Subchronic Rolipram Delivery Activates Hippocampal CREB and Arc, Enhances Retention and Slows Down Extinction of Conditioned Fear

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Rolipram, a type IV-specific phosphodiesterase inhibitor, is known to improve memory under various learning tasks. Moreover, Rolipram treatments have been shown to increase expression and phosphorylation of a key factor for hippocampal memory consolidation, the cAMP-dependent response element-binding protein, CREB. However, the exact correlation between hippocampal CREB phosphorylation and memory improvement induced by Rolipram has not yet been determined in a CREB-dependent type of hippocampal-related learning in normogenic, intact rodents. Here, we report that subchronic Rolipram delivery by using osmotic minipumps increased the basal rat hippocampal expression and phosphorylation of CREB, as well as the expression of the cAMP-dependent, memory-related protein, Arc. In parallel, the same treatment improved memory consolidation of conditioned fear. Furthermore, the increase of CREB phosphorylation and Arc expression consequent to the learning experience was enhanced in Rolipram-treated rats, compared to controls. By evaluating the time course of memory extinction over 10 days after the initial learning test, we also observed significant slowing down of the memory extinction rate in Rolipram-treated rats. This effect could be attributed to CREB phosphorylation and memory having been initially higher, as osmotic minipumps stopped to release Rolipram the first day after the initial learning test. Our data define the conditions through which the pharmacological manipulation of hippocampal CREB expression and activation result in memory amelioration in normogenic, intact animals. These results are relevant for the study of molecular correlates of memory, and may also be important in view of the efforts to design new pharmacological treatments, targeting the CREB pathway and leading to enhancement of learning and memory, even in the absence of patent neuropathology.
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INTRODUCTION

From *Aplysia* to mammals, the cellular cascade triggered by cAMP is involved in memory formation and consolidation (Poser and Storm, 2001; Wang and Storm, 2003). A prominent role for cAMP has been demonstrated in long-term potentiation (LTP) and in hippocampus-dependent memory, through activation of the cAMP-dependent protein kinase A (PKA), phosphorylation of the transcription factor cAMP-dependent response element-binding protein (CREB) and stimulation of CREB-dependent gene expression (Frey *et al*, 1993; Bourtchuladze *et al*, 1994; Lonze and Ginty, 2002; Nguyen and Woo, 2003; Ahi *et al*, 2004). In particular, the role of CREB activation through its phosphorylation on serine-133 in memory consolidation

has been studied in rodents after two types of single-trial hippocampus-dependent tasks: contextual learning and inhibitory avoidance (Impey *et al*, 1998; Taubenfeld *et al*, 1999). Recent data on transgenic mice overexpressing the type 1 adenylyl cyclase in the forebrain have suggested that the process of memory extinction also could depend on increased CREB activation (Wang *et al*, 2004).

Phosphodiesterase (PDE) inhibitors, such as the selective type IV PDE inhibitor Rolipram (Schneider, 1984; Weishaar *et al*, 1985), have been used to increase the brain level of cAMP. The consequent increase of cAMP-dependent cellular pathways has antidepressant action and reverses memory deficits caused by antagonists of NMDA receptor and other drugs (Fujimaki *et al*, 2000; Itho *et al*, 2004; Imanishi *et al*, 1997; Zhang *et al*, 2000, 2004; Zhang and O'Donnell, 2000; Sato *et al*, 2004; Vitolo *et al*, 2002). It also improves memory after ischemia and febrile seizures in a mouse model of Rubinstein–Taybi syndrome (Nagakura *et al*, 2002; Bourtchouladze *et al*, 2003; Chang *et al*, 2003). The dependence of LTP and hippocampus-dependent memories on the cAMP signaling cascade suggested that drugs enhancing this pathway could improve learning and

reinforce memory. Rolipram, indeed, induced persistent LTP in hippocampal slices and increased long-term memory retention when injected at low dosage in mice, shortly before submitting them to a hippocampus-dependent memory task (Barad et al, 1998; Navakkode et al, 2004). Noticeably, in these experiments, high Rolipram dosage delivered before training was ineffective, while both low and high doses improved memory when administered immediately after training (Barad et al, 1998). In this study, however, no attempt was performed to correlate the Rolipram-dependent memory improvement with the induction of the cAMP/PKA/P-CREB pathway in the mice hippocampus. Recent experiments on a mouse model for Alzheimer's disease not only demonstrated improvement of hippocampus-dependent memory after acute administration, but also suggested that long-term systemic administration of the drug could result in amelioration of memory deficit for at least 2 months after ending the treatment (Gong et al, 2004). In these experiments, the correlation of the memory improvement with hippocampal P-CREB was only demonstrated concerning the learning-impaired transgenic mice, as no effect of Rolipram administration was observed in wild-type mice. In other experiments, prolonged Rolipram administration increased hippocampal CREB expression at the mRNA level (Nibuya et al, 1996), as well as its phosphorylation in relation to adult neurogenesis in hippocampal dentate gyrus (Nakagawa et al, 2002a, b; Fujioka et al, 2004).

Notwithstanding the increasing importance of manipulating the cAMP-dependent cascade in studies on memory (Giorgi et al, 2004; Zhang et al, 2005), the exact correlation between Rolipram effects on CREB activation and memory consolidation/extinction has not yet been precisely determined. Furthermore, the expression of other memory-related genes as a consequence of the increased cAMP cascade activation has not been addressed using pharmacological approaches to activate CREB. Among cAMP-dependent memory-related genes, it is particularly interesting the activity-regulated cytoskeletal-associated protein Arc/Arg 3.1. Arc is an immediate early gene, whose expression has found to be altered in different hippocampus-dependent learning tasks (Guzowski et al, 2000, 2001; Montag-Sallaz and Montag, 2003; Inoue et al, 2005), and whose inhibition impairs LTP and memory (Guzowski et al, 2000). Even if Arc does not contain any CRE consensus sequence in its promoter region, it has been demonstrated that its expression is regulated by the cAMP/PKA/CREB pathway (Waltereit et al, 2001). Moreover, BDNF-induced hippocampal LTP in vivo has been shown to upregulate Arc expression, in parallel with CREB activation (Ying et al, 2002).

We report here that, at variance with acute administration, steady Rolipram delivery through osmotic minipumps increases basal CREB expression and phosphorylation in the rat hippocampus. When Rolipram-treated rats were trained in a conditioned fear task, increase of hippocampal CREB phosphorylation was significantly higher than in vehicle-treated animals and, in parallel, memory retention was improved and memory extinction slowed down. Moreover, Arc behaved similarly to CREB in its expression in response to subchronic Rolipram treatment as well as to the learning experience.

MATERIALS AND METHODS

Animals and Surgery

Male Wistar rats were purchased from Harlan Italy at 7 weeks of age and used when they were 10 weeks old. All animals were maintained on a 12 : 12 h light/dark cycle and given food and water ad libitum. The experiments were carried out in accordance with the requirements of the Italian and European Community laws on the use of animals for experimental purposes. The experimental protocols were approved by a local bioethical committee and the experiments were performed under the supervision of a veterinary commission.

Rolipram (Sigma, St Louis, MO) was delivered either through i.p. injections or through osmotic minipumps (model 2001, Alzet, Cupertino, CA), implanted subcutaneously in the back of animals under light ether anesthesia. The actual concentration of Rolipram for pump delivery was calculated on a weight basis, in such a way as to obtain a steady release of 0.5 mg/kg/day for 7 days. Vehicle-treated animals received injection of saline containing 2% DMSO, or were implanted with pumps containing the Rolipram vehicle (50% DMSO). For the pump delivery experiments, a further control group was constituted of sham-operated rats.

Apparatus and Testing Procedure

Training sessions were carried on 2 h after the last, or unique, Rolipram i.p. injection, or at the end of the fifth day after pump implantation. Learning tests were performed 24 h later. The training chamber for fear conditioning (Ugo Basile, Comerio, VA, Italy) consisted of a microprocessor-controlled unit, made by a $28 \times 21 \times 21$ cm^3 cage with the 20 stainless steel bar floor connected to a shock generator. The transparent ceiling allowed the rat to be observed. Rats were trained with a context-tone presentation which has been demonstrated to strongly involve hippocampus (Phillips and LeDoux, 1994; Rogelj et al, 2003). The conditioned stimulus consisted of a tone (70 dB, 670 Hz) and the unconditioned stimulus of an electrical foot shock (0.7 mA for 2 s) delivered immediately after the tone.

Animals were divided into two groups: the control group, in which the animals explored the context (3 min), followed by the conditioned stimulus and by another period (4 min) spent in the chamber; the conditioned group in which animals explored the chamber (3 min) before to be exposed to both the conditioned stimulus and the shock and remained there for additional 4 min. After the training, each animal was returned to its home cage and the apparatus was cleaned with 70% ethanol. To test memory retention, each animal was introduced in the same apparatus 24 h after training and, after 1 min, the same tone used for the training session was delivered.

The time spent in freezing by the animal during a fixed time after the tone was assumed as the index of conditioned fear, based on the ability to remember and associate the aversive event with both the tone and the context in which it was presented during the training (Sanders et al, 2003; Woolf et al, 1999, 2001). The freezing behavior, defined as the lack of any movement besides respiration, was scored by

two independent observers, every 5 s during a 2-min interval. At the end of testing, animals were killed by decapitation and the hippocampus was immediately dissected and frozen at $-80°C$ until used for Western blot analysis. The extinction trials were performed on groups of rats trained and tested as described above and subsequently tested again, with the same procedure, every other day during a 10-days interval (Corcoran and Maren, 2001; Fischer et al, 2004).

Western Blotting

Protein samples were homogenized in 100 mM Tris containing 200 mM NaCl, 10% glycerol, 2 mM NaF, 2 mM $Na_4P_2O_7$, 2 mM DTT, 1 mM EDTA, 1mM benzamidine, 0.1 mM Na_3VO_4, 1 mM pepstatine, 10 mg/ml trypsin inhibitor, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 10 mM PMSF at pH 7.4 (all chemicals were from Sigma Chemical, St Louis, MO, USA). After 15 min lysis in ice, samples were centrifuged at 20,500g for 15 min. After determination of protein content (Lowry et al, 1951), equal amounts of protein (30 μg) from each sample were resolved in SDS-PAGE before electroblotting. Membranes were incubated with antibodies against CREB, independently from its phosphorylation state or phosphorylated on serine-133 (Upstate Biotechnology, Lake Placid, NY, USA), or Arc (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or β-actin (Sigma), then with an HRP-linked secondary antibody (Amersham) and visualized by ECL (Amersham). The films were scanned and densitometry was performed using the software 'NIH Image'. Quantitation of CREB, P-CREB, and Arc was performed with reference to the invariant cytoskeletal protein, β-actin, as an additional loading control. Additionally, the P-CREB/CREB *ratio* was calculated in order to precisely estimate changes in the phosphorylation state of CREB in relation to the pharmacological manipulation and the learning experience.

Statistical Analysis

All results were subjected to statistical analysis with one-way and two-way ANOVA (variables: treatment and training), followed by Bonferroni's *post hoc* comparison test and Student's *t*-test, in order to evaluate the significance of the differences.

Figure 1 Time-course analysis of acute Rolipram administration (3 mg/kg) on phosphorylated CREB or total CREB and on Arc in rat hippocampus. Western Blot analysis was performed on hippocampi from rats treated with a single i.p. injection of Rolipram (3 mg/kg) and collected at different time points (from 2 to 18h) after the injection. Their relative densitometries revealed that this dose of Rolipram induced a short-lasting (2–4h), but statistically significant increase in phosphorylated CREB (a) and in Arc (c) levels, but not in total CREB, that is, independently from its phosphorylation state (b). Both P-CREB and Arc return to basal level within 6h. Data are expressed as the percentage relative to controls (vehicle-treated animals) for each protein level, after normalization for the β-actin content in each sample. Each bar represents the mean $\pm$ SEM of 3–5 animals. ***$p<0.001$, **$p<0.01$, *$p<0.05$ compared to the respective controls. Bonferroni's test after one-way ANOVA.

RESULTS

Rolipram Affects Hippocampal CREB and Arc

In order to evaluate the feasibility of obtaining a long-term increase of CREB phosphorylation by using acute Rolipram injection, we started delivering single Rolipram doses, based on the amount of drug previously demonstrated to be able to increase hippocampal levels of cAMP in rats (Giorgi et al, 2004). Acute i.p. administration of Rolipram at a dose of
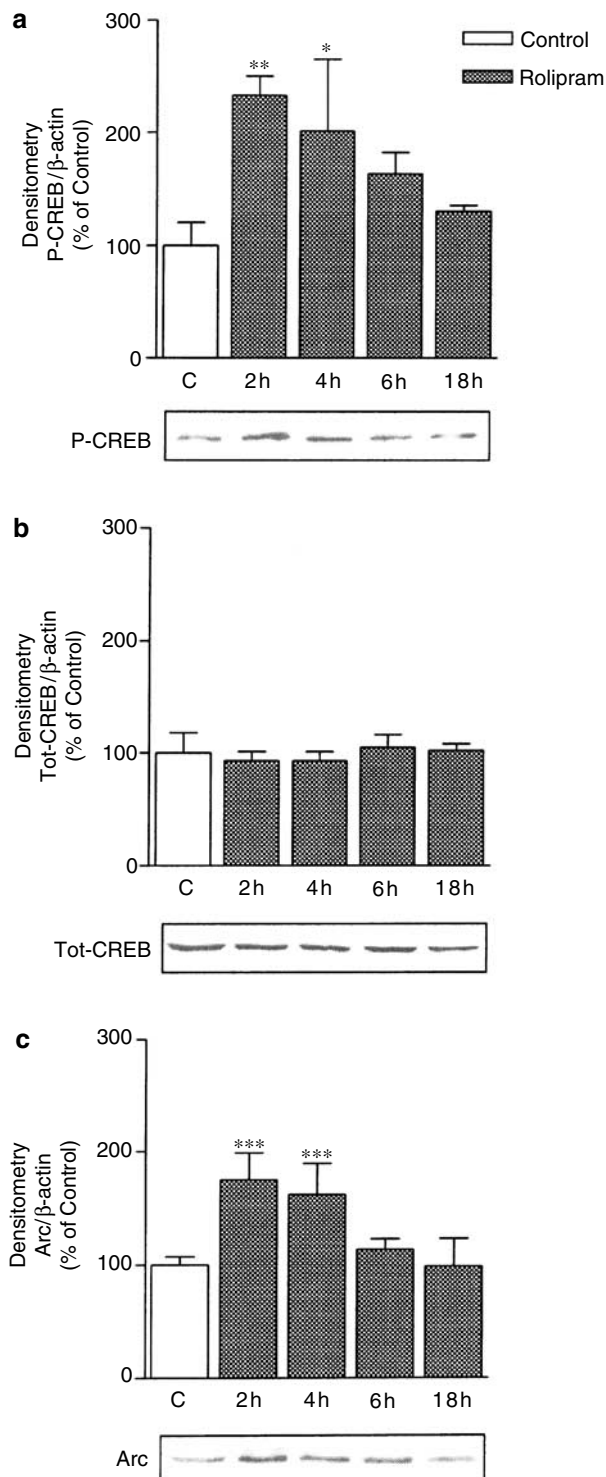
0.3 mg/kg, which was previously shown not to change hippocampal cAMP levels (Giorgi et al, 2004), had no effect on hippocampal CREB phosphorylation from 2 to 6 h after injection (data not shown). A higher (3 mg/kg) dosage of Rolipram, which was previously demonstrated to be able to increase cAMP levels up to 24 h after administration (Giorgi et al, 2004), only resulted in a short-lasting (2–4 h) increase of hippocampal P-CREB (Figure 1a) and Arc levels (Figure 1c), while the expression of CREB protein was unchanged (Figure 1b). Both levels of P-CREB and Arc returned to control values within 6 h after drug administration (Figure 1a, c). Administration of the same dosage of Rolipram split into two daily injections for 5 days gave a similar transitory induction of CREB phosphorylation and Arc expression after the last drug injection (data not shown). Both these last treatments resulted in no significant memory improvement in the fear-conditioning test, as rats trained 2 h after the unique, or the last, Rolipram injection and tested 24 h later exhibited the same freezing score of vehicle-treated animals (Table 1). Previous studies have demonstrated a sedative effect of Rolipram shortly (15–60 min from injection) after acute administration (Silvestre et al, 1999a, b; Zhang and O'Donnel, 2000). No such effect was observed in our experiments, both at the time of training (2 h after administration) and testing (24 h after administration).

In order to test whether continuous administration of Rolipram could result in long-lasting increase of CREB phosphorylation, we used controlled delivery through

osmotic minipumps. This procedure of continuous delivery of Rolipram (0.5 mg/kg/day) had no apparent side effects and significantly increased the steady level of CREB expression and phosphorylation (Figure 2a, b), as determined in the hippocampi of rats killed 5 days after pump implantation, compared to sham-operated and vehicle-treated animals. As the increase in phosphorylated protein

Table 1 Effects of Single or Repeated Rolipram Injections on Conditioned Fear Memory Retention

	Vehicle		Rolipram	
	Control	Conditioned	Control	Conditioned
Single injection	2.4 ± 3.7	$82.2 \pm 15.7^{***}$	3.8 ± 3.8	$81.8 \pm 18.2^{***}$
Repeated injections	3.6 ± 2.3	$83.5 \pm 16.2^{***}$	2.8 ± 3.9	$80.9 \pm 17.5^{***}$

Rats treated either with a single (3 mg/kg) or with repeated (twice a day for 5 days, 3 mg/kg/day) Rolipram injections were trained for conditioned fear 2 h after the unique or last injection and tested for memory retention 24 h later. No differences were observed between the conditioned groups of Rolipram- or vehicle-treated animals following either the single or the repeated treatment. Data are expressed as the mean of freezing percentage $\pm$ SEM in control and conditioned groups of vehicle- and Rolipram-treated rats. Each value represents the mean $\pm$ SEM of 3–4 animals. $^{***}p < 0.001$, compared to the respective control groups; Bonferroni's test after two-way ANOVA.
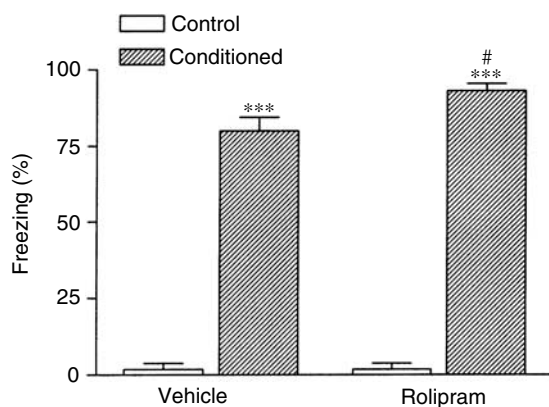

Figure 2 Effect of subchronic Rolipram (0.5 mg/kg/day) administration through osmotic minipumps on phosphorylated CREB or total CREB and on Arc levels in rat hippocampus. Western blot analysis were performed on the hippocampi from sham-operated or vehicle- or Rolipram-treated rats and collected after 5 days from minipump implant. Densitometries of rat hippocampal level of phosphorylated CREB (a), of total CREB (b) and of Arc (c) revealed that subchronic Rolipram delivery induced a significant increase in the steady level of both CREB expression and phosphorylation, as well as that of Arc expression. Data are expressed as the percentage relative to controls (sham-operated animals) of each protein level, after normalization for the β-actin content in each sample. Each bar represents the mean $\pm$ SEM of 3–5 animals. $^{*}p < 0.05$ compared to sham-operated rats. Bonferroni's test after one-way ANOVA.
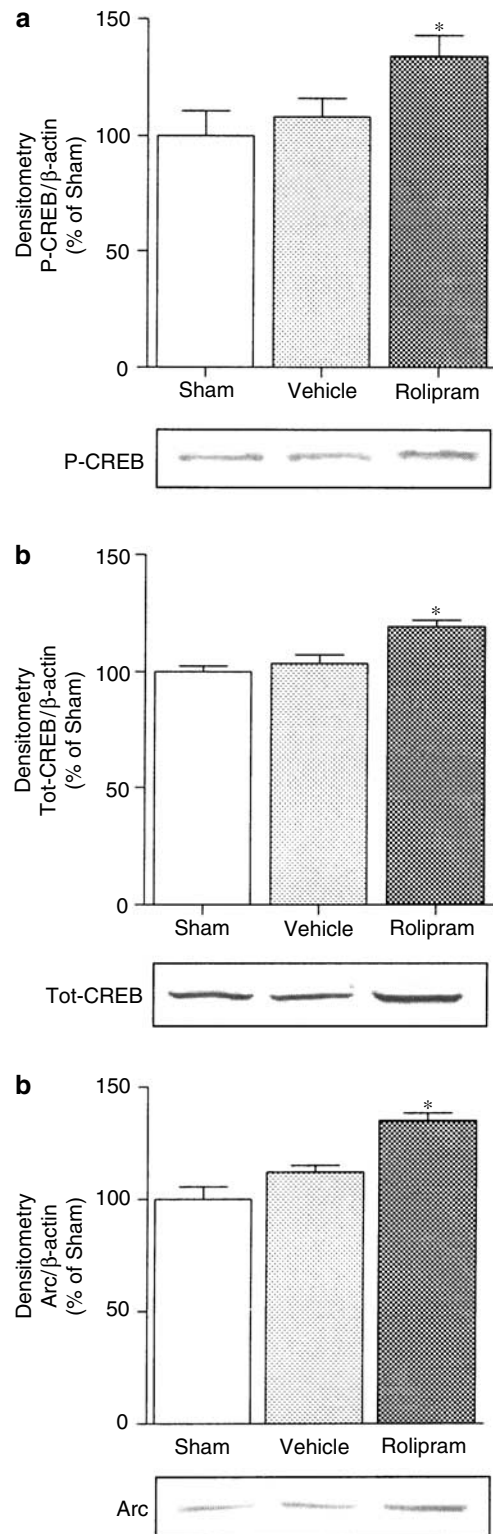
was larger than the increase in native protein expression, the *ratio* of P-CREB/CREB was also significantly increased (vehicle: $100 \pm 1.3\%$; Rolipram: $113.5 \pm 2.6\%$; $p < 0.05$). An equivalent increase was also noticed for Arc protein expression in this group of Rolipram-treated rats (Figure 2c).

Increased Hippocampal CREB Activation and Arc Expression Correlate with Stronger Memory Retention

We performed training for conditioned fear 5 days after pump implantation and tested its retention 24 h later, when the pump was still releasing Rolipram. Upon conditions of continuous delivery, Rolipram had no patent sedative effect on rats, as also demonstrated by the lack of freezing observed in Rolipram- compared to vehicle-treated animals in the absence of conditioned fear training. As expected, both vehicle- and Rolipram-treated rats of the conditioned groups exhibited much higher freezing levels, compared to controls (Figure 3). In addition, notwithstanding the nearly maximal level of the behavioral response in control animals, the freezing score of Rolipram-treated rats was slightly, but significantly increased compared to vehicle-treated animals (Figure 3). No differences in the freezing score were observed between vehicle-treated and sham-operated rats (data not shown). In the hippocampi of these rats, killed immediately after the retention test, we determined the level of CREB phosphorylation and Arc expression, both known to be induced in hippocampus-dependent memory consolidation (Impey *et al*, 1998; Taubenfeld *et al*, 1999; Guzowski *et al*, 2000, 2001). As the subchronic Rolipram treatment induced a significant increase in CREB protein level, as shown before (Figure 2), the results were expressed as the *ratio* P-CREB/total CREB, in order to isolate the

increase in CREB phosphorylation related to the combined effects of learning and pharmacological treatment. The P-CREB/CREB *ratio*, a known index of CREB activation (Gong *et al*, 2004), was significantly increased in the hippocampus of fear-conditioned rats compared to controls, thus demonstrating the expected correlation between learning and activation of hippocampal CREB (Figure 4a). In addition, the subchronic Rolipram treatment additionally activated hippocampal CREB, as demonstrated by the significant increase in the P-CREB/CREB *ratio* found in drug- compared to vehicle-treated rats (Figure 4a). Hippocampal Arc expression behaved similarly to the index of CREB activation, as it was increased in the hippocampus in relation to learning, and a further increase was shown by Rolipram-treated rats (Figure 4b). No differences in
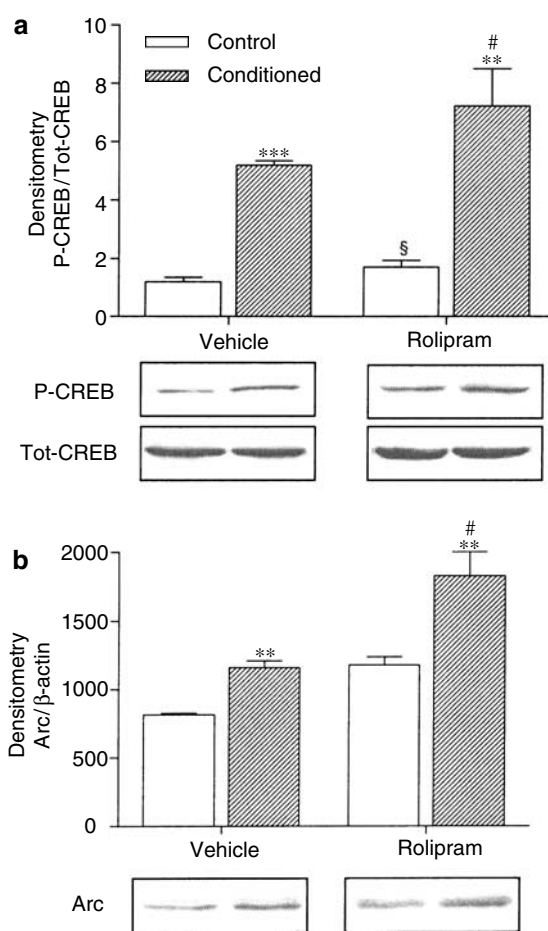
Figure 4 Effect of subchronic Rolipram treatment on CREB and Arc activation following induction of conditioned fear. Western Blot analysis was performed on the hippocampi from rats treated either with vehicle or with Rolipram (0.5 mg/kg/day) through osmotic minipumps, trained for conditioned fear 5 days after the implant and tested for memory retention 24 h later. Quantification through densitometries revealed that the phospho-CREB/total CREB (a) as well as the Arc/β-actin (b) *ratio* were significantly increased in conditioned rats compared to controls and that, in parallel with memory retention, subchronic Rolipram treatment further increased both values compared to vehicle-treated rats. Each bar represents the mean $\pm$ SEM of 4–10 animals. ***$p < 0.001$, **$p < 0.01$ compared to the respective controls; #$p < 0.05$ compared to the conditioned group of vehicle-treated rats. Bonferroni's test after two-way ANOVA.

Figure 3 Effect of subchronic Rolipram treatment through osmotic minipumps on conditioned fear. Rats subchronically treated either with vehicle or with Rolipram (0.5 mg/kg/day) through osmotic minipumps were trained for conditioned fear 5 days after the implant and tested for memory retention 24 h later. Freezing percentage was used as index of conditioned fear memory retention. The conditioned groups of both vehicle- and Rolipram-treated rats showed a much higher freezing percentage compared to their respective controls and the freezing score of the conditioned group of Rolipram-treated animals was significantly increased compared to the conditioned group of vehicle-treated rats. Each bar represents the mean $\pm$ SEM of 4–10 animals. ***$p < 0.001$, compared to the respective control groups; #$p < 0.05$ compared to the conditioned group of vehicle-treated rats. Bonferroni's test after two-way ANOVA.

P-CREB/CREB *ratio* or Arc expression were observed between vehicle-treated and sham-operated rats (data not shown).

Stronger Memory Consolidation Induced by Rolipram Slows Down Subsequent Memory Extinction

Parallel groups of rats were used to assess the effect of stronger initial memory and higher CREB activation on the rate of memory extinction, evaluated by testing the animals every other day from the second to the 10th day after the initial learning test. Although Rolipram-treated rats showed extinction of conditioned fear memory, their rate of memory extinction was significantly slowed down, compared to both sham-operated and vehicle-treated rats (Figure 5). This could not be attributed to long-term persistence of increased P-CREB/CREB *ratio* and Arc expression after the ending of the pharmacological treatment. No differences were, indeed, noticed after extinction tests in the hippocampi of previously Rolipram-treated, vehicle-treated or sham-operated rats (Figure 6a, b).

DISCUSSION

We have subchronically delivered steady amounts of Rolipram through osmotic minipumps and this resulted in increased CREB and P-CREB basal levels in rat hippocampus. Upon training in a conditioned fear task, these rats displayed an increased freezing response and an increased level of the P-CREB/total CREB *ratio* in their hippocampus. Another cAMP-dependent protein involved in hippocampal memory, Arc, was also increased in its basal and learning-induced levels in the hippocampus of Rolipram-treated rats. These concomitant effects were only observed in rats treated subchronically through osmotic minipumps, indicating that attainment of an increased steady level of expression and activity was essential in the enhancement of the behavioral response. Furthermore, when the time-course of memory extinction was studied at different times after the initial test and after the ending of drug delivery, previously Rolipram-treated rats exhibited slower extinction rate of the acquired memory.

Previous reports separately demonstrated that Rolipram or other PDE inhibitors were able to: (i) increase hippocampal CREB expression or activity (Nibuya *et al*, 1996; Nakagawa *et al*, 2002a, b; Fujioka *et al*, 2004; Itho *et al*, 2004); (ii) improve hippocampus-dependent memory (Barad *et al*, 1998; Navakkode *et al*, 2004); or (iii) reverse various types of memory deficits (Imanishi *et al*, 1997; Zhang *et al*, 2000, 2004; Zhang and O'Donnell, 2000;
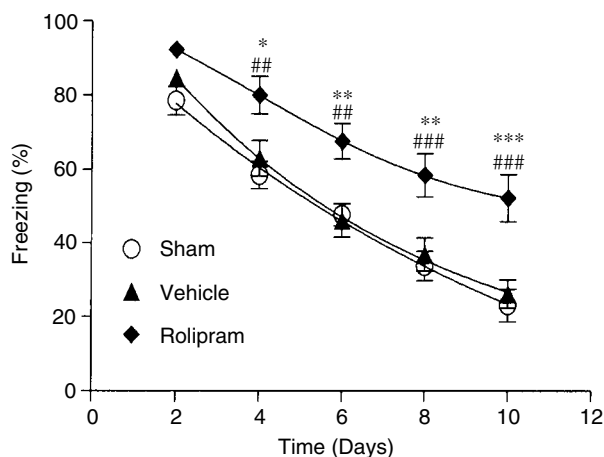
Figure 5 Effect of subchronic Rolipram treatment through osmotic minipumps on conditioned fear memory extinction rate. Rats subchronically treated with Rolipram through osmotic minipumps (0.5 mg/kg/day) were trained for conditioned fear 5 days after the implant, tested 24 h later and further tested every other day during the following 10 days. Freezing percentage at different days (2–10) after the initial learning test has been used as index of conditioned fear memory retention in the conditioned rat groups. Although subchronic Rolipram-treated rats (◆) exhibited memory extinction, its rate was significantly slowed down compared to both sham-operated (○) and vehicle-treated (▲) animals. Each point represents the mean ± SEM of five animals. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to vehicle-treated rats at the same day; $##p < 0.01$, $###p < 0.001$ compared to sham-operated rats on the same day. Bonferroni's test after two-way ANOVA.
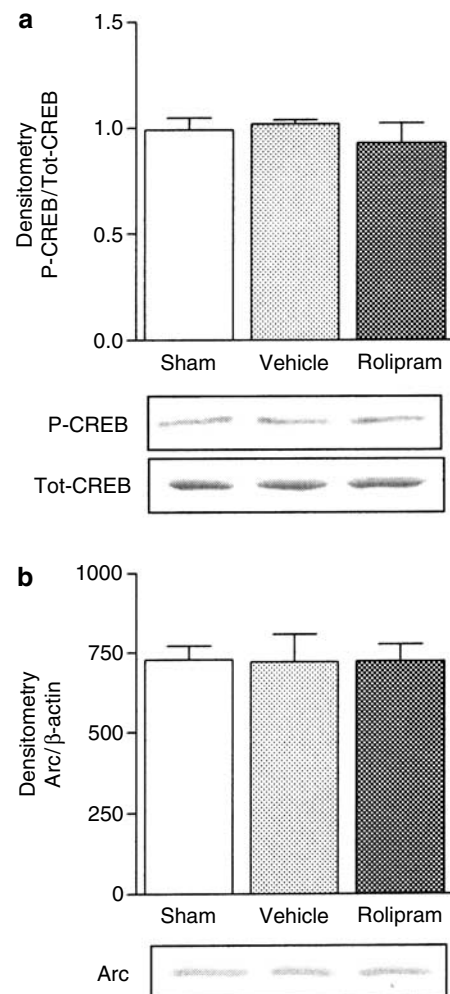
Figure 6 Effect of subchronic Rolipram treatment through osmotic minipumps on P-CREB, total-CREB, and Arc level at the end of the memory extinction experiment. Western blot analysis was performed on hippocampi from the same groups of Figure 5. Densitometries revealed that there was no difference either in the phospho-CREB/total-CREB (a) or in the Arc/β-actin (b) *ratio*. Each bar represents the mean ± SEM of three animals.

Nagakura *et al*, 2002; Bourtchouladze *et al*, 2003; Chang *et al*, 2003; Sato *et al*, 2004). Recently, it was also shown that low dosage of Rolipram administered for 3 weeks to an Alzheimer mouse model resulted in long-term recovery (2 months after the end of the treatment) of hippocampal CREB phosphorylation and memory, while having no such effects on wild-type animals (Gong *et al*, 2004). Sizeable effects temporally related to the actual drug treatment were only observed in normal mice by using much higher doses (1.25 mg/kg/day for 15 days; Nakagawa *et al*, 2002a, b; Fujioka *et al*, 2004). Concerning rats, biochemical effects on the hippocampal cAMP system and restoration of behavioral impairment in normogenic, intact animals has been only reported for substantially higher doses of Rolipram (Giorgi *et al*, 2004; Nagakura *et al*, 2002; Egawa *et al*, 1997).

Conditioned fear, while apparently a simple form of learning, is actually a rather complex phenomenon from a neurobiological point of view. The exact role of the hippocampus in the process leading to memory retention in this learning task has been discussed (Sanders *et al*, 2003). Recent data point at the hippocampus as the central memory structure for fear conditioning, not only concerning the spatial representation of the context but also the other elements used to remind the whole contextual situation, such as the tone (Quinn *et al*, 2002; Sanders *et al*, 2003). The central role of the hippocampal-related contextual recognition in our experiments was strongly supported by the fact that rats trained by exposure to tone and shock immediately after entering the training chamber, without being allowed to preventively explore the context, displayed only very low freezing, as previously described by others (Fanselow, 1986, 1990; Westbrook *et al*, 1994). We observed, in addition, that these animals showed no increase in hippocampal CREB phosphorylation when assayed 24 h after training (data not shown).

Extinction of contextual fear memory has been recently studied in transgenic mice that overexpress type-1 adenylyl cyclase in their forebrain (Wang *et al*, 2004). These authors found a trend similar to the one described here, with transgenic mice showing a slower memory extinction rate compared to wild-type animals (Wang *et al*, 2004). However, in transgenic animals, higher levels of CREB phosphorylation are constitutively present (Wang *et al*, 2004). In our model, instead, Rolipram release, and the consequent increase of CREB phosphorylation, ended shortly (approximately 24 h) after the initial memory test. Memory extinction after Pavlovian fear conditioning is not a simple process of progressive forgetting, but rather a new learning experience during which the animal shifts from conditioned stimulus–unconditioned stimulus to a new conditioned stimulus–no unconditioned stimulus association (Rescorla, 2001; Suzuki *et al*, 2004). There is also evidence that the memory extinction signaling pathways are different from those involved in memory consolidation (Lin *et al*, 2003; Szapiro *et al*, 2002; Fischer *et al*, 2004). Our data demonstrate through a novel experimental procedure of subchronic PDE inhibition the pivotal role of hippocampal CREB activation in memory consolidation (Bourtchuladze *et al*, 1994; Impey *et al*, 1998; Taubenfeld *et al*, 1999, 2001a, b; Athos *et al*, 2002). At the same time, our results do not support a direct role of CREB activation in memory extinction, as the slowing down of the extinction process

occurred in our model when Rolipram was not released any more. The pharmacological upregulation of CREB activation was, therefore, absent during the extinction period, as the subchronic Rolipram treatment did not result in any long-term increase of P-CREB/CREB *ratio* (or Arc expression as well). From these results, it could be concluded that it is the stronger initial memory, associated to higher initial levels of CREB activation, that is important in slowing down the memory extinction rate, possibly by counteracting the mechanisms of consolidation of the new learning.

Arc is an immediate early gene whose expression is highly regulated by synaptic activity, in LTP and memory consolidation (Lyford *et al*, 1995; Guzowski *et al*, 2000, 2001; Lee *et al*, 2004). The present data are in line with these previous results and strengthen the functional link between Arc expression and CREB activation. Arc has been shown to be regulated by the cAMP/PKA/CREB cascade, even if its promoter does not contain any CRE consensus elements, thus suggesting an indirect way of regulation (Waltereit *et al*, 2001). Our data not only demonstrate that Arc is induced by conditioned fear learning, but also strengthen the role of the cAMP signaling pathway in Arc induction in hippocampus-dependent memory.

In conclusion, our data define the conditions through which the pharmacological manipulation of hippocampal CREB expression and activation result in parallel enhancement of memory consolidation and slowing down of memory extinction in normogenic, intact animals. In addition to their relevance for the study of molecular correlates of memory, the present results may be important in view of the efforts to design new pharmacological treatments, targeting the CREB pathway and leading to enhancement of learning and memory, even in the absence of patent neuropathology (Barco *et al*, 2003; Jackson and Ramaswami, 2003).

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