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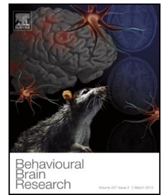

Research report

Contextual fear conditioning modulates hippocampal AMPA-, GluN1- and serotonin receptor 5-HT1$_A$-containing receptor complexes

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HIGHLIGHTS

- Phase-specific AMPA, NMDA and 5HT1A-RCs are reported in cFC.
- Receptor complexes and subunits parallel individual cFC phases.
- Receptor complexes should be examined along with receptor subunits.

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ABSTRACT

Although the roles of AMPAR (α-amino-3-hydroxyl-5-methyl-4-isoxazole propionate receptor), NMDAR (N-methyl-D-aspartate receptor) and 5HT1$_A$R (5-hydroxytryptamine sub type 1A) in contextual fear conditioning (cFC) have been studied, information about receptor-containing complexes (RC) is not available. Moreover, there are no data on membrane or endosomal NMDA-, 5HT1$_A$- or AMPA-RC levels, which would likely be indicative of the trafficking of these receptors. cFC was carried out in C57BL/6j mice and animals were sacrificed in the individual phases and hippocampi were taken for the determination of receptor complex and subunit levels using BN- and SDS-PAGE with subsequent Western blotting. GluA1-4, GluN1 (NMDAR subunit NR1)- and 5HT1$_A$-RC were differentially regulated during the individual phases and differentially regulated in the membrane and endosomal fractions. GluA1-RC levels in the membrane were increased in acquisition, consolidation and retrieval phases; GluA2-RC and GluA3-RC membrane levels were reduced and modulated in early endosomes during these phases. GluA4-RC and GluN1-RC levels as well as their subunits showed the same pattern in the membrane during consolidation while 5HT1$_A$-RC membrane and endosome levels were mainly increased during consolidation and retrieval. Taken together, the results suggest that levels of 5-HT1$_A$-RC, NMDA-RC and AMPA-RC and subunits in membrane and endosomal preparations are paralleling individual phases of cFC. The findings from the current study suggest phase-specific receptor complex and subunit formation and propose that receptor complexes should be examined in parallel with receptor subunits to aid the interpretation of previous work and to design future work on neurotransmitter receptors in memory paradigms.

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1. Introduction

Contextual fear conditioning (cFC) is one of the associative learning tasks in which a rodent learns to associate a neutral environment (context) with an aversive foot shock [1,2]. Memory acquisition occurs as the animal learns an association between a context and a shock. During consolidation, which can last from minutes to days, this memory is moved from a labile to a more fixed state. During retrieval, the animal is returned to the conditioning context, where memory for the context–shock association is assessed.

Hippocampal connection to contextual memory is well acknowledged and is essential in regulating the learning context in which a fearful event takes place [2–6]. The hippocampus has been recognized as a significant component of the system involved in the acquisition, consolidation and retrieval of fear memory [7–9].

AMPA receptors (AMPARs) play a significant role in cFC in hippocampus. It was shown that the link between context and shock

Abbreviations: cFC, contextual fear conditioning; BN-PAGE, blue-native PAGE; RC, receptor containing complexes; NMDAR, N-methyl-D-aspartate receptor; AMPAR, α-amino-3-hydroxyl-5-methyl-4-isoxazole propionate receptor; 5-HT1$_A$, 5-hydroxytryptamine subtype 1A.

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increases AMPARs in hippocampal CA1 resulting in synaptic transmission, which implies that AMPARs are important for encoding the fearful event [10]. Additionally, it was demonstrated that synaptic GluA1-trafficking in the hippocampus is required for the encoding of cFC [11,12]. During cFC in the mouse dorsal hippocampus, the retrieval of fear memory induced biphasic sequential changes in the membrane expression and synaptic strength of GluA2-containing AMPARs [13]. Moreover, it was reported that spontaneous AMPAR-mediated synaptic transmission takes place in the hippocampal CA1 region during association of context and shock [10].

NMDAR involvement in cFC is also known: Gao et al. [14] showed that the NMDAR is involved in acquisition and consolidation of cFC. Moreover, dorsohippocampal blockage of NMDARs resulted in the attenuation of acquisition of cFC [15]. An additional study provided evidence for its involvement as NMDAR antagonism blocked acquisition of fear memory [16].

The role of the 5-HT1$_A$ receptor (5HT1$_A$R) in cFC was shown and it was demonstrated that mice injected with 8-OH-DPAT (an agonist of the 5-HT1$_A$R) were unable to inform processing of short-to long-term memory [17]. Administration of the 5-HT1$_A$ agonist flesinoxan resulted in deficits in the expression of conditioned fear creating anxiolytic effects by stimulating the 5-HT1$_A$R [18].

The abovementioned studies indicate that AMPARs, NMDARs and 5-HT1$_A$Rs play a significant role in cFC. Furthermore, AMPAR trafficking and endocytosis is specifically regulated in fear conditioning [12,13]. However, little is known about the intracellular mechanisms and differential regulation of AMPARs, NMDARs and 5-HT1$_A$Rs and its trafficking that are related to different cFC phases in the hippocampus, including memory acquisition, consolidation and retrieval.

A systematic search for native protein receptor complexes in the membrane and endosome fractions during fear conditioning phases (e.g., acquisition, consolidation and retrieval) has not been carried out so far and in this study we examined receptor complexes and subunits in the membrane and early endosome fractions separated by sucrose gradient ultracentrifugation. For detection and quantification of receptor complexes blue-native (BN)-PAGE and for determination of receptor subunits SDS-PAGE followed by immunoblotting with specific antibodies was used.

And indeed, phase-dependent formation of receptor complexes was observed.

2. Materials and methods

2.1. Behavioral tests

2.1.1. Animals

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 have therefore been performed in accordance with the ethical standards.

72 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 singled housed for 1 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.

2.1.2. cFC protocol

The fear-conditioning 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 was used throughout the procedure [19].

Briefly, immediately prior to each test, the chamber was cleaned with 1% Incidin®. Animals were divided into four groups for the induction of cFC and subsequent biochemical analyses of the acquisition ($n = 12$ animals; hippocampi from two animals were pooled to provide an adequate amount of protein for the study of late, membrane and endosomal fraction), consolidation ($n = 12$) and retrieval ($n = 12$) phases. An additional foot shock control group was also used ($n = 6$) 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 the 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 "percentage of freezing" [20]. Freezing behavior, defined as lack of movement other than breathing for at least 2 s, was automatically recorded using commercial software (FREEZING, Panlab Harvard Apparatus). 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.

2.2. Biochemical analyses

2.2.1. Homogenization and sucrose gradient preparation

All procedures were performed at 4 °C. Pairs of hippocampi were homogenized together in ice-cold homogenization buffer [10 mM HEPES, 300 mM sucrose, and one complete protease inhibitor tablet (Roche Molecular Biochemicals, Mannheim, Germany) per 50 mL at pH 7.5] in a Dounce homogenizer following 15 cycles of aspiration with a syringe. After centrifugation at 1000 × g, the post-nuclear supernatant (PNS) was collected for subsequent sucrose density gradient fractionation and the pellet was discarded. The separation of the endosome and membrane fractions from the PNS was carried out as previously described with slight modifications [21]. Sucrose density gradient centrifugation solutions of 62%, 35%, 25%, and 10% (w/v) were prepared, and the concentrations of the sucrose solutions were controlled with a refractometer. The sucrose concentration of the PNS was adjusted to 40.6% via the addition of 62% sucrose solution. The resultant PNS was loaded onto the bottoms of the ultracentrifuge tubes. Sucrose solution of 35% and 25% were sequentially overloaded on the PNS, and the tubes were filled with homogenization buffer. Samples were then ultracentrifuged at 4 °C at 210,000 × g for 2 h (Beckman Coulter Inc., Brea, California, United States). The late endosomes were found at the interface between the 25% sucrose and the homogenization buffer. The early endosomes were enriched at the interface of the 35% and 25% sucrose solutions, and the heavy membranes were at the interface of the 40.6% and 35% sucrose solutions. All fractions collected from

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