Modulation of 22-kHz Postejaculatory Vocalizations by Conditioning to New Place: Evidence for Expression of a Positive Emotional State

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It has been assumed that the 22-kHz ultrasonic vocalizations (USVs) are emitted by adult rats as a result of a negative emotional state. However, emission of the 22-kHz vocalizations by male rats has been also observed following ejaculation, which has a high rewarding value as shown by a conditioned place preference test. These observations suggest that 22-kHz USVs may also occur in response to a positive emotional state. The aim of this study was to determine whether the postejaculatory 22-kHz USVs are related to conditioning processes. The 22 kHz USVs were recorded in Sprague-Dawley males in the postejaculatory refractory period during conditioning processes to a new chamber unrelated to copulation. During the first session in the clean recording chamber, males vocalized marginally and exhibited intensive rearing behavior. From the second to fourth sessions, vocalization duration increased and the number of rearing decreased. Following established conditioning process, odor cues from foreign males, but not the familiar ones, resulted in decreased duration of 22-kHz USVs and increased the number of rearing. On the other hand, in the presence of mating cues (copulatory chamber and presence of the female), males exhibited increased duration of postejaculatory 22-kHz USVs and reduced number of rearing. These results demonstrated that the conditioning to the cues, both unrelated and related to copulation, is important for evoking postejaculatory 22-kHz USVs as well as the relaxation state. Furthermore, these results confirmed the postejaculatory 22-kHz USVs' involvement in expression of the positive emotional state.

Keywords: ejaculation, CPP, 22-kHz ultrasonic vocalization, emotional state, rats

Ultrasonic vocalizations (USVs) in rodents attract attention as a model of communication, expression of emotions, or changes of arousal level during social interactions (Arriga & Jarvis, 2013; Asaba, Hattori, Mogi, & Kitkusui, 2014; Bell, 1974; Brudzynski, 2015; Holy & Guo, 2005; Sales & Pye, 1974; Willadsen, Seffer, Schwarting, & Wöhr, 2014; Wöhr & Schwarting, 2014), also including sexual behavior (Bialy, Rydz, & Kaczmarek, 2000; McGinnis & Vakulenko, 2003; McIntosh, Barfield, & Geyer, 1978; Nyby, 1983; Sachs & Bialy, 2000). On the other hand, some authors have expressed skepticism regarding the communication function of USVs in rats (Ågmo & Snoeren, 2015; Blumberg & Alberts, 1991; Snoeren & Ågmo, 2013). Despite the advancing knowledge in this field, many aspects still remain unexplained. One of the unanswered questions concerns the function of the 22-kHz vocalizations emitted by males, and specifically whether it is limited to expression of negative emotional state. The present study addressed this question in the context of conditioning to new place—environmental cues in relation to postejaculatory vocalizations. Conditioned place preference (CPP) procedure has shown, that ejaculation has the highest rewarding value of copulation (Ågmo & Berenfeld, 1990; Tenk, Wilson, Zhang, Pitchers, & Coolen, 2009).

In the adult rats, two main types of ultrasounds can be detected: 50-kHz (30-kHz–70-kHz) USVs including short calls (typically from 10 ms to 150 ms), and the second type, 22-kHz USVs including flat long-lasting (1–3 s) loud sounds, although, at this frequency, calls shorter than 300 ms can also be observed (Brudzynski, 2015). On the basis of mostly pharmacological research, Brudzynski (2007, 2015) proposed a simple and very attractive model explaining the function of these calls in expres-
sion of emotion, with the 50-kHz USVs reflecting a positive emotional state of the animals and the 22-kHz USVs a negative one.

Long-lasting 22-kHz vocalizations occurring during an aversive situation are well documented. They serve as alarm cries altering the behavior in a colony of rats (Blanchard, Blanchard, Aguillana, & Weiss, 1991), and reflect an enhanced level of anxiety but not fear (Jelen, Soltysik, & Zagrodzka, 2003). One of the most controversial points in Brudzynski’s (2015) model is the function of the 22-kHz USVs occurring following the ejaculation (Barfield & Geyer, 1972; Sachs & Bialy, 2000), because of the strong rewarding value of ejaculation, suggested by the instrumental reactions and the CPP tests. Males exhibited place preference that could be observed even after a single mating session (Ågmo & Berenfeld, 1990; Tenk et al., 2009). Also during instrumental reactions, the removal of a male after ejaculation to a neighboring compartment results in a subsequent spontaneous postejaculatory departure of a male with a preference for this compartment (Beck, 1997; Beck, Bialy & Kostowski, 2002). During postejaculatory 22-kHz vocalizations, males display little movement, and the EOG recording displays a sleep-like slow wave activity (Barfield & Geyer, 1975) consistent with the relaxation state. It has also been suggested that postejaculatory 22-kHz USVs represent a desist-contact signal directed to the estrous female to defer her sex soliciting behavior until the male is ready to resume copulation (Barfield & Geyer, 1972). This hypothesis, when tested, showed no differences in female behavior in the presence of the intact-vocalizing male, devocalized male, or devocalized male accompanied by the playback of male 22-kHz USVs (Thomas, Howard, & Barfield, 1982). Alternatively, Blumberg and Moltz (1987) suggested that such vocalizations are artifacts and the effect of a breathing pattern that serves to cool the brain after copulation.

We have previously suggested that postejaculatory 22-kHz calls serve a communication function, and/or reflect the expression of emotions related to the female presence after ejaculation: The presence of the female increased 22-kHz vocalizations, facilitated noncontact erections, and delayed the exploratory behavior (Sachs & Bialy, 2000). However, the absence of the female didn’t completely reduce such calls, which suggested a possible effect of additional cues in modulating the postejaculatory 22-kHz vocalizations.

Method

Animals

Adult Sprague-Dawley rats (12 males and 13 females; 3 months old and sexually naïve at the beginning of training) were used in the present study. All males and 10 females acquired sexual experience and were intensively acclimated to the experimental procedures for the period of at least 3 months. They were provided with water and laboratory chow ad libitum, and housed in rooms at reversed 12h light–dark cycle (lights off at 09.30), and a temperature of 23 °C, with 5 animals per cage (58 cm × 37 cm × 20 cm). Additionally, all animals were handled 2–3 times a week, and fed with enriching compounds (fresh vegetables, fruit, cereals and dairy).

Females were ovariectomized under IP injection of ketamine 10 mg/100g with Xylazine 1 mg/100g anesthesia. Ten females were housed in a separate room and were used in copulatory tests after pharmacologically induced estrus by SC injections of estradiol benzoate 50 μg/rat and progesterone 500 μg/rat, at 48h–72h and 4h–8h respectively, before the test. Three other females did not receive hormonal injections, and one week after recovery were introduced into cages with males. The males were housed in a separate room in three socially enriched groups consisting of four males with one anestrous female per cage. In such conditions, males were acclimated to cues from the female. This type of housing simulates more closely natural conditions, and communication between males and females, which facilitates full development of the communication behavior including USVs.

Behavioral Procedures

All behavioral tests were conducted between 12.30 and 17.30.

Training sessions. During the first three weeks in the laboratory, all rats were acclimated to the handling and to all chambers used during both training and experiment, for at least three sessions lasting 10 min for the first time and 5 min subsequently. Copulatory training was initiated following the habituation. All males acquired sexual experience during four 30-min sessions with estrus females by simultaneously placing four males in four copulatory cages (43 cm × 28 cm × 15 cm). Odors from estrous females were present in a copulatory chamber, as receptive females were introduced into the chamber for 10–15 min before the experiment. During both the copulatory training and subsequent experimental sessions, the male was placed into the copulatory chamber 5 min prior to the female. One female usually copulated with 2 males; individual males were always exposed to a single female. The copulatory chamber was washed only after the last male from each cohort, so a particular male during copulation was exposed to the odor of both the females and the familiar males. During the subsequent copulatory training sessions, performed several times, males were habituated to the removal procedure from the chamber after ejaculation. The sessions continued until the first ejaculation; immediately after genital grooming, the male was carried to a separate recording room and placed into the recording chamber. We also habituated all males to different conditions in the recording chamber; each of the seven times the individual male after ejaculation visited the recording chamber, the environment was different (presence of estrous female, odor of males from same/other cage, lack of any odors, different shape/color of the cage). The recording chamber was washed with alcohol and water after every male. If the male did not achieve any intromission, the session was terminated after 20 min. Failure to copulate during three sessions resulted in exclusion of the male from the study (2 males were excluded from one cage). Copulatory sessions were performed once a week.

Experimental sessions. The diagram of the experimental sessions is present in Figure 1. The surrounding visual cues in the recording room during the experimental sessions were different compared with training sessions, as far as possible. Also, the recording Plexiglas chamber (dimensions: 33 cm × 23 cm × 21 cm) was never used in training sessions. The recording chamber was washed with alcohol and water after each session. Experimental copulatory sessions were performed with a single male in a Plexiglas copulatory chamber (dimensions: 50 cm × 25 cm × 32 cm). Following, the first ejaculation, just after genital
grooming, the male was taken out from the copulatory chamber, placed in the recording chamber, and transported to the recording room (this procedure lasted about 20–25 s). Observations were continued for 300 s after ejaculation. This procedure was repeated four times (Sessions 1–4).

Subsequently, the 10 males were divided into two subgroups (each, \( N = 5 \)) with similar copulatory, behavioral, and vocalization parameters. After ejaculation, the males from the first group were recorded in a recording chamber with the presence of odor from males from another cage (unfamiliar, foreign male odor), and the males in the second group were exposed to odor from the familiar males from the same cage. During the next session, males from the first group were exposed to odor from males from the familiar, same cage and males from the second group were exposed to the odor of unfamiliar males from another cage (Sessions 5 and 6). During the last session, each individual male was removed from the copulatory chamber, the copulatory chamber with the female was moved to the recording room, the male was introduced again into the copulatory chamber, and the copulatory chamber was used for recording. In this case, the individual male was exposed to cues from a female, from odors from copulation, and from the copulatory chamber, but not cues from the copulatory room (Session 7).

**Behavioral Recording and Measurement**

Postejaculatory behavior was recorded using the Noldus Etho-Vision observation system, and the ultrasonic vocalizations simultaneously recorded by the Metris Sonotrack system equipped with spectral ultrasounds analysis. The postejaculatory behavior was analyzed in terms of the following: latency to first vocalization (VL), that is, time from ejaculation to first call at 22-kHz; vocalization duration (VD), that is, time from first to last call at 22-kHz; number of 50-kHz calls, that is, number of ultrasounds at the frequency from 30- to 80-kHz; rearing (R), that is, number of rearing. Because the recording chamber was small, movement activity was correlated with the number of rearing.

**Statistics**

Statistical analysis was performed using repeated measured analyses of variance (ANOVAs) and subsequent post hoc Fisher tests and contrast analysis. Vocalization latency does not have a normal distribution and required transformation suggested by the JMP statistical program. Data of the 5th session—in the recording chamber with the odor of the foreign males—were collected from the 5th session from first subgroup and the 6th from the second subgroup. Data of the 6th session—in the recording chamber with the odor of the familiar males—were collected from the 6th session from first subgroup and the 5th from the second subgroup. For additional analysis of the effect of foreign versus familiar male odor, ANOVAs and post hoc Fisher tests were used for subgroups (each consisting of 5 males) analysis.

**Results**

Data from experimental sessions showed that VL, VD, and R differed significantly as a function of conditioning to cues from the recording chamber. During the 1st conditioning session, males emitted 22-kHz USVs for a very short time, and the increase of VD was detected from the second session onward (Figure 2A). Exposure to odor of the foreign males affected both VD and R, with a significant decrease in USVs and an increase in R (Figures 2B and 3).

**Vocalization latency.** Individual sessions differed significantly with respect to VL (\( F_{6,4} = 2.98, p < .05 \)), with the longest VL during the 1st and the shortest during the 7th session (Figure 2A). Significant differences were detected between the 1st session and Sessions 4, 6, and 7, between the 2nd and the
7th session, and between Sessions 5 and 7. In subgroup analysis (see Figure 3), when the 4th and the 5th sessions were compared separately (using Session × Group analysis), VL did not differ significantly (F_{1,8} = 0.170, with \( p = 0.691 \)).

**Vocalization duration.** During the first conditioning session, only 3 males emitted 22-kHz USVs for a very short time (2 males emitted one call of about 300 ms duration, and one male one call of 700 ms duration). Increase in VD was detected from the 2nd session onward (Figure 2A).

Individual sessions differed significantly (F_{6,54} = 18.572, \( p < .001 \)) with respect to the VD, with the shortest VD during the 1st session and the longest during the 7th session (Figure 2A). Significant differences were detected in VD between the 1st and all the other (2nd–7th) sessions, between the 7th and all other (1st–6th) sessions, and between the 5th session (in the recording chamber with the odor of the foreign males, data collected from both subgroups) and the 6th session (in the recording chamber with the odor of the familiar males; Figure 2B). Sessions 4 and 5 did not differ significantly, and showed only a marginal difference (\( p = 0.074 \)). The subgroup analysis (see Figure 3), comparing the 4th and 5th sessions (using Session × Group analysis), revealed a significant effect (F_{1,8} = 9.217, \( p < .05 \)), with differences between subgroups at Session 5 as the effect of the odor of unfamiliar (foreign) versus familiar males (\( p < .05 \)), and between the 4th and

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**Figure 2.** A. Effect of conditioning processes on 22-kHz USVs and rearing behavior. Vocalization latency (VL), vocalization duration (VD), and rearing behavior (rearing number, R). Acquisition of conditioning to new recording chamber, 1st–4th sessions; exposition to odors of foreign (unfamiliar) males, 5th session; exposition to odors of familiar males, 6th session; exposure to female and to copulatory chamber, 7th session. B. Effect of odor of unfamiliar males on 22-kHz USVs and rearing behavior. Exposition to odors of foreign (unfamiliar) males, 5th session; exposition to odors of familiar males, 6th session (measured in an alternating arrangement).

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**Figure 3.** Effect of odor of unfamiliar (foreign) males in subgroup analysis on 22-kHz USVs. VL = vocalization latency; VD = vocalization duration; R = number of rearing. Session 4: Both subgroups of males (Unfamiliar and Familiar) exposed to clean recording cage. Session 5: One subgroup (Unfamiliar) exposed to odors of unfamiliar males; second subgroup (Familiar) exposed to odors of familiar males.
5th sessions in the subgroup exposed to the odor of unfamiliar males ($p < .05$), but not in both subgroups in the 4th session ($p = .81$), nor between the 4th and 5th sessions in the subgroup exposed to the odor of the familiar males ($p = .14$).

**Rearing.** Sessions in the recording chamber differed significantly with respect to the number of rearing ($F_{4/54} = 11.962, p < .001$), with the highest number observed during the 1st session and the lowest during the 7th session (Figure 2A). Significant differences were detected between the 1st session and all other (2nd–7th) sessions; between the 7th and 1st–5th sessions; between the 2nd and Sessions 4, 6, and 7; among the 3rd and 6th sessions; and among the 5th and 6th sessions. Sessions 4 and 5 did not differ significantly ($p = .12$). Subgroup analysis (see Figure 3) of the 4th and the 5th sessions (using Session $\times$ Group analysis) revealed a significant effect of the odor of unfamiliar (foreign) versus familiar males ($F_{1/8} = 10.356, p < .05$), but not in both subgroups in the 4th session ($p = .88$) or between the 4th and 5th sessions in the subgroup exposed to the odor of familiar males ($p = .25$).

**50-kHz vocalizations.** Only a few 50-kHz USVs were detected in experimental sessions; during the 1st session from 0 to 5 calls were recorded, and this number stayed relatively stable during the subsequent sessions: 2nd session (1–8 calls), 3rd (1–8 calls), 4th (1–12 calls), 5th (0–6 calls), 6th (0–20 calls) and 7th (0–5 calls). The difference in the number of 50-kHz calls between sessions was not significant ($F_{4/54} = 0.60, p > .05$).

**Discussion**

The results demonstrated that postejaculatory 22-kHz vocalizations depend on cues associated with the emotional state of the male after ejaculation. Ejaculation alone is not sufficient to evoke fully expressed postejaculatory vocalization; in the absence of any familiar cues related to emotional state accompanying ejaculation, vocalizations occurred only occasionally and were short in duration. Postejaculatory vocalization appears to be sensitive to conditioning to the new cues, unrelated to the copulation, and unrelated to the copulatory chamber, as suggested by a significant increase in vocalization observed in the course of subsequent sessions in the recording chamber. On the other hand, odor cues from foreign, unfamiliar males (which are related to increased anxiety levels) diminished postejaculatory 22-kHz vocalization.

CPP procedures are useful in establishing the rewarding value of contacts or drug administration. CPP occurs as the result of association between cues from the chamber with a positive emotional state, and such association has an influence on future preference to such a place (Prus, James, & Rosecrans, 2009). Ejaculation has the highest rewarding value among other elements of copulation, such as mounting and intromissions. The CPP response can be observed after a single ejaculation during the first exposure to the CPP apparatus (Ågmo & Berenfeld, 1990; Tenk et al., 2009). In the present study, during the first four sessions following ejaculation, the males were not exposed to any cues from copulation, from the female, from the copulatory chamber, or from the surrounding area, except for emotional state evoked by ejaculation. Males were exposed to a new chamber unrelated to the previous sexual experience. Only very weak vocalizations (not typical for the postejaculatory intervals) were observed during the first conditioning session. Usually, males vocalize for 1–4 min and such vocalizations consist of many calls lasting 1 s or longer per call. Our results from the first session suggested that full expression of postejaculatory 22-kHz vocalizations require familiar cues associated with ejaculation. A single conditioning session and an association process to cues from a new recording chamber are sufficient to change subsequent vocalizations and rearing behavior, as indicated by the increased vocalization duration and the decreased number of rearing observed between the 1st and the 2nd–4th sessions. However, only slight changes both in rearing behavior and in the vocalization latency were observed as the effect of subsequent (from 2nd to 4th) conditioning sessions. The increase in 22-kHz USVs shows that the postejaculatory vocalization is dependent on conditioning processes to cues from the chamber unrelated to copulation. Furthermore, conditioning processes resulted in decreased movement activity. These data suggested that ultrasonic 22-kHz USVs occur during the relaxation state and only in a familiar environment, when exploration and movement activity is very low. Bell (1974) suggested, that 22-kHz USVs correlate with a state of abrupt decrease of arousal. One may ask whether the abrupt decrease of arousal is sufficient to evoke 22-kHz vocalizations. The observation of incomplete USVs prior to conditioning processes suggested that it is not, but rather that the most important factor responsible for the full postejaculatory vocalization is the relaxation state evoked by decreased arousal in a safe and familiar environment.

The reduction of 22-kHz vocalization duration and the increase in number of rearing as the effect of exposure to the odor cues of foreign males, which we assume enhances anxiety levels, further supported the hypothesis that 22-kHz USVs reflect a positive and relaxation state rather than a negative emotional state. The effect is not due to the presence of a new element in the recording chamber in well-established experimental situations, because the odor from familiar males (also new in the experimental chamber) did not affect the postejaculatory calls.

The 22-kHz USVs were the longest, and the number of rearing were the lowest, when males following ejaculation were exposed to cues from the female and the copulatory chamber. These results were consistent with a significant role of cues derived from the female after ejaculation in 22-kHz vocalizations described previously (Sachs & Bialy, 2000). All unconditioned and conditioned stimuli related to copulation and ejaculation contribute to the achievement of the full expression of a relaxation state and 22-kHz vocalization, as the presence of the female and cues from the copulatory chamber significantly enhanced postejaculatory vocalizations.

Expression of emotion during 22-kHz vocalizations do not exclude their communication function. Among the male rats, no competition for females is observed when males and females copulate in semi-natural conditions (Chu, Guerracci, & Ågmo, 2015). We can speculate that the function of the postejaculatory vocalizations can be a signal for other members of the group, and that the male is in the relaxation state, does not analyze cues from environment properly, and may not recognize the potential danger. Our preliminary observations showed that during copulation in a group of several males and females, postejaculatory 22-kHz vocalizations inhibit movement of all members of the group, but for only tens of seconds. Following this short response, females display sex soliciting behavior, and copulation with the other members of group returns.
In contrast to the 22-kHz USVs, only limited 50-kHz USVs were observed in males following the ejaculation during all conditioning sessions. In sexually aroused rats before ejaculation, removal of the female leads to a significant increase in 50-kHz vocalizations in male (McGinnis & Vakulenko, 2003). The low level of 50-kHz vocalizations detected in the present study (especially during the first conditioning session, when nearly no 22-kHz USVs and high level of rearing were observed) suggested that ejaculation is sufficient to inhibit the neuronal network (including nucleus accumbens) involved in 50-kHz vocalizations (Bialy, Kalata, Nikolaev-Diak, & Nikolaev, 2010; Brudzynski, 2007).

It has been suggested that the postejaculatory 22-kHz USVs represent desist-contact signals, a state of social depression, or withdrawal (Anisko, Suer, McClintock, & Adler, 1978; Barfield & Geyer, 1975), and that similarly to other aversive situations reflects a negative emotional state (Brudzynski, 2015). Differences in spectral analysis of the 22-kHz vocalization in response to aversive versus postejaculatory vocalization were observed (Burgdorf et al., 2008). It has been shown that 22-kHz vocalizations, which reflect a negative emotional state, depend on muscarinic stimulation (Brudzynski, 2015). After ejaculation, elevated levels of serotonin and diminished levels of dopamine (Lorrain, Riolo, Matuszewich, & Hull, 1999), and both D2 receptor agonist and D1 receptor agonists, depress postejaculatory vocalizations (Beck et al., 2002; Cagiano, Barfield, White, Pleim, & Cuomo, 1989). Moreover, the rewarding value of ejaculation measured in CPP procedure depends on activation of an opioid system (Ågmo & Berenfeld, 1990), although the effect of naltraxone and morphine on 22-kHz vocalizations can be different in different strains of rats (Bialy et al., 2014). These data suggest that several different neurotransmitters systems may be involved in the 22-kHz vocalization associated with the negative versus positive emotional state. Postejaculatory 22-kHz vocalizations should be investigated in relation to activation of the neuronal networks involved in the rewarding value of ejaculation.

In summary, our results showed that postejaculatory 22-kHz vocalizations are related to a relaxation state in a safe, familiar environment, and express a positive emotional state.

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