Phosphorylated cAMP Response Element-Binding Protein as a Molecular Marker of Memory Processing in Rat Hippocampus: Effect of Novelty

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From mollusks to mammals the activation of cAMP response element-binding protein (CREB) appears to be an important step in the formation of long-term memory (LTM). Here we show that a 5 min exposure to a novel environment (open field) 1 hr after acquisition of a one-trial inhibitory avoidance training hinders both the formation of LTM for the avoidance task and the increase in the phosphorylation state of hippocampal Ser 133 CREB [phosphorylated CREB (pCREB)] associated with the avoidance training. To determine whether this LTM deficit is attributable to the reduced pCREB level, rats were bilaterally cannulated to deliver Sp-adenosine 3′,5′-cyclic monophosphothioate (Sp-cAMPS), an activator of PKA. Infusion of Sp-Adenosine 3′,5′-cyclic monophosphothioate Sp-cAMPS to CA1 region increased hippocampal pCREB levels and restored normal LTM of avoidance learning in rats exposed to novelty. Moreover, a 5 min exposure to the open field 10 min before the avoidance training interferes with the amnesic effect of a second 5 min exposure to the open field 1 hr after avoidance training and restores the hippocampal levels of pCREB. In contrast, the avoidance training-associated activation of extracellular signal-regulated kinases (p42 and p44 mitogen-activated protein kinases) in the hippocampus is not altered by novelty. Together, these findings suggest that novelty regulates LTM formation by modulating the phosphorylation state of CREB in the hippocampus.

Key words: phosphorylated CREB; hippocampus; avoidance training; memory processing; novelty; amnesia

It is widely accepted that long-term memory (LTM) formation requires the onset of the transcriptional and translational machinery in distributed, but selected, neuronal systems (Davis and Squire, 1984; Yin and Tully, 1996; Izquierdo and Medina, 1997; Impey et al., 1998; Silva et al., 1998). Evidence suggests that cAMP-responsive transcription, mediated by the cAMP response element-binding protein (CREB) family of proteins, is a crucial step for the establishment of LTM. Nonassociative learning in *Aplysia,* avoidance learning in *Drosophila* and rats, and Pavlovian conditioning and spatial learning in rodents have provided strong evidence that the activation of CREB plays a pivotal role in LTM formation (Bourtchuladze et al., 1994; Bernabeu et al., 1997; Guzowski and McGaugh, 1997; Lamprecht et al., 1997; Impey et al., 1998; Silva et al., 1998). 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 (Izquierdo and Medina, 1997; Taubenfeld et al., 1999), is associated with an increase in the phosphorylation state of CREB (pCREB) and CRE-mediated gene expression in the hippocampus (Bernabeu et al., 1997; Impey et al., 1998; Taubenfeld et al., 1999; Cammarota et al., 2000).

Memory is not acquired in its definitive form. It is a temporally graded process during which new information becomes consolidated and stored (McGaugh, 1966, 2000; Izquierdo and Medina, 1997; Izquierdo et al., 1998; Milner et al., 1998). We found recently that an exposure to a novel environment (open field) for 2 min, 1 hr after submitting rats to a one-trial inhibitory avoidance training, caused amnesia for the avoidance task measured 1 or several days later (Izquierdo et al., 1999). This finding is in line with the first description of retrograde interference of memory by other experiences performed a century ago (Muller and Pilzecker, 1900).

Therefore, to test whether pCREB is a molecular marker of memory processing in the rat hippocampus, we determined the phosphorylation state of Ser 133 CREB in animals trained in the

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inhibitory avoidance with or without retrograde interference induced by novelty and in rats that do not perceive the exposure to an open field as new. Here we show that the exposure to a novel environment for 5 min, 1 hr after the acquisition of a one-trial inhibitory avoidance, hinders both the formation of inhibitory avoidance memory and the associated increase in CREB phosphorylation in the hippocampus. This amnesic effect is prevented by the infusion of a PKA activator delivered into the CA1 region that increased the hippocampal pCREB levels. Furthermore, a 5 min exposure to the open field 10 min before avoidance training interferes with the amnesic effect of a second 5 min exposure to the open field 1 hr after avoidance training and restores the levels of pCREB. Therefore, we suggest that the level of pCREB in the hippocampus is a molecular marker of memory processing and that novelty modulates memory formation of avoidance training by regulating the phosphorylation state of hippocampal CREB.

MATERIALS AND METHODS

Subjects. One hundred seventy male Wistar rats (age, 2–3 months; weight, 180–250 gm) from our own breeding colony were used. The animals were housed in plastic cages, five to a cage, with water and food available *ad libitum*, under a 12 hr light/dark cycle (lights on at 7:00 A.M.) at a constant temperature of 23°C.

Behavioral procedures. Inhibitory avoidance was as follows (Bernabeu et al., 1997; Izquierdo et al., 1998). Rats were placed on a 2.5 cm high, 8.0 cm wide platform at the left of a 50.0 × 25.0 × 25.0 cm yellow acrylic training apparatus, which floor was a series of parallel 0.2 cm caliber bronze bars spaced 1.0 cm apart. Latency to step down onto the grid with all four paws was measured. In the training trial, immediately after this, the animals received a 0.4 mA, 4.0 sec scrambled foot shock. In the test session performed 24 hr after training, the procedures were similar except that the foot shock was omitted.

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 12 equal squares by black lines. Number of line crossings and rearings (Izquierdo et al., 1999) were measured for a 5 min period.

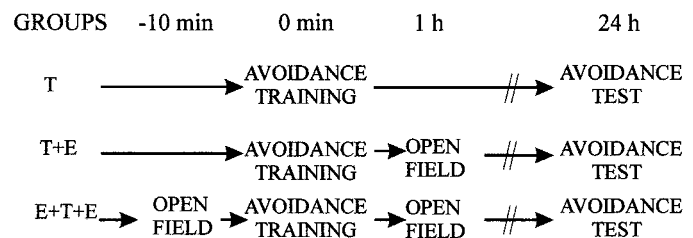
Three groups of 15 animals were trained in the avoidance task using a 0.4 mA shock (see Fig. 1*A*). The first one (trained group, T) was just submitted to this task. The second group (trained plus exposed group, T+E) was exposed for 5 min to the novel environment 1 hr after the avoidance training. The same was performed to the third group but, in addition, this group was exposed for 5 min to the open field 10 min before the avoidance training (exposed plus trained plus exposed group, E+T+E). All groups were tested at 24 hr after the avoidance training.

Surgery and infusion procedures. Seventy rats were implanted under deep thionembutal anesthesia with 30 ga guide cannulas in the dorsal CA1 region of the hippocampus at the coordinates of the atlas by Paxinos and Watson (1986): anterior, −4.3; lateral, ±4.0; ventral, 3.4. The cannulas were fixed to the skull with dental acrylic (Bernabeu et al., 1997; Izquierdo et al., 1998). After recovery from surgery, these animals were trained in inhibitory avoidance and tested 24 hr later. Three groups of cannulated rats received, 110 min after training, a bilateral infusion of either saline or the PKA activator Sp-adenosine 3′,5′-cyclic monophosphothioate (Sp-cAMPS) dissolved in saline (0.1 or 0.5 μg/side). Infusions were in all cases bilateral and had a volume of 0.5 μl. Three other groups of rats were treated as above but, in addition, were exposed to a novel environment 1 hr after training.

Histological examination of cannula placements was performed as described previously (Izquierdo et al., 1997, 1998). Only the behavioral data from animals with the cannula located in the intended site were used.

Biochemical procedures. The rest of the animals were used for biochemical measurements and divided in five experimental groups as shown in Figure 1*B*: (1) animals withdrawn from their home cages and killed immediately (naive group, N); (2) animals submitted to a 5 min session of open-field test and killed 1 hr later (group E); (3) animals trained in the inhibitory avoidance task and killed 2 hr later (group T); (4) animals trained in the inhibitory avoidance box, returned to home cage for 1 hr, subjected to a 5 min session in the open-field test, and killed 1 hr latter (group T+E); and (5) animals that received the same treatment as group 4 but, 10 min before avoidance training, they were subjected to a 5 min session in the open field (group E+T+E).

A



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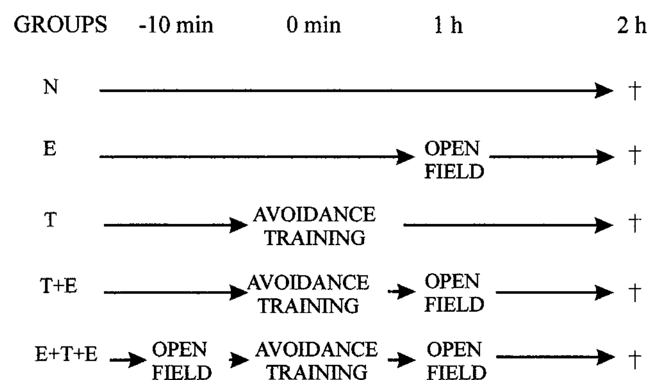

Figure 1. *A*, Scheme showing groups used for the behavioral experiments. *B*, Scheme showing groups used for the biochemical measurements. †, Killed.

The entire procedure was performed at 4°C. After the animals were killed, the brains were immediately removed, and 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 PMSF, 10 μg/ml aprotinin, 15 μg/ml leupeptin, 50 mM NaF, and 1 mM sodim orthovanadate). The homogenate was centrifuged for 10 min at 900 × *g*, and the obtained nuclear pellet was 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.

SDS-PAGE and immunoblotting. Samples of nuclear extracts (12–25 μg of protein) were subjected to SDS-PAGE (10% gels), and immunoblots was performed as described previously (Cammarota et al., 2000). Membranes were incubated with the following antibodies: anti-CREB (1:1000; New England Biolabs, Beverly, MA), anti-pCREB (1:1000; New England Biolabs), anti-p42 and -p44 mitogen-activated protein kinases (MAPKs) (1:2000; New England Biolabs), and anti-activated p42 and p44 MAPKs (1:2000; New England Biolabs). Densitometric analysis of the films was performed by using an MCID Image Analysis System (version 5.02; Imaging Research Inc., St. Catharines, Ontario, Canada). Western blots were developed to be linear in the range used for densitometry.

PKA activity. To investigate whether intrahippocampal infusion of Sp-cAMPS affects PKA activity, the phosphorylation of kemptide was determined as described previously (Bernabeu et al., 1997) using a 2-mm-thick slice taken 10 min after the infusions from the area in which the infusion cannula was placed.

Data analysis. Statistical analysis was performed by one-way ANOVA using the Duncan's test or Student's *t* test. Mann–Whitney *U* test was used for the nonparametric analysis.

RESULTS

Effect of novelty on the retention of a one-trial avoidance training

Figure 1*A* depicts the experimental protocol and the groups of rats used for the behavioral experiments. Confirming and extending recent findings from our laboratories, a 5 min exposure to an

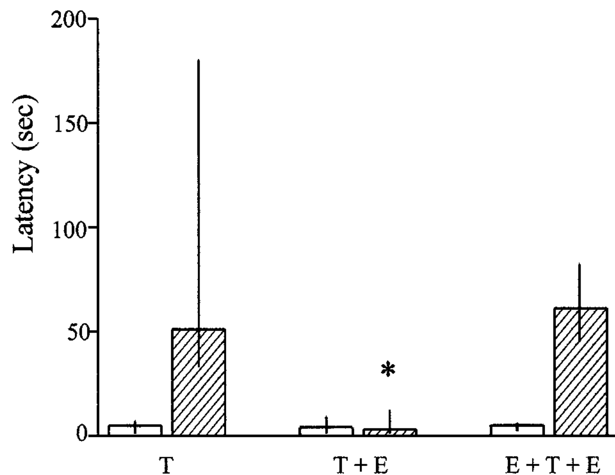

Figure 2. Novelty caused retrograde amnesia for the avoidance learning. Medians (interquartile range) of latencies to step down from the platform of the inhibitory avoidance box in the training session (*open bars*) and in the test session performed 24 hr later (*hatched bars*) in the groups of rats shown in Figure 1*A*. *$p < 0.002$; Mann–Whitney *U* test; $n = 15$ per group.

open field 1 hr after training rats in a one-trial inhibitory avoidance task caused retrograde amnesia for the avoidance learning (Fig. 2). The amnesic effect of the novelty presented 1 hr after avoidance training was totally blocked when rats were exposed to the open field 10 min before the avoidance training. In other words, pretraining exposure to the open field disrupted the amnesic effect of the post-training exposure to the open field. In this group of animals, the post-training exposure was not recognized as novelty, because the number of crossings and rearings per session were lower in the second open-field trial than in the first one (crossings, 77.3 ± 3.7 vs 53.6 ± 3.4; rearings, 24.7 ± 1.5 vs 15.6 ± 1.1, for the first and second open-field exposure, respectively; $p < 0.0001$; Student's *t* test). Therefore, the perception of novelty is associated with its deleterious effect on long-term avoidance memory.

Effect of novelty on the avoidance-induced increase in the phosphorylation state of CREB in the hippocampus

We and others have found previously that one-trial inhibitory avoidance training in rats is specifically associated with a time-dependent and NMDA receptor-dependent increase in Ser 133 pCREB in the hippocampus without changes in total CREB protein (Bernabeu et al., 1997; Impey et al., 1998; Taubenfeld et al., 1999; Cammarota et al., 2000). Given that CREB has an important role in LTM formation (for references, see Silva et al., 1998), that inhibitory avoidance training results in CRE-mediated gene expression in the hippocampus (Impey et al., 1998), and that phosphorylation of CREB at Ser 133 is associated with CREB-regulated gene expression (Montminy, 1997), we determined the levels of Ser 133 pCREB in the experimental groups shown in Figure 1*B*.

Representative immunoblots using an antibody that specifically detects Ser 133 pCREB and the densitometric analysis of the data are shown in Figure 3, *A* and *B*. Confirming previous findings, inhibitory avoidance training results in a large increase in the phosphorylation state of CREB in hippocampal extracts (+127%; $p < 0.05$; $n = 9$) 2 hr after acquisition of the avoidance training, without altering total CREB protein levels. Rats exposed to the novel environment for 5 min (group E) exhibited a modest and

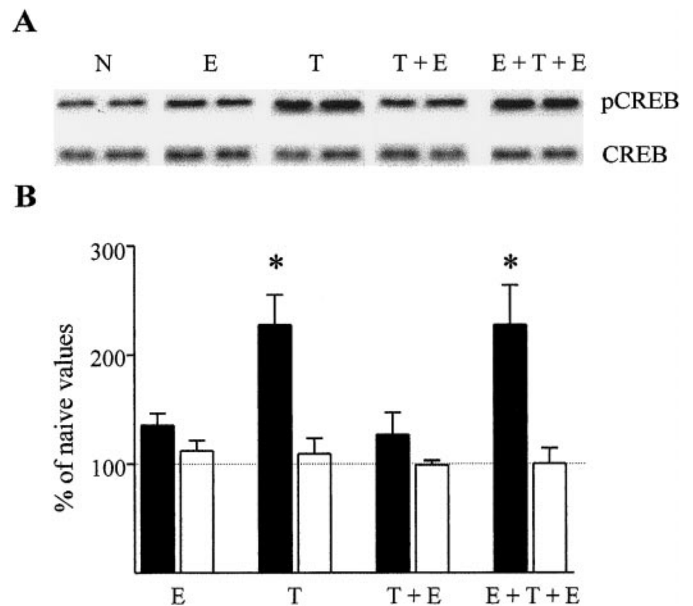

Figure 3. Novelty decreased the hippocampal pCREB levels associated with the inhibitory avoidance training. *A*, Representative Western blots with anti-pCREB and anti-CREB antibodies in hippocampal nuclear samples of rats from experimental groups shown in Figure 1*B*. *B*, Densitometric analysis of the data. Data are expressed as mean ± SEM percentage of naive control values for pCREB (*filled bars*) and CREB (*open bars*). The number of animals per group ranged between eight and nine. *$p < 0.05$ with respect to E and T+E groups; Duncan's test.

nonsignificant increase (+35%; $n = 8$) in pCREB levels. Rats exposed to the open field 1 hr after acquisition of the avoidance training (group T+E) showed a significant decrease in the phosphorylation state of CREB compared with rats subjected only to the avoidance task (+26 vs +127% of naive control values; $p < 0.05$; $n = 9$). Therefore, post-training novelty caused retrograde amnesia of the avoidance training (Fig. 2) and blocked the increase in hippocampal pCREB levels that accompanied this training (Fig. 3*B*). More importantly, a 5 min exposure to the open field 10 min before avoidance training not only abolished the amnesic effect of a second 5 min exposure to the open field 1 hr after avoidance training (Fig. 2) but also restored the levels of pCREB in the hippocampus (Fig. 3*B*). As expected, the exploration of the open field in the second session was significantly lower than in the first one (crossings, 43 ± 6.3 vs 82.4 ± 7.2; $p < 0.001$; rearings, 12.5 ± 2.2 vs 21.3 ± 1.6; $p < 0.01$; Student's *t* test). Moreover, it is important to mention here that the performance of rats in the open field given 1 hr after the avoidance training (group T+E) is similar to that observed in exposed rats (crossings, 73 ± 3.2 vs 76.7 ± 5.5; rearings, 25 ± 2.2 vs 29.7 ± 1.2; $p > 0.05$; Student's *t* test), indicating that the avoidance training did not alter the subsequent performance of rats in the open field.

There is an emerging body of evidence demonstrating that different training procedures result in activation of extracellular signal-regulated kinases (p42 and p44 MAPKs) (Atkins et al., 1998; Crow et al., 1998). Given that p42 and p44 MAPKs couples PKA and PKC to CREB phosphorylation in hippocampus (Roberson et al., 1999) and that we found recently an activation of p42 and p44 MAPKs 2 hr after acquisition of inhibitory avoidance training (Cammarota et al., 2000), we next determined whether the exposure to a novel environment 1 hr after an avoidance training modulates the avoidance-associated activation of MAPKs. For this purpose, we used immunoblot techniques to detect dually phos-

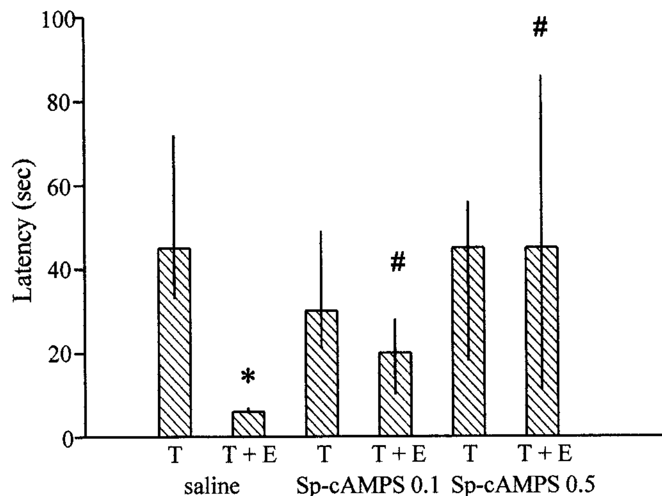

Figure 4. Infusion of Sp-cAMPS into the CA1 region of the dorsal hippocampus overcomes novelty induced-amnesia. Medians (interquartile range) of latencies to step down from the platform of inhibitory avoidance box in the test session performed 24 hr after training. Rats were trained in the avoidance task or received this training plus an exposure to the open field 1 hr later. In all cases, a bilateral CA1 injection of saline or Sp-cAMPS (0.1 or 0.5 μg/side) was administered 110 min after the avoidance training. *$p < 0.002$ versus T rats injected with saline; #$p < 0.02$ versus T+E rats injected with saline; Mann–Whitney *U* test; n = 10–11 per group.

phorylated, activated p42 and p44 MAPKs. In contrast to what happened with hippocampal Ser 133 CREB, post-training novelty did not alter the increased levels of phospho-p42 and -p44 MAPKs associated with the avoidance training (phospho-p42 MAPK: trained, 130.7 ± 11% vs trained plus exposed, 128.1 ± 9.2% with respect to naive values, $n = 8$; phospho-p44 MAPK: trained, 209 ± 39% vs trained plus exposed, 195 ± 34% with respect to naive values, $n = 8$).

Infusion of Sp-cAMPS into the CA1 region of the hippocampus blocked novelty-induced amnesia

Given that memory formation of avoidance training requires PKA activation and is associated with an increased phosphorylation of CREB and CRE-mediated gene expression (Bernabeu et al., 1997; Impey et al., 1998; Taubenfeld et al., 1999; Cammarota et al., 2000), we next determined whether *in vivo* activation of PKA and the subsequent increment of pCREB levels in the hippocampus is able to overcome the amnesic effect of novelty on avoidance training. Bilateral microinjection of the PKA activator Sp-cAMPS (0.1 and 0.5 μg/0.5 μl) in the CA1 region of the dorsal hippocampus of T+E rats, 110 min after avoidance training (i.e., 10 min before the increase in pCREB levels associated with the avoidance task; see Fig. 1*A*), reverted the amnesic effect of novelty on avoidance training (Fig. 4). Sp-cAMPS (0.5 μg) given into CA1 region 110 min after avoidance training increased by 40% hippocampal pCREB levels in T+E rats (saline, 336 ± 35.5 vs Sp-cAMPS, 471 ± 40.8 in relative units; $p < 0.03$; Student's t test; $n = 7$). As expected, Sp-cAMPS (0.1 μg) increased PKA activity by 30% (saline, 301.8 ± 22.3 vs Sp-cAMPS, 399 ± 35.3 pmol of ^{32}P-kemptide per minute per milligram of protein; $n = 5$; $p < 0.05$) 10 min after injection. Therefore, activation of hippocampal PKA blocked novelty-induced amnesia and restored normal LTM.

DISCUSSION

The main finding of the present study is that the levels of pCREB in the hippocampus parallels the behavioral index of a memory trace of a hippocampal-dependent learning task. This is based on three series of data. First, learning of the avoidance task is associated with an increase in hippocampal pCREB levels. Second, this increase was abolished by the exposure to a novel environment 1 hr after avoidance training (Fig. 3), a behavioral procedure that induces retrograde amnesia for the avoidance task (Fig. 2). Moreover, this novelty-induced amnesia of the avoidance training was overcome by the bilateral infusion of Sp-cAMPS into the CA1 region of the hippocampus (Fig. 4). As expected, Sp-cAMPS produced a 30% increase in PKA activity and was able to increase the hippocampal pCREB levels in the T+E rats. Third, a pretraining exposure to a novel environment that blocks the amnesic effect of a post-training exposure to an open field on avoidance training restored hippocampal pCREB levels. Therefore, LTM formation of an avoidance training is associated with some optimal level of pCREB in the hippocampus 2 hr after training.

What is the mechanism for the novelty-induced disruption of both pCREB increase and memory formation of the avoidance training? The molecular mechanisms of one-trial inhibitory avoidance are now known to involve a sequence of molecular events in the hippocampus, including an early NMDA- and calcium/calmodulin-dependent protein kinase II-dependent phase and a crucial late PKA- and protein synthesis-dependent phase (Bernabeu et al., 1997; Izquierdo and Medina, 1997). This late phase, which occurs 2–6 hr after acquisition, is necessary for the persistence of memory of this and other hippocampal-dependent tasks (Carew, 1996; Bernabeu et al., 1997; Bourtchuladze et al., 1998) (see also McGaugh, 2000).

This late phase is also associated with an increased phosphorylation of CREB (Bernabeu et al., 1997; Taubenfeld et al., 1999; Cammarota et al., 2000) and CRE-mediated gene expression (Impey et al., 1998; Cammarota et al., 2000). In addition, the intrahippocampal infusion of CREB antisense oligonucleotides blocks consolidation, but not acquisition, of a water-maze learning (Guzowski and McGaugh, 1997; McGaugh, 2000). Together, these findings support the hypothesis that the late phase of memory consolidation involves PKA-mediated activation of CREB (Izquierdo and Medina, 1997; Silva et al., 1998; McGaugh, 2000). Post-training novelty may affect memory consolidation of the avoidance learning because of a resetting of its underlying molecular mechanisms (Morris, 1998; Izquierdo et al., 1999). The detection of novelty depends, at least in part, on hippocampal systems (Knight, 1996; Zhu et al., 1997). Interestingly, a 4 min exposure to a novel environment caused, after 60 min, an increase in hippocampal CRE binding (Kinney and Routtenberg, 1993). In our experiments, post-training novelty is presented when the molecular mechanisms of memory formation of the avoidance task are on their way toward the crucial late PKA-dependent phase. In line with this assumption, the exposure to a novel environment before or too late (6 hr) after acquisition did not disrupt memory consolidation of the avoidance training (Izquierdo et al., 1999). Our present findings showing that the exposure to a novel environment 1 hr after acquisition of avoidance training is accompanied by a marked decrease in pCREB levels in the hippocampus (Fig. 3*B*) are consistent with this hypothesis. Moreover, when post-training exposure to an open field is not perceived as novelty, by virtue of a pretraining exposure to the novel environment, retention test performance of the avoidance task is normal and pCREB levels are restored.

It is important to stress here that phosphorylation of CREB is just one component of a complex biochemical cascade that leads

to gene expression, which also involves recruitment of CREB binding proteins and their binding to CRE sequence, in combination with other transcription factors (Montminy, 1997).

Consistent with hebbian models of synaptic plasticity and in remarkable parallel with the present findings, the exposure to a novel environment 1 hr after induction of long-term potentiation in CA1 region of the hippocampus hinders LTP expression (Xu et al., 1998).

In conclusion, our results, together with those reporting that fornix lesions disrupts both inhibitory avoidance memory and the increased pCREB levels associated with this task (Taubenfeld et al., 1999), endorse the hypothesis that pCREB is a molecular marker of memory processing in rat hippocampus. A major question arises from this study. Which of the plethora of CREB-regulated genes are specifically involved in memory consolidation? Answer to this question should give us interesting clues on the important role of CREB family of transcription factors in the establishment of long-lasting memories.

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