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Air puff-induced 22-kHz calls in F344 rats

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HIGHLIGHTS

- Air puff-induced 22-kHz calls were compared between two rat strains.
- Emission levels did not differ between inbred F344 rats and outbred Wistar rats.
- Air puff stimuli may reliably emit similar amounts of 22-kHz calls among rat strains.

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ABSTRACT

Air puff-induced ultrasonic vocalizations in adult rats, termed "22-kHz calls," have been applied as a useful animal model to develop psychoneurological and psychopharmacological studies focusing on human aversive affective disorders. To date, all previous studies on air puff-induced 22-kHz calls have used outbred rats. Furthermore, newly developed gene targeting technologies, which are essential for further advancement of biomedical experiments using air puff-induced 22-kHz calls, have enabled the production of genetically modified rats using inbred rat strains. Therefore, we considered it necessary to assess air puff-induced 22-kHz calls in inbred rats. In this study, we assessed differences in air puff-induced 22-kHz calls between inbred F344 rats and outbred Wistar rats. Male F344 rats displayed similar total (summed) duration of air puff-induced 22 kHz vocalizations to that of male Wistar rats, however, Wistar rats emitted fewer calls of longer duration, while F344 rats emitted higher number of vocalizations of shorter duration. Additionally, female F344 rats emitted fewer air puff-induced 22-kHz calls than did males, thus confirming the existence of a sex difference that was previously reported for outbred Wistar rats. The results of this study could confirm the reliability of air puff stimulus for induction of a similar amount of emissions of 22-kHz calls in different rat strains, enabling the use of air puff-induced 22-kHz calls in inbred F344 rats and derived genetically modified animals in future studies concerning human aversive affective disorders.

1. Introduction

Rats emit calls in the ultrasonic range (>20 kHz) that are known as ultrasonic vocalizations [1]. In aversive or dangerous situations such as predator exposure, fighting, or drug withdrawal, adult rats emit long ultrasonic calls (usually between 0.5 and 3.0 s per individual call) that have a relatively low peak frequency (20–30 kHz) and narrow bandwidth (1.0–4.0 kHz), and that are termed "22-kHz calls" [2–4]. In the laboratory, application of an air puff stimulus is known to reliably elicit 22-kHz calls. As such, previous research has established that comprehensive analysis of air puff-induced 22-kHz calls is a useful tool in psychoneurological and psychopharmacological studies of human aversive affective disorders regarding fear and/or anxiety [3–8].

Development of gene targeting technologies has enabled genetic modification strategies that have been indispensable for biomedical research in recent years. Although it was formerly difficult to produce genetically modified mammals other than mice, as germline-competent embryonic stem cells were available only for mice, newly developed technologies using engineered site-specific nucleases, including zinc finger nucleases, transcription activator-like effector nucleases, and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated endonucleases, have helped overcome these challenges. These nuclease technologies are considered very efficient tools for precise and multiplex genome editing for the generation of genetically modified rats [9–14]. These technologies might be needed for further advancement of biomedical experiments using air puff-induced 22-kHz calls in rats as an animal model of human aversive affective disorders. However, all previous studies using air puff-induced 22-kHz calls included only rats of Wistar [4], Sprague–Dawley (SD) [5], Long–Evans [3,7], or other outbred strains. Therefore, we considered it

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necessary to assess air puff-induced 22-kHz calls in inbred rats and to compare these with the 22-kHz calls of outbred rats.

Toward this end, this study examined air puff-induced 22-kHz calls in F344 (Fischer) rats, as inbred F344 rats have been widely used for production of genetically modified strains [10,11,13–16]. First, we assessed differences in air puff-induced 22-kHz calls between inbred male F344 rats and outbred male Wistar rats (Experiment 1). We then investigated sex differences in air puff-induced 22-kHz calls in F344 rats and compared the data with those from a recent study of Wistar rats [17] (Experiment 2).

2. Materials and methods

2.1. Animals

A total of 16 male F344 Fischer rats, 8 male Wistar rats, and 16 female F344 Fischer rats (Charles River Laboratories Japan, Kanagawa, Japan) were used in this study. All animals were housed in pairs in individual ventilated cages (360 × 260 × 160 mm; Oriental Giken, Tokyo, Japan) with paper bedding (Eco Chip; CLEA Japan, Tokyo, Japan). Rats were provided with water and food *ad libitum* and maintained on a 12-h light–dark cycle with lights extinguished at 20:00. Cages were maintained at a constant temperature (23 ± 1 °C) and humidity (45–60%) under specific-pathogen-free (SPF) conditions.

In Experiment 1, we used male F344 rats ($n = 8$, weighing from 200 to 230 g) and male Wistar rats ($n = 8$, weighing from 330 to 363 g). In Experiment 2, we used male F344 rats ($n = 8$, weighing from 205 to 228 g), first day of diestrus (D1) female F344 rats ($n = 8$, weighing from 130 to 151 g), and proestrus (PE) female F344 rats ($n = 8$, weighing from 140 to 147 g). Estrous cycle stages were determined based on the relative ratio of three cell types (nucleated epithelial cells, cornified epithelial cells, and leukocytes) observed in vaginal smears collected between 8:00 and 9:00 on the day of the experiment. Only rats that exhibited at least three consecutive normal estrous cycles were included.

2.2. Experimental apparatus and procedures

All experiments were performed when subjects were 10 weeks of age. Each animal was transferred to a wire-topped transparent experimental cage (400 × 250 × 200 mm) from each home cage and habituated to the cage for 5 min. After 5 min, the animal received air puff stimuli. A total of 30 air puffs with an interstimulus interval of 2 s was directed to the nape of each subject's neck. Puffs were delivered from a nozzle (10 mm outer diameter and 2 mm caliber) inserted through the gap in the wire mesh cage lid and held approximately 150 mm from the subject. Air puff pressure was maintained at 0.3 MPa by a pressure valve following procedures used in previous studies [5,18]. Immediately after application of the air puff stimuli we recorded 22-kHz calls for 5 min using an ultrasound microphone (Condenser Microphone CM16/CMPA; Avisoft Bioacoustics, Berlin, Germany) set 50 mm from the top of the wire lid. Data acquisition hardware (UltraSoundGate 116Hbm; Avisoft Bioacoustics) and recording software (Avisoft-Recorder version 4.0; Avisoft Bioacoustics) were operated on a personal computer. Settings included a sampling rate of 100 kHz and a 16-bit format. The previously described sequence of air puff stimuli and recording of 22-kHz calls was repeated three times per subject. Subject behavior was concurrently recorded (GZ-E765; JVC Kenwood Corporation, Kanagawa, Japan) during the audio recording of 22-kHz calls. After the experiment, the number of fecal boli excreted by each subject was counted and body weight was measured. All experimental procedures were conducted between 10:00 and 13:00. This study was approved by the Animal Care and Use Committee of Nagoya University.

2.3. Data analyses

For spectrogram generation, recordings were transferred to Avisoft-SASLab Pro (version 5.1; Avisoft Bioacoustics) and a fast Fourier transformation (FFT) was conducted. Spectrograms were generated with an FFT length of 512 points and a time window overlap of 50% (100% Frame, FlatTop window). We defined 22-kHz calls as long calls (0.1–3.0 s) in the ultrasonic range within a narrow band of peak frequencies (20–27 kHz) and with a narrow bandwidth (1–5 kHz). All calls obtained from each subject were used to calculate the total duration and total number of 22-kHz calls emitted from each subject. We also analyzed acoustic variables including mean peak frequency, mean duration, and mean bandwidth of 22-kHz calls. All analyses were performed automatically using Avisoft-SASLab Pro (Avisoft Bioacoustics). In addition, we calculated the duration of video-recorded freezing responses during the 15 min that 22-kHz calls were recorded. Freezing was defined as an immobile posture (except movement due to respiration) with cessation of skeletal and vibrissae movement.

2.4. Statistical analyses

Data were displayed as mean ± standard error. Total duration and total number of 22-kHz calls, total duration of freezing, total number of fecal boli, and the three acoustic variables of 22-kHz call mean peak frequency, mean duration, and mean bandwidth were compared. In Experiment 1, Student's *t*-test was used to compare between male F344 and male Wistar rats. In Experiment 2, analyses were performed using a one-way analysis of variance (ANOVA) followed by the post hoc Tukey–Kramer test for comparison among male, D1 female, and PE female F344 rats. The criterion for statistical significance was

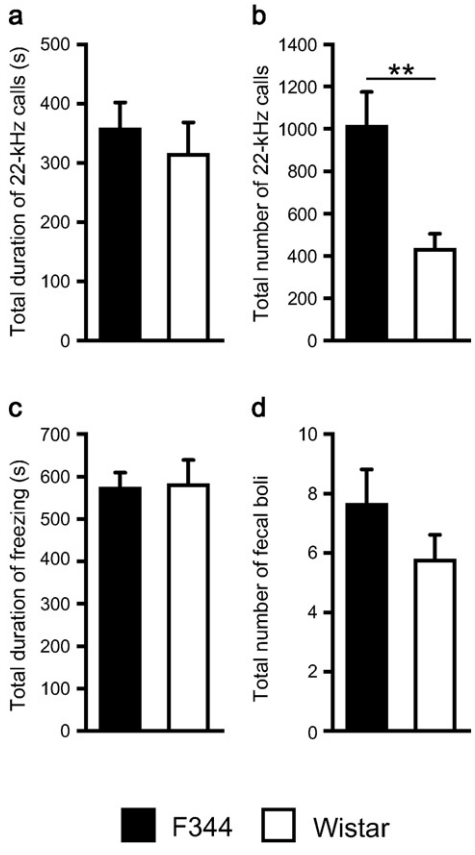

Fig. 1. Behavioral results in Experiment 1. The total duration of 22-kHz calls (a), the total number of 22-kHz calls (b), the total duration of freezing (c), and the total number of fecal boli (d) observed in male F344 rats ($n = 8$) and male Wistar rats ($n = 8$) after receiving air puff stimuli. Each bar represents the mean + standard error; $^{**}p < 0.01$.