A MELHORA DA MEMÓRIA INDUZIDA POR ESPERMIDINA ENVOLVE A FOSFORILAÇÃO DA PKC, PKA E CREB EM HIPOCAMPO DE RATOS

Gustavo Petri Guerra

UFSM

Tese de doutorado

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PPGBT

SANTA MARIA – RS – BRASIL

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por

Gustavo Petri Guerra

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica da Universidade Federal de Santa Maria, como requisito parcial para a obtenção do grau de

Doutor em Ciências Biológicas: Bioquímica Toxicológica.

PPGBT

SANTA MARIA – RS – BRASIL

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Universidade Federal de Santa Maria

Centro de Ciências Naturais e Exatas

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica

A Comissão Examinadora, abaixo assinada,

aprova a Tese de Doutorado

A MELHORA DA MEMÓRIA INDUZIDA POR ESPERMIDINA ENVOLVE A FOSFORILAÇÃO DA PKC, PKA E CREB EM HIPOCAMPO DE RATOS

elaborada por

Gustavo Petri Guerra

como requisito parcial para obtenção do grau de

Doutor em Ciências Biológicas: Bioquímica Toxicológica

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Santa Maria, 08 setembro de 2011.

“Não se pode esperar resultados diferentes

fazendo as coisas da mesma forma”.

Albert Einstein

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SUMÁRIO

LISTA DE ABREVIATURAS

L-NAME – N-nitro L-arginina metil éster

AC – Adenilato ciclase

AKAP – proteína âncora da quinase A

AMPA – Ácido α-amino-3-hidroxi-5-metil-4-isoxazol propiônico

AMPc – Adenosina monofosfato cíclica

AP-5 – Ácido D-2-amino-5-fosfonopentanóico

CaM – Calmodulina

CaMKII – Proteína quinase dependente de cálcio/calmodulina do tipo II

CPG – Ácido DL-beta-clorofenil glutâmico

CREB – Proteína ligante do elemento responsivo ao AMPc

DAG - Diacilglicerol

DAO – Diamina oxidase

GABA – Ácido δ-aminobutírico

IH – Intra-hipocampal

MgluR – Receptor glutamatérgico metabotrópico

MK-801 – (+)5-metil-10,11-dihidro-5H-dibenzo[a,b]-ciclohepteno-5-10-amino

MTA – Metiltioadenosina

NADPH – Nicotinamida adenina dinucleotídeo fosfato reduzida

NMDA – *N*-metil-D-aspartato

NO – Óxido Nítrico

NOS – Óxido nítrico sintase

ODC – L- ornitina descarboxilase

PAO – Poliamina oxidase

PDE - Fosfodiasterase

PKA – Proteína quinase A

PKC – Proteína quinase C

PKG – Proteína quinase dependente de GMPc

SNC – Sistema Nervoso Central

SPD – Espermidina

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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil.

A MELHORA DA MEMÓRIA INDUZIDA POR ESPERMIDINA ENVOLVE A FOSFORILAÇÃO DA PKC, PKA E CREB EM HIPOCAMPO DE RATOS

Autor: Gustavo Petri Guerra
Orientadora: Maribel Antonello Rubin
Co-orientador: Juliano Ferreira
Local e Data da Defesa: Santa Maria, 08 de setembro de 2011.

As poliaminas endógenas, putrescina, espermidina e espermina, são aminas alifáticas que estão presentes em altas concentrações no sistema nervoso central (SNC). As poliaminas modulam diversos canais iônicos, incluindo o subtipo de receptor glutamatérgico *N*-metil D-aspartato (NMDA). Os processos mediados pelo receptor NMDA incluem plasticidade sináptica e formação de circuitos neurais. Acredita-se que estas plasticidades ocorrendo em algumas regiões cerebrais específicas, como o hipocampo, são críticas para os processos de aprendizado e memória. Está descrito que a espermidina (SPD), assim como as proteínas quinase, esta diretamente envolvida com os processos de formação da memória. Assim, investigamos o envolvimento das proteínas quinases dependente de AMPc (PKA) e dependente de Ca2+ (PKC) sobre a melhora da memória induzida por SPD em ratos. Para isso, ratos Wistar machos foram canulados bilateralmente no hipocampo e após a recuperação cirúrgica treinados na tarefa de esquiva inibitória. Imediatamente após o treino os animais receberam através das cânulas (0,5 µl/sítio) a administração de N-[2-bromocinamilamino etil]-(5-isoquinolina sulfonamida) [H-89, 0,5 ρmol intrahipocampal (ih)], inibidor da PKA, 3-[1-(Dimetilaminopropil)indol-3-il]-4-(indol-3-il)maleimida hidrochloride [GF 109203X, 2,5 ρmol (ih)], inibidor da PKC, arcaína (0,02 nmol, ih), antagonista do sítio de ligação das poliaminas no receptor NMDA ou SPD (0,2 nmol, ih). Um grupo de animais foi eutanasiado 30 ou 180 minutos após a administração das drogas e a atividade da PKC, PKA e o elemento ligante responsivo ao AMPc (CREB), no hipocampo, foi determinada por Western blot. Os outros animais foram submetidos à sessão de teste, 24 horas depois do treino na esquiva inibitória. A administração de H-89, GF 109203X ou arcaína preveniu a melhora da memória induzida por SPD. A SPD (0,2 nmol) aumentou a fosforilação da PKC 30 min, da PKA e do CREB 180 min após a injeção e aumentou a translocação da subunidade catalítica da PKA do citosol para o núcleo. GF 109203X, (2,5 ρmol) preveniu o efeito estimulatório da SPD sobre a fosforilação da PKC, PKA e CREB. Além disso, arcaína (0,02 nmol) e H-

89 (0,5 ρmol) preveniram o efeito estimulatório da SPD sobre a fosforilação da PKA e CREB 180 min depois da injeção. Nenhuma das drogas alterou a atividade motora dos animais. Estes resultados sugerem que o efeito facilitatório da memória induzido pela administração ih de SPD envolve um cruzamento entre PKC e PKA/CREB, com a ativação inicial da PKC, seguida da ativação da cascata PKA/CREB em ratos. Assim, poderemos determinar um possível mecanismo de ação da espermidina nos processos de formação da memória, e desta forma, fornecer subsídios para o desenvolvimento de fármacos.

ABSTRACT

PhD thesis
Post Graduation Program in Biological Sciences: Toxicological Biochemistry
Federal University of Santa Maria, RS, Brazil

THE IMPROVEMENT OF THE MEMORY INDUCED BY SPERMIDINE INVOLVE THE PKC, PKA AND CREB PHOSPHORILATION IN HIPOCAMPPUS OF RAT

Author: Gustavo Petri Guerra
Advisors: Maribel Antonello Rubin
Juliano Ferreira
Place and date of defense: Santa Maria, september 08, 2011.

The endogenous poliaminas, putrescine, spermidina and spermine are aliphatics amines that are present in high concentrations in the central nervous system (SNC). The action of the poliamines involves the modulation of several ionic channels, including the subtype of glutamatergic N-methyl-D-aspartate receptor (NMDA). The processes mediated by NMDA receptor include synaptic plasticity and formation of neural circuitry. It is believed that these plasticities happening in some cerebral areas specifies, as the hippocampus, are critical for the learning and memory processes. It is described that spermidine (SPD), as well as the protein kinase are directly involved with processes of formation of the memory. Therefore, we investigated the involvement of the Ca^{2+} dependent (PKC) and cAMP-dependent (PKA) protein kinase in the facilitatory effect induced by SPD on the memory of males Wistar rats. For that, the rats were bilaterally cannulae in the hippocampus, after the surgical recovery, the animals were trained in the inhibitory avoidance task and injected (0.5 μL) bilaterally in the hippocampus. A subset of the animals was euthanized 30 or 180 min after injections and activity of PKC, PKA and cAMP response element-binding protein (CREB), in the hippocampus, was determined for Western blot. The other animals had a testing session, 24 h pos-training in the inhibitory avoidance apparatus. The post-training administration of the 3-[1-(Dimethylaminopropyl)indol-3-yl]-4-(indol-3-yl)maleimide hydrochloride [GF 109203X, 2.5 ρmol intrahippocampal (ih)], inhibitor of PKC, N-[2-bromocinnamylamino ethyl]-(5-isoquinoline sulfonamide) [H-89, 0.5 ρmol intrahippocampal (ih)], PKA inhibitor or arcaine (0.02 nmol ih), the antagonist of the NMDA receptor polyamine-binding site prevented memory improvement induced by SPD (0.2 nmol ih). The SPD (0.2 nmol), in the hippocampus, facilitated PKC 30 min, PKA and CREB phosphorylation 180 min after administration, and increased translocation of the catalytic subunit of PKA into the nucleus. GF 109203X, (2.5 ρmol) prevented the stimulatory effect of SPD on PKC, PKA

and CREB phosphorylation. Furthermore, arcaine (0.02 nmol) and H-89 (0.5 ρmol) prevented the stimulatory effect of SPD on PKA and CREB phosphorylation 180 min after administration. None of the drugs studies altered the locomotor activity of the animals. These results suggest that the facilitatory effect of the memory induced by the ih administration SPD involves the cross talk between PKC and PKA/CREB, with PKC activation follow by PKA/CREB pathways activation in rats.

I. INTRODUÇÃO

As poliaminas, putrescina, espermidina e espermina são aminas alifáticas simples, que estão presentes em todas as células vivas e são amplamente distribuídas no sistema nervoso central. Elas são solúveis em água, possuem baixo peso molecular e caráter fortemente básico (Carter, 1994; Seiler et al., 1996; Teti et al., 2002; Gugliucci, 2004).

O principal foco de estudo das poliaminas baseia-se à interação com o receptor NMDA (Coughenour and Barr, 2001). Em particular, espermina e espermidina atuam de maneira bifásica sobre o receptor NMDA, promovendo um efeito estimulatório ou inibitório sobre o receptor (Williams, 1997). A resposta bifásica das poliaminas parece também existir a nível comportamental. Neste contexto, tem sido mostrado que a administração de baixas doses de poliaminas melhoram o desempenho dos animais em tarefas cognitivas, enquanto altas doses prejudicam ou não modificam (Conway, 1998; Rubin et al., 2000; 2001; 2004).

O efeito facilitatório da SPD sobre a memória envolve o receptor NMDA uma vez que o MK-801, antagonista NMDA, e arcaína, antagonista do sítio das poliaminas no receptor NMDA, revertem a melhora da memória induzida pelas poliaminas (Rubin et al., 2000; 2001; 2004; Camera et al., 2007; Gomes et al., 2010). Além disso, a melhora da memória induzida por poliminas parece depender da atividade da enzima óxido nítrico sintase hipocampal e da produção de óxido nítrico, uma vez que a administração de L-NAME, um inibidor não específico da enzima óxido nítrico sintase, impede a melhora da memória causada por SPD na

tarefa de esquiva inibitória e o aumento dos níveis de nitrato e nitrito, induzidos por SPD (Guerra et al., 2006).

Apesar de existirem vários estudos demonstrando o envolvimento das poliaminas e do receptor NMDA sobre a memória, pouco ainda é conhecido sobre o mecanismo de ação das poliaminas sobre a memória, após a ativação do receptor NMDA.

Existem evidências demonstrando o envolvimento de proteínas quinase nos processos de aprendizado e memória (Bernabeu et al., 1997a; Izquierdo and Medina, 1997; Vianna et al., 2000a; b; Quevedo et al., 2005). A proteína quinase C (PKC) é uma enzima monomérica, ou seja, um único polipeptídio simples formado por um domínio regulatório e um domínio catalítico (Newton, 2003; Amadio et al., 2006; Sun and Alkon, 2010). Foi visto que a atividade hipocampal da PKC aumenta imediatamente depois do treino na tarefa de esquiva inibitória, alcança um pico 30 min mais tarde e retorna aos níveis normais 120 min depois do treino (Bernabeu et al., 1995; Cammarota et al., 1997). Além disso, alguns estudos relatam um cruzamento entre PKC e proteína quinase A (PKA), ou seja, é sugerido que PKC contribua para a ativação da cascata AMPc/PKA/CREB (Sugita et al., 1997; Kubota et al., 2003; Yao et al., 2008).

A PKA é uma holoenzima tetramérica formada por duas subunidades R e duas subunidades C, sendo que a subunidade R possui dois sítios de ligação para o AMPc. A PKA hipocampal pode ser ativada

pelo influxo de Ca^{2+} que estimula a adenilil ciclase, sensível a Ca^{2+}/calmodulina, a qual sintetiza AMPc (Eliot et al., 1989; Chetkovich and Sweatt, 1993). A PKA na forma ativa migra para o núcleo, fosforila a proteína de elemento ligante responsivo ao AMPc (CREB) tornando-a ativa e assim, aumenta a síntese protéica (Dash et al., 1991; Mayford et al., 1995a; Impey et al., 1998).

A atividade da PKA e os níveis de CREB fosforilada aumentam depois do treino na tarefa de esquiva inibitória em dois picos. O primeiro imediatamente depois do treino, e o segundo, 3-6 horas depois (Bernabeu et al., 1997a). O primeiro pico da atividade da PKA ocorre antes da alteração nos níveis da AMPc, provavelmente devido a preexistência de AMPc celular. O segundo pico coincide com o aumento máximo nos níveis de AMPc depois do treino, e deve ser ativado por este (Bernabeu et al., 1996).

A cascata AMPc/PKA/CREB possui um papel importante na fase final da consolidação da memória, que necessita da síntese de proteínas (Barad et al., 1998; Huang and Kandel, 1998; Pereira et al., 2001; Quevedo et al., 2005;).

No presente estudo, foi investigado o envolvimento da PKC e da cascata PKA/CREB sobre a melhora da memória induzida por SPD na tarefa de esquiva inibitória. Para isto, foi avaliado o efeito do GF 109203X, um inibidor da PKC, do H-89, um inibidor da PKA, e da arcaína, um antagonista do sitio das poliaminas no receptor NMDA, na melhora da

memória causada pela administração intra-hipocampal de SPD em ratos, na tarefa de esquiva inibitória.

II. OBJETIVOS

II.1. OBJETIVO GERAL

Avaliar o envolvimento da proteína quinase C (PKC), proteína quinase A (PKA) e do elemento ligante responsivo ao AMPc (CREB) na melhora da memória induzida pela administração intra-hipocampal de SPD na tarefa de esquiva inibitória em ratos.

II.2. OBJETIVOS ESPECÍFICOS

- Investigar o efeito da administração intra-hipocampal de H-89, de arcaína e GF 109203X sobre a melhora da memória induzida por SPD na tarefa de esquiva inibitória em ratos.

- Avaliar o efeito da administração intra-hipocampal de H-89 e de arcaína sobre o possível aumento da fosforilação da PKA e CREB induzidos pela administração intra-hipocampal de SPD.

- Verificar a possível translocação da subunidade catalítica da PKA do citosol para o núcleo, induzida pela administração intra-hipocampal de SPD.

- Avaliar o efeito da administração intra-hipocampal de GF 109203X sobre o possível aumento da fosforilação da PKC, PKA e CREB induzidos pela administração intra-hipocampal de SPD.

III. REVISÃO BIBLIOGRÁFICA

III.1 – MEMÓRIA

A memória desperta o interesse e a imaginação do homem desde a Antiguidade, contudo os primeiros estudos científicos foram realizados há pouco mais de um século. Hoje, possuímos uma razoável compreensão sobre os mecanismos de sua formação. A memória é uma das funções cognitivas mais complexas que a natureza produziu, e as evidências científicas sugerem que o aprendizado de novas informações e o seu armazenamento causam alterações estruturais no sistema nervoso (Izquierdo, 2002).

Para a formação de uma memória é necessário que ocorra antes um aprendizado, que é a aquisição de novas informações, é a modificação de um comportamento após uma experiência vivida. E a memória é a capacidade de armazenar estas novas informações e recordar aprendizados anteriores (Izquierdo, 2002).

De acordo com seu conteúdo, a memória pode ser classificada em dois diferentes tipos: a memória declarativa e a não declarativa. A memória declarativa é aquela evocada pelo consciente e a qual conseguimos verbalizar, é uma memória para fatos e eventos que ocorreram em nossa vida, como uma viagem ou um casamento. A memória não declarativa, também chamada de memória de procedimento, é aquela evocada pelo inconsciente e que não conseguimos verbalizar, é uma memória relacionada com hábitos, habilidades motoras e

comportamentos, como andar de bicicleta ou dirigir um automóvel (Squire and Zola-Morgan, 1988; Squire et al., 1993; Squire and Zola, 1996; Stickgold, 2005).

A memória também pode ser classificada quanto ao seu tempo de duração: como imediata, de curta e de longa duração. A memória imediata, também chamada memória de trabalho, mantém as informações por apenas alguns segundos, não deixando traços ou produzindo arquivos, como por exemplo, a memória de um número de telefone que consultamos na lista telefônica, e que esquecemos logo após tê-lo digitado. A memória de curta duração, que dura minutos ou poucas horas, e a memória de longa duração, que dura meses ou anos, por sua vez são armazenadas e formam arquivos de memórias. Sendo este período chamado de período de "consolidação" (McGaugh, 2000; Izquierdo, 2002; Squire and Kandel, 2003; Ranganath and Blumenfeld, 2005).

III.1.1 – A FORMAÇÃO DA MEMÓRIA

A formação de uma nova memória depende de processos neurais que iniciam com a aquisição de uma informação, seguido por um processo de consolidação (armazenamento da informação) e por fim um processo de evocação, quando a memória está pronta para ser lembrada (Abel and Lattal, 2001).

Uma seqüência de eventos bioquímicos é necessária para formação da memória. Acredita-se que a seqüência inicial envolva um aumento na liberação de neurotransmissores, principalmente glutamato (McGaugh and Izquierdo, 2000; McGaugh, 2002). O glutamato liberado se liga aos receptores glutamatérgicos ácido α-amino-3-hidroxi-5-metil-4-isoxazol propiônico (AMPA), cainato, N-metil-D-aspartato (NMDA) e metabotrópicos (mGluR), provocando o aumentando da concentração de cálcio intracelular. Como conseqüência disso, ocorre a liberação de segundos mensageiros e cascatas bioquímicas, conduzidas pelo aumento da atividade das proteínas quinases, ocasionando assim alterações nas subunidades dos receptores glutamatérgicos, aumentando a expressão dos fatores de transcrição e aumentando a transmissão de informações entre neurônios (Izquierdo and Medina, 1997; Mizuno and Giese, 2005). Tais alterações entre os neurônios têm sido denominada "plasticidade sináptica" (McGaugh and Izquierdo, 2000; McGaugh, 2000, 2002).

Entre as proteínas quinases, pelo menos quatro diferentes tipos estão envolvidas no processo de formação da memória: proteína quinase cálcio/calmodulina dependente (CaMKII) (Silva et al., 1992; Wolfman et al., 1994; Mayford et al., 1995b; Bernabeu et al., 1997b; Giese et al., 1998), proteína quinase dependente de cálcio (PKC) (Bernabeu et al., 1995; Bernabeu et al., 1997b), proteína quinase dependente de GMPc (PKG) (Bernabeu et al., 1997c) e proteína quinase dependente de AMPc

(PKA) (Eliot et al., 1989; Chetkovich et al., 1991; Chetkovich and Sweatt, 1993; Bernabeu et al., 1997a; Bevilaqua et al., 1997;).

III.1.2 – HIPOCAMPO

O hipocampo, que possui formato de “cavalo-marinho” é uma estrutura de grande importância para a consolidação da memória, formada por duas camadas de neurônios, dobradas uma sobre a outra, sendo uma chamada giro denteado e a outra corno de Amon. O corno de Amon possui três divisões: CA1, CA2 e CA3 (CA significa corno de Amon) (Miller and O'Callaghan, 2005).

A grande via de entrada de informações no hipocampo é o córtex entorrinal. O córtex entorrinal envia informações ao hipocampo por meio de um feixe de axônios chamado via perforante. Estes axônios estabelecem sinapses em neurônios do giro denteado. Os neurônios do giro denteado projetam axônios (chamados de fibras musgosas) que estabelecem sinapses em células da CA3, que por sua vez, projetam axônios, que se ramificam. Um ramo deixa o hipocampo pelo fórnix, e o outro ramo, chamado colateral de Schaffer, forma sinapses excitatórias em neurônios da CA1 (Fig. III.1). A informação neural é transmitida a partir da região CA1 a outras áreas, constituindo uma saída da informação

pré-processada no hipocampo (Bear et al., 2002; Watts and Thomson, 2005; Burke and Barnes, 2010).

O hipocampo sem dúvida é de grande importância para a formação da memória, uma vez que manipulações farmacológicas e bioquímicas nestas áreas alteram a memória em diferentes tarefas (Morris, 1989; Izquierdo and Medina, 1995; Bernabeu et al., 1996; Rubin et al., 2000; Berlese et al., 2005; Guerra et al., 2006; Bonini et al., 2011; Kornisiuk et al., 2011).

Estudos mostram que a administração intra-hipocampal (Jafari-Sabet, 2006) e intra-amigdala (Roesler et al., 2000; LaLumiere et al., 2004) imediatamente pós-treino de ácido-D-2-amino-5-fosfonopentanóico (AP5), antagonista competitivo do receptor glutamatérgico NMDA, causa prejuízo de memória na tarefa de esquiva inibitória. A administração intra-hipocampal de AP5 (1-10 μg/rato) além de causar déficit de memória, diminui o efeito facilitatório sobre a memória induzido por injeção intra-hipocampal de NMDA (10 μg/rato) na tarefa de esquiva inibitória (Jafari-Sabet, 2006).

Estudos mostram ainda que ratos com lesões no hipocampo apresentam um prejuízo no aprendizado espacial (Broadbent et al., 2004; Goodrich-Hunsaker et al., 2010; Goodrich-Hunsaker and Hopkins, 2010).

Estas evidências indicam que os receptores glutamatérgicos hipocampais estão diretamente envolvidos na formação da memória.

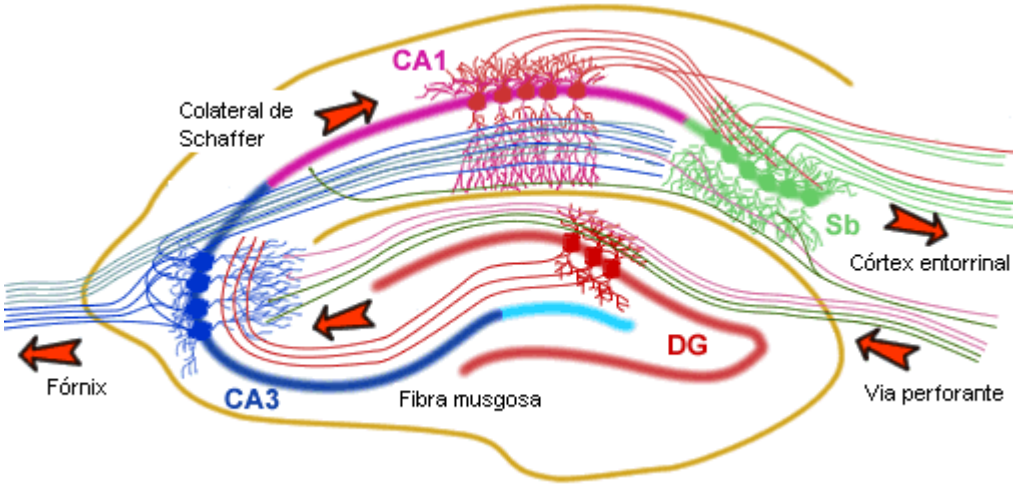

Fig. III.1 - Representação esquemática do hipocampo e seus micro circuitos. CA: corno de Amon; DG: giro denteado. (Adaptado de: Neuropsychoanalyse Journal. Disponível em: <moderne-psychoanalyse/>. Acesso em: 12 de julho de 2011).

III.2 – RECEPTOR N-METIL-D-ASPARTATO – NMDA

O receptor NMDA é um subtipo de receptor glutamatérgico amplamente distribuído no sistema nervoso central (SNC), sendo os níveis mais altos encontrados na região CA1 do hipocampo (Monaghan and Cotman, 1985). O NMDA possui várias subunidades denominadas: NR1 (onde se liga a glicina), NR2 (A-D) (onde se liga o glutamato) e NR3 (A-B) (onde se liga a glicina) (Yamakura and Shimoji, 1999; Prybylowski and Wenthold, 2004; Furukawa et al., 2005; Paoletti and Neyton, 2007), sendo que a combinação de duas subunidades NR1 associadas a duas

subunidades NR2 e/ou NR3 forma um canal iônico tetramérico (Furukawa et al., 2005).

O canal iônico formado pelas subunidades do receptor NMDA possui alta permeabilidade aos íons sódio (Na^+), potássio (K^+) e cálcio (Ca^{2+}) (MacDermott et al., 1986; Mayer and Westbrook, 1987). No potencial de repouso o canal está bloqueado por íons Mg^{2+} (Riedel et al., 2003), impedindo a passagem de outros íons. A liberação de neurotransmissores na fenda sináptica, incluindo o glutamato, o deslocamento do Mg^{2+} e a ativação do receptor NMDA, resulta no influxo de íons Na^+ e principalmente Ca^{2+} e no efluxo de íons K^+ (Scatton, 1993; Ozawa et al., 1998; Paoletti and Neyton, 2007). O influxo de cálcio promove a ativação de proteínas quinases responsáveis por algumas respostas celulares, incluindo o aumento da expressão dos fatores de transcrição, e o aumento da transmissão de informações entre neurônios (Bliss and Collingridge, 1993; Elgersma and Silva, 1999; Izquierdo and Medina, 1997; Lau et al., 2009).

O receptor NMDA é um complexo protéico com vários sítios de ligação para agonistas e antagonistas, tais como glutamato/NMDA, sítio onde se liga dizocilpina (MK-801), sítio modulatório para a glicina (co-agonista do receptor NMDA), zinco, bem como sítios de ligação para poliaminas (Ransom and Stec, 1988; Williams et al., 1991; Riedel et al., 2003) (Fig. III. 2).

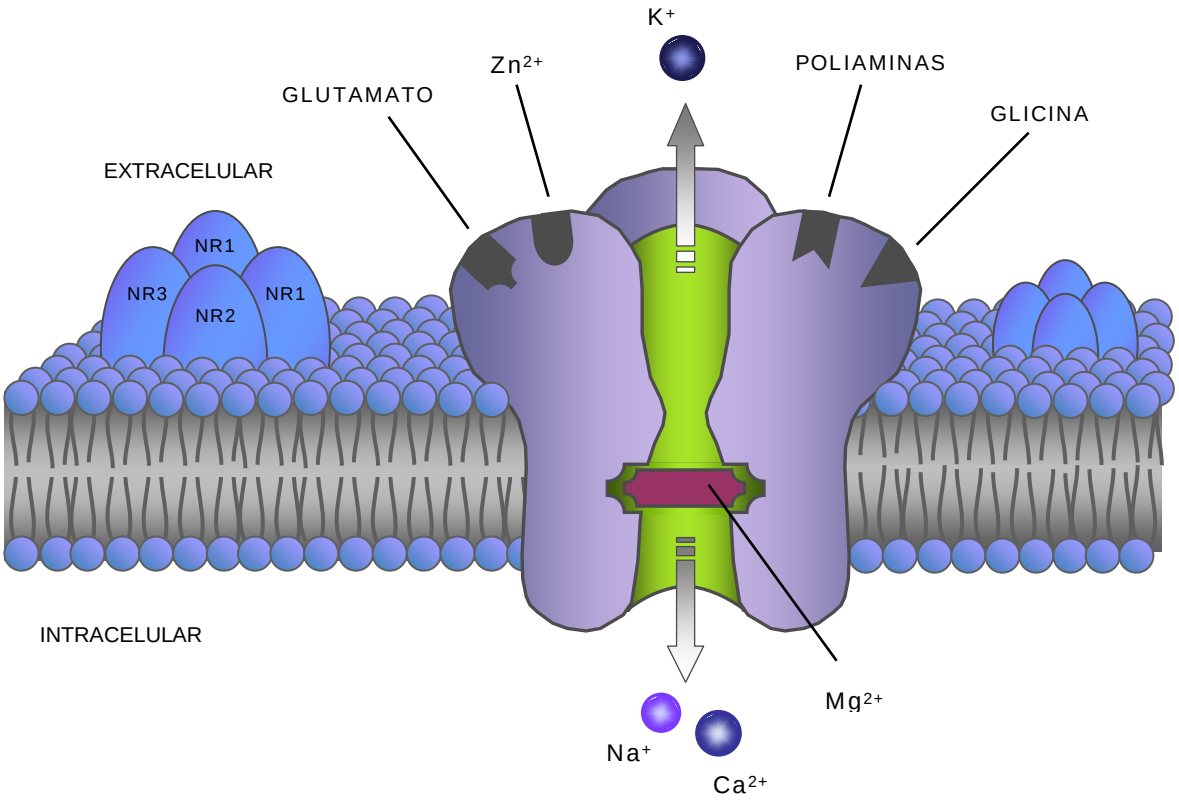

Fig. III.2 – Representação esquemática do receptor NMDA.

III.2.1 – RECEPTOR NMDA E MEMÓRIA

Evidências demonstram que o receptor glutamatérgico NMDA é fundamental para os processos de plasticidade sináptica e formação de memória para diferentes tarefas (Castellano et al., 2001; Riedel et al., 2003). Estudos com manipulações farmacológicas e/ou genéticas mostram que o receptor NMDA está diretamente envolvido na formação

de memórias aversivas (Miserendino et al., 1990; Izquierdo et al., 1992; Roesler et al., 1998; Cravens et al., 2006) e espaciais (Morris et al., 1986; Caramanos and Shapiro, 1994; Ahlander et al., 1999; Shapiro and Eichenbaum, 1999; von Engelhardt et al., 2008; Fellini et al., 2009; Hu et al., 2009; Wang et al., 2009).

A administração sistêmica ou intracerebral de antagonistas do receptor NMDA causam prejuízo no aprendizado em uma grande variedade de tarefas de memória (Izquierdo and Medina, 1995; Martin et al., 2000; Izquierdo et al., 2006; Krystal et al., 2009; van der Staay et al., 2011).

De Lima e colaboradores (2005), demonstraram que a administração sistêmica de 0,1 mg/kg de (+)5-metil-10,11-dihidro-5H-dibenzo[a,b]-cicloheptено-5-10-amino (MK-801), um antagonista não-competitivo do receptor NMDA, 20 minutos antes ou imediatamente depois do treino na tarefa de reconhecimento de objetos prejudica a memória de curta e longa duração. Roesler e colaboradores (2000), também demonstraram prejuízo da memória na tarefa de esquiva inibitória devido a administração pré ou pós-treino intra-amigdala de 5 mg de AP5, um antagonista competitivo do receptor NMDA. Enquanto que a administração de AP5 pré-teste não afeta a memória para a mesma tarefa. Efeito similar ainda foi demonstrado para administração intra-hipocampal destes compostos, na tarefa de esquiva inibitória (Jafari-

Sabet, 2006) e na tarefa do labirinto aquático de Morris (Morris, 1989; Fellini et al., 2009).

Entretanto, agonistas do receptor NMDA, como o glutamato (Izquierdo and Medina, 1995; Rubin et al., 1997) e o ácido DL-beta-clorofenil glutâmico (CPG) (Flood et al., 1990) melhoram a performance dos ratos ou camundongos na tarefa de esquiva inibitória e no labirinto em T, respectivamente.

Todas estas evidências farmacológicas estão de acordo com estudos envolvendo animais transgênicos, onde é demonstrado que a super-expressão de receptores NMDA produz um melhor desempenho destes animais em tarefas de memória (Tang et al., 1999; Tang et al., 2001; White and Youngentob, 2004; Wang et al., 2009).

Além disso, estudos demonstram que o número de receptores NMDA está reduzido no sistema nervoso central de pacientes (Sze et al., 2001) e animais geneticamente modificados que desenvolvem a doença de Alzheimer (Snyder et al., 2005; Dewachter et al., 2009;).

III.3 – POLIAMINAS

As poliaminas são aminas alifáticas simples que estão presentes em todas as células vivas, procarióticas, eucarióticas, plantas e animais (Thomas and Thomas, 2001), entretanto, apenas as poliaminas

putrescina, espermidina e espermina são sintetizadas pelos mamíferos (Coleman et al., 2004).

Os nomes putrescina e espermina provém da fonte de onde, estas poliaminas, foram inicialmente isoladas. A espermina foi descrita pela primeira vez há mais de 300 anos, quando Antoni van Leuwenhoek, em 1678, relatou a presença de cristais em amostras de esperma seco, enquanto que, em 1885, Ludwig Brieger, descobriu a putrescina na carne em putrefação (Gugliucci, 2004).

As poliaminas estão amplamente distribuídas no SNC e encontradas principalmente em regiões do encéfalo como hipotálamo, bulbo, hipocampo e cerebelo, sendo a mais alta concentração de SPD, seguida por espermina e por fim putrescina, que possui uma concentração muito pequena em comparação as outras duas (Seiler and Schmidt-Glenewinkel, 1975).

III.3.1 – ESTRUTURA DAS POLIAMINAS

As poliaminas, putrescina (1,4 – diaminobutano), espermidina [N-(3-aminopropil)-1,4- diaminobutano] e espermina [bis-N-(3-aminopropil)-1,4- diaminobutano], são constituídas por uma, duas ou três cadeias de carbono, respectivamente, conectadas por átomos de nitrogênio (fig III.3). Elas são solúveis em água, possuem baixo peso molecular e caráter

fortemente básico, devido aos grupamentos amino (Carter, 1994; Seiler et al., 1996; Teti et al., 2002; Gugliucci, 2004).

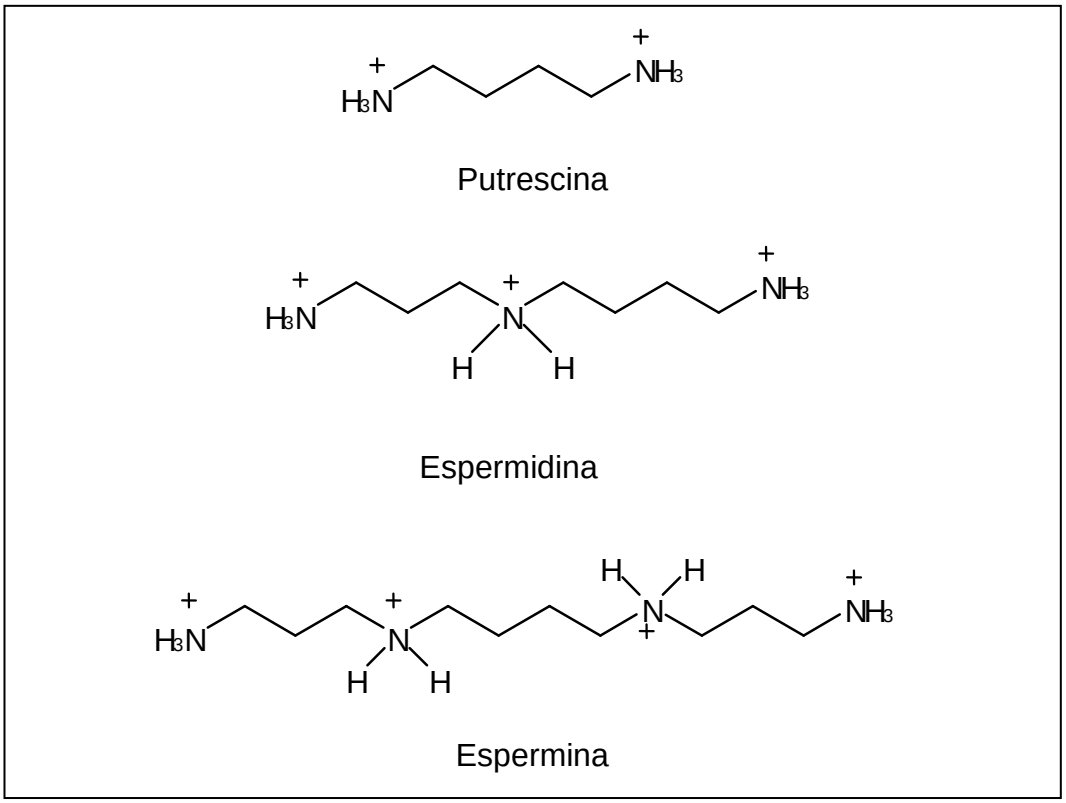

Fig. III.3 - Estrutura química das poliaminas (adaptado de Teti et al., 2002 and Gugliucci, 2004).

III.3.2 – METABOLISMO DAS POLIAMINAS

As poliaminas encontradas nos seres humanos são sintetizadas no organismo ou provém da flora gastrintestinal capaz de metabolizar aminoácidos provenientes da dieta (Seiler, 1981; Carter, 1994; Teti et al.,

2002; Larque et al., 2007). O metabolismo das poliaminas e as características das diferentes enzimas envolvidas têm sido bastante estudados. (Tabor and Tabor, 1984; Urdiales et al., 2001; Pegg, 2009).

O nível celular adequado de poliaminas é alcançado por meio de um balanço entre a sua síntese, rota de interconversão e catabolismo.

III.3.2.1 – SÍNTESE

O aminoácido ornitina é o principal precursor das poliaminas endógenas. No cérebro, a ornitina é formada a partir da clivagem hidrolítica do aminoácido arginina em uma reação catalisada pela arginase (Seiler, 1981; Yu et al., 2003).

A putrescina é sintetizada pela descarboxilação da ornitina em uma reação catalisada pela enzima ornitina descarboxilase (ODC), enzima limitante na síntese das poliaminas. A putrescina formada serve como precursor imediato da síntese de espermidina e espermina. Esta síntese requer o grupo aminopropil que é fornecido pela ação de duas enzimas: a S-adenosilmetionina descarboxilase (SAMDC), que descarboxila a S-adenosilmetionina (SAM), e a espermidina sintase, uma enzima transferase que catalisa a transferência do grupamento aminopropil da SAM para a putrescina ou espermina sintase que catalisa a transferência de um segundo grupamento aminopropil para a SPD, formando SPD e

espermina respectivamente (Tabor and Tabor, 1984; Urdiales et al., 2001; Larque et al., 2007; Pegg, 2009).

Esta rota de síntese de poliaminas é reversível, ou seja, a espermina pode ser convertida em SPD e esta em putrescina. O primeiro passo desta interconversão é a acetilação da espermina ou SPD na posição N1, catalisada pela enzima espermidina/espermina acetiltransferase (SSAT). Após este passo, a poliamina acetilada sofre quebra oxidativa, por ação da enzima poliamina oxidase (PAO), liberando os grupos aminopropil provenientes da S-adenosilmetionina descarboxilada (SAM-D) para formar SPD e putrescina. O produto destas reações permanece no ciclo e pode ser reutilizado na síntese das poliaminas (Urdiales et al., 2001; Pegg, 2009).

Estudos com a inibição da poliamina oxidase têm demonstrado que a rota de interconversão de poliaminas, no encéfalo de ratos, é responsável por 70% da putrescina formada a partir da SPD, enquanto somente 30% da putrescina é formada pela descarboxilação da ornitina (Seiler, 2004; Seiler et al., 1985).

Assim as enzimas chave na regulação da síntese de poliaminas são ornitina descarboxilase, S-adenosilmetionina descarboxilase e espermidina/espermina acetiltransferase (Urdiales et al., 2001; Pegg, 2009).

III.3.2.2 – CATABOLISMO

O catabolismo das poliaminas consiste na desaminação oxidadiva de seus grupos amino primários, sendo a reação catalisada por amino oxidases dependentes de cobre, como a diamina oxidase (DAO). Através desta desaminação cada intermediário do ciclo da interconversão pode ser transformado em um aldeído, que é posteriormente oxidado em um aminoácido, e apenas a putrescina pode ser convertida em ácido aminobutírico (GABA).

Os produtos finais do catabolismo das poliaminas, bem como as poliaminas acetiladas, podem então ser excretados na urina (Carter, 1994; Teti et al., 2002; Gugliucci, 2004; Seiler, 2004; Larque et al., 2007).

III.4 – RECEPTOR NMDA, POLIAMINAS E MEMÓRIA

A ativação do receptor NMDA está associada com diferentes formas de plasticidade sináptica, como Potencialização de Longa Duração (LTP) e aprendizado e memória (Lee and Silva, 2009). Existem vários relatos demonstrando o envolvimento das poliaminas e do receptor NMDA em processos de aprendizado e memória, tanto melhorando a memória de ratos em diferentes tarefas, como atenuando o déficit de memória induzido por diferentes agentes amnésicos (Shimada et al., 1994; Kishi et

al., 1998a; b; Rubin et al., 2000; 2001; 2004; Mikolajczak et al., 2003; Tadano et al., 2004; Berlese et al., 2005; Guerra et al., 2006; Camera et al., 2007).

As poliaminas apresentam uma resposta bifásica sobre o receptor NMDA em nível comportamental. Neste contexto, tem sido mostrado que a administração de baixas doses de poliaminas melhoram, enquanto altas doses prejudicam o desempenho dos animais em tarefas cognitivas. Assim, altas doses de espermina (125-250 nmol/5μl), administradas por via intracerebroventricular, causam dano hipocampal e déficit de aprendizado em ratos na tarefa do labirinto aquático de Morris (Conway, 1998). E administração intra-peritoneal de espermidina (80 mg/kg) potencializa a diminuição do aprendizado induzido por MK-801 no labirinto em T de 14 braços (Shimada et al., 1994).

Por outro lado, a administração intracerebral (0,2 nmol) (Rubin et al., 2000; 2001; 2004; Berlese et al., 2005; Guerra et al., 2006) e sistêmica (10 mg/kg) (Mikolajczak et al., 2002; Camera et al., 2007) de espermidina melhora o desempenho de ratos em tarefas como esquiva inibitória, medo condicionado e reconhecimento social.

Além disso, administração intra-hipocampal de SPD (10 mg/hipocampo) atenua, o número de erros para memória de trabalho induzidos por administração intra-hipocampal tanto de MK-801, um antagonista não-competitivo do receptor NMDA (Kishi et al., 1998a), quanto por escopolamina (3,2 mg/hipocampo), antagonista do receptor

muscarínico (Kishi et al., 1998b). A administração sistêmica de espermina atenua o prejuízo do aprendizado induzido por CPP, um antagonista competitivo do receptor NMDA, no labirinto em T de 14 braços (Meyer et al., 1998).

O efeito facilitatório da SPD sobre a memória parece depender da atividade da enzima óxido nítrico sintase hipocampal e da produção de óxido nítrico, uma vez que a administração intra-hipocampal de N-nitro-L-arginina metil éster (L-NAME), um inibidor não específico da enzima óxido nítrico sintase, imediatamente depois do treino previne a melhora da memória causada por SPD na tarefa de esquiva inibitória (Guerra et al., 2006). A SPD aumenta os níveis de nitratos e nitritos, e a co-administração de L-NAME previne este efeito (Guerra et al., 2006).

O efeito facilitatório da SPD sobre a memória envolve o receptor NMDA uma vez que o MK-801, antagonista NMDA, e arcaína, antagonista do sítio das poliaminas no receptor NMDA, revertem a melhora da memória induzida pelas poliaminas (Rubin et al., 2000; 2001; 2004; Camera et al 2007, Gomes et al., 2010). Outro antagonista do sítio das poliaminas no receptor NMDA, ifenprodil, administrado por via intra-amigdala e intraperitoneal prejudica o desempenho dos ratos na tarefa de medo condicionado (Rodrigues et al., 2001) e a sua administração intracerebroventricular, reverte o efeito facilitatório da SPD sobre a memória (Tadano et al., 2004). Além disso, as poliaminas estão envolvidas na facilitação da extinção do medo condicionado contextual

(Gomes et al., 2010) e na dependência de estado na tarefa de esquiva inibitória (Mariani et al., 2011).

Apesar de existirem estudos demonstrando o envolvimento das poliaminas e do receptor NMDA sobre a memória (Kishi et al., 1998a; b; Rubin et al., 2000; 2001; 2004; Camera et al., 2007), pouco ainda é conhecido sobre o mecanismo de ação das poliaminas sobre a memória, após a ativação do receptor NMDA, sendo que uma das conseqüências pode ser a ativação das proteínas quinases.

III.5 – PROTEÍNA QUINASE

Proteína quinase é um tipo de enzima que modifica outras proteínas pela transferência de grupos fosfatos de moléculas doadoras de alta energia, como o ATP, para moléculas-alvo específicas (substratos), processo que tem o nome de fosforilação. A fosforilação resulta em uma alteração funcional da proteína alvo, através de mudanças na atividade enzimática, localização celular ou associação com outras proteínas. Todas as quinases necessitam de um íon metálico divalente, como o Mg^{2+}, para transferir o grupo fosfato (Micheau and Riedel, 1999).

Uma proteína fosfatase faz exatamente a reação inversa das quinases, ela remove um grupamento fosfato, ou seja, faz a desfosforilação (Skroblin et al., 2010). Dentre as fosfatases, a calcineurina responde diretamente a um segundo mensageiro (aumento da

concentração intracelular de Ca^{2+}), enquanto outras são ativadas quando fosforiladas pelas próprias proteínas quinase (Skroblin et al., 2010).

As células contêm várias proteínas quinases e proteínas fosfatases, cada uma sendo responsável por agir em um determinado grupo ou uma determinada proteína. As células apresentam normalmente um estado de equilíbrio entre as proteínas quinase, fosfatases (ativadas ou desativadas) e os substratos protéicos (fosforilados ou desfosforilados). O genoma humano contém cerca de 500 genes de proteínas quinase, constituindo aproximadamente 2% de todo o genoma humano, sendo que 20% de todas as proteínas sintetizadas em uma célula servem como substrato para essas enzimas. As proteínas quinase são capazes de regular a maioria das cascatas celulares, especialmente as que envolvem transdução de sinal (Manning et al., 2002).

III.5.1 – PROTEÍNA QUINASE A (PKA)

A proteína quinase dependente de AMPc (PKA) foi caracterizada em 1968, é a mais comum e versátil enzima de células eucarióticas (Walsh et al., 1968). A PKA é uma quinase no qual a atividade depende dos níveis de AMPc na célula. A família da PKA consiste de quatro subunidades regulatórias (RIα, RIβ, RIIα, RIIβ) e três subunidades catalíticas (Cα, Cβ, Cγ) (McKnight et al., 1988; Doskeland et al., 1993),

expressas no encéfalo dos mamíferos (McKnight et al., 1988; Cadd and McKnight, 1989).

Cada PKA é uma holoenzima tetramérica formada por duas subunidades R e duas subunidades C, sendo que a subunidade R possui dois sítios de ligação para o AMPc. O maior controle da atividade da PKA é fornecido pelo AMPc, pois quando os níveis de AMPc estão baixos a holoenzima permanece intacta e cataliticamente inativa (Taylor et al., 1990). Entretanto, quando ocorre um aumento da concentração de AMPc, mediante a ativação da adenilato ciclase (AC), o AMPc liga-se aos dois sítios na subunidade R, liberando a subunidade catalítica. A subunidade catalítica livre pode então catalisar a transferência do grupo fosfato do ATP para os resíduos de serina/treonina do substrato. Esta fosforilação pode ocorrer diretamente em uma enzima, aumentando ou diminuindo sua atividade, (Taylor et al., 1990; Wang et al., 1991; Gibbs et al., 1992; Wang et al., 2004), ou a subunidade catalitica pode migrar para o núcleo, fosforilar a CREB e assim, aumentar a síntese protéica (Fig. III. 4) (Dash et al., 1991; Mayford et al., 1995a; Impey et al., 1998).

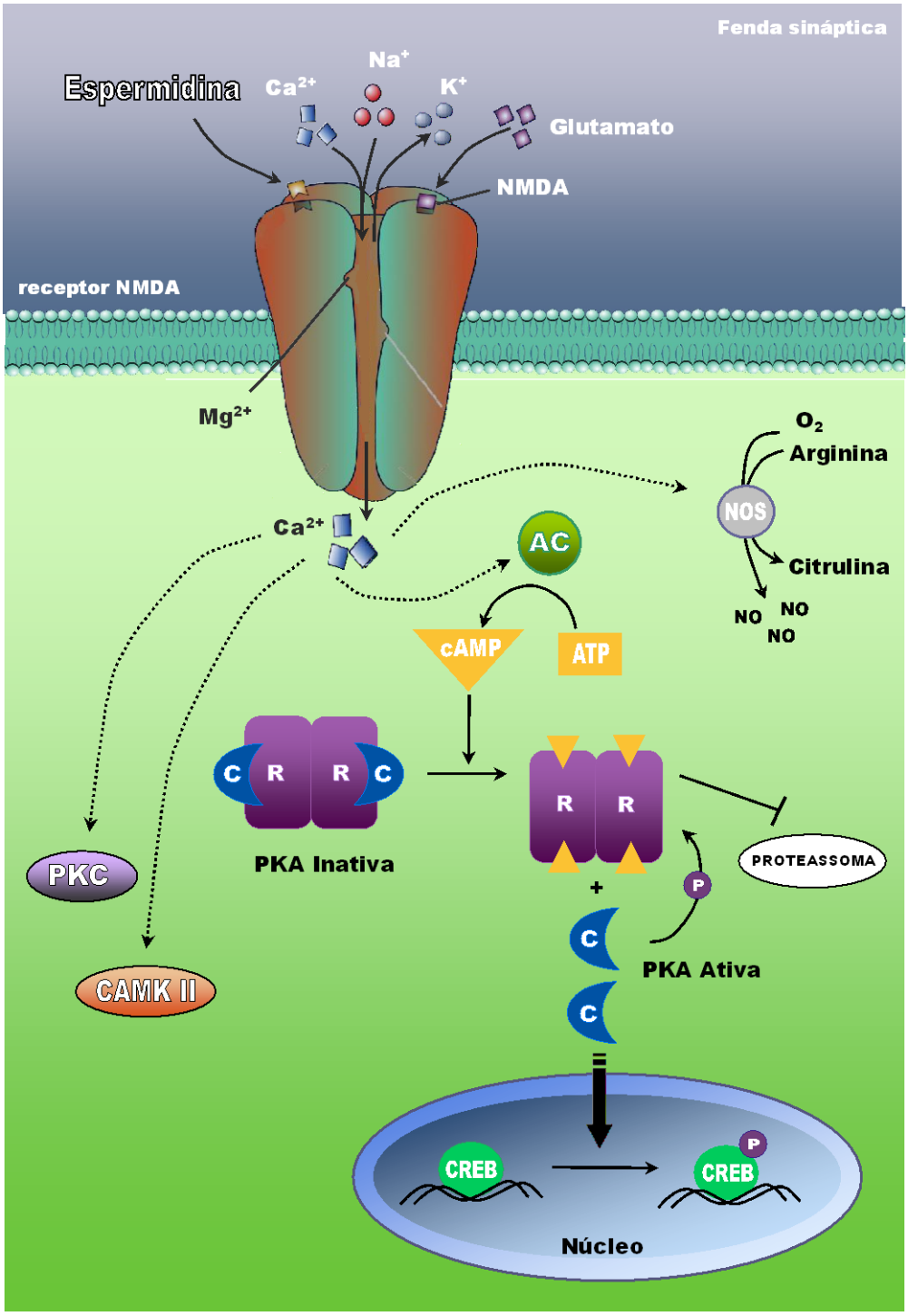

Fig. III.4 – Ativação da cascata AMPc/PKA/CREB.

III.5.1.1 – INIBIÇÃO DA PKA

Devido a composição tetramérica os inibidores da PKA podem se ligar tanto nas subunidades regulatórias como nas subunidades catalíticas. A ação dos inibidores das subunidades regulatórias ocorre através da ligação de análogos do AMPc no sitio de ligação do AMPc nestas subunidades, impedindo a dissociação e liberação da subunidade catalítica, não permitindo, assim, a ativação da PKA (Lochner and Moolman, 2006).

A ação dos inibidores das subunidades catalíticas pode ocorrer de duas maneiras: competitiva com relação ao ATP, uma vez que, os inibidores alostéricos competem pelo sítio de ligação do ATP, ou não competitiva, com relação ao substrato, uma vez que os inibidores alostéricos se ligam em sítios distintos ao do substrato, inativando a enzima. Quando os inibidores alostéricos se ligam ocorre uma alteração na conformação espacial destas enzimas e, consequentemente, bloqueiam os sítios de ligação do ATP ou dos substratos protéicos (Hidaka et al., 1990; Adams, 2001; Lochner and Moolman, 2006; Pickin et al., 2008).

III.5.1.2 –REGULAÇÃO DA PKA

A produção do AMPc, um segundo mensageiro que ativa a PKA, ocorre a partir do ATP, e é catalisada por uma família de nove isoformas da enzima adenilato ciclase (AC) (Hanoune and Defer, 2001; Cooper, 2003), enquanto que a degradação do AMPc para AMP ocorre através de uma super família de fosfodiesterases (PDE), que incluem mais de 40 diferentes isoformas da enzima (Baillie et al., 2005; Lugnier, 2006). Portanto, os níveis de AMPc na célula não são determinados apenas pela sua geração através da ativação da AC, eles dependem também, da degradação catalisada pela ativação da PDE (Baillie et al., 2005; Conti and Beavo, 2007).

A atividade da PKA é regulada pelos níveis de AMPc, através de um mecanismo de “feedback”, uma vez que, a própria PKA pode ativar a PDE (MacKenzie et al., 2002; Li et al., 2010; Skroblin et al., 2010) e inibir a AC (Willoughby and Cooper, 2007; Willoughby et al., 2010), reduzindo os níveis de AMPc e diminuindo sua atividade. Entretanto, o aumento na atividade da PKA pode ser mantido através da degradação seletiva da subunidade regulatória, pela via ubiquitina-proteassoma, mesmo na ausência dos níveis elevados de AMPc preexistentes (Hegde et al., 1993; Chain et al., 1995; 1999). A PKA pode, ainda, ser auto-regulada, ou seja, a subunidade catalítica pode fosforilar a subunidade regulatória, diminuindo afinidade entre as duas subunidades (First et al., 1988; Zelada et al., 2002). Tanto a degradação, como a fosforilação da subunidade

regulatória diminui a probabilidade do retorno da associação da mesma com a subunidade catalítica, mantendo assim, a PKA ativa.

Além disso, a composição das subunidades contribui para o perfil de ativação de diferentes isoformas da PKA. Por exemplo, as isoformas contendo RIβ são ativadas em concentrações mais baixas de AMPc do que as isoformas contendo RIα, assim, diferentes expressões de várias subunidades conferem diferentes sensibilidades para o AMPc, provocando variações na ativação da PKA (Cadd et al., 1990; Solberg et al., 1994).

III.5.1.3 – PROTEÍNA ANCORADOURA

Para que as proteínas quinase possuam maior ativação e eficiência na fosforilação de outras proteínas, elas devem estar presentes em regiões especificas na célula, como próximas das regiões produtoras de segundos mensageiros ou dos substratos a serem fosforilados (Pidoux and Tasken, 2010).

A proteína âncora da quinase A (AKAP) é uma família de proteínas ancoradouras, que tem como função determinar diferentes localizações para a PKA na célula (Bauman and Scott, 2002; Michel and Scott, 2002; Wong and Scott, 2004; Carnegie et al., 2009; Pidoux and Tasken, 2010). Funcionalmente, as AKAPs possuem três características principais:

primeira, elas possuem um domínio que ancora a PKA; segunda, ligam-se a outras enzimas, formando um complexo multi-enzimático; terceira, possuem um domínio onde ligam-se substratos específicos (Wong and Scott, 2004; Smith and Scott, 2006; Jarnaess and Tasken, 2007). A AKAP aumenta a especificidade e eficiência da PKA, uma vez que, liga-se à subunidade regulatória, e permite que a quinase esteja localizada próxima a alvos específicos de substratos, que são regulados pela fosforilação (Smith and Scott, 2002; Bauman et al., 2004; Dell'Acqua et al., 2006).

III.5.2 – PROTEINA QUINASE C (PKC)

Em 1977, foi descrita uma proteína quinase independente de nucleotídeos cíclicos para sua ativação (Takai et al., 1977). Subsequentemente, foi demonstrado que esta enzima pode ser ativada por cálcio e diacilglicerol (DAG), diferindo das quinases dependentes de nucleotídeos cíclicos (PKA e PKG), sendo então, nomeada proteína quinase dependente de Ca^{2+} (PKC) (Takai et al., 1979). Entretanto, a PKC é membro da familia AGC (PKA, PKG, PKC) das proteinas quinase que compartilham certas características estruturais básicas (Steinberg, 2008). A PKC é composta por uma família de serina/treonina quinases que apresenta alta atividade e expressão da maioria de suas isoformas no cérebro (Tanaka and Nishizuka, 1994). No SNC, a ativação da PKC está

associada com regulação da transmissão sináptica e das funções neurais em diferentes níveis, incluindo liberação de neurotransmissores (Malenka et al., 1986; Majewski and Iannazzo, 1998), funcionamento dos receptores (Macek et al., 1998; Suen et al., 1998), expressão gênica (Routtenberg et al., 2000) e processos de aprendizado e memória (Wehner et al., 1990; Alkon et al., 2005).

Em mamíferos, são descritas 10 isoformas de PKC, com base na estrutura e sensibilidade ao Ca^{2+} e DAG estas isoformas têm sido classificadas em três subfamílias: clássica ou convencional – cPKCs (α, βI, βII e γ) ativada por DAG e Ca^{2+} (Coussens et al., 1986; Parker et al., 1986); nova – nPKC (δ, ε, η e θ) ativada por DAG mas não por Ca^{2+} (Ono et al., 1987; Osada et al., 1990; Saido et al., 1992); atípica – aPKC (ζ e λ/ι) insensível a ambos DAG e Ca^{2+} (Ogita et al., 1992).

Ao contrário da PKA, a PKC é uma enzima monomérica, ou seja, apresenta uma única cadeia polipeptídica formada por um domínio regulatório e um domínio catalítico. A isoforma convencional da PKC é formada por quatro regiões conservadas (C1-C4). A metade amino-terminal, contendo as regiões C1, que interage com diacilglicerol (DAG), éster de forbol e fosfatidilserina; C2, que interage com Ca^{2+}; além de um pseudo-substrato adjacente a C1, representam o domínio regulatório. A metade carbóxi-terminal, contendo as regiões C3 e C4, responsáveis pela ligação do ATP e do substrato, respectivamente, representa o domínio catalítico (Newton, 2003; Amadio et al., 2006; Sun and Alkon, 2010).

III.5.2.1 – ATIVAÇÃO DA PKC

Para a completa ativação, a PKC necessita três etapas consecutivas: a fosforilação da enzima, a migração para a membrana e a remoção do pseudo-substrato, que bloqueia o sítio ativo (Pascale et al., 2007). A ativação da PKC inicia com a fosforilação da enzima através da proteína quinase dependente de fosfoinositídeo (Dutil et al., 1998), seguida pela autofosforilação intramolecular em dois sítios adicionais na seqüência carboxi-terminal (Bornancin and Parker, 1996; 1997). A PKC fosforilada, presente no citosol, possui uma conformação estável, cataliticamente competente e resistente a proteases, entretanto, permanece em uma conformação inativa, uma vez que, o pseudo-substrato ocupa o sítio de ligação do substrato (Ron and Kazanietz, 1999; Newton, 2001; 2003). Após uma elevação nos níveis intracelulares o Ca^{2+} liga-se ao domínio C2 da PKC e aumenta a afinidade da quinase pela fosfatidilserina, translocando a enzima para membrana. Esta ligação do domínio C2 com a membrana é uma interação de baixa afinidade (Nalefski and Newton, 2001; Schaefer et al., 2001; Steinberg, 2008), entretanto, uma vez ancorada na membrana, o domínio C1 da PKC liga-se ao DAG (produto da hidrólise de fosfatidil-inositídeos restrito a membrana). A interação dos domínios C1 e C2, aumentam a afinidade da PKC com a membrana, fornecendo energia suficiente para remover o pseudo-substrato e liberar o sítio de ligação do substrato, ativando o

domínio catalítico da PKC (Nishizuka, 1995; Toker, 1998; Newton, 2001; Steinberg, 2008).

III.6 – CREB

O elemento ligante responsivo ao AMPc (do inglês: cAMP response element-binding - CREB) é membro de uma família de proteínas que atua como fator de transcrição. Localizada no núcleo, a CREB é fundamental na transmissão da informação da sinalização celular iniciada na membrana, até as alterações na expressão dos genes (Shaywitz and Greenberg, 1999; Mayr and Montminy, 2001).

Esta cascata bioquímica inicia com a ativação de receptores que aumentam a liberação de cálcio e produção de segundos mensageiros, como AMPc, permitindo a ativação de proteínas quinase como PKA e CaMKII. As proteínas quinase, quando ativadas, translocam para o núcleo da célula, onde fosforilam CREB tornando-a ativa. A CREB, na forma ativa, liga-se a certas seqüências de DNA chamadas de elementos de resposta ao AMPc (CRE) e assim, aumenta ou diminui a transcrição gênica. Os genes cuja transcrição é regulada pelo CREB são os responsáveis pela ligação da RNA-polimerase ao DNA para a síntese de proteínas (Montminy et al., 1990; Carlezon et al., 2005).

A CREB desempenha funções em diferentes órgãos, entretanto, a maioria dos estudos tem sido realizada em encéfalo, sendo que a expressão de genes, nesta área, possui um papel fundamental tanto na sobrevivência e neuroproteção quanto na plasticidade sináptica e formação da memória (Carlezon et al., 2005; Contestabile, 2008). De fato, estudos têm mostrado que a síntese de proteínas é um processo essencial na formação de novas memórias (Bourtchouladze et al., 1998; Quevedo et al., 2004; 2005).

III.7 – PROTEÍNA QUINASE, CREB E MEMÓRIA

Existem algumas evidências, abaixo listadas, apoiando o papel da PKC no processo de aprendizado e memória. Primeiro, a atividade hipocampal da PKC aumenta imediatamente depois do treino na tarefa de esquiva inibitória, alcança um pico 30 min mais tarde e retorna aos níveis normais durante os próximos 120 min depois do treino (Bernabeu et al., 1995; Cammarota et al., 1997). Além disso, o aprendizado em uma tarefa espacial aumenta os níveis de PKC ligados a membrana (forma ativa), no hipocampo de ratos (Nogues et al., 1994; Golski et al., 1995), e a atividade da enzima está reduzida em camundongos com baixo aprendizado (Wehner et al., 1990). Adicionalmente, tem sido demonstrado que o aumento da neuromodulina (GAP-43, B50), substrato da PKC,

depois da tarefa de esquiva inibitória, coincide com o pico de atividade da PKC e pode ser bloqueado, por inibidores desta quinase (Cammarota et al., 1997). Segundo, infusão de ativadores da PKC, como forbol 12,13-dibutirato e 1-oleoil-2-acetil glicerol (análogo sintético do DAG), melhora a formação da memória em ratos (Paylor et al, 1991; Yang and Lee, 1993) e camundongos (Nogues et al., 1996), respectivamente. Terceiro, camundongos trangênicos que superexpressam GAP-43, substrato da PKC, melhoram o aprendizado e LTP (Routtenberg et al., 2000). Quarto, administração de inibidores da PKC, como estausporina, CGP 41231, Go 6976 (inibidor seletivo α- e βI-PKC) e Go 7874, provoca amnésia retrógrada quando infundidos 1-2 h após o treinamento da esquiva inibitoria (Jerusalinsky et al, 1994;. Vianna et al, 2000a; Bonini et al. , 2007). A coincidência nos tempos de alteração da atividade da PKC hipocampal e nos efeitos amnésicos dos inibidores da PKC, sugere um claro envolvimento desta quinase nos processos de formação da memória. Quinto, camundongos mutantes que não expressam PKCγ, PKCβ ou substratos da PKC convencional, como neurogranina e GAP-43, apresentam déficits de aprendizado e formação de memória em diferentes tarefas (Abeliovich et al., 1993; Pak et al., 2000; Weeber et al., 2000; Miyakawa et al., 2001; Rekart et al., 2005; Huang et al., 2006).

Assim como a PKC, a cascata AMPc/PKA/CREB também está envolvida nos processos de formação da memória, possuindo um papel importante na fase final da consolidação da memória (Abel et al., 1997;

Bernabeu et al., 1997a; Barad et al., 1998; Huang and Kandel, 1998; Vianna et al., 2000b; Pereira et al., 2001; Quevedo et al., 2005). Existem evidências que reforçam o envolvimento do AMPc no aprendizado e memória. Primeiro, os níveis de AMPc hipocampal aumentam, lentamente, 60 min depois do treino na tarefa de esquiva inibitória, e alcançam um pico 180–360 min depois do treino (Bernabeu et al., 1996; Bernabeu et al., 1997a; b). Segundo, a infusão de agentes que aumentam os níveis intracelulares de AMPc, como inibidor da fosfodiesterase (Vitolo et al., 2002; Zhang et al., 2000; 2004; Puzzo et al., 2009), análogo da AMPc, ativador da adenilato ciclase (Bernabeu et al., 1997a), e a super expressão da adenilato ciclase (Wang et al., 2004), facilitam a formação da memória. Terceiro, camundongos com expressão reduzida para a adenilato ciclase, apresentam diminuição nos níveis de AMPc e déficit na formação da memória em diferentes tarefas (Wu et al., 1995; Wong et al., 1999). Além disso, a atividade da PKA aumenta depois do treino na tarefa de esquiva inibitória em dois picos: o primeiro, imediatamente depois do treino; e o segundo, 3-6 horas mais tarde, que coincide com o aumento máximo nos níveis de AMPc depois do treino (Bernabeu et al., 1997a).

A importância da PKA na memória tem sido reforçada por estudos demonstrando que inibidores específicos desta enzima prejudicam a memória (Bernabeu et al., 1997a; Bourtchouladze et al., 1998; Vianna et al., 2000b; Wallenstein et al., 2002; Ahi et al., 2004; Quevedo et al., 2004; Sharifzadeh et al., 2005) e que camundongos trangênicos R (AB),

expresando uma forma inibitória da subunidade regulatória da PKA, exibem deficit de memória (Abel et al., 1997; Bourtchouladze et al., 1998; Isiegas et al., 2006).

O próximo passo na cascata AMPc/PKA/CREB, após a ativação da PKA, é fosforilação da CREB (Gonzalez and Montminy, 1989; Bernabeu et al., 1997a). Estudos têm demonstrado que os níveis de CREB fosforilada no hipocampo aumentam depois do treino na tarefa de esquiva inibitória em dois picos, que correlacionam com os picos de aumento da atividade da PKA (Bernabeu et al., 1997a; b).

Assim, o aumento coincidente da forma fosforilada da CREB em períodos específicos, junto com a sensibilidade da memória para inibidores da síntese de proteínas durante períodos em que a PKA está ativa, sugerem que a cascata AMPc/PKA/CREB contribua para a síntese de novas proteínas, evento fundamental para a formação da memória de longa duração. Além disso, alguns estudos relatam um cruzamento entre PKC e PKA, ou seja, é sugerido que PKC contribua para a ativação da cascata AMPc/PKA/CREB (Sugita et al., 1997; Kubota et al., 2003; Yao et al., 2008).

Apesar de todos os estudos já existentes sobre proteínas quinases e memória, o envolvimento da ativação destas proteínas na melhora da memória induzida por espermidina ainda não foi investigado. Este estudo poderá determinar um possível mecanismo de ação da espermidina nos processos de formação da memória, e desta forma, fornecer subsídios

para desenvolver fármacos com potencial para serem utilizados para tratar pacientes com patologias, como a doença de Alzheimer, em que os pacientes afetados apresentam déficit de memória.

IV. RESULTADOS

IV.1 – ARTIGO

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Hippocampal PKA/CREB pathway is involved in the improvement of memory induced by spermidine in rats

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ABSTRACT

Spermidine (SPD) is an endogenous polyamine that modulates N-methyl-D-aspartate (NMDA) receptor function, and has been reported to facilitate memory formation. In the current study we determined whether or not the PKA/CREB signaling pathway is involved in SPD-induced facilitation of memory of inhibitory avoidance task in adult rats. The post-training administration of the cAMP-dependent protein kinase (PKA) inhibitor, N-[2-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide [H-89, 0.5 pmol intrahippocampal (ih)] or the antagonist of the NMDA receptor polyamine-binding site (arcaine, 0.02 nmol ih) with SPD (0.2 nmol ih) prevented memory improvement induced by SPD. Intrahippocampal administration of SPD (0.2 nmol) facilitated PKA and cAMP response element-binding protein (CREB) phosphorylation in the hippocampus 180 min, but not 30 min, after administration, and increased translocation of the catalytic subunit of PKA into the nucleus. Arcaine (0.02 nmol) and H-89 (0.5 pmol) prevented the stimulatory effect of SPD on PKA and CREB phosphorylation. These results suggest that memory enhancement induced by the ih administration of SPD involves the PKA/CREB pathways in rats.

1. Introduction

The polyamines putrescine, spermine, and spermidine (SPD) are a group of aliphatic amines that are present at high concentrations in cerebral structures involved in learning and memory (Carter, 1994). Polyamine concentrations change with age (Liu, Gupta, Jing, & Zhang, 2008), and it has been suggested that region-specific changes in polyamine levels may be causally related to age-associated memory impairment.

Polyamines modulate learning and memory by interacting with the polyamine-binding site at the N-methyl-D-aspartate receptor (NMDAr) (Izquierdo & Medina, 1997; Kishi, Ohno, & Watanabe, 1998a, 1998b; Rubin et al., 2000; Rubin et al., 2001; Rubin et al., 2004; Shimada, Spangler, London, & Ingram, 1994). Accordingly, the systemic (Camera, Mello, Ceretta, & Rubin, 2007), intrahippocampal (ih) (Berlese et al., 2005; Gomes et al., 2010; Guerra et al., 2006; Rubin et al., 2000), and intra-amygdalar (Rubin et al., 2001; Rubin et al., 2004) administration of SPD improves memory. While there is evidence that NMDAr activation is involved in the facilitation of memory induced by SPD (Guerra et al., 2006), the role of downstream kinases in SPD-induced memory improvement has not been defined. This is particularly relevant considering that NMDAr-associated intracellular signaling results in the activation of multiple protein targets, such as PKA (Bernabeu et al., 1997a; Bevilaqua et al., 1997; Chetkovich, Gray, Johnston, & Sweatt, 1991; Chetkovich & Sweatt, 1993; Eliot, Dudai, Kandel, & Abrams, 1989), protein kinase C (PKC) (Bernabeu, Cammarota, Izquierdo, & Medina, 1997b; Bernabeu, Izquierdo, Cammarota, Jerusalinsky, & Medina, 1995), calcium–calmodulin-dependent protein kinase II (CaMKII) (Bernabeu et al., 1997b; Giese, Fedorov, Filipkowski, & Silva, 1998; Mayford, Abel, & Kandel, 1995a; Silva, Stevens, Tonegawa, & Wang, 1992; Wolfman et al., 1994), and protein kinase G (PKG) (Bernabeu et al., 1997c).

The cAMP/PKA signaling pathway seems to play a role in the final phases of memory consolidation, which require protein synthesis (Abel et al., 1997; Barad, Bourtchouladze, Winder, Golan, & Kandel, 1998; Bernabeu et al., 1997a; Huang & Kandel, 1998; Pereira, Ardenghi, Mello e Souza, Medina, & Izquierdo, 2001; Quevedo et al., 2005; Vianna et al., 2000). There are lines of evidence supporting a role for adenosine 3′,5′ monophosphate (cAMP) in learning and memory. First, hippocampal cAMP levels slowly increase beginning 60 min after inhibitory avoidance training, reaching a peak 180–360 min after training (Bernabeu, Schmitz, Faillace, Izquierdo, & Medina, 1996; Bernabeu et al., 1997a, 1997b).

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Second, infusion of agents that increase intracellular cAMP levels, such as a phosphodiesterase inhibitor (Puzzo et al., 2009; Vitolo et al., 2002; Zhang, Crissman, Dorairaj, Chandler, & O'Donnell, 2000; Zhang et al., 2004) a cAMP analog, an adenylyl cyclase activator (Bernabeu et al., 1997a), and the overexpression of type I adenylyl cyclase (Wang, Ferguson, Pineda, Cundiff, & Storm, 2004), facilitates memory formation. Third, mice lacking type I adenylyl cyclase present diminished cAMP levels and memory formation deficits in different tasks (Wong et al., 1999; Wu et al., 1995).

The PKA family consists of four regulatory (R) subunits (RIα, RIβ, RIIα, and RIIβ) and three catalytic (C) subunits (Cα, Cβ, and Cγ) (Doskeland, Maronde, & Gjertsen, 1993; McKnight et al., 1988), which are expressed in mammalian brain (Cadd & McKnight, 1989). Each R subunit contains two binding sites for cAMP (Taylor, Buechler, & Yonemoto, 1990), which activate PKA (Gibbs, Knighton, Sowadski, Taylor, & Zoller, 1992; Taylor et al., 1990). PKA activity increases after inhibitory avoidance training in the following two peaks: the first, that occurs immediately after training; and the second peak, that occurs 3–6 h later, and correlates with the maximum rise of cAMP levels after training (Bernabeu et al., 1997a).

A role for PKA in memory has been supported by the findings that specific inhibitors impair memory (Ahi, Radulovic, & Spiess, 2004; Bernabeu et al., 1997a; Bourtchouladze et al., 1998; Quevedo et al., 2004; Sharifzadeh, Sharifzadeh, Naghdi, Ghahremani, & Roghani, 2005; Vianna et al., 2000; Wallenstein, Vago, & Walberer, 2002) and that R (AB) transgenic mice, which express an inhibitory form of the regulatory subunit of PKA, exhibit memory deficits (Abel et al., 1997; Bourtchouladze et al., 1998; Isiegas, Park, Kandel, Abel, & Lattal, 2006).

CREB is a transcription factor whose phosphorylation on Ser133 by PKA causes its activation (Bernabeu et al., 1997a; Gonzalez & Montminy, 1989). Phosphorylated CREB (p-CREB) levels in the hippocampus increase after inhibitory avoidance training in two peaks that correlate with the peaks of increased PKA activity (Bernabeu et al., 1997a,b). The coincident increase in the nuclear phosphorylated form of CREB at these specific periods, together with memory sensitivity to inhibitors of gene transcription and protein synthesis during PKA active periods, suggest that this signaling pathway may contribute to the synthesis of new proteins, a crucial event for long-term memory establishment. Therefore, in the current study we investigated whether PKA/CREB signaling pathways are activated by SPD in rats subjected to inhibitory avoidance training.

2. Materials and methods

2.1. Animals

All animal experiments reported in this study were conducted in accordance with Brazilian law No. 11.794/2008 in agreement with the Policies on the Use of Animals and Humans in Neuroscience Research, which were revised and approved by the Society for Neuroscience Research in January 1995, and with the Institutional and National Regulations for Animal Research (process 0206). Male Wistar rats (230–250 g; $n = 222$) were bred in the Animal House of the Federal University of Santa Maria, housed 5 to a cage, and maintained on a natural day/night cycle at a temperature of 21 °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).

2.2. Surgery

Rats were anaesthetized by intraperitoneal (ip) injection of a mixture of ketamine (80 mg/kg) and xylazine (8 mg/kg), and were implanted with two 27-gauge guide cannulae placed 1 mm above the CA1 region of the dorsal hippocampus at the following coordinates: A, 4 mm; L, 3.0 mm; and V, 2.0 mm (Paxinos & Watson, 1986). Placement of injections was histologically-verified, as described elsewhere (Rubin et al., 1997). Only data from the animals with correct cannula placement were analyzed.

2.3. Behavioral and infusion procedures

One week after surgery, the animals were subjected to a single training session in a step-down inhibitory avoidance apparatus, consisting of a 25 × 25 × 35-cm box with a grid floor and the left portion covered by a 7 × 25-cm platform, measuring 2.5 cm in height. The rat was placed gently on the platform facing the rear left corner. Once the rat stepped down with all 4 paws on the grid, a 3-s, 0.3-mA shock was applied to the grid. Immediately post-training, vehicle, one drug, or a combination of the following drugs were injected bilaterally into the hippocampus (0.5 μl/brain hemisphere over 1 min): spermidine [N-(3-aminopropyl)-1,4-butanediamine trihydrochloride (SPD); Sigma–Aldrich Co., St. Louis, MO, USA]; arcaine (1,4-diguanidinobutane sulfate; Sigma–Aldrich Co.); and N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89; Sigma–Aldrich Co.). H-89 is an isoquinolinesulfonamide which inhibits PKA by competing with ATP for the catalytic subunit of enzyme (Chijiwa et al., 1990; Hidaka, Hagiwara, & Chijiwa, 1990). SPD and arcaine were dissolved in 50 mM phosphate buffer saline (PBS; pH 7.4), and H-89 was dissolved in 0.01% DMSO in 50 mM PBS (pH 7.4). The injections were performed via an infusion pump 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 CA1 region in the dorsal hippocampus. The injection needles were left in place for an additional 60 s to minimize backflow. After the injection, the animals were returned to their home cages and tested for retention 24 h later. A step-down latency test was taken as a measure of retention, with a cut-off time of 600 s.

Immediately after the inhibitory avoidance test session, the animals were transferred to a 50 × 60-cm open field, with the floor divided into 12 squares. During the 5-min open field session, the number of crossing and rearing responses was recorded. The open field was used to identify motor disabilities which might influence inhibitory testing avoidance performance.

Step-down latency data were analyzed with the Kruskal–Wallis test or the Scheirer–Ray–Hare extension of the Kruskal–Wallis test. Crossing and rearing responses were analyzed by one- or two-way ANOVA. Significance was considered at a $p < 0.05$.

2.4. Preparation of tissues and Western blot analysis

Western blot analysis was carried out, as described previously (Casu et al., 2007) with minor modifications. Rats were decapitated, and the hippocampi were rapidly removed, dissected, homogenized in 300 μl of ice-cold A buffer (10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM NaF, 10 μg/ml aprotinin, 10 mM β-glycerolphosphate, 1 mM PMSF, 1 mM DTT, and 2 mM of sodium orthovanadate in 10 mM HEPES, pH 7.9), incubated for 15 min on ice, and centrifuged at 16 000×*g* for 45 min at 4 °C. The supernatant (S1), denominated cytosolic fraction, was reserved for posterior processing. The pellet (P1) was resuspended in 150 μl of ice-cold buffer B (10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM NaF, 10 μg/ml aprotinin, 10 mM β-glycerolphosphate, 1 mM PMSF, 1 mM DTT, 2 mM sodium orthovanadate, and 1% Triton-X in 10 mM HEPES, pH 7.9), incubated for 15 min on ice, and centrifuged at 16 000×*g* for 45 min at 4 °C. The supernatant (S2) was discarded and the pellet (P2) was resuspended in 100 μl of ice-cold buffer C (50 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM NaF,

10 μg/ml aprotinin, 10 mM β-glycerolphosphate, 1 mM PMSF, 1 mM DTT, 2 mM sodium orthovanadate, 420 mM NaCl, and 25% glycerol in 20 mM HEPES, pH 7.9), incubated for 15 min on ice, and centrifuged at 16,000×*g* for 45 min at 4 °C. The supernatant (S3) was considered the nuclear fraction (Medeiros et al., 2007). The protein concentration in the cytosolic and nuclear fractions was determined using the Bradford method (1976). Equivalent amounts of protein (80 μg or 20 μg for cytosolic or nuclear fractions, respectively) were added to 0.2 volumes of concentrated loading buffer (200 mM Tris, 10% glycerol, 2% SDS, 2.75 mM β-mercaptoethanol, and 0.04% bromophenol blue) and boiled for 10 min. Proteins were separated in 12% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) and transferred to polyvinilidene difluoride membranes. Ponceau staining (data not shown) served as a loading control (Romero-Calvo et al., 2010). Western blot analysis of PKA regulatory subunit and CREB was carried out in the cytosolic and nuclear fractions, respectively. PKA catalytic subunit immunoreactivity was measured in the nuclear and cytosolic fractions, in order to determine whether PKA catalytic subunit translocated to the nucleus, as described below. Membranes were processed using a SNAP i.d. system (Millipore, Billerica, MA, USA). First, the membrane was blocked with 1% BSA in 0.05% Tween 20 in Tris-borate saline (TBS-T), then incubated for 10 min with specific primary antibodies diluted 1:150 in TBS-T (anti-phospho-PKA RIIα, anti-total-PKA RIIα, anti-phospho-CREB-1, anti-total-CREB-1 and anti-phospho-PKA Cα/β/γ polyclonal antibodies; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Blots were washed three times, with TBS-T followed by incubation with adjusted alkaline phosphatase-coupled secondary antibody (1:3000, anti-rabbit IgG; Santa Cruz Biotechnology, Inc.) for 10 min. Protein bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate and *p*-nitro blue tetrazolium (BCIP/NBT; Millipore). Membranes were dried, scanned, and quantified with the Scion Image PC version of NIH image. The results were normalized for the control group (PBS/PBS) densitometry values and expressed as the relative amount of phosphorylated and non-phosphorylated forms, and the phosphorylated/total ratio. Statistical analyses were performed using a two-way ANOVA or a Student's *T*-test. *F* and *p* values are shown only if $p < 0.05$.

2.5. Experiment 1 – Effect of a PKA inhibitor (H-89) on inhibitory avoidance task performance

A dose–response curve for H-89 was performed to define the ih dose of H-89 for the subsequent experiments. Immediately post-training, the animals were injected with vehicle (0.01% DMSO in 50 mM PBS, pH 7.4) or H-89 (0.5–5 pmol/hippocampus). Initial dose range was selected based on previous studies (Sharifzadeh et al., 2006). The animals were subjected to a step-down inhibitory avoidance session, open field test, and histologic examination, as described above.

2.6. Experiment 2 – Effect of a PKA inhibitor on memory improvement and phosphorylation of the PKA and CREB induced by SPD

Once it was determined that H-89 at a dose of 0.5 pmol (ih) did not alter memory, the effect of H-89 on SPD-induced improvement of memory and phosphorylation of PKA and CREB were determined. Animals were trained in the step-down inhibitory avoidance apparatus and injected immediately post-training with vehicle (0.01% DMSO in 50 mM PBS, pH 7.4), H-89 (0.5 pmol/hippocampus), SPD (0.2 nmol/hippocampus), or H-89 (0.5 pmol/hippocampus) combined with SPD (0.2 nmol/hippocampus). The dose of SPD (0.2 nmol/hippocampus) was selected because it improves memory of the inhibitory avoidance task (Rubin et al., 2000). A subset of the animals was sacrificed 30 and 180 min after injections and the hippocampi were dissected for Western blot analysis of PKA and CREB. The other animals had a testing session in the inhibitory avoidance apparatus and an open field test. It has been reported previously that autophosphorylation of the PKA regulatory subunit occurs when PKA is activated by cAMP (Tasken & Aandahl, 2004). Thus, the detection of phosphorylation of the PKA regulatory subunit is useful evidence for PKA activation.

2.7. Experiment 3 – Effect of arcaine on memory improvement and phosphorylation of PKA and CREB induced by SPD

Animals were trained in a step-down inhibitory avoidance apparatus and injected immediately post-training with vehicle (50 mM PBS, pH 7.4), arcaine (0.02 nmol/hippocampus), SPD (0.2 nmol/hippocampus) or arcaine (0.02 nmol/hippocampus) combined with SPD (0.2 nmol/hippocampus). The dose of arcaine (0.02 nmol/hippocampus) did not alter memory (Rubin et al., 2000). A subset of the animals was sacrificed 180 min after injection and the hippocampi were dissected for Western blot analysis of phosphorylated forms of PKA and CREB. The other animals had a testing session in the inhibitory avoidance apparatus and an open field test, as described above.

2.8. Experiment 4 – Effect of SPD on PKA catalytic subunit translocation

Animals were trained in the step-down inhibitory avoidance apparatus and injected immediately post-training with vehicle (50 mM PBS, pH 7.4) or SPD (0.2 nmol/hippocampus). Three hours after the injection, the animals were sacrificed and the hippocampi were dissected for Western blot analysis of translocation of the PKA catalytic subunit (PKA C) to the nucleus.

Table 1
Effect of the post-training intrahippocampal administration of H-89 (0.5–5 pmol) on the inhibitory avoidance task performance of rats (measured as the test step-down latency) and on the behavior of rats (number of crossing and rearing responses) in the open field immediately after the inhibitory avoidance testing session.

Group	Latency (s)	Crossing	Rearing	*N*
PBS	129.0 (51.5–355.5)	15.6 ± 1.3	6.7 ± 0.7	13
H-89 (0.5 pmol)	91.0 (63.5–382.5)	15.5 ± 2.0	7.0 ± 1.6	13
H-89 (5 pmol)	41.0 (20–102.5)	15.7 ± 1.6	6.6 ± 0.9	13
Statistical analysis	$H_2 = 6.72$, $p < 0.05$	$F_{2,36} = 0.005$, $p > 0.05$	$F_{2,36} = 0.042$, $p > 0.05$	

Data are the median (interquartile ranges) or means ± SEM; *N*, number of animals in each group.

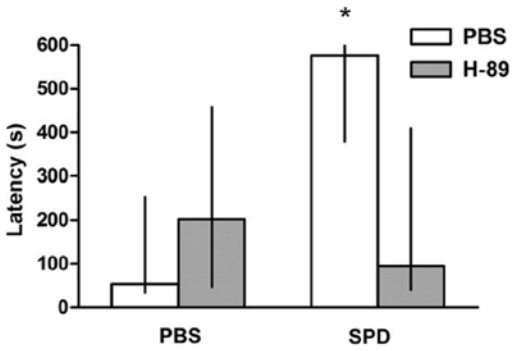

Fig. 1. Co-administration of H-89 (0.5 pmol intrahippocampus) immediately after training prevents the improvement in memory induced by spermidine (SPD, 0.2 nmol). Phosphate-buffered saline (50 mM PBS, pH 7.4) was used as a vehicle. Data are the median and interquartile range for 16 animals in each group. *$P<0.05$ as compared with control group values (PBS/PBS).

3. Results

3.1. Experiment 1

Table 1 shows the effect of bilateral ih injection of H-89 (0.5–5 pmol) immediately after training on test step-down latencies. Statistical analysis (Kruskal–Wallis test) revealed that H-89, in the dose of 5 pmol, significantly decreased step-down latencies at testing (*F* values shown in Table 1), revealing that injection of the selective PKA inhibitor impaired the memory in the inhibitory avoidance task. Table 1 also shows the effect of H-89 on exploratory behavior in the open field immediately after the inhibitory avoidance testing session. Statistical analysis of open-field data (one-way ANOVA) revealed that H-89 injection did not alter the number of crossing or rearing responses in a subsequent open-field testing session (*F* values shown in Table 1), suggesting that its injection, immediately after training, did not cause gross motor disabilities during testing. The dose of H-89 to be used in the subsequent experiments (0.5 pmol) was chosen based on the lack of effect on memory in this experiment.

3.2. Experiment 2

Figs. 1–3 show the effect of the ih administration of H-89 (0.5 pmol), SPD (0.2 nmol), and the co-administration of H-89 and SPD immediately after training on step-down latencies during testing (Fig. 1) and on Western immunoblotting and densitometry analyses of PKA (Fig. 2) and CREB phosphorylation (Fig. 3). Statistical analysis of step-down latencies during testing (nonparametric two-way ANOVA) showed a significant SPD or PBS vs H-89 or PBS interaction [$H_1 = 6.84$, $p < 0.05$], revealing that co-injection of the selective PKA inhibitor prevented facilitation of memory

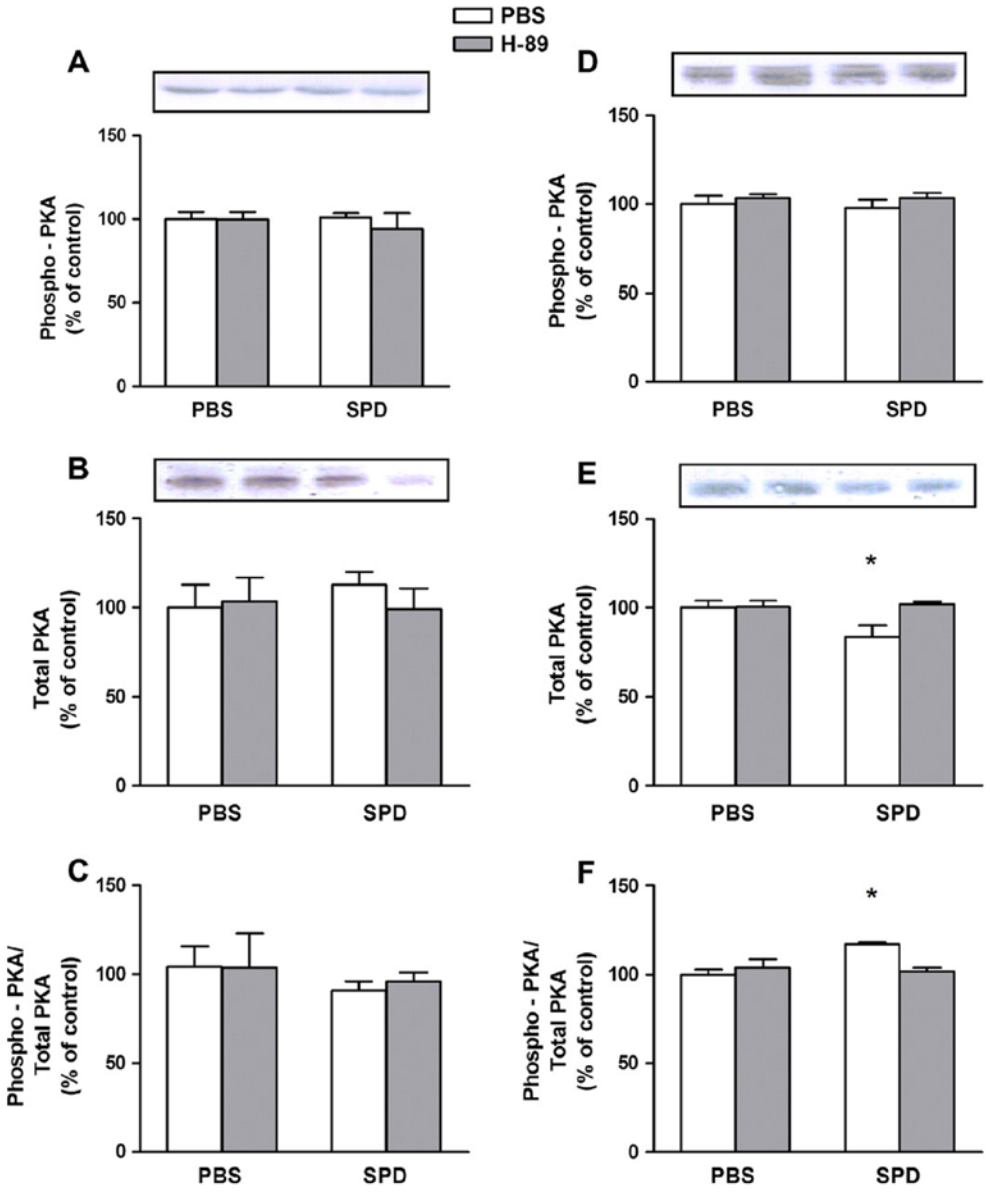

Fig. 2. Effect of the post-training intrahippocampal co-administration of H-89 (0.5 pmol) and spermidine (SPD, 0.2 nmol) on the representative images of Western immunoblotting and densitometry analyses of phospho-PKA (A, D), total-PKA (B, E), and the ratio of phospho- PKA/total-PKA (C, F). In Fig. A, B, and C, and D, E, and F rats were killed 30 and 180 min after the injections of H-89 and SPD, respectively. The results were normalized by arbitrarily setting the densitometry of the control group (PBS/PBS). Ponceau staining served as a loading control. Data are the mean ± SEM for 3–4 animals in each group. *$P < 0.05$ as compared with control group values (PBS/PBS).

induced by SPD (Fig. 1). This result suggests that memory improvement induced by SPD depends on PKA activation. The open field data demonstrated that pharmacologic treatment did not alter the number of crossing or rearing responses in a subsequent open field testing session (data not shown). These data indicate that none of the compounds tested caused gross motor disabilities during testing.

Statistical analysis (two-way ANOVA) also revealed that bilateral hippocampal injection of H-89, SPD, or a combination of H-89 and SPD did not alter phospho-PKA immunoreactivity at 30 or 180 min (Fig. 2A and D). However, a significant SPD or PBS versus H-89 or PBS interaction for total PKA levels [$F_{1,11} = 7.60$, $p < 0.05$, Fig. 2E] and the phospho-PKA/total PKA ratio at 180 min [$F_{1,11} = 8.82$, $p < 0.05$, Fig. 2F], but not 30 min (Fig. 2B and C) after the injections. These results suggest that H-89 prevents the SPD-induced increase in the phospho-PKA/total PKA ratio and decrease in the total PKA level. The administration of H-89, SPD, or a combination of H-89 and SPD did not alter the total CREB levels (Fig. 3B and E) at 30 and 180 min; however, a significant SPD or PBS versus H-89 or PBS interaction for phospho-CREB levels [$F_{1,12} = 11.54$, $p < 0.05$, Fig. 3D] and the phospho-CREB/total-CREB ratio at 180 min [$F_{1,12} = 13.78$, $p < 0.05$, Fig. 3F], but not at 30 min (Fig. 3A and C) after injections was found, indicating that H-89 prevented the SPD-induced increase of the phospho-CREB level and phospho-CREB/total-CREB ratio.

3.3. Experiment 3

Figs. 4–6 show the effect of the ih administration of arcaine (0.02 nmol), SPD (0.2 nmol), and the co-administration immediately after training on step-down latencies during testing and on Western immunoblotting and densitometry analyses of PKA and CREB phosphorylation. Step-down latencies had a significant PBS or SPD versus PBS or arcaine interaction [$H_1 = 5.08$; $p < 0.05$],

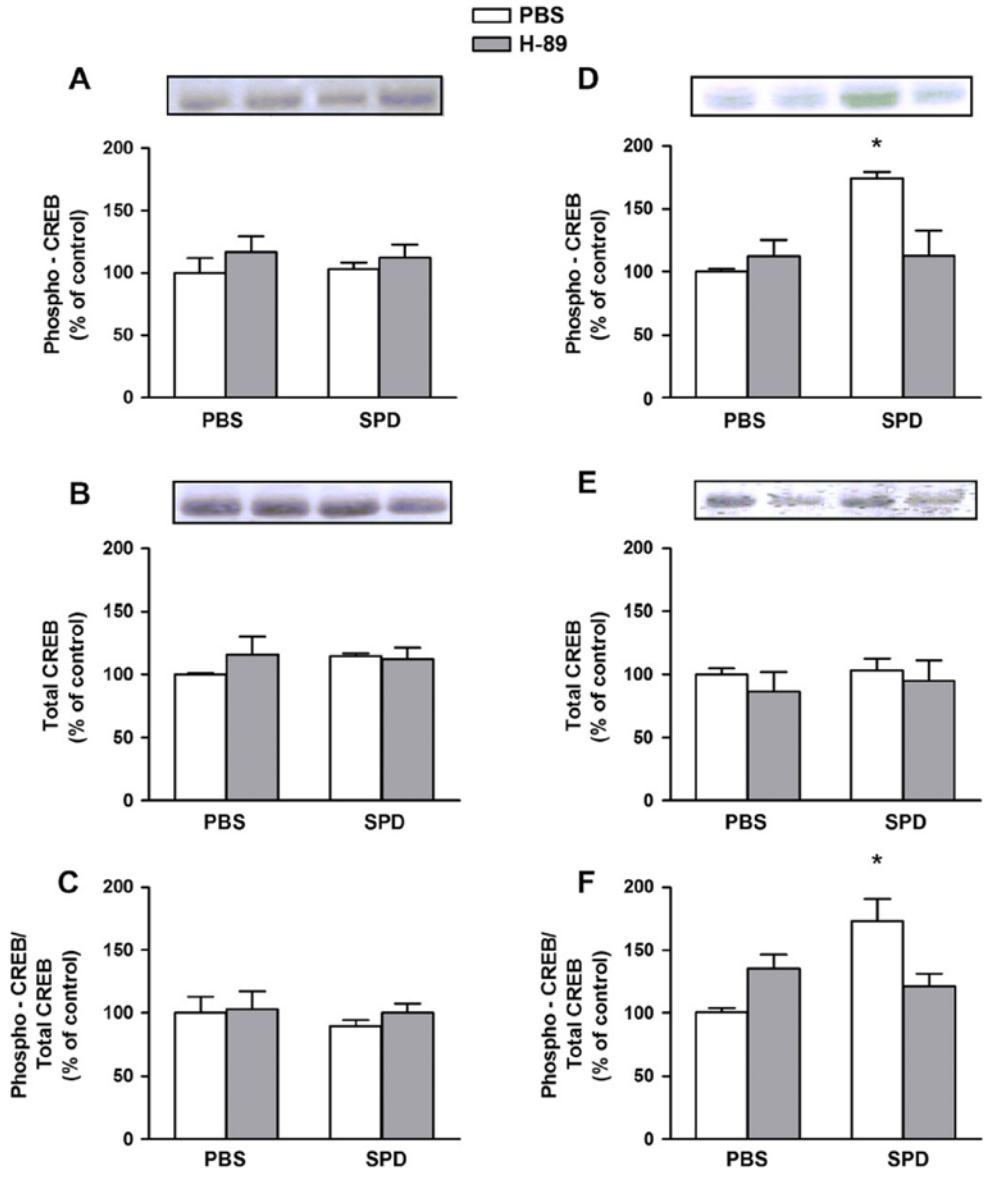

Fig. 3. Effect of the intrahippocampal co-administration of H-89 (0.5 pmol) and spermidine (SPD, 0.2 nmol) on the levels of phospho-CREB (A, D), total-CREB (B, E), and the phospho-CREB/total-CREB ratio (C, F). A, B, and C, and D, E, and F show the 30- and 180-min data, respectively. The results were normalized by arbitrarily setting the densitometry of the control group (PBS/PBS). Ponceau staining served as a loading control. Data are the mean ± SEM for 3–4 animals in each group. *$P < 0.05$ as compared with control group values (PBS/PBS).

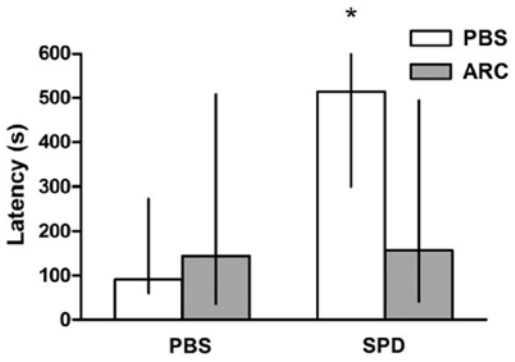

Fig. 4. Co-administration of arcaine (ARC, 0.02 nmol, ih) immediately after training prevents the improvement in memory induced by spermidine (0.2 nmol). Phosphate-buffered saline (50 mM PBS, pH 7.4) was used as a vehicle. Data are the median ± interquartile ranges for 14 animals in each group. $^{*}P < 0.05$ as compared with control group values (PBS/PBS).

suggesting that co-injection of an antagonist of the polyamine-binding site at the NMDA receptor prevents the facilitation of memory induced by SPD (Fig. 4). The open field data demonstrated that pharmacologic treatment did not alter the number of crossing or rearing responses in a subsequent open field testing session (data not shown), indicating that the tested compounds do not cause gross motor disabilities during testing.

Arcaine, SPD, or a combination of arcaine and SPD did not significantly modify the phospho-PKA and total-PKA levels (Fig. 5A and B); however, we showed a significant polyamine (PBS or SPD) versus NMDA antagonist (PBS or arcaine) interaction [$F_{1,12} = 7.03$, $p < 0.05$] for the phospho-PKA/total-PKA ratio (Fig. 5C). This result suggests that arcaine prevents the SPD-induced increase in the phospho-PKA/total-PKA ratio.

The administration of arcaine, SPD, or a combination of arcaine and SPD did not significantly alter the total-CREB levels (Fig. 6B). However, we found a significant SPD or PBS versus arcaine or PBS interaction for the phospho-CREB levels [$F_{1,12} = 4.85$, $p < 0.05$, Fig. 6A] and the ratio of phospho-CREB/total-CREB [$F_{1,12} = 5.34$, $p < 0.05$, Fig. 6C]. This data suggests that arcaine prevents the SPD-induced increase in phospho-CREB levels and the ratio of phospho-CREB/total-CREB, and that the PKA/CREB pathway underlies the facilitatory effects of SPD on memory.

3.4. Experiment 4

Fig. 7 shows the effect of ih administration of SPD (0.2 nmol) immediately after training on immunocontent of phospho-PKA catalytic subunits to cytosolic and nuclear fraction SPD did not significantly modify the catalytic subunits of phospho-PKA levels in the cytosolic fraction (Fig. 7A); however, SPD increased the catalytic subunits of phospho-PKA levels in the nucleus fraction ($t = 1.926$, df = 14, $p < 0.05$, Fig. 7B). This data suggests that SPD facilitates the translocation of PKA catalytic subunits from the cytosol to the nucleus.

4. Discussion

In this study we showed that H-89, an inhibitor of PKA, and arcaine, an antagonist of the polyamine-binding site at the NMDA receptor, prevent the facilitatory effect of SPD on the memory of an inhibitory avoidance task. The injection of H-89 or arcaine also prevented SPD-induced phosphorylation of PKA and CREB in the hippocampus. While H-89 has been reported to inhibit protein kinases other than PKA (Davies, Reddy, Caivano, & Cohen, 2000), the currently reported reversal of known PKA-mediated responses,

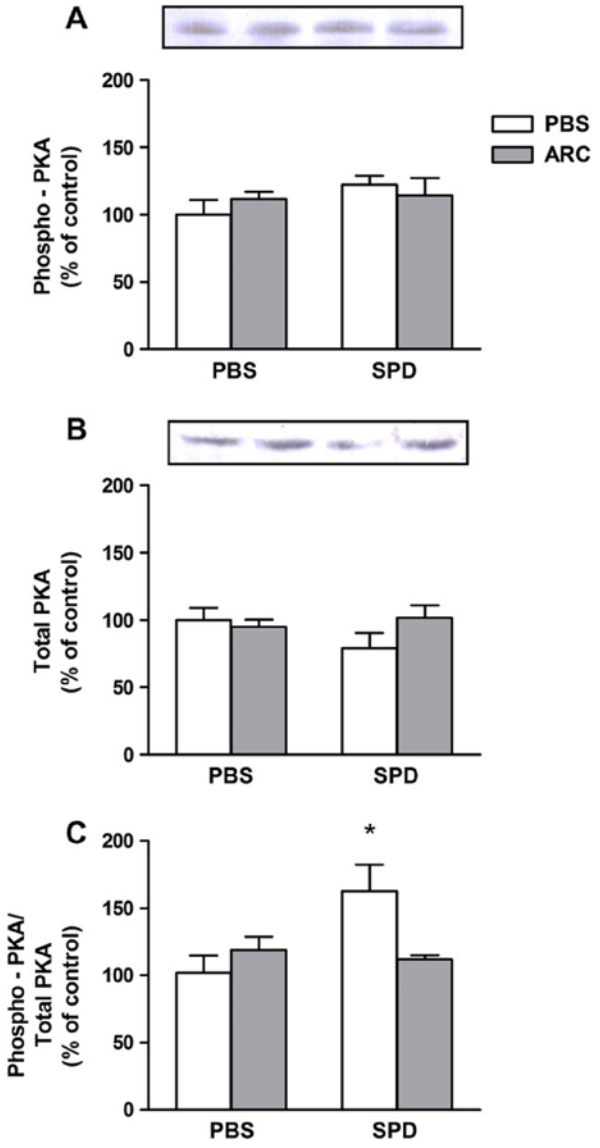

Fig. 5. Effect of the post-training intrahippocampal co-administration of arcaine (ARC, 0.02 nmol) and spermidine (SPD, 0.2 nmol) on representative images of Western immunoblotting and densitometry analyses of phospho-PKA (A), total-PKA (B), and the ratio of phospho-PKA/total-PKA (C) 180 min after the injection of SPD and ARC. The results were normalized by arbitrarily setting the densitometry of the control group (PBS/PBS). Ponceau staining served as a loading control. Data are the mean ± SEM for four animals in each group. $^{*}P < 0.05$ as compared with control group values (PBS/PBS).

such as PKA and CREB phosphorylation by H-89, suggests that the current effects of SPD on PKA and CREB phosphorylation as well as their reversal by a PKA inhibitor (H-89) are not due to nonspecific effects. The reversal of facilitatory effects of ih-injected SPD on step-down inhibitory avoidance by a PKA inhibitor and a NMDA receptor polyamine-binding site antagonist implicate the NMDA receptor and PKA activation in SPD-induced facilitation of memory (Figs. 1 and 4, respectively). This finding was corroborated by the demonstration that SPD activates PKA, and that H-89 prevents SPD-induced PKA activation (Fig. 2). SPD-induced activation of PKA occurred 180 min after the injection of the polyamine, which likely reflects the PKA activity peak 3–6 h after training. Furthermore, this peak correlates with the maximum rise in cAMP levels after training (Bernabeu et al., 1997a). In agreement with this view,

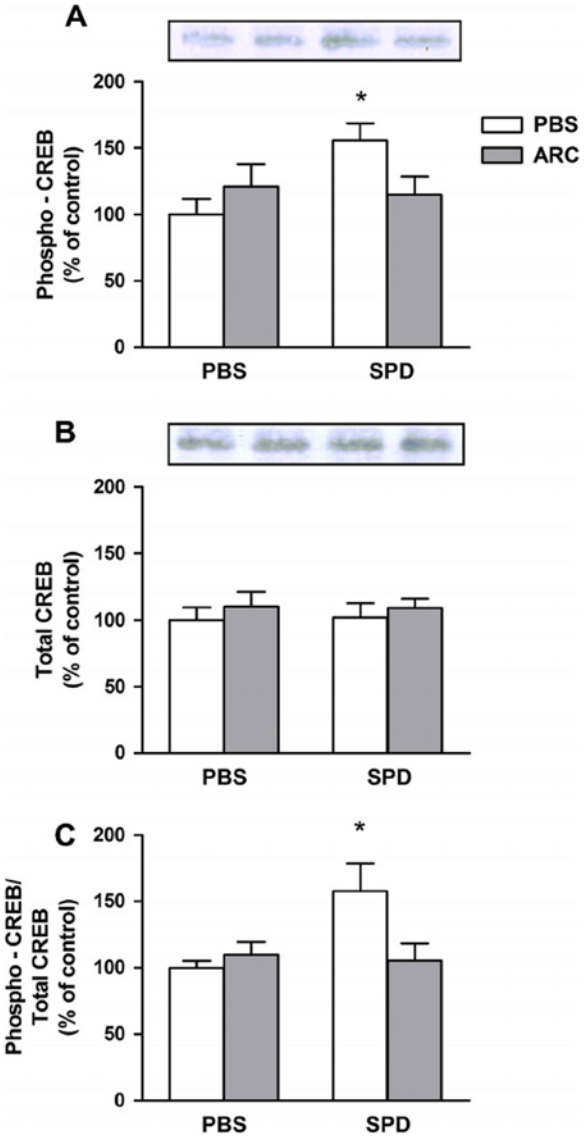

Fig. 6. Effect of the post-training intrahippocampal co-administration of arcaine (ARC, 0.02 nmol) and spermidine (SPD, 0.2 nmol) on representative images of Western immunoblotting and densitometry analyses of phospho-CREB (A), total-CREB (B), and the ratio of phospho-CREB/total-CREB (C) 180 min after the injection of SPD and ARC. The results were normalized by arbitrarily setting the densitometry of the control group (PBS/PBS). Ponceau staining served as a loading control. Data are the mean ± SEM for four animals in each group. $^{*}P < 0.05$ as compared with control group values (PBS/PBS).

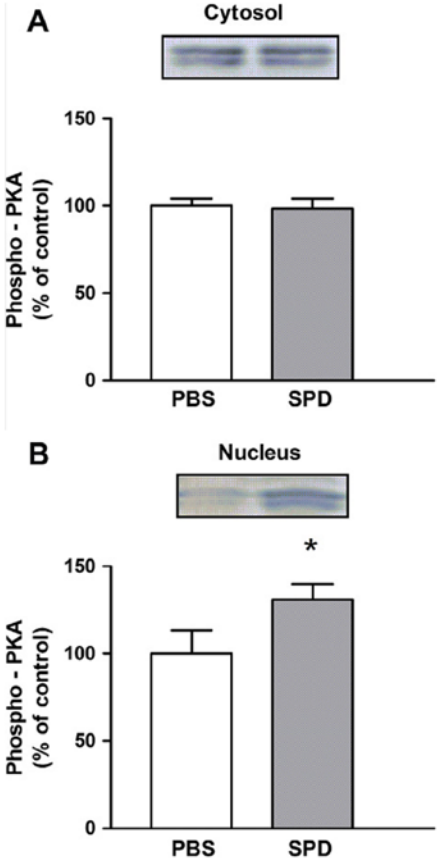

Fig. 7. Effect of the post-training intrahippocampal administration of spermidine (SPD, 0.2 nmol) on representative images of Western immunoblotting and densitometry analyses of the cytosolic (A) and nuclear (B) fractions of phospho-PKA catalytic subunits 180 min after the injection of SPD. The results were normalized by arbitrarily setting the densitometry of the control group (PBS/PBS). Ponceau staining served as a loading control. Data are the mean ± SEM for eight animals in each group. $^{*}P < 0.05$ as compared with control group values.

arcaine prevented the facilitatory effect of SPD on PKA phosphorylation, supporting the involvement of NMDA receptors in SPD-induced PKA activation (Fig. 6).

Because CREB phosphorylation depends on PKA activity (Bernabeu et al., 1997a; Gonzalez & Montminy, 1989), we extended our investigation to phospho-CREB levels. The ih administration of SPD increased CREB phosphorylation, and as expected, H-89 and arcaine prevented SPD-induced CREB phosphorylation (Figs. 3 and 6, respectively). In addition, SPD injection increased catalytic phospho-PKA immunoreactivity in the nuclear fraction of the hippocampus (Fig. 7). Because the hippocampal cAMP/PKA pathway is activated by NMDA receptor-mediated Ca^{2+} influx (Chetkovich et al., 1991), it is very possible that Ca^{2+} influx sequentially stimulates adenylate cyclase (AC) activity, cAMP accumulation, and PKA and CREB phosphorylation (Eliot et al., 1989; Poser & Storm, 2001). Indeed, it has been shown that cAMP binds to R PKA subunits, resulting in the release of the monomeric C active subunit of PKA (Gibbs et al., 1992; Taylor et al., 1990; Wang, Salter, & MacDonald, 1991; Wang et al., 2004). The free PKA C subunits translocate to the nucleus, where they phosphorylate CREB (Dash, Karl, Colicos, Prywes, & Kandel, 1991; Impey et al., 1998; Mayford, Wang, Kandel, & O'Dell, 1995b). Interestingly, ih administration of SPD increased p-PKA/PKA ratio at 180 min, mainly due to a decrease in the total levels of the R subunit of PKA (Fig. 2E and F). Accumulating evidence suggests that the PKA R subunits are degraded by ubiquitin–proteasome-mediated proteolysis in animal (Hegde, Goldberg, & Schwartz, 1993) and human brain (Liang, Liu, Grundke-Iqbal, Iqbal, & Gong, 2007). Furthermore, lactacystin, a specific proteasome inhibitor, completely blocks the forskolin-induced down-regulation of the R subunit in differentiated human neuroblastoma SH-SY5Y cells (Boundy, Chen, & Nestler, 1998). It has been suggested that PKA R (RIIα and RIIβ) and C subunit (Cβ) deficits decrease enzyme activity, and consequently, memory impairment in patients with Alzheimer's disease (Liang et al., 2007). In contrast, degradation of R subunits by the proteasome gives rise to an excess of C subunits. In support of this view, inhibitory avoidance training causes a 33% NMDA receptor-dependent increase in the amount of C subunits of PKA in nuclear fractions of hippocampal neurons (Cammarota et al., 2000) and the ih injection of a proteasome inhibitor blocks long-term memory (LTM) formation (Lopez-Salon

et al., 2001). Further, it is interesting to note that the time course of the effect of proteasome inhibitors on LTM formation parallels those observed with two different specific inhibitors of PKA delivered into the same brain region (Bernabeu et al., 1997a; Vianna et al., 2000) and coincides with the period during which hippocampal PKA activity is increased (Izquierdo & Medina, 1997).

In summary, this study showed that SPD-induced memory facilitation depends on a sequence of biochemical events in the rat hippocampus which is triggered by NMDA receptor, followed by PKA/CREB signaling activation.

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IV.2 – MANUSCRITO

Submetido para “HIPOCAMPPUS”

Spermidine-induced improvement of memory involves a cross-talk between protein kinases C and A

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ABSTRACT

Spermidine (SPD) is an endogenous polyamine that modulates N-methyl-D-aspartate (NMDA) receptor function, and has been reported to facilitate memory formation. Recent evidence suggests that PKA and CREB play a role in SPD-induced improvement of memory. In the current study, we determined whether the PKC signaling pathway is involved in SPD-induced facilitation of memory of inhibitory avoidance task in adult rats. The post-training administration of the calcium-dependent protein kinase (PKC) inhibitor, 3-[1-(dimethylaminopropyl)indol-3-yl]-4-(indol-3-yl)maleimide hydrochloride [GF 109203X, 2.5 ρmol, intrahippocampal (ih)] with SPD (0.2 nmol, ih) prevented memory improvement induced by SPD. Intrahippocampal administration of SPD (0.2 nmol) facilitated PKC phosphorylation, in the hippocampus, 30 min after administration. GF 109203X prevented not only the stimulatory effect of SPD on PKC, but also PKA and CREB phosphorylation. These results suggest that memory enhancement induced by the ih administration of SPD involves the cross talk between PKC and PKA/CREB, with sequential activation of PKC and PKA/CREB pathways, in rats.

Keywords PKC, CREB, Polyamine, Memory, Spermidine, GF109203X

1. Introduction

Polyamines putrescine, spermine, and spermidine (SPD) are a group of aliphatic amines present in almost all cells, but at particularly high concentrations in the vertebrate nervous system, including cerebral structures involved in learning and memory, such as the amygdala and hippocampus (Carter, 1994; Morrison et al., 1995). Polyamines modulate learning and memory by interacting with the polyamine-binding site at the N-methyl-D-aspartate receptor (NMDAr) (Izquierdo and Medina, 1997; Kishi et al., 1998a, b; Rubin et al., 2004; Rubin et al., 2000; Rubin et al., 2001; Shimada et al., 1994). Accordingly, the systemic (Camera et al., 2007), intrahippocampal (ih) (Berlese et al., 2005; Gomes et al., 2010; Guerra et al., 2006; Rubin et al., 2000), and intra-amygdalar (Rubin et al., 2004; Rubin et al., 2001) administration of SPD improves memory. Recent evidence suggests the participation of PKA/CREB pathway cascade in the facilitation of memory induced by SPD (Guerra et al., 2011). However, there are other well known kinases, such as Ca^{2+}-dependent protein kinase (PKC), which are also activated by NMDA receptor-mediated Ca^{2+} influx (Jimenez and Tapia, 2004; Wang et al., 2006; Wu et al., 2003). Since PKC can also phosphorylate CREB (Mao et al., 2007; Selcher et al., 2002), this kinase would naturally arise as a candidate to mediate the memory improvement induced by SPD.

Protein kinase C comprises a family of serine/threonine kinases with 10 known isozymes, divided into three major subsets based on structure and regulation. These include the conventional isoforms dependent on calcium (α, β, and γ), novel (δ, ε, η, and θ), and atypical (λ and ζ) types, which are structurally homologous but can be regulated independently of calcium (Ohno and Nishizuka, 2002; Parekh et al., 2000; Weeber et al., 2000). Protein kinase C cascade regulates neuronal efficacy at different levels, including neurotransmitter release, neurotransmitter receptor function and gene expression (Ben-Ari et al., 1992; Kleschevnikov and Routtenberg, 2001; Macek et al., 1998; Malenka et al., 1986; Manseau et al., 1998; Meberg et al., 1993), and it seems to play a role in memory formation process (Alkon et al., 2007; Alvarez-Jaimes et al., 2005; Bonini et al., 2007; Izquierdo and Medina, 1997; Lorenzetti et al., 2008; Nelson et al., 2008; Nogues, 1997; Serrano et al., 2008). There are lines of evidence supporting a role for PKC in learning and memory. First, PKC activity is increased in the hippocampus immediately after step-down avoidance training, reaching a peak 0.5 h later, and returning to normal values over the next 2 h (Bernabeu et al., 1995; Cammarota et al., 1997). Moreover, it has been shown that learning of a spatial-discrimination task increases membrane-bound PKC levels (and, therefore, activated) in the hippocampus of rats (Golski et al., 1995; Nogues et al., 1994), and PKC activity is reduced in the hippocampus of poor learner mice (Wehner et al., 1990). Furthermore, it has been shown that by increasing PKC substrate,

neuromodulin (GAP-43, B50), matches with the PKC activity peak and can be blocked by inhibitors of this protein kinase (Cammarota et al., 1997). Second, infusion of the PKC activators phorbol 12,13-dibutyrate (PDBu) and 1-oleoyl-2-acetyl glycerol (DAG synthetic analog) improves memory formation in rats (Paylor et al., 1991; Yang and Lee, 1993) and mice (Nogues et al., 1996), respectively. Third, transgenic mice overexpressing conventional PKC substrate, GAP-43, demonstrate enhanced learning and LTP (Routtenberg et al., 2000). Fourth, injection of the PKC inhibitors, such as staurosporin, CGP 41231, Go 6976 (selective α- and βI-PKC inhibitor) and Go 7874 causes retrograde amnesia when infused into CA1 in the first 1–2 h after training (Bonini et al., 2007; Jerusalinsky et al., 1994; Vianna et al., 2000b). The coincidence of the time course of the change of hippocampal PKC activity with that of the amnestic effects of the PKC inhibitors points to a clear and crucial involvement of this enzyme in the posttraining memory processing. Fifth, studies with mutant mice lacking PKCγ, PKCβI or conventional PKC substrates, such as neurogranin and GAP-43, present learning and memory formation deficits in different tasks (Abeliovich et al., 1993; Huang et al., 2006; Miyakawa et al., 2001; Pak et al., 2000; Rekart et al., 2005; Weeber et al., 2000).

Numerous studies have shown that consolidation of different types of memory in rodents requires phosphorylation/activation of the transcription factor CREB by cAMP- or Ca^{2+}-dependent protein kinase (Bernabeu et al., 1997; Brightwell et al., 2007; Cammarota et al., 2000; Cammarota et al.,

2005; Countryman et al., 2005; Desmedt et al., 2003; Impey et al., 1998; Izquierdo and Medina, 1997; Izquierdo et al., 2001; Kida et al., 2002; Mao et al., 2007; Stanciu et al., 2001; Taubenfeld et al., 1999; Trifilieff et al., 2006; Vianna et al., 2000a; Zhang et al., 2003). Recently, we have shown that SPD induces PKA and CREB phosphorylation in the hippocampus of rats (Guerra et al., 2011). Furthermore, the PKA-induced CREB phosphorylation requires PKC activity in striatal neurons (Zanassi et al., 2001). However, no study has addressed whether PKC cascade is involved in the memory improvement induced by SPD and whether the SPD-induced PKA/CREB phosphorylation requires PKC activity. Thus, in the present study, we investigated whether PKC signaling pathway is activated by SPD in rats subjected to inhibitory avoidance training.

2. Material and methods

2.1. *Animals*

All experiments reported in this study were conducted in accordance with Brazilian law No. 11.794/2008 in agreement with the Policies on the Use of Animals and Humans in Neuroscience Research, which were revised and approved by the Society for Neuroscience Research in January 1995, and with the Institutional and National Regulations for Animal Research (process 0206). Male Wistar rats (230–250 g; n = 164) were bred in the Animal House of the Universidade Federal de Santa

Maria, housed 5 to a cage, and maintained on a natural day/night cycle at 21°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 (from 9:00 a.m. to 5:00 p.m.).

2.2. Surgery

Rats were anaesthetized by using intraperitoneal (ip) injection of a mixture of ketamine (80 mg/kg) and xylazine (8 mg/kg), and were implanted with two 27-gauge guide cannulae placed 1 mm above the CA1 region of the dorsal hippocampus at the following coordinates: A = 4 mm; L = 3.0 mm; and V = 2.0 mm (Paxinos and Watson, 1986). Placement of injections was histologically-verified, as described elsewhere (Rubin et al., 1997). Only data from the animals with correct cannula placement were analyzed.

2.3. Behavioral and Infusion Procedures

One week after surgery, the animals were subjected to a single training session in a step-down inhibitory avoidance apparatus, consisting of a 25 × 25 × 35-cm box with a grid floor and the left portion covered by a 7 × 25-cm platform, measuring 2.5 cm in height. The rat was placed gently on the platform facing the rear left corner. Once the rat stepped down with all 4 paws on the grid, a 3-s, 0.3-mA shock was applied to the grid.

Immediately post-training, vehicle, one drug, or a combination of the following drugs were injected bilaterally into the hippocampus (0.5 µl/ brain hemisphere for 1 min): spermidine [N-(3-aminopropyl)-1,4-butanediamine trihydrochloride (SPD); Sigma-Aldrich Co., St. Louis, MO, USA]; and 3-[1-(dimethylaminopropyl)indol-3-yl]-4-(indol-3-yl)maleimide hydrochloride (GF 109203X; Sigma-Aldrich Co.). SPD were dissolved in 50 mM phosphate buffered saline solution (PBS; pH 7.4), and GF 109203X was dissolved in 0.01% DMSO in 50 mM PBS (pH 7.4). The injections were performed by using an infusion pump 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 CA1 region in the dorsal hippocampus. The needles were left in place for an additional 60 s to minimize backflow. After the injection, the animals were returned to their home cages and tested for retention 24 h later. A step-down latency test was taken as a measure of retention, with a cut-off time of 600 s.

Immediately after the inhibitory avoidance test session, the animals were transferred to a 50 X 60-cm open field, with the floor divided into 12 squares. During the 5-min open field session, the number of crossing and rearing responses was recorded. The open field was used to identify motor disabilities which might influence inhibitory testing avoidance performance.

Step-down latency data were analyzed with the Kruskal–Wallis test or the Scheirer–Ray–Hare extension of the Kruskal–Wallis test. Crossing and

rearing responses were analyzed by one- or two-way ANOVA. Significance was considered at $p<0.05$.

2.4. Preparation of tissues and Western blot analysis

Western blot analysis was carried out, as described previously (Casu et al., 2007) with minor modifications. Rats were decapitated, and the hippocampi were rapidly removed, dissected, homogenized in 300 μl of ice-cold A buffer (10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM NaF, 10 μg/ml aprotinin, 10 mM β-glycerolphosphate, 1 mM PMSF, 1 mM DTT, and 2 mM of sodium orthovanadate in 10 mM HEPES, pH 7.9), incubated for 15 min on ice, and centrifuged at 16 000 x g for 45 min at 4°C. The supernatant (S1), denominated cytosolic fraction, was reserved for posterior processing. The pellet (P1) was resuspended in 150 μl of ice-cold buffer B (10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM NaF, 10 μg/ml aprotinin, 10 mM β-glycerolphosphate, 1 mM PMSF, 1 mM DTT, 2 mM sodium orthovanadate, and 1% Triton-X in 10 mM HEPES, pH 7.9), incubated for 15 min on ice, and centrifuged at 16 000 X g for 45 min at 4°C. The supernatant (S2) was discarded and the pellet (P2) was resuspended in 100 μl of ice-cold buffer C (50 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM NaF, 10 μg/ml aprotinin, 10 mM β-glycerolphosphate, 1 mM PMSF, 1 mM DTT, 2 mM sodium orthovanadate, 420 mM NaCl, and 25% glycerol in 20 mM HEPES, pH 7.9), incubated for 15 min on ice, and

centrifuged at 16 000 X g for 45 min at 4^{o}C. The supernatant (S3) was considered the nuclear fraction. The protein concentration in the cytosolic and nuclear fractions was determined by using the Bradford method (1976). Equivalent amounts of protein (80 μg or 20 μg for cytosolic or nuclear fractions, respectively) were added to 0.2 volumes of concentrated loading buffer (200 mM Tris, 10% glycerol, 2% SDS, 2.75 mM β-mercaptoethanol, and 0.04% bromophenol blue) and boiled for 10 min. Proteins were separated in 12% sodium dodecyl sulphate–polyacrylamide gels (SDS–PAGE) and transferred to polyvinilidene difluoride membranes. Ponceau staining (data not shown) served as a loading control (Romero-Calvo et al., 2010). Western blot analysis of PKA and PKC was carried out in the cytosolic fractions, and CREB was carried out in the nuclear fractions. Membranes were processed by using a SNAP i.d. system (Millipore, Billerica, MA, USA). First, the membrane was blocked with 1% BSA in 0.05% Tween 20 in Tris-borate saline (TBS-T), then incubated for 10 min with specific primary antibodies diluted 1:150 in TBS-T (anti-phospho-PKCα, anti-total-PKCα, anti-phospho-PKA RIIα, anti-total-PKA RIIα, anti-phospho-CREB-1 and anti-total-CREB-1 polyclonal antibodies; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Blots were washed three times, with TBS-T followed by incubation with adjusted alkaline phosphatase-coupled secondary antibody (1:3000, anti-rabbit IgG; Santa Cruz Biotechnology, Inc.) for 10 min. Protein bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate and p-nitro blue tetrazolium

(BCIP/NBT; Millipore). Membranes were dried, scanned, and quantified with the Scion Image PC version of NIH image. The results were normalized for the control group (PBS/PBS) densitometry values and expressed as the relative amount of phosphorylated and non-phosphorylated forms, and the phosphorylated/total ratio. Statistical analyses were performed by using a two-way ANOVA or a Student's T-test. F and p values are shown only if $p<0.05$.

2.5. Experiment 1 – Effect of a PKC inhibitor (GF 109203X) on inhibitory avoidance task performance

A dose-response curve for GF 109203X was performed to define the dose for the subsequent experiments. Immediately after training, the animals were injected with vehicle (0.01% DMSO in 50 mM PBS, pH 7.4) or GF 109203X (1.25 – 2.5 ρmol/ hippocampus). The animals were subjected to a step-down inhibitory avoidance session, open field test, and histologic examination, as described above.

2.6. Experiment 2 – Effect of a PKC inhibitor on memory improvement and phosphorylation of the PKC induced by SPD

Once it was determined that GF 109203X at a dose of 2.5 ρmol (ih) did not alter memory, the effect of GF 109203X on SPD-induced

improvement of memory and phosphorylation of PKC were determined. Animals were trained in the step-down inhibitory avoidance apparatus and injected immediately after training with vehicle (0.01% DMSO in 50 mM PBS, pH 7.4), GF 109203X (2.5 ρmol/hippocampus), SPD (0.2 nmol/hippocampus), or GF 109203X (2.5 ρmol/hippocampus) combined with SPD (0.2 nmol/hippocampus). The dose of SPD (0.2 nmol/ hippocampus) was selected because it improves memory of the inhibitory avoidance task (Rubin et al., 2000). A subset of the animals was sacrificed 30 min after injections and the hippocampi were dissected for Western blot analysis of PKC. The other animals had a testing session in the inhibitory avoidance apparatus and an open field test. PKC phosphorylation is an event that regulates the maturation of the enzyme in addition to PKC activation. The autophosphorylation is fundamental for the localization of PKC isozymes and for obtaining a catalytically competent conformation (Ron and Kazanietz, 1999).

2.7. Experiment 3 – Effect of a PKC inhibitor on phosphorylation of the PKA and CREB induced by SPD

To assess a possible cross talk between PKC and PKA, we investigated PKA modulation when the PKC pathway was blocked. The effect of GF 109203X, a PKC inhibitor, at a dose of 2.5 ρmol (ih) on SPD-induced phosphorylation of PKA and CREB were determined. Animals

were trained in the step-down inhibitory avoidance apparatus and injected immediately after training with vehicle (0.01% DMSO in 50 mM PBS, pH 7.4), GF 109203X (2.5 ρmol/hippocampus), SPD (0.2 nmol/hippocampus), or GF 109203X (2.5 ρmol/hippocampus) combined with SPD (0.2 nmol/hippocampus). The animals were sacrificed 180 min after injections and the hippocampi were dissected for Western blot analysis of PKA and CREB. It has been reported previously that autophosphorylation of the PKA regulatory subunit occurs when PKA is activated by cAMP (Tasken and Aandahl, 2004). Thus, the detection of phosphorylated PKA regulatory subunit is useful evidence for PKA activation.

3. Results

3.1. Experiment 1

Table 1 shows the effect of bilateral ih injection of GF 109203X (1.25 – 2.5 ρmol) immediately after training on test step-down latencies. Statistical analysis (Kruskal– Wallis test) revealed that GF 109203X injection did not alter the step-down latencies at testing (F values are shown in Table 1), suggesting that injection of the selective PKC inhibitor, did not impair the memory in the inhibitory avoidance task. Table 1 also shows the effect of GF 109203X on exploratory behavior in the open field immediately after the inhibitory avoidance testing session. Statistical

analysis of open-field data (one-way ANOVA) revealed that GF 109203X injection did not alter the number of crossing or rearing responses in a subsequent open-field testing session (F values shown in Table 1), suggesting that its injection, immediately after training, did not cause gross motor disabilities during testing. The dose of GF 109203X to be used in the subsequent experiments (2.5 ρmol) was chosen based on the lack of effect on memory in this experiment.

3.2. Experiment 2

Figures 1-2 show the effect of the ih administration of GF 109203X (2.5 ρmol), SPD (0.2 nmol), and the co-administration of GF 109203X and SPD immediately after training on step-down latencies during evaluation (Fig. 1) and on Western immunoblotting and densitometry analyses of PKC (Fig. 2). Statistical analysis of step-down latencies during testing (nonparametric two-way ANOVA) showed a significant SPD or PBS vs GF 109203X or PBS interaction [$H_{(1)}$ = 6.84; $p<0.05$], revealing that co-injection of the selective PKC inhibitor prevented facilitation of memory induced by SPD (Fig. 1). This result suggests that memory improvement induced by SPD depends on PKC activation. The open field data demonstrated that pharmacologic treatment did not alter the number of crossing or rearing responses in a subsequent open field testing session (data not shown). These data indicate that none of the compounds tested caused gross motor disabilities during testing.

Statistical analysis (two-way ANOVA) also revealed that bilateral hippocampal injection of GF 109203X, SPD, or a combination of GF 109203X and SPD did not alter the total-PKC levels (Fig. 2B); however, a significant SPD or PBS versus GF 109203X or PBS interaction for phospho-PKC levels [$F_{(1,12)} = 5.86$; $p < 0.05$; Fig. 2A] and the phospho-PKC/total-PKC ratio [$F_{(1,12)} = 5.62$; $p < 0.05$; Fig. 2C] at 30 min after injections was found. These results suggest that GF 109203X prevented the SPD-induced increase of the phospho-PKC level and phospho-PKC/total-PKC ratio.

3.3. Experiment 3

Figures 3-4 show the effect of the ih administration of GF 109203X (2.5 ρmol), SPD (0.2 nmol), and the co-administration of GF 109203X and SPD immediately after training on Western immunoblotting and densitometry analyses of PKA (Fig. 3) and CREB phosphorylation (Fig. 4). Statistical analysis (two-way ANOVA) revealed that bilateral hippocampal injection of GF 109203X, SPD, or a combination of GF 109203X and SPD did not alter total-PKA immunoreactivity (Fig. 3B). However, a significant SPD or PBS versus GF 109203X or PBS interaction for phospho-PKA levels [$F_{(1,8)} = 5.47$; $p < 0.05$; Fig. 3A] and the phospho-PKA/total PKA ratio [$F_{(1,8)} = 6.95$; $p < 0.05$; Fig. 3C], at 180 min after the injections. These results suggest that GF 109203X prevented the SPD-induced increase of the phospho-PKA level and phospho-PKA/total-PKA ratio. Statistical

analysis (two-way ANOVA) also revealed that bilateral hippocampal injection of GF 109203X, SPD, or a combination of GF 109203X and SPD did not alter total-CREB immunoreactivity (Fig. 4B); however, a significant SPD or PBS versus GF 109203X or PBS interaction for phospho-CREB levels [$F_{(1,8)} = 23.16$; $p < 0.05$; Fig. 4A] and the phospho-CREB/total-CREB ratio [$F_{(1,8)} = 16.42$; $p < 0.05$; Fig. 4C] was found, indicating that GF 109203X prevented the SPD-induced increase of the phospho-CREB level and phospho-CREB/total-CREB ratio.

4. Discussion

In this study, we showed that GF 109203X, an inhibitor of PKC, prevents the facilitatory effect of SPD on the memory of the inhibitory avoidance task. The injection of GF 109203X also prevented SPD-induced phosphorylation of PKC, PKA and CREB in the hippocampus. The reversal of facilitatory effects of ih-injected SPD on step-down inhibitory avoidance by a PKC inhibitor implicates PKC activation in SPD-induced facilitation of memory (Fig. 1). This finding was corroborated by the demonstration that SPD increases PKC phosphorylation, which is prevented by GF 109203X (Fig. 2). SPD-induced activation of PKC was observed 30 min after the injection of the polyamine. Interestingly, such an increase of PKC phosphorylation coincides with the known activity peak

that occurs after inhibitory avoidance training, which correlates with the increased phosphorylation of the PKC substrate GAP-43, and is blocked by preferential PKC inhibitors (Cammarota et al., 1997). The currently reported results, therefore, support an early role for PKC in the SPD-induced memory improvement.

There is a body of evidence suggesting a cross-talk between PKC and PKA pathways (Kubota et al., 2003; Sugita et al., 1997; Yao et al., 2008). Moreover, it has been shown recently that SPD administration increases PKA and CREB phosphorylation in the hippocampus at 180 min, but not 30 min after injection (Guerra et al., 2011). Therefore, one might reasonably ask whether PKC and PKA/CREB pathways were sequentially activated in the presence of SPD. If these pathways were sequentially activated, they should take place at different intervals of time and be prevented by the inhibition of early events. In line with this view, ih administration of GF 109203X prevented SPD-induced phosphorylation of PKA and CREB in the hippocampus, suggesting that the activation of PKA/CREB pathway depends on PKC activation. Moreover, SPD-induced activation of PK is an early event (observed 30 min after SPD injection), whereas SPD-induced activation of PKA occurs later (180 min after SPD injection (Guerra et al., 2011) further indicating a sequential interaction between these pathways.

Although our results indicate that early PKC and late PKA/CREB activation are involved in SPD-induced memory improvement, the exact

mechanisms by which such interaction occurs remain obscure. A likely mechanism by which PKC could activate PKA involves neurogranin. Current evidence suggests that activation of PKC cause the phosphorylation of neurogranin (also named RC3), a PKC substrate that binds calmodulin (CaM) at low Ca^{2+} levels (Baudier et al., 1991; Diez-Guerra, 2010; Gerendasy et al., 1994; Huang et al., 1993). It has been suggested that calcium influx induces PKC-mediated neurogranin phosphorylation, reducing its affinity for CaM. This would increase CaM availability and the formation of Ca^{2+}/CaM complexes in the cell (Li et al., 1999; Sheu et al., 1996; Wu et al., 2003). Increased Ca^{2+}/CaM levels are known to activate Ca^{2+}/CaM-sensitive adenylyl cyclase type I and VIII, thereby enhancing the cAMP level (Ferguson and Storm, 2004; Mons et al., 1999; Poser and Storm, 2001; Wang and Storm, 2003). In line with this view, deletion of neurogranin gene impairs PKA activation, either by NMDA (Wu et al. 2003) or by forskolin (Wu et al., 2002), and the subsequent signaling involved in hippocampus-dependent learning and memory.

It has also been shown that PKC may directly activate adenylyl ciclase (Bell et al., 1985; Choi et al., 1993; Cooper et al., 1995; Jacobowitz et al., 1993; Lustig et al., 1993; Pieroni et al., 1993; Yoshimura and Cooper, 1993). Activators of PKC seem to cause, by these means, an increase in the excitability of the sensory neuron of Aplysia, (Sugita et al., 1997)and increase cAMP synthesis by type II adenylyl ciclase (Yoshimura

and Cooper, 1993). In line with this view, staurosporine, a potent PKC inhibitor, attenuates the stimulatory effect of activator on type II activity, and an inactive phorbol ester (4α-PMA) has no effect (Yoshimura and Cooper, 1993). These observations support the notion that PKC may increase cAMP levels by the direct and indirect activation of Ca^{2+}/CaM-insensitive adenylyl cyclase and Ca^{2+}/CaM-sensitive adenylyl cyclase, respectively.

In summary, this study showed that SPD-induced memory facilitation involves the sequential activation of PKC and PKA/CREB signaling in the hippocampus of rats.

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Figure legends

Fig. 1. Co-administration of GF 109203X (2.5 ρmol intrahippocampus) immediately after training prevents the improvement in memory induced by spermidine (SPD, 0.2 nmol). Phosphate-buffered saline (50 mM PBS, pH 7.4) was used as a vehicle. Data are the median and interquartile range for 13 animals in each group. *P<0.05 as compared with control group values (PBS/PBS).

Fig. 2. Effect of the post-training intrahippocampal co-administration of GF 109203X (2.5 ρmol) and spermidine (SPD, 0.2 nmol) on the representative images of Western immunoblotting and densitometry analyses of phospho-PKC (A), total-PKC (B), and the ratio of phospho- PKC/total-PKC (C). The rats were killed 30 min after the injections of GF 109203X and SPD. The results were normalized by arbitrarily setting the densitometry of the control group (PBS/PBS). Ponceau staining served as a loading control. Data are the mean ± SEM for 4 animals in each group. *P<0.05 as compared with control group values (PBS/PBS).

Fig. 3. Effect of the intrahippocampal co-administration of GF 109203X (2.5 ρmol) and spermidine (SPD, 0.2 nmol) on the levels of phospho-PKA (A), total-PKA (B), and the phospho-PKA/total-PKA ratio (C). The rats were killed 180 min after the injections of GF 109203X and SPD. The

results were normalized by arbitrarily setting the densitometry of the control group (PBS/PBS). Ponceau staining served as a loading control. Data are the mean ± SEM for 3 animals in each group. **P*<0.05 as compared with control group values (PBS/PBS).

Fig. 4. Effect of the intrahippocampal co-administration of GF 109203X (2.5 ρmol) and spermidine (SPD, 0.2 nmol) on the levels of phospho-CREB (A), total-CREB (B), and the phospho-CREB/total-CREB ratio (C). The rats were killed 180 min after the injections of GF 109203X and SPD. The results were normalized by arbitrarily setting the densitometry of the control group (PBS/PBS). Ponceau staining served as a loading control. Data are the mean ± SEM for 3 animals in each group. **P*<0.05 as compared with control group values (PBS/PBS).

Table 1 Effect of the post-training intrahippocampal administration of GF 109203X (1.25 – 2.5 ρmol) on the inhibitory avoidance task performance of rats (measured as the test step-down latency) and on the behavior of rats (number of crossing and rearing responses) in the open field immediately after the inhibitory avoidance testing session

Group	Latency (s)	Crossing	Rearing	N
PBS	179.5 (106.5 – 333)	21.5 ± 3.3	9.7 ± 1.6	12
GF (1.25 ρmol)	116.5 (35 – 183)	20.4 ± 2.3	13.1 ± 2.0	12
GF (2.5 ρmol)	114.5 (27 – 370)	18.3 ± 2.8	9.4 ± 1.3	12
Statistical analysis	H (2) = 2.55, $p > .05$	$F_{(2,33)} = 0.32$, $p > .05$	$F_{(2,33)} = 1.49$, $p > .05$	

Data are the median (interquartile ranges) or means ± SEM; N, number of animals in each group

Figure 1

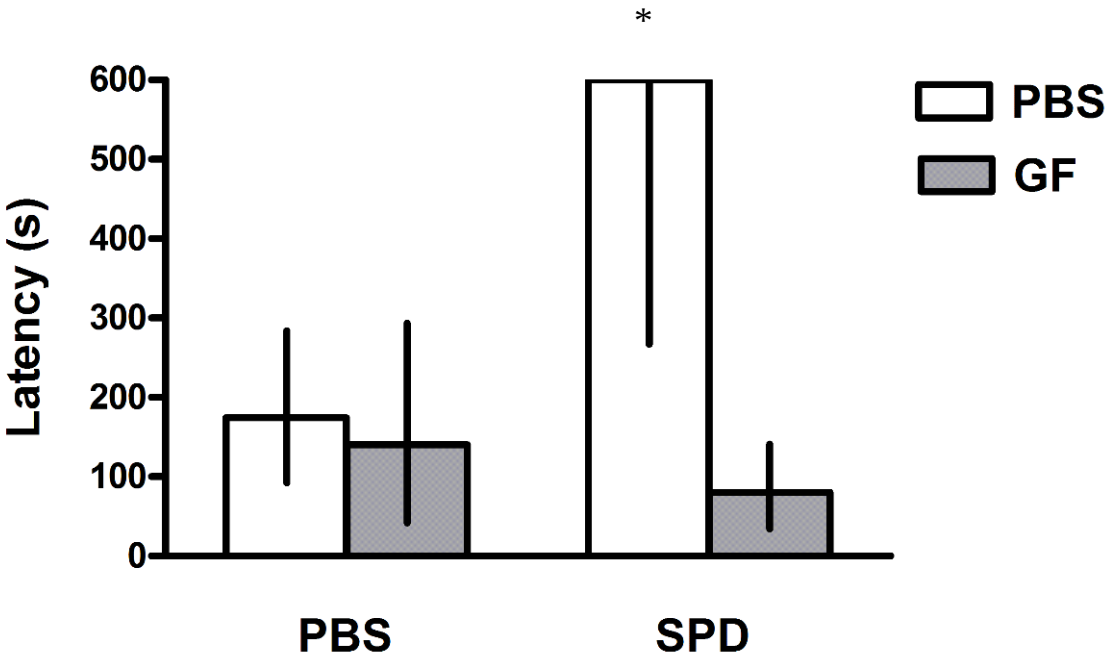
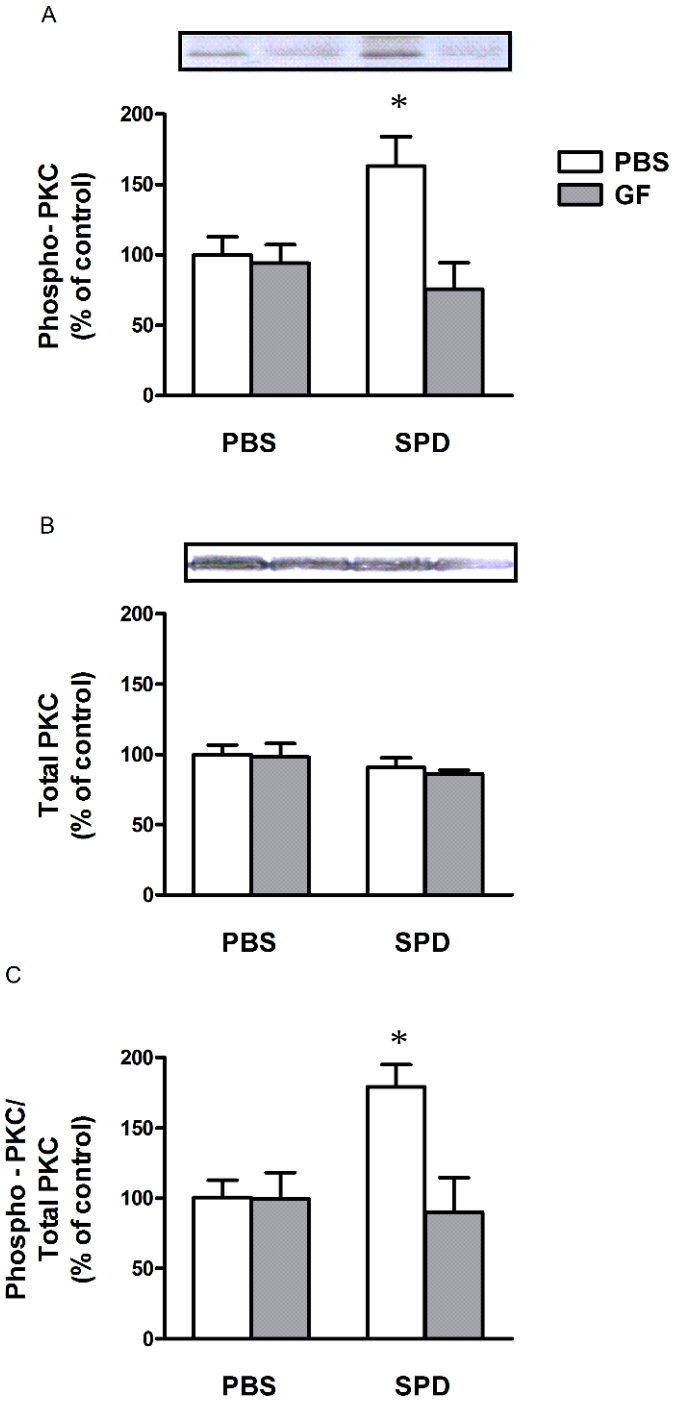


Figure 2


A
Phospho- PKC
(% of control)
200
150
100
50
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*
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SPD
B
Total PKC
(% of control)
200
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100
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PBS
SPD
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Phospho - PKC/
Total PKC
(% of control)
200
150
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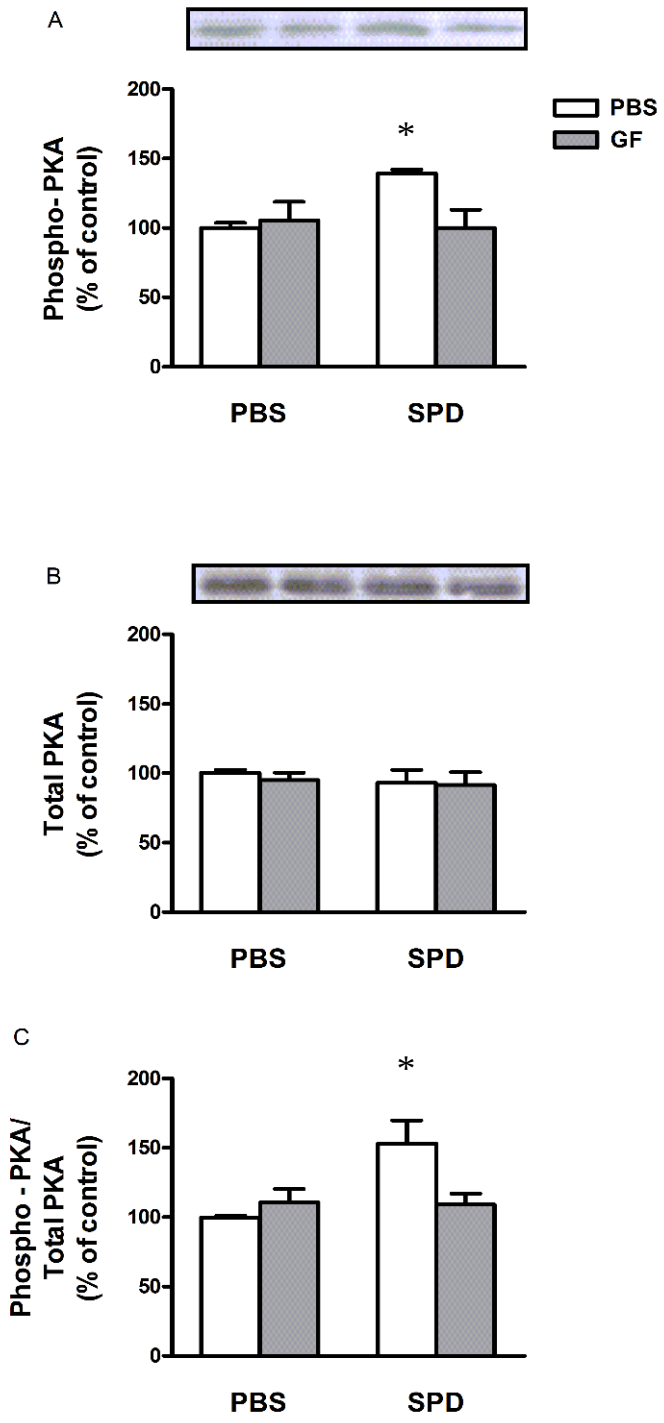
*
PBS
SPD

Figure 3


A
Phospho- PKA
(% of control)
200
150
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PBS
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B
Total PKA
(% of control)
200
150
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50
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PBS
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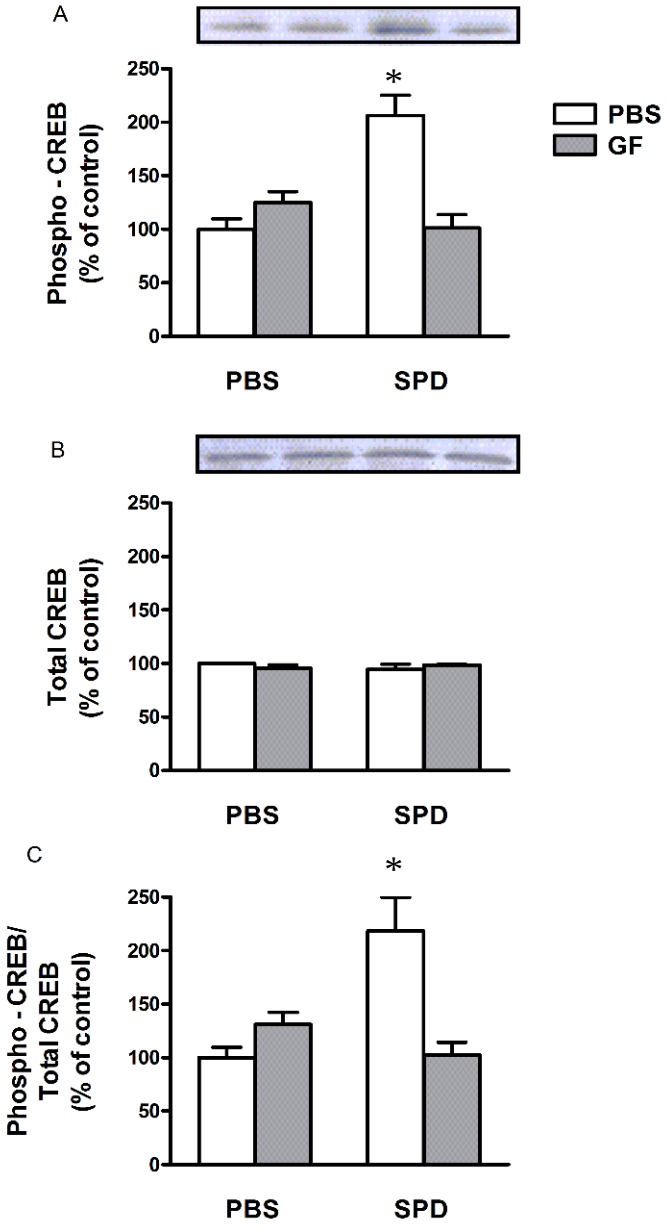
C
Phospho - PKA/
Total PKA
(% of control)
200
150
100
50
0
*
PBS
SPD

Figure 4


A
Phospho - CREB
(% of control)
250
200
150
100
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0
*
PBS
GF
PBS
SPD
B
Total CREB
(% of control)
250
200
150
100
50
0
PBS
SPD
C
Phospho - CREB/
Total CREB
(% of control)
250
200
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50
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PBS
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V. DISCUSSÃO

Neste estudo nós demonstramos que H-89, um inibidor da PKA, arcaína, um antagonista do sítio das poliaminas no receptor NMDA, e GF 109203X, inibidor da PKC, preveniram a melhora da memória da tarefa de esquiva inibitória induzida por SPD em ratos. A injeção de H-89 ou arcaína preveniu a fosforilação da PKA e CREB, enquanto a injeção de GF 109203X preveniu a fosforilação da PKC, PKA e CREB, induzida por SPD no hipocampo. A reversão do efeito facilitatório da injeção intrahipocampal de SPD sobre a memória por um inibidor da PKA, um antagonista do sítio das poliaminas no receptor NMDA e um inibidor da PKC (Fig. 1 e 4 – artigo e Fig. 1 – manuscrito, respectivamente), implica na ativação do receptor NMDA e das quinases PKA e PKC na melhora da memória induzida por SPD. Estes resultados foram corroborados, por demonstrarem que SPD ativa a PKC e a PKA, e que GF 109203X e H-89, respectivamente, previnem a ativação da PKC (Fig. 2 – manuscrito) e da PKA (Fig. 2 – artigo) induzida por SPD. A ativação da PKC induzida por SPD foi observada 30 min depois da injeção da poliamina. Interessantemente, este aumento na fosforilação da PKC coincide com o pico de atividade da PKC que ocorre depois do treino na esquiva inibitória, que é correlacionado com o aumento na fosforilação do substrato da PKC, GAP-43, e pode ser bloqueado por inibidores desta quinase (Cammarota et al., 1997). Por outro lado, a ativação da PKA induzida por SPD ocorre 180 min depois da injeção da poliamina, o que provavelmente reflete o pico de atividade da PKA, que ocorre 3-6 horas

depois do treino. Além disso, este pico é correlacionado com o aumento máximo nos níveis de AMPc após o treino (Bernabeu et al., 1997a). De acordo com essa visão, a arcaína impediu o efeito facilitatório da SPD sobre a fosforilação da PKA, apoiando o envolvimento dos receptores NMDA na ativação da PKA induzida por SPD (Fig. 6 – artigo).

Como a fosforilação da CREB depende da atividade da PKA (Gonzalez e Montminy, 1989; Bernabeu et al., 1997a;), a investigação foi ampliada para os níveis de CREB fosforilada. A administração intrahipocampal de SPD aumentou a fosforilação da CREB, e como esperado, H-89 e arcaína preveniram a fosforilação da CREB induzida por SPD (Fig. 3 e 6 – artigo, respectivamente). Além disso, a injeção de SPD aumenta a imunorreatividade da subunidade catalítica da PKA fosforilada na fração nuclear do hipocampo (Fig. 7 – artigo). Uma vez que, a cascata hipocampal AMPc/PKA é ativada pelo influxo de Ca^{2+} proveniente do receptor NMDA (Chetkovich et al., 1991), é possível que o influxo de Ca^{2+}, sequencialmente, estimule a atividade da adenilato ciclase, acumulando AMPc, e fosforilando a PKA e CREB (Eliot et al., 1989; Poser and Storm, 2001). De fato, foi demonstrado que o AMPc liga-se a subunidades regulatória da PKA, resultando na liberação da subunidade monomérica catalítica ativa da PKA (Taylor et al., 1990; Gibbs et al., 1992; Wang et al., 1991; 2004). A subunidade catalítica da PKA livre, transloca para o núcleo, onde pode fosforilar a CREB (Dash et al., 1991; Mayford et al., 1995a; Impey et al., 1998).

Surpreendentemente, H-89 por si só, não causou efeito sobre a atividade da PKA ou CREB. É possível que não tenha sido revelado qualquer efeito do H-89 devido a janela de tempo selecionada para executar o ensaio. Por exemplo, Viana e colaboradores (2000b) demonstraram que a Rp-cAMPS inibe a atividade da PKA 15 min após a injeção do inibidor, mas não possui efeito em 90 min. Curiosamente, Rp-cAMPS, em uma dose que não altera a atividade da PKA em 90 min, impede o aumento da atividade da PKA induzida pelo treino na tarefa de esquiva inibitória em 180 min. Os resultados de Viana e colaboradores (2000b) são muito semelhantes aos obtidos no presente estudo, em que o inibidor impediu o aumento da fosforilação de PKA induzida por espermidina, 180 min, apoiando a visão de que um inibidor da PKA, em dose que não possua efeito por si só, pode impedir o aumento da atividade PKA induzida pelo treino na tarefa de esquiva inibitória ou, no caso do presente estudo, induzida pela administração de SPD.

Interessantemente, a administração intrahipocampal de SPD aumentou a razão p-PKA/PKA 180 min depois da injeção, provavelmente, devido a diminuição nos níveis da subunidade regulatória total da PKA (Fig. 2E e 2F – artigo). Evidências sugerem que a subunidade regulatória da PKA é degradada por proteólise mediada por ubiquitina-proteassoma no encéfalo de animais (Hegde et al., 1993) e humanos (Liang et al., 2007). Tem sido sugerido, que déficits na subunidade regulatória (RIIα e RIIβ) e na subunidade catalítica (Cβ) diminuem a atividade da enzima e,

conseqüentemente, prejudicam a memória em pacientes com doença de Alzheimer (Liang et al., 2007). Em contraste, lactacistina, um inibidor específico de proteassoma, bloqueia completamente a “down-regulation” da subunidade regulatória induzida por forskolina em células de neuroblastoma humano SH-SY5Y (Boundy et al., 1998). Além disso, a degradação proteassomal das subunidades regulatórias da PKA gera um excesso da subunidade catalítica. Corroborando com estas evidências, um estudo mostra que o treino na tarefa de esquiva inibitória provoca um aumento na quantidade de subunidade catalítica da PKA dependente dos receptores NMDA em frações nucleares de neurônios hipocampais (Cammarota et al., 2000) e, além disso, a injeção intrahipocampal de um inibidor de proteassoma bloqueia a formação da memória de longa duração (Lopez-Salon et al., 2001). Adicionalmente, é interessante notar paralelos na evolução temporal entre o efeito de inibidores de proteossoma sobre a formação da memória de longa duração e inibidores específicos da PKA, distribuídos na mesma região do encéfalo (Bernabeu et al., 1997a; Vianna et al, 2000b) sendo que, ainda coincide com o período durante o qual a atividade da PKA hipocampal está aumentada (Izquierdo e Medina, 1997).

Existem evidências sugerindo um cruzamento entre a PKC e a ativação da PKA (Sugita et al., 1997; Kubota et al., 2003; Yao et al., 2008). Além disso, nós encontramos que a administração de SPD induz a fosforilação da PKA e CREB no hipocampo de ratos, somente 180 min,

mas não 30 min depois da injeção, então, nós investigamos os níveis de fosfo-PKA e fosfo-CREB, depois da injeção do inibidor da PKC. A administração intrahipocampal de GF 109203X preveniu a fosforilação da PKA e CREB induzida por SPD no hipocampo (Fig. 3 e 4 – manuscrito, respectivamente).

Embora os resultados do presente estudo indiquem que a ativação da PKC em períodos iniciais e da PKA em períodos mais tardis estão envolvidos na melhora da memória induzida por SPD, o exato mecanismo pelo qual esta interação ocorre permanece incerto. Um provável mecanismo pelo qual a PKC pode ativar a PKA involve a neurogranina. Evidências sugerem que a ativação da PKC cause a fosforilação da neurogranina (também chamada RC3), um substrato da PKC que liga-se a calmodulina (CaM) em baixas concentrações de Ca^{2+} (Baudier et al., 1991; Huang et al., 1993; Diez-Guerra, 2010). Após a ativação de receptores, como o NMDA, ocorre aumento do influxo de Ca^{2+}, induzindo a fosforilação da neurogranina mediada pela PKC. A neurogranina quando fosforilada reduz a afinidade da ligação para a CaM. Isto aumenta a disponibilidade de CaM e formação do complexo Ca^{2+}/CaM (Sheu et al., 1996; Li et al., 1999; Wu et al., 2003) (Fig. V.1). Um aumento nos níveis de Ca^{2+}/CaM ativa a adenilato ciclase tipo I e VIII, aumentando os níveis de AMPc (Mons et al., 1999; Poser and Storm, 2001; Wang and Storm, 2003; Ferguson and Storm, 2004;). Neste sentido, a falta do gene para a neurogranina prejudicaria a ativação da PKA induzida por NMDA (Wu et

al., 2003) ou por forskolina (Wu et al., 2002), e a subsequente sinalização envolvida na formação da memória.

Tem sido demonstrado também que a PKC pode ativar diretamente a adenilato ciclase (Bell et al., 1985; Choi et al., 1993;; Jacobowitz et al., 1993; Lustig et al., 1993; Pieroni et al., 1993; Yoshimura and Cooper, 1993; Cooper et al., 1995). Estudos demonstram que ativadores da PKC, aumentam a síntese de AMPc através da adenilato ciclase tipo II (Yoshimura and Cooper, 1993) e causam um aumento na excitabilidade do neurônio sensorial de *Aplysia,* (Sugita et al., 1997). Enquanto que, a estaurosporina, um inibidor da PKC, atenua o efeito estimulatório do éster de forbol sobre a adenilato ciclase tipo II (Yoshimura and Cooper, 1993).

Estas observações sugerem que a ativação da PKC aumenta os níveis de AMPc, por uma ativação direta e indireta da adenilato ciclase insensível a Ca^{2+}/CaM (tipo II) e adenilato ciclase sensível a Ca^{2+}/CaM (tipo I e VIII), respectivamente.

Uma vez que, a cascata AMPc/PKA hipocampal é ativada pelo acúmulo de AMPc mediante a ativação da adenilato ciclase (Eliot et al., 1989; Poser and Storm, 2001), podemos sugerir que a fosforilação da PKC induzida por SPD pode estar aumentando tanto a fosforilação da neurogranina, quanto a atividade da adenilato ciclase, contribuindo para um acúmulo de AMPc e conseqüentemente a ativação da PKA e CREB.

Assim, este estudo demonstrou que a melhora da memória induzida por SPD depende de uma seqüência de eventos bioquímicos no

hipocampo dos ratos desencadeada pelo receptor NMDA, seguida pela ativação da PKC e da cascata de sinalização PKA/CREB.

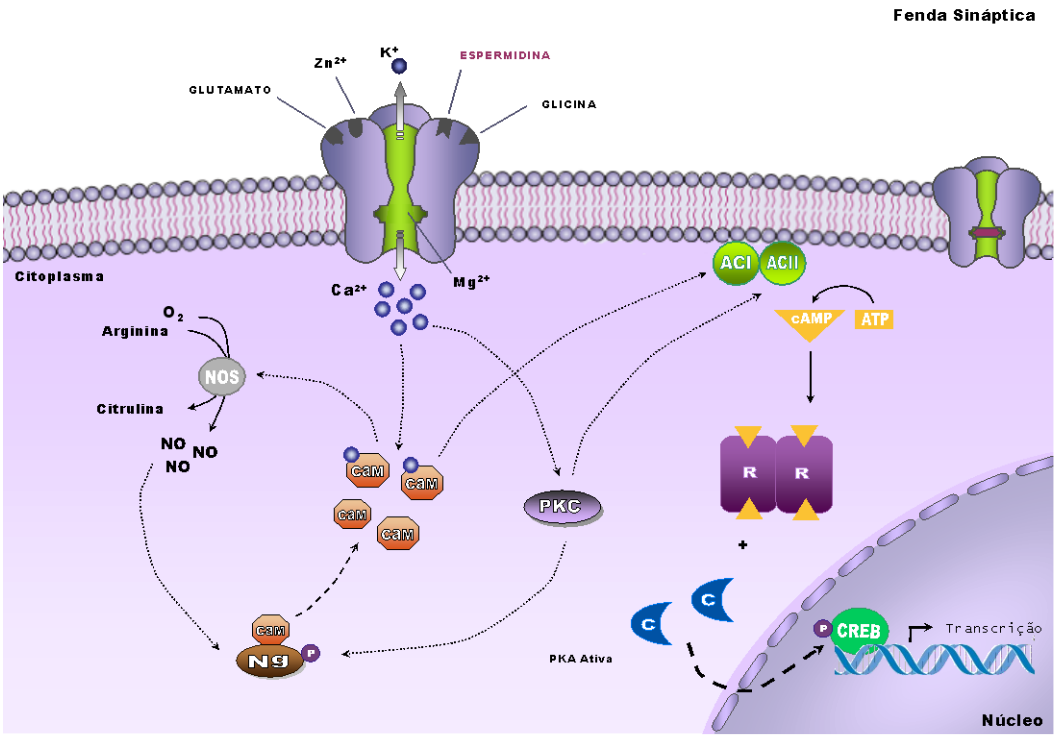

Fig. V.1 – Ativação da PKC, PKA e CREB na melhora da memória induzida por SPD. Neurogranina (Ng).

VI. CONCLUSÕES

Com base nos resultados obtidos, concluiu-se que:

- A administração intra-hipocampal de GF 109203X, um inibidor da PKC, H-89, um inibidor da PKA, e arcaína, um antagonista do sítio das poliaminas no receptor NMDA, preveniram a melhora da memória induzida por SPD. Sugerindo que a ativação do receptor NMDA e das proteínas quinase A e C estão envolvidos na melhora da memória induzida por SPD.

- A administração intra-hipocampal de H-89 e arcaína, preveniram o aumento da fosforilação da PKA e CREB induzidos pela administração intra-hipocampal de SPD. Sugerindo que SPD aumenta a ativação da PKA e CREB através da ativação do receptor NMDA.

- A administração intra-hipocampal de SPD aumentou os níveis da subunidade catalítica da PKA no núcleo. Sugerindo que SPD aumentou a migração da subunidade catalítica da PKA do citosol para o núcleo.

- A administração intra-hipocampal de GF 109203X, preveniu o aumento da fosforilação da PKC, PKA e CREB induzidos pela administração intra-hipocampal de SPD. Sugerindo que SPD aumenta a ativação da PKC, PKA e CREB. Sendo que a ativação da PKA e CREB necessita da ativação previa da PKC.

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