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Research report

Chronic unpredictable mild stress alters an anxiety-related defensive response, Fos immunoreactivity and hippocampal adult neurogenesis

J.S. de Andrade[a], I.C. Céspedes[a], R.O. Abrão[a], T.B. dos Santos[a], L. Diniz[b], L.R.G. Britto[b], R.C. Spadari-Bratfisch[a], D. Ortolani[a], L. Melo-Thomas[a], R.C.B. da Silva[a], M.B. Viana[a,*]

[a] Department of Biosciences, Federal University of São Paulo (UNIFESP), Santos, SP, Brazil
[b] Institute of Biomedical Sciences, Department of Physiology and Biophysics, University of São Paulo (USP), São Paulo, SP, Brazil

HIGHLIGHTS

- Chronic stress facilitates elevated T-maze avoidance, not altering escape.
- Avoidance increases Fos-ir in the cortex, amygdala, hippocampus.
- Escape increases Fos-ir in the dorsolateral periaqueductal gray and locus ceruleus.
- Chronic stress activates avoidance but not escape-related structures.
- Chronic stress decreases doublecortin cells and increases corticosterone levels.

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ABSTRACT

Previous results show that elevated T-maze (ETM) avoidance responses are facilitated by acute restraint. Escape, on the other hand, was unaltered. To examine if the magnitude of the stressor is an important factor influencing these results, we investigated the effects of unpredictable chronic mild stress (UCMS) on ETM avoidance and escape measurements. Analysis of Fos protein immunoreactivity (Fos-ir) was used to map areas activated by stress exposure in response to ETM avoidance and escape performance. Additionally, the effects of the UCMS protocol on the number of cells expressing the marker of migrating neuroblasts doublecortin (DCX) in the hippocampus were investigated. Corticosterone serum levels were also measured. Results showed that UCMS facilitates ETM avoidance, not altering escape. In unstressed animals, avoidance performance increases Fos-ir in the cingulate cortex, hippocampus (dentate gyrus) and basomedial amygdala, and escape increases Fos-ir in the dorsolateral periaqueductal gray and locus ceruleus. In stressed animals submitted to ETM avoidance, increases in Fos-ir were observed in the cingulate cortex, ventrolateral septum, hippocampus, hypothalamus, amygdala, dorsal and median raphe nuclei. In stressed animals submitted to ETM escape, increases in Fos-ir were observed in the cingulate cortex, periaqueductal gray and locus ceruleus. Also, UCMS exposure decreased the number of DCX-positive cells in the dorsal and ventral hippocampus and increased corticosterone serum levels. These data suggest that the anxiogenic effects of UCMS are related to the activation of specific neurobiological circuits that modulate anxiety and confirm that this stress protocol activates the hypothalamus–pituitary–adrenal axis and decreases hippocampal adult neurogenesis.

1. Introduction

Stress was originally defined by Hans Selye [1] as a non-specific response of the body to any demand for change. The stimulus that initiates the stress response, the so-called stressor, is aversive and potentially harmful to the organism and may either elicit an acute or a chronic stress response [2]. Stress responses include a series of physiological alterations, one of the most important being the activation of the hypothalamus–pituitary–adrenal axis (HPA) [3,4] with release of stress hormones in the blood stream. Furthermore, exposure to stress also results in a series of important behavioral changes, i.e. inhibition of exploratory activity, aversive conditioning and anxiety-related responses. Although responses to short-term or acute stress are adaptive and prepare the organism to deal with an infection or injury, the stress response might be potentially hazardous if the stressor is too intense or continuous. Thus, chronic stress has been associated with a greater risk for

* Corresponding author at: Av. Ana Costa, 95, 11060-001 Santos, SP, Brazil. Tel.: +55 11 38683203; fax: +55 11 38683203.
E-mail addresses: mviana@unifesp.br, milenabv@gmail.com (M.B. Viana).

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different clinical conditions, including psychiatric disorders, such as depression and anxiety [5].

To better study the consequences of stress on behavior, animal models have been developed. Nevertheless, results from studies testing anxiety-related reactions induced by prior exposure to stress are quite ambiguous. While some authors have reported increases in anxiety, others have found no effects or even a decrease in anxiety-related responses after exposure to different kinds of stressors [6–15].

In a prior study [16] we investigated the consequences of previous exposure to acute restraint in the elevated T-maze (ETM) model of anxiety (for details on this experimental model see [17–21]). The ETM was developed as an attempt to establish a correspondence between specific behavioral tasks and different subtypes of anxiety disorders. The model allows the measurement, in the same rat, of two defensive responses: avoidance and escape. Based on extensive behavioral and pharmacological validations these defensive responses have been related in terms of psychopathology to generalized anxiety and panic disorders, respectively [17,19–21]. Corroborating previous observations [22] our results showed that rats exposed to acute restraint displayed anxiogenic-like behavior, evidenced by facilitation of ETM avoidance responses. In contrast, ETM escape was unaltered by restraint. Since these tasks seem to activate distinct sets of brain structures, we further suggested that the differences observed were due to particularities in the neurobiological mechanisms, which modulated these defensive responses [16].

However, another possible explanation for the effects observed [16] might rely on the magnitude of the stressor used. It is possible, for instance, that acute restraint was not strong enough to alter ETM escape. In this sense it remained to be investigated how chronic stress alters ETM avoidance and escape responses. Thus, in the present study, animals were either unstressed or exposed for 2 weeks to an unpredictable chronic mild stress paradigm (UCMS) and subsequently tested in the avoidance or in the escape task of the ETM (Experiment 1). The UCMS model presents good face validity and has been broadly used to investigate some of the physiological and behavioral consequences of chronic stress [14,23–26]. Briefly, in this test, rodents are exposed to a variety of relatively mild stressors (i.e. restriction, inversion of the light–dark cycles, water/food deprivation, damp sawdust) intermittently, usually for two to three weeks [14,23–26].

The behavior of unstressed and stressed animals submitted to the ETM was further compared to the behavior of animals exposed to an enclosed T-maze, an apparatus already used in our earlier study [16] and that possesses all three arms enclosed by walls. As previously shown, exposure of animals to this apparatus controls the effects of handling by the experimenter, the novelty of the exposition and the locomotor activity of the animals while exploring the maze [16].

In order to understand the neurobiological mechanisms responsible for the behavioral effects obtained, in the present study Fos protein immunoreactivity (Fos-ir) was also used to map areas activated by previous UCMS exposure (Experiment 2). For comparison reasons, the same regions previously analyzed in response to acute stress [16] were investigated in the present study. As formerly noted, the product of the immediate-early gene c-*Fos* is expressed throughout the brain in response to a variety of tasks, thus making it a powerful instrument to study intracellular responses of neurons to different stimuli [27]. Since previous evidence shows that stress is a potent inhibitor of adult neurogenesis (for a review see [28]) and taking into account that adult neurogenesis seems to be involved in several behavioral alterations, including certain types of anxiety-related reactions [29], we also evaluated the effects of UCMS on the number of cells expressing the marker of migrating neuroblasts doublecortin (DCX) in the dorsal and ventral hippocampus (Experiment 3). It has been previously shown that quantification of DCX-expressing cells provides an accurate measurement of adult neurogenesis [30]. In order to verify the effectiveness of the UCMS procedure in activating the HPA axis, corticosterone serum levels were measured in an independent group of unstressed and stressed animals (Experiment 4).

2. Materials and methods

2.1. Animals

One day after their arrival to the laboratory, male Wistar rats (250–300 g) were assigned either to the unstressed or to the UCMS group. Unstressed animals were housed in groups of 4–6 per cage (50 cm × 60 cm × 22 cm). Room temperature was maintained at 22 ± 1 °C with lights on from 0700 to 1900 h. Food and water were freely available throughout the experiments. UCMS animals were housed under the same conditions except during the periods they were exposed to some of the mild stressors (i.e. food and water restriction/deprivation, inversion of the light/dark cycle). All procedures were conducted in conformity with the Brazilian Society of Neuroscience and Behavior Guidelines for Care and Use of Laboratory Animals, which are also in compliance with international laws and policies. All efforts were made to minimize animal suffering.

2.2. Apparatus

The ETM was made of wood and had three arms of equal dimensions (50 cm × 12 cm). One arm, enclosed by walls of 40 cm high, was perpendicular to two opposed open arms. To avoid falls, a 1 cm high Plexiglas rim surrounded the open arms. The whole apparatus was elevated 50 cm above the floor.

The enclosed T-maze used as control for the effects of handling, novelty and locomotion had all three arms (50 cm × 12 cm) surrounded by 40 cm high wooden walls.

Luminosity at the level of the mazes' arms was 60 lux. After each experimental session, the mazes were cleaned with a 10% ethanol solution.

2.3. Unpredictable chronic mild stress (UCMS) procedure

The UCMS protocol was performed as described previously [23,24] (see Table 1). Rats were subjected to different kinds of stressors, which varied from day to day, for 14 consecutive days. There were a total of seven stressors: (1) periods of 1 h of restraint in a small acrylic cylinder (16 cm × 7.5 cm × 7.0 cm), as previously described [23,24]; (2) lights on overnight; (3) food deprivation overnight, followed by 2 h access to restricted food (resulting from the scattering of pellets of 45 mg in the cage); (4) water deprivation overnight, followed by 1 h contact with an empty water bottle; (5) food restriction for 2 h; (6) damp sawdust overnight; (7) inversion of the light/dark cycle from Friday night to Monday morning.

2.4. Elevated T-maze (ETM) and enclosed T-maze procedure

Twenty-four hours after the end of the UCMS protocol (or 15 days after their arrival to the laboratory for unstressed rats), all animals were pre-exposed to one of the open arms of the ETM for 30 min. A wooden barrier mounted on the border of the open arm, between the maze's central area and the arm's proximal end, isolated this arm from the rest of the maze. It has been shown that this pre-exposure procedure shortens escape latencies, rendering it more sensitive to the effects of treatment [20,21,31].

On the next day, independent groups of unstressed and stressed animals ($N = 8$ per treatment group) were tested in the avoidance or escape tasks of the ETM or in the enclosed T-maze.

For ETM avoidance measurements, each rat was placed at the end of the enclosed arm, and the time taken to exit this arm with all four paws was recorded (baseline latency). The same measurement was then repeated in two subsequent trials (avoidance 1 and 2) at 30 s intervals. The time rats remain in the enclosed arm, in a second and third exposure to this arm, usually increases due to these animals' innate fears to height and openness [17,18,20,32]. For escape measurements, rats were placed on the open arm on which they had been pre-exposed and the latency to leave this arm with the four paws was recorded for three consecutive times (escape 1, 2 and 3), again at 30 s intertrial intervals. A cutoff time of 300 s was established for the avoidance and escape latencies.

The same procedure described above was employed for the enclosed T-maze, except that animals were placed either at the distal end of the stem arm or at the distal end of the transversal arm of the maze.

2.5. Fos protein immunohistochemistry

At 2 h after the tests with the ETM or enclosed T-maze, six randomly chosen animals from each of the treatment groups described above were anesthetized with ketamine/xylazine 2:1 (1 ml/kg) and perfused with ≈100 ml of 0.9% saline for approximately 1 min, followed by 500–700 ml of 4% formaldehyde (from