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Short communication

High-frequency ultrasonic vocalizations in rats in response to tickling: The effects of restraint stress

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HIGHLIGHTS

- High-frequency ultrasonic vocalizations indicate positive affect of rats.
- High-frequency vocalizations can be induced by manual stimulation (tickling).
- Prolonged restraint stress diminished tickling-induced high-frequency vocalizations.
- Reduction of tickling-induced high-frequency calls suggests decreased positive affect.

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ABSTRACT

We tested the hypotheses that (a) the propensity to emit high-frequency (~50 kHz) ultrasonic vocalizations in response to manual "tickling" by an experimenter, may serve as a behavioral marker of positive affect in rats and, (b) that tickling may reduce the severity of stress. Group-housed adult rats were subjected to the 15-s tickling procedure daily, and their ultrasonic vocalization response was measured over a period of two weeks, until it has stabilized. The animals were then subjected to the restraint stress lasting for one week. The experimental groups were exposed to stress 1 h before or 1 h after tickling and the controls were tickled without stressing. Rats that were stressed 1 h before tickling demonstrated a decreased number of the high-frequency calls as compared with the non-stressed controls. Stressing 23 h before tickling reduced the call response less effectively. The propensity to emit high-frequency calls has normalized 7 and 12 days following the end of stressing. In addition, stressed groups showed a diminution of sucrose preference, which in the case of rats stressed 23 h before tickling persisted even for 12 days following the end of restraint. The present data suggest that repeated stress may decrease the propensity to produce high-frequency vocalizations, and that this measure may serve as a biomarker of the depressive state of animals.

1. Introduction

Since prolonged stress has been regarded as a major risk factor for depressive disorder [9], the models based on stressed animals represent a useful way to induce and study depressive-like symptoms [2,8,14,16] and to characterize novel antidepressants [22]. However, the direct investigation of infra-human animals' well-being is restricted to a tiny number of measures, mostly reflecting anhedonia and represented by sucrose preference and/or the threshold of electrical self-stimulation [15,21,24]. While these measures are clearly altered in the stressed animals, it appears that they reflect the *sensitivity to the reward* rather than the *positive affect* of animals. Characterization of the meaningful and relevant measures of the animal's affect is challenging, yet it may help in describing an emotional state of the subject, purportedly being compromised in depressed animals, or at least in animals subjected to the procedures thought to induce depressive-like symptoms.

In this regard, the discovery of the "laughing rats" [19] has offered a novel way for neurobiological analysis of human emotions [17]. An accumulating body of evidence indicates that frequency-modulated 50-kHz calls accompany play behavior of the rats and may reflect positive emotional feelings (see Brudzynski [3] for the review). Interestingly, when the rats are stimulated ("tickled") in a playful way by the human, they emit a similar pattern of 50-kHz calls [19]. Other studies summarized in Section 4 have shown that these vocalizations could be rewarding and/or joyful for the animals [4].

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The present experiment was designed to investigate whether the rat's positive affect, as assessed by the propensity to emit high-frequency ultrasonic vocalizations in response to the manual tickling by an experimenter, could be altered by the exposure to the prolonged restraint stress. Because the clinical diagnosis of depression relies not only on a decreased mood, but also on the incidence of anhedonia, in addition, we measured the preference for sucrose solution as an independent additional factor.

2. Methods

2.1. Animals

Male Sprague–Dawley rats (Charles River, Germany) weighting 250–280 g on the arrival were used in this study. They were group-housed (4 rats/cage) in the temperature (21 ± 1 °C) and humidity (40–50%) A/C controlled colony room under 12/12-h light/dark cycle (lights on at 06:00 h). Rats were allowed to acclimatize for at least 7 days before the start of the experimental procedure. Behavioral testing was carried out during the light phase of the light/dark cycle. The experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the Ethics Committee for Animal Experiments, Institute of Pharmacology.

2.2. Apparatus

Ultrasonic vocalizations were made audible in the headphones by the use of ultrasound heterodyne bat detector microphone (Ultrasound Advice, UK) set to reflect the high-frequency ~50 kHz "happy" calls. The same vocalizations were online visualized by a PC computer running Raven Pro 1.4 interactive sound analysis software (Cornell Lab of Ornithology, Bioacoustics Research Program, USA) connected via the A/D converter DAQ device (USB-6251 1.25 MS/s M Series) to another ultrasound microphone (Avisoft, Germany). Thus, the calls were recorded by investigators who simultaneously listened to, and observed the vocalizations on the computer screen.

2.3. Procedure

During the experiment, investigators recorded the number of high-frequency ~50 kHz calls. While as indicated by the visualization, 50-kHz vocalizations contained both frequency modulated (bandwidth > 20 kHz; trills and step calls) as well as constant frequency (i.e., flat) calls [1,5], no attempt was made to differentiate between them in the present study. The 22-kHz "alarm" calls were observed too sporadically to create a powerful set of data.

The animals were investigated for the propensity to produce high-frequency calls in response to skin stimulation (tickling) in an experimental room adjacent to the stress room. Tickling was done according to the method described by Panksepp and Burgdorf [18] and consisted of gentle holding of the rat on its back with the left hand of the investigator, and rapid right-hand finger movements across the ventral body surface of the animal, followed by its release after 15 s of stimulation. Due to the purportedly subjective nature of tickling efficiency, two 15-s stimulations were carried out one after another, by two investigators whose results were subjected to the correlation analysis and then, averaged for statistical analyses. Since the initial rate of vocalizations was low as compared with the literature data using isolated rats [13,18] and was highly variable, the daily measurements continued until the response has stabilized (14 days); see also [14]. To be included in the analyses, the rat had to meet the arbitrary criterion of 15 or more calls per 15 s during each of the last 5 days of the pre-stress period. Twenty-nine out of 40 rats (72.5%) have met this criterion; the other cagemates were left intact until the end of the experiment to avoid additional variation due to removal of group members. Thus, each cage housing 4 rats, contained both the tested and untested (1–3) cagemates.

A day before the introduction of stress, for each rat the number of calls registered during the last 5 days of pre-stress period was averaged, and based on this measure, the cages were randomly divided into the three experimental groups. This was done to assure that within a given cage, all of the subjects received the same treatment. To investigate the immediate effects of stress on the propensity to call, the first group composed of three cages was subjected to stress 1 h before tickling. To investigate the delayed effects of restraint, and to assess whether the tickling affects the severity of stress, another group (three cages) was exposed to stress 23 h before tickling (i.e., was tickled 1 h before stressing). The non-stressed controls (four cages) were tickled randomly at the time when the stressed subjects were tickled.

The stress paradigm consisted of 1 h daily restraint stress for seven consecutive days [16]. Rats were transferred from a housing facility to the stress-room, separate from the test room. Animals were placed into perforated plastic tubes (6.5 cm inner diameter) of adjustable length. The restraint allowed for normal breathing and limited movements of the head and limbs. The rats were restrained between 10:00 and 12:00 h. The non-restrained controls remained undisturbed in their home cages in the housing facility.

Subsequently, the subjects were investigated for the propensity to produce high-frequency calls, as described above, for three more times: on day 1, 7 and 12 following the end of stressing. In addition, these animals were tested for the preference of 10% sucrose solution over water. On the night following the last day of restraint stress, the rats were familiarized with the bottles containing 10% sucrose for 2 h in order to avoid food neophobia. Two hours after the tickling, the non-tested rats were removed from cages and the remaining "active" subjects were offered a free choice between two bottles containing tap water and 10% sucrose for 2 h. Testing the groups of rats rather than individual animals was done to avoid the transfer to single cages, implying additional stress due to short-time isolation, investigation of novel cages and other factors purportedly affecting sucrose preference. To prevent possible effects of side preference in drinking behavior, the position of the bottles was randomized [21]. No previous food or water deprivation was applied before the test. The consumption of water and sucrose solution was assessed in control and stressed cages by weighing the bottles. The intake was calculated as the amount of consumed fluid in grams per mean weight of rats tested. The design of the experiment is shown in Table 1.

Table 1
The description of the experiment.

14 days	7 days	Day 1, 7 and 12 following the end of stress
	Restraint stress	
Initial measurement of vocalizations in response to the tickling	The measurement of vocalizations in response to the tickling: 1 or 23 h following 1-h restraint	The measurement of vocalizations in response to the tickling, followed 2 h later by 10% sucrose preference test lasting for 2 h

2.4. Statistics

The results are presented as the mean number of vocalizations per 15 s, averaged from the readout of two investigators, and analyzed with a series of 2-way mixed design ANOVA (the day of test × group). The mean amount of consumed fluids in g/kg was subjected to a 3-way ANOVA (the day × group × fluid). Duncan test was used as a post hoc (SPSS 16 for Windows). The alpha value was set at $P < 0.05$ level. The data fulfilled criteria of normal distribution.

3. Results

3.1. The effects of tickling on the emission of high-frequency vocalizations

Fig. 1 shows a typical pattern of ~50 kHz ultrasonic calls produced in response to the tickling. Based on 696 pairs of measurements (29 rats × 24 days), a significant correlation was noted between the number of calls registered by two investigators ($r^2 = 0.4655$, $P < 0.0001$). The auditory output produced by a relatively inexpensive ultrasound heterodyne bat detector complemented a more sophisticated interactive sound analysis software providing on-screen display of the calls.

During the pre-stress period, the number of calls increased gradually (Fig. 2 left panel). There were no differences among groups but a 2-way ANOVA showed significant effects of the day: $F(13,338) = 24.0267$, $P < 0.001$.

3.2. Immediate effects of stress on the emission of high-frequency vocalizations due to the tickling

The restraint stress did not produce any visible effects on behavioral responses to being handled and tickled. Fig. 2 middle panel shows that stress decreased the number of calls mostly in the rats tickled 1 h after stressing. In this group the number of calls increased along with the day of stressing and on days 5–7 it became non-significantly different from the controls. In the rats tickled 23 h after stressing, at no time point the number of calls was significantly different from the controls. However it appeared that also in this group the stress affected the response, because on days 2, 4, 6 and 7