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Detection of Novelty, But Not Memory of Spatial Habituation, Is Associated With an Increase in Phosphorylated cAMP Response Element-Binding Protein Levels in the Hippocampus

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ABSTRACT: There is a growing body of evidence showing that the formation of associative memories is associated with an increase in phosphorylated cAMP response element-binding protein (pCREB) levels. We recently reported increased pCREB levels in the rat hippocampus after an exploration to a novel environment. In the present work, we studied whether this increment in CREB activation is associated with the formation of memory of habituation to a novel environment or with the detection of novelty. Rats were submitted to consecutive open field sessions at 3-h intervals. Measurement of the hippocampal pCREB level, carried out 1 h after each training session, showed that (1) it did not increase when rats explored a familiar environment; (2) it did not increase after a reexposure that improves the memory of habituation; (3) it increased after a brief novel exploration unable to form memory of habituation; and (4) it increased in amnesic rats for spatial habituation. Taken as a whole, our results suggest that the elevated pCREB level after a single open field exploration is not associated with the memory formation of habituation. It is indeed associated with the detection of a novel environment. © 2003 Wiley-Liss, Inc.

KEY WORDS: spatial novelty; nonassociative learning; pCREB; open field; multiple trials

INTRODUCTION

Several lines of evidence support involvement of the activation of cAMP response element-binding protein (CREB) in the formation of long-term memory. A series of genetic and pharmacological manipulations along different phyla (*Aplysia*, *Drosophila*, and rodents) demonstrated that CREB is required for memory formation (Silva et al., 1998). Injections of double-stranded oligonucleotides comprising a CRE site into the nuclei of *Aplysia* neurons blocked long-term facilitation (Dash et al., 1990), and the administration of CREB antisense oligonucleotides into the hippocampus of rats before training impaired spatial long-term memory (Guzowski and McGaugh, 1997). Moreover, transgenic flies overexpressing a CREB activator or CREB repressor proteins showed improved or impaired long-term olfactory conditioned memory, respectively (Yin et al., 1994, 1995). The generation of knockout mice added information about the requirement of CREB in the water maze spatial task, object recognition, and fear conditioned memories (Bourtchuladze et al., 1994; Kida et al. 2002; Pittenger et al., 2002). In this context, we and others have shown that memory processing of a one-trial inhibitory avoidance training in rats, a hippocampal-dependent associative learning, is associated with an increase in phosphorylated CREB (pCREB) levels in the hippocampus (Bernabeu et al., 1997; Taubenfeld et al., 1999, Cammarota et al., 2000; Viola et al., 2000). In addition, pharmacological or behavioral induced-amnesia for this task abolished the increase of pCREB in hippocampal nuclear extracts (Cammarota et al., 2000; Viola et al., 2000).

In contrast, limited data are available about the involvement of pCREB in memory formation of nonassociative learning tasks. In a seminal work, Kinney and Routtenberg (1993) showed an increase in the specific binding to CRE consensus sequence, 30–120 min after a 4-min radial maze exploration in the hippocampus of rats. Also, we reported that hippocampal pCREB levels increased 1–2 h after an open field exposure, returning to basal levels 3 h after training (Vianna et al., 2000).

Nonassociative behavioral habituation provides one of the most elementary forms of learning, both in animals and in humans. Rats submitted for the first time to an open field display higher spatial exploration than in successive exposures. Thus, the decrement in the response to successive exposures is taken as an index of memory of habituation. However, the exposure to a novel environment also triggers the processing of novelty detection. The detection of a novel stimulus involves an increased level of attention and the matching of stored memories of places previously explored with the new spatial information to judge its novelty (Montag-Sallaz et al., 1999).

In humans, the hippocampal region is an essential component of the network that detects and responds to novel stimuli (Knight, 1996; Grunwald et al., 1998). In rats, the hippocampus is involved in novelty-associated biochemical and plastic changes (Acquas et al., 1996; Xu et al., 1998; Manahan-Vaughan and Braunewell, 1999; Giovannini et al., 2001; Sacchetti et al., 2002), as well as in the formation of several spatial learning tasks, including the memory formation of spatial habituation (Riedel et al., 1999; Vianna et al., 2000; Pittenger et al., 2002).

Taking these considerations into account, the objective of the present work was to determine whether the pCREB level increment in the hippocampus of rats submitted to a nonassociative spatial exploration is associated with memory formation of habituation or to novelty detection. To that end, we carried out different experiments for behavioral dissection of both processes.

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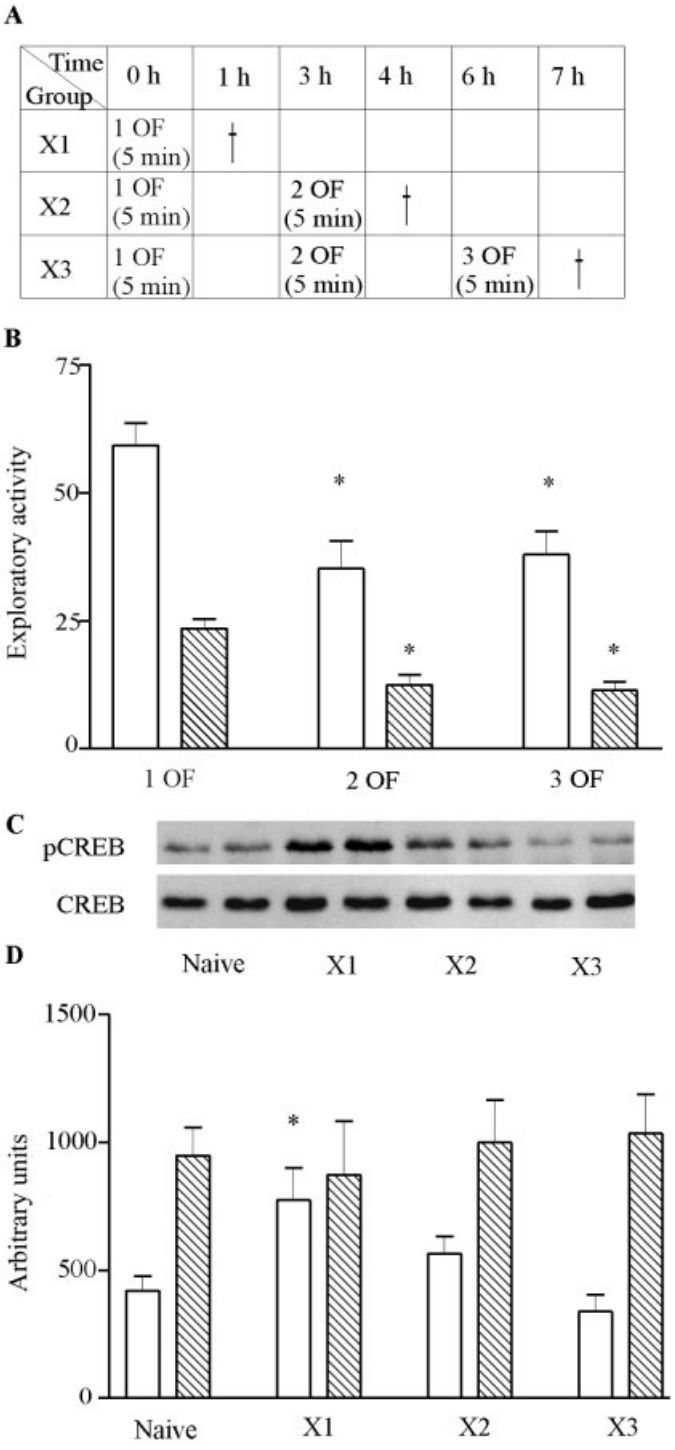
MATERIALS AND METHODS

Subjects

Male Wistar rats (age, 2 months; weight, 180–210 g) from our own breeding colony were used. The animals were housed in plastic cages, three to a cage, with water and food ad libitum, under a 12-h light/dark cycle (lights on at 7:00 A.M.) at a constant temperature of 23°C.



Behavioral Procedures

The novel environment was a 50-cm-high, 50-cm-wide, and 39-cm-deep open field with black plywood walls and a brown floor divided into nine equal squares by black lines. The number of line crossings and rearings (Izquierdo et al., 2001) was measured; their decrement was an index of memory of habituation.

We carried out four different experiments. In experiment I, animals were submitted to three 5-min exploration sessions to the open field, separated by 3-h intervals (Fig. 1A). Experiment II differed from I only in that the second training session lasted 20 min (Fig. 2A). In experiment III, the rats were submitted to a brief 20-s exploration to the novel environment; 3-h later, the animals explored the open field during a 5-min session (Fig. 3A). In all experiments, to perform biochemical assays, different groups of animals were sacrificed 1 h after each training sessions (Figs. 1A, 2A, and 3A).

In experiment IV, 15 rats were implanted under deep thionembutal anesthesia with 30-g guide cannulae in the dorsal CA1 region of the hippocampus at coordinates A −4.3, L ± 4.0, V 2.6, according to the atlas by Paxinos and Watson (1986). The cannulae were fixed to the skull with dental acrylic (Bernabeu et al., 1997). After recovery from surgery, the animals were submitted to one exploration session to the open field; immediately after training, they were injected into the CA1 region with saline or with CaMKII blocker (KN62, 3.6 ng/side); 1 h later, the animals were sacrificed (Fig. 4A). In all cases, infusions were bilateral and had a volume of 0.5 μl.

Biochemical Procedures

All procedures was carried out at 4°C. After sacrifice, the brains were immediately removed, the hippocampi were dissected out, pooled, and homogenized in ice-chilled buffer (20 mM Tris-HCl, pH 7.4, 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg/ml aprotinin, 15 μg/ml leupeptin, 50 mM NaF, and 1 mM sodium orthovanadate). The homogenate was centrifuged 10 min at 900*g* and the obtained nuclear pellet resuspended in buffer (20 mM Tris-HCl, pH 7.4, 1 mM PMSF, 50 mM NaF, and 1 mM sodium orthovanadate). The samples were stored at −70°C until used.

FIGURE 1. Hippocampal phosphorylated cAMP response element-binding protein (pCREB) level increased after the first, but not after the second or third, consecutive 5-min training sessions in an open field. A: Schematic representation of groups used for the behavioral and biochemical experiments. The duration of each OF session is indicated between parenthesis. B: Effect of three consecutive 5-min training sessions (X3 group, A) on exploratory activity in rats. Data are expressed as mean ±SEM of crossings (open bars) and rearings (hatched bars). n = 15, *$P < 0.001$ with respect to the first session (1 OF), Tukey-Kramer test after repeated-measures analysis of variance (ANOVA). C: Representative Western blots with anti-pCREB and anti-CREB antibodies in hippocampal nuclear samples from experimental groups shown in A. D: Densitometric analysis of the data. Data are expressed as mean ±SEM values (arbitrary units) for pCREB (open bars) and for CREB (hatched bars). Immunoblots were carried out 1 h after the last training session for each group. n = 7–15 per group. *$P < 0.05$ with respect to naive group; Dunnett's test after ANOVA. †Sacrificed; OF, open field.

SDS-PAGE and Immunoblotting

Samples of nuclear fractions (30 μg of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% gels). Immunoblots were performed as previously described (Cammarota et al., 2000). Membranes were incubated with the following antibodies: anti-CREB (1:1,000; New England BioLabs) and anti-pCREB (1:1,500; New England BioLabs). Densitometric analysis of the films was performed by using a MCID Image Analysis System (5.02v, Image Research, Ontario, Canada). Western blots were developed for linearity within the range used for densitometry.

Data Analysis

The Tukey-Kramer multiple comparison test was applied after repeated-measures one-way analysis of variance (ANOVA) for statistical analysis of the behavioral data. Dunnett's multiple comparison test after ANOVA was performed for analysis of the biochemical data. The nonpaired Student's *t*-test was used when two independent groups were compared.

RESULTS

Experiment I

In this experiment, rats were subjected to three consecutive 5-min open field sessions at 3-h intervals (Fig. 1A). A 5-min exposure to a novel environment induced memory formation of spatial habituation expressed as a decrease in the number of crossings and rearings carried out by an animal in the second 5-min open field session performed 3 h later ($P < 0.001$; Fig. 1B). However, the spatial exploration displayed in the third training session did not differ from the second one (Fig. 1B). Thus, in this experimental procedure, memory formation of habituation occurs only after the first visit to the arena.

Confirming and extending our previous results (Vianna et al., 2000), an increase in hippocampal pCREB levels was observed 1 h after the first training session ($P < 0.05$, Fig. 1D), without changes in the total amount of CREB protein. Phosphorylated CREB levels returned to basal values after 4 and 7 h of a single open field training (naive = 419 ± 57.8; 4 h = 468 ± 157; 7 h = 378 ± 97, n = 6-9; $P > 0.05$).

Neither the second nor the third 5-min open field exposures modified the phosphorylation state of CREB measured 1 h after the corresponding session ($P > 0.05$; Fig. 1D). Given that the first exploration to the open field involves both the detection of a novel environment and the formation of memory of habituation, we

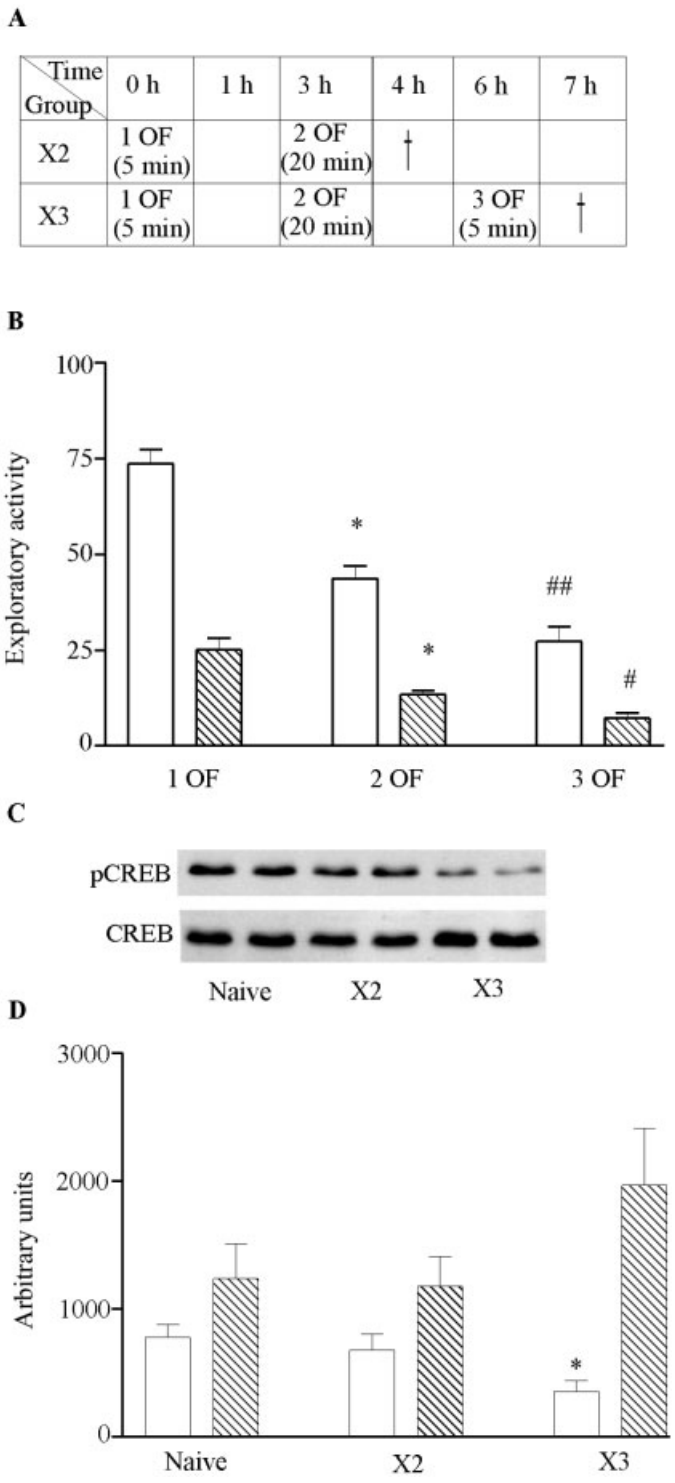
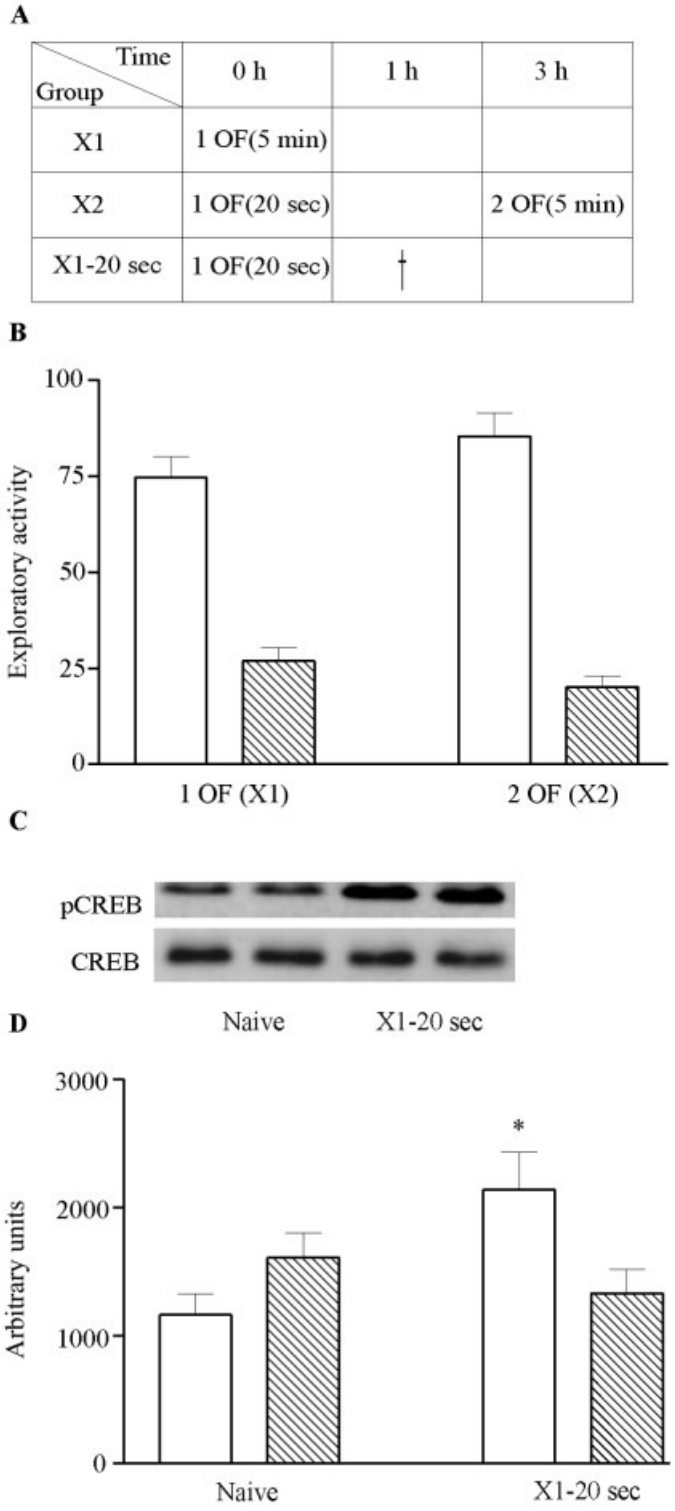


FIGURE 2. Hippocampal phosphorylated cAMP response element-binding protein (pCREB) level did not increase after a familiar session that enhances the memory of habituation. A: Schematic representation of groups used for the behavioral and biochemical experiments. The duration of each OF session is indicated in parentheses. B: Memory of habituation after consecutive 5-min and 20-min OF successive sessions (X3 group, A). Exploratory activity data are expressed as mean ±SEM of crossings (open bars) and rearings (hatched bars). It is shown the initial 5 min of the 20-min second OF exposure. n = 11. *$P < 0.01$ vs 1 OF; #$P < 0.05$; ##$P < 0.01$ vs 2 OF, Tukey-Kramer test after repeated-measures analysis of variance (ANOVA). C: Representative Western blots with anti-pCREB and anti-CREB antibodies in hippocampal nuclear samples from experimental groups shown in A. D: Densitometric analysis of the data. Data are expressed as mean ±SEM values (arbitrary units) for pCREB (open bars) and for CREB (hatched bars). Immunoblots were carried out 1 h after the last training session. n = 5–7 per group. *$P < 0.05$ with respect to the naive group; Dunnett's test after ANOVA. †Sacrificed; OF, open field.

performed experiments II and III to dissect these two hippocampal-dependent processes.

Experiment II

It is conceivable that if the exposure to the environment is long-lasting, the spatial habituation should be also strengthened. When the second exposure to the open field lasted 20 min instead of 5 min (Fig. 2A), an enhanced memory of habituation was observed in a subsequent third open field session ($P < 0.05$, Fig. 2B). Unexpectedly, hippocampal increments in pCREB levels did not parallel behavioral memory of habituation (Fig. 2C,D). In fact, rats exhibiting an improved memory of habituation showed a decrease in the phosphorylation state of CREB with respect to naive animals ($P < 0.05$, Fig. 2D). This deactivation of CREB was dependent on the presence of the third open field session, because no changes in pCREB levels were found 4 h after the second 20-min exploration (naive = 1843 ± 628 vs exposed = 1746 ± 349, n = 4–7, $P > 0.05$). Because the activation of CREB dos not appear to be associated with a second exploration, we suggest that the detection of novelty is the underlying process that activates CREB. Experiment III was designed to support this hypothesis.



Experiment III

A brief exposure of 20 s in the open field led animals to take contact with the novel environment but did not allow memory formation of habituation (Fig. 3A,B). Thus, Fig. 3 B showed that the number of crossings and rearings displayed during a 5-min open field training session was similar in the presence or in the absence of a previous brief exposure to the arena ($P > 0.05$). Importantly, an increment in hippocampal nuclear pCREB levels was still observed 1 h after the brief novel event, without changes in the total CREB protein amount ($P < 0.05$, Fig. 3D).

The results from experiments I–III are consistent with the idea that pCREB levels increase in the hippocampus after a novel environment exploration. Finally, to further substantiate this hypothesis, in experiment IV we measured hippocampal pCREB levels in amnesic animals for spatial habituation.

Experiment IV

Recent evidence demonstrated that the immediately posttraining infusion of KN62, a CaMKII blocker, into the CA1 region of the dorsal hippocampus induced amnesia for spatial habituation (Wolfman et al., 1999; Vianna et al., 2000). In the present study, saline-injected and KN62-injected rats were sacrificed 1 h after an open field training session (Fig. 4A). The infusion of KN62 did not

FIGURE 3. Hippocampal phosphorylated cAMP response element-binding protein (pCREB) level increased after a brief exposure to a novel environment. A: Schematic representation of groups used for the behavioral and biochemical experiments. The duration of each OF session is indicated in parentheses. B: Brief 20-s exposure to the open field did not alter the exploratory behavior in a subsequent session. Data are expressed as mean ±SEM of crossings (open bars) and rearings (hatched bars). The graph shows the exploratory activity during a 5-min OF session in the absence (1OF X1) or in the presence (2OF X2) of a 20-s preexposure to the novel environment. n = 6 per group. $P > 0.05$; nonpaired Student's *t*-test. C: Representative Western blots with anti-pCREB and anti-CREB antibodies in hippocampal nuclear samples from naive and X1-20-s groups shown in A. D: Densitometric analysis of the data. Data are expressed as mean ±SEM values (arbitrary units) for pCREB (open bars) and for CREB (hatched bars). Immunoblots were carried out 1 h after the 20-s session in the X1-20-s group. n = 8 per group. *$P < 0.05$; Student's *t*-test. †Sacrificed; OF, open field.

A

Group \ Time	0 h	1 h
S	OF ↓ (saline)	†
K	OF ↓ (KN62)	†

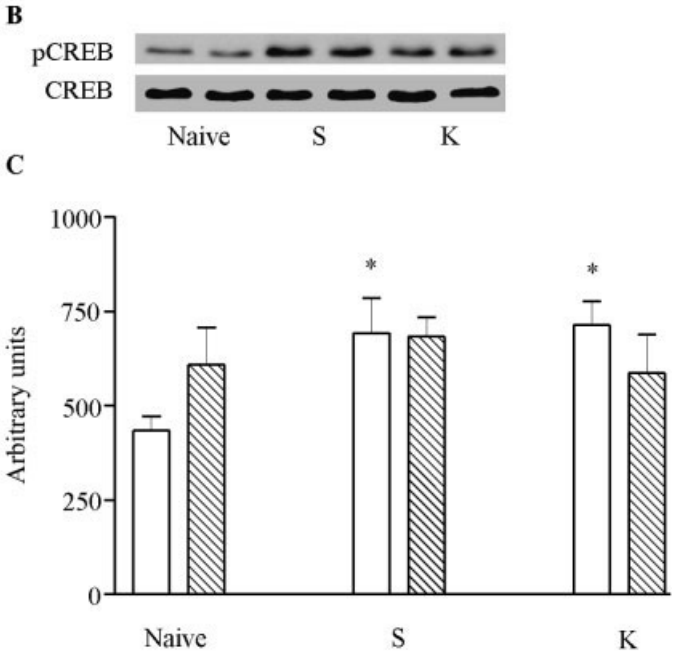


FIGURE 4. Hippocampal phosphorylated cAMP response element-binding protein (pCREB) level increased in amnesic animals for spatial habituation. A: Schematic representation of groups used for the biochemical experiments. S (saline-injected animals), K (KN62-injected animals). B: Representative Western blots with anti-pCREB and anti-CREB antibodies in hippocampal nuclear samples from experimental groups shown in A. C: Densitometric analysis of the data. Data are expressed as mean ±SEM values (arbitrary units) for pCREB (open bars) and for CREB (hatched bars). Immunoblots were carried out 1 h after saline or KN62 infusions into the CA1 region. n = 5 per group. *$P < 0.05$ vs naive group; Dunnett's test after ANOVA. †Sacrificed; OF, open field.

block the training-induced increase in pCREB levels (as shown in Fig. 4B,C; $P < 0.01$), indicating that the phosphorylated state of CREB in the hippocampus is not correlated with memory formation of a nonassociative spatial learning task.

DISCUSSION

The exploration of a novel environment triggers at least two processes: detection of spatial novelty and memory formation of habituation. In the present study, we dissected both processes, employing different behavioral and pharmacological approaches. Table 1 summarizes the results:

1. The exposure of rats to a novel environment is associated with high levels of pCREB measured in hippocampal nuclear fractions 1 h after the event. This phenomenon was independent of the animal ability to form memory of habituation (Figs. 1, 3, 4; see also the first open field columns in Table 1).
2. Memory of habituation induced by a familiar 20-min exposure to the open field was not associated with the activation of CREB (Fig. 2; see also Table 1, experiment II). In this context, Berman et al. (1998) showed that the consumption of a novel taste resulted in phosphorylation of ERK1-2 and activation of the transcription factor ELK-1 in the insular cortex, whereas the consumption of a familiar taste had no effect. In contrast, using fluorescence in situ hybridization (FISH), Guzowski et al. (1999) measured the mRNA levels of the immediate-early gene Arc in CA1 neurons after two sequential 5-min explorations of a novel environment. These investigators demonstrated that after reexposure to the environment, most of the cells that were responsive at the first exposure (~40% of the population cells) were activated.
3. We reported that the infusion of KN62 into the CA1 region of the dorsal hippocampus immediately after the exposure to a novel environment abolished the memory formation of habituation (Wolfman et al., 1999; Vianna et al., 2000). In this study, we found a high level of pCREB in hippocampus of amnesic rats for spatial habituation (Fig. 4). This finding, together with the increase in pCREB levels observed after a 20-s exposure to the open field (Fig. 3), suggests that pCREB activation is not sufficient to form memory of habituation.

Taken as a whole, our results suggest that the activation of CREB after a new exploration is related to the detection of novelty, and is not correlated with the formation of memory of habituation. Alternatively, given that the behavioral procedure involved exploration of a complex environment, we cannot rule out the possibility that the increase in CREB phosphorylation is, in fact, related to the formation of some type of associative new learning triggered by the novel environment.

TABLE 1.

*Effects of Open Field Exploration on Memory Formation of Habituation (MH) and pCREB Levels Measured in Hippocampal Nuclear Fractions 1 h After Each Session**

	1st open field		2nd open field	
Experiment[a]	MH	pCREB	MH	pCREB
I (5 min–5 min–5 min)	+	+	=	=
II (5 min–20 min–5 min)	+	+	+	=
III (20 s–5 min)	=	+	ND	ND
IV (KN62-injected rats)	=	+	ND	ND

ND, not determined; pCREB, phosphorylated cAMP response element-binding protein.
[a]The time of exploration in consecutive visits to the open field is shown in parentheses (see Materials and Methods). MH +, this open field exploration formed MH (expressed as a decrement of spatial exploration in the following visit); MH, the performance of animals in the following exposure to the environment had similar characteristic to the previous one; +, an increase in the pCREB levels (compared with naive rats).

It has been shown that after exposure to a novel environment, several transcription factors are activated in the hippocampus (Kinney and Routenberg, 1993; Zhu et al., 1997; Guzowski et al., 2001). However, little is known about the transcription factor response after consecutive training sessions. Guzowski et al. (2001) measured the mRNA levels of two transcription factors (zif268 and c-fos) and Arc in the dorsal hippocampus of rats subjected to seven sessions in a spatial water maze. High levels of mRNA for the three immediate-early genes were found 30 min after the first training session. However, this response decreased in the seventh training session, in comparison with the first session, probably due to the lost of attention to the surrounding environment (Guzowski et al., 2001; Guzowski, 2002). Surprisingly, when the platform was moved to another location in a seven-reversal session, the response of the transcription factors was unaltered, but Arc mRNA levels were again increased (Guzowski et al., 2001).

In the present study, reexposure of rats to an environment was not associated with increased levels of pCREB in the hippocampus. In contrast, there was a clear-cut decrease in the phosphorylated state of CREB after the third exploration to the open field (Fig. 2). We suggest that CREB deactivation is probably caused by the environmental familiarity and could be related to the expression of the memory of habituation. Kinney and Routtenberg (1993) found that the increment in specific binding to CRE consensus sequence registered after a 4-min exploration to a radial maze was not observed when the exploration lasted 15 min, probably reflecting the behavioral habituation that induces deactivation of CREB.

Several reports measured the level of CREB phosphorylation on the critical residue Ser-133 that serves to recruit the coactivator CBP (CREB-binding protein) to the promoter facilitating gene transcription. However, CREB function relays on a complex post-translational regulations, including the phosphorylation of CREB Ser-142/Ser-143 and methylation of CBP that disrupt CRE-CBP interactions (for review, see Lonze and Ginty, 2002). In addition, CREB binds to its DNA target sequence as a homodimer, but different members of the CREB/activating transcription factor-1 (ATF-1)/CRE modulator (CREM) subgroup might be able to form heterodimers modulating the duration of the transcriptional event (for review, see Shaywitz and Greenberg, 1999). Thus, a complex combination of specific covalent modifications, cell-type transcriptional activators and coactivators, and the different protein dimerizations will determine CREB transcriptional activity.

What are the molecular mechanisms involved in spatial novelty-induced phosphorylation of CREB in Ser-133? We ruled out the involvement of CaMKII in CREB phosphorylation, because the administration of KN62 immediately after a novel exploration did not impair the activation of CREB in the hippocampus (Fig. 4). Given that the activation of ERK1/2 occurs after an open field exploration (Vianna et al., 2000), but not after a second one (Izquierdo et al., 2001), we propose that ERK1/2 cascade in the hippocampus may participate in novelty-induced CREB activation. This assumption is consistent with recent findings showing that ERK1/2 signaling pathway is involved in the detection of a novel taste (Berman et al., 2000).

In conclusion, our results indicate that, in contrast to associative memories, a spatial nonassociative memory is not associated with activation of CREB in the hippocampus. This activation is indeed associated with the detection of novelty.

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