

# Effects of infrasound at 8 Hz 90 dB/130 dB on NMDAR1 expression and changes in intracellular calcium ion concentration in the hippocampus of rats

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**Abstract.** In the present study, we investigated the effect of infrasound on the expression of N-methyl-D-aspartate (NMDAR)1 as well as changes in intracellular calcium ion concentration ( $[Ca^{2+}]_i$ ) in the hippocampus of rats. Sprague-Dawley (SD) rats were exposed for 2 h daily to infrasound at 8 Hz 90 dB or 130 dB, and NMDAR1 expression was examined on days 1, 7, 14, 21 and 28. The expression of NMDAR1 in the rat hippocampus upon exposure to infrasound at 8 Hz 90 dB sound pressure level (SPL) showed an initial decrease on day 1, an increase on days 7 and 14, a further decrease on day 21, and a return to normal levels on day 28. The peak level was observed on day 14 in every examined subregion of the hippocampus. By contrast, exposure to infrasound at 8 Hz 130 dB SPL had opposite effects, showing an increase on day 1, a decrease on day 7, a decrease to the lowest point on days 14, another increase on day 21 and a return to normal levels on day 28. The lowest expression of NMDAR1 was found in the CA1 and CA3 regions on day 14 and in the DG region on day 7 with exposure at 130 dB. There were significant differences in  $[Ca^{2+}]_i$  concentration on days 14 and 21 with infrasonic exposure at both 8 Hz 90 dB and 130 dB, but no significant differences in  $[Ca^{2+}]_i$  concentration on days 1, 7 and 28 compared to the control group. The highest  $[Ca^{2+}]_i$  level was noted on day 14 with infrasound exposure at 8 Hz 130 dB. These changes suggest that 8 Hz 90 dB/130 dB infrasound

exposure induced certain reversible changes in NMDAR1 expression and  $[Ca^{2+}]_i$  concentration in hippocampal cells, which may influence mnemonic functions related to the hippocampus.

## Introduction

The effect of infrasound at a given frequency and sound pressure level (SPL) may affect the structure and function of the central nervous system (1-4). Research on the mechanism of this effect has involved the receptors of N-methyl-D-aspartate (NMDA), and focused on different aspects of its function (5). When a receptor is activated, the ion channel on the receptor is rapidly opened, mainly due to an increase in the permeability of  $Ca^{2+}$ , and results in an inflow of  $Ca^{2+}$ . The NMDA receptor is closely associated with the learning and memory functions of the nervous system, and the plasticity of synaptic development (6,7). Infrasound exposure can damage the ultra-microscopic tissue of the brain, including the membranous structure (1,2). Our aim was to ascertain whether the effect of infrasound at 90 and 130 dB alters the concentration of  $[Ca^{2+}]_i$  and the expression of NMDAR1 in hippocampal nerve cells. We propose further significant research on the mechanism of the effect of infrasound and the development of sanitary protection zones.

## Materials and methods

**Subjects.** One hundred and thirty-two healthy male Sprague-Dawley rats weighing  $200 \pm 10$  g were provided by the Experimental Animal Center of the Fourth Military Medical University, China. Rats were maintained at a constant humidity ( $60 \pm 5\%$ ) and temperature ( $23 \pm 1^\circ C$ ) under a 12-h light/dark cycle with free access to food and water. All procedures were carried out in accordance with the guidelines of the National Committee for the Care and Use of Laboratory Animals, and the number and suffering of the animals were minimized. The study protocol was approved by the Medical Ethics Commission of the Fourth Military Medical University, Xi'an, China. The animals were randomly divided into three groups:

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rats exposed to infrasound at 8 Hz 90 dB, rats exposed to infrasound at 8 Hz 130 dB, and control rats. Groups exposed to infrasound were placed in an infrasonic pressure chamber (8) and exposed to 8 Hz at either 90 or 130 dB for 2 h once daily. The control group was maintained under the same conditions, but with no exposure to infrasound. On days 1, 7, 14, 21 and 28, respectively, rats in each group were randomized into two subgroups for the separate detection of NMDAR1 expression and  $[Ca^{2+}]_i$  concentration.

**Isolation of hippocampal cells.** Rats used for the detection of  $[Ca^{2+}]_i$  were sacrificed, and both sides of the hippocampus were removed, scissored into pieces ( $\sim 1\text{ mm}^3$ ) and placed in a cell culture dish containing 1 ml D-Hank's  $Ca^{2+}$ -free saline. The suspension was sequentially incubated with 0.125% of trypsin for 10 min at  $37^\circ\text{C}$ , and subsequently with 1 mg/ml of soybean trypsin inhibitor for 10 min on ice (9). Cells were resuspended in D-Hank's  $Ca^{2+}$ -free solution before centrifugation at  $800 \times g$  for 5 min, and then rewashed in D-Hank's  $Ca^{2+}$ -free solution.

**Measurement of  $[Ca^{2+}]_i$  in hippocampal cells.** Membrane-permeable Fluo3/AM was hydrolyzed by endogenous acetoxymethyl esterase to form Fluo3. Fluo3 specifically binds to intracellular  $Ca^{2+}$ , and the Fluo3- $Ca^{2+}$  complex shows a certain fluorescence intensity as an indicator (10). Concentration changes in intracellular  $Ca^{2+}$  were determined according to the fluorescence intensity in terms of their direct ratio. Separated hippocampal cells were resuspended in  $30\text{ }\mu\text{l}$  D-Hank's saline and incubated with  $30\text{ }\mu\text{l}$  Fluo3/AM solution ( $10\text{ }\mu\text{g/ml}$ ) at  $37^\circ\text{C}$  for 30 min.  $Ca^{2+}$  fluorescence intensity and distribution were monitored by a laser scanning confocal microscope (LSCM) at an excitation wavelength of 488-nm, and analyzed by image analysis software (Bio-Rad, USA).

**Immunohistochemical staining for NMDAR1 in the hippocampus.** Rats were anesthetized with 1% Nembutal ( $20\text{ mg/kg}$ ) and intracardially perfused with 100 ml saline followed by 500 ml 4% paraformaldehyde ( $4^\circ\text{C}$ , pH 7.4) for  $\sim 30$  min. The rat brains were then harvested and fixed with 4% paraformaldehyde for another 6 h. The samples were submerged in 0.1 M 20% sucrose phosphoric buffer until falling to the bottom of a jar. Frozen sections ( $35\text{-}\mu\text{m}$ ) were cut on a Leica (Germany) cryostat through the hippocampal level on the coronal plane. Rabbit anti-rat NMDAR1 polyclonal antibody (1:200; Promega, USA) was incubated at  $4^\circ\text{C}$  for 48 h, then the secondary antibody, biotin-labeled goat anti-rabbit IgG (1:500; Sigma, USA) was added at room temperature ( $24\pm 1^\circ\text{C}$ ) for 2 h. After the addition of ABC (1:500; Sigma, USA) for 2 h at room temperature, positive cell expression was detected by diaminobenzidine (DAB) with the blue-color dyeing method. The gray scale of NMDAR1-positive neurons was measured under a Leica microscope at a magnification of  $\times 400$ . Three randomized sections were taken from every rat, and the positive-stained neurons were respectively counted in the CA1, CA3 or DG subregions in each  $\times 400$  magnified visual field with two continuous microscopic visual fields of each section. The average gray scale was determined for each three sections and used for statistical analysis and the plotting of the graphs.

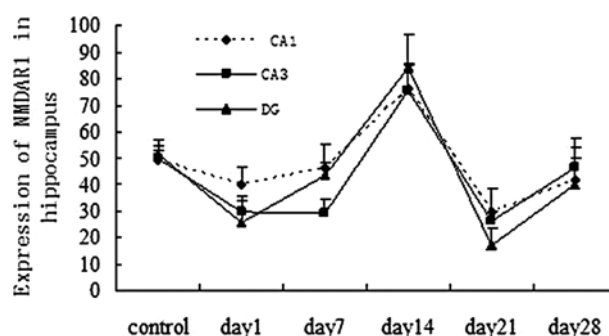


Figure 1. Effect of infrasound exposure at 8 Hz 90 dB on the expression of NMDAR1 in different subregions of the hippocampus of rats at the specified time periods. Data are presented as the means  $\pm$  SD ( $n=6$ ).

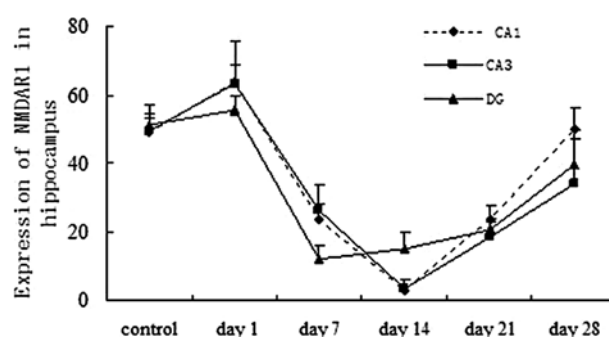


Figure 2. Effect of infrasound exposure at 8 Hz 130 dB on the expression of NMDAR1 in the different subregions of the hippocampus of rats at the specified time periods. Data are presented as the means  $\pm$  SD ( $n=6$ ).

**Statistical analysis.** Data are presented as the means  $\pm$  standard deviation (SD). Statistical analyses were performed with SPSS software using one-way ANOVA followed by LSD for *post hoc* multiple comparisons. A statistical significance level of  $\alpha=0.05$  and  $P<0.05$  was applied to all the tests.

## Results

**Effect of infrasound at 8 Hz 90 dB on the expression of NMDAR1.** With exposure to infrasound at 8 Hz 90 dB, NMDAR1 expression detected in every region of the hippocampus showed non-linear changes (decrease  $\rightarrow$  increase  $\rightarrow$  marked increase  $\rightarrow$  decrease  $\rightarrow$  return to the normal level), reaching its peak on day 14. NMDAR1 expression in the neurons showed similar regularity in the CA1, CA3 and DG subregions of the hippocampus at each time point: on day 1, NMDAR1 expression was decreased in the CA3 and DG regions ( $p<0.01$  vs. control); on day 7, it was increased to normal levels in the CA1 and DG regions, while a low level of expression was noted in the CA3 region ( $p<0.05$  vs. control). NMDAR1 expression was notably increased on day 14 in every examined hippocampal subregion (CA3,  $p<0.01$ ; CA1 and DG,  $p<0.05$  vs. control). This level was again decreased on day 21 (CA3 and DG,  $p<0.01$ ; CA1,  $p<0.05$  vs. control). On day 28, NMDAR1 expression was returned to normal levels in all subregions of the hippocampus (Figs. 1 and 3).

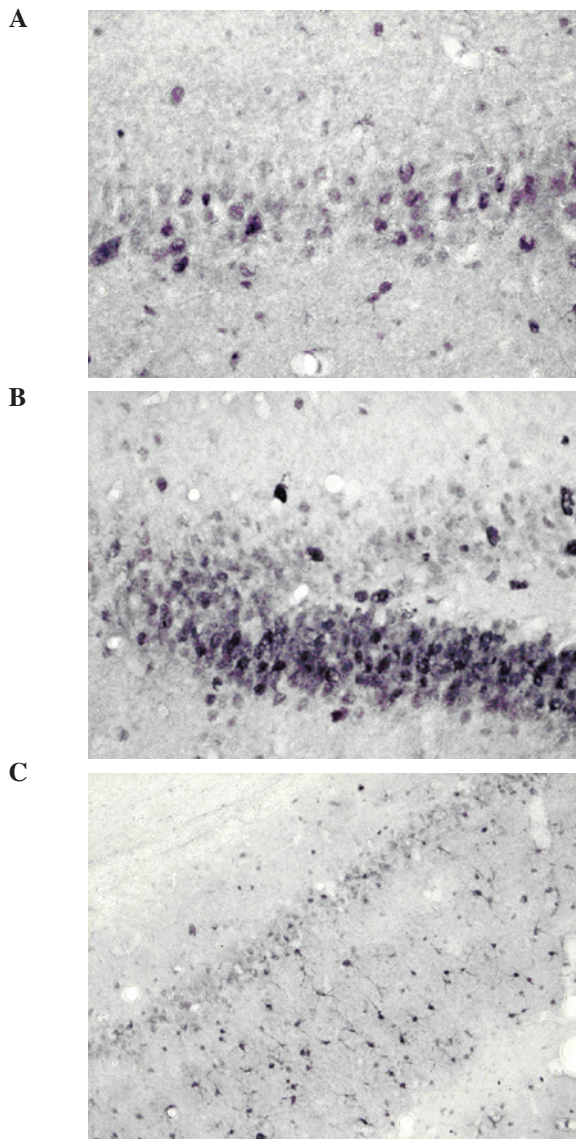


Figure 3. NMDAR1-positive expression observed in the hippocampal CA1 subregion (10x40 magnification). On day 14, strong expression of NMDAR1 was noted in the hippocampal CA1 region in the group exposed to 90 dB (B), whereas in the 130 dB group NMDAR1 positively-expressed pyramidal neurons in the CA1 region were sharply decreased and exhibited significantly weak staining (C). (A) Control (x400), (B) 90 dB group on day 14 (x400), and (C) 130 dB group on day 14 (x400).

**Effect of infrasound at 8 Hz, 130 dB on the expression of NMDAR1.** When subjected to infrasound at 130 dB, NMDAR1 expression in the CA1 and CA3 subregions of the hippocampus showed a similar trend, increasing markedly on day 1 ( $p < 0.01$  vs. control), decreasing markedly on day 7 and reaching its lowest point on day 14 ( $p < 0.01$  vs. control). On day 21, NMDAR1 expression increased somewhat but remained lower than in the control group ( $p < 0.01$  vs. control). On day 28, it returned to normal levels in the CA1 region, but not in the CA3 region ( $p < 0.01$  vs. control). In the DG region, NMDAR1 expression was not significantly changed on day 1; on day 7, it reached its lowest point ( $p < 0.01$  vs. control), then increased somewhat but still remained lower than the control group on days 14 and 21 ( $p < 0.01$  vs. control). On day 28, it returned to normal levels (no significant difference compared

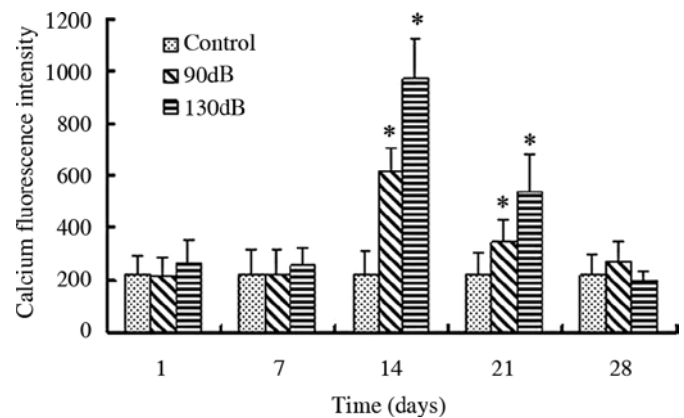


Figure 4.  $\text{Ca}^{2+}$  fluorescence intensity in the hippocampal cells of rats. Data are presented as the means  $\pm$  SD ( $n=6$ ). Rats were exposed to infrasound at 8 Hz 90 dB and 130 dB for 2 h once daily, and assessed on days 1, 7, 14, 21 and 28. Significant changes in  $[\text{Ca}^{2+}]_i$  concentration were observed on days 14 and 21 with 8 Hz infrasonic exposure at both 90 dB and 130 dB ( $*p < 0.01$  vs. control). The peak  $[\text{Ca}^{2+}]_i$  level was observed on day 14 in the group exposed to 8 Hz 130 dB SPL infrasound.

to the control). Changes in the expression of NMDAR1 at the different time points with exposure to infrasound at 8 Hz 130 dB SPL induced opposite effects compared to exposure at 90 dB SPL. The lowest expression of NMDAR1 was noted on day 14 in the CA1 and CA3 subregions and on day 7 in the DG region (Figs. 2 and 3).

**Changes in  $\text{Ca}^{2+}$  fluorescence intensity in hippocampal cells.** Changes in  $[\text{Ca}^{2+}]_i$  concentration after exposure to infrasound at 8 Hz 90 dB and 130 dB are shown in Fig. 4. There were significant changes in  $[\text{Ca}^{2+}]_i$  on day 14 and 21 in both the 8 Hz 90 dB and 130 dB SPL groups ( $p < 0.01$  vs. control), but no significant differences in  $[\text{Ca}^{2+}]_i$  on days 1, 7 or 28 at either SPL compared to the control group. The  $[\text{Ca}^{2+}]_i$  concentration reached its peak on day 14 in the group exposed to 8 Hz 130 dB infrasound (Fig. 4).

Changes in  $[\text{Ca}^{2+}]_i$  concentration in the hippocampal cells exhibited a significant positive correlation when exposed to 90 dB infrasound and a negative correlation when exposed to 130 dB infrasound compared to changes in NMDAR1 expression in each hippocampal subregion at the different time points.

## Discussion

Dynamic changes in  $\text{Ca}^{2+}$  concentrations in cells play an important role in maintaining the function of the nervous system, such as the excitability of nerve cells and the promotion of neurotransmitter release, and affect synaptic plasticity, gene expression the toxicity of neurons (11,12). Increases in  $\text{Ca}^{2+}$  concentration in nerve cells may involve the voltage gated calcium channel on the cell membrane and the receptor sensitivity calcium channel (13), including NMDAR, leading  $\text{Ca}^{2+}$  to flow inwards. The effect may also be mediated by the RyRs on the ER membrane and the inositol triphosphate receptors (IP3Rs), which increase the release of  $\text{Ca}^{2+}$  in the cells (13,14). NMDA receptor cloned out of the rat brain is a hybrid oligomeric protein consisting of one NMDA1 receptor



subunit and four NMDA2 receptor subunits, with each subunit peptide chain possessing four M<sub>1</sub> to M<sub>4</sub> transmembrane function domains and five subunits grouped into a cation channel on the cell membrane, which is highly permeable to Ca<sup>2+</sup> (12). Among the five subunits, the NMDA1 receptor subunit plays a key role in realizing the function of the receptors.

In the present study, NMDAR1 expression in the hippocampus showed a markedly sensitive reaction to exposure to infrasound at 8 Hz 90 dB. Positive expression in each hippocampal region on day 14 markedly increased NMDA receptor-induced excitatory amino acid, glutamic acid (Glu), aspartic acid and Asp-induced postsynaptic effect. When the NMDA receptor was activated, the ion channel of the receptor opened, increasing the permeability of Ca<sup>2+</sup> and causing Na<sup>+</sup> to flow inwards, K<sup>+</sup> to flow outwards and postsynaptic membranes to produce a rapid excitatory postsynapse potential (EPSP). Any mechanism leading to extraordinary stimulus to the Glu or Asp receptor, such as excessive synapse release, intake deletion or receptor supersensitivity, may induce excitotoxicity in the central neurons. The resulting excessive amount of glutamic acid acts on the NMDA receptor present on the cell membrane, causing an increase in cell membrane permeability to Ca<sup>2+</sup> and Na<sup>+</sup>, an enormous inflow of Ca<sup>2+</sup> and an increase in Na<sup>+</sup> in the cell. The passage of glutamic acid through the Na<sup>+</sup>/Ca<sup>2+</sup> exchange system also enables a further increase in Ca<sup>2+</sup>, thus causing an overload of Ca<sup>2+</sup> in the cell. We therefore suggest that the Ca<sup>2+</sup> overload in the hippocampal cells and correlated high NMDAR1 expression observed on day 14 after exposure to infrasound at 8 Hz 90 dB may have been due to excessive activation of the NMDA receptor, which led to an increase in Ca<sup>2+</sup> concentration in the cells, thereby triggering a series of biochemical processes, including the activation of Ca<sup>2+</sup>-controlled protease, phospholipase and endonuclease. The activation of these enzymes destroys the cellular skeleton and membrane lipid, leading to the formation of arachidonic acid metabolic products, free radicals and mitochondrial function obstruction. A serious case may result in energy exhaustion and cell death, in which Ca<sup>2+</sup> plays a key role in the mechanism of damage caused by glutamic acid nervous excitatory toxicity (15). In addition, by increasing Ca<sup>2+</sup>, NMDA receptor activation may activate NOS and produce NO. Excessive production of NO has a direct killing effect on the neuron (16).

In the present study, 8 Hz 130 dB infrasound initially had a stimulatory effect on NMDAR1 expression in each region of the hippocampus. Thus, in the short term, it was possible for infrasound at 130 dB to affect hippocampal tissue, leading to an increase in Ca<sup>2+</sup> concentration in hippocampal cells and resulting in a further series of biological effects. Yuan *et al* reported that a marked increase in Glu content in the hippocampal region of rats was detected upon exposure to infrasound at 8 Hz 120 dB for 2 h (4). Cao *et al* reported that there was high expression of the metabolic glutamic acid receptor mGluR1 $\alpha$  on days 1 and 7 in rats exposed to ultrasound at 8 Hz 130 dB 2 h/day. The expression of mGluR1 $\alpha$  was found to return to normal levels on day 14 (17). This suggests that notable changes occur in hippocampal Glu and its receptor upon exposure to infrasound at 8 Hz 120-130 dB applied daily for 2 h. Regarding the effect of 8 Hz 130 dB infrasound on the increase in Ca<sup>2+</sup> concentration, it was

demonstrated that not only the ion glutamic acid receptor (iGluR), but also the metabolic glutamic acid receptor (mGluR), participated in its increase. However, infrasound at 8 Hz 130 dB resulted in the inhibition of NMDAR1 expression on day 7, leading to high mGluR1 expression. In this case, the effect on the increase in Ca<sup>2+</sup> concentration was mainly mediated by mGluR1. Infrasound at 8 Hz 130 dB was found to have an inhibitory effect on NMDAR1 and mGluR1 expression on day 14; this coincided with a Ca<sup>2+</sup> overload symptom, accompanying distinct damage in the ultramicroscopic structure of the cells, such as mitochondrial swelling and pyknosis (1,17). This indicates that the effect of 8 Hz 130 dB infrasound on day 14 directly resulted in damage to the structure of the cell membrane and the opening of various types of calcium channels, thus enabling extracellular Ca<sup>2+</sup> to flow inwards and Ca<sup>2+</sup> in the mitochondria and endoplasmic network to be released; this in turn caused intracellular Ca<sup>2+</sup> to overload, mitochondrial energy to undergo metabolic inhibition, protein synthesis to be blocked and NMDAR1 expression to be inhibited. Upon initiation of the repair mechanism and the function regulation after damage to the hippocampal tissue, NMDAR1 expression was gradually recovered.

The hippocampus plays an important role in learning and memory function. The physiology of neuroelectricity has confirmed that LTP is one of the vital nerve foundations for learning and memory. The induction of LTP depends mainly on the role of NMDAR. In the majority of synapses that support LTP (in the hippocampus and elsewhere), the postsynaptic increase in calcium is mediated through activation of the NMDA receptor. Genetic disruption of the NR1 subunit leads to impairments in LTP and spatial learning (18). As discussed above, infrasound at 8 Hz 130 dB causes an abnormal incessant increase in the Ca<sup>2+</sup> concentration in the cells, markedly inhibiting NMDAR1 expression, which subsequently hinders the learning and memory function of the hippocampus. Wang *et al* reported that the cognitive function of mice was markedly retarded of 14 days of exposure to 8 Hz 90 dB/100 dB/110 dB or 120 dB infrasound (19). In the present study, it was noted that an obvious decrease in NMDAR1 expression caused by the abnormal increase or overload in Ca<sup>2+</sup> concentrations in the hippocampal cells resulting from the effect of 8 Hz 130 dB infrasound may play an important role in the cognitive function of human beings and animals caused by a high SPL of infrasound.

Consequently, the application of a Ca<sup>2+</sup> antagonist or receptor antagonist may aid in protecting against damage to brain function caused by the effect of 8 Hz 90 dB infrasound. For protection against damage to brain function caused by exposure to 8 Hz 130 dB infrasound, an NMDAR1 agonist should be applied in addition to a Ca<sup>2+</sup> antagonist. It was previously confirmed that the mGluR antagonist MCPG effectively attenuated the amount of damaged neurons after exposure for 7 and 14 days to 8 Hz 130 dB infrasound (17), and that the NMDA antagonist can block the loss of hippocampal tissue caused by ischemia (20).

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