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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 ρmol 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 ρmol) 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.
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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 ρmol/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 ρmol (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 ρmol/hippocampus), SPD (0.2 nmol/hippocampus), or H-89 (0.5 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 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 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	129.0 (51.5–355.5)	15.6 ± 1.3	6.7 ± 0.7	13
H-89 (0.5 ρmol)	91.0 (63.5–382.5)	15.5 ± 2.0	7.0 ± 1.6	13
H-89 (5 ρmol)	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.

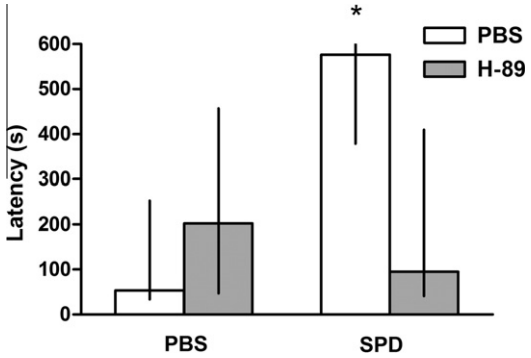

Fig. 1. Co-administration of H-89 (0.5 ρmol intrahippocampus) immediately after training prevents the improvement in memory induced by spermidine (SPD, 0.2 nmol). Phosphate-buffered saline (50 mM PBS, pH 7.4) was used as a vehicle. Data are the median and interquartile range for 16 animals in each group. *$P<0.05$ 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 ρmol) immediately after training on test step-down latencies. Statistical analysis (Kruskal–Wallis test) revealed that H-89, in the dose of 5 ρmol, 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 ρmol) 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 ρmol), 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

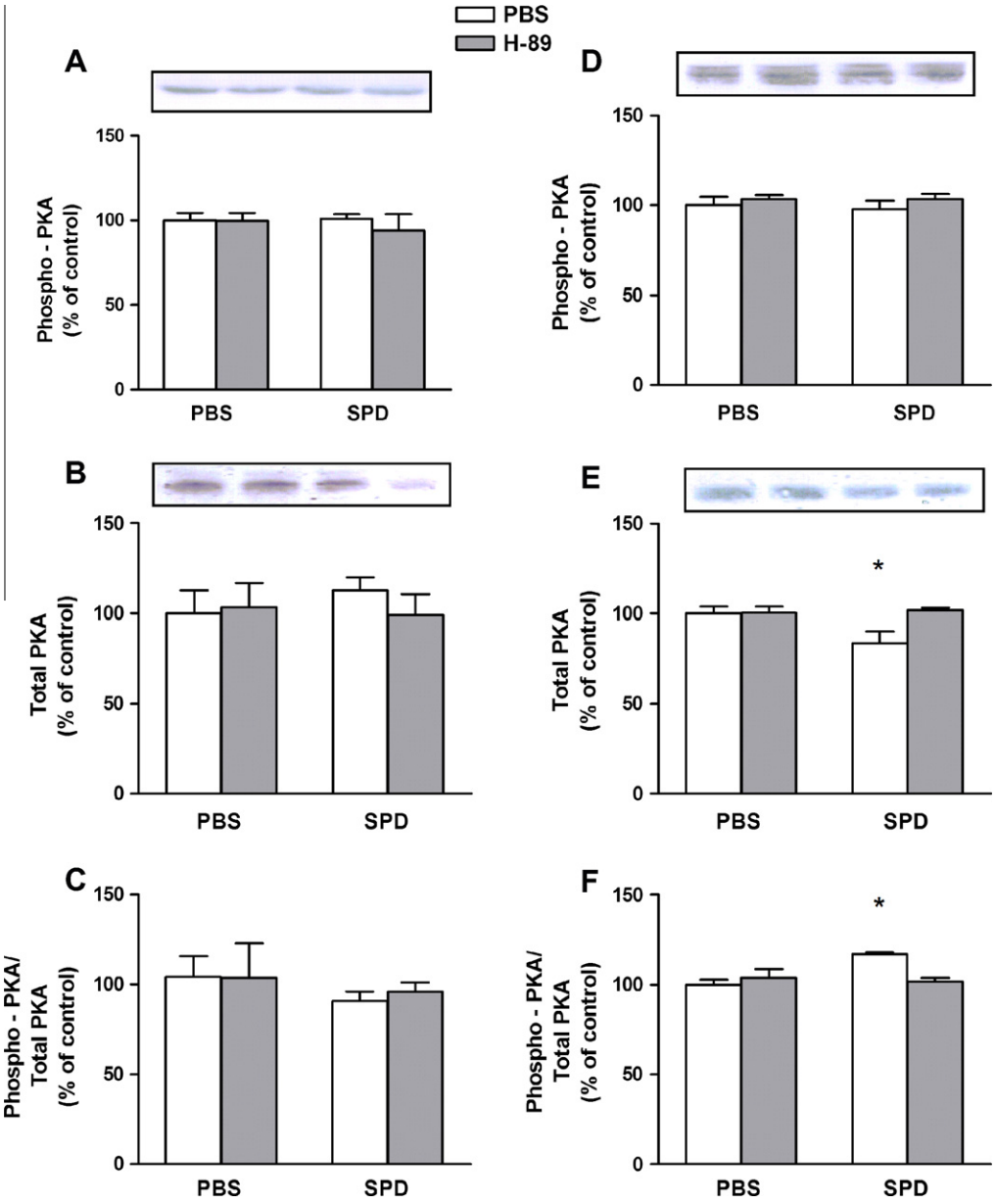

Fig. 2. Effect of the post-training intrahippocampal co-administration of H-89 (0.5 ρmol) and spermidine (SPD, 0.2 nmol) on the representative images of Western immunoblotting and densitometry analyses of phospho-PKA (A, D), total-PKA (B, E), and the ratio of phospho- PKA/total-PKA (C, F). In Fig. A, 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 [$H_1 = 5.08$; $p < 0.05$],

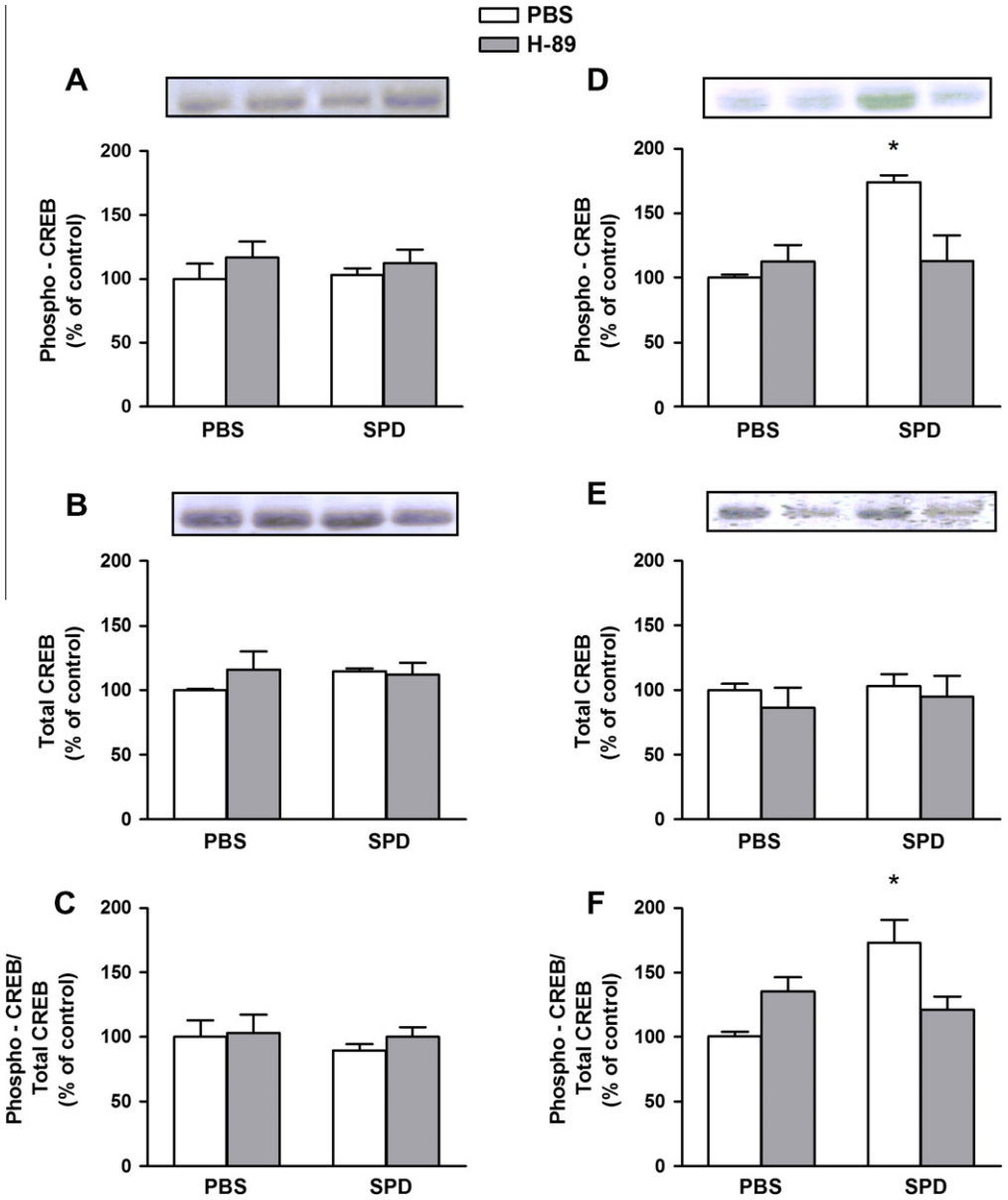

Fig. 3. Effect of the intrahippocampal co-administration of H-89 (0.5 ρmol) and spermidine (SPD, 0.2 nmol) on the levels of phospho-CREB (A, D), total-CREB (B, E), and the phospho-CREB/total-CREB ratio (C, F). A, B, and C, and D, E, and F show the 30- and 180-min data, respectively. The results were 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).

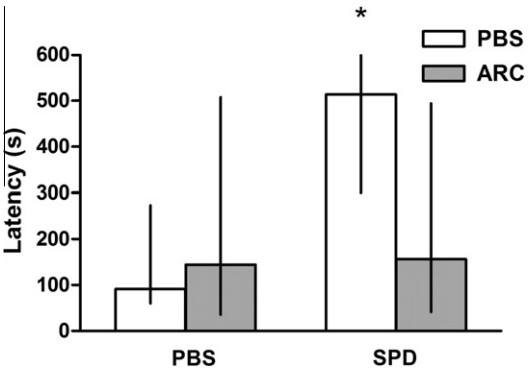

Fig. 4. Co-administration of arcaine (ARC, 0.02 nmol, ih) immediately after training prevents the improvement in memory induced by spermidine (0.2 nmol). Phosphate-buffered saline (50 mM PBS, pH 7.4) was used as a vehicle. Data are the median ± interquartile ranges for 14 animals in each group. $^{*}P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

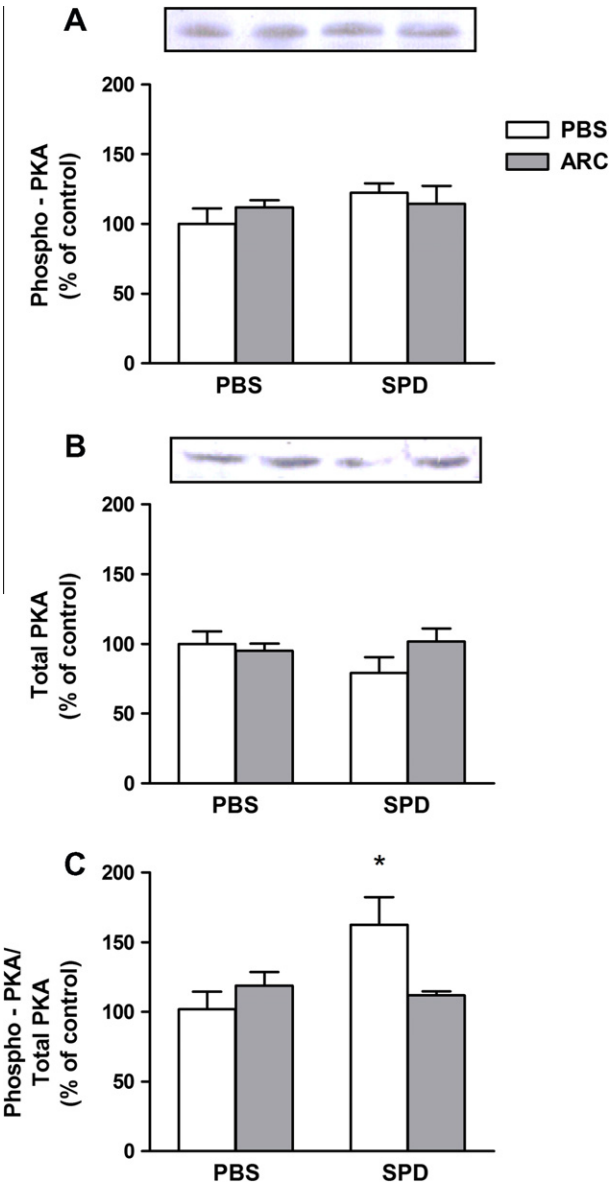

Fig. 5. Effect of the post-training intrahippocampal co-administration of arcaine (ARC, 0.02 nmol) and spermidine (SPD, 0.2 nmol) on representative images of Western immunoblotting and densitometry analyses of phospho-PKA (A), total-PKA (B), and the ratio of phospho-PKA/total-PKA (C) 180 min after the 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,

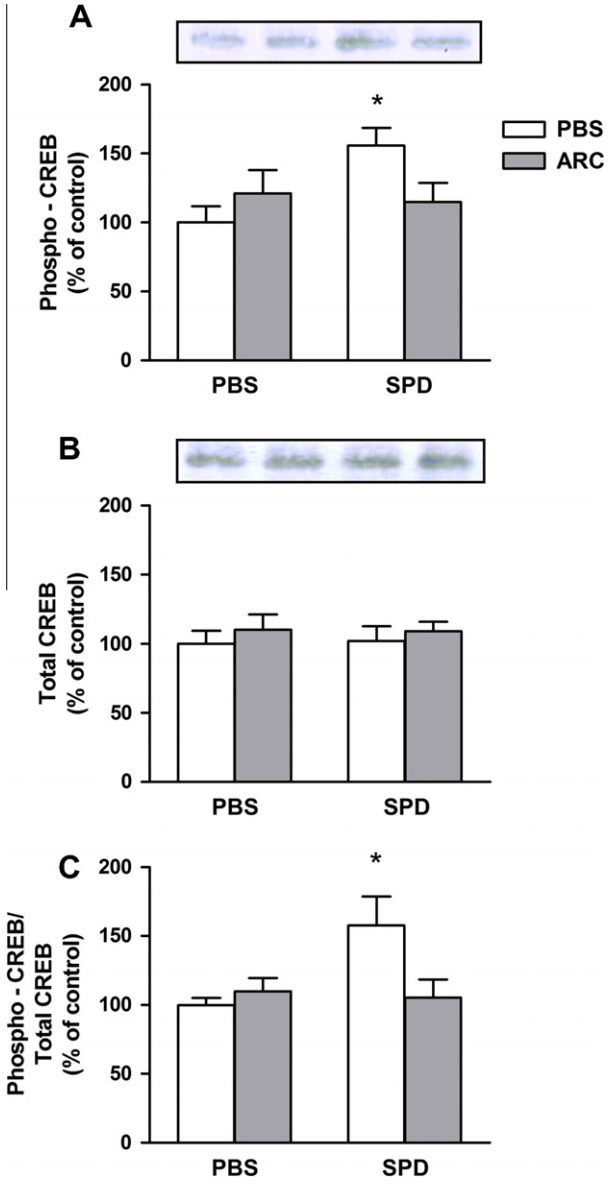

Fig. 6. Effect of the post-training intrahippocampal co-administration of arcaine (ARC, 0.02 nmol) and spermidine (SPD, 0.2 nmol) on representative images of Western immunoblotting and densitometry analyses of phospho-CREB (A), total-CREB (B), and the ratio of phospho-CREB/total-CREB (C) 180 min after 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).

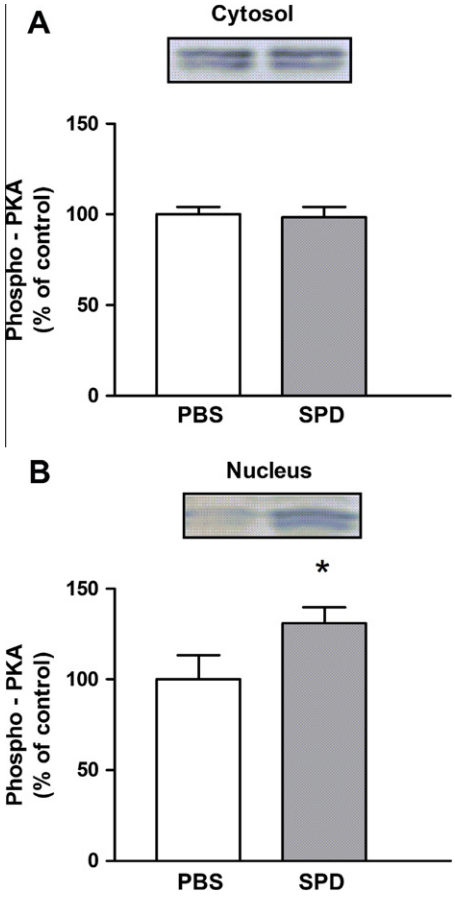

Fig. 7. Effect of the post-training intrahippocampal administration of spermidine (SPD, 0.2 nmol) on representative images of Western immunoblotting and densitometry analyses of the cytosolic (A) and nuclear (B) fractions of phospho-PKA catalytic subunits 180 min after the injection of SPD. The results 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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References

Abel, T., Nguyen, P. V., Barad, M., Deuel, T. A., Kandel, E. R., & Bourtchouladze, R. (1997). Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. *Cell, 88*, 615–626.

Ahi, J., Radulovic, J., & Spiess, J. (2004). The role of hippocampal signaling cascades in consolidation of fear memory. *Behavioural Brain Research, 149*, 17–31.

Barad, M., Bourtchouladze, R., Winder, D. G., Golan, H., & Kandel, E. (1998). Rolipram, a type IV-specific phosphodiesterase inhibitor, facilitates the establishment of long-lasting long-term potentiation and improves memory. *Proceedings of the National Academy of Sciences of the United States of America, 95*, 15020–15025.

Berlese, D. B., Sauzem, P. D., Carati, M. C., Guerra, G. P., Stiegemeier, J. A., Mello, C. F., et al. (2005). Time-dependent modulation of inhibitory avoidance memory by spermidine in rats. *Neurobiology of Learning and Memory, 83*, 48–53.

Bernabeu, R., Bevilaqua, L., Ardenghi, P., Bromberg, E., Schmitz, P., Bianchin, M., et al. (1997a). Involvement of hippocampal cAMP/cAMP-dependent protein kinase signaling pathways in a late memory consolidation phase of aversively motivated learning in rats. *Proceedings of the National Academy of Sciences of the United States of America, 94*, 7041–7046.

Bernabeu, R., Cammarota, M., Izquierdo, I., & Medina, J. H. (1997b). Involvement of hippocampal AMPA glutamate receptor changes and the cAMP/protein kinase A/CREB-P signalling pathway in memory consolidation of an avoidance task in rats. *Brazilian Journal of Medical and Biological Research, 30*, 961–965.

Bernabeu, R., Izquierdo, I., Cammarota, M., Jerusalinsky, D., & Medina, J. H. (1995). Learning-specific, time-dependent increase in [3H]phorbol dibutyrate binding to protein kinase C in selected regions of the rat brain. *Brain Research, 685*, 163–168.

Bernabeu, R., Schmitz, P., Faillace, M. P., Izquierdo, I., & Medina, J. H. (1996). Hippocampal cGMP and cAMP are differentially involved in memory processing of inhibitory avoidance learning. *NeuroReport, 7*, 585–588.

Bernabeu, R., Schroder, N., Quevedo, J., Cammarota, M., Izquierdo, I., & Medina, J. H. (1997c). Further evidence for the involvement of a hippocampal cGMP/cGMP-dependent protein kinase cascade in memory consolidation. *NeuroReport, 8*, 2221–2224.

Bevilaqua, L., Ardenghi, P., Schroder, N., Bromberg, E., Schmitz, P. K., Schaeffer, E., et al. (1997). Drugs acting upon the cyclic adenosine monophosphate/protein kinase A signalling pathway modulate memory consolidation when given late after training into rat hippocampus but not amygdala. *Behavioural Pharmacology, 8*, 331–338.

Boundy, V. A., Chen, J., & Nestler, E. J. (1998). Regulation of cAMP-dependent protein kinase subunit expression in CATH a and SH-SY5Y cells. *Journal of Pharmacology and Experimental Therapeutics, 286*, 1058–1065.

Bourtchouladze, R., Abel, T., Berman, N., Gordon, R., Lapidus, K., & Kandel, E. R. (1998). Different training procedures recruit either one or two critical periods for contextual memory consolidation, each of which requires protein synthesis and PKA. *Learning and Memory, 5*, 365–374.

Cadd, G., & McKnight, G. S. (1989). Distinct patterns of cAMP-dependent protein kinase gene expression in mouse brain. *Neuron, 3*, 71–79.

Camera, K., Mello, C. F., Ceretta, A. P., & Rubin, M. A. (2007). Systemic administration of polyaminergic agents modulate fear conditioning in rats. *Psychopharmacology (Berl), 192*, 457–464.

Cammarota, M., Bevilaqua, L. R., Ardenghi, P., Paratcha, G., Levi de Stein, M., Izquierdo, I., et al. (2000). Learning-associated activation of nuclear MAPK, CREB and Elk-1, along with Fos production, in the rat hippocampus after a one-trial avoidance learning: Abolition by NMDA receptor blockade. *Brain Research and Molecular Brain Research, 76*, 36–46.

Carter, C. (1994). *The Neuropharmacology of Polyamines*. London: Academic Press.

Casu, M. A., Sanna, A., Spada, G. P., Falzoi, M., Mongeau, R., & Pani, L. (2007). Effects of acute and chronic valproate treatments on p-CREB levels in the rat amygdala and nucleus accumbens. *Brain Research, 1141*, 15–24.

Chetkovich, D. M., Gray, R., Johnston, D., & Sweatt, J. D. (1991). N-methyl-D-aspartate receptor activation increases cAMP levels and voltage-gated Ca^{2+} channel activity in area CA1 of hippocampus. *Proceedings of the National Academy of Sciences of the United States of America, 88*, 6467–6471.

Chetkovich, D. M., & Sweatt, J. D. (1993). NMDA receptor activation increases cyclic AMP in area CA1 of the hippocampus via calcium/calmodulin stimulation of adenylyl cyclase. *Journal of Neurochemistry, 61*, 1933–1942.

Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., et al. (1990). Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. *Journal of Biological Chemistry, 265*, 5267–5272.

Dash, P. K., Karl, K. A., Colicos, M. A., Prywes, R., & Kandel, E. R. (1991). CAMP response element-binding protein is activated by Ca^{2+}/calmodulin- as well as cAMP-dependent protein kinase. *Proceedings of the National Academy of Sciences of the United States of America, 88*, 5061–5065.

Davies, S. P., Reddy, H., Caivano, M., & Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochemical Journal, 351*, 95–105.

Doskeland, S. O., Maronde, E., & Gjertsen, B. T. (1993). The genetic subtypes of cAMP-dependent protein kinase–functionally different or redundant? *Biochimica et Biophysica Acta, 1178*, 249–258.

Eliot, L. S., Dudai, Y., Kandel, E. R., & Abrams, T. W. (1989). Ca^{2+}/calmodulin sensitivity may be common to all forms of neural adenylate cyclase. *Proceedings of the National Academy of Sciences of the United States of America, 86*, 9564–9568.

Gibbs, C. S., Knighton, D. R., Sowadski, J. M., Taylor, S. S., & Zoller, M. J. (1992). Systematic mutational analysis of cAMP-dependent protein kinase identifies unregulated catalytic subunits and defines regions important for the recognition of the regulatory subunit. *Journal of Biological Chemistry, 267*, 4806–4814.

Giese, K. P., Fedorov, N. B., Filipkowski, R. K., & Silva, A. J. (1998). Autophosphorylation at Thr286 of the alpha calcium–calmodulin kinase II in LTP and learning. *Science, 279*, 870–873.

Gomes, G. M., Mello, C. F., da Rosa, M. M., Bochi, G. V., Ferreira, J., Barron, S., et al. (2010). Polyaminergic agents modulate contextual fear extinction in rats. *Neurobiological Learning and Memory, 93*, 589–595.

Gonzalez, G. A., & Montminy, M. R. (1989). Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell, 59*, 675–680.

Guerra, G. P., Mello, C. F., Sauzem, P. D., Berlese, D. B., Furian, A. F., Tabarelli, Z., et al. (2006). Nitric oxide is involved in the memory facilitation induced by spermidine in rats. *Psychopharmacology (Berl), 186*, 150–158.

Hegde, A. N., Goldberg, A. L., & Schwartz, J. H. (1993). Regulatory subunits of cAMP-dependent protein kinases are degraded after conjugation to ubiquitin: A molecular mechanism underlying long-term synaptic plasticity. *Proceedings of the National Academy of Sciences of the United States of America, 90*, 7436–7440.

Hidaka, H., Hagiwara, M., & Chijiwa, T. (1990). Molecular pharmacology of protein kinases. *Neurochemical Research, 15*, 431–434.

Huang, Y. Y., & Kandel, E. R. (1998). Postsynaptic induction and PKA-dependent expression of LTP in the lateral amygdala. *Neuron, 21*, 169–178.

Impey, S., Obrietan, K., Wong, S. T., Poser, S., Yano, S., Wayman, G., et al. (1998). Cross talk between ERK and PKA is required for Ca^{2+} stimulation of CREB-dependent transcription and ERK nuclear translocation. *Neuron, 21*, 869–883.

Isiegas, C., Park, A., Kandel, E. R., Abel, T., & Lattal, K. M. (2006). Transgenic inhibition of neuronal protein kinase A activity facilitates fear extinction. *The Journal of Neuroscience, 26*, 12700–12707.

Izquierdo, I., & Medina, J. H. (1997). Memory formation: The sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. *Neurobiological Learning and Memory, 68*, 285–316.

Kishi, A., Ohno, M., & Watanabe, S. (1998a). Concurrent activation of hippocampal glycine and polyamine sites of the N-methyl-D-aspartate receptor synergistically reverses working memory deficits in rats. *Neuroscience Letters, 257*, 131–134.

Kishi, A., Ohno, M., & Watanabe, S. (1998b). Spermidine, a polyamine site agonist, attenuates working memory deficits caused by blockade of hippocampal muscarinic receptors and mGluRs in rats. *Brain Research, 793*, 311–314.

Liang, Z., Liu, F., Grundke-Iqbal, I., Iqbal, K., & Gong, C. X. (2007). Down-regulation of cAMP-dependent protein kinase by over-activated calpain in Alzheimer disease brain. *The Journal of Neurochemistry, 103*, 2462–2470.

Liu, P., Gupta, N., Jing, Y., & Zhang, H. (2008). Age-related changes in polyamines in memory-associated brain structures in rats. *Neuroscience, 155*, 789–796.

Lopez-Salon, M., Alonso, M., Vianna, M. R., Viola, H., Mello e Souza, T., Izquierdo, I., et al. (2001). The ubiquitin-proteasome cascade is required for mammalian long-term memory formation. *European Journal of Neuroscience, 14*, 1820–1826.

Mayford, M., Abel, T., & Kandel, E. R. (1995a). Transgenic approaches to cognition. *Current Opinion in Neurobiology, 5*, 141–148.

Mayford, M., Wang, J., Kandel, E. R., & O'Dell, T. J. (1995b). CaMKII regulates the frequency-response function of hippocampal synapses for the production of both LTD and LTP. *Cell, 81*, 891–904.

McKnight, G. S., Clegg, C. H., Uhler, M. D., Chrivia, J. C., Cadd, G. G., Correll, L. A., et al. (1988). Analysis of the cAMP-dependent protein kinase system using molecular genetic approaches. *Recent Progress in Hormone Research, 44*, 307–335.

Medeiros, R., Prediger, R. D., Passos, G. F., Pandolfo, P., Duarte, F. S., Franco, J. L., et al. (2007). Connecting TNF-alpha signaling pathways to iNOS expression in a mouse model of Alzheimer's disease: Relevance for the behavioral and synaptic deficits induced by amyloid beta protein. *The Journal of Neuroscience, 27*, 5394–5404.

Paxinos, G., & Watson, C. (1986). *The Rat Brain in Stereotaxic Coordinates* (2nd ed.). Sydney; Orlando: Academic Press.

Pereira, P., Ardenghi, P., Mello e Souza, T., Medina, J. H., & Izquierdo, I. (2001). Training in the step-down inhibitory avoidance task time-dependently increases cAMP-dependent protein kinase activity in the entorhinal cortex. *Behavioural Pharmacology, 12*, 217–220.

Poser, S., & Storm, D. R. (2001). Role of Ca^{2+}-stimulated adenylyl cyclases in LTP and memory formation. *International Journal of Developmental Neuroscience, 19*, 387–394.

Puzzo, D., Staniszewski, A., Deng, S. X., Privitera, L., Leznik, E., Liu, S., et al. (2009). Phosphodiesterase 5 inhibition improves synaptic function, memory, and amyloid-beta load in an Alzheimer's disease mouse model. *The Journal of Neuroscience, 29*, 8075–8086.

Quevedo, J., Vianna, M. R., Martins, M. R., Barichello, T., Medina, J. H., Roesler, R., et al. (2004). Protein synthesis, PKA, and MAP kinase are differentially involved in short- and long-term memory in rats. *Behavioural Brain Research, 154*, 339–343.

Quevedo, J., Vianna, M. R., Roesler, R., Martins, M. R., de-Paris, F., Medina, J. H., et al. (2005). Pretraining but not preexposure to the task apparatus prevents the memory impairment induced by blockade of protein synthesis, PKA or MAP kinase in rats. *Neurochemical Research, 30*, 61–67.

Romero-Calvo, I., Ocon, B., Martinez-Moya, P., Suarez, M. D., Zarzuelo, A., Martinez-Augustin, O., et al. (2010). Reversible Ponceau staining as a loading control alternative to actin in Western blots. *Analytical Biochemistry, 401*, 318–320.

Rubin, M. A., Berlese, D. B., Stiegemeier, J. A., Volkweis, M. A., Oliveira, D. M., dos Santos, T. L., et al. (2004). Intra-amygdala administration of polyamines modulates fear conditioning in rats. *The Journal of Neuroscience, 24*, 2328–2334.

Rubin, M. A., Boemo, R. L., Jurach, A., Rojas, D. B., Zanolla, G. R., Obregon, A. D., et al. (2000). Intrahippocampal spermidine administration improves inhibitory avoidance performance in rats. *Behavioural Pharmacology, 11*, 57–61.

Rubin, M. A., Jurach, A., Zanolla, G. R., Boemo, R. L., Souza, D. O., & de Mello, C. F. (1997). Intrahippocampal GMP administration improves inhibitory avoidance performance through GABAergic and glutamatergic mechanisms in rats. *NeuroReport, 8*, 3713–3716.

Rubin, M. A., Stiegemeier, J. A., Volkweis, M. A., Oliveira, D. M., Fenili, A. C., Boemo, R. L., et al. (2001). Intra-amygdala spermidine administration improves inhibitory avoidance performance in rats. *European Journal of Pharmacology, 423*, 35–39.

Sharifzadeh, M., Haghighat, A., Tahsili-Fahadan, P., Khalaj, S., Zarrindast, M. R., & Zamanian, A. R. (2006). Intra-hippocampal inhibition of protein kinase AII attenuates morphine-induced conditioned place preference. *Pharmacology, Biochemistry and Behavior, 85*, 705–712.

Sharifzadeh, M., Sharifzadeh, K., Naghdi, N., Ghahremani, M. H., & Roghani, A. (2005). Posttraining intrahippocampal infusion of a protein kinase AII inhibitor impairs spatial memory retention in rats. *The Journal of Neuroscience Research, 79*, 392–400.

Shimada, A., Spangler, E. L., London, E. D., & Ingram, D. K. (1994). Spermidine potentiates dizocilpine-induced impairment of learning performance by rats in a 14-unit T-maze. *European Journal of Pharmacology, 263*, 293–300.

Silva, A. J., Stevens, C. F., Tonegawa, S., & Wang, Y. (1992). Deficient hippocampal long-term potentiation in alpha-calcium–calmodulin kinase II mutant mice. *Science, 257*, 201–206.

Tasken, K., & Aandahl, E. M. (2004). Localized effects of cAMP mediated by distinct routes of protein kinase A. *Physiological Reviews, 84*, 137–167.

Taylor, S. S., Buechler, J. A., & Yonemoto, W. (1990). CAMP-dependent protein kinase: Framework for a diverse family of regulatory enzymes. *Annual Review of Biochemistry, 59*, 971–1005.

Vianna, M. R., Izquierdo, L. A., Barros, D. M., Ardenghi, P., Pereira, P., Rodrigues, C., et al. (2000). Differential role of hippocampal cAMP-dependent protein kinase in short- and long-term memory. *Neurochemical Research, 25*, 621–626.

Vitolo, O. V., Sant'Angelo, A., Costanzo, V., Battaglia, F., Arancio, O., & Shelanski, M. (2002). Amyloid beta -peptide inhibition of the PKA/CREB pathway and long-term potentiation: Reversibility by drugs that enhance cAMP signaling. *Proceedings of the National Academy of Sciences of the United States of America, 99*, 13217–13221.

Wallenstein, G. V., Vago, D. R., & Walberer, A. M. (2002). Time-dependent involvement of PKA/PKC in contextual memory consolidation. *Behavioural Brain Research, 133*, 159–164.

Wang, H., Ferguson, G. D., Pineda, V. V., Cundiff, P. E., & Storm, D. R. (2004). Overexpression of type-1 adenylyl cyclase in mouse forebrain enhances recognition memory and LTP. *Natural Neuroscience, 7*, 635–642.

Wang, L. Y., Salter, M. W., & MacDonald, J. F. (1991). Regulation of kainate receptors by cAMP-dependent protein kinase and phosphatases. *Science, 253*, 1132–1135.

Wolfman, C., Fin, C., Dias, M., Bianchin, M., Da Silva, R. C., Schmitz, P. K., et al. (1994). Intrahippocampal or intraamygdala infusion of KN62, a specific inhibitor of calcium/calmodulin-dependent protein kinase II, causes retrograde amnesia in the rat. *Behavioral and Neural Biology, 61*, 203–205.

Wong, S. T., Athos, J., Figueroa, X. A., Pineda, V. V., Schaefer, M. L., Chavkin, C. C., et al. (1999). Calcium-stimulated adenylyl cyclase activity is critical for hippocampus-dependent long-term memory and late phase LTP. *Neuron, 23*, 787–798.

Wu, Z. L., Thomas, S. A., Villacres, E. C., Xia, Z., Simmons, M. L., Chavkin, C., et al. (1995). Altered behavior and long-term potentiation in type I adenylyl cyclase mutant mice. *Proceedings of the National Academy of Sciences of the United States of America, 92*, 220–224.

Zhang, H. T., Crissman, A. M., Dorairaj, N. R., Chandler, L. J., & O'Donnell, J. M. (2000). Inhibition of cyclic AMP phosphodiesterase (PDE4) reverses memory deficits associated with NMDA receptor antagonism. *Neuropsychopharmacology, 23*, 198–204.

Zhang, H. T., Zhao, Y., Huang, Y., Dorairaj, N. R., Chandler, L. J., & O'Donnell, J. M. (2004). Inhibition of the phosphodiesterase 4 (PDE4) enzyme reverses memory deficits produced by infusion of the MEK inhibitor U0126 into the CA1 subregion of the rat hippocampus. *Neuropsychopharmacology, 29*, 1432–1439.