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Research report

A single session of emotional stress produces anxiety in Wistar rats

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Abstract

The sensorial stimulation arising from a physically stressed (PS) subject may produce emotional stress in a witnessing partner (WP). Both members of the pair develop functional changes. We tested changes in locomotor activity (crossing) and in the defensive burying test in WP, and PS adult male Wistar rats having been submitted to a single 10 min session in a two-compartment cage. During this session, the WP rats received auditory and olfactory stimulation coming from a PS pair submitted to unavoidable electric footshocks (1 mA, dc, 0.5 s, 0.5 c/s, 10 min). This experiment was replicated in other groups pre-treated with vehicle or diazepam, and their urine was collected and analyzed by the static Head-Space and Gas Chromatography–Mass Spectrometry (HS-GC/MS) techniques. The WP group displayed a significantly higher crossing [$F_{(2,45)} = 4.31$, $P < 0.01$] and more cumulative burying time [$F_{(2,22)} = 4.73$, $P < 0.01$] than the control or PS groups. Diazepam (1 mg/kg) reverted these changes. Our results indicate that the conspecific sensorial communication coming from the PS group produces anxiety probably mediated by 2-heptanone, since the HS-GC/MS analyses showed the highest amount of 2-heptanone in the urine from the PS group [$F_{(2,42)} = 5.17$, $P < 0.009$].

Keywords: Anxiety; Alarm substances; Burying test; Diazepam; Emotional stress; 2-Heptanone

1. Introduction

When a group of mice or rats shares an uncomfortable experience, they develop functional alterations [9,31], regardless of whether they receive unavoidable physical stress (PS) or only participate as witnessing partners (WP). Under these conditions, the WP may develop hyperthermia [19], suppression of T-killer and B-cells [8], stomach lesions and high corticosterone plasma levels [15]. It is noteworthy that rats and mice are macrosmatic species [13]; consequently, the participation of odors as cues in conspecific communication must be explored [45]. In fact, PS rats submitted to unavoidable electric footshocks excrete certain substances in their urine and faeces which, upon being perceived by a WP, increase locomotion and other activities such as rearing and climbing of the walls in the WP [24,25,54].

The open field activity test is widely employed since locomotion means spatial exploration and motivation, and is affected by stress [18]. WP rats maintained in relative safety situation, once being exposed to odors and ultrasonic vocalizations coming from PS rats, increase their locomotion in an attempt to find a way of escaping from the disturbing environment [29]. Likewise, PS rats having received unavoidable low-intensity electric footshocks during 5 days in a two-compartment cage decrease their time of ambulation, rearing and sniffing in the open field test, whereas WP displays increased locomotion [34–37,50]. Also, by using Treit's defensive burying test [47–49], Pijlman and van Ree [34] found that, although the total cumulative burying is similar between the WP and PS groups, the peak of burying varies through the 10 min test, depending on WP or PS condition. Therefore, the effects of stress on locomotion and anxiety may depend on the elapsed time between the stress and the test sessions, and on the severity of the stress.

Most of the stressor substances reputed as alarm cues are volatile six to eight carbon chains containing a ketonic group

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[40]. Among other ketones, 2-heptanone is normally present in the urine from *Mus musculus* female mice and from strain C57BL/10 [2,44] as well as in human beings [1]. It is possible that emotional stress constitutes a sufficient stimulus to produce anxiety in WP rats, perhaps through some alarm substance excreted in the urine from PS animals. Therefore, in the present study we evaluated: (a) the locomotion and the time spent in burying as indicators of anxiety in WP, PS and control Wistar rats; (b) the effect of diazepam; (c) the content of ketones in their urine through static Head-Space and Gas Chromatography–Mass Spectrometry (HS-GC/MS).

2. Material and methods

2.1. Animals and housing

All experiments followed the principles of animal care published by the National Institute of Health. We included 121 male Wistar rats aged 3 months and weighing 300–350 g at the beginning of the study. They were maintained inside housing facilities, in acrylic translucent boxes (45 cm × 30 cm × 30 cm) in groups of 8 animals per box, with a 12-h light:12-h darkness cycle (light ON at 7:00 a.m.) and ad lib access to water and food. All the experiments were carried out during the period of light between 9:00 and 11:00 a.m.

2.2. Apparatus

We constructed a two-compartment glass box (30 cm × 25 cm × 30 cm) with three opaque walls, a transparent frontal wall and a grid floor (stainless steel bars: 0.5 cm diameter, separated by 1.3 cm). The box was internally divided into two halves (15 cm × 25 cm × 30 cm, each) by an opaque plate (0.2 cm, thickness). With these dimensions of the cage, they can move with minimal restrictions in their respective compartment. A Plexiglas plate covered the floor of the safe-compartment (right) to prevent any electric shock to the WP group. The grid of the shocks-compartment (left) was electrified by an electronic stimulator (Grass Instruments S44, Quincy, MA, USA) coupled in series to a stimulus isolation unit (Grass Instruments SIU5, Quincy, MA, USA) and a constant current unit (Grass Instruments CCUIA, Quincy, MA, USA SIU5) for applying unavoidable electric footshocks (1 mA, dc, 0.5 s, 0.5 c/s, 10 min: 300 shocks) to the PS group. The open field test or the probe-burying test were applied immediately after their experience in the two-compartment box.

To evaluate locomotion, we used an acrylic box (44 cm × 33 cm) with walls 20 cm in height and with the floor divided into 12 squares (11 cm × 11 cm). Lastly, the defensive burying test was done in an acrylic box (27 cm × 17.5 cm × 15.5 cm) with the floor covered with a fine sawdust bed 5 cm deep. From one of the walls of the box (17.7 cm × 15.5 cm), an electrode (7 cm in length and 1 cm in diameter) protruded horizontally 2 cm above the sawdust bed. The electrode delivered constant intensity current (0.3 mA, dc) through an electronic stimulator (Grass Instruments S44) coupled in series to a stimulus isolation unit (Grass Instruments SIU5) and a constant-current unit (Grass Instruments CCUIA). We selected 0.3 mA as the shock intensity based on its proven sensitivity to anxiolytics [12,27,33], and because higher intensities produce freezing [10].

After each experimental session, the two-compartment cage, the open field or the defensive burying test boxes were carefully cleaned and deodorized with a cleaning solution (ammonia 0.5%, ethanol 15%, extran 10%, isopropyl alcohol 5%, pinol 19% and water 50.5%).

2.3. Physical and emotional stress

Firstly, each rat from the control, WP or PS group was placed alone in its corresponding side of the two-compartment box during a 5 min habituation session. The 10 min video-taped sessions began 24 h later. The rats from the PS and WP groups were individually but simultaneously placed in their corresponding compartment. While a PS rat received unavoidable electric footshocks, its WP pair supposedly perceived odors and vocalizations. Devoid of physical or emotional stress the control subjects (Ctrl) were individually placed in the safe-compartment.

2.4. Experimental groups

One set of animals (Ctrl, $n = 16$; WP, $n = 16$; PS, $n = 16$) was submitted to the open field test to evaluate their locomotor activity. A second independent group of rats ($n = 25$) underwent the defensive burying test (Ctrl, $n = 8$; WP, $n = 9$; PS, $n = 8$), and a third independent group was injected with diazepam (1.0 mg/kg, i.p.: Hoffman-Roche, Basel, Switzerland) or vehicle (Vh) 30 min before their experience in the two-compartment box and then submitted to the defensive burying test (Ctrl–Vh = 9, Ctrl–DZP = 7; WP–Vh = 8; WP–DZP = 8; PS–Vh = 8; PS–DZP = 8). In the last set, we collected a sample of about 0.1 ml urine of each rat from the floor or the two-compartment box with 1 ml syringes (Pisa®) and urine samples were placed in sealed vials for immediate HS-GC/MS analyses.

2.5. Behavioral tests

2.5.1. Locomotor activity: open field test

A 5 min pretest habituating session, discarded from the statistical analysis, was conducted 24 h before the 5 min video-taped test. Two independent observers counted the number of squares that each rat completely crossed in the open field box (crossing). No other behaviors, such as rearing or sniffing, were evaluated.

2.5.2. Anxiety test: burying behavior

After each rat had been isolated in an individual cage during 72 h and had undergone the two-compartment cage experience, we evaluated the latency and the total time spent in burying during 10 min. Habitually, when a rat explored the box, it incidentally received a shock; then it began to cover the electrode with the sawdust [38]. The time elapsed between the first shock received and the first attempt at burying (latency) is inversely indicating the rat's reactivity [41]. Likewise, the total amount of time spent in burying (cumulative burying) is directly related to their anxiety [38,49]. In the diazepam group, the rats were tested in open field activity immediately after the defensive burying behavior test.

2.6. HS-GC/MS analyses

Analyses of volatile compounds in urine were carried out using a 7694E Headspace sampler. The vial was sealed with a septum lined with PTFE and heated to 100 °C with an equilibrium time of 25 min for an injection time corresponding to 1 min. The analyses were performed using a Hewlett-Packard G1800B GCD System (Agilent Technologies, México) gas chromatograph. After headspace, the volatile components were injected and separated using fused silica HP-5 crosslinked capillary columns, 5% phenyl-methylpolysiloxane as non-polar stationary phase siloxane (30 m × 0.25 mm i.d. and 0.25 μm film thickness), supplied by Agilent Technologies. The gas chromatograph was programmed so that the oven temperature was kept at 40 °C for 3 min and increased to a final temperature of 180 °C at a rate of 20 °C/min with helium as the carrier gas at a flow rate of 1 ml/min. All compounds were initially identified based on an NIST mass spectra library search. Once the most constant ketone had been detected, its identification was confirmed by comparing the spectra and retention times with an authentic standard (2-heptanone, Sigma Chemical Co., P.O., St. Louis, MO, USA), and its concentration was determined by comparing the ratio of the peak area in the samples with a known concentration of this compound in a calibration curve. The ion m/z 43.15 was used for quantifying and the ion m/z 58.15 for qualifying ion identification.

2.7. Statistical analysis

For testing differences among Ctrl, WP and PS groups in locomotor activity and in the defensive burying test, we used the one-way ANOVA for independent groups and Dunnett's test as post hoc. For testing drug treatment differences in the other Ctrl, WP and PS groups, we used the two-way ANOVA and the Student–Newman–Keuls test. The criterion of statistical