Chem. Senses 29: 35–40, 2004 DOI: 10.1093/chemse/bjh004

Alarm Pheromones with Different Functions are Released from Different Regions of the Body Surface of Male Rats

Yasushi Kiyokawa, Takefumi Kikusui, Yukari Takeuchi and Yuji Mori

Laboratory of Veterinary Ethology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

Correspondence to be sent to: Takefumi Kikusui, Laboratory of Veterinary Ethology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan. e-mail: akikus@mail.ecc.u-tokyo.ac.jp

Abstract

Our previous study suggested that the alarm pheromones in rats could be divided into at least two functionally different categories: one evoking autonomic responses and the other evoking behavioral responses, and the present study was conducted to test this hypothesis. Four regions of the body surface, i.e. the whisker pad, neck, rump and perianal region, of an anesthetized male Wistar rat were electrically stimulated (donor) and, after removal of the donor, the recipient rat was introduced into the same box and its behavioral and autonomic changes were recorded. Electrical stimulation of the perianal region of anesthetized donor rats provoked the release of odor that subsequently augmented core body temperature in other awake male rats. By contrast, electrical stimulation of the whisker pad of anesthetized donor males provoked the release of odor that augmented sniffing, rearing and locomotor activity in other awake male subjects. These results suggest that the alarm pheromone released from the face modifies behavior and that from the anal area induces autonomic stress responses in recipients.

Key words: alarm pheromone, anal gland, piloerection, regional stimulation, stress-induced hyperthermia, whisker pad

Introduction

Chemical communication plays important roles in various social interactions among mammals, including sexual (Vandenbergh, 1973), territorial (Eichmann and Holst, 1999) and maternal behaviors (Leon and Moltz, 1971). Alarm chemosignals, which alert animals to the proximity of conspecific individuals (Abel and Bilitzke, 1990; Vieuille-Thomas and Signoret, 1992; Boissy *et al.*, 1998), are considered to be used widely in the animal kingdom. In rodent species, it has been reported that rats could distinguish between the odors released from stressed and non-stressed conspecifics (Valenta and Rigby, 1968) and that these odors changed behavior (Mackay-Sim and Laing, 1980, 1981; Abel and Bilitzke, 1990) and immune responses of recipients (Cocke *et al.*, 1993).

We previously reported that the alarm pheromones released from male donor rats by receiving foot shocks evoked both behavioral and autonomic (i.e. stress-induced hyperthermia, SIH) responses, as well as increased Fos protein expression in the mitral cell layer of the accessory olfactory bulb, in another male rat (Kikusui *et al.*, 2001; Kiyokawa *et al.*, 2004). Castration of the donor rats reduced their ability to release the alarm pheromone that affected behavioral responses, whereas testosterone implantation restored this ability. In contrast, neither castration nor testosterone replacement affected SIH responses in recipient rats (Kiyokawa *et al.*, 2004). Based on these results, we hypothesized that the alarm pheromones in rats could be divided into at least two functionally different categories: a group that provokes autonomic responses in a testosterone-independent manner and a group that evokes behavioral responses in a testosterone-dependent manner. However, we could not exclude the alternative possibility that both behavioral and autonomic responses are mediated by the same or similar components of an alarm pheromone to which the thresholds may differ between behavioral and autonomic responses.

When animals confront threatening or stressful stimuli, they usually show several species-specific defense reactions. Piloerection is one of the defensive reactions seen in many species, including rodents (Fernandez-Espejo and Mir, 1990; Levine *et al.*, 1990; Li *et al.*, 1997; Dettling *et al.*, 1998). When rats received electrical foot shocks, they showed piloerection as was reported in mice (Ichimaru and Gomita, 1987). Piloerection is induced by the muscle contractions associated with the hair follicles and these contractions also induces odor release by expressing secretion from the hair follicles or the surrounding sebaceous glands (Flood, 1985). In addition, the stiff erect hairs permit more efficient air

circulation on the skin surface (Flood, 1985). The expression of secretions by the anal gland, which is specialized for the purpose of producing an odorous substance, is another well-known defensive reaction. This gland exists beneath the anal canal epithelium in many species, including rats (Montagna and Noback, 1947; Montagna and Parks, 1948; Crump, 1980; Zhang *et al.*, 2002) and its secretion was anecdotally known to be used for defensive and/or alarming purposes in many species, such as skunk or fox (Blackman, 1911; Donovan, 1967; Albone and Fox, 1971). Taking these facts into account, it is conceivable that there are some relations between the release of the alarm pheromone and these two defensive reactions.

To test our hypothesis, we here attempted to induce the release of only one of the two possible alarm pheromones. We electrically stimulated various regions of the body surface of anesthetized rats in a test box, namely the whisker pad, neck and rump, in order to induce odor release from these regions accompanied by piloerection. Electrical stimulation was also given to the perianal region to induce excretion of the anal gland content. We then replaced the donor rat with the recipient rat and measured behavioral and autonomic changes of the recipient as indices of the amount of pheromones remaining in the box.

Materials and methods

Recipient animals

Experimentally naïve male Wistar rats were purchased from Clea Japan (Tokyo, Japan) and were used at the age of 10 weeks. They were housed three or four animals per cage under constant temperature (24 ± 1°C) and humidity (45 ± 5%) until they were implanted with a telemetry transmitter. Food and water were available *ad libitum* and they were kept under a 12 h light/12 h dark cycle (lights on at 08.00) throughout the experiment. The animals were cared for in accordance with the 'Policies Governing the Use of Live Vertebrate Animals' of the University of Tokyo, based on 'The Public Health Service Policy on Human Care and Use of Laboratory Animals' of the Awardee Institution (revised 1985) and 'The National Institutes of Health Guide for the Care and Use of Laboratory Animals' (revised 1985). The recipient animals were implanted with a telemetry transmitter (TA10TA-F40; Data Sciences International, St Paul, MN) i.p. under anesthesia with ether 10–11 days before the experiment. After the surgery, they were housed individually in a soundproof chamber located in another room. All animals were handled for 5 min each day, beginning 6 days before the experiment and then exposed to the test box for 10 min each day beginning 3 or 4 days before the experiment to minimize the effects of novelty stress.

Procedures

The general procedures were basically the same as those in our previous study (Kikusui *et al.*, 2001; Kiyokawa *et al.*, 2004). We used intact male rats as both the odor donors and the recipient animals. We prepared three male rats as the odor source for each recipient rat whose responses were recorded. In order to mimic the experimental condition of our previous studies where alarm pheromones existed in addition to conspecific's general odor, two conscious male rats were first placed in the box for producing general body odor and the one anesthetized donor rat was used for producing regional odors by electrical stimulation to various body parts. Namely, two adult male rats were placed in a test box (27.5 × 20 × 27 cm) for 7 min without any disturbance and then were replaced by an anesthetized donor rat carrying two intra-dermal needles (27G) for electrical stimulation at either the whisker pad, neck, rump, or perianal region. The needles for perianal regions were placed at the edge of both sides of the anal canal. Anal glands were seen as numbers of macroscopic tubercles surrounding the anal canal. The donor rat was placed in the box for 8 min and during this period it received eight electrical stimulations (10 V for 1 s) at 1 min intervals. Electrical stimulation parameters were chosen based on preliminary observations so that this stimulation could induce odor release, which even an experimenter could smell, without any visible damages to the skin. It should be noted that the pricking needles and electrical stimulation did not evoke any bleeding or apparent damage to the skin; in fact, after the removal of needles we were unable to locate the stimulated area with the naked eye. A box in which a pricked donor was introduced but not electrically stimulated was used as a control.

After the stimulation, the donor rat was removed and the empty box was brought into another room kept at a constant temperature (22 ± 1°C) under a 12 h light/12 h dark cycle (lights on at 08.00). In this room, the test box was installed on an antenna board in a soundproof chamber (36 × 54 × 35 cm; Muromachi Kikai, Tokyo, Japan) for recording autonomic and behavioral responses of the recipient rat. To ensure stable baselines for body temperature (BT), recipient rats were only introduced into the test box upon showing a BT level between 37.0 and 37.5°C for at least 5 min before the experiment and then they were held there for the subsequent 45 min. The recipient rats were randomly assigned to one of five groups according to the stimulated body region of the donor, i.e. Whisker (n = 6), Neck (n = 7), Rump (n = 7), Anal (n = 7) and Control (n = 7) groups. After the introduction to the test box, the behavior of recipient rat was video-recorded (DCR-TRV18; Sony, Tokyo, Japan) through a window on the wall of the chamber to be analyzed later. Simultaneously, the BT was transmitted via an antenna board (RLA1020 RPC-1; Data Sciences International, St Paul, MN) placed under the test box and the signals were recorded and analyzed by a data acquisition system (Dataquest® LabPRO 3.10; Data Sciences International). After the experiment, the test box was washed in hot water with a cleanser to remove any odors that might influence the next measurement. All experiments

were conducted between 09.00 and 18.00. Donor rats were used three or four times with an interval of at least 1 week, while recipient rats were used only once.

Data analysis and statistical procedure

Data analysis was performed with Stat View J 5.0 software (Abacus Concepts, Berkeley, CA) and values of $P < 0.05$ were considered to indicate statistical significance for all tests. The analysis of the behavioral and autonomic parameters was conducted as described in our previous study (Kiyokawa *et al.*, 2004). Briefly, the behavioral response of the recipient was classified using six parameters: sniffing, freezing, resting, walking, rearing and grooming and the durations (for sniffing, freezing, resting, rearing and grooming) or frequency (number of steps taken with the hind paws, for walking) were statistically analyzed by MANOVA (Hotelling's trace) followed by Dunnett's *post hoc* test. The BT was recorded continuously and stored as an average for 5 s in each minute. An individual baseline was defined as the average of the BT values recorded in the home cage for 5 min before introduction to the test box. The change from the baseline was expressed as the area under the curve (AUC) for group comparison and AUC was statistically analyzed by one-way ANOVA followed by Dunnett's *post hoc* test.

Results

All the subject animals (recipients) showed autonomic responses to the novel test box and Figure 1 (top) shows how the SIH response was modified by the odor released from the perianal region of donors. The magnitude of the SIH responses to the odor released from different body regions are indicated by the area under the curve (AUC) values in Figure 1 (bottom). This response differed significantly according to the body regions stimulated [$F(4,29) = 3.84$, $P < 0.05$] and the odor released from the perianal region, but not other areas, resulted in a significantly ($P < 0.05$) greater SIH response in the recipient than in the control animals. Behavioral responses to the novel environment were also different depending on the body region from which the odor was released [$F(24,90) = 3.73$, $P < 0.0001$]. A *post hoc* test revealed that, unlike the autonomic responses, behavioral responses were triggered only by odor release from the whisker pad; these responses consisted of elongated duration of sniffing ($P < 0.05$) and rearing ($P < 0.05$), increased frequency of walking ($P < 0.05$) and shortened duration of resting ($P < 0.05$) as compared to the control animals (Figure 2). The duration of grooming and freezing were not significantly altered by odor release from any of the regions tested (grooming: Whisker, 1034 ± 132; Neck, 918 ± 90; Rump, 989 ± 66; Anal, 827 ± 62; Control, 858 ± 134; freezing: Whisker, 10.6 ± 3.0; Neck, 24.1 ± 9.2; Rump, 8.4 ± 3.1; Anal, 17.3 ± 5.6; Control, 10.3 ± 2.1; mean ± SEM).

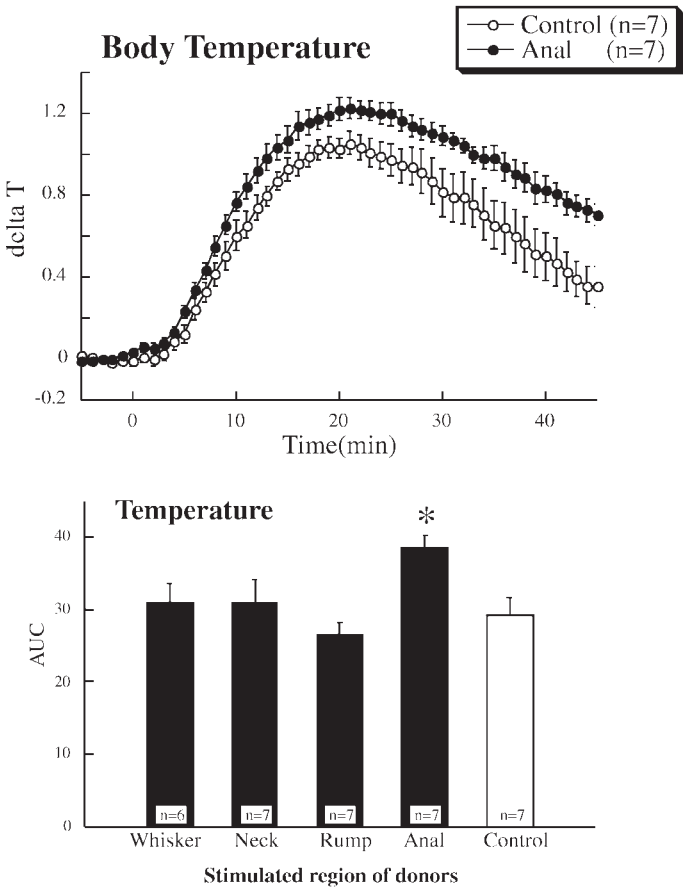

Figure 1 Top: time-dependent changes in the body temperature of recipient rats introduced into a test box containing either the control odor (Control) or the odor released from the perianal region of an anesthetized donor (Anal) by regional electrical stimulation (mean ± SEM). Bottom: autonomic responses of recipient rats exposed to the odor released from the whisker pad (Whisker), neck (Neck), rump (Rump), or perianal region (Anal) of an anesthetized donor by regional electrical stimulation. The recipients exposed to the odor released from an anesthetized donor without stimulation were used as controls (Control). The change from the baseline was expressed as the area under the curve (AUC) for group comparison. *$P < 0.05$ as compared to the control group by ANOVA followed by Dunnett's post hoc test (mean ± SEM).

Discussion

In the present study, the odor released from the whisker pad and the perianal region of the donor by electrical stimulation specifically evoked behavioral and autonomic responses, respectively, as compared to those in the control group. In contrast, the odors released from the neck and rump regions were shown to affect neither behavioral nor autonomic responses. The pattern and intensity of changes in behavioral parameters induced by the whisker pad odor were almost identical to those evoked by alarm pheromones released from conscious male rats given foot shocks (Kiyokawa *et al.*, 2004). Similarly, the profile of changes in SIH responses induced by the odor of the perianal region

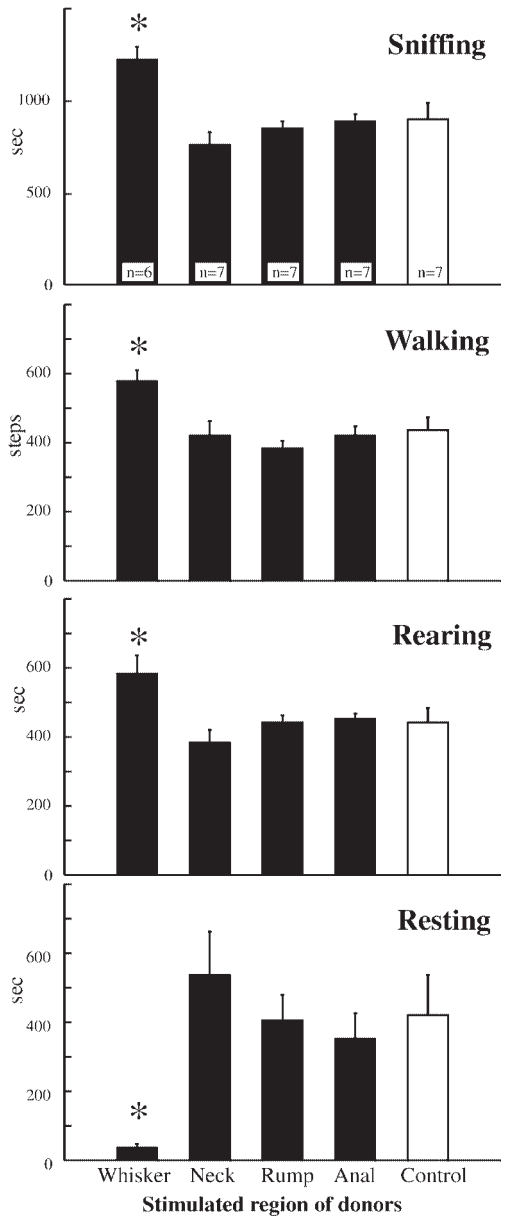

Figure 2 Behavioral responses of recipient rats exposed to the odor released from the whisker pad (Whisker), neck (Neck), rump (Rump), or perianal region (Anal) of an anesthetized donor by regional electrical stimulation. The recipients exposed to the odor released from an anesthetized donor without stimulation were used as controls (Control). *$P < 0.05$ as compared to the control group by MANOVA followed by Dunnett's *post hoc* test (mean ± SEM).

was similar to that observed in the same study (Kiyokawa *et al.*, 2004). These results suggest that the odors released from the whisker pad and those released from the perianal region may represent the two previously postulated categories of alarm pheromones, i.e. those evoking behavioral and those evoking autonomic responses (Kiyokawa *et al.*, 2004). Previous findings that alarm pheromones were released from the body surface support this view (Rottman and Snowdon, 1972; Hornbuckle and Beall, 1974; Mackay-Sim and Laing, 1980; Abel and Bilitzke, 1990; Abel, 1991, 1992).

One of the two types of alarm pheromones, i.e. those evoking behavioral responses, might have been emitted from the sebaceous gland under the control of hormones such as testosterone and ACTH (Abel, 1994; Kiyokawa *et al.*, 2004), since both testosterone and ACTH are necessary for maintaining the physiological function of this gland (Lecaque and Secchi, 1982; Chen *et al.*, 1997). Production of several male pheromones have been shown to be dependent on circulating levels of testosterone (Gawienowski *et al.*, 1976; Novotny *et al.*, 1990) and castration of the donor diminished the ability to release the alarm pheromone responsible for behavioral changes (Kiyokawa *et al.*, 2004). ACTH, like testosterone, mediated the efficiency of the male pheromone (Nowell *et al.*, 1980; Caldwell and Lepri, 2002), especially that of alarm pheromones evoking the behavioral response (Abel, 1994). Taking these findings into account, it is conceivable that the sebaceous gland is involved in the release of an alarm pheromone that intensifies behavioral vigilance under the control of testosterone and ACTH as co-mediators.

This alarm pheromone might be produced specifically in the whisker pad region and released by piloerection. This view is supported by the finding that pheromone production in the sebaceous glands of male goat skin is limited to specific parts of the body (Iwata *et al.*, 2000; Wakabayashi *et al.*, 2000), as well as by the observation that the warning odor in porcupines is released as a consequence of piloerection when the animals encounter a predator or agonistic conspecific (Li *et al.*, 1997). In rats, sebaceous glands exist in the skin of the whisker pad and other body areas (Rice *et al.*, 1993; Fundin *et al.*, 1997) and piloerection is one of the defensive behaviors (Fernandez-Espejo and Mir, 1990). In light of these previous findings, it seems plausible that the alarm pheromone evoking behavioral responses is produced specifically in the sebaceous glands at the whisker pad and is released in association with the piloerection when the rat exhibits defensive behavior.

Regarding the other proposed category of alarm pheromones, those evoking an SIH (autonomic) response in the recipient rat, it seems reasonable that these pheromones might be released from the perianal region, possibly from the anal gland, because of its testosterone independence (Kiyokawa *et al.*, 2004). The anal gland is not remarkably different between male rats and female rats (Montagna and Noback, 1947) and it was much less affected by circulating levels of testosterone as compared to other glands (Jannett, 1978). Moreover, we previously showed that the alarm pheromone released from castrated donor rats could evoke the SIH response, but not behavioral responses, in recipient rats (Kiyokawa *et al.*, 2004). Although some previous studies dealt with the chemical nature of the perianal gland secretion in various species (Aldrich, 1896; Albone and Fox, 1971; Andersen and Bernstein, 1975; Crump, 1980; Zhang *et*

al., 2002), there have been only a few reports that investigated their biological significance (Zhang *et al.*, 2003). In earlier studies by Donovan (1967, 1969), it was reported that mature dogs were deterred by anal gland secretions voluntarily expressed by a frightened dog, while involuntarily expressed secretions acted as sexual attractants. In addition, young puppies were not deterred by the secretions expressed by a frightened dog. Considering the ontogeny of response that young rats do not respond to alarm pheromones until they reach 26 days of age (Abel, 1993), the anal gland secretion could be a source of this category of the alarm pheromone in rats.

In conclusion, we found that the odor released by electrical stimulation of the perianal region and whisker pad in anesthetized rats evoked autonomic and behavioral responses, respectively, in recipients and these responses were indistinguishable from those evoked by the alarm pheromones released by conscious male rats (Kiyokawa *et al.*, 2004). These results provide further evidence for our hypothesis that the alarm pheromones can be divided into at least two functionally different categories, which are released from different parts of the body under differing regulatory mechanisms.

Acknowledgements

This study was supported by grants-in-aid for Scientific research (14760187 and 15GS0306) from the Japan Society for the Promotion of Science.

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Accepted November 9, 2003