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Translocation and activation of Rac in the hippocampus during associative contextual fear learning

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Abstract

Small G proteins including Rac are mediators of changes in neuronal morphology associated with synaptic plasticity. Previous studies in our laboratory showed that Rac is highly expressed in the adult mouse hippocampus, a brain area that exhibits robust synaptic plasticity and is crucial for the acquisition of memories. In this study, we investigated whether Rac was involved in NMDA receptor-dependent associative fear learning in the area CA1 of adult mouse hippocampus. We found that Rac translocation and activation was increased in the hippocampus following associative fear conditioning in mice, and that these increases are blocked by intraperitoneal injection of the NMDA receptor channel blocker MK801 at the acquisition stage. Our data indicate that NMDA receptor-dependent associative fear learning alters Rac localization and function in the mouse hippocampus.

Keywords: Learning and memory; Context fear; Hippocampus; MK-801; Small GTPases

1. Introduction

There is substantial evidence that connects the hippocampus to learning and performance of contextual tasks (Kim, Rison, & Fanselow, 1993; Phillips & LeDoux, 1992; Young, Bohenek, & Fanselow, 1994) with NMDA receptor involvement. Activation of the NMDA receptor in hippocampus-dependent associative learning leads to an increase in intracellular Ca^{2+} (Fanselow & Kim, 1994), and eventually to changes in gene expression (Davis, Vanhoutte, Pages, Caboche, & Laroche, 2000). It has been reported that NMDA receptor blockade with 5-amino-phosphovaleric acid (APV) impairs acquisition of context fear (Fanselow & Kim, 1994; Kim, DeCola, Landeira-Fernandez, & Fanselow, 1991; Young et al., 1994).

But learning and memory formation is a much more complex process that triggers many signaling cascades resulting in changes in neuronal morphology as well as gene expression. Over the past several years it has become clear that the Rho family of GTPases plays an important role in neuronal development (Govek, Newey, & Van Aelst, 2005). These proteins function as molecular switches that are inactive when they are bound to GDP and active when they are bound to GTP (Smith & Rittinger, 2002). Upstream signals that activate guanine nucleotide-exchange factors (GEFs), remove GDP and permit GTP loading, thereby activating Rho proteins to promote the interaction with specific downstream effectors. Inactivation of the Rho GTPases is mediated by GTPase-activating proteins (GAPs), which stimulate their intrinsic GTPase enzymatic activity (Foreman et al., 2003). It is well established that a specific blueprint of neuronal associations is critical for the function of the adult nervous system. The dynamics and morphology of lamellipodia and filopodia are coupled to a coordinated assembly and disassembly flow of actin filaments (Lin & Forscher, 1995), processes in which GTPases such as Rac are inherently involved (Harwood & Braga, 2003; Luo, 2000).

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Rac is an important molecule that regulates cytoskeletal reorganization and gene expression (Ridley, Paterson, Johnston, Diekmann, & Hall, 1992). Previous studies in our laboratory showed that Rac is highly expressed in the adult mouse hippocampus (Tejada-Simon, Villasana, Serrano, & Klann, 2006), a brain area that exhibits robust synaptic plasticity and is crucial for the acquisition of associative memories (Gruart, Munoz, & Gado-Garcia, 2006). Stimulation of hippocampal slices with NMDA caused the translocation of Rac from the cytosolic to the membrane fraction increasing the amount of Rac at the post-synaptic density (Tejada-Simon et al., 2006). Moreover, NMDA increased the amount of GTP-bound Rac, indicating that activation of NMDA receptors induced Rac activation. Taken together these results suggest that NMDA receptor activation alters Rac localization and function in hippocampal slices *in vitro*.

In the present study, we investigate the effects of hippocampus-dependent contextual fear conditioning on translocation and activation of Rac in adult mice. As previously reported in rats (Bolles & Collier, 1976), using our protocol mice learned to fear the context in which they received a shock and froze when they were returned to the chamber 1 and 24 h later. Pairing three shocks with the context resulted in significant freezing that was associated with the NMDA receptor-dependent translocation and activation of Rac. Together, our data suggest that as reported *in vitro*, similar alterations in Rac localization and function are associated with NMDA receptor activation following hippocampus-dependent learning in the behaving animal.

2. Materials and methods

2.1. Test animals

The animals used in all experiments were male C57Bl/6 (Charles River Laboratories, Wilmington, MA). Separate groups of mice were used for each behavioral experiment. Animals were handled and sacrificed in compliance with institutional and national guidelines and policies. The protocols used were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine.

2.2. Contextual fear conditioning test

Associative memory was assessed using a contextual fear conditioning paradigm. Animals were moved from their home room to the behavioral core facility at least 30 min prior to fear conditioning training. The equipment consisted of a standard mouse conditioning chamber (13 × 10.5 × 13 cm, Med Associates/Actimetrics chamber system; Med Associates, Georgia, VT) equipped with a house light (28 V), and a floor consisting of 19 equally spaced metal rods (2.8 mm diameter). The fear conditioning chambers were housed inside sound-attenuating cubicles (56 × 50 × 41 cm, Med Associates) equipped with a background noise-generating fan to overshadow extraneous sounds. Each mouse initially was placed into the chamber and left undisturbed for 2 h before the training session (latent inhibition group), or immediately subjected to training (context, saline, MK-801 groups). Naïve mice were exposed to neither the fear conditioning chamber nor the foot shock. The training session for contextual fear conditioning consisted of a 2-min exploratory period followed by a foot shock (2 s, 0.75 mA). The foot-shock was again presented at 4 and 6 min after session onset. The session ended 1 min after the third shock was received. Freezing behavior was measured every 10 s throughout training using a computer software (FreezeFrame, Med Associates/Actimetrics). The animals were removed immediately and returned to their home cages. To test for fear conditioning to contextual cues, the same animals were returned to the training context at 1 and 24 h after training for a 7-min test session. No shocks were presented during the test session. Freezing behavior was measured every 10 s throughout testing using the same computer software.

In experiments where animals were given an injection of either saline (100 μl/injection) or MK-801 (0.1 mg/kg bw, 100 μl/injection), injections were administered 1 h prior to fear conditioning (acquisition group), immediately after the training session (consolidation group), or 23 h after training session (retrieval group). Age-matched naïve animals that were handled but did not receive any experimental manipulations were used as controls in all fear conditioning experiments. The lowest effective dose of MK-801 was chosen for our experiments (0.1 mg/kg bw; Hlinak & Krejci, 2003; Levenson et al., 2004).

2.3. Hippocampus extraction

Male C57Bl/6 mice (6 weeks old) were sacrificed by cervical dislocation under gentle anesthesia. The brain was removed and hippocampus was dissected. Area CA1 was isolated immediately and homogenized in buffer (HBC; 10 mM Hepes, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μM microcystin-LR, and 200 nM calyculin A).

2.4. Fractionation

The homogenates were centrifuged at 1500*g* for 10 min at 4 °C to eliminate debris. Homogenates then were centrifuged at 100,000*g* for 45 min using an air driven ultracentrifuge (Beckman Coulter, Palo Alto, CA) and separated into membrane and cytoplasmic fractions. Total protein concentrations were determined (Bradford, 1976). Presence of Na^+/K^+ ATPase was tested to determine the purity of the cytoplasmic and membrane fractions.

2.5. Rac activation assay

Ten μg of GST tagged PAK-PBD protein beads (Upstate Biotechnology, Lake Placid, NY) were added to 150–200 μg protein of hippocampal CA1 homogenates containing protease and phosphatase inhibitor cocktail (Sigma, St. Louis, MO). Homogenates were incubated at 4 °C on a rocker for 1 h. PAK-PBD-GST beads then were pelleted by centrifugation at 14,000*g* for 10 min at 4 °C. The supernatant was removed and the pelleted beads were washed with HBC. PAK-PBD-GST beads were pelleted again by centrifugation at 14,000*g* for 10 min at 4 °C. The supernatant was removed again followed by two more washes and centrifugations. The final pellet was resuspended in 50 μl Laemmli buffer and analyzed by SDS–PAGE and Western blot analysis.

2.6. Western blot analysis

Equivalent amounts of protein were resolved via electrophoresis on 12% SDS-PAGE gels, transferred to polyvinylidene difluoride (PVDF) membranes, and incubated in TBS with 0.1% Tween 20 (TTBS) containing 5% non-fat milk or I-Block (for phospho-antibodies, Applied Biosystems, Foster City, CA) for 1 h at room temperature. Blots were incubated with the corresponding primary antibody (Rac [1:10,000], Na^+/K^+ ATPase [1:1000], from Upstate Biotechnology, Lake Placid, NY; p-PAK1/2 [1:1000] and PAK 1/2/3 [1:1000] from Cell Signalling Technology, Beverly, MA), for 2 h at room temperature under shaking, washed three times for 10 min in TTBS, followed by incubation with a horseradish peroxidase-conjugated goat anti-mouse or rabbit IgG (1:10,000 dilution, Promega, Madison, WI). Blots were visualized using enhanced chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ). The bands of each