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AMYGDALAR ROLES DURING EXPOSURE TO A LIVE PREDATOR AND TO A PREDATOR-ASSOCIATED CONTEXT

R. C. MARTINEZ,[a1] E. F. CARVALHO-NETTO,[a1] É. R. RIBEIRO-BARBOSA,[a] M. V. C. BALDO[b] AND N. S. CANTERAS[a]*

[a]*Department of Anatomy, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP 05508-000, Brazil*

[b]*Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP 05508-000, Brazil*

Abstract—The amygdala plays a critical role in determining the emotional significance of sensory stimuli and the production of fear-related responses. Large amygdalar lesions have been shown to practically abolish innate defensiveness to a predator; however, it is not clear how the different amygdalar systems participate in the defensive response to a live predator. Our first aim was to provide a comprehensive analysis of the amygdalar activation pattern during exposure to a live cat and to a predator-associated context. Accordingly, exposure to a live predator up-regulated Fos expression in the medial amygdalar nucleus (MEA) and in the lateral and posterior basomedial nuclei, the former responding to predator-related pheromonal information and the latter two nuclei likely to integrate a wider array of predatory sensory information, ranging from olfactory to non-olfactory ones, such as visual and auditory sensory inputs. Next, we tested how the amygdalar nuclei most responsive to predator exposure (i.e. the medial, posterior basomedial and lateral amygdalar nuclei) and the central amygdalar nucleus (CEA) influence both unconditioned and contextual conditioned anti-predatory defensive behavior. Medial amygdalar nucleus lesions practically abolished defensive responses during cat exposure, whereas lesions of the posterior basomedial or lateral amygdalar nuclei reduced freezing and increased risk assessment displays (i.e. crouch sniff and stretch postures), a pattern of responses compatible with decreased defensiveness to predator stimuli. Moreover, the present findings suggest a role for the posterior basomedial and lateral amygdalar nuclei in the conditioning responses to a predator-related context. We have further shown that the CEA does not seem to be involved in either unconditioned or contextual conditioned anti-predatory responses. Overall, the present results help to clarify the amygdalar systems involved in processing predator-related sensory stimuli and how they influence the expression of unconditioned and contextual conditioned anti-predatory responses.

Key words: medial amygdalar nucleus, basomedial amygdalar nucleus, lateral amygdalar nucleus, central amygdalar nucleus, unconditioned fear, conditioned fear.

The amygdala is a heterogeneous structure consisting of several nuclei and is widely accepted as playing an essential role in unconditioned and conditioned fear. Most of the knowledge regarding the roles played by the amygdalar sites during fear processing derives from studies using Pavlovian fear conditioning to a context or to a neutral stimulus, using noxious stimuli, usually electric foot shock, as unconditioned stimulus. Accordingly, associative learning between conditioned and unconditioned stimuli is likely to occur in the basolateral amygdalar complex, where lesions or pharmacological inactivation prevent acquisition and expression of fear conditioning (Phillips and LeDoux, 1992; Fanselow and LeDoux, 1999; Davis, 2000; LeDoux, 2000; Maren, 2001; McGaugh, 2004). The basolateral complex, in turn, projects to the central nucleus, which, via projections to the hypothalamus and brainstem, is currently regarded as a major amygdalar output to brain regions controlling the expression of fear, and central amygdalar lesions have been shown to disrupt freezing, along with the autonomic reactions observed during cue or contextual conditioned fear behavior (Kapp et al., 1979; Phillips and LeDoux, 1992).

Over the last years, a great deal has been learned about the neural systems involved in processing innate fear response to a predator or its odor. The amygdala occupies a central role in integrating predator-related sensory cues, and large amygdalar lesions have been shown to practically abolish innate defensiveness to a predator (Blanchard and Blanchard, 1972). Lesions of the basolateral amygdalar complex have been shown to disrupt unconditioned fear responses elicited by cat fur (Vazdarjanova et al., 2001) or cat odor (Li et al., 2004; Takahashi et al., 2007); and recent studies have shown that the basolateral amygdalar complex is involved in contextual fear memory consolidation to a cat odor-related environment (Takahashi et al., 2007). In contrast, central amygdalar nucleus (CEA) lesions had no significant effects on unconditioned fear responses to cat odor (Li et al., 2004),

[1] These authors contributed equally.

*Corresponding author. Tel: +55-11-3091-7628; fax: +55-11-3091-7285.
E-mail address: newton@icb.usp.br (N. S. Canteras).

Abbreviations: AAA, anterior amygdalar area; ANOVA, analysis of variance; BLAa, basolateral amygdalar nucleus, anterior part; BLAp, basolateral amygdalar nucleus, posterior part; BMAa, basomedial amygdalar nucleus, anterior part; BMAp, basomedial amygdalar nucleus, posterior part; CEA, central amygdalar nucleus; CEAc, central amygdalar nucleus, capsular part; CEAl, central amygdalar nucleus, lateral part; CEAm, central amygdalar nucleus, medial part; COAa, cortical amygdalar nucleus, anterior part; COApl, cortical amygdalar nucleus, posterior part, lateral zone; COApm, cortical amygdalar nucleus, posterior part, medial zone; EPd, endopiriform nucleus, dorsal part; IA, intercalated nuclei amygdala; LA, lateral amygdalar nucleus; MEA, medial amygdalar nucleus; MEAad, medial amygdalar nucleus, anterodorsal part; MEAav, medial amygdalar nucleus, anteroventral part; MEApd, medial amygdalar nucleus, posterodorsal part; MEApv, medial amygdalar nucleus, posteroventral part; NLOT, nucleus of the lateral olfactory tract; NMDA, N-methyl-D-aspartate; opt, optic tract; PA, posterior amygdalar nucleus; PAA, piriform-amygdalar area; PIR, piriform area; TMT, trimethylthiazoline.

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and, to our knowledge, its role in predator-related conditioning fear responses remains to be established.

In contrast to what has been learned from electric shock-based fear conditioning, the medial amygdalar nucleus (MEA) has a central role in anti-predatory defense. Predator odor seems to be processed as a pheromone by prey species in the accessory olfactory system, and up-regulates Fos expression in a distinct region of the MEA, its posteroventral part (Dielenberg et al., 2001; McGregor et al., 2004). Importantly, medial amygdalar lesions disrupt unconditioned fear responses to cat odor, and the nucleus seems to have a role in the retrieval of predator odor fear memory during cat odor contextual conditioning (Li et al., 2004; Takahashi et al., 2007).

Notably, the vast majority of studies investigating the amygdalar role in anti-predatory defense have used essentially predator odor as the unconditioned stimulus, which certainly yields a rather fragmentary view on how the amygdala processes the entire array of predator-derived sensory information. Therefore, in the present study, we used a live cat as the unconditioned stimulus; and our first aim was to provide a comprehensive analysis of the amygdalar activation pattern during cat exposure and exposure to cat-associated context by examining the amygdalar Fos expression in response to these situations. Next, we tested how the amygdalar nuclei most responsive to predator exposure (i.e. the medial, posterior basomedial and lateral amygdalar nuclei) and the CEA (critically involved in the expression of contextual fear responses in classical fear conditioning studies) influence both unconditioned and contextual conditioned anti-predatory defensive behavior, by placing relatively circumscribed bilateral cytotoxic lesions with iontophoretic deposits of N-methyl-D-aspartate (NMDA) in these amygdalar targets. Overall, the present results serve to clarify the amygdalar circuits involved in processing predator-related sensory stimuli and how these circuits influence the expression of unconditioned and contextual conditioned anti-predatory responses.

EXPERIMENTAL PROCEDURES

Animals and housing

Adult male Wistar rats (n=75), weighing about 250 g and obtained from the local breeding facilities, were used in the present study. The animals were kept under controlled temperature (23 °C) and illumination (12 h cycle) in the animal quarters, and had free access to water and standard laboratory diet.

Experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, 1996). All experimental procedures had been previously approved by the Committee on The Care and Use of Laboratory Animals of the Institute of Biomedical Sciences—University of São Paulo, Brazil (Protocol number 084/2005). In the present study, we attempted to minimize the number of animals used and their suffering.

Experimental apparatus and procedure

The experimental apparatuses were made of clear Plexiglas. Each consisted of a 25×25×25 cm^3 home cage connected to another 25×25×25 cm^3 chamber (the food compartment) by a hallway 12.5 cm wide and 100 cm long, with 25 cm high walls. Between the home cage and the hallway, there was a sliding door (12.5 cm wide and 26 cm high), which was opened when the animals were allowed to explore the rest of the apparatus. During 9 days, each animal was isolated in the home cage, and, at the beginning of the dark phase, the animals were allowed to explore the rest of the apparatus and obtain food pellets stored in the food compartment.

The testing procedure consisted of three phases of a 10 min observation period, during the beginning of the dark phase of the light/dark cycle.

Phase 1. After the habituation period, on the 10th day, animals were allowed to explore the familiar environment, providing a low-defense baseline.

Phase 2. On the 11th day, a neutered 2-year-old male cat was placed and held in the food compartment by an experimenter, and, as the rat's home cage door was opened, they were exposed to a live cat during a 10 min period. During this period, the cat was held by an experimenter and remained relatively calm and quiet without attempting to attack the rat. After the cat was removed at the end of the 10 min period, the hallway and food compartment were cleaned with 5% alcohol and dried with paper towels.

Phase 3. On the day after cat exposure, the sliding door was opened and the animals were exposed to the environment where the predator had been previously encountered, providing high levels of contextual conditioned fear responses.

For all testing periods, the pellets in the home cage were removed 3 h before the beginning of the dark phase, and, after the testing periods, placed back into the home cage. During the tests, the animals were recorded using a horizontally mounted video camera, under 50 W red light illumination.

Experiment 1

In experiment 1, we made a systematic analysis of the amygdalar expression of Fos immunoreactivity in the animals after each one of the three different phases of the testing procedure, that is, exposure to a safe environment (Phase 1), direct exposure to a predator (Phase 2), and exposure to a predator-associated context (Phase 3).

Ninety min after the testing procedures, five animals in each different phase of the testing procedure were deeply anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and perfused transcardially with a solution of 4.0% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4; the brains were removed and left overnight in a solution of 20% sucrose in 0.1 M phosphate buffer at 4 °C. The brains were then frozen and four series of 30 μm sections were cut with a sliding microtome in the frontal plane. One series of sections was processed for immunohistochemistry with anti-Fos antiserum raised in rabbit (Ab-5; Calbiochem, San Diego, CA, USA; lot # D09803) at a dilution of 1:10,000. The primary antiserum was localized using a variation of the avidin–biotin complex system (ABC; Hsu and Raine, 1981). In brief, sections were incubated for 90 min at room temperature in a solution of biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA), and then placed in the mixed avidin–biotin horseradish peroxidase (HRP) complex solution (ABC Elite Kit; Vector Laboratories) for the same period of time. The peroxidase complex was visualized by a 10 min exposure to a chromogen solution containing 0.02% 3,3' diaminobenzidine tetrahydrochloride (DAB; Sigma, St Louis, MO, USA) with 0.3% nickel-ammonium sulfate in 0.05 M Tris–buffer (pH 7.6), followed by incubation for 10 min in chromogen solution with hydrogen peroxide (1:3000) to produce a blue-black product. The reaction was stopped by extensive washing in potassium phosphate-buffered saline (KPBS; pH 7.4). Sections were mounted on gelatin-coated slides, and then dehydrated and coverslipped with DPX (Sigma). An adjacent series was always