The Journal of Veterinary Medical Science

FULL PAPER

Physiology

Relationship between motor function and ultrasonic vocalizations induced by maternal separation in rat pups

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ABSTRACT. When rat pups are isolated from their mothers, they emit ultrasonic vocalizations (USVs). Although previous studies have reported that USVs are related to anxiety, others have reported that they are related to simple, nonemotional factors, such as physiological reactions to coldness. In this study, we examined the influence of three maternal separations on rat pups. The number of USVs during 5 min of USV test under maternal separation, latency in the righting reflex as motor function, and body temperature were recorded twice (the first and second tests) before and after the pups were put in various environments for 10 min. The environments were no maternal separation (Control: CON), maternal separation with littermates (LMS), and single maternal separation with a heater (SMS). In the second test, the SMS pups had fewer USVs, a lower body temperature, and a more rapid righting reflex than the CON and LMS pups. In addition, there was no strong correlation between USVs and righting reflex. As a result, pups undergoing 10 min of SMS while being kept warm by the heater showed rapid righting reflex. Thus, by a single maternal separation, the number of USVs decreased but the decrease was unrelated to decrease in motor function.

KEY WORDS: anxiety-like behavior, maternal separation, motor function, rat pup, ultrasonic vocalization

J. Vet. Med. Sci.
81(2): 287–293, 2019
doi: 10.1292/jvms.18-0604

Received: 19 October 2018
Accepted: 23 December 2018
Published online in J-STAGE: 15 January 2019

Rat pups emit 20- to 60-kHz ultrasonic vocalizations (USVs) when they are isolated from their mothers and littermates [8]. These USVs are a rodent pups-specific response shown before weaning. Postnatal rat pups emit USVs to tell their mothers where they are and to induce maternal behaviors that are essential for their survival [3, 11].

Previous studies have reported that rat pups' USVs just after maternal separation were reduced by anxiolytic and antidepressant agents [4, 8]. This suggests that pups' USVs are anxiety-like behaviors that are induced by maternal separation. However, previous studies have reported that USVs are related to nonemotional factors, such as physiological reactions to coldness [1, 2, 9].

The aim of this study was to examine whether pups' USVs after maternal separation were related to nonemotional factors, such as body temperature and motor function. A previous study has reported that pups' USVs are reduced by the presence of anesthetized littermates [5]. Therefore, this study examined both single pups separated from their mothers (single maternal separation, SMS) and pups separated from their mothers but kept with their littermates (littermate maternal separation, LMS). Based on a previous study [4], the righting reflex test was employed to assess motor function in these pups.

MATERIALS AND METHODS

Animals

Pregnant female Wistar–Imamichi rats were purchased from the Institute for Animal Reproduction (Ibaraki, Japan) and kept under standard ambient lighting (10:00–22:00 hr) at room temperature (25 ± 2°C) and humidity 55 ± 10%. The mothers and pups were housed in 200 × 410 × 250-mm plastic cages and had *ad libitum* access to food and water. The day of birth was counted as postnatal day (PND) 0. The litters were adjusted to eight per mother with at least three males at PND1. All procedures in this study were performed in accordance with the Guidelines of the Animal Care and Use Committee of Meiji University.

USV test and measurement of body temperature

The USV measuring apparatus (Fig. 1) comprised a glass petridish (Tokyo Glass Kiki, Tokyo, Japan), microphone (CO-100K,

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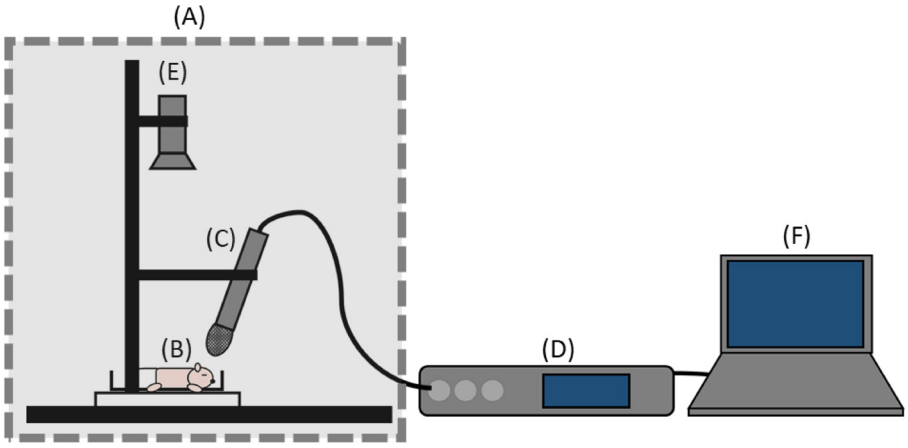

Fig. 1. Pattern diagram of the ultrasonic vocalizations (USVs) measuring apparatus. (A) A soundproof box. (B) A pup is isolated in a glass petridish. (C) A microphone to record USVs. (D) An amplifier to amplify USVs. (E) A compact camera to observe pups. (F) A personal computer to visualize USVs on the screen.

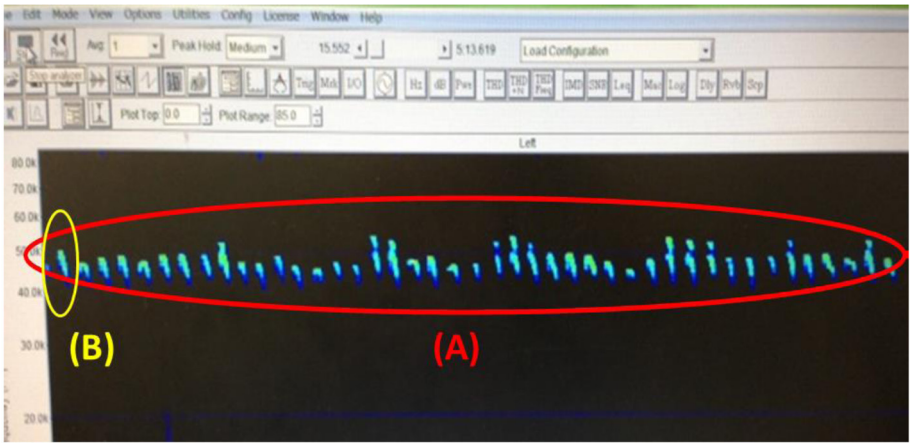

Fig. 2. PC screen of visualization of individual USVs. (A) Pups' USVs. (B) It is counted as 1 USV.

Sanken Microphone, Tokyo, Japan), and compact camera (TAV308DC, SPACECOM, Tochigi, Japan). The equipment was connected to an amplifier (OCTA-CAPTURE, Roland Corp., Shizuoka, Japan) to amplify and visualize the pups' USVs on the computer screen using the ultrasonic measuring software, Spectra PLUS 5.0 (Pioneer Hill Software, Poulsbo, WA, U.S.A.) (Fig. 2). The equipment was placed in a 160 × 100 × 110-cm soundproof box. Body surface temperature was measured on the backs of pups using an infrared thermometer (400-TST805, Sanwa Supply, Okayama, Japan) immediately before each USV test.

Motor function test

Righting reflex test was conducted after USV tests to assess motor function. To assess righting reflex latency, the pups were placed flat on their backs and the latency to reach an upright position with all four paws flat on the surface was recorded (Fig. 3). The pups were given a maximum of 10 sec to complete the task.

Experimental schedule

Effects of maternal separation on pups' USVs, body temperature, and motor function were examined. The experimental schedule is shown in Fig. 4. All procedures were conducted between 10:00–18:00 hr. Experiments were performed in the following four steps: Step 1. The first USV test; Step 2. A 3 hr interval with the pup in the home cage with its mother and littermates; Step 3. 10 min of conditioning with or without maternal separation; and Step 4. The second USV test. Body temperature was measured before the first and second USV tests, and motor function was measured after the first and second USV tests. The pups were assigned to three groups. Pups in the control group were not isolated during the 10 min of conditioning at step 3 (control: CON pups); pups in the maternal separation with littermates group were separated from their mother but kept with their littermates during the 10 min of conditioning (maternal separation with littermates: LMS pups) (Fig. 5a); and pups in the single maternal

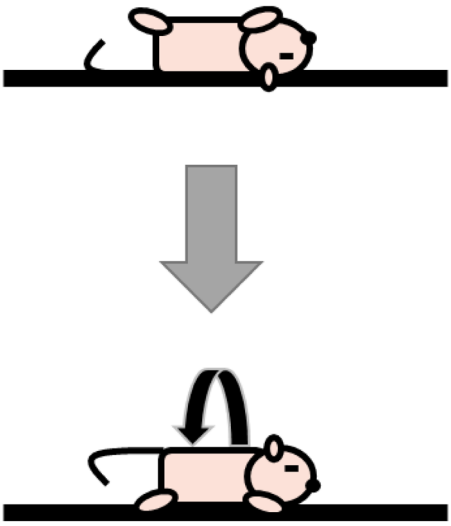

Fig. 3. Righting reflex test. Righting reflex test was conducted after USV tests to assess motor function. To assess righting reflex latency, pups were placed flat on their backs and, latency to reach an upright position with all four paws flat on the surface was recorded. Pups were given a maximum of 10 sec to complete the task.

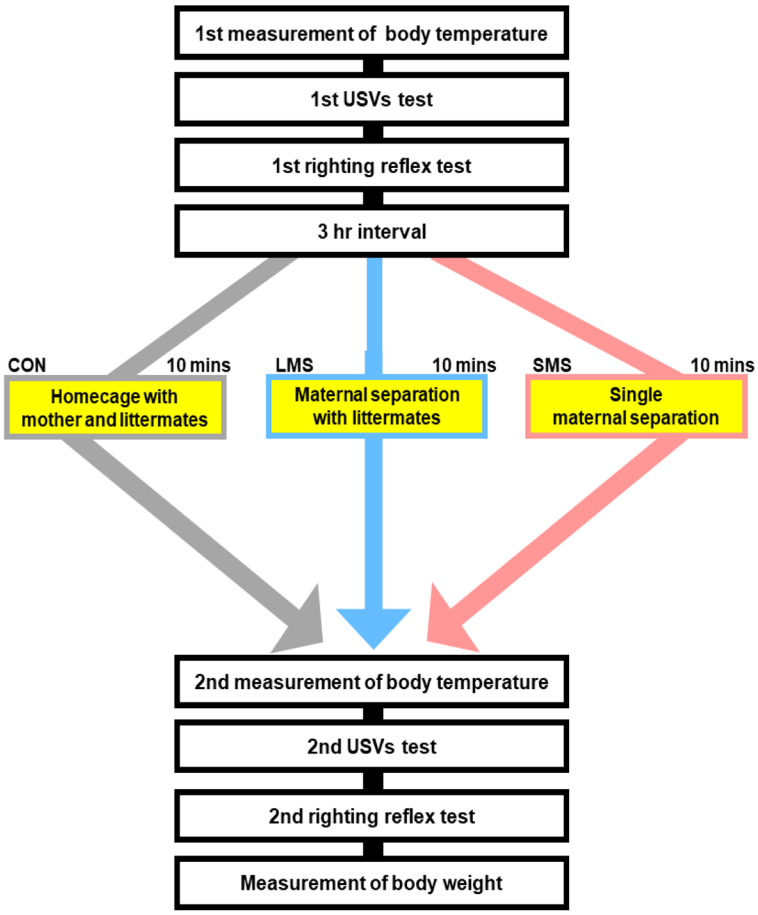

Fig. 4. Experimental schedules. USVs, ultrasonic vocalizations; CON, no maternal separation (control); LMS, maternal separation with littermates; SMS, single maternal separation.

separation group were separated from their mothers and littermates and kept alone during the 10 min of conditioning (single maternal separation: SMS pups) (Fig. 5b). The number of mothers in each group was unified to 9 or 10 to ensure that there was no big difference between the groups (mothers of CON and LMS, n=10; mothers of SMS, n=9). Further, the number of pups used for the experiments was unified to 3 or 4 per litter.

According to unpublished data (Maiko Kawaguchi), Wistar-Imamichi rat pups emit the most frequencies of USVs at PND5. This study aimed to examine the effect of maternal separation on the USVs of pups, therefore, PND5 was identified to be an appropriate age to observe the impact on the USV. At PND5, male pups took the USV test twice, designated as the first and second USV tests. The first USV test was to evaluate basic ability in the number of USVs produced by the pup, and the second USV test examined whether maternal separation, with or without littermates, affected the number of USVs produced by the pup. In the first USV test immediately after measurement of body temperature, each pup was taken from the home cage and kept in the petridish of the measuring equipment for 5 min at room temperature. During the first USV test, the home cage was kept warm by a heater (RH-101, Marukan, Osaka, Japan) and covered by a blackout curtain to suppress the mother's stress. After the first USV test, the pups were tested for their righting reflex and then returned to their home cages. After a 3-hr interval, the CON pups were kept in their home cages until the second USV test. The LMS pups were separated from their mothers and placed in the petridish on the heater for 10 min. The SMS pups were placed alone in the petridish on the heater for 10 min. Subsequently, the measurement of body temperature, the second USV test, and righting reflex tests were conducted, as in the first test. Body weight was measured after each procedure. After each USV test, the petridish was cleaned with 70% ethanol and tap water and wiped with a paper towel to eliminate odor and stains. During the USV test, the pups were monitored using a compact camera. USVs were recorded for 5 min and measured using Spectra PLUS 5.0. The number of USVs emitted by each pup was counted using a counter by visual judgment.

Statistical analysis

All results are expressed as mean ± standard error of the mean. All analyses were conducted using IBM SPSS Statistic 22 (IBM Corp., Armonk, NY, U.S.A.). The number of USVs and body temperature were compared among the CON, LMS, and SMS pups by repeated-measures analysis of variance (ANOVA) using Tukey-Kramer test. One-way ANOVA was used for comparisons of

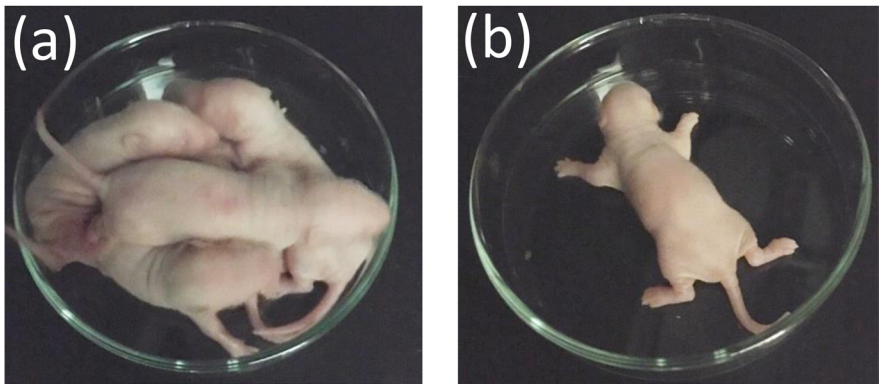

Fig. 5. Environments during 10 min of conditioning. (a) The LMS pup was put with 3 or 4 littermates in a petri dish. (b) The SMS pup was put in a petridish on the heater alone.

Table 1. Body weights, body temperature and the number of USVs

	CON (*n*=32)	LMS (*n*=31)	SMS (*n*=27)
Body weights (g)	11.782 ± 0.2	12.238 ± 0.2	11.727 ± 0.2
Body temperature before the first USV test	36.2 ± 0.1	36.4 ± 0.1	36.1 ± 0.1[b)]
Body temperature before the second USV test	36.4 ± 0.1	36.6 ± 0.1	36.2 ± 0.1[a,b)]
Change in body temperature	0.25 ± 0.1	0.23 ± 0.1	0.14 ± 0.1
The number of USVs in the first USV test	455.5 ± 27.9	498.9 ± 23.8	438.6 ± 23.7
The number of USVs in the second USV test	363.3 ± 35.0	382.6 ± 28.0	218.4 ± 29.3[a,b)]

Values are means ± standard error of the mean. Body temperature change means the value of body temperature before the second USV test minus body temperature before the first USV test. a) and b) indicate significant differences (a) $P<0.05$ versus CON pups, b) $P<0.05$ versus LMS pups).

body weight and change in body temperature. Kaplan–Meier survival analysis was used for comparisons among the CON, LMS, and SMS pups in the righting reflex test. Differences were considered significant when the *P*-value was <0.05.

RESULTS

Number of USVs and body weight

Results of USVs and body weights are shown in Table 1. There were no significant differences among the three groups in the results of the first USV test. In the second USV test, the SMS pups emitted significantly fewer USVs than the CON and LMS pups (CON vs. SMS, $P=0.002$; LMS vs. SMS, $P<0.001$). Furthermore, in each group, the number of USVs in the second USV test was less than that in the first USV test (all groups, $P<0.001$). Statistical values were as follows: main effect of stage, F[1.87]=73.643, $P<0.001$; interaction of stage and group, F[2.87]=5.366, $P=0.006$; main effect of group, F[2.87]=52.257, $P=0.007$. There were no significant differences among the three groups in body weight.

Motor function

The results of motor function are shown in Fig. 6. There were no significant differences among the three groups in the results of the righting reflex test after the first USV test (Fig. 6a). However, in the righting reflex test after the second USV test, the SMS pups showed significantly shorter latency than the CON and LMS pups (Fig. 6b, CON vs. SMS, $P<0.001$; LMS vs. SMS, $P=0.006$).

Body temperature

The results of body temperature are shown in Table 1. Before the first USV test, the SMS pups had significantly lower body temperatures than the LMS pups (LMS vs. SMS, $P=0.009$). Before the second USV test, the SMS pups had significantly lower body temperatures than the CON and LMS pups (CON vs. SMS, $P=0.026$; LMS vs. SMS, $P<0.001$). Statistical values were below: main effect of stage, F[3.261]=236.448, $P<0.001$; interaction of stage and group, F[6.261]=0.611, $P=0.721$; main effect of group, F[2.87]=7.707, $P=0.001$. The values of body temperature before the second USV test minus body temperature before the first USV test were compared as the change in body temperature. As a result, there was no significant difference (F[2.87]=0.470, $P=0.626$, CON vs. LMS, $P=0.986$; CON vs. SMS, $P=0.630$; LMS vs. SMS; $P=0.730$).

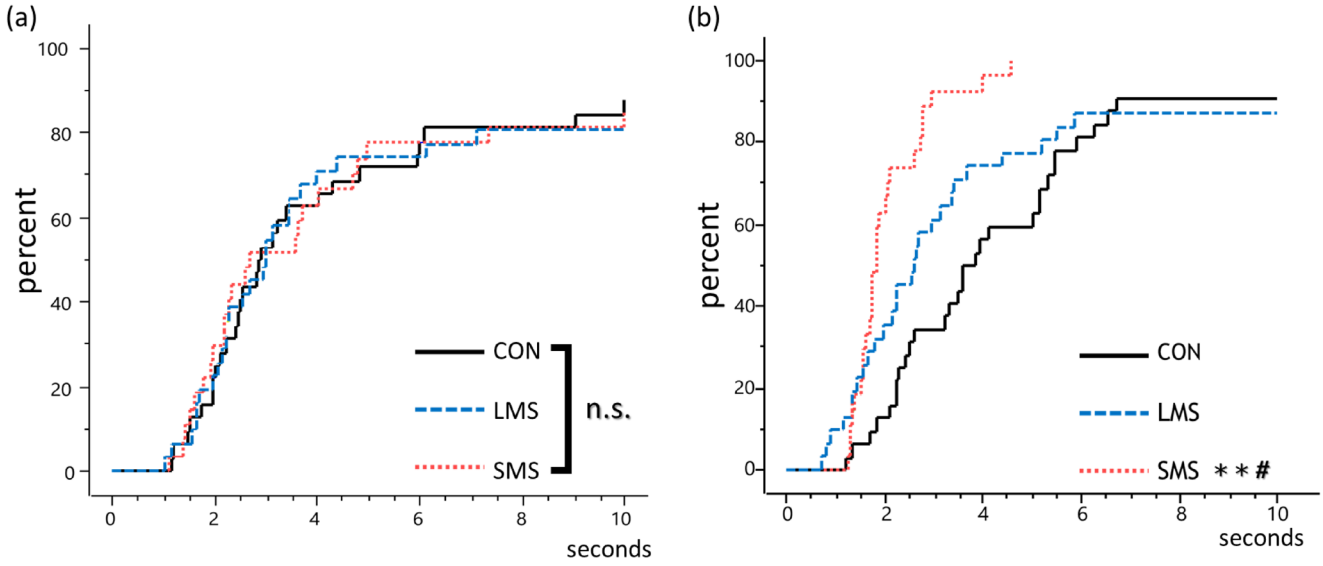

Fig. 6. (a) Latency in the first righting reflex test. CON, *n*=32; LMS, *n*=31; SMS, *n*=27. (b) Latency in the second righting reflex test. CON, *n*=32; LMS, *n*=31; SMS, *n*=27. CON, no maternal separation (control); LMS, maternal separation with littermates; SMS, single maternal separation. ** and # indicate significant differences (**P<0.001 vs. CON pups, #P<0.05 vs. LMS pups).

Table 2. Correlation about motor functions and USVs

	ALL (*n*=90)	CON (*n*=32)	LMS (*n*=31)	SMS (*n*=27)
The number of USVs between the first and second USV tests	r=0.509[a)] P<0.001	r=0.547[a)] P=0.001	r=0.698[a)] P<0.001	r=0.163 P=0.417
The number of USVs in the first USV test and latency in the first righting reflex test	r=0.097 P=0.361	r=0.451[a)] P=0.010	r=−0.304 P=0.097	r=0.078 P=0.698
The number of USVs in the second USV test and latency in second righting reflex test	r=−0.123 P=0.246	r=−0.296 P=0.100	r=−0.461[a)] P=0.009	r=0.139 P=0.488

Significant strong correlation: a) P<0.01.

Correlations

Results of correlations are shown in Table 2. In the CON and SMS pups, there were no strong correlations between the results of the second USV and second motor function tests. However, in the LMS pups, a correlation was observed (P=0.009, r= −0.461), indicating that the LMS pups that emitted several USVs in the second USV test showed a short latency in the righting reflex test. Between the CON and LMS pups, there was a strong correlation between the number of USVs measured in the first and second tests (CON, P=0.001, r=0.547; LMS, P<0.001, r=0.698). This result indicates that the CON and LMS pups that emitted several USVs in the first test emitted several USVs in the second test. This correlation was not found in the SMS pups.

DISCUSSION

In this study, we examined the influence of maternal separation environments on subsequent USVs, motor function, body temperature, and their correlations in rat pups at PND5. Three types of environments were prepared, namely, a group without isolation (CON pups), a group of maternal separation with littermates (LMS pups), and a group of single maternal separation with heater (SMS pups). As a result, in the second test, the SMS pups had fewer USVs, a lower body temperature, and a more rapid righting reflex than the CON and LMS pups. In addition, there was no strong correlation between USVs and righting reflex.

There were no significant differences among the three groups in the number of USVs in the first USV test. However, in the second USV test, the SMS pups emitted significantly fewer USVs than the CON and LMS pups. This indicates that SMS can reduce the number of later USVs. This result is inconsistent with previous studies that have shown that the number of USVs increased by maternal separation [5, 12]. In the present study, a heater was used during the 10 min of SMS. Because the SMS pups were kept warm for 10 min while separated from their mothers before the second USV test, they may have shown fewer USVs in the second test as a result of the lack of need for USVs. Also, this study used PND5 rat pups, unlike the previous studies [5, 12] that used PND14 mouse pups. The differences between the results of this study and previous studies may have been due to differences in the age and species of the pups. In the second USV test, there was no significant difference between the CON and LMS pups. The LMS pups were also isolated similar to the SMS pups during the 10 min of conditioning. However, their USVs

were not affected because of maternal separation with littermates. Thus, we have concluded that because other rats (mothers and/or littermates) were housed with test pups, the LMS pups may have shown the results similar to those of the CON pups in the second USV test. Further, this result is similar to that observed in a previous study [5].

Correlation analysis of the ALL, CON, and SMS pups found that the number of USVs was not related to righting reflex in the second test except for the LMS pups. This result indicates that decreased number of USVs in the second test in the SMS pups was not due to decreased motor function. Moreover, there was a strong correlation between the number of USVs in the first and second USV tests in the CON and LMS pups but not in the SMS pups. Further, the individual ability to emit USVs was more important than environmental factors in the CON and LMS pups but not in the SMS pups.

The righting reflex was examined to consider relationships between motor function and USVs in rat pups. There were no significant differences among the three groups in the results of the first righting reflex test, indicating that there were no intergroup differences in the basic activity related to the righting reflex test. In the second righting reflex test, the SMS pups had shorter latency than the CON and LMS pups. According to the SMS pups' fewer second USVs and rapid righting reflex indicating increased motor function, fewer USVs in SMS pups were not caused by decreased motor function. Previous studies have assessed the relationship between pups' motor activity and maternal separation [6, 7]. Although one day of maternal separation reduced motor activity in rat pups at PND12 [7], rat pups at PND14 became active after maternal separation [6]; this finding is consistent with that of the second righting reflex test in the SMS pups in the present study.

Before the first USV test, the SMS pups had lower body temperatures than the LMS pups. There was a possibility that the SMS mothers' maternal behaviors, including heat insulation, were not active than the others, therefore, the SMS pups could not be kept sufficiently warm. Also, before the second USV test, the SMS pups had lower body temperatures than the CON and LMS pups. This is possibly because the SMS pups were separated from their mothers and littermates for 10 min just before the second USV test, and it may not possible to receive enough heat insulation. However, there was a significant difference in body temperatures between the SMS and LMS pups before the first test. Moreover, there was no difference in the change body temperature among the CON, LMS, and SMS pups, indicating that the change in body temperature does not differ depending on the separation environment. Therefore, the original difference in body temperature may most likely be reflected on the body temperature before the second USV test. Previous studies [1, 2, 9] have stated that rat pups' USVs are emitted simply as byproducts of physical reflexes to hypothermia. Therefore, the problem that the intergroup differences in second USVs are not by emotional difference of separation environment but by differences in the body temperatures is encountered. However, in the present study, although the body temperature of the LMS and SMS pups differed before the first USV test, there was no difference between these two groups in the number of USVs in the first test. Moreover, the SMS pups with lower body temperature than the other pups emitted fewer USVs in the second test. According to previous studies [1, 2, 9], we predicted that the SMS pups showing lower body temperature than the CON and LMS pups would show more USVs in the second USV test. However, we found that the group differences in body temperature might not always be reflected in the group differences of USV. Low body temperature is surely a major factor in pups' USVs, but the difference in the number of USVs is based not only on differences in body temperature but also on other factors, such as anxiety, activity, and the presence of littermates.

Generally, rat pups' USVs induce maternal behavior, such as nursing, heat incubation, anogenital licking, and retrieving [3, 11]. In the present study, the SMS pups did not experience maternal behaviors for 10 min, and the number of USVs in the second test decreased. The lack of response from mothers may have reduced emitting USVs to call mothers and/or habituation to maternal separation in the SMS pups. This habituation may have been promoted by the presence of the heater in the absence of the mother. The greater number of USVs in the second test in the CON and LMS pups than in the SMS pups could have been caused by the deprivation of familiar rats, their mothers, and littermates. A previous study has reported that rat pups emit several USVs after being deprived of their mothers [10]. In addition, pups in all groups emitted significantly fewer USVs in the second test than in the first test. Because this decrease occurred in all pups, it is highly likely that the decrease in the second USV test was caused by the experience of the first USV test rather than by 10 min of conditioning.

In conclusion, a 10-min separation of rat pups at PND5 from their mothers and littermates while keeping them warm resulted in a decrease in USVs. Moreover, the SMS pups showed rapid righting reflex, indicating that single maternal separation could activate the motor function of pups related to righting reflex.

REFERENCES

1. Blumberg, M. S. and Sokoloff, G. 2001. Do infant rats cry? *Psychol. Rev.* **108**: 83–95. [Medline] [CrossRef]
2. Blumberg, M. S., Sokoloff, G., Kirby, R. F. and Kent, K. J. 2000. Distress vocalizations in infant rats: what's all the fuss about? *Psychol. Sci.* **11**: 78–81. [Medline] [CrossRef]
3. Brunelli, S. A., Curley, J. P., Gudsnuk, K., Champagne, F. A., Myers, M. M., Hofer, M. A. and Welch, M. G. 2015. Variations in maternal behavior in rats selected for infant ultrasonic vocalization in isolation. *Horm. Behav.* **75**: 78–83. [Medline] [CrossRef]
4. Hodgson, R. A., Guthrie, D. H. and Varty, G. B. 2008. Duration of ultrasonic vocalizations in the isolated rat pup as a behavioral measure: sensitivity to anxiolytic and antidepressant drugs. *Pharmacol. Biochem. Behav.* **88**: 341–348. [Medline] [CrossRef]
5. Hofer, M. A. and Shair, H. 1978. Ultrasonic vocalization during social interaction and isolation in 2-weeek-old rats. *Dev. Psychobiol.* **11**: 495–504. [Medline] [CrossRef]
6. Hofer, M. A. and Shair, H. N. 1987. Isolation distress in two-week-old rats: influence of home cage, social companions, and prior experience with littermates. *Dev. Psychobiol.* **20**: 465–476. [Medline] [CrossRef]
7. Hofer, M. A., Brunelli, S. A. and Shair, H. N. 1993. The effects of 24-hr maternal separation and of litter-size reduction on the isolation-distress

response of 12-day-old rat pups. *Dev. Psychobiol.* **26**: 483–497. [Medline] [CrossRef]
8. Iijima, M. and Chaki, S. 2005. Separation-induced ultrasonic vocalization in rat pups: further pharmacological characterization. *Pharmacol. Biochem. Behav.* **82**: 652–657. [Medline] [CrossRef]
9. Kromkhun, P., Katou, M., Hashimoto, H., Terada, M., Moon, C. and Saito, T. R. 2013. Quantitative and qualitative analysis of rat pup ultrasonic vocalization sounds induced by a hypothermic stimulus. *Lab. Anim. Res.* **29**: 77–83. [Medline] [CrossRef]
10. Shair, H. N., Masmela, J. R., Brunelli, S. A. and Hofer, M. A. 1997. Potentiation and inhibition of ultrasonic vocalization of rat pups: regulation by social cues. *Dev. Psychobiol.* **30**: 195–200. [Medline] [CrossRef]
11. Wöhr, M., Borta, A. and Schwarting, R. K. 2005. Overt behavior and ultrasonic vocalization in a fear conditioning paradigm: a dose-response study in the rat. *Neurobiol. Learn. Mem.* **84**: 228–240. [Medline] [CrossRef]
12. Yin, X., Chen, L., Xia, Y., Cheng, Q., Yuan, J., Yang, Y., Wang, Z., Wang, H., Dong, J., Ding, Y. and Zhao, X. 2016. Maternal Deprivation Influences Pup Ultrasonic Vocalizations of C57BL/6J Mice. *PLoS One* **11**: e0160409. [Medline] [CrossRef]