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Inaudible airborne ultrasound affects emotional states in the olfactory bulbectomized rat depression model

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Abstract

Recently, exposure to sounds including ultrasound (US) components has been shown to modulate brain activity. However, the detailed US effects on emotional states remain unclear. We previously demonstrated that olfactory bulbectomized (OBX) rats, a widely used depression model are suitable for examining the effects of high-frequency audible sound on emotionality. Here we investigated the impact of inaudible US exposure on the emotional state of OBX rats. Exposure to 100 kHz US for one h did not affect the number of c-Fos-positive cells in auditory-related cortical areas, suggesting that this frequency is inaudible to rats. However, 12- and 24-h exposures to 100 kHz US improved hyperemotionality (HE) scores in OBX rats accompanied by a decrease in the plasma corticosterone levels, suggesting ameliorative effects on depression-like symptoms and stress. In contrast to HE scores, US exposure did not influence anxiety-like behaviors in the elevated plus maze. In conclusion, we demonstrated that exposure to 100 kHz US could alleviate depressive-like symptom in the OBX rat depression model. This is the first study to show that airborne US alone exposure produced the changes in emotional states in animal model. Future research is necessary to elucidate the underlying mechanisms and determine the optimal treatment regimens.

1. Introduction

The effects of airborne ultrasound (US) on brain function have garnered substantial interest since Oohashi and colleagues first reported that nonstationary sounds containing a wealth of inaudible high-frequency components above the human audible range evoke various physiological, psychological, and behavioral responses (hypersonic effect; Oohashi 2000)[1]. The sound with high-frequency components was subjectively preferred over sounds without these components, enhanced occipital alpha-EEG signals, and activated deep-lying brain structures including reward-related neural circuitry. Kuribayashi et al.[2] also showed that subsequently provided further evidence for the impact of inaudible high-frequency components on human brain activity. Using a double-blind study design, these authors found that excerpts from J. S. Bach's French Suite No. 5 with inaudible high-frequency components induced greater high-frequency α-EEG power (10.5–13 Hz) than excerpts without these components, indicating change in brain activity induced by these inaudible components.

Despite several studies show airborne US could affect brain activity non-invasively, the detailed effect of US exposure on emotional states are still unclear. Further, whether audible components are necessary, or US alone could affect brain activity has not been sufficiently investigated. Ascone et al.[3] exposed participants to 22.4 kHz US alone or to sham stimuli for 28 nights in the home bedroom and assessed subjective, behavioral change and magnetic resonance imaging (MRI). In the study, they found differences regional grey matter volume in exposure groups compared to sham group by MRI study. However, participants in the sham group also self-reported somatic changes, suggesting a nocebo effect. This indicates that experiments on the effect of US exposure alone on emotional states in human subjects may be significantly influenced by placebo or nocebo effects. Therefore, it is crucial to

investigate the effects of US alone under well-controlled laboratory conditions using objective and quantitative methods that eliminate the impacts of these confounding factors.

We recently reported that a 24-h exposure to high-frequency ultrasonic vocalizations (50 kHz USVs) or artificial 50 kHz US reduced hyperemotionality in the olfactory bulbectomized (OBX) rat depression model, suggesting that this animal model is an appropriate for investigating the psychological effects of high-frequency sound[4]. However, since USVs are audible to rats, the observed emotional changes may have resulted from listening to the speaker's USVs or sounds of similar frequency. In the current study, we investigated the effects of inaudible US exposure on emotional states using OBX rat depression model in order to clarify whether the exposure US alone could affect emotional states. Animal models like the OBX rat may allow for a clear demonstration of US exposure effects on emotional state without associated placebo or nocebo effects. If the OBX rat could also be used to examine the effects of inaudible US exposure, it could provide a novel method for studying US exposure.

2. Results

2.1 Effects of US on c-Fos positive cells in the auditory cortex

We first confirmed that the US used in this study was inaudible for rats by immunostaining for c-Fos, a marker of neuronal activity, following exposure to audible white noise (WN; Fig. 1a, b) or inaudible 100 kHz US (Fig. 1c, d). The typical immunostained image is shown in Fig. 2a, b, c. The numbers of c-Fos-immunopositive cells were significantly greater in the WN group compared to the no sound control group in the primary auditory area(Au1) ($F_{(2, 8)}$ = 29.1, P < 0.001 by one-way ANOVA and post hoc Holm–Sidack test; Fig. 2d), in dorsal secondary auditory area (AuD) ($F_{(2, 8)}$ = 7.00, P = 0.0199 by one-way ANOVA and post hoc Holm–Sidack test; Fig. 2e), and ventral secondary auditory area (AuV) ($F_{(2, 8)}$ = 7.19, P = 0.0116 by one-way ANOVA and post hoc Holm–Sidack test; Fig. 2f) but did not differ between no sound and US groups in Au1(P = 0.122 by post hoc Holm–Sidack test), AuD (P = 0.9321 by post hoc Holm–Sidack test), AuV (P = 0.304 by post hoc Holm–Sidack test) suggesting that 100 kHz US was inaudible to rats. Additionally, no significant lateralization in the expression levels of c-Fos was observed in Au1, AuD, and AuV (data not shown). These results suggest that rats cannot perceive 100 kHz US in the auditory system. Therefore, 100 kHz US was used as an inaudible sound stimulus for subsequent behavioral assessments.

2.2 Effects of 100 kHz ultrasound exposure on hyperemotionality in OBX rats

OBX rats were exposed to 100 kHz US or no sound in a soundproof box and hyperemotionality was assessed before and after US exposure. The total mean hyperemotionality scores of OBX rats did not differ from baseline after 12 and 24 h in the soundproof box without delivered sound stimuli (Wilcoxon signed rank test: P > 0.05; Fig. 3a, b). In contrast, mean hyperemotionality scores of OBX rats were

significantly lower compared to baseline following 12 and 24 h of exposure to 100 kHz US (Wilcoxon signed rank test: $P = 0.001$; Fig. 3c, P = 0.0195; Fig. 3d, respectively). However, no significant changes in hyperemotionality scores were observed after 48 h of exposure ($P = 0.0781$; Fig. 3e).

2.3 Effects of 100 kHz ultrasound exposure in Elevated Plus Maze (EPM) tests

Given that 12 and 24 h of 100 kHz US exposure suppressed hyperemotionality in OBX rats, we performed EPM tests to evaluate effects on anxiety-like behavior. The time spent in the open arms was significantly lower in OBX rats compared to sham-operated rats after the 12-h no sound intervention ($F_{(2, 17)} = 5.21$, $P = 0.0255$ by one-way ANOVA and post hoc Holm–Sidak test; Fig. 4a) and the 24-h no sound intervention ($F_{(2, 29)} = 3.63$, $P = 0.0483$ by one-way ANOVA and post hoc Holm–Sidak test; Fig. 4b). Anxiety-like behavior, as indicated by the decreased time spent in the open arms, was not alleviated by 100 kHz US exposure for 12 h compared to the no sound group ($P = 0.754$ by post hoc Holm–Sidak test), indicating greater basal anxiety (Fig. 4a). Similarly, anxiety was not alleviated by 24 h of US compared to the no sound group ($P = 0.562$ by Holm–Sidak post hoc test) (Fig. 4b). In addition, there were no significant differences in total distance traveled among groups after either the 12-h intervention ($F_{(2, 17)} = 1.45$, $P > 0.05$; Fig. 4c) or the 24-h intervention ($F_{(2, 29)} = 1.07$, $P > 0.05$ by one-way ANOVA followed by post hoc Holm–Sidak test; Fig. 4d). These results suggest that while 100 kHz US exposure can reduce hyperemotionality, it does not appear to mitigate anxiety-like behaviors in OBX rats as measured by the EPM test.

2.4 Effect of ultrasound exposure on blood corticosterone levels

Trunk blood samples were collected immediately after hyperemotionality evaluation in groups exposed to 100 kHz US for 12, 24, and 48 h. Corticosterone concentration was significantly lower in all 100 kHz US-exposed groups compared to the no sound control group ($F_{(3, 27)} = 3.614$, P = 0.0328, 0.0328, 0.0226, respectively, by one-way ANOVA and post hoc Holm–Sidak tests, Fig. 5). These findings suggest that 100 kHz US exposure can relieve physiological stress level in OBX rats.

3. Discussion

Exposure to 100 kHz US significantly reduced hyperemotionality in OBX rats (Fig. 3) despite the absence of auditory-related cortex activation (as indicated by the absence of greater c-Fos immunopositive cell numbers). While exposure to 100 kHz US did not impact anxiety-like behavior as measured in the EPM test (Fig. 4), plasma corticosterone levels were significantly reduced following 100 kHz US exposure (Fig. 5). These results demonstrating that inaudible US can modulate the emotional state of OBX rats suggest therapeutic potential against mood disorders.

It is generally accepted that rats cannot perceive elastic vibrations above 80 kHz. as sound. In this study, there were no change in c-Fos immunopositive cell numbers in bilateral Au1, AuD, and AuV, indicating

that rats could not perceive 100 kHz US within the known auditory system. It was previously reported that inaudible high-frequency sounds (> 20 kHz) can influence human brain activity, even though humans cannot perceive a frequency range above 20 kHz. This effect occurs only when sounds are presented through speakers and reach to a large area of the body surface area, suggesting the involvement of other (nonauditory) sensory systems[1,5]. Although the sound used in this study was a specific frequency sound without audible components, it appeared to modulate emotional state in accord with previous human experiments. To the best of our knowledge, this is the first study to demonstrate that airborne US beyond the audible range can produce an ameliorating effect in a rat model of depression. For more reliability, future studies are warranted to examine the effects of this inaudible US exposure on other depression models and depressive-like symptoms such as anhedonia, hypoactivity, and learned helplessness.

Hyperemotionality was reduced by both 12 and 24 h of 100 kHz US exposure, but not by 48 h. The US exposure was conducted in a soundproof box to control sound from environment. It is possible that this prolonged duration in a soundproof box induced environmental stress and thereby counteracted the effects of US exposure. Although the hyperemotionality in OB rats were significantly decreased following 12 and 24 h of exposure, there were no significant effects on anxiety-like behavior in the EPM test. The EPM test assesses innate anxiety, while hyperemotionality responses assess irritability to external stimuli as manifested by attack, startle, and struggle responses[5]. While the underlying neural circuits for these behaviors are not well understood, our findings suggest that US ameliorates hyperemotionality more effectively than innate anxiety-like behaviors. We suggested that the methods or components of US effective for depression may differ from those targeting anxiety. Further studies are necessary to understand the specific effects and limitations of 100 kHz US exposure on depression and anxiety, as well as the underlying mechanisms driving these differential effects.

While the results of the behavioral assessments varied with exposure time, plasma corticosterone concentrations were significantly reduced in all exposure groups. Kawai and colleagues[6] reported that human whole-body exposure to sounds with inaudible high-frequency components above 20 kHz (HFC), which induces hypersonic effects, suppressed the increase in blood glucose levels in the oral glucose tolerance test among healthy humans. Exposure to HFC sounds activates deep brain regions such as the midbrain, thalamus, and hypothalamus[1]. Further, it is well experienced that stress hormones have a significant impact on glycemic control. Thus, Kawai et al. suggested that the observed suppression of glucose elevation was due to a reduction in stress hormones induced by deep brain activation. They also suggested that sounds rich in inaudible US components acting on the hypothalamus and brainstem may have physiological effects that lower stress hormones rather than primarily reducing subjective stress. Therefore, we speculate that exposure to 100 kHz US may have reduced hyperemotionality in OBX rats via deep brain stimulation and ensuing suppression of corticosterone levels. However, these behavioral changes did not occur in the 48-h exposure group despite similarly reduced plasma corticosterone, again suggesting that the physiological and emotional changes following US exposure are mediated through distinct mechanisms.

In this work, we tested the effects of inaudible US exposure on emotional state using the OBX rat depression model. The World Health Organization estimates that approximately 322 million people currently suffer from depression, and available treatments, including antidepressants and cognitive behavioral therapy (CBT), have limited efficacy in a subpopulation of patients. For instance, monoamine antidepressants do not lead to remission in approximately 30% of patients and significant clinical effects usually require several weeks even among responsive patients[7,8]. Additionally, a substantial proportion of patients receiving CBT do not achieve remission and the drop-out rate is relatively high[9,10]. To overcome these challenges, it is necessary to develop novel treatments for major depressive disorder. Recently, Ito and colleagues investigated potential applications of hypersonic effect in the treatment of major depressive disorders[11]. Similar to hypersonic effect, investigating whether US exposure affects depressive-like behavior in OBX rat might potentially lead to the development of new method of depression treatment.

Our study provides evidence that exposure to inaudible 100 kHz US can alleviate hyperemotionality and reduce plasma corticosterone concentrations in the OBX rat depression model, suggesting that US exposure alone can modulate emotional state. Moreover, we suggest that the OBX rat appears suitable for further investigations on the therapeutic efficacy of US against specific depressive traits. If inaudible US could alleviate depressive symptom, it may be useful for treating depression in various environments. Such studies could lead to the development of alternative noninvasive treatments for depression.

4. Materials and methods

4.1 Animals

Male Wistar/ST rats (7–9 weeks old) were purchased from Sankyo Labo Service Corporation (Tokyo, Japan) and housed under controlled temperature (23°C ± 1°C), relative humidity (55% ± 5%), and 12-h/12-h light/dark cycle (lights on at 8:00 a.m.) with food and water *ad libitum*. All animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee at the Tokyo University of Science (Approval No. Y22014) and conducted in compliance with National Institutes of Health and Japan Neuroscience Society guidelines. The study design and all procedures followed the ARRIVE Guidelines.

4.2 Sound exposure for immunohistochemical assessment

Artificial 100-kHz US (30 ms pulses at 60 ms intervals) and 35–100 kHz WN were produced as intervention and positive control stimuli using SASLab Pro software 5.2.09 (Avisoft Bioacoustics, Glienicke/Nordbahn, Germany) (Fig. 1) and delivered using an UltraSoundGate Player 116 (Avisoft Bioacoustics). All rats used in this assessment were not bulbectomized operated. Rats were randomly divided into three groups (no sound, audible sound stimulation with white noise, and inaudible sound stimulation with 100 kHz US) and placed in cages individually. Before sound exposure, all rats were

habituated silently for at least 1 h without sound, then exposed to no sound, WN, or 100 kHz US for 1 h according to group designation.

4.3 Immunohistochemistry

Immediately after the sound exposure or equivalent no sound period, all rats were anesthetized by intraperitoneal injection of a medetomidine (2.4 mg/kg), midazolam (0.45 mg/kg), and butorphanol (3.0 mg/kg) mixture dissolved in 0.9% saline. Rats were then perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.2M phosphate buffer (PB) (PFA; Sigma-Aldrich, Tokyo, Japan). Brains were excised, post fixed overnight in 4% PFA, immersed in 30% sucrose dissolved in 0.2M PB for cryoprotection, embedded in an Optimal Cutting Temperature compound (Sakura-Finetek, Tokyo, Japan), frozen at − 80˚C, and sliced at 40-μm thickness. Brain sections were stored at − 20˚C in cryoprotection solution (30% ethylene glycol, 25% glycerol in 1 × PBS).

For c-Fos immunostaining, six sections per animal − 3.14−−5.60 mm from the bregma were incubated with 0.3% hydrogen peroxide in 40% methanol/PBS for 5 min to quench endogenous peroxidase activity, washed three times with PBS plus Triton X (PBST), blocked with 3% bovine serum albumin (BSA, Jackson ImmunoResearch, West Grove, Pennsylvania, USA) in PBST for 1 h, and then incubated with c-Fos antibody (anti-c-Fos (C-10), SCB Santa Cruz Biotechnology, Dallas, TX, USA) diluted 1:1000 in blocking solution for overnight. After washing with PBST, sections were incubated with a biotinylated horse antimouse antibody (BA-2000; Vector Laboratories, Newark, CA, USA) diluted in a blocking solution for 90 min, washed three times with PBST, incubated with AB solution (Vector Laboratories) for 60 min, rewashed three times with PBST, incubated with DAB (Vector Laboratories) for six min, washed three times with PBST, mounted on glass slides, and mounted using Eukitt (ORSAtec GmbH, Bobingen, Germany). Digital images were obtained using NDP viewer (HAMAMATSU PHOTONICS, Shizuoka, Japan) and processed using Fiji software by investigators blinded to the animal group. Briefly, after converting the images to 8 bits, noise was reduced using the Subtract Background tool. The regions of interest (ROIs) within auditory cortex were set according to the Paxinos and Watson atlas (fourth edition). The number of c-Fos-positive cells within each ROI was counted using the Analyze Particles tool. The number of c-Fos per unit area was determined by dividing the number of positive cells by the total ROI area of the ROI for each section, and averaged for each rat.

4.4 Surgical procedure of olfactory bulbectomy

Olfactory bulbectomy was performed as previously described[4,12]. Briefly, rats were anesthetized using a mixed solution of medetomidine (2.4 mg/kg), midazolam (0.45 mg/kg), and butorphanol (3.0 mg/kg) dissolved in 0.9% saline and fixed in a stereotactic apparatus. The skull covering the olfactory bulbs was exposed, and burr holes were drilled 7.0 mm anterior to the bregma and ± 1.8 mm lateral to the midline. Both olfactory bulbs were removed by aspiration. Blood loss was prevented by filling the burr holes with hemostatic sponges (Spongel, Astells Pharma Inc., Tokyo, Japan). All animals received antibiotics and analgesics on the day of surgery. Postoperatively, animals were housed in single cages (W: 15 × D: 20 × H: 16 cm) for two weeks. Upon completion of the behavioral experiments, OBX rats were decapitated

and the accuracy of bulbectomy was visually verified. Data obtained from animals with incomplete removal of the olfactory bulbs or incidental frontal cortex damage were discarded. The sham operations were performed similarly, but the olfactory bulbs were left intact.

4.5 Ultrasound exposure for behavioral assessment

For the behavioral assessment, OBX rats were exposed to 100 kHz US (Fig. 1c, d) produced using SASLab Pro software 5.2.09 (Avisoft Bioacoustics) or to no sound as a control. The cage containing an individual rat was placed in a soundproof box (20 × 20 × 33 cm, LabDesign, Ibaraki, Japan; at 23°C and 10 Lux) and exposed to US generated by an UltraSoundGate Player 116 (Avisoft Bioacoustics) or placed in the same soundproof box without US exposure for 12, 24, or 48 h.

4.6 Evaluation of olfactory bulbectomy-induced hyperemotionality (HE)

The hyperemotionality of OBX rats at baseline and following the indicated intervention was evaluated using a hyperemotionality score[4,12,13] derived from the following behaviors: (1) attack response to a rod held in front of the snout, (2) startle response to air blown on the snout, (3) struggle response to handling with a gloved hand, and (4) fight response to a tail pitch delivered with mosquito forceps. Each response was graded as 0 (no reaction), 1 (slight), 2 (moderate), 3 (marked), or 4 (extreme). Vocalizations during the test were also scored as 0 (no vocalization), 1 (occasional), or 2 (marked), and the vocal score was added to the respective emotional response score. The total emotional response score was calculated as the sum of these scores. The maximum score of emotional responses was set to 24 in total. We conducted the experiment across multiple Lots to verify reproducibility.

4.7 Elevated Plus Maze test

The EPM test was conducted following 12 or 24 h in the aforementioned soundproof box with or without US exposure and 1 h of habituation. Tests were conducted as described in our previous study[4,14]. Briefly, the EPM apparatus was constructed of plastic and consisted of four 10 cm wide arms projecting in a cross pattern from a neutral central square and elevated 50 cm above the floor. Two of the opposing arms were enclosed by vertical walls (closed arms) while the other two had unprotected edges (open arms). The entire maze was placed under indirect light (50 Lux). At the beginning of the 5-min test session, each rat was positioned in the central neutral zone facing one of the closed arms. The total distance traveled and time spent on the open arms was then recorded using a video camera system and analyzed using Smart 3.0 (Harvard Apparatus, Holliston, MA, USA). The experiment was conducted across multiple sessions to verify reproducibility.

4.8 Corticosterone assay

Immediately after the HE evaluation, trunk blood was collected into heparin-containing tubes and centrifuged for 15 min at 15,000 rpm and 4°C to isolate the plasma fraction. Plasma samples were then aliquoted and stored at − 30°C until analysis of corticosterone levels. Concentrations were measured in

duplicate using a corticosterone enzyme-linked immunosorbent assay kit (Cayman Chemical, Ann Arbor, MI, USA) following the manufacturer's instructions.

4.9 Data analysis

All data are presented as the mean ± standard error of the mean (S.E.M.). Results were compared among groups using the Wilcoxon signed rank test or one-way ANOVA followed by post hoc Holm–Sidak tests as indicated. The threshold for statistical significance was set at $p < 0.05$ (corrected for multiple comparisons). In figures, levels of significance are indicated as follows: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. All statistical analyses were performed using GraphPad Prism7 (GraphPad Software, Inc., San Diego, CA, USA).

Declarations

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Author contributions

S.Miyazaki, S.Irriyama, K.Yoshizawa and A.Saitoh conceptualized the study. All authors contribute to design the experiments. T.Yamauchi and K.Takahashi acquired and analyzed the data. S.Kasai and Y.Nakano aided in interpreting the results.S.Nishino contributed funding acquisition. T.Yamauchi, K.Takahashi T.Yoshioka, D.Yamada and A.Saitoh wrote the paper, which was critically reviewed by all authors. All authors read and agreed to the published version of the manuscript and contributed significantly to the study.

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Competing interests

There are no conflicts of interest to declare.

Data availability statement

The raw data supporting the conclusions of this study are available from the corresponding author on reasonable request.

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Figures

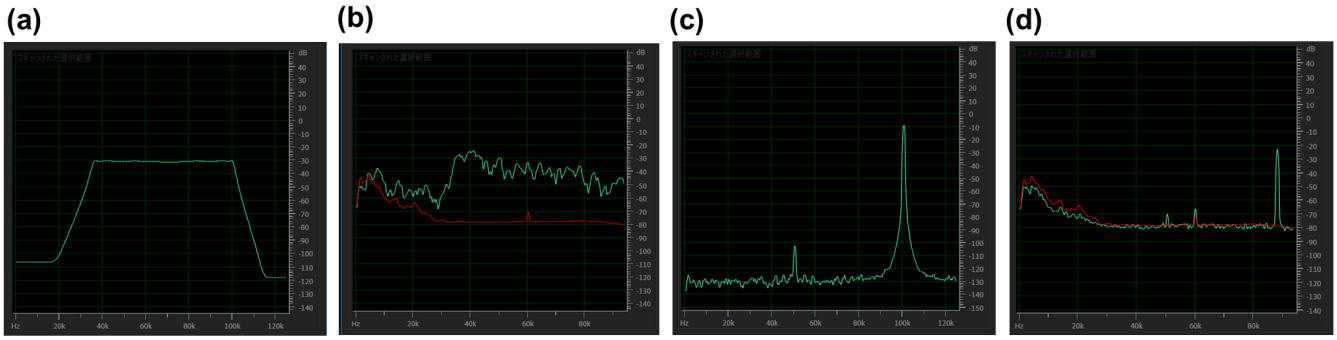


Figure 1

Power spectra of the ultrasound (US) and white noise (WN) delivered to experimental rats.

(**a**, **c**) Power spectra of the WN (**a**) and 100 kHz US (**c**) sound source signals generated using Adobe Audition (Adobe Inc. USA). (**b**, **d**) The power spectra of WN (**b**) and 100 kHz US (**d**) recorded inside the soundproof box (green line). The red line represents the recorded sound inside the soundproof box with no sound played (baseline)

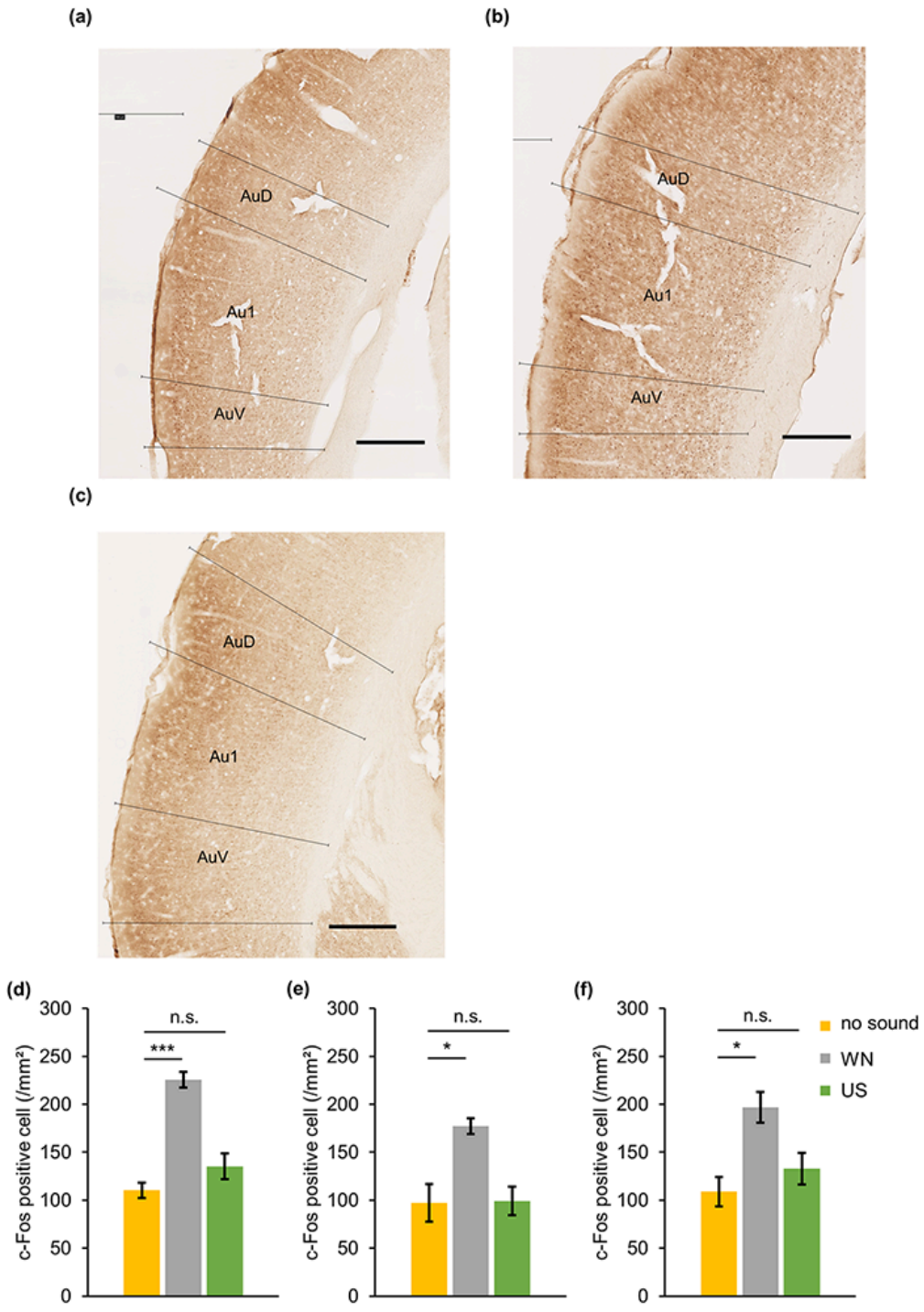


Figure 2

Exposure to white noise (WN) increases the number of activated (c-Fos immunopositive) neurons in auditory-related cortex but not 100 kHz ultrasound (US).

(**a**, **b**, **c**) Representative photomicrographs of c-Fos immunoreactivity in response to (**a**) no sound, (**b**) WN, and (**c**) 100 kHz US for 1 h. Scale bar = 0.5 mm. (**d**, **e**, **f**) Number of c-Fos immunopositive cells in (**d**)

Au1, (**e**) AuD, (**f**) AuV. All results are presented as mean ± S.E.M. (n = 4 for no sound group, n = 3 for WN group, n = 4 for US group). *P < 0.05 and ***P < 0.001 by one-way ANOVA with post hoc Holm–Sidak multiple comparison tests

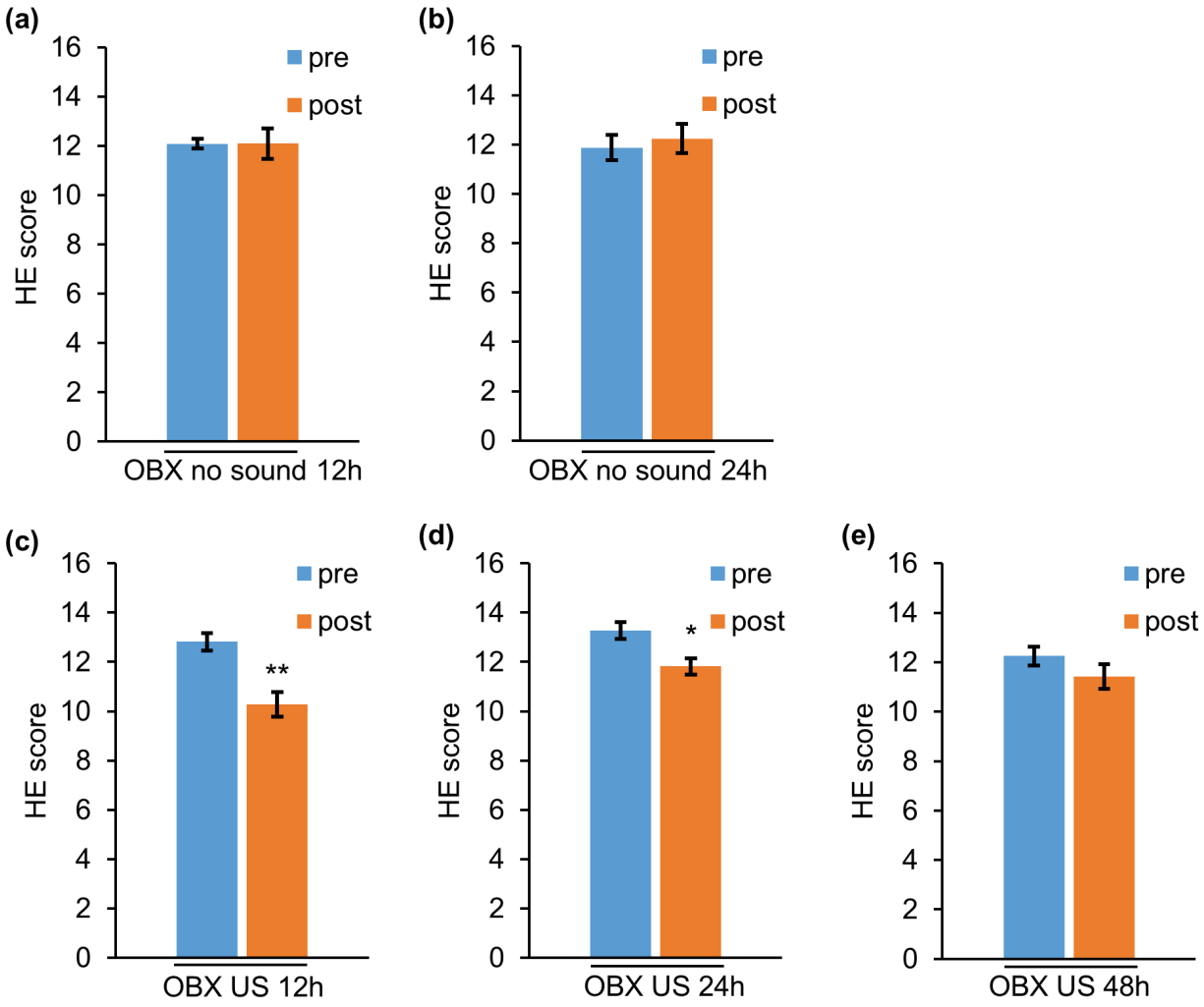


Figure 3

Exposure to 100 kHz US reduces hyperemotionality in olfactory bulbectomized (OBX) rats.

Hyperemotionality (HE) scores were measured before (blue) and after (orange) ultrasound or no sound (control) exposure. (**a-e**) HE scores in the (**a**) no sound 12 h group (n = 12), (**b**) no sound 24 h group (n = 8), (**c**) US 12 h group (n = 11), (**d**) US 24 h group (n = 11), and (**e**) US 48 h group (n = 12). All results are expressed as mean ± S.E.M. *P < 0.05 and **P < 0.01 by Wilcoxon matched-pairs signed rank test

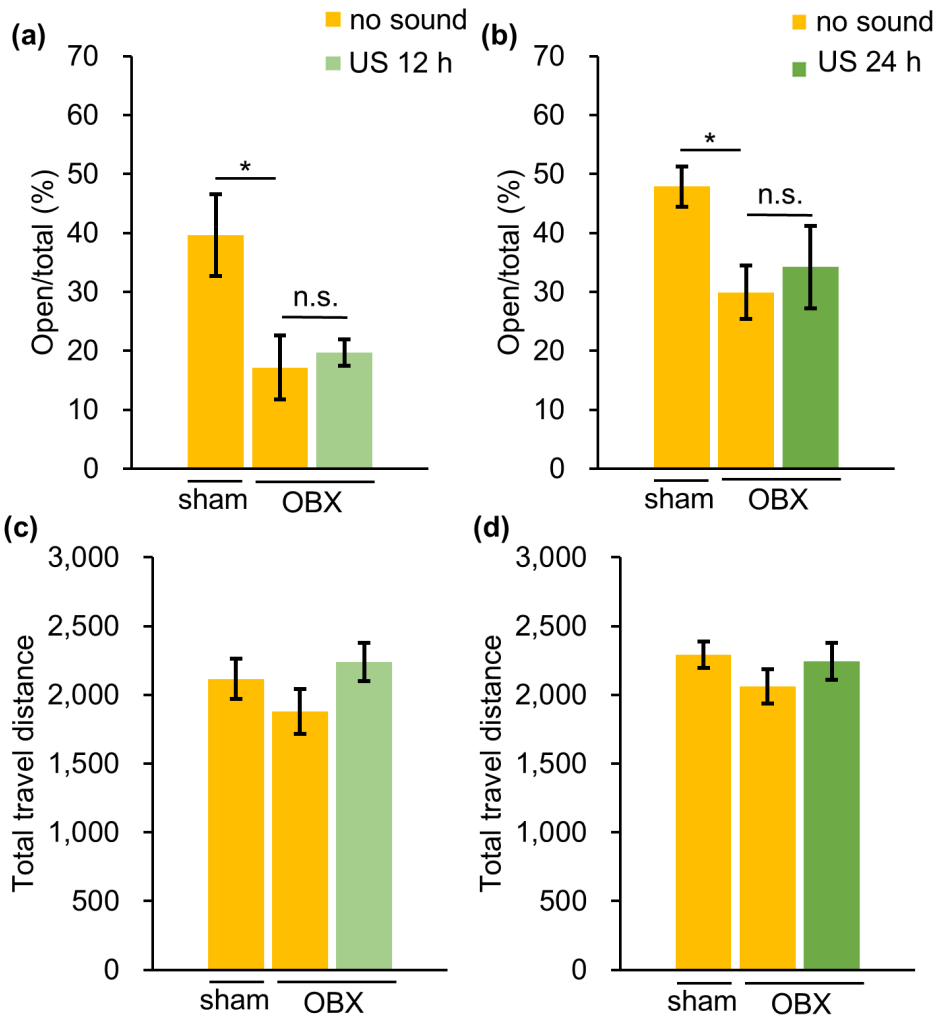


Figure 4

Exposure to 100 kHz US has no effect on anxiety as measured by the elevated plus maze (EPM) test.

(**a**, **b**) Proportion of time spent in the open arms by sham controls and OBX rats during the EPM test conducted after 12 h (**a**) or 24 h (**b**) in a soundproof box with no sound (yellow) or US (green). (**c**, **d**) Total distance traveled during these same EPM tests. Group sizes for the 12 h (**c**) exposure: sham rats (n = 7), OBX rats with no sound (n = 7), and OBX rats 100 kHz US (n = 6). Group sizes for the 24 h (**d**) exposure: sham rats (n = 12), OBX rats with no sound (n = 10), and OBX rats 100 kHz US (n = 10). All data are presented as mean ± S.E.M. *P < 0.05 by one-way ANOVA with post hoc Holm–Sidak multiple comparison tests

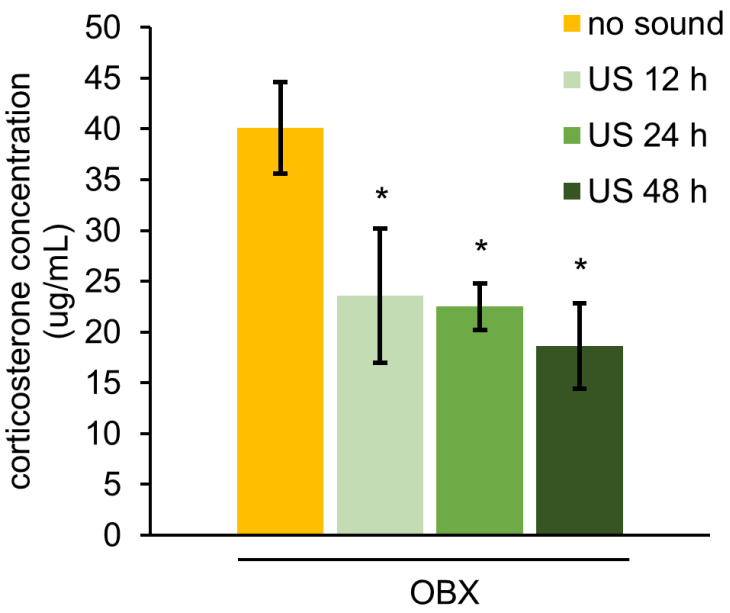


Figure 5

Exposure to 100 kHz US reduces plasma corticosterone concentrations in OBX rats.

Trunk blood was collected immediately after the hyperemotionality evaluation. Group sizes were as follows: no sound group (n = 8), 12-h group (n = 9), 24-h group (n = 8), and 48-h group (n = 6). Data are represented as mean ± S.E.M. *P < 0.05 by one-way ANOVA with post hoc Holm–Sidak multiple comparison tests