Naloxone blocks social buffering of conditioned fear responses in rats

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Abstract

Social buffering is the phenomenon in which the presence of an affiliative conspecific mitigates stress responses. We previously demonstrated that social buffering completely ameliorates conditioned fear responses in rats. The present study explored the neurochemical background of this social buffering. In Experiment 1, fear-conditioned subjects first received an intraperitoneal injection of either naloxone (non-selective opioid receptor antagonist), haloperidol (dopamine D2 receptor antagonist), SR49059 (vasopressin V1A receptor antagonist), atosiban (oxytocin receptor antagonist), or saline. The subjects were then exposed to a conditioned stimulus with an unfamiliar non-conditioned rat. Naloxone, but not the other three antagonists, blocked social buffering. In Experiment 2, we assessed the effect of naloxone on locomotor activity during an open-field test. Naloxone did not affect walking steps during the test. Therefore, it is unlikely that the results of Experiment 1 were due to decreased activity by naloxone. In Experiment 3, we assessed Fos expression in 16 brain regions accompanied by the blockade of social buffering by naloxone. Consistent with the results of Experiment 1, Fos expression was increased in the paraventricular nucleus of the hypothalamus. In addition, Fos expression was decreased in the nucleus accumbens shell, anterior cingulate cortex, and insular cortex and tended to be decreased in the nucleus accumbens core. Naloxone thus appears to affect these four regions and/or act upstream of these regions during blockade of social buffering. Based on these results, we conclude that naloxone blocks social buffering of conditioned fear responses in rats.

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Running title: Naloxone blocks social buffering

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Conflicts of interest
The authors declare that they have no conflict of interest.

**Keywords:** Hypothalamic-pituitary-adrenal axis, Freezing, Social buffering, Opioid, C-Fos mapping, Neurochemical background

**CRediT authorship contribution statement**

Takumi Yamasaki: Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. Yasushi Kiyokawa: Conceptualization, Methodology, Validation, Formal analysis, Resources, Data curation, Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition. Arisa Munetomo: Writing – Review & Editing, Funding acquisition. Yukari Takeuchi: Writing – Review & Editing, Funding acquisition.

**Abstract**

Social buffering is the phenomenon in which the presence of an affiliative conspecific mitigates stress responses. We previously demonstrated that social buffering completely ameliorates conditioned fear responses in rats. The present study explored the neurochemical background of this social buffering. In Experiment 1, fear-conditioned subjects first received an intraperitoneal injection of either naloxone (non-selective opioid receptor antagonist), haloperidol (dopamine D2 receptor antagonist), SR49059 (vasopressin V1A receptor antagonist), atosiban (oxytocin receptor antagonist), or saline. The subjects were then exposed to a conditioned stimulus with an unfamiliar non-conditioned rat. Naloxone, but not the other three antagonists, blocked social buffering. In Experiment 2, we assessed the effect of naloxone on locomotor activity during an open-field test. Naloxone did not affect walking steps during the test. Therefore, it is unlikely that the results of Experiment 1 were due to decreased activity by naloxone. In Experiment 3, we assessed Fos expression in 16 brain regions accompanied by the blockade of social buffering by naloxone. Consistent with the results of Experiment 1, Fos expression was increased in the paraventricular nucleus of the hypothalamus. In addition, Fos expression was decreased in the nucleus accumbens shell, anterior cingulate cortex, and insular cortex and tended to be decreased in the nucleus accumbens core. Naloxone thus appears to affect these four regions and/or act upstream of these regions during blockade of social buffering. Based on these results, we conclude that naloxone blocks social buffering of conditioned fear responses in rats.

1. **Introduction**

The presence of an affiliative conspecific (or cues associated with a conspecific) reduces stress responses to a wide variety of stimuli (Davitz & Mason, 1955; Lyons et al., 1988; da Costa et al., 2004; Bowen et al., 2013; Kanitz et al., 2014). This phenomenon is known as “social buffering” (Kiyokawa, 2017; 2018; Kiyokawa & Hennessy, 2018). Numerous studies in a variety of species have demonstrated that, in addition to social buffering by the mother or mate (Hennessy et al., 2009; Smith & Wang, 2014), social buffering can be elicited by other conspecifics (Lyons et al., 1993; Winslow et al., 2003; Kiyokawa et al., 2004; Hennessy et al., 2008). Given that these three classes of conspecifics induce social buffering via distinct neural mechanisms, specification of the type of conspecific is important when discussing the neural mechanisms underlying social buffering (Kiyokawa & Hennessy, 2018).

We demonstrated in rats that social buffering induced by a conspecific other than the mother or mate can ameliorate stress responses to an aversively conditioned stimulus (CS). When a fear-conditioned rat was exposed to the CS alone, conditioned fear responses, including increased freezing and hypothalamic-pituitary-adrenal axis activation, were observed. However, the presence of a non-conditioned unfamiliar same-sex rat (associate) completely blocked these responses (Kiyokawa et al., 2004; Kiyokawa et al., 2007; Kiyokawa & Takeuchi, 2017). Subsequent studies revealed a number of characteristics (Kiyokawa et al., 2004; Ishii et al., 2016; Mikami et al., 2016; Kiyokawa et al., 2018; Kiyokawa et al., 2019; Mikami et al., 2020) and a possible neural pathway underlying social buffering of conditioned fear responses (Kiyokawa et al., 2009; Kiyokawa et al., 2012; Takahashi et al., 2013; Kiyokawa et al., 2014; Fuzzo et al., 2015; Minami et al., 2019). However, little information is available regarding the neurochemical background of social buffering.
We propose opioids, dopamine, oxytocin, and vasopressin as candidates responsible for social buffering because they play an important role in affiliative behavior toward conspecifics other than the mother or mate. For example, play behavior between same-sex adolescent rats is reduced by treatment with an opioid receptor antagonist (Beatty & Costello, 1982). Another study using social play as an unconditioned stimulus demonstrated that opioid receptor antagonists block the establishment of conditioned place preference (Trezza et al., 2011). Dopamine is an additional candidate because treatment with a dopamine receptor antagonist was shown to reduce intrinsic play behavior between same-sex adolescent rats (Beatty et al., 1984; Niesink & Van Ree, 1989) and optogenetically induced social investigation between female mice (Gunaydin et al., 2014). In addition to opioids and dopamine, oxytocin and vasopressin should be included among the list of candidates based on their established roles in the formation of attachment to one’s children and mates. For example, after giving birth, ewes exhibit rejection behavior toward alien lambs. However, oxytocin infusion reverses such rejection behavior (Kendrick et al., 1992). Similarly, in monogamous prairie voles, males and females show a preference for their mates over unfamiliar opposite-sex conspecifics. However, this partner preference is blocked by treatment with vasopressin antagonists in males (Winslow et al., 1993) and treatment with oxytocin antagonists in females (Insel & Hulihan, 1995). Consistent with these findings, an oxytocin antagonist was found to block social buffering induced by bonded males in female prairie voles (Adam, 2014). In addition, some studies have suggested possible roles for oxytocin and vasopressin in affiliative behavior toward same-sex conspecifics. For example, treatment with an oxytocin antagonist was shown to reduce intermale social investigation both in rats and mice (Lukas et al., 2011). A similar effect was observed in vasopressin gene–knock-down male mice (Rigney et al., 2022). Based on these findings, we hypothesized that antagonists of one or more of these neurochemicals would block social buffering of conditioned fear responses in rats.

To elucidate the neurochemical background of social buffering, it is important to identify the sites at which responsible neurochemicals act. The presence of opioids (Stein, 2016) or dopamine (Einhorn et al., 1991) decreases the intrinsic excitability of neurons, whereas the presence of oxytocin (Rogers-Carter et al., 2018) or vasopressin (Liu et al., 2003) increases the intrinsic excitability of neurons. Therefore, during antagonist-mediated blockade of social buffering, the antagonist would modulate neural activity at the site of action within the neural pathway related to social buffering and/or affiliative behavior. One methodological approach for identifying the site of modulation would be to observe the changes in Fos expression throughout the brain. We expect that Fos expression would be altered at the sites at which responsible neurochemicals act.

In this study, a series of experiments were performed to explore the neurochemical background of social buffering in rats. In Experiment 1, fear-conditioned subject rats were first injected intraperitoneally with either naloxone (a non-selective opioid receptor antagonist), haloperidol (a dopamine D2 receptor antagonist), atosiban (a oxytocin receptor antagonist), SR49059 (a vasopressin V1a receptor antagonist), or saline. The subjects were then exposed to the CS with an associate. The effect of each antagonist was evaluated by observing the behavioral responses. Experiment 2 examined the effect of naloxone on locomotor activity during an open-field test. In Experiment 3, we analyzed Fos expression in 16 brain regions of the second cohort of subjects during naloxone-mediated blockade of social buffering.

2. Materials and methods

All experiments were approved by the Institutional Animal Care and Use Committee, Graduate School of Agricultural and Life Sciences, University of Tokyo, based on guidelines adapted from the Consensus Recommendations on Effective Institutional Animal Care and Use Committees by the Scientists Center for Animal Welfare.

2.1. Animals

Experimentally naïve male Wistar rats (aged 7-8 weeks) were purchased from Charles River Laboratories Japan (Kanagawa, Japan). Upon arrival, the rats were housed at two to three per cage in a temperature (24 ± 1°C)- and humidity (45 ± 10%)-controlled room on a 12-h light-dark cycle (lights on from 8:00-20:00).
Food and water were available ad libitum. In order to maintain unfamiliarity between the subjects and associates in Experiments 1 and 3, all rats in the same cage were assigned as either a subject or an associate (rats accompanying the subjects during the fear-expression test). From 3 days before fear conditioning or the open-field test, all animals were housed individually and handled for 5 min per day for 3 days. All behavioral procedures were performed during the light phase of the light-dark cycle.

2.2. Antagonists

Haloperidol (1.0 mg/kg; Sigma Aldrich, MO, USA), atosiban (1.0 mg/kg; Cayman Chemical Company, MI, USA), SR49059 (1.0 mg/kg; AOBIOUS, MA, USA), and Naloxone (4.0 mg/kg; Sigma Aldrich) were used in this study. The doses of antagonists were determined based on previous studies (Bardo et al., 1989; Coria-Avila et al., 2008; Ramos et al., 2016; da Cruz et al., 2017). Sterile saline was used as a vehicle, except for SR49059, for which a mixture of DMSO (15%; Sigma Aldrich), Tween 80 (2%; Tokyo Chemical Industry, Tokyo, Japan), and sterile saline (83%) was used. Test substances were prepared in a volume of 0.3 ml and administered intraperitoneally. After injections, the subjects were returned to their home cage and left undisturbed until the fear expression test or open-field test.

2.3. Procedures

2.3.1. Experiment 1

Fear conditioning was performed in an illuminated room, as described in our previous studies (Kodama et al., 2011; Kiyokawa et al., 2013). Each subject was placed in an acrylic conditioning box (28 × 20 × 27 cm) for 20 min. During this period, the subject received seven repetitions of a 3-s tone (8 kHz, 75 dB) that was terminated concurrently with a foot shock (0.5 s, 0.6 mA). Intertrial intervals varied randomly from 120 to 240 s. After fear conditioning, the subject was returned to its home cage.

Twenty-four hours after fear conditioning, a fear expression test was performed as described in our previous studies (Kiyokawa et al., 2012; Takahashi et al., 2013). The subjects were first intraperitoneally administered either haloperidol (Hal group: n = 8), SR49059 (SR group: n = 6), atosiban (Ato group: n = 8), naloxone (Nal group: n = 6), or saline (Sal group: n = 6). Twenty-five minutes after administration, the subjects were placed in one of two rectangular enclosures (25 × 25 × 35 cm) situated on an acrylic board (45 × 60 cm) in a dark room illuminated by dim red light. An associate was simultaneously placed in the other enclosure. Each enclosure had three acrylic walls, one wire mesh wall, and a wire mesh ceiling. The wire mesh wall was constructed of 1-cm square mesh in the lower part (20 cm) and vertical bars spaced at 1-cm intervals in the upper part (15 cm), which prevented the rats from climbing up to the ceiling. The two enclosures were positioned such that their wire mesh walls faced one another with a 5-cm intervening distance. The acrylic board within the enclosures was covered in clean bedding. We also prepared an additional control group in which the subjects were intraperitoneally administered saline and tested alone (Alo group: n = 7). After a 5-min acclimation period, a 3-s CS tone was presented five times at 1-min intervals during the first half of the 10-min experimental period. The behaviors of the subjects during the acclimation and experimental periods were recorded using a video camera (HDR-HC9, Sony, Tokyo, Japan) and an HDD-BD recorder (DMR-BW770; Panasonic, Osaka, Japan).

2.3.2. Experiment 2

The subjects were first intraperitoneally administered either naloxone (Nal group: n = 8) or saline (Sal group: n = 8). Twenty-five minutes after administration, each subject was gently placed in the center of a polypropylene box (44 × 66 × 39.8 cm) located in a dark room illuminated by dim red light. Behaviors were recorded for 15 min using a video camera (HDR-HC9, Sony) and an HDD-BD recorder (DMR-BW770; Panasonic).

2.3.3. Experiment 3

Fear conditioning and the fear expression tests were performed as described above, with the exception that the subjects were intraperitoneally administered either naloxone (Nal group: n = 9) or saline (Sal group: n
After the fear-expression test, each subject was returned to its home cage and kept in the colony room. At 50 min after the fear-expression test (i.e., 60 min after the first tone delivery), each subject was deeply anesthetized using sodium pentobarbital and perfused intracardially with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brain was dissected, immersed overnight in the same fixative, and then placed in 30% sucrose/phosphate buffer for cryoprotection. The avidin-biotin-peroxidase method was used to identify immunoreactive (ir) cells, as described previously (Kobayashi et al., 2013; Kobayashi et al., 2015). Briefly, six serial 30-μm sections containing the prelimbic cortex (PL), infralimbic cortex (IL), and posterior complex of the anterior olfactory nucleus (AOP; Bregma + 3.24 mm), anterior cingulate cortex (ACC), nucleus accumbens (NAc) shell, and NAc core (Bregma + 2.28 mm), dorsomedial bed nucleus of the stria terminalis (dmBNST) and ventral bed nucleus of the stria terminalis (vBNST; Bregma - 0.12 mm), insular cortex (IC), paraventricular nucleus of the hypothalamus (PVN), and anterior cortical amygdala (aCoA; Bregma - 1.80 mm), anterior subdivision of the medial amygdala (MeA; Bregma - 2.04 mm), posterodorsal subdivision of the medial amygdala (MePD), and posteroverentral subdivision of the medial amygdala (MePV; Bregma -3.12 mm), or posterolateral cortical amygdala (plCoA) and posteromedial cortical amygdala (pmCoA; Bregma -4.44 mm) were collected (Fig. 1). After incubation in 0.3% H2O2, the free-floating sections were incubated in citrate buffer (B442, LSI Medience Corporation, Tokyo, Japan) at 60°C for 2 h. The sections were then incubated with anti-c-Fos antibody (1:7500, RRID: AB224721, Cat# 2250, Cell Signaling Technology, Danvers, MA, USA) for 65 h at 4°C, followed by incubation with VECTASTAIN Elite ABC reagent (PK-6100, Vector Laboratories). Finally, the sections were developed using diaminobenzidine solution with nickel intensification.

2.4. Data and statistical analyses

All data are expressed as mean ± standard error of the mean (SEM). All statistical analyses were performed using JMP software (version 16.2.0, SAS Institute, Cary, NC, USA). The significance criterion for all statistical tests was set at P < 0.05. A researcher blinded to the experimental conditions recorded behavioral responses using a Microsoft Excel–based Visual Basic program that recorded the duration and number of key presses (Kiyokawa et al., 2015; Kiyokawa et al., 2016). In Experiments 1 and 3, the duration of freezing (an immobile posture with cessation of skeletal and vibrissae movements, except for those related to respiration) and investigation (sniffing towards the other enclosure at a nose-wire mesh distance of less than 1 cm, including poking of the snout into the wire mesh and climbing up the wire mesh), and frequency of walking (number of steps taken with the hind paws) of the subjects were recorded during the acclimation and experimental period. The data were analyzed using multiple analysis of variance (MANOVA) followed by Dunnett’s post hoc test. In Experiment 2, the frequency of walking in each 5-min bin was recorded. After Mauchly’s test of sphericity, the data were analyzed by two-way repeated analysis of variance (ANOVA).

For immunohistochemical analyses, six sections of each region were captured at 4× (the dmBNST, MePD, plCoA, and pmCoA) or 10× (the other 12 brain regions) magnification using a microscope equipped with a digital camera (DP30BW, Olympus, Tokyo, Japan). A researcher blinded to the experimental conditions randomly selected three sections of the left hemisphere and three sections of the right hemisphere for each nucleus. The number of Fos-ir cells was determined within a 0.5 × 0.5 mm square of each nucleus using ImageJ software (version 1.53t). When the designated area was smaller than the boundaries of a 0.5-mm square, only the cells within the nucleus were counted. Although some sections were lost due to technical issues, more than 5 subjects in every group were analyzed. In all regions, the density (Fos-ir cells/0.25 mm²) was analyzed using the non-directional Student’s t-test.

3. Results

3.1. Experiment 1

The behavioral responses during the acclimation period differed significantly among the groups (F(15,92) = 2.88, P = 0.0010) (Table 1). However, a post hoc test revealed that the behavioral responses in each group did not differ from those in the Sal group. The behavioral responses during the experimental period differed
significantly among the groups ($F_{15,92} = 3.07, P = 0.0005$) (Fig. 2). A post hoc test revealed that both the Nal and Alo groups showed increased freezing (Nal: $P = 0.0076$, Alo: $P < 0.0001$) and decreased walking (Nal: $P = 0.016$, Alo: $P = 0.013$) and investigation (Nal: $P = 0.0067$, Alo: $P = 0.0005$) as compared to the Sal group.

### 3.2. Experiment 2

Mauchly’s test indicated that the assumption of sphericity had not been violated ($\chi^2_{22} = 0.097, P = 0.99$). Two-way repeated ANOVA revealed that walking was significantly affected by time ($F_{2,28} = 116, P < 0.0001$) (Fig. 3). However, the effects of group ($F_{1,14} = 0.36, P = 0.55$) and the interaction between the two factors ($F_{2,28} = 0.03, P = 0.96$) were not significant.

### 3.3 Experiment 3

The behavioral responses during the acclimation period were not significantly different between the groups ($F_{3,15} = 3.08, P = 0.059$) (Table 2). In contrast, the behavioral responses during the experimental period differed significantly between the groups ($F_{3,15} = 4.75, P = 0.016$) (Table 2). A post hoc test revealed that the Nal group showed increased freezing ($P = 0.0013$) and decreased walking ($P = 0.0031$) and investigation ($P = 0.015$) as compared to the Sal group.

We analyzed Fos expression in 16 brain regions (Table 3). Statistical analyses revealed that the Nal group showed increased density of Fos-ir cells in the PVN ($t_{17} = -2.43, P = 0.026$) and decreased density of Fos-ir cells in the NAc shell ($t_{17} = 2.76, P = 0.013$), ACC ($t_{17} = 4.11, P = 0.0007$), and IC ($t_{16} = 2.17, P = 0.045$) compared to the Sal group (Fig. 4). In addition, the Nal group tended to show decreased density of Fos-ir cells in the NAc core ($t_{17} = 2.09, P = 0.052$).

### 4. Discussion

In the present study, a series of experiments were performed to explore the neurochemical background of social buffering of conditioned fear responses in rats. In Experiment 1, pretreatment with naloxone, but not haloperidol, SR49059, or atosiban, increased freezing and decreased walking and investigation behaviors. These results suggest that naloxone blocks social buffering, whereas the other three antagonists do not. In Experiment 2, naloxone did not affect walking steps during the test. Therefore, the results of Experiment 1 are less likely due to decreased activity of naloxone. In Experiment 3, Fos expression was increased in the PVN, supporting the blockade of social buffering by naloxone. In addition, Fos expression was decreased in the NAc shell, ACC, and IC. Furthermore, Fos expression in the NAc core tended to be decreased. Naloxone thus appears to affect these four regions and/or upstream regions during the blockade of social buffering. From these collective results, we conclude that naloxone blocks social buffering of conditioned fear responses in rats.

Based on the present findings, we suggest that certain strains of rats can induce social buffering because they stimulate opioid receptors. Animals show distinct social behaviors when they evaluate an individual as to whether it is a member of the same group and recognize social similarity to that individual. In rats, the strain can serve as the group with regard to recognizing social similarity (Ben-Ami Bartal et al., 2014; Han et al., 2019; Kogo et al., 2021; Kiyokawa et al., 2022). We previously found that social buffering is affected by the strain of associates (Nakamura et al., 2016). Specifically, social buffering in Wistar rats can be induced by unfamiliar Wistar, Sprague-Dawley, Long-Evans, or Lewis rats, but not by unfamiliar Fischer344 or Brown-Norway rats. In addition, autoradiography data suggested that opioid receptors are stimulated when rats interact with other rats of the same strain (Panksepp & Bishop, 1981). Furthermore, the stimulation of opioid receptors is known to ameliorate stress responses. For example, although rats show an augmented anxiety response after undergoing acute restraint, this augmentation is blocked by morphine, an opioid receptor agonist (Joshi et al., 2014). These findings suggest that the recognition of social similarity stimulates opioid receptors, which in turn induces social buffering as a part of ingroup favoritism. This conclusion is further supported by the finding that naltrexone, an opioid receptor antagonist, reduces feelings of social connection in humans (Inagaki et al., 2016).
We also found in the present study that reduced Fos expression in the NAc, ACC, and IC accompanied the blockade of social buffering by naloxone. Based on these results, we hypothesize that opioid receptors in the NAc play an important role in social buffering. It is known that the benefit of social interaction is produced by the stimulation of opioid receptors in the NAc. For example, rats prefer to stay in a compartment where they had previously interacted with conspecifics (Tzschentke, 1998; Thiel et al., 2008). This socially conditioned place preference was blocked by the administration of the mu-opioid receptor antagonist CTAP into the NAc immediately before social interaction (Trezza et al., 2011). Given that social buffering is one of the benefits of social interaction, it is possible that social buffering is also induced by the stimulation of opioid receptors in the NAc. However, the observed decrease in Fos expression was unexpected. Stimulation of opioid receptors is known to decrease neuronal excitability (Stein, 2016). Therefore, Fos expression would be expected to increase when an antagonist binds to opioid receptors. An alternative possibility is that opioid receptors responsible for social buffering are located in brain regions upstream of the NAc. For example, the ventral tegmental area (VTA) is a region that projects into the NAc and expresses opioid receptors (Le Merrer et al., 2009). In addition, microinjection of the mu-opioid receptor agonist DAMGO into this region was shown to increase Fos expression in the NAc (Bontempi & Sharp, 1997). Therefore, it is possible that opioid receptors in the VTA, rather than the NAc, are responsible for social buffering. Furthermore, it is also possible opioid receptors in the ACC and IC or their upstream regions contribute to social buffering because the ACC and IC are known to contribute to empathy (Apps et al., 2016) and stress identification (Rogers-Carter et al., 2018; Djerdjaj et al., 2022), respectively, in rats. Further research is needed to determine the location of the opioid receptors responsible for social buffering.

5. Conclusion

In summary, the present study revealed that naloxone blocks social buffering of conditioned fear responses in rats. Further analyses to determine the location of opioid receptors responsible for social buffering would broaden our understanding of the neural mechanism underlying social buffering. Such analyses would enhance understanding of the neural mechanisms of affiliative social behavior and contribute to improving the well-being of social animals.

Data availability

Data are available from the corresponding author upon request.

References


### Tables

**Table 1.** Behavioral responses during the acclimation period in Experiment 1.

<table>
<thead>
<tr>
<th></th>
<th>Sal</th>
<th>Hal</th>
<th>SR</th>
<th>Ato</th>
<th>Nal</th>
<th>Alo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freezing</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0.2 ± 0.1</td>
<td>0 ± 0</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td>Walking</td>
<td>99 ± 8</td>
<td>110 ± 8</td>
<td>127 ± 12</td>
<td>131 ± 7</td>
<td>77 ± 8</td>
<td>90 ± 11</td>
</tr>
<tr>
<td>Investigation</td>
<td>131 ± 12</td>
<td>141 ± 9</td>
<td>131 ± 15</td>
<td>121 ± 8</td>
<td>105 ± 9</td>
<td>83 ± 13</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM.

Freezing and investigation are shown in seconds.

Walking is shown as the number of steps.

**Table 2.** Behavioral responses during the acclimation and experimental periods in Experiment 3.

<table>
<thead>
<tr>
<th></th>
<th>Sal</th>
<th>Nal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acclimation period</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Freezing</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Walking</td>
<td>117 ± 8</td>
<td>81 ± 8</td>
</tr>
<tr>
<td>Investigation</td>
<td>120 ± 9</td>
<td>122 ± 8</td>
</tr>
<tr>
<td>Experimental period</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Freezing</td>
<td>102 ± 15</td>
<td>34 ± 13*</td>
</tr>
<tr>
<td>Walking</td>
<td>124 ± 18</td>
<td>53 ± 20*</td>
</tr>
<tr>
<td>Investigation</td>
<td>28 ± 13</td>
<td>196 ± 44*</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM.

Freezing and investigation are shown in seconds.

Walking is shown as the number of steps.

*P < 0.05 compared to the Sal group in the corresponding period.

**Table 3.** Number of Fos- immunoreactive cells per 0.25 mm² in various brain regions in Experiment 3.
<table>
<thead>
<tr>
<th></th>
<th>Sal</th>
<th>Nal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Prelimbic cortex</td>
<td>66.0 ± 6.7</td>
<td>50.4 ± 7.2</td>
</tr>
<tr>
<td>2 Infrolimbic cortex</td>
<td>56.3 ± 6.8</td>
<td>53.8 ± 5.6</td>
</tr>
<tr>
<td>3 Posterior complex of the anterior olfactory nucleus</td>
<td>62.8 ± 3.2</td>
<td>55.2 ± 6.2</td>
</tr>
<tr>
<td>4 Anterior cingulate cortex</td>
<td>117.8 ± 6.3</td>
<td>78.8 ± 7.1*</td>
</tr>
<tr>
<td>5 Nucleus accumbens shell</td>
<td>78.0 ± 7.4</td>
<td>53.0 ± 4.6*</td>
</tr>
<tr>
<td>6 Nucleus accumbens core</td>
<td>81.1 ± 4.7</td>
<td>67.2 ± 4.5#</td>
</tr>
<tr>
<td>7 Dorsomedial bed nucleus of the stria terminalis</td>
<td>9.4 ± 1.5</td>
<td>8.8 ± 1.1</td>
</tr>
<tr>
<td>8 Ventral bed nucleus of the stria terminalis</td>
<td>12.4 ± 1.4</td>
<td>10.1 ± 1.8</td>
</tr>
<tr>
<td>9 Insular cortex</td>
<td>10.3 ± 1.8</td>
<td>5.6 ± 0.7*</td>
</tr>
<tr>
<td>10 Paraventricular nucleus of the hypothalamus</td>
<td>39.3 ± 4.7</td>
<td>60.7 ± 7.6*</td>
</tr>
<tr>
<td>11 Anterior cortical amygdala</td>
<td>32.5 ± 2.5</td>
<td>25.0 ± 5.6</td>
</tr>
<tr>
<td>12 Anterior subdivision of the medial amygdala</td>
<td>35.3 ± 3.1</td>
<td>27.0 ± 2.9</td>
</tr>
<tr>
<td>13 Posterodorsal subdivision of the medial amygdala</td>
<td>17.2 ± 4.7</td>
<td>16.6 ± 3.0</td>
</tr>
<tr>
<td>14 Posteroventral subdivision of the medial amygdala</td>
<td>23.9 ± 6.2</td>
<td>20.0 ± 3.4</td>
</tr>
<tr>
<td>15 Posterolateral cortical amygdala</td>
<td>20.6 ± 1.5</td>
<td>18.6 ± 2.2</td>
</tr>
<tr>
<td>16 Posteromedial cortical amygdala</td>
<td>21.6 ± 2.8</td>
<td>19.7 ± 2.1</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM.

*P < 0.05 and #P = 0.052 compared to the Sal group, as determined using the Student’s t-test.

**Figure legends**

**Fig. 1.** Schematic diagrams showing the location of brain regions in which Fos-immunoreactive cells were counted (open square). For simplicity, the locations are shown only on the right side. For an explanation of abbreviations, see the corresponding numbers in Table 3.

**Fig. 2.** Duration of freezing, duration of investigation, and frequency of walking during the test period of Experiment 1. Fear-conditioned subjects received an intraperitoneal administration of either haloperidol (Hal, n = 8), SR49059 (SR, n = 6), atosiban (Ato, n = 8), naloxone (Nal, n = 6), or saline (Sal, n = 6) and were tested in the presence of an associate (Social: open bars). Fear-conditioned subjects received an intraperitoneal administration of saline and were tested alone (Alo: filled bars, n = 7). *P < 0.05 as determined using MANOVA, followed by Dunnett’s post hoc test.

**Fig. 3.** Frequency of walking (mean ± SEM) in each 5-min bin in Experiment 2. The subjects received an intraperitoneal administration of either naloxone (Nal, n = 8) or saline (Sal, n = 8) and underwent the open-field test.

**Fig. 4.** Representative photomicrographs showing Fos-immunoreactive cells in the nucleus accumbens (NAc) shell, anterior cingulate cortex (ACC), insular cortex (IC), and paraventricular nucleus of the hypothalamus (PVN) of fear-conditioned subjects that received an intraperitoneal administration of saline (Sal) or naloxone (Nal) and were tested in the presence of an associate. Scale bars indicate 200 μm.

**Graphical Abstract Text**

The presence of a non-conditioned unfamiliar same-sex rat completely ameliorates conditioned fear responses to an auditory conditioned stimulus in rats. The present study demonstrated that naloxone (but not haloperidol, SR49059, or atosiban) blocks this social buffering of conditioned fear responses. The blockade of social buffering was accompanied by decreased c-Fos expression in the nucleus accumbens, anterior cingulate cortex, and insular cortex.