Ultrasonic vocalizations – Novel seizure-related manifestation in rats

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ABSTRACT

Objective: Seizures of frontal or temporal lobe origin can associate with vocalizations in humans. Our objective was to assess whether rats emit specific seizure-related patterns of ultrasonic vocalizations (USVs) during seizures and epileptiform activity.

Methods: Adult male Sprague-Dawley rats were treated with a single administration of pentylenetetrazol (PTZ, 50 mg/kg, i.p.) and monitored with simultaneous USV and video-electroencephalogram recordings for up to 15 min. USVs were detected using a deep learning algorithm (DeepSqueak-Screener) and manually annotated into the 15 previously described subcategories. The number, frequency, duration, sonographic structure, and temporal relationship of the USVs to seizures and epileptiform activity were assessed.

Results: A total of 2147 USVs were recorded in 12 rats that expressed a total of 22 PTZ-induced seizures. Of the USVs, 77% were in the 50-kHz range (i.e., appetitive state) and 23% in the 22-kHz (i.e., aversive state) range. More than a third (37%) of the USVs could be classified into 1 of the 15 call subcategories; the remaining 63% belonged to a novel “multiform” USV category with a complex sonographic structure. Of the 2147 USVs, 23% occurred during the PTZ-induced seizures and 77% during other types of PTZ-induced epileptiform activity. Almost all (19/22) of seizures were associated with USVs. In each rat, the first seizure was always associated with a USV. The shorter the latency to the first USV, the shorter the latency to the onset of the first electrographic seizure (r = 0.995, p < 0.001). The greater the number of USVs, the greater the number of seizures (r = 0.916, p < 0.001) and the longer the total seizure duration in a given rat (r = 0.750, p < 0.05).

Significance: Like in humans, vocalizations are a seizure-related behavioral feature in rats and recording USVs provides a novel noninvasive tool for detecting experimental seizures. Further studies are needed to explore USV occurrence during spontaneous seizures and their potential for screening novel anti-seizure drugs.

1. Introduction

Phonatory expression is one seizure-related manifestation of human frontal and temporal lobe epilepsy (Geier et al., 1977; Yen et al., 1996) that may present as a broad range of repetitive and non-repetitive speech or non-speech vocalizations (Gabr et al., 1989; Janszky et al., 2000; Lebrun, 1994). Vocalizations have also been described in non-human primates (Macaca mulatta) during complex partial seizures induced by alumina gel injection in the temporal lobe (Ribak et al., 2000) and in baboons (Papio papio) during seizures induced by light stimulation (Fischer-Williams et al., 1968).

Rodents communicate in the ultrasonic range by emitting ultrasonic vocalizations (USVs) (Brudzynski, 2005). Detection of the USVs requires the appropriate equipment for recording and play-back (Wöhr and Schwarting, 2013). In normal adult rats, 2 broad families of USVs are described (Simola and Brudzynski, 2018; Wöhr, 2018). The 22-kHz call type is associated with negative or aversive emotional states (Litvin et al., 2007), whereas the 50-kHz call type is associated with positive or appetitive emotional states (Knutson et al., 2002). These 2 broad categories can be additionally subdivided according to their acoustic
properties. In the 22-kHz category, the calls are usually long-lasting (up to 300 ms) and range from 18 to 32 kHz without considerable frequency modulation. In the 50-kHz category, the calls are shorter in duration, range from 35 to 90 kHz, and can be frequency-modulated (but can also lack modulation) (Brudzynski, 2005; Simola and Granon, 2019). The 50-kHz category can be further subdivided into 14 call categories, depending on the sonographic structure and frequency modulation features (Wright et al., 2010).

Mice and rats are the most commonly used animal species in experimental epilepsy research (Löschner, 2017). Although less diverse, the spectrum of behavioral seizure manifestations in rodents shares features with that observed clinically, particularly the somatomotor manifestations of clonic, tonic, and tonic-clonic seizures (Velíšková and Velíšek, 2017). Seizure manifestations from other functional outputs such as vocalizations have been noted in only a few studies. Pinel and Rovner (1978) found that the seizure behavior in fully kindled rats was “almost always accompanied by a loud squealing”. Leite et al. (1990) reported squeak-like sonic vocalizations during pilocarpine-induced seizures. Vocalizing is also observed in rat pups with severe kindling stimulation-induced seizures (Haas et al., 1990). No systematic data on seizure-related higher-frequency vocalizations, however, have been reported.

Our objective was to assess whether seizures in rodents are associated with the expression of USVs. We hypothesized that like in humans, rats will emit USVs at least during some seizures. Moreover, emission of USVs will (a) vary during the evolution of seizure-related epileptiform activity and (b) depend on the type of epileptiform activity and seizure severity. Seizures were induced by administering pentylenetetrazol (PTZ), followed by immediate USV-video-electroencephalogram (EEG) co-recordings. This first systematic analysis of seizure-related USVs revealed specific types of calls as components of seizure manifestations in rats, expanding the spectrum of rodent seizure symptomatology.

2. Methods

2.1. Study designs are summarized in Fig. 1A-B

2.1.1. Animals

Adult male Sprague-Dawley rats (n = 15; 358.4 ± 10.5 g; Envigo Laboratories B.V., Melderslo, The Netherlands) were used (14 ± 12 weeks old at the time of the experiments). All animals were individually housed in a controlled environment with temperature 22 ± 1 °C, humidity 50–60%, 12-h light/dark cycle (lights on at 7:00 A.M.) with access to food and water ad libitum. All animal procedures were approved by the Animal Ethics Committee of the Provincial Government of Southern Finland and performed in accordance with the guidelines of the European Community Council Directives 2010/63/EU. All experiments were conducted during the light phase of the circadian cycle.

2.1.2. Recording of ultrasonic vocalizations

2.1.2.1. Recording chamber. The day of the experiment, naïve rats (n = 3) were individually placed in a custom-made plexiglass cage (40 cm wide × 40 cm deep × 30 cm high; Fig. 1C). To minimize locomotion-related noise artifacts, the cage contained no bedding, food, or water. The cage was cleaned with 70% alcohol and dried between every recording session to reduce odors. The cage was placed inside a soundproof recording chamber (SmartChamber, Series-2; Metris B.V., Hoofddorp, The Netherlands) for USV recording and video monitoring.

2.1.2.2. Microphones and Software. Sonotrack Recording System and Software (Version 2.4.0, Metris B.V.) were used to continuously record USVs at a 200-kHz sampling rate and a 16-bit sample resolution. Two additional ultrasonic microphones (Model ST-B200, Metris B.V.) were placed 35 cm above the cage floor together with the default microphone position of the SmartChamber (~55 cm above the floor). The behavior of the rat was monitored using a color video camera (Polaroid Cube+ HD Action Camera) placed in the center above the uncovered custom-made plexiglass cage (Fig. 1C).

2.1.2.3. Analysis. Recorded files were first exported to Waveform Audio File Format (WAV) and then analyzed with “DeepSqueak-Screener” (Lara-Valderrabano and Ciszek, 2020). During the course of the study, we modified the DeepSqueak (Coffey et al., 2019) software to facilitate the screening and manual re-classification of missed or incorrectly detected and measured USVs by automatic detection. In particular, the DeepSqueak-Screener was modified to: (i) enable free backward-forward movement along the sonogram of a record, (ii) display an additional larger sonogram over a time window of several seconds for improved USV contextualization, (iii) increase the number of Custom Labels to 30 and press Ctrl + Left Click on the USV to categorize it. The complete list of modifications, software source code, and executable binary for “DeepSqueak-Screener” are freely available from https://github.com/UEFepilepsyAVI/DeepSqueak.

For the analysis (a) the slider option in the software was set to “High Precision”, (b) the “Multi Detect” feature was enabled, and (c) the networks for “Long Rat Call” (15-s chunk length) and “Short Rat Call” (3-s Chunk Length) were selected. Then, recordings were visually browsed. The automatically detected regions of interest (ROIs) pinpointing a call were visually inspected and corrected when necessary (e.g., ROI area was expanded to completely contain a given call or 2 calls with an interval >20 ms within a single ROI were separated). If an undetected call was found (<2% of all calls), a ROI was added manually to annotate it for the analysis.

2.1.2.4. Classification of USVs. Calls were classified manually into 15 categories previously described (Wright et al., 2010) using the DeepSqueak-Screener. Of these, 14 categories belong to the 50-kHz range and 1 to the 22-kHz range. Calls occurring at the 50-kHz level that did not fit into any of the 14 categories were classified as multiformal USVs as their sonographic structure was widely variable. In animals with PTZ injection (see below), calls were considered to have a temporal association with generalized periodic discharges (GPDs)/myoclonus if (a) the USV occurred within the 300-ms time window before or after the GPD with or without myoclonus or (b) the USV occurred during the PTZ-induced seizure. Calls with noise artifact interference related to locomotor activity were excluded from the analysis. Finally, the call subtype, duration, frequency, bandwidth, and power were exported to a spreadsheet for further analysis.

2.1.2.5. Induction of seizures with PTZ. To monitor seizure-related USVs, unhabituated rats were administered a single dose (50 mg/kg, i.p.) of PTZ (1,5-pentamethylene-tetrazole; Sigma-Aldrich YA-Kemia Oy, Finland) dissolved in sterile 0.9% NaCl (1 ML/kg) (Fig. 1B) and immediately placed into the USV-video-EEG monitoring custom-made cage.

To assess possible effects of the handling, recording chamber and intraperitoneal injection procedure on the number and type of USVs, we performed a preliminary analysis in 3 naïve rats with a single injection of vehicle (0.9% NaCl, 1 ML/kg, i.p.). In this animal group, USVs were recorded for 30 min immediately after the injection. The next day, the same animals were treated with PTZ (50 mg/kg) and recorded for another 30 min under the same conditions (Fig. 1A).

2.1.3. Video-EEG monitoring

2.1.3.1. Electrode implantation. To monitor the evolution and duration of PTZ-induced electrographic seizures, epidural screw electrodes were implanted in 12 rats as previously described (Andrade et al., 2019) (Fig. 1E). Briefly, animals were deeply anesthetized with 5% isoflurane, and then mounted in a stereotaxic frame with lambda and bregma at the...
same horizontal level. The temperature was continuously monitored and regulated with a heating pad (set to a maximum of 38 °C). Anesthesia was maintained with 1.9–2.0% isoflurane during the surgery with SomnoSuite (SomnoSuite Small Animal Anesthesia System # SS6069B; Kent Scientific Corporation, Torrington, CT, USA). Of the 12 rats, 6 were implanted with 4 stainless steel epidural screw electrodes (EM12/20/SPC; P1 Technologies, Roanoke, VA, USA) in the skull in the left (C3; AP: 1.7; ML: 2.5) and right (C4; AP: 1.7; ML: 2.5) frontal cortex as well as in the left (O1; AP: 7.6; ML: 2.5) and right (O2; AP: 7.6; ML: 2.5) parieto-occipital cortex. The remaining 6 animals underwent similar epidural screw electrode implantations. In addition, however, they were implanted with 3 intracerebral tungsten bipolar electrodes (EM12/3–2TW/SPC, 1.0 mm tip separation, P1 Technologies): 1 in the left fronto-parietal cortex (Y1,2 = AP: 1.7; ML: 4.0; DV: −1.8), 1 in the left parieto-occipital cortex (X1,2 = AP: 1.7; ML: 4.0; DV: −1.8), and 1 in the left hippocampus (H1,2 = AP: 3.0; ML: −1.4; DV: −3.6). In all animals, 2 epidural stainless-steel screw electrodes were placed posterior to lambda, 1 on the left (ground) and 1 on the right (reference). The electrodes were connected to a multi-pin connector (MS363; P1 Technologies). Finally, dental acrylic (Selectaplus, DeguDent GmbH) was used to fix the electrode assembly to the skull.

Immediately after electrode implantation and 24 h later, the rats were treated with buprenorphine (0.05 mg/kg, s.c.; Orion Pharma, Finland).

2.1.3.2. Simultaneous USV and video-EEG recordings. To prevent stress or pain related to the electrode headset or the surgery procedure,
recordings were started 18–28 d after electrode implantation (Fig. 1B). The home cage of each rat (without bedding, water, or food) was placed into a custom-made plexiglass cage (29 cm wide × 44 cm deep × 50 cm high) (Fig. 1D). Then, immediately after PTZ administration, rats were connected to a 12-pin swivel commutator (SL12C, P1 Technologies) via a flexible shielded cable (M12C-363/2; P1 Technologies) and placed inside its home cage. EEG recordings were performed using a 320-channel Digital Lynx 16SX amplifier (Neuralynx, Bozeman, MT, USA) with a 10 kHz sampling rate in high definition. The amplifier was configured with an analog bandwidth between 0.01.01 Hz and 80 kHz. The data from each channel was converted individually into 24 bits. During the EEG recording, each rat was monitored using a single high-resolution camera (Basler acA1300-75gm GigE; Basler, Germany) configured to record 30 frames per second (fps; maximum 75 fps) with a resolution of 1.3 megapixels and compressed using H.264. For simultaneous ultra

sound, video and EEG recordings, an ultrasonic microphone was placed 45 cm above the cage floor and connected to a Sonotrack Recording System with the same parameters as described above. All recordings were started when the rat displayed no electrographic seizures during 5 consecutive minutes or after 15 min (mean 10 min 47 s ± 2 min 29 s, range 8 min 19 s - 15 min). Simultaneous USV and video-EEG recordings were performed in an electrically shielded room.

2.1.3.3 Data analysis. Electrographic seizures were defined as described previously by Kharatishvili et al. (2006). Briefly, seizure onset corresponded to an increase in high-amplitude rhythmic discharges distinct from the baseline (repetitive spikes, spike-and-wave discharges, and slow waves) that lasted at least 5s. Epileptic events occurring with an interval of less than 5s without the EEG returning to baseline were defined as belonging to the same seizure. GPDs were defined as waveforms that were repeated and generalized at nearly regular intervals (see Fig. 4C, D). They present with a relative uniform morphology and duration, and with a quantifiable interdischarge interval between events (Hirsch et al., 2021).

Behavioral seizure severity was scored according to the scoring proposed for generalized seizures induced by convulsants that affect gamma-aminobutyric acid neurotransmission (Velísková and Velíšek, 2017). Briefly, seizure stages were scored as follows: 0 - no changes in behavior; 0.5 - abnormal behavior (e.g., sniffing, extensive washing or orientating); 1 - isolated myoclonic jerks; 2 - atypical (unilateral or incomplete) clonic seizures; 3 - fully developed bilateral forelimb clonus, 3.5 - forelimb clonus with a tonic component and twist of body; 4 - tonic-clonic seizures with suppressed tonic phase (only clonus of all limbs); 5 - fully developed tonic–clonic seizures.

2.1.3.4 Co-analysis of USVs and video-EEG. Ultrasonic spectrograms, and video and EEG recordings were imported to Spike2 (Version 9.06; Cambridge Electronic Design, Cambridge, UK) for visualization and offline synchronization. The latencies from the PTZ injection to the first PTZ-induced seizure and to the first USV (or cluster of USVs) were measured from the video-EEG recordings synchronized with the spectrogram.

2.1.4. Statistical analysis All statistical analyses were performed with GraphPad Prism Software (Version 8.0; GraphPad Inc, La Jolla, CA, USA). Statistical differences between the groups were analyzed using nonparametric Mann–Whitney test. Correlations were calculated using Pearson’s test (r). A p value of less than 0.05 was considered significant. All values are expressed as mean ± standard deviation of the mean (SD).

3. Results

3.1. Preliminary study

In the preliminary study including 3 rats, we first investigated whether intraperitoneal injection of PTZ induced any changes in the USV pattern, and whether the pattern differed from that evoked by an intraperitoneal injection of saline. The experiment was performed under video monitoring (no EEG). Each rat received saline on day 1 and PTZ on day 2. PTZ induced behavioral seizures in all 3 animals. Altogether, 4 behavioral seizures were detected, and all occurred during the first 15 min after PTZ injection. The seizure onset latency and duration are summarized in Table 1. The temporal distribution of USVs during the first 15 min after vehicle and PTZ administration in each rat is shown in Fig. 2A-B.

3.1.1. Prevalence and number of USVs after saline and PTZ injections

The total number of USVs emitted by the 3 rats after vehicle injection was 32. We observed no particular pattern or USV bouts during the 15-
Fig. 3. Temporal distribution of ultrasonic vocalizations (USVs) after pentylenetetrazol (PTZ)-induced seizures in implanted animals. (A) USV-video-EEG co-registration in electrode-implanted rats (y-axis) after PTZ-injection (x-axis). The USVs (blue vertical rasters 50-kHz and red rasters 22-kHz) started to occur at the time of the onset 106 ± 28 s (median 104s after PTZ injection) of the first electrographic seizure (light blue bar). In 3 of the 12 rats, the USV occurred within 1 s before or after the electrographic seizure onset, in 3 animals within 5–7 s before the onset. Interestingly, in 6 animals, the USVs occurred within a second of the onset of the electrographic seizure. Of the 12 rats, 9 emitted USVs during postictal suppression (pink bars). All rats emitted USVs during the postictal and interictal epileptiform activity (yellow bars). (B) A bar graph showing the percentage of USVs that occurred during electrographic seizures vs. postictal and interictal epileptiform activity. Note the similarity to Fig. 1, panel C. (C) The longer the delay to the onset of the first electrographic seizure, the longer the delay to the emission of the first USV after PTZ injection ($r = 0.995$, $p < 0.001$). (D) The greater the number of electrographic seizures, the greater the total number of USVs ($r = 0.916$, $p < 0.001$).
min post-injection follow-up. Rather, the calls appeared in an isolated and apparently random fashion (Fig. 2A). The frequency range (50-kHz vs 22-kHz), and total number of calls are summarized in Table 1.

During the first 15 min after PTZ administration, a total of 672 USVs were emitted by the 3 rats, representing 94% of all USVs recorded during the 30-min post-injection follow-up. Importantly, the first USV and the
The percentage of USVs emitted during behavioral seizures vs. postictal and interictal periods was 35% and 65%, respectively (Fig. 2C). The total number of USVs, latency to the first USV, number of USVs at different frequency ranges (22-kHz vs 50-kHz), and latency to the first behavioral seizure are summarized in Table 1.

### Table 1

Characteristics of ultrasonic vocalizations (USVs) in naïve rats injected with 0.9% NaCl or pentylenetetrazol (PTZ 50 mg/kg, i.p., dissolved in 0.9% NaCl) without or with electrode implantation (PTZ only).

| Rats without electrode implantation (n = 3) | Treatment | Parameter | Total Number | Mean ± SD (per animal) | Median | Min | Max |
|-------------------------------------------|-----------|-----------|--------------|------------------------|--------|-----|-----|
| 0.9% NaCl | USVs (number/30 min) | 32 | 10.6 ± 6.8 | 13 | 3 | 16 |
| | Frequency (calls/min) | n/a | 0.3 ± 0.2 | 0.4 | 0.1 | 0.5 |
| | USV 50-kHz (number/30 min) | 22 | 7.3 ± 4.6 | 10 | 2 | 10 |
| | USV 22-kHz (number/30 min) | 10 | 3.3 ± 2.5 | 3 | 1 | 6 |
| | Latency to the first USV (s) | n/a | 100.0 ± 110.9 | 40 | 32 | 228 |
| PTZ | USVs (number/15 min) | 672 | 224.0 ± 266.7 | 130 | 17 | 525 |
| | Frequency (calls/min) | n/a | 7.5 ± 6.6 | 4.3 | 0.6 | 17.5 |
| | USV 50-kHz (number/15 min) | 649 | 216.3 ± 253.8 | 130 | 17 | 502 |
| | USV 22-kHz (number/15 min) | 23 | 7.6 ± 13.3 | 0 | 0 | 23 |
| | Latency to first USV (s) | n/a | 112.3 ± 89.8 | 63 | 58 | 216 |
| | Latency to first behavioral seizure (s) | n/a | 111.0 ± 90.1 | 63 | 59 | 217 |
| | Behavioral seizures (total number) | 4 | 1.3 ± 0.6 | 1 | 1 | 2 |
| | Behavioral seizures (duration, s) | n/a | 32.2 ± 18.4 | 26 | 18 | 59 |

| Rats with electrode implantation (n = 12) | Treatment | Parameter | Total Number | Mean ± SD (per animal) | Median | Min | Max |
|------------------------------------------|-----------|-----------|--------------|------------------------|--------|-----|-----|
| PTZ | USVs | 2147 | 178.9 ± 247.2 | 50 | 22 | 745 |
| | Frequency (calls/min) | n/a | 14.1 ± 15.9 | 6.4 | 2.2 | 50.3 |
| | USV 50-kHz total number | 1965 | 167 ± 244.2 | 47 | 5 | 741 |
| | USV 22-kHz total number | 182 | 15.1 ± 23.5 | 0.5 | 0 | 65 |
| | Latency to the first USV (s) | n/a | 104.5 ± 25.8 | 104 | 75 | 169 |
| | Latency to the first electrographic seizure (s) | n/a | 106.0 ± 27.6 | 103.5 | 75 | 174 |
| | Electrographic seizures (total number) | 22 | 1.83 ± 1.26 | 1 | 1 | 5 |
| | Electrographic seizures (duration, s) | n/a | 33.8 ± 22.6 | 26.5 | 12 | 100 |

Abbreviations: max, maximum; min, minimum; n/a, not applicable; s, second. Values are shown as mean ± standard deviation (SD).

The percentage of USVs emitted during behavioral seizures vs. postictal and interictal periods was 35% and 65%, respectively (Fig. 2C). The total number of USVs, latency to the first USV, number of USVs at different frequency ranges (22-kHz vs 50-kHz), and latency to the first behavioral seizure are summarized in Table 1.
Table 2
Acoustic properties and types of ultrasonic vocalizations (USVs) emitted after vehicle (0.9% NaCl) or pentylenetetrazol (PTZ) administration in rats without or with electrode implantations. Annotated USVs were manually classified into the 15 previously described categories (Wright et al., 2010) or to a new multiform category.

| Treatment Call category | Number | Duration (ms) | Frequency (kHz) | Bandwidth (kHz) | Power (dB) |
|-------------------------|--------|---------------|-----------------|-----------------|------------|
| 0.9% NaCl 50-kHz calls |        |               |                 |                 |            |
| Downward ramp           | 2      | 10.8 ± 8.5    | 63.5 ± 17.9     | 18.1 ± 5.5      | -78.5 ± 4.7|
| Flat                    | 1      | 27.6          | 52              | 40.1            | -72.1 ± 0.6|
| Short                   | 3      | 7.7 ± 1.3     | 52.8 ± 9.1      | 14.0 ± 17.5     | -78.6 ± 0.6|
| Multiform               | 16     | 50.6 ± 36.9   | 50.1 ± 18.7     | 23.1 ± 15.4     | -78.9 ± 2.5|
| 22-kHz calls            | 10     | 43.9 ± 33.1   | 23.7 ± 4.9      | 6.7 ± 4.0       | -86.3 ± 2.3|

| PTZ Call category | Number | Duration (ms) | Frequency (kHz) | Bandwidth (kHz) | Power (dB) |
|-------------------|--------|---------------|-----------------|-----------------|------------|
| 50-kHz calls      |        |               |                 |                 |            |
| Complex           | 2      | 21.6 ± 9.6    | 65.5 ± 7.2      | 2.9 ± 2.1       | -76.1 ± 2.8|
| Upward ramp       | 4      | 59.4 ± 48.4   | 58.8 ± 9.5      | 8.9 ± 4.0       | -74.0 ± 3.5|
| Downward ramp     | 1      | 18.4          | 70.86           | 0.82            | -80.2 ± 0.2|
| Flat              | 1      | 30            | 61.71           | 0.38            | -81.26 ± 0.7|
| Short             | 15     | 9.3 ± 2.3     | 60.3 ± 13.9     | 2.2 ± 1.9       | -80.1 ± 0.6|
| Split             | 5      | 47.4 ± 19.8   | 50.8 ± 10.4     | 27.9 ± 2.3      | -72.2 ± 0.6|
| Step up           | 0      | 0             | 0               | 0               | -          |
| Step down         | 3      | 69.2          | 42.4 ± 0.2      | 26.6 ± 1.1      | -69.6 ± 0.4|
| Multi-step        | 9      | 54.5 ± 13.4   | 49.3 ± 11.1     | 27.2 ± 4.3      | -73.6 ± 0.4|
| Trill             | 4      | 55.1          | 67.0 ± 2.5      | 17.3 ± 6.2      | -80.0 ± 0.4|
| Flat/trill combo  | 1      | 140.4         | 58.4            | 13.3            | -77.0 ± 0.4|
| Trill with jumps  | 36     | 67.0 ± 23.9   | 66.9 ± 3.0      | 21.5 ± 9.6      | -79.8 ± 2.4|
| Inverted U        | 6      | 76.7          | 61.7 ± 5.1      | 6.4 ± 3.9       | -72.3 ± 0.7|
| Composite         | 75     | 804.8 ± 333.5 | 33.5 ± 9.9      | 43.1 ± 7.3      | -73.2 ± 0.6|
| Multiform         | 358    | 84.4 ± 102.5  | 63.2 ± 6.5      | 13.1 ± 10.4     | -76.5 ± 0.4|
| 22-kHz calls      | 23     | 487.1 ± 578.7 | 22.6 ± 5.9      | 7.5 ± 10.2      | -83.2 ± 8.3|

| PTZ Call Category | Other USVs (during epileptiform activity, suppression, between spikes or between myoclonus) | USVs during electrographic seizures and associated with spikes/myoclonus complex (during post-ictal and interictal activity) |
|-------------------|-------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|
| 50-kHz calls      | Number | Duration (ms) | Frequency (kHz) | Bandwidth (kHz) | Power (dB) | Number | Duration (ms) | Frequency (kHz) | Bandwidth (kHz) | Power (dB) |
| Complex           | 13     | 48.5 ± 27.2   | 53.7 ± 6.4      | 6.9 ± 4.6       | -77.1 ± 5.3| 5      | 35.7          | 56.7 ± 5.0       | 7.8 ± 2.8       | -70.9 ± 5.7|
| Upward ramp       | 6      | 33.1 ± 16.8   | 48.2 ± 6.8      | 5.3 ± 1.0       | -81.1 ± 1.7| 8      | 38.7          | 53.9 ± 5.2       | 5.1 ± 2.4       | -77.3 ± 5.2|
| Downward ramp     | 3      | 59.4 ± 31.5   | 49.2 ± 1.2      | 2.7 ± 0.4       | -76.9 ± 1.7| 6      | 42.5          | 58.7 ± 15.0      | 10.3 ± 9.3      | -75.0 ± 1.6|
| Flat              | 12     | 78.6 ± 36.8   | 47.9 ± 9.3      | 3.1 ± 2.1       | -76.4 ± 5.0| 14     | 59.9          | 42.9 ± 11.6      | 2.1 ± 2.9       | -71.5 ± 7.2|
| Short             | 54     | 7.5 ± 3.5     | 59.1 ± 9.1      | 2.5 ± 2.1       | -79.5 ± 2.5| 45     | 95.1          | 57.6 ± 10.4      | 2.3 ± 1.9       | -76.8 ± 3.9|
| Split             | 13     | 132.2 ± 52.3  | 47.1 ± 8.0      | 32.8 ± 9.9      | -70.0 ± 4.9| 26     | 127.4         | 45.8 ± 8.2       | 30.2 ± 8.5      | -67.3 ± 5.8|
| Step up           | 0      | 0             | 0               | 0               | -          | 6      | 95.8          | 40.8 ± 5.9       | 23.6 ± 4.2      | -68.4 ± 4.0|
| Step down         | 1      | 49.5          | 59.6            | 17.7            | -65.5 ± 0.4| 5      | 49.7          | 47.0 ± 8.4       | 26.0 ± 3.7      | -72.0 ± 3.9|
| Multi-step        | 16     | 62.0 ± 9.1    | 22.4 ± 3.7      | 0               | -          | 5      | 53.9 ± 7.9    | 21.3 ± 3.5      | -          | -          |
3.1.2. USV Categories

Calls were classified into 15 sub-categories according to Wright et al. (2010). After saline injection, 16/32 (50%) calls could be classified into 4 of the 15 sub-categories. The remaining 16/32 (50%) were classified as multiform.

After PTZ injection, 194/672 (29%) calls could be classified into 13 of the 15 sub-categories. The remaining 478/672 (71%) calls were classified as multiform (Table 2).

3.1.3. Acoustic properties of USVs

Of the USVs emitted by saline-treated animals 22/32 (69%) were in the 50-kHz frequency range and 10/32 (31%) were in the 22-kHz frequency range. Of the 672 USVs emitted by the PTZ-injected rats, 649/672 (97%) were in the 50-kHz frequency range and only 23/627 (3%) were in the 22-kHz frequency range (Table 1).

The acoustic parameters of each sub-category (duration, frequency, bandwidth, and power) after saline or PTZ injection are summarized in Table 2.

Based on the preliminary data suggesting a PTZ-induced emission of specific patterns of USVs, we next assessed the temporal link between the PTZ-induced seizures and/or epileptiform activity and the different types of USVs by time-locked USV-video-EEG co-registrations.

3.2. USV-video-EEG co-registration after PTZ administration

3.2.1. Prevalence and number of PTZ-induced electrographic seizures

To confirm that at least some of the USVs were related to the PTZ-induced seizures, we next assessed the temporal link between the PTZ-induced seizures and/or epileptiform activity and the different types of USVs by time-locked USV-video-EEG co-registrations.

**Table 1.**

| Treatment | Call category | Number | Duration (ms) | Frequency (kHz) | Bandwidth (kHz) | Power (dB) |
|-----------|---------------|--------|---------------|-----------------|----------------|-----------|
| Rats without electrode implantation (n = 3) | | | 41.9 ± 12.5 | -75.0 ± 4.1 | | 43.8 ± 29.6 |
| Trill     |               | 25     | 48.2 ± 13.4  | 65.4 ± 8.9      | 14.5 ± 9.0  | -76.9 ± 4.1 |
| Flat/trill combination | 7       | 42.1 ± 13.4 | 64.4 ± 9.3 | 22.9 ± 4.7 | -74.9 ± 4.1 | 48.0 ± 28.7 |
| Trill with jumps | 211   | 51.1 ± 17.3 | 63.1 ± 7.8 | 18.7 ± 7.5 | -77.7 ± 5.1 | 48.0 ± 29.5 |
| Inverted U | 6           | 62.3 ± 19.9 | 53.5 ± 2.7 | 6.7 ± 2.1 | -73.3 ± 3.3 | 0 ± 3.3 |
| Composite | 1            | 392.4 ± 20.7 | 50.7 ± 30.8 | 30.8 ± 70.8 | 35.7 ± 10.3 | 56.7 ± 5.0 | 7.8 ± 2.8 |
| Multiform | 749          | 59.3 ± 34.5 | 56.8 ± 6.9 | 12.5 ± 8.7 | -75.2 ± 5.1 | 595 ± 64.7 | 55.6 ± 10.2 | 16.4 ± 11.8 | -71.8 ± 5.9 |
| 22-kHz calls | 15       | 563.7 ± 268.3 | 23.9 ± 0.8 | 13.9 ± 12.6 | -64.4 ± 6.1 | 167 ± 191.1 | 24.5 ± 27.0 | 7.0 ± 8.8 | -71.9 ± 7.9 |

Abbreviations: dB, decibel; kHz, kilohertz; PTZ, pentylenetetrazol; ms, millisecond. Values are shown as mean ± standard deviation (SD).

**Fig. 5.** Temporal distribution of ultrasonic vocalizations (USVs) during individual seizures and postictal suppression. Time course of USV emissions during individual seizures in each of the 12 animals with electrode implantations (1–5 seizures per rat). Light blue bars indicate electrographic seizures and pink bars indicate postictal suppression (x-axis). Blue vertical rasters mark the occurrence of a single 50-kHz USV and red rasters denote 20 kHz USVs. Scale bar is shown in the right lower corner.
induced seizures, we implanted 12 rats and recorded USVs and video-EEG simultaneously. Electrographic seizures occurred in all 12 rats. Of the 12 rats, 5 had more than 1 seizure (i.e., 2–5). A total of 22 seizures were recorded, of which 21 occurred during the first 10 min post-injection. The number of electrographic seizures, latency to onset of the first seizure, and seizure duration are summarized in Table 1. The temporal distribution of the occurrence of electrographic seizures and epileptiform activity in each animal are shown in Fig. 3A.

3.2.2. Prevalence and number of USVs

Total number, frequency range of the calls, and latency to the first call are summarized in Table 1. The electrode-implanted animals emitted a total of 2151 USVs during up to 15-min USV-video-EEG follow-up. Of the 2151 USVs, 4 calls were excluded because of noise interference caused by seizure-related movement, leaving 2147 calls for analysis. The rats with 6 electrodes emitted 162 ± 286 (range 25 – 745) and the rats with 12 electrodes 196 ± 228 (range 22 – 623) USVs (p > 0.05).

The mean number of calls recorded per rat was 179 ± 247 (range 22 – 745). The latency to the first USV after PTZ-injection was quite consistent between animals (106 ± 25 s, range 75 – 169 s). The higher the number of electrographic seizures, the higher the number of USVs (r = 0.916, p < 0.001) (Fig. 3C). The mean behavioral seizure score was 3.38 ± 0.25 (range 1 – 5). The behavioral seizure score did not correlate with the total number of USVs emitted during a given seizure (r = −0.088, p = 0.695). During the 17 generalized seizures (Score 3–5), we recorded 295 USVs (on average 17.4 ± 23.5 /seizure), of which 96% (283/295) were in the 50-kHz and 4% (12/295) in the 22-kHz range. During the 5 partial seizures (Score 0.5–2), we recorded 93 USVs (on average 21.0 ± 8.5 /seizure), of which 95% (88/93) were in the 50-kHz range and 5% (5/93) in the 22-kHz range. Also, the percentages of emitted USVs belonging to different call categories were comparable between animals (106 – 254, range 75 – 169 s). In contrast, 182/2147 (8%) of the USVs were in the lower frequency range (22-kHz), which is commonly associated with a positive emotional state, varying from 11% to 100% between animals. In contrast, 182/2147 (8%) of the USVs were in the lower frequency range (22-kHz), which is associated with negative emotional states, varying from 0% to 89% between animals (Table 1). All rats emitted USVs at the 50-kHz range and only 5/12 rats emitted calls at the 22-kHz range. The behavioral seizure score did not correlate with the total number of 50-kHz USVs (r = 0.010, p = 0.656) or 22-kHz USVs (r = 0.075, p = 0.216) calls emitted to a given seizure.

3.2.3. USV categories

Based on visual analysis, we were able to classify 803 of the 2147 USVs (37%) into some of the 15 categories described by Wright et al. (2010). Although the remaining 1344/2147 (63%) USVs had some resemblance to the typical USVs annotated into the 15 categories, they had clear sonographic structure anomalies. Consequently, we annotated them to a novel “multiform” category. Specifically, multiform USVs showed instantaneous changes such as sudden frequency jumps (Fig. 4B, 1st, 4th, 7th panel at the bottom), 1 or more random directional changes in frequency (Fig. 4A, bottom panel), and/or random 3- to 8-ms interruptions within the sonographic structure (Fig. 4C, 1st and 4th bottom panel). As summarized in Table 2, multiform USVs widely varied in their duration, bandwidth, sonographic structure, and temporal distribution throughout the recording period (e.g., during electrographic seizure and epileptiform activity) (Supplementary Figure 1).

3.3. Occurrence of USVs during seizures and during postictal and interictal activity

3.3.1. Seizures

PTZ induced a total of 22 electrographic seizures, lasting on average for 34 s (33.8 ± 22.6 s, median 26.5, range 12 – 100 s). Representative examples of USVs during two electrographic seizures are shown in Fig. 4A–B. Altogether, 388/2147 (18%) USVs occurred during electrographic seizures (Fig. 3 and Fig. 5). The mean number of vocalizations per seizure was 17.6 ± 24 (median 7, range 0 – 75). Of the 22
electrographic seizures, 4 included more than 55 USVs and 3 included no vocalizations (see also Video 1). The 3 seizures without vocalizations lasted for 18.3 ± 8.5 s (median 15 s, range 12–28 s), and they were the shortest of the seizures expressed by a given rat. The remaining 19 seizures included USVs. As summarized in raster plots (Fig. 5), 15/22 (69%) of the electrographic seizures were associated with ≤ 10 USVs (mean 5.0 ± 3.3 s, median 6, range 0–10). The remaining 7 USV-rich seizures co-occurred with ≤ 10 USVs (mean 44.7 ± 27.2 s, median 56, range 13–75). The longer the seizure duration, the greater the number of USVs during the seizure (r = 0.750, p < 0.05).

The temporal occurrence of USVs during electrographic seizures (beginning, middle, end) varied between seizures and between animals. The temporal distribution of USVs during seizures also varied between seizures in a given animal (Fig. 5). Importantly, the occurrence of the first USV (or USV bout) occurred within the preceding 7 s relative to the onset of the first electrographic seizure (mean 1.5s before, median 0, range 7s before to 1s after), which often already revealed some epileptiform activity and discrete behavioral manifestations. Correlation analysis demonstrated that the shorter the latency to the first USV, the shorter the latency to the seizure onset (r = 0.995, p < 0.001; Table 1 and Fig. 3D).

Interestingly, 96% (331/388) of the USVs detected during seizures were at 50-kHz frequency range and 4% (17/388) were at the 22-kHz frequency range. Of the 388 50-kHz USVs detected during seizures, the majority (309, 80%) were categorized as multiform USVs. Particularly, the first USV at the beginning of the first seizure was typically a multiform USV (11/12).

3.3.2. Postictal suppression
Of the 22 seizures, 21 had clear postictal suppression lasting for 17.4 ± 13.3 s (median 13.7, range 3–50.5 s). Of the 21 postictal suppressions, 12 (57%) were associated with at least 1 USV (mean 6.4 ± 5.6, median 4, range 1–19 USVs). Of the 2147 USVs, 78 (4%) were associated with postictal suppression. Temporal occurrence was variable as the USVs occurred in the beginning, middle, and end of the postictal suppression (Fig. 5).

The 74 50-kHz calls associated with postictal suppression belonged to different categories, including 2 flat, 40 multiform, 17 multiform associated with myoclonus, 1 trill, 13 trills with jumps, or 1 trill with jumps associated with myoclonus. We also identified 4 calls in the 22-kHz frequency range.
### 3.3.3. Postictal or interictal GPDs/myoclonus

All PTZ-injected animals expressed GPDs/myoclonus-associated USVs. Of the 2147 USVs, 654 (31%) were classifiable (Wright et al., 2010) and associated with postictal or interictal GPDs (Fig. 6A). Particularly, GPDs with or without a myoclonic jerk were associated with a vocalization in 44% of all classifiable USVs (Fig. 6B and Video 1).

Notably, the vast majority of all 22-kHz calls, 167 of the 654 USVs (92%) were associated with a GPD with or without myoclonus or with myoclonus only (Table 2).

Of the 654 USVs, 504 (77%) were in the 50-kHz range and 150 (23%) were in the 22-kHz range (Fig. 6C). The USV sub-types associated with GPDs are shown in Table 2.

#### 3.3.4. Types of vocalizations related to other epileptiform activity

The multiformal USVs displayed a striking synchrony with the GPDs (Fig. 4C and Video 1) and also occurred during the intervals between successive GPDs (Fig. 4D and Video 1). In 3 animals (3/12), 87% of the 22-kHz USVs occurred during the continuous epileptiform discharges (Fig. 7A), occurring approximately 2 min (or more) after the last electrographic seizure [Fig. 3A, red rats]. Interestingly, the USVs at the 22-kHz band fluctuated over time within a relative broad frequency bandwidth (range 16.5–44.6 kHz), sometimes starting in the higher frequencies and ending in the lower frequencies (Fig. 7B, 1st call in the bottom panel).

#### 3.3.5. Concatenated USVs — a novel USV subtype

Three implanted rats emitted 64 (mean 16 ± 29/rat, median 2, range 1–59) distinct elaborate USVs (Fig. 7B), which were previously categorized into the “composite” sub-category. That is, “calls other than flat/trill combinations, comprising 2 or more categories” (Wright et al., 2010). We also found 52 such USVs in 1 naïve PTZ-injected animal in the preliminary study (Fig. 7C). The composite USVs were long (427 ± 361 ms, median 244, range 35–1571 ms), usually repetitive and stereotyped, and emitted in bouts of 3–9 USVs. They comprised a combination of 4 call categories in a single utterance, always in the same order: (1) the first component being a complex, short, upward ramp or inverted U, (2) followed by a longer 22-kHz component with harmonics, (3) a short or complex transitory component, (4) and finally, a trill or trill with jumps (Fig. 7B–C, Video 1, and Supplementary Figure 2).

### 4. Discussion

The present study was designed to evaluate whether seizures in rodents are associated with the expression of USVs, as in humans. The present study presents (i) the first evidence of seizure-associated USVs, (ii) novel USV types, and (iii) an improved version of the open-access software for analyzing USVs in rodents.

#### 4.1. PTZ-induced seizures trigger multiple types of USVs

The current study demonstrated that rats emit a large variety of USVs during PTZ-induced epileptiform activity. Most of these USVs could be categorized into the 15 previously described USV subtypes emitted by Wistar (Wright et al., 2010) and Sprague-Dawley (Avvisati et al., 2016; Riede et al., 2020) rats during normal inter-subject communication. Importantly, vehicle administration did not induce USVs in naïve rats, suggesting that the observed USVs were not related to, e.g., injection-related pain. Moreover, PTZ induced a comparable USV production pattern in non-electrode and electrode-implanted animals, indicating that electrode implantation did not cause any discomfort that compromised the data interpretation.

Our study is the first description of seizure-associated USVs in both the 22-kHz and 50-kHz frequency ranges, representing the 2 major frequency bands used by rats in their communication (Brudzynski, 2013). Interestingly, only 23% of the USVs recorded were classified as 22-kHz calls, which are usually described as aversive or pain-induced (Litvin et al., 2010, 2007). Most of the 22-kHz bouts after PTZ injection were emitted at the later phase of our 15-min recordings. Further, the first 22-kHz call immediately after GPDs presented a broader frequency range than the subsequent ones. This uncommon USV feature has previously been described in rats as a response to a mild air puff applied to the nape of the neck (Brudzynski and Holland, 2005), suggesting that the GPD was perceived as aversive. Importantly, the 22-kHz calls did not show sudden frequency jumps or random directional frequency changes as the multiformal 50-kHz calls. In addition, a previous study reported that when PTZ was injected as an anxiogenic agent at very low non-convulsive doses of 1–5 mg/kg, the number of 22-kHz USVs was increased; importantly, administration of diazepam suppressed the 22-kHz USVs (Jelen et al., 2009). Whether or not the 22-kHz USVs report on physical discomfort on the experience of seizures or GPDs remains to be further explored as they intermingled with several 50-kHz calls during the same seizure (or GPD)(see Fig. 4B).

Detailed call subtype analysis revealed that in addition to previously characterized USVs, PTZ-induced epileptiform activity also triggered rats to emit “multiform” USVs, a new USV type characterized by erratic sonographic structures. As these USVs have not been reported previously in normal rats, they may represent a specific seizure-related USV-singature. The generation of the novel multiformal USV subtype could be explained by the loss of neuromuscular control in the laryngeal-respiratory coordination during seizures. Moreover, uncontrolled seizure spread to motor areas controlling phonatory expression could explain the instantaneous changes in the frequency, erratic sonographic structure, and repetitive utterances of USVs.

In 4 PTZ-treated rats, we also detected another previously undescribed USV — a composite USV characterized by a combination of 4 calls of different subtypes in a single utterance. These call subtypes occurred in a repetitive and stereotyped pattern of 3–6 USVs during the intervals between the GPDs. A similar pattern was described previously in rats during exposure to females where males concatenate 22-kHz and 50-kHz calls into a single vocalization (Hernandez et al., 2017). Further studies are needed to better understand the composite USVs and whether they represent a novel call category. Particularly, whether they are emitted also by naïve animals and whether their expression depends on the socio-affective state. Furthermore, it would be particularly interesting to test if the USVs with an erratic sonographic structure or the elaborate composite USVs are capable of inducing a particular motivational state in conspecifics. Also, whether other seizure-inducing drugs, and particularly, whether unprovoked seizures trigger the emission of normal, multiformal and/or composite USVs remains to be studied.

#### 4.2. USVs are emitted during various types of PTZ-induced epileptiform activity

Of the 2147 USVs recorded, 77% were related to epileptiform activity. Of these 23% were related to seizures. The seizure-associated USVs typically appeared at the time of the electrographically verified seizure onset. Our recent functional magnetic resonance study showed that PTZ administration activates widespread areas of the cerebral cortex and subcortical areas bilaterally (Huttunen et al., 2018). The cortical area proposed to be critical for the initiation of USVs is the posterior prelimbic cortex (Bennett et al., 2019). Thus, it is possible that activation of the posterior prelimbic cortex contributes to the expression of USVs induced by PTZ administration. The seizure-onset related USVs could resemble the “ictal cry” in humans, a fragmented guttural
utterance signaling the onset of a seizure (Elzawahry et al., 2010). In humans, seizure-onset related vocalizations are proposed to be caused by the respiratory muscles contracting and forcing air to the vocal cords (Elzawahry et al., 2010). In rats, USVs are produced by a “whistle” mechanism (diaphragm and laryngeal neuromuscular complex interaction (Riedo et al., 2015)) and not by the vibration of vocal folds as in humans (Maht et al., 2016; Riedo et al., 2017). We also observed a low number of USVs at the later phase of electrographic seizures, which may be related to the increased muscle tone, preventing the pharyngeal muscles to properly emit USVs.

Alignment of the sonogram with the EEG revealed a conspicuous temporal association between the myoclonic jerk and/or GPDs and the USV emission, suggesting activation of the neuromuscular circuits responsible for the USV production. Rats emitted myoclonus-associated USVs in a repetitive and uncontrolled manner, which has also been observed in humans (Lebrun, 1994). Interestingly, Rizzo et al. (2018) reported in a rat model of Tourette’s syndrome induced by microinjection bicuculline into the striatum that the resulting tics and stereotypes behavior were associated with rhythmically repeating USVs. It is possible that the low dose of striatal bicuculline induced the myoclonus that generated USVs. Interestingly, USV production is modulated in some mouse models of genetic epilepsy, including Angelman’s syndrome (Jiang et al., 2010; Mandel-Brein et al., 2015). Also, hyperthermic seizures in rat pups affects USV emissions (Keller et al., 2004). Mikulecká et al. (2011) reported that an anxiolytic and anti-seizure drug, clonazepam, suppresses the maternal isolation-induced number of USVs and prolonged their inter-call interval. It remains to be assessed if anticonvulsant treatment affects USV production during seizures.

What generates the wide variety of different types of seizure-related USVs in rats, and do these USVs carry meaningful information? Also in humans, seizure-related vocalizations are frequent (Horvath et al., 2009), variable and can present as non-speech vocalizations, characterized by repetitive or non-repetitive sounds that are not clearly understandable (Janszky et al., 2006; Patra et al., 2011). Seizure-related verbal deficiencies can present as speech arrest, aphasia, or neologisms (Dussaule et al., 2017); vary from simple vocalizations to intelligible speech (Daniel and Scott Perry, 2016); and even “groaning” sounds have been reported (Rego et al., 2006). They can be emotionally colored, irrelevant, or recurrent utterances (Serafetindes and Falconer, 1963). Further studies are needed to explore the mechanisms of seizure-related USVs and their potential to serve as noninvasive indicators of epileptiform activity and treatment effects.

5. Conclusions

Our data show for the first time that PTZ-induced epileptiform activity is associated with a wide variety of USVs, some of which were previously associated with positive and negative emotional states, and reproductive behavior. We also found novel USV types. It remains to be explored whether USVs are a common feature for seizures induced by different electrical or chemical stimuli. Also, whether USVs occur during spontaneous seizures in various genetic and acquired rodent models of epilepsy, not to forget the sex and strain dependency. Also, whether the seizure onset zone affects the number or type of USVs should be assessed. Further studies are needed to evaluate the effect of anti-seizure drugs on USVs. Finally, the present study opens up an exciting scenario of using USVs as noninvasive quantitative and qualitative indicators of seizure or epileptiform activity occurrence as well as indicators of anti-seizure efficacy of anti-seizure and anti-epilepticogenic compounds.

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Data Availability

“DeepSqueak-Screener” software source code, and executable binary are publicly available from https://github.com/UFepilepsyAI/DeepSqueak. A new merged version of the “Screener” and original DeepSqueak is now publicly available at https://github.com/DrCoffey/DeepSqueak as DeepSqueak V3. Datasets generated are available upon reasonable request.

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Declarations of Interest

The authors declare no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.eplepsyres.2022.106927.

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