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Hippocampal Mek/Erk signaling mediates extinction of contextual freezing behavior

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Abstract

Fear memories elicit multiple behavioral responses, encompassing avoidance, or behavioral inhibition in response to threatening contexts. Context-specific freezing, reflecting fear-induced behavioral inhibition, has been proposed as one of the main risks factors for the development of anxiety disorders. We attempted to define the key hippocampal mediators of extinction in a mouse model of context-dependent freezing. Nine-week-old male C57BL/6J mice were trained and tested for contextual fear conditioning and extinction. Freezing behavior scored by unbiased sampling, was used as an index of fear. Proteomic, immunoblot, and immunohistochemical approaches were employed to identify, verify, and analyze the alterations of the hippocampal extracellular signal-regulated kinases 1 and 2 (Erk-1/2). Targeted pharmacological inhibition of the Erk-1/2 activating kinase, the mitogen activated and extracellular signal-regulated kinase (Mek), served to establish the role of Mek/Erk signaling in extinction. When compared to acquisition, extinction of contextual freezing triggered a rapid activation of Erk-1/2 showing a distinctive time-course, nuclear localization, and subcellular isoform distribution. These differences suggested that the upstream regulation and downstream effects of this pathway might be specific for each process. Dorsohippocampal injections of the Mek inhibitors U0126 (0.5 μg/site) and PD98059 (1.5 μg/site) immediately after the nonreinforced trials prevented Erk-1/2 activation and significantly impaired extinction. This effect was dissociable from potential actions on memory retrieval or reconsolidation. On the basis of these findings, we propose that hippocampal Mek/Erk signaling might serve as one of the key mediators of contextual fear extinction.

Keywords: Extinction; Erk; Proteome; Context; Freezing; Hippocampus; Mice

1. Introduction

Anxiety disorders are thought to result from abnormal emotional responses associated with memories of aversive events. Most forms of anxiety are accompanied by exaggerated and persistent fear in response to threatening environments that can be modeled by contextual fear conditioning (Grillon, 2002). Rodent studies have significantly advanced our understanding of the neurobiology of contextual fear by establishing the essential biochemical pathways within the hippocampal brain area mediating fear conditioning (Kandel, 2001; Sweatt, 2001). However, persistent fear may be also caused by impaired extinction, reflecting a learned reduction of fear responses after subsequent nonreinforced contextual exposures (reviewed by Myers & Davis, 2002).

The molecular basis of extinction and its relevance for anxiety disorders has gained significant attention by discovering the roles of the basolateral amygdala (Lu, Walker, & Davis, 2001; Walker, Ressler, Lu, & Davis, 2002) and

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prefrontal cortex (Morgan, Romanski, & LeDoux, 1993; Morgan & LeDoux, 1995; Milad & Quirk, 2002; Sotres-Bayon, Cain, & Ledoux, 2006) in extinction of cue-dependent fear. On the basis of the prominent role of the amygdala in different aspects of the fear response such as acquisition, expression, extinction (Davis, Rainnie, & Cassell, 1994; LeDoux, 2000), it could be expected that this brain area would also significantly affect extinction of contextual fear. Such an indiscriminative involvement of the amygdala in fear regulation cannot explain the greater resistance to extinction of contextual rather than cue-dependent fear both in rodent experimental models (Stiedl et al., 1999) and human anxiety disorders (Grillon, 2002). On the other hand, the prefrontal cortex does not seem to be involved in the extinction of contextual fear responses (Morgan & LeDoux, 1999) despite its significant role in extinction of tone-dependent fear (Santini, Ge, Ren, Pena de Ortiz, & Quirk, 2004). Thus, the neuroanatomical and molecular substrates of contextual fear extinction remain scarce.

It is increasingly recognized that the hippocampus may contribute to the extinction of fear-motivated behaviors, such as inhibitory avoidance, triggered by environmental contexts (Szapiro, Vianna, McGaugh, Medina, & Izquierdo, 2003). Nevertheless, its role in extinction of contextual freezing, reflecting a generalized behavioral inhibition in response to a context associated with an aversive stimulus, has not been extensively investigated. Notably, among multiple responses elicited by fear states, context-specific freezing has been proposed as one of the main risks factors for the development of anxiety disorders (Buss, Davidson, Kalin, & Goldsmith, 2004). Impaired extinction of freezing to contextual stimuli has been observed in mouse strains with reduced hippocampal commissure (Schimanski, Wahlsten, & Nguyen, 2002). Supporting this view, we have recently shown that hippocampal mechanisms encompassing actin rearrangement are required for contextual fear extinction (Fischer, Sananbenesi, Schrick, Spiess, & Radulovic, 2004) in this paradigm. Because cytoskeletal rearrangement regulates the activation and distribution of a number of intracellular signaling molecules, the underlying mediators remained to be characterized in more detail. In addition to the recently demonstrated role of phosphatidylinositol 3 kinase (PI3; Chen et al., 2005), we identified in the present study the Mek/Erk-1/2 signaling pathway as one of the key mediators of extinction of contextual freezing.

2. Materials and methods

2.1. Animals

Nine-week-old male C57BL/6J mice (Centre d'Elevage Janvier and Jackson Laboratories) were individually housed after 8 weeks of age and maintained in enclosed animal cubicles with their own ventilation system (15 air exchanges/h), at a 12/12 dark light cycle (7 am–7 pm), 40–50% humidity, and 20 ± 2 °C. All studies were approved by the Animal Care and Use Committee of Northwestern University in compliance with National Institutes of Health standards.

2.2. Drugs and antibodies

Immunoblot and immunohistochemical analyses were performed using antibodies specific for Erk-1/2, histone H1, (1:1000, Santa Cruz), Erk 1/2 phosphorylated at Thr-183/Tyr-185 (1:5000, mouse monoclonal IgG_1, Sigma), anti-pThr-Erk-1/2 (1:8000, mouse monoclonal IgG_1, Sigma) and anti-pTyr-Erk-1/2 (1:8000, mouse monoclonal IgG_1, Santa Cruz), CREB phosphorylated at Ser-133 (pCREB; rabbit polyclonal IgG, 1:2000, Calbiochem), lactate dehydrohenase (LDH; 1:1000, rabbit polyclonal IgG, Rockland Immunochemicals), and cFos (1:12000, rabbit polyclonal IgG, Oncogene). All antibodies gave clear signals at the predicted molecular sizes of the investigated proteins in total hippocampal lysates.

The Mek inhibitors U0126 (Promega) and PD98059 (2′-amino-3′methoxyflavone, Calbiochem) were employed for treatment.

2.3. Contextual fear conditioning and extinction

Fear conditioning was performed in an automated system (TSE Inc.) and consisted of a single exposure to context (3 min) followed by footshock (2 s, 0.7 mA, constant current) as described previously (Radulovic, Kammermeier, & Spiess, 1998). Context-dependent freezing was measured 24 h later every 10 s over 180 s by two observers unaware of the experimental conditions and expressed as % of total number of observations. The extinction trials were performed in 24 h intervals and consisted of nonreinforced 3-min exposures to the context (Fischer et al., 2004).

2.4. Proteomics

Cytoplasmic and nuclear hippocampal lysates were prepared as described below, from naïve mice (N), mice exposed to fear conditioning (FC), or mice exposed to 4 consecutive extinction trials (E4). Hippocampi of the FC and E4 groups were obtained 1 h after training and last extinction trial, respectively, and pooled from 6 mice/group. Two-dimensional electrophoresis was performed as described in detail previously (Stannard, Soskic, & Godovac-Zimmermann, 2003). Briefly, proteins from hippocampal cytoplasmic and nuclear fractions were precipitated with 10% trichloroacetic acid, pellet washed twice in ethanol:ether (1:1; vol/vol) and solubilized in immunoelectrophoresis buffer. Proteins were separated according to their isoelectric point using 18 cm immobilized pH-gradient strips (pH 5.5–6.7, Amersham Biosciences). The proteins were then additionally separated by SDS–PAGE and visualised by silver staining. The resultant protein patterns were analyzed by Melanie software (GenBio SA).

Protein spots were excised from the gel, and processed automatically by Progest (Genomic Solutions). Tryptic digests (0.7 μl) were applied to the target plate and allowed to air-dry, then 0.7 μl of saturated solution of α-cyano-4-hydroxycinnamic acid in 33% acetonitrile and 0.1% TFA (v/v) was overlaid. Mass spectra of the tryptic digests were obtained on an Autoflex matrix-assisted laser desorption ionization-time of flight mass spectrometer (MALDI-TOF MS; Bruker Daltonics, Billerica, MA), calibrated with a mixture of seven standard peptides. Monoisotypic peptide masses were assigned and the mass lists were used for protein identification in the NCBI nonredundant (nr) protein database by Mascot search algorithm (Matrix Science Inc. London, UK).

2.5. Protein extraction and immunoblot

After training or indicated extinction trials (E1-4), the dorsal and ventral hippocampi were dissected according to neuroanatomical coordinates (Franklin and Paxinos, 1997). The rostral 2.5 mm septal pole and caudal 2.5 mm of the temporal pole were employed to prepare dorso- and ventohippocampal lysates, respectively. Hippocampal extracts for dorsal and ventral hippocampi were prepared separately because we expected maximal drug effects in the injected (dorsohippocampal) area. Nuclear extracts were prepared with the modified high salt extraction method (Dignam et al., 1983) as described in detail by Thiels, Kanterewicz, Norman, Trzaskos, and Klann (2002). To control for the purity of the nuclear and