relativas ao mecanismo de ação envolvido apontam para um possível envolvimento da via de sinalização da PKA. A confirmação da participação desta via requer a determinação do estado de ativação (fosforilação) das proteínas PKA e CREB em animais que receberam forscolina e/ou Morfina e 8 Br-AMPc e/ou Morfina. A metodologia e os resultados parciais referentes a este experimentos são descritos abaixo:

Metodologia:

Quatro grupos de animais receberam uma injeção via intrahipocampal de PBS ou forscolina (na dose 0,13 μg/μl, via intrahipocampal) 11:30 horas

após o treinamento e salina ou morfina (10 mg/kg) nos 30 minutos seguintes. Outros quatro grupos receberam uma injeção via intrahipocampal de PBS ou 8-Br-AMPc (na dose 7,5 μg/μl, via intrahipocampal) 11:30 horas após o treinamento e salina ou morfina (10 mg/kg) nos próximos 30 minutos. Nos tempos de 1 hora e 180 minutos após a administração do último tratamento, os animais foram submetidos a eutanásia e tiveram o hipocampo dissecado para ser utilizado nas análises de Western Blot.

Para a realização das análises de *western blot* a estrutura foi rapidamente homogeneizada em 300 μL de tampão "A" contendo HEPES 10 mM, pH 7,9, KCl 10 mM, $MgCl_2$ 2 mM, EDTA 1 mM, NaF 1mM, 10 μg/mL aprotinina, β-glicerolfosfato 10 mM, fenilmetilsulfonil fluoreto 1 mM, DDT 1 mM e 2 mM ortovanadato de sódio. Após a centrifugação (16000 g por 45 min a 4°C), o sobrenadante (S1) denominado fração citosólica, foi reservado para posterior processamento. O precipitado (P1) foi resuspendido em 150 μL de tampão "B" resfriado no gelo (HEPES 10 mM, pH 7,9, KCl 10 mM, $MgCl_2$ 2 mM, EDTA 1 mM, NaF 1 mM, 10 μg/mL aprotinina, β-glicerolfosfato 10 mM, fenilmetilsulfonil fluoreto 1 mM, DDT 1 mM, 2 mM ortovanadato de sódio e Triton-X a 1%), incubado por 15 minutos no gelo, e centrifugado a 16000 *g* por 45 min a 4°C. O sobrenadante (S2) foi descartado e o precipitado (P2) foi resuspendido em 100 μL de tampão "C" (HEPES 20 mM, pH 7,9, KCl 50 mM, $MgCl_2$ 2 mM, EDTA 1 mM, NaF 1mM, 10 μg/mL aprotinina, β-glicerolfosfato 10 mM, fenilmetilsulfonil fluoreto 1 mM, DDT 1 mM, ortovanadato de sódio 2 mM, NaCl 420 mM e glicerol 20%), incubado por 15 min no gelo e centrifugado a 16000 *g* por 45 min. O sobrenadante (S3) foi considerado a fração nuclear (Medeiros, Prediger *et al.,* 2007). A concentração de proteína nas frações citosólicas e nuclear foram determinadas usando o método de Bradford (1976). Quantidades equivalentes de proteínas (80 μg ou 20 μg para fração citosólica ou nuclear, respectivamente) foram adicionadas para 0,2 volumes de tampão concentrado (Tris 200 mM, glicerol a 10%, SDS a 2%, beta-mercaptoetanol 2,75 mM e 0,04% azul de bromofenol) e fervidas por 10 minutos. As proteínas foram separadas em gel SDS-poliacrilamida a 12% e transferidas para uma membrana de fluoreto de polivinilideno (PVDF). As análises de Western Blot da subunidade catalítica da PKA foram realizadas

nas frações citosólica e nuclear. A imunoreatividade a CREB foi medida na fração nuclear. As membranas foram processadas usando o Sistema SNAP i.d. (Millipore, Billerica, MA, USA) e foram bloqueadas com 1% de BSA em 0,05% de Tween-20 em Tris–borato salina (TBS-T). Posteriormente, foram incubadas por 10 minutos com os anticorpos primários específicos diluídos 1:150 em TBS-T (anti-fosfo-PKA Cα/β/γ, anti-total-PKA Cα/β/γ, anti-fosfo-CREB-1, anti-total-CREB-1 anticorpos policlonais - Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA). As membranas foram lavadas 3 vezes com TBS-T por 5 min e incubadas com anticorpo secundário acoplado a fosfatase alcalina (anticorpo policlonal de coelho; em uma diluição de 1:3000 in TBS-T) por 10 minutos. As bandas de proteína foram visualizadas com 5-bromo-4-cloro-3-indolil fosfato e p-nitro tetrazólio azul (BCIP/NBT; Millipore). As membranas foram secas, escaneadas, e quantificadas com a versão Scion Image PC de NIH imagem. Os resultados foram expressos como a quantidade relativa das formas fosforilada e não fosforilada, e a razão fosforilada/total.

Resultados esperados:

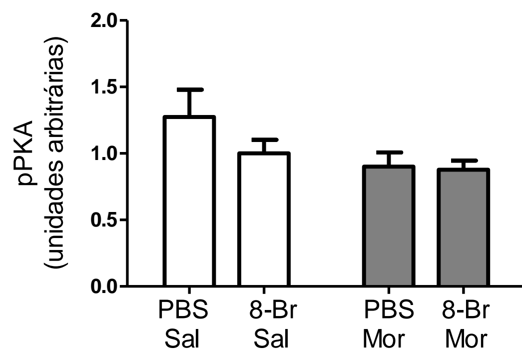
Como resultado, esperava-se que em algum ou, em ambos os tempos, as injeções de forscolina e 8-Br-AMPc realizadas no hipocampo no período de 11:30 min após o treinamento de medo contextual, fossem capazes de:

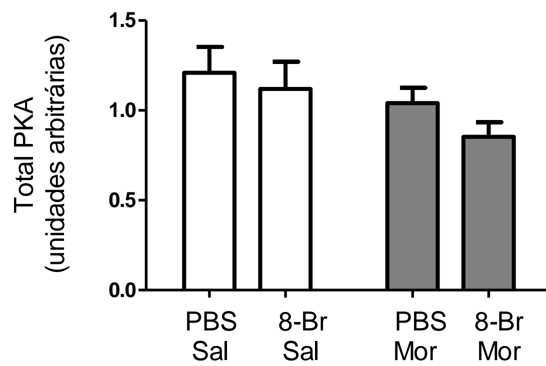
- Prevenir a redução da razão entre as formas fosforilada e total das subunidades catalíticas da PKA, na fração citosólica e nuclear, causada pela administração de morfina (10 mg/kg, via intraperitoneal) 12 horas após o treinamento;
- Prevenir a redução da razão entre as formas fosforilada e total da proteína CREB no núcleo, decorrente da administração de morfina 12 horas após o treinamento;

A confirmação destes resultados esperados comprovaria que a morfina reduz a persistência da memória e que tal efeito está associado a uma diminuição da ativação da via de sinalização da PKA e do fator de transcrição CREB. Abaixo são apresentados os resultados parciais do experimento referente ao

pré-tratamento dos animais que receberam morfina 12 horas após o treino, com 8-Br-cAMP. A eutanásia dos animais foi realizada uma hora após a injeção de morfina:

Resultados:





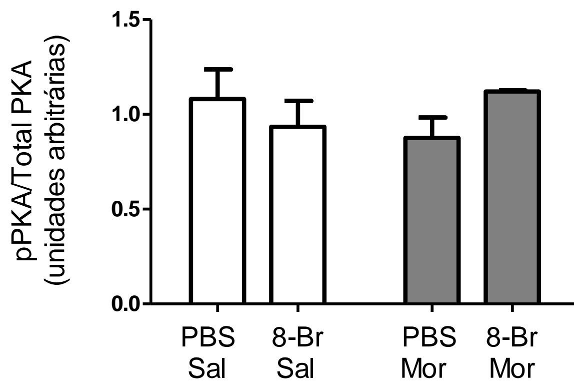


Figura 4 – Efeito da administração de 8-Br AMPc sobre o efeito da morfina 12 horas após o treino em imagens representativas de *western blot* e análises densitométricas da pPKA, PKA total e razão pPKA/PKAtotal na fração citosol 1 hora após a injeção de morfina. Dados são media ± erro padrão da media para 3 -4 animais em cada grupo.

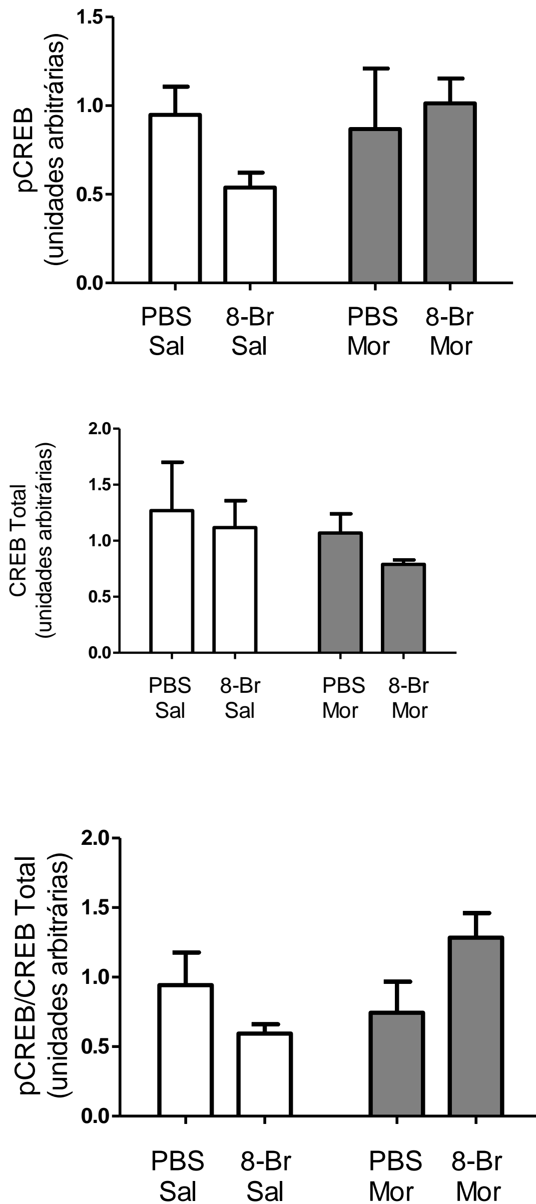


Figura 5 – Efeito da administração de 8-Br AMPc sobre o efeito da morfina 12 horas após o treino em imagens representativas de *western blot* e análises densitométricas da pCREB, CREB total e razão pCREB/CREB total na fração nuclear 1 hora após a injeção de morfina. Dados são média ± erro padrão da media para 3 -4 animais em cada grupo.