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Exchange Protein Activated by Cyclic AMP 2 (Epac2) Plays a Specific and Time-Limited Role in Memory Retrieval

Anghelus Ostroveanu,¹ Eddy A. van der Zee,¹ Ulrich L.M. Eisel,¹
Martina Schmidt,² and Ingrid M. Nijholt^{1*}

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. Intra-hippocampal 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. Down-regulation 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. © 2009 Wiley-Liss, Inc.

KEY WORDS: fear; conditioning; learning; mouse; hippocampus

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 and Nguyen, 2008). However, fairly recently, a new effector of cAMP signaling has been identified named exchange protein directly activated by cAMP (Epac). In independ-

ent 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 multidomain 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 and Neher, 2003; Zhong and Zucker, 2005; Gekel and 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 and Shewan, 2008). In spinal cord tissue Epac advances neurite regeneration (Murray and Shewan, 2008).

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. Gelinis and colleagues reported that Epac activation enhances the maintenance of LTP in area CA1 of mouse hippocam-

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

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Anghelus Ostroveanu is currently at Department of Metabolic and Endocrine Diseases, University Medical Center Utrecht, The Netherlands.

Ingrid M. Nijholt is currently at Department of Neuroscience, Section Anatomy, University Medical Center Groningen, The Netherlands.

*Correspondence to: Ingrid M. Nijholt, Department of Neuroscience, Section Anatomy, University Medical Center Groningen, Antonius Deusinglaan1, Groningen, The Netherlands. E-mail: i.m.nijholt@rug.nl

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pal slices (Gelinas et al., 2008) and coapplication of a selective PKA and a selective Epac activator was shown to rescue the memory retrieval impairment observed in dopamine- β -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), 9–12 weeks old, were individually housed in standard macrolon cages. Subjects were maintained on a 12-h 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 stereotactic holder during 1.2% avertin anesthesia (0.02 ml/g, i.p.) under aseptic conditions (Nijholt et al., 2008a,b). The cannulae were placed into both dorsal hippocampi (intrahippocampal; i.h.), AP -1.5 mm, lateral 1 mm, depth 2 mm (Franklin and Paxinos, 1997). The animals were allowed to recover for 6–7 days 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 μ l/min (final volume: 0.3 μ l 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. 8-pCPT-2'-O-Me-cAMP was shown to activate both Epac1 and Epac2 in vitro (Enserink et al., 2002; H. Rehmann and J. Bos, personal communication). A separate set of animals was injected with vehicle (ACSF pH = 7.4). Untreated animals without cannula served as controls for possi-

ble cannulation and injection effects. Initial pilot experiments with intracerebroventricular injections of 8-pCPT-2'-O-Me-cAMP and vehicle 1 h or 20 min before the training or the retention test showed that, only when 8-pCPT-2'-O-Me-cAMP was injected 20 min before the retention test, freezing was increased ($85.5 \pm 2.0\%$) in comparison to vehicle ($73.9 \pm 1.9\%$) and untreated animals ($64.6 \pm 3.8\%$; $F_{(2,28)} = 13.85$, $P < 0.001$). All other treatments did not lead to a significant change in memory performance in the retention test when compared to untreated animals. Therefore, 20 min was taken as the time period before training/retrieval for the activator or the vehicle to be injected.

Fear Conditioning

Fear conditioning was performed as described before (Nijholt et al., 2008a,b) in a Plexiglas cage (44 \times 22 \times 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 s followed by a scrambled footshock (0.7 mA, 2 s, constant current) delivered through a stainless steel grid floor. The mouse was removed from the fear conditioning box 30 s after shock termination to avoid an aversive association with the handling procedure. Memory tests were performed 24 h, 72 h, or 17 days after fear conditioning. Contextual memory was tested in the fear conditioning box for 180 s 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 (also known as inhibitory avoidance) were performed in a plexiglas cage (44 \times 22 \times 44 cm) consisting of a dark compartment (22 \times 22 \times 20 cm) equipped with a stainless steel grid floor and a light compartment (22 \times 22 \times 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 h before the training session. During habituation sessions, the mouse was introduced into the light compartment facing the closed guillotine door. After 60 s 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 s. During the training session, the mouse was again introduced into the light compartment, and the guillotine door was opened after 60 s. 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 scrambled footshock (0.3 mA, 2 s, constant current) was delivered to the

mouse. The mouse was removed from the apparatus 30 s after shock termination to avoid an aversive association with the handling procedure. Memory tests were performed 24 h after training. During the memory test the guillotine door was opened 60 s after introducing the mouse into the light compartment and left opened for maximally 480 s. 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 s.

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 s. 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 behavior, with a higher ratio being indicative of lower anxiety levels.

Immunohistochemistry

Thirty micrometer thick coronal sections of C57Bl/6J mice perfused with 4% paraformaldehyde, were preincubated with 0.3% H₂O₂ to reduce endogenous peroxidase. Nonspecific 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 1:1,000, several batches [1C8, 4D9, and 5D3] kindly provided by J. Bos, University Utrecht, The Netherlands and Epac1 A5, sc-28360, Santa Cruz) or Epac2 (1:1,000) (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).

Semiquantitative 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). About 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 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 10 min. Ten microliters of each sample was removed every three 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 h 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. Ten microliters of each sample was removed every three 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'-GTTGTCGACCCACAGGAAGT-3' and reverse 5'-ACCCA GTACTGCAGCTCGTT-3', for Epac 2 were: forward 5'-CAT GAGGGGAACAAGACGTT-3' and reverse 5' GGCCTTCGA GGCTCTAATCT 3', and for HPRT forward primer 5'-CCT GCTGGATTACATTAAGCACTG-3' and reverse 5'-CCTGA AGTACTCATTATAGTCAAGG-3'.

In Vivo siRNA Transfection

Mice injected i.h. with 50 ng siGLO Green (25 ng/hippocampus; D-001630-01-05, Dharmacon, Inc. Lafayette, CO), were sacrificed 6, 24, or 48 h postinjection. The brain hemispheres were placed in a 4% PFA solution for 24 h, followed by 48 h 30% sucrose immersion. Afterwards, 30 µm thick coronal sections were stained with DAPI (1:5,000) 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 RAPGEF4 (Epac2 siRNA) probes were purchased from Dharmacon. The target sequences for the mouse-specific Epac2 siRNAs mixture were: sense: CGAAAGACCUGGCGUACCAU (J-057784-05); sense: CAA-GUUAGCUCUAGU-GAACUU (J-057784-06); sense: GACA-GAAAGUACCACCUAAU (J-057784-07) and sense: GGAG-GAACUGUGUUGUUUAAU. ON-TARGETplus Nontargeting Pool siRNA (D-001810-10) was used as control (Dharmacon). 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., NY) according to the transfection protocol of the manufacturer. Fifty nanograms siRNA was injected i.h. on 3 consecutive days 3, 24, and 48 h after training or on the 3 days before 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 statis-

tical significance of appropriate multiple comparisons. Statistical comparisons for the passive avoidance results were made by the non parametric Kruskal-Wallis one-way analysis of variance. Data were expressed as mean \pm S.E.M. Significance was determined at the level of $P < 0.05$.

RESULTS

Epac Expression in the Mouse Brain

First, 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. 1A). 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. 1B).

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. 1C). 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 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 h after training in comparison to vehicle-injected and untreated mice (one-way ANOVA: $F_{(2,25)} = 1.110$; $P = 0.312$, Fig. 2A). 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. 2B).

To determine the effect of Epac activation on the retrieval of fear memory, mice were injected i.h. with 8-pCPT-2'-O-Me-

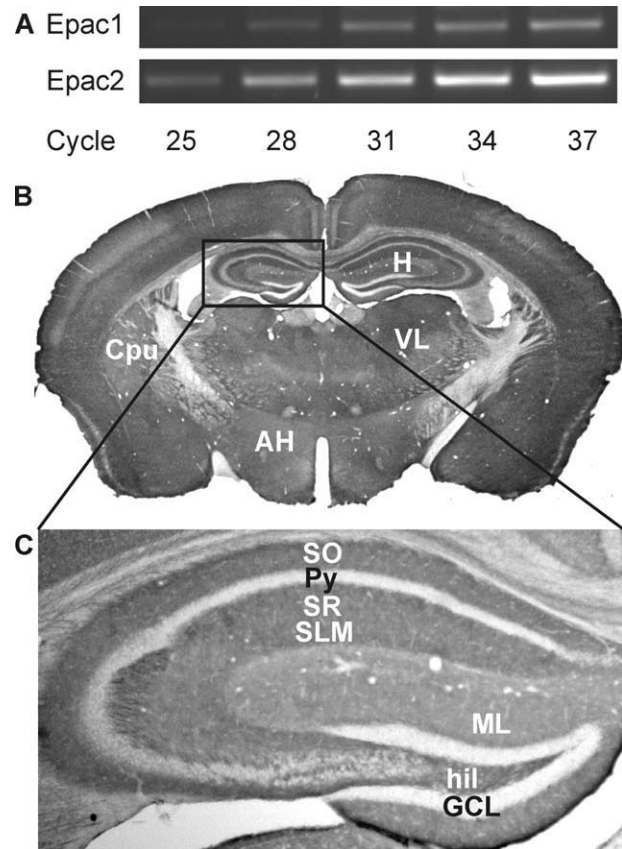


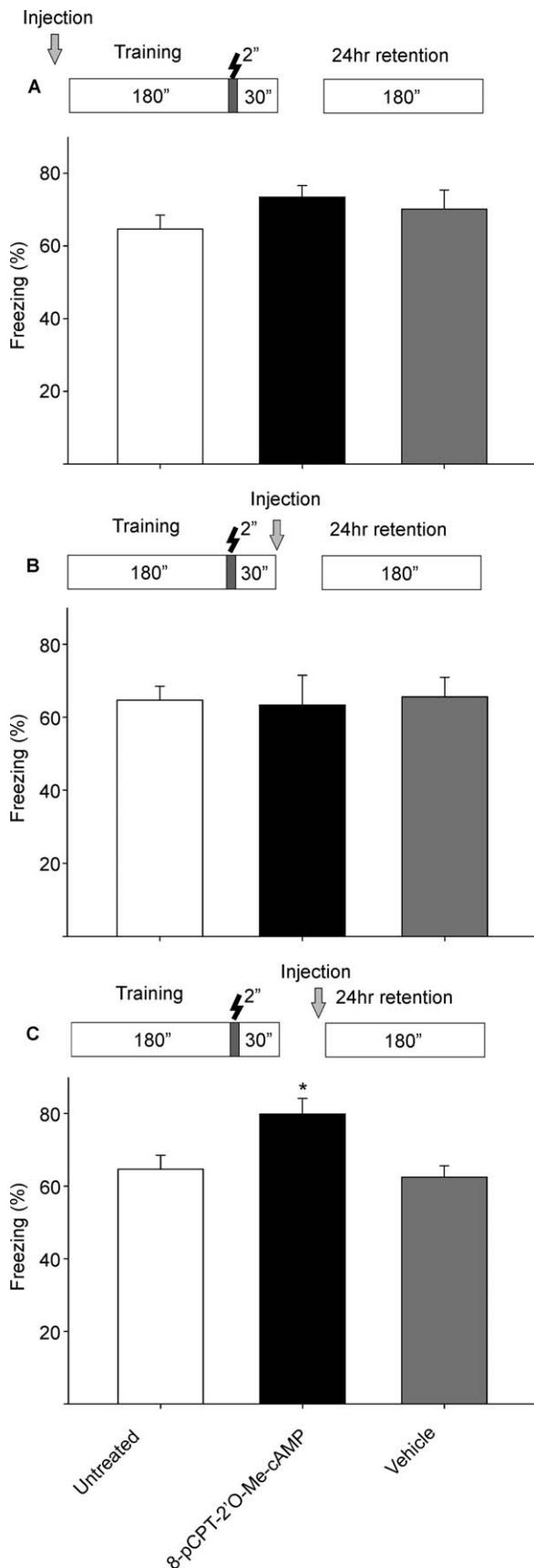
FIGURE 1. Detection of Epac1 and Epac2 in mouse brain. (A) Semiquantitative RT-PCR experiment for Epac1 and Epac2. Epac2 mRNA could be detected after a lower amount of cycles as Epac1 mRNA. A representative experiment is presented. (B) Epac2 staining in the mouse brain: caudate putamen (Cpu), hippocampus (H), ventrolateral thalamic nucleus (VL), anterior hypothalamic area (AH). (C) Epac2 Immunoreactivity in the hippocampus: stratum pyramidale (Py), stratum oriens (SO), stratum radiatum (SR), stratum lacunosum moleculare (SLM) and molecular layer (ML) granule layer (GCL), hilus (hil).

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. 2C).

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 acquisition, consolidation and 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 experi-



mental set-up during three sessions before the training session. We did not observe any difference between groups in their latencies to enter the dark compartment during the training session (Kruskal Wallis test, $P = 0.38$, Fig. 3A). 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 (Kruskal Wallis test, $P = 0.02$, Fig. 3B). 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. When 8-pCPT-2'O-Me-cAMP (1 mM) was injected 20 min before the training or immediately after the training session, the latency to enter the dark compartment during the retention test did not differ from the control groups (before training: vehicle latency 149.2 ± 43.4 s vs. 8-pCPT-2'O-Me-cAMP 186.8 ± 60.4 s; after training: vehicle latency 152.2 ± 28.9 s vs. 8-pCPT-2'O-Me-cAMP 146.4 ± 38.3 s). Thus, also in the passive avoidance task Epac activation only promotes memory retrieval whereas memory acquisition and consolidation remained unaffected.

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 4). 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 before 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, increased anxiety can be excluded as an explanation for the effect of Epac activation by 8-pCPT-2'O-Me-cAMP in the fear-motivated learning tasks. The enhanced freezing in the memory test can thus most likely be ascribed to enhanced memory retrieval.

Intrahippocampal Epac2 siRNA Injection Impairs Fear Memory Retrieval

To investigate the role of hippocampal Epac2 in memory acquisition, consolidation, or retrieval, we specifically down-

FIGURE 2. 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 behavior was measured in the memory test 24 h after training. Error bars indicate standard error of the mean. Statistically significant differences: * $P < 0.05$ vs. control groups.

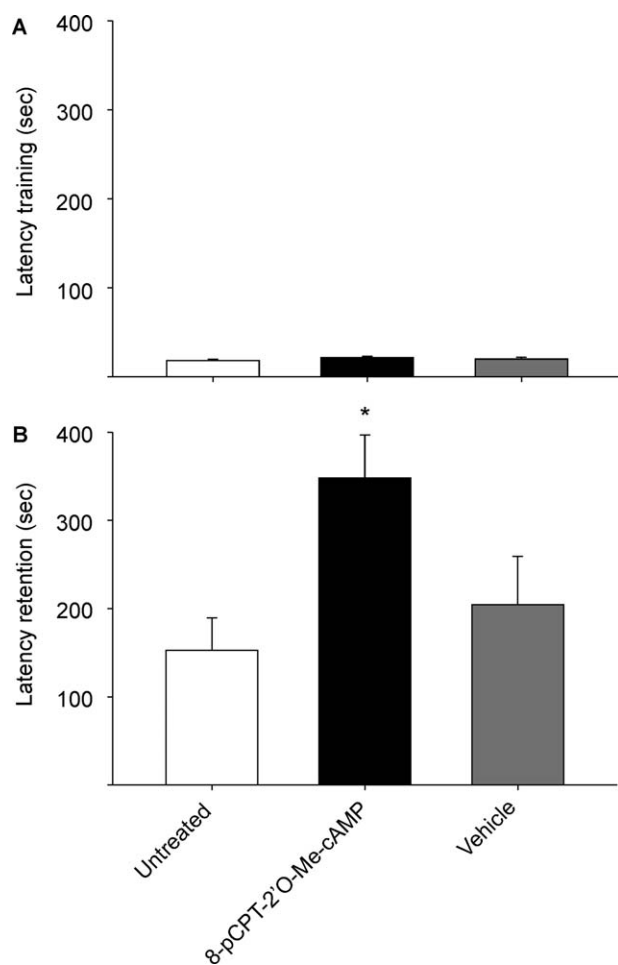


FIGURE 3. Intrahippocampal injection of the Epac activator 8-pCPT-2'O-Me-cAMP (1 mM) facilitates memory retrieval in the passive avoidance paradigm. Mice were habituated to the experimental set-up during three sessions. Mice were injected with 8-pCPT-2'O-Me-cAMP or vehicle 20 min before the retention 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: * $P < 0.05$ vs. control groups.

regulated Epac2 expression before the training session or the memory test using in vivo lipid mediated siRNA gene silencing. A previous study already showed the efficient down-regulation of Epac2 expression by these siRNA probes in in vitro neuronal cell cultures (Nijholt et al., 2008a,b). To check for siRNA transfection efficiency in the in vivo mouse brain, we first injected mice i.h. with fluorescent siGLO green (Figs. 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 h after injection. The signal lasted at least up to 48 h after injection. Other brain areas were not affected by the treatment.

Down-regulation of Epac2 expression by i.h. injection of specific siRNA probes on three consecutive days was verified by semiquantitative RT-PCR on the fourth day. Injection of Epac2 siRNA resulted in a 47% reduction of hippocampal Epac2

mRNA (Figs. 5C,D). The low level of Epac1 mRNA was not affected by the transfection with Epac2 siRNA.

In the behavioral experiments, mice were injected i.h. with Epac2 siRNA (50 ng/brain) 72, 48, and 24 h before the training or 3, 24, and 48 h after training in a contextual fear conditioning paradigm (Figs. 6A,B). Epac2 siRNA injection before the training did not affect memory performance in the retention test (one way ANOVA: $F_{(2,26)} = 0.326$; $P = 0.725$, Fig. 6A) whereas Epac2 siRNA injection after the training completely abolished the 8-pCPT-2'O-Me-cAMP-induced enhancement of retrieval and already caused a significant decrease in freezing behavior by itself during the first retention test (one-way ANOVA: $F_{(4,37)} = 9.187$; $P = 0.001$, Fig. 6B). In animals that received control siRNA injections after training, 8-pCPT-2'O-Me-cAMP injection again improved memory retrieval (Fig. 6B). 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 before this delayed retention test also did not affect freezing in any of the groups (Fig. 6C).

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

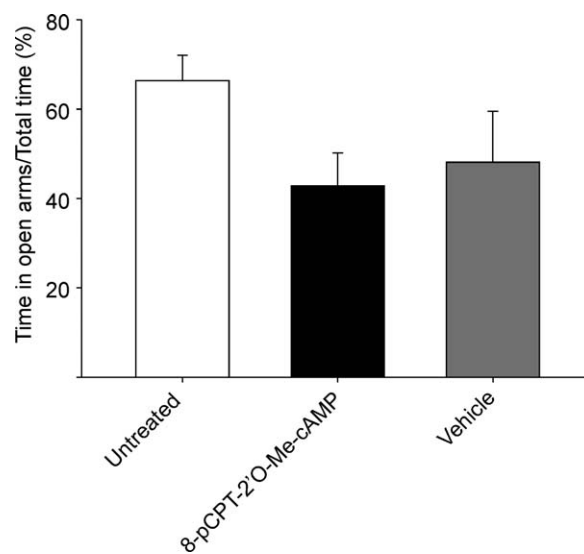


FIGURE 4. Intrahippocampal injection of 8-pCPT-2'O-Me-cAMP (1 mM) does not affect anxiety. Mice were injected with 8-pCPT-2'O-Me-cAMP or vehicle 20 min before the test. Untreated and vehicle-injected mice served as controls. Time spent in the different compartments of the maze was measured during 480 s and ratio between time in open arms and total time in maze was taken as a measure of anxiety. Error bars indicate standard error of the mean.

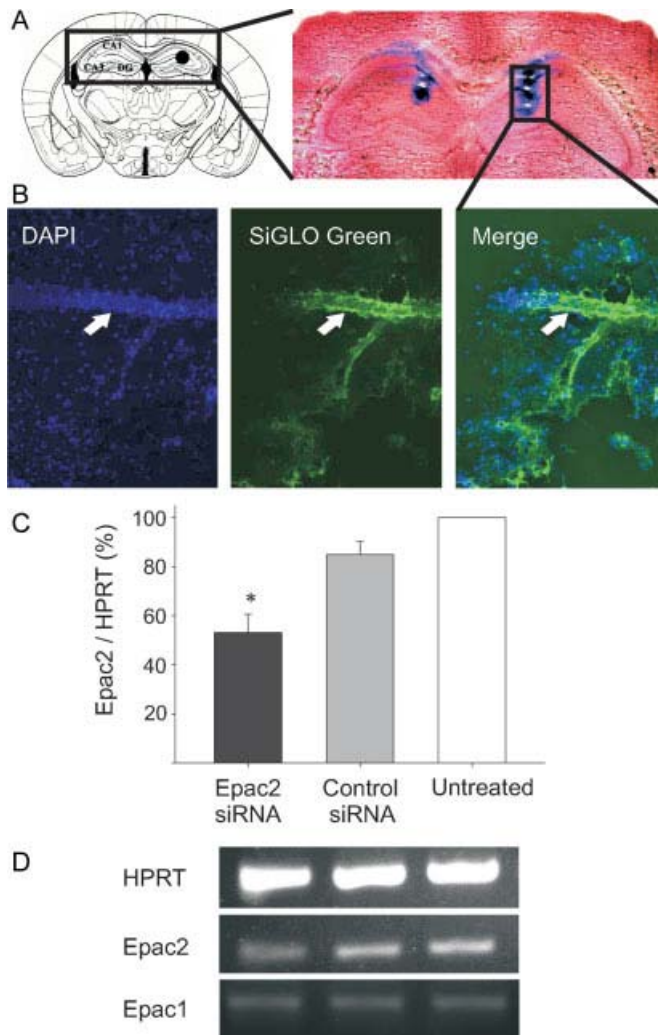


FIGURE 5. Efficient down-regulation of hippocampal Epac2 expression by in vivo siRNA transfection. (A) Left panel, coronal brain section of the mouse brain atlas (Franklin and Paxinos, 1997), black dot indicates the injection site; right panel, photomicrograph showing a representative detail of a coronal 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: $*P < 0.05$ vs. control groups. (D) Bands reflect the levels of Epac1 and Epac2 mRNA expression after 34 cycles. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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 sig-

naling 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- β -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 down-regulation 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 β -adrenergic signaling and Epac has already been established (Jensen, 2007; Métrich et al., 2008).

There are two possible explanations for 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. The memory at 17 days could be Epac2 independent but still stored in hippocampal assemblies or the memory could be no longer stored in the hippocampus and this would make it Epac2 independent. Although we can not distinguish between these two possibilities the second explanation is probably the most likely one. Several studies already reported that retrieval may become independent of the hippocampus over time (McClelland et al., 1995; Squire et al., 2001; Wiltgen et al., 2004; Morris, 2006).

The subcellular mechanism by which Epac2 modulates the retrieval of fear memory still remains to be elucidated.

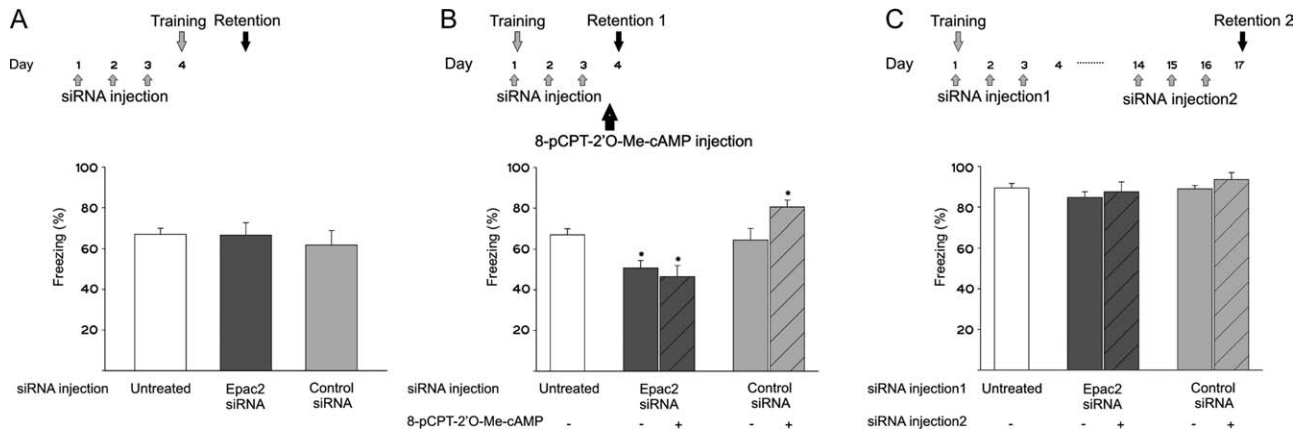


FIGURE 6. Intrahippocampal injection of Epac2 siRNA impairs memory retrieval in contextual fear conditioning. Mice were injected with Epac2 or control siRNA either 72, 48, and 24 h before the training (A) or 3, 24, and 48 h after training (B). Untreated mice served as additional controls. Freezing behavior was assessed as a measure of memory performance. (C) Freezing

behavior was again assessed during a second retention test 14 days after the first retention test. Where indicated this retention test was preceded by Epac2 siRNA injections (injection 2) on 3 consecutive days. Error bars represent standard error of the mean. Statistically significant differences: * $P < 0.05$ vs. control groups.

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 nondiseased 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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REFERENCES

- Abel T, Nguyen PV. 2008. Regulation of hippocampus-dependent memory by cyclic AMP-dependent protein kinase. *Prog Brain Res* 169:97–115.
- Bos JL. 2006. Epac proteins: Multi-purpose cAMP targets. *Trends Biochem Sci* 31:680–686.
- de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL. 1998. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* 396:474–477.
- Enserink JM, Christensen AE, de Rooij J, van Triest M, Schwede F, Genieser HG, Doskeland SO, Blank JL, Bos JL. 2002. A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. *Nat Cell Biol* 4:901–906.

- Franklin KBJ, Paxinos G. 1997. *The Mouse Brain in Stereotaxic Coordinates*. San Diego: Academic Press.
- Gekel I, Neher E. 2008. Application of an Epac activator enhances neurotransmitter release at excitatory central synapses. *J Neurosci* 28:7991–8002.
- Gelinas JN, Banko JL, Peters MM, Klann E, Weeber EJ, Nguyen PV. 2008. Activation of exchange protein activated by cyclic-AMP enhances long-lasting synaptic potentiation in the hippocampus. *Learn Mem* 15:403–411.
- Hucho TB, Dina OA, Levine JD. 2005. Epac mediates a cAMP-to-PKC signaling in inflammatory pain: An isolectin B4(+) neuron-specific mechanism. *J Neurosci* 25:6119–6126.
- Izquierdo LA, Barros DM, Ardenghi PG, Pereira P, Rodrigues C, Choi H, Medina JH, Izquierdo I. 2000. Different hippocampal molecular requirements for short- and long-term retrieval of one-trial avoidance learning. *Behav Brain Res* 111:93–98.
- Jensen J. 2007. More PKA independent beta-adrenergic signaling via cAMP: Is Rap1-mediated glucose uptake in vascular smooth cells physiologically important? *Br J Pharmacol* 151:423–425.
- Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, Housman DE, Graybiel AM. 1998. A family of cAMP-binding proteins that directly activate Rap1. *Science* 282:2275–2279.
- Lin SL, Johnson-Farley NN, Lubinsky DR, Cowen DS. 2003. Coupling of neuronal 5-HT7 receptors to activation of extracellular-regulated kinase through a protein kinase A-independent pathway that can utilize Epac. *J Neurochem* 87:1076–1085.
- McClelland JL, McNaughton BL, O'Reilly RC. 1995. Why there are complementary learning systems in the hippocampus and neocortex: Insights from the successes and failures of connectionist models of learning and memory. *Psychol Rev* 102:419–457.
- McPhee I, Gibson LC, Kewney J, Darroch C, Stevens PA, Spinks D, Cooreman A., MacKenzie SJ. 2005. Cyclic nucleotide signaling: A molecular approach to drug discovery for Alzheimer's disease. *Biochem Soc Trans* 33:1330–1332.
- Métrich M, Lucas A, Gastineau M, Samuel JL, Heymes C, Morel E, Lezoualc'h F. 2008. Epac mediates beta-adrenergic receptor-induced cardiomyocyte hypertrophy. *Circ Res* 102:959–965.
- Morris RG. 2006. Elements of a neurobiological theory of hippocampal function: The role of synaptic plasticity, synaptic tagging and schemas. *Eur J Neurosci* 23:2829–2846.
- Murchison CF, Zhang XY, Zhang WP, Ouyang M, Lee A, Thomas SA. 2004. A distinct role for norepinephrine in memory retrieval. *Cell* 117:131–143.
- Murray AJ, Shewan DA. 2008. Epac mediates cyclic AMP-dependent axon growth, guidance and regeneration. *Mol Cell Neurosci* 38:578–588.
- Nijholt IM, Dolga AM, Ostroveanu A, Luiten PG, Schmidt M, Eisel UL. 2008a. Neuronal AKAP150 coordinates PKA, Epac mediated PKB/AKT phosphorylation. *Cell Sign* 10:1715–1724.
- Nijholt IM, Farchi N, Kye M, Sklan EH, Shoham S, Verbeure B, Owen D, Hochner B, Spiess J, Soreq H, Blank T. 2004. Stress-induced alternative splicing of acetylcholinesterase results in enhanced fear memory and long-term potentiation. *Mol Psychiatry* 9:174–183.
- Nijholt IM, Ostroveanu A, Scheper WA, Penke B, Luiten PG, Van der Zee EA, Eisel UL. 2008b. Inhibition of PKA anchoring to A-kinase anchoring proteins impairs consolidation and facilitates extinction of contextual fear memories. *Neurobiol Learn Mem* 87:693–696.
- Ouyang M, Zhang L, Zhu JJ, Schwede F, Thomas SA. 2008. Epac signaling is required for hippocampus-dependent memory retrieval. *Proc Natl Acad Sci USA* 105:11993–11997.
- Roscioni SS, Elzinga CR, Schmidt M. 2008. Epac: Effectors and biological functions. *Naunyn Schmiedebergs Arch Pharmacol* 377:345–357.
- Sakaba T, Neher E. 2003. Direct modulation of synaptic vesicle priming by GABA(B) receptor activation at a glutamatergic synapse. *Nature* 424:775–778.
- Squire LR, Clark RE, Knowlton BJ. 2001. Retrograde amnesia. *Hippocampus* 11:50–55.
- Ster J, De Bock F, Guérineau NC, Janossy A, Barrère-Lemaire S, Bos JL, Bockaert J, Fagni L. 2007. Exchange protein activated by cAMP (Epac) mediates cAMP activation of p38 MAPK and modulation of Ca²⁺-dependent K⁺ channels in cerebellar neurons. *Proc Natl Acad Sci USA* 104:2519–2524.
- Szapiro G, Izquierdo LA, Alonso M, Barros D, Paratcha G, Ardenghi P, Pereira P, Medina JH, Izquierdo I. 2000. Participation of hippocampal metabotropic glutamate receptors, protein kinase A, mitogen-activated protein kinases in memory retrieval. *Neurosci* 99:1–5.
- Wiltgen BJ, Brown RA, Talton LE, Silva AJ. 2004. New circuits for old memories: The role of the neocortex in consolidation. *Neuron* 44:101–108.
- Zhong N, Zucker RS. 2005. cAMP acts on exchange protein activated by cAMP/cAMP-regulated guanine nucleotide exchange protein to regulate transmitter release at the crayfish neuromuscular junction. *J Neurosci* 25:208–214.