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# Inhibition of PKA anchoring to A-kinase anchoring proteins impairs consolidation and facilitates extinction of contextual fear memories

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ABSTRACT

Both genetic and pharmacological studies demonstrated that contextual fear conditioning is critically regulated by cyclic AMP-dependent protein kinase (PKA). Since PKA is a broad range protein kinase, a mechanism for confining its activity is required. It has been shown that intracellular spatial compartmentalization of PKA signaling is mediated by A-kinase anchoring proteins (AKAPs). Here, we investigated the role of PKA anchoring to AKAPs in different stages of the memory process (acquisition, consolidation, retrieval and extinction) using contextual fear conditioning, a hippocampus-dependent learning task. Mice were injected intracerebroventricularly or intrahippocampally with the membrane permeable PKA anchoring disrupting peptides St-Ht31 or St-superAKAP-IS at different time points during the memory process. Blocking PKA anchoring to AKAPs resulted in an impairment of fear memory consolidation. Moreover, disrupted PKA anchoring promoted contextual fear extinction in the mouse hippocampus. We conclude that the temporal and spatial compartmentalization of hippocampal PKA signaling pathways, as achieved by anchoring of PKA to AKAPs, is specifically instrumental in long-term contextual fear memory consolidation and extinction, but not in acquisition and retrieval.



## 1. Introduction

Contextual fear conditioning is a form of associative learning in which animals learn to fear a new environment because of its temporal association with an aversive unconditioned stimulus (US), usually an electrical footshock. The neuroanatomical systems and neurochemical basis underlying conditioned fear have been extensively investigated. It affects multimodal sensory information processing of continuously present (tonic) stimuli and it depends on a time-limited function of the hippocampus (see for review e.g. Sanders, Wiltgen, & Fanselow, 2003).

Studies investigating the intracellular signal transduction pathways involved have shown a crucial role for cAMP-dependent protein kinase (PKA) in contextual fear conditioning. Abel and colleagues generated transgenic mice which express R(AB), an inhibitory form of the regulatory subunit of PKA, only in forebrain regions such as the hippocampus. In these mice hippocampal PKA activity is reduced, which is paralleled by behavioral deficits in long-term but not short-term memory for contextual fear conditioning (Abel et al., 1997). The time course of amnesia in these transgenic mice is similar to the time course observed in mice treated with inhibitors of PKA (Bourtchouladze et al., 1998). Other studies using pharmacological approaches also reported that PKA inhibitors impair contextual fear conditioning (Ahi, Radulovic, & Spiess, 2004; Schafe, Nadel, Sullivan, Harris, & LeDoux, 1999; Wallenstein, Vago, & Walberer, 2002).

Although much is known about the mechanisms involved in the storage of contextual fear memories, the processes underlying the extinction of fear memories are far less understood. Recently, a role for PKA in fear extinction was proposed. Transgenic mice which express R(AB) show facilitated extinction of both recent and remote contextual fear memories (Isiegas, Park, Kandel, Abel, & Lattal, 2006) whereas increased PKA activity was found to impair extinction (McNally, Lee, Chiem, & Choi, 2005; Wang, Ferguson, Pineda, Cundiff, & Storm, 2004). In general these studies suggest that the PKA signal transduction pathway is important in the consolidation and extinction of contextual fear memories.

However, PKA is a multifunctional enzyme with a broad substrate specificity and thus coordinated control of PKA signaling is required. This is partly achieved by association of the enzyme with so called A-kinase anchoring proteins (AKAPs) (Rubin, 1994). AKAPs are a group of more than 50 identified functionally related proteins. Although they share little primary structure similarities, they all have the ability to bind the regulatory subunits of PKA,

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and therefore to coordinate specific cAMP signaling pathways by sequestering PKA to a particular subcellular location (Beene & Scott, 2007; Wong & Scott, 2004). Up to 75% of the total cellular PKA is believed to be associated with some member of the AKAP family. Compartmentalization of individual AKAP–PKA complexes occurs through specialized targeting domains that are present on each anchoring protein.

Interestingly, several AKAPs bind more than one signaling enzyme simultaneously. These multivalent AKAPs serve as scaffolds for the assembly of signaling complexes consisting of several kinases and phosphatases. Compartmentalization of both kinases and phosphatases to the same location may provide a coordinated activity of two enzymes with opposite catalytic activities.

Previous studies mainly focused on the effect of changes in PKA activity on learning and memory processes. However, recent findings suggest that positioning of PKA at its proper subcellular location by AKAPs is crucial for its efficient catalytic activation and accurate substrate selection and may thus be important in learning and memory processes. Hitherto knowledge on the importance of PKA anchoring to AKAPs in learning and memory processes is limited. In an initial study Moita and colleagues showed that local inhibition of PKA anchoring in the rat lateral amygdala impaired memory consolidation of auditory fear conditioning (Moita, Lamprecht, Nader, & LeDoux, 2002). More recent studies in *Drosophila* reported an important role for AKAPs in olfactory memory processing (Lu, Lu et al., 2007; Schwaerzel, Jaeckel, & Mueller, 2007). Furthermore, data from genetically modified mice that conditionally express Ht31, an inhibitor of PKA anchoring to AKAPs, showed that an anchored pool of PKA is important in theta-burst LTP and hippocampus-dependent spatial memory storage (Nie, McDonough, Huang, Nguyen, & Abel, 2007). In aplysia sensory neurons Ht31 was found to prevent both short- and long-term facilitation (Liu, Hu, Schacher, & Schwartz, 2004).

In the present study, we investigated the importance of PKA anchoring in the distinct stages of the memory process during contextual fear conditioning.

## 2. Materials and methods

### 2.1. Animals

All experiments were performed with 9–12 weeks old male C57BL/6J mice (Harlan, Horst, The Netherlands). Individually housed mice were maintained on a 12 h light/dark cycle (lights on at 7.00 a.m.) with food (Hopefarm® standard rodent pellets) and water ad libitum. A layer of sawdust served as bedding. The animals were allowed to adapt to the housing conditions for 1–2 weeks before the experiments started. 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 (DEC4174C).

### 2.2. Fear conditioning

Fear conditioning was performed in a plexiglas cage (44 × 22 × 44 cm) with constant illumination (12 V, 10 W halogen lamp, 100–500 lux). The training (conditioning) consisted of a single trial. Before each individual mouse entered the box, the box was cleaned with 70% ethanol. The mouse was exposed to the conditioning context for 180 s followed by a 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 1 or 24 h 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). In some experiments, animals were exposed to an alternative context 24 h after the training session. This alternative context consisted of a white plastic chamber (39 × 29 × 19 cm) which was exposed to 500–1000 lux, did not have a rod floor and was washed with 1% acetic acid, before each individual mouse entered the chamber.

To assess fear extinction mice underwent a daily re-exposure to the conditioning chamber for 3 min after the retention test. During these extinction trials freezing behavior and mean activity was measured.

### 2.3. Animal surgery

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 as previously described (Nijholt et al., 2004) into both lateral brain ventricles (i.c.v.) with anteroposterior (AP) coordinates zeroed at Bregma AP 0 mm, lateral 1 mm, depth 3 mm or directed toward both dorsal hippocampi (i.h.), AP −1.5 mm, lateral 1 mm, depth 2 mm (Franklin & Paxinos, 1997). Each double guide cannula with inserted dummy cannula and dust cap was fixed to the skull with dental cement (3M ESPE AG, Germany). Administration of 1 mg/ml finadyne (0.005 ml/g i.p.) before the surgery served as pain killer. The animals were allowed to recover for 6–7 days before the behavioral experiments started.

### 2.4. Brain injections

Bilateral injections were performed during a short isoflurane anesthesia using a Hamilton microsyringe fitted to a syringe pump unit (TSE systems, Bad Homburg, Germany) at a constant rate of 0.5 μl/min (final volume: 1 μl per side) for the i.c.v. injections and 0.34 μl/min (final volume: 0.3 μl per side) for the i.h. injections.

PKA anchoring to AKAPs was inhibited by intracerebroventricular (i.c.v.) or intrahippocampal (i.h.) injection of the peptide Ht31 (InCELLect® AKAP St-Ht31 inhibitor peptide (Promega, Madison, WI)) or superAKAP-IS. These peptides inhibit the interaction between the regulatory subunits of PKA and AKAP (Gold et al., 2006; Vijayaraghavan, Goueli, Davey, & Carr, 1997). SuperAKAP-IS was synthesized by solid phase peptide synthesis using BOC-chemistry and purified after cleavage from the matrix by preparative HPLC. Purity was controlled by analytical HPLC and mass spectrometry. The stearated form of Ht31 and superAKAP-IS was used to enhance the cellular uptake of the peptide through the membrane. St-Ht31 was injected in a final concentration of 10 mM (i.c.v. 20 nmol/mouse and i.h. 6 nmol/mouse) and St-superAKAP-IS in a final concentration of 5–500 μM (i.h. 0.003–0.3 nmol/mouse per injection). Unfortunately, it was not possible to prepare concentrations of St-superAKAP-IS higher than 500 μM. 50 mM Tris–HCl (pH 7.5) served as vehicle. To test the specificity of the observed effects another set of animals was injected with either InCELLect® St-Ht31P, a proline-substituted derivative which does not inhibit PKA anchoring (control peptide; final concentration 10 mM in 50 mM Tris–HCl, pH 7.5; i.c.v. 20 nmol/mouse and i.h. 6 nmol/mouse), or vehicle alone (50 mM Tris–HCl, pH 7.5). Untreated animals without cannula served as controls for possible cannulation and injection effects. The number of animals per group varied from 6 to 18.

### 2.5. Histology

Immediately after the behavioral test mice were injected during 1.2% avertin anesthesia (0.02 ml/g, i.p.) with methylene blue solution i.c.v., or i.h. Brains were removed and serially sectioned at 50 μm, collecting the sections on glass slides. Sections were stained on glass for 5 min in 0.1% nuclear fast red solution. To identify the location of the injection, sections were analyzed using light microscopy (Fig. 1).

Only data from animals in which the exact site of injection was confirmed after the behavioral experiments were evaluated. The methylene blue injections in the dorsal hippocampus did not show a diffusion of the solution to other brain or hippocampal areas.

### 2.6. Immunoprecipitation

One hour after intrahippocampal injection of PKA anchoring disruptor peptide or vehicle solution, the dorsal hippocampus was excised and mechanically homogenized in 10 volumes of homogenization buffer [50 mM Hepes (pH 7.4), 150 mM NaCl, 0.2% NP-40, 4 mM EGTA, 10 mM EDTA, 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM PMSF, and Complete Mini Protease Inhibitor Cocktail (Roche)]. The homogenate was centrifuged at 20,000*g* for 10 min at 4 °C, and the resulting supernatant was used for AKAP150 immunoprecipitation.

Per sample 100 μl of Dynabeads protein A (Dynal Biotech) was washed twice with Na-phosphate buffer (0.1 M, pH 8.1). Ten micrograms of goat anti-AKAP150C-20 antibody (1:2500, sc-6445 Santa Cruz, CA, USA) was incubated with the beads for 10 min. Afterwards the beads were washed three times with Na-phosphate buffer (0.1 M, pH 8.1) and twice with triethanolamine (0.2 M). IgGs were crosslinked with dimethyl pimelimidate (20 mM in 0.2 M trietholamine) for 30 min. The beads were washed for 15 min with Tris (50 mM, pH 7.5) and three times with phosphate buffered saline. Unbound IgG was removed by washing twice for 30 min with Na-citrate (0.1 M, pH 2–3). The dorsal hippocampus homogenate was incubated for 1 h with the beads. Bound proteins were eluted by denaturation at 95 °C for 5 min. The immunoprecipitated sample was stored at −80 °C until use. All the steps of the immunoprecipitation procedure were performed at room temperature.