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Research report

Midazolam reduces the selective activation of the rhinal cortex by contextual fear stimuli

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ABSTRACT

Independent brain circuits appear to underlie different forms of conditioned fear, depending on the type of conditioning used, such as a context or explicit cue paired with footshocks. Several clinical reports have associated damage to the medial temporal lobe (MTL) with retrograde amnesia. Although a number of studies have elucidated the neural circuits underlying conditioned fear, the involvement of MTL components in the aversive conditioning paradigm is still unclear. To address this issue, we assessed freezing responses and Fos protein expression in subregions of the rhinal cortex and ventral hippocampus of rats following exposure to a context, light or tone previously paired with footshock (Experiment 1). A comparable degree of freezing was observed in the three types of conditioned fear, but with distinct patterns of Fos distribution. The groups exposed to cued fear conditioning did not show changes in Fos expression, whereas the group subjected to contextual fear conditioning showed selective activation of the ectorhinal (Ect), perirhinal (Per), and entorhinal (Ent) cortices, with no changes in the ventral hippocampus. We then examined the effects of the benzodiazepine midazolam injected bilaterally into these three rhinal subregions in the expression of contextual fear conditioning (Experiment 2). Midazolam administration into the Ect, Per, and Ent reduced freezing responses. These findings suggest that contextual and explicit stimuli endowed with aversive properties through conditioning recruit distinct brain areas, and the rhinal cortex appears to be critical for storing context-, but not explicit cue-footshock, associations.

1. Introduction

Emotional memory research in animals has widely employed paradigms of aversive learning as tools for evaluating the neural mechanisms of memory acquisition, consolidation, storage, and retrieval. In Pavlovian fear conditioning, an aversive unconditioned stimulus (US), usually an electrical footshock, is paired with an initially neutral cue, such as a tone, light, or background context. After a few pairings, the conditioned stimulus (CS) comes to evoke a conditioned fear response that consists of a number of behavioral and autonomic responses, including freezing, urination, and increased arterial blood pressure [6,11,25,27,44]. This form of associative learning has been crafted by evolution to promote survival in the face of present and future threats, also being an essential component of many mammalian defensive behavior systems [28].

The freezing response—defined as the suppression of all visible movements, with the exception of respiration—is the most commonly used measure of fear conditioning in rats and mice [11,25]. Although this defensive behavior appears similar across distinct tests, different types of freezing, which do not always correlate with other behavioral measures of fear, have been proposed [12]. Indeed, strong evidence indicates that independent brain circuits underlie different forms of memory, and that the involvement of specific structures within a particular aversive memory domain may depend on the type of stimulus used for fear conditioning, such as an explicit cue or the background context [33,46,49,59,60,69].

Accumulating evidence suggests that the hippocampus plays an important role in stimulus selection during learning [80] and that it is also involved in the acquisition [59,79] and expression [43,54,75,81] of associative fear memories evoked by contextual stimuli. Additionally, the ventral hippocampus (VH) has also been implicated in expression of fear in general, including conditioned

Abbreviations: CA2, subfield CA2 of the ventral hippocampus; CA3, subfield CA3 of the ventral hippocampus; CS, conditioned stimulus; D, different context-conditioned group; Ect, ectorhinal cortex; Ent, entorhinal cortex; GABA, γ-aminobutyric acid; L, light-conditioned group; MTL, medial temporal lobe; N, non-conditioned group; PAG, periaqueductal gray; PBS, phosphate-buffered saline; Per, perirhinal cortex; RC, rhinal cortex; S, same context-conditioned group; T, tone-conditioned group; US, unconditioned stimulus; VH, ventral hippocampus.

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fear to explicit CS [15]. In all cases, however, the hippocampus appears not to be the site of permanent memory storage, although the time course of consolidation is not completely consistent across studies [43,47,62]. It has been shown that hippocampal damage 1 day after training impairs freezing responses, but damage after 7, 14, 28 [43], 50 [4], or 100 days [47] results in significant retention of contextual fear. Researchers have also proposed that long-term memories are stabilized and stored outside the hippocampus, possibly in the neocortex [5,24,43,50,56]. Thus, although emotional memories are temporally stable, the neuroanatomical basis of these memories is dynamic, and the hippocampus appears to play a time-limited role in memory storage [4,43,47].

Within the neurobiology of learning and memory literature is a well founded agreement that some subsets of structures within the medial temporal lobe (MTL)—which includes the rhinal cortex (RC), hippocampal formation, and parahippocampal cortex [82]—mediate long-term memory representations in several animal species, including humans (for review, see [74]). The RC—composed by the ectorhinal (Ect), perirhinal (Per), and entorhinal (Ent) subareas—has also been recruited in stressful situations, such as audiogenic stress [21] and cued fear conditioning [20,22,66,68,71,73]. However, considerable disagreement subsists on which regions are involved and the degree to which each one participates in different models of stress and learning. More precisely, although some studies have demonstrated the involvement of the RC in the paradigm of contextual fear conditioning [8,81], few of them have examined the role played by the RC subregions and VH in the expression of conditioned fear involving different CSs.

To address this issue, the present study investigated the involvement of these areas in aversive information storage and retrieval processes in different types of conditioned fear. Experiment 1 assessed freezing responses and Fos protein distribution in the RC (Ect, Per and Ent cortices) and VH (CA2 and CA3 subfields) during the expression of conditioned fear in response to a context, light, or tone in unrestrained Wistar rats. Experiment 2 assessed the effects of local administrations of the benzodiazepine midazolam into these RC subregions on the expression of contextual fear responses. In contrast to previous studies that investigated the role of the RC in fear conditioning using lesion procedures [13,14,17,22,46,48,61], the present study used a noninvasive protocol (i.e., Fos immunohistochemistry) associated with local drug injections, which does not produce nonspecific effects in cells and fibers of passage.

2. Materials and methods

2.1. Subjects

A total of 94 male Wistar rats from the animal house of the University of São Paulo, *campus* Ribeirão Preto, weighing 280–300 g, were used. They were housed in groups of four per cage with food and water available *ad libitum* in a temperature-controlled room (23 ± 1 °C) under a 12 h/12 h light/dark cycle (lights on at 07:00 AM) for 72 h. These animals were transported to the experimental room in their home cages and left undisturbed for 1 h prior to testing. The experiments reported in this article were performed in accordance with the recommendations of the Brazilian Society of Neuroscience and Behavior and complied with the United States National Institutes of Health Guide for Care and Use of Laboratory Animals. The procedures were approved by the Committee for Animal Care and Use, University of São Paulo (No. 06.1.123.8.53.9).

2.2. Fear conditioning

In Experiment 1, rats were subjected to fear conditioning using the context, a light, or a tone as the CS. In Experiment 2, rats were subjected to contextual fear conditioning 7 days after surgery. These procedures have been routinely used in our laboratory [57,67,78]. Two distinctive chambers, A and B, served as the experimental boxes. Chamber A was the training cage. Chamber B served as a "shifted" context for testing CS-elicited freezing. Both chambers were illuminated with a dim and indirect ambient fluorescent light from a ceiling panel, with a background ambient sound level of 57 dB.

2.2.1. Chamber A (40 cm × 25 cm × 25 cm)

The side and back walls were constructed of white acrylic, and the ceiling and front door were made of transparent Plexiglas. The grid consisted of stainless steel rods spaced 1.2 cm apart. It had a loudspeaker and a lamp (15 W) mounted in the rear panel. A sound generator produced a 1 kHz tone (72 dB). This chamber was enclosed in a sound-attenuating box. The footshocks were delivered through the test cage floor by a constant current generator built with a scrambler and the application of the stimuli was controlled by a computer and an input/output board (Insight Instruments, Ribeirão Preto, Brazil). The chamber was thoroughly cleaned with 20% alcohol for the group subjected to contextual fear conditioning or 2% acetic acid for all other groups after each test.

2.2.2. Chamber B (50 cm × 30 cm × 25 cm)

The side and back walls were constructed of black acrylic, and the ceiling and front door were made of transparent Plexiglas. The grid consisted of stainless steel rods spaced 1.0 cm apart. It had a sound generator, loudspeaker, and light bulb mounted in different positions compared to chamber A. This chamber was also enclosed in a sound-attenuating box. Application of the stimuli was controlled by a computer and an input/output board (Insight Instruments). The chamber was thoroughly cleaned with 20% alcohol after each test.

2.2.3. Training

All animals were placed individually in chamber A, and after 5 min of habituation, they received 10 light- or tone-paired footshocks. In each pairing, a light (15 W) or tone (72 dB, 1 kHz) was presented for 10 s, and a footshock (0.6 mA, 1 s) was delivered at the end of each CS presentation. The intertrial interval varied randomly between 30 and 120 s. In the contextual conditioning session, no explicit cue was presented between footshocks. Each animal was removed 3 min after the last shock and returned to its homecage. The training session lasted 16 min. The non-conditioning group was submitted to the same contextual conditioning procedure, with the exception of the electrical stimulation having been switched off during the session.

2.2.4. Testing

Twenty-four hours later, the test session was conducted similarly to the training session, but without the presentation of footshocks. With the exception of the animals in the same context-exposed group, all groups were tested in chamber B. The criterion used to assess fear conditioning was the duration of the freezing response during a 16 min session. Freezing was operationally defined as the total absence of movement of the animal, with the exception of respiration [11,25]. All experiments were monitored in real-time by a trained investigator that, through a video camera mounted 12 cm above the door and situated at the upper side of the box, scored conditioned freezing manually.

2.3. Experiment 1

This experiment compared the duration of freezing in rats subjected to the context, light, or tone previously paired with footshock and examined the involvement of the RC and VH in the expression of conditioned fear elicited by these three CSs using Fos protein immunohistochemistry. Thirty-four male Wistar rats were randomly assigned to one of five groups, according to the type of fear conditioning: non-conditioned group (N, $n = 7$), different context-conditioned group (D, $n = 6$), same context-conditioned group (S, $n = 8$), light-conditioned group (L, $n = 7$), and tone-conditioned group (T, $n = 6$). Two hours after the test session, the animals were deeply anesthetized with chloral hydrate (500 mg/kg, i.p.) and intracardially perfused with 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were removed and immersed for 2 h in paraformaldehyde and then stored for 72 h in 30% sucrose in 0.1 M PBS for cryoprotection. The brains were quickly frozen in isopentane (−40 °C) and sliced in a cryostat (−19 °C).

2.3.1. Fos-protein immunohistochemistry

Brain slices (40 μm) were collected in 0.1 M PBS and subsequently processed free-floating according to the avidin–biotin system, using the Vectastain ABC Elite peroxidase rabbit IgG kit (Vector Laboratories, Burlingame, CA, USA). All reactions were performed under agitation at 23 ± 1 °C. The sections were first incubated with 1% H_2O_2 for 10 min, washed four times with 0.1 M PBS (5 min each), and then incubated overnight with primary Fos rabbit polyclonal IgG (Santa Cruz, CA, USA) at a concentration of 1:4000 in 0.1 M PBS enriched with 0.2% Triton-X and 0.1% bovine serum albumin (PBS+). Sections were again washed three times (5 min each) with 0.1 M PBS and incubated for 1 h with secondary biotinylated anti-rabbit IgG (H+L; Vectastain, Vector Laboratories) at a concentration of 1:400 in PBS+. After another series of three 5 min washes in 0.1 M PBS, the sections were incubated for 1 h with the avidin–biotin-peroxidase complex (A and B solution of the kit ABC, Vectastain, Vector Laboratories) in 0.1 M PBS at a concentration of 1:200 in 0.1 M PBS and again washed three times in 0.1 M PBS (5 min each). Fos immunoreactivity was revealed by the addition of the chromogen 3,3′-di-aminobenzidine (0.02%, Sigma, St. Louis, MO, USA) to which H_2O_2 (0.04%) was added before use. Finally, tissue sections were washed twice with 0.1 M PBS, mounted on gelatin-coated slides, and dehydrated.