



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

Tsugumi Yamauchi<sup>1,7</sup>, Kou Takahashi<sup>2,7</sup>, Toshinori Yoshioka<sup>1</sup>, Daisuke Yamada<sup>1</sup>, Yoshio Nakano<sup>3</sup>, Satoka Kasai<sup>4</sup>, Satoshi Iriyama<sup>5</sup>, Kazumi Yoshizawa<sup>4</sup>, Shoichi Nishino<sup>6</sup>, Satoru Miyazaki<sup>3</sup> & Akiyoshi Saitoh<sup>1</sup>✉

Recently, exposure to sounds with ultrasound (US) components has been shown to modulate brain activity. However, the effects of US on emotional states remain poorly understood. We previously demonstrated that the olfactory bulbectomized (OBX) rat depression model is suitable for examining the effects of audible sounds on emotionality. Here, we investigated the impact of US exposure on the emotional state of OBX rats. In naive rats, exposure to 100 kHz US for 1 h did not increase the number of c-Fos-positive cells in auditory-related cortical areas, and US, as a tone cue, did not elicit a conditioned fear response in the auditory fear conditioning test. These results indicate that the frequency of 100 kHz is hard to hear for rats. However, US improved hyperemotionality (HE) scores and decreased plasma corticosterone levels in OBX rats, 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 airborne US can alleviate depressive-like symptoms in the OBX rat depression model. This is the first study to show that exposure to airborne US alone produces changes in emotional states in an animal model.

**Keywords** Ultrasound, Olfactory bulbectomized rats, Depression, Anxiety, Antidepressant therapy

The effects of airborne ultrasound (US) on brain function have garnered substantial interest since Oohashi et al. in 2000 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 known as the hypersonic effect<sup>1</sup>. The sounds with high-frequency components were subjectively preferred over those without these high-frequency components, as they enhanced occipital alpha-EEG signals and activated deep brain structures, including reward-related neural circuitry. Kuribayashi et al.<sup>2</sup> also demonstrated the effect 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  $\alpha$ -EEG power (10.5–13 Hz) than excerpts without these components, indicating a change in brain activity induced by these inaudible components.

Despite several studies demonstrating that airborne US can non-invasively affect brain activity, the effect detail of US exposure on emotional states remain unclear. Furthermore, whether audible components are necessary or US alone can affect brain activity has not been sufficiently investigated. Ascone et al.<sup>3</sup> exposed participants to 22.4 kHz US alone or sham stimuli for 28 nights in their home bedrooms and assessed subjective and behavioral changes as well as structural magnetic resonance images. The study found differences in regional gray matter volume between the exposure and sham groups. However, participants in the sham group also self-reported somatic changes, suggesting a placebo effect. This indicates that experiments investigating the effects of

<sup>1</sup>Laboratory of Pharmacology, Department of Pharmacy, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Chiba, Japan. <sup>2</sup>Department of Neurobiology and Anatomy, Kochi Medical School, Kochi University, Kochi, Japan. <sup>3</sup>Laboratory of Bioinformatics, Department of Pharmacy, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Chiba, Japan. <sup>4</sup>Laboratory of Pharmacology and Therapeutics, Department of Pharmacy, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Chiba, Japan. <sup>5</sup>Laboratory of Quantum information dynamics, Department of Information Sciences, Faculty of Science and Technology, Tokyo University of Science, Chiba, Japan. <sup>6</sup>FUJIMIC, Inc., Tokyo, Japan. <sup>7</sup>Tsugumi Yamauchi and Kou Takahashi contributed equally. ✉email: akiyoshi\_saitoh@rs.tus.ac.jp

US exposure alone on emotionality in human participants may be substantially 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 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 appropriate for investigating the psychological effects of high-frequency sound<sup>4</sup>. However, since USVs and 50 kHz US 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 present study, we investigated the effects of inaudible US exposure on emotional states using the OBX rat depression model to clarify whether exposure to US alone could affect emotional states. The hyperemotionality (HE) score in OBX rats improves with chronic antidepressant treatment<sup>5–7</sup>, thus this model has high predictive validity. Therefore, the OBX rat is suitable for the purpose of this study, confirming the effectiveness of US exposure. Moreover, animal models, such as the OBX rats, may allow for a clear demonstration of US exposure effects on emotional states without associated placebo or nocebo effects. If the OBX rat model can also be used to examine the effects of inaudible US exposure, it may provide a novel method for studying US exposure.

## Results

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

We first confirmed that the US used in this study was inaudible to rats by c-Fos immunostaining, a marker of neuronal activity, following exposure to either audible white noise (WN) (Fig. 1a and c, and 1e) or inaudible 100 kHz US (Fig. 1b and d, and 1f). Representative immunostained images are shown in Fig. 2a–c. The number of c-Fos-immunopositive cells was greater in the WN group ( $n=3$ ) than in the no-sound control group ( $n=4$ ) in the primary auditory area (Au1) ( $P=0.0038$  via Kruskal–Wallis test;  $P=0.011$ , via post hoc Dunn's multiple comparisons test; Fig. 2d), dorsal secondary auditory area (AuD) ( $P=0.04$ ; post hoc  $P=0.0598$ ; Fig. 2e), and ventral secondary auditory area (AuV) ( $P=0.030$ ; post hoc  $P=0.036$ ; Fig. 2f); however, the number did not differ between the no-sound and US groups ( $n=4$ ) in Au1 ( $P=0.40$ ), AuD ( $P>0.99$ ), and AuV ( $P>0.99$ ), indicating 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).

### Behavioral response to 100 kHz US

A behavioral test was conducted to further validate that the ultrasound was inaudible. The experimental design is shown in Fig. 3a, b. During the conditioning session, naive rats were conditioned with foot shock paired with either no-sound ( $n=6$ ), WN ( $n=6$ ), or 100 kHz US ( $n=6$ ). Figure 3c shows that the group conditioned with no-sound exhibited an average freezing time of 8 s when exposed to WN during the test session. Statistical analyses revealed that the freezing time in the WN group was significantly higher than that in the no-sound group ( $F_{(2,15)}=8.62$ ,  $P=0.0032$ , via one-way ANOVA;  $P=0.015$  via post hoc Holm–Sidak test; Fig. 3c). However, there is no significant difference between the 100 kHz US group and the no-sound group ( $P=0.41$ ). The immunostaining and behavioral results indicate that the 100 kHz US used in this study is beyond the hearing range of rats.

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

We confirmed that the HE scores were sufficiently high, consistent with our previous studies<sup>4,8</sup>.

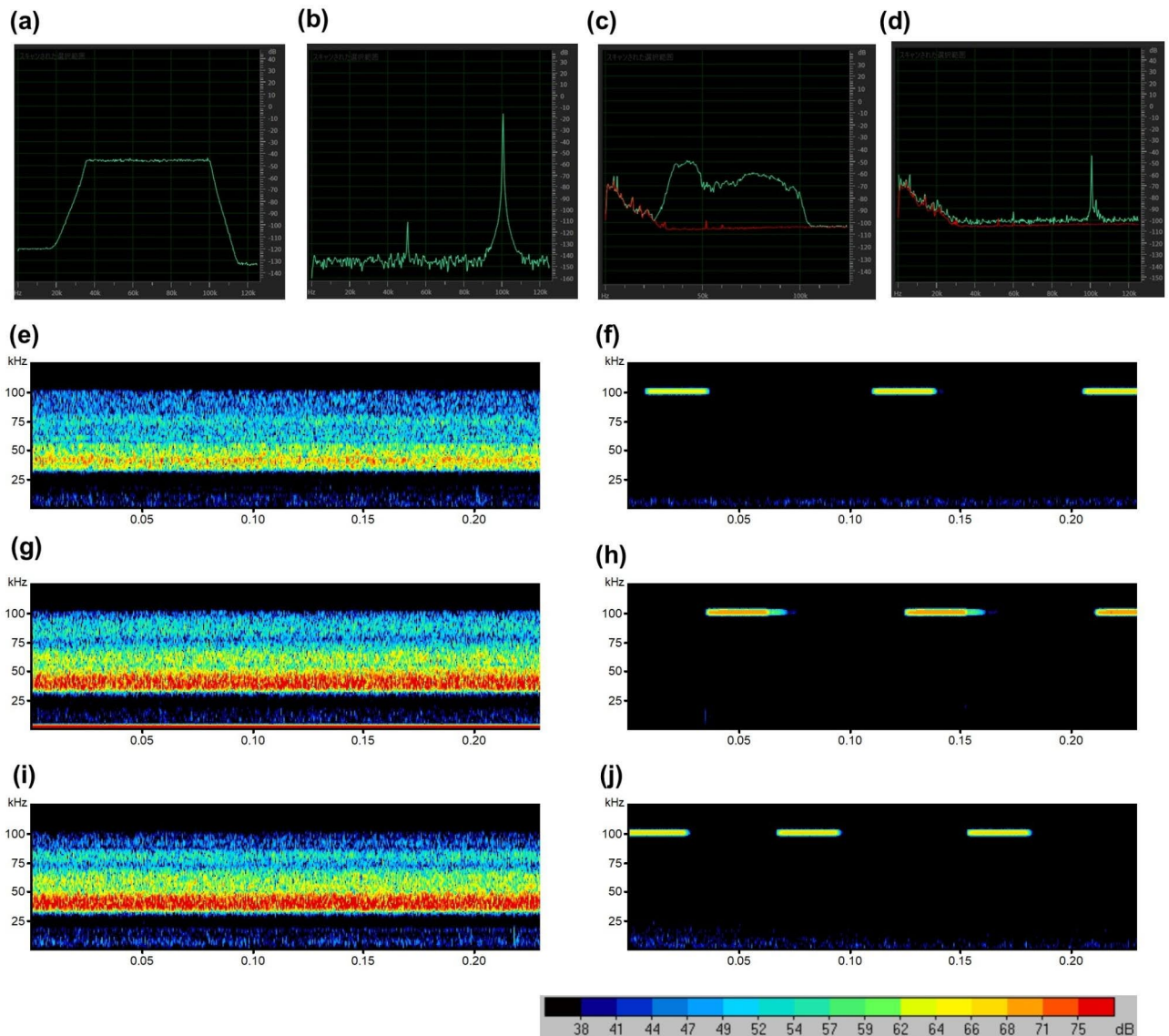
OBX rats were exposed to 100 kHz US (power spectra and spectrograms are shown in Fig. 1b and d, and 1f) and no-sound in a soundproof box and hyperemotionality was assessed before and after US exposure. The ratio of HE scores before and after exposure to ultrasound for 12 ( $n=11$ ) and 24 h ( $n=11$ ) was significantly lower than that of the group exposed to no-sound for the same duration (for 12 h,  $n=12$ ; for 24 h,  $n=8$ ;  $P=0.0018$ , 0.023 respectively, via Mann–Whitney U test; Fig. 4a and b). However, no significant changes in hyperemotionality scores were observed after 48 h of exposure ( $n=12$ ;  $P=0.078$  via Wilcoxon matched-pair signed-rank test; Fig. 4c).

### 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 the effects on anxiety-like behavior. The time spent in the open arms was significantly lower in OBX rats than sham-operated rats after the 12-h no-sound intervention (sham group,  $n=7$ ; OBX no-sound group,  $n=7$ ;  $F_{(2,17)}=5.21$ ,  $P=0.017$ , via one-way ANOVA;  $P=0.025$ , via post hoc Holm–Sidak test; Fig. 5a) and the 24-h no-sound intervention (sham group,  $n=12$ ; OBX no-sound group,  $n=10$ ;  $F_{(2,29)}=3.63$ ,  $P=0.039$ ; post hoc  $P=0.048$ ; Fig. 5b). The decrease in time spent in the open arms was not alleviated by 100 kHz US exposure for 12 h ( $n=6$ ) compared with the no-sound group ( $P=0.75$ ), indicating that anxiety levels in OBX rats, which were higher than those in sham rats, were not improved by exposure to ultrasound (Fig. 5a). Similarly, anxiety was not alleviated in the 24-h US group ( $n=10$ ) compared with the no-sound group ( $P=0.56$ ; Fig. 5b). Additionally, there were no significant differences in the total distance traveled among the groups after the 12-h intervention ( $F_{(2,17)}=1.45$ ,  $P=0.26$  and post hoc  $P>0.05$ ; Fig. 5c) and the 24-h intervention ( $F_{(2,29)}=1.07$ ,  $P=0.35$  and post hoc  $P>0.05$ ; Fig. 5d). 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 via the EPM test.

### Effect of ultrasound exposure on blood corticosterone levels

Trunk blood samples were collected immediately after hyperemotionality evaluation in the groups exposed to 100 kHz US for 12 ( $n=9$ ), 24 ( $n=8$ ), and 48 h ( $n=6$ ). The corticosterone concentration was significantly lower in all 100 kHz US groups compared with the no-sound control group ( $n=8$ ) ( $F_{(3,27)}=3.61$ ,  $P=0.026$ ; one-way



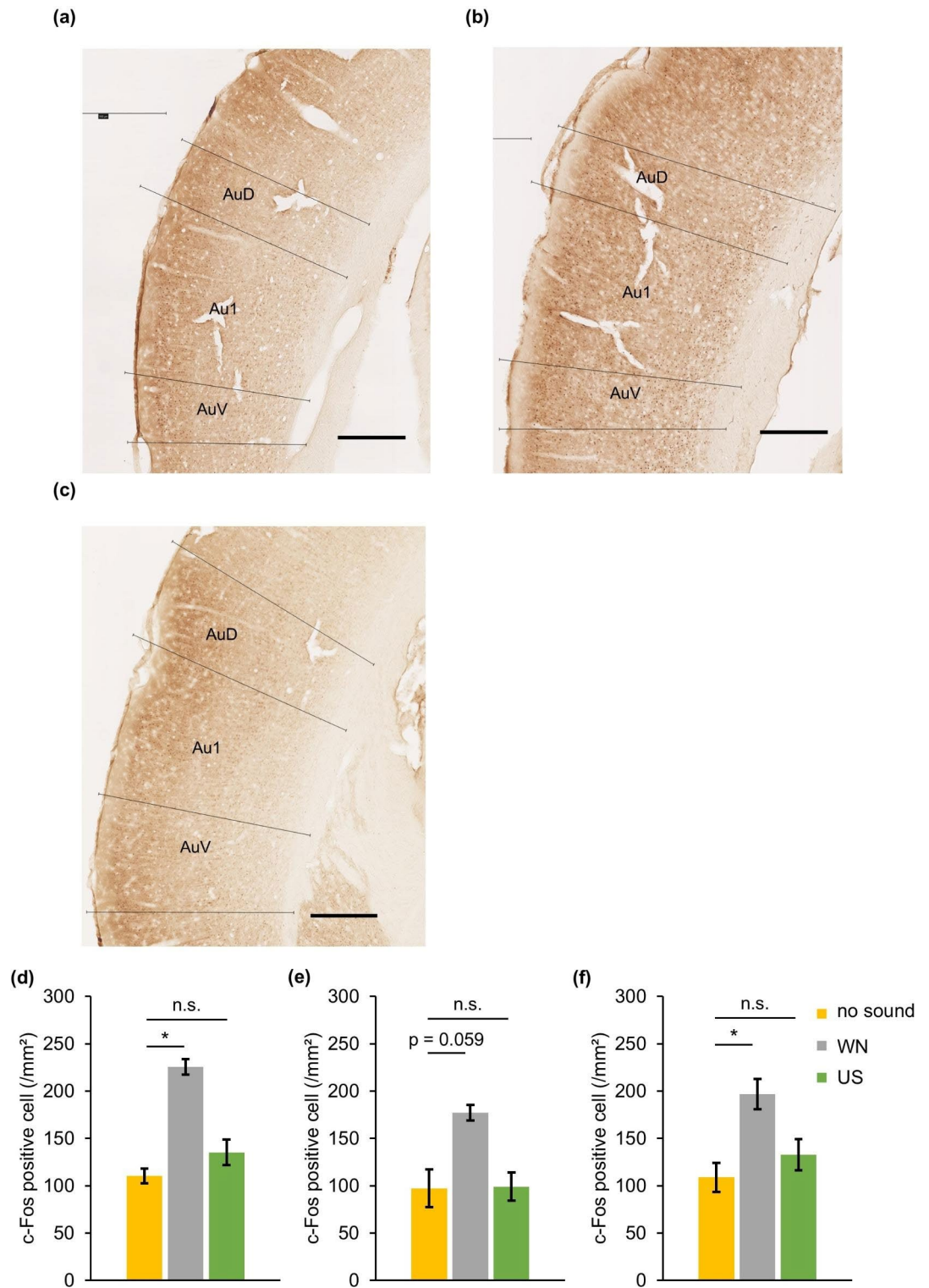
**Fig. 1.** Power spectra and spectrograms of the 100 kHz ultrasound (US) and white noise (WN) delivered to experimental rats. **(a and b)** Power spectra of the WN **(a)** and 100 kHz US **(b)** sound source signals generated using Adobe Audition (Adobe Inc. USA). **(c and d)** The power spectra of WN **(c)** 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). **(e and f)** The WN **(e)** and 100 kHz US **(f)** spectrogram in the experimental environment for immunohistochemical assay, HE evaluation, and EPM test. **(g, h)** The WN **(g)** and 100 kHz US **(h)** spectrogram in the conditioning session in tone fear conditioning test. **(i and j)** The WN **(i)** and 100 kHz US **(j)** spectrogram in the test session in tone fear conditioning test.

ANOVA;  $P=0.032, 0.032, 0.022$ , respectively, post hoc Holm–Sidak tests, Fig. 6). These findings suggest that 100 kHz US exposure can reduce physiological stress levels in OBX rats.

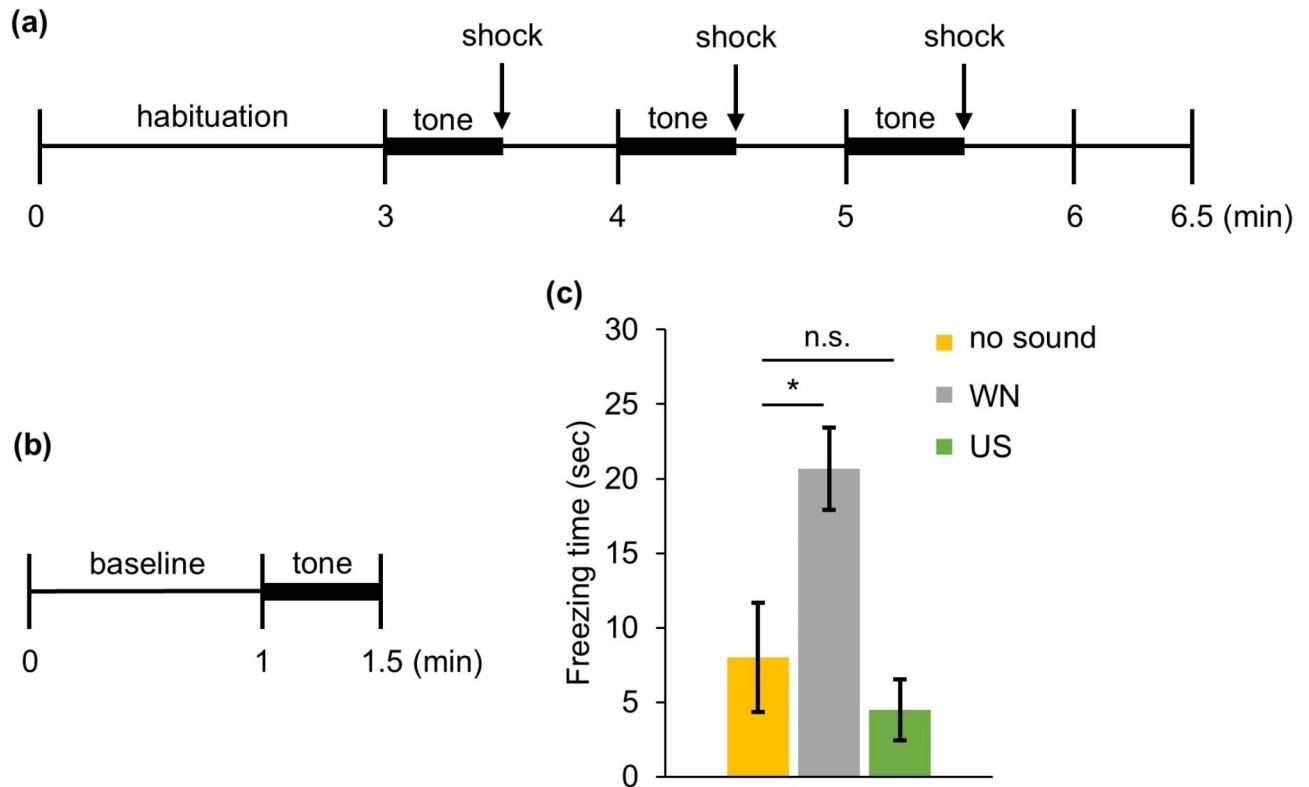
## Discussion

Exposure to 100 kHz US significantly reduced hyperemotionality in OBX rats, even though the US used in this study might be inaudible to rats, indicated by *c-Fos* immunopositive cell numbers and the auditory fear conditioning test. Although exposure to 100 kHz US did not impact anxiety-like behavior as measured in the EPM test, plasma corticosterone levels were significantly reduced following the 100 kHz US exposure. These results demonstrate that inaudible US can modulate the emotional state of OBX rats, suggesting its therapeutic potential against mood disorders.

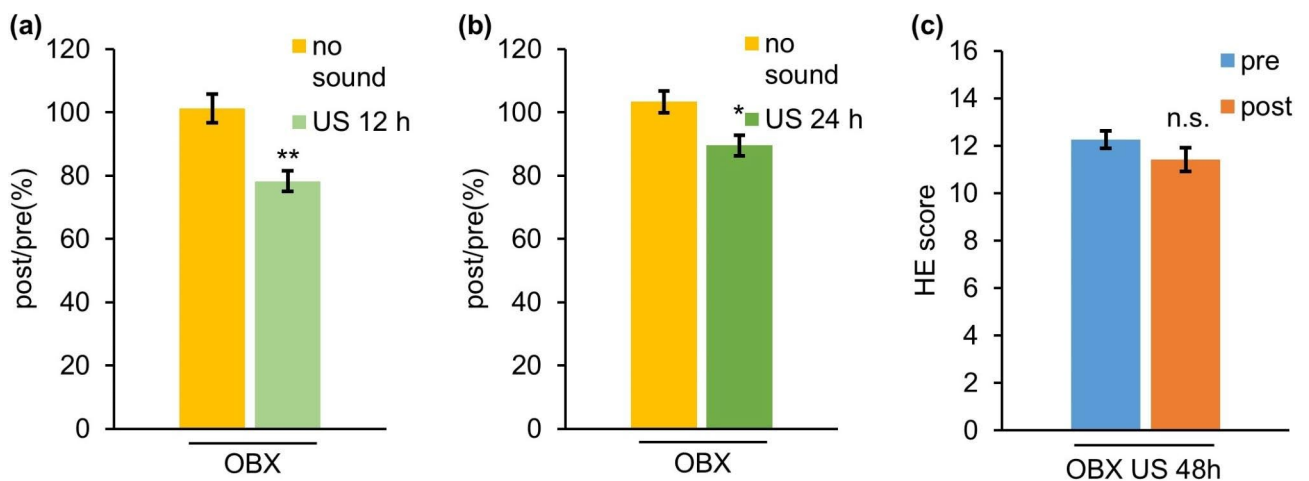
Previous experiments have shown that elastic vibrations above 80 kHz may be less audible or even inaudible to rats<sup>9–12</sup>. In this study, there was no change in the number of *c-Fos* immunopositive cells in the bilateral Au1, AuD, and AuV. Additionally, rats did not exhibit freezing behavior when 100 kHz US was presented as a conditioned stimulus in the auditory fear conditioning test, indicating that rats do not perceive 100 kHz US



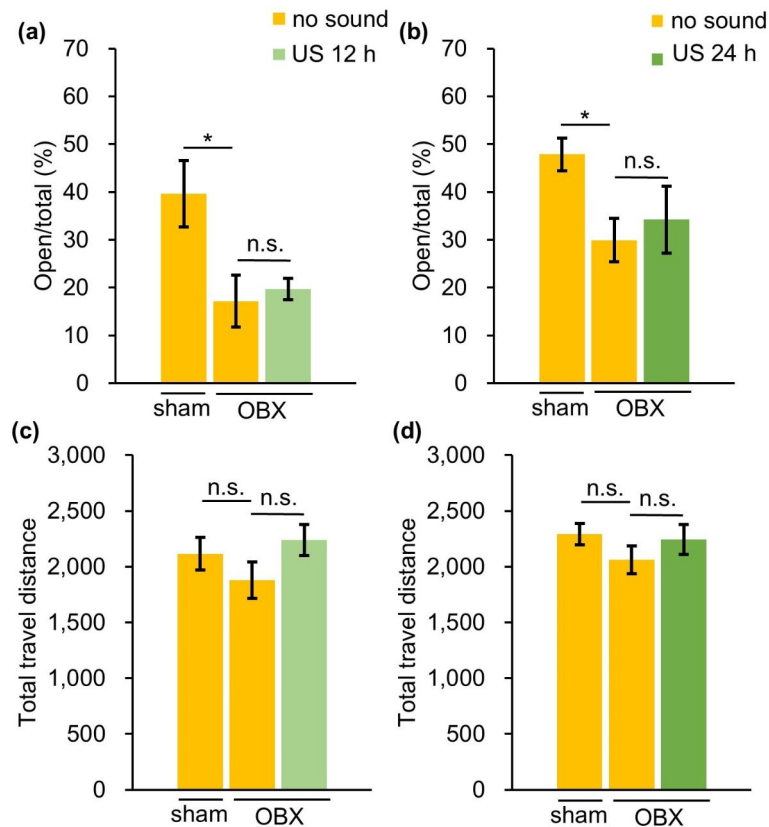
**Fig. 2.** Exposure to white noise (WN) increases the number of activated (c-Fos immunopositive) neurons in the auditory-related cortex, but 100 kHz ultrasound (US) does not. **(a, b, and c)** Representative photomicrographs of c-Fos immunoreactivity in response to **(a)** no-sound, **(b)** WN, and **(c)** 100 kHz US for 1 h. Scale bar = 0.5 mm. **(d, e, and f)** Number of c-Fos immunopositive cells in **(d)** Au1 (CV = 0.14, 0.06, and 0.19 from left to right), **(e)** AuD (CV = 0.41, 0.08, and 0.30 from left to right), **(f)** AuV (CV = 0.28, 0.14, and 0.25 from left to right). All results are presented as mean ± standard error of the mean (SEM) ( $n = 4$  for the no-sound group,  $n = 3$  for the WN group, and  $n = 4$  for the US group). \* $P < 0.05$  by Kruskal–Wallis test with post hoc Dunn’s multiple comparisons test.



**Fig. 3.** Exposure to WN, but not 100 kHz US, conditioned with foot shock, increases the freezing response. (a and b) Experimental design. Rats were randomly divided into a no-sound group ( $n=6$ ), WN group ( $n=6$ ), and 100 kHz US group ( $n=6$ ). Twenty-four hours after the conditioning session (a), a fear memory conditioned with tones was tested in a different context (test session; b). (c) Freezing time of rats during the tone in the test session. CV = 1.12, 0.33, 0.29, and 1.11 from left column to right. All results are presented as mean  $\pm$  standard error of the mean (SEM). \* $P < 0.05$  by one-way ANOVA with post hoc Holm–Sidak multiple comparison tests.



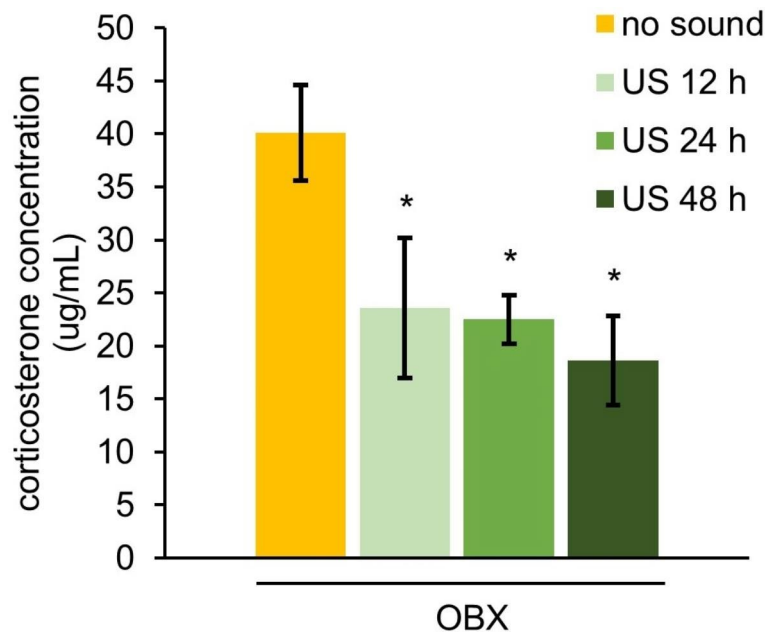
**Fig. 4.** Exposure to 100 kHz US reduces hyperemotionality in olfactory bulbectomized (OBX) rats. (a and b) The HE score ratio before and after exposure to no-sound or US. (a) Exposure to no-sound ( $n=12$  and CV = 0.15) or US ( $n=11$  and CV = 0.13) for 12 h. (b) Exposure to no-sound ( $n=8$  and CV = 0.09) or US ( $n=11$  and CV = 0.12) for 24 h. \* $P < 0.05$  and \*\* $P < 0.01$  by Mann–Whitney U test. (c) HE scores were measured pre- (blue) and post-exposure (orange) to 48 h of ultrasound ( $n=12$ , CV = 0.11 for pre-assessment, and 0.15 for post-assessment) and n.s. via Wilcoxon matched-pairs signed-rank test. All results are expressed as mean  $\pm$  standard error of the mean (SEM).



**Fig. 5.** Exposure to 100 kHz US has no effect on anxiety as measured by the elevated plus maze (EPM) test. **(a and b)** Proportion of time spent in the open arms by sham controls and OBX rats during the EPM test conducted after **(a)** 12 h (CV = 0.46, 0.83, and 0.27 from left to right) or **(b)** 24 h (CV = 0.25, 0.48, and 0.65 from left to right) with no-sound (yellow) or US (green). **(c and d)** Total distance traveled during these same EPM tests, **(c)** 12 h (CV = 0.18, 0.23, and 0.15 from left to right) and **(d)** 24 h (CV = 0.15, 0.19, and 0.19 from left to right). Group sizes for the 12 h **(a and c)** exposure: sham rats ( $n = 7$ ), OBX rats with no-sound ( $n = 7$ ), and OBX rats exposed to 100 kHz US ( $n = 6$ ). Group sizes for the 24 h **(b and d)** exposure: sham rats ( $n = 12$ ), OBX rats with no-sound ( $n = 10$ ), and OBX rats exposed to 100 kHz US ( $n = 10$ ). All data are presented as mean  $\pm$  standard error of the mean (SEM). \* $P < 0.05$  by one-way ANOVA with post hoc Holm–Sidak multiple comparison tests.

with auditory attention under these experimental conditions. It has been previously reported that inaudible high-frequency sounds ( $> 20$  kHz) can influence human brain activity, even though humans cannot perceive frequencies  $> 20$  kHz. This effect occurs only when sounds are presented through speakers and reach a large area of the body surface, indicating the involvement of other (non-auditory) sensory systems<sup>1,13</sup>. Although the sound used in this study was of a specific frequency without audible components, it appeared to modulate the emotional state in accordance with previous human experiments. To the best of our knowledge, this is the first study to demonstrate that airborne US can produce an ameliorating effect in a rat model of depression without auditory attention. For more reliability, further studies are needed to examine the effects of inaudible US exposure on other depression models and depressive-like symptoms, such as anhedonia, hypoactivity, and learned helplessness.

Hyperemotionality was reduced after 12 and 24 h of 100 kHz US exposure but not after 48 h. The US exposure was conducted in a soundproof box to control environmental sound. It is possible that this prolonged duration in a soundproof box induced environmental stress and thereby counteracted the effects of US exposure. Although hyperemotionality in OBX rats significantly decreased following 12 and 24 h of exposure, there were no significant effects on anxiety-like behavior in the EPM test. We previously showed that exposure to 50 kHz USVs, which are associated with pleasant emotions in rats significantly reduced anxiety-like behavior in the EPM test in OBX rats, accompanied with significant decrease of HE score and plasma corticosterone levels. Additionally, artificial 50 kHz US at a frequency similar to that of USVs also significantly decreased the HE score<sup>4</sup>. The primary difference between the results of this study and our previous study is whether anxiety-like behavior improves in the EPM test. Appetitive 50 kHz USVs is emitted in rewarding situations<sup>14,15</sup>. It has been reported that these USVs communicate positive affective states to conspecific receivers and alter behavioral responses<sup>16,17</sup>. Furthermore, Wöhr et al.<sup>16</sup> demonstrated that frequency characteristics are important for the communicative values of ultrasonic vocalizations in rats. They used artificial 50 kHz sine wave tones, a frequency similar to 50 kHz USVs, and showed that approach behavior was induced by the artificial tone. Our previous



**Fig. 6.** Exposure to 100 kHz US reduces plasma corticosterone concentrations in OBX rats. Trunk blood was collected immediately after the hyperemotionality evaluation. CV = 0.32, 0.84, 0.29, and 0.55 from left to right. Group sizes were as follows: no-sound group ( $n=8$ ), 12-h group ( $n=9$ ), 24-h group ( $n=8$ ), and 48-h group ( $n=6$ ). All data are represented as mean  $\pm$  standard error of the mean (SEM). \* $P < 0.05$  by one-way ANOVA with post hoc Holm–Sidak multiple comparison tests.

findings are consistent with the result. These audible USVs induce Fos-like immunoreactivity in auditory areas and regions associated with emotion, indicating that they may influence emotions through auditory perception<sup>18</sup>. Conversely, the 100 kHz US used in this study did not increase c-Fos positive cell counts in the auditory cortex. Therefore, the mechanism through which 100 kHz US improves the HE score and reduces plasma corticosterone levels may differ from our previous study with USVs.

Additionally, the EPM test assesses innate anxiety, whereas hyperemotionality responses assess irritability to external stimuli as manifested by attack, startle, and struggle responses<sup>4–8</sup>. Although 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. Thus, we propose that the methods or components of the US that are effective against depression may differ from those that target 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. The results of the present study indicate that the OBX rat model is suitable for investigating the effects of audible high-frequency sounds and ultrasonic frequencies beyond the audible range.

While the results of the behavioral assessments varied with exposure time, plasma corticosterone concentrations were significantly reduced in all exposure groups. Kawai et al.<sup>19</sup> 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 healthy humans' oral glucose tolerance test. Exposure to HFC sounds activates deep brain regions, such as the midbrain, thalamus, and hypothalamus<sup>1</sup>. Furthermore, it is known that stress hormones have a significant impact on glycemic control. Kawai et al.<sup>19</sup> suggested that glucose elevation was suppressed because of reduced 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 reduces hyperemotionality in OBX rats by inducing changes in deep brain activity, which in turn lowers corticosterone levels. Additionally, previous studies using ultrasound stimulation have shown that transcranial ultrasound stimulation modulates neuroinflammatory activity and BDNF release<sup>20–22</sup>. These factors are also known to be closely related to stress and corticosterone levels<sup>23–25</sup>, indicating that the neural modulation of these stress-related factors by ultrasound stimulation might be related to the present results regarding the reduction of corticosterone levels. Moreover, the fact that behavioural changes did not occur within the 48-hour exposure group despite similarly reduced plasma corticosterone levels, might suggest that the physiological and emotional changes following the US exposure are mediated through distinct mechanisms. Further investigations are necessary to compare our findings with previous studies, such as quantifying neuroinflammatory factors and assessing activation in deep brain regions, including the midbrain, thalamus, and hypothalamus.

In this study, 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<sup>1,26,27</sup>. Additionally, a substantial proportion of patients receiving CBT fail to achieve remission and their drop-out rate is relatively high<sup>28,29</sup>. To overcome these challenges, it is necessary to develop novel treatments for major depressive disorder. Recently, Ito et al. investigated the potential application of the hypersonic effect in the treatment of major depressive disorders<sup>30</sup>. Similar to the hypersonic effect, investigating whether US exposure affects depressive-like behavior in OBX rats could potentially lead to the development of a new method to treat depression.

Our study provides evidence that exposure to 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 model is suitable for further investigations on the therapeutic efficacy of US against specific depressive traits. If inaudible US can alleviate depressive symptoms, it may be useful for treating depression in various environments. Such studies could lead to the development of alternative noninvasive treatments for depression.

This study has several limitations. First, the discussion is based on a comparison with previous studies that focused on the effects of US components on the psychophysiological states of humans. However, the effects of US are still debatable and under investigation. Oohashi et al.<sup>1,13</sup> showed that high-frequency components affect subjective impressions, whereas Kuribayashi et al.<sup>31</sup> found no difference in subjective impressions depending on the US components. Each previous study used different sound stimuli. Thus, considering their varying results, the effect of the US might depend on the experimental conditions. The results of the current are not directly comparable to those of previous studies as we used only US sounds and, moreover, used rats instead of human participants. Rats may be more receptive to higher tones in the cochlea than humans. Several studies have suggested that rats respond to high-frequency sound<sup>32</sup> although the auditory cortex was not activated in our results. Conversely, Oohashi et al.<sup>13</sup> reported that hypersonic effects could be induced when ultrasound was exposed to the skin surface, and based on this finding, they hypothesized the existence of unknown mechanisms for sensing and transducing high-frequency elastic vibrations distinct from the conventional air-conducting auditory nervous system<sup>13</sup>. Indeed, it has been reported that receptors in the skin respond to kHz-level acoustic stimuli<sup>33</sup>. Therefore, ultrasound at not-too-high sound pressures, detected by the skin or other organs, could potentially impact emotional states. There is also the possibility that the eardrum, the most sensitive organ specialized for sound detection, detects ultrasound without subjective auditory attention. Previous studies have focused on the acoustic signatures of activated neurons, such as the tonal frequencies that neurons in the AuD, AuI, and AuV respond to, or the tonotopy related to activation in the inferior colliculus<sup>34–36</sup>. These experiments could provide more specific insights into our previous study. Thus, further studies are needed to elucidate the underlying mechanisms of responses to US using the model proposed in this study.

Second, we used only male rats. Previous reports indicate that stress susceptibility and the onset of psychiatric disorders are related to sex-based differences<sup>37,38</sup>. Thus, it is preferable to include male and female subjects for therapeutic applicability. Additionally, this study investigated the response to ultrasound using only naive animals. It remains unclear whether similar results would be found in OBX rats. Thus, further studies incorporating female and OBX rats are warranted.

Moreover, this study confirmed c-Fos expression levels only in the auditory cortex. Previous studies have shown that the function of brain regions related to emotion, such as the frontal cortex, hippocampus, and amygdala, changes in the OBX rat depression model<sup>6,39</sup>. Therefore, it is important to examine the activity of other brain regions, including the limbic system. Furthermore, the HE evaluation was non-blind and may be subjective. In addition to the corticosterone levels shown in this study, examining the activity of the limbic system would provide more evidence to support our results.

## Methods

### Animals

Male Wistar/ST rats (7–9 weeks old, 200–320 g) 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 the National Institutes of Health and Japan Neuroscience Society guidelines. The study design and all procedures adhered to the ARRIVE Guidelines.

### Sound materials and presentation system

The sound stimuli were produced using SASLab Pro version 5.2.09 (Avisoft Bioacoustics, Glienicke/Nordbahn, Germany). Artificial 100 kHz US (30 ms pulses at 60 ms intervals) and 35–100 kHz WN were created at a sampling rate of 250,000 Hz in 16-bit format. To deliver the sound stimuli, ultrasonic dynamic speaker Vifa (Avisoft Bioacoustics) was used; this speaker was capable of sufficiently driving the frequency range of the acoustic stimuli used in this study from 1 to 120 kHz. To produce the sounds, a PC was connected to an UltraSoundGate Player 116 (Avisoft Bioacoustics), and the sound files were converted to analog signals and played through the speakers. The distance between the speaker and the rodent was 12 cm in the cage (w 18 × d 30 × h 13 cm), 40–52 cm in context A (w 32 × d 31 × h 31.5 cm), and 40–50 cm in context B (w 30 × d 30 × h 35 cm). Because the distances between the speaker and the rats differed in each experimental condition, and the Avisoft speaker shows attenuation at high frequencies, the sound pressure at the rat's position was adjusted by increasing the sound level. All sounds were recorded using an ultrasonic condenser microphone (CM16/CAMPA; Avisoft Bioacoustics, Berlin, Germany). The ultrasound microphone was connected via an UltraSoundGate 416 H USB

audio device (Avisoft Bioacoustics) to a PC, where acoustic data were recorded at a sampling rate of 500 kHz and 16-bit format using RECORDER USGH (Avisoft Bioacoustics). The power spectra and spectrograms of the two sounds in each exposure environment are shown in Fig. 1. The power spectra were calculated using the Audition program (Adobe, San Jose, USA), and the spectrogram and sound pressure levels (SPLs) were analyzed using Avisoft SASLab Pro software version 5.2.0. The maximum SPLs for each experiment were within  $83 \pm 5$  dB (WN) and  $70 \pm 3$  dB (US). The SPL conditions for WN were based on our previous study<sup>4</sup>.

### Immunohistochemical assessment

All rats used in this assessment were not bulbectomized. The rats were randomly divided into three groups (no-sound, audible sound stimulation with WN, and inaudible sound stimulation with 100 kHz US) and placed individually in cages. Before sound exposure, all rats were habituated for at least 1 h without sound in the cages and then exposed to no-sound, WN (spectrogram in a cage shown in Fig. 1e), or 100 kHz (Fig. 1f) for 1 h according to group designation.

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 0.2 M phosphate buffer (PB) (PFA; Sigma-Aldrich, Tokyo, Japan). Brains were excised, post-fixed overnight in 4% PFA, immersed in 30% sucrose dissolved in 0.2 M PB for cryoprotection, embedded in an Optimal Cutting Temperature compound (Sakura-Finetek, Tokyo, Japan), frozen at  $-80^{\circ}\text{C}$ , and sliced at 40- $\mu\text{m}$  thickness. Brain sections were stored at  $-20^{\circ}\text{C}$  in cryoprotection solution (30% ethylene glycol, 25% glycerol in 0.2 M PB).

For c-Fos immunostaining, six sections per animal, ranging from  $-3.14$  to  $-5.60$  mm anteroposterior to the bregma, according to the Paxinos and Watson rat brain atlas (fourth edition) were incubated with 0.3% hydrogen peroxide in 40% methanol/PBS for 5 min to quench endogenous peroxidase activity, washed thrice 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 overnight with c-Fos antibody (anti-c-Fos (C-10), SCB Santa Cruz Biotechnology, Dallas, TX, USA) diluted 1:1,000 in blocking solution. 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 thrice with PBST, incubated with AB solution (Vector Laboratories) for 60 min, rewashed thrice with PBST, incubated with DAB (Vector Laboratories) for 6 min, washed thrice 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. After converting the images to 8 bits, the Subtract Background tool reduced the noise. The regions of interest (ROIs) within the 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-positive cells per unit area was determined by dividing the number of positive cells by the total area of the ROI for each section and then averaging for each rat.

### Auditory fear conditioning test

All rats used in this assessment were not bulbectomized. The rats were randomly divided into three groups (no-sound, WN, and 100 kHz US), and the auditory fear conditioning test was performed as previously described<sup>40</sup> with slight modification. The experimental design is shown in Fig. 3a, b. The conditioning session was conducted in the conditioning chamber (context A), consisting of gray walls and a grid floor ( $w 32 \times d 31 \times h 31.5$  cm; Muromachi Kikai, Tokyo, Japan). After the rats were placed in context A for 180 s, three 30 s tones, each co-terminating with a 1 s foot shock (0.8 mA), were presented every 1 min. The animal returned to its home cage 1 min after the last foot shock. The spectrograms of the presented tones are shown in Fig. 1g (35–100 kHz WN at  $86 \pm 1$  dB) and Fig. 1h (100 kHz US at  $70 \pm 2$  dB). Twenty-four hours after the conditioning sessions, a test was conducted in a different chamber named context B ( $w 30 \times d 30 \times h 35$  cm); the chamber was a box with striped walls with a flat floor with sawdust bedding. In the test session, a rat was placed in context B for 60 s (baseline period) and then presented with a 30 s tone. The group conditioned with no-sound or WN was exposed to WN (the spectrogram in context B is shown in Fig. 1i). The group conditioned with 100 kHz US was exposed to the US (Fig. 1j). All rats were monitored for freezing behavior using a monitor connected to a video camera system mounted over the experimental chamber. The freezing time was measured by a well-trained scorer who was blinded to the experimental conditions.

### Surgical procedure of olfactory bulbectomy

Olfactory bulbectomy was performed as previously described<sup>14,5</sup>. Briefly, the 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 stereotaxic apparatus. The skull covering the olfactory bulbs was exposed, and burr holes were drilled 7.0 mm anterior to the bregma and  $\pm 1.8$  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 ( $w 15 \times d 20 \times h 16$  cm) for 2 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, incidental frontal cortex damage, or insufficient hyperemotionality (total score  $< 10$ ) were discarded; 9 rats were excluded. The sham operations were performed similarly, but the olfactory bulbs were left intact.

### Ultrasound exposure for behavioral assessment

For the behavioral assessment, OBX rats were exposed to 100 kHz US (Fig. 1f) or no-sound as a control in the cage. The cage containing an individual rat was placed in a soundproof box (20 × 20 × 33 cm, LabDesign, Ibaraki, Japan; at 23 °C and 10 lx) and exposed to US or placed in the same soundproof box without US exposure for 12, 24, or 48 h.

### Evaluation of olfactory bulbectomy-induced hyperemotivality (HE)

The hyperemotivality of OBX rats at baseline (pre evaluation) and following (post evaluation) the no-sound or US exposure was evaluated using a hyperemotivality score<sup>4,5,8</sup> derived from the following behaviors: (1) attack response to a rod held in front of the snout—0 (no reaction), 1 (interest in the rod), 2 (evasive action), 3 (aggressive behavior, such as biting), or 4 (intense aggressive behavior); (2) startle response to air blown on the snout—0 (no reaction), 1 (slight body movement), 2 (startle response), 3 (marked startle response without jumping), or 4 (marked startle response with jumping); (3) struggle response to handling with a gloved hand—0 (no reaction and muscular relaxation), 1 (easy to handle), 2 (mild muscle tension), 3 (difficult to handle, with muscle tension), or 4 (extremely difficult to handle, with substantial muscle tension); and (4) fight response to a tail pinch delivered with mosquito forceps—0 (no reaction), 1 (interest in the mosquito forceps), 2 (evasive action), 3 (aggressive behavior, such as biting), or 4 (intense aggressive behavior). 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 for emotional responses was set to a total of 24. The experimenters were well-trained in measuring hyperemotivality. Moreover, we experimented with multiple lots, with surgical treatments performed on different days (2–5 lots for each group) to verify reproducibility.

### EPM test

The EPM test was conducted following 12–24 h in the before mentioned (Methods 4.7) soundproof box with or without US exposure and 1 h of habituation. The tests were conducted as described in our previous study<sup>4,41</sup>. 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), whereas the other two had unprotected edges (open arms). The entire maze was placed under indirect light (50 lx). 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 traveled distance, and time spent on the open arms was recorded using a video camera system and analyzed using Smart 3.0 (Harvard Apparatus, Holliston, MA, USA). The experiment was conducted across multiple lots with surgical treatments conducted on different days (3 or 4 lots for each group) to verify reproducibility.

### Corticosterone assay

Blood samples were collected from a part of animals after HE evaluation. Rats exposed to no-sound or 100 kHz US for 12, 24–48 h had their trunk blood samples collected immediately after the post-HE evaluation into heparin-containing tubes. All blood samples were collected between 16:00 and 16:30. Subsequently, the blood samples were 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. Per the manufacturer's instructions, concentration was measured in duplicate using a corticosterone enzyme-linked immunosorbent assay kit (Cayman Chemical, Ann Arbor, MI, USA). The specificity of this kit was 100% for corticosterone, and sensitivity units were 30 pg/ml. Detailed specifications are available at the manufacturer's website (<https://cdn.caymanchem.com/cdn/seawolf/insert/501320.pdf>).

### Data analysis

All data are presented as the mean ± standard error of the mean (SEM.). The Kruskal–Wallis test with post hoc Dunn's multiple comparisons test was used to analyze the immunohistochemical assay. The Mann–Whitney U test or Wilcoxon signed-rank test was used to compare the HE scores within each group. Additionally, the results of the EPM and plasma corticosterone levels were compared between the groups using one-way ANOVA followed by post hoc Holm–Sidak tests. The threshold for statistical significance was set at  $p < 0.05$  (corrected for multiple comparisons). In the figures, the 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).

### Data availability

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

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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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## Declarations

## Competing interests

The authors declare no competing interests.

## Additional information

**Correspondence** and requests for materials should be addressed to A.S.

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