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Relationship between 22-kHz calls and testosterone in male rats

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ABSTRACT

Ultrasonic calls in rats induced by the presence of a predator, referred to as "22-kHz calls," are mainly emitted by socially dominant male rats. Testosterone levels are closely related to social dominance in male rats. In the present study, we investigated the relationship between the emission of stress-induced 22-kHz calls and circulating testosterone levels in male rats, using a combination of surgery (castration or sham operation) and chronic steroid administration (testosterone or cholesterol) to modify circulating testosterone levels. We also assessed the effects of androgen and/or estrogen receptor antagonists on the emission of 22-kHz calls in male rats. An air puff stimulus, known to reliably induce 22-kHz calls in rats, was used as a stressor. Castrated rats with cholesterol implants exhibited significantly fewer 22-kHz calls than rats that had received a sham operation and cholesterol implants, and there was no significant difference between castrated rats with testosterone implants and rats that had received a sham operation and cholesterol implants. Only male rats pretreated with a binary mixture of androgen and estrogen antagonists exhibited significantly fewer 22-kHz calls than controls. These results show that testosterone in male rats has a positive effect on the emission of stress-induced 22-kHz calls, and the calls may be regulated by the activation of both androgen and estrogen receptors.

Introduction

Rats emit social vocalizations in the ultrasonic range (above 20 kHz) in a variety of aversive or appetitive situations (Brudzynski, 2009). Adult rats emit long (usually between 0.5 and 3.0 s for a single call) ultrasonic bouts of 20–30 kHz with a narrow bandwidth of 1–4 kHz, referred to as "22-kHz calls". These ultrasonic calls are typically responses to potentially harmful or life-threatening situations or to the expectation of a known unpleasant stimulus even if the rat lacks exact information about when it will happen. For example, rats call in the presence of a predator (Blanchard et al., 1991), when they confront a dominant and aggressive rat (Panksepp et al., 2004), and even when they receive a light but sudden and unpredictable air puff (Knapp and Pohorecky, 1995) or tactile stimulus (Inagaki et al., 2005). Moreover, it is widely accepted that 22-kHz calls serve as alarm calls to warn conspecifics of external danger; namely, 22-kHz calls are considered to be one of social communicative behaviors (Litvin et al., 2007).

A male rat's emission of 22-kHz calls can be influenced by the rat's social hierarchical position; when a predator (such as a cat) is presented to groups that include several male and female rats in seminatural visible burrow systems, it is usually the socially dominant males that emit 22-kHz calls (Blanchard et al., 1991). It is also well known that social status is closely related to testosterone levels in male rats (Bernhardt, 1997); for example, testosterone removal (castration) causes socially dominant rats to become submissive (Albert et al., 1986), and chronic administration of testosterone to subordinate rats significantly increases their dominant behaviors (Bonson and Winter, 1992). On the basis of this information, we hypothesized that the testosterone can influence the emission of 22-kHz calls in male rats, although no systematic analysis regarding such relationship has been reported.

To test our hypothesis, we investigated the relationship between the total number and duration of stress-induced 22-kHz calls and circulating testosterone levels in male rats using a combination of surgery (castration or sham operation) and chronic steroid administration (testosterone or cholesterol) (Experiment 1). We also assessed the effects of androgen and/or estrogen receptor antagonists on the emission of 22-kHz calls in male rats (Experiment 2).

Materials and methods

Animals

A total of 98 male Wistar rats (Clea Japan, Tokyo, Japan) were used in this study. All animals were housed in pairs in wire-topped transparent cages (400 × 250 × 180 mm) with wood shavings for bedding. They were provided with water and food *ad libitum* and maintained on a 12-h light–dark cycle (lights turned off at 20:00). The cages were maintained at a constant temperature (24 ± 1 °C) and humidity (40–45%).

Pretreatment before experiments

In Experiment 1, 40 rats aged 8 weeks received one of three treatments. A total of 12 rats were castrated and implanted with silicon

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tubes (1.57-mm inner diameter, 3.18-mm outer diameter; Kaneka Medix Corporation, Osaka, Japan) containing cholesterol (Wako Pure Chemical Industries, Osaka, Japan). A total of 14 rats were castrated and implanted with silicon tubes containing testosterone (Wako Pure Chemical Industries), and 14 rats received sham operations and were implanted with silicon tubes containing cholesterol. We packed a 10-mm length of each tube with cholesterol or testosterone and closed each end of the tube with a glass bead (3 mm in diameter). Each subject received a single implantation of either tube. All operations were performed under inhalation anesthesia with ethyl ether.

In Experiment 2, 58 rats aged 10 weeks received one of four types of treatment featuring subcutaneous injections once a day for 7 consecutive days. A total of 14 rats received an androgen receptor antagonist, flutamide (Wako Pure Chemical Industries; 50 mg/kg, 50 mg flutamide dissolved in 1 ml of vehicle); 12 rats received an estrogen receptor antagonist, tamoxifen (MP Biomedicals, Solon, OH, USA; 1 mg/kg, 1 mg tamoxifen dissolved in 1 ml of vehicle); 16 rats were treated with a binary mixture of the two drugs (flutamide 50 mg/kg and tamoxifen 1 mg/kg, 50 mg flutamide and 1 mg tamoxifen dissolved in 1 ml of vehicle), and 16 rats received corn oil as a control (Wako Pure Chemical Industries; 1 ml/kg).

Experimental apparatus and procedures

All experiments were performed when subjects were 11 weeks of age. At the beginning of the experiment they were moved to a soundproofed experimental room and remained in their own cages for at least 60 min. Each animal was then transferred to a wire-topped transparent experimental cage (400 × 250 × 200 mm) and habituated to the cage for 5 min. After 5 min, the wire lid was removed and the animal received an air puff stimulus, which is known to reliably induce 22-kHz calls in rats (Brudzynski and Holland, 2005; Inagaki et al., 2012; Knapp and Pohorecky, 1995). A total of 30 air puffs with an interstimulus interval of 2 s were directed to the nape of each subject's neck, delivered from a nozzle (10 mm outer diameter and 2 mm caliber) at a distance of approximately 50 mm from the subject. The pressure of the air puff was maintained at 0.3 MPa by a pressure valve, following procedures used in earlier studies (Brudzynski and Holland, 2005; Knapp and Pohorecky, 1995). Immediately after the air puff stimuli, we placed the wire lid on the experimental cage and recorded 22-kHz calls for 5 min using an ultrasound microphone (Condenser Microphone CM16/CMPA; Avisoft Bioacoustics, Berlin, Germany) set at a distance of 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) on a personal computer were used. Settings included a sampling rate of 100 kHz and a 16-bit format. The abovementioned sequence of air puff stimuli and the recording of 22-kHz calls was repeated three times for each subject. During the recording of 22-kHz calls, the behaviors of subjects were simultaneously video-recorded (HDR-SR12; Sony, Tokyo, Japan). After the experiment, the number of fecal boli excreted by each subject was counted. All experimental procedures were conducted between 10:00 and 15:00. For Experiment 1 only, 7 days after the experiment, we anesthetized each subject with ethyl ether and collected blood (6 − 8 ml, for euthanasia) from the abdominal aorta into a heparinized conical tube. Each blood sample was maintained at 4 °C and centrifuged to obtain plasma, which was then frozen at −80 °C. We collected all blood samples between 13:00 and 15:00. This study was approved by the Animal Care and Use Committee of the Faculty of Agriculture, The University of Tokyo.

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 (0.5 − 3.0 s) in the ultrasonic range within a narrow band of peak frequencies (20 − 23 kHz) with a narrow bandwidth (1 − 4 kHz) according to an earlier report (Brudzynski, 2009).We recorded the total number of 22-kHz calls and also added together all calls from each subject to generate the total duration of calls. The calls were measured automatically using Avisoft-SASLab Pro. In addition, we calculated the duration of video-recorded freezing responses during the time that 22-kHz calls were recorded (15 min). Freezing in this study was defined as an immobile posture with cessation of skeletal and vibrissae movement except for respiration. Plasma testosterone levels in Experiment 1 were measured by enzyme immunoassay (Testosterone EIA kit; Cayman Chemical Company, Ann Arbor, MI, USA).

Statistical analyses

All data were displayed as the mean ± standard error. Statistical comparisons were performed using a one-way analysis of variance (ANOVA) followed by the post hoc Dunnett test. The criterion for statistical significance was $p < 0.05$ for all comparisons. In experiment 2, effect sizes (η^2 for the one-way ANOVA and Cohen's d for the post hoc test) were calculated to evaluate magnitudes of effects of drug treatments on emission of 22-kHz calls (η^2: large = 0.14, medium = 0.06, small = 0.01; Cohen's d: large = 0.80, medium = 0.50, small = 0.20).

Results

Experiment 1: Relationship between the emission of 22-kHz calls and circulating testosterone levels

The total duration of air puff-induced 22-kHz calls was significantly affected by treatment ($F_{2,37} = 6.65, p < 0.01$). The post hoc test indicated that castrated rats with cholesterol implants exhibited significantly shorter 22-kHz calls than rats that had received a sham operation and cholesterol implants ($p < 0.01$). There was no significant difference between castrated rats with testosterone implants and rats that had received a sham operation and cholesterol implants (Fig. 1a). The total number of air puff-induced 22-kHz calls was also significantly affected by treatment ($F_{2,37} = 4.48, p < 0.05$). The post hoc test indicated that castrated rats with cholesterol implants exhibited significantly fewer 22-kHz calls than rats that had received sham operations and cholesterol implants ($p < 0.05$). There was no significant difference between castrated rats with testosterone implants and rats that had received sham operations and cholesterol implants (Fig. 1b). In contrast, the total duration of freezing and the total number of fecal boli produced were not significantly affected by treatment (freezing: $F_{2,37} = 1.94$, $p = 0.14$, fecal boli: $F_{2,37} = 0.33, p = 0.72$) (Fig. 1c, d). Plasma testosterone levels were significantly affected by treatment ($F_{2,37} = 30.5$, $p < 0.01$). The post hoc test indicated that castrated rats with cholesterol implants had significantly lower plasma testosterone levels ($1.60 \pm 0.11 \times 10^{-2}$ ng/ml) than rats that had received a sham operation and cholesterol implants (2.42 ± 0.34 ng/ml) ($p < 0.01$). There was no difference between castrated rats with testosterone implants (2.11 ± 0.17 ng/ml) and rats that had received a sham operation and cholesterol implants.

Experiment 2: Effects of sex hormone receptor antagonists on the emission of 22-kHz calls

The total duration of air puff-induced 22-kHz calls was significantly affected by treatment ($F_{3,54} = 3.08, p < 0.05$). The effect size was large ($\eta^2 = 0.15$). The post hoc test indicated that only rats pretreated with a binary mixture of flutamide and tamoxifen exhibited significantly shorter 22-kHz calls than control rats ($p < 0.05$) (Fig. 2a), and the effect size for paired comparisons was larger (Cohen's $d = 1.10$) than those of