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Brief communication

The volatility of an alarm pheromone in male rats

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ABSTRACT

The volatility of an alarm pheromone in male rats. PHYSIOL BEHAV 00(0) 000-000, 2008. We previously reported that an alarm pheromone released from the perianal region of male rats is perceived by the vomeronasal organ and evokes stress-induced hyperthermia and defensive and risk assessment behavior. In addition, we recently reported that the alarm pheromone enhances the acoustic startle reflex (ASR). However, in contrast to our knowledge about such biological aspects of the pheromone, information concerning the physical character of the alarm pheromone is extremely limited. In this study, we investigated the volatility of the alarm pheromone using enhancement of the ASR as an index of the pheromone effect. The alarm pheromone enhanced the ASR when it was presented at a distance of 10 mm but not at 200 mm. In addition, the pheromone effect was observed even after the pheromone was trapped in the adsorbent (Tenax) and then extracted using purified water. These results suggest that the alarm pheromone is both volatile and water soluble.

1. Introduction

Many organisms communicate intraspecific information using chemosensory signals, in which alarm pheromones communicate the presence of danger among conspecifics [1]. Many studies have shown that stressed animals release the alarm pheromone, which evokes responses in recipients to avoid the source of danger [2–7].

We previously reported that foot-shocked male Wistar rats release an alarm pheromone that aggravates a rise in body temperature (stress-induced hyperthermia) [8]. This pheromone was produced in a testosterone-independent manner [9] and was released from the perianal region of the donor rat [10]. Pheromone recipient rats perceived this pheromone by the vomeronasal organ [11] and showed anxiety-related responses such as aggravated stress-induced hyperthermia in the home cage [12], increased defensive and risk assessment behaviors in a modified open-field test [13], and an enhanced acoustic startle reflex (ASR) [14].

In contrast to our knowledge about biological aspects of the alarm pheromone, information concerning the physical character of the pheromone is limited to the finding that it is soluble in water [12]. However, based on our previous finding that the alarm pheromone was trapped in water droplets on the ceiling of the box in which the alarm pheromone was released into ambient air [12], we hypothesize that the alarm pheromone is volatile, although we cannot exclude the possibility that the pheromone is a non-volatile and water-soluble substance, such as peptides, which is released as an invisible mist and merged into the water droplets on the ceiling.

To assess this hypothesis, we indirectly presented the alarm pheromone to recipient rats and observed the ASR as an index of the pheromone effect [14] (Experiment 1). In addition, we trapped the alarm pheromone in an adsorbent for volatile substances and assessed whether the effect of the pheromone was retained after extraction (Experiment 2).

2. Materials and methods

2.1. Animals

Sixty-three experimentally naïve male Wistar rats were purchased (Clea Japan, Tokyo, Japan) at 8 weeks old. They were housed singly in wire-topped, transparent cages (410×250×180 mm) with wood shavings for bedding. The animals were provided with water and food ad libitum and kept on a 12-h light–dark cycle with the lights turned off at 20:00. The vivarium was maintained at a constant temperature (24 °C) and humidity (40–45%). All subjects were handled in an experimental room (temperature: 22 °C, humidity: 50–55%) for 5 min and were habituated to the animal holder (see below) for 5 min per day beginning 2 days before the experiment. Each subject rat was used only once as a pheromone recipient. This study was approved by the Animal Care and Use Committee of the Faculty of Agriculture, The University of Tokyo.

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2.2. Experimental apparatus

The startle apparatus and software used in this study (StartleReflexSystem 2004; O'Hara & Co., Tokyo, Japan) were described in detail in our previous study [14]. Briefly, we used an animal holder to obtain ASR data from each subject rat. The holder consisted of an acrylic cylinder (200×60 mm, 56 mm diameter, 2 mm thickness), front and rear stoppers (acrylic plates, 100×45 cm, 2 mm thickness), and an acrylic bottom sheet (230×120 mm, 2 mm thickness) to support the cylinder. The subject rat was kept inside the cylinder using the two stoppers, the front of which had a total of 42 perforations (2 mm in diameter). The animal holder was fixed on a platform in a soundproof test chamber (480×350×370 mm) during experiments. Startle responses were elicited by 105-dB and 100-ms white noise auditory stimuli delivered through a high-frequency speaker on the ceiling of the test chamber located 150 mm above the top of the animal holder. All auditory stimuli were made by an interface (WP-1020; O'Hara & Co.) under the control of the software on a personal computer (OptiPlex GX270; Dell, Round Rock, TX). Background noise (70 dB wideband) was produced by a speaker located in the rear of the soundproof chamber ceiling. Animal movements in the holder resulted in the displacement of an accelerometer (linearity: ±5% FS) affixed to the bottom of the platform. The voltage output of the accelerometer was digitized and recorded via the personal computer software. The startle amplitude was defined as the maximal peak-to-peak voltage that occurred during the first 200 ms after the onset of the startle-eliciting auditory stimulus. A calibration system was used to ensure comparable startle magnitudes across the experiments. The test chamber was equipped with two white fluorescent bulbs (10 W each) on the ceiling.

2.3. General procedure

On the day of the experiment, each subject was moved to the experimental room and kept in its home cage for about 60 min before the experiment. It was then placed inside the animal holder and fixed on the platform in the soundproof test chamber. The experiment consisted of three consecutive sequences, i.e., the baseline trial, sample presentation, and the test trial.

In the baseline trial, the subject was first acclimatized for 5 min and exposed to the 20 auditory stimuli at an interstimulus interval of 30 s. Immediately after the baseline trial, we took the animal holder containing the subject outside the test chamber and set a sheet of filter paper (50×50 mm, folded in two) on the holder across the perforated front animal stopper, 10 mm or 200 mm (Experiment 1) and 10 mm (Experiment 2) from the rat's nose, enabling the rat to sniff the filter paper without direct contact. Each water sample (600 µl; see below) was dropped onto the paper. The size of holder in which the animal was kept during the test had been adjusted to fit the body size of male Wistar rats at 8 weeks of age, and thus animals' movement during the ASR test was restricted to the extent that they could slightly move their heads, which resulted in a relatively consistent distance (within than 5 mm variation) between the filter paper and the nose tip of subject animal. After 1–2 min of the sample presentation procedure, we returned the animal holder to its place on the platform in the test chamber. Then, each subject was exposed to 30 auditory stimuli with interstimuli intervals of 30 s for the test trial after the 5-min acclimation period. The filter paper containing each water sample was left at a distance of 10 mm or 200 mm from the rat during the test trial. We considered that the volatility of the alarm pheromone would be confirmed if the pheromone effect was observed when it was presented at a distance of 10 mm or more, because earlier studies demonstrated that non-volatile pheromones required direct contact between the pheromone and the nose of the recipient to induce the pheromone effect [15,16].

Baseline trials and test trials were conducted under the illumination of the fluorescent bulbs on the ceiling of the test chamber, and all experimental procedures were conducted between 11:30 and 16:30.

2.4. Experiment 1: effect of alarm pheromone presented indirectly

For Experiment 1, we prepared water samples according to an established method that has been previously described [14]. Basically, we prepared adult male Wistar rats (12–16 weeks old) as pheromone donors and sprayed purified water (5 ml) on the ceiling of an acrylic box (200×200×100 mm, 2 mm thickness). Each donor rat was anesthetized (50 mg/kg, i.p., Nembutal, Abbott Laboratories, North Chicago, IL), and intradermal needles (27 G) for electrical stimulation were placed in the neck or perianal region. Each rat was placed in the box for 5 min and was given 15 electrical stimulations (10 V for 1 s) at 20-s intervals to either the neck or perianal region. The electrical stimulation of the perianal region induced the release of the alarm pheromone; stimulation of the neck region was conducted as a control, as stimulation of this area does not release the alarm pheromone. After being stimulated in this manner, the donor rat was removed, and the water droplets on the ceiling containing either the alarm pheromone or neck odor were collected in a polypropylene conical tube using a glass bar and Pasteur pipette. We collected approximately 4 ml of water as samples from 5 ml of sprayed water on the ceiling of the acrylic box at the end of the 5-min electric stimuli; the volume of samples collected was consistent and reproducible. Water droplets collected from a box in which no animal had been placed were used as the vehicle control. Each 4-ml sample was used for five to six subjects (recipient rats) after having been stored at 4 °C for 1 to 5 h. The pheromone box was washed in hot water with a cleanser and wiped with a paper towel before each use. The donor rats were used 2–3 times as donors with at least 2 weeks between uses.

We divided 36 subject rats into four groups depending on the type and distance of water sample they were exposed to, i.e., control-10 mm (n=9), neck-10 mm (n=9), pheromone-10 mm (n=9), or pheromone-200 mm (n=9).

2.5. Experiment 2: effect of alarm pheromone trapped in the adsorbent then extracted using purified water

For Experiment 2, we trapped volatile substances of the alarm pheromone or the neck odor using an adsorbent, Tenax (TENAX-TA; Buchem B.V., Apeldoorn, Netherlands), which is a porous polymer based on 2,6-diphenyl-p-phenyleneoxide and is an excellent material for trapping volatiles [17,18]. An anesthetized male adult donor Wistar rat (12–16 weeks old) was electrically stimulated at its perianal region or neck skin as described in Experiment 1 (10 V for 1 s, 20-s intervals, 5 min), although not in the acrylic box but in the air-conditioned clean room air. During the electrical stimulation, released substances were sucked into a glass tube (200×6 mm, 1 mm thickness) containing 0.25 g adsorbent (10 mm from the tip of the glass tube) with a conical glass funnel (45 mm diameter, 40 mm height) connected at the donor side of the tube, using a suction pump (MP-2N; SIBATA SCIENTIC TECHNOLOGY, Tokyo, Japan; 1000 ml/min). The funnel was set at a distance of 10 mm from the perianal region without direct contact. The adsorbed substances were extracted with 5-ml purified water. The glass tube containing absorbent was kept upright and 1-ml water was passed through by gravity 5 times and collected into a polypropylene conical tube set underneath as water samples for the ASR test. In addition, for the preparation of a control water sample, clean air was pumped into the adsorbent-filled glass tube and then extracted by purified water in the same way as described above. Each 5-ml sample was used for five to six subjects (recipient rats) after being stored in a refrigerator at 4 °C for 1 to 5 h. The donor rats were used 2–3 times as donors with at least 2 weeks between uses.

We divided 27 subject rats into three groups depending on the type of water sample they were exposed to, i.e., clean air (n=9), neck odor (n=9), or pheromone (n=9). All water samples were presented at a distance of 10 mm from subjects.