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Research report

Postnatal exposure to synthetic predator odor (TMT) induces quantitative modification in fear-related behaviors during adulthood without change in corticosterone levels

R. Hacquemand, G. Pourie, L. Jacquot, G. Brand *

Laboratoire de Neurosciences, Université de Franche-Comté, Place Leclerc, 25,000 Besançon, France

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ABSTRACT

Environmental stimuli and adverse experiences in early life may result in behavioral and physiological changes in adulthood. In several animal species, the odors cues are crucial in the setting of adaptive behaviors, especially towards predators. However, little is known about the effects of postnatal exposure to predator odor on the later physiological and behavioral responses to this natural stressor. Thus, the aim of this study was to *investigate* the effects of a postnatal exposure to synthetic predator odor (TMT) *in mice pups* on later adult fear-related behaviors and corticosterone levels in response to this specific stimulus. Pups postnatally exposed to only water showed later in adult life behavioral responses when exposed to TMT that were statistically different from mice that were exposed as neonates to TMT. In addition, mice exposed as neonates to TMT showed a decrease of fear-related behaviors while no differences occurred in the corticosterone levels between both groups.

1. Introduction

Adult behavioral and physiological responses are partly dependent on neonatal experiences. In a wide variety of mammals, neonatal period is very sensitive to chemosensory signals and early postnatal experiences may play a significant role in determining how an animal deals with predatory threats later in life. In rodents, several studies have examined the effects of a novel or unfamiliar environment, adverse experience such as "handling" or stressors (e.g. noise, electric shock or proximity to an unfamiliar adult male) during early postnatal periods on later adult life [1–7]. Stress in adulthood is usually evaluated in response to predatory odors, especially the synthetic 2,4,5-trimethythiazoline (TMT), a component of natural fox feces. Surprisingly, no study had investigated the effects of a postnatal inhalation exposure to TMT on physiological and behavioral responses to this molecule in adulthood, while the interest for fear-related studies is increasing in neuroscience.

Fear is a crucial physiological and behavioral response for animal species allowing to resist against environmental pressures like predation. Two kinds of fear are described in laboratory condition: conditioned and unconditioned fears. Fear conditioning is a process that plays an adaptative role in generating defensive behaviors during threatening situations and that renders neutral stimuli reminiscent of a threatening situation able to generate inappropriate fear responses in non-threatening situations [8]. Unconditioned fear is an innate response without learning in a threatening situation. Among unconditioned stimuli used to generate fear, electric shocks, restraint, tail-flick, hot plate or formalin tests are common. However, most of them can simultaneously induce pain which involves another neural circuitry. This can be avoided using other unconditioned stimuli which are more appropriate to induce fear, especially predator cues.

Predator cues like feces or collar soaked with fur/skin odor are very efficient to induce robust fear behaviors [9]. However, since approximately 10 years an increasing number of studies have used synthetic fox anal gland secretion named 2,4,5-trimethylthiazoline (or TMT) isolated by Vernet-Maury [10,11]. Overall, these studies showed that TMT induced various fear-related behavioral responses such as a longer time to enter in an open-field, more defecation and miction, less motor activity and less approach to the center of the open-field. Concerning neurophysiological consequences, TMT exposure is able to enhance dopamine release in the medial prefrontal cortex and amygdala, and leads to elevated plasma corticosterone levels [12,13] as well as an inactivation of the bed nucleus of the stria terminalis blocks freezing, a specific fear-like response [14]. Comparatively, TMT does not induce the same responses as cat odor [15,16]. The possibility that TMT acts more as an unpleasant and nocive substance than as a predator cue is currently being discussed, probably due to the high concentration used which is not in accordance with environmental

* Corresponding author. Tel.: +33 381 66 57 52; fax: +33 381 66 57 46.
E-mail address: gerard.brand@univ-fcomte.fr (G. Brand).

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conditions. Recently, authors [17] showed that 10% TMT induces the same avoidance response *as* butyric acid, a strong trigeminal nerve activator [18], but only TMT produced a robust freezing indicating that TMT acts more like a predator cue, only at weak concentrations. Additionally, it has been shown [19] that the avoidance is higher with either pure or 50% TMT as compared to natural fox feces, whereas the difference is slight with 10% TMT.

In the field of fear-related behavior to predator odor, although it is well known that early postnatal stress can profoundly affect subsequent adult behaviors, no investigations have measured the influence of a juvenile exposition to TMT during the adulthood. For instance, adult Wistar male rats exposed to a repeated social stress presented an increasing social anxiety at adulthood with an unfamiliar male rat [20], and a daily separation of their mother provokes in 2-week-old mice pups an increase of anxiety in the elevated plus-maze test at adulthood, especially if dam was stressed during the separation time [21]. From a neurobiological point of view, it has been shown that foot shocks in 3-week-old pups attenuated extinction of contextual fear conditioning under the dependence of serotoninergic mechanisms [22].

Thus, the aim of the present work in mice was to study the effects of a 3 weeks postnatal exposure to synthetic predator odor TMT on subsequent adult behaviors and corticosterone levels in response to a short TMT inhalation exposure. Concerning the behavioral measures, several parameters in relation to avoidance, motor activity and stress are considered. Corticosterone is the major glucocorticoid in mice [23] showing a robust response to TMT exposure [12].

2. Materials and methods

2.1. Animals

The animals were offspring of 4 female Crl:OF1 mice. Pregnant females were purchased from Charles River (France). When females delivered, they were housed with their own pups in home polycarbonate cages (type E: depth, 40.5 cm; width, 25.5 cm; height, 19.7 cm; floor area, 1,032.75 cm^2) with open stainless steel wire lids (Charles River, France). The animals were *kept* in *a* room at constant temperature (22 ± 1 °C), constant humidity (45–55%) and at constant luminosity (350 ± 30 lux) under a 24 h cycle with light phase of 12 h from 8 p.m to 8 a.m. Animals had free access to food pellets and water. The study was carried out in accordance with the "Guide for the care & Use of Laboratory Animals" (National Institute of Health, USA, 1985).

2.2. Postnatal exposures

At postnatal day 1, two females with their pups were exposed to 1% TMT and two other females with their pups were exposed to distilled water (30 min per day, 5 days per week during 3 weeks). During the exposure periods, mice were placed in an inhalation chamber (long: 80 cm; wide: 42 cm; deep: 41 cm; volume: 0.13 m^3). 150 μl of 1% TMT ($C_6H_{11}NS$, PheroTech, Britannic Colombia, Canada) diluted with an agitator in distilled water were placed on a piece of cotton in a open glass in the exposure chamber. Two silicone hoses (1 m length, 1 cm diameter) placed into two sides of the exposure chamber allowed passive ventilation during 1% TMT exposure periods. When mice were 12 weeks old, two groups of 10 females were constituted: one exposed to 1% TMT (named *TMT group*) and another exposed to distilled water (named *control group*). The study considered only females because differences have been shown in relation to sex in response to predator odor, i.e. females are more sensitive than males [9,19]. Moreover, females were housed ten per cage in order to induce a homogeneous hormonal state in the group, i.e. anestrus, while adult males cannot be housed in the same conditions.

2.3. Behavioral tests

The ten mice of each group performed all the behavioral tests. The test order of the three experimental conditions (corridor, open-field and elevated plus-maze) was randomized and the experimental condition tests were realized in separated sessions on the same day. Corridor, open-field and elevated plus-maze were carefully washed with alcohol and dried between each animal passage.

In accordance with previous studies [19,24], preference/avoidance response to odorant was evaluated in a corridor maze (60 cm in length, 7 cm in breadth, and 7 cm in height). Both ends of the corridor contained a watch glass with a filter paper soaked with 5 μl of either 1% TMT or distilled water (control/water zone). TMT odor and water were randomly distributed in the right and the left side at each test. Each mouse was placed at the middle of the corridor and then allowed to move freely for 3 min. At each end of the corridor, a hydraulic exhaust fan (2 l/min air) prevented the diffusion of odors beyond the middle of the corridor. Each test lasted for 3 min to prevent habituation. The movements of the mice were video recorded and analyzed with the EthoVision video tracking system for automation of the behavioral experiments (Noldus Technology, Wageningen, The Netherlands). Data collected concerned the total duration spent by each mouse in each half part of the corridor.

Distance to odorant, velocity of movement, immobility and freezing behavior were measured in a black circular open-field (36 cm in diameter, 20 cm in height). The odorant stimulus was placed in the center of the open-field and each test of 3 min was video recorded and analyzed with the EthoVision system. Data collected with EthoVision concerned the total time of immobility (in seconds) and the velocity of movement (in 'cm/second), two parameters considered to be indexes of general activity, while the mean distance to odorant (in centimeters) was recorded as an index of avoidance. At the same time, freezing behavior (in seconds) was also checked by the experimenter on a control screen (as distinguished from immobility recorded by the EthoVision system).

Stress behavior was measured in a classical elevated plus-maze test. The arms were arranged in a cross, with two opposite arms enclosed (closed arms) and the other two arms left open (open arms). The arms intersected at a central 6 cm × 6 cm square platform. Each arm was 24 cm long and 6 cm wide. The closed arms had walls along the sides and at the end that were 15 cm high. Immediately after a 5 min exposure period in a closed chamber containing the odorant stimulus, the mouse was placed on the center platform facing a closed arm and allowed to move freely in the elevated plus-maze for 3 min. An arm entry was counted when the hind paws of the mouse were completely within the arm. Two parameters are classically recorded in the elevated plus-maze, the duration spent in the open arms (in seconds) as an index of stress level [25], i.e. a decrease of stress level corresponding to an increase of the duration spent in open arms, and the number of entries in the closed arms as an index of general activity [26], which was not considered in the present study.

2.4. Estimation of corticosterone level

At the beginning of the active phase, adult mice were placed during 30 min in a chamber with 1% TMT or with distilled water. After that, animals were anesthetized and blood was collected using the eye bleed technique and centrifuged (3000 rpm, 10 min, 4 °C). Plasma was stored at −20 °C. Corticosterone level for each plasma sample was determined with a Corticosterone DA ^{125}I Test kit obtained from MP Biomedicals (LLC, Diagnostics Division, Eschwege, Germany).

2.5. Statistical analyses

Data were statistically evaluated with Statview 5.0 software using 2 (group) × (experimental condition) ANOVAs and Scheffé *post hoc* tests. Data were expressed as means ± standard errors. The significant level was set at 0.05. The non-significant results were noted as ns.

3. Results

3.1. Behavioral experiments

Results of preference/avoidance tests in a corridor are reported in Fig. 1. The ANOVA showed a group effect ($F_{1,19} = 5.96$, $p < 0.01$) and an experimental condition effect ($F_{1,19} = 64.54$, $p < 0.0001$). *Post hoc* Scheffé tests showed that mice spent more time in the water zone than in the TMT zone, for both the *TMT group* ($F = 8.47$, $p < 0.01$) and for the *control group* ($F = 16.09$, $p < 0.001$). Moreover, mice of the *TMT group* spent more time in the TMT zone than the control group ($F = 4.51$, $p < 0.05$) while there are no statistical differences between both groups concerning the water zone ($F = 1.87$, ns).

Concerning distance to odorant, velocity of movement, immobility and freezing parameters collected in an open-field, results are reported in Fig. 2(a–d). For distance to odorant, the ANOVA showed a group effect ($F_{1,19} = 17.14$, $p < 0.001$) and an experimental condition effect ($F_{1,19} = 7.62$, $p < 0.01$). *Post hoc* Scheffé tests showed no significant difference between both groups in the water experimental condition. In contrast, significant differences occurred between both groups in the TMT experimental condition, i.e. mice of the *TMT group* "stood closer to" 1% TMT ($F = 18.52$, $p < 0.001$) than mice of the *control group*. For the velocity of movement, the ANOVA showed a group effect ($F_{1,19} = 11.24$, $p < 0.001$) and an experimental condition effect ($F_{1,19} = 24.20$, $p < 0.0001$). *Post hoc* Scheffé tests showed no significant difference between both groups in the water experimental condition. In contrast, significant differences occurred between