Research Article

Animal Behavior

Title:

Sex differences in vocalizations to familiar or unfamiliar females in mice

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Abstract

Mice, both wild and laboratory strains, emit ultrasounds to communicate. The sex differences between male to female (male–female) and female to female (female–female) ultrasonic vocalizations (USVs) have been discussed for decades. In the present study, we compared the number of USVs emitted to familiar and unfamiliar female by both males (male–female USVs) and females (female–female USVs). We found that females vocalized more to unfamiliar than to familiar females. In contrast, males exhibited more USVs to familiar partners. This sexually dimorphic behavior suggests that mice change their vocal behavior in response to the social context, and their perception of the context is based on social cognition and memory. In addition, because males vocalized more to familiar females, USVs appear to be not just a response to novel things or individuals, but also to be a social response.

Keywords: ultrasonic vocalizations, social behavior, affiliation, social cognition, and memory

1. Introduction

Social animals utilize signals for some modalities to communicate with other individuals. Among such signals, vocal communications are widely observed in animals and are superior in many aspects [1]. Recently, vocal communication in mice has attracted considerable academic attention because of its power and utility in the investigation of molecular and neural mechanisms for social behaviors and their deficits [2–4]. In mice, ultrasonic vocalizations (USVs) are used for communication, and they can be widely observed in both wild and laboratory strains [5]. There are two types of USVs commonly known in mice: pupUSVs and courtship vocalizations [4]. Mice pups in the first several days after birth emit ultrasounds (pupUSVs), mainly when they are outside their nest. A state of arousal due to the perception of cold, perception of unusual tactile stimulation, or loss of social contact triggers the emission of pupUSVs, which enhance the mother's approach to the sound source [6]. The mother's attention and movement lead to the expression of maternal behavior. On the other hand, adult male mice emit ultrasounds in the presence of adult females or her urine [7]; these are known as courtship vocalizations or courtship songs [8]. Activational effects of sex hormones

on male vocalizations have been reported [9,10]. Furthermore, the number of male USVs is enhanced by sociosexual experience with females [11], and usage of syllable types (call patterns) is modulated according to the progressive phase of sexual behaviors [12]. Thus, USVs from males to females are thought to be male-specific precopulatory behaviors [11,13].

In this regard, vocalizations in mice involved in these two contexts have been well investigated since they were first reported about fifty years ago [14–16]. In the process of such research history, adult females were initially not thought to vocalize either to males or females [17]. However, using several strains, Maggio and Whitney thoroughly revealed that adult females emit USVs to females (female–female), where they rarely emit to males (female–male) and that the number of female–female USVs is comparable to male–female courtship USVs [18]. Thereafter, a study observing female–female USVs in albino NMRI female mice indicated that females vocalize more to unfamiliar females than to familiar ones [19]. This finding suggests that female–female USVs reflect social cognition and memory. In fact, administration of a classical cholinergic antagonist (scopolamine) disrupted this social memory [19]. Although

female–female USVs are known to be related to this social memory, the functions of these USVs is poorly understood.

In the study by D'Amat et al. [19] described above, familiar individuals were those that were previously presented only several minutes in advance (while simultaneously recording ultrasound). Therefore, we decided to use female individuals that were reared in the same cage after weaning as familiar individuals. In other words, we hypothesized that female–female USVs reflect social affiliation if females emit USVs more to familiar females than to unfamiliar ones under such conditions. If this hypothesis is correct, the results of the study by D'Amat et al. and our expected results could be explained as follows: females express high levels USVs to establish an affiliative relationship with a stranger of the same sex first, and, at the same time, females also emit more USVs to familiar social members. In the present study, we also compared female–female vocalizations and male–female vocalizations using the B6 strain, which is currently the most commonly used laboratory strain. Social cognition and memory in male mice has not yet been measured in this context, but it is important to measure the sociality of both sexes using the same methods because mouse models

and USVs have been recently investigated to clarify the basic mechanisms of developmental disorders [2–4].

2. Materials and Methods

(a) Animals

We used an inbred strain of B6 (C57BL/6J) mice. Mice were purchased from Japan SLC (Hamamatsu, Japan). Subject animals were used when they were 8–10 weeks-old for recording tests.

For recordings of male–female USVs, a male and an age-matched unfamiliar female mouse were cohoused in a standard cage (182 × 260 × 128 mm, CREA Japan) immediately following arrival from the supplier until experiments began.

For recordings of female–female USVs, females that were bred together in the same cage after weaning were purchased, and two individuals were housed in each cage immediately after arrival from the supplier until experiments began. One was used as the subject, and the other was used as the familiar partner (see Fig. 1A).

For both recordings, age-matched, unfamiliar adult females were also purchased, and 3–4 animals were housed in the same cage. These animals were used repeatedly as an unfamiliar intruder for recordings (see below and Fig. 1A).

Food and water were supplied *ad libitum*, and the animals were kept under a standard 12-h:12-h light-dark cycle. The environment was maintained at a constant temperature (22–25°C) and humidity (50 ± 5%). All experimental procedures were approved by the Institutional Animal Use Committee of Kagoshima University (#L18004 and #L19003).

(b) Experimental design and general procedures.

The subject male was cohoused with an unfamiliar female, and the subject female was cohoused with a female that was bred together with the subject female in the same cage after weaning, as described above. These male–female and female–female were cohoused for 7 days without exchange of beddings. At the 7th day (Day 7 shown in Fig. 1A) after the pairing of male–female and female–female began (Day 0 shown in Fig. 1A), the partner female was removed. Recordings of USVs were conducted twice

(recording 1 and 2) for all subjects, and two experimental conditions were tested in both male–female and female–female contexts; some subjects were used for recording 1 30 min after the removal of the partner, and the other subjects were used 1 day (24–25 h) after the removal. Recording 2 was conducted 30 minutes after recording 1 for all subjects. USVs were induced by introducing a female mouse. The subjects were exposed to the familiar partner and an unfamiliar female once each, and the order of encountering the familiar or unfamiliar partner was counter-balanced between recordings 1 and 2.

(c) Settings and procedures for ultrasound recordings

The microphone was hung 16 cm above the floor of a sound-attenuating chamber. Immediately prior to recording, the home cage of the subjects was moved into the chamber where a red dim light was placed. The ultrasound recording was performed for 100 s after the intruder was introduced into the home cage.

According to previous studies [12,20,21], we conducted recordings without the devocalization of female encounters under natural conditions. Previous experiments

suggested that most vocalizations are produced by the resident subjects and that the vocal contribution of female encounters is very limited in the male–female and female–female contexts [22–24].

(d) Ultrasound analysis

Most of the settings for the USV recordings followed those of our previous study [11], with some modifications. Ultrasonic vocalizations were recorded using a CM16/CMPA condenser microphone (Avisoft Bioacoustics, Berlin, Germany), ultrahigh-speed ADDA converter BSA768AD-KUKK1710 and its software SpectoLibellus2D (Katou Acoustics Consultant Office, Kanagawa, Japan) with a sampling rate of 384 kHz (to measure 20-192 kHz).

Recorded sounds were saved on a computer as .wav files using SpectoLibellus2D. These sound files were analyzed with the GUI-based software USVSEG (implemented as MATLAB scripts) recently developed by us [25]. Continuous sound signals with frequencies ranging from to 40–160 kHz and durations ranging from 3 to 300 ms were analyzed and detected as syllables. Each syllable was

segmented and saved as a jpeg image file, and the noise data (false positives) were manually excluded. Finally, the number of vocalizations (syllables) was quantified.

(e) Statistics

Statistical analyses were performed using JMP 14 software (SAS Institute, Cary, NC, USA). In the present study, most of the data seemed not to be distributed normally; therefore, nonparametric tests were used. To compare two conditions within an individual, Wilcoxon signed-rank tests were applied (see Fig. 1), and Kruskal–Wallis tests were used for comparison among groups, followed by post-hoc Steel–Dwass tests (see Fig. 2). Values are reported as dot plots with boxplots. Differences with p values $P < 0.05$ were considered significant.

3. Results

(a) Female–female vocalization

Under the 30 min isolation condition, the number of USVs emitted from

subject females to unfamiliar female was significantly higher than to the familiar partner (Fig. 1B; Wilcoxon signed-rank test, $W_{(14, 14)} = 39.0$, $P = 0.02$). On the other hand, there was no significant difference between the number of USVs emitted to the unfamiliar and familiar partner after 1 day isolation (Fig. 1C; Wilcoxon signed-rank test, $W_{(11, 11)} = 3.0$, $P = 0.9063$).

(b) Male–female vocalization

Under the 30 min isolation condition, there was no significant difference between the number of USVs emitted from males to unfamiliar and familiar partners (Fig. 1D; Wilcoxon signed-rank test, $W_{(15, 15)} = 26.0$, $P = 0.1758$). On the other hand, the number of USVs emitted to the familiar partner was significantly higher than that of the unfamiliar female after 1 day isolation (Fig. 1E; Wilcoxon signed-rank test, $W_{(15, 15)} = -47.5$, $P = 0.0067$).

(c) Total comparison among groups

Differences between the number of USVs emitted to familiar and unfamiliar

partners ([#. familiar] - [#. unfamiliar]) were calculated from the data shown in Fig.1 and were compared among the 4 groups (Fig. 2). The Kruskal–Wallis test indicated significant differences between groups (χ^2 = 19.4288, *df* = 3, *P* = 0.0002) and the Steel–Dwass test (performed *post-hoc*) showed that the value of males under the 1 day isolation condition was significantly higher than that of males or females under the 30 min isolation condition (*P* = 0.0085 and *P* = 0.0025, respectively), or females under the 1day isolation condition (*P* = 0.0337).

4. Discussion

As described in the Introduction, we prepared female individuals with affiliative relations. Nevertheless, contrary to our expectation, the result of the previous study of D'Amat et al. [19] was replicated in our 30 min isolation condition (Fig. 1B); here we found that females vocalized more to an unfamiliar female than to familiar females. It is unclear as to why females rarely vocalized under the 1day isolation condition, but no difference was found between the number of USVs to the unfamiliar

and familiar females under such conditions, as was the case in D'Amat et al's studies. We believe that it is safe to conclude that B6 females vocalize more to unfamiliar females.

On the other hand, it was surprising that males exhibited an opposite tendency toward females. It has been reported that male courtship USVs are enhanced by sociosexual experience [11] and that vocal usage is altered according to the progressive phase of sexual behavior [12]. These results indicate that male USVs directed towards females express sexual motivation. We expected males to show more vocalization to unfamiliar females; however, this was not a "flirtatious"' tendency, as males made many vocalizations to the familiar partner. Even though different experimental conditions, such as length of cohousing, could possibly have led to different results, it appears that males exhibited more sexual motivation toward the familiar partner (cohoused for 1 week) under the present conditions.

Although it is not clear why the optimal conditions for the observation of differential vocal responses to the familiar or unfamiliar females differ between males and females, the important finding of this study is that there is a sex difference in such

vocal responses. For decades, the sex differences between male–female and female–female USVs have been discussed. The amount of USVs between male–female and female–female is comparable [18]. In addition, the vocal repertoire between sexes is also reported to be comparable [23]. In contrast, the sex differences shown in the present study indicate that mice change their vocal behavior in response to the context of whom they are dealing with; their perception of the context is based on social cognition and memory. In addition, because males vocalized more to familiar females, USVs are thought to be not just a response to novel things or individuals but also to be a social response. These findings with wild-type mice provide a practical behavioral assay for the disease model mice, focusing on their social impairment.

Although such sex differences in USVs have been revealed, the past and present studies have yet to determine the biological significance and function of female–female USVs. Since Maggio and Whitney [18] hypothesized that female vocalization contributes to the formation of social hierarchy among females, this hypothesis has been referred to in some studies [19,23]. However, to our knowledge, this hypothesis has never been investigated experimentally in mice. A recent study made

an important point about sex differences in acoustic features, with more diversity (inter-individual difference) observed in the vocal repertoire in males than in females [26]. Nonetheless, the biological significance and function of female USVs remain unclear, and further studies, including investigation of the associated neural mechanisms, are needed.

Ethics. All experimental procedures were approved by the Institutional Animal Use Committee of Kagoshima University (#L18004 and #L19003).

Data accessibility. The dataset supporting this article are available from the figshare Digital Repository: XXXXXXXXX

Authors' contributions K.K. contributed to the experimental design. E.S. and Y.T. conducted the experiments. E.S., Y.T., and K.K. contributed to the interpretation of data and writing of the manuscript.

Competing interests. There are no conflicts of interest to declare.

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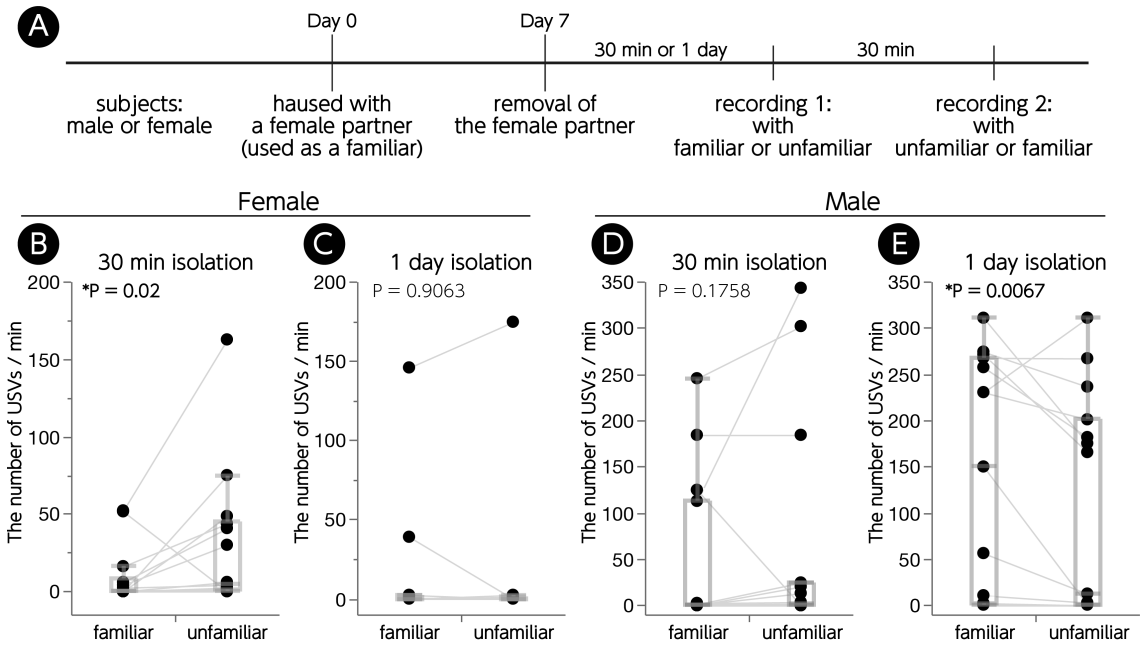
1 **Legends for figures**



2 **Fig. 1. Male and female USVs to familiar or unfamiliar females.** (A) Schematic

3 diagram of the experimental procedure. (B) Female USVs to the familiar or unfamiliar

4 females under the condition of 30 min isolation before recording 1. (C) Females' USVs

5 to the familiar or unfamiliar females under the condition of 1day isolation before

6 recording 1. (D) Male USVs to the familiar or unfamiliar females under the condition of

7 30 min isolation before recording 1. (E) Male USVs to familiar or unfamiliar females

8 under the condition of 1day isolation before recording 1. *$P < 0.05$, Wilcoxon

9 signed-rank test; B, n = 11; C, n = 14, D and E, n = 15.

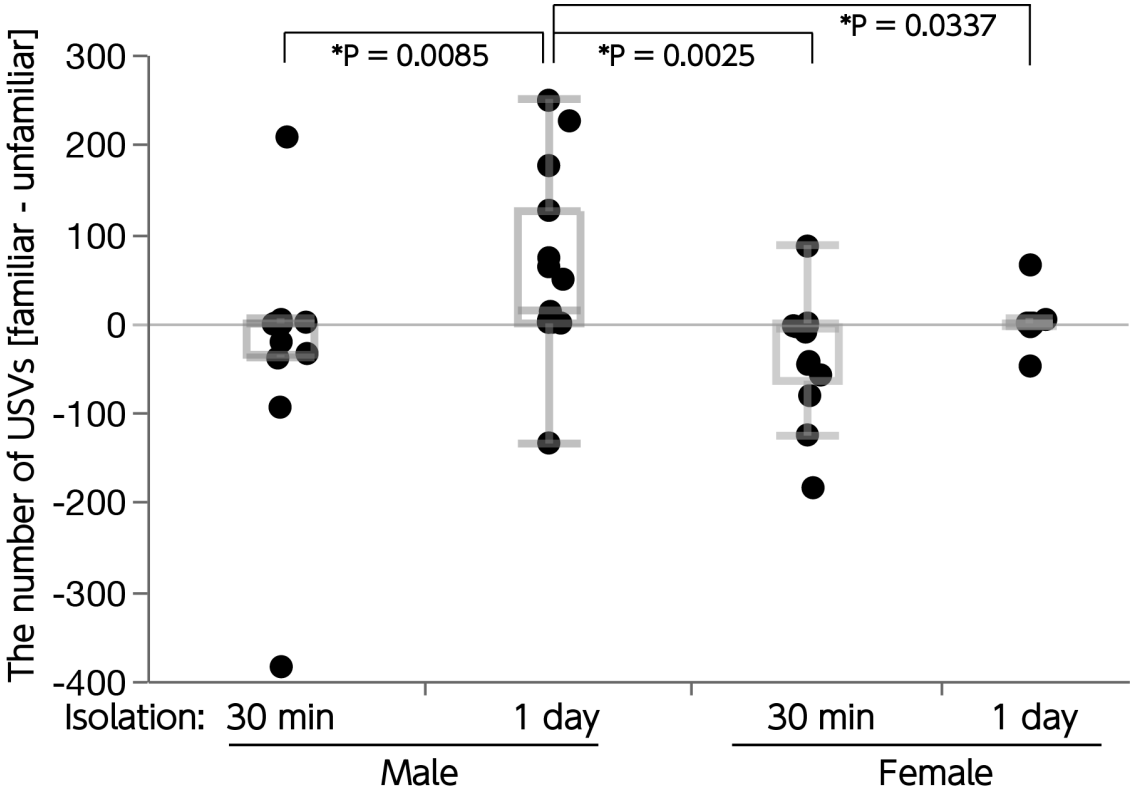


Fig. 2. Sex differences in vocal response to familiar or unfamiliar females. Total comparison among groups is shown in Fig. 1. *P < 0.05, Steel–Dwass test after Kruskal–Wallis test; male 30 min and 1day isolation, n = 15; female 30 min isolation, n = 11, female 1day isolation, n = 14.