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The alarm pheromone in male rats as a unique anxiety model: Psychopharmacological evidence using anxiolytics

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ABSTRACT

Previously, we demonstrated that an alarm pheromone released from male donor Wistar rats evoked anxiety-related physiological and behavioral responses in recipient rats. Thus, we believe that this pheromone may increase anxiety levels in rats. In the current study, we evaluated the predictive validity of this alarm pheromone-induced anxiogenic effect in detail by investigating whether six types of human anxiolytics, each of which has a different mechanism of action, were efficacious in reducing anxiety, using changes in the acoustic startle reflex (ASR) as an index. The alarm pheromone-enhanced ASR was not affected by vehicle pretreatment but was dose-dependently attenuated by pretreatment with midazolam, phenelzine, propranolol, clonidine, and CP-154,526—although not buspirone. These results may reflect some aspects of the predictive validity of the alarm pheromone-induced anxiety in rats as an animal model of human anxiety.

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1. Introduction

Anxiety disorders are the most commonly observed mental illnesses in humans. The prevalence of anxiety disorders is 18% in the general population (ages 18 years and older) (Bienvenu and Ginsburg, 2007). Although several sophisticated methods are used to investigate the causes, correlates, and consequences of psychopathological disorders, animal models are an important tool for understanding the nature of such abnormal states (e.g., anxiety disorders) because they permit the control of genetic and environmental variables, the use of invasive and toxic techniques, and detailed studies of mechanisms (Sher and Trull, 1996). Therefore, it is extremely important to develop reliable and effective animal models for anxiety in humans.

Previous studies from our lab demonstrated that pheromone donor rats produce a water-soluble (Kiyokawa et al., 2005a) and volatile (Inagaki et al., 2009) alarm pheromone testosterone-independently (Kiyokawa et al., 2004a) and release it from the perianal region (Kiyokawa et al., 2004b). The pheromone recipient rat perceives this pheromone via the vomeronasal organ (Kiyokawa et al., 2007) and shows anxiety-related responses such as an aggravated stress-induced hyperthermia (Kikusui et al., 2001), increased defensive and risk-assessment behaviors in a modified open-field test (Kiyokawa et al., 2006), and an enhanced acoustic startle reflex (ASR) (Inagaki et al., 2008). These responses may be induced by the activation of the amygdala and other limbic regions; pheromone exposure increases Fos expression in these regions (Kiyokawa et al., 2005b). Based on these observations, we predict that alarm pheromone exposure increases anxiety in rats and may possibly be a useful animal model of human anxiety.

To serve as an animal model of human anxiety, the following three criteria are considered to be important (Belzung and Griebel, 2001; Fendt et al., 2005): (1) face validity—that behavioral and physiological signs of the anxiety model should be similar to those of humans; (2) construct validity—that brain structures processing and/or inducing these anxiety-related changes should be the same in the animal model and in humans; and (3) predictive validity—that anxiolytic drugs for human treatment should also work in the animal model. Our previous studies indicate that alarm pheromone exposure fulfills two of three of these criteria. The face validity criterion was met; increased ASR responses are reported in both our animal model (Inagaki et al., 2008) and human anxiety (Grillon et al., 1997; Ludewig et al., 2005; Prehn et al., 2006). In addition, the construct validity criterion was also fulfilled; alarm pheromone exposure in rats increased Fos expression in the amygdala, (Kiyokawa et al., 2005b) and this same brain region is involved in human anxiety (Rauch et al., 2000; Stein et al., 2007). As for predictive validity, however, little information is available. The only potential information regarding predictive validity is that pretreatment

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with the anxiolytic diazepam attenuates the enhancement of the ASR (Inagaki et al., 2008). Therefore, it is necessary to evaluate the predictive validity of the alarm pheromone in detail.

In this study, we examined the predictive validity of alarm pheromone-induced anxiety by pretreatment with six types of human anxiolytics using ASR as a bioassay parameter. We used the following anxiolytics: midazolam, a benzodiazepine; phenelzine, a nonselective monoamine oxidase (MAO) inhibitor; propranolol, a nonselective β-adrenergic receptor antagonist; clonidine, an α2-adrenergic receptor agonist; CP-154,526, a corticotropin-releasing factor subtype 1 receptor (CRF1) antagonist; and buspirone, a serotonin-1A ($5\text{-}HT_{1A}$) receptor agonist.

2. Materials and methods

2.1. Animals

Two hundred and seventy-five experimentally naive male Wistar rats were purchased (Clea Japan, Tokyo, Japan) at 7 weeks of age. Animals were provided with water and food *ad libitum* and kept on a 12-h light–dark cycle (lights turned off at 20:00). The vivarium was maintained at a constant temperature (24 ± 1 °C) and humidity (40–45%). Animals were housed in pairs for 9 days in wire-topped, transparent cages (410 × 250 × 180 mm) with wood shavings for bedding. Each rat was then housed singly in the same type of cage. Three days after being housed singly, these rats were used as pheromone recipients in the experiment. All rats 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 prior to the experiment. Each 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.

2.2. Experimental apparatus

The startle apparatus and software used in this study (StartleReflexSystem 2004; O'Hara & Co., Tokyo, Japan) are described in detail in a previous study (Inagaki et al., 2008). Briefly, we used an animal holder to obtain ASR data from each 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 rat was kept inside the cylinder using the two stoppers. 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 through 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 within the holder resulted in displacement of an accelerometer 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.

2.3. Preparation of water samples

Before the experiment, we prepared water samples according to an established method that has been previously described (Inagaki et al., 2008). 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 pentobarbital sodium, intraperitoneally; Nembutal: Abbott Laboratories, North Chicago, IL or Somnopentyl: Schering–Plough Animal Health, Harefield, UK), 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. The stimulation of the neck region was conducted as a control because stimulation of this area does not release the alarm pheromone (Inagaki et al., 2008; Kiyokawa et al., 2004b, 2005a). After being stimulated in this manner, the donor rat was removed, and the water droplets on the ceiling that contained either the alarm pheromone or neck odor were collected in a polypropylene conical tube using a glass bar and Pasteur pipette. Water droplets collected from a control box (in which no animal was present) were used as the vehicle control. Each sample of water was stored at 4 °C for 1–5 h and then used for five to six recipient rats. The pheromone box was washed in hot water with a cleanser and wiped with a paper towel prior to each use. The donor rats were used two or three times as donors, with at least 2 weeks between uses.

2.4. Drugs

The following drugs, each of which was dissolved in a vehicle (saline containing 0.5% tragacanth gum powder; Wako Pure Chemical Industries, Osaka, Japan), were prepared and used in the experiment: midazolam (0, 0.4, and 1.0 mg/kg; Wako Pure Chemical Industries); phenelzine (0, 15, and 30 mg/kg; Sigma Chemical, St. Louis, MO); propranolol (0, 10, and 20 mg/kg; Wako Pure Chemical Industries); clonidine (0, 1.0, and 5.0 μg/kg; Wako Pure Chemical Industries); CP-154,526 (0, 10, and 30 mg/kg; Pfizer, New York, NY); and buspirone (0, 2.0, and 5.0 mg/kg; Sigma Chemical). We referred to earlier studies to determine the doses of each drug (Lorrain et al., 2005; McGregor et al., 2002; Paslawski et al., 1996; Soderpalm and Engel, 1988; Walker and Davis, 1997, 2002).

2.5. Experimental 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. The vehicle or a single dose of each drug was then administered intraperitoneally. Forty-five minutes after the drug (or control) injection, each subject was placed inside the animal holder and fixed on the platform in the soundproof test chamber. The experiment consisted of three consecutive sequences: 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 30 auditory stimuli at an interstimulus interval of 30 s. Immediately after the baseline trial, we took the animal holder containing the subject to outside the test chamber and set a sheet of filter paper (50 × 50 mm, folded in two) on the front animal stopper. Each water sample (600 μl; see above) was dropped onto the paper. After 1–2 min of the sample presentation procedure, we returned the animal holder with the filter paper to the test chamber. Then, each subject was exposed to 30 auditory stimuli with interstimuli intervals of 30 s, after the 5-min acclimation period for the test trial. Baseline and test trials were conducted under the illumination of fluorescent bulbs (10 W) on the ceiling of the test chamber, and all experimental procedures were conducted between 11:30 and 16:30.

We divided the 275 subjects into six groups depending on the type of drug administered: midazolam ($n = 45$), phenelzine ($n = 50$), propranolol ($n = 45$), clonidine ($n = 50$), CP-154,526 ($n = 45$), and buspirone ($n = 40$). In each drug group, the subjects were divided equally into five subgroups, on the basis of the treatment (dose of the drug and water sample).

2.6. Data analysis

Individual baseline data were defined as the mean amplitude of the last 20 responses to each sound in the baseline trial. The baseline data were analyzed using a one-way analysis of variance (ANOVA) to compare different treatments.

The test data were defined as the mean amplitude of all 30 responses in the test trial. We individually calculated the difference in amplitude between the test data (T) and the baseline data (B) as $T-B$. Differences in amplitude were then statistically analyzed using a one-way ANOVA followed by Dunnett's *post hoc* test.

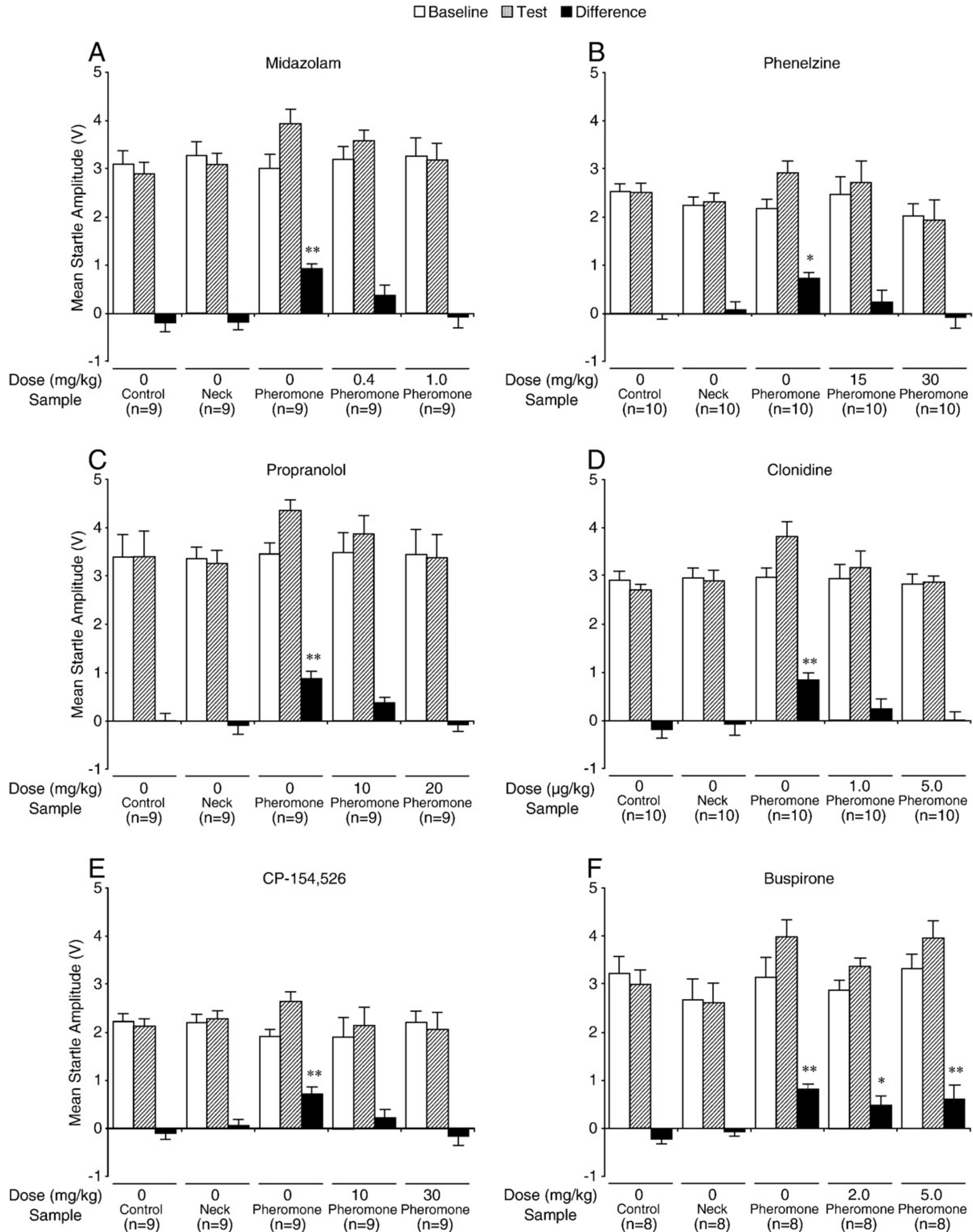

Fig. 1. Baseline data (Baseline, white bars), test data (Test, striped bars), and differences in amplitude between the baseline and test data (Difference, black bars) are shown for the acoustic startle reflex (ASR) evoked by audio stimuli using sound bursts 105 dB in intensity. Rats were pretreated with a vehicle or drug 45 min before the experiment and were presented with samples between the baseline trial and the test trial. The drug dose (mg/kg or μg/kg), type of presented sample (control water: Control; neck odor water: Neck; or pheromone water: Pheromone), and the number of subjects (n) are described under each graph for the drug administration groups (A: midazolam, B: phenelzine, C: propranolol, D: clonidine, E: CP-154,526, and F: buspirone). Also given are p values ($^{**}p<0.01$ and $^{*}p<0.05$) for drug-treated subjects versus those pretreated with vehicle (no drug administration) and presented with control water (one-way ANOVA followed by *post hoc* Dunnett's test).

All data are displayed as the mean ± standard error. The criterion for statistical significance was $p<0.05$ for all comparisons.

3. Results

The baseline data did not differ among treatments in any drug administration group [midazolam: $F(4,40)=0.14$, $p=0.97$ (Fig. 1A); phenelzine: $F(4,45)=0.77$, $p=0.55$ (Fig. 1B); propranolol: $F(4,40)=0.02$, $p=0.99$ (Fig. 1C); clonidine: $F(4,45)=0.06$, $p=0.99$ (Fig. 1D); CP-154,526: $F(4,40)=0.47$, $p=0.76$ (Fig. 1E); buspirone: $F(4,35)=0.58$, $p=0.68$ (Fig. 1F)].

Differences in amplitude between the test data and the baseline data were significantly affected by treatment in all administration groups [midazolam: $F(4,40)=7.55$, $p<0.01$ (Fig. 1A); phenelzine: $F(4,45)=3.26$, $p<0.05$ (Fig. 1B); propranolol: $F(4,40)=8.41$, $p<0.01$ (Fig. 1C); clonidine: $F(4,45)=5.14$, $p<0.01$ (Fig. 1D); CP-154,526: $F(4,40)=5.49$, $p<0.01$ (Fig. 1E); buspirone: $F(4,35)=7.48$, $p<0.01$ (Fig. 1F)]. *Post hoc* tests indicated that exposure to the alarm pheromone, but not to neck odor, significantly enhanced the ASR (phenelzine group: $p<0.05$; others: $p<0.01$) as compared with those in the control group. This alarm pheromone effect was blocked by pretreatment with 0.4 and 1.0 mg/kg doses of midazolam (Fig. 1A), 15 and 30 mg/kg doses of phenelzine (Fig. 1B), 10 and 20 mg/kg doses of propranolol (Fig. 1C), 1.0 and 5.0 µg/kg doses of clonidine (Fig. 1D), and 10 and 30 mg/kg doses of CP-154,526 (Fig. 1E). In contrast, pretreatment with any dose of buspirone did not antagonize the effects of the alarm pheromone (Fig. 1F).

4. Discussion

Consistent with our previous study (Inagaki et al., 2008), alarm pheromones enhanced the ASR in recipient rats. These pheromone effects were dose-dependently blocked by pretreatment with midazolam, phenelzine, propranolol, clonidine, and CP-154,526. In contrast, pretreatment with buspirone did not antagonize the pheromone effect. These results are evidence that the alarm pheromone exposure model fulfills the predictive validity criterion for human anxiety.

This and previous (Inagaki et al., 2008) studies reveal a specific response to human anxiolytics in alarm pheromone-induced anxiety in rats, indicating the predictive validity of this model. In this study, pretreatment with buspirone did not block the pheromone effect. In contrast, buspirone is an anxiolytic in many animal models of anxiety. For example, pretreatment with buspirone reduces burying behavior against an electrified prod in the shock-probe burying test (Fernandez-Guasti et al., 2005; Lopez-Rubalcava et al., 1999), decreases the latency period for leaving the enclosed arms in an elevated T-maze test (Graeff et al., 1998; Poltronieri et al., 2003), increases time spent in social interactions in a social interaction test (Dunn et al., 1989; Louis et al., 2008), decreases ultrasonic vocalizations (USVs) in rat pups in an isolation-induced USVs test (Iijima and Chaki, 2005; Olivier et al., 1998), and enhances the ASR in a light-enhanced startle test (Walker and Davis, 1997). Therefore, it is possible that the alarm pheromone exposure model is a novel and unique model of anxiety as compared to the other reported models.

In addition to these acute effects, knowledge of the chronic effect of drugs is also required for the alarm pheromone exposure model to fulfill predictive validity because selective serotonin reuptake inhibitors (SSRIs) are widely used treatments for all types of human anxiety disorders and are clinically effective after chronic long-term administration (Baldwin et al., 2005; Bandelow et al., 2008). However, chronic SSRI treatments are only effective in the novelty-suppressed feeding model (Bodnoff et al., 1989) and defensiveness to cat odor model (Dielenberg and McGregor, 2001). In other well-known models, chronic treatment did not exert anxiolytic effects, such as the elevated plus maze test (Durand et al., 1999; File et al., 1999; Griebel et al., 1999; Silva and Brandao, 2000), the light–dark transition test (Kshama et al., 1990; Sanchez and Meier, 1997), the social interaction test (Bristow et al., 2000; Duxon et al., 2000; File et al., 1999; To et al., 1999), and conditioned freezing (Li et al., 2001). Therefore, it is both necessary and of interest to assess whether the alarm pheromone exposure model is sensitive to chronic administration in future studies.

Based on the present results, we cannot exclude the possibility that increased HPA axis activity was due to restraint stress and that it masked the anxiolytic effect of buspirone. However, this explanation appears less likely because of the habituating procedure used before the experiment to reduce restraint stress as much as possible. In addition, the same dose of buspirone used here (5.0 mg/kg, i.p.) attenuated the light-enhanced startle response, in which restraint mesh cages (150 × 150 × 80 mm) were used for animal holders (Walker and Davis, 1997).

These results suggest some differences between anxiety-related mechanisms evoked by intra- and interspecies communications. Earlier studies have shown that the benzodiazepine midazolam reduces anxiety (reduction of defensiveness) in rats in response to a cat odor, but not to the main chemical in fox odor (2,5-dihydro-2,4,5-trimethylthiazole [TMT]) (McGregor et al., 2002). Moreover, both diazepam and the CRF1 antagonist antalarmin are effective against increased anxiety to cat odor, but not to TMT, in the staircase test, which is used to compare the contact times between a brush to which cat odor or TMT has been added and a control brush with no odor, each of which is placed on the top stair (Blanchard et al., 2003). Thus, on the basis of these observations and the results of this study, it is conceivable that the neural mechanisms processing alarm pheromone signals are more closely related to those used for cat odor rather than those used for TMT. Supporting this idea, the vomeronasal system is most likely the main pathway involved in anxiety-related responses evoked by both cat odor (McGregor et al., 2004) and the alarm pheromone (Kikusui et al., 2001; Kiyokawa et al., 2005b, 2007). In contrast, the main olfactory system may be involved in the emergence of TMT-induced anxiogenic effects (Staples et al., 2008). Nevertheless, the effects of cat odor can be suppressed by pretreatment with buspirone (Blanchard et al., 2003), which in this study had no efficacy against the alarm pheromone-mediated enhancement of the ASR. These findings suggest that neural mechanisms involved in anxiogenic olfactory communications via cat odor differ from those via alarm pheromone. However, further studies are needed to fully clarify these issues.

In conclusion, the present study provides further evidence for the predictive validity of alarm pheromone-induced responses in rats as an animal model of human anxiety. Future studies should examine whether certain subtypes of human anxiety disorders are adequately modeled by rats exposed to the alarm pheromone. This can be accomplished by evaluating not only the predictive validity via chronic drug administration studies, but also the face validity and construct validity in much more detail.

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