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SHORT REPORT

Ultrasonic vocalisations during rapid eye movement sleep in the rat

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Summary

Rats are known to use a 22-kHz ultrasonic vocalisation as a distress call to warn of danger to other members of their group. We monitored 22-kHz ultrasonic vocalisation emissions in rats (lean and obese) as part of a sleep deprivation study to detect the eventual presence of stress during the procedure. Unexpectedly, we detected ultrasonic vocalisation emission during rapid eye movement (REM) sleep, but not during non-REM (NREM) sleep, in all the rats. The event occurs during the expiratory phase and can take place singularly or as a train. No difference was detected in the number or duration of these events in lean versus obese rats, during the light versus the dark period, and after sleep deprivation. As far as we know, this is the first report showing that rats can vocalise during REM sleep.

KEYWORDS
diaphragm EMG, high-fat diet, sleep deprivation

1 | INTRODUCTION

Rats are highly social animals and vocal communication is a regular feature of their social interaction. Rats can emit audible sounds (squeals, 2–4 kHz) (Nitschke, 1982), which can express either physical pain/discomfort or the affective dimension of pain or anticipated pain (Borszcz, 2006). However, they mainly communicate in the range of ultrasonic frequencies apparently in order to tell their emotional state and affect the emotional state of other members of the social group (Brudzynski, 2013).

As ultrasonic calls at 22 kHz have been shown to express negative (aversive) states, and serve as warning and alarm calls (Brudzynski, 2013), we have recorded these calls in a study in which wake–sleep regulation was assessed in lean and obese rats kept under a sleep-deprivation protocol, as a possible indicator of discomfort and stress of the rats (Luppi et al., 2014, 2017).

Unexpectedly, ultrasonic emissions at 22 kHz were found not only occasionally in Wake, but also during rapid eye movement (REM) sleep. The occurrence of such REM sleep-related vocalisations was not significantly influenced by the weight of the rat, the time of the day, or sleep intensity, and appears to be a constitutive phenomenon of this sleep phase.

2 | MATERIALS AND METHODS

Methods are briefly summarised, as they have been extensively published previously (Luppi et al., 2017).

2.1 | Animals

In total, 15 adult male Sprague–Dawley rats (Charles River) were used. The rats were housed under normal laboratory conditions: food and water ad libitum, ambient temperature 24.0 ± 0.5 °C, 12-h:12-h light:dark cycle (light period: 9:00 a.m. to 9:00 p.m.; 100 lux at cage level). The experiments were approved by the National Health Authority (186/2013-B) and were carried out under the supervision of the Central Veterinary Service of the University of Bologna in accordance with the European Union Directive 2010/63/EU. All efforts were made to reduce the number of rats used and their possible pain and distress.

2.2 | Experimental protocol

After their arrival at the laboratory, all the rats were fed a standard normocaloric (NC) laboratory diet (D12450B: 3% fat, 10% calories from fat, Mucedola). Starting from the end of the sixth week of life the rats were randomly separated in two groups: one group (seven rats) continued to be fed the standard NC diet, while the second group (eight) received a high-fat hypercaloric (HC) diet (D12492:35% fat, 60% calories from fat, Mucedola). According to this protocol, two experimental groups were studied:

i. NC diet (NC group, seven rats)
ii. High-fat HC diet (HC group, eight rats)

After at least a 1-week recovery from surgery, all rats were studied for 3 consecutive days: the first 2 days under baseline conditions, while during the third day they were totally sleep deprived by gentle-handling during the 12-h light period and were allowed to recover during the following 12-h dark period (post-handling recovery).

2.3 | Surgery

While under deep general anaesthesia (diazepam, ValiumRoche, 5 mg/kg intramuscular; ketamine-HCl, Ketalar, Parke-Davis, 100 mg/kg intraperitoneal), all rats were implanted with:

i. two stainless-steel electrodes for frontal–parietal recording of the electroencephalogram (EEG);
ii. electrodes for nuchal and diaphragmatic recording of the electromyogram (EMG);
iii. a catheter placed into the femoral artery for the telemetric recording of arterial pressure (AP);
iv. a thermistor mounted inside a stainless-steel needle (21 G), which was stereotaxically implanted above the left anterior hypothalamus (from Bregma: 2 mm posterior, 2 mm lateral, and 6 mm ventral) to record the deep brain temperature (Tbrain).

Immediately after surgery rats received 20 mL/kg of saline subcutaneously and a wide spectrum antibiotic intramuscularly. The rats recovered from surgery for at least 1 week, while adapting to the recording apparatus in individual Plexiglas cages kept in a thermoregulated and sound-proof box.

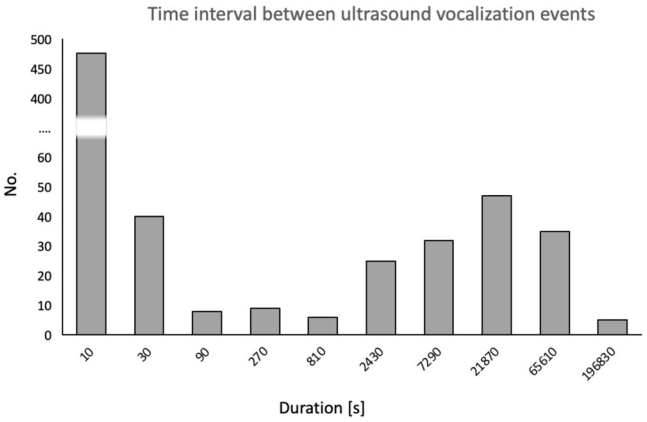

FIGURE 1 Frequency distribution of the duration (s) of the time interval between two consecutive ultrasonic vocalisation events in in either lean or obese rats kept under the different experimental conditions of the study. Classes have been calculated on a logarithmic bases and the upper limit of each class has been indicated.

2.4 | Signal recording and data analysis

After recovery from surgery, each rat was recorded continuously for 3 days, starting at the onset of the light period. The only exception was a brief period every day from 9:00 to 9:15 a.m., during which bedding, food and water were changed.

All bioelectrical signals recorded (EEG, EMG, Tbrain) were first amplified (Grass 7P511L, Astro-Med Inc, West Warwick, RI, USA) and filtered (EEG: high-pass 0.3 Hz, low-pass 30 Hz; EMG: high-pass 100 Hz, low-pass 1 kHz; Tbrain: high-pass 0.5 Hz), then converted to a digital format (Micro MK 1401 II, CED, Cambridge, UK; acquisition rate: EEG: 1 kHz; EMG: 1 kHz; Tbrain: 100 Hz) and stored on a personal computer. The signal of the AP was recorded telemetrically, and heart rate was automatically derived from AP peak detection.

The sound emission was detected through a 'Bat Detector' (BatBox III D, Steyning, UK), a device used to make ultrasound audible by heterodyning the input signal, set for the acquisition of frequencies at 22 ± 8 kHz. The signal was converted to digital format (Micro MK 1401 II, CED, Cambridge, UK; acquisition rate: ultrasonic vocalisations: 1 kHz) and stored on a PC. Sound detection was originally introduced in the study in order to have a gross index of the stress levels in the rats during the gentle handling procedure, but not to make a fine analysis of vocalisations in terms of spectral frequencies analysis of emitted sounds. So, both the range of frequency collection and sampling rate cannot allow a fine discrimination of the differences, if any, in the frequency components of the vocalisations emitted in the different wake–sleep states.

Sound emissions occurred in the form of single events or in that of rapid sequences (series) of events. In fact, as shown in Figure 1, as the frequency distribution of the duration of the time interval

between two consecutive events was bimodal on a logarithmic basis with a minimum at the 31–90 s class, two consecutive events were considered to be part of a series when separated by an interval lasting < 30 s.

In each rat, the following parameters were assessed separately for the light and dark periods:

i. Number of the series
ii. Duration of the series
iii. Average number of the events per each series
iv. Duration of the events

The duration of the series was calculated as the time passed from the beginning of the first to the end of the last event of each series. Thus, the time interval between each event was considered to be part of the series. The number of the series has been normalised on the amount of REM sleep and expressed as *n*/h of REM sleep. Such an amount of REM sleep is reported in the original paper (Luppi et al., 2017) for the different experimental conditions. The analysis of the baseline light (BLL) conditions was carried out on both Day 1 and 2.

2.5 | Statistical analysis

Statistical analysis was carried out using two-way analysis of variance (ANOVA; SPSS version 21.0) with the factors 'body weight' and 'experimental time'. The factor 'body weight' had two levels, NC or HC diet, while the factor 'experimental time' had three levels: BLL, baseline dark (BLD), recovery period (REC) after sleep deprivation. The time 'recovery light' condition was not considered as, of course, no REM sleep occurred during the total sleep deprivation period. The modified *t* test with Bonferroni's correction (Holm, 1979) was used to compare means. For all comparisons, statistical significance was set at $p < 0.05$.

3 | RESULTS

3.1 | Qualitative analysis

Ultrasonic emissions at 22 kHz were found to specifically occur during either Wake or REM sleep, and just once during non-REM (NREM) sleep (see below). In Figure 2, a comparison between a vocalisation series occurring in one rat during Wake or REM sleep is shown. In both cases, the vocalisations occur during the phase of suppression of diaphragmatic activity following an inspiratory act (diaphragmatic contraction). The event can take place singularly or, more often, within a series of consecutive events (up to 31) during subsequent expiratory acts. Events were never observed to occur during an inspiratory act, and the diaphragmatic efforts that preceded vocalisations did not apparently seem to be consistently different from those that were not followed by a vocalisation. Neither a single nor a series of events

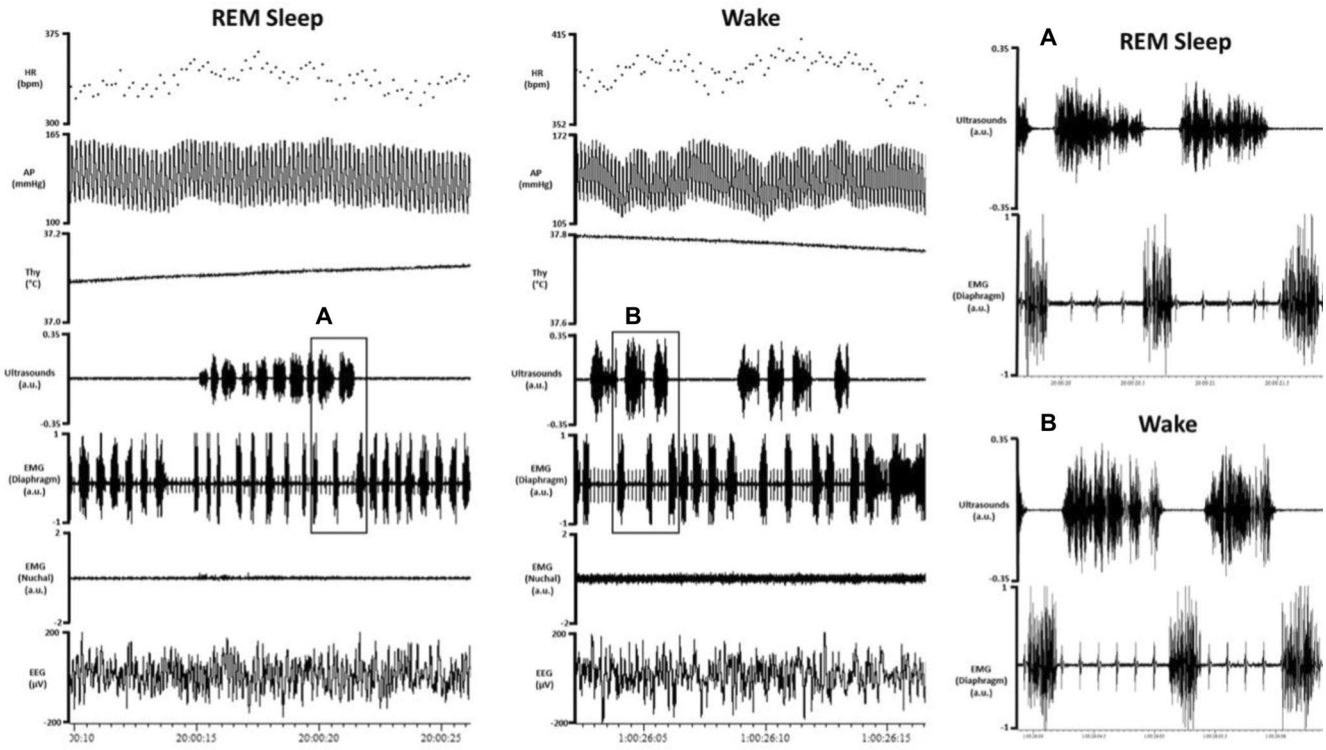

FIGURE 2 An example of a vocalisation event during rapid eye movement (REM) sleep or Wakefulness. The solid box A is expanded in panel A; the solid box B is expanded in panel B. AP, arterial pressure; EEG, electroencephalogram; EMG, electromyogram; HR, heart rate; Thy, hypothalamic temperature.

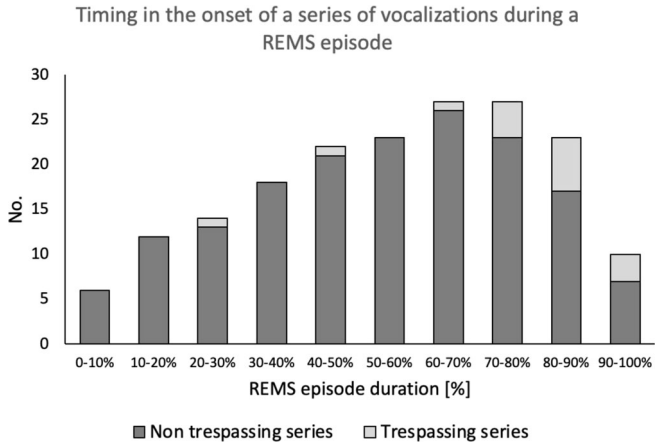

FIGURE 3 Timing in the onset of a series of vocalisations during a rapid eye movement (REM) sleep episode in in either lean or obese rats kept under the different experimental conditions of the study. Bars indicate the number of series of vocalisations starting in each of the 10 consecutive 10% of duration of the different REM sleep episodes. Non-trespassing series (i.e., series starting and ending during a REM sleep episode) are shown in dark grey and are separated by trespassing series (i.e., series starting in REM sleep, but ending in the subsequent Wake or non-REM sleep episode), which are shown in light grey.

were observed to start during a NREM sleep episode, although in ~9% of cases it happened that a series briefly trespassed from REM sleep to the subsequent Wake or, in just one case, even to the following NREM sleep episode.

As shown in Figure 3, the probability for a series of events to start during the REM sleep episode was the lowest at the beginning of the episode, and constantly increased during its occurrence, reaching a maximum at around the beginning of the last quarter of the episode. Moreover, it is clear from the figure that the probability for a series to trespass from REM sleep to the subsequent Wake or even NREM sleep episode was larger for series that started at the end of the REM sleep episode.

3.2 | Quantitative analysis

As shown in Figure 4, overall, the mode in the frequency distribution of the duration of the events occurred at ~600 ms, while ~75% of series were rather short, as they were made by less than five subsequent events. In fact, > 70% of the series lasted < 15 s. The continuity in the frequency distributions of the duration of both events and series suggest that they constitute a single population of cases. Finally, a more detailed analysis of the series that trespassed from REM sleep to Wake or, in one case, to NREM sleep showed that the average duration of the time out of REM sleep for these series was ~3.6 s.

As shown in Figure 5, the series of vocalisations (expressed as *n*/h of REM sleep) were rather rare events and occurred with a similar rate in either the light or dark period of the baseline, or during the 12-h recovery period that followed a 12-h total sleep deprivation,

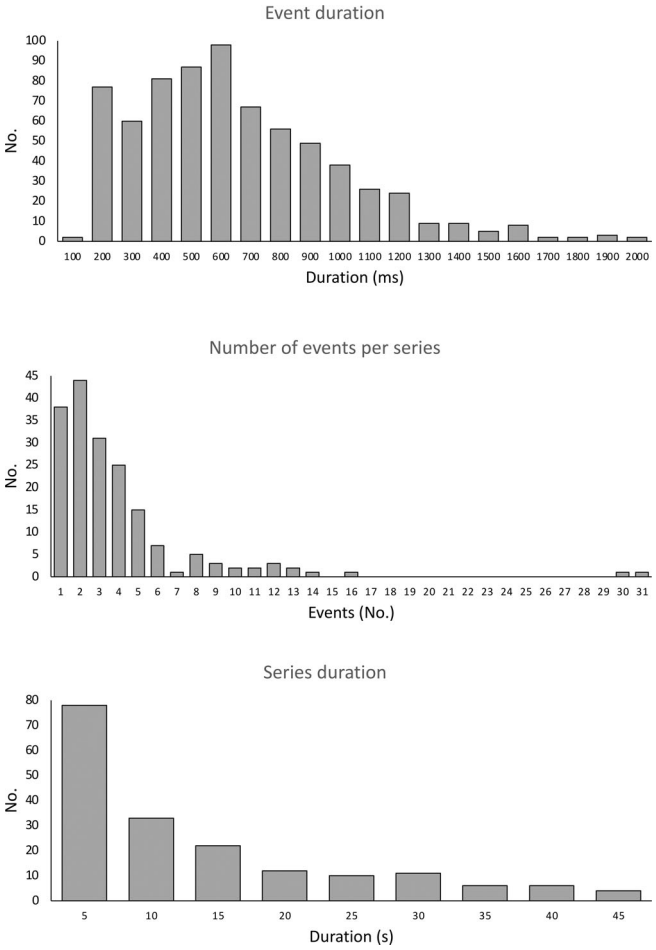

FIGURE 4 Frequency distribution of (a) ultrasound vocalisation events duration (s); (b), number of events/series, and (c) series duration (s) in either lean or obese rats kept under the different experimental conditions of the study. The upper limit of each class is shown for each histogram. The upper limit of each class has been indicated.

without apparent differences between rats kept under a NC or a HC diet leading to obesity. The average duration of the series was also similar among groups, so as the average number of events within each series. Finally, there were also no substantial differences concerning the average duration of the events among the different experimental conditions). The statistical analysis (two-way ANOVA) of the results showed the absence for all parameters of any significant difference due to the different experimental condition (BLL, BLD, REC) or to the diet/weight of the rat (NC diet, HC diet).

4 | DISCUSSION

To the best of our knowledge, this is the first time that the occurrence of repeated 22-kHz ultrasonic vocalisation during sleep resembling those observed during Wake (i.e., sequences of vocalisations occurring during expiration) has been reported in the rat. However, we are aware that the recorder that we used in the present study did not

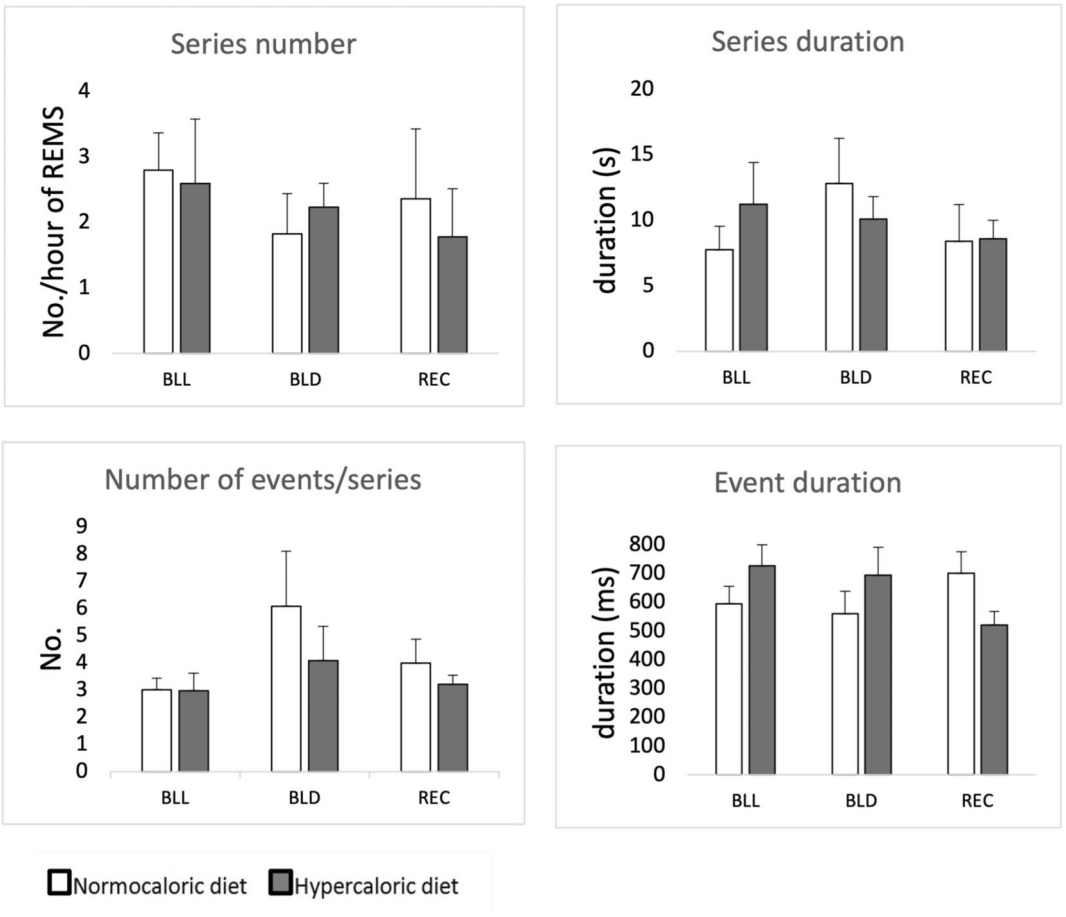

FIGURE 5 Group data comparison for the number of series of vocalisation (top left), duration of the series (top right), number of events per series (bottom left), duration of the event (bottom right) during the light period of baseline (BLL), the dark period of baseline (BLD), and the recovery period (REC) after sleep deprivation.

allow us to make a precise analysis of the physical features of the sounds emitted within the whole range of ultrasonic frequencies, and the possibility that the rat also emits calls at different frequencies during REM sleep cannot be excluded.

Based on the absence of any contraction at the diaphragmatic level during sound emission, it is possible to surmise that during both Wake and REM sleep the 22-kHz calls were produced with a similar modality during a prolonged expiration. Audible sounds (squeals, 2–4 kHz) (Nitschke, 1982) are produced in the rat, as it happens in most other species, by the vibration of the vocal folds. Conversely, when ultrasound vocalisations are emitted, the larynx is stabilised and used as a whistle with a very small orifice created by the vocal folds (Sanders et al., 2001), which are tightly constricted and cannot vibrate (Nyby, 2001). Animals build up considerable abdominal pressure and push air through the orifice, and the vibrating air column produces the ultrasonic sound. In future studies, the components in frequency of ultrasonic vocalisations will need to be better assessed by using more appropriate recording devices and signal acquisition rates. Also, the dynamics of sound generation would be worth investigating by simultaneous recording from the lingual and oral floor muscles, which have been shown to be phasically active during REM sleep (Megirian et al., 1978; Rukhadze et al., 2011). This would allow us to make a better comparison of the quality of the ultrasonic vocalisations emitted in the different wake–sleep states. In humans, sound emission during sleep is mostly linked to snoring. Loud snoring may occur in concomitance with the strong inspiratory effort aimed at overcoming, at least partially, the obstruction of upper airways occurring in patients, often obese, with obstructive sleep apnea (OSA) (Pevernagie et al., 2010). Although recent studies have shown that obese mice can develop conditions of flow limitation in upper airways leading to increases in the inspiratory effort (Fleury Curado et al., 2018), there is no compelling data yet that rodents exhibit OSA spontaneously, even when they are obese. Furthermore, the absence of any diaphragmatic contraction during sound emission leads us to exclude that the sounds that we have recorded were produced during an inspiratory act, suggesting that they were not produced by snoring.

Sound emission during expiration has been described in patients with catathrenia, a very peculiar and rare parasomnia in which, especially during REM sleep, brief inspirations are followed by prolonged expiratory acts during which the subject groans loudly (Vetrugno et al., 2007). The rarity of this disease in humans supports the hypothesis of a different genesis of the two phenomena in humans and rats

and makes it unlikely that physiological vocalisation in rats may work and be used as an animal model for this condition.

As the frequency and the duration of the series of ultrasonic vocalisations during REM sleep seem not to be influenced by the time of the day in which sleep occurs, by sleep intensity, or by the weight of the rat, such a vocalisation seems to be a constitutive phenomenon of this sleep phase. This suggests for this unexpected phenomenon a specific physiological or ethological meaning, although the possible link with pathophysiological respiratory problems needs to be further explored.

An analysis of the meaning of the 22-kHz ultrasonic vocalisation during Wake was made by Brudzynski (2015), suggesting that the 'short' 22-kHz vocalisations, lasting < 300 ms are associated with an internal discontent, while the 'long' one, lasting > 300 ms, express the presence of an external danger or threat. By assuming, with extreme caution, that the same concept may be transferred to REM sleep, it may be speculated that as in our recordings the average duration of the single vocalisation was always > 500 ms, REM sleep vocalisation relates to an external stimulus coming from the laboratory setting or to the occurrence of a standard 'dream' content (whatever it means for a rat) rather than to a reaction of the rat to perceived pain or discomfort as the consequence, e.g., of the presence of a surgical implant for the recording of physiological variables.

In conclusion, beyond the causes and the evolutionary meaning of this phenomenon—that are still far from being understood—REM sleep vocalisations seem to be a constitutive phenomenon of this sleep phase.

AUTHOR CONTRIBUTIONS

Designed the research: Matteo Cerri, Roberto Amici. Performed the research: Fabio Squarcio, Matteo Cerri, Marco Luppi, Timna Hitrec. Analysed data: Alessandra Occhinegro, Emiliana Piscitiello, Davide Martelli, Domenico Tupone, Fabio Squarcio, Ludovico Taddei, Matteo Cerri, Roberto Amici. Wrote the paper: Matteo Cerri, Roberto Amici.

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CONFLICT OF INTEREST STATEMENT

No conflict of interest to report from the authors.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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