Alarm pheromone increases defensive and risk assessment behaviors in male rats

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Running Head: BEHAVIORAL RESPONSE TO ALARM PHEROMONE

Abstract

KIYOKAWA, Y., T. KIKUSUI, Y. TAKEUCHI AND Y. MORI. Alarm pheromone increases defensive and risk assessment behavior in male rats. PHYSIOL BEHAV 85(3) 000-000, 2005.-Previously, we reported that alarm pheromone released from the perianal region of male rats aggravated stress-induced hyperthermia and increased Fos expression in the vomeronasal pathway and stress-related nuclei in pheromone-recipient rats. However, the alarm property of this pheromone in terms of behavior modification is still unclear. We recently found that this alarm pheromone could be trapped in water. Based on this finding, we developed an experimental paradigm to assess the effect of alarm pheromone on recipient behavior. Male Wistar rats were acclimatized for 5 min to an open field, where two pieces of filter paper soaked with 750 μl of either pheromone-containing water or vehicle water were attached to the wall. Then, a small ″hiding box″ was placed in one corner of the field, and the behavioral responses of the subject rat were recorded for 10 subsequent minutes. Exposure to alarm pheromone significantly increased defensive and risk assessment behaviors and decreased exploratory and grooming behaviors compared to the vehicle control group, indicating

the alarm property of the pheromone. In addition, the comparison with previous results suggests that the alarm pheromone released from the perianal region of the male rat increases anxiety in recipients, rather than evoking a stereotyped autonomic response.

Key words: Alarm pheromone; Defensive behavior; Risk assessment behavior; Anxiety.

Introduction

Chemical communication plays an important role in various social interactions among individuals of the same mammalian species and affects sexual [1], territorial [2], and maternal behavior [3]. When produced by a conspecific, alarm pheromone communicates the presence of danger [4] and possibly plays important role in increasing overall species fitness.

Previously, we reported that stressed male Wistar rats released alarm pheromone, which enhanced behavioral responses (increased freezing, sniffing and walking and decreased resting behaviors), and caused increased body temperature (stress-induced hyperthermia: SIH) and Fos expression, an index of neural activation [5], in the mitral/tufted cell layer of the accessory olfactory bulb in recipient rats [6]. We found that donor rats released two types of pheromone responsible for behavioral or autonomic responses in recipients: pheromone that modified recipient behavior was produced in a testosterone-dependent manner and was released by the electrical stimulation to the whisker pad, and pheromone that aggravated SIH was produced in a testosterone-independent manner and was released by the stimulation to the perianal

region of the anesthetized donor rat [7,8]. Subsequently, we have focused on the latter type of alarm pheromone, because the intensity of SIH was thought to reflect the animal′s anxiety status [9,10]. This type of alarm pheromone increased Fos expression in the vomeronasal pathway and in several stress-related nuclei in the brains of recipient animals [11]. Moreover, we recently reported that the pheromone is water soluble, as water droplets collected from the ceiling of a box in which alarm pheromone was released reproduced all the responses seen in recipients exposed to the pheromone directly [12]. Despite an apparent influence on autonomic function, clear evidence for the alarm property of this pheromone is still lacking. This is partly due to our experimental paradigm, which measured the SIH of animals in a small box which limited the recipient's behavioral responses.

Along with responses elicited by intraspecies chemical communication, interspecies chemical communication can also elicit a fear or anxiety response, *e.g.*, on exposure to predator odor. When a rat was exposed to cat odor in an apparatus where it had a choice of escape to a safe place, it showed defensive (*e.g.*, escaped into a burrow or small hiding box) and risk assessment behaviors (*e.g.*, flat back approach and typical

″head out″ behavior) to the stimuli [13,14]. These behavioral responses were thought to result from increased anxiety, as several anxiolytics attenuated the responses [15-20]. However, as mentioned above, our previous studies used a small, inescapable box for technical reasons. Our recent finding that alarm pheromone could be trapped in water [12] led us to develop an improved apparatus, which enabled investigation of pheromone effects on behavior. We hypothesized that alarm pheromone has an alarm property and that the aggravated SIH seen in recipients results from increased anxiety. If so, alarm pheromone should also elicit defensive and risk assessment behaviors.

To test this hypothesis, we prepared a modified open-field apparatus based on those constructed by Dielenberg *et al*. [19], which allowed rats to choose between being in an open arena in the presence of a test substance or hiding in a small box located in the corner of the apparatus opposite to the test substance.

Material and Methods

Animals

Experimentally naïve male Wistar rats were purchased from Clea Japan

(Tokyo, Japan) at 8 weeks of age and were housed two animals per cage under constant temperature (24 ± 1°C) and humidity (45 ± 5%). Food and water were available *ad libitum*, and the animals were kept under a 12-h light/12-h dark cycle (lights on at 0800) throughout the experiment. The animals were cared for in accordance with ″Policies Governing the Use of Live Vertebrate Animals,″ set by the University of Tokyo, and based on ″The Public Health Service Policy on Humane Care and Use of Laboratory Animals″ (revised in 1985) and the ″National Institutes of Health′s Guide for the Care and Use of Laboratory Animals″.

Preparation of water samples

The procedures used for preparing water samples were the same as those used in our previous study [12]. Briefly, we prepared adult male Wistar rats as pheromone donors and an acrylic box (20 × 20 × 10 cm) as the pheromone box. Approximately 5 ml of purified water were sprayed on the ceiling of the pheromone box. An anesthetized donor rat (50 mg/kg, i.p., Nembutal, Abbott Laboratories, Chicago, IL) with two intradermal needles (27G) placed at the edge of both sides of the anal canal for

electrical stimulation of the perianal region was placed in the box for 15 minutes. During this period, the donor rats received 15 electrical stimuli of 10 V (1.5 mA approximately) for 1 second at 1-minute intervals to the perianal region, which induced the release of alarm pheromone and aggravated SIH in other rats [8]. The pricking needles and electrical stimulation did not evoke any bleeding or apparent damage to the skin and we were unable to locate the stimulated site with the naked eye after the removal of needles. After being stimulated in this manner, the donor rat was removed, and the water droplets on the ceiling containing alarm pheromone were collected in a polypropylene conical tube using a glass bar and Pasteur pipette. Water droplets collected from a box in which no animal had been placed were used as vehicle control. After collection, the pheromone-containing and control water were stored in a refrigerator at 4°C for 3~6 hours until use. The pheromone donors were used two to three times, with at least a one-week interval between uses, and the pheromone box was washed in hot water with a cleanser and wiped with a paper towel before each use.

Procedure

Experiments were conducted in a plastic open field (64.3 × 44.7 × 23 cm). We attached two pieces of filter paper (5 × 5 cm) soaked with 750 μl of either pheromone-containing or control water each in one corner. Our previous study showed that the rats could perceive alarm pheromone under such circumstances [12]. Each subject rat was exposed to new filter papers soaked with either type of water that was prepared by the independent preparation procedure. The entire experimental room was lit, and the center of the field was illuminated at about 115 lux. We placed a subject rat in the center of the arena and left it there for 5 min for acclimation; during this time, all the subject rats explored the arena and perceived the stimuli. After acclimation, we placed a small polycarbonate box (17.5 × 24.5 × 12.5 cm) with a punctured metal board as the ceiling, called the ″hiding box,″ in the corner opposite to the stimuli. The behavior of the subject was video-recorded using a camera (SE-2000NV, Daiwa Industry, Tokyo, Japan) mounted about 95 cm above the arena (Figure 1). The hiding box had a small round hole (7.5 cm diameter) in the center of one wall that allowed just enough space for a rat to enter. Subject rats had been habituated to the box in their home

cage for about 20 hours since the day before the experiment. The subject rats were assigned to one of two groups according to the type of water sample they were exposed to: Alarm pheromone (n = 10) and Control (n = 10) groups. The subject rats kept in the same home cage were assigned to the same treatment group to avoid contamination of the water sample via the hiding box. The box was used for the two subjects and was cleaned with a paper towel before and between experiments if a subject urinated or defecated in the box. After the experiments, we counted the number of feces, cleaned the open field with ethanol and paper towels and washed the hiding box thoroughly in hot water with a cleanser for subsequent uses. All experiments were conducted between 1530 and 1730 to reduce the effects of circadian rhythm.

Enzyme immunoassay

Within 3 min of the end of the experimental period, blood samples were collected in heparin-coated hematocrit-capillaries (Hirschmann Laborgeräte, Eberstadt, Germany) after making a small incision in the tail. After sampling, the blood was centrifuged at 4°C, and the plasma was stored at –20°C for subsequent enzyme

immunoassay (EIA). The EIA for corticosterone [21] was performed on a single plate with HRP-corticosterone (FKA419, Cosmo Bio, Tokyo, Japan) and specific anti-corticosterone serum (FKA420E, Cosmo Bio), which cross-reacted with deoxycorticosterone (8%), progesterone (2.1%), 11-dehydrocorticosterone (0.23%), cortisol (0.2%), and other steroids (<0.05%). The minimum detectable level of corticosterone was 9.9 pg/well, and the intra-assay coefficient of variation was 11.6%.

Data analysis and statistical procedures

The data were analyzed using Stat View J 5.0 software (SAS Institute, Cary, NC; no longer available) and expressed as means ± SEM. The significance level was set at $P = 0.05$ for all statistical tests.

A researcher who was blind to the experimental conditions analyzed the behavior of the subject using Microsoft Excel-based Visual Basic software for recording the frequency and the duration of each parameter. The parameters were chosen based on a previous study [13]. The number of steps taken with the hind paws (walking) in the open arena and the durations of outside, head out, conceal, rearing, grooming, near the

stimuli, and flat back approach behaviors were recorded during 10-minute experimental period. ″Outside″ was defined as the time the rats spent in the open arena; similarly, ″head out″ was defined as the rat poking its head, or head and shoulders, out of the hiding box entrance with their hind paws remaining inside the box; ″conceal″ was defined as the rat being entirely inside the hiding box; ″near the stimuli″ was defined as the rat spending time within the 10-cm square near the corner where the stimuli were attached; and ″flat back approach″ was defined as the rat approaching the stimuli with a flattened body with its head oriented towards the stimuli. The durations of rearing and grooming were analyzed when these behaviors were observed in the open arena; these behaviors were defined in our previous studies [6,22]. The ″near the stimuli″, ″rearing″ and ″grooming″ were expressed as the ratio to the time in the open arena (%) for each animals, and all behavioral data were analyzed statistically using ANOVA. Note that the ″outside″, ″head out″ and ″conceal″ were not counted together and that the ″flat back approach″ was not observed and was subsequently excluded from the statistical analyses. For the number of feces and plasma corticosterone levels in subjects, we used Mann-Whitney′s U-test to compare group means.

Results

All the subject rats entered the box without hesitation and also escaped into the box when the experimenter was removing the subject from the arena, indicating that the hiding box served as a safe area for the subjects. The existence of alarm pheromone in the open arena significantly increased the head out ($F(1, 18) = 9.77$, $P < 0.01$) and conceal ($F(1, 18) = 6.31$, $P < 0.05$) behaviors, whereas it decreased the outside ($F(1, 18) = 10.5$, $P < 0.01$) and grooming ($F(1, 18) = 4.61$, $P < 0.05$) behaviors in subject rats (Figure 2). Alarm pheromone did not affect the other behaviors (rearing; $F(1, 18) = 0.851$, $P = 0.368$, near the stimuli; $F(1, 18) = 1.14$, $P = 0.301$) although it tended to decrease the walking ($F(1, 18) = 4.38$, $P = 0.0507$) behavior. Statistical analyses also revealed that there were no differences in behaviors between the first and the second subjects from each cage (data not shown).

In contrast to its effects on behavioral responses, alarm pheromone had no influence on the number of feces excreted (Alarm pheromone: 1.2 ± 0.5; Control: 1.5 ± 0.6) or corticosterone levels after the experiments (Alarm pheromone: 374 ± 30;

Control: 393 ± 31 ng/ml).

Discussion

In this study, rats exposed to alarm pheromone in the modified open field showed increased defensive (conceal) and risk assessment (head out) behaviors accompanied by decreased exploratory (outside) and grooming behaviors. The rats exposed to cat odor showed similar responses to those seen in here [13,14], indicating that the alarm pheromone used in our experimental paradigm has clear alarm properties for recipient rats.

At this moment, we cannot exclude the possibility that the increases of hiding and risk assessment behaviors were due to the existence of novel odors released either from other rats or from filter papers, but were not caused by alarm pheromone. However, this appears less likely, as we reported previously that the exposure to the filter paper containing other male′s odor released by electrical stimulation to the neck skin attenuated tachycardiac response evoked by the filter paper presenting procedure, which indicates that other individual′s odor may serve as anxiolytic stimuli rather than

anxiogenic one [12]. In addition, laboratory rats, unlike wild rats, have a tendency to show interest in or attention to novel stimuli [23], which is utilized for cognitive behavioral tests such as habituation/dishabituation test [24].

The exposure to alarm pheromone influenced neither corticosterone levels nor the number of feces, an indicator of autonomic response, in recipient rats. One possible explanation is that the time of blood sampling (15 min) was not adequate for observing endocrine response because File *et al*. reported that the rats exposed to cat odor showed increased corticosterone levels 35 min after the initial exposure [25]. Another explanation of our results is that the subject rats regulated their stress levels by escaping into the hiding box. Although alarm pheromone increased stress levels in recipients, the increased conceal behavior seen in these animals might have attenuated their stress level to that of the control animals. Further research is required to analyze the effects of alarm pheromone on HPA axis activity.

Comparison with previous results

These results further support our previous hypothesis that alarm pheromone

increases anxiety in recipients, rather than evoking a stereotyped response [12]. In this study, the presence of alarm pheromone on two pieces of filter paper increased defensive and risk assessment behavior in exposed rats, whereas the same pheromone stimulus aggravated SIH in rats if it was presented in their home cage [12]. Both of these responses might be mediated by anxiety in rats. For example, rats exposed to cat odor showed increased defensive and risk assessment behaviors, which were attenuated by pretreatment with several anxiolytics [15-20]. In addition, although rats showed increased anxiety in the elevated plus-maze after exposure to cat odor, this response was absent in the rats that did not show increased hiding behavior after repeated habituation [26]. As for SIH, its intensity is thought to reflect the anxiety status of an animal based on several pharmacological studies in which various anxiolytic drugs were shown to attenuate the intensity of SIH in a dose-dependent manner [9,10]. The two anxiety-related responses evoked by the same stimulus suggest that the primary effect of alarm pheromone is to increase anxiety and that the responses evoked by alarm pheromone are secondary to increased anxiety in pheromone-exposed rats. However, no information is currently available if the responses evoked by the exposure to alarm

pheromone share common mechanisms with those evoked by other stimuli, and so we need to examine, for example, if the behavioral effects of alarm pheromone are reversed by various anxiolytic drugs.

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Figure legends

Figure 1. Schematic diagram of the test apparatus used in this study.

Figure 2. Behavioral responses of rats that were either exposed to alarm pheromone-containing water (Alarm pheromone) or control water (Control) in the test apparatus. The near the stimuli, rearing and grooming were expressed as the ratio to the time in the open arena (%) for each animals. *$P < 0.05$ as compared to the Control group by ANOVA (mean + SEM).

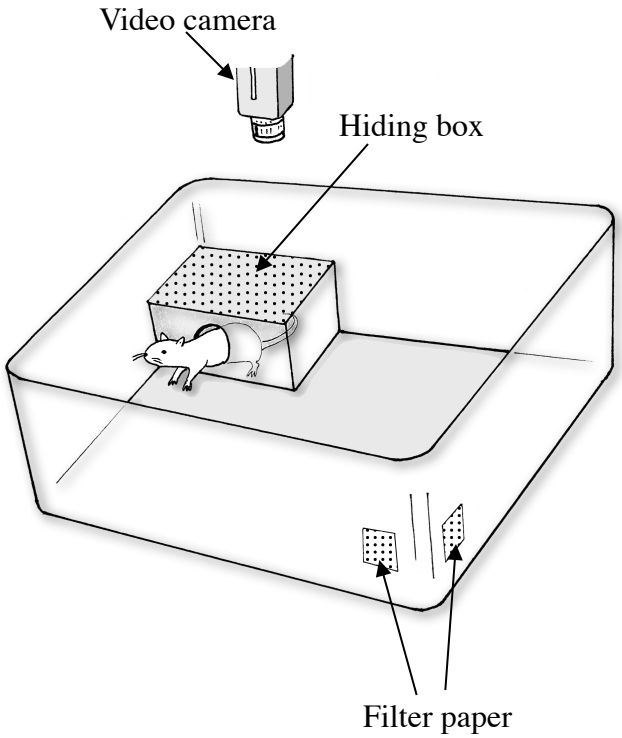

Figure 1.

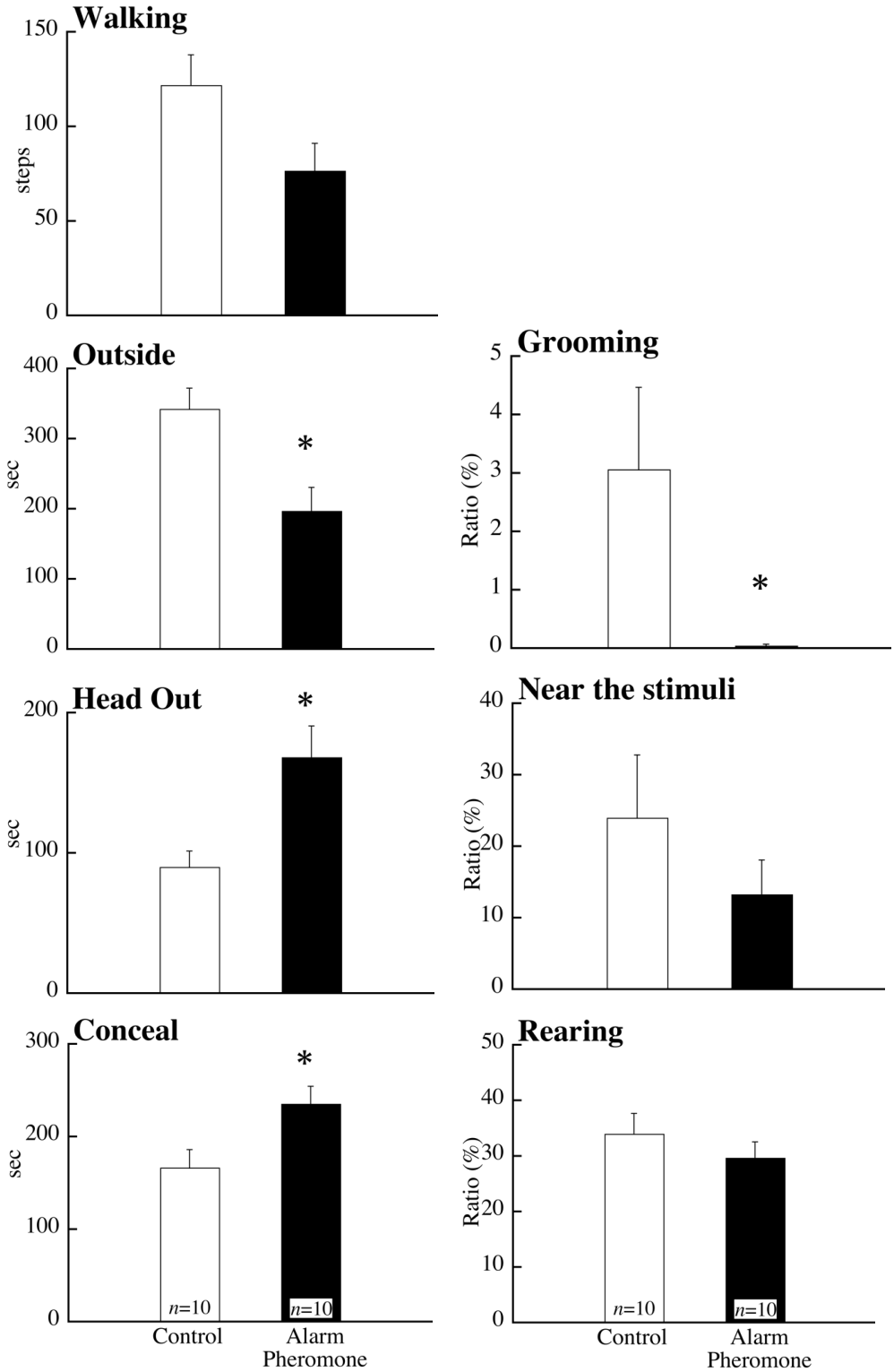
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Figure 2.