Olfactory signals mediate social buffering of conditioned fear responses in male rats

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Abstract

In social animals, the presence of an affiliative conspecific alleviates acute stress responses, and this is called social buffering. We previously reported that social buffering mitigates the fear responses of male rats to auditory conditioned stimuli that had been paired with foot shocks. Subsequent studies revealed that signals that are perceived by the main olfactory epithelium are important for social buffering. Because olfactory signals are the signal perceived by the main olfactory epithelium, we hypothesized that we could induce the social buffering of conditioned fear responses by presenting olfactory signals that were derived from a conspecific. In order to test this hypothesis, we exposed fear-conditioned subjects to a conditioned stimulus either in a clean test box or in a test box that was odorized by keeping a conspecific in it as an odor donor beforehand. When the subjects were tested in the clean test box, they showed behavioral fear responses and enhanced Fos expression in the paraventricular nucleus. In contrast, the presence of conspecific olfactory signals blocked these fear responses and Fos expression. These results suggested that olfactory signals suppress conditioned fear responses. Fos expression in the posteromedial region of the olfactory peduncle and

amygdala suggested that this suppression involves the same neural mechanisms as those of social buffering. Taken together, we concluded that olfactory signals mediate the social buffering of conditioned fear responses.

Key words: Social buffering, Fear conditioning, Appeasing pheromone, Posterior complex of the anterior olfactory nucleus, Amygdala

Abbreviations: AOP, posterior complex of the anterior olfactory nucleus; BA, basal amygdala; BNST, bed nucleus of the stria terminalis; BNSTl, anterior division lateral group of the bed nucleus of the stria terminalis; BNSTm, anterior division medial group of the bed nucleus of the stria terminalis; CeA, central amygdala; CS, conditioned stimulus; LA, lateral amygdala; MOB, main olfactory bulb; mOC, medial olfactory cortex; MOE, main olfactory epithelium; pmOP, posteromedial region of the olfactory peduncle; PVN, paraventricular nucleus; TMT, 2,4,5-trimethyl-3-thiazoline; Tu, olfactory tubercle.

1. Introduction

In social mammals, signals from conspecific animals influence stress responses. For example, autonomic [1] and behavioral [2] responses to a novel environment or startle responses to a loud noise [3] are aggravated in rats by olfactory signals that are released from stressed conspecifics. These findings indicate the existence of alarming olfactory signals that have also been reported for several other species [4-7]. In contrast, signals from conspecifics can mitigate stress responses because the presence of an affiliative conspecific animal has been shown to alleviate stress responses in a variety of social species [8-13]. These phenomena are part of what is called social buffering [14]. However, in contrast to the identification of the responsible visual signals in the social buffering of behavioral, autonomic, and endocrine responses to a novel environment in sheep [8], the responsible signal in other experimental models remains unclear.

We previously reported that the presence of a conspecific animal blocked the fear responses of a male rat to a contextual and auditory conditioned stimulus (CS) that had been paired with foot shocks as well as the associated Fos expression in the

paraventricular nucleus (PVN) of the hypothalamus [15,16]. This social buffering of conditioned fear responses occurred even when the dyad was separated by 2 wire-mesh screens that were placed 5 cm apart [17]. In addition to the separation, lesions of the main olfactory epithelium (MOE) of the subject also blocked social buffering [17], suggesting that signals perceived at the MOE are important for social buffering. Because olfactory signals are the signals that are perceived at the MOE, we hypothesized that we could induce the social buffering of conditioned fear responses by presenting olfactory signals that were derived from a conspecific.

In addition, we analyzed the neural mechanisms that underlie the social buffering of conditioned fear responses. After being perceived at the MOE, the signals responsible for social buffering are transmitted to the main olfactory bulb (MOB) because anatomical evidence suggests that all of the signals that are detected at the MOE are sent to the MOB [18]. Subsequently, the signals that are responsible for social buffering are transmitted to the lateral amygdala (LA) and/or central amygdala (CeA), which play pivotal roles in the behavioral and endocrine stress responses to auditory CS [19-21], and suppress their activations [16]. The posteromedial region of the olfactory

peduncle (pmOP), which comprises the posterior complex of the anterior olfactory nucleus (AOP), olfactory tubercle (Tu), and medial olfactory cortex (mOC) (Fig. 1), appears to be a relay point in the transmission from the MOB to the amygdala because the pmOP is anatomically connected to the MOB and amygdala and because lesions of the pmOP or disconnections between the pmOP and amygdala block social buffering [22]. Therefore, if the presentation of olfactory signals induces the social buffering of conditioned fear responses, it should activate the pmOP and suppress the activation of the amygdala to the CS.

In order to address these issues, we prepared a clean test box and an identical test box that was odorized by keeping a male rat that was an odor donor in it for the preceding 3 h. Then, the fear-conditioned subjects were exposed to the CS while they were in 1 of the 2 boxes. The effectiveness of the olfactory signals for social buffering was assessed by observing the behavioral responses and Fos expression in the PVN. We further observed the Fos expression in the pmOP and amygdala, as well as in the bed nucleus of the stria terminalis (BNST), in order to assess the underlying neural mechanisms.

2. Material and methods

2.1 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 male Wistar rats (aged 8 weeks) were purchased from Charles River Laboratories Japan (Kanagawa, Japan). They were housed 3 animals per cage in an ambient temperature of 24 ± 1°C and a humidity of 45 ± 5% in a controlled colony room with food and water available *ad libitum*. The animals were maintained under a 12-h light/12-h dark cycle (lights switched on at 08:00). Each rat was assigned to either the subject or the odor-donor group, and cage mates were assigned to the same group in order to maintain unfamiliarity between the subject and the donor rats. All rats were housed separately and were handled for 5 min per day from 3 days before the conditioning day.

2.2 Fear conditioning

Fear conditioning was performed in an illuminated room between 09:00 and 17:00, as described in our previous studies [16,17,23]. A subject in the paired group was placed in an acrylic conditioning box (28 × 20 × 27 cm) for 20 min, and it received 7 repetitions of a 3-s tone (8 kHz, 80 dB) that terminated concurrently with a foot shock (0.5 s, 0.65 mA). We prepared the unpaired group by presenting the CS and foot shock separately during a 20-min period. The intertrial interval randomly varied between 30 and 180 s. Each rat was returned to its home cage after the fear conditioning.

2.3 Fear-expression test

A fear-expression test was performed 24 h after the fear conditioning in a dark room that was illuminated with dim red light [16,17]. Preceding the fear-expression test, an odor-donor rat was placed and kept in an acrylic test box (24 × 44 × 18 cm) with clean bedding for 3 h in a sound-proof chamber (42 × 57 × 41 cm) in order to odorize the test box. We prepared the control box by keeping a test box with clean bedding in a

sound-proof chamber without placing a donor rat in it. Donor rats were acclimatized to the test box for 1 h on the conditioning day and on a preceding day and were used only once for the odorization.

After the odorization, the donor rat was removed, and the test box was placed on an experimental table. The subjects were then placed either in the odorized box (odor situation, n = 9 in the paired and unpaired group) or control box (control situation, n = 9 in the paired and unpaired group). After a 2-min acclimation period, a CS was presented 5 times for 3 s each at 1-min intervals during the first half of the 10-min experimental period. We recorded the behavior of the subjects during the acclimation and experimental periods with a video camera (DCR-TRV18; Sony, Tokyo, Japan) and an HD-DVD recorder (DVR-77H; Pioneer, Kanagawa, Japan). After the fear-expression test, the subject was returned to its home cage and kept in a colony room.

2.4 Immunohistochemistry for c-Fos

Each subject was deeply anesthetized with sodium pentobarbital (Somnopentyl, Schering-Plough Animal Health, Harefield, UK) and perfused

intracardially with 0.9% saline, which was 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. The avidin-biotin-peroxidase method was used for detection of the immunohistochemistry, as previously described [16,17]. Briefly, 6 successive 30-μm sections were collected, and the first and fifth sections were stained with cresyl violet in order to confirm the location of the nucleus, while the remaining sections were used for free-floating immunohistochemistry. The sections were incubated with primary antibody to Fos protein (PC38, Merck, Darmstadt, Germany) for 65 h and anti-rabbit secondary antibody (PK-6101, VECTASTAIN ABC kit, Vector Laboratories, Burlingame, CA) for 2 h, and the sections were then processed with the ABC kit and developed using a diaminobenzidine solution with nickel intensification.

2.5 Data analyses and statistical procedures

The data are expressed as means ± standard error of means, and significance was set at $P < 0.05$ for all statistical tests. A researcher who was blind to the experimental conditions recorded the duration of the behaviors of freezing (immobile posture, with cessation of skeletal and vibrissae movement except in respiration) and sniffing (regular movement of vibrissae with exploring) and the frequency of walking (number of steps taken with the hind paws) by the subjects with Microsoft Excel-based Visual Basic software that records the duration and number of pressing keys, as was done in our previous studies [16,17,23]. The behavioral data during the initial acclimation and experimental period of the subjects were analyzed by two-way MANOVA, which was followed by Fisher's PLSD post hoc test.

We analyzed the expression of Fos, as described in our previous studies [16,24], in the AOP, Tu, mOC, BNST, including the anterior division medial group (BNSTm) and anterior division lateral group (BNSTl), PVN, LA, CeA, and basal amygdala (BA), as shown in Figure 1. The regions of interest were confirmed by the adjacent sections that were stained with Cresyl Violet and were evaluated according to a brain atlas [25]. We were not able to obtain sections containing the pmOP of 1 animal in

the unpaired group in the control situation due to technical problems. Four sections of each region were captured using a microscope equipped with a digital camera (DP30BW, Olympus, Tokyo, Japan). The numbers of Fos-immunoreactive cells in a 0.5-mm square was counted unilaterally with ImageJ 1.41 software by an experimenter who was blind to the experimental groups. When the designated area was smaller than the boundaries of a 0.5-mm square, only the cells in the region of interest were counted. The mean numbers of cells in the 4 sections in each region were analyzed by two-way ANOVA, which was followed by Fisher's PLSD post hoc test.

3. Results

3.1 Conditioned fear response to the CS

The behavioral responses during the acclimation period were significantly affected by the test situation [$F_{(3,30)}$=3.39; P<0.05]. However, the effects of the conditioning procedure and the interaction between the 2 factors were not significant. A post hoc test revealed that the behavioral responses were not different between the paired and unpaired group in the same situation (Table 1).

The behavioral responses during the experimental period were significantly affected by the test situation [$F_{(3,30)}$=5.88; P<0.01] and the conditioning procedure [$F_{(3,30)}$=6.62; P<0.01]. The interaction between the 2 factors was also significant [$F_{(3,30)}$=5.52; P<0.01]. A post hoc test revealed that the paired group showed an increased duration of freezing (P<0.01), a decreased duration of sniffing (P<0.01), and a decreased frequency of walking (P<0.01) compared to the unpaired group in the control situation (Fig. 2A). However, when the fear-expression test was conducted in the odor situation, no behavioral responses were different between the paired and unpaired group (Fig. 2A).

In the PVN, the test situation [$F_{(1,32)}$=17.1; P<0.01] and the conditioning procedure [$F_{(1,32)}$=11.4; P<0.01] significantly affected Fos expression. The interaction between the 2 factors was also significant [$F_{(1,32)}$=16.3; P<0.01]. A post hoc test revealed that the paired group showed increased Fos expression compared to the unpaired group (P<0.01) in the control situation, whereas Fos expression did not differ between the paired and unpaired group in the odor situation (Fig. 2B).

3.2 Fos expression in the pmOP, amygdala, and BNST

In the pmOP, we examined the levels of Fos expression in the AOP, Tu, and mOC in order to determine the area that was responsible for social buffering (Fig. 3A). The test situation significantly affected the levels of Fos expression in the AOP [$F_{(1,31)}$=8.10; P<0.01] and Tu [$F_{(1,31)}$=11.9; P<0.01]. In addition, the conditioning procedure affected the levels of Fos expression in the AOP [$F_{(1,31)}$=8.04; P<0.01]. The interaction between the 2 factors was not significant in all areas. A post hoc test revealed that the paired group in the odor situation showed increased levels of Fos expression in the AOP (P<0.01) compared to the other 3 groups (Fig. 3B, top). In the Tu, both the paired and unpaired group in the odor situation showed increased levels of Fos expression compared to both the paired and unpaired group in the control situation (P<0.05) (Fig. 3B, middle). In contrast, the levels of Fos expression in the mOC did not differ among the groups (Fig. 3B, bottom).

In the amygdala, the test situation did not affect the levels of Fos expression. In contrast, the conditioning procedure affected the levels of Fos expression in the LA [$F_{(1,32)}$=8.24; P<0.01], CeA [$F_{(1,32)}$=7.34; P<0.05], and BA [$F_{(1,32)}$=10.8, P<0.01]. In

addition, the interaction between the 2 factors was significant in the LA [$F_{(1,32)}$=9.10, P<0.01] and BA [$F_{(1,32)}$=4.88; P<0.05]. A post hoc test revealed that the paired group showed increased levels of Fos expression in the LA (P<0.01), CeA (P<0.05), and BA (P<0.01) in the control situation compared to the unpaired group, whereas the levels of Fos expression did not differ between the paired and unpaired group in the odor situation (Fig. 3C).

In the BNST, the effects of the test situation, the conditioning procedure, and the interaction between the 2 factors were not significant for the levels of Fos expression in the BNSTm and BNSTl (Table 2).

4. Discussion

When fear-conditioned subjects were exposed to the CS in a clean test box, they showed fear responses, including increased freezing and decreased sniffing and walking, as well as increased levels of Fos expression in the PVN. Olfactory signals from a conspecific suppressed these behavioral and neural responses, as was seen in our previous studies [16,17]. These results suggested that olfactory signals suppress

conditioned fear responses. We further observed Fos expression in the pmOP and amygdala. In the pmOP, the levels of Fos expression were increased in the AOP when the suppression of the conditioned fear response was observed, suggesting that the pmOP, and especially the AOP, was activated during the suppression of the conditioned fear responses. In the amygdala, the suppression of the conditioned fear responses was accompanied by the blockade of Fos expression in the LA, the CeA, and the BA, suggesting that the suppression was achieved by the blockade of the activation of the amygdala. These results suggested that olfactory signals suppressed the conditioned fear responses through the same neural mechanisms that underlie social buffering. Taken together, we concluded that olfactory signals mediate the social buffering of conditioned fear responses.

Since, we did not include an additional control group that was presented with an odor that did not evoke fear, we cannot rule out the possibility that the presence of any odor can suppress the conditioned fear responses. However, on the basis of our previous finding that the presence of a heterospecific, a male guinea pig, did not induce social buffering in male rats [17], we think that this possibility is less likely. Therefore,

we believe that social buffering is specifically induced by olfactory signals derived from conspecifics.

According to the criteria, olfactory signals that mediate social buffering can be classified as appeasing pheromones. Pheromones were first proposed by Karlson and Luscher [26] as “substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behaviour or a developmental process.” In the present study, we demonstrated that olfactory signals from a conspecific mitigated behavioral and neural fear responses. In addition, such olfactory signals have also been reported to mitigate tachycardia in response to a novel environment [27]. Based on these stress-buffering effects, olfactory signals that are released from a conspecific and that alleviate stress responses can be classified as appeasing pheromones. Therefore, these findings suggested that social buffering is a phenomenon in which appeasing pheromones mitigate stress responses. However, it remains unclear whether the present olfactory signals fulfill the second criteria that “The principle of minute amounts being effective holds [26].” In addition, in the present study, we placed the donor and the

subject in the same test box. Therefore, we cannot rule out the involvement of low volatile compounds in the odorized box, which is in contrast to previous studies in which only volatile compounds were transmitted from a donor because the donor and subject were separated by 2 wire-mesh screens [17]. Future studies are needed to clarify these points.

Olfactory signals derived from the mammary or facial areas are commercially used to reduce stress responses in several species [28-31]; this further supports our notion that olfactory signals can suppress stress responses in animals. These signals fulfill the first criteria of being classified as pheromones; however, whether these olfactory signals fit the second criteria for pheromones has not been investigated yet. Further, the neural mechanisms underlying these stress-buffering effects have not been investigated. Such investigations would increase our understanding of how olfactory signals control stress in animals.

Another important finding in this study is that the levels of Fos expression in the AOP were increased only when social buffering was observed within the pmOP. Although we have identified the pmOP as a relay point in social buffering based on

lesion studies, we could not specify the responsible area within the pmOP. Therefore, we examined Fos expression in the AOP, Tu, and mOC within the pmOP in order to clarify this point. Among these 3 areas, the AOP was the only area that showed increased levels of Fos expression during social buffering. In contrast, the levels of Fos expression in the Tu were increased when the subjects were tested in the odorized test box, suggesting that the Tu was activated in response to general olfactory signals. The levels of Fos expression in the mOC were not affected by any of the conditions. These results suggested that the AOP is the area within the pmOP that is responsible for social buffering. Because it has not been studied much previously, almost no information is available regarding the AOP, unlike the well-studied anterior portions of the anterior olfactory nucleus [32-34]. In this study, olfactory signals alone did not increase the levels of Fos expression in the AOP, even if olfactory signals appear to be transmitted to the pmOP [22]. Therefore, it is possible that the activated amygdala in the fear-conditioned subject affected the response of the AOP to the olfactory signals from a donor. A previous study showing that rats increased the levels of Fos expression in the AOP after exposure to cat odors, but not to fox-derived 2,4,5-trimethyl-3-thiazoline

(TMT) or formaldehyde [35], supports the close relationships between the AOP and the amygdala. Although both cat odors and TMT induce freezing in rats, freezing in response to cat odors is amygdala dependent [36,37], whereas the BNST plays a more important role in the freezing response to TMT [38-40]. It is possible that formaldehyde does not induce freezing because a similar repugnant odor, butyric acid, also failed to induce freezing in rats [40-42]. Therefore, the AOP appears to be activated only when olfactory signals are related to the activity of the amygdala. However, further research is necessary in order to clarify why the activated AOP suppressed the activation of the amygdala during social buffering but not in response to cat odors.

In the present study, the CS increased the levels of Fos expression in the amygdala but not in the BNST. These results were consistent with the prevailing notion that the BNST is not involved in the freezing to an auditory CS, whereas it participates in the freezing to a contextual CS or in the potentiation of acoustic startle responses by the long duration of an auditory CS [20,43,44]. In addition, neither the presentation of olfactory signals nor social buffering increased the levels of Fos expression in the BNST. Therefore, the BNST is less likely to be involved in our experimental model.

5. Conclusion

In conclusion, we showed that olfactory signals from a donor suppressed conditioned fear responses in male rats. In addition, the levels of Fos expression in the pmOP and amygdala suggested that the suppression of conditioned fear responses has the same neural mechanisms as those that underlie social buffering. Fos expression in the pmOP also suggested that the AOP is the responsible area within the pmOP. Taken together, we concluded that olfactory signals mediate the social buffering of conditioned fear responses in male rats. Additional research that builds upon these results will enable us to further our understanding of how olfactory signals control amygdala activities.

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Table 1. Behavioral responses during the acclimation period of subjects

Situation	Control		Odor	
Group	Unpaired	Paired	Unpaired	Paired
Freezing	6.8 ± 3.3	10.1 ± 4.9	0 ± 0	0 ± 0
Sniffing	93.3 ± 6.0	90.3 ± 5.3	102 ± 3	105 ± 4
Walking	51.9 ± 4.9	44.6 ± 5.2	58.7 ± 3.3	57.8 ± 7.0

Data are expressed as mean ± SEM.

Nine animals in each group.

Table 2. Number of Fos-immunoreactive cells/0.5-mm square in the BNST

Situation	Control		Odor	
Group	Unpaired	Paired	Unpaired	Paired
Anterior division medial group of the bed nucleus of the stria terminalis (BNSTm)	3.8 ± 0.5	5.4 ± 0.7	5.1 ± 0.8	5.9 ± 0.9
Anterior division lateral group of the bed nucleus of the stria terminalis (BNSTl)	7.0 ± 0.9	7.7 ± 1.3	7.5 ± 0.7	8.8 ± 1.4

Data are expressed as mean ± SEM.

Nine animals in each group.

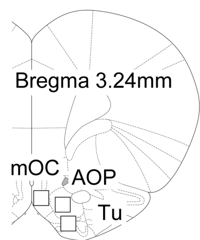
Figure legends

Fig. 1. Schematic diagrams showing the location of the brain regions (open square) in which Fos-immunoreactive cells were counted. Abbreviations: AOP: posterior complex of the anterior olfactory nucleus; BA: basal amygdala; BNSTl: anterior division lateral group of the bed nucleus of the stria terminalis; BNSTm: anterior division medial group of the bed nucleus of the stria terminalis; CeA: central amygdala; LA: Lateral amygdala; mOC: medial olfactory cortex; PVN: paraventricular nucleus, Tu: olfactory tubercle.

Fig. 2. Conditioned fear responses to the auditory conditioned stimulus (CS) of the subjects. Duration of freezing and sniffing and frequency of walking (mean + SEM) (panel A) and the mean numbers of Fos-immunoreactive cells (mean + SEM) in the paraventricular nucleus (PVN) (panel B) of the fear-conditioned (paired group) or nonconditioned (unpaired group) subjects that underwent the fear-expression test either in an test box odorized by a donor rat (odor situation) or in a clean test box (control

situation). The letters indicate the significant differences ($P<0.05$) according to ANOVA followed by Fisher's PLSD post hoc test.

Fig. 3. The expression of Fos in the posteromedial region of the olfactory peduncle (pmOP) and amygdala. Representative photomicrographs of Fos-immunoreactive cells in the pmOP (panel A), Fos expression in the posterior complex of the anterior olfactory nucleus (AOP), olfactory tubercle (Tu), and medial olfactory cortex (mOC) within the pmOP (panel B), and in the lateral amygdala (LA), central amygdala (CeA), and basal amygdala (BA) within the amygdala (panel C) of the fear-conditioned (paired group) and nonconditioned (unpaired group) subjects that underwent the fear-expression test either in a test box that was odorized by a donor rat (odor situation) or in a clean test box (control situation). The horizontal bar indicates 500 μm. The letters indicate significant differences ($P<0.05$) with ANOVA followed by Fisher's PLSD post hoc test.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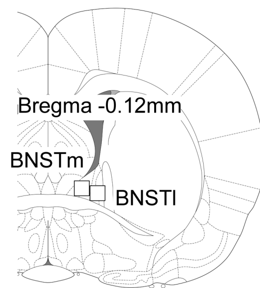
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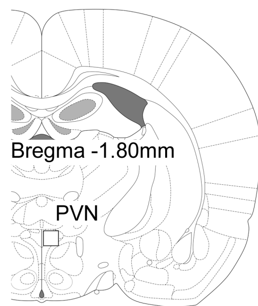
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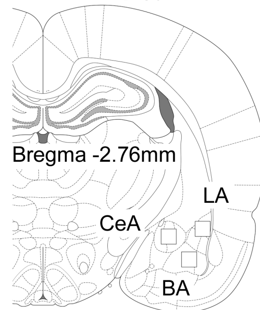
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CeA
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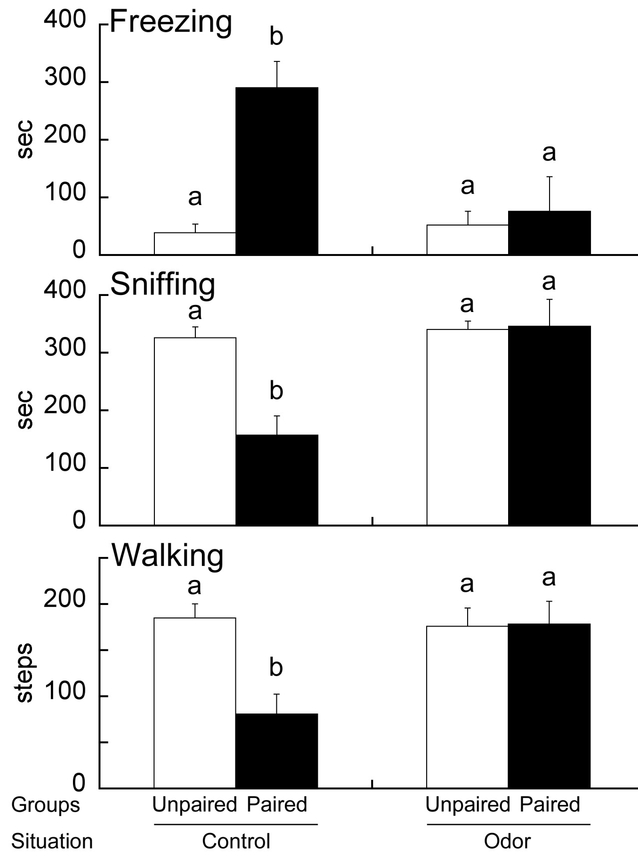
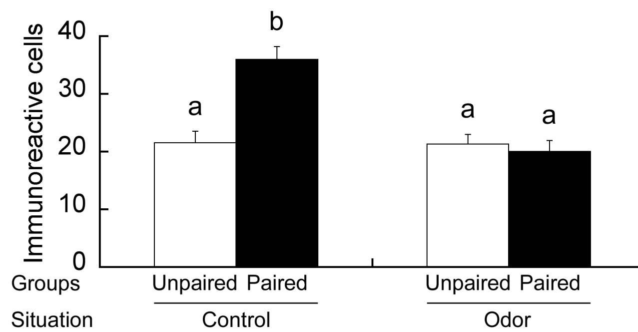
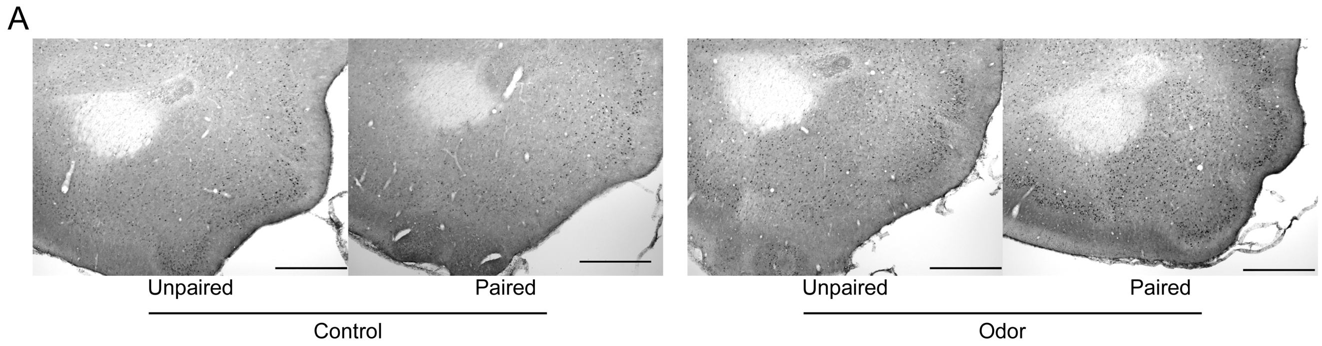
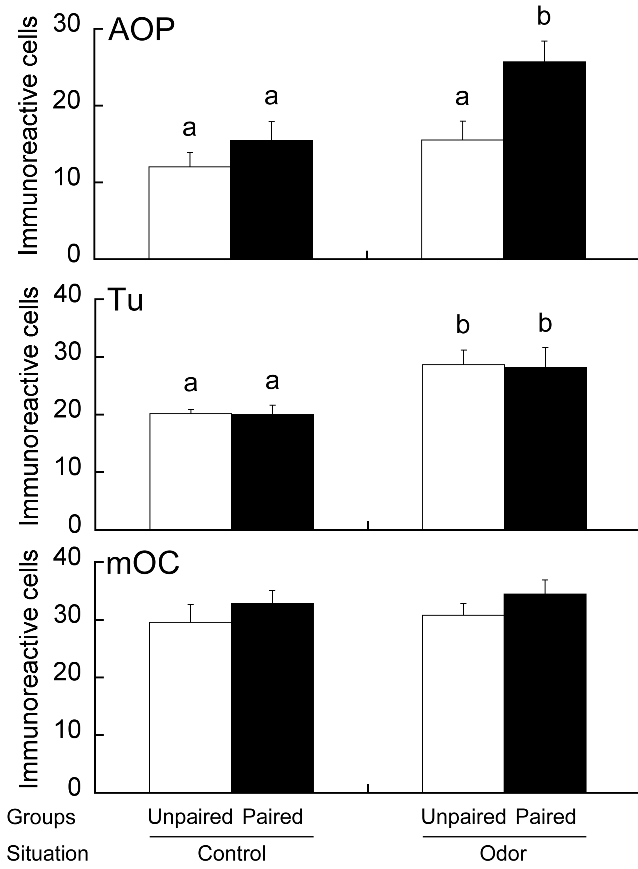

A
Behavioral responses to the CS
Freezing
sec
400
300
200
100
0
a
b
a
a
Sniffing
sec
400
300
200
100
0
a
b
a
a
Walking
steps
200
100
0
a
b
a
a
Groups
Unpaired Paired
Unpaired Paired
Situation
Control
Odor


B
Fos expression in the PVN
Immunoreactive cells
40
30
20
10
0
a
b
a
a
Groups
Unpaired Paired
Unpaired Paired
Situation
Control
Odor

A



B



C