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Playback of 22-kHz and 50-kHz ultrasonic vocalizations induces differential c-fos expression in rat brain

Monika Sadananda *, Markus Wöhr, Rainer K.W. Schwarting

Experimental and Physiological Psychology, Philipps University, Gutenbergstr. 18, D-35032 Marburg, Germany

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Abstract

Rodent ultrasonic vocalizations, which serve as sensitive measures in a number of relevant individual and social behaviours, have become increasingly interesting for biopsychological studies on emotion and motivation. Of these, high frequency (50-kHz) ultrasonic vocalizations can index a positive emotional state, and induce approach, whereas low frequency (22-kHz) ultrasonic vocalizations can induce avoidance and may index anxiety, since they are emitted during various unconditioned and conditioned aversive situations. While cholinergic and dopaminergic systems have been implicated, specific neural substrates that sub-serve these vocalization-dependent states remain to be elucidated. Using c-fos immunocytochemistry, we revealed neural activity in brain areas of naïve male Wistar rats in response to playback of 22-kHz and flat and frequency-modulated 50-kHz ultrasonic vocalizations. Presentation of background noise or no acoustic stimulus at all constituted the controls. Playback of 50-kHz ultrasonic vocalizations led to approach behaviour. Acoustically stimulated animals demonstrated differential activation in auditory areas, with a frequency-dependent activation in the auditory cortex. Specific forebrain, thalamic, hypothalamic and brainstem areas were also activated differentially. While 50-kHz playback induced sparse fos-like immunoreactivity in frontal association cortex, nucleus accumbens, thalamic parafascicular and paraventricular nuclei, 22-kHz playback elicited c-fos expression in the perirhinal cortex, amygdalar nuclei and the periaqueductal gray. This study unveils neural substrates that are activated during ultrasonic playback perception, which could sub-serve the affective states elicited by these vocalizations.

Keywords: Ultrasonic vocalization (USV); Playback; Aversive; Appetitive; Fos expression

The growing research interest in mammalian vocalization is concomitant with increasing interest in the underlying neural mechanisms, since vocalizations can index a great deal about brain, behaviour and the general state of the organism. In the rat, the impact that 22-kHz ultrasonic vocalizations (USVs) have on the receiver in terms of behaviour [27,5] and its neural substrates [1,2] have been studied. On the other hand, the effect of 50-kHz call presentation on behaviour has also been studied [9,33] and neural substrates have been suggested [17]. Playback of 22-kHz USVs can lead to avoidance or locomotor inhibition [5,10,33, but see 14,22], while 50-kHz calls can be appetitive [9], induced approach [33] and enhanced self-administration [9].

By far, as pharmacological studies have shown, it is the cholinergic [4] and dopaminergic [8] pathways that seem to affect overt behaviour and vocalization emission to a great extent, though other neurotransmitter systems also play a role [32]. While cholingeric pathways have been shown to underlie 22-kHz vocalization and the overt behaviour associated with a negative state, the dopaminergic system in the shell region of the nucleus accumbens is said to underlie 50-kHz calling and the positive state associated with it [8]. However, other studies have shown that the neural substrates involved in the initiation and production of these vocalizations are more complex [17].

Here, we use immediate early gene expression to screen for active brain regions in response to the playback of recorded ultrasonic calls. Immediate early genes are known to induce downstream cascades of gene-induction and represent cellular activity leading to protein synthesis. C-fos immunocytochemistry has served as a powerful tool for anatomical mapping of functional characteristics in complex systems such as the auditory brainstem pathways [15], in response to novel and familiar sounds [31], and in response to auditory stimuli that attain behavioural significance [28]. This would indicate that

* Corresponding author. Tel.: +49 6421 2823694.
E-mail address: monika.sadananda@staff.uni-marburg.de (M. Sadananda).

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the response is not just reflecting auditory features of the stimulus, but also the salience of the stimulus, and this should be seen not only in auditory-relevant regions, but also regions associated with withdrawal and/or aversive behaviour, such as the periaqueductal gray and parts of the amygdala, and regions associated with positive affects, such as the ventral striatum.

Sixteen naïve male Wistar rats (HsdCpb:WU, Harlan-Winkelmann, Germany) weighing 100–124 g were procured, housed in groups of four in cages (Macrolon type IV) on Tapvei peeled aspen bedding (indulab ag, Gams, Switzerland), and maintained in 12:12 h light/dark cycle (21–25 °C; 49–59% humidity). The animals were handled for 5 min on 3 consecutive days. On the 4th day, they were randomly assigned to four groups corresponding to the type of acoustic stimulus presented: no playback (arena-only); background noise, 22-kHz calls and 50-kHz calls. Then, they were removed from their home cages, isolated for 1 h, after which they were habituated to the test arena under red light (approx. 8 lux) for 1 h.

On the 5th day, animals were isolated for 1 h and then placed in the test arena, with playback of acoustic stimuli presented for 30 min. The testing arena (38 cm × 60 cm × 35 cm) consisted of two compartments (38 cm × 24 cm × 35 cm) joined by a central alley (38 cm × 12 cm × 35 cm). The two compartments had one side-wall replaced with a grid in front of which the loudspeaker was placed. The arena was wiped clean and the floor covered with fresh bedding each time. The recording room was devoid of any sound other than that from the recording equipment.

Acoustic stimuli, using hardware and frequency settings as described [33], were presented through an ultrasonic speaker (ScanSpeak, Avisoft Bioacoustics, Germany), placed 20 cm away from the test apparatus, with its position being changed from one compartment to the other for each animal. The calls presented had been recorded from a male Wistar rat while exploring a cage with scents from a cage mate (50-kHz), or from a rat that had received foot shocks (22-kHz). All stimuli [(a) 50-kHz of both, flat and frequency-modulated types [29], (b) long 22-kHz calls, and (c) background noise] were presented with a sampling rate of 192 kHz in 16-bit format, at ∼69 dB, with background noise presented at ∼50 dB, which corresponds to the background noise during playback of the other stimuli. Number of entries into the compartments and USVs emitted were recorded and analysed.

The animals remained in the testing arena for another 30 min, after which they were deeply anaesthetised and perfused transcardially with 0.9% saline and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed, post-fixed and cryo-protected. Coronal sections of 30 μm were cut on a cryostat and subsequently processed for immunocytochemistry.

Briefly, sections were washed in 0.01 M phosphate buffered saline (PBS), rinsed in 0.2% Triton (PBS-T) detergent, endogenous peroxidase activity blocked with 0.3% hydrogen peroxide (H_2O_2), incubated in 5% normal goat serum (NGS-Vector S-1000), then transferred to c-fos antiserum (sc-52; Santacruz Biotech., 1:1000; 1% NGS) for 36–48 h. Sections were subsequently incubated in biotinylated goat anti-rabbit antiserum (1:100) followed by avidin–biotin–horseradish-peroxidase complex (Vector Elite PK-6101), and bound peroxidase visualised with 0.025% diaminobenzidine tetrahydrochloride (Sigma) and 0.06% H_2O_2.

Fos expression was screened qualitatively on a BX 61 Olympus microscope. Fos-positive cells were then quantified using Stereoinvestigator® (6.00-MicroBrightField Inc.) according to histologically defined criteria of the rat atlas [25]. Counting was done in a stipulated 0.25 mm × 0.25 mm square area on randomly selected sections from each brain. Photomicrographs were made using an Optronics digital camera MicroFire™ and worked on using Corel Draw (Corel Corp., 2000).

Experiments were carried out in accordance with the European Communities Council Directives, and permitted by the local animal ethics committee.

Behavioural results show that the 50-kHz group demonstrated significantly more locomotor activity during playback (total entries—arena-only: 53.25 ± 14.77; background: 56.25 ± 8.07; 22-kHz: 58.25 ± 11.18; 50-kHz: 98.75 ± 7.98; group means ± S.E.M.; $p = 0.049$; Kruskal–Wallis H-test), which was mainly directed to the compartment with the loud speaker (number of entries—arena-only: 14.00 ± 4.02; background: 13.75 ± 2.02; 22-kHz: 14.00 ± 2.80; 50-kHz: 27.75 ± 2.87; $p = 0.032$). Entries into the compartment without the loud speaker did not differ significantly (arena-only: 12.75 ± 3.50; background: 14.50 ± 2.02; 22-kHz: 15.50 ± 2.90; 50-kHz: 20.75 ± 2.78; $p = 0.336$). While no 22-kHz calls were emitted by any of the groups, some 50-kHz calls were detected in all groups—arena-only: 0.41 ± 0.25; background: 0.075 ± 0.028; 22-kHz: 0.083 ± 0.052; 50-kHz: 0.21 ± 0.088 (means calls/min ± S.E.M.; $p = 0.210$).

Fos-like immunoreactivity was confined to the nuclei of activated cells, which could be easily distinguished from background (Fig. 1). Basal expression was observed in arena-only animals in the olfactory lobes, piriform cortex, dorsal thalamus, lateral habenular nuclei (Fig. 1D), septal areas and some hypothalamic nuclei. Since differential fos-like immunoreactivity was observed in the four groups, 35 regions of interest (Fig. 2) were selected to further quantify the differences (Table 1).

Compared to the arena-only condition, an upregulation of fos-like immunoreactivity was observed in the acoustically stimulated groups in various cortical areas, such as the auditory, motor, frontal association, temporal association and ectorhinal cortices. Activation was also detected in the nucleus accumbens shell region, in the lateral septum and in the dorso-medial periaqueductal gray. Significant differences between 22-kHz and 50-kHz groups were observed in the frontal and perirhinal cortices, basolateral and lateral amygdala, paraventricular thalamic nucleus and dorso-medial periaqueductal gray. While activation in the 22-kHz group was observed in the basolateral, lateral and medial parts of the amygdala and the perirhinal cortex, the 50-kHz group demonstrated some activation in the accumbens core and shell regions, the anterior cingulate and frontal association cortices.

Areas in the auditory pathway were labelled to varied extents, with sparse labelling in the inferior colliculus and moderate to dense labelling in the primary and secondary areas of the auditory cortex (AC). In the 22-kHz group, cells in the central nucleus of the inferior colliculus were

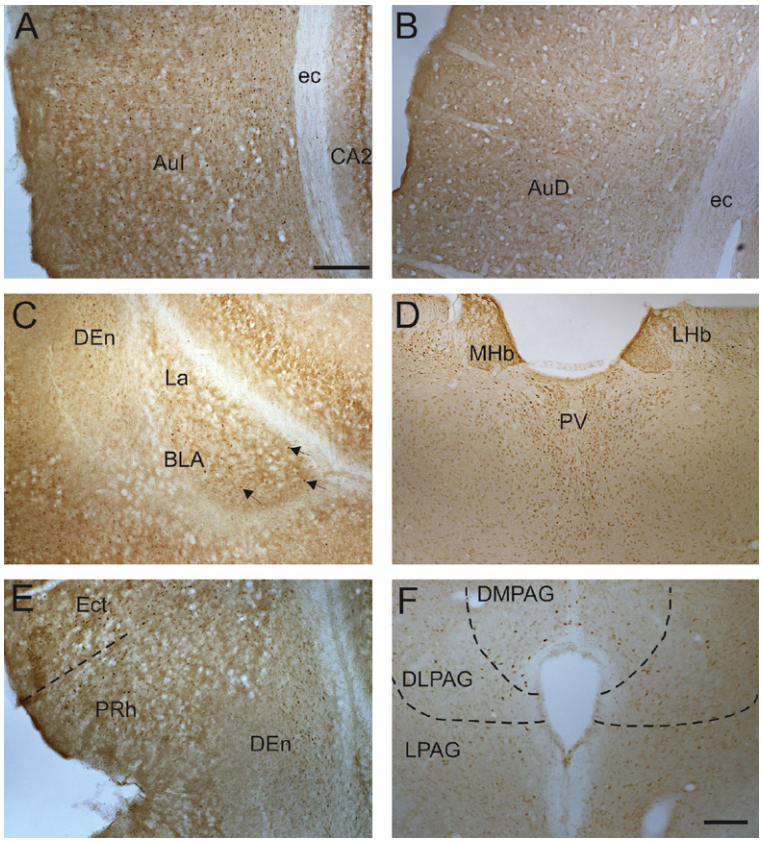

Fig. 1. Representative photomicrographs of fos-like immunoreactivity in response to playback of 22-kHz (left panel) and 50-kHz (right panel) ultrasonic vocalizations in male rats. (A) Fos expressing cells in the primary auditory area (AuI); (B) fos activation in the secondary auditory area (AuD); (C) few labelled cells in the lateral (La) and basolateral (BLA) amygdala, an expression not observed in the 50-kHz playback condition; (D) lateral habenular (LHb) and paraventricular (PV) nuclei; (E) fos labelling in the ectorhinal (Ect) and perirhinal (PRh) cortices; (F) activated cells in different sub-divisions of the periaqueductal gray (PAG; DM, dorso-medial; DL, dorso-lateral; L, lateral); fos expression seen in (D) and (F) was observed to varied extents in all four groups. Scale bar = 250 μm (*F*: 200 μm). Other abbreviations: CA2, field CA2 of hippocampus; ec, external capsule; DEn, endopiriform nucleus; MHb, medial habenular nucleus.

observed to be obliquely labelled across the nucleus, while there was very sparse fos-like immunoreactivity in the 50-kHz group.

Differential fos expression in AC was observed in response to playback of vocalizations of different frequencies. Labelling appeared either in discrete clusters in frontal AuD and AuV, or outspread through layers II–VI in AuI (Fig. 3). Hemispheric lateralization was also evident in the AC, with the left hemisphere showing higher activation. 22-kHz animals demonstrated dense fos expression in the primary auditory area (AuI, Fig. 1A) and in ventral (AuV) and dorsal (AuD) secondary auditory areas, while 50-kHz showed more c-fos activation in the frontal AuD (Fig. 1B) and AuV areas, and less in the AuI area. The temporal association cortex, ectorhinal cortex and to a certain extent the perirhinal cortex (Fig. 1E) were labelled in response to 22-kHz calls. The expression was lower in the 50-kHz group, except in the temporal association cortex, where it was on comparable levels.

In the amygdala, the basolateral and lateral nuclei contained a few scattered labelled cells in the 22-kHz group (Fig. 1C). In the 50-kHz group, sparse fos expression was observed in the central amygdala. Few fos expressing cells were observed in the medial shell region of the nucleus accumbens in the 50-kHz group, a pattern also observed in the arena-only group. In more caudal sections, few scattered nuclei were observed in the ventral core region.

The hypothalamus demonstrated differential fos expression in all groups. Parts of the pre-optic and lateral hypothalamus were labelled. In addition, the medial forebrain bundle, the ventral pallidum, and the parafascicular nuclei located just dorsal to the fornix in the thalamus demonstrated fos-positive cells in the 50-kHz group, but not in any other. In the rest of the brain stem, activation in sub-regions of the periaqueductal gray (Fig. 1F) was evident in all four groups to varied extents. While arena-only animals showed the least followed by 50-kHz animals, background and 22-kHz groups showed comparable expression. The pontine nuclei demonstrated comparable activation in response to 22- and 50-kHz playback.

Arena-only controls showed some fos expression. This activation is not due to novelty, as the animals had been habituated. It represents basal fos expression that exists in olfactory regions, visual cortex and a few other areas. All groups also demonstrated some thalamic, hypothalamic, and septal activation.

In the playback groups, cortical auditory regions were activated more in the left than the right hemisphere. This result is in line with previous evidence obtained in mice, where hemispheric lateralization in auditory cortex processing [13], and left hemisphere dominance in auditory perception and recognition

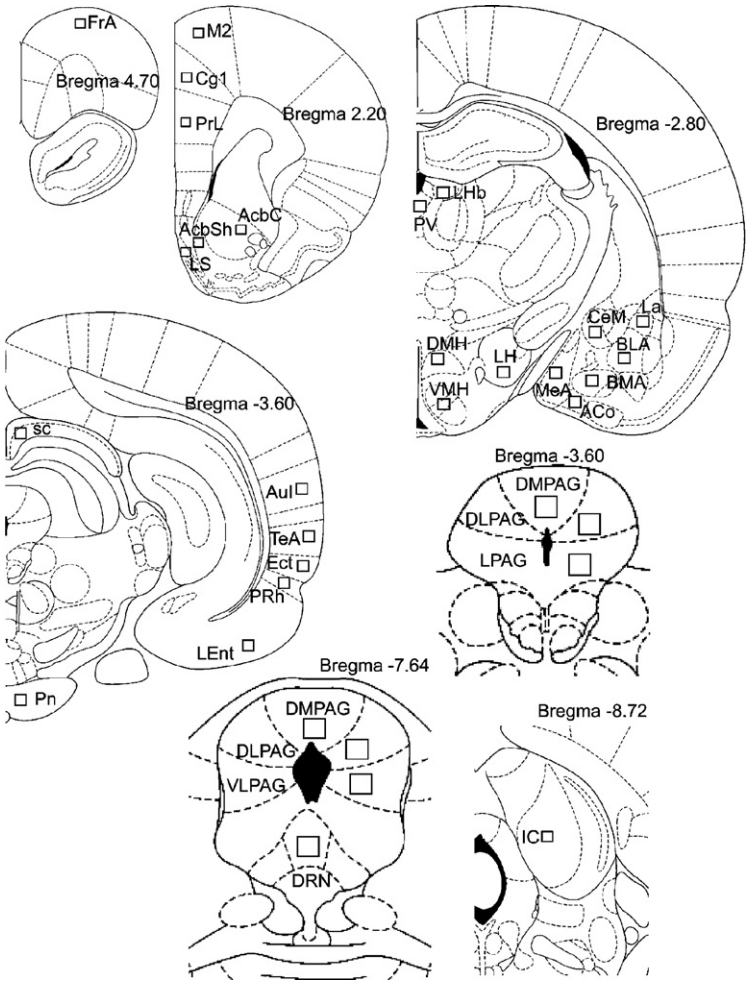

Fig. 2. Schematic diagrams of frontal sections of the rat brain from the Paxinos and Watson atlas, showing the 35 areas in which fos expression was quantified (Table 1). Open squares indicate the position of the 0.25 mm × 0.25 mm grid drawn to scale within which cell nuclei stained with fos were counted. For abbreviations, see Table 1.

was shown using c-fos mapping [16]. The fact that differential fos expression was observed here in the AuI, AuD and AuV areas could reflect a representation of the different frequencies perceived. While tonotopic fields AI and AAF with a high to low frequency gradient constitute the core [23,12], dorsally-, ventrally- and posteriorly located fields constitute the belt [23,26] of the auditory cortex. The more frontal fos expression observed in response to 50-kHz calls fits well with the topography of the high frequency area in AI and AAF [12,26], while fos expressing neurons found in clusters in the belt or secondary auditory areas could indicate processing at a higher level [18]. The increase in fos activation in the 22-kHz group could be due to the intensity, and the duration of the aversive acoustic stimulus, which can produce a spread of neuronal activation and an enlarging of tonotopic bands [28].

Functionally, 22-kHz calls are said to play an important role as alarm calls [3], and previous work has shown that such calls can lead to avoidance behaviour [5,10,33]. Such avoidance could not be detected here, which may be due to differences in the type of environment or behavioural measures. Nevertheless, presentation of 22-kHz calls led to neuronal activation in parts of the amygdala, albeit sparsely. While the lateral, basolateral and

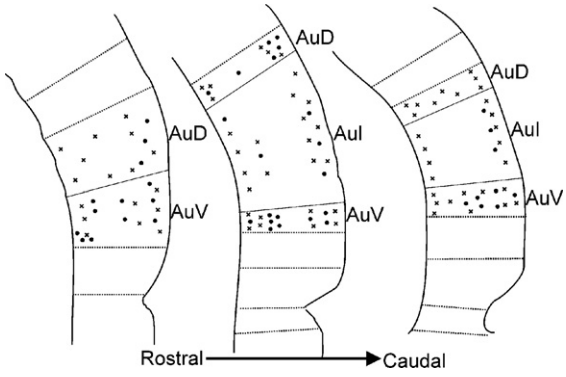

Fig. 3. Schematic representation of fos-like immunoreactivity in frontal sections of the auditory cortex. Crosses denote c-fos induction in response to playback of 22-kHz and circles to 50-kHz vocalizations. Shown is the activation in the left auditory cortex. The activation is attenuated in the right auditory cortex, cells being confined to the dorsal sub-division (AuD) in the 50-kHz group, while the activation is more spread out in the 22-kHz group. Rostral, Bregma −3.14 mm; caudal, Bregma −5.60 mm according to the Paxinos and Watson atlas.

Table 1
Number of fos-positive cells (mean ± S.E.M.) counted within a 0.25 mm × 0.25 mm square in 35 brain regions

Region	Bregma (mm)	Arena ($n=4$)	Background ($n=4$)	22-kHz ($n=4$)	50-kHz ($n=4$)	p values (H-test)
Auditory system						
Inferior colliculus (IC)	−7.80 to −8.80	8.05 ± 0.25	8.6 ± 1.5	4.3 ± 0.57*	4.5 ± 0.53*	0.005
Primary aud. cortex L (AuI)	−3.60 to −6.30	4.8 ± 1.12	7.35 ± 0.94	17.2 ± 0.96*,#	5.1 ± 0.66#	0.002
Primary aud. cortex R (AuI)	−3.60 to −6.30	5.15 ± 1.31	9.3 ± 0.97	14.3 ± 2.5	7.9 ± 2.78	0.108
Forebrain						
Frontal cortex (FrA)	3.70 to 1.70	0 ± 0	0.45 ± 0.33	0.35 ± 0.35#	2.3 ± 0.13*,#	0.003
Perirhinal cortex L (PRh)	−3.60 to −6.30	1.8 ± 1.2	0.95 ± 0.56	2.0 ± 0.54	0.85 ± 0.53	0.544
Perirhinal cortex R (PRh)	−3.60 to −6.30	0.30 ± 0.30	4.45 ± 0.68*	2.15 ± 0.33*,#	0.3 ± 0.24#	0.000
Motor cortex (M2)	3.70 to 1.70	0.35 ± 0.35	2.35 ± 0.31	1.1 ± 0.80	1.7 ± 0.69	0.105
Cingulate cortex (Cg1)	3.70 to 1.70	0.7 ± 0.41	1.0 ± 0.75	0.15 ± 0.15	1.85 ± 0.79	0.339
Temporal cortex (TeA)	−3.60 to −6.30	1.7 ± 0.98	5.95 ± 1.43	6.35 ± 0.59	6.45 ± 1.65	0.075
Ectorhinal cortex (Ect)	−3.60 to −6.30	1.25 ± 0.63	5.8 ± 2.15	4.5 ± 0.75*	2.15 ± 0.77	0.047
Prelimbic (PrL)	3.70 to 2.20	0.10 ± 0.10	0.90 ± 0.66	1.55 ± 0.94	2.1 ± 1.2	0.433
Entorhinal cortex (LEnt)	−5.20 to −6.30	1.2 ± 0.73	2.1 ± 2.1	0 ± 0	1.0 ± 0.87	0.107
Amygdala						
Ant. cortical amygdala (ACo)	−2.30 to −3.30	0.40 ± 0.28	1.8 ± 1.8	0 ± 0	0 ± 0	0.543
Medial amygdala (MeA)	−2.30 to −3.30	1.0 ± 0.51	4.0 ± 2.3	0.95 ± 0.95	0 ± 0	0.315
Basolateral amygdala (BLA)	−2.30 to −3.30	1.4 ± 0.36	0 ± 0*	3.9 ± 0.46*,#	0.9 ± 0.52#	0.000
Basomedial amygdala (BMA)	−2.30 to −3.30	0.7 ± 0.34	1.4 ± 1.4	0.25 ± 0.25	0 ± 0	0.235
Lateral amygdala (La)	−2.30 to −3.30	0.65 ± 0.65	0 ± 0	3.5 ± 0.75#	0.10 ± 0.10#	0.008
Central amygdala (CeM)	−2.30 to −3.30	2.9 ± 0.37	0 ± 0*	0.4 ± 0.4*	1.5 ± 0.29*	0.000
Basal ganglia						
Nuc. accumbens core (AcbC)	1.70 to 0.70	1.25 ± 0.36	0.45 ± 0.45	0.45 ± 0.45	2.55 ± 1.02	0.127
Nuc. accumbens shell (AcbSh)	1.70 to 0.70	0.8 ± 0.2	1.3 ± 0.60	1.4 ± 1.04	2.2 ± 1.2	0.783
Septum and hypothalamus						
Lateral septum (LS)	1.70 to 0.20	0.95 ± 0.95	4.2 ± 0.90	3.3 ± 3.3	1.5 ± 1.5	0.253
Lateral hypothalamus (LH)	−1.30 to 2.30	3.5 ± 1.57	7.0 ± 1.25	4.45 ± 1.13	2.45 ± 1.43	0.164
Ventromedial nucleus (VMH)	−2.80 to −3.30	6.75 ± 2.3	4.0 ± 2.4	1.8 ± 1.8	2.7 ± 1.6	0.317
Dorsomedial nucleus (DMH)	−2.80 to −3.30	5.35 ± 1.02	5.15 ± 0.94	6.4 ± 2.7	4.65 ± 1.73	0.986
Thalamus						
Paraventricular nucleus (PV)	−2.80 to −3.80	16.8 ± 2.64	11.95 ± 1.77	6.85 ± 0.45*,#	11.00 ± 1.75#	0.014
Lateral habenular nucleus (LHb)	−2.80 to −3.80	17.4 ± 3.49	10.8 ± 1.32	7.8 ± 1.81	7.4 ± 1.19*	0.023
Tectum						
Superior colliculus (SC)	−6.30 to −7.30	4.15 ± 1.35	6.15 ± 1.16	8.25 ± 0.66	4.10 ± 0.78	0.066
Periaqueductal gray						
Rostral dorso-medial (DMPAG)	−5.60 to −7.30	2.15 ± 0.26	3.9 ± 0.34*	4.6 ± 0.48*,#	2.5 ± 0.31#	0.001
Rostral dorso-lateral (DLPAG)	−5.60 to −7.30	2.7 ± 0.13	4.8 ± 0.74	4.0 ± 0.75	2.6 ± 0.53	0.096
Rostral lateral (LPAG)	−5.60 to −7.30	2.95 ± 0.78	1.85 ± 0.26	2.55 ± 0.58	1.95 ± 0.33	0.500
Caudal dorso-medial (DMPAG)	−7.64 to −8.72	2.35 ± 0.46	2.85 ± 0.92	3.25 ± 0.40	2.0 ± 0.49	0.506
Caudal dorso-lateral (DLPAG)	−7.64 to −8.72	2.2 ± 0.55	3.05 ± 0.41	4.1 ± 0.77	2.55 ± 0.3	0.206
Caudal ventro-lateral (VLPAG)	−7.64 to −8.72	1.95 ± 0.40	1.5 ± 0.13	2.05 ± 0.56	2.5 ± 0.17	0.281
Tegmentum						
Dorsal raphe nuclei (DRN)	−7.64 to −8.72	1.45 ± 0.26	3.75 ± 0.54*	0.20 ± 0.20*	0.4 ± 0.16*	0.000
Pontine nuclei (Pn)	−6.72 to −7.30	5.8 ± 0.50	3.75 ± 0.84	6.2 ± 2.19	6.55 ± 0.17	0.104

Group means were tested using the non-parametric analysis of variance Kruskal–Wallis test (H-test). Individual group differences were further tested using the Mann–Whitney U-test. Abbreviations: L, left; R, right; aud, auditory; ant, anterior; Nuc, nucleus; all others are mentioned in brackets. For the exact location of each of the densitometric sites, see Fig. 2.

* $p<0.05$ relative to arena-only group.

# $p<0.05$ between 22-kHz and 50-kHz playback groups.

central amygdalar nuclei have been implicated in fear conditioning, central amygdalar lesions specifically appear to block production of 22-kHz USVs and freezing [11]. Another cortical structure implicated in 22-kHz perception [1,22] is the perirhinal cortex, which was also activated here. This multimodal cortex has reciprocal connections with the amygdala [22], and the fact that some neurons respond with a different firing pattern to USVs than to continuous control tones, indicates that neurons in the perirhinal cortex respond to complex 22-kHz USVs [1,22].

The periaqueductal gray, which was activated to varied extents in all four groups, seems to play a central role in coordination of different subsystems required to produce emotional vocalizations [21]. While the lateral sub-division is said to play

an important role in defensive responses and in the production of USVs, the ventro-lateral sub-division, which showed fos expression only in the 22-kHz group, is said to be important for submission, but has no known role in the emission of USVs. In previous work [2], c-fos expression was more pronounced than what is observed here, which could be due to differences in signal presentation, or the type of antibodies used there.

50-kHz USVs are elevated by food rewards, sexual behaviour, rough-and-tumble play, experimenter-induced "tickling", drugs of abuse, and anticipation of rewarding electrical brain stimulation [24,7,32,29]. This led to the hypothesis that 50-kHz calls index positive affective states associated with specific brain sites, including ventral striatum and pallidum [20,8]. Interestingly, the 50-kHz group was the only one which demonstrated sparse to moderate fos expression in the ventral striatum, ventral pallidum, medial forebrain bundle and in the parafascicular thalamic nucleus. While the latter has been specifically implicated in juvenile play in rats [30], other brain areas, such as the inferior colliculus, dorsal periaqueductal gray, ventromedial hypothalamus, ventral striatum activated here also demonstrate enhanced c-fos mRNA during play behaviour in juvenile rats [17], a situation during which the rate of 50-kHz calls is increased [19,6].

Taken together, this study demonstrates differential early gene expression in diverse brain areas in response to playback of 22- and 50-kHz vocalizations. Some of these activations may index negative and positive affective states elicited by these different vocalizations, while others may indicate stimulus-specific processing, though it is clear that more studies are required to completely unravel the brain circuitries that underlie responses to conspecific calling in rodents.

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