Changes in acoustic startle reflex in rats induced by playback of 22-kHz calls

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HIGHLIGHTS

- Playback of 22-kHz calls enhanced the acoustic startle reflex (ASR) in rats.
- Synthesized 25-kHz, not 60-kHz, sine tones also enhanced the ASR in rats.
- 22-kHz calls possess anxiogenic effect as alarm signals in rats.
- Ultrasonic frequency may be critical in vocal alarm signals in rats.

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ABSTRACT

In aversive or dangerous situations, adult rats emit long characteristic ultrasonic calls, often termed "22-kHz calls," which have been suggested to play a role of alarm calls. Although the playback experiment is one of the most effective ways to investigate the alarming properties of 22-kHz calls, clear behavioral evidence showing the anxiogenic effects of these playback stimuli has not been directly obtained to date. In this study, we investigated whether playback of 22-kHz calls or synthesized sine tones could change the acoustic startle reflex (ASR), enhancement of which is widely considered to be a reliable index of anxiety-related negative affective states in rats. Playback of 22-kHz calls significantly enhanced the ASR in rats. Enhancement effects caused by playback of 22-kHz calls from young rats were relatively weak compared to those after calls from adult rats. Playback of synthesized 25-kHz sine tones enhanced ASR in subjects, but not synthesized 60-kHz tones. Further, shortening the individual call duration of synthesized 25-kHz sine tones also enhanced the ASR. Accordingly, it is suggested that 22-kHz calls induce anxiety by socially communicated alarming signals in rats. The results also demonstrated that call frequency, i.e., of 22 kHz, appears important for ultrasonic alarm-signal communication in rats.

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1. Introduction

In aversive or dangerous situations such as predator exposure, fighting, or drug withdrawal, adult rats emit long calls (usually between 0.5 and 3.0 s per individual call) in the ultrasonic range (>20 kHz) that have a relatively low peak frequency (20–30 kHz) and narrow bandwidth (1.0–4.0 kHz) [1–3]. These are often termed "22-kHz calls."

Such 22-kHz calls have been suggested to play the role of alarm calls, warning conspecifics in the colony about the presence of predators and informing other rats of the vocalizing rat's anxiety and negative state [1]. It has been therefore suggested that the production of 22-kHz calls is socially contagious [4]. One of the most effective ways to support the observation that 22-kHz calls have alarming function would be playback experiments using recorded natural 22-kHz calls or appropriately

synthesized 22-kHz sine tones. Through such playback studies, it has been demonstrated that activation of brain areas regulating behavioral responses related to anxiety is induced by playback of 22-kHz calls or appropriately synthesized 22-kHz sine tones [5,6]. However, clear behavioral evidence showing the anxiogenic effects of these playback stimuli have not been obtained to date [5–11]. Therefore, it has been suggested that limited behavioral responses to playback of 22-kHz calls indicates that these signals are not recognized innately as alarm calls, but they can obtain the alarming signal value as a consequence of associative learning.

The acoustic startle reflex (ASR) is a contraction of facial and skeletal muscles with eyelid closure, which is regulated by a simple reflexive neural pathway in response to an abrupt auditory stimulus [12]. The ASR is a reliable index of anxiety in rats, because the magnitude of ASR is enhanced in anxiogenic situations [13–15]. Additionally, brain areas regulating anxiety responses are associated with such enhancement of ASR [16], and anxiolytic drugs attenuate the enhanced ASR

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[13–15,17]. Indeed, in a previous study, the ASR used as a bioassay successfully identified two main alarm pheromones in rats [18]. We hypothesized that assessment of changes in the ASR would allow for evaluation of the anxiogenic effects of the replay of 22-kHz calls in rats in a more direct way.

To test this hypothesis, we recorded 22-kHz calls of young and adult male rats and investigated changes in the ASR induced by playback of these 22-kHz calls. Additionally, we artificially synthesized several types of sine tones of different frequencies and durations, to investigate whether such stimuli could influence the magnitude of the ASR in rats.

2. Materials and methods

2.1. Animals

A total of 52 male Wistar rats (CLEA Japan, Tokyo, Japan) were used in this study. All animals were housed in pairs in separate 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. This study was approved by the Animal Care and Use Committee of Nagoya University.

2.2. Recording and analysis of 22-kHz calls in young and adult male rats

The subjects were 6 young (4 weeks of age) and 6 adult (11 weeks of age) male rats. Each rat was placed inside an animal holder. The holder consisted of an acrylic cylinder (for young rats: 200 mm length, 50 mm outside diameter, 46 mm inside diameter, 2 mm wall thickness; for adult rats: 200 mm length, 60 mm outside diameter, 56 mm inside diameter, 2 mm wall thickness), stainless mesh (for young rats: 1 mm diameter wire, 10 mm mesh, 23 mm width × 89 mm height; for adult rats: 1 mm diameter wire, 10 mm mesh, 34 mm width × 89 mm height) as the front barrier, an acrylic plate (for young rats: 36 mm width × 90 mm height, 2 mm wall thickness; for adult rats: 44 mm width × 100 mm height, 2 mm wall thickness) as the rear barrier, and an acrylic bottom plate (230 mm length × 120 mm width, 2 mm wall thickness) to support the cylinder. We inserted the subject into the cylinder of the animal holder head first, and each subject was kept inside the cylinder between the front and rear barriers (young rats: 120 mm length; adult rats: 180 mm length). After a 5-min acclimation period, each subject received air-puff stimuli through a square hole (20 mm length, 10 mm width) situated at the top of the cylinder of the animal holder, immediately above the nape of the neck of the subject. Thirty air puffs with an interstimulus interval of 2 s were directed to the nape of each subject's neck. Puffs were delivered from a nozzle (10 mm outer diameter and 2 mm caliber) held approximately 150 mm from the subject's nape. Air puff pressure was maintained at 0.3 MPa by a pressure valve, following procedures used in previous studies [19,20]. After application of the air puff stimuli we recorded 22-kHz calls for 5 min using an ultrasound microphone (Type40BE; G.R.A.S. Sound & Vibration, Holte, Denmark) placed 10 mm from the wire mesh front barrier. An amplifier (SR-2200; Ono Sokki, Kanagawa, Japan), data acquisition hardware (Avisoft-UltraSoundGate 116Hbm; Avisoft Bioacoustics, Berlin, Germany) and recording software (Avisoft-RECORDER USGH; Avisoft Bioacoustics) were operated on a personal computer. Settings included a sampling rate of 192 kHz and a 16-bit format. The sequence of air-puff stimuli and recording was repeated at least three times per subject until 22-kHz calls were emitted. All experimental procedures were conducted between 15:00 and 18:00.

For spectrogram generation, recordings were transferred to Avisoft-SASLab Pro (version 5.1; Avisoft Bioacoustics) and a fast Fourier transformation (FFT) was applied. 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 (young rats: 49–216 calls, adult rats: 89–216 calls) were used to analyze the acoustic characteristics of 22-kHz calls, including mean peak frequency (kHz), mean call duration (s), mean bandwidth (kHz), and mean peak amplitude (dB). All analyses were performed automatically using Avisoft-SASLab Pro (Avisoft Bioacoustics). A reference sound source (Type2126; Aco, Tokyo, Japan) was used for the analysis of mean amplitude of 22-kHz calls. Data were displayed as mean ± standard error. Statistical analyses were performed using Mann-Whitney tests. The criterion for statistical significance was set at $p < 0.05$ for all comparisons.

2.3. Acoustic startle reflex test

We conducted the ASR test using startle apparatus and software (Startle Reflex System 2004; O'Hara & Co., Tokyo, Japan) described in previous studies [15,17,18]. The subjects were 40 adult (9 weeks of age) male rats.

The experiment consisted of 5 consecutive days. On days 1 and 2, each subject was handled for 7 min in the experimental room (temperature 22 °C, humidity 45–55%). Then, the subject was acclimatized to the animal holder, which was very similar to the apparatus used for recording 22-kHz calls in adult rats (see above). In ASR tests, an acrylic plate (44 mm width × 100 mm height, 2 mm wall thickness) with 42 perforations (2 mm diameter) was used as the front barrier instead of the wire mesh. Each subject was placed between the front and rear barrier (170 mm length) of the animal holder. Immediately after that, the animal holder including the subject was attached to a platform in a dark, soundproof test chamber (480 mm length, 350 mm width, 370 mm height) with background noise (65-dB white noise), and maintained there for 10 min. On experimental day 3, subjects were acclimatized to the entire ASR test procedure. In the experimental room, each rat was placed inside the same individual animal holder used on experimental days 1 and 2. Then, the animal holder containing each subject was attached to the platform in the dark soundproof test chamber with background noise, as in experimental days 1 and 2. Following this, the ASR test, consisting of a baseline trial and a test trial, was initiated. During the baseline trial, after an initial 300-s acclimation period, each subject was exposed to 30 auditory stimuli (105 dB, 0.1 s white noise) with an interstimulus interval of 30 s. Subsequently, the test trial was conducted in the same manner as the baseline trial. On experimental days 4 and 5, the subjects underwent the ASR test, which consisted of the presentation of ultrasound stimuli or no sound during the test trial, in a counterbalanced order. During the trials, the subject's movements within the holder resulted in displacements of an accelerometer affixed to the platform. The voltage output of the accelerometer was digitized and recorded. The startle amplitude was defined as the maximal peak-to-peak voltage that occurred during the first 0.2 s after the onset of the startle-eliciting auditory stimulus.

All experimental procedures were conducted between 15:00 and 18:00. For data analyses, we defined individual baseline data as the mean amplitude of the last 20 responses in the baseline trial. The test data were defined as the mean amplitude of all responses in the test trial. The increase in amplitude between the test data (T) and the baseline data (B) was calculated as $T–B$ for each subject. Data on days 4 and 5 were statistically compared within each experimental group using a paired *t*-test. The criterion for statistical significance was set at $p < 0.05$ for all comparisons.

2.4. Presentation of ultrasound stimuli

Throughout the ASR test trial on experimental days 4 or 5, ultrasound stimuli were presented through an ultrasonic speaker (Trb-001; Katou Acoustics Consultant Office, Kanagawa, Japan) placed 50 mm in