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

Hideaki Inagaki [a,*], Takahiro Ushida [b]

[a] Center for Animal Research and Education, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan
[b] Multidisciplinary Pain Center, Aichi Medical University, Yazakokarimata, Nagakute, Aichi 480-1195, Japan

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.

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

* Corresponding author.
E-mail address: ahina@aichi-med-u.ac.jp (H. Inagaki).

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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

front of the front barrier of the animal holder attached to the stage in a soundproof ASR test chamber. The stimuli were delivered using a portable ultrasonic power amplifier (Avisoft-UltraSoundGate Player 216H; Avisoft Bioacoustics) and playback software (Avisoft-RECORDER USGH; Avisoft Bioacoustics). All ultrasound stimuli were presented with a sampling rate of 192 kHz in 16-bit format. One of the following ultrasound stimuli was presented during each 15-min test trial in ASR test: the pre-recorded young male 22-kHz calls (119-times-repeated presentation of 6-calls sequence; mean peak frequency: 24.6 kHz, mean individual duration: 0.402 s, mean bandwidth: 1.80 kHz, total summed duration: 287 s, total number: 714 calls; Fig. 1a), the pre-recorded adult male 22-kHz calls (91-times-repeated presentation of 6-calls sequence; mean peak frequency: 21.8 kHz, mean individual duration: 0.644 s, mean bandwidth: 1.90 kHz, total summed duration: 352 s, total number: 546 calls; Fig. 1b), synthesized 25-kHz long-duration sinewave tones (91-times-repeated presentation of 6-tones sequence; total summed duration: 352 s, total number: 546 tones; Fig. 1c), synthesized 60-kHz long-duration sinewave tones (91-times-repeated presentation of 6-tones sequence; total summed duration: 352 s, total number: 546 tones; Fig. 1d), or synthesized 25-kHz short-duration sinewave tones (91-times-repeated presentation of 30-tones sequence; total summed duration: 352 s, total number: 2730 tones; Fig. 1e). All these ultrasound were presented at between 57.1 and 63.2 dB, as measured in the cylinder of ASR animal holder, at the location of the subject's ears. The individual duration and order of the 6 long sine tones in the repeatedly presented sequence were the same as those of the 6 adult male 22-kHz calls (Fig. 1b–d). The individual duration of the short-duration sine tones (0.129 s) was defined as the equal division of the total duration of 6 long-duration sine tones in the repeatedly presented sequence (3.86 s) into 30 parts; therefore, the total duration of the 30 short sine tones was the same as the total duration of 6 long sine tones (Fig. 1c, e). Sine tones in this study were synthesized by computer software (Audacity 2.1.1 and WaveGene 1.40, freeware).

3. Results

3.1. Analysis of 22-kHz calls in young and adult male rats

Similar to a previous study [21], the mean peak frequency of 22-kHz calls in young rats was significantly higher ($p < 0.05$) and the mean call duration significantly shorter than adult rats ($p < 0.01$) (Table 1). There was no significant difference in the bandwidth of 22-kHz calls between young and adult rats, nor in the peak call amplitude (Table 1).

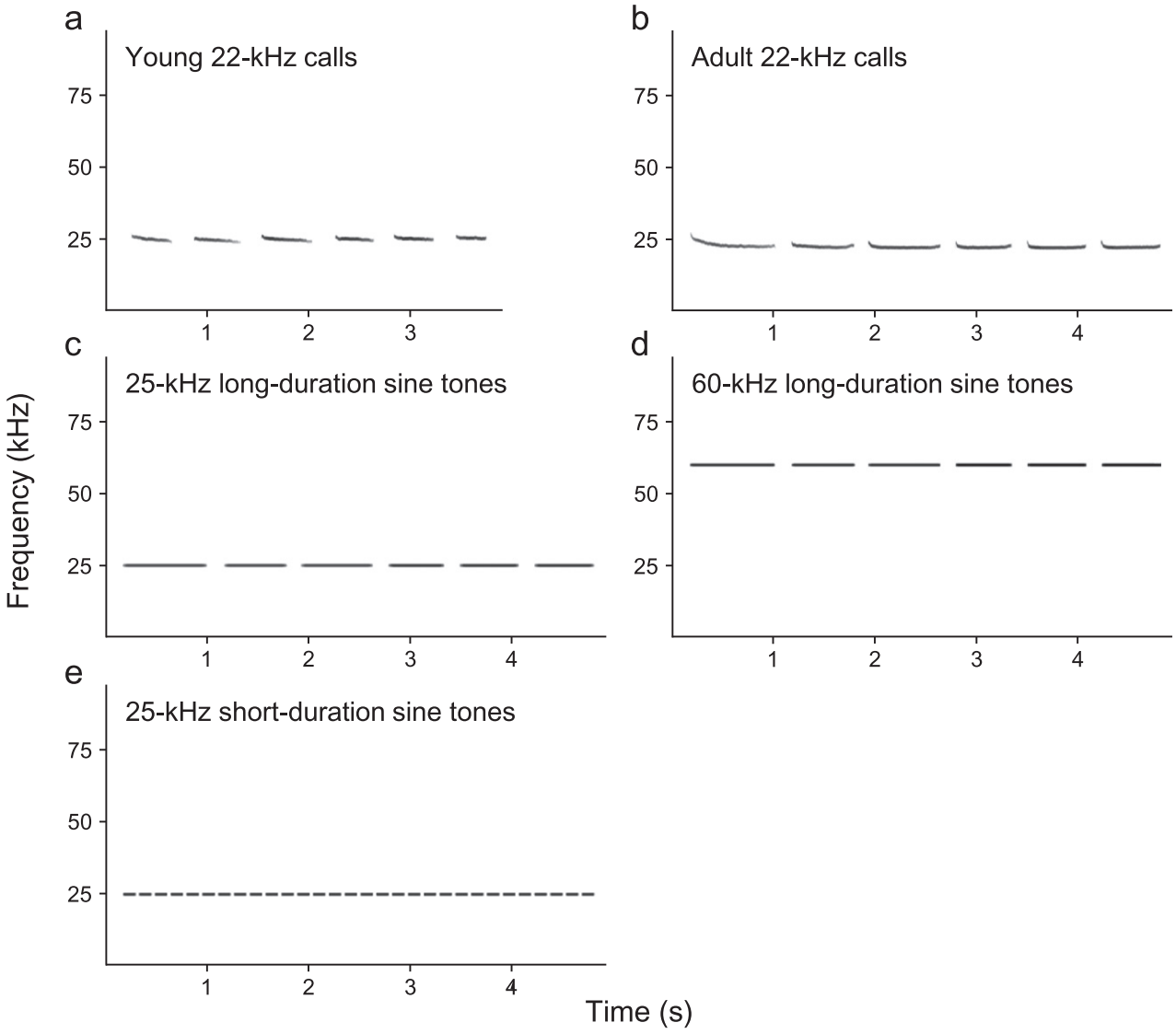

Fig. 1. Spectrograms of recorded (a) young and (b) adult rat 22-kHz calls and synthesized sine tones (c: 25-kHz long-duration sine tones, d: 60-kHz long-duration sine tones, e: 25-kHz short-duration sine tones) used for playback experiments in this study.

Table 1
Acoustic characteristics of recorded 22-kHz calls in male rats aged 4 weeks (4 w, $n = 6$) and 11 weeks (11 w, $n = 6$).

	Peak frequency (kHz)	Duration (s)	Bandwidth (kHz)	Peak amplitude (dB)
4 w	25.1 ± 0.250*	0.350 ± 0.0419**	2.11 ± 0.179	65.7 ± 2.86
11 w	23.5 ± 0.447	0.642 ± 0.0562	2.07 ± 0.121	70.5 ± 1.69

The mean ± standard error; $^{**}p < 0.01$, $^{*}p < 0.05$, vs. 11 w.

3.2. Acoustic startle reflex test

3.2.1. Playback of young and adult rat 22-kHz calls

Although the tendency to the increased ASR amplitude was observed during playback of young rat 22-kHz calls, the difference was not significant ($t = 1.53$, $p = 0.17$; Fig. 2a). Playback of adult 22-kHz calls significantly enhanced the ASR ($t = 2.91$, $p < 0.05$; Fig. 2b).

3.2.2. Playback of synthesized sine tones

During playback of 25-kHz long-duration sine tones, a significant increase in the amplitude of ASR was observed ($t = 2.48$, $p < 0.05$; Fig. 3a). In contrast, presentation of 60-kHz long-duration sine tones did not enhance the ASR ($t = 1.25$, $p = 0.25$; Fig. 3b). Presentation of 25-kHz short-duration sine tones significantly enhanced the ASR ($t = 2.47$, $p < 0.05$; Fig. 3c).

4. Discussion

In this study, we assessed changes in ASR induced by playback of 22-kHz calls or synthesized sine tones. Although both young and adult 22-kHz calls enhanced ASR in subjects, the latter change was significant and the former caused relatively weak effect not reaching the level of significance. Spectrogram analysis of these recorded 22-kHz calls indicated significantly higher peak frequency and shorter individual call duration of young-rat calls compared to adult calls. We synthesized 25-kHz sine tones, in which individual tone duration, the total number of tones, the summed total duration of all presented tones, and the amplitude of tones, were almost the same as those of the aforementioned recorded adult 22-kHz calls (see Fig. 1b, c). The result of the replay experiment was that presentation of these 25-kHz long-duration sine tones significantly enhanced the ASR in subjects. In contrast, playback of synthesized 60-kHz long-duration sine tones that possessed the same duration, number, and amplitude as the 25-kHz long-duration sine tones induced no enhancement of the ASR in subjects. However, replay of synthesized 25-kHz short-duration sine tones significantly enhanced the ASR in subjects.

The ASR experiment in this study indicates that replaying 22-kHz calls may have anxiogenic properties for rats. In contrast to the result of this study, earlier studies have demonstrated weak or no behavioral responses to such stimuli [5–11]. One possible explanation for this discrepancy is that a considerable and stable quantity of ultrasound stimuli could reach subjects' ears from a short distance and promptly induce anxiety in this study. The ASR system used in this study incorporated an animal holder attached to each subject's platform, such that the subject could be exposed to sufficient number of recorded 22-kHz calls from a stable distance. However, in earlier studies, free movement of subjects in open field systems, conditioning chambers, or maze systems could have prevented them from receiving enough 22-kHz stimuli to evoke anxiety. An additional suggestion is that sound pressure levels used in this study were adequate to evoke anxiety in rats, because sound pressure levels of natural 22-kHz calls are as high as those of ultrasound presented in this study [22].

The ASR test using synthesized sine tones in this study suggests that tone frequency, i.e., approximately 22-kHz, is one of the most important elements for an ultrasonic stimulus to have anxiogenic properties. In support of this conclusion, earlier studies reported that 20-kHz tones induced anxiety-related responses (e.g., a reduction in locomotor activity) in rats [23–28]. This study further showed that shortening the individual tone duration of sine tones may have a small role in ultrasound-induced anxiety in rats. The importance of tone frequency has been demonstrated in research on 50-kHz calls, which are induced by appetitive situations in rats, such as rough-and-tumble play [29], mating [30], and injection of amphetamine [31], and which are considered as social contact calls possessing a pro-social communicative function. Behavioral experiments using a radial maze system showed that playback of 50-kHz calls or synthesized 50-kHz sine tones induced social approach behavior and emission of 50-kHz calls in subjects, whereas playback of 22-kHz calls did not [10]. Accordingly, that 60-kHz sine tones in the current study produced no anxiogenesis, and instead had rather

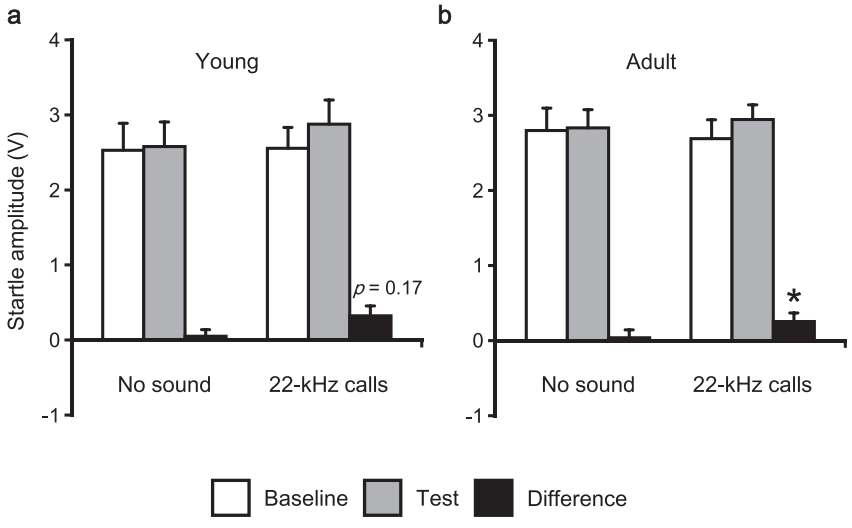

Fig. 2. Changes in acoustic startle reflex in subjects ($n = 8$ each) induced by playback of (a) young and (b) adult rat 22-kHz calls. Baseline data (Baseline, white bars), test data (Test, gray bars), and differences in amplitude between the baseline and test data (Difference, black bars) are shown for acoustic startle reflex. Each bar represents the mean + standard error; $^{*}p < 0.05$ vs. no-sound control (paired t-test).

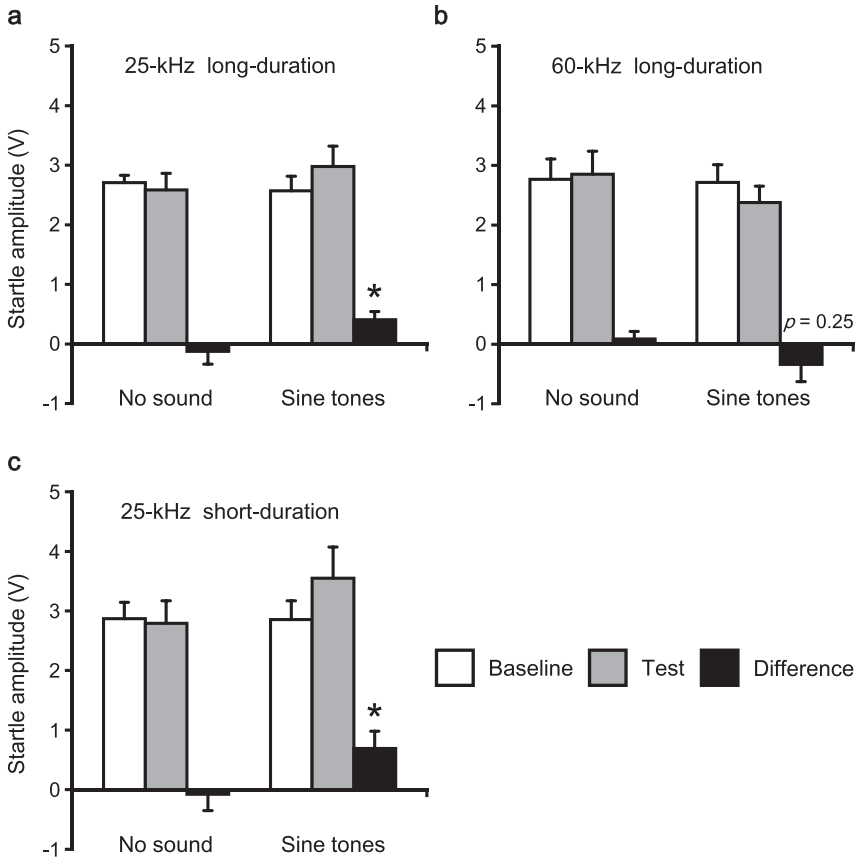

Fig. 3. Changes in acoustic startle reflex in subjects ($n = 8$ each) induced by playback of (a) 25-kHz long-duration sine tones, (b) 60-kHz long-duration sine tones, and (c) 25-kHz short-duration sine tones. Baseline data (Baseline, white bars), test data (Test, gray bars), and differences in amplitude between the baseline and test data (Difference, black bars) are shown for acoustic startle reflex. Each bar represents the mean + standard error; * $p < 0.05$ vs. no-sound control (paired t-test).

anxiolytic properties, as suggested by the slightly reduced ASR in this study (Fig. 3b), might be ascribed to higher peak frequency of the sine tones.

The ASR test using playback of 22-kHz calls in this study shows that adult 22-kHz calls seem to be a little more effective in inducing anxiety than the 22-kHz calls of young rats. One possible explanation is that group size of subjects used in this study was small and with an increased n-value, it would reach the significance level. Another possibility is that 22-kHz calls by young rats might have a less alarming effect on receiver rats compared to calls emitted by adult rats. A similar phenomenon was reported in ground squirrels, which warn conspecifics of potential predators through alarm calls; they show reduced responsiveness to alarm calls made by young squirrels versus adults, presumably because of the lower predictive value of calls produced by young animals [32,33]. In this study, 22-kHz calls of young rats possessed a higher peak frequency and shorter individual duration compared to those of adult rats, and the same results were obtained as in a previous study [21]. Taken together, such quantitative acoustic features of young rats' 22-kHz calls may signal lesser urgency to an adult receiver animal. Further studies are necessary to clarify this issue.

5. Conclusion

This study showed that playback of 22-kHz calls enhanced ASR in rats, suggesting anxiogenesis induced by 22-kHz calls as socially communicative alarming signals of rats. It was also demonstrated that call frequency, i.e., approximately 22-kHz, might be important in conveying alarming ultrasonic communication in rats. In ASR tests using playback of 22-kHz calls, we maintained a comparable quality and quantity of presented sound stimuli. Accordingly, we strongly suggest that changes in the ASR induced by playback of 22-kHz calls could become one of the most useful tools to study anxiety-related negative affective states evoked by alarming vocal communication in rats.

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