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ORIGINAL ARTICLE

Involvement of hippocampal CAMKII/CREB signaling in the spatial memory retention induced by creatine

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Abstract Although Creatine (Cr) and Phosphocreatine (PCr) systems play a key role in cellular energy and energy transport in neuronal cells, its implications for learning and memory are still controversial. Thus, we decided to investigate the involvement of cAMP-dependent protein kinase A (PKA), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and cAMP responsive element binding protein (CREB) in the spatial consolidation after an intra-hippocampal injection of Cr. Statistical analysis revealed that Cr (2.5 nmol/hippocampus) (post-training) decreased the latency for escape and the mean number of errors on Barnes maze test. Post-training co-administration of the PKA inhibitor (H-89 25 ρmol/hippocampus) did not alter the facilitatory effect of Cr in this memory test. On the other hand, Cr-induced spatial retention was reverted by co-administration of the CaMKII inhibitor (STO-609 5 nmol/hippocampus). Neurochemical analysis revealed that intrahippocampal injection of Cr, when analyzed after 30 min rather than after 3 h, increased the levels of pCREB and pCaMKII but not pPKA levels. Statistical analysis also revealed that the post-training co-administration of STO-609 but not H-89 reversed the increase of pCREB levels induced by Cr. The results presented in this report suggest that intracellular CaMKII/CREB pathway plays a key role in the Cr-induced spatial retention. Thus, it is plausible to propose that Cr plays a putative role as a neuromodulator in the brain, and that at least some of its effects may be mediated by intracellular CaMKII/CREB pathway.

Keywords Creatine · Spatial memory · Hippocampus · Protein kinases · CREB

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Introduction

Creatine (*N*-[aminoiminomethyl]-*N*-methyl glycine) is a guanidine compound endogenously synthesized from glycine, arginine and S-adenosylmethionine in kidneys, liver, pancreas and the brain (Wyss and Kaddurah-Daouk 2000). Initially, was popularized as a performance-enhancing supplement used to increase high-intensity athletic performance by increasing the intracellular stores of Cr and its phosphorylated form, PCr (Persky and Brazeau 2001). Recently, supplementation of this nutrient has been shown to improve the cognitive function by increasing brain Cr

(9 %) and PCr (4 %) levels (Ajilore et al. 2007; Dechent et al. 1999). Furthermore, Cr enhances intelligence test scores, reduces mental fatigue and protects against cerebral oxygenated hemoglobin decrease when subjects repeatedly perform a mathematical calculation (Valenzuela et al. 2003; Rae et al. 2003; Watanabe et al. 2002). In recent tests, Cr supplementation prior to 18–36 h of sleep deprivation led to significant improvements in the performance of complex central executive tasks (McMorris et al. 2006, 2007) suggesting that Cr enhances brain function under normal and stress conditions.

Nevertheless, the view that Cr exerts its functions exclusively via effects in intracellular energy metabolism (Wyss and Kaddurah-Daouk 2000) cannot explain a number of recently reported findings. As a result, a direct modulatory role for Cr in the central transmission processes has been proposed (Almeida et al. 2006; Andres et al. 2008). Accordingly, we have shown that intrahippocampal Cr administration leads to spatial retention improvement by a mechanism that depends partially on its interaction with the extracellular polyamine binding site at the *N*-methyl-D-aspartic acid (NMDA) receptor (Oliveira et al. 2008). Nonetheless, the complete mechanism by which Cr improves memory, as well as the neuronal conditions in which this guanidino compound could be effective in spatial consolidation, needs to be further investigated.

Some studies have shown that spatial memory formation is associated with increased CREB phosphorylation/activation within the hippocampus (Colombo et al. 2003; Mizuno et al. 2002; Porte et al. 2008). Indeed, CREB activation, through its phosphorylation of Serine^{133} (pCREB) by protein kinases, controls the induction of the regulation of immediate-early genes that, in turn, induce the transcription of late downstream genes, and activate effector proteins, such as structural proteins, signaling enzymes or growth factors, that are essential for learning and memory (Alberini 2009).

Consistent with the view that PKA/CREB signaling pathway activation in hippocampus plays an important role in long-term memory (LTM) consolidation, (Abel et al. 1997; Bernabeu et al. 1997) pharmacological inhibition of PKA blocks LTM (Vianna et al. 1999, 2000). Hence, PKA activation is thought to be associated with changes in gene expression required for consolidation of information in the hippocampus (Vazquez et al. 2000).

Likewise, several lines of evidence suggest that CaMKII is one of the best candidates for being a molecular component of learning and memory machinery in the mammalian brain (Rongo 2002). Studies with knockout mice for NMDA receptors (Sakimura et al. 1995; McHugh et al. 1996) and CaMKII (Silva et al. 1992) support the hypothesis that CaMKII signaling is required to initiate the formation of new spatial memories in hippocampus. Furthermore, the increase in autophosphorylated (active) form of CaMKII reinforces the assumption that activation of this pathway is closely related to spatial memory (Tan and Liang 1996; Rodrigues et al. 2004; Miyamoto 2006).

Therefore, considering that PKA, CaMKII and CREB signaling activation in the hippocampus is important for spatial memory formation (Mizuno et al. 2002) and that Cr has been considered as a prototype drug to sustain brain function (Wyss and Kaddurah-Daouk 2000), we decided to investigate whether PKA, CaMKII and CREB activation are involved in the effect exerted by this guanidino compound in spatial memory model of Barnes maze test.

Materials and methods

Animals

Adult male Wistar rats (270–300 g, *n*= 289) were used in the present study. Rats were housed five to a cage, light and temperature were controlled (12 h light/dark cycle, 24 ± 1 °C, 55 % relative humidity), and rats had free access to food (Guabi, Santa Maria, Brazil) and water. All experimental protocols were designed to keep the number of animals used to a minimum, as well as their suffering. All experimental protocols were conducted in accordance with national and international legislation (guidelines of Brazilian College of Animal Experimentation (COBEA) and of US Public Health Service's Policy on Human Care and Use of Laboratory Animals-PHS Policy), and approved by the Ethics Committee for animal research at the Federal University of Santa Maria. Behavioral tests were conducted during the light phase of the cycle (between 9:00 a.m. and 1:00 p.m.).

Surgery

The rats were anesthetized with Equithesin (1 % phenobarbital, 2 % magnesium sulfate, 4 % chloral hydrate, 42 % propylene glycol, 11 % ethanol; 3 ml/kg, i.p.) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, rats were implanted with two 27-guage guide cannulas, which were placed 1 mm above the CA1 region of the dorsal hippocampus (coordinates relative to bregma: AP 4 mm, ML 3 mm, V2 mm from the dura, Fig. 1; Paxinos et al. 1985). Chloramphenicol (200 mg/kg, i.p.) was administered immediately before the surgical procedure. Injection placements were histologically verified, as described elsewhere (Rubin et al. 1997). Only data from the animals with correct cannula placement were analyzed.

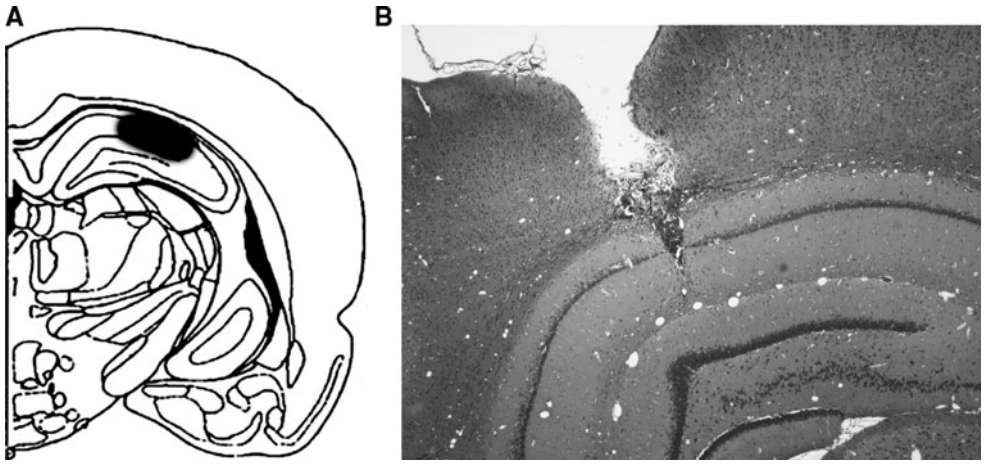

Fig. 1 **a** Drawing adapted from Paxinos and Watson 1985 showing the area (*black*) where the infusions were considered correctly placed. **b** Micrograph (×40) showing the lesion caused by the cannula and injection needle in the dorsal hippocampus. Note that infusions were bilateral

Experimental design

To evaluate the spatial memory and locomotor activity the same set of animals were submitted to Barnes maze and open field arena.

Another set of animals were used to evaluate the effects of Cr on another memory task and determine its effects on anxiety.

Western Blot analyses of PKA, CaMKII and CREB was carried out in a set of animals that was trained in the Barnes maze paradigm and killed after 30 or 180 min.

Barnes maze and exploratory behavior assay

One week after surgery, animals were trained to solve the Barnes maze. The Barnes maze is a validated test often used for the assessment of spatial learning and memory in rodents (Barnes 1979). The Barnes maze paradigm exploits the natural inclination of small rodents to seek escape to a darkly lit, sheltered environment when placed in an open arena under bright, aversive illumination. Our maze consists of a 120 cm diameter circular wooden table, 3.5 cm-thick and elevated 90 cm above the floor.

Twenty holes, 6 cm diameter, were equidistantly located around the perimeter and centered 5 cm from it. The apparatus was located in a 4 m × 4 m test room where four visuospatial cues made of rigid black paper (rectangle, circle, cross, triangle) were affixed to the walls but not directly over any one maze hole; this increases the spatial component of the Barnes maze during training (Bach et al. 1995). A black wooden escape tunnel (15 cm × 10 cm × 30 cm) was placed beneath one hole, selected randomly for each rat but remained constant throughout the training sessions for a given rat. The remaining 19 holes led only to a false escape box (15 cm × 10 cm × 10 cm) which, from the platform, appeared indistinguishable from an escape box but was too small to be entered. False boxes removed visual cues that might be observed through an open hole. There was a bright illumination of 300 lux over the maze.

On the first day of the experiment, the rats were moved to a testing room and left undisturbed for 60 min. Following this habituation period, the rats were trained to find the escape hole; they were placed in the escape box for 1 min, then into a cylindrical opaque chamber (start box) in the center of the maze. With light on, the start box was removed and the rats were allowed to explore freely and find the escape box. A maximum of 180 s to find it was allowed. Each rat was given three trials per day, over four consecutive days. In each trial, we recorded the time to reach the escape tunnel and the number of wrong holes visited. A visited hole was considered when the animal poked at a hole. The arena, as well as the boxes was wiped clean using distillated water both between each training session for a given rat and between each rat.

Immediately after the second training session on Barnes maze, the animals were transferred to an open-field measuring 50 × 60 cm, with the floor divided into 12 squares measuring 12 × 12 cm each. The open-field test was carried out to identify motor disabilities and lasted for 5 min and during this time, an observer, who was not aware of pharmacological treatments, recorded the number of crossing and rearing responses manually.

Object exploration task (OET) and elevated plus maze

To evaluate the effects of Cr on another memory task and determine whether it improved the performance by decreasing anxiety we performed OET as a paradigm to investigate spatial change (object configuration) and non-spatial change (object recognition) and Elevated Plus Maze to evaluate to analyze the anxiety.

One week after surgery, animals were trained to solve the OET paradigm. The OET field consisted of a square

box with an open top, painted black, 75 cm wide × 50 cm tall. The arena was dimly illuminated and surrounded by a black curtain so that the environment was visually uniform. The test, adapted from (Cippitelli et al. 2010) consisted of 10 min habituation in the empty open field (session 1). In the session 2 (24 h after session 1) the animal was exposed to a diagonal configuration of two identical objects in the field for 10 min. The objects were cleaned and counterbalanced between animals.

In session 3 (24 h after session 2), object 2 was displaced and moved to a linear configuration in relation to object 1 to test recognition of spatial change. The objects were cleaned and counterbalanced between animals. In session 4 (10 min after session 3), object 1 was replaced by a novel object (object 3), to test recognition of the new, non-displaced object. The objects were cleaned and only object 2 was counterbalanced with object 1 between animals. All objects were located at the same distance from the wall of the apparatus. The apparatus and the objects were cleaned with water and dried after every session to avoid that animal's guide by odor.

The percentage of the total exploration time that the animal spent investigating the novel object (percentage new of total) was the measure of recognition index of memory (defined by RI). It was calculated using the following formula: [(novel object investigation time − known object investigation time)/(total investigation time of both objects)] × 100. This measure takes into account individual differences in the total amount of exploration time (Todd Roach et al. 2004).

After OET animals were tested on the elevated plus-maze apparatus that consisted of a wooden structure elevated 50 cm from the floor and comprising two opposite open arms, 50 × 10 cm, crossed at right angles by two arms of the same dimensions enclosed by 40 cm high walls, with an open roof. Initially, subjects were placed on the center platform of the maze facing an enclosed arm (File and Gonzalez 1996). The behaviors recorded were the: total number of entries, the percentage of time spent on either arm, and percentage of time spent on the middle. The apparatus was cleaned thoroughly between the 5 min observation sessions with a 30 % ethanol solution.

Drugs and microinjections

Cr monohydrate, *N*-[2-bromocinnamylamino) ethyl]-5-isoquinoline sulfonamide (H-89), which is a selective inhibitor of PKA, and 7H-Benzimidazo[2,1-a]benz[de]isoquinoline-7-one-3-carboxylic acid (STO-609), a selective inhibitor of CaMKII, were obtained from Sigma Saint Louis, MO. Cr monohydrate and H-89 were dissolved in sterile phosphate-buffered saline (PBS) pH 7.4. STO-609 was dissolved in 15 % dimethyl sulfoxide (DMSO).

In order to investigate the effect of Cr on spatial learning, the animals were bilaterally injected with Cr monohydrate (2.5 nmol/hippocampus) or vehicle (PBS), immediately after the end of the first day of the Barnes maze (Oliveira et al. 2008). To investigate whether Cr improves different spatial and non-spatial memory the animals were bilaterally injected with Cr (2.5 nmol/hippocampus) or PBS, immediately after session 2 in OET.

The involvement of PKA and CaMKII pathways in the effect exerted by Cr in Barnes Maze model of spatial learning was tested by injecting the animals with an inhibitor of PKA (H-89, 0.25; 2.5 and 25 ρmol/hippocampus) and an inhibitor of CaMKII (STO 609; 0.5 and 5 nmol/hippocampus) immediately following the first day of training. The doses of the PKA and CaMKII inhibitors used in the present study were based in a dose–response curve for H-89 and STO-609 to define the dose for the subsequent experiments. Initial dose range for H-89 was selected based on previous studies (Sharifzadeh et al. 2005) and for STO-609 on Ki values (Tan and Liang 1996; Tokumitsu et al. 2002). The animals were not previously adapted to the microinjection procedure. The injections were performed using a 10 µl Hamilton syringe and a 30-gauge needle that fit into the guide cannula, with the tip of the infusion needle protruding 1.0 mm beyond that of the guide cannula and, therefore, aimed at the CA1 in the dorsal hippocampus. The infusions (0.5 µl/side) were carried out over 60 s and the infusion cannulas were left in place for 60 additional seconds to minimize backflow.

Preparation of tissues and Western blot analyses

Immediately after training in the Barnes maze, the animals received a single bilateral injection of pharmacological treatments. The experimental protocol used was the same as described in the experiments above, except that, the animals were killed either 30 or 180 min after the injections (Izquierdo et al. 2006). 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 MgCl2, 1 mM EDTA, 1 mM NaF, 10 lg/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 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 sulfatepolyacrylamide 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 CaMKII was carried out in cytosolic fractions and CREB was carried out in the nuclear fractions. 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

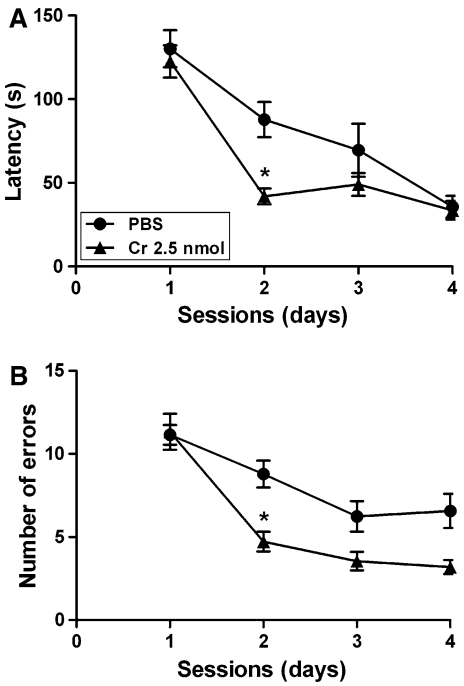

Fig. 2 Effect of the intrahippocampal administration of Cr (2.5 nmol/side) immediately after the end of the first session on escape latency (**a**) and number of errors (**b**) in the Barnes maze. PBS represents vehicle treatment. $*P < 0.05$ compared with PBS treated group by the Student–Newman–Keuls test. Data are the mean ± SEM. $n = 11$–12 animals in each group

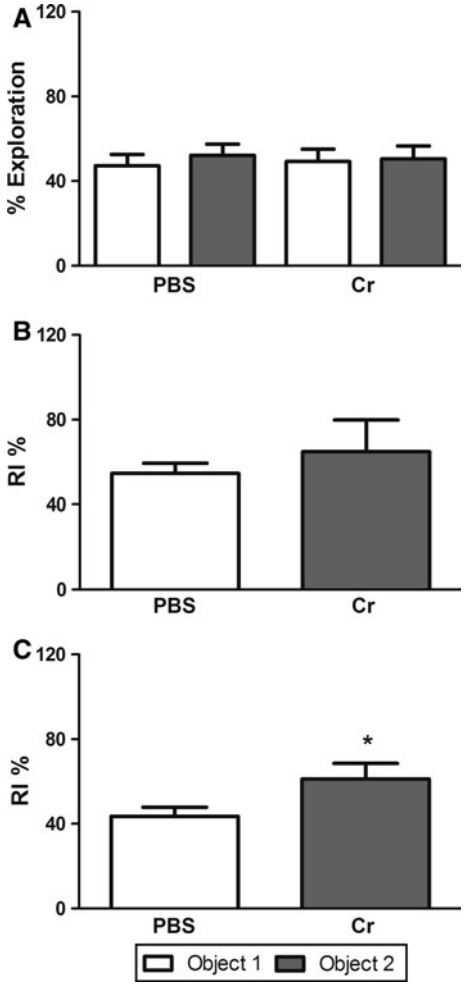

Fig. 3 Effect of the intrahippocampal administration of Cr (2.5 nmol/side) immediately post-training (session 2) on Object exploration task. **a** % of exploration on training session, **b** Recognition Index on non-spatial and **c** spatial version of Object exploration task. PBS represents vehicle treatment. $*P < 0.05$ compared with PBS treated group by the Student–Newman–Keuls test. Data are the mean ± SEM. $n = 7$–9 animals in each group

Table 1 The effect of Creatine on anxiolitic like behavior

Group	PBS	Creatine	*N*
%T.O	14 ± 1.6	13 ± 3.4	7–9
%No.E.O	39.7 ± 4.6	34 ± 6.2	7–9
%T.E	79.8 ± 1.8	77.4 ± 4.9	7–9
%No.E.E	60 ± 4.6	65 ± 6.2	7–9
%T.M	6.07 ± 1.07	9 ± 2.1	7–9

%T.O percent of time spend on open arms; %No.E.O percent of number of entries on open arms; %T.E percent of time spend on enclosed arms; %No.E.E percent of number of entries on enclosed arms; %T.M percent of time spend on the middle

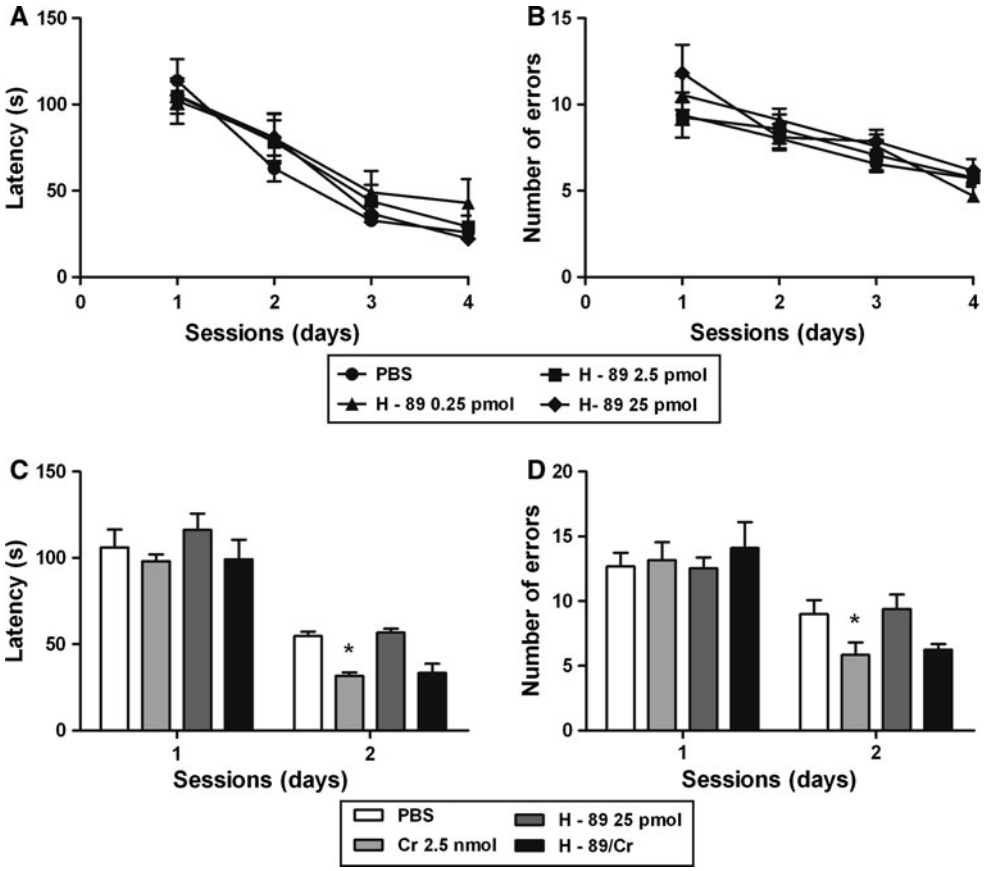

Fig. 4 Effect of the intrahippocampal administration of H-89 (0.25–25 ρmol/side) immediately after the end of first session on escape latency (**a**) and number of errors (**b**) in the Barnes maze. Figure 4c, d shows the effect of the co-administration of Cr (2.5 nmol/side) and H-89 (25 ρmol/side) immediately after the end of first session on escape latency and number of errors, respectively. PBS represents vehicle treatment. $^*P < 0.05$ compared with vehicle treated group by the two-way ANOVA Student–Newman–Keuls test. Data are the mean ± SEM. $n = 10$–14 animals in each group

RIIα, anti-total-PKA RIIα, anti-phospho-CREB-1 (Ser 133), anti-total-CREB-1, anti-phospho-CaMKIIα (Thr 286) and anti-total CaMKIIα 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 (1:3,000, 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) densitometry values and expressed as the relative amount of phosphorylated and non-phosphorylated forms, and the phosphorylated/total ratio.

Statistical analysis

Statistical analysis was carried out by t test; one-, two-, or three-way analysis of variance (ANOVA) and only F values of $P < 0.05$ are presented. Post hoc analysis was carried out, when appropriate, by the Student–Newman–Keuls test. All data were expressed as mean ± SEM. Statistical analyses were performed utilizing the SPSS software in a PC-compatible computer.

Results

Effect of Cr on spatial memory

All animals decreased the latency for escape [$F(3.63) = 49.88$; $P < 0.05$; Fig. 2a] and the mean number of errors [$F(3.63) = 30.96$; $P < 0.05$; Fig. 2b], indicating that they have learned the task. In addition, statistical analysis (two-way ANOVA) showed that intrahippocampal injection of Cr (2.5 nmol/side) decreased the latency for escape [$F(3.63) = 3.13$; $P < 0.05$; Fig. 2a] and the mean number of errors [$F(3.63) = 3$; $P < 0.05$; Fig. 2b] when compared with control group. Considering that Cr only presented effects in the second day of spatial memory testing, the following experiments conducted were limited to 2 days.

Effect of Cr on spatial and non-spatial retention memory

Figure 3 shows the effect of Cr on object exploration task (OET). Statistical analysis showed no difference between groups concerning object 1 and 2 exploration [$F(1.14) = 0.17$; $P > 0.05$; Fig. 3a] as well as the recognition index for object exploration [$F(1.14) = 0.05$; $P > 0.05$; Fig. 3b]. On the other hand, the intrahippocampal administration of

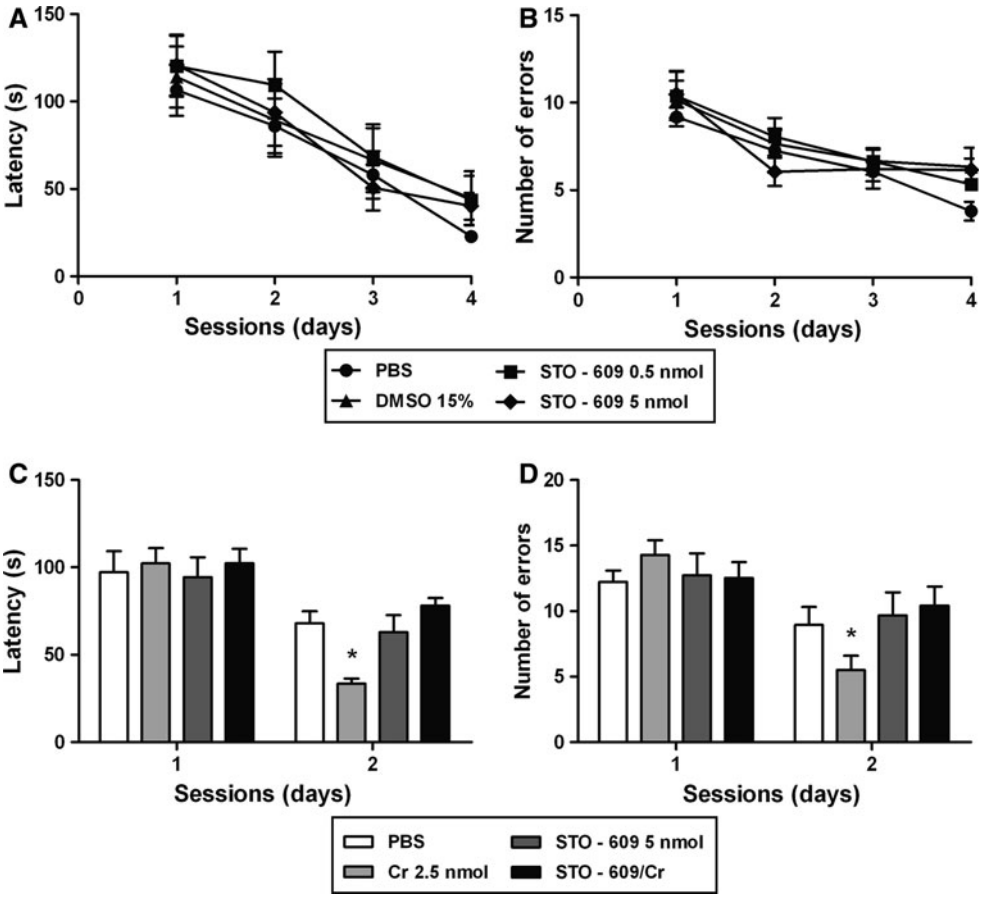

Fig. 5 Effect of the intrahippocampal administration of STO-609 (0.5 and 5 nmol/side) immediately after the end of first session on escape latency (**a**) and number of errors (**b**) in the Barnes maze. Figure 5c, d shows the effect of the co-administration of Cr (2.5 nmol/side) and STO-609 (5 nmol/side) immediately after the end of first session on escape latency and number of errors, respectively. PBS represents vehicle treatment. $^{*}P < 0.05$ compared with vehicle treated group by two- or three-way ANOVA Student–Newman–Keuls test. Data are the mean ± SEM. $n = 9$–13 animals in each group

Cr (2.5 nmol/hippocampus) (post-training; session 2) improved the spatial version of OET [$F(1.14) = 5.83$; $P < 0.05$; Fig. 3c].

Effect of Cr on anxiety

The intrahippocampal administration of Cr did not alter percent of time [$F(1.14) = 0.04$; $P > 0.05$] and percent of entries in open arm [$F(1.14) = 0.517$; $P > 0.05$] in the elevated plus maze. In addition, statistical analysis revealed that intrahippocampal Cr administration did not alter percent of time [$F(1.14) = 0.248$; $P > 0.05$], number of entries into the enclosed arm [$F(1.14) = 0.485$; $P > 0.05$] and time spent on middle [$F(1.14) = 1.740$; $P > 0.05$] indicating that this treatment had no effect on anxiety-like behavior (Table 1).

Participation of PKA and CaMKII on memory improvement induced by Cr on Barnes maze

In order to study the role of PKA and CaMKII activation in the Cr-induced spatial memory improvement, the PKA (H-89) and CaMKII (STO-609) inhibitors were co-administrated with Cr in the hippocampus. Statistical analysis revealed that in Barnes maze test, all the animal decreased latency for escape [$F(3.13) = 74.7$; $P < 0.05$; Fig. 4a] and mean number of errors [$F(3.13) = 24.20$; $P < 0.05$; Fig. 4b] along the days. The intrahippocampal injection of H-89 (0.25; 2.5 and 25 ρmol/0.5 µl) did not alter the latency [$F(9.135) = 0.82$; $P > 0.05$; Fig. 4a] and number of errors [$F(9.135) = 0.79$; $P > 0.05$; Fig. 4b] when compared with control group. In addition, co-administration of H-89 (25 ρmol/0.25 µl) did not revert spatial retention induced by intrahippocampal injection of Cr (2.5 nmol/0.25 µl) characterized by decrease of latency for escape [$F(1.38) = 0.19$; $P > 0.05$; Fig. 4c] and the mean number of errors [$F(1.38) = 0.19$; $P > 0.05$; Fig. 4d].

The effect of post-training intrahippocampal administration of STO-609 on Cr-induced spatial consolidation is shown in Fig. 5. Statistical analysis showed that the animals decreased latency for escape [$F(9.138) = 32.66$; $P < 0.01$; Fig. 5a] and mean number of errors [$F(9.138) = 19.85$; $P < 0.01$; Fig. 5b] along the days. In addition, statistical analysis (two-way ANOVA) revealed that intrahippocampal injection of STO-609 (0.5 and 5 nmol/0.5 µl) had no effect per se on latency for escape [$F(9.138) = 0.28$; $P > 0.05$; Fig. 5a] and mean number errors [$F(9.138) = 0.64$; $P > 0.05$; Fig. 5b] when compared with control group. However, co-administration of STO-609 (5 nmol/0.25 µl) reverted the effect of Cr

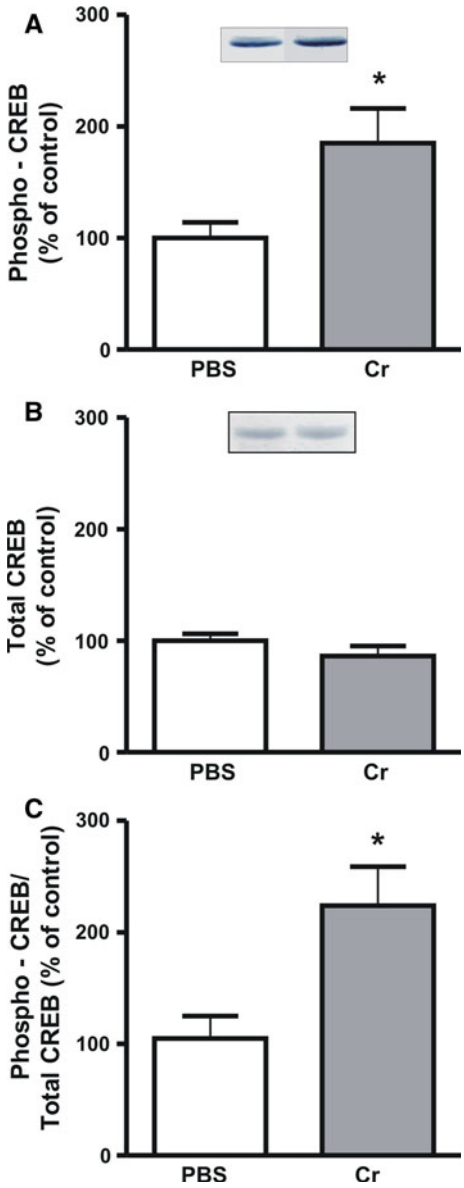

Fig. 6 Hippocampal levels of pCREB (**a**), total CREB (**b**) and pCREB/CREB ratio (**c**), 30 min after the intrahippocampal administration of creatine (2.5 nmol/side) $^{*}P < 0.05$ compared with PBS treated group by two-way ANOVA Student–Newman–Keuls test. Data are the mean ± SEM. $n = 5$–7 animals in each group

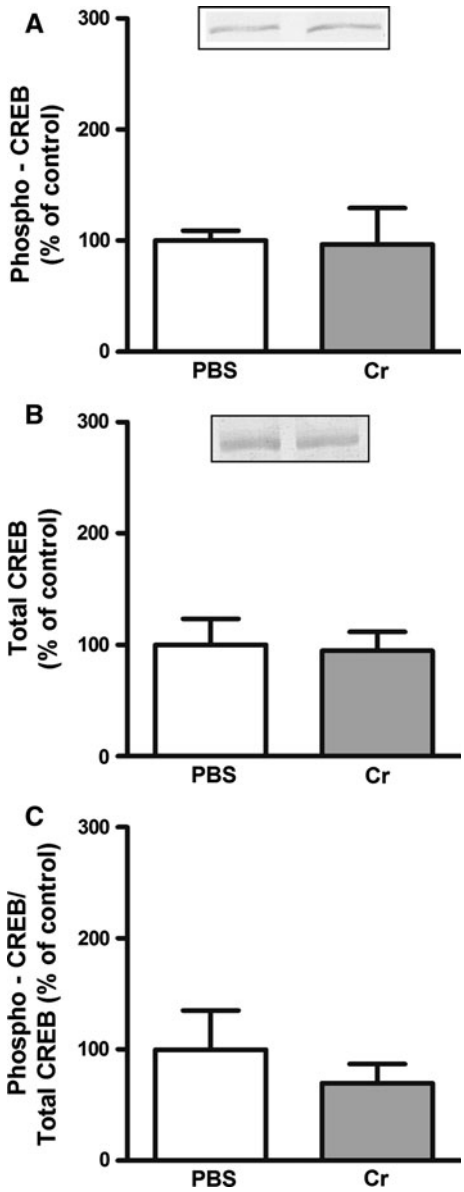

Fig. 7 Hippocampal levels of pCREB (**a**), total CREB (**b**) and pCREB/CREB ratio (**c**), 180 min after the intrahippocampal administration of creatine (2.5 nmol/side). Data are the mean ± SEM. $n = 4$ animals in each group

(2.5 nmol/0.25 μl) on latency for escape [$F(1.36) = 5.40$; $P < 0.05$; Fig. 5c] and the mean number of errors [$F(1.36) = 4.11$; $P < 0.05$; Fig. 5d].

Effect of Cr on PKA, CaMKII e CREB phosphorilation

Since CREB pathway has an essential role in memory formation (Mizuno et al. 2002), we decided to analyze the levels of pCREB in the hippocampus, 30 and 180 min after Cr administration on the first day of experiments (Izquierdo et al. 2006). Statistical analyses showed significant differences in pCREB/CREB levels when analyzed 30 min [$F(1.10) = 6.89$; $P < 0.05$; Fig. 6c], but not 180 min [$F(1.7) = 0.51$; $P > 0.05$; Fig. 7c]. In subsequent experiments, we analyzed the involvement of PKA and CaMKII pathways in increased levels of Cr-induced pCREB. Statistical analysis revealed that Cr had no effect on PKA phosphorylation [$F(1.6) = 0.07$; $P > 0.05$; Fig. 8c]. On the other hand, the intrahippocampal injection of Cr induced a significant increase of CaMKII phosphorylation [$F(1.14) = 5.42$; $P < 0.05$; Fig. 9b] and in pCaMKII/CaMKII [$F(1.14) = 6.07$; $P < 0.05$; Fig. 9c] when compared

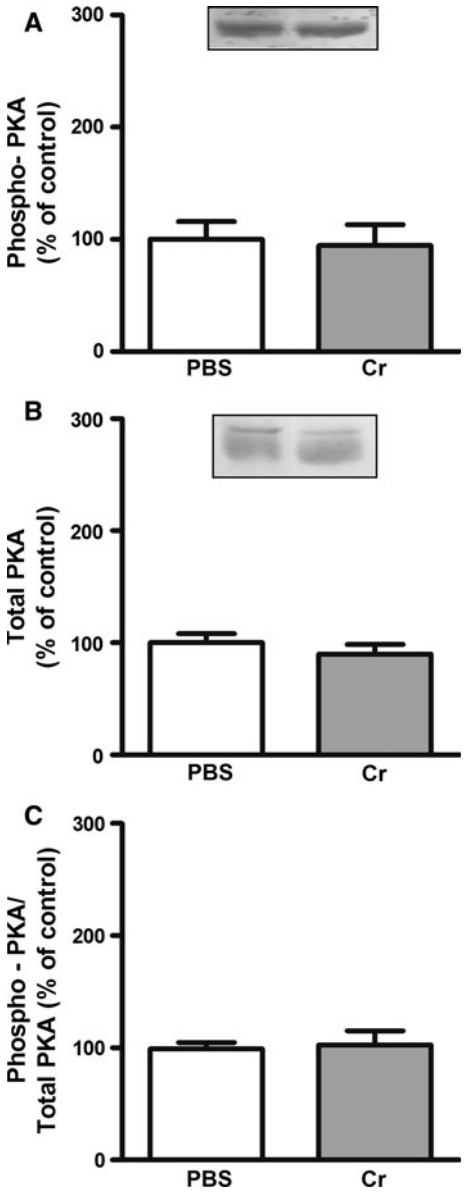

Fig. 8 Hippocampal levels of pPKA (**a**), total PKA (**b**), pPKA/PKA ratio (**c**), 30 min after the intrahippocampal administration of creatine (2.5 nmol/side) PBS represent vehicle treatment. Data are the mean ± SEM. $n = 4$ animals in each group

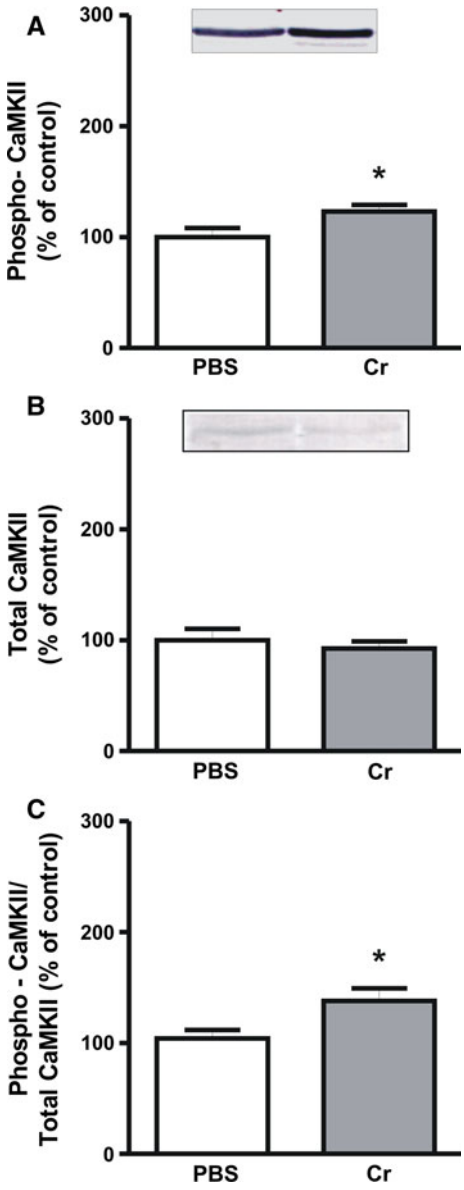

Fig. 9 Hippocampal levels of pCaMKII (**a**), total CaMKII (**b**), pCaMKII/CaMKII ratio (**c**), 30 min after the intrahippocampal administration of creatine (2.5 nmol/side). *$P < 0.05$ compared with PBS treated group by one-way ANOVA Student–Newman–Keuls test. Data are the mean ± SEM. $n = 6$–8 animals in each group

with the vehicle group. In addition, statistical analyses showed that intrahippocampal co-administration of H-89 (25 ρmol/0.25 µl; Fig. 10) did not change the effect of Cr [$F(1.12) = 0.09$; $P > 0.05$; Fig. 10c] but STO-609 (5 nmol/0.25 µl) reverted the Cr-induced pCREB levels increase [$F(1.18) = 9.15$; $P < 0.05$; Fig. 11c].

Effect of Cr and PKA and CaMKII inhibitors on locomotor activity

Table 2 shows the effect of Cr, STO-609, H-89 as well as their association on the exploratory behavior in an open field test after the second training session on Barnes maze. Statistical analysis revealed that neither the compounds alone, nor their association altered the number of crossing or rearing responses in a subsequent open-field testing session, indicating that none of the compounds tested caused gross motor disabilities during testing.

Discussion

In the present study, we revealed that Cr caused spatial retention but not alter object recognition. Cr also increased

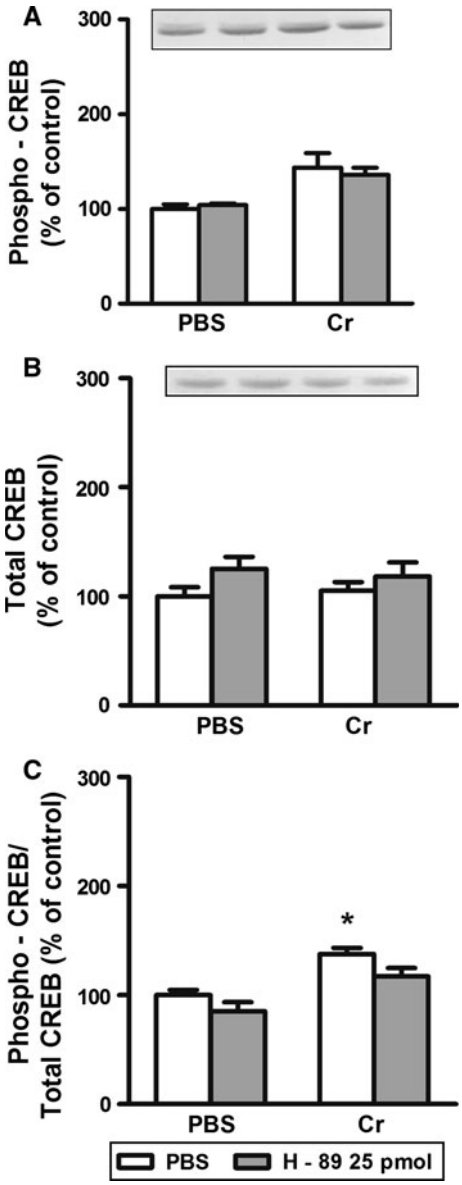

Fig. 10 Hippocampal levels of pCREB (**a**), total CREB (**b**), pCREB/CREB ratio (**c**), 30 min after the intrahippocampal co-administration of creatine (2.5 nmol/side) and H-89 (25 ρmol/side). $^*P < 0.05$ compared with PBS treated group by two-way ANOVA Student–Newman–Keuls test. Data are the mean ± SEM. $n = 6$ animals in each group

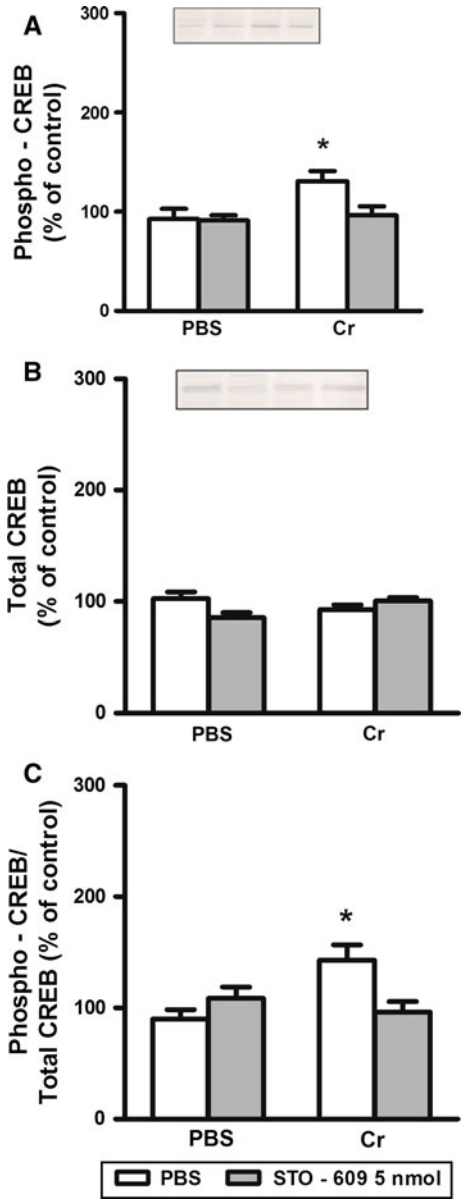

Fig. 11 Hippocampal levels of pCREB (**a**), total CREB (**b**), pCREB/CREB ratio (**c**), 30 min after the intrahippocampal co-administration of creatine (2.5 nmol/side) and STO-609 (5 nmol/side). $^*P < 0.05$ compared with PBS treated group by two-way ANOVA Student–Newman–Keuls test. Data are the mean ± SEM. $n = 5$–6 animals in each group

pCREB levels and co-administration of STO-609 (inhibitor of CaMKII) but not H-89 (inhibitor of PKA) decreased the facilitatory effect of Cr on Barnes maze. The results presented in this report also showed, for the first time, that Cr had no effect on PKA phosphorylation but induced a significant increase of CaMKII phosphorylation suggesting that intracellular CaMKII/CREB pathway plays a key role in the Cr-induced spatial retention. In fact, intrahippocampal STO-609 co-administration but not H-89 attenuated the Cr-induced pCREB levels increase. In the present study we also demonstrate that Cr did not alter anxiety measures or motor ability of the animals indicating that Cr affects spatial memory in Barnes maze and OET task and does not alter the motivational aspects of learning.

Over the last century, new ideas and concepts of Cr function have been described, particularly in the area of brain and muscle biochemistry (Andres et al. 2008). In this context, the facilitatory effect of Cr on Barnes maze test, at least in part, in agreement with studies demonstrating that Cr supplementation improves intelligence test scores (Rae

Table 2 Effects of treatments on locomotor and exploratory activity

Group	Crossing	Rearing	*N*
PBS	44.4 ± 3.2	16.4 ± 1.8	11
DMSO	38.5 ± 4.4	10.08 ± 1.8	12
Cr (2.5 nmol/side)	39.5 ± 2.7	14.27 ± 1.5	12
H-89 (25 nmol/side)	46 ± 4.1	10.36 ± 1.2	11
H-89 (25 nmol/side) + Cr (2.5 nmol/side)	40.35 ± 2.8	12.58 ± 2.0	12
STO-609 (5 nmol/side)	37.15 ± 4	10.61 ± 4.7	13
STO (5 nmol/side) + Cr (2.5 nmol/side)	38.6 ± 3.1	9.8 ± 1.2	10

et al. 2003), attenuates the cognitive impairment elicited by sleep deprivation (McMorris et al. 2007) and neurological status in Cr deficient patients (Braissant et al. 2011).

It is widely believed that memory is mediated by a medial temporal lobe memory system consisting of several distinct structures, including hippocampus and perirhinal cortex (Mishkin 1978; Zola-Morgan et al. 1991). However, it is well know that lesions restrict to the perirhinal cortex is a crucial factor in the severe object recognition memory impairment (Murray and Mishkin 1998). In line of this view, Winters et al. (2004) revealed that rats with hippocampal lesions were impaired relative to controls and those with peripostrhinal cortex lesions on the spatial memory task, whereas rats with peripostrhinal lesions were impaired relative to the hippocampal and control groups in object recognition. Accordingly, it has been demonstrated that Perirhinal lesions yielded no deficit in the spatial location task suggesting that this cortical area does not participate in spatial memory unless the stimuli have overlapping features (Bachevalier and Nemanic 2008). Considering that hippocampus is important but not essential for object recognition task (Winters et al. 2008), the lack of effect on object recognition task elicited by Cr could be explained by the region of infusion. However, this explanation remains speculative in nature, and further studies are necessary to determine the role of Cr in object place preference task.

In the present study, the increase of pCREB levels when analyzed 30 min after Cr administration agree with assumption that hippocampal-learning specificity may be better reflected by duration, than amplitude of CREB phosphorylation (Porte et al. 2008). Considering that Cr had an effect just in the second day of the Barnes maze, it is plausible to propose that an early CREB activation is critical for hippocampus-dependent memory (Colombo et al. 2003; Trifilieff et al. 2006).

Our neurochemical analysis also revealed that, the intrahippocampal injection of Cr induced significant increase of pCaMKII. The genetic analysis of αCaMKII isoform in mice supports the idea that CaMKII signaling is required to initiate the formation of new spatial memories in hippocampus (Rongo 2002). Moreover, CaMKII phosphorylates a number of postsynaptic proteins including AMPA and NMDA receptors, substrates responsible for the physiological changes in the postsynaptic cell needed for LTM (Mizuno and Giese 2005). Considering that Cr leads to spatial retention by modulation of NMDA receptor (Oliveira et al. 2008) and that activation of this receptor leads to subsequent activation of CaMKII (Miyamoto 2006), the results presented in this report suggest that Cr-induced spatial retention involves a downstream cascade of NMDA such as CaMKII and CREB phosphorylation.

The results presented in this study showed that intrahippocampal Cr injection did not alter pPKA levels and that H-89 co-administration had no effect on spatial consolidation. Although it is well established that PKA regulates biological processes through its phosphorylation of proteins (Izquierdo et al. 2006; Mizuno et al. 2002; Vazquez et al. 2000), the high number of connections within and among various brain structures in LTP and the memory formation is a reason why it is difficult to schematize them. For example, CREB is a PKA substrate clearly involved in LTM (Bernabeu et al. 1997) but not in short-term memory (STM) (Igaz et al. 2002). In addition, PKA activation is thought to be associated with changes in gene expression required for consolidation of information in the hippocampus (Vazquez et al. 2000). Thus, our results suggest that PKA pathway is not involved, at least in the first moment, in the spatial retention improvement elicited by Cr.

In conclusion, the current study reports that the intrahippocampal injection of Cr induced spatial memory retention and involves intracellular CaMKII/CREB pathway activation. Therefore, considering that Cr enhances brain function in normal and stress conditions (Watanabe et al. 2002; Rae et al. 2003) and if the effects detected in this study also occur in clinical studies in humans, the future of this compound in health and disease prevention may be even brighter than as an ergogenic aid. However, further in-depth studies are necessary to establish a definitive mechanism for Cr action on the spatial retention facilitation.

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carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) revised 1996, and that the University Ethics Committee approved the respective protocols.

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