Unpleasant Sound Elicits Negative Emotion and Reinstates Drug Seeking

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Abstract

Although previous studies have suggested an association between unpleasant sounds and the use of drugs, scientific evidence supporting this is lacking. This study investigated in rats (male Sprague-Dawley rats) if aversive sounds modulate dopamine (DA) transmission in the mesolimbic reward system and cocaine reinforcement. For sound stimulation, we used artificial low-frequency ultrasound (ALFUS) in the frequency ranges (22–38 kHz) which produces an aversive response in rats. Rats displayed increased anxiety-like behaviors, 22-kHz ultrasonic vocalizations (USVs), and stress responses with ALFUS. In vivo extracellular recording and immunohistochemistry revealed that ALFUS stimulation activated central amygdalar neurons and amygdalar GABAergic neurons. Amygdalar lesions prevented an increase of 22-kHz USVs by ALFUS. Dopamine levels in NAc decreased during ALFUS stimulation. In rats self-administering cocaine, ALFUS caused reinstatement of cocaine seeking after a period of extinction. Thus, ALFUS stimulation induced negative emotional states in association with a decrease in mesolimbic DA function and reinstatement of cocaine-seeking behaviors, suggesting that exposure to unpleasant sounds enhances negative emotional states and may induce relapse in addicts.

Keywords Unpleasant sound · Negative emotion · Drug seeking · Cocaine · Anxiety · Stress

Introduction

Certain sounds, such as unpleasant noises, a baby crying, machine noise at work, a female scream, and a knife on a bottle, are often perceived as highly unpleasant in humans. The unpleasant sounds, particularly in the frequency range of 2–5 kHz, can evoke negative emotions by activating the amygdala, a brain region important for processing emotions [1, 2], and provoke several kinds of stress responses [3]. While

unpleasant sounds are known to interfere with complex task performance, continuous exposure to these sounds frequently produces serious psychological effects (i.e., anxiety) and, possibly, negative emotional states [4–6] that are characteristic of withdrawal from alcohol and other drugs of abuse. For example, a clinical study revealed that subjects exposed to a baby crying displayed elevated levels of aversion, arousal, distress, and increased alcohol drinking [7].

Chronic administration of drugs of abuse is thought to elicit negative emotions (e.g., anxiety, dysphoria, and irritability) during abstinence that contribute to drug cravings and relapse [8], although it remains controversial [9]. Human addicts experience negative emotional states such as enhanced anxiety, depressed mood, and strong cravings during abstinence from chronic drug use [10]. Rats undergoing withdrawal from chronic cocaine reveal anxiety- and depression-like behaviors [11] and increased thresholds for intracranial electrical self-stimulation [12], implying enhanced negative emotions in cocaine abuse. In turn, negative emotions can promote drug craving and relapse susceptibility through interactions between amygdala and dopamine (DA) pathways in the brain. Such emotions can disturb homeostasis and thus lead to an allostatic hedonic state that defines chronic addiction [8, 13],

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suggesting that negative emotions and drug relapse are closely linked.

Although previous studies have suggested an association between the unpleasant sounds and the use of drugs [14, 15], scientific evidence supporting this and the underlying mechanism are lacking. Unfortunately, there have also been limitations because there are no established sounds perceived as unpleasant in rats. One of the most commonly used paradigms for studying the relationship between reinstatement of drug seeking and aversive stressful events is the use of electric foot shocks [16]. While this method has proven to be reliable, it not only is apart from the types of many aversive events that humans encounter in their natural environment but also elicits tactile pain. The study of unpleasant sounds will make it possible to evaluate mechanisms of drug relapse that might be maintained through relatively natural stressful events rather than stress induced by activation of pain pathways. The present study aimed to investigate whether unpleasant sounds promote relapse to drug seeking by inducing negative emotional states in association with a decrease in mesolimbic DA function. For sound stimulation to adult rats, we utilized a simple device (an ultrasonic pest repeller) producing low-frequency ultrasound in the 20–40 kHz range to which rats are sensitive and aversive. The present study examined in rats whether artificial low-frequency ultrasound (ALFUS) stimulation elicits negative emotional responses such as anxiety-like behaviors and 22-kHz ultrasonic vocalizations and induces stress responses. Since it is known that central amygdala (CeA) is critical for emotional processing [1, 17], relapse to drug seeking [18], and the processing of emotional auditory information [19], we investigated whether ALFUS activates central amygdalar neurons, especially gamma-aminobutyric acid (GABA) neurons. Furthermore, it was explored whether ALFUS affects DA release in nucleus accumbens (NAc) and reinstates cocaine-seeking behaviors after extinction in a rat cocaine self-administration model.

Materials and Methods

Animals

Male Sprague-Dawley rats (weight 270–320 g, Daehan Animal, Seoul, Korea) were housed in groups of two to three animals per cage on a 12-h light-dark cycle and freely accessed to food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Daegu Haany University (DHU2017-040) and conducted in accordance with National Institutes of Health guideline. Each group consisted of five to eight rats, unless described. No animals were used for more than one experiment.

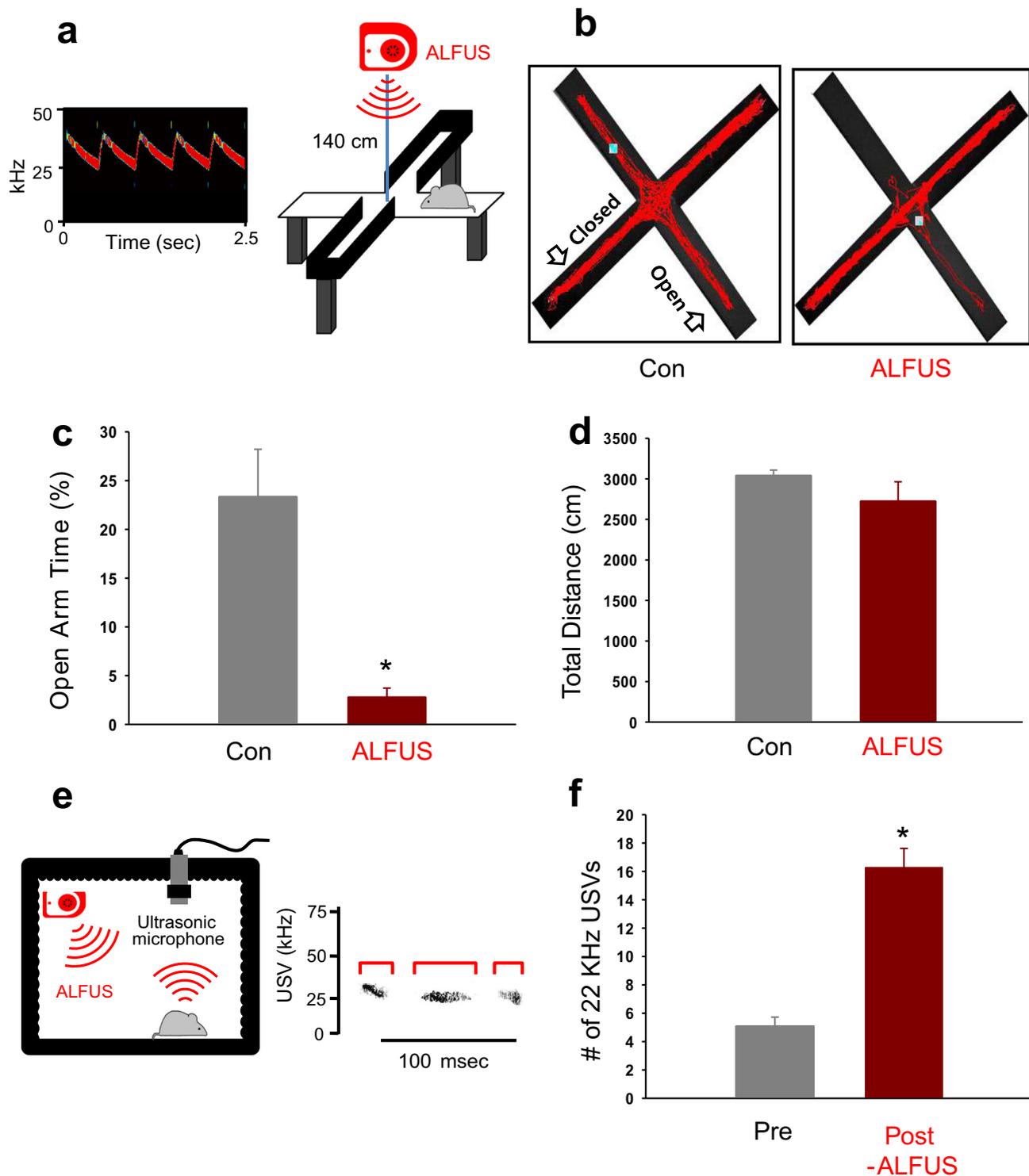### Artificial Low-Frequency Ultrasound (ALFUS)

Artificial low-frequency ultrasound (ALFUS) was generated by ultrasonic pest repellers that are designed to continuously sweep an ultrasound frequency range between 20 and 40 kHz and 50~110 dB in order to repel household pests, such as rodents and insects. A preliminary study was performed to select a suitable ultrasound generator among three different repellers (Model UDR-020, HDT Korea Inc., Korea; Model TY-300, Taeyang Inc., Korea; and, Model TY-200, Taeyang Inc., Korea) by examining avoidance behaviors from the ALFUS, as previously described [20] but with a slight modification. When the repellers turned on, the animals ($n = 8$) displayed escape responses from the compartment installed with the ALFUS speaker and its effect was relatively well observed in Model TY-200. Thus, this device was used for ALFUS stimulation throughout the study. The actual frequency of ALFUS emitted by the pest repeller was measured with condenser ultrasound microphones (Ultramic 250K, DoDotronic, Italy) at the supposed position of the rats in the chamber used for Fig. 1e. The microphone signals were fed into an UltraSoundGate 416H data acquisition device (Avisoft Bioacoustics, Germany) with a sampling rate of 250 kHz and 16-bit resolution and recorded using Avisoft-SASLab Pro (version 4.2, Avisoft Bioacoustics). Spectrograms were generated with a fast Fourier transform length of 512 points and an overlap of 75% (Flat Top window, 100% frame size). In the following experiments, control (con) rats were exposed to white noise corresponding to the sound intensity of ALFUS, unless described.

Elevated plus Maze

Anxiety-like behavior was measured using the elevated plus maze (EPM) as previously described [21] with a slight modification. Briefly, the EPM apparatus consisted of two closed arms ($50 \times 10 \times 40$ cm) and two open arms (50×10 cm) extending from a central platform (5×5 cm) located 50 cm above the floor and illuminated with a low-intensity light (1.5 to 2.0 lx). An ALFUS generator was set-up 140 cm over the center of maze. While ALFUS was generated at an intensity of 50–60 dB, each rat was placed into the center. The time spent in the open arms and the total distance traveled were measured for 5 min using a video tracking system (Ethovision, Nodus Information Technology BV, Netherlands). The percentage of time spent in the open arms and the total distance traveled were used as indices of anxiety and general motor behavior, respectively.

Recordings of Ultrasonic Vocalizations (USVs)

Ultrasonic vocalizations emitted by rats in response to ALFUS stimulation were recorded using customized sound-

Fig. 1 *Effect of artificial low-frequency ultrasound on anxiety-like behavior and ultrasonic vocalizations.* **a** Ultrasound waves recorded from the artificial low-frequency ultrasound (ALFUS) generator (left). The generator was installed 140 cm over center of elevated plus maze (right). **b–d** Effect of ALFUS in elevated plus maze test. Representative activity traces from naïve (Con) and ALFUS-treated rats (ALFUS; **b**). Effect of ALFUS on the percentage of time spent in open arms of the maze (**c**). ALFUS significantly decreased time spent in the open arms, compared to control group (ALFUS vs. Con; *$p = 0.003$, $n = 5$/group). Effect of ALFUS on total distance traveled (**d**). **e, f** Effect of ALFUS on 22-kHz ultrasonic vocalizations (USVs). **e** Schematic for USVs recordings (left) and representative examples of 22 kHz USVs (right). **f** The numbers of USVs before (Pre) and after 30-s stimulation of ALFUS (Post-ALFUS, $n = 6$). Pre vs. Post-ALFUS, *$p = 0.001$

attenuating chambers as previously described [22]. The chamber consisted of two boxes to minimize exterior noise (inside box: $60 \times 42 \times 42$ cm, outside box: $68 \times 50 \times 51$ cm). The ultrasonic microphone was positioned at the center of the ceiling of the chambers, and an ALFUS speaker was placed about 20 cm from the microphone. Ultrasonic vocalizations (USVs) were recorded using the ultrasonic microphone with the Avisoft-RECORDER software (Avisoft Bioacoustics). For 22 kHz USVs, the signals were band-filtered between 18 and 32 kHz and analyzed using Avisoft-SASLab Pro (version 4.2, Avisoft Bioacoustics). Animals were habituated for at least 30 min in the chambers prior to experiments. The USVs were recorded for 2 min as baseline and then recorded for 4 min after exposure to ALFUS for 30 s. Data are expressed as the numbers of 22-kHz USVs/min and compared before and after ALFUS stimulation.

Measurement of Systolic Blood Pressure

Systolic blood pressure (BP) was measured non-invasively with a tail cuff blood pressure monitor (Model 47, IITC, USA), as performed previously in our laboratory [23]. Briefly, animals were placed in a chamber kept at 27 °C and an occluding cuff and a pneumatic pulse transducer were positioned on the base of rat tail. A programmed electrosphygmomanometer (Narco Bio-Systems Inc., USA) was inflated and deflated automatically, and the tail cuff signals from the transducer were automatically collected using the IITC apparatus. The mean of two readings was taken at each blood pressure measurement. After basal BP was recorded for at least 30 min, the animals were subjected to ALFUS for 90 min and the BP was measured every 10 min up to 90 min after initiation of ALFUS in awake. Control rats were exposed to white noise (50–60 dB).

Measurement of Serum Corticosterone Level

Immediately after the last measurement of BP, the rats were anesthetized with isoflurane and the blood samples (about 3 ml) were then collected via cardiac puncture. Serum was separated after centrifugation ($5500 \times g$, 10 min) and stored at −80 °C until use. Serum corticosterone levels were quantified using an ELISA kit (Enzo Life Sciences, USA).

In Vivo Extracellular Single-Unit Recordings in Central Amygdala (CeA) Neurons

To evaluate whether ALFUS affects CeA neuronal activity, single-unit discharges of CeA neurons during ALFUS were recorded as described previously [24]. Briefly, under intraperitoneal (i.p.) pentobarbital anesthesia (50 mg/kg), animals were positioned on a stereotaxic apparatus and burr holes were drilled into skull to accommodate recording electrodes. The body temperature was kept constant at 37.5 °C using a feedback-controlled heating pad. During recordings, anesthesia was maintained by administering isoflurane (1.5~2%) in 100% oxygen. A carbon-filament glass microelectrode were stereotaxically advanced to the CeA (stereotaxic coordinates: posterior, −1.92~−2.64 mm; lateral, +3.6~+4.6 mm; deep, 7.8~8.6 mm), according to the atlas of Paxinos and Watson [25]. Single-unit activity was amplified and filtered at 0.1–10 kHz (ISO-80; World Precision Instruments, USA) and discriminated from noise, binned at 1-s intervals, and analyzed via a CED 1401 Micro3 device and Spike2 software (Cambridge Electronic Design, UK). We recorded only the CeA neurons which had spontaneous basal firing rates in the ranges reported previously [26]. After recording stable baseline, the rats were subjected to ALFUS and the mean firing rate for 5 min before, 10 min during, and 5 min after ALFUS exposure were compared. In order to verify the location of recording sites, the rats were sacrificed at the end of experiments and perfused with 4% formaldehyde. Brains were removed, stored in 30% sucrose solution, and cryosectioned 30 μm thick. The slices were stained with toluidine blue and examined under a light microscope.

Immunohistochemistry for c-Fos and Glutamic Acid Decarboxylase 67 (GAD67) in CeA

Immunolabeling for c-Fos and GAD in CeA was carried out as described previously [24]. Briefly, a separate group of animals was sacrificed after ALFUS exposure of 1 h and the brains were removed, post-fixed with 4% paraformaldehyde for 2 h, and cryoprotected in 30% sucrose for at least 48 h. The brains were cryosectioned into 30 μm thick and incubated with a blocking solution containing 0.3% Triton X-100 and 5% normal donkey serum in phosphate-buffered saline (PBS) for 1 h. The sections were incubated with anti-c-Fos rabbit polyclonal antibodies (1:500; Santa Cruz, USA) overnight at 4 °C, followed by a biotinylated donkey anti-rabbit Alexa Fluor 488 (green; 1:500; Invitrogen, Oregon, USA). For double-staining of c-Fos with glutamic acid decarboxylase (GAD), the brain slices were incubated with anti-c-Fos rabbit polyclonal antibodies (1:500; Santa Cruz, USA) and anti-GAD67 mouse monoclonal antibodies (1:500; Santa Cruz, USA), followed by a biotinylated donkey anti-rabbit Alexa Fluor 594 (red; 1:500; Invitrogen, USA) and anti-mouse Alexa Fluor 488 (green; 1:500; Invitrogen, USA), respectively. The slices were then mounted onto gelatin-coated slides, photographed, and examined under a confocal laser scanning microscope (LSM700, Carl Zeiss, Germany). The number of c-Fos–positive cells was blindly counted in the bilateral CeA and 4~6 slices per animal were analyzed.

Chemical Lesion of Central Amygdala

For central amygdalar lesions, ibotenic acid (0.5 μl/site; 5 mg/ml saline; Sigma, USA) was bilaterally microinjected as described previously [27]. Under pentobarbital anesthesia (50 mg/kg, i.p.), two holes were drilled in the skull to access the following coordinates: (posterior, −1.92 to −2.64 mm; lateral, +3.6 to 4.6 mm; deep, 7.8 to 8.6 mm). A 26-gauge Hamilton syringe (Hamilton, USA) filled with either ibotenic acid or saline was infused at a rate of 0.5 μl/min using a microinjection pump (Pump 22, Harvard Apparatus, USA). The syringe was left in place for at least 5 min to facilitate diffusion after injection. The rats were allowed to recover for at least 7 days. After basal recordings of USVs for 2 min, the rats were given a 30-s stimulation of ALFUS and the numbers of 22-kHz USVs were measured for another 4 min. At the termination of the experiments, all rats were sacrificed for histological confirmation of lesions. The animals were perfused with phosphate-buffered saline (PBS) and then with 4% paraformaldehyde. The brains were removed, post-fixed in 4% paraformaldehyde, and cryoprotected in 30% sucrose. The tissue was then cryosectioned into 30-μm-thick sections and stained with toluidine blue. Only those rats with correctly placed lesions were included for data analysis.

Measurements of NAc DA Release by In Vivo Fast-Scan Cyclic Voltammetry

Electrically evoked DA release in the NAc was measured by fast-scan cyclic voltammetry (FSCV) in vivo, as performed in our laboratory [24, 28]. Briefly, a 7.0-μm-diameter carbon fiber electrode (CFE) was inserted into a capillary tubing (1.2 mm o.d., A-M Systems, USA) and pulled with a pipette puller (Sutter Instrument, Model P-97, USA) and cut under microscopic control with 150~200 μm of bare fiber protruding from the end of the glass micropipette. The tip of the CFE was dipped in cyanoacrylate, allowed to dry, and back-filled with 3 M KCl. The electrode potential was linearly scanned with a triangular waveform from −0.4 to 1.3 V and back to −0.4 V versus an Ag/AgCl reference electrode using a scan rate of 400 V/s. Cyclic voltammograms were recorded at the CFE every 100 msec by means of a ChemClamp voltage clamp amplifier (Dagan Corporation, USA). Voltammetry recordings were performed and analyzed using LabVIEW-based (National Instruments, USA) customized software (Demon Voltammetry). Bipolar, coated stainless steel electrodes were stereotaxically implanted into the medial forebrain bundle (stereotaxic coordinates: posterior, −2.5 mm; lateral, +1.9 mm; deep, −8.0~−8.3 mm) and a capillary glass-based CFE in the NAc (stereotaxic coordinates: anterior, +1.6 mm; lateral, +1.9 mm; deep, −6.5~−8.0 mm). The medial forebrain

bundle was stimulated with 60 monophasic pulses at 60 Hz (4 msec pulse width) at 2-min intervals. After basal recordings were stable (less than 10% variability in peak heights of three consecutive collections), the effects of ALFUS on DA release in NAc were evaluated. Control rats (Con) were exposed to white noise corresponding to the sound intensity of ALFUS.

Procedures for Cocaine Self-Administration and Reinstatement

Food Training and Surgical Procedure

Self-administration was carried out in operant chambers (32 × 25 × 34 cm; Med Associate, USA) equipped on one wall with two levers (active and inactive levers), a white house light, and a stimulus light, as described previously [28]. To facilitate the acquisition of operant responding in administration chambers, rats were subjected to mild food restriction (approximately 16 g of lab chow a day) and trained to lever press 45-mg food pellets (Bio-Serv, USA) under a fixed ratio 1 (FR1) schedule until they achieved a criterion of 100 food pellets for three consecutive days. Two days after the last training, an indwelling catheter (Dow Corning, USA) was surgically implanted into the right jugular vein under sodium pentobarbital anesthesia (50 mg/kg, i.p.) and flushed with 0.2 ml heparinized (30 U/ml) saline containing gentamicin (0.33 mg/ml) to prevent clotting and infection. The animals were allowed to recover for at least 7 days.

Cocaine Self-Administration Procedure

Rats were trained to self-administer cocaine during a 2-h daily session under an FR1 schedule over 2–3 weeks, as described previously [28]. Each active lever press produced a 0.1-ml infusion of cocaine (0.5 mg/kg) over 5 s while lever responses on inactive lever did not result in cocaine infusion. Each infusion was followed by an additional 15-s time-out, while the house light remained off.

Extinction and Reinstatement Testing

After establishment of stable cocaine self-administration (less than 15% in variation for cocaine intake for three consecutive sessions), the rats underwent daily 2-h extinction sessions for a minimum of 3 weeks. During daily 2-h extinction sessions, active lever presses were recorded but did not produce cocaine infusion. The extinction sessions continued until the animals responded with less than 10 active lever presses. When this criterion was met, cocaine-seeking behavior was considered extinguished. One day after the extinction criterion was established,

reinstatement testing was performed. During reinstatement testing, the same conditions as those of the cocaine self-administration were maintained, except that cocaine was replaced with saline. To examine whether ALFUS reinstates cocaine-seeking behaviors, rats were placed in an operant conditioning chamber equipped with an ALFUS generator and given ALFUS stimulation for the first 60 min of the 2 h session. The responses on the active or inactive levers were recorded for 2 h after initiation of ALFUS. Since a background white noise of approximately 65 dB produced by a ventilation fan mounted in the operant chamber was present throughout experiments, the rats were not given additional white noises.

Data Analysis

All data are presented as mean ± SEM (standard error of the mean) and analyzed by one or two-way repeated measures analysis of variance (ANOVA) with Tukey post-hoc tests or paired or unpaired t test, where appropriate. Statistical significance was considered at $P < 0.05$.

Results

Artificial Low-Frequency Ultrasound Induced Anxiety-like Behaviors

For artificial low-frequency ultrasound (ALFUS), we utilized a simple device that was designed to produce ultrasonic noise in order to repel pests including rats. When the frequency range and amplitude were measured through an ultrasonic microphone, the device emitted ultrasonic sounds repeatedly in a form of sawtooth wave in frequency range of 22–38 kHz and amplitude of 50–60 dB (Fig. 1a). To determine if ALFUS might elicit negative emotions, particularly anxiety, we evaluated rat behavior in the elevated plus maze (EPM). Exposure of ALFUS significantly decreased the percentage of time spent in the open arms of the elevated plus maze, compared to that of naïve controls (ALFUS vs. Con; t test, $p = 0.003$; Fig. 1b, c). On the other hand, ALFUS stimulation did not affect total distance traveled in open and closed arms compared to the control group (Fig. 1d). Neither sudden movements nor startled responses were observed on video recording when the ALFUS turned on (data not shown). ALFUS increased anxiety-like behavior without affecting general locomotor behaviors. To further examine whether ALFUS increases negative emotional states, we measured 22-kHz ultrasonic vocalizations (USVs) emitted by rats after ALFUS stimulation (Fig. 1e). Rats emitted 22-kHz USVs in response to ALFUS, which was about threefold greater during ALFUS

stimulation than under control conditions before ALFUS (Pre vs. Post-ALFUS; paired t test, $p = 0.001$; Fig. 1f).

ALFUS Elicited Stress Responses

To examine whether exposure to ALFUS also causes stress responses, systolic blood pressures (BP) and serum corticosterone levels were measured in rats following ALFUS exposure (Fig. 2a, b). Twenty minutes after exposure to ALFUS, systolic BP began to significantly increase by about 149 ± 5 mmHg, compared to the control group (Con; white noise exposed), and the elevated BP was maintained during the exposure to ALFUS (two-way repeated ANOVA: group factor $F = 99.414$, $p = 0.002$; time factor $F = 4.297$, $p < 0.001$; interaction $F = 3.936$, $p = 0.001$; Fig. 2c). In blood samples collected after the last measurement of BP, serum corticosterone levels in the ALFUS group increased significantly compared to controls (Con, no ALFUS), suggesting the induction of stress responses by ALFUS (Con vs. ALFUS; t test, $p < 0.05$; Fig. 2d).

ALFUS Enhanced Neuronal Activity of Central Amygdala

To explore if ALFUS activates neurons in the central amygdala (CeA), a key brain region for emotional processing [1, 17], in vivo extracellular single-unit recordings were performed in the CeA (Fig. 3A, B) in rats ($n = 8$). CeA neurons had spontaneous basal firing rates of 2.49 ± 0.21 Hz, similar to what has been reported previously [26]. Once exposed to ALFUS, the firing rates of CeA neurons significantly increased to 9.13 ± 0.59 Hz immediately after the onset of ALFUS, then it was maintained during 10 min of stimulation and quickly returned to baseline after the offset of ALFUS (one-way ANOVA; $F(2,21) = 99.545$, $p < 0.001$; Fig. 3C, D). To further confirm the activation of CeA neurons by ALFUS stimulation, we examined c-Fos expression in the CeA following ALFUS in a separate group of animals. The number of c-Fos–positive cells in the CeA increased about eightfold after treatment with ALFUS (Con, 2.19 ± 0.75; ALFUS, 17.8 ± 1.26; t test, $p < 0.05$; Fig. 3E, F). To examine if GABAergic neurons, the most abundant cell type in CeA [29], are excited by ALFUS, immunostaining for c-Fos and GAD67 was performed. Many c-Fos/GAD67 double-labeled neurons were found in the CeA of ALFUS-treated rats (Fig. 3G1–3). Taken together, the results showed the activation of CeA neurons by ALFUS.

To evaluate if the increase of negative emotional states by ALFUS is associated with CeA activation, CeA was destroyed by bilaterally injections of ibotenic acid 7 days prior to recording (Fig. 3H3), and the numbers of 22 kHz USVs following a 30-s stimulation of ALFUS were measured for another 4 min (Fig. 3H1). ALFUS stimulation
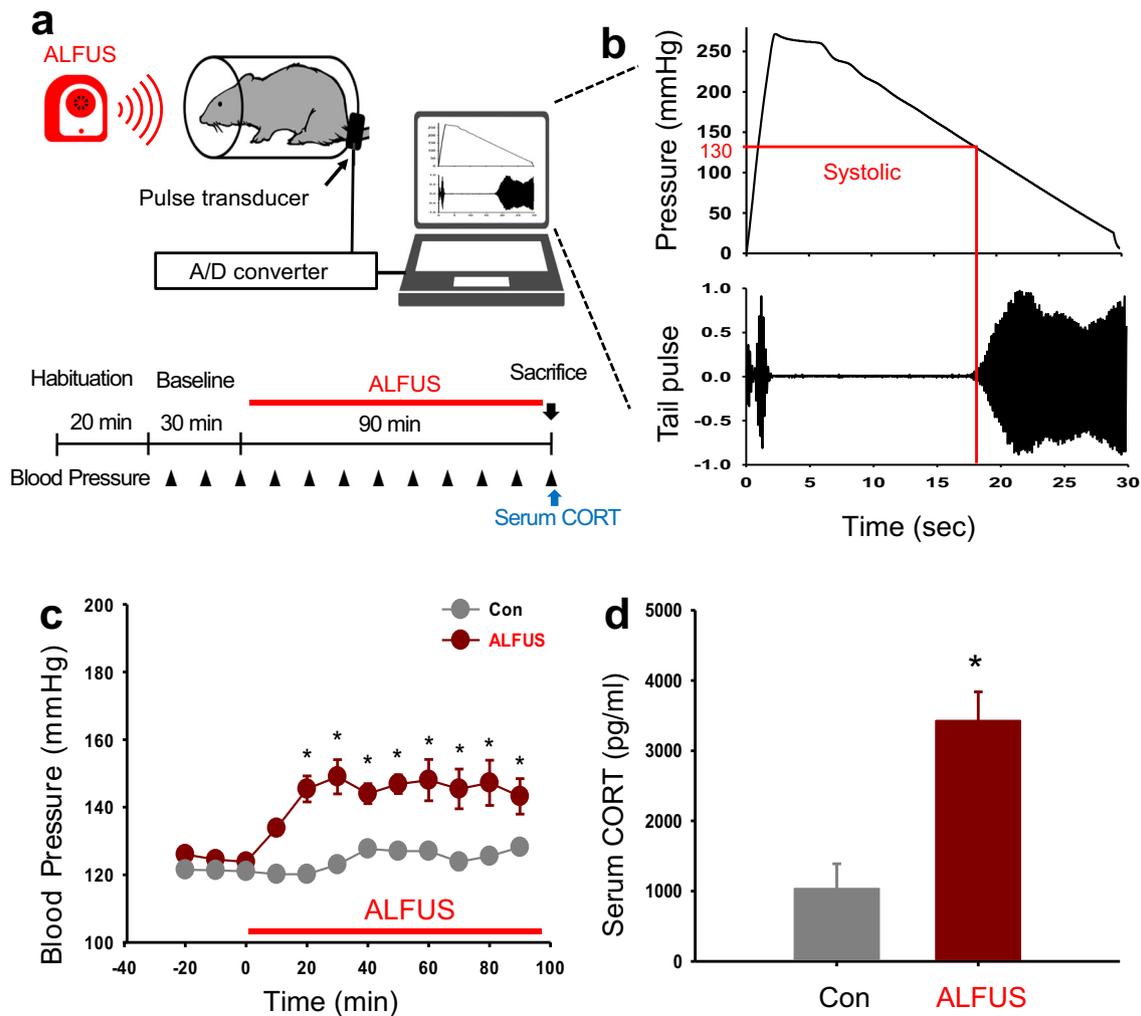
Fig. 2 *Effect of artificial low-frequency ultrasound on systolic blood pressure and serum corticosterone levels.* **a–c** Effect of exposure to artificial low-frequency ultrasound (ALFUS) on systolic blood pressure. Schematic for experimental procedure (**a**). A representative example for measurement of systolic blood pressure. The systolic blood pressure was recorded when the first pulse in the tail (**b**, lower) detected via pulse transducer during gradual reduction of the cuff pressure (**b**, upper).

ALFUS significantly increased systolic blood pressure 20 min after exposure to ALFUS ($n = 5$–6/group; **c**). Con vs. ALFUS, $*p < 0.05$. **d** Change of serum corticosterone levels following ALFUS exposure. The rats given ALFUS revealed increased corticosterone levels in serum, compared to control rats (Con; t test, $*p = 0.002$, $n = 5$/group). CORT, corticosterone

failed to increase the number of 22-kHz USVs per minute in the CeA-lesioned rats, compared to the values before ALFUS stimulation (Pre; Fig. 3H2). There was no significant difference in the numbers of basal 22-kHz USVs before ALFUS in ibotenic acid-injected (Pre, Fig. 3H2) vs naïve rats (Pre, Fig. 1f), suggesting the mediation of CeA in ALFUS-induced behaviors.

ALFUS Reduced Extracellular Dopamine Release in Nucleus Accumbens

To explore whether ALFUS affects NAc DA release and thus leads to negative emotions like anxiety, we measured electrically evoked DA release in the NAc of anesthetized

rats using in vivo FSCV (Fig. 4a). Voltammetric recordings in NAc revealed a stable baseline in response to electrical stimulation of the VTA, corresponding to about 8.82 ± 1.55 nA. Figure 4b, c shows a representative voltammogram following exposure to either ALFUS or white noise (Con). When exposed to ALFUS, the NAc DA release rapidly dropped to 7.98 ± 1.82 nA (about 13% from the values of pretreatment) and remained low during stimulation (two-way repeated ANOVA: group factor $F = 26.908$, $p = 0.004$; time factor $F = 0.324$, $p = 0.938$; interaction $F = 1.156$, $p = 0.352$; Fig. 4d). When compared to control group (Con), ALFUS significantly decreased MFB-stimulated DA release in the NAc (Con vs. ALFUS; t test, $p = 0.002$; Fig. 4e).
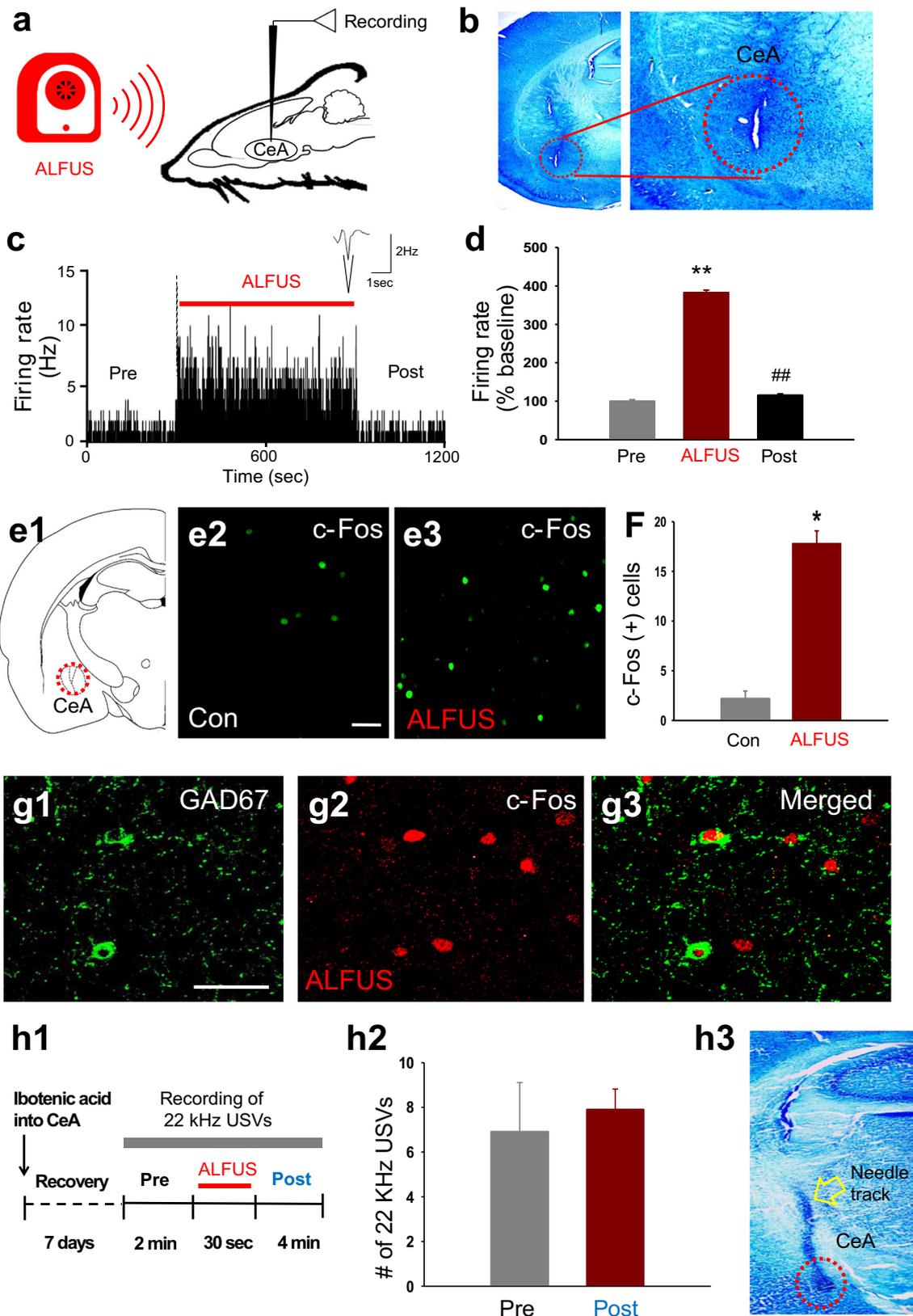
◀ **Fig. 3** *Effect of artificial low-frequency ultrasound on neuronal activation in central amygdala*. **A** Schematic for in vivo extracellular recordings in CeA (central amygdala) during artificial low-frequency ultrasound (ALFUS) stimulation. The effects of ALFUS on the spontaneous activity of CeA were investigated in eight neurons from eight rats. **B** Histological verification of recording locations. The section was stained with toluidine blue after termination of experiment. **C**, **D** In vivo extracellular single unit recording in CeA. Peristimulus time histogram (**C**) and mean values of firing rates/sec before (Pre), during (ALFUS) and after (Post) ALFUS, expressed as percentage of the pretreatment values (Pre) (**D**). Single-unit activities of central amygdala neurons were evoked during ALFUS stimulation. **p < 0.001 vs. Pre; ##p < 0.001 vs. Post. **E**, **F** Immunohistochemistry for c-Fos (green) expression in CeA. Increased numbers of c-Fos–positive cells in CeA (**E1**) are seen in ALFUS-treated group (n = 5; **E3**, **F**), compared to untreated naïve group (**E2**, Con; n = 5). Con vs. ALFUS, t test, *p < 0.001, Bar = 20 μm. **G** Immunofluorescence double-staining of c-Fos and GAD67 in CeA. Double-labeled cells with GAD67 (green; **G1**) and c-Fos (red; **G2**) were observed in CeA. Bar = 20 μm. **H** Effect of CeA lesion on 22-kHz ultrasonic vocalizations (USVs) following ALFUS stimulation. Experimental procedure (**H1**). The numbers of 22-kHz USVs per minute before (Pre) and after 30-s stimulation of ALFUS (Post) in the CeA-lesioned rats (n = 6; **H2**). Toluidine blue staining of injection site of ibotenic acid (**H3**). A needle track is seen

ALFUS Reinstated Cocaine-Seeking Behaviors after Extinction

During the last 3 days of cocaine self-administration, active and inactive lever presses were about 55.9 ± 6.3 and 9.9 ± 1.2, respectively (data not shown). The rats were then subjected to daily 2-h extinction sessions over 2–3 weeks (about 18 days; Fig. 5a), during which the number of active lever presses steadily decreased and eventually stabilized to less than 10 lever presses per session (Fig. 5b). On the other hand, the numbers of inactive lever presses were not altered during extinction (Fig. 5c). When 60 min of ALFUS was applied, the rats exhibited significant increases in responding on the active lever for the 2-h session (t test, p < 0.05; Fig. 5d), but not the inactive lever (Fig. 5e), compared to their preceding 3-day baseline (Baseline). When analyzed at 1-h intervals, responding significantly increased during 60 min exposure to ALFUS (early 1-h: Baseline, 3.4 ± 0.51; ALFUS, 13.6 ± 3.83; one-way ANOVA; $F(3,19) = 4.270$, $p = 0.021$) but tended to return to the control level after termination of ALFUS (late 1-h: Baseline, 3.4 ± 0.6; ALFUS, 7.4 ± 2.54; Fig. S1). In another set of animals, ALFUS did not affect cocaine self-administration (Fig. S2).

Discussion

ALFUS increased anxiety-like behavior in the EPM paradigm and enhanced blood pressure and corticosterone levels in serum of naïve rats. ALFUS stimulation activated central amygdala neurons with in vivo extracellular recordings and amygdalar GABAergic neurons on c-Fos/GAD67 double-

immunolabeling. The increase of 22-kHz USVs by ALFUS was prevented by chemical lesion of the CeA. Dopamine levels in NAc decreased during ALFUS stimulation. In rats self-administering cocaine, ALFUS produced reinstatement of cocaine seeking after a period of extinction. Our findings suggest that ALFUS serves as a stressful unpleasant sound in rats and induces negative emotional states in association with a decrease in mesolimbic DA function and elicits a reinstatement of drug-seeking behaviors in this rat model of cocaine reinstatement. This is the first study to demonstrate that unpleasant sound induces drug relapse and suggest a mechanism by which amygdalar activation by these sounds reinstates drug-seeking behaviors.

ALFUS Serves as an Unpleasant Sound and its Acoustic Stimulation Induces Negative Emotions-Associated Responses in Rats

Adult rats produce two different types of ultrasonic vocalization (USV): low and high frequency USV. Low-frequency USV in 18–32 kHz range ("called as 22 kHz USV") are emitted by rats as a result of negative emotional states, under various distress conditions such as aversive stimulations, conditioned fear stress, or foot-shock stimulation [30, 31]. In turn, playback of the recorded USV induces fear- and anxiety-related behaviors and social transmission of fear in rats [32, 33]. In the present study, the ultrasonic audio stimuli were in the frequency ranges of 22–38 kHz, similar to those of the 22-kHz USV emitted by rats under negative emotional states [31]. The rats exposed to the ALFUS spent less time in the open arms of the elevated plus maze and emitted 22-kHz USVs, indicating expression of anxiety and negative emotion states [21]. Furthermore, ALFUS induced stress responses, as indicated by elevation of systolic blood pressure and serum corticosterone levels [34]. Previous studies have suggested a close relationship between stress and negative emotions [35, 36]. For example, stress increases the symptoms of anxiety in a study on 130 women [36]. Perceived stress was positively correlated with anger in high school students [35] and seriously affected by anger and depression in cancer patients [37]. Our data suggest that ALFUS generates negative emotional states associated with stress in rats. It is further supported by the present data showing excitation of central amygdala neurons and elevated corticosterone levels by ALFUS. Considerable evidence indicates that amygdala plays a critical role in the processing of negative emotions in response to unpleasant sounds. Functional magnetic resonance imaging studies have shown an interaction between brain areas involved in auditory processing (e.g., auditory cortex) as well as in the amygdala, which is active in the processing of negative emotions when subjects are subjected to
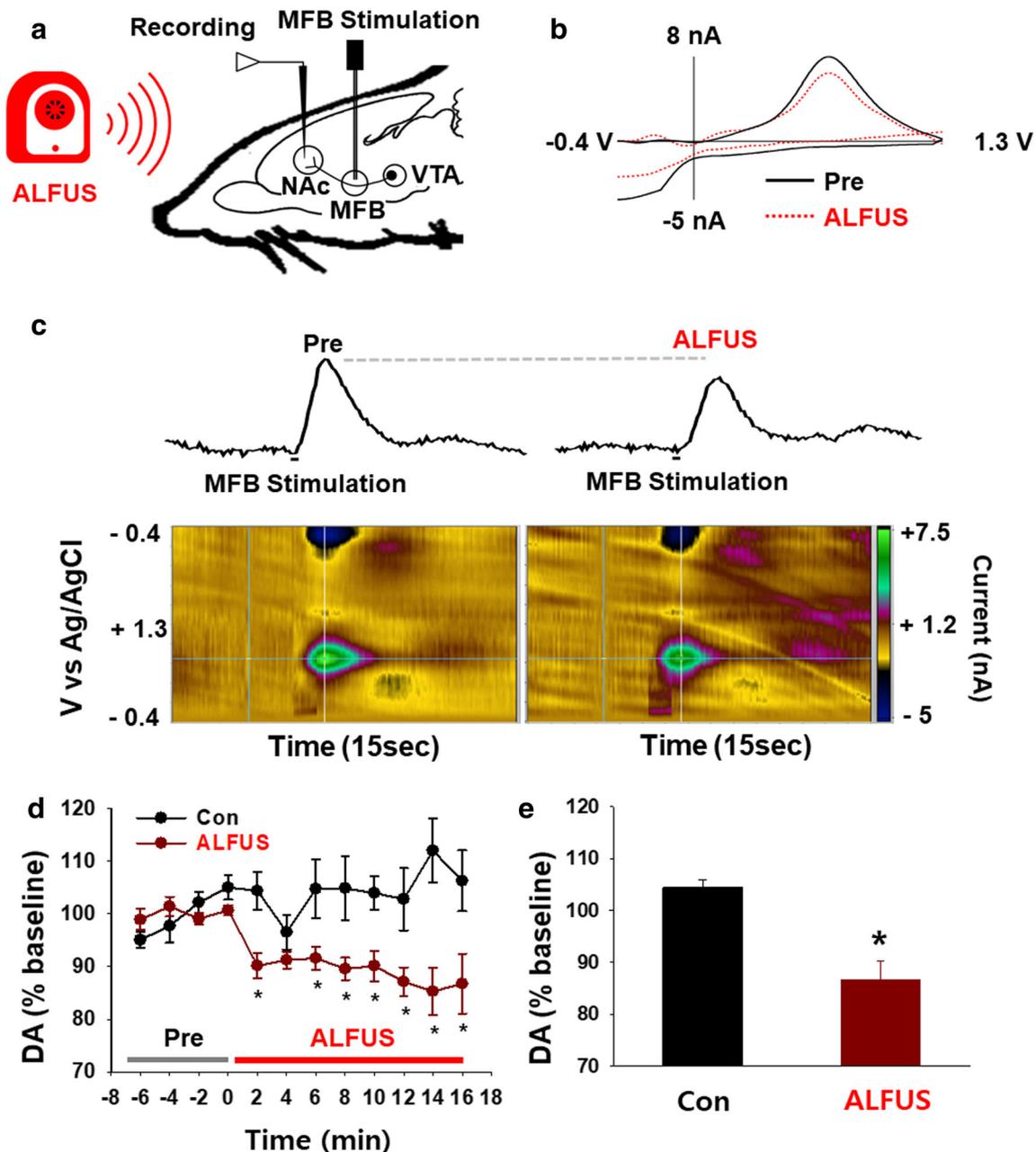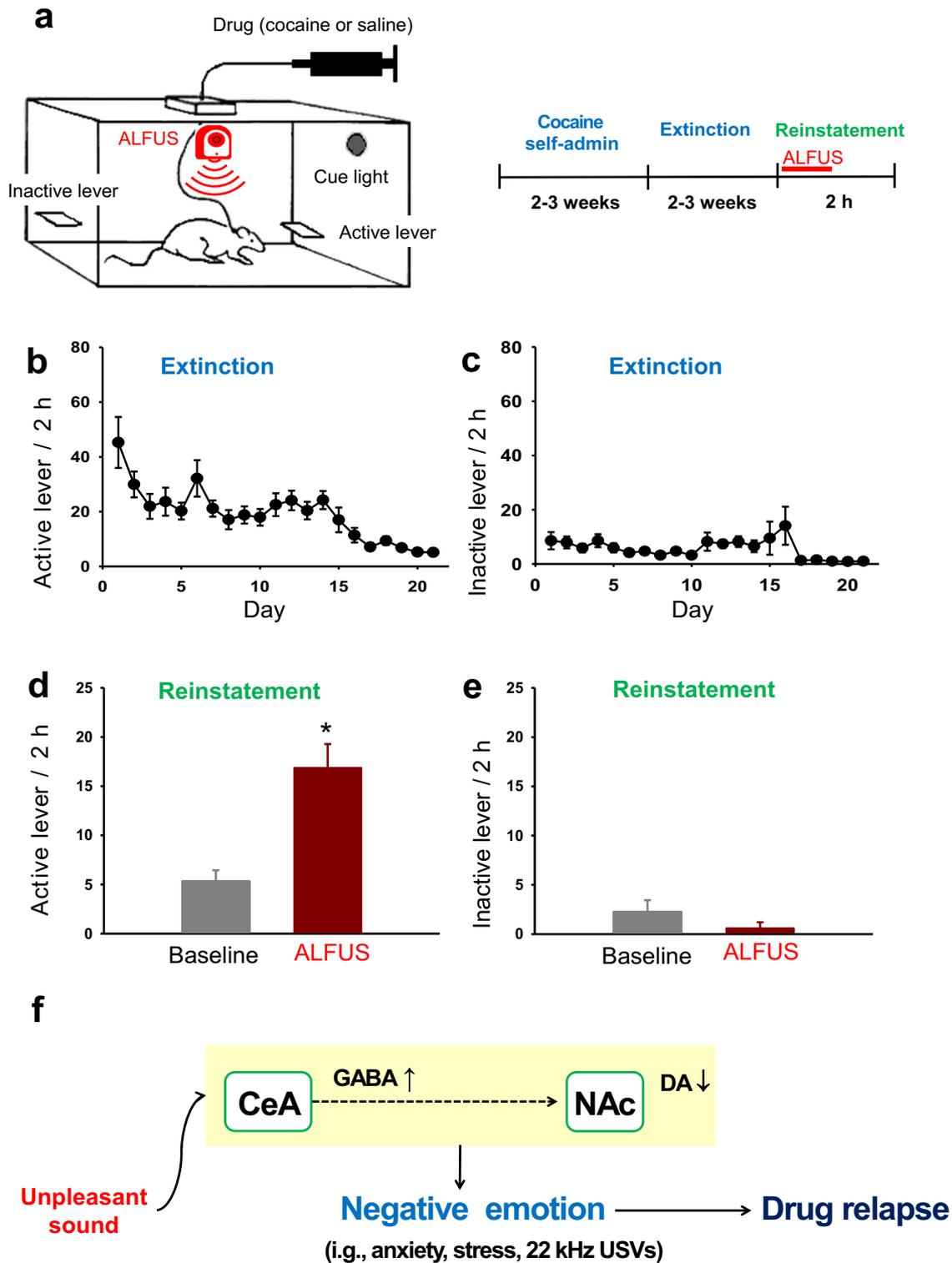
Fig. 4 *Artificial low-frequency ultrasound induced reduction of electrically evoked-DA release in the NAc by ALFUS.* **a** Schematics of in vivo fast-scan cyclic voltammetry (FSCV) for real-time measurement of DA release in NAc. MFB, medial forebrain bundle. **b**, **c** Representative voltammograms (current versus voltage plots; **b**) and pseudo-color graphic images (**c**) before (Pre) and during artificial low-frequency ultrasound stimulation (ALFUS). **d**, **e** Effect of ALFUS on electrically evoked-DA release in the NAc. Extracellular DA levels in the NAc significantly decreased during ALFUS stimulation, compared to those of pretreatment (Pre, $n = 8$) and control (Con, $n = 5$; **d**). Two-way repeated ANOVA, $*p < 0.05$ vs. Con. The mean values of dopamine levels during ALFUS (16 min) are expressed as percentage of baseline (Pre) (**e**). *t* test, $*p = 0.002$ vs. Con

unpleasant sounds [1, 2]. Corticotropin-releasing factor (CRF), released during stress, is enriched in the CeA and an infusion of the CRF into NAc causes anxiety-like behavior in rats [38]. In addition, ALFUS increased 22-kHz USVs, known to be emitted by rats under negative emotional states [31], and the increase was abolished by chemical lesion of the CeA, suggesting that ALFUS induced negative emotions and stress responses via activation of the CeA.

In the present study, ALFUS reduced DA release in NAc during ALFUS stimulation. It is well known that DA plays a crucial role in reward and euphoria; elevated DA activity in the mesolimbic systems leads to the experience of pleasure, while decreased dopaminergic activity is associated with

Fig. 5 *Effect of artificial low-frequency ultrasound stimulation on cocaine-seeking behavior after extinction.* **a** Schematic of cocaine self-administration (left) and experimental procedure (right). Cocaine self-admin, cocaine self-administration. **b**, **c**. The numbers of active (**b**) and inactive (**c**) lever presses during the extinction session. The active lever response was steadily decreased during the extinction session. **d**, **e** Effect of artificial low-frequency ultrasound (ALFUS) stimulation on active and inactive lever presses in cocaine relapse model. Exposure to ALFUS significantly increased active lever responses (**d**), but not inactive lever presses (**e**), compared to the preceding 3-day baseline (Baseline). Baseline vs. ALFUS, *$p = 0.025$, $n = 9$/group. **f** Overall hypothesis. Unpleasant sound increases negative emotional states by activating amygdalar GABAergic neurons and subsequent decrease of NAc dopamine release, which leads to drug relapse. CeA, central amygdala; NAc, nucleus accumbens; DA, dopamine; 22-kHz USVs, 22-kHz ultrasonic vocalizations in rats

unpleasant feelings and dysphoria [8, 39]. Our present data showing that ALFUS lowered mesolimbic DA release suggest that ALFUS serves as an unpleasant stimulus and can induce negative emotions experimentally in rats via emotional processing of brain regions including the amygdala.

Unpleasant Sounds Reduce DA Release and Reinstate Drug-Seeking Behaviors

Negative emotional states as well as stress drive drug seeking and promote relapse in addiction [8]. In support of this, pronounced elevations of negative emotions are found in cocaine addicts [40]. Chronic depression increases the risk of relapse during and after treatment in alcohol-dependent patients [41], and rapid increases in negative affect tend to precede nicotine relapse [42]. Exposure to such stressors as foot-shock stimulation and social defeat stress results in an increase of cocaine self-administration behavior [43] and induces reinstatement of heroin- or cocaine-seeking behaviors in rats [44, 45]. In the present study, ALFUS stimulation evoked stress responses and negative emotional states (i.e., enhanced anxiety and 22-kHz ultrasonic vocalizations) and produced reinstatement of drug-seeking behaviors during cocaine withdrawal in self-administering rats. While previous studies have suggested a possible association between the use of drugs and unpleasant sounds [14, 15], our findings provide direct experimental evidence that unpleasant sounds induce a stress response and negative emotion and promote relapse to drug seeking. While the neural circuit involved in drug relapse by unpleasant sounds remains elusive, our study further shows activation of CeA GABAergic neurons by ALFUS. The CeA, a part of extended amygdala, acts as an integrative hub for converting emotion-relevant sensory information (i.e., emotive auditory stimuli and stressful stimuli) into behavioral and physiological responses [1, 17]. Previous auditory fear conditioning experiments have revealed that the auditory signals are relayed to the amygdala from thalamic and cortical regions of the auditory systems and then transmitted to CeA, thereby leading to the acquisition and expression of fear from negative emotions [46, 47]. The CeA is also known to play a crucial role in cocaine addiction. Activation of CeA intensifies motivation to take cocaine or induces cocaine craving, while optogenetic or chemical inhibition of CeA suppresses cocaine intake [48]. The CeA contains 95% GABAergic medium-sized neurons and modulates mesolimbic DA transmission, which regulate anxiety-like behaviors as well as drug craving [8, 29, 49], suggesting that unpleasant sounds like ALFUS elevated stress and negative emotional states via increased GABA transmission from the central amygdala and subsequent decrease of NAc DA release, which results in reinstatement of cocaine-seeking. Also, it is known that basolateral amygdala (BLA) responds to ultrasonic vocalization of 22 kHz, mediates many

features of emotional responses to sensory stimuli, and plays an integral role in the pathophysiology of addiction [50–52]. Given that the BLA sends dense glutamatergic projections to the CeA [17], the actions of ALFUS may be associated with the BLA-CeA pathway.

Dopamine neurons in the VTA are responsive to aversive stimuli and stress, although there is a conflict on the direction of DA neuron responses by which aversive stimuli elevates or inhibits DA signaling under certain conditions [53, 54]. In support of our study, electrophysiological and electrochemical studies have shown that the activity of DA neurons in the VTA is reduced rapidly in response to aversive stimuli. This reduction in DA activity is mediated by stress response mediators such as CRF [54, 55]. Given that ALFUS induced a stress response and decreased NAc DA release, it is probable that NAc DA release by unpleasant sound such as ALFUS is associated with stress mechanisms in rats. As another possible explanation for DA reduction by ALFUS, a complex circuit of CeA-VTA may be considered. It has been proposed that the CeA could regulate NAc DA release via a GABAergic projection to the VTA [56]. The CeA is composed most of local and projecting GABAergic neurons and anatomically divided into medial (CeM) and lateral (CeL) division of CeA, with substantial unidirectional connections between the CeL and the CeM. The primary output of the CeL to CeM is also GABAergic. Axons from CeA neurons synapse onto a local GABAergic interneuron network in the VTA [57]. Therefore, activating axons from the CeL could inhibit the CeM via GABA release, resulting in disinhibition of VTA GABA neurons and thus inhibition of DA neurons projecting to the nucleus accumbens [56]. In the present study, ALFUS activated CeA GABAergic neurons and attenuated electrically evoked DA release in the NAc in rats. We propose that ALFUS activates local GABAergic neurons in CeL and disinhibits VTA GABA neurons, which results in decreased DA release in the NAc.

Previous studies have shown that stressful events such as foot shock increase DA release in the NAc and induce a robust relapse of drug-seeking behaviors [8, 43]. In contrast, the present data suggested that ALFUS causes a mild relapse of cocaine-seeking behaviors by decreasing NAc DA release. Similar to our results, a recent study using fast-scan cyclic voltammetry in freely moving animals demonstrated that aversive stressful stimuli by quinine as a bitter taste rapidly reduce DA signaling and have mild reinstating effects on cocaine-seeking behaviors, which is reversed by blocking CRF [54]. It suggests that ALFUS may recruit similar mechanisms as that of aversive stimuli by quinine, but different from that of foot shock. In this study, it is also questionable how DA reduction by ALFUS promotes relapse to drug seeking. It was reported that reduced DA activity is found in drug abusers and associated with a greater likelihood of relapse [58]. As NAc DA levels decrease, animals resume cocaine self-

administration and titrate cocaine intake to maintain the desired DA level in brain [59, 60]. We assume that ALFUS would lower NAc DA level by inducing negative emotion in rats and the animals might reinstate cocaine seeking to avoid a state of the lowered DA in the NAc. Future studies will be necessary to verify the brain circuitry involved in modulation of DA signaling by aversive stressful stimuli.

In conclusion, the present study revealed that ALFUS induced negative emotions as well as stress response, activated central amygdala, lowered DA release in the NAc, and reinstated cocaine-seeking behaviors in rats. These findings suggest that unpleasant sounds can elicit drug relapse in addicts by enhancing negative emotion states (Fig. 5f).

Compliance with Ethical Standards

Conflict of Interest   The authors declare no competing financial interests.

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