The right central amygdala shows greater activation in response to an auditory conditioned stimulus in male rats

Yasushi KIYOKAWA[1)]*, Daichi TAKAHASHI[1)], Yukari TAKEUCHI[1)] and Yuji MORI[1)]

[1)]*Laboratory of Veterinary Ethology, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan*

ABSTRACT. Pavlovian fear conditioning is an experimental procedure in which a conditioned stimulus (CS) acquires an ability to elicit fear responses. This type of conditioning depends on the basolateral complex of the amygdala (BLA) and/or central amygdala (CeA). We previously found that rats showed reduced fear responses to an auditory CS when they were subjected to a pre-training chemical lesion of the entire right amygdala as compared with the left amygdala. Based on this finding, we hypothesize that the BLA and/or CeA in the right hemisphere will be more strongly activated by an auditory CS than those in the left hemisphere. To test this hypothesis, we re-exposed fear-conditioned and non-conditioned rats to an auditory CS 1 day after fear conditioning. We assessed Fos expression in the BLA and CeA in each hemisphere. We found that fear-conditioned subjects showed fear responses, such as increased freezing and decreased walking, as well as increased Fos expression in the BLA and CeA. When we compared Fos expression between hemispheres, Fos expression in the CeA, but not the BLA, was greater in the right hemisphere compared with the left hemisphere. These results suggest that the right CeA is more strongly activated by the auditory CS.
KEY WORDS: amygdala, auditory fear conditioning, fear responses, Fos expression, lateralization

Lateralized behavior has been observed in many species. One of the most obvious examples of this phenomenon is lateralized handedness. The predominant use of one hand has been reported in many species, including mice [29], rats [9], dogs [28, 30] and humans [8]. Similarly, lateralized brain function appears to occur in many species. For example, asymmetry in striatal dopamine levels appears to determine side preference in rats. Specifically, when rats received foot shocks in the long arm of a T-maze, they preferentially escaped to the side of the short arm contralateral to the striatum containing a higher level of dopamine [32]. In addition, head-dipping in the hole-board test was reduced when lidocaine was injected into the left, but not right, basolateral complex of the amygdala (BLA) in rats [1]. Furthermore, avoidance of a black compartment in which rats had previously received a foot shock was antagonized when the right, but not left, BLA was inactivated [6, 7].

Pavlovian fear conditioning, or threat conditioning, is an experimental procedure in which a conditioned stimulus (CS) acquires an ability to elicit fear responses. This type of conditioning depends on the activation of the BLA and/or CeA (central amygdala) [21, 22]. Using an auditory CS as a stressor [18], we are analyzing the phenomenon known as social buffering in male rats, in which the presence of an associate male rat ameliorates fear and stress responses in a subject rat [13]. During our analyses of social buffering,

we unexpectedly obtained results suggesting a lateralized role of the amygdala in auditory fear conditioning. In our previous study, we subjected rats to a unilateral pre-training chemical lesion of the entire amygdala. Rats that received the lesion in the right hemisphere showed reduced fear responses compared with rats that received the lesion in the left hemisphere [14]. Given the importance of the BLA and CeA in fear responses, we hypothesized that the BLA and/or CeA in the right hemisphere would show greater activation to an auditory CS compared with those in the left hemisphere.

To test this hypothesis, we re-exposed fear-conditioned and non-conditioned rats to an auditory CS one day after fear conditioning. We assessed Fos expression in the BLA and CeA in each hemisphere. We also observed behavioral responses to the CS, such as freezing and walking, to confirm successful fear-conditioning.

MATERIALS AND METHODS

Animals: All experiments were approved by the Animal Care and Use Committee of the Faculty of Agriculture at The University of Tokyo, according to guidelines 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 (Yokohama, Japan). They were housed with three animals per cage in a room with an ambient temperature of 24 ± 1 °C and a humidity of 45 ± 5%. The room had a 12-hr light /12-hr dark cycle (lights were switched on at 8:00). Food and water were available *ad libitum*. All rats were housed separately and were handled for 3 min twice daily, commencing 3 days before the conditioning day.

*Correspondence to: Kiyokawa, Y., Laboratory of Veterinary Ethology, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan. e-mail: akiyo@mail.ecc.u-tokyo.ac.jp

Fear conditioning: Fear conditioning was performed in an illuminated room between 9:00 and 18:00, as described in our previous studies [11, 23]. A subject from the conditioned group was placed in an acrylic conditioning box (28 × 20 × 27 cm) for 20 min, where it received 7 repetitions of a 3-sec tone (CS, 8 kHz, 70 dB) that terminated concurrently with a foot shock (0.5 sec, 0.75 mA). We also prepared the non-conditioned group by presenting the tone and foot shock separately during a 20-min period. The intertrial interval randomly varied from 30 to 240 sec. The subjects were returned to their home cage after fear conditioning.

Fear-expression test: We performed a fear-expression test as described in our previous studies [11, 14]. The test took place between 9:00 and 18:00, 24 hr after fear conditioning. A test box (25 × 25 × 35 cm) was placed in a dark room illuminated with dim red light. The box had three acrylic walls, one wire mesh wall and a wire mesh ceiling. The wire mesh wall was constructed with 1-cm^2 gauge mesh in the lower section (20 cm) and vertical bars spaced 1 cm apart in the upper section (15 cm), which prevented the rats from climbing up to the ceiling. The floor of the box was covered in clean bedding.

The subject was placed in the box and kept undisturbed during a 3-min acclimation period. Then, a CS was presented five times for 3 sec each at 1-min intervals during the first half of the 10-min experimental period. The behavior of the subjects during the acclimation and experimental periods was recorded with a video camera (HDR-HC9; Sony, Tokyo, Japan) and an HDD-BD recorder (DMR-BW770; Panasonic, Osaka, Japan). After the test, the subjects were returned to their home cage.

Sixty minutes after the beginning of the acclimation period, each subject was deeply anesthetized with sodium pentobarbital and intracardially perfused with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brain was removed and immersed overnight in the same fixative, and then placed in 30% sucrose/phosphate buffer for cryoprotection. We used the avidin-biotin-peroxidase immunohistochemistry method to detect Fos expression, as described previously [15, 16, 19]. Briefly, we collected successive 30-μm sections from Bregma −2.16 mm through Bregma −3.24 mm. Half of the sections were stained with cresyl violet to confirm the location of the nucleus. The remaining sections were incubated with a primary antibody to c-Fos protein (1:7,500; PC38, Merck Millipore, Billerica, MA, U.S.A.) for 65 hr and a biotinylated anti-rabbit secondary antibody (BA-1000, Vector Laboratories, Burlingame, CA, U.S.A.) for 2 hr. The sections were then processed using the ABC kit (Vector Laboratories), and staining was developed by incubating the tissue in a diaminobenzidine solution with nickel intensification.

Data analyses and statistical procedures: The data are expressed as means ± standard error of the means (SEM). The significance level was set at $P<0.05$ for all statistical tests. A researcher who was blind to the experimental conditions recorded the duration of freezing (immobile posture, with cessation of skeletal and vibrissae movement, except for that associated with respiration) and the frequency of

Table 1.　Behavioral responses during the acclimation period

Group	Freezing (sec)	Walking (steps)
Non-conditioned	10.9 ± 5.5	45.9 ± 5.4
Conditioned	3.1 ± 1.2	54.6 ± 6.0

Data are expressed as means ± standard error of the mean.

walking (number of steps taken with the hind paws) using Microsoft Excel-based Visual Basic software to record the duration and number of key presses, as in our previous studies [17, 19, 24]. The behavioral data for the conditioned and non-conditioned subjects during the acclimation and experimental periods were compared using a MANOVA followed by Fisher's PLSD post hoc test.

For immunohistochemical analyses of the BLA and CeA, we counted the number of Fos-immunoreactive cells in each bilateral nucleus in all sections. We then compared the total number of immunoreactive cells between the conditioned and non-conditioned groups using a Student's *t* test. When we detected activation of the nucleus, we compared the total number of immunoreactive cells between the right and left sides in each group using a paired *t* test.

In order to assess the relationship between fear and neural responses, the correlation between duration of freezing and number of Fos-immunoreactive cells in the BLA and CeA was analyzed using Pearson's correlation analysis.

RESULTS

Rats were either fear-conditioned (conditioned group: $n = 10$) or non-conditioned (non-conditioned group: $n = 11$) to an auditory CS on the conditioning day. Then, 24 hr after the conditioning procedure, each subject was placed in the test box and kept undisturbed for 3 min as an acclimation period. Then, during a subsequent 10-min experimental period, five CS tones were presented.

As summarized in Table 1, we found no difference between the conditioned and non-conditioned groups during the initial acclimation period ($F_{(2,18)}$=0.964, P=0.400). In contrast, behavioral responses during the experimental period were significantly affected by the conditioning procedure ($F_{(2,18)}$=14.3, $P<0.01$). A post hoc test revealed that the conditioned group showed increased freezing ($P<0.01$) and decreased walking ($P<0.01$) compared with the non-conditioned group (Fig. 1A). Along with these behavioral responses, the conditioned group showed increased Fos expression in the BLA (t_{19}= −3.29, $P<0.01$) and CeA (t_{19}= −2.30, $P<0.05$) compared with the non-conditioned group (Fig. 1B). Therefore, we decided to compare BLA and CeA activation between the hemispheres.

In the BLA (Fig. 2A and 2B), Fos expression was similar between the right and left hemispheres, both in the conditioned (t_9= −1.25, P=0.244) and non-conditioned groups (t_{10}= 0.698, P=0.501) (Fig. 3). In the CeA (Fig. 2C and 2D), we found that Fos expression in the conditioned group was greater in the right hemisphere compared with that in the left hemisphere (t_9= −2.75, $P<0.05$) (Fig. 3). Fos expression in
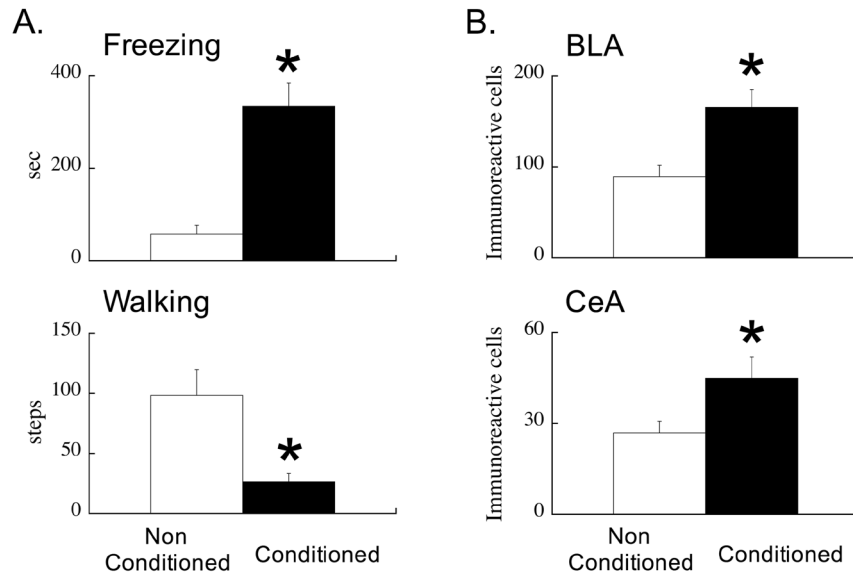
Fig. 1. Conditioned fear responses to the auditory conditioned stimulus. (A) Duration of freezing and frequency of walking (mean + SEM) and (B) the number of Fos-immunoreactive cells (mean + SEM) in the basolateral complex of the amygdala (BLA) and central amygdala (CeA) of fear-conditioned and non-conditioned subjects. *$P<0.05$ according to a MANOVA followed by Fisher's PLSD post hoc test for behavioral results, and according to a Student's t test for Fos expression in the BLA and CeA.
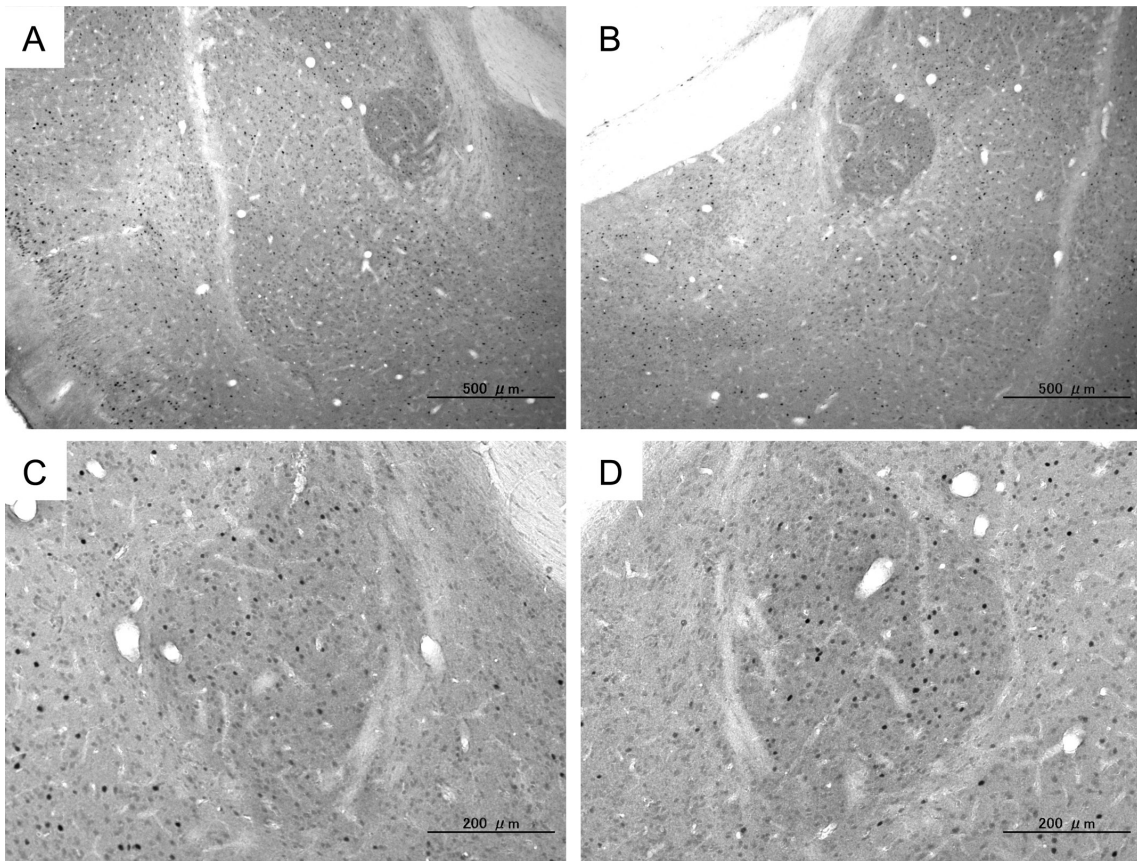

Fig. 2. Representative photomicrograph showing Fos immunoreactive cells in the left (A) and right (B) basolateral complex of the amygdala and in the left (C) and right (D) central amygdala of fear-conditioned subjects.
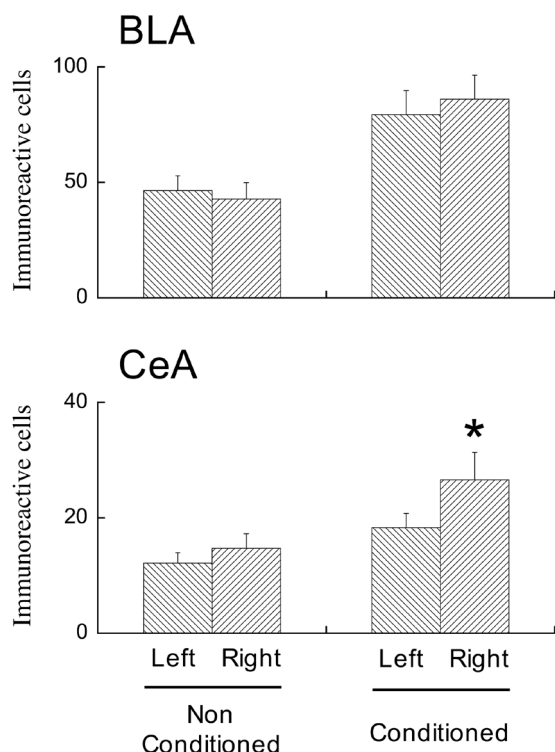
BLA

CeA

Fig. 3.   Number of Fos-immunoreactive cells (mean + SEM) in the right and left basolateral complex of the amygdala (BLA) and central amygdala (CeA) of fear-conditioned and non-conditioned subjects. *$P<0.05$ according to a paired t test.

the non-conditioned group was similar between the right and left hemispheres ($t_{10}= -1.25$, $P=0.241$) (Fig. 3).

In the fear-conditioned group, we further assessed the correlation between the duration of freezing during the experimental period and Fos expression in the bilateral BLA ($P=0.265$), bilateral CeA ($P=0.683$), right BLA ($P=0.445$), left BLA ($P=0.160$), right CeA ($P=0.557$) and left CeA ($P=0.979$). However, we did not find significant correlation.

DISCUSSION

When fear-conditioned subjects were re-exposed to the auditory CS, they showed fear responses, such as increased freezing and decreased walking. Along with these behavioral responses, fear-conditioned subjects exhibited activation of the BLA and CeA, as assessed by Fos expression. These results confirmed that the fear-conditioning was successful in our subject group. When we compared Fos expression between the hemispheres, Fos expression in the CeA, but not in the BLA, was greater in the right hemisphere compared with the left hemisphere. These results suggest that the right CeA is more strongly activated in response to the auditory CS.

In the present study, fear-conditioned subjects showed fear responses and an increment of Fos expression in the amygdala simultaneously. Given that the amygdala plays a critical role in fear responses [21, 22], we assume a causal relation-

ship between these 2 phenomena. However, we cannot deny an alternative possibility that the observed difference in Fos expression was ascribed to the activation of the neurons that are not related to fear responses, because we did not confirm the type of neurons that showed Fos expression. To clarify this, we assessed the correlation between these phenomena. However, the correlation was not significant possibly due to the small number of the subjects. Therefore, further analyses are crucial to clarify this point.

Based on the findings in the present and previous studies, we hypothesize that the CeA is the region of the amygdala that plays a lateralized role in auditory fear conditioning. In the present study, we found stronger activation in the right hemisphere in the CeA, but not the BLA. In addition, in our previous study in which we obtained results suggesting a lateralized role of the amygdala, the lesioned brain area included the CeA [14]. This hypothesis may also explain why previous studies have not reported a lateralized role of the amygdala. In rats with pre-training [2] or post-training [2, 20] electrical lesion of the unilateral amygdala, differences in fear responses did not occur according to the lesioned side. However, the lesions in these studies appear to have been placed mainly on the BLA, such that the damage to the CeA was likely minimal. Given that the CeA mediates fear responses to the auditory CS independently from the BLA [31], it is possible that the residual CeA mediated fear responses, which prevented the observation of a lateralized role of the amygdala in these studies.

In contrast to auditory fear conditioning, contextual fear conditioning has been reported to be right amygdala dominant. This may be attributable to the facts that an intact basal amygdala is indispensable for contextual fear conditioning. In an above-mentioned study, freezing in response to a contextual CS was reduced when a post-training, but not pre-training, electrical lesion was placed mainly on the right as compared with the left BLA [2]. Another study also implied right amygdala dominance in contextual fear conditioning. Specifically, rats received an electrical lesion of the entire amygdala in one hemisphere and a small chemical lesion of each sub-nucleus of the amygdala in the contralateral hemisphere. Then, these rats underwent fear conditioning and were re-exposed to the contextual CS. The authors found that rats showed reduced freezing when the left amygdala and right BLA were lesioned as compared with when the right amygdala and left BLA were lesioned [10]. Given that the CeA requires an intact BLA, specifically an intact basal amygdala [4], to mediate fear responses to a contextual CS [25], it is possible that lesioning the BLA was sufficient to impair the function of the ipsilateral CeA, which enabled us to observe the lateralized role of amygdala in the present study. Consistent with this notion, Fos expression in the basal amygdala and CeA in response to the contextual CS was greater in the right hemisphere than in the left hemisphere [27].

One possible explanation for the lateralized role of the amygdala is that the conditioning procedure induces more synaptic plasticity in the right CeA than in the left CeA. Fear conditioning is established by the following mechanisms.

In the CeA, some of the neurons that induce fear responses receive both synapses transmitting foot shock information and synapses transmitting the CS information. The foot shock information and CS information strongly and weakly activate the neurons, respectively. When these activations occur simultaneously during fear conditioning, the synapses transmitting the CS information are strengthened, which enable the CS alone to strongly activate the neuron. As a result, the CS can induce fear responses in the absence of a foot shock [3]. Previous findings suggest that this synaptic plasticity can be more easily established in the right CeA than in the left CeA. For example, neurons in the right CeA show greater activation to pain than those in the left CeA [12]. In addition, pain activates the extracellular signal-regulated kinases required for synaptic plasticity associated with auditory fear conditioning [26] in the right, but not the left, CeA [5]. Therefore, during fear conditioning, it is possible that the pain caused by foot shocks activates a greater number of neurons that induce fear responses in the right compared with the left CeA, which increases the number of the neurons that receive CS information simultaneously. As a result, the number of strengthened synapses in the right CeA becomes higher than that in the left CeA, leading to right hemisphere dominance in fear responses to the CS.

In summary, we found that Fos expression in the CeA, but not the BLA, was greater in the right hemisphere compared with the left hemisphere. These results suggest that the right CeA shows a greater degree of activation in response to an auditory CS. Although lateralized brain function has been reported for both fear conditioning and other phenomena, the biological significance of lateralization has yet to be clarified. Further analyses may benefit from using the present experimental model.

REFERENCES

1. Alvarez, E. O. and Banzan, A. M. 2011. Functional lateralization of the baso-lateral amygdala neural circuits modulating the motivated exploratory behaviour in rats: role of histamine. *Behav. Brain Res.* **218**: 158–164. [Medline] [CrossRef]
2. Baker, K. B. and Kim, J. J. 2004. Amygdalar lateralization in fear conditioning: evidence for greater involvement of the right amygdala. *Behav. Neurosci.* **118**: 15–23. [Medline] [CrossRef]
3. Blair, H. T., Schafe, G. E., Bauer, E. P., Rodrigues, S. M. and LeDoux, J. E. 2001. Synaptic plasticity in the lateral amygdala: a cellular hypothesis of fear conditioning. *Learn. Mem.* **8**: 229–242. [Medline] [CrossRef]
4. Calandreau, L., Desmedt, A., Decorte, L. and Jaffard, R. 2005. A different recruitment of the lateral and basolateral amygdala promotes contextual or elemental conditioned association in Pavlovian fear conditioning. *Learn. Mem.* **12**: 383–388. [Medline] [CrossRef]
5. Carrasquillo, Y. and Gereau, R. W. 4th. 2007. Activation of the extracellular signal-regulated kinase in the amygdala modulates pain perception. *J. Neurosci.* **27**: 1543–1551. [Medline] [CrossRef]
6. Coleman-Mesches, K. and McGaugh, J. L. 1995. Muscimol

7. injected into the right or left amygdaloid complex differentially affects retention performance following aversively motivated training. *Brain Res.* **676**: 183–188. [Medline] [CrossRef]
7. Coleman-Mesches, K. and McGaugh, J. L. 1995. Differential involvement of the right and left amygdalae in expression of memory for aversively motivated training. *Brain Res.* **670**: 75–81. [Medline] [CrossRef]
8. Frayer, D. W., Lozano, M., Bermúdez de Castro, J. M., Carbonell, E., Arsuaga, J. L., Radovčić, J., Fiore, I. and Bondioli, L. 2012. More than 500,000 years of right-handedness in Europe. *Laterality* **17**: 51–69. [Medline]
9. Glick, S. D. and Jerussi, T. P. 1974. Spatial and paw preferences in rats: their relationship to rate-dependent effects of d-amphetamine. *J. Pharmacol. Exp. Ther.* **188**: 714–725. [Medline]
10. Goosens, K. A. and Maren, S. 2001. Contextual and auditory fear conditioning are mediated by the lateral, basal, and central amygdaloid nuclei in rats. *Learn. Mem.* **8**: 148–155. [Medline] [CrossRef]
11. Ishii, A., Kiyokawa, Y., Takeuchi, Y. and Mori, Y. 2016. Social buffering ameliorates conditioned fear responses in female rats. *Horm. Behav.* **81**: 53–58. [Medline] [CrossRef]
12. Ji, G. and Neugebauer, V. 2009. Hemispheric lateralization of pain processing by amygdala neurons. *J. Neurophysiol.* **102**: 2253–2264. [Medline] [CrossRef]
13. Kiyokawa, Y. 2015. Social Odors: Alarm Pheromones and Social Buffering. *Curr. Top. Behav. Neurosci.* [Medline] [CrossRef]
14. Kiyokawa, Y., Wakabayashi, Y., Takeuchi, Y. and Mori, Y. 2012. The neural pathway underlying social buffering of conditioned fear responses in male rats. *Eur. J. Neurosci.* **36**: 3429–3437. [Medline] [CrossRef]
15. Kiyokawa, Y., Kodama, Y., Takeuchi, Y. and Mori, Y. 2013. Physical interaction is not necessary for the induction of housing-type social buffering of conditioned hyperthermia in male rats. *Behav. Brain Res.* **256**: 414–419. [Medline] [CrossRef]
16. Kiyokawa, Y., Honda, A., Takeuchi, Y. and Mori, Y. 2014. A familiar conspecific is more effective than an unfamiliar conspecific for social buffering of conditioned fear responses in male rats. *Behav. Brain Res.* **267**: 189–193. [Medline] [CrossRef]
17. Kiyokawa, Y., Ishida, A., Takeuchi, Y. and Mori, Y. 2016. Sustained housing-type social buffering following social housing in male rats. *Physiol. Behav.* **158**: 85–89. [Medline] [CrossRef]
18. Kiyokawa, Y., Mikami, K., Mikamura, Y., Ishii, A., Takeuchi, Y. and Mori, Y. 2015. The 3-second auditory conditioned stimulus is a more effective stressor than the 20-second auditory conditioned stimulus in male rats. *Neuroscience* **299**: 79–87. [Medline] [CrossRef]
19. Kobayashi, T., Kiyokawa, Y., Takeuchi, Y. and Mori, Y. 2015. Neural correlates underlying naloxone-induced amelioration of sexual behavior deterioration due to an alarm pheromone. *Front. Neurosci.* **9**: 52. [Medline] [CrossRef]
20. LaBar, K. S. and LeDoux, J. E. 1996. Partial disruption of fear conditioning in rats with unilateral amygdala damage: correspondence with unilateral temporal lobectomy in humans. *Behav. Neurosci.* **110**: 991–997. [Medline] [CrossRef]
21. LeDoux, J. 2012. Rethinking the emotional brain. *Neuron* **73**: 653–676. [Medline] [CrossRef]
22. LeDoux, J. E. 2014. Coming to terms with fear. *Proc. Natl. Acad. Sci. U.S.A.* **111**: 2871–2878. [Medline] [CrossRef]
23. Mikami, K., Kiyokawa, Y., Takeuchi, Y. and Mori, Y. 2016. Social buffering enhances extinction of conditioned fear responses in male rats. *Physiol. Behav.* **163**: 123–128. [Medline] [CrossRef]
24. Muyama, H., Kiyokawa, Y., Inagaki, H., Takeuchi, Y. and Mori,

Y. 2016. Alarm pheromone does not modulate 22-kHz calls in male rats. *Physiol. Behav.* **156**: 59–63. [Medline] [CrossRef]

25. Pitts, M. W., Todorovic, C., Blank, T. and Takahashi, L. K. 2009. The central nucleus of the amygdala and corticotropin-releasing factor: insights into contextual fear memory. *J. Neurosci.* **29**: 7379–7388. [Medline] [CrossRef]

26. Schafe, G. E., Atkins, C. M., Swank, M. W., Bauer, E. P., Sweatt, J. D. and LeDoux, J. E. 2000. Activation of ERK/MAP kinase in the amygdala is required for memory consolidation of pavlovian fear conditioning. *J. Neurosci.* **20**: 8177–8187. [Medline]

27. Scicli, A. P., Petrovich, G. D., Swanson, L. W. and Thompson, R. F. 2004. Contextual fear conditioning is associated with lateralized expression of the immediate early gene c-fos in the central and basolateral amygdalar nuclei. *Behav. Neurosci.* **118**: 5–14. [Medline] [CrossRef]

28. Tan, U. 1987. Paw preferences in dogs. *Int. J. Neurosci.* **32**:

825–829. [Medline] [CrossRef]

29. Waters, N. S. and Denenberg, V. H. 1994. Analysis of two measures of paw preference in a large population of inbred mice. *Behav. Brain Res.* **63**: 195–204. [Medline] [CrossRef]

30. Wells, D. L. 2003. Lateralised behaviour in the domestic dog, Canis familiaris. *Behav. Processes* **61**: 27–35. [Medline] [CrossRef]

31. Wilensky, A. E., Schafe, G. E., Kristensen, M. P. and LeDoux, J. E. 2006. Rethinking the fear circuit: the central nucleus of the amygdala is required for the acquisition, consolidation, and expression of Pavlovian fear conditioning. *J. Neurosci.* **26**: 12387–12396. [Medline] [CrossRef]

32. Zimmerberg, B., Glick, S. D. and Jerussi, T. P. 1974. Neurochemical correlate of a spatial preference in rats. *Science* **185**: 623–625. [Medline] [CrossRef]