Effects of experience and context on 50-kHz vocalizations in rats

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Abstract

Rats can emit ultrasonic 50-kHz vocalizations which are generally assumed to reflect the animals' positive emotional state. However, some aspects question the reliability of 50-kHz calls as indicators of positive affective states. Firstly, rats also emit them in novel environments containing scents of other rats, or even while being victims of intra-species aggression. Secondly, huge inter-individual variability in call production can be observed. The present two studies were conducted to further determine factors other than reward, which may influence or even induce calling. Experiment A showed that 50-kHz calls were emitted in relatively high numbers during short isolation in test cages, and, to a lesser extent, also during testing in an open field and an elevated plus maze. Despite inter-individual variability, calling behavior was individually stable over days and occurred irrespective of whether rats were tested in a cage with or without familiar rat scents. These data indicate that 50-kHz calling is not necessarily a response to the presence of pleasurable or social stimuli. Additionally, it was observed that call emission during isolation is strongly affected by prior experience. Rats that had been trained repeatedly in an appetitive discrimination task emitted only few calls during short isolation in test cages, whereas naïve rats emitted high numbers of 50-kHz calls which decreased over time. The most likely explanation is that rats call in response to separation from the cage mate, as the first group was trained before the recordings, while the naïve rats were recorded immediately after separation. This explanation was supported by Experiment B, which showed that the rats that remained alone in the home cage also called at 50 kHz after separation from the cage mates. In both experiments, most of the 50-kHz calls were not frequency modulated, which lend support for the suggestion that this subtype has a social-coordinating function. The present findings urge sophisticated spectrographic analysis of ultrasonic vocalizations and caution when interpreting 50-kHz vocalizations, since specific subtypes of these calls can occur in contexts that are not necessarily pleasurable to rats, and are affected by prior experience and huge individual differences.

1. Introduction

Rats emit distinct types of ultrasonic vocalizations, which differ depending on the animal's age, its current state and environmental factors [1–3]. Juvenile and adult rats mainly produce two different types of ultrasonic vocalizations, representing distinct affective states.

Low frequency vocalizations, often termed 22-kHz calls, are emitted when rats are exposed to predators [4], foot shocks [5–7], during inter-male aggression [8], drug withdrawal [9], handling [10], and social isolation [11]. Furthermore, anxio-lytic drugs can reduce such vocalizations [12,13]. Accordingly, it was assumed that 22-kHz calls reflect a negative affective state akin to anxiety and sadness [5,6].

Conversely, high-frequency vocalizations, often termed 50-kHz calls, occur during or in anticipation of juvenile play [14,15], tickling [16–19], mating [20–24], food consumption [25], electrical self-stimulation of the brain [25], and addictive drugs [26–29]. On the other hand, aversive stimuli like cat scents [2], bright light [15], and the presence of a foot shock cue [25] can inhibit 50-kHz calling in otherwise rewarding situations. Recently, detailed spectrographic analysis of 50-kHz calls has revealed two call subtypes, of which predominantly the frequency modulated variety is emitted during tickling [30]. Remarkably, this call type resembles the squirrel monkey trill call which is related to appetitive behavior [31]. Based on a bulk

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of evidence, it was suggested that 50-kHz calls may serve as sensitive markers of unconditioned and conditioned states of reward [2], representing a rat homologue of human joy [18].

There is other experimental evidence, however, which shows that 50-kHz calls also occur in situations that are not necessarily appetitive to rats. For instance, 50-kHz calls were detected in various experimental controls, for example, in naïve rats that were placed into a test arena containing fresh bedding [24,32], or in saline-injected rats in drug studies [27–29]. Most intriguingly, rats emit 50-kHz calls during aggressive encounters such as in resident–intruder tests [8,13,33–39], with devocalization studies implicating the intruder as the source of high-frequency calls [40,41]. Rats even emit bursts of 50-kHz calls when entering an area associated with the potential presence of an offender [36,37]. Interestingly, the number of 50-kHz calls emitted by the intruder increased with the number of aggressive encounters in this context [37], which could be effectively decreased by anxiolytics, like diazepam [36]. 50-kHz calls have also been recorded when animals were restrained in positions resembling submissive postures [42]. Finally, rats undergoing morphine-withdrawal emit high-frequency calls [43]. All these results indicate that 50-kHz calls can occur also in non-rewarding or even aversive contexts. Indeed, Berridge [44] has questioned the interpretation that 50-kHz calls represent a rat homolog for human joy.

Secondly, a huge inter-individual variability in call production has been observed repeatedly [15,16,19,29]. Recently, Burgdorf and Panksepp ([45], p. 180) reported that only half of their adult rats show "reasonably high levels of tickle induced 50-kHz ultrasonic vocalizations but the remaining half remain very unresponsive". Schwarting et al. [19] provided evidence that such a variability results at least in part from dispositions or traits that are characteristic to the subject under study. Apart from these studies, variability in the emission of 50-kHz calls has received little specific attention, except, that the propensity to emit tickling-induced 50-kHz calls was used to breed rat lines with high and low call rates [17,46].

The present two studies were conducted mainly to determine which contexts and factors other than reward may influence or even induce calling. In Experiment A, calling behavior during solitary exposure to a novel cage was compared between naïve rats versus rats that had prior experience with a food-reinforced discrimination task. It was expected that the latter, due to their experience of an appetitive testing situation, would be less anxious and therefore emit more calls during the housing cage test than their naïve cage mates. In order to screen for potential individual differences in calling, the rats were recorded on three consecutive days. Recording was done in cages with scents of other rats in order to assure high calling rates [32]. The effect of the appetitive and social value of the additional rat scents was tested on the second day, when half of the rats were placed in clean cages without rat scents. In line with Brudzynski and Pniak [32], it was expected that the absence of scents would reduce call rate, and affect qualitative aspects of calling. Finally, in order to identify behavioral measures that might correlate with ultrasonic calling, rats were tested in an open field to determine their reactivity to novelty and their anxiety on the elevated plus maze. These tests were applied since it was shown repeatedly that male Wistar rats, although identical in breeder, age and housing conditions, can show stable individual differences in their locomotor activity in response to novelty [47–51] and anxiety-related behavior [52–55]. As the results of Experiment A were difficult to explain by the affective hypothesis on 50-kHz calling, it was hypothesized alternatively that rats call for their mates, that is, that 50-kHz calls may also serve to establish or keep social contact. This expectation was tested in Experiment B.

2. Experiment A

2.1. Methods

2.1.1. Subjects and housing

Twenty-four naïve male Wistar rats (HsdCpb:WU, Harlan, The Netherlands), weighing 280–310 g on delivery, were used. They were housed in pairs in Makrolon type IV cages (1815 cm^2; height: 20 cm; Tecniplast, Milan, Italy) with a heightened metal wire lid. The cages, which contained bedding material type LTE E-001 (Abedd, Dominik Mayr KEG, Köflach, Austria), were enriched with a shelter, two gnawing sticks and paper towels. Lab chow (RMH-B®, Hope Farms, Woerden, The Netherlands) and water were available ad libitum. Animals were housed in temperature-controlled rooms (22±1 °C) under a reversed dark/light cycle (dim red light; 50 lx: 7:00–19:00; bright white light; 285 lx: 19:00–7:00) with background music (56 dB) throughout. Cages were cleaned and the animals weighed once a week after the day's experimental tests.

2.1.2. General procedure

Here, two groups of rats were compared, namely experimentally naïve rats versus subjects which had repeatedly been tested in an appetitive discrimination task before being monitored for ultrasonic vocalization. Initially, all animals were allowed to adjust to the housing and light conditions for 5 days and were handled on two days during this period. Thereafter, one rat of each cage pair was trained daily in a T-maze discrimination task (termed: experienced rats, $n=12$). The cage mate stayed in the home cage during this time (termed: naïve rats, $n=12$). To enhance motivation in the T-maze, food in the home cage was removed in the early morning of each training day. Recordings of the ultrasonic vocalizations began three weeks after the start of training, when animals were approximately 12 weeks old (body weight: 355±19 g). Experienced and naïve rats were recorded individually on three consecutive days (termed: housing cage test). Two weeks thereafter, training in the discrimination task ended. One week later, all animals were screened in an open field. Finally, they were tested in the elevated plus maze one day later. All training and testing was conducted within the dark phase between 9:00 and 16:00 h (without background music).

All experimental procedures were performed according to the legal requirements of The Netherlands, and had been approved by the Ethical Committee of the Utrecht University.

2.1.3. Appetitive discrimination task

A wooden T-maze was used which consisted of a start box ($l \times w \times h$: $30 \times 25 \times 30$ cm) and two goal boxes ($30 \times 30 \times 60$ cm), connected by arms ($70 \times 30 \times 60$ cm; for details see: [56]). After two 10 min habituation sessions without reward, the daily training sessions started, each consisting of 10 trials and lasting about 30 min. In the first phase, the rats were trained to discriminate the arm that contained a saucer with syrup from the arm with an empty saucer. At the time of the first housing cage test (see below) rats had experienced 7 training sessions. After reaching a criterion of 8/10 correct choices per session, they entered a second phase of 4 sessions in which they had to discriminate between syrup with and without capsaicin. Only 7 rats reached this phase. The preference for syrup was assessed again in the third phase, which was similar to the first phase. By the end of the training, all rats had experienced between 8 and 21 sessions. A Makrolon type III cage (840 cm^2; height: 18 cm; Tecniplast) was used to transport rats to and from the T-maze apparatus.

2.1.4. Housing cage test

For ultrasonic recording, rats were tested individually in a separate experimental room. Recordings were made under dim red light (50 lx) in a Makrolon type III cage with a flat metal wire lid and bedding material type LTE E-001 (Abedd, Dominik Mayr KEG). Experienced rats were trained in the T-maze task before being placed into the recording cage, whereas naïve rats were immediately transferred to the cage. Both groups were tested on three consecutive days for 15 min. On the first day, the cages contained soiled bedding from the home cage, i.e. bedding with scents of both rats. On the second day, half of the animals of each group were exposed to a cage with fresh bedding, whereas the other half was exposed again to a soiled cage. On the third day, all rats were placed into soiled cages again. During testing, only one animal was present in the experimental room.

2.1.5. Open field test

This test was used to measure individual levels of locomotor activity and anxiety-related behavior. The apparatus, which consisted of a round arena (75 cm diameter) with a 33 cm high wall, was made of dark grey plastic and was located in the middle of a separate experimental room held under dim red light illumination (32 lx). Behavior was monitored with a video camera (Panasonic, NV DS29) suspended 260 cm above the open field. Before each trial, the apparatus was cleaned with warm water and soap, and then dried off with paper towels. Each animal was placed into the open field, facing the wall. Immediately thereafter, sound recording and the observation program (EthoVision; EVCP 3.0, Noldus Information Technology, Wageningen, The Netherlands) were started and the animal was observed for 5 min. Subjects were tested in a randomised order. For behavioral analysis, two concentric areas were defined using EthoVision: centre (25 cm diameter) and outer zone (25 cm width, measured from the wall). As measures of anxiety, latency to enter the centre and time spent in the centre were considered. An entry was defined as the animal's centre of gravity being within a specific area of the open field. Locomotion, that is, the total distance traveled in cm, and rearing behavior were considered as indices of activity. Rearing was measured by an experienced observer.

2.1.6. Elevated plus maze test

The plus-shaped apparatus was made of black plastic and consisted of two closed ($l \times w \times h$: $40 \times 10 \times 27$ cm), two opposite open arms (40×10 cm), and a central square (30×30 cm). The maze was elevated 74 cm above the floor and was located in a separate room with bright white light illumination (260 lx). For behavioral recording, a video camera (Panasonic, NV DS29) was suspended 186 cm above the maze. Before each trial, the apparatus was cleaned as described above. Each animal was placed into the centre, facing one of the open arms. Immediately thereafter, sound recording and data collection with the EthoVision program began. Animals were observed for 5 min in a randomised order. Parameters recorded were the duration and frequency of entries to each of the five areas. To measure anxiety-related behavior, the total frequency of open arm entries and the total time spent on the open arms were measured, along with the latency to enter an open arm.

2.1.7. Ultrasonic recording and analysis

Rat calls were recorded using an UltraSoundGate Condenser Microphone CM16 (Avisoft Bioacoustics, Berlin, Germany), which was positioned 30 cm above the floor of the cage and 60 cm above the elevated plus maze and the centre of the open field. This microphone was sensitive to frequencies of 15–180 kHz, with a flat frequency response (± 6 dB) between 25 and 140 kHz. It was connected via an UltraSoundGate 416 USB Audio device (Avisoft Bioacoustics) to a computer. Acoustic data were displayed in real time by the Avisoft RECORDER, a multi-channel triggering hard-disk recording software (version 2.95; Avisoft Bioacoustics), and were recorded at a sampling rate of 214,285 Hz in 16 bit format.

For acoustic analysis, recordings were transferred to Avisoft SASLab Pro (version 4.34; Avisoft Bioacoustics) and a fast Fourier transform was conducted (512 FFT-length, 100% Frame, Hamming window and 75% time window overlap). Accordingly, the spectrograms were produced at 488 Hz of frequency resolution and 0.512 ms of time resolution. A lower-cut-off-frequency of 20 kHz was used to reduce background noise outside the relevant frequency band to 0 dB. Each call was manually marked by a section label to be included in the automated parameter measurement. Then, various parameters were determined automatically, including peak frequency and peak amplitude, which were derived from the average spectrum of the entire element. Peak amplitude was defined as the point with the highest energy within the spectrum, and peak frequency was defined as the frequency at the location of the peak amplitude. As temporal parameters, latency to call, mean call duration, and total calling time were determined. Finally, the total number of calls emitted was measured. On the basis of their shape, calls were classified into flat and frequency modulated ones according to Burgdorf and Panksepp [45]. Calls containing both types (see Fig. 1c and d) were classified as
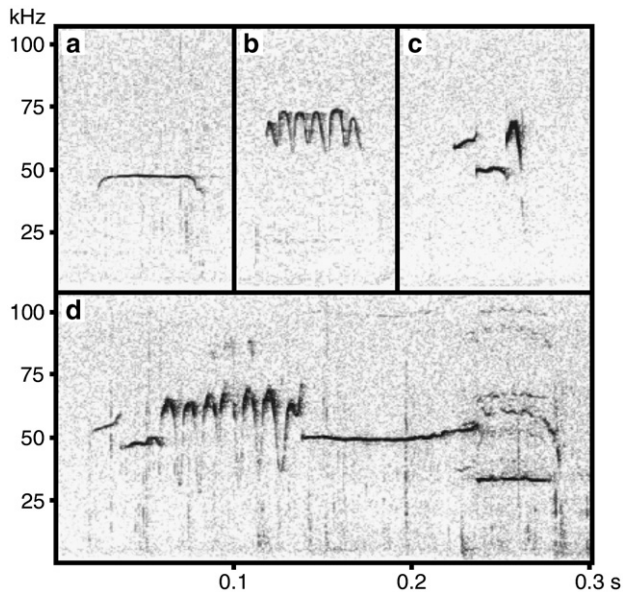
Fig. 1. Spectrograms of four exemplary calls emitted during the housing cage test. Part (a) shows a flat 50-kHz call with one element. Part (b) shows a frequency modulated 50-kHz call with one element. Part (c) shows a 50-kHz frequency step call with three elements. Elements one and two are flat, whereas element three is frequency modulated. Part (d) shows a 50-kHz call with five elements where only element three is frequency modulated.

frequency modulated. Furthermore, calls emitted during the first housing cage test were analysed in more detail by an experienced observer. Here, the extent of frequency modulation, i.e. the difference between the lowest and the highest peak frequency within each specific call, and the number of call elements were measured (see Fig. 1a–d).

2.1.8. Statistical analysis

For each subject, the mean of each call parameter served as the statistical unit. Unpaired two-tailed t-tests were used to determine whether experienced and naïve animals differ in call production during housing cage test. p-values were corrected for unequal variances when appropriate. An ANOVA for repeated measurements was used to test whether the time course of ultrasonic calling differed between both groups in the first housing cage test. Subsequent paired t-tests were used to compare call emission in the first minute versus the last minute. Due to the relatively small number of animals, Mann–Whitney U-tests were used to determine the effect of scents [57]. Mann–Whitney U-tests were also used for comparisons between experienced and naïve rats in calling and overt behavior during open field testing and elevated plus maze screening. Pearson's correlation coefficient was used to correlate call behavior over days and to explore the relationship between overt behavior and vocalization.

2.2. Results

2.2.1. Housing cage test

During the housing cage test, calls in the range of 22 kHz and 50 kHz occurred. Out of these, 22-kHz calls were emitted only

rarely, that is, on the last two days of the housing cage test, one and two experienced animals, respectively, emitted 22-kHz calls. Naïve rats did not display 22-kHz calls. Predominantly, 50-kHz vocalizations were detected. These were characterized by high variability within and between subjects, especially with respect to their shapes (for examples see Fig. 1).

2.2.1.1. Preceding experiences. 50-kHz calling in the housing cage test was largely affected by preceding experiences (see Fig. 2). On the first day in the housing cage test, T-maze trained rats ($n=9$, since 3 rats had to be excluded due to partial data loss) vocalized with a mean rate of 12.79 ± 4.62 calls, whereas naïve rats ($n=12$) produced 608.81 ± 102.59 calls ($t_{11.045}=5.803$, $p<.001$). Although call rate gradually decreased over minutes ($F_{6,14}=14.091$, $p<.001$), the group difference was detectable at every time point ($F_{1,19}=25.546$, $p<.001$; subsequent t-tests: all p-values $<.050$; see Fig. 3). Also, the time course of call emission differed between both groups ($F_{6,14}=8.690$, $p<.001$). Subsequent t-tests showed that call rates declined from 76.87 ± 17.53 calls during the first minute to 11.01 ± 3.69 during the last in naïve animals ($t_{11}=3.949$, $p=.002$), whereas in experienced rats, the decrease from the first (2.90 ± 1.63) to the last minute (0.32 ± 0.24) was not significant ($t_7=1.793$, $p=.111$). Similar to call rate, total calling time was higher in naïve rats (20.91 ± 5.56 s) than in experienced ones (0.29 ± 0.13 s; $t_{11.012}=3,700$, $p=.003$).

Regarding call types, naïve rats emitted both, more flat ($t_{11.064}=6.015$, $p<.001$) and more frequency modulated calls ($t_{11.020}=4,993$, $p<.001$) than experienced rats. Notably, the ratio between flat and frequency modulated calls differed ($t_{11}=-5.362$, $p<.001$), since experienced rats emitted mostly flat calls ($91.57 \pm 11.57\%$), as compared to $66.75 \pm 9.64\%$ in naïve rats. The mean number of call elements was equal in both groups ($t_{9.748}=3.226$, $p=.681$), but call length was longer in naïve (31.09 ± 2.54 ms) than experienced rats (19.03 ± 2.68 ms; $t_{19}=3.226$, $p=.004$). Also, naïve rats started calling after 4.42 ± 3.62 s as compared to 218.60 ± 105.10 s in experienced rats ($t_{8.019}=-2.037$, $p=.076$).

Additional group differences were found in peak amplitude and peak frequency. Firstly, peak amplitude was lower in experienced (46.10 ± 1.17 dB) than naïve rats (52.90 ± 0.68 dB; $t_{19}=5.305$, $p<.001$). Secondly, calls uttered by experienced rats showed a mean peak frequency of 46.06 ± 2.84 kHz, which was lower than that of naïve animals (55.53 ± 1.06 kHz, $t_{19}=3.470$, $p=.003$). Finally, frequency modulation was less broad in experienced (5.89 ± 1.03 kHz) than in naïve animals (9.73 ± 0.86 kHz; $t_{19}=2.880$, $p=.010$).

On the second and third days, call rates of experienced rats remained low in the housing cage test (27.31 ± 15.24 and 22.89 ± 11.30 calls, respectively). These rats were therefore excluded from further analysis concerning the effect of scents and the stability of ultrasonic calling over days.

2.2.1.2. Scents. On the second day, the previously naïve rats were exposed singly to a cage which contained either familiar scents again or fresh bedding ($n=6$ each; Table 1). Apart from a difference in the very first minute ($U=5.0$, $p=.041$), animals
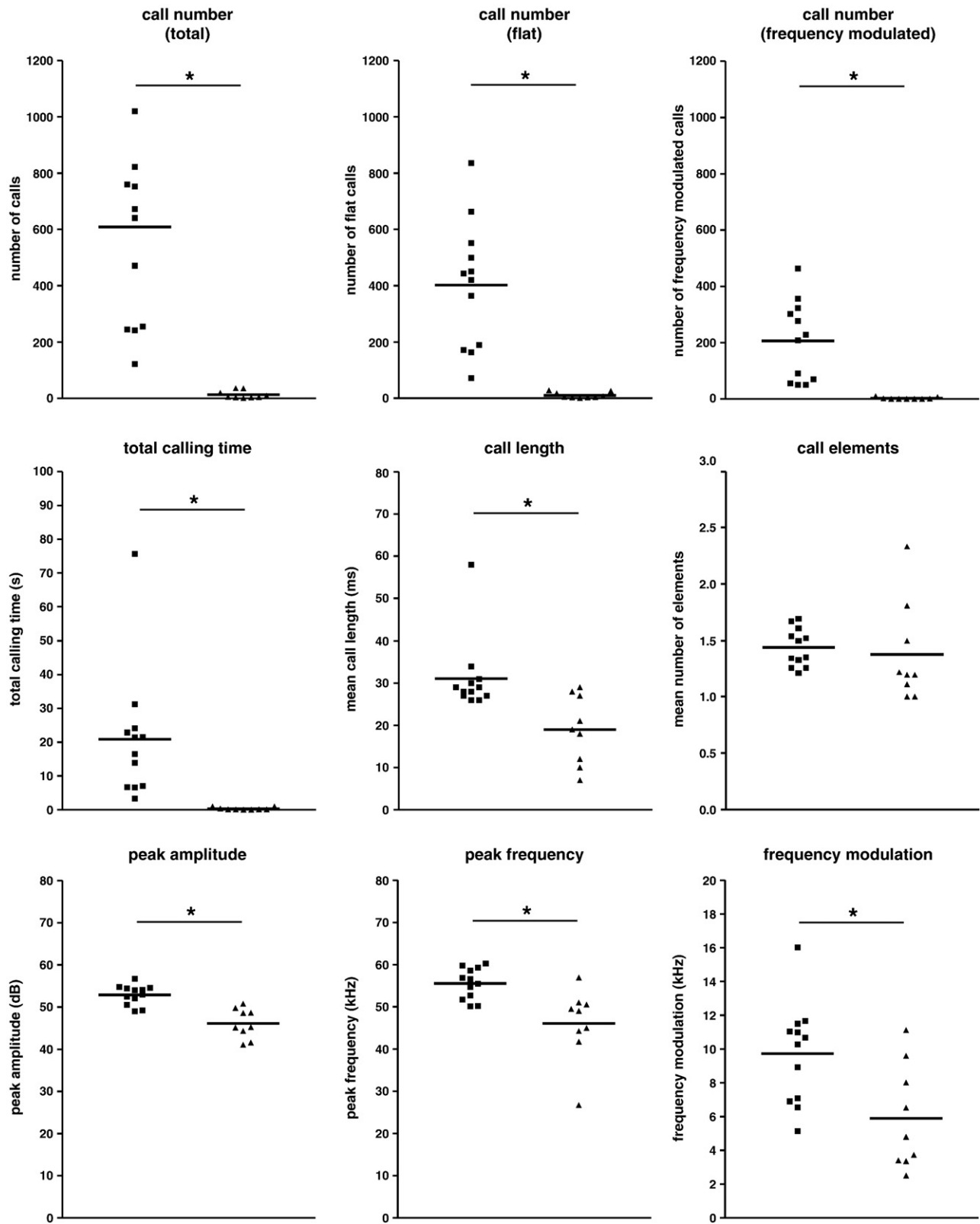
Fig. 2. Column graphs depicting the effect of prior experience on call number (total, flat, and frequency modulated), total calling time (s), call length (ms), number of call elements, peak amplitude (dB), peak frequency (kHz), and frequency modulation (kHz) during housing cage test 1. Symbols reflect individual averages (except for call number and total calling time) of rats which were naïve (squares; $n=12$) or experienced (triangles, $n=9$). Significant group differences are marked with asterisks: $*p<.05$.
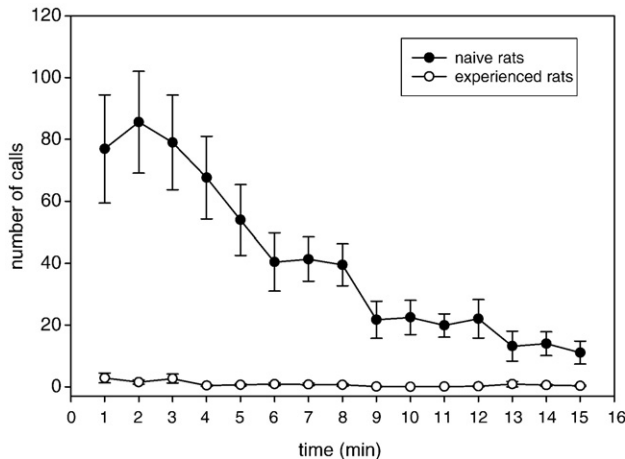
Fig. 3. Time course of call number per minute during housing cage test 1. Symbols reflect group averages for rats which were naïve (black circles; $n=12$), or experienced (open circles, $n=9$).

which were tested in a soiled cage did not emit more calls than animals tested in a clean cage ($U=11.0$, $p=.310$). Also, there were no differences in total calling time ($U=11.0$, $p=.310$), mean call duration ($U=14.0$, $p=.589$), amplitude ($U=15.0$, $p=.699$), peak frequency ($U=15.0$, $p=.699$), or latency to call ($U=7.0$, $p=.093$). An unexpected a-priori difference between both groups was detected on the first testing day, since animals, which were exposed to the soiled cage, had emitted significantly more calls on the first day of the housing cage test than animals which were exposed to the clean cage ($U=5.0$, $p=.041$).

2.2.1.3. Individuality. On day 3, the naïve animals were again tested in a cage containing fresh bedding. Here, they still emitted rather high rates of 50-kHz calls, although a reduction from day 1 to day 3 was observed (608.81 ± 102.59 versus 358.01 ± 58.98 calls; $t_{11}=5.933$, $p<.001$). Furthermore, call rate decreased over minutes in a pattern similar to that of day 1. Call rates did not differ between animals which had been either exposed to a cage containing familiar scents or fresh bedding the day before ($U=19.0$, $p=.699$).

Between days, stable individual differences in call emission were detected. For one, call rates were significantly correlated (day 1 versus 3: $r=.719$, $p=.008$). Similar correlations were obtained in case of call duration ($r=.970$, $<.001$) and total calling time ($r=.792$, $p=.002$), and a trend in case of latency to call ($r=.530$, $p=.076$). Apart from those temporal parameters, peak frequency ($r=.670$, $p=.017$) and amplitude ($r=.577$, $p=.050$) were also significantly correlated between days 1 and 3.

2.2.2. Open field test

Behavioral parameters in the novel open field were not affected by the preceding experience of T-maze training, since experienced and naïve animals did not differ in any behavioral parameter (all p-values$>.100$). Compared to the housing cage test, 50-kHz calls were detected only rarely. This time, however, naïve animals emitted less calls (2.17 ± 0.93) than experienced ones (6.08 ± 1.77; $U=35.0$, $p=.028$). This effect disappeared when only calling animals were considered for analysis

($U=17.0$, $p=.160$), since only 6 out of 12 naïve, but 10 of 12 experienced animals, vocalized. Overall, animals emitted mostly flat calls ($63.66\pm8.68\%$). Despite the fact that groups did not differ in the relative number of frequency modulated and flat calls ($U=21.50$, $p=.192$), the higher proportion of flat calls was only evident in experienced rats ($73.95\pm6.36\%$) and not in naïve rats ($46.53\pm19.67\%$). Notably, none of them displayed 22-kHz calls.

Since naïve, but not experienced, rats had shown substantial call rates in the housing cage test, only the former were used to correlate calls in the housing cage test with behavior in the open field. These animals entered the centre 9.75 ± 1.72 times and spent 13.90 ± 2.34 s there. The first entry into the centre occurred after 32.48 ± 12.08 s. Furthermore, the animals traveled a total of 2395.86 ± 101.59 cm (centre: 164.10 ± 27.24 cm) and showed 35.50 ± 2.44 times of rearing. Pearson's correlation revealed that the distance moved in the open field was positively correlated with total calling time ($r=.582$, $p=.047$) and mean call length ($r=.608$, $p=.036$) in housing cage test 1. Also, a negative correlation with peak frequency was obtained ($r=-.719$, $p=.008$). None of the other parameters yielded substantial correlations (all p-values$>.100$).

2.2.3. Elevated plus maze test

Similar to the open field, no 22-kHz and only a few 50-kHz calls were recorded in the elevated plus maze. Again, experienced animals predominately emitted 50-kHz calls (experienced rats: 8.27 ± 1.97; naïve rats: 3.08 ± 0.98; $U=30.0$, $p=.025$). This effect was again largely due to call likelihood, since no difference ($U=30.0$, $p=.152$) was observed when only vocalizing animals were analysed (naïve: 9/12; experienced: 11/11 — one animal was excluded from further analysis since it fell off the plus maze). Remarkably, both groups emitted primarily flat calls (experienced rats: $79.79\pm5.36\%$; naïve rats: $62.84\pm13.77\%$; $U=38.50$, $p=.412$). Apart from ultrasonic calling, groups did not differ significantly in behavioral parameters (all p-values$>.100$). The number of calls emitted in the open field was positively correlated with that emitted in the plus maze ($r=.581$, $p=.004$).

Again, only naïve rats were used to compare vocalization in the housing cage test with plus maze behavior. These animals spent most of the time in the enclosed arms (249.65 ± 7.55 s), entered the open arms rarely in comparison to enclosed arms (0.50 ± 0.26 and 7.75 ± 1.27, respectively), and first entered the open arm after 240.12 ± 29.91 s. Plus maze behavior was only

Table 1
Comparison between rats placed in soiled and unsoiled cages

	Unsoiled ($n=6$)	Soiled ($n=6$)	
Latency to first call (s)	16.36 ± 5.93	5.04 ± 3.67	$p=.093$
Number of calls	256.50 ± 67.83	379.17 ± 70.78	$p=.310$
Number of calls in first min	30.50 ± 18.96	74.67 ± 18.69	$p=.041$
Total calling time (s)	7.71 ± 1.86	13.06 ± 3.33	$p=.310$
Mean call length (ms)	30.20 ± 1.40	33.21 ± 3.52	$p=.589$
Peak amplitude (dB)	51.45 ± 1.41	50.86 ± 0.83	$p=.699$
Peak frequency (kHz)	53.52 ± 1.86	53.86 ± 1.45	$p=.699$

Given are means$\pm$SEM. p-values reflect the results of Mann–Whitney U-test.

weakly associated with ultrasonic calling during the housing cage test, since only the latency to utter the first call was negatively correlated with the latency to enter an open arm ($r = -.726$, $p = .007$; all other p-values $> .100$).

3. Experiment B

Experiment A had shown that rats which were experienced in appetitive discrimination tasks barely vocalized, while naïve rats vocalized at surprisingly high levels. This indicates that preceding experience affects calling, although it remains unclear why. Apart from the training experience, the rats of both groups differed in another manner: the naïve rats were recorded immediately after separation from their cage mate, while the cage mate was recorded about 30 min later, as it was being trained first. The steep decline in calling rate of the naïve rats suggests that the motivation to call is strongest upon entering the test cage. An explanation for these findings could be that the rats call after separation from their cage mate to keep contact, and reduce calling when they get no response. If this hypothesis is true, then one would expect that rats which remain in the home cage also call after separation. This prediction is tested in Experiment B.

3.1. Methods

3.1.1. Subjects and housing

10 Wistar (HsdCpb:WU, Harlan, The Netherlands) and 16 Long-Evans (HsdBlu:LE, Harlan, The Netherlands) housed in groups of 2–3 in Makrolon type IV cages under the same conditions as in Experiment A were used. Since surplus rats that had been part of earlier experiments were used, the strains differed in age and experience at the time of recording.

Long-Evans rats were tested at the age of about 12 months. At about 2 months, they had learned to associate a sound with oncoming reward (for details see: [58]). During that period, which lasted 4–6 weeks, they were housed in either standard ($n = 8$) or enriched ($n = 8$) Makrolon IV cages comparable to the ones used in Experiment A. Until ultrasonic testing, the rats were handled regularly, but remained otherwise relatively undisturbed in their home cage.

Wistar rats were tested at an age of about 6 months. At about 2 months, they had been used for the training of aggressive rats that served as residents in a resident–intruder test. Each rat experienced aggressive resident behavior during 2–3 training sessions over a period of 2 weeks. They were housed like enriched Long-Evans rats, and were handled regularly.

3.1.2. Procedure

Two rats of the same cage were recorded at the same time, but in separate rooms with no other rats present. One rat of each cage was isolated in its home cage, its cage mate in a clean Makrolon IV cage with fresh sawdust. If there was a third cage mate, it was temporarily housed in a separate cage in the storage room. Recording started immediately after both rats had been placed in the recording rooms, and lasted 6 min. All recordings were performed between 9:30 and 14:00 h. The order of

recording and the assignment of rats to treatment were done according to an a-priori assessed semi-random schedule.

For ultrasonic recording, two UltraSoundGate Condenser Microphones CM16 (Avisoft Bioacoustics) were used. They were placed 30 cm above the centre of the cage floor and were connected to the Avisoft devices as described under Experiment A. Ultrasonic calls were recorded with a sampling rate of 166,666 Hz in 16 bit format. Acoustical analysis was performed as in Experiment A, and the number of 50-kHz calls was determined. On the basis of their shape, calls were classified into flat and frequency modulated ones as described above.

3.1.3. Statistical analysis

Since calling in the Long-Evans strain did not differ between enriched and standard housed rats ($t_6 > 1.34$, $p > .05$), their results were collapsed across housing conditions. In order to assess whether rats in the home cage called more or less than their cage mates in a novel cage, the number of 50-kHz calls of both were compared using a GLM for repeated measures (recording in home versus novel cage) with strain as the between-subject factor. A GLM for repeated measurements was also used to compare the ratio between flat and frequency modulated calls. To assure normal distribution and homogeneity of variance of the data, the log transformed values plus 1 were used ([59], p. 378).

3.2. Results

As in Experiment A, rats emitted 50-kHz calls in a novel clean cage when separated from their cage mates (see Fig. 4). In line with the prediction, it was found that rats, which stayed alone in the home cage, also emitted 50-kHz calls. Surprisingly, they called even more than the rats in the novel cages (factor test condition: $F_{1,13} = 15.17$, $p = .002$). This effect was found in both strains (no interaction between strain and test condition: $F_{1,13} = 4.08$, $p = .062$), although Wistar rats called more than Long-Evans rats (factor strain: $F_{1,11} = 16.67$, $p = .001$). As in Experiment A, calls emitted were mostly flat ($74.82 \pm 3.86\%$), and this was found irrespective of whether rats were tested alone
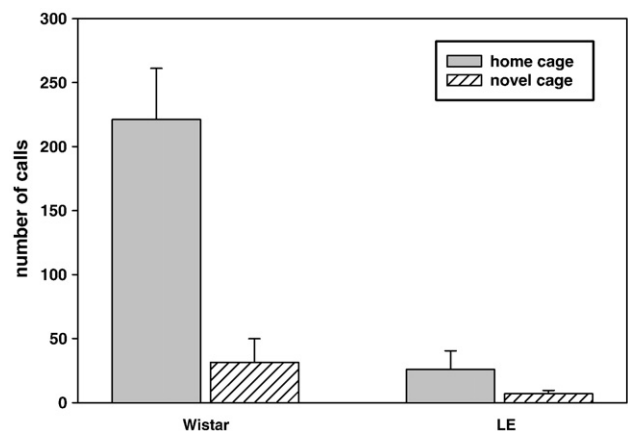

Fig. 4. The number of 50-kHz calls emitted either by Wistar (left) or Long-Evans (LE, right) rats in the home cage (grey bar) or in a novel cage (dashed bar). Data reflect means ± SEM.

in the home cage ($70.83 \pm 4.74\%$) or in a novel cage ($77.53 \pm 5.75\%$, factor test condition: $F_{1,11} = .64$, $p = .440$). However, Long-Evans rats emitted more flat calls ($82.58 \pm 2.44\%$) than Wistar rats ($65.77 \pm 2.64\%$; strain: $F_{1,11} = 20.221$, $p = .001$; no interaction between strain and test condition: $F_{1,11} = 2.53$, $p = .140$).

4. General discussion

The present studies show that male adult rats have rather high rates of 50-kHz calling during transient isolation in a housing cage with bedding containing rat scents from the home cage. Rats even showed high numbers of 50-kHz calls when tested in clean cages containing fresh bedding. During open field and elevated plus maze testing, 50-kHz calls were also recorded, but at very low rates. Importantly, 50-kHz calling was strongly affected by prior experience, since calling in the housing cage test barely occurred in rats that had undergone appetitive discrimination training. Experiment B gave evidence that this effect of experience could be explained by the time since separation from the cage mate. It was found that separation also induced 50-kHz calling in the cage mates that remained alone in the home cage. Finally, evidence was provided that 50-kHz calling is dependent on individual factors, since call production varied substantially between rats, but was highly stable over days and was related to other behavioral measures, as obtained in the open field.

4.1. 50-kHz calling occurred in contexts that are not necessarily appetitive to rats

A bulk of evidence from ethological, pharmacological and brain stimulation studies, shows that 50-kHz calling is positively related to reward and negatively related to aversion, as indicated by place preference, self-administration, and instrumental approach [2,18,27]. The present findings, however, indicate that high rates of 50-kHz calling can also occur in contexts that are not necessarily pleasurable and appetitive to rats. Although the highest rate of 50-kHz calling found in the present study (76.9 calls/min by the naïve rats during the first minute of day 1) is not as high as the highest rates found during tickling (about 200 calls/min, [18], Fig. 2), it is much higher than the highest rate reported for a similar context of scented novel cages (about 17 calls/min, [32], p. 75). Rats in unscented cages on day 2 also had higher calling rates (30.5 calls/min during the first minute). The low call rates reported by Brudzynski and Pniak [32], but also the low rates (about 6 calls/min) found by Schwarting et al. ([19], Fig. 2) in test cages with fresh bedding, could probably be due to the fact that these rats had no cage mate. In fact, social experience [43] and housing conditions, whether singly or in groups [27], are known to modulate calling behavior.

The current results now clearly consolidate earlier suggestions by Schwarting et al. [19] that olfactory cues from other rats are not a prerequisite for substantial calling. The alternative explanation that calling in unsoiled cages on day 2 is solely based on Pavlovian association of scents and context on day 1 is implausible, since animals also emitted 50-kHz calls in Experiment B without such association. Nevertheless, in line with Brudzynski and Pniak [32], calling rates in the unscented cages were lower, albeit only in the first minute. This result, however, is flawed by the fact that the two subgroups had shown unexpected a-priori differences on the day before, where rats to be tested in a soiled cage had higher call rates.

When compared to the housing cage tests, 50-kHz call rates in the open field and the plus maze were rather low in the present study, but this could be explained by the possible aversiveness of these tests [15]. Accordingly, anxiety-related behavior was observed, namely avoidance of the centre and open arms. Nevertheless, the present data, especially the calls during plus maze testing under bright white light, add to other examples of 50-kHz calling in potentially aversive situations, like anticipation of an attacking opponent [36,37], aggressive encounters [8,13,33–41], and states of drug withdrawal [43].

4.2. 50-kHz calling was strongly affected by prior experience

In Experiment A, it was also found that the emission of 50-kHz ultrasonic calls was substantially affected by preceding experience. Rats which had been repeatedly handled and tested in other environments not only emitted fewer 50-kHz calls than the naïve rats, but their calls were also shorter, lower in amplitude and frequency, and were less frequency modulated. When tested in the open field and plus maze after termination of the appetitive training one week later, these rats vocalized more than naïve rats. These results show the importance of preceding experiences, which seem to have distinct and even inversed short- and long-term consequences on 50-kHz calling. Since treatment of the 'experienced' rats differed in a number of ways from that of naïve rats, which included extra handling and repeated exposures to T-mazes with food rewards, the effects cannot be attributed to a specific factor of experience, and will require specific testing in future studies. Furthermore, the possibility that the difference in calling rates between the experimental groups was related to some unknown reward aspect cannot be excluded, since the appetitive value of the housing cage test was not specifically tested.

4.3. The social function of 50-kHz calling

Several hypotheses have been proposed regarding the possible function of 50-kHz calls, like echo-location and others (for some early hypotheses see [60]). Currently, two major hypotheses regarding 50-kHz calls exist, which are not mutually exclusive: 1) calling as an affective expression [2], and 2) calling as a social signal [32,35,60]. The present findings are more in line with the social hypothesis that 50-kHz calls serve to maintain or (re-)establish social contact ("to call for somebody"). The occurrence of 50-kHz calls in non-rewarding situations and the high but decreasing rates of calling in the housing cage test can be explained by the

assumption that rats call for their cage mates. It also would explain why trained rats vocalized much less — they had been taken out of their home cage earlier and might have already reduced vocalization during that period. The corollary prediction that rats remaining alone in the home cage would also vocalize after separation was confirmed in Experiment B. Moreover, these rats vocalized even more than their cage mates which were placed in a novel cage. Therefore, the 50-kHz calls emitted in Experiment A cannot simply be attributed to features of the novel cage, e.g. scents of other rats or joy of exploration. Remarkably, similar effects were obtained in Long-Evans rats and Wistar rats, although call rates and inter-individual variability were higher in Wistar rats. This is not necessarily due to strain differences, since the two groups also differed in age and prior experience. Either way, these results show that it is important to take into account factors like strain, age and experience.

:The social hypothesis is not necessarily in contrast with the affective hypothesis, since 50-kHz calls can serve several purposes. Firstly, communicating a positive affective state by 50-kHz calls may have a function in inducing playfulness [18,61], i.e. "come on and play" [17]. Likewise, it has been suggested that rats may call to signal that they are "approaching in a friendly manner" [32]. Indeed, rats that enter an environment where social contact can be expected emit 50-kHz calls [20,32,36,37]. Secondly, vocalizations can reveal several types of information at the same time, e.g. the species, sex, condition, intention, location and identity of the sender, and especially 50-kHz calls potentially harbor many possible ways to convey subtle types of information. In fact, 50-kHz calls are known for their intra-individual variation, and accumulating evidence indicates that there are actually several call subtypes [8,21–23]. Recently, Burgdorf and Panksepp [45], who divided 50-kHz calls into flat and frequency modulated ones, showed that tickle responders primarily emitted frequency modulated calls. Further evidence that only the frequency modulated variety reflects a positive affective state, is provided by the fact that playback of frequency modulated calls is self-administered, whereas playback of flat calls is not [30]. Finally, Burgdorf et al. [62] have shown that the disruption of the mesolimbic dopamine system either by lesions or pharmacological blockade specifically reduce frequency modulated calls. Thus, it seems to be likely that the conflicting findings of 50-kHz calls in rewarding and non-rewarding or even mildly aversive contexts can be solved by using the distinction between flat and frequency modulated calls. Indeed, during natural behaviors that are clearly rewarding, i.e. rough-and-tumble play and mating, about 90% of the calls were frequency modulated, whereas during aggressive behavior the majority of the 50-kHz calls (about 65%) were flat [30]. Since the proportion of call subtypes in the present study more closely match the findings during aggressive behavior, it seems plausible that flat 50-kHz calls have a more social-coordinating function. Such a distinction may find parallels in humans where the unfelt social smile is a communicative gesture, and the Duchenne smile one that is affectively veridical [63]. At the very least, the present data show that social separation may be a useful and easy method for eliciting this barely understood call variant for further study.

4.4. Individuality

As found in several other studies (e.g. [15,16,19,29,45]), huge inter-individual variability in call emission was observed. Part of this variability can be explained by consistent individual differences, as not only call number and total calling time, but also additional acoustic parameters, like mean call duration, peak frequency and amplitude were significantly correlated between housing cage tests 1 and 3. This consistent inter-individual variety may therefore reflect dispositions or traits of the subject [19]. Moreover, individual differences in call production were related to differences in overt behavior, i.e. locomotion in an open field as tested three weeks later. Individual differences in undrugged locomotion in an unfamiliar open field are thought to gauge the expression of a sensation-seeking trait in the rat [49], which is related to striatal dopamine [50,51], which, in turn, is related to the production of 50-kHz calls [26,28,62]. In contrast to open field behavior, plus maze behavior was not related to ultrasonic calling (but see [19]), possibly due to a ceiling effect based on a rather anxiogenic testing procedure, which may have masked individual differences.

5. Conclusion

The present finding of substantial 50-kHz calling rates in non-rewarding situations shows that the occurrence of 50-kHz calls is apparently not restricted to appetitive contexts. The finding that separation from the cage mates also induced 50-kHz calling in rats remaining in the home cage, suggests that the calls are also used to (re)establish or maintain contact. This also holds for the reduced calling in the experienced rats which were separated for a longer period before being tested. These data show that (social) experience is an important factor to consider when interpreting vocalization data. The finding of the relatively high proportion of flat 50-kHz indicates that this call variant may have primarily a social-coordinating function.

Acknowledgements

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