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Social buffering ameliorates conditioned fear responses in female rats

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ABSTRACT

The stress experienced by an animal is ameliorated when the animal is exposed to distressing stimuli along with a conspecific animal(s). This is known as social buffering. Previously, we found that the presence of an unfamiliar male rat induced social buffering and ameliorated conditioned fear responses of a male rat subjected to an auditory conditioned stimulus (CS). However, because our knowledge of social buffering is highly biased towards findings in male subjects, analyses using female subjects are crucial for comprehensively understanding the social buffering phenomenon. In the present studies, we assessed social buffering of conditioned fear responses in female rats. We found that the estrus cycle did not affect the intensity of the rats' fear responses to the CS or their degree of vigilance due to the presence of a conspecific animal. Based on these findings, we then assessed whether social buffering ameliorated conditioned fear responses in female rats without taking into account their estrus cycles. When fear conditioned female rats were exposed to the CS without the presence of a conspecific, they exhibited behavioral responses, including freezing, and elevated corticosterone levels. By contrast, the presence of an unfamiliar female rat suppressed these responses. Based on these findings, we conclude that social buffering can ameliorate conditioned fear responses in female rats.

Introduction

The stress experienced by a subject can be ameliorated when the subject is exposed to distressing stimuli while in the company of a conspecific animal. This phenomenon is called social buffering and can be induced by a mother, mate, or a same-sex or opposite-sex conspecific animal in a nonsexual relationship (Kiyokawa, in press). The phenomenon elicited by the last type of conspecific has been observed in a wide variety of social species, including sheep (da Costa et al., 2004), guinea pigs (Hennessy et al., 2006, 2008), and rats (Terranova et al., 1999).

Previously, we conducted a series of experiments focused on the social buffering of conditioned fear responses in rats. In this model, male subject rats exhibited robust freezing and activation of the hypothalamic-pituitary-adrenal (HPA) axis in response to an auditory conditioned stimulus (CS), when the CS was paired with a foot shock during fear conditioning. The presence of an unfamiliar non-conditioned male rat (an associate) ameliorated these responses (Kiyokawa et al., 2007). Subsequently, we found that this social buffering occurred even when the subject and associate were separated by wire-mesh or double wire-mesh partitions (Kiyokawa et al., 2009, 2014a). Given that social buffering was inhibited by lesioning the main olfactory epithelium (Kiyokawa et al., 2009) and that associate-derived olfactory cues alone are able to induce social buffering (Takahashi et al., 2013; Kiyokawa et al., 2014b), we suggest that olfactory signaling mediates social buffering of conditioned fear responses. Recent studies have shed light on the neural mechanisms underlying social buffering, such as the suppression of the basolateral complex of the amygdala (Fuzzo et al., 2015) and the involvement of the posterior complex of the anterior olfactory nucleus as a relay point for signaling from the olfactory bulb to the amygdala (Kiyokawa et al., 2012; Takahashi et al., 2013).

Our knowledge of social buffering is highly biased towards findings in male subjects, because all our previous studies, as well as most studies in the literature, have been conducted using males. Although some studies have reported the social buffering phenomenon in female subjects, the phenomenon was induced by a mate (Kaiser et al., 2003; Hennessy et al., 2008; Smith and Wang, 2014). Given that the neural mechanisms underlying social buffering differ depending on the type of conspecific animal, i.e., a mother, mate, or conspecific without sexual relationships, it would be appropriate to understand each phenomenon individually (Kiyokawa, in press). To the best of our knowledge, only one study using female guinea pigs reported a clear social buffering phenomenon by a conspecific animal without sexual relationships (a female guinea pig) (Hennessy et al., 2008). In addition, sex differences are thought to play a large role in stress responses. For example, males tend to show a "fight and flight" response to distressing stimuli, while females tend to exhibit a "tend and befriend" response (Taylor et al., 2000). Therefore, analyses using female subjects seem to be necessary to obtain a more comprehensive understanding of the social buffering phenomenon, especially that induced by conspecifics without sexual relationships.

Changes in ovarian hormones that are dependent on the estrus cycle, such as estrogen and progesterone, appear to be one of the

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major challenges in analyzing social buffering of conditioned fear responses in female rats. First, these hormones affect the intensity of conditioned fear responses (Morgan and Pfaff, 2001). Given that the observed responses in the presence of a conspecific are residual intrinsic CS-induced responses after suppression by social buffering, we cannot appropriately evaluate the degree of social buffering if the intensity of intrinsic CS-induced responses fluctuates according to the estrus cycle. Second, these hormones can affect the degree of vigilance (Petersson et al., 1998), which may cause difference in the intensity of vigilance attributable to the presence of a conspecific. Fluctuations in vigilance due to the estrus cycle would also prevent us from appropriately evaluating social buffering, because vigilance can affect behavioral measures that require stillness and/or physiological measures related to metabolism, such as freezing and/or HPA axis activity, respectively. Therefore, it was necessary to assess the effects of the estrus cycle on these two factors.

In the present study, we conducted a series of experiments to assess social buffering of conditioned fear responses in female rats. In Experiment 1, we assessed the effects of the estrus cycle on the intensity of conditioned fear responses. Fear conditioned and non-conditioned female subjects were exposed to an auditory CS while alone in the testing apparatus (solitary situation). Behavioral responses, including freezing, were compared across the stages. In Experiment 2, we assessed the effects of the estrus cycle on vigilance. Female subjects at all stages of the estrus cycle encountered unfamiliar female associates who were also in all stages of the cycle. Their behavioral responses were analyzed, including locomotor activity. In Experiment 3, we assessed whether social buffering ameliorated conditioned fear responses in female rats. Fear conditioned or non-conditioned female subjects were exposed to the auditory CS either alone (solitary situation) or with an unfamiliar female associate (dyad situation). Their behavioral responses, including freezing, and corticosterone levels were measured in order to evaluate the presence of social buffering. We conducted parallel experiments using male rats in order to evaluate sex differences in social buffering.

Material and methods

Animals

All experiments were approved by the Animal Care and Use Committee of the Faculty of Agriculture of The University of Tokyo and were based on guidelines that were adapted from the Consensus Recommendations on Effective Institutional Animal Care and Use Committees by the Scientists Center for Animal Welfare.

Experimentally naïve female and male Wistar rats were purchased from Charles River Laboratories Japan (Kanagawa, Japan) at 7.5 weeks of age. Animals were housed 2–3 rats per cage upon arrival and under conditions of controlled temperature (24 ± 1 °C) and humidity (45 ± 5%). The housing room had a 12-h light/12-h dark cycle (lights on at 8:00). Water and food were available ad libitum. All behavioral procedures in Experiments 1 and 2 were performed between 8:00 and 15:00. The behavioral procedures in Experiment 3 were performed between 8:30 and 13:00 (see Experiment 3).

Experiment 1

Female subjects were housed individually 3 days before fear conditioning until the test day and were handled for 3 min twice daily until the day of conditioning. A vaginal smear was taken every morning beginning on the day after arrival to evaluate the stage of the estrus cycle.

Fear conditioning was performed under white-light conditions, as previously described (Kiyokawa et al., 2009). Subjects were placed in an acrylic conditioning box (28 × 20 × 27 cm) for 20 min. During fear conditioning, paired subjects were exposed to 7 pairings of a 3-s auditory CS (8 kHz, 70 dB) and a 0.5 s foot shock (0.80 mA), which ended simultaneously. Unpaired subjects received the same number of CS and foot shocks, but these were presented separately. The inter-trial interval varied randomly between 60 and 180 s. After conditioning, subjects were returned to their home cages.

The fear-expression test was performed 24 h after conditioning under dim red light, as previously described (Kiyokawa et al., 2009). Two rectangular enclosures (25 × 25 × 35 cm) were placed on an acrylic board (45 × 60 cm). Each enclosure consisted of 3 acrylic walls, 1 wire mesh wall, and a wire mesh ceiling. The inside of the enclosure was covered with clean bedding. The enclosures were placed side-by-side so that the wire mesh walls were facing each other, separated by a 5 cm gap. The wire mesh wall was composed of a 1 cm^2 grid in the lower half (20 cm) and vertical bars in the upper half (15 cm) to prevent subjects from climbing the wire-mesh wall. Subjects were placed randomly in one of the two enclosures. After a 3-min acclimation period, the 3-s CS was presented 5 times at intervals of 1 min during the first 5 min of the 10-min test period. The subjects' behavior during the fear-expression test was recorded with an HDD-BD recorder (DMR-BW770; Panasonic, Osaka) and a video camera (DCR-SR 300; Sony, Tokyo).

The paired subjects were divided into 4 groups according to their estrus cycle at the time of the fear-expression test (diestrus 1, $n = 10$; diestrus 2, $n = 10$; proestrus, $n = 8$; estrus, $n = 12$). Because the estrus cycle did not alter the behavioral responses of the unpaired female subjects during the fear-expression test, all the unpaired subjects (diestrus 1, $n = 4$; diestrus 2, $n = 4$; proestrus, $n = 5$; estrus, $n = 3$) were combined into 1 group, regardless of their estrus cycle.

All data are presented as the mean ± standard error of the mean (SEM). The significance level was set at $p < 0.05$ for all statistical tests. Behavioral parameters during the acclimation and test period were measured using Visual Basic software in Microsoft Excel, which recorded the duration and number of pressed keys. The duration of freezing (the lack of any movement except that which is required for respiration), the duration of investigation (sniffing towards the wire mesh wall within 1 cm or poking of the snout towards the wire mesh), and the frequency of walking (number of steps taken with the hind paws) during the preceding acclimation period and during the test period were analyzed by multivariate analysis of variance (MANOVA), followed by Fisher's PLSD post-hoc test. Effect sizes were further estimated by calculating the value of multivariate η^2 and Cohen's d for data analyzed by MANOVA and Fisher's PLSD post-hoc test, respectively.

Experiment 2

The encounter test was conducted in the same enclosures under dim red light as described above. In the encounter test, a female rat was placed in each enclosure for 5 min to observe the behavior to a novel animal, which was recorded with an HDD-BD recorder and a video camera. Each rat served both as the subject and also as an associate for the other. Based on the subject's and the associate's estrus cycle, we prepared 16 groups ($n = 4$–6 per group). Rats were housed individually until the test day, and were handled for 3 min twice daily for 3 days.

The duration of freezing, the duration of investigation, and the frequency of walking were analyzed by two-way MANOVA. Effect sizes were further estimated by calculating the value of multivariate η^2 for data analyzed by MANOVA.

Experiment 3

Fear conditioning was performed as described in Experiment 1. Female and male rats assigned as subjects underwent fear conditioning, while rats assigned as associates remained in their home cages during fear conditioning. Subjects were further assigned as either paired or unpaired subjects. Because we found that the estrus cycle did not affect the intensity of the rats' fear responses to the CS or their degree of vigilance due to the presence of a conspecific animal, we did not assess the stage