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Positive and Negative Ultrasonic Social Signals Elicit Opposing Firing Patterns in Rat Amygdala

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Abstract

Rat ultrasonic vocalizations (USVs) are ethologically-essential social signals. Under natural conditions, 22 kHz USVs and 50 kHz USVs are emitted in association with negative and positive emotional states, respectively. Our first experiment examined freezing behavior elicited in naïve Sprague-Dawley rats by a 22 kHz USV, a 50 kHz USV, and frequency-matched tones. None of the stimuli elicited freezing, which is the most commonly-used index of fear. The second experiment examined single-unit responses to these stimuli in the amygdala (AM), which is well-known for its role in innate and acquired fear responses. Among 127 well-discriminated single units, 82% were auditory-responsive. Elicited firing patterns were classified using a multidimensional scheme that included transient (phasic) responses to the stimulus onsets and/or offsets as well as sustained (tonic) responses during the stimulus. Tonic responses, which are not ordinarily evaluated in AM, were 4.4-times more common than phasic responses. The 22 kHz stimuli tended to elicit tonic increases in the firing rates, whereas the 50 kHz stimuli more often elicited tonic decreases in firing rates. These opposing tonic responses correspond with the ethological valence of USVs in the two frequency bands. Thus, a relatively-small sample of single-unit responses in AM furnished a more sensitive index of emotional valence than freezing behavior. Latency analysis suggested that stimuli in the two frequency bands are processed through different pathways to AM. One possible interpretation is that phasic responses in AM reflect the detection of a stimulus change, whereas tonic responses indicate the valence of the detected stimulus.

Keywords

ultrasonic vocalizations; single-unit recording; amygdala; freezing

1.0 Introduction

Rat ultrasonic vocalizations (USVs) are ethologically-essential social signals [1–6]. Adult rats innately emit USVs in two frequency ranges, which are identified as either “22 kHz USVs” or “50 kHz USVs”. The two types of USVs are distinguishable both by their acoustic parameters [7] and by the environmental situations in which they are emitted [6]. Twenty-

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two kHz USVs, which actually range from 18–32 kHz, are characterized by relatively long call durations (300–3400 ms; [7, 8]). The individual calls are described as being “flat” because they have a narrow bandwidth (1–6 kHz; [7, 8]). Typical 50 kHz USVs, which actually range from 35–72 kHz, consist of relatively short calls (30–50 ms). The individual calls can either be flat (5–7 kHz bandwidth) or they can contain complex frequency-modulations called “trills” [7, 9]. There are several published spectrograms of 22 kHz USVs [10–14] and 50 kHz USVs [9, 10, 15, 16].

Rats emit 22 kHz USVs during negative affective circumstances, such as predator encounters [17], defeat by a conspecific [18, 19], and anticipation of aversive stimulation [11, 20]. Twenty-two kHz USVs are positively correlated with freezing behavior and other indices of fear [11, 20]. By contrast, 50 kHz USVs are emitted during positive affective circumstances, such as conspecific play [9, 21, 22], mating [9], and anticipation of rewarding stimuli [23, 24]. Fifty-kHz USVs are positively correlated with increased locomotor activity, exploration, and approach behaviors [25, 26]. Thus, 22 kHz USVs are viewed as markers of a negative affective state, whereas 50 kHz USVs are considered indicative of a positive state [6, 26]. Twenty-two kHz USVs have been identified as social “alarm signals” [17, 27], whereas 50 kHz USVs have dubbed “rat laughter” [28, 29].

Whether behavioral responses to USVs are innate remains an open question [2]. Some studies have reported that playback of 22 kHz USVs induces freezing and avoidance responses [17, 30, 31], whereas playback of 50 kHz USVs induces approach responses [32, 33]. However, these reports do not demonstrate that the behavioral responses are innately-generated. In fact, other research suggests that rats do not innately exhibit defensive responses to 22 kHz USVs [10, 32, 34]. Emerging evidence [14], discussed later, indicates that a prior aversive experience is necessary for 22 kHz USVs to elicit freezing. There has been no analogous research on 50 kHz USVs.

The present study examined both behavioral and neurophysiological responses to USVs in experimentally-naïve Sprague-Dawley rats. Experiment 1 re-examined whether 22 kHz USVs elicit freezing behavior--this time using methods designed to more sensitive than ones employed in a previous study [10] that failed to find evidence of USV-elicited freezing. To understand the neurophysiological processing of these ethologically-important calls, Experiment 2 characterized single-unit responses in the amygdala (AM) to a 22 kHz USV, a 50 kHz USV, and two, frequency-matched continuous tones. AM was selected because it is essential for processing both innate [35, 36] and acquired fear responses [11, 37–40]. AM is also involved in reward anticipation [41–43] and social/affective interactions [44–47]. Furthermore, neuroimaging studies reveal that negative emotional stimuli increase amygdala activity [45–50], whereas positive stimuli decrease amygdala activity [46, 47, 51–53].

2.0 Materials and Methods

2.1 Basic statistics

Analysis of continuous variables used two-tailed t-tests and analysis of variance (ANOVA, F-tests). Significant F-tests were followed by a Fisher’s LSD post hoc test. Categorical data analysis used log-likelihood ratio tests (G-tests) rather than chi-square tests [54]. Correlations were based on Pearson’s *r*.

2.2 Experiment 1

Subjects were adult male Sprague-Dawley rats (*N* = 32; 250–350 g, Charles-River Laboratories; Wilmington, MA). Upon arrival to the colony, subjects were singly-housed, on a 12 hr light/dark cycle, and had *ad libitum* access to food and water. All procedures were in strict compliance with the Yale University’s Institutional Animal Care and Use Committee.

Subjects were handled for 3–5 d before testing. Rats were physically but not acoustically isolated from conspecifics for 3–5 d before experimentation began.

Subjects were randomly assigned to one of four groups, based on the stimulus type. The stimuli were a 22 kHz USV, a 22 kHz tone, a 50 kHz USV, and a 50 kHz tone. The 22 kHz USV was recorded from a naïve adult male Sprague-Dawley rat that was given multiple unsignaled footshocks (1–1.2 mA for 1 s). The USV had a root frequency of 19 kHz, a “bout” duration of 7.92 s, and contained 11 calls with a mean call duration of 0.55 s (Fig. 1A). The 50 kHz USV was recorded from two male juvenile Sprague-Dawley rats engaged in rough-and-tumble play. This USV had a root frequency of 53 kHz, a bout duration of 6.74 s and contained 26 calls with a mean call duration of 0.06 s (Fig. 1B). Thus, the duty cycle was much lower for the 50 kHz USV compared to the 22 kHz USV. Continuous tones were matched to their USV counterparts in terms of root frequency and bout duration (Fig. 1C and 1D).

Stimuli were presented free-field with an Enhanced Real-Time Processor and an Electrostatic Speaker Driver (both from Tucker Davis Technologies, Alachua, FL). The frequency-response characteristics of the ES1 speaker (shown in supplemental Fig. 1), had a nonlinearity that, if uncorrected, would have favored the 50 kHz stimuli. This nonlinearity was circumvented by matching the four stimuli on loudness (65 dB SPL; measurements taken at floor level with an Ultraprobe 9000, UE Systems Inc., Elmsford, NY).

Ultrasonic stimuli were presented through a speaker placed on top of an octagonal Plexiglas® chamber (35 cm diameter × 50 cm height), which was enclosed in a larger sound-attenuating Faraday chamber. The shape of the Plexiglas chamber was optimal for detecting freezing rather than escape or avoidance responses. The distance between the ultrasonic speakers and the rat was approximately 50 cm.

After a 120 s baseline period, stimuli were presented 20 times, with a mean inter-stimulus interval (ISI) of 10 ± 5 s. By contrast, in Bang et al [10] each animal received only 60 s of stimulus presentation. The procedural change was envisioned to furnish a more sensitive measure of any stimulus-elicited freezing. The session ended 60 s after the stimulus presentations. Behavior was recorded with an IR-CCD camera (Circuit Specialists, mesa, AZ). Freezing and immobility were measured using custom video-analysis software [12]. Movement was detected by comparing consecutive video images, sampled at 5 Hz. Freezing was defined as cessation of movement lasting ≥ 3 s, excluding respiratory movements (See supplementary Figs. 1–2 in [12]). Immobility was defined as cessation of movement lasting ≥ 1 s. Machine-scored freezing data is almost perfectly correlated with human-scored data ($r = 0.97$; $p < 0.005$; [12]).

Three different analyses were done to detect possible stimulus-elicited freezing behavior and/or possible changes in stimulus-elicited freezing, which might occur if the animals habituated to the ultrasonic stimuli. Repeated measures ANOVAs were conducted for the three analyses. In the first analysis, freezing was assessed in successive 1-min time bins and converted to a percentage score. This analysis included the entire 8-min experimental session, which consisted of the 2-min baseline period, the 20 stimulus presentations, and the 19 ISIs. The second analysis focused specifically on possible stimulus-elicited freezing across the 20 stimulus presentations. This trial-level analysis excluded the baseline and the ISI periods. Finally, immobility was evaluated on a second-by-second time scale during the first stimulus presentation. This analysis sought to reveal possible rapid habituation that might have been obscured in the previous two analyses.

2.3 Experiment 2

Animals were adult male Sprague Dawley rats (N = 22). Housing conditions were the same as in Experiment 1. Rats were physically but not acoustically isolated from conspecifics for 6–12 d. Each animal was handled at least 3 d before surgery and 3 d before the stimulus presentations. Under ketamine (100 mg/kg, i.p.) and xylazine (20 mg/kg, i.p.) anesthesia, animals were implanted with an 8-wire electrode bundle directed at the right lateral amygdala (LA). Using previously-described methods [55, 56], electrode bundles were implanted from the lateral surface of the skull. A micromanipulator drove the electrode bundle tip 2.5 mm into LA at a 45° angle (−3.24 A/P; −5.4 M/L; −8.2 D/V). The electrode bundle, which was connected to a headset on the dorsal skull, was fixed in place using dental cement.

The auditory stimuli and the experimental apparatus were the same as in Experiment 1. Subjects received 20 randomly-interleaved presentations of each of the four auditory stimuli, for a total of 80 presentations (mean ISI = 10±5 s). Single-unit activity was monitored and digitized in awake, freely-behaving rats before and during the stimulus presentations. Procedures for electrophysiological recording and offline sorting are described in detail elsewhere [55, 56]. Accepted units had a signal-to-noise ratio of 5:1 (peak-to-peak signal divided by root mean square of noise, as in previous studies of PR [55, 56]).

Graphical and statistical analyses were performed with NeuroExplorer (Plexon, Inc.), customized Microsoft Excel spreadsheets (Seattle, WA), and SPSS 15.0 (Chicago, IL). Stimulus-elicited firing patterns were classified based on combinations of three attributes, which defined the “elemental” firing patterns [55–57]. The first attribute was whether the stimulus caused the firing rate to increase or decrease, denoted by (+) and (−), respectively. The second was whether the change in firing was sustained or transient, denoted by tonic and phasic, respectively. The third attribute—applicable only to transient responses—was whether the firing change was triggered by the onset or offset of the auditory stimulus. “Compound” firing patterns contained two or more of the elemental patterns (See Fig. 3).

Firing latencies were defined by the first 10 ms time bin, after the stimulus presentation, to show a statistically significant change in firing rate compared with the rate during the pre-exposure stage of the experiment (two-tailed t-test for repeated measures, $p < 0.05$). The latency value for each unit was taken as the midpoint of the time bin in which a significant firing change was detected. To facilitate comparisons with similar studies [56, 58, 59], time-series plots of firing rates used averaged Z-scores (Fig. 5 and 6) as follows. First, the firing rate of each unit was converted to a Z-score:

$$Z = (R_A - R_B) / S_B,$$

where R_A is the firing rate in a given time bin after the presentation of the auditory stimulus, R_B is the mean firing rate averaged across the 5 s baseline period immediately before the presentation of the auditory stimulus, and S_B is the standard deviation of the firing rate during the baseline period. Next, Z-scores for each unit were averaged across 20 trials for each stimulus and each time bin. Finally, the time course of the mean Z-score was plotted as a function of time.

Within a day of single-unit recording, subjects were sacrificed with an overdose of Isoflurane (30% V/V). A small marking lesion was made by passing current between two of the recording wires (5 s, 50 μ A DC; Lesion Maker, Grass Instruments, Quincy, MA). The animals were transcardially perfused with 100 ml of 0.1 M physiological buffered saline and 100 ml of 4% paraformaldehyde. The brain was extracted and submerged in 4%

paraformaldehyde for 24 h and 30% sucrose solution for at least 72 h. Coronal sections (70 μm) were obtained with a freezing microtome and Nissl-stained with Cresyl Violet. Slides were examined with a Zeiss Axioskop light microscope (Thornwood, NY) and compared with a rat brain atlas [60] to determine the location of the lesion.

3. Results

3.1 Experiment 1

In Experiment 1, naïve Sprague Dawley rats were exposed to 20 presentations of either a 22 kHz USV, a 22 kHz tone, a 50 kHz USV, or a 50 kHz tone (Fig. 1). Three analyses were used to detect possible stimulus-elicited freezing and/or habituation of freezing across time or trials. First, freezing during the entire 8-min session was analyzed in 1-min time bins. There was no significant within-subjects effect of time ($F(7) = 1.71$, $p > 0.05$), no significant time $\times$ group interaction, ($F(21) = 0.71$, $p > 0.05$), and no significant between-subjects effect of stimulus type ($F(3,28) = 0.56$, $p > 0.05$). Figure 2A plots mean freezing levels across the 8-min session as a function of time. The graph shows that there is no hint of a stimulus-associated increase in freezing and no hint of a trend toward reduced freezing across time.

Second, stimulus-elicited freezing was assessed as a function of the trial number instead of time. The baseline period and ISI periods were excluded from this analysis in order to focus on stimulus-associated freezing. There was no within-subjects effect of trial number ($F(19) = 0.78$, $p > 0.05$), no trial $\times$ group interaction ($F(57) = 0.80$, $p > 0.05$), and no between-subjects effect of trial ($F(3,28) = 1.95$, $p > 0.05$). Thus, the 20 stimulus presentation trials did not cause the freezing response to habituate. Figure 2B plots freezing as a function of trial number. For improved visualization, the x-axis shows blocks of 2 trials. As indicated, there is no trend that might be suggestive of habituation.

Third, stimulus-elicited immobility was evaluated on a second-by-second time scale during the very first stimulus presentation. This was done to detect rapid habituation that might have been obscured in the preceding analyses. There was no significant within-subjects effect of time ($F(5) = 0.5$, $p > 0.05$), no time $\times$ group interaction ($F(15) = 0.86$, $p > 0.05$), and no between-subjects effect of time ($F(3,28) = 1.06$, $p > 0.05$). This result means that there was no evidence of habituation during the very first stimulus presentation. Figure 2C plots percent immobility as a function of time in seconds. The response is termed “immobility” because “freezing” is defined here as a lack of movement lasting at least 3 s (see Methods). This analysis did not include measurements during the last 0.92 s of the 22 kHz USV and tone and during the last 0.74 s of the 50 kHz USV and tone because “immobility” was defined as cessation of movement lasting at least 1 s. The immobility time series shows no trend across the first few seconds of the first presentations of any of the four stimuli.

3.2 Experiment 2

Histological examination revealed that 19 of the 22 electrode markings were confined to LA. Twelve of the 19 markings were restricted to dorsolateral LA (Fig. 3). Three of the 22 markings were in the posterior region of basolateral amygdala (BLA). All of the marking lesions were located between -2.80 and -3.30 mm (A/P) relative to Bregma, corresponding to plates 31–33 [60]. As expected [55, 56], the lateral surgical approach caused minimal damage to surrounding areas (Fig. 3).

A total of 127 well-isolated single-units were recorded from 22 rats. The mean spontaneous firing rate was 5.3 ± 0.3 Hz, which is comparable to some values reported in rat LA [39], but higher than others [58, 61]. The mean baseline firing rate did not differ significantly among

stimuli, $F(3, 503) = 0.21$, $p > 0.05$. There was no significant difference between the mean baseline firing rate in LA and BLA, $t(125) = 1.19$, $p > 0.05$.

Eighty-two percent of units (104/127) were responsive to one or more stimuli. Responsiveness was defined as a significant difference (two-tailed, paired t-test, $p < 0.05$) between the mean firing rate during the baseline period and one of three pre-determined time intervals: the phasic onset period (the first 200 ms after the stimulus onset); the tonic period (entire stimulus duration); and the phasic offset period (the first 200 ms after the stimulus offset). One goal of the experiment was to determine and compare the overall responsiveness of AM units to the four ultrasonic stimuli. All four stimuli were similar in terms of the probability of eliciting a response, $G(3) = 0.06$, $p > 0.05$. Among 104 stimulus-responsive units, 37% responded to the 22 kHz USV, 57% responded to the 22 kHz tone, 41% responded to the 50 kHz USV, and 36% responded to the 50 kHz tone. Some units (40/127) were responsive to two and only two auditory stimuli. A significantly higher proportion of these units (63%) responded to stimuli of the same frequency rather than different frequencies (38%), $G(1) = 6.32$, $p < 0.05$. Among single-units that responded to two stimuli of different frequencies, 22 kHz USVs and tones always elicited a tonic (+) response, and 50 kHz USVs and tones always elicited a tonic (−) response.

A second goal was to discover whether the four types of ultrasonic stimuli elicited different firing patterns. Four elemental and four compound firing patterns were observed. The distribution of firing patterns as a function of stimulus type is shown in Table 1. Representative examples of firing patterns are shown in Figures 4. The most commonly-observed firing pattern was a tonic response (Table 1; Fig. 4C). Tonic responses were 4.4-fold more frequent than phasic responses (Table 1; Fig 4A, 4B). Different proportions of units exhibited tonic (+) versus tonic (−) responses to the four auditory stimuli, $G(3) = 49.3$, $p < 0.01$. The effect size was large (Cohen's $w = 0.59$; Cohen, 1988). The 22 kHz USV and tone ("22 kHz stimuli") elicited a significantly greater proportion of tonic (+) responses than the 50 kHz stimuli, $G(3) = 33.6$, $p < 0.05$. By contrast, the 50 kHz stimuli elicited a significantly greater proportion of tonic (−) responses than the 22 kHz stimuli, $G(3) = 40$, $p < 0.05$. More specifically, the 22 kHz stimuli elicited significantly more tonic (+) than tonic (−) responses, $G(3) = 15.7$, $p > 0.01$; whereas the 50 kHz stimuli elicited significantly more tonic (−) than tonic (+) responses, $G(3) = 57.3$, $p < 0.01$. Differences in tonic firing patterns were evidently determined by the principle frequency rather than the spectrotemporal structure of the eliciting stimuli. Note that the preceding analysis was done exclusively on cells that exhibited an elemental tonic response. Figure 5 plots the aggregate peristimulus time histogram (PSTH) for the four elemental firing patterns (Table 1) using averaged Z-scores. The magnitude of firing rate change of the aggregate phasic onset (+) and aggregate phasic offset (+) histograms ($z = 2.5 - 3.0$) is higher than that of the tonic (+) ($z = 1.5 - 2.00$) and tonic (−) responses ($z = -0.5$; Fig. 5).

Among the single-units that exhibited a phasic onset (+) response to the 22 kHz USV, 2 of them exhibited what has been termed a "call-related firing pattern" [55, 56]. In these units, individual calls within the 22 kHz USV bout elicited a series of phasic onset (+) responses. Figure 6 plots the peristimulus time histogram and a three-dimensional contour plot of one of the two single-units that exhibited this type of firing pattern. This firing pattern was more commonly-observed in PR (29/142, 20%; [55]).

Overall, there were no significant differences ($p > 0.05$) between LA and BLA units in terms of elicited firing patterns ($G(4) = 2.17$ for 22 kHz USV; $G(4) = 1.69$ for 22 kHz tone; $G(2) = 0.54$ for 50 kHz USV; $G(4) = 2.49$ for 50 kHz tone). The phasic onset (+) and compound response categories were excluded from the 50 kHz USV contingency test because this stimulus failed to elicit these firing patterns (Table 1). An ANOVA compared the

magnitudes of firing-rate changes elicited by the four stimuli during the onset, tonic, and offset periods. All of the phasic-onset responses were firing-rate increases (Table 1). The mean firing-rate change during the onset period differed significantly among stimuli, $F(3, 420) = 8.79$, $p < 0.001$. The 22 kHz tone elicited a larger rate change than the 22 kHz USV ($p < 0.05$), the 50 kHz USV ($p < 0.01$), and the 50 kHz tone ($p < 0.01$). The 22 kHz USV elicited a larger rate-change than the 50 kHz USV ($p < 0.05$).

The mean firing-rate changes during the tonic period also differed significantly among stimuli, $F(3, 420) = 10.5$, $p < 0.01$. Tonic changes entailed both increases and decreases in mean firing rate. The 22 kHz tone elicited a larger firing rate increase than the 50 kHz USV and 50 kHz Tone ($p < 0.01$ for both). The 22 kHz USV also elicited a larger rate change than the 50 kHz USV and the 50 kHz Tone ($p < 0.01$ for both comparisons). The mean firing-rate changes during the offset period also varied significantly among stimuli, $F(3, 420) = 3.13$, $p < 0.05$. The 22 kHz tone elicited a greater mean firing-rate change than the 50 kHz USV, $p < 0.01$. In every case, the elicited firing change was a phasic (+) response.

The firing latency, defined as the first 10 ms time-bin after the stimulus onset to show a statistically-significant change in firing-rate (paired two-tailed t-test, $p < 0.05$; $n = 60$), was calculated for each unit that exhibited a phasic onset (+) response ($n = 60$). Such responses were observed for the 22 kHz USV ($n = 19$), 22 kHz Tone ($n = 32$), and 50 kHz Tone ($n = 9$). The 50 kHz USV failed to elicit phasic onset (+) responses (Table 1). There were no significant differences between LA and BLA units in terms of the firing latency to the 22 kHz tone ($t(30) = 1.51$, $p > 0.05$) and the 50 kHz tone ($t(7) = 2.23$, $p > 0.05$). Region-specific latency differences to the 22 kHz USV could not be examined because only one BLA unit displayed a phasic onset (+) response to this stimulus. There were no region-specific differences in the mean latency to the peak firing rate to the 22 kHz tone ($t(30) = 1.24$, $p > 0.05$) and 50 kHz tone ($t(7) = 2.15$, $p > 0.05$). Units from LA and BLA were combined in the remainder of the analysis.

The mean firing latencies were 16.6 ± 3.1 ms for the 22 kHz tone ($n = 32$), 23.4 ± 5.2 ms for the 22 kHz USV ($n = 19$), and 32.8 ± 9.3 ms for the 50 kHz tone ($n = 9$). The mean firing latencies were significantly different among the stimuli, $F(2, 57) = 7.56$, $p < 0.01$. The 22 kHz USV and tone elicited shorter mean firing latencies than the 50 kHz tone, $p < 0.001$. The mean latency to the peak firing rate occurred at 40.3 ± 6.2 ms for 22 kHz tone, 51.8 ± 8.3 ms for 22 kHz USV, and 67.3 ± 14.8 ms for 50 kHz tone, $F(2, 57) = 1.96$, $p > 0.05$.

Figure 7 shows the time course of the mean Z-scores (see Methods). The arrows point to the time bins containing the earliest significant increase in the firing rate and the latency to the peak firing rate. The earliest significant increase in firing occurred between 10–20 ms for the 22 kHz tone, 10–20 ms for the 22 kHz USV and 20–30 ms for the 50 kHz tone. The latency to the peak firing rate fell in the interval between 30–40 ms for the 22 kHz Tone, 50–60 ms for the 22 kHz USV, and 50–60 ms for the 50 kHz Tone.

A subset of units (8/60, 12%) was phasically activated by both a 50 kHz stimulus (the tone) and a 22 kHz stimulus (the USV and/or the tone). Within this group, the earliest significant increase in firing occurred at 21.2 ± 11.1 ms for the 22 kHz stimulus and at 33.3 ± 9.7 ms for the 50 kHz stimulus, $t(14) = 0.82$, $p > 0.05$. The mean latency to the peak firing rate occurred at 45 ± 15 ms for the 22 kHz stimulus and 46.5 ± 9.1 ms for the 50 kHz stimulus, $t(14) = 0.09$, $p > 0.05$. The two latency measures were compared between this subset of units and units that were responsive to only one frequency. Four t-tests, based on 2 principal frequencies and 2 latency measures, revealed no significant differences between dual-frequency and single-frequency responsive units ($p > 0.05$ for all comparisons).

4. Discussion

4.1 Overall Summary of Results

The present study quantified behavioral and neurophysiological responses to rat ultrasonic vocalizations (USVs) and frequency-matched tones. In Experiment 1, naïve Sprague-Dawley rats were exposed to multiple presentations of one of four ultrasonic stimuli, including a 22 kHz USV (Fig. 1). None of the stimuli elicited significant freezing relative to the pre-stimulus baseline levels (Fig. 2A). The reason for failing to detect stimulus-elicited freezing was not because freezing behavior rapidly habituated (see Fig 2 and related discussion).

In Experiment 2, single-unit responses to the same four stimuli were recorded in AM (Fig. 3). Stimulus-elicited firing patterns were classified using a multidimensional scheme, which furnished new insights into auditory processing. Perhaps surprisingly, most units in AM (82%) were auditory-responsive. There was no significant difference in the overall responsiveness of AM units to any the four ultrasonic stimuli. Four “elemental” firing patterns were observed: phasic onset (+), phasic offset (+), tonic (+), and tonic (−) (Table 1, Figs. 4 and 5). Also observed were four “compound” firing patterns, which consisted of two or more elemental patterns (Table 1, Fig. 4B).

Tonic responses, which are not ordinarily quantified in rat AM, were 4.4-fold more prevalent than phasic responses. Stimuli in the two frequency bands tended to produce opposite tonic responses. In particular, 22 kHz stimuli produced significantly more tonic (+) responses, whereas 50 kHz stimuli produced significantly more tonic (−) responses. When single units responded to two stimuli, they tended to be of the same principal frequency. Among single-units that responded to different frequencies, the 22 kHz stimuli always elicited a tonic (+) response, and the 50 kHz stimuli always elicited a tonic (−) response. The opposing tonic responses corresponded to the affective valence of these USVs under natural conditions. The spectrotemporal structure of the USVs was evidently not a factor, since frequency-matched tones had the same effect on the elicitation of tonic responses.

Latency analysis was performed on all stimuli except the 50 kHz USV, which did not elicit phasic onset responses (Table 1). Only the phasic onset responses can furnish the earliest latencies, which in turn can help set limitations on the underlying neuronal circuits. Across all units that exhibited a phasic onset response, the earliest significant increase in the firing rate occurred in the interval between 10–30 ms after the stimulus onset (Fig. 7). The latency to the peak firing rate occurred between 30–60 ms after the stimulus onset (Fig. 7). The mean latency associated with the 50 kHz tone was 2–4 times longer (Fig. 7C) than the latencies associated with the 22 kHz USV (Fig. 7B) and 22 kHz tone (Fig. 7A).

4.2 Are Behavioral Responses to USVs Innate?

The results convincingly demonstrate that, under the conditions of the present experiments, 22 kHz USVs do not elicit freezing behavior (Fig. 2), consistent with several previous studies [10, 32, 34]. The pertinent literature on this topic was recently reviewed by Wohr and Schwarting [2], who concluded that 22 kHz calls are not innately recognized as alarm calls, but can reach alarm signal value as a consequence of associative learning. They suggest that there is a biological preparedness to associate 22 kHz calls with aversive events and that the perirhinal cortex (PR) may furnish part of the “neural template” responsible for this preparedness.

Since that review was published, there is now direct evidence [14], based on a study of pair-housed male rats, that a prior aversive experience is necessary for 22 kHz USVs to elicit freezing behavior. One member of each pair (the “sender”) was given 10 tone-shock

pairings, while the other member (the “receiver”) experienced either 3 unsignaled footshocks or remained experimentally-naïve. When the pair was placed in a novel chamber and presented with the tone, the sender rat exhibited conditioned fear responses, including freezing and 22 kHz USV emission. The conditioned fear response of sender rats induced freezing in receiver rats that had previously experienced an aversive event, but did not affect receiver rats that were experimentally-naïve.

These findings clearly document the essential role of an aversive experience in determining behavioral reactivity to 22 kHz USVs. Simultaneously, the results demonstrate that the mere presence of a conspecific, or even the presence of a fearful conspecific, is not a sufficient condition for 22 kHz USVs to elicit freezing.

The fact that the 50 kHz USV did not elicit freezing behavior (Fig. 2) was not surprising, since these calls are typically emitted in association with a positive affective state [9, 21–24]. Fifty kHz USVs tend to induce approach and investigative behaviors, both in sexual [62, 63] and non-sexual situations [32, 33]. Notably, the production of 50 kHz USVs by males is necessary for normal courtship and mating (for a review, see [2]). It remains to be seen whether approach responses to 50 kHz USVs are innate.

4.3 Significance of Phasic versus Tonic Responses in AM

The use of a multidimensional classification scheme revealed three novel facts about single-unit responses in AM to auditory stimuli (see Table 1 and associated discussion). First, the preponderance of AM units showed firing changes to at least one of the four stimuli. Second, tonic responses were more common than phasic responses. Previous studies of single-unit responses in rat AM to auditory stimuli have focused exclusively on phasic onset (+) responses. Third, the main difference in single-unit responses to 22 kHz and 50 kHz stimuli was in terms of the proportions of elicited tonic (+)/(–) responses. The role of experience in controlling these response tendencies remains to be determined.

The present results invite speculation about the neurophysiological and behavioral significance of phasic versus tonic responses in AM. One possibility is that phasic onset/offset (+) responses mark the detection of a stimulus change, whereas tonic (+/–) responses reflect the valence of the stimulus. Phasic onset (+) responses in AM may play an important role in triggering the motor circuits that control freezing, while tonic activity may set or modulate the emotional tone. It remains to be seen whether, for example, aversive conditioning converts tonic (–) responses to tonic (+) responses and/or converts unresponsive units to phasically-activated units.

4.4 Relationships to Other Measures of AM Activity

The expression of c-Fos protein has been commonly used to quantify increases in gross neural activity (for a review, see [64]). In rats, the playback of 22 kHz USVs and tones has been reported to induce c-Fos expression in AM [32, 65, 66], PR [32], and the periaqueductal gray (PAG; [32]). These findings are perhaps not surprising, given that AM is critical for fear conditioning [11, 20, 35, 37, 39, 58], PR is essential for fear conditioning to 22 kHz USVs [12, 13, 16], and PAG mediates the behavioral expression of fear (for a review, see [37]). Moreover, 22 kHz USVs elicit more c-Fos expression in AM compared to 50 kHz USVs [32] or no sound [65, 66]. This may suggest that AM is predisposed to respond to negative stimuli. The increased c-Fos expression could be associated with increases in either phasic (+) and/or tonic (+) single-unit responses. At the present, little is known in AM about the causal relationships between the cellular neurophysiology and c-Fos expression.

In humans, functional magnetic imaging (fMRI) has been used to evaluate the relationship between emotion and global AM activity. Several studies report that negative stimuli, such as fearful facial expressions, enhance AM activity [46–50, 67]. This could reflect the aggregate effect of phasic (+) and/or tonic (+) responses. On the other hand, decreases in AM activity have been found in response to pleasant music [51], a loved one [53], and sexual arousal [52]. These changes could be caused by the aggregate effect of an increase in tonic (–) responses or a reduction in phasic (+) responses. Unfortunately, the imaging results are not entirely consistent. In particular, some studies have report decreased AM activity to happy facial expressions [46, 47], whereas others failed to see this effect [48–50]. The discrepancy has been suggested to result from the mediating effects of personality traits [68–70].

4.5 Circuitry Underlying Cue-Specific Differences in Firing Latencies

The overall firing latencies observed in the present study (Fig. 7) are comparable to values reported elsewhere [58, 71]. The short-latency responses to 22 kHz stimuli are consistent with activation via the thalamo-amygdala pathway, which may have evolved to support rapid fight/flight reactions [58, 71, 72]. By contrast, the longer-latency responses to the 50 kHz tone presumably reflect cortical projections to AM. The cortical projections are likely to originate from either PR [73, 74] or the auditory cortex [75], since both of these structures are connected to AM and furnish it with rich auditory inputs. Both PR [55, 56] and the auditory cortex [76, 77] are known to process ultrasonic stimuli.

4.6 Comparisons with Single-Unit Responses in PR

A previous study [55] examined single-unit responses to 22 kHz USVs, frequency-matched continuous tones, and temporally-matched tone segments in PR using the same multidimensional classification system as the present study. PR is a periallocortical region that receives rich unimodal and polymodal sensory inputs [74]. Phasic onset (+) responses were the most commonly-observed elicited firing pattern in PR [55], in contrast to AM units (Table 1). Call-related firing patterns (e.g., Fig. 6) were more common in PR (29/142, 20%) than in AM (2/127, 2%) [55], presumably due to the greater prevalence of phasic (+) responses in PR.

Among PR single-units that responded to both a USV and a frequency-matched tone, the majority (18/20, 90%) exhibited a call-related firing pattern to the USV and elemental phasic onset (+) or offset (+) responses to the continuous tone [55]. Among PR units that responded to two different 22 kHz USVs (which differed in terms of root frequency, call duration, and number of calls within a bout), the majority (20/29, 69%) exhibited call-related firing patterns. When units responded to a 22 kHz USV with a call-related firing pattern, they gave a “congruent” response to a temporally-matched discontinuous tone [55]. Specifically, if the USV elicited phasic onset responses to each call, then the discontinuous tone similarly elicited onset responses to each tone segment. On the other hand, if the USV elicited offset responses to each call, the tone segments also elicited offset responses.

In sum, unlike AM, PR single-units tended to exhibit more selective responses based on the temporal structure of the stimulus. These neurophysiological responses are consistent with the fact that damage to PR robustly impairs fear conditioning to USVs and discontinuous tones, but not continuous tones [12, 13, 16]. As mentioned earlier, c-Fos expression is increased in PR in response to playback of 22 kHz USVs. This increase happens even in the absence of an avoidance response [32].

4.6 Final Conclusions

The present study was motivated by five goals. The first was to re-examine whether 22 kHz USVs elicit freezing behavior. Sensitive testing demonstrated that experimentally-naïve rats did not freeze to 22 kHz USVs. One possible reason is that normal reactivity to these social signals requires certain prior experiences [14] that were missing in our subjects. The second goal was to determine the overall responsiveness of AM units to 22 kHz versus 50 kHz stimuli. Surprisingly, the preponderance of AM units was responsive to these ultrasonic stimuli. Furthermore, there were no significant differences among the stimuli in the probability of eliciting a response.

The third goal was to discover whether these stimuli elicited distinct firing patterns. Twenty-two kHz USVs and tones elicited more tonic (+) than 50 kHz USVs and 50 kHz tones. In contrast, 50 kHz USVs and tones elicited more tonic (−) responses than 22 kHz USVs and tones. The results dovetail with findings of bidirectional amygdala activations in humans to positive and negative facial expressions [46, 47]. A small fraction of the units responded to the 22 kHz USV with a unique call-related firing pattern.

The fourth goal was to evaluate whether differences in firing patterns were based on the principal frequency or some other acoustic feature of the stimuli. Artificial tones elicited the same tonic firing patterns as their USV counterparts, suggesting that stimulus-specificity in AM is based primarily on the principal frequency of the stimulus rather its temporal structure. The fact that only two units exhibited a call-related firing pattern to the 22 kHz USV reinforces this inference. The final goal was to assess whether 22 and 50 kHz stimuli are processed via distinct pathways in AM. Based on firing latencies, it seems that 22 kHz stimuli are processed through the thalamo-amygdala pathway [58, 71, 72]. On the other hand, the longer firing latencies associated with the 50 kHz tone are consistent with a thalamo-cortico-amygdala pathway.

Perhaps surprisingly, the results show that single-unit responses in AM may furnish a more sensitive index of emotional valence than freezing behavior. The bidirectional tonic activation to negative and positive social signals is consistent with several neuroimaging studies in humans to emotionally-salient stimuli [46–52, 67, 70, 78]. We suggest that rodents offer extraordinary opportunities for foundational studies of the neurobiology of social/ affective auditory interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research Highlights

- We examined single-unit responses in amygdala to rat ultrasonic vocalizations
- Naïve rats did not freeze to ultrasonic vocalizations or frequency-matched tones
- Amygdala neurons displayed sustained activation to calls associated with fear
- Vocalizations associated with positive affect decreased amygdala activity
- Tonic changes in amygdala were a more sensitive index of valence than freezing

Figure 1. Spectrograms of the four ultrasonic stimuli

(A) A pre-recorded 22 kHz USV elicited from a male Sprague-Dawley rat that was given multiple footshocks. (B) A pre-recorded 50 kHz USV recorded from two juvenile rats engaged in rough-and-tumble play. (C) A 22 kHz continuous tone matched to its USV counterpart in frequency, duration and amplitude. (D) A 50 kHz continuous tone matched to its USV counterpart in frequency, duration, and amplitude. (E) Amplitude plot of the 22 kHz USV. (F) Amplitude plot of the 50 kHz USV.

Figure 2. Freezing elicited by 22 kHz and 50 kHz USVs and tones

In Experiment 1, experimentally-naïve subjects were exposed to 20 presentations of one of four ultrasonic stimuli. A) Freezing as a function of time (measured in 1 min time bins over the 8-min experimental session). After 2 min of silence, which defined the baseline period, one of four ultrasonic stimuli was presented 20 times with an interstimulus interval (ISI) of 10 ± 5 s. The total stimulus presentation period, including ISIs, was 6 min. The horizontal dotted line represents the mean freezing across all groups during the 2-min baseline period ($16.5 \pm 6\%$). The downward arrow indicates the beginning of the stimulus presentation period. Subjects did not freeze significantly more during the stimulus presentation period relative to the baseline period. B) Stimulus-elicited freezing over 20 trials (shown as blocks

of 2 consecutive trials). The four ultrasonic stimuli elicited uniform freezing levels over 20 trials. There was no evidence of habituation or stimulus-specific differences in freezing. C) Second-by-second analysis of immobility (%) to the first trial of stimulus presentation. The last 0.74 s of the 50 kHz stimuli and the last 0.92 s of the 22 kHz stimuli were excluded from this analysis because immobility is defined as cessation of movement for at least 1 s. There was no evidence of habituation to any of the four ultrasonic stimuli. Error bars are SEM for all plots.

Figure 3. Histologically-verified locations of electrodes mapped onto stereotaxic plates
A) The recording sites were confined to -2.80 to -3.30 mm (plates 31–33) from Bregma (Paxinos & Watson, 2005). B) A representative Nissl-stained coronal brain section (70 μm) with a marking lesion in LA. Implanting the electrodes from the lateral surface minimized cortical damage. LA: lateral amygdala; BLA: basolateral amygdala.

Figure 4. Representative examples of firing patterns elicited by the four ultrasonic stimuli
 Gray shading represents the stimulus presentation. Each time bin is 50 ms wide. Not all possible combinations of firing patterns are shown. A) A phasic onset (+) response elicited by the 22 kHz USV. B) A compound firing pattern, elicited by the 22 kHz Tone, consisting of a phasic onset (+), a tonic (+), and a phasic offset (+) response. C) A tonic (+) response to the 22 kHz USV. D) A tonic (-) response to the 50 kHz USV.

Figure 5. Aggregate histograms of four elemental firing patterns

Firing rates for each unit and time bin were transformed to Z-scores (see Methods) and then averaged. The Z-scores compared the firing rate in each time bin to the firing rate during the 5 s baseline period that preceded the stimulus. The vertical-dashed line on each histogram represents the stimulus onset. A) Aggregate phasic onset (+) responses ($n = 26$) in 10 ms bins. For graphical purposes, only 250 ms before and 250 ms after the stimulus onset are shown. B) Aggregate phasic offset (+) responses ($n = 16$) in 10 ms bins. Due to the small sample size of elemental phasic offset (+) responses ($n = 8$), compound responses that contained a phasic offset (+) component were also included ($n = 8$). For graphical purposes, 250 ms before the stimulus onset and 250 ms after the stimulus offset are shown. C) Aggregate histogram of tonic (+) responses ($n = 57$) in 100 ms bins. The x-axis shows the 5 s baseline period and the tonic period (entire stimulus duration). D) Aggregate histogram of tonic (-) responses ($n = 92$) in 100 ms bins. The x-axis shows the 5 s baseline and the tonic periods.

Figure 6. Call-related firing pattern elicited by a 22 kHz USV

A) A raster plot and peristimulus time histogram shows that the onset of each call within the 22 kHz USV bout elicited a phasic onset (+) response. B) A three-dimensional contour plot of the same single-unit as panel A. The increase in firing rate is higher at the beginning and end of the USV bout, which gives the contour plot a saddle shape.

Figure 7. Earliest and peak latencies to fire to ultrasonic stimuli

Firing rates for each unit and 10 ms time bin were transformed to Z-scores and averaged (see Methods). The mean Z-scores are plotted as a function of time. In each plot, the first arrow indicates the earliest latency to fire, and the second arrow indicates the earliest latency to the peak firing rate. (A) For the 22 kHz tone, the earliest significant increase in the firing rate occurred between 10–20 ms and the earliest latency to peak firing occurred between 30–40 ms. (B) For the 22 kHz USV, the earliest significant increase in firing fell in the 10–20 ms bin and the earliest latency to peak firing fell in the 50–60 ms bin. (C) For 50 kHz tone, the earliest significant increase in firing occurred between 20–30 ms and the earliest latency to peak firing occurred between 50–60 ms. The 50 kHz USV was excluded from the latency analysis since this stimulus failed to elicit phasic onset (+) responses (Table 1).

Distribution of stimulus-elicited firing patterns in the lateral and basal nuclei of the amygdala to four ultrasonic stimuli.

Table 1

Firing Pattern	Auditory Stimulus			
	22 kHz USV	22 kHz Tone	50 kHz USV	50 kHz Tone
Elemental Firing Patterns	Phasic Onset (+)	14/127 (11%)	0%	7/127 (6%)
	Phasic Offset (+)	1/127 (1%)	2/127 (2%)	4/127 (3%)
	Tonic (+)	20/127 (16%)	7/127 (6%)	2/127 (2%)
	Tonic (-)	8/127 (6%)	43/127 (34%)	31/127 (24%)
Compound Firing Patterns	Phasic onset (+)/tonic (+)	11/127 (9%)	0%	0%
	Phasic onset (+)/tonic(-)	1/127 (1%)	0%	0%
	Phasic onset (+)/phasic offset(+)	1/127 (1%)	0%	2/127 (2%)
	Phasic onset (+)/phasic offset (+)/tonic (+)	0%	0%	0%
Total		47/127 (37%)	72/127 (57%)	46/127 (36%)