Amplitude modulation pattern of rat distress vocalisations during fear conditioning

Eugenia Gonzalez-Palomares*[1], Julie Boulanger-Bertolus[2], Maryne Dupin[2], Anne-Marie Mouly*[2], Julio C. Hechavarria*[1]

*Corresponding authors: gonzalezpalomares@bio.uni-frankfurt.de, annemarie.mouly@cnrs.fr, hechavarria@bio.uni-frankfurt.de

[1]Institute for Cell Biology and Neuroscience, Goethe University, 60438 Frankfurt am Main, Germany

[2] Université Claude Bernard Lyon 1, CNRS, INSERM, Centre de Recherche en Neurosciences de Lyon CRNL U1028 UMR5292, CMO, F-69500, Bron, France

Abstract

In humans, screams have strong amplitude modulations (AM) at 30 to 150 Hz. These AM correspond to the acoustic correlate of perceptual roughness. In bats, distress calls can carry AMs, which elicit heart rate increases in playback experiments. Whether amplitude modulation occurs in fearful vocalisations of other animal species beyond humans and bats remains unknown. Here we analysed the AM pattern of rats' 22-kHz ultrasonic vocalisations emitted in a fear conditioning task. We found that the number of vocalisations decreases during the presentation of conditioned stimuli. We also observed that AMs do occur in rat 22-kHz vocalisations. AMs are stronger during the presentation of conditioned stimuli, and during escape behaviour compared to freezing. Our results suggest that the presence of AMs in vocalisations emitted could reflect the animal's internal state of fear related to avoidance behaviour.

Introduction

Juvenile and adult rats emit two basic types of ultrasonic vocalisations (USVs), with carrier frequencies around 22 and 50 kHz, expressing negative and positive emotional arousal, respectively (Brudzynski, 2019; Wöhr and Schwarting, 2013). 50-kHz USVs are emitted in positive interactions, such as playing (Burgdorf et al., 2008; Panksepp and Burgdorf, 2000), mating (Barfield et al., 1979; van der Poel and Miczek, 1991) or as response to drugs of abuse (Knutson et al., 1999). On the other hand, 22-kHz USVs are emitted in aversive situations, such as confrontation with predators (Blanchard et al., 1991), submission in inter-male fighting (Kaltwasser, 1990), social isolation (Francis, 1977), and aversive stimuli (Brudzynski and Ociepa, 1992; Jelen et al., 2003; Wöhr et al., 2005), including unconditioned and conditioned stimuli. These USVs serve as "alarm calls" to other conspecifics (Brudzynski, 2001; Kim et al., 2010) and are part of the rats' defensive behaviour repertoire.

Defensive behaviours have been extensively studied in the Pavlovian fear conditioning paradigm, in which an aversive unconditioned stimulus (US), e.g., a foot-shock, is presented following a neutral conditioned stimulus (CS), e.g., a tone or an odour. After a few trials, exposure to the CS alone elicits conditioned fear responses. Fear conditioning is widely used in studies of memory and associative learning (Bertolus et al., 2016; LeDoux, 2000; Maren, 2001; Raber et al., 2019). The strength of the fear memory is commonly assessed using overt behaviour analysis, such as freezing responses. However, other responses can give further insights into the animal's fear response. Among them, the duration of USVs and the rate at which these calls are produced correlates well with the freezing rate (Boulanger-Bertolus et al., 2017; Hegoburu et al., 2011; Wöhr et al., 2005). Additionally, these parameters follow a dose-response curve, i.e., the call rate, total call duration and call amplitude increase with increasing shock intensities, while latency of the first call and inter-call length decrease (Hegoburu et al., 2011; Wöhr et al., 2005).

In human screams –a vocalisation type used to express discomfort and fear–, a key feature in transmitting an alarming intentionality is through amplitude modulations (AM). These have been related to a meaning of urgency or distress, in both natural and artificial sounds (Arnal et al., 2015). The perceptual correlate of these AM is the so-called "roughness". In humans the amplitude modulations that evoke a rough percept span between 30 and 150 Hz.

Roughness and its acoustic correlates have been investigated in other animal species besides humans. A study in passive listening mice reported that, in contrast to humans, mice seemed to perceive dissonant sounds (two tones played simultaneously with high roughness levels) more pleasant than consonant sounds (Postal et al., 2020). In bats, fast-AM between 1.15 and 2.45 kHz have been described in 48 % of the distress calls studied (González-Palomares et al., 2021; Hechavarría et al., 2020). Bats distress vocalisations characterised by fast-AM enhances the activity in the hypothalamic-pituitary-adrenal axis (Mariappan et al., 2013) and the dopaminergic system (Mariappan et al., 2016), hence suggesting that fast-AMs could be a correlate for roughness perception in bats.

In rats, a commonly used model in laboratory experiments, whether acoustic roughness occurs in response to stress has not been investigated. A possible hypothesis is that amplitude modulated sounds (putative roughness) occur more often when rats are subject to stressful conditions. The reasoning behind this hypothesis is that amplitude modulation could be a generalised trait in vocal animals to signal distress. In this article we characterised the AM pattern of 22-kHz USVs uttered by rats during a fear conditioning task. The idea was that AM in 22-kHz vocalisations could indicate urgency and stress, as previously described in bats and humans (Arnal et al., 2015; Hechavarría et al., 2020). We report that, as hypothesised, rat 22-kHz USVs have amplitude modulations. In rats, the AM power is highest in vocalisations uttered during the odour presentation and in late vs. early trials of the fear conditioning paradigm.

Results

General characteristics of vocalisations emitted during fear conditioning

We analysed the vocalisations emitted by rats while undergoing an odour fear conditioning paradigm (Fig. 1). The trials started with a 30-s long pre-odour period, followed by the odour presentation. There were two groups of rats, the first one received 20 s of odour presentation (n = 11), and the second, 30 s (n = 9). During the last second of odour presentation the rats received a foot shock. The following period is referred to as the post-shock period, from which we considered for analyses the first 30 s. In total, each trial lasted 4 min. All animals underwent 10 trials with shock presentation in total, but only the first three and last three trials were analysed. For simplicity, the first and last 3 trials were termed as early and late trials. The reason for

focussing on early and late trials only was that we were interested in possible differences that could occur between early and late stages of the task. The vocalisations analysed were at least 300-ms long and their peak frequency was between 18 and 32 kHz. In total 11956 vocalisations were analysed. An example of a trial recording is given in Fig. 1A, showing two zoomed-in segments with 7 and 15 vocalisations, respectively, and examples of two individual vocalisations in Fig. 1B.

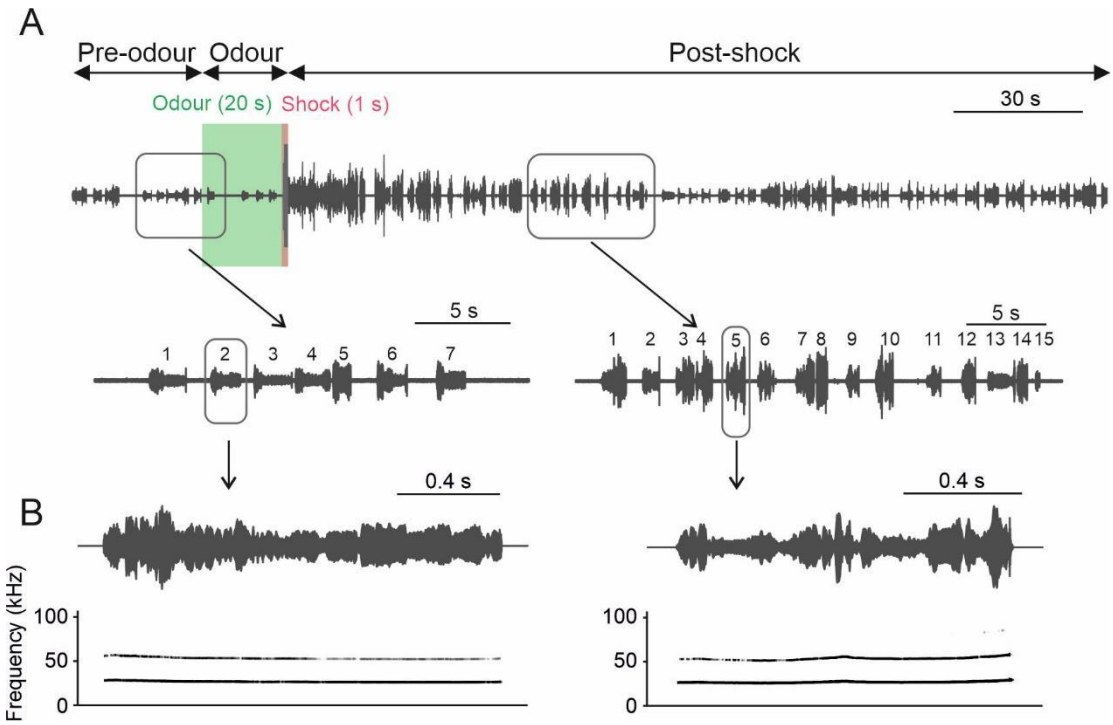

Figure 1. Example of USV recordings during a trial. A) Top: USV were recorded during the 4-min trial divided into three periods: pre-odour, odour, and post-shock. The shock was applied during the last second of odour presentation. Bottom, zoom-ins of the recording on the time intervals signalled on the traces above. Each number indicates individual vocalizations. B) Top, oscillograms of two individual vocalisations (zoom-in of A). Bottom, spectrograms of the two vocalisations.

Several parameters of the 22-kHz calls were analysed over the time course of the trials. The idea was to identify the influence of the pre-odour, odour, and post-shock periods on the acoustic parameters of the calls emitted by the rats. Because USVs emission strongly impacts respiratory rate (Boulanger-Bertolus et al., 2017; Frysztak and Neafsey, 1991; Hegoburu et al., 2011; Sirotin et al., 2014), the respiratory rate was also analysed. For the analysis presented in the main text,

the first three and last three conditioning trials from both groups of rats were merged together (group 1: 20 s of odour presentation (n = 11), group 2: 30 s odour presentation (n = 9)).

The first striking result we observed was that the number of vocalizations changed dramatically between pre-odour, odour, and post-shock periods (Fig. 2A, Chi-squared test: $p = 3.5 \cdot 10^{-30}$; see Methods for more details). Specifically, it was during the odour presentation that the rats vocalized the least (Bonferroni-corrected chi-squared tests: $p = 9.2 \cdot 10^{-20}$; $p = 1.0 \cdot 10^{-30}$, compared to pre-odour and post-shock, respectively). Rats vocalized the most during the post-shock period ($p = 0.04$, compared to pre-odour). The duration of the vocalizations during the odour presentation was significantly longer than pre-odour and post-shock periods (Fig. 2B; Kruskal-Wallis test: $p = 6.7 \cdot 10^{-14}$, Bonferroni-corrected multiple comparison test; $p = 5.0 \cdot 10^{-10}$; $p = 1.5 \cdot 10^{-13}$, respectively; pre-odour vs post-shock, $p = 0.19$). The vocalizations during odour have the smallest root mean square amplitude (RMS, relative measure of loudness; Fig. 2C; Kruskal-Wallis test: $p = 2.2 \cdot 10^{-23}$, Bonferroni-corrected multiple comparison test; $p = 2.2 \cdot 10^{-5}$; $p = 1.3 \cdot 10^{-22}$, vs. pre-odour and post-shock) and post-shock USVs the largest ($p = 1.1 \cdot 10^{-11}$, vs pre-odour). The peak frequency was not significantly different between the periods (Fig. 2D; Kruskal-Wallis test: $p = 0.07$). Regarding the respiration rate, it was highest during the post-shock period (Fig. 2E; Kruskal-Wallis test: $p = 1.8 \cdot 10^{-16}$, Bonferroni-corrected multiple comparison test; $p = 6.1 \cdot 10^{-17}$; $p = 4.0 \cdot 10^{-5}$, vs pre-odour and odour, respectively), followed by the odour period ($p = 0.015$, vs pre-odour).

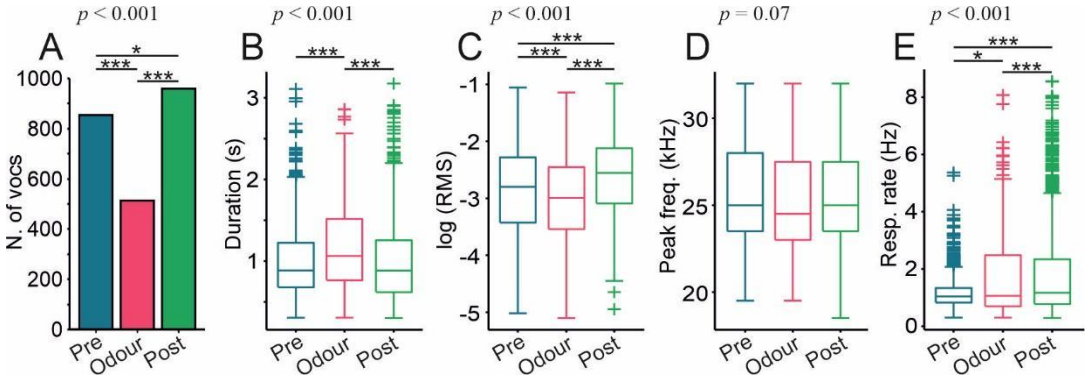

Figure 2. Analyses of 22-kHz USVs and respiration rate during the fear-conditioning task. A) Histogram of number of 22-kHz USVs uttered in each period (pre-odour, odour, and post-shock). Chi-squared tests with number of vocalisations in 20 randomly selected 1-s bins. B-D) Boxplots of the call duration, root mean square (RMS) and peak frequency of the same vocalisations, respectively. E) Boxplot of respiration rate calculated during 1-s bins. Kruskal-Wallis tests for

data shown in B-E (*p* values shown on top of each panel; n = 1300, n = 606, n = 1447, for pre-odour, odour and post-shock, respectively). Stars and horizontal black lines indicate between group comparisons (Bonferroni-corrected rank-sum tests, *$p < 0.05$, *** $p < 0.001$).

Changes in acoustic parameters across trials

In order to investigate the effect of previous exposures to the CS-US pairs, the acoustic parameters mentioned in the preceding text were analysed trial by trial (considering trials 1 to 3 and 8 to10; Fig. 3). This analysis was done for each trial period separately (i.e., pre-odour, odour, and post-shock). Again, here both experimental groups (20 and 30 s odour presentation) were merged together. We observed the following:

1- Number of vocalisations (Fig. 3A, F, K): The number of vocalisations increased in both pre-odour and odour periods in the first two trials reaching a stable level in the third trial, which was maintained in later trials (Chi-square tests, first and second trials compared to the rest, $p < 0.05$). In the post-shock period, the stable number of vocalisations is reached in the second trial.
2- Duration (Fig. 3B, G, L): The duration of the 22-kHz USVs in the pre-odour and post-shock periods was constant to some extent. During the odour presentation, the vocalisations were slightly shorter in the first trial than in the rest (Holm-Bonferroni corrected Wilcoxon rank-sum tests, $p < 0.05$).
3- Loudness (Fig. 3C, H, M): The vocalisations were mainly louder (measured as uncalibrated RMS) in the first trials compared to the last ones before and during the odour presentation. In the post-shock period, the vocalisations were loudest in the second and third trials, reaching in later trials comparable levels to the first trial.
4- Peak frequency (Fig. 3D, I, N): The peak frequency increased gradually and significantly in the first three trials in all periods relative to odour presentation. Peak frequency was even higher in the last trials, where it remained more stable.
5- Respiration rate (Fig. 3E, J, O): The respiration rate is higher in the first trial compared to the rest in all three periods reaching the lowest level in the third trial. The later trials have comparable levels to the second trial. These data point towards an inverse relationship between respiration rate and number of vocalisations.

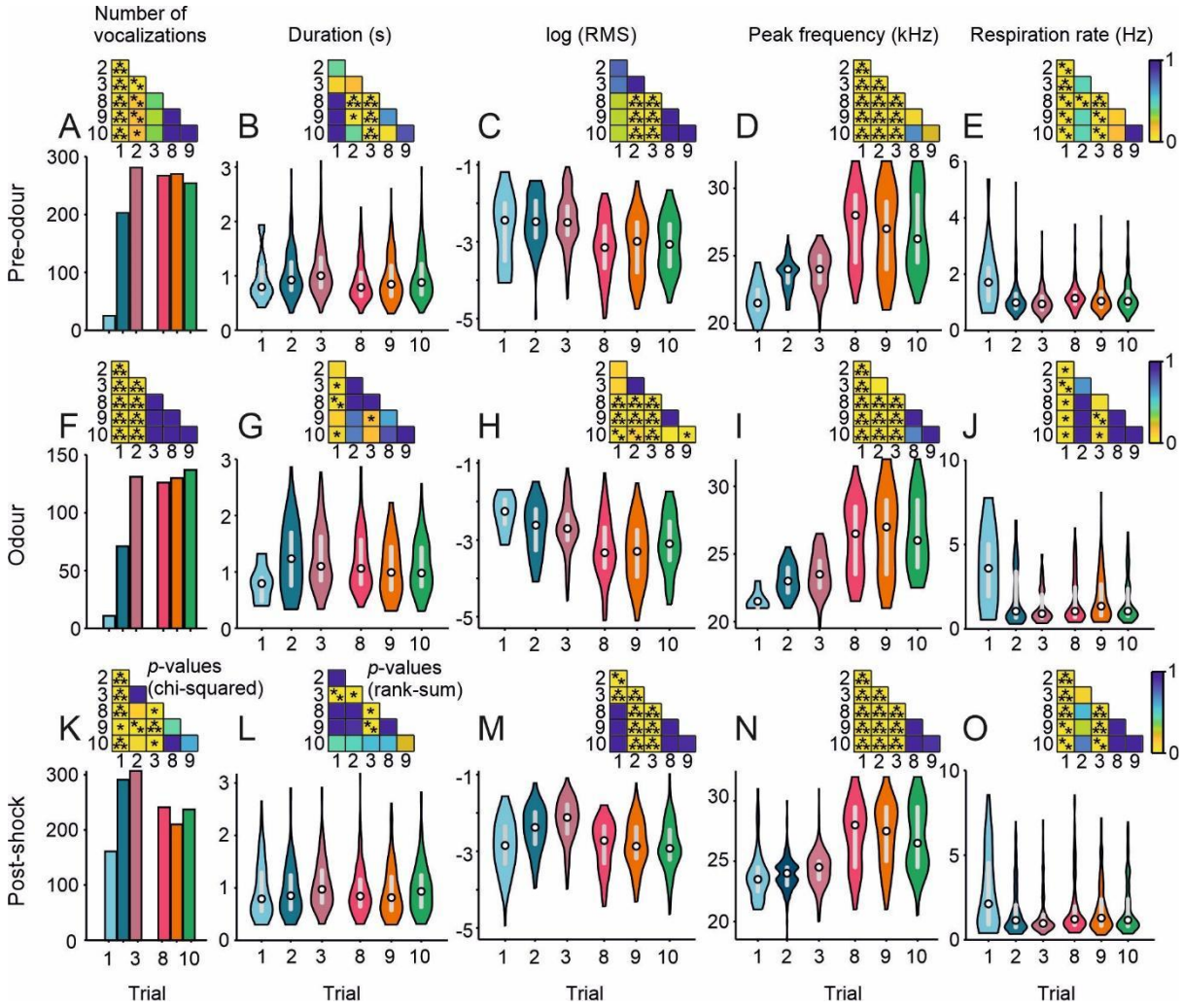

Figure 3. Analyses of 22-kHz USVs as a function of trial number. Histogram of number of vocalisations (A, F, K) and violin plots of duration (B, G, L), root-mean square (RMS; C, H, M), peak frequency (D, I, N) and respiration rate (E, J, O). Insets: statistical significance; number of vocalisations: Holm-Bonferroni corrected chi-squared test; duration, RMS, peak frequency, and respiration rate: Holm-Bonferroni corrected Wilcoxon rank-sum tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

22-kHz USVs have fast amplitude modulations

In the following, we describe the analysis pertaining to the amplitude modulation pattern of rat 22-kHz vocalisations. We were interested in this parameter since in other species, amplitude modulation is correlated with the production of distress and alarm sounds. For example in bats, ~48 % of distress vocalisations uttered have fast amplitude modulations (fAM) at around 1.7

kHz (Hechavarría et al., 2020). Amplitude modulation is also present in human screams, although in humans AMs span the range from 30-100 Hz (as opposed to kHz in bats). Since rats utter 22-kHz USVs in distressful situations (i.e., fear conditioning, this study), we wanted to determine if the rat calls carry AMs as described in previous studies for bats and humans. We reasoned that calls emitted in different trials and/or trial timings (pre, post and odour presentation) could have different AM patterns.

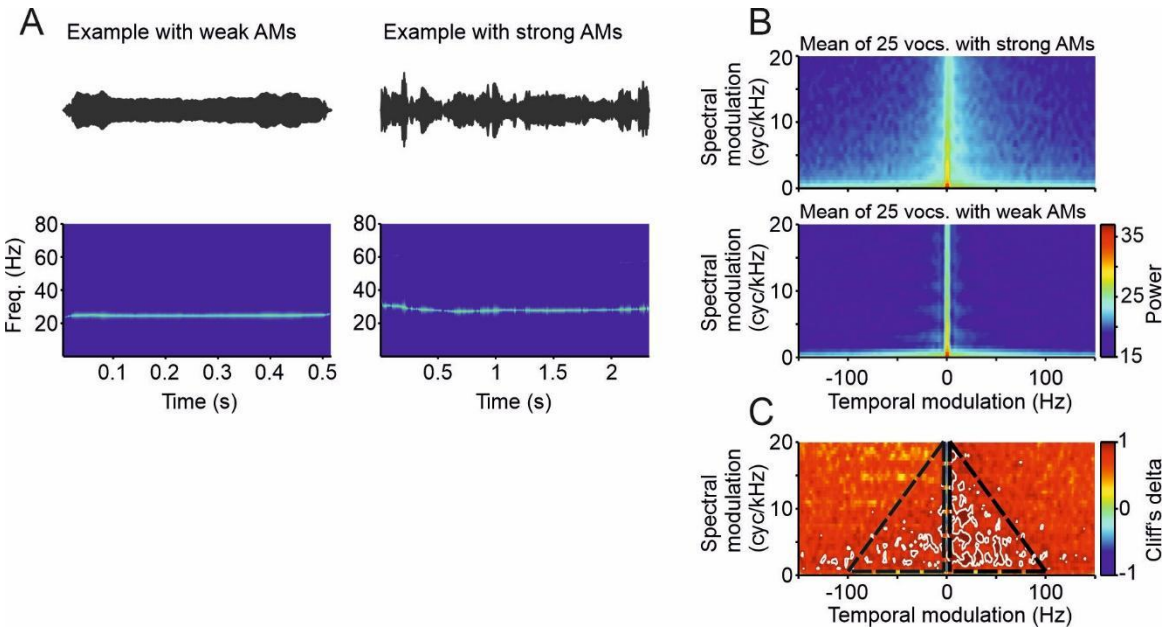

Figure 4. A) Oscillograms (top) and spectrograms (bottom) of two examples of 22-kHz rats' vocalisations, one with weak (left) and one with strong (right) amplitude modulations (AMs), . B) Mean of the modulation power spectra (MPS) of 25 manually selected vocalisations with few (A, top) and many (A, bottom) amplitude modulations. Only the first 300 ms were considered and all were RMS-normalized. C) Cliff's delta of the MPS in A). White lines designating areas with values > 0.948 (double the threshold of what is considered as large effect size). Dashed black lines show the area within which the mean of the MPS values were calculated for all vocalisations (what is considered as AM scores in this manuscript).

The method used to evaluate amplitude modulations was the modulation power spectrum (MPS). The same method has been used to study AMs in humans, bats and birds ((Arnal et al., 2015; Elliott and Theunissen, 2009; Hechavarría et al., 2020; Theunissen et al., 2000)). The MPS allows the analysis of both temporal and spectral modulations simultaneously. Positive and negative temporal modulations display downward and upward frequency modulations, respectively.

For the initial MPS analysis, we hand-picked 50 example rat vocalisations across trials and trial periods: 25 vocalisations that displayed strong AM in their oscillogram, and 25 that did not (Fig. 4B). The objective was to compute the MPS of the example calls, and to compare fast AM vs slow AM to identify regions of interest (ROIs) in the MPS. The ROIs would indicate MPS regions where slow and fast modulated vocalisations differ from each other. We thought that once identified, these ROIs could be carried over to all future analysis. At the methodological level, for computing the MPS we focussed on the first 300 ms from all vocalisations (RMS-normalised). This approach let us equalise the spectro-temporal resolution of the analysis across vocalisations, and to focus on AM differences rather than on RMS differences.

Figure 4A shows the average MPS of the 25 hand-selected fast AM rat calls and the 25 slow AM calls (top and bottom, respectively). As it can be noted, the MPS of the fast AM calls is broader than that of the slow AM vocalisations. For comparison purposes, we calculated the Cliff's delta of these two groups of calls and marked the regions which were higher than 0.948: double the absolute value that is typically used to define a large effect size (Romano et al., 2006). These regions indicate very high differences between the MPS of fast and slow AM calls and are indicated with white lines in Fig. 4B. Differences with very large effect sizes occurred consistently between the two groups at frequencies between 3 and 100 Hz, mostly on the right side of the MPS that corresponds to downward frequency modulations. Based on the results obtained, we triangular ROIs (dashed black lines in Fig. 4B) from which we could calculate an AM score. The AM-score was computed for each vocalisation as the mean value of the regions of interest defined in Fig 4B.

When considering all the calls, we observed that the AM score was significantly highest during the odour period (Fig. 5A; Kruskal-Wallis test: $p = 2.5 \cdot 10^{-6}$, Bonferroni-corrected multiple comparison test; pre-odour vs odour, $p = 2.2 \cdot 10^{-6}$; pre-odour vs post-shock, $p = 0.96$; odour vs post-shock, $p = 6.5 \cdot 10^{-5}$). When considering the trials separately, the AM score was higher in most cases in late trials during the pre-odour and odour periods (Fig. 5B; Holm-Bonferroni corrected Wilcoxon rank-sum tests, $p < 0.05$), although during the post-shock period this effect was not clear. When the rats received the shock, they displayed escape behaviours rapidly followed by freezing (Dupin et al., 2019). In order to look for a possible effect of the defence behaviour displayed by the animal (freezing or escape), we compared the AM score between

USVs emitted during the 10 s after the shock. We found that the USVs emitted during escape had stronger fast AMs than those emitted during freezing (Fig. 5C; Wilcoxon rank-sum test, $p = 2.7 \cdot 10^{-10}$).

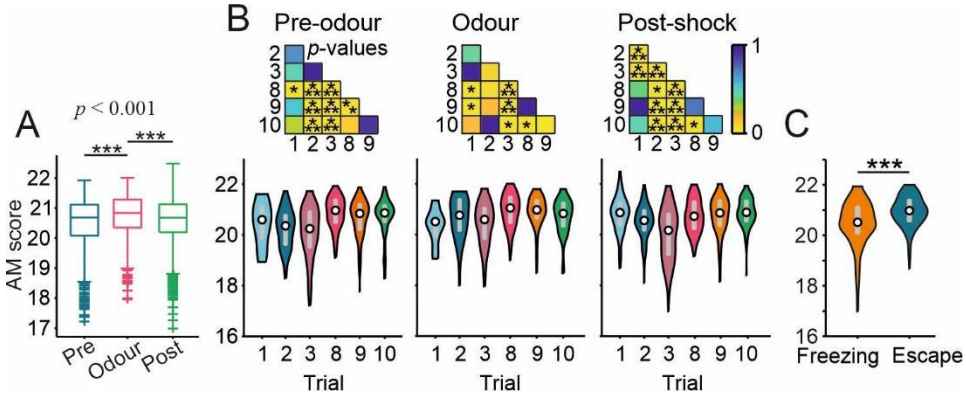

Figure 5. Amplitude modulation score. A) Boxplot of the AM score in each for the three periods during the first three and last three trials for both groups. B) Violin plots of the amplitude modulation score (see Methods) for each period and trial. Insets: *p* values of Wilcoxon rank-sum tests. C) AM score of the vocalisations emitted right after the shock (10 s post-shock period) and classified into freezing (n = 145) or escape (n = 141) depending on the rat's behaviour. Holm-Bonferroni-corrected Wilcoxon rank-sum test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The amplitude modulation score is higher in longer and fainter vocalisations

The acoustic analyses hitherto showed the presence of "fast" amplitude modulations, which are stronger during the odour presentation of fear conditioning trials. To determine how the acoustic parameters may interact between them, we performed correlational analyses considering all 22kHz USVs measured (Fig. 6; Pearson's correlation coefficient and linear regression). The AM score is moderately and negatively correlated with the call loudness (measured as logarithmic RMS), and weakly correlated with the rest of the variables, respiratory rate (positively), number of vocalisations (negatively), duration (positively) and peak frequency (positively; for the *r*'s interpretation see (Schober and Schwarte, 2018)). The number of vocalisations is moderately positively correlated with the loudness and peak frequency and weakly negatively correlated with the call duration and freezing behaviour. The loudness is weakly negatively correlated with the peak frequency, duration, and respiration rate (at vocalisation onset). The peak frequency is weakly negatively correlated with call duration. Longer calls are weakly correlated with higher respiration rate.

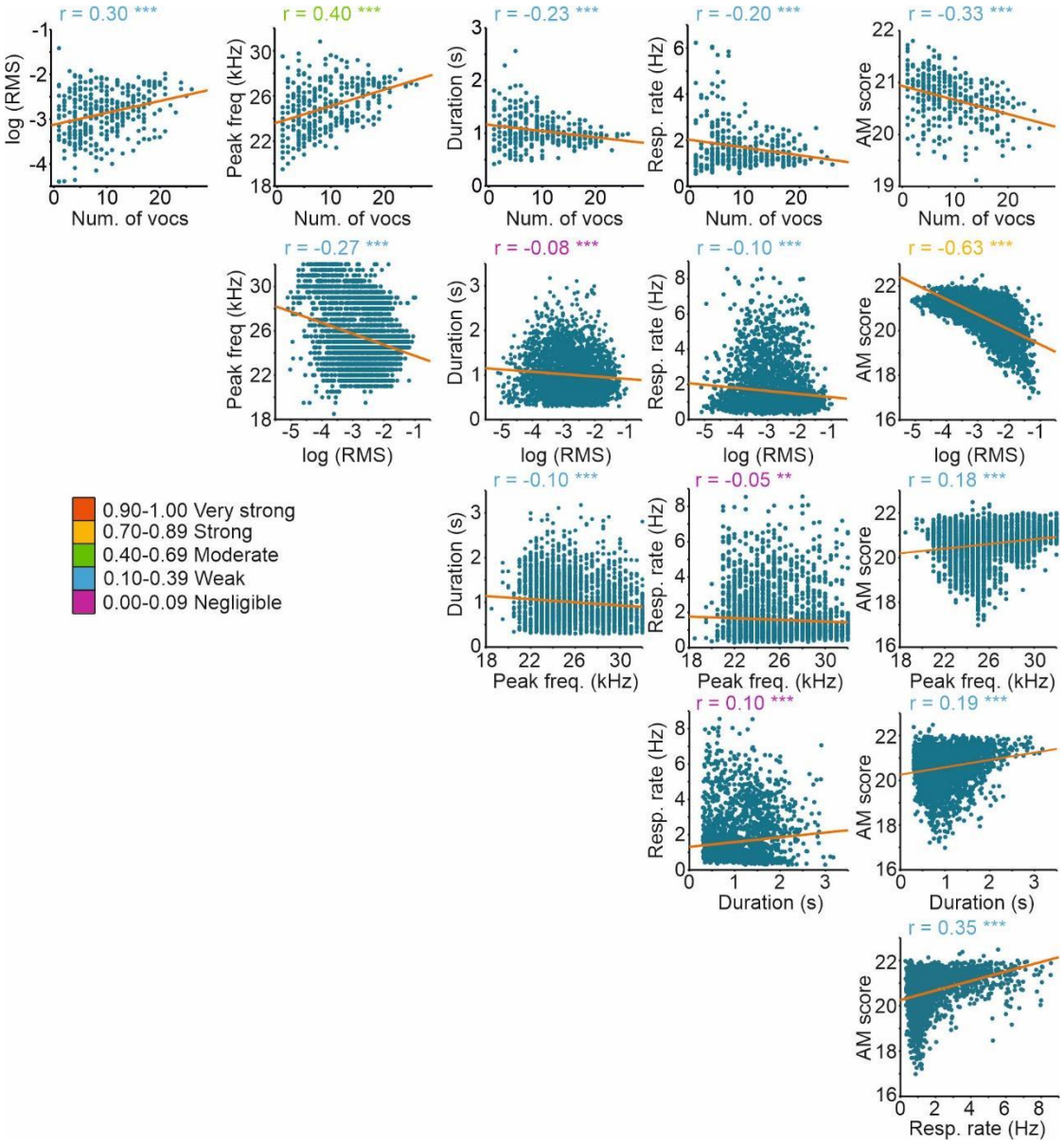

Figure 6. Correlations between variables of 22-kHz vocalisations. Variable names of the y-axes are on top of each column, variable names of x-axes are in the first plot of each row. For the number of vocalisations, it was considered the mean values of the corresponding variable of USVs emitted in 5-s bins for each rat. Fitted linear regression (red lines). Pearson's correlation coefficient (*r*) shown for each plot. ** $p < 0.01$, *** $p < 0.001$.

Discussion

The main goal of this study was to analyse the temporal modulations of rat 22-kHz USVs emitted while undergoing a fear conditioning task, together with the animals' respiration rate. A

specific aim of the study was to investigate whether amplitude modulated sounds (putative roughness) can be detected in rat vocalisations as the level of stress increases in a fear conditioning task.

We found that: (1) during the odour compared to the pre-odour period, the number of 22-kHz vocalisations decreases together with the loudness, whereas the calls become longer and the respiratory rate increases; (2) during the post-shock, the number of calls increases (more than in pre-odour) and call duration becomes shorter (as in pre-odour) and the respiratory rate increases (more than during odour). (3) In late trials (compared to early), the number of vocalisations increases, the calls' peak frequency is higher, whereas call loudness is reduced. (4) Concerning fast amplitude modulations, we found their presence in rats' 22-kHz USVs, mostly during the odour, and generally more during late trials and during escape behaviour.

Comparison with previous studies

Rats emit most of the 22-kHz USVs when immobile (Hegoburu et al., 2011), and call emission occurs together with increases in heart rate and blood pressure (Antoniadis and Mcdonald, 1999; Frysztak and Neafsey, 1991; Walker and Carrive, 2003). The majority of the situations in which rats emit 22-kHz USVs have clear aversive components, e.g., confrontation with a predator, during intermale social defeat (Sales, 1972; Takahashi and Lore, 1982) and during fear conditioning (Choi and Brown, 2003; Jelen et al., 2003; Schwarting et al., 2007; Wöhr et al., 2005). However, the rats did not emit USVs while in an elevated plus maze (Borta et al., 2006), despite its broad usage in anxiety research. During the CS (odour in our case) the call rate decreases, also in line with past literature which shows that USVs are more frequently uttered during the intertrial interval compared to the CS presentation (Frysztak and Neafsey, 1991; Jelen et al., 2003; Shionoya et al., 2013). It has been suggested that context cues between the CS-US pairings may signal anxiety, whereas the CS prior to the US may indicate acute fear, and that USVs may specifically reflect the anxiety state (Jelen et al., 2003). While freezing is often similar in fear and anxiety states, sustained 22-kHz USV have been shown to occur preferentially between trials, whereas acute fear induced by the CS as an imminent danger signal resulted in a decrease of the number of USVs (Frysztak and Neafsey, 1991; Jelen et al., 2003). The present results are consistent with prior findings suggesting suppression of context supported USV by the CS. Additionally, in line with previous studies, we have shown here that the number of

vocalisations, as well as the peak frequency, increase with the situation's aversiveness (Borta et al., 2006). Shock delivery restores USV emission levels (to pre-odour levels) and increases the loudness. With increasing number of trials, the number of utterances and their peak frequency increase as well. This is in consonance with previous data showing that call rate increases with the aversiveness level (Borta et al., 2006; Hegoburu et al., 2011).

Amplitude modulations

A specific aim of the present work was to investigate whether amplitude modulations can be found in rats' USVs. We brought evidence that such modulations are indeed present especially during odour presentation. According to our results, the amplitude modulation score is negatively correlated with the vocalisation's loudness and number of vocalisations and positively correlated with the peak frequency, duration and respiration rate. The AM score also increases with the number of trials. According to Morton's motivational rules, broadband low-frequency sounds are associated more with hostile situations (Morton, 1977). Since the 22-kHz are very narrow spectrally, the amplitude modulations would be a useful way to increase the information they carry, in a similar way that amplitude modulations generate rough-like sounds in humans (Arnal et al., 2015). This is a hypothesis that needs to be researched in the future to assess whether rats perceive roughness from others and whether they make use of it to modify their own behaviour. Altogether, rats' AMs are modulated by external conditions. This evidence hints that the AMs reflect the animal's internal state. Albeit whether this is a result of other changes in the vocal production or is something that can be willingly controlled and useful for the listeners remains unclear.

The defensive responses triggered by the foot shock can be passive (freezing) or active (escape). In the published article using the same data as here (Dupin et al., 2019), the authors analysed the USVs emitted during the first minute after the foot shock during the two situations (freezing and escape). During escape, the call duration was shorter, and the peak amplitude and frequency were higher than during freezing. Here, we analysed the AM score of the vocalisations uttered 10 s after the shock as well, and found that during escape, the USVs have stronger AMs. These observations suggest that AMs of USVs take a greater role in the rats' active defence behaviour. In that sense, AM constitutes a useful bioacoustical marker that could allow to distinguish

between USVs signalling acute fear and those reflecting anxiety, and between passive and active defence behaviours.

Final remarks

Amplitude modulations in human screams have been proposed as the correlate of acoustic roughness (Arnal et al., 2015). In bat distress calls (González-Palomares et al., 2021; Hechavarría et al., 2020), fast amplitude modulations occur in ~48 % of the cases at rates ~1.7 kHz, also have been suggested to express a higher degree of urgency. We investigated here if amplitude modulations occur in rat vocalisations. We analysed the 22-kHz USVs recorded during a fear conditioning paradigm. We found that AMs are strongest during the odour presentation, and that AM strength shows a trend to increase after the first shock application, denoting a relation with aversiveness. Nonetheless, we noticed that the spectral range of the AM in rats is not as delimited and high frequency as in bats (AMs centred at 1.7 kHz; (Hechavarría et al., 2020)), but rather covers the same broad range of frequencies that characterises perceptual roughness in humans (~ 30-100 Hz; (Arnal et al., 2015)).

We propose two possible, not mutually exclusive, functions for the AMs found in 22 kHz rat vocalisations. First, they may reflect the internal affective state of the animals such as the fear for a potential threat and/or related to the avoidance behaviour. This is supported by the fact that the AM score increases after the first shock delivery during odour presentations and that is higher during escape than during freezing after the shock. They may be modulated quantitatively and/or qualitatively by the emotional state (fear and/or avoidance). Second, they might be used as a communication signal to indicate a potential danger to other conspecifics.

Further research should clarify which of these functions (if any) holds true. More research could be done in the vocal production mechanisms, in the response to AM USVs in playback experiments. Additionally, investigating the AMs in other rat vocalisation types, or in 22-kHz but in different contexts could also shed light into the role amplitude modulations play within the rat soundscape.

Material and methods

The data used here were recorded during experiments directed to the investigation of the respiration and brain neural dynamics during odour fear conditioning. Those results are published elsewhere (Dupin et al., 2019, 2020). The present article shows analyses of the acoustic recordings, including new insights to amplitude modulations. For a more detailed description of the paradigm, experimental apparatus or data acquisition, please refer to the aforementioned papers (Dupin et al., 2019, 2020).

Animals. The data have been obtained from twenty male Long Evans rats (290-375 g, at the start of the experiments, Janvier Labs). The animals were housed individually at 23 °C, 12 h dark-light cycle, with food and water available *ad libitum*. All procedures were performed in accordance with the European Union guidelines and regulations (Directive 2010/63/EU of the European Parliament and of the Council of the European Union regarding the protection of animals used for scientific purposes), they have been approved by the French Ethical Committee N° 055 and the project is referenced by the French Ministry of Research as APAFIS #10606-2017071309472369v2.

Experimental apparatus. A detailed description of the experimental apparatus can be found here (Hegoburu et al., 2011). Briefly, it consisted of a whole-body customised plethysmograph (diameter 20 cm, height 30 cm, emka TECHNOLOGIES, France) inside a sound-attenuating cage (L 60 cm, W 60 cm, H 70 cm, 56 dB background noise). The bottom of the chamber was equipped with a shock floor connected to a programmable Coulbourn shocker (Bilaney Consultants GmbH, Düsseldorf, Germany). On top of the plethysmograph three Tygon tubing were connected to an olfactometer. Deodorized air flowed constantly through the cage (2 L/min). An odour (McCormick Pure Peppermint; 2 L/min; 1:10 peppermint vapor to air) was introduced when programmed. A condenser ultrasound microphone (Avisoft Bioacoustics CM16/CMPA, Berlin, Germany, sampling frequency 214285 Hz, 16 bit) placed on top of the plethysmograph was used to record the vocalizations, that were analysed offline. Two cameras recorded the animals' movement. The freezing percentage per 1-s bin was automatically detected from using a Labview software and verified by a researcher.

Odour fear conditioning. The animals were placed individually in the apparatus for 30 min per day during 3-4 days before the start of the experiments to get familiarised with the device. Before the recording started, the animals were allowed 4 min for exploration, then the odour was

applied during 20 or 30 s, the last second of which overlapped with the foot-shock (0.4 mA). The animals underwent a total of 10 trials with an intertrial interval of 4 min. Each trial consists of three periods: 30 s "pre-odour", 20 or 30 s "odour", and 190 or 180 s "post-shock", of which only the first 30 s were considered. Eleven rats always received a 20 s odour delivery and the other nine, 30 s. For the current article, the vocalisations from both groups of animals were analysed together while keeping track of the period in which they were uttered. A total of 6 trials were analysed, early trials (# 1, 2 and 3) and late trials (# 8, 9 and 10). This was resolved to allow us to observe a potential effect of the animal's expectation in the vocalisation parameters, as the rats become familiar with the paradigm.

Data analyses. The vocalisations were first detected automatically in Avisoft SAS Lab Pro software (v. 5.2 Avisoft Bioacoustics, Germany) using an amplitude threshold of 1.9 %, minimum duration of 1 ms and hold time of 45 ms. Subsequently, all detections were manually revised and modified when necessary. All further analyses were done in custom-written scripts in Matlab (R2020a, The MathWorks, Massachusetts, USA). Vocalisations that were longer than 300 ms and that had a peak frequency between 18 and 32 kHz (i.e., the so-called 22-kHz vocalisations) were considered for further analyses (11956 from 13614 detected calls). The spectrogram shown in Fig. 1 was calculated using *mtspecgramc* function from Chronux (2 tapers of 50-Hz bandwidth and 0.2-s duration, 5-ms long windows at 4-ms steps). The loudness was calculated as the root-mean square (RMS) and the peak frequency using *mtspectrumc* (2 tapers).

An amplitude modulation score was calculated from the modulation power spectrum (MPS) of the first 300 ms of all vocalisations. First, the vocalisations RMS normalised. Second, the MPS were calculated as the 2D fast Fourier transform of the spectrograms (Short time Fourier transform, window length = 2048, number of FFT points = 512, hop =1). Subsequently, the MPS were 2D interpolated for averaging purposes (temporal and spectral resolutions: 1 Hz and 0.5 cyc/kHz, respectively, "spline" method). Finally, the score is calculated as the mean power two triangular regions with vertices in (3 Hz, 1 cyc/kHz), (100 Hz, 1 cyc/kHz) and (3 Hz, 20 cyc/kHz) for one triangle, and (-3 Hz, 1 cyc/kHz), (-100 Hz, 1 cyc/kHz) and (-3 Hz, 20 cyc/kHz) for the second one. In order to compare the score in the two defence behaviours (freezing or escape), USVs emitted during the 10 s after the foot shock were analysed. USVs emitted during

freezing were those whose onset was in time bin (1-s long) with ≥ 50 % of freezing, and the rest was considered as escape.

Statistical analysis. Statistical analyses were done in Matlab. The normality of data distributions was assessed by the Kolmogorov-Smirnoff test. Since none of them were normal, non-parametric tests were used. To compare the number of vocalisations (Fig. 2A) between the different periods (pre-odour, odour, and post-shock), twenty random bins of 1-s duration were selected for each period so that they had the same number of bins for chi-squared tests (significance at $p < 0.05$). *P* value shown is the average after one hundred repetitions. For comparisons between two periods, the same procedure was performed with Holm-Bonferroni-corrected chi-squared tests. Statistical differences for the duration, log (RMS), peak frequency, respiration frequency (Fig. 2B-E) and amplitude modulation score (Fig. 5A), were calculated using Kruskal-Wallis tests (significance at $p < 0.05$). If significant, a multiple comparison test was performed (Bonferroni corrected; *multcompare* in Matlab). For comparisons between different trials, Wilcoxon rank sum tests (significance at $p < 0.05$) were used.

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Competing interests

The authors declare no competing interests.

Data availability statement

The original data of this study are available from A.-M.M. upon reasonable request.

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Author contributions

M.D., J.B.B. and A.-M.M. conceived the experiments. M.D. carried out the experiments; E.G.P., J.B.B., A.-M.M. and J.C.H. designed the study; E.G.P. analysed the data; E.G.P. wrote the first draft of the manuscript; all authors revised the manuscript.