

Brief Communication

Exchange protein activated by cAMP enhances long-term memory formation independent of protein kinase A

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It is well established that cAMP signaling within neurons plays a major role in the formation of long-term memories—signaling thought to proceed through protein kinase A (PKA). However, here we show that exchange protein activated by cAMP (Epac) is able to enhance the formation of long-term memory in the hippocampus and appears to do so independent of PKA, thus demonstrating the importance of Epac-mediated signaling in memory consolidation.

The molecular mechanisms underlying long-term memory consolidation mediated by cyclic adenosine monophosphate (cAMP) signaling have been extensively studied. It is thought that cAMP regulates memory formation mainly by activating the cAMP-sensitive PKA. Once activated, PKA can then phosphorylate various downstream kinases and transcription factors required for memory formation (Abel and Nguyen 2008). However, with the discovery of another major target of cAMP known as Epac (de Rooij et al. 1998; Kawasaki et al. 1998), there may be reason to reconsider the precise nature of cAMP in memory formation. Although Epac-mediated signaling has been previously studied in various *in vitro* preparations, hippocampal long-term potentiation studies (Gelinac et al. 2008; Holz et al. 2008), and now in memory retrieval paradigms (Ouyang et al. 2008; Kelly et al. 2009), its role in long-term memory formation or consolidation has yet to be addressed. In the present study, we examine the effects of Epac activation within the hippocampus on memory consolidation for contextual fear.

The following experiments were conducted with singly housed 2–4-mo-old male C57BL/6J mice (Jackson Laboratories). Animals were provided with water and rodent chow *ad libitum* and maintained on a 12-h light/dark cycle. To study the role of Epac in the hippocampus, pharmacological manipulations were conducted through targeted microinfusions into the dorsal hippocampus. First, mice were anesthetized with isoflurane, placed in a stereotaxic frame (Kopf Instruments), and implanted with a 22-gauge guide cannulae (Plastics One) (stereotaxic coordinates: 1.7 mm posterior to bregma, ± 1.5 mm from midline, and -1.5 mm from the skull surface). Animals were allowed to recover for 1 wk, then handled for 1–2 min on three consecutive days before contextual fear conditioning. Contextual fear memory was examined by placing mice in a standard conditioning chamber (Med Associates) for 2 min, 28 sec after which a single 2-sec footshock was given. Mice were left in the chamber for an additional 30 sec before being returned to their home cage. Long-term memory was assessed by measuring freezing behavior (defined as complete immobility, except for breathing) during a 5-min test session in the same chamber 24 h after training. Drugs were injected bilaterally (0.25 μ L/min for 2 min) through injection cannulae that were connected to Hamilton microsyringes via polyethylene tubes

using a microinfusion pump (Harvard Apparatus). One minute was allowed for diffusion before the injection cannulae were removed. All experiments were conducted in accordance with the policies of the Institutional Animal Care and Use Committee of the University of Pennsylvania and the National Institutes of Health.

To begin our examination of Epac in memory formation, we tested whether a single intrahippocampal infusion of the PDE-resistant cAMP analog Sp-cAMPS (Yusta et al. 1988) would enhance the consolidation of contextual fear memory. Although Sp-cAMPS is commonly used as an activator of protein kinase A (PKA), it is also capable of activating Epac (Christensen et al. 2003). In this experiment, mice were fear conditioned with a single low-intensity footshock (0.75 mA) to better detect drug-related increases in freezing. This procedure is often used to avoid ceiling effects in freezing levels that might be observed if higher footshock intensities are used (Tronson et al. 2006). Immediately after training, mice received 0, 4.5, or 9 μ g of Sp-cAMPS (Sigma-Aldrich) bilaterally into the dorsal hippocampus. Long-term memory was assessed 24 h later by measuring freezing behavior in the training context. Mice that received 4.5 μ g of Sp-cAMPS showed significantly enhanced long-term memory relative to both the vehicle and high-dose groups ($F_{(2,25)} = 4.11$, $P = 0.03$, ANOVA) resulting in an inverted U-shaped dose-response curve (Fig. 1A). Hormetic or biphasic responses of this type are characteristic of a broad range of physiological and behavioral responses and may represent an overcompensation in response to disruptions in homeostasis (Calabrese and Baldwin 2001). Post-hoc analysis revealed that the low-dose groups froze significantly more than either the vehicle or high-dose groups ($P_s < 0.05$, Student–Newman–Keuls). These data and others (Souza et al. 2002) implicate, but cannot distinguish between Epac and PKA signaling in memory consolidation. Indeed, since Epac and PKA recruit, at least to some extent, different downstream effectors (Holz et al. 2008), such a distinction is critical to further understand the nature of memory consolidation.

To address this issue, we repeated the above experiment using the Epac-specific agonist 8-(4-chlorophenylthio)-2'-O-methyladenosine-3', 5'-cyclic monophosphate (8-pCPT-2'-O-Me-cAMP) (Enserink et al. 2002). In designing this cAMP analog, specific chemical group substitutions were made at positions critical for high-affinity binding of cAMP to PKA, thereby generating a potent and highly selective agonist for Epac (Enserink et al. 2002; Christensen et al. 2003; Bos 2006; Ster et al. 2007). Mice were

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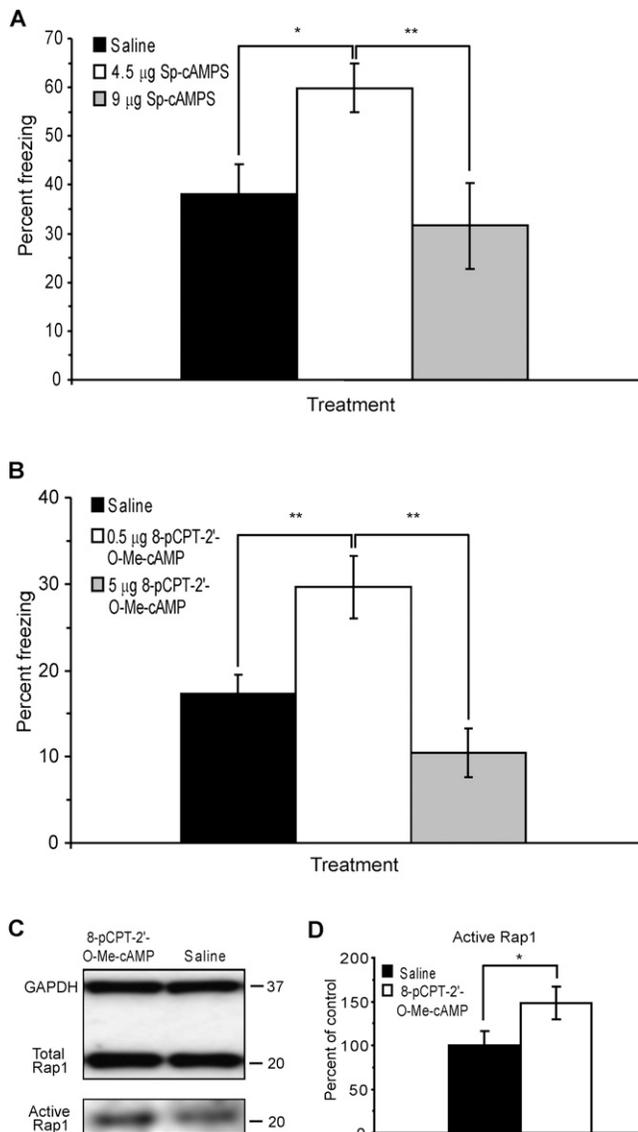


Figure 1. Epac activation in the hippocampus enhances long-term contextual fear memory. Effect of a single post-training intrahippocampal infusion of either (A) the PKA/Epac activator Sp-cAMPS (0 μg, $n = 10$; 4.5 μg, $n = 8$; 9 μg, $n = 10$) (B) or the Epac-specific activator 8-pCPT-2'-O-Me-cAMP (0 μg, $n = 10$; 0.5 μg, $n = 10$; 5 μg, $n = 9$). In both cases, inverted U-shaped dose response curves were obtained where the low doses significantly enhanced memory measured 24 h after fear conditioning. (C) A representative immunoblot of active Rap1 in the hippocampus 5 min after infusion of 0.5 μg 8-pCPT-2'-O-Me-cAMP. (D) Quantitative analysis of active Rap1 immunoreactivity. Significantly higher hippocampal GTP-Rap1 levels were observed in the hemisphere that received 8-pCPT-2'-O-Me-cAMP infusions. Data are expressed as mean ± SEM. * $P < 0.05$, ** $P < 0.01$.

infused with 0, 0.5, or 5 μg of 8-pCPT-2'-O-Me-cAMP (Axxora) and conditioned as described above. ANOVA revealed a significant effect of drug treatment on memory formation ($F_{(2,26)} = 10.68$, $P = 0.0004$), again resulting in an inverted U-shaped dose-response curve (Fig. 1B). Post-hoc analysis showed that this difference was due to enhanced freezing in the low-dose group relative to both the vehicle and high-dose groups ($P_s < 0.01$, Student–Newman–Keuls). These data suggest that the activation of Epac is sufficient to enhance memory consolidation. However, further experiments

were needed to test the behavioral and molecular specificity of the Epac agonist in vivo, and to minimize any confounding effects of PKA.

Since Epac has previously been reported to activate the neuroplasticity-related G protein Rap1 in vitro (de Rooij et al. 1998), we tested whether 8-pCPT-2'-O-Me-cAMP would also activate Rap1 in the hippocampus. Using a within-subject design, separate groups of mice were infused with 8-pCPT-2'-O-Me-cAMP (0.5 μg) in one hippocampus and saline in the other. The hemisphere that received drug was counterbalanced. Hippocampi were then removed and assayed for Rap1 activity. Hippocampal extracts containing 1500 μg of protein were then assayed with a Rap1 activation kit (Upstate). Briefly, lysates were incubated with glutathione–agarose beads precoupled with glutathione-S-transferase (GST) fusion protein, which contains a Rap1-binding domain from the human guanine nucleotide exchange factor specific for the Ras protein Ral. The GST fusion protein precipitates active Rap1 (GTP-Rap1) from the extracts. Samples were then analyzed by Western blotting using a Rap1 antibody (Upstate). As expected, agonist-infused hippocampi had significantly higher

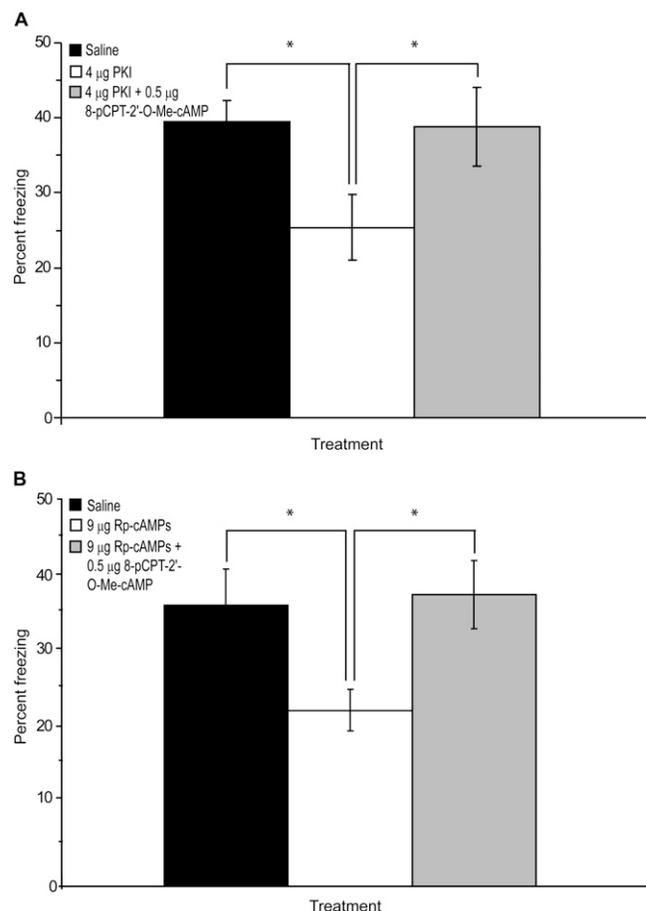


Figure 2. Coinfusion of the Epac-specific activator with a PKA inhibitor leaves fear memory intact. (A) Inhibition of PKA with PKI significantly impaired contextual fear memory relative to saline-treated animals, whereas the combination of PKI and 8-pCPT-2'-O-Me-cAMP blocked this effect (saline, $n = 10$; PKI, 4 μg, $n = 10$; PKI [4 μg] + 8-pCPT-2'-O-Me-cAMP [0.5 μg], $n = 9$). (B) The coinfusion of 8-pCPT-2'-O-Me-cAMP with Rp-cAMPS-enhanced long-term memory relative to Rp-cAMPS infusion alone (saline, $n = 9$; Rp-cAMPS, 9 μg, $n = 9$; Rp-cAMPS [9 μg] + 8-pCPT-2'-O-Me-cAMP [0.5 μg], $n = 10$). Data are expressed as mean ± SEM. * $P < 0.05$.

levels of active Rap1 relative to saline-infused hippocampi ($P = 0.05$, paired t -test; Fig. 1C). Together with previous findings linking Rap1 to hippocampus-based memory formation (Morozov et al. 2003), these results indicate that Epac-mediated memory enhancements may act through an up-regulation in Rap1 signaling.

Due to the possibility that 8-pCPT-2'-O-Me-cAMP might also activate PKA, we examined the effect of the Epac agonist when coinjected with specific inhibitors of PKA. This approach has also been used to validate the action of this agonist in hippocampal long-term potentiation studies (Gelinas et al. 2008) and in various *in vitro* studies (Holz et al. 2008). First, we examined the effects of the Epac agonist when coinjected with the PKA inhibitor PKI-(14-22)-amide (PKI, Assay Designs). This form of PKI is myristoylated on the N terminus to allow cell membrane permeability, and selectively inhibits the free catalytic subunit of PKA (Glass et al. 1989). In this experiment, a single 1.5-mA footshock was used to induce high enough levels of freezing, such that PKA-related memory deficits would be detectable. Figure 2A shows that the infusion of PKI (4 μ g) alone significantly impaired the consolidation of contextual fear memory. However, these deficits were overcome when 8-pCPT-2'-O-Me-cAMP (0.5 μ g) was coinjected with PKI (4 μ g). ANOVA revealed an overall effect of treatment group on freezing ($F_{(2,26)} = 4.71$, $P = 0.02$), where post-hoc analysis demonstrated significant differences in freezing between the PKI group relative to both the vehicle and the Epac agonist + PKI groups ($P_s < 0.05$, Student-Newman-Keuls).

We also examined whether memory deficits induced by the well-characterized PKA inhibitor Rp-cAMPS (Dostmann 1995) could be rescued with the Epac agonist. As expected, mice receiving intrahippocampal infusions of Rp-cAMPS (9 μ g) after a 1.5-mA footshock showed impaired contextual fear memory (Sananbenesi et al. 2002), but displayed normal memory when the Epac agonist (0.5 μ g) and PKA inhibitor were coinjected. ANOVA revealed an overall effect of treatment group on freezing ($F_{(2,25)} = 4.04$, $P = 0.03$), where post-hoc analysis demonstrated significant differences in freezing between the Rp-cAMPS group relative to both the vehicle and the Epac agonist + Rp-cAMPS groups ($P_s < 0.05$, Student-Newman-Keuls). Thus, the data provide strong behavioral evidence that Epac can modulate memory independent of PKA and can rescue deficits caused by reduced PKA signaling.

Next, we determined the specificity of the Epac agonist on a molecular level. To do so, we took advantage of the fact that PKA is specifically required to phosphorylate the GluR1 subunit of AMPA-type glutamate receptors at Ser845 (Lee et al. 2000; Whitlock et al. 2006). Thus, pSer845 levels can be assayed to measure PKA activity and the specificity of the Epac agonist. A within-subjects design was used to measure pSer845 levels after

intrahippocampal infusion of either vehicle, Sp-cAMPS (4.5 μ g), 8-pCPT-2'-O-Me-cAMP (0.5 μ g), or 8-pCPT-2'-O-Me-cAMP (0.5 μ g) + PKI (4 μ g). Mice were sacrificed 15 or 45 min after drug infusion, after which the hippocampi were removed and assayed by Western blotting for phospho- and total GluR1 levels. These time points were chosen to evaluate whether the Epac agonist might activate PKA directly (i.e., with kinetics similar to cAMP) or indirectly, requiring a longer period of time to alter pSer845 levels. Figure 3A shows that mice sacrificed 15 min after Sp-cAMPS infusions had significantly greater pSer845 levels ($P = 0.04$, paired t -test) in the drug-infused side relative to the saline-treated side. Similarly, Sp-cAMPS-treated mice sacrificed 45 min later showed a strong trend toward a significant increase in pSer845 levels ($P = 0.06$, paired t -test), possibly due to the long half-life of Sp-cAMPS. Interestingly, pSer845 levels appear to be increased in the vehicle-treated hippocampi of mice that also received Sp-cAMPS relative to other vehicle groups. This could be due to the propagation of

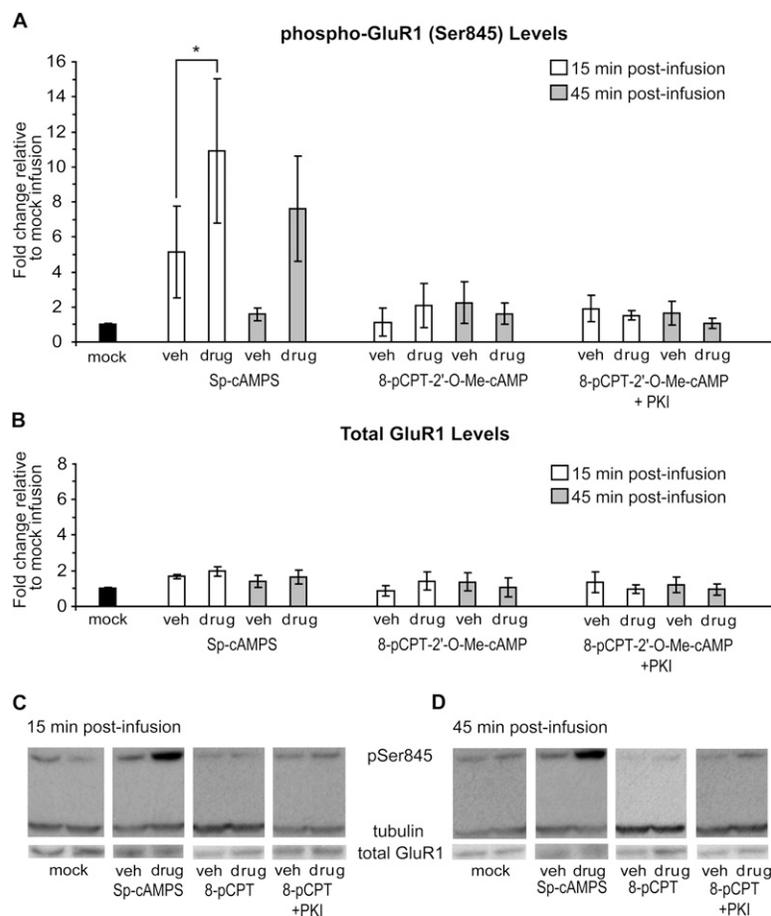


Figure 3. Infusion of the Epac-specific agonist does not change PKA-dependent phosphorylation of GluR1 receptor subunits in the hippocampus. Phospho-GluR1 (pSer845) (A) and total GluR1 (B) levels in the hippocampus 15 or 45 min post-drug infusion (open and shaded bars, respectively). (A) pSer845 levels were significantly increased 15 min after unilateral infusion of Sp-cAMPS (4.5 μ g) relative to the saline-treated side ($*P = 0.04$). Infusions of the Epac agonist or the combination of the agonist and PKI had no effect at either time point relative to their respective vehicle-treated hippocampi (8-pCPT-2'-O-Me-cAMP, 0.5 μ g; PKI [4 μ g] + 8-pCPT-2'-O-Me-cAMP [0.5 μ g]). A strong trend ($P = 0.06$) toward an increase in pSer845 levels was observed for the Sp-cAMPS-infused hippocampi at 45 min. $n = 5$ for all treatment groups and time points. (B) Total GluR1 levels were unaffected by drug treatments at either time point. (C,D) Representative immunoblots of pSer845 (1:2000, Millipore), β -tubulin (1:20000, Sigma), and total GluR1 (1:2000, Millipore) levels 15 min (C) or 45 min (D) after infusion. All values were normalized to β -tubulin levels within individual samples to control for differences in total protein loaded, and then to mice that received mock injections (black bars in A and B, $n = 4$). Data are expressed as mean \pm SEM.

activity induced in the drug-treated hemisphere to the contralateral side via commissural connectivity. Nevertheless, the Epac agonist alone had no effect on pSer845 levels, nor did the agonist in combination with PKI at either time point ($P_s > 0.05$, paired t -tests). Finally, total GluR1 levels were not significantly altered in any of the treatment groups or time points ($P_s > 0.05$, paired t -tests; Fig. 3B). Thus, the memory enhancements observed after treatment with the Epac agonist and the ability of the agonist to rescue PKA-related memory deficits were not due to increases in PKA activity and suggest that Epac can, indeed, influence memory consolidation independent of PKA.

The purpose of the present study was to determine whether the cAMP-sensitive molecule Epac can influence memory formation previously thought to occur mainly through the activity of PKA. Our findings demonstrate that Epac is able to modulate memory consolidation, thereby broadening our understanding of cAMP signaling in memory formation. While there may be some interaction between the Epac and PKA signaling pathways (Nijholt et al. 2008), the nature of this interaction and its effect on memory consolidation remains to be determined. It is worth noting that cAMP-dependent PKA signaling does not have uniform effects on memory formation within all brain regions (Arnsten et al. 2005). Likewise, the effects of Epac activation in different regions will require further investigation. Importantly, these results provide the basis for the development of novel cognition-enhancing drugs that target specific components of the cAMP–Epac signaling pathway, rather than broad activators of cAMP (e.g., rolipram) (Bach et al. 1999). Indeed, such an approach might be warranted in that mutations in the Epac gene and altered levels of expression have been linked to autism (Bacchelli et al. 2003) and Alzheimer's disease (McPhee et al. 2005), respectively.

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