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Research report

Decreased anxiety-like behavior and locomotor/exploratory activity, and modulation in hypothalamus, hippocampus, and frontal cortex redox profile in sexually receptive female rats after short-term exposure to male chemical cues

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ABSTRACT

Chemical cues are widely used for intraspecific social communication in a vast majority of living organisms ranging from bacteria to mammals. As an example, mammals release olfactory cues with urine that promote neuroendocrine modulations with changes in behavior and physiology in the receiver. In this work, four-month-old Wistar (regular 4-day cyclic) virgin female rats were utilized in the proestrus-to-estrus phase of the reproductive cycle for experimental exposure. In an isolated room, female rats were exposed for 90 min to male-soiled bedding (MSB). Elevated plus-maze assay, open field test, and light/dark box task were performed to analyze behavioral alterations on females after exposure. For biochemical assays, female rats were killed and the hypothalamus, hippocampus, and frontal cortex were isolated for further analysis. Antioxidant enzyme activities (superoxide dismutase, catalase and glutathione peroxidase), non-enzymatic antioxidant defense measurements (TRAP and TAR), and the oxidative damage parameters (TBARS, Carbonyl and SH content) were analyzed. In behavioral analyses we observe that female rats show decreased anxiety and locomotory/exploratory activities after MSB exposure. In biochemical assays we observed an increase in both enzymatic and non-enzymatic antioxidant defenses in different central nervous system (CNS) structures analyzed 30 and 90 min after MSB exposure. Furthermore, hippocampus and frontal cortex showed diminished free radical oxidative damage at 180 and 240 min after exposure. These results provide the first evidence that oxidative profile of female CNS structures are altered by chemical cues present in the MSB, thus suggesting that pheromonal communication is able to modulate radical oxygen species production and/or clearance in the female brain.

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1. Introduction

The sense of smell is fundamentally important to many animals. Some species release chemical substances into their surroundings to influence the behavior or physiology of members of the same species [55]. Chemical cues are widely used in intraspecific social communication in a vast majority of living organisms ranging from bacteria to mammals [3,8,26]. In a variety of species, sexual and reproductive behaviors are closely dependent on communication through chemical signals [40,52]. As an example, mammals release olfactory cues with urine that promote neuroendocrine modulations with changes in behavior and physiology in the receiver [32,35]. In addition, some behavioral and physiological responses are induced by airborne signals, while other organisms require physical contact between the receiver and the chemical cue [45]. Male and female-soiled bedding contain both volatile and non-volatile compounds that are known to be important in intraspecific communication, for instance promoting mate selection and copulatory activity [10,37,43]. Recent developments have led to an appreciation of the diversity of chemosensory systems and their complementary roles in influencing vertebrate physiology and behavior [9], thus indicating that pheromone activated hormonal systems play a wide range of neuroendocrine functions within the brain.

Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are pivotal enzymes for the normal function of several important biological processes in mammals, with known

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importance in brain tissue physiology/pathology [22]. SODs are highly efficient in the catalytic dismutation of $O_2^{\bullet-}$, generating H_2O_2 that can be removed by other enzymes, such as CAT and other peroxidases (e.g. GPx). Recently, novel insights into the role of intracellular redox state in cell survival, division and differentiation of central nervous system (CNS) were discussed [46,47], and the role of redox signaling in neurotransmission was also described [47]. Chemical cues are a primary factors to induce disturbances in behavior for a variety of species but, to our knowledge, there is no information on the modulation of reactive species production and antioxidant defenses in the female CNS structures by male chemical cues. Here, we analyzed oxidative stress parameters in the hypothalamus, hippocampus and frontal cortex of receptive female rats induced by male-soiled bedding (MSB) exposure. In addition, a modulation on elevated plus-maze anxiety-like behavior test was also observed after MSB exposure, suggesting a decrease in anxiety-like behavior in female rats after MSB exposure. The differences observed on both open field and light/dark box tests suggest a decreased locomotory and/or exploratory activities in receptive female rats after MSB short-term exposure.

2. Methods

2.1. Animals

Four-month-old Wistar (regular 4-day cyclic) virgin female rats (250–300 g) were obtained from our breeding colony. Animals were housed in standard cages, five per cage, in controlled temperature room (23 ± 1 °C), with a 12 h light:12 h dark cycle, lights on at 7:00 a.m. Standard laboratory chow and water were available ad libitum. Fifteen days after delivery (early before puberty) pups were sexed, males were removed (kept in male–female common room), and the females were brought to a room in complete absence of adult male chemical cues or pregnant female rats. All experimental procedures were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1985) and were carried out according to the determinations of the Brazilian College of Animal Experimentation, COBEA.

2.2. Experimental design

To collect soiled bedding from male rats housing cage, forty virgin male rats (120 days old, 300–350 g) were kept in cages (five per cage) with 50 g of wood shavings for 48 h, according to a previous reported protocol [43]. These males were housed in a room with other adult males and females. To ensure a homogeneous composition throughout the experiment, MSB from eight cages were thoroughly mixed, and utilized just before the female exposure. Control female rats were exposed to saline-soiled bedding as previously reported protocol [58].

We used sexually naive female rats in the proestrus-to-estrus phase of the reproductive cycle for experimental exposure to MSB. Vaginal smear cytology analyses are used for the determination of the estrous cycle phases [38]. Three groups of rats were utilized for behavior analysis. Twenty-four rats were subjected to the elevated plus-maze test (n = 12 for control and MSB groups), twenty-two were subjected to the open field test (n = 11 for each group), and eighteen were subjected to the light/dark box test (n = 9 for each group), not being used for any other further biochemical/behavioral analysis. Another thirty-six rats were utilized for biochemical analysis (n = 6 for four treated groups, and n = 3 for respective control groups). In an isolated room, female rats were exposed (five or four per cage, in a random distribution) for 90 min to soiled bedding previously collected. All treated female rats were exposed to the same mixed MSB. After the exposure females were housed in clean cages with naive-soiled bedding. Control- and MSB-exposed females were housed in different rooms under equivalent conditions.

For biochemical assays, female rats were killed by decapitation at 30, 90, 180, and 240 min after the end of the 90-min-period of exposure to either MSB or saline-soiled bedding. Rats exposed to the MSB were considered the treated group (T30, T90, T180 and T240), while respective animals exposed to the saline-soiled bedding were considered control groups (C30, C90, C180 and C240). After exposure, the animals were sacrificed and the brain regions hypothalamus (groups HptT30, HptT90, HptT180 and HptT240), hippocampus (groups HpcT30, HpcT90, HpcT180 and HpcT240), and frontal cortex (CtxT30, CtxT90, CtxT180 and Ctx240), as well as the respective control groups were isolated. Sample aliquots were immediately stored at −80 °C to further analysis.

2.3. Behavior tasks

All behavior tasks were performed in the morning after the predicted night of ovulation. Proestrus-to-estrus estrous cycle phase was confirmed at 6:00 a.m. We performed the exposition to MSB at 7:00 am (90 min of exposition), followed by 30 min of ambiance in experimental behavior room without MSB presence, and behavior tests observation initiate at 9:00 a.m. The observation was initiated 30 min after exposure and was terminated close to 240 min after exposure. Control and MSB female rats were observed in an alternating pattern, and all the apparatus utilized were thoroughly cleaned after each test. There was no difference in plus-maze activity, open arm and light/dark box exploration among control subgroups, as well as MSB subgroups, so they were pulled in two single groups, named "control" and "treated with MSB". A video-tracking system with a digital video camera was utilized for data collection. Females utilized for behavior assays were not utilized for biochemical analysis.

2.3.1. Elevated plus-maze test

The elevated plus-maze, a well described assay of anxiety-related behavior in rodents, was performed for behavioral analysis [63]. The elevated plus-maze used here consisted of two open arms (45 cm long × 10 cm wide, with 5 mm high railing) and two enclosed arms of equal length and width (45 × 10 cm with 40 cm high walls) forming a square cross with a 10 cm square center piece (60 cm from the floor). Female rats were placed in the center of the maze facing an open arm of the maze at the start of the test. The mice were then returned to their home cages at the end of the 5 min test. The plus-maze experimental room was illuminated with only one red overhead light (14-W) to permit videotaping. Light intensity was immeasurable at the floor of the testing apparatuses. The time spent in the open arms and the number of entries into the open and closed arms was analyzed.

2.3.2. Open field test

The open field is the oldest of anxiety tests that are based on the spontaneous exploration of an apparatus [50]. The apparatus consisted of a circular arena surrounded by 40-cm high walls. It was situated in a separate brightly lit room illuminated with two, 40-W fluorescent overhead lights each. Two black circumferences divided its white floor into three concentric circles, diameters of which were 20 cm, 50 cm, and 80 cm. Several radial lines crossed the outer circles dividing them into sixteen equal cells in the periphery, eight in the medial circle, and four in the center. All the animals were gently placed in the periphery of the arena to freely explore it for 5 min. Then, they returned to their home cages. The number of visits to the center of arena, the number of crossings from one region to another in the periphery and in the center, and the number of rearings were registered.

2.3.3. Light/dark exploration test

The animals were allowed to explore a light/dark arena 30 min after the end of MSB exposure. The light/dark box [6,16] consists of two compartments: one light compartment (45 cm × 40 cm × 25 cm), with a transparent acrylic panel on one side to facilitate observation of the rat, also the surrounding walls and floor were painted white, and the dark compartment (34 cm × 40 cm × 25 cm) with the surrounding walls and floor painted black. The light compartment was illuminated under an 80-W light. The dark one received only part of the room illumination (at 20-W). The floor of the light compartment was divided into 12 equal squares by black lines and the dark compartment was divided into 9 equal squares by white lines. The two compartments were separated by a partition with an opening to allow passage from one compartment to the other. The rats were gently placed in the corner of the light compartment facing the wall opposite to the opening. The following parameters were analyzed (5 min of behavioral observation prior to testing): (1) latency time for the first crossing to the dark compartment, (2) the number of transitions between the light and the dark compartment, (3) the total time spent in the illuminated part of the cage, (4) number of crossings (horizontal activity) in each compartment, (5) number of rearings (vertical activity), and (6) the risk assessment behavior index (RA, i.e., the number of times the animal in the dark compartment explored the light compartment).

2.4. Redox profile on CNS structures

2.4.1. Antioxidant enzyme activities quantification

Superoxide dismutase (SOD, E.C. 1.15.1.1) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm, as previously described [42]. Results are expressed as Units SOD/mg protein. Catalase (CAT, E.C. 1.11.1.6) activity was assayed by measuring the rate of decrease in H_2O_2 absorbance in a spectrophotometer at 240 nm [1]. CAT activity is expressed as Units CAT/mg protein. Glutathione peroxidase (GPx, E.C. 1.11.1.9) activity was determined by measuring the rate of NADPH oxidation in a spectrophotometer at 340 nm, as previously described [66]. GPx activity was expressed as Units (nmol NADPH oxidized/min)/mg protein.

2.4.2. Non-enzymatic antioxidant defenses measurement

The non-enzymatic antioxidant potential of the reproductive tract structures was estimated by the total antioxidant potential (TRAP) and total antioxidant reactivity (TAR) [33]. The reaction is initiated by adding luminol (5-Amino-2,3-dihydro-1,4-phthalazinedione, 4 mM) – an external probe to monitoring radical production–and AAPH (2,2'-Azobis–2-methylpropionamidine–dihydrochloride, 10 mM) – a free radical source that produces peroxyl radical at a constant rate – in glycine buffer (0.1 M) pH 8.6 at room temperature, resulting in a steady luminescence emission (system counts). Chemiluminescence was read in a liquid scintillation counter (Wallace

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