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Research report

Networks of protein kinases and phosphatases in the individual phases of contextual fear conditioning in the C57BL/6J mouse

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HIGHLIGHTS

- Protein kinases and phosphatases differ between individual phases of fear conditioning.
- Protein arrays were used to screen different signalling protein levels between phases.
- Protein phosphatase 2A was linked to the retrieval phase of cFC.

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ABSTRACT

Although protein kinases and phosphatases have been reported to be involved in fear memory, information about these signalling molecules in the individual phases of contextual fear conditioning (cFC) is limited. C57BL/6J mice were tested in cFC, sacrificed and hippocampi were used for screening of approximately 800 protein kinases and phosphatases by protein microarrays with subsequent Western blot confirmation of threefold higher or lower hippocampal levels as compared to foot shock controls. Immunoblotting of the protein kinases and phosphatases screened out was carried out by Western blotting. A network of protein kinases and phosphatases was generated (STRING 9.1). Animals learned the task in the paradigm and protein kinase and phosphatase levels were determined in the individual phases acquisition, consolidation and retrieval and compared to foot shock controls. Protein kinases discoidin containing receptor 2 (DDR2), mitogen activated protein kinase kinase kinase 7 (TAK1), protein phosphatases dual specificity protein phosphatase (PTEN) and protein phosphatase 2a (PP2A) were modulated in the individual phases of cFC. Phosphatidyl-inositol-3,4,5-triphosphate 3-phosphatase and phosphatidylinositol-3 kinase (PI3K) that is interacting with PTEN were modulated as well. Freezing time was correlating with PP2A levels in the retrieval phase of cFC. The abovementioned protein kinases, phosphatases and inositol-signalling enzymes were not reported so far in cFC and the results are relevant for interpretation of previous and design of future studies in cFC or fear memory. Protein phosphatase PP2A was, however, the only signalling compound tested that was directly linked to retrieval in the cFC.

1. Introduction

Although protein kinases are key elements in memory mechanisms, published data on hippocampal protein kinases and protein phosphatases in contextual fear conditioning (cFC) is limited:

It has been shown that conditional deletion of the *erk 5* gene results in impaired contextual fear memory consolidation but not in acquisition and retrieval. Moreover, mitogen-activated protein kinase 7 (Erk5 MAP) may play a role in the establishment of remote contextual fear memory [1]. Atkins et al. have shown that hippocampal phosphorylated mitogen-activated protein kinase 1 (MAPK 1) but not mitogen-activated protein kinase 3 (MAPK3) increased one hour following cFC. In addition, the authors have shown that inhibition of dual specificity mitogen-activated protein kinase kinase 1(MEK), which is responsible for the activation of mitogen-activated protein kinase (MAPK), blocks associative learning in fear conditioning. Many protein kinases such as MAPK, protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase type II subunit alpha (α-CaMKII) are activated in the hippocampus after fear conditioning, however, not at the same time [2] and the role of this protein kinase for the formation of a variety of fear memories was described [3].

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Sindreu et al. have shown that MAPK activation is dependent on Ca^{2+}-stimulated adenylyl cyclase activity and that the co-activation of MAPK and protein kinase A (PKA) in CA1 neurons may be necessary for contextual fear memory consolidation [4].

Preethi et al. have demonstrated that Erk/MAPK is needed for cFC memory consolidation, while protein phosphatases: protein phosphatase 2 a (PP2A) and protein phosphatase 1 alpha (PP1α) hamper ETS domain-containing protein by activation of downstream nuclear targets of Erk-1/2 [6].

Young and co-workers studied levels and translocation of several PKC isoforms. At early times after cFC PKCα and PKCγ translocated to the membrane while PKCβII and PKCε moved to the cytosol and these changes were specific to cFC trained animals and no changes were observed in a non-associative fashion [7]. Isosaka et al. observed that the freezing response in tyrosine kinase Fyn-deficient mice was impaired 24 h after conditioning; the authors observed that Fyn peaked as early as 5–10 min after conditioning and persisted for at least 40 min indicating that this protein kinase may be involved in cFC mechanisms [8] and Fischer et al. demonstrated that CDK5 is involved in fear conditioning/associative learning [9]. In a review by Tronson et al., it is stated that elevated levels of regulatory subunit-associated protein 2 (CDK5) and ERK are observed when CS and US are presented in pair to the rodent. Moreover, activation of regulators of the CaMKII pathway are activated only with the combined presence of CS and US [10].

Chen et al. have measured the activity of phosphoinositide 3-kinase (PI3K) and ERK/MAPK in the hippocampus 24 h after training in the retrieval phase. In order to see PI3K activation; they monitored levels of pAKT since RAC-alpha serine/threonine-protein kinase (AKT) is the target of PI3K and a significant increase in PI3K activity after retrieval in the dentate gyrus, C1 and CA3 regions was observed. Involvement of PI3K in contextual memory retrieval was suggested by the use of an inhibitor 15 min via infusion in the CA1 region of the hippocampus before retrieval testing [11].

In the current study approximately 800 hippocampal protein kinases and phosphatases were screened by protein arrays and protein kinase and protein phosphatase levels were verified by Western blotting when threefold increased or decreased as compared to foot shock controls. The results from herein are extending knowledge on the complex network of signalling compounds in fear memory: a series of protein kinases were paralleling the individual phases of cFC and protein phosphatase PP2A was directly linked to the retrieval phase.

2. Materials and methods

2.1. cFC

The cFC experiments were carried out at the Institute of Biology, Otto von Guericke University Magdeburg, Germany. All studies were conducted in accordance with European and German regulations for animal experiments and were approved by the local animal committee.

53 adult male C57B/6J (Charles River, Germany) were obtained at the age of 8 weeks and housed in groups in the animal facility for 2 weeks and single housed for one week prior to experiments under a 12 h light/dark cycle (lights off at 19:00 h) with food and water available ad libitum. At the beginning of the experiments male C57BL/6J mice were 10–12 weeks old and weighed 19–21 g.

2.2. Contextual fear conditioning protocol

cFC was performed in a fear-conditioning apparatus (StartFear, Panlab Harvard Apparatus). The apparatus consisted of a test chamber (25 cm height × 30 cm width × 25 cm depth), the ceiling, front and back of which were transparent, and the floor was made of a removable grid stainless steel rods (3.2 mm diameter, 4.7 mm apart). Automated fear conditioning software (FREEZING, Panlab Harvard Apparatus) that controlled the foot shocks, recorded video images of the chamber and monitored the activity of mice used throughout the procedure [12,13].

Briefly, immediately prior to each test, the chamber was cleaned with 1% Incidin®. Animals were divided into four groups for the induction of cFC: acquisition ($n = 11$ animals), consolidation ($n = 11$) and retrieval ($n = 11$) phases. An additional foot shock control group was also used ($n = 11$) and euthanized after 6 h. The animals were placed in the center of the grid floor and left to explore the test chamber for 2 min. Next, three consecutive foot shocks of 0.4 mA at 20 s intervals were administered for conditioning and the activities of mice were recorded as the baseline activities. Two minutes later, animals were removed from the chamber and returned to their home cages. Animals in the acquisition group were euthanized 6 h later. The mice in the consolidation group were euthanized 24 h after this process. On the second day, the animals in the retrieval group were returned to the same chamber in which they had previously been administered a shock, freezing was monitored in 5 min intervals for every 1 min in the absence of foot shocks, and mice were euthanized 6 h later. The activity of each animal was evaluated by estimating its mean freezing activity (i.e., the absence of movements excluding involuntary respiratory movements) during exposure to the shock context and expressed as the time of freezing [14]. Freezing behaviour, defined as lack of movement other than breathing for at least 2 s, was automatically recorded using commercial software (FREEZING, Panlab Harvard Apparatus). The freezing response is a reliable measure of conditioned fear in rodents. The mice in the foot shock control group were placed in the chamber and three consecutive foot shocks of 0.4 mA at 20 s intervals were administered without the 2 min pre- or post-habituation period. Animals were euthanized by cervical dislocation. The hippocampi were rapidly dissected and the tissues were snap-frozen in liquid nitrogen and stored at −80 °C until used for biochemical analyses.

2.3. Screening for protein kinases and phosphatases

Hippocampal tissues of mice were homogenized using a glass dounce homogenizer and suspended in ice-cold lysis buffer from Kinexus (20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 1 tablet Complete™ from Roche Diagnostics, phosphatase inhibitors from Calbiochem [cat. no. 524624, Phosphatase Inhibitor Cocktail Set I] and 1 mM DTT, pH: 7.2). Sonication in ice to shear DNA was done for 4 × 10 s. Subsequently, the sheared homogenate was centrifuged at 20,000 × *g* for 30 min at 4 °C.

Concentration of the supernatant was carried out with an Ultrafree-4 centrifugal filter unit with a cut-off molecular weight of 10,000 Da (Millipore, Bedford, MA) at 3000 × *g* at 4 °C until the eluted volume was about 4 mL and the remaining volume reached 100–200 μL. The protein content of the supernatant was determined by the Bradford assay [15].

The amount of protein required for the Kinexus® Protein Kinase Microarray (KPKM) was 100 μg per sample at a minimum concentration of 2 mg/mL. The samples were frozen and shipped to Kinexus on dry ice immediately after protein quantification.

Protein microarrays for detection and semiquantification of 800 protein kinases and protein phosphatases were carried out at Kinexus according to their standard operation protocols. The protein microarrays were carried out by Kinexus according to the information provided in the homepage (http://www.kinexus.ca/pdf/