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Short Communication

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Involvement of cannabinoid receptors in infrasonic noise-induced neuronal impairment

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Abstract

Excessive exposure to infrasound, a kind of low-frequency but high-intensity sound noise generated by heavy transportations and machineries, can cause vibroacoustic disease which is a progressive and systemic disease, and finally results in the dysfunction of central nervous system. Our previous studies have demonstrated that glial cell-mediated inflammation may contribute to infrasound-induced neuronal impairment, but the underlying mechanisms are not fully understood. Here, we show that cannabinoid (CB) receptors may be involved in infrasound-induced neuronal injury. After exposure to infrasound at 16 Hz and 130 dB for 1–14 days, the expression of CB receptors in rat hippocampi was gradually but significantly decreased. Their expression levels reached the minimum after 7- to 14-day exposure during which the maximum number of apoptotic cells was observed in the CA1. 2-Arachidonoylglycerol (2-AG), an endogenous agonist for CB receptors, reduced the number of infrasound-triggered apoptotic cells, which, however, could be further increased by CB receptor antagonist AM251. In animal behavior performance test, 2-AG ameliorated the infrasound-impaired learning and memory abilities of rats, whereas AM251 aggravated the infrasound-impaired learning and memory abilities of rats. Furthermore, the levels of proinflammatory cytokines tumor necrosis factor alpha and interleukin-1β in the CA1 were upregulated after infrasound exposure, which were attenuated by 2-AG but further increased by AM251. Thus, our results provide the first evidence that CB receptors may be involved in infrasound-induced neuronal impairment possibly by affecting the release of proinflammatory cytokines.

Key words: infrasound, cannabinoid receptors, neuronal impairment, proinflammatory cytokines, vibroacoustic disease

Introduction

Infrasound is a kind of low-frequency noise with the frequency below 20 Hz, which is below the human hearing threshold and thus not audible to the human ear [1]. It can be generated by various means of transportation such as automobile, aircraft, and rail traffic, and also by numerous industrial sources such as heavy machinery and air compressor [2,3]. Therefore, infrasonic noise ubiquitously exists around us in our daily life. Usually, temporary exposure to infrasonic noise does not affect our health. However, people in some special occupations such as aeronautical technicians, military pilots, cabin crewmembers, and disc jockeys, who are often exposed to the high sound pressure level (SPL) infrasound (over 100 dB, even up to

130 dB) for long time, can develop vibroacoustic disease [4]. Vibroacoustic disease finally causes a series of central nervous system (CNS) symptoms such as dizziness, irritability, aggression, short-term memory loss, and late-onset epilepsy [4,5]. However, the mechanisms of how the vibroacoustic disease affects the CNS are still largely unknown.

The CNS is vulnerable to infrasonic insults. Our previous experiments showed that exposure to a certain frequency and SPL of infrasound could impair learning and memory abilities in animals [6,7], interfere with adult neurogenesis in the hippocampus [8], cause apoptosis of the hippocampal cells [9], and induce intracellular Ca^{2+} overload [9–12]. Our recent studies revealed that infrasound activated microglia and astrocytes in rat brain [12–14], and the activated glial cells could release proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1β, consequently leading to neuronal impairment [12]. However, the full knowledge about the molecular mechanism(s) underlying infrasound-induced neuronal injury is still lacking.

Cannabinoid (CB) receptors, including CB1 and CB2, belong to the endocannabinoid system [15]. CB1 is primarily expressed in the presynaptic membranes of the CNS and plays a prominent role in synaptic neurotransmission [16,17]. CB2 is expressed predominantly in the cells of the immune system, such as lymphocytes and neutrophils, and has the immunomodulatory properties, such as inhibiting the activity of antigen-presenting cells and reducing cytokine production during inflammatory responses [18–21]. Recent evidence showed that glial cells that are involved in inflammatory process within the CNS also express CB receptors, especially CB2, and that their activation negatively regulates the production of proinflammatory cytokines in glial cells [22]. In the present study, we showed the involvement of CB receptors in infrasound-induced neuronal impairment. Infrasound induced a decrease in the expression of CB receptors but an increase in the number of apoptotic cells and in the expression of TNF-α and IL-1β in rat hippocampus. Activation of CB receptors could attenuate infrasonic noise-induced proinflammatory cytokine release and neuronal apoptosis.

Materials and Methods

Infrasound device

The infrasound device was used as described previously [13]. This device includes a low-frequency signal generator, a four-loudspeaker-contained chamber, a sensor for infrasound, and a data collection system. Our infrasound system can generate standard infrasonic oscillatory waves with frequencies ranging from 1 to 20 Hz and SPL from 90 to 130 dB. The sensor that is connected to the infrasonic data collection system can monitor the changes in infrasound parameters. The frequencies and SPL used were held steady during 2 h of exposure.

Animals and grouping

Male Sprague-Dawley rats (250–280 g) were obtained from the Center of Experimental Animal in the Fourth Military Medical University (Xi'an, China) and housed under controlled conditions of temperature (24°C–25°C), humidity (50%–60%), and 12-h light/dark cycle. Food and water were provided *ad libitum*. Animals were allowed to adapt to laboratory environment for at least 7 days prior to experiments. For experiments, the rats were exposed to 16 Hz and 130 dB infrasound for 2 h, once a day for 14 days, according to our previous procedure [12].

The animals were randomly divided into four groups: (1) the control group, in which the rats were just placed into the chamber without infrasound exposure; (2) the infrasound group, in which the rats were exposed to infrasound as described above; (3) the infrasound plus AM251-treated group, in which the rats received 5 mg/kg AM251 (Sigma, St Louis, USA); and (4) the infrasound plus 2-arachidonoylglycerol (2-AG)-treated group, in which the rats received 3 mg/kg 2-AG (Sigma). AM251 and 2-AG were administered intraperitoneally (i.p.) 30 min before the infrasound exposure, once a day thereafter for 14 days. All procedures used in this study were approved by the Institution Review Board and were performed according to the Guidelines of Institutional Animal Care and Use Committee at the Fourth Military Medical University.

Animal behavior test

Rats in each group ($n = 5$) were subject to behavior tests daily after 14 days of infrasound exposure. Animal spatial learning and memory abilities were assessed by the Morris water maze test, and memory acquisition and consolidation abilities were assessed by passive avoidance test. All tests were performed according to our previous study [12]. For the Morris water maze test, the escape latency, the time spent in target quadrant, and the swimming speed were recorded. For passive avoidance test, the step-through latency and the percentage of non-step-through in 24-h retention trials were recorded.

Immunohistochemistry and apoptosis analysis

The rats that had been exposed to infrasound for 1, 3, 5, 7, or 14 days ($n = 5$ for each subgroup) were deeply anesthetized with 50 mg/kg sodium pentobarbital (i.p.) and perfused transcardially with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) (pH 7.4). The brains were immediately removed, postfixed for 2 h in the same fixative, and placed in 25% sucrose at 4°C until they sank. Coronal sections (30 μm thickness) through the hippocampus (Bregma: from −2.3 to −5.0 mm) were cut on a Leica CM1900 cryostat (Leica, Wetzlar, Germany). After being washed in 0.01 M PBS, the sections were incubated with polyclonal rabbit anti-CB1 antibody (1:500; Abcam, Cambridge, UK) or with polyclonal rabbit anti-CB2 antibody (1:300; Abcam) at 4°C overnight. After three times wash with PBS, the sections were incubated with Alexa Fluor® 488 conjugated anti-rabbit IgG (1:500; Abcam) for 2 h. Omission of the primary antibodies was served as the negative control. The labeled sections were examined under a FluoView300 confocal microscope (Olympus, Tokyo, Japan). The intensities of immunoreactivity of CB1 or CB2 were quantified by an Image J software (NIH, Bethesda, USA) and further normalized to the control. For apoptosis analysis, the sections were subject to TUNEL staining according to the instructions of TUNEL kit (Roche, Indianapolis, USA). $TUNEL^+$ cells in rat hippocampal CA1 region were counted in two sections, and the averages were calculated for each group.

Western blot analysis

Rat hippocampi were collected, and total proteins were extracted using radio immunoprecipitation assay lysis buffer [PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.25 mM phenylmethanesulfonyl fluoride, 5 μg/ml aprotinin, and 1 mM sodium orthovanadate]. The extracts of brain tissues in each group ($n = 5$) were boiled for 5 min and then centrifuged at 15,000 *g* for 5 min. Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, USA). The proteins (30 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto PVDF membranes (Millipore, Bedford, USA). After incubation in blocking buffer (0.1% Tween-20 and 5% non-fat-dried milk in Tris-buffered saline, pH 7.4) at room

temperature for 60 min, PVDF membranes were incubated with anti-CB1 antibody (1:2000), anti-CB2 antibody (1:1000), or anti-β-actin antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, USA). After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:2000; Amersham, Piscataway, USA) for 1 h at room temperature. Detection was performed using an Alpha GDS8000 electrochemiluminescence (ECL) system (Ultra-Violet Products Ltd., Cambridge, UK). All band signals were quantified using the Image J software. The data were normalized to the expression of β-actin.

Cytokine detection

Hippocampal tissues were homogenized using a glass homogenizer with 1 ml of ice-cold PBS (pH 7.2) containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, and 1 μg/ml leupeptin, and centrifuged at 12,000 *g* for 20 min at 4°C. The supernatant was collected, and the concentrations of IL-1β and TNF-α were measured using a rat enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, USA) following the instructions supplied by the manufacturer. The results were shown as pg cytokine per 100 mg tissue.

Statistical analysis

All data are expressed as the mean ± SEM. The significance of differences between groups was calculated by analysis of variance (ANOVA), followed by post hoc testing for individual differences by the Bonferroni or Dunnett's test, depending on whether the data were normally distributed or not. Data management and statistical analyses were performed in SPSS v13.0. Statistical significance was set at $P < 0.05$.

Results

Infrasound exposure decreases the expression of CB receptors

To investigate the possible role of CB receptors in infrasound-induced injury, we first examined the changes in the expression of CB1 and CB2 in rat hippocampus after infrasound exposure. Immunohistochemistry using an anti-CB1 antibody revealed a strong CB1 immunoreactivity in the hippocampal CA1 region of the control rats (**Fig. 1A**). The negative control, in which the primary antibody was omitted, showed no CB1 immunoreactivity (data not shown). The expression of CB1 was gradually decreased, and a significant reduction in CB1 immunoreactivity was observed after 5-, 7-, and 14-day exposures to infrasound (**Fig. 1B–F**). It was found that CB1 immunoreactivity reached the minimum after 14 days of exposure (**Fig. 1F**). Quantification of the intensity of CB1 immunoreactivity confirmed the immunostaining results (data not shown). Immunoblotting analysis for CB1 was consistent with immunostaining results (**Fig. 1G**). Similar to that of CB1, the expression of CB2 was also downregulated after infrasound exposure, as revealed by immunostaining (data not shown) and immunoblotting (**Fig. 1H**) assays. Thus, our results indicate a probable involvement of CB receptors in infrasound-induced injury.

Infrasound-induced cell death concurs with reduced CB receptor expression

Our previous study showed that infrasound could induce cell death in the hippocampal CA1 [12]. Consistently, in this study, our TUNEL staining results showed that after 7 or 14 days of exposure, the numbers of TUNEL$^+$ apoptotic cells were significantly increased in the

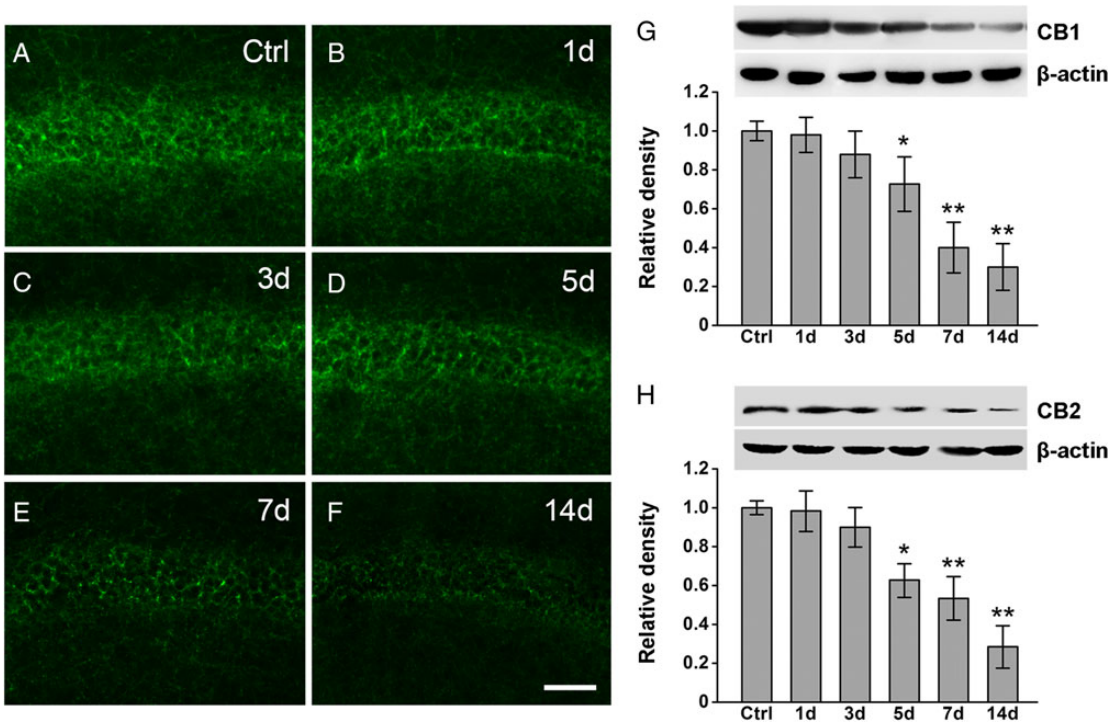

Figure 1. Infrasound exposure decreases the expression of CB receptors CB1 immunofluorescence stain in hippocampal CA1 region of the control rats (A) and those exposed to infrasound for 1 (B), 3 (C), 5 (D), 7 (E), or 14 days (F). Scale bar: 50 μm. Immunoblot analysis showed a gradual decrease in protein expression of CB1 (G) and CB2 (H) following infrasound exposure. β-Actin was used as an internal control. *$P < 0.05$, **$P < 0.01$ vs. the control group.

CA1; however, apoptosis was not obvious when rats were exposed to infrasound for 1, 3, and 5 days, when compared with the control (**Fig. 2A–D,G**). It was found that the time period during which the cell death occurred coincided with the time period during which the expression of CB receptors was decreased (**Fig. 1**).

CB receptor activation protects against infrasound-induced injury

The downregulation of CB receptor expression and the simultaneous apoptosis after infrasound exposure promoted us to test whether pharmacological activation of CB receptors could rescue the infrasound-induced cell death. As expected, using a CB receptor agonist 2-AG, the number of infrasound-triggered TUNEL$^+$ apoptotic cells was significantly reduced in the CA1 after 14 days of exposure to infrasound (**Fig. 2F,G**). Furthermore, we examined whether CB receptor antagonist could exacerbate infrasound-induced injury. Considering the fact that the expression of CB1 receptor is more predominant than that of CB2 receptor in the hippocampus [15,23], we applied CB1 receptor antagonist AM251 in the study. Our results showed that the inhibition of CB1 receptor by AM251 further elevated the number of TUNEL$^+$ cells induced by infrasound (**Fig. 2E,G**). Additionally, it was also found that 2-AG or AM251 did not affect the expression of CB receptors (data not shown), indicating that it is the changes in the activation but not in the expression of CB receptors that are involved in cell death. These results suggest that CB receptor activation may protect against infrasound-induced neuronal impairment.

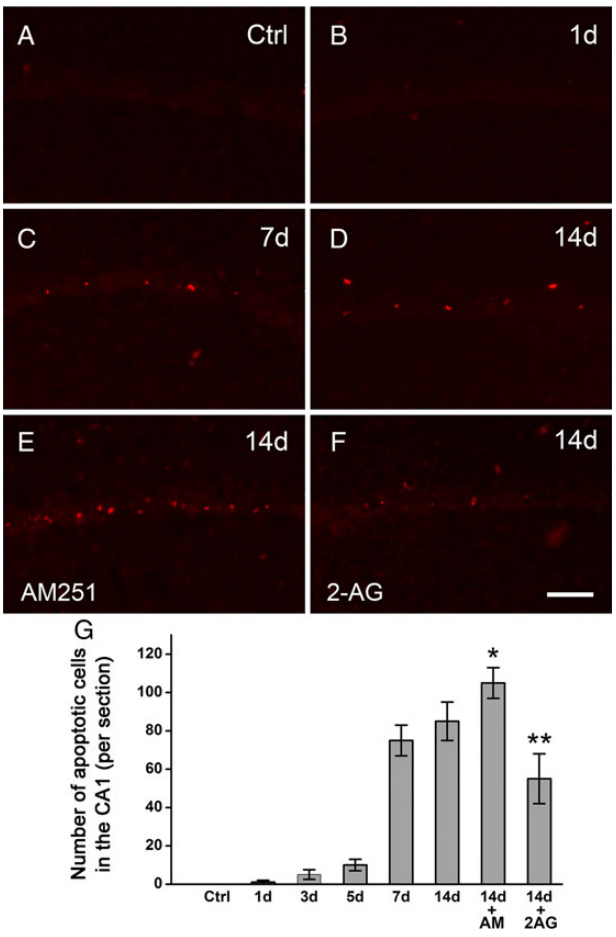

Figure 2. Infrasound exposure induces cell death in hippocampal CA1 region
Apoptotic cells in the CA1 were examined by TUNEL staining in the controls rats (A) and those exposed to infrasound for 1 (B), 7 (C), or 14 days (D). CB receptor antagonist AM251 (E) and their agonist 2-AG (F) increased and decreased the number of infrasound-triggered TUNEL$^+$ apoptotic cells in the CA1, respectively. Scale bar: 50 μm. (G) Quantitative analysis of the number of TUNEL$^+$ apoptotic cells induced by infrasound. *$P < 0.05$, **$P < 0.01$ vs. the rats exposed to infrasound for 14 days.

CB receptor activation ameliorates infrasound-impaired learning and memory abilities

To further study the protective effects of CB receptors on infrasound-induced injury, we investigated the changes in learning and memory abilities in rats with the treatment of 2-AG or AM251. The Morris water maze was used to assess hippocampus-dependent learning and memory abilities, and escape latencies were recorded in rats exposed to infrasound for 14 consecutive days. Exposure to infrasound for 1 or 3 days did not affect the escape latencies of rats, when compared with the controls (**Fig. 3A**). However, from day 4 on, the escape latencies of infrasound-exposed rats were significantly longer than the controls (**Fig. 3A**), and these rats also spent less time in the target quadrant than the controls when the platform was removed during the probe trials (**Fig. 3B**). 2-AG shortened the escape latencies, but AM251 further prolonged the escape latencies, as compared with the infrasound group (**Fig. 3A**). Consistently, rats treated with 2-AG spent more time while those with AM251 spent less time in the target quadrant than those in the infrasound group (**Fig. 3B**). In addition, swimming speeds did not differ significantly among the four groups (data not shown).

Then, the passive avoidance test was used to investigate the acquisition and consolidation of memory. Twenty-four hours after foot shock, the retention latencies of rats to enter the dark chamber (step-through) were measured. The baseline latencies of rats to enter the dark chamber were not different between groups. The step-through latencies of infrasound-exposed rats for 7 and 14 days were much shorter than the controls (**Fig. 3C**), and the percentages of infrasound-exposed rats that did not enter the dark compartment (non-step-through) were correspondingly reduced (**Fig. 3D**). 2-AG increased the step-through latencies, but AM251 further decreased the step-through latencies, as compared with the infrasound group (**Fig. 3C**). Consistently, treatment with 2-AG or AM251 increased or reduced the percentages of non-step-through, respectively, as compared with the infrasound group (**Fig. 3D**). Taken together, the above results suggest that CB receptor activation might protect against infrasound-induced injury and ameliorate infrasound-impaired rat behavior performance.

CB receptor activation reduces infrasound-elevated proinflammatory cytokine levels

Our previous studies have demonstrated that glial cell-released proinflammatory cytokines contribute to infrasound-induced neuronal impairment [12,14]. Since CB receptors are expressed in the astrocytes and microglia, and their stimulation can inhibit glial cells to produce proinflammatory cytokines [14,22,24–28], we tested whether CB receptor activation could affect the expressions of proinflammatory cytokines induced by infrasound. Consistent with our previous *in vitro* study [12], the ELISA results showed that the 14 days exposure to infrasound increased the expression levels of proinflammatory cytokines TNF-α (120.63 ± 15.23 pg/ml) and IL-1β (78.86 ± 10.33 pg/ml) in rat hippocampus (**Fig. 4**). Treatment with 2-AG reduced the levels of TNF-α and IL-1β to 90.65 ± 11.21 pg/ml ($P < 0.05$) and 58.02 ± 9.01 pg/ml

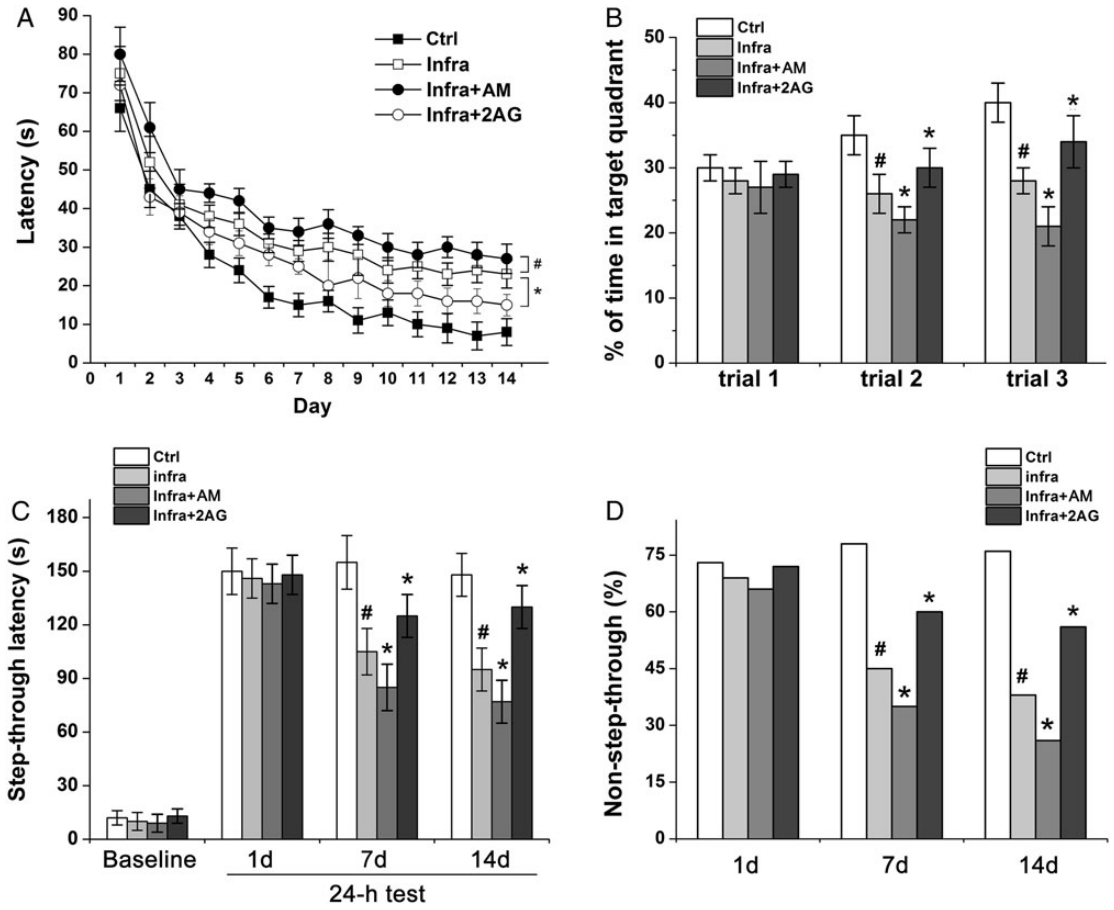

Figure 3. Activation of CB receptors ameliorates infrasound-impaired learning and memory in rats The Morris water maze test showed the effects of infrasound on escape latency (time to find the hidden platform) (A) and the percentage time spent in target quadrant during three probe trials (B) when the rats were exposed to infrasound. Activation of CB receptors by 2-AG reduced the escape latency elevated by infrasound (A) but increased the percentage time spent in target quadrant reduced by infrasound (B). Inhibition of CB receptors by AM251 had the reverse effects. Passive avoidance test showed the effects of infrasound on the step-through latency (C) and the percentage of non-step-through (D) in control rats and those exposed to infrasound for 1, 7, or 14 days. 2-AG elevated the step-through latencies and the percentages of non-step-through reduced by infrasound, but AM251 had the reverse effects (C and D). The baseline latencies were not significantly different between groups. $^{\#}P < 0.05$ vs. the control group; $^{*}P < 0.05$ vs. the infrasound group.

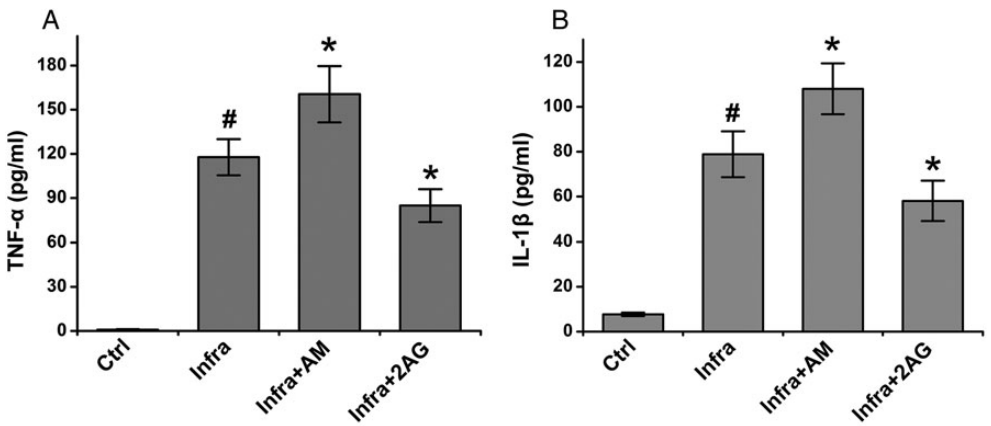

Figure 4. Activation of CB receptors reduces the expression levels of infrasound-induced proinflammatory cytokines ELISA assay showed an increase in the expression levels of TNF-α (A) and IL-1β (B) in rat hippocampal tissues after 14 days of exposure to infrasound. 2-AG reduced, but AM251 further elevated, the levels of TNF-α and IL-1β. $^{\#}P < 0.05$ vs. the control group; $^{*}P < 0.05$ vs. the infrasound group.

($P < 0.05$), respectively (**Fig. 4**). However, AM251 further elevated the levels of TNF-α and IL-1β to 160.53 ± 19.13 pg/ml ($P < 0.05$) and 108.02 ± 11.34 pg/ml ($P < 0.05$), respectively (**Fig. 4**). These results indicate that CB receptor activation may inhibit the production of proinflammatory cytokines and protect against infrasound-induced neuronal impairment.

Discussion

In this study, we showed that CB receptors, the components of the endocannabinoid system, played an important role in infrasound-induced neuronal impairment. Infrasound at a certain frequency and SPL induced the decrease in the expression of CB receptors in hippocampal CA1 region. CB receptor activation could attenuate infrasound-triggered cell death in the CA1 and learning and memory deficit, which, however, were exacerbated by CB receptor inhibition. This protective effect of CB receptors against infrasound-induced injury may be attributed to their capability of inhibiting the expression of proinflammatory cytokines (e.g. IL-1β and TNF-α).

Noise pollution is a kind of environmental pollution, and together with water pollution, atmospheric pollution, and light pollution, it has become one of the four environmental hazards in public health. Noise is composed of a spectrum of acoustic oscillation, among which low-frequency wave, such as infrasound, is particularly detrimental to the human CNS, often causing vibroacoustic disease [4,5]. A body of evidence has demonstrated that infrasound can impair the functions of the human brain [4,5,29]. Our previous studies also showed that infrasound was harmful to animal CNS [8–11,13,30]. Consistently, in the present study, we demonstrated that infrasound with 16 Hz frequency and 130 dB SPL could cause prominent cell death in hippocampal CA1 after 7–14 days of exposure, during which the most obvious deficit in animal learning and memory occurred (**Figs. 2** and 3). Moreover, it was also found that the levels of proinflammatory cytokines IL-1β and TNF-α were elevated in the hippocampus, which might be responsible for the infrasound-induced cell death (**Fig. 4**).

CB receptors, including CB1 and CB2, are G-protein-coupled receptors that can response to a number of physiological and pathological stressors and are involved in maintaining homeostasis in the face of these stressors [27]. CB receptors were initially found to be expressed at high level throughout the brain by many different classes of neurons, which are involved in a number of physiological and behavioral events [28]. Recent evidence showed that CB receptors were also expressed in the astrocytes and microglia, and their activation could inhibit glial cells to produce proinflammatory cytokines [14,22,24–28]. Consistently, a growing body of evidence showed that CB receptors and their endogenous agonists played important roles in neurodegenerative and neuroinflammatory disorders [24–26].

Our recent studies revealed that glial cell-mediated inflammation contributed to infrasound-induced neuronal impairment [12–14]. These facts promoted us to explore whether CB receptors were involved in infrasound-triggered injury. As expected, in this study, we found that the expressions of CB receptors CB1 and CB2 were significantly downregulated after infrasound exposure (**Fig. 1**). 2-AG, an agonist of CB receptors, could reduce infrasound-induced apoptosis in the CA1 and learning and memory deficit. On the contrary, AM251, an antagonist of CB1 receptor predominantly expressed in the hippocampus, aggravated infrasound-triggered injury (**Figs. 2** and 3). Moreover, 2-AG decreased, but AM251 further increased the expression of proinflammatory cytokines (e.g. IL-1β and TNF-α) induced by infrasound (**Fig. 4**). Combined with the fact that CB receptors are expressed in glial cells, we proposed that infrasound may induce the downregulation of CB receptor expression in glial cells, which derepressed glial cell-mediated inflammation, causing IL-1β and TNF-α release and consequently neuronal cell death. Admittedly, our present study is still unable to provide direct evidence that glial cell-expressed CB receptors are involved in infrasound-induced neuronal impairment, and we cannot exclude the possibilities that the effects of 2-AG or AM251 may be resulted from the modulation of auditory sensation of infrasound or from the release of proinflammatory cytokines. Therefore, further gene knockdown and overexpression assays should be carried out to address this issue. Additionally, we still lack the knowledge about how infrasound could affect the expression of CB receptors. Based on our previous study [12], which revealed that infrasound could enhance TPRV4-mediated NF-κB activation, and the reports that NF-κB was closely associated with CB receptors [31,32], we proposed that there might be a crosstalk between NF-κB and CB receptors after infrasound exposure.

In summary, our results provide the first evidence that infrasound-induced injury may involve CB receptors. CB receptor activation could attenuate infrasound-induced neuronal impairment probably by decreasing the expression of harmful proinflammatory cytokines. Therefore, CB receptors may serve as the promising targets for the treatment of noise-triggered vibroacoustic disease.

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