Infrasound increases intracellular calcium concentration and induces apoptosis in hippocampi of adult rats

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Abstract. In the present study, we determined the effect of infrasonic exposure on apoptosis and intracellular free Ca^{2+} ($[Ca^{2+}]_i$) levels in the hippocampus of adult rats. Adult rats were randomly divided into the control and infrasound exposure groups. For infrasound treatment, animals received infrasonic exposure at 90 (8 Hz) or 130 dB (8 Hz) for 2 h per day. Hippocampi were dissected, and isolated hippocampal neurons were cultured. The $[Ca^{2+}]_i$ levels in hippocampal neurons from adult rat brains were determined by Fluo-3/AM staining with a confocal microscope system on days 1, 7, 14, 21 and 28 following infrasonic exposure. Apoptosis was evaluated by Annexin V-FITC and propidium iodide double staining. Positive cells were sorted and analyzed by flow cytometry. Elevated $[Ca^{2+}]_i$ levels were observed on days 14 and 21 after rats received daily treatment with 90 or 130 dB sound pressure level (SPL) infrasonic exposure ($p<0.01$ vs. control). The highest levels of $[Ca^{2+}]_i$ were detected in the 130 dB SPL infrasonic exposure group. Meanwhile, apoptosis in hippocampal neurons was found to increase on day 7 following 90 dB SPL infrasound exposure, and significantly increased on day 14. Upon 130 dB infrasound treatment, apoptosis was first observed on day 14, whereas the number of apoptotic cells gradually decreased thereafter. Additionally, a marked correlation between cell apoptosis and $[Ca^{2+}]_i$ levels was found on day 14 and 21 following daily treatment with 90 and 130 dB SPL, respectively. These results demonstrate that a period of infrasonic exposure induced apoptosis and upregulated $[Ca^{2+}]_i$ levels in hippocampal neurons, suggesting that infrasound may cause damage to the central nervous system (CNS) through the Ca^{2+}-mediated apoptotic pathway in hippocampal neurons.

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Introduction

Infrasonic noise is characterized by acoustic oscillation with a frequency below 20 Hz, and it is difficult for the human ear to detect (1). However, high levels of infrasound have become a new public health hazard. Accumulating evidence has demonstrated that infrasound of a certain sound pressure level (SPL), usually given in decibels (dB), may lead to dysfunction of the human body (2). Moreover, exposure to infrasound has been shown to induce damage to the central nervous system (CNS) in animal models (3-6). However, the underlying mechanism remains to be clarified.

The effects of infrasonic exposure in neuronal cell death have been suggested in a number of studies. Enhanced necrotic cell death, with condensed nuclear, swollen mitochondria, and abnormal neuron ultrastructures, is observed in the rat hippocampus after 8 Hz 130 dB infrasonic exposure (6,7). However, necrosis is not the only method that leads to cell death following infrasonic exposure. Apoptosis, a type of programmed cell death (PCD), also contributes to neuronal death during acute and chronic CNS injury (8). Following infrasound treatment, a dramatic upregulation of the mRNA levels of p53, a key apoptosis regulator, is found in the hippocampus of mice (9), suggesting that apoptosis may be involved in infrasound-induced cell death. In addition, the intracellular free calcium ($[Ca^{2+}]_i$) is thought to be involved in various processes in the CNS during neural development as well as during cell death (10). Dysfunction of the CNS results in the release of Ca^{2+} from the cellular membrane or mitochondria and the endoplasmic reticulum (ER) Ca^{2+} stores. Therefore, it is possible that the Ca^{2+}-mediated pathway contributes to infrasonic exposure-induced apoptosis.

To prove this hypothesis, we investigated the potential effect of infrasonic exposure on apoptotic cell death and the

$[Ca^{2+}]_i$ in rat hippocampi. In this study, we demonstrated a marked positive correlation between infrasonic exposure-induced apoptosis and the level of $[Ca^{2+}]_i$, thus providing a theoretical basis for protection of hippocampal cells against infrasound.

Materials and methods

Reagents and instruments. Annexin V-FITC, propidium iodide (PI) and the fluorescent probe fluo-3 acetoxymethyl ester-AM (Fluo-3/AM) were purchased from Sigma (Santa Clara, CA, USA). The infrasound device, consisting of a generator and a detector, was designed by the Fourth Military Medical University (FMMU, Xi'an, China). The electric-actuated infrasound generator had a wide range of frequencies, between 1-20 Hz at 65-130 dB. A real-time ultra-low frequency signal acquisition system was applied to collect and analyze the frequency and intensity of infrasound. Images were captured using a Leica microscope (Leica Co Ltd., Germany).

Animals. Male Sprague-Dawley rats were obtained from the Center of Experimental Animals in the FMMU. These animals, weighing 200±10 g, were maintained in normal conditions with a constant humidity (60±5%) and temperature (23±1˚C). The rats were housed on a normal 12-h light/dark cycle and had free access to food and water. The experimental procedures used in this study were carried out in accordance with the 'Handbook for the Use of Animals in Neuroscience Research'. The procedures were also approved by the Committee of Animal Use for Research and Education in the FMMU. Every effort was made to minimize the number and suffering of animals used in the following experiments. A total of 66 rats were randomly divided into three groups: a control group (n=6), a 90 dB (8 Hz) infrasound exposure group (n=30) and a 130 dB (8 Hz) infrasound exposure group (n=30). In the infrasound exposure groups, animals were maintained in an infrasonic pressure chamber and were exposed to infrasound with different frequencies for 2 h per day. Samples were collected from 6 rats per group on days 1, 7, 14, 21 and 28 following exposure. In the control group, rats were maintained for 2 h in the same chamber without infrasound exposure.

Isolation of hippocampal neurons. After being exposed to infrasound treatment, hippocampal *cornu ammonis* (CA) regions of the rats were removed and chopped with scissors into small sections of 1 mm^3 in a cell culture dish containing 1 ml D-Hank's Ca^{2+}-free saline. The tissues were then incubated with 0.125% of trypsin for 10 min at 37˚C. The reaction was stopped by the addition of 1 mg/ml soybean trypsin inhibitor for 10 min on ice. After discarding the supernatant, cells were resuspended in D-Hank's Ca^{2+}-free solution prior to centrifugation at 800 x g for 5 min. Neurons were suspended in D-Hank's Ca^{2+}-free solution at a density of $2x10^6$ cells/ml. A yield of 90% viability was obtained as determined by trypan blue exclusion staining. Hippocampal neurons were maintained in a 5% CO_2 incubator at 37˚C until use.

Analysis of apoptotic cell death. Apoptosis was evaluated by Annexin V-FITC and PI double staining. Briefly, cells were incubated with Annexin V-FITC and PI at room temperature for 15 min and were then analyzed by flow cytometry (Elite, Beckman-Coulter, Inc., USA) with the excitation set at 488 nm and emission at 525 (FITC green fluorescence) and 610 nm (PI red fluorescence). Annexin V co-labeling with PI was used to discriminate between intact cells (Annexin V^-/PI^-), early apoptotic (Annexin V^+/PI^-), and late apoptotic or necrobiosis cells (Annexin V^-/PI^+). Necrotic cells was recognized as positive for Annexin V-FITC and PI staining (V^+/PI^+)(11). Then the early apoptotic cells fluorescence intensity of Annexin V^+/PI^- was analyzed by LMD software to obtain the rate of apoptosis.

Measurement of intracellular Ca^{2+} levels in hippocampal neurons. The $[Ca^{2+}]_i$ was monitored using the membrane-permeable Ca^{2+}-sensitive fluorescent dye Fluo-3/AM. The Fluo-3/AM is converted to Fluo-3 upon deacetylation within the cells, and Fluo-3 increases green fluorescence upon Ca^{2+} binding. Hippocampal neurons were incubated with 30 μl Fluo-3/AM solution (10 μg/ml) at 37˚C for 30 min. Microscopic images were captured with a Bio-Rad MRC-1024 confocal system (Bio-Rad, Hercules, CA, USA). The intensity of Ca^{2+} fluorescence and distribution was monitored by LSCM (Bio-Rad) at 488 nm excitation wavelengths. Data were then analyzed by image analysis software (Bio-Rad) and expressed by intensity of Fluo-3/AM.

Statistical analysis. The data were analyzed using the Statistical Package for Social Sciences (SPSS) 11.0 software and were plotted as the mean ± SD. Statistically significant differences were carried out by one-way analysis of variance (ANOVA), followed by post-hoc comparisons (least-significant difference, LSD or Dunnett's T3). For analysis of $[Ca^{2+}]_i$ and apoptotic rates in different experiment groups, a two-tailed t-test was used. $P<0.05$ was considered to indicate statistically significant differences. The correlation between apoptotic cell death and $[Ca^{2+}]_i$ concentration under the same SPL was calculated by bivariate correlation followed by the Pearson test. $P<0.01$ was considered to indicate statistically significant differences.

Results

Infrasonic exposure increased the levels of $[Ca^{2+}]_i$ in hippocampal neurons. To evaluate the potential effect of infrasonic exposure on the levels of $[Ca^{2+}]_i$ in hippocampal neurons, the changes in the levels of $[Ca^{2+}]_i$ were examined after rats were exposed to 8 Hz, 90 or 130 dB infrasound on different days. The membrane-permeable Ca^{2+}-sensitive fluorescent dye Fluo-3/AM was used to determine the $[Ca^{2+}]_i$ levels. As shown in Fig. 1, no significant changes in the intensity of Fluo-3/AM were found on days 1 and 7 after both 90 and 130 dB exposure. However, the intensity was significantly enhanced by nearly 5-fold on day 14 after rats received daily treatment with 130 dB SPL infrasonic exposure, when compared with the control group ($P<0.01$, 130 dB SPL vs. control). The intensity gradually declined but was still significantly higher (2.5-fold increase) than that in the control group after 21 days of infrasonic exposure ($P<0.01$); the intensity was then reduced to the control level after 28 days of exposure to 90 or 130 dB. In addition, it should be noted that the highest intensity was detected on day 14 in the 130 dB (8 Hz) infra-

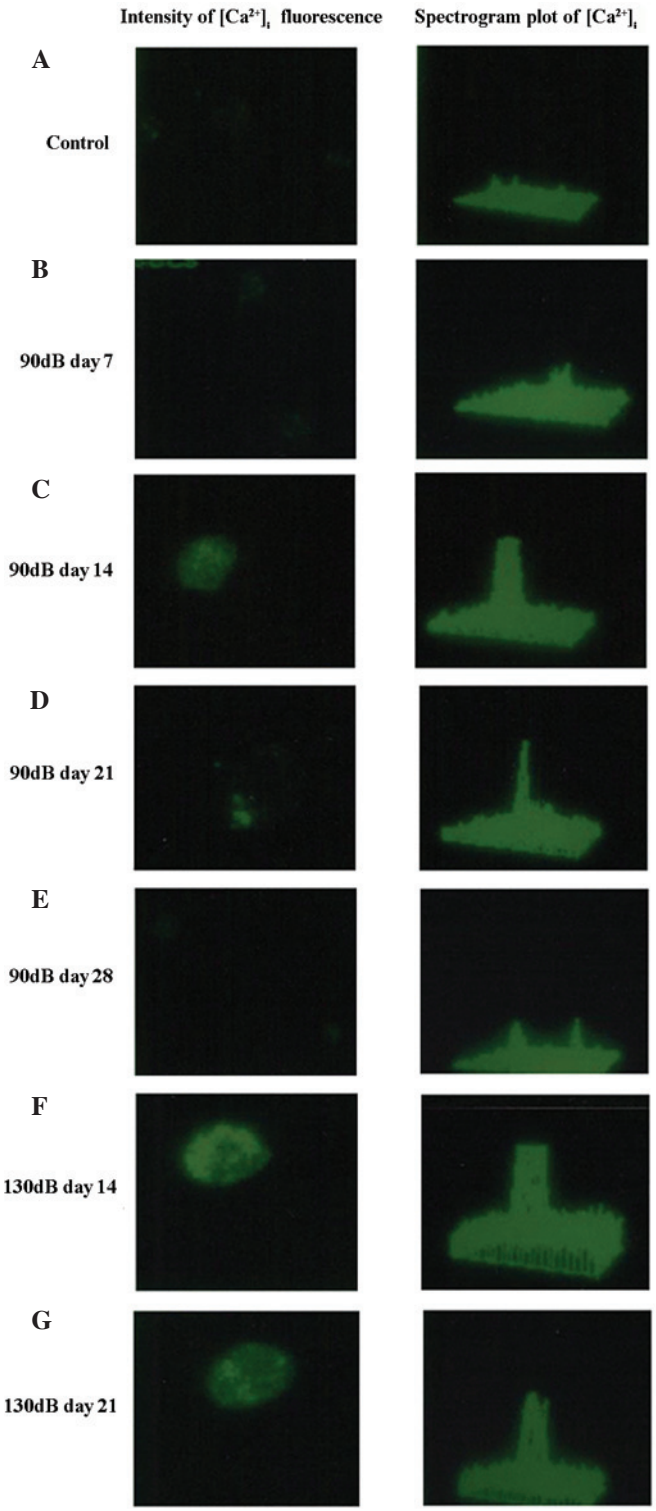

Figure 1. The intracellular levels of Ca^{2+} ($[Ca^{2+}]_i$) in rat hippocampal neurons. Rats were exposed to 8 Hz 90 dB or 130 dB infrasound for 2 h once daily. Hippocampal neurons were isolated on day 1, 7, 14, 21 or 28, and were stained with the Ca^{2+}-sensitive fluorescent dye Fluo-3/AM. Fluorescent intensity was analyzed to reflect the levels of $[Ca^{2+}]_i$ as described in the Materials and methods section. After 8 Hz 90 dB infrasonic exposure, there were no significant changes of $[Ca^{2+}]_i$ both on day 1 and (B) on day 7 (both $P>0.05$ vs. control); however, a significant increase in the intensity on (C) day 14 was observed ($P<0.01$), which remained at high levels after (D) 21 days of exposure, then returned to normal on (E) day 28. Following 8 Hz 130 dB infrasonic exposure, there were no significant differences of intensity on day 1 and day 7 (both $P>0.05$, vs. control). (F) The most marked increase in the fluorescent intensity was observed on day 14 ($P<0.01$), then it decreased but remained at a high level of intensity on (G) day 21, returning to normal levels on day 28.

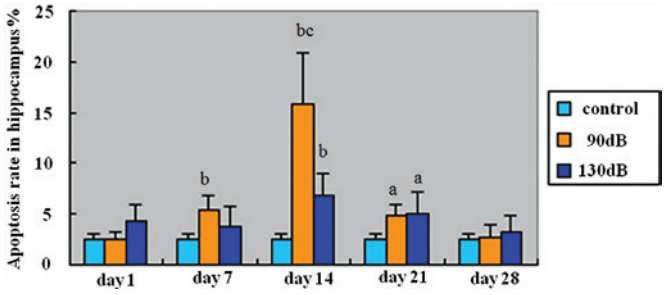

Figure 2. Percentage of the apoptotic cells in the rat hippocampus measured by flow cytometry following exposure to infrasound. Hippocampal cell apoptosis was quantified by double staining with annexin V and PI on days 1, 7, 14, 21 or 28 following infrasonic exposure. The percentage of apoptotic cells was quantified and expressed as the mean ± SD. [a]$P<0.05$ compared with the control group; [b]$P<0.01$ compared with the control group; [c]$P<0.01$ compared with all of other groups.

sound exposure group (Fig. 1). Moreover, the intensity in the 130 dB exposure group was significantly greater than that in the 90 dB group ($P<0.01$, 90 dB SPL vs. 130 dB SPL). These results indicate that infrasonic exposure with 130 dB significantly increased the levels of $[Ca^{2+}]_i$.

Effect of infrasonic exposure on apoptotic cell death. To demonstrate whether infrasonic exposure leads to cell death through apoptosis, Annexin V and PI double staining were applied. We found that an elevation in the percentage of early apoptotic cells was detected from day 7 following 90 dB infrasound exposure ($P<0.01$, 5.39±1.40% vs. control 2.45±0.61%). Significant enhancement of apoptosis was observed on day 14 (15.83±5.00%, $P<0.01$, 90 dB SPL compared with samples of all other groups). Following exposure to 130 dB (8 Hz) infrasound, the proportion of apoptotic hippocampal cells increased significantly on day 14 (6.80±2.14%, $P<0.01$, vs. control), while the number of apoptotic cells gradually decreased thereafter (Fig. 2). Notably, no detectable differences were found after 28 days of infrasonic exposure ($P>0.05$). Representative distribution of the Annexin V/PI flow cytometry results are partly shown in Fig. 3. These results suggest that infrasonic exposure induces apoptotic cell death in the rat hippocampus.

Apoptotic cell death correlated with the elevation in the levels of $[Ca^{2+}]_i$. To examine whether there is a correlation between apoptosis and the levels of $[Ca^{2+}]_i$, the Pearson correlation (r) was used to determine the association between apoptotic cell death and the elevation of $[Ca^{2+}]_i$ in the pattern of differences. We found that the apoptosis of hippocampal neurons was markedly correlated with the levels of $[Ca^{2+}]_i$ on each time point examined (90 dB infrasonic exposure, $r=0.756$, $P<0.01$; 130 dB infrasonic exposure, $r=0.674$, $P<0.01$), revealing the positive correlation between these two variables.

Discussion

Previous studies have shown that brain cognitive functions are significantly affected by infrasound (2,12,13). Similarly, impairment was also verified in the cortex and hippocampus induced by infrasound with 90 and 130 dB SPL (3,6,7). To further understand the effects of infrasound on humans and

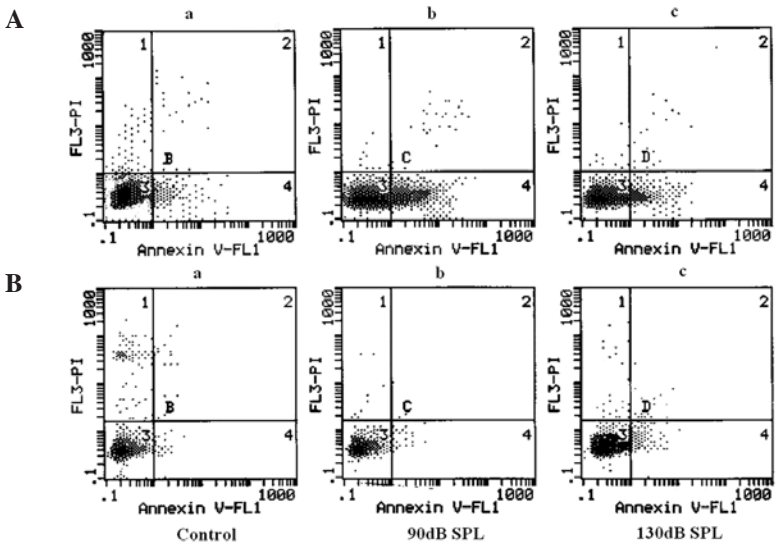

Figure 3. Representative analysis of an Annexin V vs. PI contour plot. Hippocampal cell apoptosis (Annexin V^+/PI^-) at (A) 14 or (B) 28 days after infrasonic exposure was quantified by staining with Annexin V and PI. The apoptosis rate increased significantly on day 14 with 90 and 130 dB SPL; however, no significant difference was found in the proportion of apoptotic cells between the control and exposure groups following exposure to 90 or 130 dB on day 28 (both $P>0.05$). The proportion of early apoptotic cells (plot 4) was as follows, control 2.45±0.61% (Fig. 3 Aa and Ba), apoptotic cells 15.83±5.00% on day 14 with 90 dB SPL (Fig. 3Ab, $P<0.01$ vs. control, $P<0.01$ compared with samples all of other groups), apoptotic cells 6.80±2.14% on day 14 with 130 dB SPL (Fig. 3Ac); apoptotic cells 2.71±1.28% on day 28 with 90 dB SPL (Fig. 3Bb), apoptotic cells 3.16±1.63% on day 28 with 130 dB SPL(Fig. 3Bc).

animals, we applied the same infrasonic exposure parameters once daily from 1 to 28 days for 2 h on adult rats. The present study shows that apoptosis in the hippocampus significantly increased on day 14 after rats had been exposed to 90 dB (8 Hz) infrasound 2 h daily, as demonstrated by the increased levels of $[Ca^{2+}]_i$. Moreover, following 130 dB (8 Hz) of infrasound treatment, hippocampal neurons exhibited apoptosis from day 14. Notably, in the two groups, the number of apoptotic cells gradually decreased to normal levels following 28 days of infrasonic exposure. These results suggest that 90 or 130 dB infrasound induces apoptotic cell death in the rat hippocampus, whereas the effect of infrasound on cell apoptosis depends on the exposure duration as well as SPL. Although the 90 dB infrasonic exposure induced a greater increase in apoptotic cell death on day 14 than the 130 dB infrasonic exposure, it may not be easy to conclude that 90 dB infrasound promotes cell death to a greater extent than 130 dB infrasound, since apoptosis is not the only pathway that leads to cell death. Previous studies have also revealed that 8 Hz 130 dB exposure dramatically induces necrotic cell death in the rat hippocampus (6,7). Additionally, a marked positive correlation between cell apoptosis and the levels of $[Ca^{2+}]_i$ was found during infrasound exposure on day 14 and day 21 following treatment with 90 and 130 dB SPL.

Apoptosis is one of the most important types of PCD, and it has been observed among diverse cell types in the process of non-traumatic cell death. The molecular basis of the apoptotic pathway has been defined, and genes including bcl-2, c-myc, p53, ice and fas/apo-1 have been proposed to be involved in apoptosis (14). The effect of infrasound on apoptotic cell death has been suggested in numerous studies. The infrasonic exposure at 8 or 16 Hz may induce changes in the membrane permeability of red blood cells, the activities of enzymes and serum catecholamine levels (15-17). Furthermore, a previous study has shown that 16 Hz, 90 or 130 dB infrasonic exposure markedly upregulates the levels of p53 mRNA in mice hippocampi (9). These results indicate that infrasound influences apoptotic cell death via different pathways.

Importantly, Ca^{2+} has a major function in triggering mitotic division in numerous cell types and, conversely, in the regulation of cell death (18). Low cytoplasmic Ca^{2+} concentration is critical to maintain normal cell functions, while the cellular Ca^{2+} overload is known to be highly toxic, causing massive activation of proteases and phospholipases, leading to cell death (19,20). ATPase pumps and mitochondria are closely associated with Ca^{2+} release and play significant roles in the maintenance of $[Ca^{2+}]_i$ level (20). Accumulating evidence has shown that infrasonic exposure induces abnormal ultrastructures in cortical and hippocampal neurons (6,7) and promotes blood-brain barrier permeability (5), leading to severe damage to brain tissue (16). Liu *et al* have shown that necrotic death occurs in hippocampal CA1 cells, and condensed nuclear, swollen mitochondria and vacuolar structures have been observed in these cells on days 7 or 14 following 8 Hz 130 dB infrasonic exposure (5). These abnormal structures may result in the release of Ca^{2+} from the damaged mitochondria and from the ER. Meanwhile, impaired brain energy metabolism may cause dysfunction in Ca^{2+}/Mg^{2+}-ATPase or Ca^{2+}/H^+-ATPase, resulting in cytoplasmic Ca^{2+} overload and a series of cytotoxic events.

In this study, the concentration of $[Ca^{2+}]_i$ in hippocampal neurons increased following infrasonic exposure; it peaked on day 14 (90 dB), and gradually decreased to normal levels after 28 days of infrasonic exposure, suggesting a self-repairing capability occurred in the cells. A similar time-dependent pattern has also been observed in hippocampal neuron injury as described in previous studies (4,6). It has been shown that the increased expressions of 70-kDa heat-shock protein (Hsp70) and glial fibrillary acidic protein are detected following a period of infrasonic exposure, suggesting the critical role of

Ca^{2+} overload and the activation of self-protective mechanisms during CNS damage.

In the present study, the marked positive correlation between apoptotic cell death and elevation of the $[Ca^{2+}]_i$ following infrasonic exposure was observed, suggesting that Ca^{2+} overload may trigger apoptotic cell death in the rat hippocampus. Moreover, the release of intracellular Ca^{2+} from mitochondria or ER, or increased Ca^{2+} influx may act as apoptotic signals, leading to cell apoptosis. On the other hand, released Ca^{2+} may cause the breakdown of cellular organelles, activate apoptosis-related transcription factors, and therefore trigger apoptosis (10). Ca^{2+} may act as a regulator and effector and may play a prominent role during apoptotic cell death. Future studies may aim to investigate the potential effect of the Ca^{2+} antagonist on prevention of infrasonic exposure-induced brain damage. Nevertheless, in the present study, enhanced apoptotic cell death was observed on day 7 following 8 Hz 90 dB infrasonic exposure, whereas no detectable difference was found in the level of $[Ca^{2+}]_i$ at the same time point compared with the control group, suggesting that other pathways may be involved in infrasound-induced cell apoptosis.

The hippocampus is one of the most critical regions for learning and memory. In conclusion, the present study demonstrated that a period of infrasonic exposure with an SPL of 90 or 130 dB induced apoptosis and upregulation of the concentration of $[Ca^{2+}]_i$ in the rat hippocampus, suggesting that infrasound causes CNS injury via a Ca^{2+}-mediated apoptotic pathway in hippocampal neurons. A better understanding of the molecular mechanisms of neuronal cell death in nervous system development, injury and disease would lead to new therapeutic approaches for the prevention of brain damage induced by infrasonic exposure.

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