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Short Communication

Environmental enrichment prevents acute restraint stress-induced anxiety-related behavior but not changes in basolateral amygdala spine density

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ABSTRACT

Previous studies showed that acute restraint stress or transient elevation of glucocorticoid (GC) stress hormones produces emergent changes in both anxiety behavior and dendritic branches in the basolateral amygdala complex (BLA) of rats. In this work, we demonstrate that exposure to environmental enrichment (EE) prevented stress-induced increases in anxiety (emerging 10 days post-stress) in adult rats without blocking stress-induced dendritic branch remodeling in the BLA nor stress-induced enhancement of GC serum levels.

1. Introduction

Either acute restraint stress (2 h) or systemic injection of corticosterone (CORT, a rodent GC) in rats leads to anxiety-like behavior and dendritic branches remodeling in the BLA 10–12 days later (Mitra et al., 2005; Mitra and Sapolsky, 2008). In a previous study, we found that exposure to EE prevented anxiety-related behavior in adult rats observed immediately after acute restraint stress, but it is not yet known whether EE affects the persistent effects of acute stress (Novaes et al., 2017). Recent studies showed that EE reverses the effects of early life or repeated stress on anxiety-like behavior, BLA hypertrophy, and CORT serum levels (Koe et al., 2016; Ashokan et al., 2016). Hence, this study sought to determine whether EE can prevent acute restraint stress from enhancing anxiety-related behavior and whether this protection might be attributed to its influence over BLA spine remodeling and CORT release.

2. Methods

2.1. Environmental enrichment

Experiments were carried out in accordance with the guidelines of the Brazilian National Council for the Control of Animal Experimentation (CONCEA), under the Brazilian National Law number 11794 from 10/08/2008. All experimental procedures were approved by the Ethical Committee for Animal Use of the Institute of Biomedical Sciences, University of São Paulo, Brazil (protocol number 028/2013). A total of 114 adult male *Wistar* rats (60 days of age upon arrival) were used for this study. All animals were randomly pair-housed in standard polypropylene cages for ten days (habituation period), after which half of the pairs were transferred to EE (where they remained for 14 consecutive days, pair-housed). The other half remained pair-housed in standard cages (SC) until the end of the experiment. For details about EE and standard housing conditions, see Novaes et al. (2017). Animals were kept in a controlled temperature room (21 ± 2 °C) on a 12-hour light/dark cycle (lights on at 07:00 h) with free access to food and water.

2.2. Acute restraint stress

On the last EE day, half of the EE animals and half of the non-EE animals were stressed (to avoid indirect stress, both animals of each pair were stressed), while the others remained in their cages, undisturbed. The rats were transferred at 09:00 a.m. to the experimental room and restrained in ventilated PVC tubes for 2 h. Immediately after the stress session, all animals were returned to pair-housing in standard cages. Unstressed EE animals were also transferred to standard cages.

2.3. Behavioral tests

Ten days after the acute restraint stress, animals were subjected to the elevated plus maze (EPM) or open field (OF) tests to measure

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anxiety-like behavior. All trials were videotaped and the apparatuses were cleaned with 5% (vol/vol) ethanol after each trial. All behavioral tests were performed between 08:00 a.m. and 12:00 p.m. under low-light conditions (8–10 lux). Stressed and unstressed animals from both housing conditions were run on the EPM. To confirm the EPM results, a separate group of stressed and unstressed animals from both housing conditions were run on the OF test.

2.4. Elevated plus maze

The EPM apparatus consisted of two open arms (50 × 10 cm) and two enclosed arms (50 × 10 cm, surrounded by a 40-cm-high wall), forming a plus-shape. The arms were connected by a central area (10 × 10 cm), and the maze was elevated 50 cm from the floor. Animal were placed in the center of the maze, facing one of the open arms, and were permitted to explore the maze for 5 min. Anxiety-like behavior was assessed as a function of decreased open arm exploration, measured by the total percentage of entries into the open arms and the total percentage of time spent on the open arms. An anxiety index was calculated as follows: 1 − [(time spent in open arms/total time on the maze) + (number of entries to the open arms/Total exploration on the maze)/2]. Anxiety index values range from 0 to 1, where an increase in the index expresses greater anxiety-like behavior (Cohen et al., 2008). General locomotor activity was assessed by measuring the total distance travelled in the maze (EthoVision software; Noldus, Netherlands).

2.5. Open field

The open field apparatus consisted of a circular arena (60 cm diameter) surrounded by a wall (50 cm-high). The arena was divided into four quadrants. A smaller circle (30 cm diameter) was drawn in the center of the arena, which further subdivided the arena into central (15 cm far from the wall, with 4 sections) and peripheral (with 8 sections) compartments. At the beginning of each trial, animals were placed in the peripheral compartment. Each trial lasted 5 min and the amount of time spent in the central compartment and the total percentage of squares crossed in the central compartment (in relation to the total squares crossed in the arena) were used as parameters to assess anxiety-related behavior. General locomotor activity was assessed by measuring the total squares crossed in the arena.

2.6. Euthanasia and CORT analysis

Immediately after stress or after behavior tests, rats were exposed to isoflurane anesthesia for 30 s and decapitated. Blood was collected from the body trunk in non-heparinized 2 ml tubes, allowed to clot at room temperature for 30 min and then centrifuged at 4000 rpm for 10 min. We estimate that blood was collected within 1 min of removal from the home cage, thus minimizing any contribution to CORT from either isoflurane exposure or euthanasia. Serum was collected and stored at −80 °C. Concentrations of CORT serum were quantified using the Corticosterone EIA Kit® (Enzo Life Sciences). Serum samples were diluted 1:30 and processed following manufacturer's instructions.

2.7. Dendritic arborization

Brains from animals subjected to the EPM test were processed for Golgi staining of the BLA. Following decapitation, the brain was removed and prepared using the Rapid Golgi Kit (FD NeuroTechnologies, Inc.) according to the manufacturer's instructions. Brains were incubated in impregnation solution for 14 days followed by incubation in cryoprotectant solution for 48 h, coronally sliced (120 μm) using a Leica CM3050-S cryostat (Leica Biosystems), and mounted on gelatin coated slides. Brain sections were dehydrated in ethanol, diaphonized in xylene, and coverslipped. Images of the whole BLA were captured in bright field with the slide scanner Zeiss Axio Scan.Z1 (Zeiss), using a 40X objective. A z-stack was created with a step size of 410 nm. The images were analyzed in ZEN 2 (Zeiss) software with magnification of 600×. A total of 80 neurons (5 neurons per animal, 4 animals per group) were analyzed; only neurons with the soma completely localized in the BLA, heavily impregnated, without truncated dendrites, were included. After neuronal soma identification, we classified each dendritic segment as primary (originating from the soma), secondary (originating from the primary branch), tertiary (originating from the secondary branch), or quaternary (originating from the tertiary branch). All protrusions originating from a dendrite, regardless of the morphologic classification, were considered spines. After we determined the origin and the end of each branch, the dendritic branch length was calculated by the ZEN 2 software.

2.8. Statistical analysis

For results with more than 2 experimental groups, the statistical analyses were performed using two-way ANOVA, in which housing conditions (standard or EE) and stress (unexposed or exposed) were factors. Post hoc Tukey's multiple comparison test examined differences between individual groups. The OF test was analyzed with an unpaired two-tailed Mann-Whitney test. GraphPad Prism software 7.0 was used for the statistical analyses and the level of statistical significance was $p < 0.05$. Data are presented as mean ± SEM. Additional details of all analyses are presented in Supplementary Table 1.

3. Results

EE protects against the late anxiety-like behavior triggered by 2 h of acute restraint stress, as shown in Fig. 1. Acute restraint stress reduced open arm entries (Fig. 1A) and time spent in the open arms (Fig. 1B) in standard-housed but not in enriched-housed animals. The anxiety index corroborates these results; a higher anxiety index was found in SC, Stressed animals compared to each of the other groups (Fig. 1C). Finally, there were no differences in the distance travelled in the EPM across the experimental groups, ensuring that neither housing conditions nor stress influenced locomotor activity (Fig. 1D). The OF test confirmed that stress promotes anxiety-like behavior in standard-housed but not in enriched-housed animals. The time spent in the central compartment was lower in SC, stressed animals compared to each of the other groups (Fig. 1E), with no differences in the animals' motor activity between the groups (Fig. 1F).

Next, we evaluated dendritic morphology in the BLA. As depicted in Fig. 2A, acute restraint stress increased, 10 days later, the spine density in all dendritic segments of both SC and EE animals compared to Unstressed animals. We also investigated differences in two other dendritic arborization parameters, with no differences in both total dendritic length (Fig. 2B) and number of branch points (Fig. 2C) among all experimental groups. Multiple studies have linked stress-induced changes in CORT to subsequent hypertrophy of BLA neurons (Kim et al., 2014; Mitra and Sapolsky, 2008; Rao et al., 2012). Thus, we examined CORT serum levels in both EE and SC animals immediately after the restraint stress and 10 days later. We found significantly higher levels of CORT in both the Stressed SC and EE groups immediately following stress (Fig. 2D), with no differences in CORT 10 days later (Fig. 2E).

4. Discussion

Confirming previous findings (Mitra et al., 2005; Rao et al., 2012), our results showed that 2 h of restraint stress promotes, 10 days later, anxiety-related behavior and increases the spine density in the BLA of the rats. Furthermore, in agreement with a previous report (Mitra et al., 2005), this acute restraint stress did not lead to changes in dendritic length or in the number of branch points, effects that are attributed to repeated restraint stress or exogenous administration of CORT (Mitra et al., 2005; Mitra and Sapolsky, 2008; Vyas et al., 2002). Moreover, we