Exchange protein activated by cyclic AMP 2 (Epac2) plays a specific and time-limited role in memory retrieval

Anghelus Ostroveanu¹, Eddy A. van der Zee¹, Ulrich L.M. Eiselt¹, Martina Schmidt², Ingrid M. Nijholt¹

¹ Department of Molecular Neurobiology, ² Department of Molecular Pharmacology, Graduate School of Behavioral and Cognitive Neurosciences, University of Groningen, The Netherlands

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Abstract

Knowledge on the molecular mechanisms involved in memory retrieval is limited due to the lack of tools to study this stage of the memory process. Here we report that exchange proteins activated by cAMP (Epac) play a surprisingly specific role in memory retrieval. Intrahippocampal injection of the Epac activator 8-pCPT-2’O-Me-cAMP was shown to improve fear memory retrieval in contextual fear conditioning whereas acquisition and consolidation were not affected. The retrieval enhancing effect of the Epac activator was even more prominent in the passive avoidance paradigm. Downregulation of Epac2 expression in the hippocampal CA1 area impaired fear memory retrieval when the memory test was performed 72 h after training, but not when tested after 17 days. Our data thus identify an important time-limited role for hippocampal Epac2 signaling in cognition and opens new avenues to investigate the molecular mechanisms underlying memory retrieval.

Key words: fear, conditioning, learning, cognition, hippocampus, mouse
Introduction

To date significant advances have been made in understanding the neurophysiological basis of learning and memory. In particular, cyclic adenosine monophosphate (cAMP) signaling was shown to play a pivotal role. Originally cAMP-dependent protein kinase (PKA) was thought to be the major if not the sole effector of cAMP and its importance in memory consolidation is now widely acknowledged (Abel & Nguyen, 2008). However, fairly recently, a new effector of cAMP signaling has been identified named exchange protein directly activated by cAMP (Epac). In independent studies, two variants of the Epac protein, namely Epac1 (also called cAMP-GEF-I) and Epac2 (also called cAMP-GEF-II), were characterized (de Rooij et al., 1998; Kawasaki et al., 1998). Both Epac proteins are multi-domain proteins that function as guanine-nucleotide-exchange factors (GEFs) for Rap1 and Rap2, members of the Ras superfamily of small GTPases. Activation of Epac by cAMP leads to activation of Rap1 and Rap2, which then act as molecular switches on downstream signaling cascades. While Epac1 has one cAMP binding domain, Epac2 possesses a similar additional domain, the biological function of which is still unknown (Bos, 2006). The two Epac variants also differ in their expression patterns. Epac1 has been found to be expressed ubiquitously, whereas expression of Epac2 was found mainly in adrenal glands and brain tissue (Kawasaki et al., 1998).

Since their discovery, Epac proteins have been found to control key cellular processes, including cellular calcium handling, integrin-mediated cell adhesion, gene expression, cardiac hypertrophy, inflammation, and exocytosis (Roscioni et al., 2008). However, the exact nature of any involvement that Epacs have in neuronal function, has only recently begun to be investigated. Epac was shown to enhance neurotransmitter release in glutamatergic synapses (Sakaba & Neher, 2003; Zhong & Zucker, 2005; Gekel & Neher, 2008), whereas in cerebellar granule cells it can modulate neuronal excitability (Ster et al., 2007). In dorsal root ganglion Epac mediates the translocation and activation of protein kinase C (PKC)ε leading to the establishment of inflammatory pain (Hucho et al., 2005) and promotes neurite outgrowth (Murray & Shewan, 2008). In spinal cord tissue Epac advances neurite regeneration (Murray & Shewan, 2008).
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Thus far, evidence for a role of Epac in the process of learning and memory is limited. However, since Epac is a cAMP-responsive enzyme and cAMP signaling is established to be of critical importance in learning and memory, an involvement of PKA-independent cAMP signaling through Epac proteins can be expected. Indeed the first indications for a role of Epac in hippocampus-dependent learning and memory came from very recent studies. Gelinas and colleagues reported that Epac activation enhances the maintenance of LTP in area CA1 of mouse hippocampal slices (Gelinas et al., 2008) and co-application of a selective PKA and a selective Epac activator was shown to rescue the memory retrieval impairment observed in dopamine-beta-hydroxylase deficient mice whereas application of the Epac activator alone had no effect (Ouyang et al., 2008). In the current study we investigated the role of Epac signaling in the different phases of the memory process; acquisition, consolidation and retrieval. Epac signaling via Epac2 was shown to play a specific and time-limited role in memory retrieval.

Materials and methods

Animals and housing conditions
Male C57BL/6J mice (Harlan, Horst, the Netherlands), 9 to 12 weeks old, were individually housed in standard macrolon cages. Subjects were maintained on a 12 hour light/dark cycle (lights on at 7.30 a.m.) with food (hopefarm® standard rodent pellets) and water ad libitum. A layer of sawdust served as bedding. The procedures concerning animal care and treatment were in accordance with the regulations of the ethical committee for the use of experimental animals of the University of Groningen (DEC 4174I-K).

Cannulation
Double guide cannulae (C235, Plastics One, Roanoke, VA) were implanted using a stereotactotic holder during 1.2 % avertin anesthesia (0.02 ml/g, i.p.) under aseptic conditions (Nijholt et al., 2008). The cannulae were placed into both dorsal hippocampi (intra hippocampal; i.h.), AP –1.5 mm, lateral 1 mm, depth 2 mm (Franklin & Paxinos, 1997). The animals were allowed to recover for 6-7 d before the experiments started.
Bilateral injections were performed during a short anesthetic period of isoflurane inhalation using a syringe pump (TSE systems, Bad Homburg, Germany) at a constant rate of 0.33 µl/min (final volume: 0.3 µl per side). The exact site of injection was confirmed after the behavioral experiments by injection of methylene blue solution into each hemisphere and subsequent histological evaluation (Fig. 5A). Data were evaluated only from those mice that received an injection at the correct target site.

**Drug treatment**

The Epac activator 8-pCPT-2’O-Me-cAMP (Biolog, Bremen, Germany) was injected in a final concentration of 1 mM (300 ng/brain) in artificial cerebrospinal fluid (ACSF) solution of the following composition (in mM): 130 NaCl, 3.5 KCl, 1.25 NaH₂PO₄, 1.5 MgSO₄, 2 CaCl₂, 24 NaHCO₃, and 10 glucose (pH 7.4). 8-pCPT-2’O-Me-cAMP was stored as a 100 mM stock solution in H₂O. A separate set of animals was injected with vehicle (ACSF pH=7.4). Untreated animals without cannula served as controls for possible cannulation and injection effects.

**Fear Conditioning**

Fear conditioning was performed as described before (Nijholt et al., 2008) in a Plexiglas cage (44 x 22 x 44 cm) with constant illumination (12 V, 10 W halogen lamp, 100-500 lux). The training (conditioning) consisted of a single trial. The mouse was exposed to the conditioning context for 180 sec followed by a footshock (0.7 mA, 2 sec, constant current) delivered through a stainless steel grid floor. The mouse was removed from the fear conditioning box 30 sec after shock termination to avoid an aversive association with the handling procedure. Memory tests were performed 24 hr, 72 h or 14 days after fear conditioning. Contextual memory was tested in the fear conditioning box for 180 sec without footshock presentation. Freezing, defined as the lack of movement except for respiration and heart beat, was assessed as the behavioral parameter of the defensive reaction of mice by a time-sampling procedure every 10 s throughout memory tests. In addition, mean activity of the animal during the training and retention test was measured with the Ethovision system (Noldus, The Netherlands).
Passive avoidance
Passive avoidance experiments were performed in a plexiglas cage (44 x 22 x 44 cm) consisting of a dark compartment (22 x 22 x 20 cm) equipped with a stainless steel grid floor and a light compartment (22 x 22 x 44 cm) with a plastic floor. Both compartments were separated by a guillotine door. The light compartment was brightly illuminated by a 100 W bulb. Mice were habituated to the experimental set-up during three sessions 30, 24 and 6 hr prior to the training session. During habituation sessions, the mouse was introduced into the light compartment facing the closed guillotine door. After 60 sec the door was opened and the mouse was allowed to enter the dark compartment. Upon entering the dark compartment the door was closed and the mouse was allowed to explore the compartment for 60 sec. During the training session, the mouse was again introduced into the light compartment, and the guillotine door was opened after 60 sec. Latency (defined as the time between the opening of the door and the mouse entering the dark compartment with all four paws) was recorded for each animal. Upon entering the dark chamber the door was closed and a single footshock (0.3 mA, 2 sec, constant current) was delivered to the mouse. The mouse was removed from the apparatus 30 sec after shock termination to avoid an aversive association with the handling procedure. Memory tests were performed 24 hr after training. During the memory test the guillotine door was opened 60 sec after introducing the mouse into the light compartment and left opened for maximally 480 sec. During this time period, latency to enter the dark compartment was recorded and assessed as the behavioral parameter. If a mouse did not enter the dark compartment, latency was set to 480 sec.

Elevated plus maze
Elevated plus maze experiments were performed using a plus maze (50 cm above the floor) with two opposite closed and two opposite open arms (50 cm long, 5 cm wide). The mouse was placed in the central zone of the plus maze, facing an open arm and allowed to explore the maze for 480 sec. Time spent in dark arms, open arms and center compartment were recorded for each animal with the Ethovision system (Noldus, The Netherlands). The ratio of time spent in the open arms to total time spent in the maze was calculated for each group.
of mice and taken as a measure of anxiety-related behaviour, with a higher ratio being indicative of lower anxiety levels.

**Immunohistochemistry**

30 µm thick coronal sections of C57Bl/6J mice perfused with 4 % paraformaldehyde, were preincubated with 0.3 % H2O2 to reduce endogenous peroxidase. Non-specific binding sites were blocked by preincubating the sections with 5 % normal goat serum in 0.01 M PBS for 30 min. Subsequently, sections were probed with antibodies specific for Epac1 (from 1:300 to 1:1000, several batches [1C8, 4D9 and 5D3] kindly provided by J. Bos, University Utrecht, the Netherlands and Epac1 A5, sc-28360, Santa Cruz) or Epac2 (1:1000) (2B12, provided by J. Bos) in 0.01 M PBS containing 5 % normal goat serum and 0.3 % Triton X-100 for 2 h at room temperature (RT) and subsequently for 72 h at 4 °C. After several washing steps, sections were incubated with biotin SP-Conjugated AffiniPure goat anti mouse secondary antibody overnight at 4 °C (1:400) (115-065-166, Jackson Laboratories INC) followed by the ABC complex (Vector ABC kit). For visualization, DAB was used as a chromogen (Sigma fast tablet set). Sections were examined using light microscopy. The specificity of Epac antibodies was assessed by parallel staining without primary antibodies. In these sections we could not observe any staining (data not shown). Photographs were taken with a DM1000/DFC280 Leica image analysis system (Leica, Cambridge, UK).

**Semi-quantitative RT-PCR**

To determine Epac1 and Epac2 mRNA levels in the mouse hippocampus, total RNA was extracted from a single hippocampus of a naive mouse (n=5). Total RNA was isolated according to the manufacturer’s protocol (NucleoSpin RNA II kit, Macherey-Nagel, 740955.250). RT-PCR was performed using Superscript III One-Step RT-PCR with Platinum Taq DNA Polymerase (Invitrogen, 12574) as described before (Nijholt et al., 2004). 120 ng of RNA was used for each RT-PCR reaction. The reverse transcriptase reaction was performed at 55 °C for 30 min. PCR cycling was at 94 °C for 15 sec, annealing at 55 °C for 30 sec, extension at 72 °C for 30 sec and a final extension at 72 °C
for 10 min. 10 µl of each sample was removed every 3 cycles from 25 to 37 cycles in each reaction to amplify Epac1 and Epac2.

To test the efficiency of the siRNA probes, hippocampi were collected 24 hr after the last siRNA injection and the injection site excised. Total RNA isolation and the reverse transcriptase reaction were performed as described above. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) served as control housekeeping gene. 10 µl of each sample was removed every 3 cycles from 27 to 50 cycles in each reaction to amplify Epac1, Epac2 and HPRT fragments.

Amplified PCR products were separated on 2 % agarose gels with Tris-borate EDTA buffer and stained with SYBR Green (Invitrogen). Gels were captured as a digital image and quantified by densitometry. Primer sequences for Epac1 were: forward 5’-GTTGTCGACCACAGGAAGT-3’ and reverse 5’-ACCCAGTACTGCAGCTCGTT-3’, for Epac 2 were: forward 5’-CATGAGGGGAACAAGACGT-3’ and reverse 5’ GGCC-TTCGAGGCTCTAATCT 3’ and for HPRT forward primer 5’-CCTGACTGATTACATT-AAAGCACTG-3’ and reverse 5’-CCTGAAGTACTCATTATAGTCAAGG-3’.

In vivo siRNA transfection
Mice injected i.h. with 50 ng siGLO Green (25 ng/hippocampus; D-001630-01-05, Dharmaco, Inc. Lafayette, CO, USA), were sacrificed 6, 24 or 48 hr post injection. The brain hemispheres were placed in a 4 % PFA solution for 24 hr, followed by 48 hr 30 % sucrose immersion. Afterwards, 30 µm thick coronal sections were stained with DAPI (1:5000) in PBS 0.01 M. After a quick washing step in PBS 0.01 M, sections were mounted, dried and analyzed under a Leica fluorescent microscope.

ON-TARGET plus SMART pool mouse RAPGEF3 (Epac1 siRNA) and RAPGEF4 (Epac2 siRNA) probes were purchased from Dharmacon, (Dharmacon, USA). The target sequences for the mouse-specific Epac1 siRNAs mixture were as follows: sense: CCAGGCAGGAACCGGUAUAU (J-057800-09); sense: GAUCUUUGUUCACGGC-CAAU (057800-10); sense: GGUCAAUUCUGCCGGUGAUAU (057800-11) and sense: CCACCAUCAUCCUUGAAGAU (057800-12). The target sequences for the mouse-specific Epac2 siRNAs mixture were: sense: CGAAGACUUGCAGGAACAU (J-057784-05); sense: CAAGUUAGCCUCUAGGAACAU (J-057784-06); sense: GACAGA-
AAGUACCACCUAUU (J-057784-07) and sense: GGAGGAACUGUGUUGUUUUAAUU. ON-TARGETplus Non-targeting Poll siRNA (D-001810-10) was used as control (Dharmacon, USA). siRNAs were resuspended in RNAse free water. In vivo siRNA brain delivery was performed using jetSI 10 mM cationic polymer transfection reagent (Polyplus transfection Inc., New York) according to the transfection protocol of the manufacturer. 50 ng siRNA was injected i.h. on three consecutive days 3, 24 and 48 hr after training or on the three days prior to the second retention test.

**Statistics**
Statistical comparisons were made by analysis of variance (ANOVA). For each significant $F$ ratio, Fisher's protected least significant difference (PLSD) test was used to analyze the statistical significance of appropriate multiple comparisons. Data were expressed as mean ± s.e.m. Significance was determined at the level of $p < 0.05$.

**Results**

**Intrahippocampal Epac activation facilitates memory retrieval in contextual fear conditioning**
The role of Epac in the different stages of the memory process was investigated using one trial contextual fear conditioning. Contextual fear conditioning is a hippocampus-dependent form of associative learning in which animals learn to fear a new environment because of its temporal association with an aversive mild electrical footshock. When injected intrahippocampally (i.h.) 20 min before training, the specific Epac activator 8-pCPT-2’O-Me-cAMP caused no significant change in freezing behavior during the retention test 24 hr after training in comparison to vehicle-injected and untreated mice (one-way ANOVA: $F(2,25) = 1.110; p = 0.312$, Fig. 1A). Injection of 8-pCPT-2’O-Me-cAMP or vehicle did not result in changes in mean activity or shock reactivity during training (data not shown). Moreover, no significant difference in freezing behavior was observed between groups during the retention test when 8-pCPT-2’O-Me-cAMP was injected immediately after training (one-way ANOVA: $F(2,18) = 0.032; p = 0.969$, Fig. 1B).
To determine the effect of Epac activation on the retrieval of fear memory, mice were injected i.h. with 8-pCPT-2′O-Me-cAMP or vehicle 20 min before the retention test. Injection of 8-pCPT-2′O-Me-cAMP resulted in a significant increase in freezing behavior during the retention when compared to vehicle-injected and untreated animals (one-way ANOVA: F(2,24) = 5.550, p = 0.010; Fig. 1C).

Fig. 1. Intrahippocampal injection of Epac activator 8-pCPT-2′O-Me-cAMP (1 mM) facilitates the retrieval of contextual fear memory. Mice were injected either 20 min before training (A, immediately after training (B), or 20 min before retention (C) with 8-pCPT-2′O-Me-cAMP (1 mM) or vehicle. Untreated and vehicle-injected mice served as controls. Freezing behaviour was measured in the memory test 24 h after training. Error bars indicate standard error of the mean. Statistically significant differences: *p < 0.05 versus control groups.

Taken together, these data show that Epac activation in the hippocampus modulated the retrieval of contextual fear memory, but not acquisition or consolidation.

**Intrahippocampal Epac activation facilitates memory retrieval in passive avoidance**

The effect of i.h. 8-pCPT-2′O-Me-cAMP injection on memory retrieval was also tested in the passive avoidance task. In this one trial fear-motivated avoidance task the animal learns to refrain from stepping through a door to an apparently safer but previously punished dark compartment. It is considered to be more complex than fear conditioning due to the combination of classical Pavlovian conditioning with the manifestation of an active response. Mice were habituated to the experimental set-up during three sessions prior to the
training session. We did not observe any difference between groups in their latencies to enter the dark compartment during the training session (one-way ANOVA: $F(2,23) = 0.917, p = 0.414$, Fig. 2A). The next day, one group of mice was injected i.h. with 8-pCPT-2’O-Me-cAMP (1 mM) 20 min before the retention test. Untreated and vehicle injected mice served as controls. Mice injected with 8-pCPT-2’O-Me-cAMP showed a significantly longer latency to enter the dark compartment when compared to the control groups (one-way ANOVA: $F(2,23) = 4.650, p = 0.020$, Fig. 2B). Overall, the memory retrieval enhancing effect of 8-pCPT-2’O-Me-cAMP in the passive avoidance paradigm was even more prominent than in fear conditioning.

Fig. 2. Intrahippocampal injection of the Epac activator 8-pCPT-2’O-Me-cAMP (1 mM) facilitates memory retrieval in the passive avoidance paradigm. Mice were habituated to the experimental set-up during three sessions. Mice were injected with 8-pCPT-2’O-Me-cAMP or vehicle 20 min before the retention test. Untreated and vehicle-injected mice served as controls. Latency to enter the dark compartment during training (A) and the retention test (B). Error bars indicate standard error of the mean. Statistically significant differences: *p < 0.05 versus control groups.

**Intrahippocampal Epac activation does not affect anxiety**

The performance of the mice in the retention tests may be influenced by the level of anxiety the animal experiences. Therefore, we tested the effect of 8-pCPT-2’O-Me-cAMP on anxiety behavior in an elevated plus maze. Intrahippocampal 8-pCPT-2’O-Me-cAMP (1 mM) injection 20 min before exposure to the elevated plus maze test did not specifically affect anxiety behavior (one-way ANOVA: $F(2,19) = 1.741; p = 0.202$, Fig 3). Cannulated
animals, i.e. 8-pCPT-2’O-Me-cAMP-injected and vehicle-injected mice, did show slightly, but not significantly higher levels of anxiety, which can be explained by the surgery procedure these animals underwent 6-7 days prior to testing in the elevated plus maze.

Since injection of 8-pCPT-2’O-Me-cAMP did not affect anxiety in the elevated plus maze test, the effect of Epac activation by 8-pCPT-2’O-Me-cAMP in the fear-motivated learning tasks can be ascribed to enhanced memory retrieval of the association between the electric footshock and the context.

**Epac expression in the mouse brain**

Next we determined the distribution of Epac1 and Epac2 in the mouse brain. Epac2 was shown to be abundantly expressed throughout the entire mouse brain (Fig. 4A).

High levels were detected in the cortex, hippocampus and thalamus. In the hippocampal cellular layers such as the stratum pyramidale (Py) and the granule layer (GCL) immunoreactivity was rather low whereas the basal and apical dendrites [stratum oriens (SO), stratum radiatum (SR), the stratum lacunosum moleculare (SLM) and molecular layer (ML)] showed a high Epac2 expression (Fig. 4B). For Epac1 staining, four different Epac1 antibodies (up to antibody saturation levels) were tested. Although positive Epac1 staining was observed with these antibodies in lung and heart tissue (M. Schmidt, unpublished data), no positive Epac1 staining could be detected in the brain (data not shown). Thus, it appears that Epac1 expression is very low in mouse brain. Moreover, semi-quantitative RT-PCR for
Epac1 and Epac2 with mRNA isolated from the hippocampus, showed that Epac2 mRNA could be detected much earlier as Epac1 mRNA (Fig. 4C).

Our data are consistent with a previous study from Kawasaki and colleagues who also reported a high expression of Epac2 in the rat brain whereas Epac1 was barely detectable (Kawasaki et al., 1998).

**Intrahippocampal Epac2 siRNA injection impairs fear memory retrieval**

To investigate the role of hippocampal Epac2 in memory retrieval, we specifically downregulated Epac2 expression before the memory test using in vivo lipid mediated siRNA gene silencing. A previous study already showed the efficient downregulation of Epac2 expression by these siRNA probes in in vitro neuronal cell cultures (Nijholt et al., 2008). To check for siRNA transfection efficiency in the in vivo mouse brain, we first injected mice i.h. with fluorescent siGLO green (Fig. 5A,B). A single bilateral injection of siGLO green resulted in a strong fluorescent signal in the pyramidal cell layer of the CA1
area already as early as 6 hr after injection. The signal lasted at least up to 48 h after injection. Other brain areas were not affected by the treatment.

Downregulation of Epac2 expression by i.h. injection of specific siRNA probes on three consecutive days was verified by semi-quantitative RT-PCR on the fourth day. Injection of Epac2 siRNA resulted in a 47 % reduction of hippocampal Epac2 mRNA (Fig 5C,D). The low level of Epac1 mRNA was not affected by the transfection with Epac2 siRNA.

Fig. 5. Efficient downregulation of hippocampal Epac2 expression by in vivo siRNA transfection. A. left panel: coronal brain section of the mouse brain atlas (Franklin and Paxinos, 1997), black dot indicates the injection site, right panel: photomicrograph showing a representative detail of a coronal brain section after hippocampal injection of methylene blue counterstained with nuclear fast red. DG, dentate gyrus; CA1, CA1 area of the hippocampus; CA3, CA3 area of the hippocampus. B. Representative fluorescent microphotographs showing siGLO green transfection into the pyramidal neurons of the CA1 area indicated by an arrow. A DAPI staining was used to identify nuclear staining. C. Bar graphs show the ratio of Epac2 mRNA band intensities verified to be within the linear range of product accumulation, divided by those of the coamplified HPRT product after 34 cycles. Statistically significant difference: *p < 0.05 versus control groups. D. Bands reflect the levels of Epac1 and Epac2 mRNA expression after 34 cycles.

In the behavioral experiments, mice were injected i.h. with Epac2 siRNA (50 ng/brain) 3 hr, 24 hr and 48 hr after training in a contextual fear conditioning paradigm (Fig. 6A). Epac2 siRNA injection completely abolished the 8-pCPT-2’O-Me-cAMP-induced enhancement of retrieval and already caused a significant decrease in freezing behavior by
илось during the first retention test (one-way ANOVA: \( F(4,37) = 9.187; p = 0.001 \), Fig. 6B). In the scrambled siRNA injected animals 8-pCPT-2’O-Me-cAMP injection again improved memory retrieval. Interestingly, when the Epac2 siRNA injected animals were re-exposed to the conditioning box 14 days after the first retention test, they showed high freezing levels that were comparable to untreated or control siRNA injected mice (one-way ANOVA: \( F(4,35) = 0.862; p = 0.496 \), Fig. 6C). Injection of Epac2 siRNA on three consecutive days prior to this delayed retention test also did not affect freezing in any of the groups (Fig. 6C).

Fig. 6. Intrahippocampal injection of Epac2 siRNA impairs memory retrieval in contextual fear conditioning. Mice were injected with Epac2 or control siRNA 3 hr, 24 hr and 48 hr after training. Untreated mice served as additional controls. A. Experimental protocol B. Freezing behaviour was measured in the memory test 24 h after the last siRNA injection. C. Freezing behavior assessed during the second retention test 14 days after the first retention test. Where indicated this retention test was preceded by Epac2 siRNA injections on three consecutive days. Error bars represent standard error of the mean. Statistically significant differences: *\( p < 0.05 \) versus control groups.
Discussion

In contrast to memory formation, the knowledge about the molecular mechanisms of memory retrieval is surprisingly limited due to the lack of tools to study this phase of the memory process. Most studies on memory using brain lesion and/or gene manipulation techniques cannot distinguish between effects on the molecular mechanisms of acquisition or consolidation of memories and those responsible for their retrieval from storage. Using the specific Epac activator 8-pCPT-2’O-Me-cAMP, we observed a surprisingly specific role of Epac signaling in associative fear memory retrieval whereas acquisition and consolidation were not affected.

Ouyang and colleagues also recently reported a role for Epac signaling in memory retrieval (Ouyang et al., 2008). However, their design did not allow the investigation of Epac signaling in the different phases of the memory process. In their study, the memory retrieval impairment observed in dopamine-beta-hydroxylase deficient mice could be rescued by i.h. injection of a selective PKA activator together with a selective Epac activator whereas injection of one of the activators alone did not overcome the retrieval deficit. From these data they concluded that cAMP signaling via both Epac and PKA is required for retrieval (Ouyang et al., 2008). We report here that Epac activation alone can significantly improve memory retrieval in contextual fear conditioning. This retrieval-enhancing effect was even stronger in a passive avoidance paradigm.

Since 8-pCPT-2’O-Me-cAMP activates both Epac1 and Epac2 (Enserink et al., 2002), it was not possible to distinguish between the contribution of both Epac variants to the facilitation of memory retrieval. However, the finding in our and other studies that Epac2 is abundantly expressed in mouse brain whereas Epac1 is hardly detectable (Kawasaki et al., 1998) together with our finding that downregulation of Epac2 expression in the hippocampus impairs memory retrieval, strongly suggests a role for Epac2 in memory retrieval. Interestingly, Epac2 silencing only led to impaired memory retrieval 3 days after conditioning whereas Epac2 silencing during the retention test 17 days after conditioning had no effect on memory retrieval indicating a time-limited function of Epac2 signaling after conditioning. These data are consistent with earlier data showing that signaling by
norepinephrine through the beta1-adrenergic receptor is also only required for an intermediate term of memory retrieval (Murchison et al., 2004). Since beta1-adrenergic receptors couple to cAMP via Gs, it is likely that this result is at least in part mediated by Epac2. In line with these findings activation of beta adrenergic receptors and 8-pCPT-2’O-Me-cAMP were shown to recruit similar mechanisms to facilitate long-lasting hippocampal LTP (Gelinas et al., 2008). Also in several other cell systems such as the heart and vascular smooth muscle cells, a strong connection between beta-adrenergic signaling and Epac has already been established (Jensen, 2007; Métrich et al., 2008).

From our finding that freezing was low in the Epac2 siRNA injected animals 3 days after conditioning but comparable to untreated and control siRNA injected animals 17 days after conditioning it can be concluded that Epac2 silencing in the hippocampus transiently affects memory retrieval instead of having a long-lasting effect on consolidation. Overall our data strengthen the hypothesis that retrieval may become independent of the hippocampus over time (McClelland et al., 1995; Squire et al., 2001; Wiltgen et al., 2004; Morris, 2006). However we cannot completely exclude the possibility that hippocampal signaling mechanisms other than Epac2 are involved in the delayed memory retrieval.

The subcellular mechanism by which Epac2 modulates the retrieval of fear memory still remains to be elucidated. Although little information is available on downstream molecules of Epac signaling in the hippocampus, Epacs are known to function as cAMP-mediated guanine nucleotide-exchange factors (GEFs) activating the small GTP-ase proteins Rap1 and Rap2 (Bos, 2006). Indeed, Ouyang and colleagues reported on unpublished data that the expression of a dominant-negative Rap construct in the dorsal hippocampus impairs memory retrieval in a manner identical to antagonists of beta1-adrenergic receptors, cAMP and PKA (Ouyang et al., 2008). Epac-Rap signaling has been reported to activate p42/p44 mitogen-activated protein kinases (p42/p44 MAPK; ERK1/2) in cultured rat hippocampal neurons (Lin et al., 2003). Moreover, application of 8-pCPT-2’O-Me-cAMP leads to a transient increase in p42/44 MAPK immunoreactivity in hippocampal slices of the CA1 area (Gelinas et al., 2008). Overall, MAPKs may have an important contribution in the memory enhancing effect of the Epac activator. Indeed, MAPK activation was observed to be increased in the hippocampus during memory retrieval (Szapiro et al., 2000), whereas
MAPK inhibition by intrahippocampal injection of the MAPK kinase inhibitor PD098059 was shown to impair retrieval of an one-trial step-down avoidance task (Izquierdo et al., 2000).

Impaired retrieval is generally a sensitive measure of memory impairment in age-associated memory impairment (AAMI) and the early stages of Alzheimer’s disease. From our data it can be speculated that enhancing Epac2 signaling might at least in part overcome the memory retrieval deficits reported. In this respect it is interesting to note that the Epac2 expression is reduced in brains showing Alzheimer’s pathology when compared to non-diseased control brains. These changes were restricted to those regions of the brain associated with Alzheimer’s disease such as the frontal cortex and the hippocampus but not in the cerebellum, a region resistant to this pathology (McPhee et al., 2005). On the contrary, post-traumatic stress disorder (PTSD) is characterized by traumatic memories that can manifest as daytime recollections, traumatic nightmares, or flashbacks in which components of the event are relived. These symptoms reflect excessive retrieval of traumatic memories that often retain their vividness and power to evoke distress for decades or even a lifetime. It can be hypothesized that such conditions may benefit from reduced Epac2 signaling.

Considering both the lack and the need of drugs proven to be effective in modulating memory retrieval, the specific effect of hippocampal Epac signaling on retrieval we observed is of particular interest and warrants further research into the role of Epac signaling in cognitive processes under physiological and pathological conditions.

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