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Main olfactory system mediates social buffering of conditioned fear responses in male rats

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Abstract

We previously reported that the presence of a conspecific animal blocked freezing of a male rat in response to an auditory conditioned stimulus that had been paired with foot shocks, as well as associated Fos expression in the paraventricular nucleus. Here we investigated how this 'social buffering' is mediated by examining the contributions of both physical contact and the main olfactory system. Fear-conditioned rats exposed to the conditioned stimulus alone responded by freezing and increased Fos expression in the paraventricular nucleus. However, the presence of another rat, but not a guinea pig, dramatically mitigated these responses, even if the dyad was separated by a wire mesh or a pair of wire meshes 5 cm apart. In contrast, social buffering was absent when a transparent acrylic board was inserted between the double wire mesh. Lesioning of the main olfactory epithelium by injection of $ZnSO_4$ intranasally also abolished social buffering. Thus, we conclude that the main olfactory system is essential for the social buffering but does not require physical contact between the dyad.

Introduction

In social mammals, conspecific animals influence the stress response. Signals from fearful or stressed conspecific animals aggravate stress responses (Kiyokawa *et al.*, 2004a; Inagaki *et al.*, 2008), whereas signals from non-fearful ones alleviate stress responses (Davitz & Mason, 1955; Taylor, 1981; Gust *et al.*, 1994; Terranova *et al.*, 1999). This phenomenon is termed 'social buffering'.

We previously reported that the presence of a conspecific animal blocked freezing of a male rat in response to a contextual and auditory conditioned stimulus (CS) that had been paired with foot shocks, as well as associated Fos expression in the paraventricular nucleus (PVN) of the hypothalamus (Kiyokawa *et al.*, 2004b, 2007b). On the basis of these findings, we have hypothesized that social buffering mitigates the conditioned fear responses in male rats. However, we cannot exclude the possibility that the observed phenomena are simply the consequence of a physical disturbance of stress responses by the accompanying conspecific animal.

If social buffering rather than a physical disturbance mitigates conditioned fear responses, the subject should receive some signals from the accompanying animal. According to currently available studies, the species-specific cues appear to play an important role in the stress-buffering signal. For example, a picture of the face of another sheep induced social buffering in other sheep (da Costa *et al.*, 2004), whereas a signature vocalization mediated 'mated-partner buffering' (stress buffering by mated partner) in marmosets (Rukstalis & French, 2005). Therefore, we hypothesized that olfaction may mediate social buffering in rats, a species with a highly developed olfactory system.

To test this hypothesis, we first examined whether or not physical contact between the dyad was necessary by separating the subject and associate with a wire mesh during presentation of the auditory CS. We also tested double wire mesh separated by 5 cm with or without a transparent acrylic board placed between them. We assessed the effectiveness of social buffering by measuring both freezing behavior and Fos expression in the PVN. Second, to determine whether the infralimbic region of the prefrontal cortex (IL) was involved in this phenomenon, we stained this region for Fos and glutamate decarboxylase (GAD) 67, as IL has been proposed to be a key site for suppressing conditioned freezing (Milad & Quirk, 2002; Quirk *et al.*, 2006) and thus is probably involved in the social buffering of freezing. Last, we examined the role of the main olfactory system (MOS) in social buffering by lesioning the main olfactory epithelium by injection of $ZnSO_4$ intranasally.

Materials and methods

Animals

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

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Experimentally naïve male Wistar rats were purchased at 8 weeks of age from Charles River Laboratories Japan (Kanagawa, Japan). They were housed two to four animals per cage in a temperature-controlled (24 ± 1 °C) and humidity-controlled (45 ± 5%) room. Food and water were available *ad libitum*, and the animals were kept under a 12-h light/12-h dark cycle (lights on at 08:00 h). Experimentally naïve male Hartley guinea pigs were purchased from Japan SLC (Shizuoka, Japan) at 4 weeks of age, at age at which their body size was equivalent to that of 8-week-old rats. Guinea pigs were housed two to four animals per cage in the same room for several days after arrival. Subsequently, both rats and guinea pigs were each housed individually. At this moment, rats were assigned to either the subject group or the associate group, with cage mates always being assigned to the same group, to ensure that subject and associate rats were unfamiliar with one another. All animals were handled for 5 min per day for 3 days before the fear conditioning.

Experiments to assess the role of physical contact

Fear conditioning was performed in an illuminated room between 09:00 and 13:00 h. A cup of acetic acid was placed in one corner of the conditioning room, and 1% acetic acid solution was sprayed into an acrylic box with a metal grid floor (28 × 20 × 27 cm). An animal was placed in this conditioning box for 20 min. Animals in the paired group received seven pairings of a 3-s tone (8 kHz, 80 dB) that co-terminated with a foot shock (0.5 s, 0.7 mA). We also prepared the unpaired group by presenting the CS and foot shock separately over a 20-min period. The intertrial interval varied randomly between 30 and 180 s. After fear conditioning, each rat was returned to its home cage. The conditioning box was thoroughly washed in hot water with a cleanser and wiped dry with a paper towel before each use.

A fear expression test was performed 24 h after the fear conditioning, using two rectangular enclosures (25 × 25 × 35 cm) placed on an acrylic board (45 × 60 cm) in a dark room illuminated with dim red light (Fig. 1). Each enclosure was constructed of three acrylic walls, one demountable wire mesh wall, and a wire mesh ceiling. Clean bedding was spread to cover the floor encircled by the wall. The mesh of the demountable wire mesh wall consisted of 1-cm^2 gauge in the lower part (20 cm) and of 1-cm-interval vertical bars in the upper part (15 cm), which prevented rats from climbing up to the ceiling. Two enclosures were placed side-by-side so that the wire mesh wall sides were facing each other. Two neighboring enclosures were separated with either a single wire mesh wall, with double wire mesh walls that were 5 cm apart, or with 5-cm-apart double wire mesh walls and a transparent acrylic board between them. After a 2-min acclimation period, a CS was presented for 3 s five times at 1-min intervals during the first half of the 10-min experimental period. The behavior of the animals during acclimation and experimental periods was videotaped (DCR-TRV18; Sony, Tokyo, Japan). After the fear expression test, rats were returned to their home cage and all equipment was washed in hot water with a cleanser and wiped dry with a paper towel.

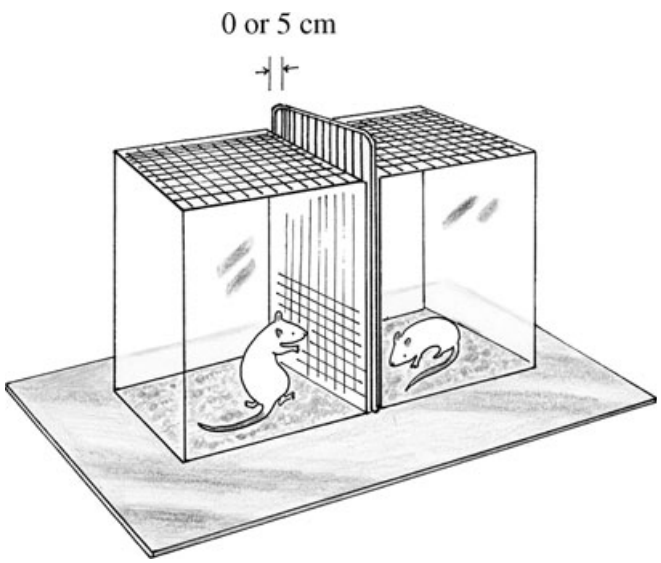

FIG. 1. Schematic diagram of the test apparatus used in this study.

Animals underwent the fear expression test in one of the four following situations (Fig. 2A): in the solitary situation, two enclosures were placed side-by-side and the subject was placed in one enclosure; in the mesh situation, two enclosures were placed side-by-side and the subject and associate rat were placed into the enclosures; in the double mesh situation, two enclosures were separated by 5 cm and the subject and associate rat were placed into the enclosures; and in the board situation, two enclosures were separated by 5 cm, a transparent acrylic board (2 mm in thickness, 30 × 35 cm) was placed between them, and the subject and associate rats were placed into the enclosures.

In order to observe Fos expression in the PVN and Fos/GAD67 expression in the IL, animals were deeply anesthetized with sodium pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL, USA) and perfused intracardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer 48 min after the fear expression test, that is, 60 min after the beginning of the acclimation period. The brain was removed, immersed overnight in the same fixative, and then placed in 30% sucrose/phosphate buffer for cryoprotection.

Experiments to assess the role of the MOS

The subject received intranasal injection of either saline or $ZnSO_4$ solution 2 days before fear conditioning, according to the method described in the previous study (Margolis *et al.*, 1974). Animals were lightly anesthetized with sodium pentobarbital (20 mg/kg, Nembutal; Abbott Laboratories) and placed supine with the head lower than the rest of the body. Polyethylene tube (SP10; Natume seisakusho, Tokyo, Japan) was inserted about 15 mm into one nostril, and 0.2 mL of $ZnSO_4$ solution (0.17 M; Wako Pure Chemical Industries, Osaka, Japan) was injected into the nostril. The excess solution was drained with an aspirator. This was repeated with the other nostril. Control animals received saline by the same procedure. Following the treatment, the animal was returned to its home cage.

Fear conditioning was performed as described above, with one exception; acetic acid was neither placed nor sprayed in the conditioning box. The fear expression test was conducted 24 h after conditioning as described, in apparatus that was identical to that of the double mesh situation in the first set of experiments. Animals were tested either alone or with a conspecific associate separated by double wire mesh.

The habituation–dishabituation test was conducted 2–3 h after the fear expression test. This test assessed the ability of an animal to discriminate two olfactory stimuli by utilizing the tendency of laboratory rodents to show interest in or be attracted to a novel stimulus (Johnston *et al.*, 1993; Kiyokawa *et al.*, 2007a). As an odor becomes more familiar, the time spent investigating the odor stimulus

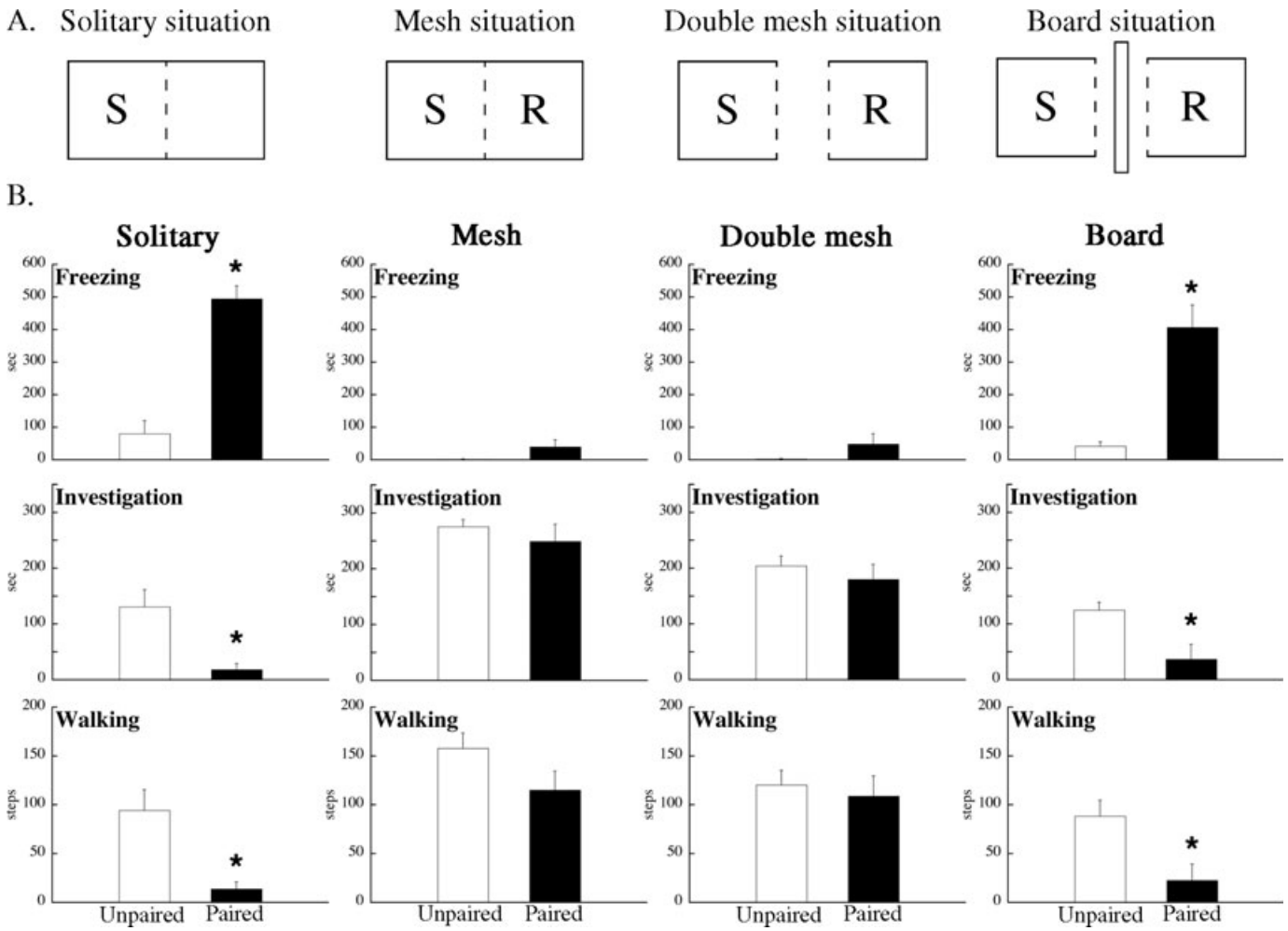

FIG. 2. The behavioral responses during the fear expression test assessing the role of the physical contact in social buffering. (A) Schematic diagram of the four situations. S, subject rat; R, associate rat. (B) Duration of freezing, duration of investigation and frequency of walking of fear-conditioned (paired) and non-conditioned (unpaired) rats that underwent a fear expression test in one of four situations (mean ± standard error of the mean). *$P < 0.05$, with MANOVA (Hotelling's trace) followed by Fisher's protected least significant difference *post hoc* test.

will decrease over successive presentations. The subsequent presentation of a different stimulus will result in a longer investigation time if the new stimulus can be discriminated from the first. The increased investigation time indicates the ability of the subject to discriminate the two odor stimuli. In the present study, we used purified water and 5% cocoa solution as odor stimuli.

A water bottle on a stainless steel cage top was removed, and each animal was transported in its home cage to another shelf in the colony room. After a 30-s acclimation period, the first odor stimulus was presented by pipetting 50 μL onto one-half of a folded filter paper (5 × 5 cm) attached to the edge of the ceiling, such that the rat was unable to make physical contact with the filter paper and only volatile odors from the stimulus were available at body level. After three consecutive 2-min presentations of purified water, the cocoa solution was presented for 2 min, at 30-s intervals. The behavior of the subject was video-recorded (DCR-DVD403; Sony) for later analysis.

A day after the fear expression test, the rat was placed in a soiled cage in which two adult female rats had been kept for about a week. Sixty minutes after the placement, the animal was killed and perfused, and the brain was removed for immunohistology to examine Fos expression in the mitral/tufted cell layer of the accessory olfactory bulb (AOB). Male rats comprising a separate negative control group were picked up and placed back into the home cage to determine the baseline level of Fos expression.

TABLE 1. Behavioral responses during the acclimation period of the fear expression test for assessing the role of physical contact

Situation and conditioning	Freezing	Investigation	Walking
Solitary			
Unpaired (8)	8.1 ± 5.3	29.7 ± 5.7	27.8 ± 5.0
Paired (8)	20.0 ± 11.5	19.6 ± 5.2	23.9 ± 6.3
Mesh			
Unpaired (8)	7.6 ± 7.5	68.4 ± 7.5	39.3 ± 5.2
Paired (8)	0 ± 0	76.2 ± 3.0	37.1 ± 1.8
Double mesh			
Unpaired (8)	0 ± 0	56.3 ± 4.5	40.5 ± 3.7
Paired (8)	0 ± 0	55.2 ± 5.5	39.5 ± 3.5
Board			
Unpaired (8)	7.0 ± 6.7	29.0 ± 5.6	20.3 ± 4.1
Paired (8)	19.3 ± 9.6	27.6 ± 8.6	19.3 ± 7.4

Data are expressed as means ± standard error of the mean. The number of animals is provided in parentheses.

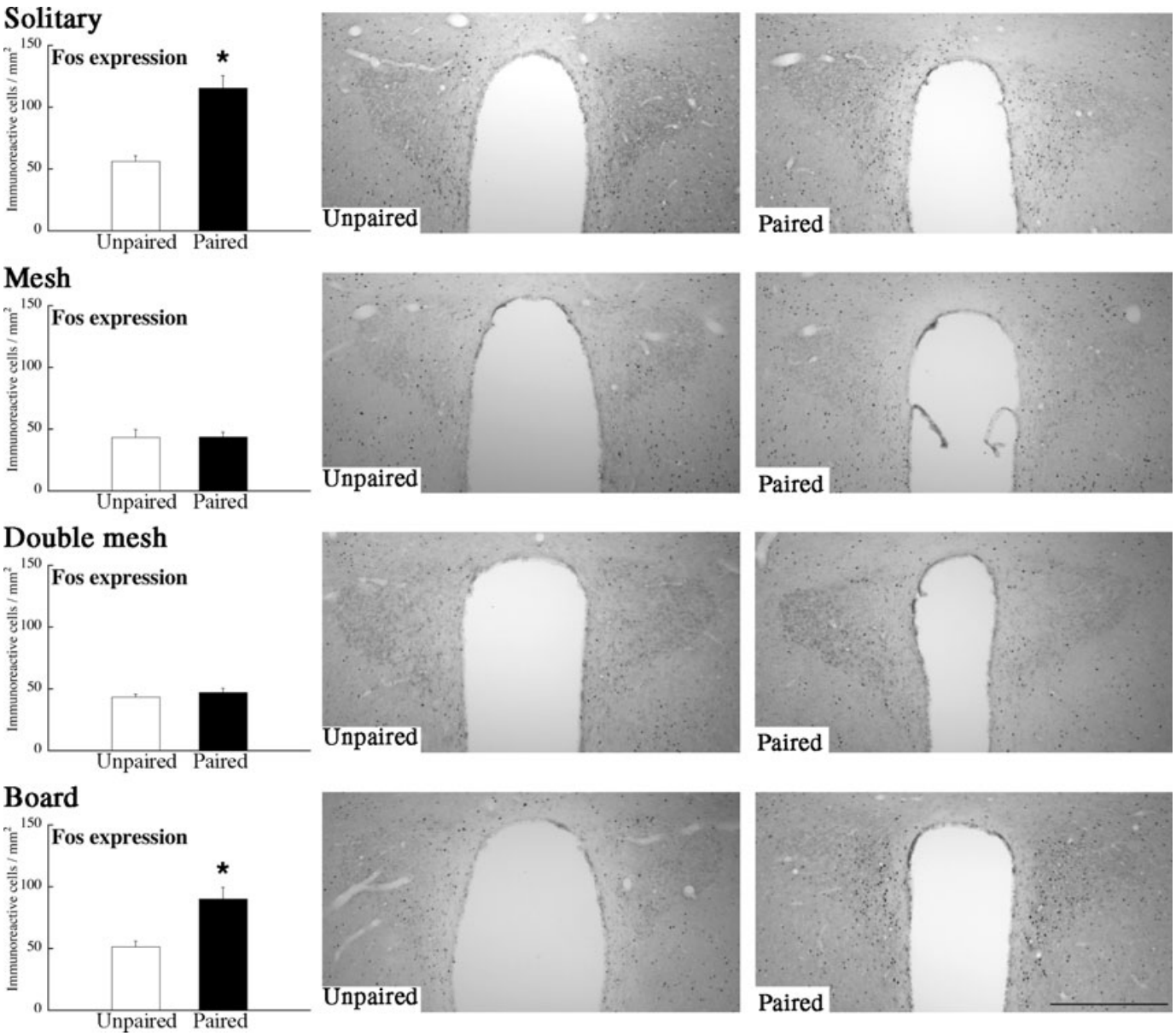

FIG. 3. The mean density and representative photomicrographs of the Fos-immunoreactive cells in the paraventricular nucleus of fear-conditioned (paired) and non-conditioned (unpaired) rats that underwent a fear expression test in one of four situations (mean ± standard error of the mean). Horizontal bar indicates 500 μm. *$P < 0.05$, with ANOVA.

Immunohistochemistry

The avidin–biotin–peroxidase method was used for immunohistochemical detection as previously described (Kiyokawa *et al.*, 2005b). Six successive coronal 30-μm sections containing the IL (bregma 3.24 mm) and PVN (bregma −1.80 mm), and nine successive sagittal 30-μm sections containing the AOB, were prepared. The second, fifth and eighth sagittal sections were stained with cresyl violet to confirm the location of the AOB, and the remaining sections were used for free-floating immunohistochemistry. The sections were incubated with a primary antibody directed against Fos (Ab-5, diluted 1 : 7500; Calbiochem, La Jolla, CA, USA) for 65 h, and then incubated with an anti-rabbit secondary antibody (VECTASTAIN elite ABC kit; Vector Laboratories, Burlingame, CA, USA) for 2 h. Subsequently, sections were processed with the elite ABC kit and developed using a diaminobenzidine solution with nickel intensification. The sections containing the IL were further incubated with a primary antibody for GAD67 (MAB5406, diluted 1 : 4000; Chemicon, Billerica, MA, USA) for 18 h, and then incubated with an anti-mouse secondary antibody (VECTASTAIN elite ABC kit; Vector Laboratories) for 2 h, processed with the elite ABC kit, and developed using a diaminobenzidine solution.

The regions of interest were confirmed in adjacent sections stained with cresyl violet and evaluated using a brain atlas (Paxinos & Watson, 2007). The IL-containing, PVN-containing and AOB-containing sections were photographed using a microscope equipped with a digital camera (DP30BW and DP70; Olympus, Tokyo, Japan).

Data analyses and statistical procedures

The data are expressed as means ± standard error of the mean, and significance was set at $P < 0.05$ for all statistical tests. A researcher blind to the experimental conditions analysed the duration of freezing

(immobile posture, with cessation of skeletal and vibrissae movement except in respiration) and investigation (sniffing towards another enclosure within 1 mm from the wire mesh, including climbing up the wire mesh), and the frequency of walking (number of steps taken with the hind paws), using MICROSOFT EXCEL-based Visual Basic software to record the data. The behavioral data during the initial acclimation and experimental period of each situation in the first set of experiments were analysed by MANOVA (Hotelling's trace) followed by Fisher's protected least significant difference (PLSD) *post hoc* test. It should be noted that the freezing during the acclimation period in the double mesh situation was excluded from the analysis because none of the rats exhibited freezing behavior. To achieve a between-situation comparison, the behavioral data during the acclimation period were also analysed by MANOVA (Hotelling's trace) followed by Dunett's *post hoc* test. The behavioral data in the second set of experiments were analysed by MANOVA (Hotelling's trace) followed by Fisher's PLSD *post hoc* test.

The duration of the investigation time for each odor stimulus was recorded in the habituation–dishabituation test using the same software. The investigation time was defined as the time that the rat spent sniffing towards the stimulus, poking its nose into the ceiling. The investigation time between the third water presentation and cocoa solution was analysed using Wilcoxon's signed-ranks test.

For immunohistochemical analyses, the numbers of Fos-immunoreactive cells in the PVN were counted, and the area of the nucleus was measured bilaterally with IMAGEJ 1.38x software (downloaded from http://rsb.info.nih.gov/ij/). The mean density (number of cells/mm^2) of Fos-immunoreactive cells was analysed by one-way ANOVA. The Fos expression in the mitral/tufted cell layer of the AOB was also calculated in the same way, and analysed by one-way ANOVA followed by Fisher's PLSD *post hoc* test. In the IL, the numbers of Fos-immunoreactive, GAD67-immunoreactive and double-immunoreactive cells in a 0.5-mm square were counted and analysed by one-way ANOVA.

Results

Physical contact is not necessary for social buffering

Animals were either fear-conditioned (paired) or not fear-conditioned (unpaired) to an auditory CS on the conditioning day. Then, 24 h after the conditioning procedure, the rats were re-exposed to the CS in the test apparatus alone (solitary situation: unpaired, $n = 8$; paired, $n = 8$), with an associate separated by a wire mesh (mesh situation: unpaired, $n = 8$; paired, $n = 8$), with an associate separated by double wire mesh (double mesh situation: unpaired, $n = 8$; paired, $n = 8$), or with an associate separated by double mesh and a transparent acrylic board (board situation: unpaired, $n = 8$; paired, $n = 8$) (Fig. 2A). As summarized in Table 1, no difference between the unpaired and paired groups was observed during the initial acclimation period in any of these situations.

Additional between-situation analyses revealed that the behavior during the acclimation period was significantly affected by the situation ($F_{9,158} = 13.3$, $P < 0.01$). Although the effects of the conditioning procedure and the interaction between the two factors were not significant, a *post hoc* test revealed that both investigation and walking increased in the mesh and double mesh situations as compared to the solitary situation ($P < 0.05$).

In the solitary situation, behavioral responses during the fear expression test were significantly affected by the conditioning procedure ($F_{3,12} = 18.9$, $P < 0.01$), and a *post hoc* test revealed that the conditioning procedure increased freezing ($P < 0.01$) and decreased both investigation ($P < 0.01$) and walking ($P < 0.01$) by the subject (Fig. 2B). In contrast, the conditioning procedure had no effects on the behavioral responses in the mesh or double mesh situations. However, in the board situation, the conditioning procedure significantly affected the behavior of the subject ($F_{3,12} = 8.32$, $P < 0.01$), and a *post hoc* test revealed that the conditioning procedure significantly increased freezing ($P < 0.01$) and decreased both investigation ($P < 0.05$) and walking ($P < 0.05$).

The presence of a conspecific associate also attenuated Fos expression in the PVN (Fig. 3). Although the conditioning procedure significantly increased Fos expression in the solitary situation ($F_{1,14} = 30.1$, $P < 0.01$), this increase of Fos expression was not observed in the mesh or double mesh situations. However, the conditioning procedure did increase Fos expression in the board situation ($F_{1,14} = 14.3$, $P < 0.01$).

The conditioning procedure significantly increased Fos expression in the IL in the solitary situation ($F_{1,14} = 4.66$, $P < 0.05$) (Table 2). The conditioning procedure also increased Fos expression in the board situation ($F_{1,14} = 12.8$, $P < 0.01$), whereas Fos expression in the mesh or double mesh situation was unaltered. Whereas GAD67 expression was not affected by the conditioning procedure in any situation, the number of double-labeled cells increased both in the solitary situation ($F_{1,14} = 4.76$, $P < 0.05$) and in the board situation ($F_{1,14} = 13.0$, $P < 0.01$).

TABLE 2. Number of immunoreactive cells/0.25 mm^2 in the infralimbic region of the prefrontal cortex after the fear expression test for assessing the role of physical contact

Situation and conditioning	Fos	GAD67	Double labeled
Solitary			
Unpaired (8)	15.5 ± 2.3	39.6 ± 1.0	1.7 ± 0.3
Paired (8)	23.2 ± 2.7*	39.4 ± 1.7	2.9 ± 0.5*
Mesh			
Unpaired (8)	19.9 ± 3.1	37.9 ± 2.5	2.6 ± 0.5
Paired (8)	26.8 ± 1.6	38.6 ± 1.7	2.5 ± 0.3
Double mesh			
Unpaired (8)	15.9 ± 2.2	41.9 ± 0.9	2.0 ± 0.3
Paired (8)	14.8 ± 1.8	39.8 ± 1.3	1.7 ± 0.3
Board			
Unpaired (8)	13.1 ± 2.2	43.4 ± 2.1	1.5 ± 0.2
Paired (8)	24.1 ± 2.2*	42.8 ± 1.3	2.7 ± 0.2*

Data are expressed as means ± standard error of the mean. The number of animals is provided in parentheses. *$P < 0.05$ as compared to unpaired group in the same situation (ANOVA).

The lack of behavioral response to the CS is not attributable to distraction by the presence of another animal

To examine whether the lack of behavioral responses might be attributable to distraction caused by the presence of another animal, we ran an additional experiment. The rats were either fear-conditioned (paired: $n = 8$) or not fear-conditioned (unpaired: $n = 7$) to an auditory CS on the conditioning day. Then, 24 h after the conditioning procedure, subjects were re-exposed to the CS with an animal of a different species, guinea pig, in the neighboring cage, separated by a wire mesh.

Although the behavioral responses during the acclimation period were not different between the groups (freezing, unpaired, 7.6 ± 2.3; freezing, paired, 11.1 ± 5.2; investigation, unpaired, 57.4 ± 6.2; investigation, paired, 57.8 ± 6.0; walking, unpaired, 15.4 ± 2.9; walking, paired, 19.1 ± 4.3), the conditioning procedure significantly affected the behavior of the subject during the fear expression test

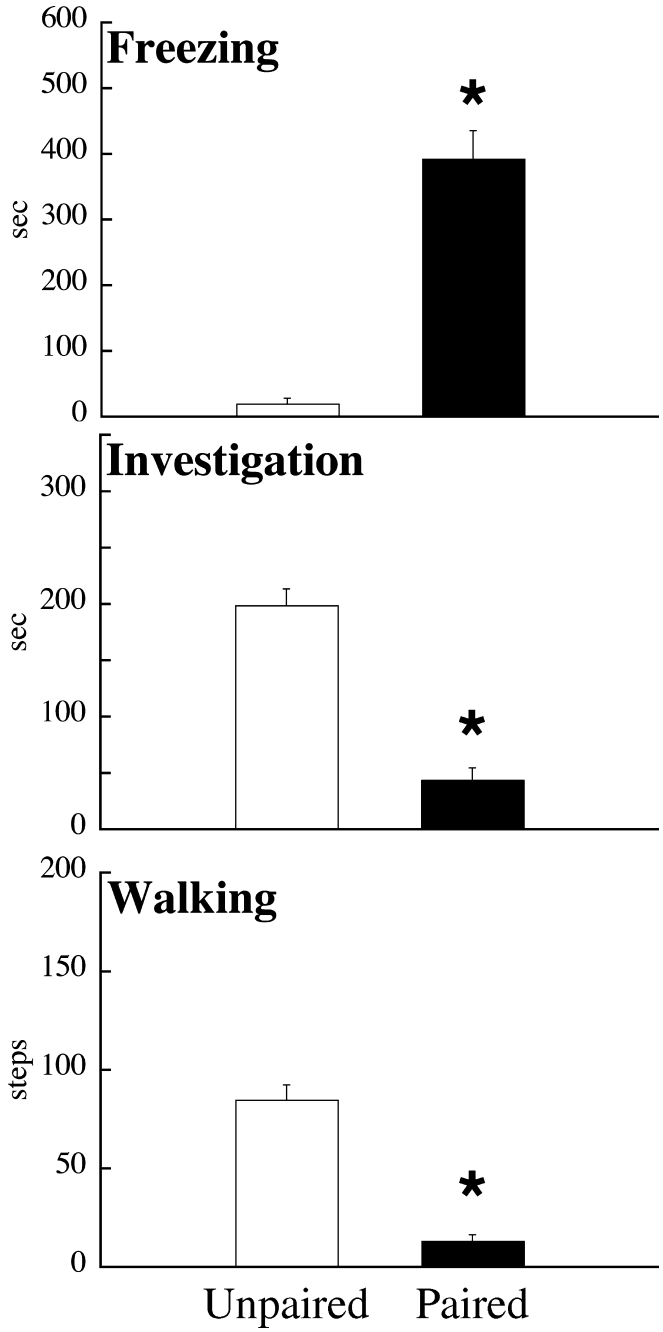

FIG. 4. Duration of freezing, duration of investigation and frequency of walking of fear-conditioned (paired) and non-conditioned (unpaired) rats that underwent a fear expression test with a guinea pig separated by a wire mesh. *$P < 0.05$, with MANOVA (Hotelling's trace) followed by Fisher's protected least significant difference *post hoc* test.

($F_{3,11} = 34.5$, $P < 0.01$). A *post hoc* test revealed that the conditioning procedure significantly increased freezing ($P < 0.01$) and decreased both investigation ($P < 0.01$) and walking ($P < 0.01$) (Fig. 4). Accordingly, the lack of behavioral response to the CS cannot be attributed to distraction caused by the presence of another animal during the fear expression test.

The MOS mediates the social buffering

Results from the initial set of experiments demonstrated that physical contact is not necessary for social buffering, and that the signal mediating social buffering was blocked by a transparent acrylic board, but not by wire mesh. Considering the well-developed olfactory sense in rodents, we hypothesized that the MOS mediates social buffering. To test this hypothesis, the main olfactory epithelium was lesioned with an intranasal injection of $ZnSO_4$. In this experiment, rats received a single intranasal injection of either $ZnSO_4$ (Zn) or saline 2 days before the conditioning day. Then, they were either fear-conditioned (paired) or not fear-conditioned (unpaired) to an auditory CS. Twenty-four hours later, they were re-exposed to the CS in the test apparatus either alone (solitary) or with a rat associate separated by double wire mesh (dyad). We assessed the role of the MOS by comparing the conditioning effects across different situations of the content of the intranasal injection and the presence of an associate during the fear expression test (saline–solitary, unpaired, $n = 7$; saline–solitary, paired, $n = 8$; saline–dyad, unpaired, $n = 8$; saline–dyad, paired, $n = 8$; Zn–solitary, unpaired, $n = 8$; Zn–solitary, paired, $n = 8$; Zn–dyad, unpaired, $n = 8$; Zn–dyad, paired, $n = 8$).

TABLE 3. Behavioral responses during the acclimation period of the fear expression test for assessing the role of the main olfactory system

Situation and conditioning	Freezing	Investigation	Walking
Saline injection			
Solitary			
Unpaired (7)	2.1 ± 2.1	21.7 ± 4.0	36.3 ± 7.3
Paired (8)	1.5 ± 1.0	22.5 ± 5.4	28.2 ± 7.0
Dyad			
Unpaired (8)	0 ± 0	45.9 ± 5.3	57.7 ± 3.4
Paired (8)	0 ± 0	50.7 ± 3.6	57.3 ± 3.8
ZnSO4 injection			
Solitary			
Unpaired (8)	0 ± 0	19.5 ± 2.1	37.6 ± 4.7
Paired (8)	0.1 ± 0.1	17.2 ± 1.4	37.6 ± 5.7
Dyad			
Unpaired (8)	0 ± 0	25.3 ± 2.1	37.3 ± 4.6
Paired (8)	0 ± 0	31.1 ± 2.7	39.0 ± 3.5

Data are expressed as means ± standard error of the mean. The number of animals is provided in parentheses.

The behavior of the subjects during the acclimation period were affected by the intranasal injection ($F_{3,53} = 13.4$, $P < 0.01$) and presence of an associate ($F_{3,53} = 18.1$, $P < 0.01$), but not by the conditioning procedure. Interactions between the two factors or three factors were not significant except for between the intranasal injection and presence of an associate ($F_{3,53} = 3.98$, $P < 0.05$). However, a *post hoc* test revealed that the behavioral responses were not different between the unpaired and paired groups in all situations (Table 3).

The behavioral responses during the fear expression test were affected by the intranasal injection ($F_{3,53} = 36.8$, $P < 0.01$), presence of an associate ($F_{3,53} = 52.7$, $P < 0.01$), and conditioning procedure ($F_{3,53} = 165$, $P < 0.01$). The interactions between the two factors (intranasal injection × conditioning procedure, $F_{3,53} = 17.7$, $P < 0.01$; intranasal injection × presence of an associate, $F_{3,53} = 19.9$, $P < 0.01$; and conditioning procedure × presence of an associate, $F_{3,53} = 28.8$, $P < 0.01$) and all three factors ($F_{3,53} = 9.90$, $P < 0.01$) were also significant. *Post hoc* tests revealed that the conditioning procedure increased freezing ($P < 0.01$) and decreased investigation ($P < 0.01$) and walking ($P < 0.01$) in both the saline–solitary and Zn–solitary situations (Fig. 5A). In contrast, the conditioning procedure increased freezing ($P < 0.01$) and decreased investigation ($P < 0.01$) and walking ($P < 0.01$) in the Zn–dyad situation, whereas the behavioral responses between the unpaired and paired group were not different in the saline–dyad situation.

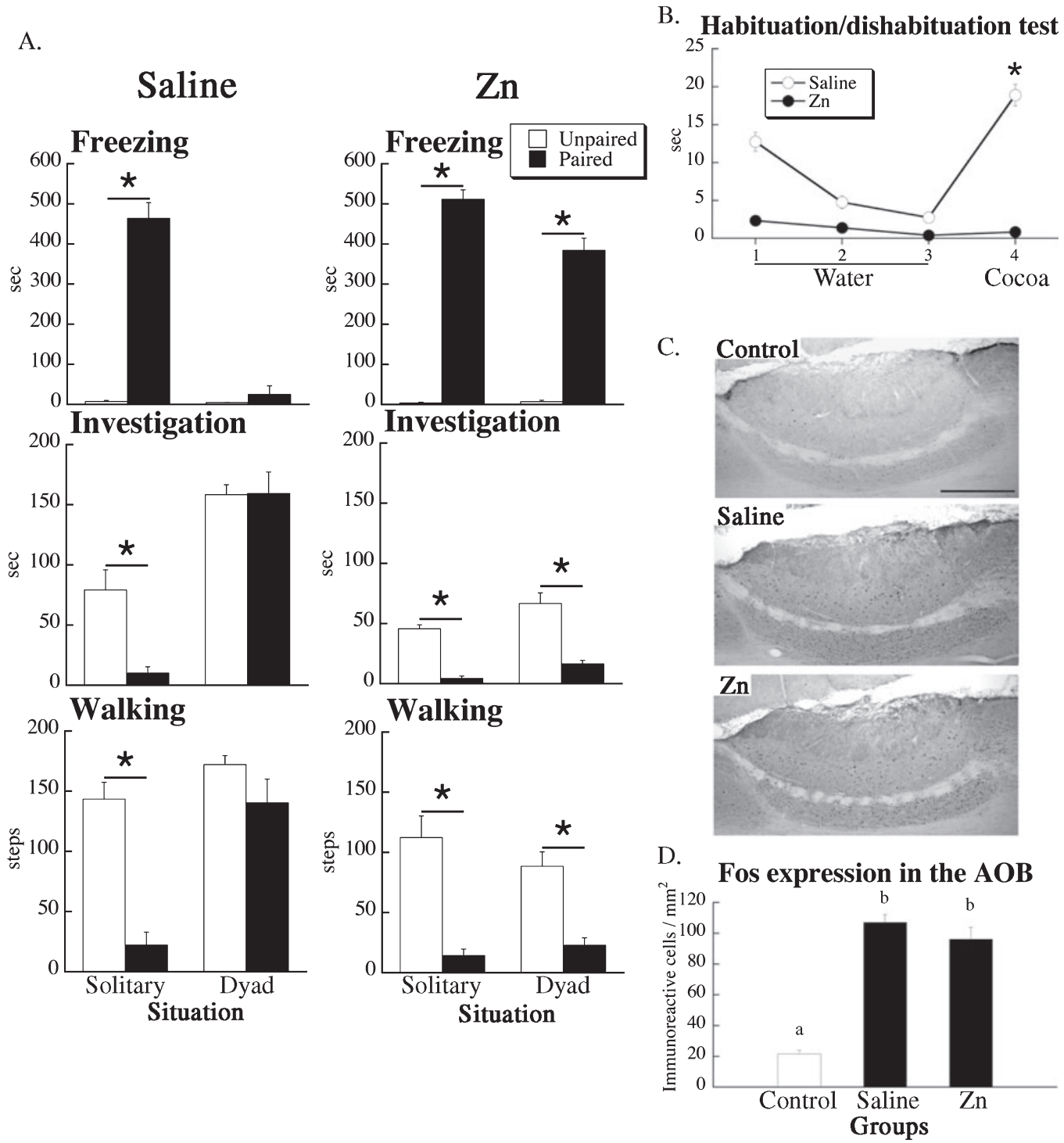

FIG. 5. The role of the main olfactory system in social buffering. (A) Duration of freezing, duration of investigation and frequency of walking [mean ± standard error of the mean (SEM)] during a fear expression test (*$P < 0.05$), (B) the investigation time (mean ± SEM) for cocoa solution presented in the habituation–dishabituation test, and (C) representative photomicrographs and (D) the mean density (mean ± SEM) of Fos-immunoreactive cells in the mitral/tufted cell layer of the accessory olfactory bulb (AOB) after the exposure to the female-soiled cage of fear-conditioned (paired) and non-conditioned (unpaired) rats that received either saline (saline) or $ZnSO_4$ (Zn) by intranasal injection beforehand and underwent the fear expression test either in the solitary condition (solitary) or with an associate rat separated by double wire meshes (dyad). A control group of male rats was examined to determine the baseline level of Fos expression in the AOB (control). Horizontal bar indicates 500 μm. *$P < 0.05$ in (A), with MANOVA (Hotelling's trace) followed by Fisher's protected least significant difference (PLSD) *post hoc* test. (B) Wilcoxon's signed-ranks test comparisons with the third presentation of the previous stimulus. (D) The letters 'a' and 'b' indicate significant differences according to ANOVA followed by Fisher's PLSD test.

The habituation–dishabituation test was performed to confirm the completeness of the MOS lesion. The sniffing behavior of the saline-treated rats was decreased by the consecutive presentations of purified water and increased when the cocoa solution was presented after the third water presentation ($P < 0.01$) (Fig. 5B). In contrast, the sniffing behavior of the $ZnSO_4$-treated subjects did not

increase when the odor stimulus was changed from water to cocoa solution.

Finally, we confirmed the effectiveness of the vomeronasal system after the intranasal injection by determining Fos expression levels in the mitral/tufted cell layer of the AOB (Fig. 5C). These were compared to a control group comprising male rats that were picked up and placed in their home cage. Fos expression in the mitral/tufted cell layer of the AOB was significantly different between the groups ($F_{2,68} = 20.5$, $P < 0.01$), and *post hoc* tests revealed that the levels of Fos expression of the saline-treated and $ZnSO_4$-treated subjects were increased as compared to controls ($P < 0.05$) after the exposure to the female-soiled cage but did not differ from each other (Fig. 5D).

Discussion

When fear-conditioned rats were exposed to the CS alone, they exhibited a stereotypic freezing behavioral response and hypothalamic–pituitary–adrenal axis activation that was indicated by increased Fos expression in the PVN. The presence of a conspecific associate mitigated conditioned fear responses, even if the dyad was separated by a wire mesh or double wire mesh. Therefore, physical contact between the dyad is not necessary for social buffering. In contrast, the lesion of the MOS with intranasal injection of $ZnSO_4$ blocked social buffering of conditioned fear responses, although the vomeronasal system was intact. We conclude from these findings that the MOS mediates social buffering in male rats.

This is the first evidence for the involvement of the MOS in social buffering between male rats. Considering the variety of species-specific signals mediating stress-buffering phenomena in other species, such as visual cues in sheep (da Costa *et al.*, 2004) and auditory signals in marmosets (Rukstalis & French, 2005), recognition of a conspecific animal by species-specific signals appears to be necessary for the stress-buffering phenomenon. We propose that the MOS plays an important role in these signals in rats. Rats may be able to recognize an associate as conspecific through olfactory signals, as they can discriminate individuals by olfactory signals (Hopp *et al.*, 1985). In addition, the importance of the recognition of individuals in the stress-buffering phenomenon is also supported by the findings that stress-buffering effects are limited to a specific animal in 'mother buffering' (stress buffering by mother) (Graves & Hennessy, 2000; Hennessy *et al.*, 2002) and mated-partner buffering (Sachser *et al.*, 1998; Kaiser *et al.*, 2003; Hennessy *et al.*, 2008) paradigms. Therefore, we propose that conditioned fear responses are mitigated in rats following recognition of an associate as a conspecific animal. However, another possibility is that the olfactory signal itself mitigated conditioned fear responses by acting as an 'appeasing pheromone'. This possibility is supported by the observation that an olfactory signal from conspecific animals attenuated the heart rate response to a novel environment in male rats (Kiyokawa *et al.*, 2005a). Further research is required to distinguish between these possibilities.

The results of the present study do not agree with those of a previous study reporting the importance of physical contact between the dyad in social buffering (Wilson, 2001). In this previous study, a 0.64-cm-thick Plexiglas partition containing 126 holes (0.32 cm in diameter, 1.27 cm apart) was used to separate two juvenile rats. Therefore, the partition may have been too thick, with too few small holes, to permit the transmission of the olfactory signals from an associate. In support of this interpretation, the presence of an associate increased investigation time in the present study, whereas the time spent beside the partition was not increased, but was rather decreased, by the presence of another rat in the previous study (Wilson, 2001).

The results of the immunohistochemistry do not support a predominant role for IL in social buffering. The IL is the only region reported to suppress the conditioned freezing in response to an auditory CS in the paradigm of fear extinction (Milad & Quirk, 2002; Quirk *et al.*, 2006). In our previous study, it was unclear whether the IL also contributed to social buffering of freezing, because both the conditioned fear responses and the social-buffering phenomenon were accompanied by the increase in Fos expression in the IL (Kiyokawa *et al.*, 2007b). Therefore, we hypothesized that this increase in Fos reflected the activity of distinct populations of neurons in the IL. To differentiate between increases in Fos expression that were a consequence of the conditioned fear response and those related to social buffering, we applied double immunohistochemistry to this region. However, and somewhat unexpectedly, social buffering was not accompanied by Fos expression in the IL in this study. Therefore, the increase in Fos expression in the IL accompanying social buffering in our previous study may have been a consequence of physical contact with a conspecific. We conclude from both our current and previous work that the IL is not a key site for social buffering, and that freezing behavior in response to a tone CS is suppressed by a different neural mechanism from those involved in the fear extinction paradigm.

In summary, the results of the present study demonstrate that the MOS mediates social buffering of conditioned fear responses in male rats. We propose that future research using this experimental model will reveal the neural circuit of social buffering and will also shed light on the neural mechanism of positive emotion in animals.

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Abbreviations

AOB, accessory olfactory bulb; CS, conditioned stimulus; GAD, glutamate decarboxylase; IL, infralimbic region of the prefrontal cortex; MOS, main olfactory system; PLSD, protected least significant difference; PVN, paraventricular nucleus.

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