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Fear-like behavioral responses in mice in different odorant environments: Trigeminal versus olfactory mediation under low doses

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Odors can have repulsive effects on rodents based on two complementary adaptive behaviors: the avoidance of predator odors (potentially dangerous) and the avoidance of trigeminal stimulants (potentially noxious). The present study aimed to compare the behavioral effects on mice of odors according to their trigeminal properties and ecological significance. We used three different odors: 2,4,5-trimethylthiazoline (TMT: a fox feces odor frequently used to elicit fear-induced behaviors), toluene (a strong stimulant of the trigeminal system) and phenyl ethyl alcohol (PEA: a selective stimulant of the olfactory system). First, we checked preference and avoidance behaviors in mice with and without anosmia towards these odors to ensure their olfactory/trigeminal properties. Secondly, we used a standard test (open-field and elevated plus-maze) to assess the behaviors of mice when exposed to these odors. The results show that the anosmic and control mice both avoided TMT and toluene odors. In the open-field and the elevated plus-maze, mice exhibited "anxious" behaviors when exposed to TMT. Conversely, exposure to PEA induced "anxiolytic" effects confirmed by low blood corticosterone levels resulting from completion of the elevated plus-maze. Compared with TMT exposure, toluene exposure induced moderate "anxious" effects.

1. Introduction

Olfactory cues are common sensorial stimuli that enable animals to collect information from the environment to guide their basic behaviors. In this context, odors are essential stimuli for most mammals, inducing environment exploration, feeding, sexual or social behaviors and also emotional behaviors such as fear, anxiety, etc. (Doty, 1986; Lledo et al., 2005). Animals can detect various olfactory cues that may be crucial to their survival, including pungent and predator odors. Animals show avoidance behaviors to pungent odors to avoid potential toxicity and exhibit an innate escape response to preserve their life when they detect a predator odor (Blanchard et al., 2001; Capone et al., 2005). In recent years, a large number of studies have used 2,4,5-trimethylthiazoline (TMT), a component of red fox feces odor, as a fear-inducing stimulus. Indeed, most odorants are able to simultaneously stimulate olfactory and trigeminal systems in the nasal cavity, at least at high concentrations (Brand, 2006). The trigeminal system has long been considered as essential in the defense systems of many species against potentially toxic or noxious molecules. Pungent odors are typically selective trigeminal stimuli. When considering predator odors, TMT may also act as a trigeminal stimulant and may not be mediated only by the olfactory system per se (Hacquemand et al., 2010). This point of view seems to be congruent with the fact that TMT can cause nausea in humans (Fendt et al., 2005). Several studies have used very large TMT volumes as high as 35 μl (Morrow et al., 2000a, 2000b; Day et al., 2004) and up to 100 μl (Falconer and Galea, 2003). These experimental conditions could explain the activation of both the trigeminal and olfactory systems (Brand, 2006; Boyle et al., 2007). Furthermore, Day et al. (2004) showed that TMT (volume: 35 μl) can activate the external lateral parabrachial nucleus, which is an essential structure of the trigeminal sensorial system. However, a behavioral study by Buron et al. (2007) showed that low doses of TMT can produce similar effects to natural conditions with fox feces. Using low doses of TMT seems to correspond more closely to natural conditions than high doses (over 30 μl) and fear-induced behavior, such as freezing, is more common with these lower doses (Blanchard et al., 2003; Endres et al., 2005). Moreover, such volumes also induce defensive behaviors (Wallace and Rosen, 2000; Blanchard et al., 2003; Endres et al., 2005; Fendt and Endres, 2008). This raises the question of whether TMT acts as a usual pungent odor, mediated by trigeminal nerve fibers, or as a specific stimulus due to its ecological significance (escape from predators).

The aim of the present study was, firstly, to evaluate which chemical sensorial systems (trigeminal and/or olfactory) are involved in TMT detection in rodents and, secondly, to assess the behavioral effects of this odorant compared with other odorants that have no ecological significance but that are known to be

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selective stimulants of either the olfactory or trigeminal system. For this purpose, we used phenyl ethyl alcohol (PEA: a synthetic rose odor), which activates only the olfactory system in humans, and toluene, which has a strong effect on the trigeminal system in humans (Doty et al., 1978). Firstly, we used an intranasal treatment with a solution of zinc sulfate ($ZnSO_4$), which is known to elicit a transient anosmia in mice (Ducray et al., 2002) without impacting the trigeminal system (Hansen et al., 1994). This enabled us to study withdrawal or attraction in response to the different odors in order to evaluate their olfactory/trigeminal properties with defined volumes. Secondly, we tested mice in an open-field and an elevated plus-maze (EPM), using the different odorant environments to assess their behaviors. Thirdly, blood samples were collected at the end of the elevated plus-maze test to assess plasma corticosterone levels.

2. Materials and methods

2.1. Animals

Adult female OF1 mice (Charles River, France) were used in this study. They were group-housed in standard-size Plexiglas cages, allowing them free access to food and water and maintaining them in a climate-controlled environment (22 °C) on a 12 h light/dark cycle (light onset at 7 a.m.). The animals were 3 months old at the beginning of the experiment and had no previous experience in any behavioral study. Different groups of mice were used for each test.

2.2. Odors

Apart from TMT (PheroTech, Delta, Canada), two odors were selected from the literature for their selective trigeminal or olfactory properties in rodents or humans. We used toluene (Sigma–Aldrich®, Steinheim, Germany), which activates the trigeminal system, and PEA (undiluted solution, Acros Organics®, France), a rose-like odor that is a selective stimulant of the olfactory nerve (Doty et al., 1978; Elsner, 2001; Jacquot et al., 2006). Distilled water was used for control conditions.

2.3. Behavioral tests

2.3.1. Preference/avoidance test

We carried out preliminary tests in order to check attraction and withdrawal behaviors in relation to the different olfactory cues. Preference/avoidance responses to odorants were evaluated in a corridor maze as already described (Buron et al., 2007). This apparatus was 60 cm long, 7 cm wide and 7 cm high. Both ends of the corridor contained a watch glass with a filter paper soaked with 5 μl of one of the three odorants or with distilled water. We also added a control condition with both filter papers soaked with distilled water. Odors and water were randomly distributed on the right and left sides in each test. Each mouse was placed in the middle of the corridor and then allowed to move freely for 3 min. At each end of the corridor, a hydraulic exhaust fan (1.5 L/min air) prevented the diffusion of odors beyond the middle of the corridor. The corridor was carefully washed with alcohol and dried between each animal passage. The corridor was divided into three virtual zones (of equal area), a medial zone, in the center of the corridor, and two lateral zones, at each end of the corridor. The presence or absence of odor was verified in the two lateral zones by gas chromatography and thus further analyses concern only these two zones, mentioned either as an odor zone or as a no-odor zone.

Intranasal perfusion of zinc sulfate leads to transient anosmia in mice (McBride et al., 2003) without any effect on trigeminal sensitivity (Hansen et al., 1994). This treatment allows the effect of trigeminal stimulation on preference/avoidance to be investigated in mice. A bilateral intranasal perfusion with 16 μl of 10% $ZnSO_4$ was performed to destroy olfactory neurons after light anesthesia with a 0.3 M chloral hydrate solution (5 mg/g body weight). This treatment induces a transient anosmia which appears 3 days after $ZnSO_4$ treatment (Ducray et al., 2002). This anosmia results from the specific destruction of mature olfactory neuroreceptors without impacting progenitor cells, subsequently leading to neurogenesis. 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, Netherlands). Data was collected concerning the total duration of time spent by the mice in each of the two lateral zones of the corridor. Fifteen untreated mice and 11 anosmic mice were used in each condition (control, PEA, toluene and TMT) of this preference test.

2.3.2. Open-field test

The locomotor activity of the mice was assessed in a circular open-field arena (48 cm in diameter, walls 15 cm high), divided into virtual central (30 cm in diameter) and peripheral (9 cm from the edge) zones. The mice were placed individually in the center of the open-field and their behavior was recorded for 5 min. Peripheral and central locomotion was recorded and the total duration of time spent in each of the two zones was calculated. 1.25 μl of odorants (PEA, TMT or toluene) were deposited on four filter paper strips fixed to the top of the wall and arranged according to the four virtual quadrants, in order to allow a homogenous diffusion of odors. A control condition was also performed with the filter papers soaked with distilled water. The arena was covered with glass to avoid odorant dissipation into the room. The maze was cleaned with 50% ethanol and the filter papers were changed after each trial. Data was collected with Ethovision concerning the time spent in each zone (peripheral and central). Freezing was defined as continuous inactivity lasting at least 3 s, and any behavior that yielded an inactivity of less than 3 s was recorded as general activity. The freezing percentage (total duration of freezing/total duration of observation), the distance moved and the velocity of movement were calculated. This test was performed using four groups of 10 naïve mice each.

2.3.3. Elevated plus-maze

The EPM was used to assess the anxiety-like behaviors of mice in the different odorant environments. The arms were arranged in a cross, with two opposite arms enclosed by walls (15 cm high) and the two other arms open. The arms intersected at a central 6 cm × 6 cm square platform. Each arm was 24 cm long and 6 cm wide. 5 μl of the three different odorants were placed on each of the four arms of the maze by rubbing with soaked filter paper. Another experiment was conducted with the paper filter soaked with distilled water as a control. The maze was cleaned with 50% ethanol and the filter papers were changed after each trial. The mice were individually placed in the center of the maze and their behavior was observed for 5 min. We took into account the percentage number of entries into the open arms compared with the total number of entries into an arm. This test was performed using four groups of 10 naïve mice each.

2.3.4. Corticosterone level

Immediately after the elevated-plus maze, 5 mice per group were randomly selected and anesthetized (chloral 0.2 g/kg). In addition, 5 mice exposed to TMT for 10 min and 5 mice exposed to water in standard-size Plexiglas cages were also anesthetized. These two supplementary conditions were selected as useful controls. Blood samples were collected using the retro-orbital bleeding technique and centrifuged (3000 rpm, 10 min, 4 °C). Plasma was