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Changes in amygdala neural activity that occur with the extinction of context-dependent conditioned fear stress

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ABSTRACT

The purpose of the present study was to characterize functional changes in the amygdala that accompany the extinction of context-dependent conditioned fear stress in a rat, an animal model of anxiety. Specifically, the effect of extinction of conditioned fear-induced cyclic AMP responsive element-binding protein (CREB) phosphorylation in the amygdala was investigated using immunohistochemistry. Experiments demonstrated that CREB phosphorylation in the basal nucleus of the amygdala decreased with the extinction of context-dependent conditioned fear-induced freezing behavior. These data suggest that the basal nucleus of the amygdala plays an essential role in the expression of context-dependent conditioned fear. Further, this is the first study to demonstrate that CREB phosphorylation in the basal nucleus of the amygdala changes in parallel with the extinction of context-dependent conditioned fear.

1. Introduction

Past studies have demonstrated that the amygdala plays a crucial role in anxiety and fear (Ono and Nishijo, 1992; LeDoux, 2000) and that the amygdala may be a target for the action of various kinds of anxiolytic drugs (Beck and Fibiger, 1995; Menard and Treit, 1999; Inoue et al., 2004). We recently reported that conditioned fear stress (CFS), an animal model of anxiety in rats, specifically induced c-Fos expression in the basal nucleus of the amygdala and that the administration of citalopram, a selective reuptake inhibitor, attenuated this increase in c-Fos expression (Izumi et al., 2006).

Conditioned fear stress is a type of classical conditioning (Fanselow, 1980) distinguished by acquisition, expression, and extinction (Myers and Davis, 2002). Acquisition occurs when a sensory stimulus (CS, conditioned stimulus), such as light, tone, or exposure to the test box (context), is paired with an aversive stimulus (US, unconditioned stimulus), such as footshock. Expression occurs when the animal is re-exposed to the CS without the US, and it elicits a variety of autonomic, hormonal, and behavioral conditioned responses. Extinction occurs when the CS is repeatedly presented in the absence of the US, and it decreases the amplitude of conditioned responses.

Extinction is thought to be an active learning process (Myers and Davis, 2002). Several studies have attempted to characterize the effect of a prefrontal cortex lesion on extinction, but the results have varied (Gewirtz et al., 1997; Morgan and LeDoux, 1999; Quirk et al., 2000). Further, administration of a *N*-methyl-D-aspartate (NMDA) receptor glycine site agonist facilitated extinction, while administration of a NMDA receptor antagonist, benzodiazepine receptor agonist, benzodiazepine receptor inverse agonist, muscarinic receptor antagonist, or dopamine-1 receptor agonist inhibited extinction (reviewed by Myers and Davis, 2002; Davis and Myers 2002).

The goal of the present study was to characterize changes in the amygdala neural activity that occur with the extinction of context-dependent CFS, using cAMP responsive element-binding protein (CREB) phosphorylation as an index of cellular activity.

2. Methods

This study was approved by the Hokkaido University School of Medicine Animal Care and Use Committee, and all protocols complied with the Guide for the Care and Use of Laboratory Animals of the Hokkaido University School of Medicine.

2.1. Animals

Male Sprague–Dawley rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan), weighing 250–300 g, were used. Four rats were housed per cage (38×33×17 cm), in a 12-h light:12-h dark cycle and a temperature-controlled environment (22±1 °C) with free access to food and water. Experiments were initiated after a 14-day adaptation period.

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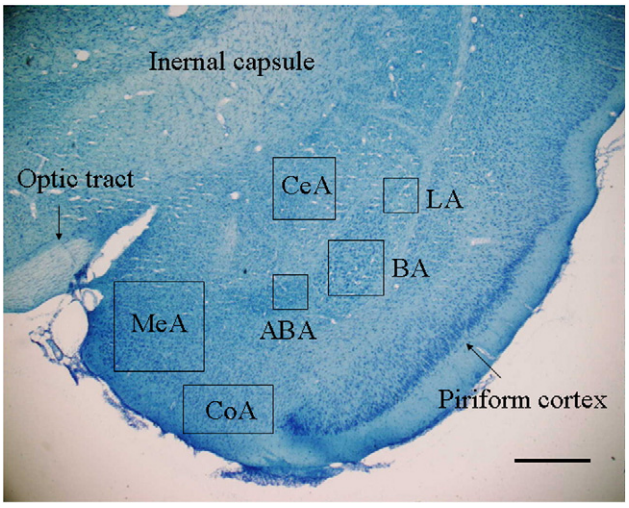

Fig. 1. Nissl staining of the amygdala (−3.14 mm to Bregma). BA, basal nucleus of the amygdala; ABA, accessory basal nucleus of the amygdala; CeA, central nucleus of the amygdala; CoA, cortical nucleus of the amygdala; LA, lateral nucleus of the amygdala; MeA, medial nucleus of the amygdala. Bar=500 μm.

2.2. CFS-induced freezing

Each rat was placed in a shock chamber (19×22×20 cm) and underwent 5 min of inescapable electric shocks (scrambled shocks of 0.2-mA intensity and 30-s duration, five times at variable intervals). Twenty-four hours after the footshock, the rats were again placed in the shock chamber and observed for 5 min without any shock application. During the 5-min observation period, freezing behavior was recorded using a time-sampling procedure (Fanselow, 1980), in which the animal behavior was classified as either "freezing" or "activity" at every 10-s interval. Freezing was defined as the lack of any observable movement of the body and the vibrissae, with the exception of movements related to respiration. Percentage scores for freezing were calculated for a 5-min observation period. Analysis of the freezing behavior was performed by an investigator who was blinded to the treatment.

2.3. Immunohistochemistry

Rats were anesthetized by pentobarbital injection (40 mg/kg, intraperitoneally) and perfused with saline and then by 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. The brains were sectioned at 30-μm thickness. Immunohistochemistry was performed on free-floating coronal sections (Umino et al., 1995). After 24-h incubation in 0.01 M phosphate-buffered saline and normal goat serum, the sections were incubated for 48 h in 0.01 M phosphate-buffered saline containing 0.2% Triton X-100 and rabbit anti-phospho-CREB antibody (Upstate Biotechnology, NY, 1:1000 dilution). The sections were incubated for 1 h in 0.01 M phosphate-buffered saline containing 0.2% Triton X-100 and biotinylated goat anti-rabbit IgG (Vector Labs) and then were incubated for 1 h in 0.01 M phosphate-buffered saline and avidin–biotinylated horseradish peroxidase complex (Vector Labs, Vectastain Elite ABC Kit). The reaction product was visualized by transferring the sections to a 50 mM Tris–HCl buffer (pH 7.6) containing 0.05% diaminobenzidine, 0.6% nickel ammonium sulfate and 0.01% H_2O_2.

2.4. Semiquantitative cell counting

According to the atlas of Paxinos and Watson (1997), the section that was located −3.14 mm posterior from the bregma was selected for semiquantitative evaluation of phospho-CREB (pCREB) immunoreactivity with a densitometric video image analysis system (MCID system, Imaging Research, CA, USA), according to the method of Bilang-Bleuel et al. (2002). The unit areas (200×200 μm) of the lateral nucleus, basal nucleus, accessory basal nucleus, central nucleus, medial nucleus, and cortical nucleus of the amygdala (Fig. 1) were digitally recorded by a CCD camera (CCD-IRIS, Sony, Japan) connected to a photomicroscope (B× 50, Olympus, Japan). The number of pCREB positive cells was assessed by automated selection of those cells within the unit areas that satisfied the following criteria: (1) the gray value of the cell nucleus was higher than the threshold value (threshold gray

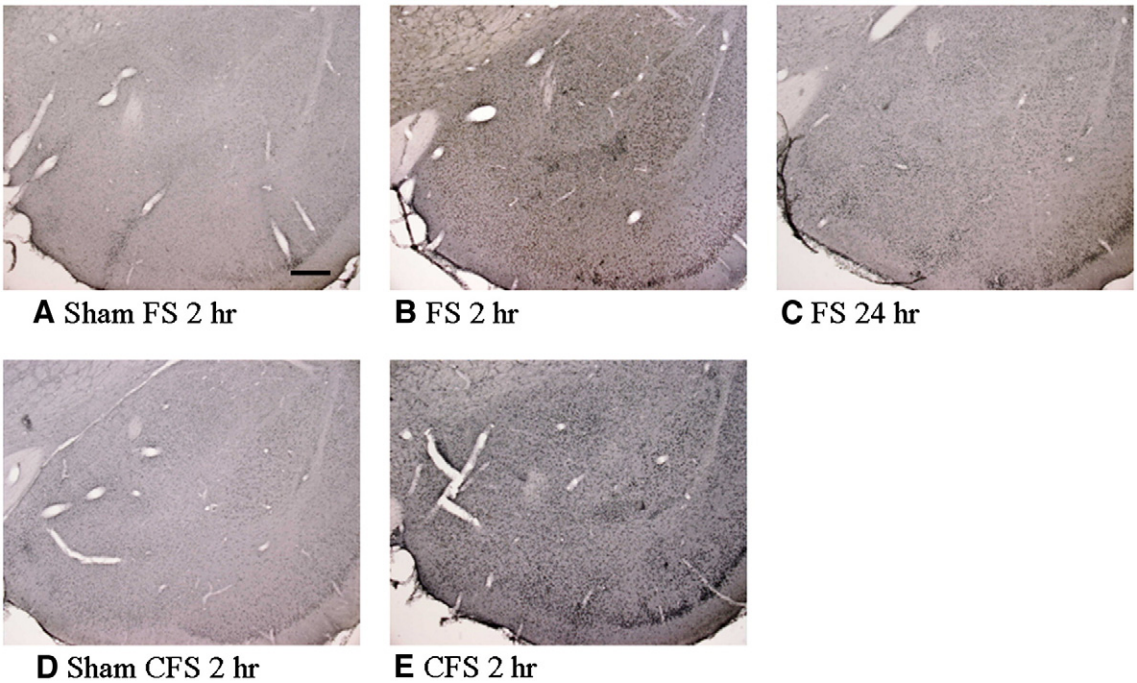

Fig. 2. Photomicrographs of the amygdala showing the expression of footshock and conditioned fear stress-induced phosphorylated CREB-like immunoreactivity. (A) 2 h after sham FS; (B) 2 h after FS; (C) 24 h after FS; (D) 2 h after sham CFS; (E) 2 h after CFS. FS, footshock; CFS, conditioned fear stress. Bar=200 μm.