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Original Article

The effect of acupuncture stimulation on alleviating emotional changes due to acute alcohol administration and the possibility of sigma$_1$ receptor involvement

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ABSTRACT

Background: Most ETOH addiction preclinical studies have focused on the rewards of chronic ETOH self-administration or the ETOH reinstatement model. Acute ETOH administration studies are scarce despite the potential of ETOH to cause sedation, intoxication and reduced acute functional tolerance. Here, we established a rat model of acute ETOH administration induced by an intraperitoneal injection of 1 g/kg ethanol and assessed the similarities in physiological and behavioral effects between acupuncture and Sigma1 R antagonists.

Methods: Male Wistar rats (300–330 g) received pretreatment with (1) saline injection, (2) saline + mechanical stimulation using a mechanical acupuncture instrument (MAI) for acupuncture at the Shenmen (HT7), (3) ETOH (1 g/kg) injection, (4) ETOH + HT7, or (5) the selective σ_1 R antagonist BD 1047 (3, 10, or 30 mg/kg, intraperitoneal (IP) injection). ETOH (1 g/kg) or saline was IP injected after 10 min. Then, ETOH-induced immobility was evaluated in an open field arena, ultrasonic vocalizations (USVs) indicating ethanol-induced emotional changes were recorded in a recording chamber, and the rats were sacrificed for the analysis of protein levels of σ_1 R in several regions of the brain.

Results: Acute ethanol exposure increased the immobile time, 22-kHz USVs, and protein levels of σ_1 R in the ventral tegmental area (VTA). However, pretreatment with acupuncture at HT7 induced recovery of immobile time, reduced 22-kHz USVs, and regulated the protein levels of σ1 R in the VTA. These effects have similarities with IP injection of BD 1047 (10 mg/kg).

Conclusion: This study showed that acupuncture at HT7 regulates immobility and 22-kHz USVs via Sigma$_1$ R in the VTA upon acute ETOH exposure.

1. Introduction

According to the World Health Organization,[1] the number of fatalities due to ethanol intoxication is 3.3 million worldwide. Ethanol, similar to cigarettes, is a high-demand commodity throughout the world and an addictive substance that causes many problems in society. A low dose of ethanol can be a psychological stimulant, but a high dose causes depression and sedation and suppresses or impairs motor activity.[2] Ethanol users commonly have comorbid affective disorders such as depression and anxiety.[3] As many as 80% of alcoholics complain of depressive symptoms at some time in their lives.[4] Most ETOH addiction preclinical studies have focused on the rewards of chronic ETOH self-administration or the ETOH reinstatement animal model. Acute ETOH administration studies are scarce despite the potential of ETOH to cause sedation, intoxication and reduced acute functional tolerance.

Acupuncture has been widely used for over 2500 years across Asia, and its effects have been proven in clinical studies. Among acupuncture points, the Shenmen (HT7) acupuncture point is the source point of the heart meridian and has frequently been used to treat mental disorders, including drug addiction, anxiety, and depression.[5,6] In the ETOH self-administration rat models, the application of acupuncture at HT7 reduces lever pressing.[7] Stimulation of HT7 is known to regulate the activity of GABAergic neurons of the ventral tegmental area (VTA), and the mechanism of ethanol is also thought to be related to this area.[7] According to

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recent studies, HT7 stimulation attenuates anxiety-like behavior during ethanol withdrawal through regulation of the neuroendocrine system and amygdaloid neuropeptide Y reversals.[9,10] In addition, HT7 simulation modulating mesolimbic DA release can be regulated through the endorphinergic input of NAc in the alcohol self-model.[11] Furthermore, in a recent follow-up study these NAc endorphinergic inputs were found to be derived from the arcuate nucleus.[12]

Sigma receptors include two subtypes of receptors: $Sigma_1$ and $Sigma_2$ receptors. In particular, the $Sigma_1$ receptor ($Sigma_1$ R) has been shown to play a role in learning and memory, responses to stress and depression, psychostimulant-induced sensitization, and vulnerability to addiction.[13] The activity of $Sigma_1$ R has been observed upon ethanol treatment in the rodent brain.[14] The regional distribution of $Sigma_1$ R determined in rats using immunohistochemistry showed moderate-to-intense staining in most dopaminergic neurons, including neurons in the VTA, the nucleus accumbens (NAc), the amygdala (Amg), and the prefrontal cortex (PFC).[15] However, studies on the effects of $Sigma_1$ R activation and acupuncture treatment on acute ETOH administration are insufficient.

Ultrasonic vocalizations (USVs) emitted by rats are considered to be a reflection of their real-time emotional state and are widely accepted as animal models of affect. Rodents emit USVs in the 50–55 kHz and 22–28 kHz ranges, which are reliably associated with positive and negative emotional states, respectively.[16–18] According to recent ethanol addiction research, escalated levels of ETOH consumed by ETOH-dependent rats are significantly correlated with ETOH anticipatory 50–55 kHz FM USVs, and ETOH-dependent rats in a state of withdrawal are more easily provoked to emit negative affect-associated 22–28-kHz USVs in response to mildly aversive stimuli.[17–19] In the acute ETOH model, studies have focused on behavioral changes such as locomotor activity and FST, but few studies have evaluated the changes in real-time emotional state through USV analysis.

Here, we established a rat model of acute ETOH administration induced by an intraperitoneal injection of 1 g/kg ethanol and assessed the similarities in physiological and behavioral effects between acupuncture and $Sigma_1$ R antagonists. This present study aimed to investigate whether acupuncture at HT7 and treatment with the selective $Sigma_1$ R antagonist BD 1047 would (1) block ethanol-induced immobility, (2) attenuate ethanol-induced negative or positive emotional states, and (3) regulate the ethanol-induced increases in protein levels of $Sigma_1$ R in several regions of the rat brain.

2. Methods

2.1. Animals

Adult male Wistar rats weighing 300−330 g were used. After delivery from the supplier, rats (3–4/cage) were housed in standard polycarbonate cages in a vivarium at a controlled temperature of 21−23 °C and humidity of 45–60% on a 12:12 h light/dark cycle (lights on at 07:00). Food and water were freely available except during the experiments. All experiments were conducted in accordance with the Animal Care and Use Committee at the Korea Institute of Oriental Medicine (KIOM) with reference number #18-076.

2.2. Mechanical acupuncture instrument stimulation

A mechanical acupuncture instrument (MAI) was able to apply quantitative mechanical stimulation consistently compared with manual acupuncture and significantly reduced the time for stimulation compared with electroacupuncture. Since the MAI was developed to mimic the vibrations produced by manual acupuncture stimulation, it has been applied in a number of oriental medicine studies.[8,20,21] The MAI was developed by Daegu Hanny University and KIOM in the Republic of Korea.[8] The device comprises a cell phone vibrator (MB-0412V or MB-1203V, Motor Bank) attached with an alligator clip to an acupuncture needle. The MAI was used at an intensity of 1.3 m/s^2 and a frequency of 85 Hz to apply vibration to the needle. HT7 is located transversely on the wrist of the forepaw radial to the tendon of the flexor carpi ulnaris muscle (Sup. 1).

2.3. Pretreatment and post-injection procedure

Preliminary experiments confirmed that anesthesia did not affect immobility and USVs (Sup. 2). Based on this result, we administered anesthesia (3% isoflurane in a mixture of N_2O/O_2 gas) on the acupuncture-stimulated rats to stimulate the correct acupuncture points for the correct time. In the case of other groups without acupuncture (Sal, ETOH, BD 1047), IP injection was performed after handling. The acupuncture group received bilateral acupuncture (0.18 mm diameter, 20 mm length, Dongbang Medical) at HT7 or at a non-acupuncture point at a depth of 3 mm from the skin surface and underwent vibration of the acupuncture needle with the MAI for 30 s, after which the placement of the needle was maintained for an additional 1 min. After 10 min, the rats received IP injections of ETOH (1 g/kg, 16 w/v, Merck Millipore) or saline (0.9% NaCl, vehicle). The volume of injected ETOH was 2.38–2.61 mL per rat.

2.4. Open field test (OFT)

Rats were placed individually in a rectangular test arena (600 × 600 × 300 mm, high) for 40 min for habituation. After the 40-min habituation period in the test arena, the rats were subjected to the pretreatment and post-injection procedures. They were then positioned at the center of the test arena and tracked for 40 min during the test session. The arena was cleaned with 70% ETOH and water after each session. Video tracking was incorporated using Smart 3.0 video tracking software.

2.5. Ultrasonic vocalizations (USVs)

The emission of USVs by the subject animal was recorded by an ultrasound gate condenser microphone CM16/CMPA (Avisoft Bioacoustics, Berlin, Germany), which is sensitive to frequencies between 10 and 200 kHz. This apparatus was mounted 45 cm above the observation cage and connected to a computer with the recording software Avisoft Recorder (Avisoft SASLab Pro Version 3.8; R. Specht, Berlin). Experiments were performed under 40 l× illumination, with USVs being recorded for 60 min immediately after the injection of ETOH or stimulation of HT7. To decrease the aversiveness of the test situation and facilitate the detection of USV, the observation cage contained fresh bedding. Analysis of the USVs emitted by the experimental rat was made afterwards using Avisoft SAS Lab Pro software (version 4.34; Avisoft Bioacoustics). For the manual counting and classification of USVs, spectrograms from the recordings were generated at a sampling frequency of 250 kHZ, frequency resolution of 244 Hz, fast Fourier transform-length of1024 points, and a time resolution of 0.512 ms. USVs of 22 kHz are calls in the 18–32 kHz range of frequency emitted either in bouts or individually. The separation between one call and another was defined by an inter-call interval of at least 190–320 ms, which is the time for inhalation between two voiced calls. Then, 50 kHz USVs were filtered with 40∼70 kHz and 30 ms; their classification into subtypes was based on their spectrographic shapes, and they were

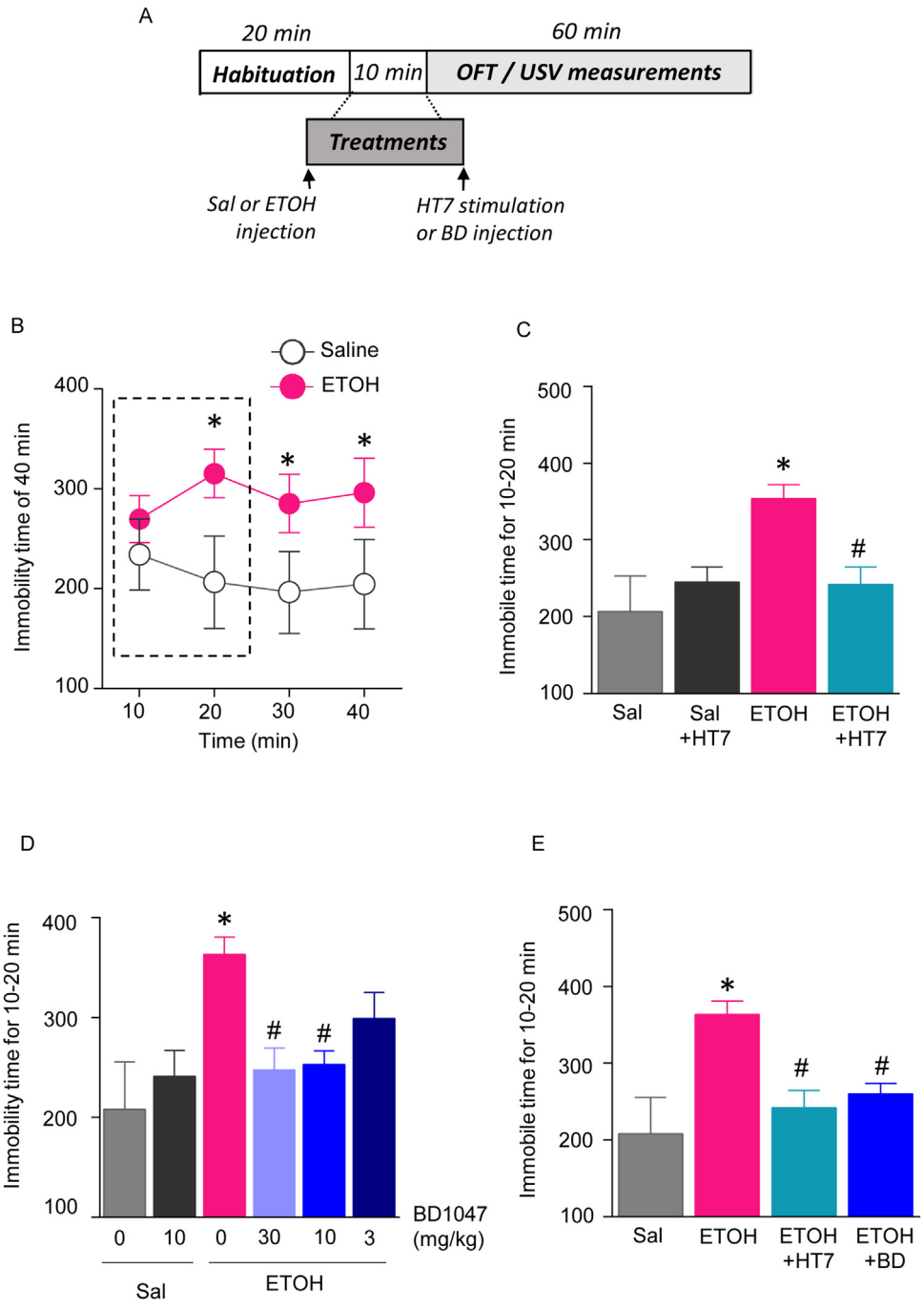

Fig. 1. Effects of acupuncture stimulation and BD 1047 treatment on immobility behavior. Experimental schedules used in this work (A). Quantification of the total immobile time during the 40-min period after acute ETOH injection (n = 12 for each group) and the time course of the immobile time over 10–20 min (n = 8 for each group) (B). Quantification of the total immobile time during the 10–20 min period after BD1047 injection (n = 8∼10 for each group). (C). Quantification of the total immobile time between the HT7 stimulation and BD1047 injection groups (n = 8∼10 for each group) (D). The data were analyzed using a repeated measures ANOVA followed by Tukey's test. $^{*}p < 0.05$ vs. Sal group; $\#p < 0.05$ vs. ETOH group. Values are expressed as the mean ± SEM.

deemed flat when the peak frequency changes within a single call were equal or lower than 5 kHz [22] After habituation for 20 min in the recording chamber, the baseline session was recorded for 20 min; following the pretreatment and post injection procedures, the treatment session was recorded for 40 min (Sup. 3).

2.6. Western blot

Rats were sacrificed by decapitation 20 min after they underwent the pretreatment and post-injection procedures. The brains were removed and sectioned coronally in a rat brain matrix, and the PFC, NAc, hippocampus (HIP), Amg and VTA regions were dissected out and placed on ice. Samples of the brain regions were immediately frozen by immersion in liquid nitrogen and subsequently stored at −80 °C until processing for Western blotting. Samples were homogenized with a microsonicator in lysis buffer and centrifuged at 12,000 × *g* for 20 min at 4 °C. The supernatant was transferred to new tubes and stored at −80 °C until electrophoresis. The protein concentrations of the samples were determined by the Bradford technique using a protein assay kit with bovine serum albumin (BSA, Bio-Rad) as the standard. Following protein quantification, 20 μg of protein was diluted in an equal volume with

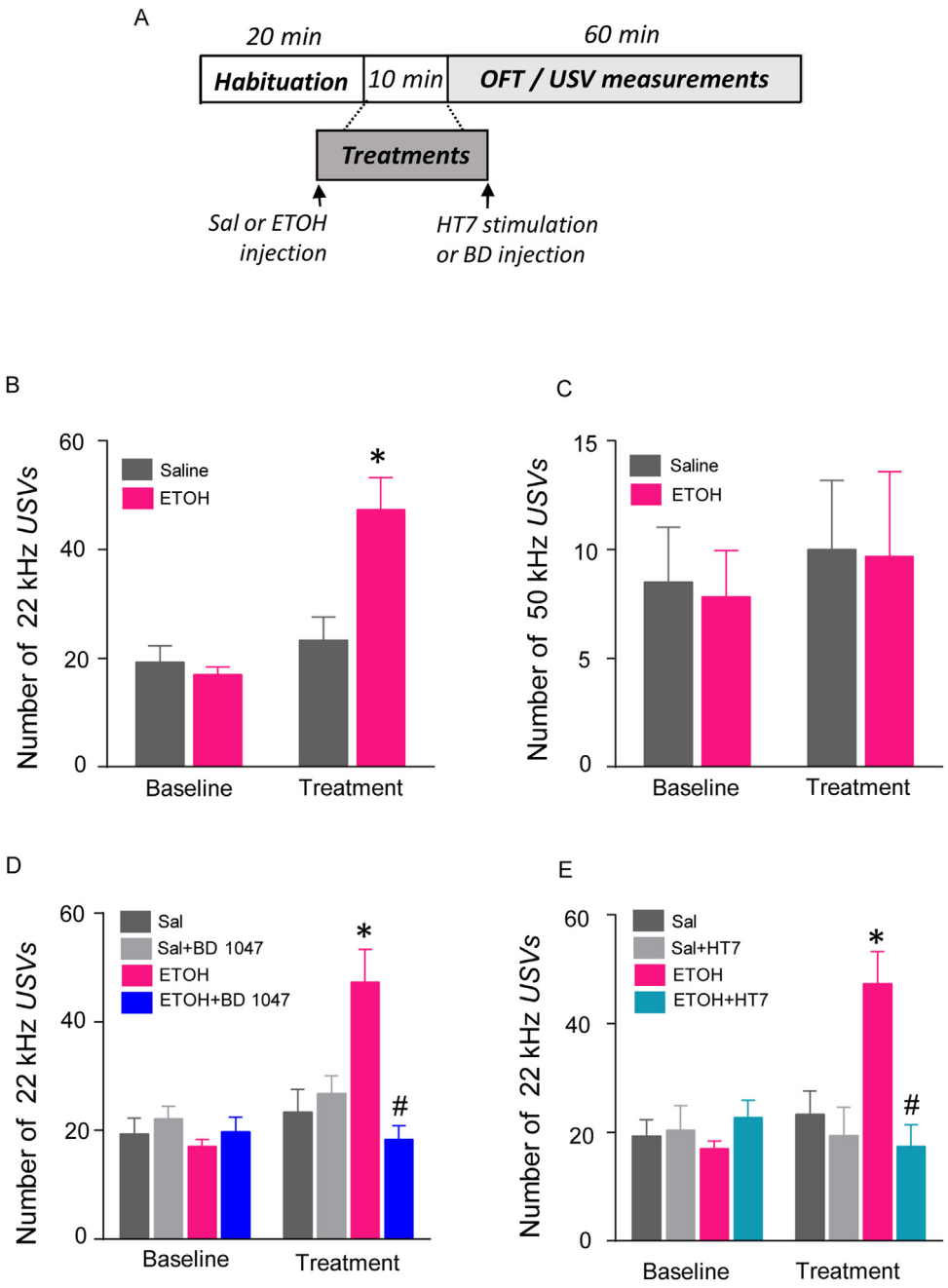

Fig. 2. Effects of acupuncture stimulation and BD 1047 treatment on USVs. Experimental schedules used in this work (A). Quantification of 22-kHz USVs associated with acute ETOH administration before and after (B). Quantification of 50-kHz USVs associated with acute ETOH administration before and after (C). Quantification of 22-kHz USVs after BD1047 injection (D). Quantification of 22-kHz USVs after HT7 stimulation (E). All sample sizes are 5~6 per group. The data were analyzed using a repeated measures ANOVA followed by Tukey's test. *$p < 0.05$ vs. Sal group; #$p < 0.05$ vs. ETOH group. Values are expressed as the mean ± SEM.

2× Laemmli sample buffer (Bio-Rad) and boiled for 5 min at 95 °C. Proteins were separated electrophoretically on Mini-protean TGX precast gels (Bio-Rad). The separated proteins were transferred to nitrocellulose blotting membranes, and the membranes were then treated for 30 min at room temperature with 5% non-fat dry milk to block nonselective binding in Tris-buffered saline-added Tween 20 (TBST). Membranes were incubated overnight at 4 °C with either anti-Sigma$_1$ R rabbit polyclonal antibody (1:500, Cat. # ab53852, Abcam) or anti-β-actin mouse monoclonal antibody (1:2000, Cat. # A5316, Sigma Aldrich). After three washes for 10 min in TBST, the membranes were incubated for 60 min at room temperature with the respective secondary antibodies (1:2000, Cat. # SC2004, SC2005, Santa Cruz) and then washed three times for 10 min in TBST. Blots were developed using the West Pico or Femto Detection System according to the manufacturer's instructions (Thermo Scientific) and analyzed by Image J software. The expression levels of target proteins are reported as percentages relative to the expression of β-actin.

2.7. Statistics

All measures (OFT, USVs, Western blot) are expressed as the mean ± SEM. OFT and USVs were analyzed using a two-way analysis of variance (ANOVA), and a post hoc analysis was performed using the Student-Newman-Keuls (SNK) and Tukey methods. Western blot data were analyzed by paired *t*-tests and one-way ANOVA or t-

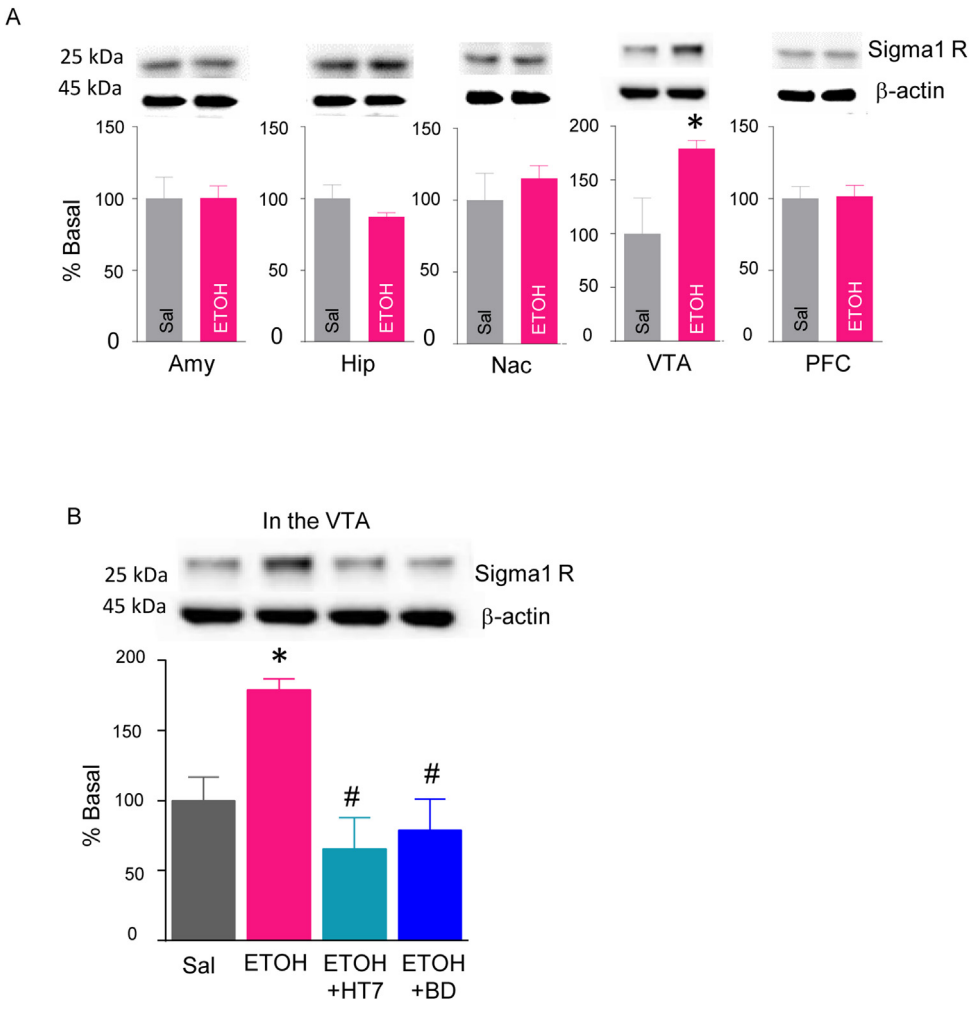

Fig. 3. Effects of acupuncture stimulation and BD 1047 treatment on the expression levels of Sigma$_1$ R.
Protein levels of Sigma$_1$ R in the PFC, NAc, HC, Amg and VTA after IP injection of ETOH or saline (A). Protein levels of Sigma$_1$ R after IP injection of HT7 stimulation and BD1047 administration in the VTA (B). All sample sizes are 4∼5 per group. The data were analyzed using repeated measures ANOVA followed by Tukey's test. *$p < 0.05$ vs. Sal group; #$p < 0.05$ vs. ETOH group. Values are expressed as the mean ± SEM.

tests followed by Tukey's honestly significant difference test using Graph Pad Prism 6 (GraphPad Software Incorporation, San Diego, CA, USA). The criterion for statistical significance was $P < 0.05$.

3. Results

3.1. Acupuncture at HT7 and BD 1047 treatment rescued ETOH-induced immobility

Post-injection of acute ETOH (1 g/kg, IP) increased the immobility time in the OFT group compared with the Sal group during the 20–40 min time period (Fig. 1A, B). In particular, the ETOH group showed significantly increased immobility time compared with the Sal group and the Sal + HT7 group, but in the ETOH + HT7 group recovery of the immobility time occurred at 10–20 min (Fig. 1C). To compare the effects of sigma receptor antagonist (BD1047) administration and HT7 stimulation, ETOH groups were pretreated with an IP injection of different doses of BD1047 (3, 10 and 30 mg/kg) The immobility time of the BD1047 10 and 30 mg/kg groups were significantly reduced compared with that in the ETOH group (Fig. 1D). As a result, the increased immobility time of the ETOH-treated group decreased with HT7 stimulation and BD1047 treatment. (Fig. 1E).

3.2. Acupuncture at HT7 and BD 1047 treatment reduced ETOH-induced 22-kHz USVs

According to the results of the USVs measured 20 min before and after ETOH treatment, 22-kHz emissions were significantly increased after ETOH treatment compared with before ETOH treatment (Fig. 2A, B). However, 50-kHz USVs were not increased compared with saline treatment for 20 min during the treatment session or compared with the baseline session (Fig. 2C). Pretreatment with an IP injection of BD 1047 (10 mg/kg) attenuated ETOH-induced 22-kHz USVs for 20 min during the treatment session (Fig. 2D). Similarly, pretreatment with acupuncture in the HT7 group significantly decreased ETOH-induced 22-kHz USVs for 20 min during the treatment session (Fig. 2E).

3.3. Acupuncture at HT7 and BD 1047 treatment regulated ETOH-induced increases in protein levels of sigma$_1$ R

We measured the level of sigma1 R expression in each region of the rat brain when immobility and 22-kHz emissions were increased due to ETOH administration. Compared with saline treatment, acute injection with ETOH (1 g/kg, IP) significantly increased the protein levels of Sigma$_1$ R in the VTA but not in the PFC, the

NAc, the Hip, or the Amg compared with saline (Fig. 3A). Pretreatment with acupuncture at HT7 or IP injection of BD 1047 (10 mg/kg) reduced ETOH-induced increases in the protein levels of $Sigma_1$ R in the VTA (Fig. 3B).

4. Discussion

This study showed that acupuncture at HT7 regulated ETOH-induced (1 g/kg) immobility and 22-kHz USVs via $Sigma_1$ R expression in the VTA. ETOH-induced immobility was observed in the OFT, whereas pretreatment with acupuncture at HT7 and IP injection with BD 1047 (10 mg/kg) led to recovered immobility for 20 min after ETOH administration. Furthermore, ETOH-induced 22-kHz USVs were increased, but pretreatment with acupuncture at HT7 and IP injection of BD 1047 reduced the number of 22-kHz USVs in the treatment session for 20 min after ETOH administration. In addition, the Western blot analysis showed that an ETOH-induced increased level of $Sigma_1$ R expression was present in the VTA, but pretreatment with acupuncture at HT7 and IP injection of BD 1047 were found to decrease the levels of $Sigma_1$ R in the VTA after IP injection of ETOH. These results suggest that stimulation of HT7 regulates ETOH-induced immobility and 22-kHz USVs via $Sigma_1$ R in the VTA.

According to a recent study, ETOH is a sedative drug that causes motor impairments such as ataxia, incoordination and immobility.[23] In a locomotion experiment, Chuck et al. showed that 1 and 2 g/kg IP injections of ETOH have motor suppressant effects for 30 min compared with vehicle administration.[24] Consistent with these findings, our results showed that the administration of 1 g/kg ETOH induced immobility for 40 min compared with saline administration. Based on this result, the induction time for immobility caused by ETOH was determined to be 10−20 min, and acupuncture was performed during this time period. The role of sigma receptors in the rewarding and reinforcing effects of ETOH has also been recently proposed, suggesting that hyperactivity of these receptors may result in excessive ETOH consumption. These receptor antagonists block cocaine-induced c-fos expression, locomotion, place preference conditioning, seizures, and lethality in rodents.[25] Likewise, the BD1047 dose dependently attenuated ETOH-induced locomotion and blocked ETOH-induced place and taste conditioning.[26] Consistent with these findings, our results showed that HT7 stimulation mitigates the immobility induced by acute ETOH administration. Injection of the $Sigma_1$ R antagonist BD 1047 (10 and 30 mg/kg, i. p.) also reduced the immobility caused by acute ETOH administration.

A recent study showed that rats with high ETOH consumption emit 22−28-khz USVs.[27,28] Our data also showed that after administration of 1 g/kg ETOH, 22-kHz USVs were significantly different at the 20-min measurement time point, but 50-kHz USVs were not significantly different during the treatment session compared with the baseline session. Additionally, our results showed that pretreatment with acupuncture at HT7 and IP injection of BD 1047 (10 mg/kg) effectively reduced 22-kHz USVs, which represent the negative emotional state caused by ETOH. This result represents a novel finding that the acute injection of ETOH induces negative emotions associated with abnormal mobility.

In rodents, 22–28-kHz USVs are initiated by activity of the ascending mesolimbic cholinergic pathway, whereas 50–55 kHz FM USVs are initiated by activation of the mesolimbic dopaminergic pathway.[29,30] DA neuron activation by ETOH administration alters the activity of several downstream regions including the PFC, Amg, thalamic structures, and NAc and VTA.[31] Moreover, activated sigma receptors stimulate DA in a brain area critical for reinforcing the effects of addicted drugs.[32] In acupuncture, recent research has shown that HT7 stimulation has the effect of relieving addiction through the regulation of GABA neuronal activity. Yang et al. showed that acupuncture inhibits GABA neuronal activity in the VTA and reduces ETOH self-administration.[25] The activation of GABA neurons are regulated by $Sigma_1$ R activation, which has been implicated in actions of psychostimulants.[26] We also found that acute ETOH administration increased sigma receptor expression in the VTA, and increased sigma receptor levels were decreased by stimulation of the HT7 acupuncture point. This finding suggests that HT7 stimulation regulates immobility and 22-kHz USVs via $Sigma_1$ R in the VTA upon acute ETOH exposure. In general, the protein levels of $Sigma_1$ R are considered to be increased by acute ETOH exposure, which leads to endoplasmic reticulum stress that causes programmed cell death and apoptosis. Our last study suggested that acupuncture stimulation mitigates this endoplasmic reticulum stress in ovariectomized rats.[21] For this reason, the efficacy of sigma receptor actions may occur through ER stress relief.

In summary, the previous results have suggested that (1) immobility is a reaction due to the effects of acute ETOH administration, (2) 22-kHz USVs reflect negative emotional responses caused by acute ETOH and (3) $Sigma_1$ R is activated by acute ETOH. These responses are inhibited by acupuncture at HT7 mediated via $Sigma_1$ R in the VTA of rats.

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Author contribution

Conceptualization: SYS and SPK; Methodology: SYS; Software: SPK; Validation: SPK; Formal analysis: SJC; Investigation: KHC; Resources: SYK; Data curation: SKB; Writing - original draft: SYS and SPK; Writing - review & editing: YR; Visualization: SYS; Supervision: YR; Project administration: SJC; Funding acquisition: BB.

Conflict of interest

The authors declare that they have no competing interests.

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Ethical statement

Animal Care and Use Committee at the Korea Institute of Oriental Medicine (KIOM) with reference number #18-076.

Data availability

The data will be made available upon reasonable request..

Supplementary material

Supplementary material related to this article can be found in the online version, at doi:https://doi.org/10.1016/j.imr.2020.100497.

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