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Research Article

Sound affects the neuronal maturation of neuroblastoma cells and the repair of damaged tissues

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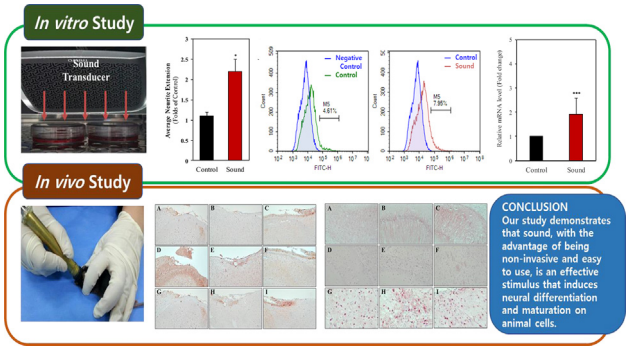
GRAPHICAL ABSTRACT



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ABSTRACT

Background: Sound is a kind of mechanical stimulus and has various effects on the growth and metabolism of plants and animal cells. In previous studies, it was confirmed that sound stimulation promotes the neurodifferentiation process of mesenchymal stem cells. In this study, we examined the effect of sound on the maturation of neuroblastoma cells, SH-SY5Y cells, and investigated its effect on an ischemic mouse stroke model. In the *in vitro* study, SH-SY5Y cells were exposed to the sound for 3 days and then performed rt-PCR, FACS, and western blot for analysis. In the *in vivo* study, mesenchymal stem cells were injected into the injured area, and then rats were exposed to sound for 4 weeks. Then, immunohistochemical staining and western blotting were performed.
Results: Sound upregulated the expression of presynaptic proteins synaptophysin and postsynaptic density protein 95, as well as neuronal-related proteins such as NFL, Tau, and MAP2. T-type calcium channels such as *CACNA1G* and *CACNA1I* were also induced by sound. In an experiment using the brain of ischemic mice, the expression of proteins involved in neuronal differentiation such as MAP2, NF200, and S100 was increased, while the inflammation-related proteins IFNγ, MMP9, and TNFα were decreased. In this neuronal differentiation process, both ERK and CREB, which are proteins involved in the initial signal transduction process, were activated.

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Conclusions: Our study demonstrates that sound, with the advantage of being non-invasive and easy to use, is an effective stimulus that induces neural differentiation and maturation on animal cells.

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1. Introduction

Sound is an acoustic energy of a wave propagated through gases, liquids, or solids and transfers energy at the same time. It exists everywhere in nature, is identified by its hertz (or wavelength), intensity (or decibel), velocity and direction [1]. Sound technology has been applied to plant growth, photosynthesis, hormones, etc. Sound can increase disease resistance of plants and reduce requirements for chemical fertilizers and biocides [2,3]. The effects of sound stimulation in animals were reported. The size and number of nerve cells were increased in fertilized chick eggs, and the expression of cAMP response element binding protein (CREB) and brain-derived neurotrophic factor (BDNF) and Ca^{2+} influx was changed in chick hippocampus [4,5,6]. In a study of patch-clamp recordings in hippocampal neurons in awake mice, sound almost induced hyperpolarization in hippocampal neurons [7].

Mechanical stimulation was shown to have a crucial role in cell growth and differentiation *in vitro,* because those are an essential regulator in tissue homeostasis, which is required for normal functions *in vivo* [8]. Mechanical stimulation, controlled by bioreactors in the field of tissue engineering and regenerative medicine, is widely used to mimic the mechanical environment that cells, especially connective tissue cells, are exposed to. Therefore, the type of mechanical stimulation is dependent on the type of cell, tension to tendon fibroblasts, ligament fibroblasts [9,10], skeletal muscle cells [11], chondrocytes [12], and vascular cells [13]. The effect of mechanical stimulation depends on its frequency, magnitude, and duration. When a mechanical stimulus is delivered to a cell, it produces a biochemical output known as a mechanochemical signal [14]. Human bone marrow-derived mesenchymal stem cells (hBM-MSCs) were induced to undergo neuronal differentiation by sound waves (1 kHz, 81 dB) via protein tyrosine kine 2 (PYK2) and cAMP response element-binding protein (CREB) phosphorylation, and calcium release [15]. Sounds, including acoustic vibrations, induced cell death or growth arrest in breast cancer cell lines [16] and affected cytoskeletal molecules via coherent changes in their spatial organization and mechano-transduction signaling [17].

We previously reported the effect of mechanical stimulation on cell differentiation. Briefly, sub-sonic vibration (SSV) inhibited preadipocyte growth and induced their maturation to adipocytes by upregulating lipid accumulation-related genes [18]. Furthermore, umbilical cord and adipose tissue derived mesenchymal stem cells were induced to differentiate into neural cells by SSV, partly through extracellular signal-regulated kinase (ERK) activation [16,19]. In that study, SSV delivered a stimulus directly to the cells. However, few studies have investigated the effect of indirectly transmitted sound on cells.

In this study, the effect of sound transmitted indirectly through the air on SH-SY5Y cells *in vitro*, a model of neuronal function and maturation, was examined. In the *in vivo* study, we used hBM-MSCs instead of SH-SY5Y cells for cell transplantation and magnetic nanoparticle (MNP) incorporated cells were used for cell localization. We have confirmed the effect of sound in a mouse model of ischemic stroke.

2. Materials and methods

2.1. Exposure of SH-SY5Y cells to sound

Neuroblastoma SH-SY5Y cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, NY, USA) containing 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% (v/v) fetal bovine serum (Lonza, Basel, Switzerland). Culture medium was changed to high glucose DMEM supplemented with 5% (v/v) FBS and 5 μM retinoic acid (Sigma-Aldrich, St. Louis, MO, USA) and divided into 2 groups: control (no exposure) and sound (1 kHz, 81 dB, 0.125 $\mu W/cm^2$). The space between the cells and the transducer was 5 cm and they were exposed for 15 min every 12 h and the exposure was terminated on the third day (Fig. 1).

2.2. Cell toxicity assay

General toxicity was performed by an LDH assay modified by Ho et al. [20]. LDH activity was performed using an LDH-LQ kit (Asan Pharmaceutical, Seoul, Korea). The ratio of medium: working solution was 2:1, and incubated in the dark condition for 30 min. Then, the reaction was terminated by adding stop solution, and the absorbance was measured at 490 nm. Neurites were counted by microscopy.

2.3. Western blot analysis

SH-SY5Y cells were extracted with sample buffer (pH 6.8) consisting of 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 0.1 mg/mL bromophenol blue dissolved in Tris-HCl and incubated at 95 °C for 5 min. Protein concentrations were determined by Bicinchoninic acid assay (Thermo Scientific, Rockford, IL, USA). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% (v/v) acrylamide gel and then transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% (v/v) skim milk solution at room temperature, followed by incubation with primary antibodies against Microtubule-associated protein 2 (MAP2), Neuronal Differentiation 1 (NEUROD1), phosphorylated extracellular signal-regulated kinase (p-ERK), phosphorylated cAMP response element-binding protein (p-CREB) (Cell Signaling Technology, Beverly, MA, USA), microtubule-associated protein (TAU) (Invitrogen, Carlsbad, CA, USA), as well as β-actin (Sigma-Aldrich, St. Louis, MO, USA). After washing, the membrane was incubated with anti-mouse and anti-rabbit secondary antibodies (Cell Signaling, Beverly, MA, USA) at room temperature. Protein detection was performed with Lumi Femto (Daeil Laboratory Service, Seoul, Korea) and the Molecular Imager ChemiDoc XRS + station. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to analyze and quantify the western blot images.

2.4. Fluorescence-activated cell sorting (FACS)

Anti-NeuN and anti-DCX (doublecortin) antibodies were purchased from Abcam (1:100, Cambridge, UK). A total of 1×10^6cells

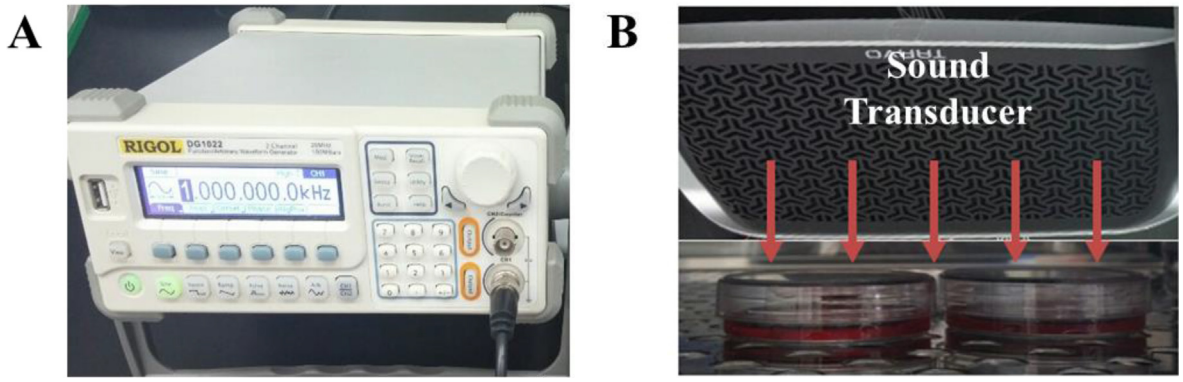

Fig. 1. Photo images of the machine used in this experiment. (A) A sound generator installed outside the CO_2 incubator (B) Sound transducer installed inside. The sound generator produced a sound at 1 kHz, 81 dB (0.125 μW/cm^2). Cells were exposed to sound twice (during 15 min every 12 h) per day for 3 d.

were re-suspended in 500 μL ice-cold phosphate buffered saline with 3% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) and incubated with antibodies. After washing, the cells were incubated with human secondary antibodies conjugated with Alexa Fluor 488 and Alexa Fluor 555 (Cell Signaling Technology, Boston, MA, USA) for 1 h at 4 °C in the dark. Anti-NeuN was conjugated as Alexa Fluor 555 secondary antibody (Phycoerythrin, PE-conjugated) and Anti-Doublecortin was conjugated as Alexa Fluor 488 secondary antibody (Fluorescein isothiocyanate, FITC-conjugated). The flow cytometry performed a FACScan (BD Sciences, San Jose, CA, USA), and the data were analyzed using CELLQUEST software (BD Sciences, San Jose, CA, USA).

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

1×10^6 cells were treated with 1 mL of Trizol (Thermo Fisher Scientific, USA). Homogenized samples in Trizol were mixed with of chloroform. After centrifugation, the supernatant containing RNA was transferred to a new tube, after which isopropanol was added for precipitation of RNA. After 10 mins, centrifugation was performed. The pellet was washed with 75% (v/v) ethanol, centrifuged, and the pellet dried. The amount of total RNA was determined by a NanoDrop device (Thermo Fisher Scientific, USA). Polymerase chain reaction (PCR) was conducted to synthesize cDNA from 1 μg of total RNA using an Advantage RT-PCR kit (Clontech, Palo Alto, CA, USA). Real-time qPCR was performed using the SimpliAmp Thermal Cycler (Applied Biosystems, Foster City, CA, USA), and SYBR green was bound to double-stranded DNA to enable real-time quantitative detection of PCR products based on SYBR green fluorescence. Results were analyzed with a comparative cycle threshold (CT) method for quantification of relative gene expression for housekeeping genes (GAPDH). Primers used for PCR are listed in Table 1.

2.6. Immunohistochemical staining

Cells on cover slides were fixed with 4% (v/v) paraformaldehyde for 10 min and then washed with 10 mM Tris-HCl buffer. Next, the slides were slightly shaken with postsynaptic density protein 95 (PSD 95, 1:200, abcam, Cambridge, UK), neurofilament-L (NF-L, 1:100, Cell Signaling Technology, Boston, MA, USA), microtubule associated protein (TAU, 1:200, Invitrogen, Carlsbad, CA, USA), and synaptophysin (1:200, Cell Signaling Technology, Boston, MA, USA) antibodies, followed by a dextran polymer system, EnVision$^+$ kit (DAKO, Copenhagen, Denmark).

2.7. Photothrombotic (PT) ischemic mouse model

2.7.1. Animals

All *in vivo* protocols in this study were performed as required by the Animal Care and Use Committee of Dongguk University (IACUC2013-022). We induced a mouse stroke model by using photothrombosis. Mice were anesthetized with Zoletil and Rompun, and Rose Bengal was injected through the penis at 10 μl/g body weight. Then, 10 min later, the mouse brain was exposed for 15 min with a 150 W intensity cold light illuminator (Schott KL 1500 LCD, Mainz, Germany) (Fig. 2). A total of 30 mice were used in the *in vivo* experiment. Three days after injury, 5 mice without stroke formation were excluded. The remaining mice were used in the final experiment.

2.7.2. Cell injection

hBM-MSCs were purchased from Lonza (Walkersville, MD, USA). Cells were maintained in a nonhematopoietic (NH) stem cell medium (Miltenyi Biotech, Bergisch Gladbach, Germany) with penicillin 100 unit/mL and streptomycin 100 μg/mL (Invitrogen, Carlsbad, CA, USA). For cell separation, accutase (Innovative Cell Tech., San Diego, CA, USA) was used and cells were passaged in a 1:4 plate ratio.

There are three groups in the *in vivo* study. Stroke only group (cell-free control, $N = 9$), stroke with hMSC injection group (hBM-MSC injection after stroke, $N = 8$), stroke with both hMSC injection and sound exposure group (sound exposure after hBM-MSC injection after stroke induction, $N = 8$). After induction of ischemia, 1×10^5 hBM-MSCs cells were injected. Cells were not differentiated, but cells treated with Fe_3O_4 magnetic nanoparticles (MNP) were injected 24 h before. The Fe_3O_4 nanoparticles were used to determine cell localization within the mouse brain [21].

Table 1
Primer sequences used for RT-PCR.

Genes	Upstream primer sequence	Downstream primer sequence
PSD95	5′-GAA TTT AGT GGG GAG AAG CA-3′	5′-TCA CCT GCA ACT CAT ATC CT-3′
CACNA1E	5′-GTT CGG CCG CGA TCA CCT TTG T-3′	5′-GGC GGC CAA TCG ATG AGC TTC T-3′
CACNA1G	5′-CGG CAA CTA CGT GCT CTT CA-3′	5′-GTG ACT TCA TCT CGT GGG CC-3′
CACNA1I	5′-CGT TGT CAT AGC GAC CCA GTT-3′	5′-CAC AGC TCT CTT CCC GAG TGA-3′
GAPDH	5′-ACC ACA GTC CAT GCC ATC AC-3′	5′-TCC ACC ACC CTG TTG CTG TA-3′

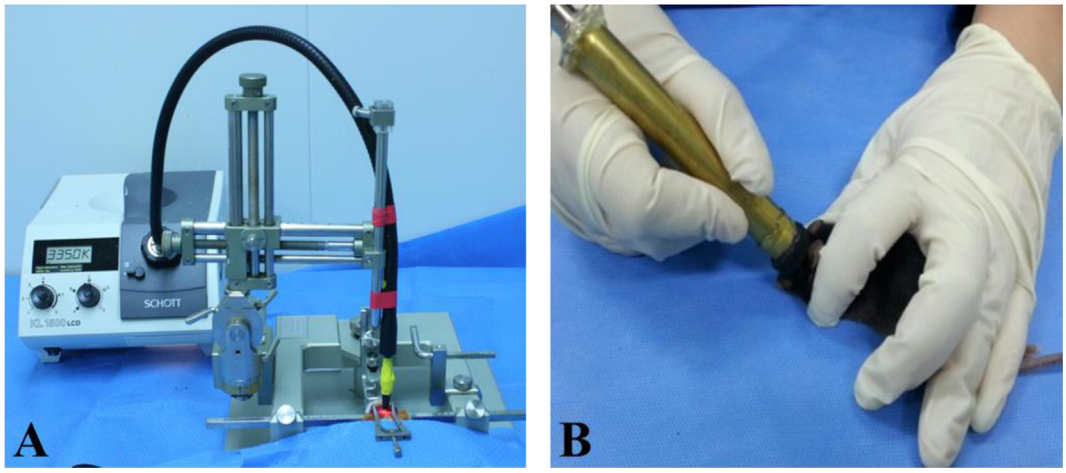

Fig. 2. Photos of instruments used in the in vivo study. (A) Cold laser for ischemic injury of mouse brain. (B) Sound generator. Ischemic mouse brains were exposed to sound for 20 min per day.

2.7.3. Exposure to sound

Sound exposure in ischemic stroke mice started 1 d after cell injection. Sound with 1 kHz frequency and 81 dB (0.125 μW/cm^2) intensity continued for 20 min per day for 4 weeks. During sound exposure, the probe was positioned approximately 50 mm from the ischemic lesion. As shown in Fig. 2B, the mouse was held, and this prevented the mouse from moving out of the sound exposure area.

2.8. Histological and immunohistochemical analyses

Hematoxylin and eosin staining was performed 10 d after ischemia and the brains in each group were removed and embedded in paraffin. The brain tissues were dissected laterally into 2-mm slices, including the infarct core on the mouse brain matrix, with a surgical blade. For staining, slices including the perilesional ischemic core were stained with 4% (v/v) paraformaldehyde for 1 h and were then washed three times. The sections were stained with Mayer's Hematoxylin, washed in tap water to mature the hematoxylin staining, and then stained with Eosin in subsequent eosin maturation baths containing 95% (v/v) ethanol and 100% (v/v) ethanol, followed by xylene baths, and then mounted using permanent mounting media (Richard-Allan Scientific, Kalamazoo, MA, USA).

In Prussian blue staining, mouse brain sections were washed with PBS three times and incubated with 5% (w/v) potassium ferrocyanide in 5% (v/v) hydrochloric acid solution. After 30 min, they were washed with PBS, counterstained with nuclear fast red, and examined under a light microscope.

For immunohistochemical analysis, we fixed the sections with 4% (v/v) paraformaldehyde for 10 min, then washed them with 10 mM Tris-HCl buffer (pH 7.2) three times. These sections were incubated with anti-MAP2, anti-NF200, anti-S100, anti-IFNγ (interferon gamma), anti-MMP9 (matrix metallopeptidase 9) and anti-TNFα (Tumour Necrosis Factor alpha) antibodies (Abcam, Cambridge, UK) and developed with the Envision + kit (DAKO, Copenhagen, Denmark).

2.9. Western blotting for in vivo studies

Brains were isolated from the skull and infarcted brain tissue was biopsied (diameter 2 mm × height 1.5 mm) from the wound in the mouse brain cortex using a 2-mm biopsy punch. The ischemic brain tissue was dissolved using sample buffer. Primary and secondary antibodies were used in the same manner as in vitro Western blotting.

2.10. Statistical analysis

Data are presented as mean ± SEM of three independent experiments performed in triplicate. A one-way analysis of variance (ANOVA) followed by Tukey's HSD (Honestly Significant Difference) multiple comparisons test was performed with Prism (GraphPad, San Diego, CA, USA). Mean differences were significant at $P < 0.05$ ($^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.005$).

3. Results

3.1. Effect of sound on cell morphology, growth, and damage

SH-SY5Y cells cultured in 10% FBS did not respond to sound. It is thought that high concentrations of FBS mask the effects of sound. When the concentration of FBS was reduced to 5% and retinoic acid (RA) was added at 5 μM, the cells responded well to sound. Control cells showed a clustered morphology with few neurites whereas sound exposed cells showed a scattered morphology (Fig. 3 A–D) with many elongated neurites (Fig. 3 E). Of note, there was no statistical difference in cell number or relative LDH activity among groups 3 days after exposure. This result indicates that sound did not damage the cells (Fig. 4).

Conflicting studies have reported cell growth is either stimulated or inhibited by mechanical stimulation. Ultrasound is known to enhance cell survival and proliferation by promoting membrane permeability followed by an increase in mass transfer; however, we did not consider this effect because sound did not improve cell proliferation in our study.

3.2. Effect of sound on neuronal maturation

Based on morphological changes, we analyzed whether sound had a neuronal maturation effect. MAP2 and TAU proteins are cytoskeleton-related proteins that stabilize the neuronal shape of cells. Doublecortin (DCX) is a type of neuronal migration-related protein expressed during maturation and is used as a neurogenesis marker. In this experiment, we confirmed an increase in MAP2, NFL, TAU and DCX proteins by sound (Fig. 5). Immunohistochemical staining showed the same results for NF and TAU proteins, and synaptophysin, used as a presynaptic vesicle protein, was also well

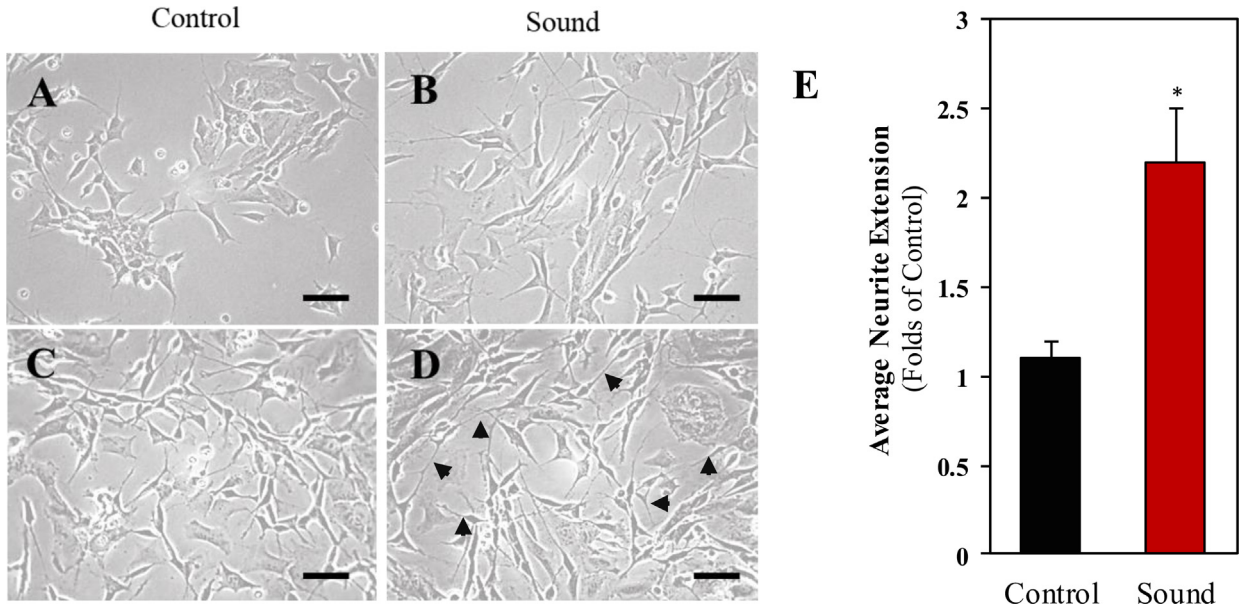

Fig. 3. Morphology of the SH-SY5Y cells after stimulation with sound for 2–3 d and neurite extension folds compared with controls. All cells were cultured in media that induced neuronal differentiation. (A, C) Control, (B, D) exposed to sound, (A, B) after 2 d, (C, D) after 3 d. (E) Average neurite outgrowth folds compared with controls. Original magnification: ×100, bar = 100 μm. Each bar represent the mean ± SD of independent experiments performed in triplicate (n = 3). *p < 0.01.

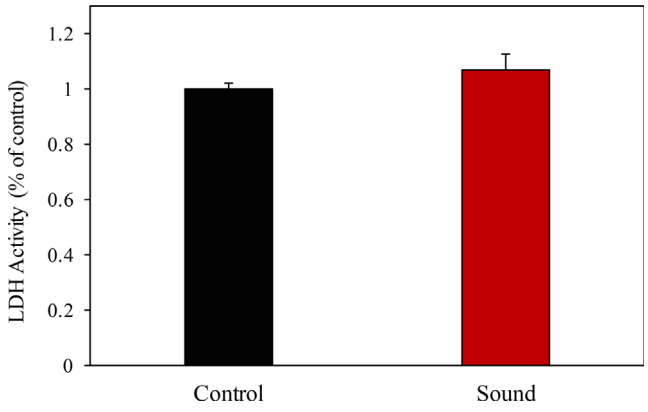

Fig. 4. Cytotoxicity of sound on SH-SY5Y cells. Cells were exposed to sound twice (15 min every 12 h) per day for 3 d. Each bar represents mean ± SD of independent experiments performed in triplicate (n = 3).

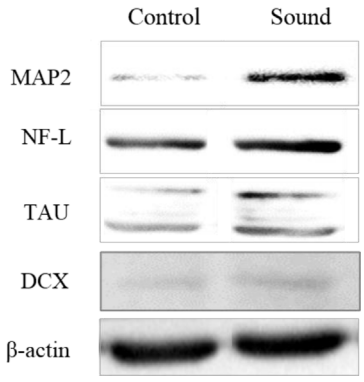

Fig. 5. The changes of neuronal maturation-related proteins in SH-SY5Y cells by sound.

expressed (Fig. 6). When DCX protein is continuously expressed in neuronal progenitor cells for 2–3 weeks, neuronal progenitor cells reach maturity, and at this time, neuronal nuclear antigen, NeuN, is expressed. We performed FACS analysis to compare the expression of DCX and NeuN induced by sound in SH-SY5Y cells. DCX protein expression increased from 4.61% to 7.95% by sound, but there was no change in NeuN protein expression (Fig. 7). We could hardly detect any NeuN positive cells for DCX in this analysis (Data are not shown).

3.3. Effect of sound on synaptic vesicles

Synaptophysin is an integral membrane glycoprotein localized to synaptic vesicles. Although its exact function is unknown, it interacts with synaptobrevin, an essential synaptic vesicle protein. In neurons, synaptic vesicles are released at the ends of synapses, which are regulated by voltage-dependent calcium channels. PSD95 proteins may interact at the postsynaptic sites to form associated signaling proteins. As shown in Fig. 8, *PSD95* gene expression was increased 1.9 folds compared to the control, and it was confirmed that protein expression was also increased through immunohistochemical staining.

Calcium channels are divided into two groups, high and low voltage-dependent channels. *CACNA1G* and *CACNA1I* are voltage-dependent T-type calcium channels activated by low voltage and are present during the early stages of brain development [22]. *CACNA1E* is a voltage-dependent R-type calcium channel, which is activated by intermediate voltage and is primarily expressed in neurons [23]. Fig. 9 shows that only T-type voltage-dependent calcium channels were upregulated by sound whereas R-type calcium channels were not affected.

3.4. Effect of sound on the expressions of neural proteins in ischemic mouse brains

For the *in vivo* study, ischemic lesions were induced using the cold-laser exposure PT method. We used hMSCs for cell transplantation to mouse brains because they help the recovery of spinal cord injury in rats induced by mechanical stimulation such as extremely low frequency electromagnetic fields [24]. We described the synergistic effect of sound in ischemic injured mouse brain. To identify ischemic lesions, H & E staining was performed. The

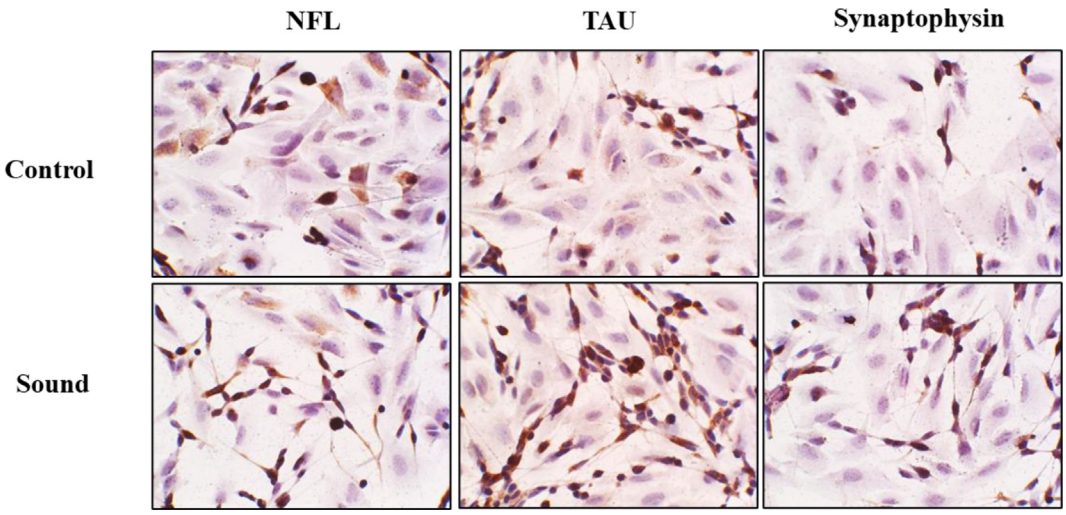

Fig. 6. Neuron-related protein expression after sound exposure. Immunohistochemical staining of neuronal cell markers in SH-SY5Y cells. Cells were fixed and labeled with anti-NFL, anti-TAU, and anti-synaptophysin antibodies. Original magnification 200×.

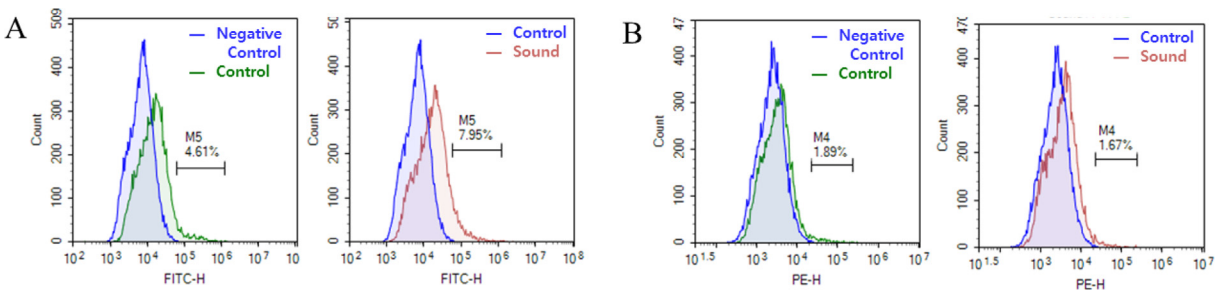

Fig. 7. The changes of neuronal markers NeuN and Doublecortin in SH-SY5Y cells by sound using FACS analysis. (A) FITC-conjugated anti-DCX. (B) PE-conjugated anti-NeuN. A negative control means that the cells do not express both NeuN and DCX proteins in the cell surface.

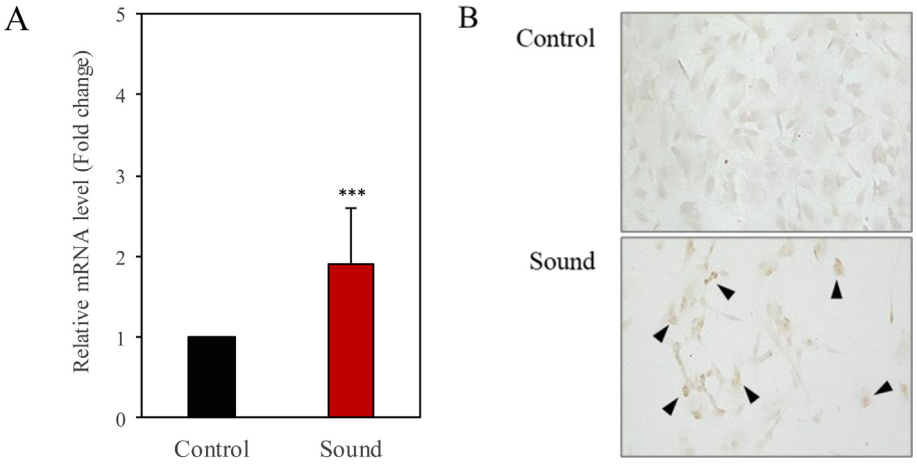

Fig. 8. The changes of postsynaptic-related proteins PSD95 in SH-SY5Y cells by sound. (A) Real-time PCR. (B) Immunohistochemical staining. Cells were fixed and labeled with anti-PSD95. Arrowheads indicate expressed PSD95 protein. Original magnification 200×. Column heights correspond to the mean values and the error bars correspond to the standard deviations (n = 3). ***p < 0.005.

ischemic lesions were clearly detected in all groups (Fig. 10). In this study, we used MNP for cell tracking, which was incorporated into the hMSCs before injection. Cells were detected by Prussian blue staining in the damaged lesions (Fig. 10 E,F); therefore, we concluded that hMSCs migrated to the injured lesions in the mouse brain. When exposed to sound for 1 month, the size of the wound area did not increase compared to the stroke with hMSC injection group (Fig. 10 B,C). We performed the immunohistochemical staining of neural proteins including MAP2, NF200, and S100 to determine the effect of sound on injured lesions in mouse brain. As shown in Fig. 11, the stroke with both hMSC injection and sound exposure groups had an increase in the filamentous shape of MAP2 and NF200 in ischemic lesions, and S100, a neural protein-related astrocyte, was expressed at higher levels than those in

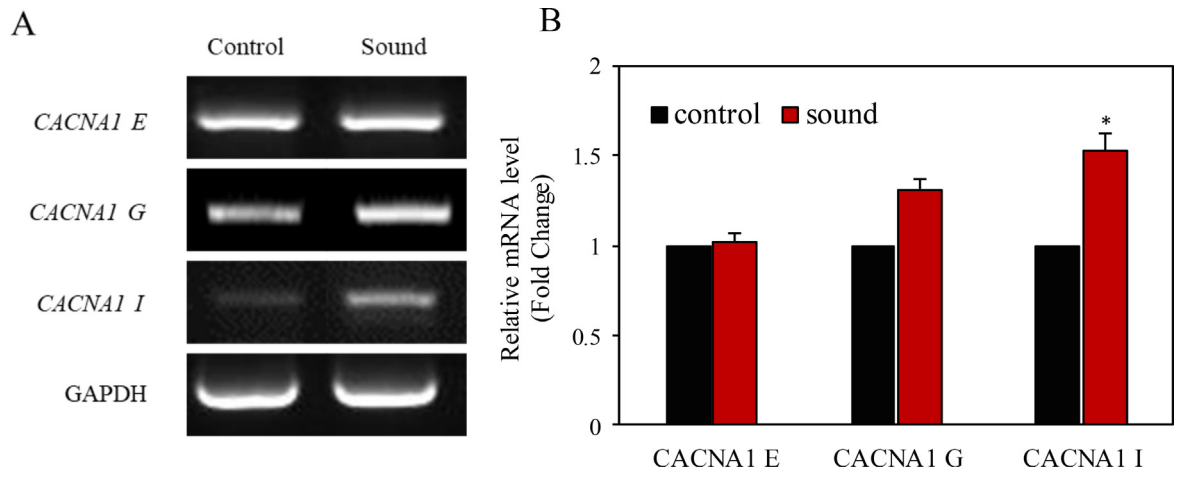

Fig. 9. The effect of voltage-dependent calcium channel levels in SH-SY5Y cells by sound. (A) Gene expression of calcium channels subtypes using PCR. (B) Fold expression of each genes using real-time polymerase chain reaction analysis. Column heights correspond to the mean values and the error bars correspond to the standard deviations (n = 3). *p < 0.01.

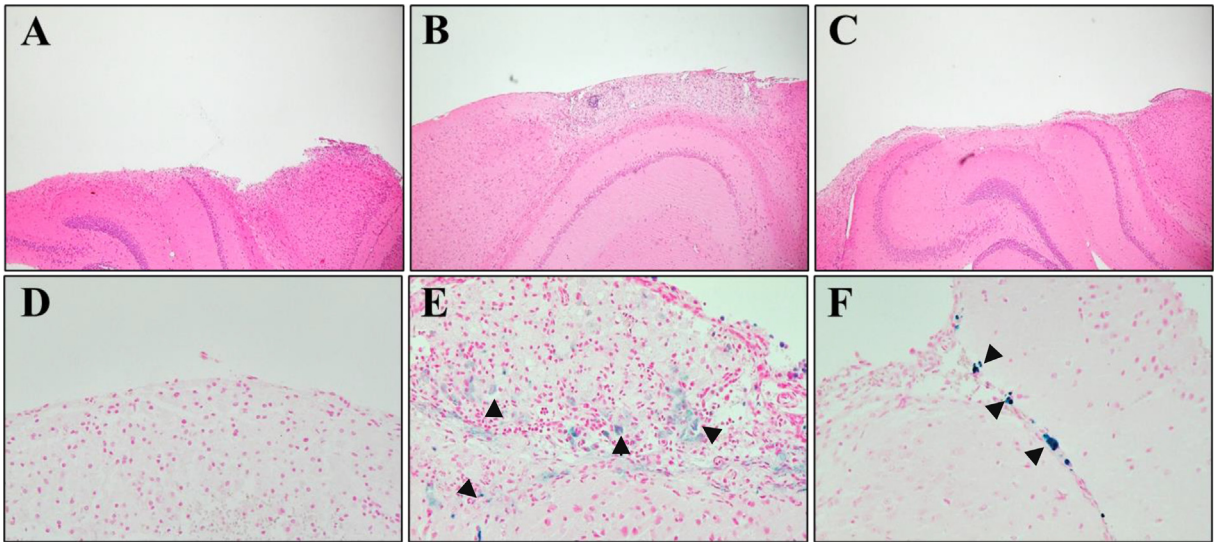

Fig. 10. Histological staining of the ischemic mouse injury lesion. (A–C) H&E staining of injured lesions. (D–F) Prussian Blue staining. A, D; stroke only, B, E; stroke with hMSC injection, C, F; stroke with both hMSC injection and sound exposure. Arrowheads indicate hMSCs containing MNP. Original magnification, A-C: 40× and D-F: 200×.

the control group. As a result, sound induced the expressions of neural proteins in the injured lesions and inflammatory responses were reduced (Fig. 12).

3.5. Effect of sound on neuronal differentiation and signaling activation proteins in ischemic mouse brains

To investigate the neurogenesis process induced by sound in the mouse ischemic model, we performed western blotting for neuronal and signaling activation proteins in injured brain tissues. As shown in Fig. 13, compared with the first group (stroke only), the third group (stroke with both hMSC injection and sound exposure) showed MAP2 protein expression was increased 4.4-fold, NEUROD1 protein expression was increased 3.6-fold, and TAU protein expression was slightly increased 1.3-fold. The neural related proteins were more highly expressed in the third group than in the second (stroke with hMSC injection) group. The expression of ERK and CREB was confirmed among the signaling proteins expressed in the early stages of differentiation of neurons. Fig. 13 shows that the levels of p-ERK and p-CREB increased in the third group, and about 11-fold increase in the case of CREB phosphorylation.

4. Discussion

From the viewpoint of differentiation, sound effect is known to promote the native specialized characteristics of various cells through cytoskeleton expression, growth factor production, cell alignment, and extracellular matrix deposition [9]. Aggio et al. [25] reported that an increase in yeast cell growth affects metabolic pathways by sonic vibration.

We previously reported that SSV promoted the maturation of preadipocytes or the neural differentiation of mesenchymal stem cells. Specifically, SSV is a mechanical stimulation with only subsonic frequency because it transfers energy by solid contact with cells. Therefore, the effect of SSV is considered similar to other

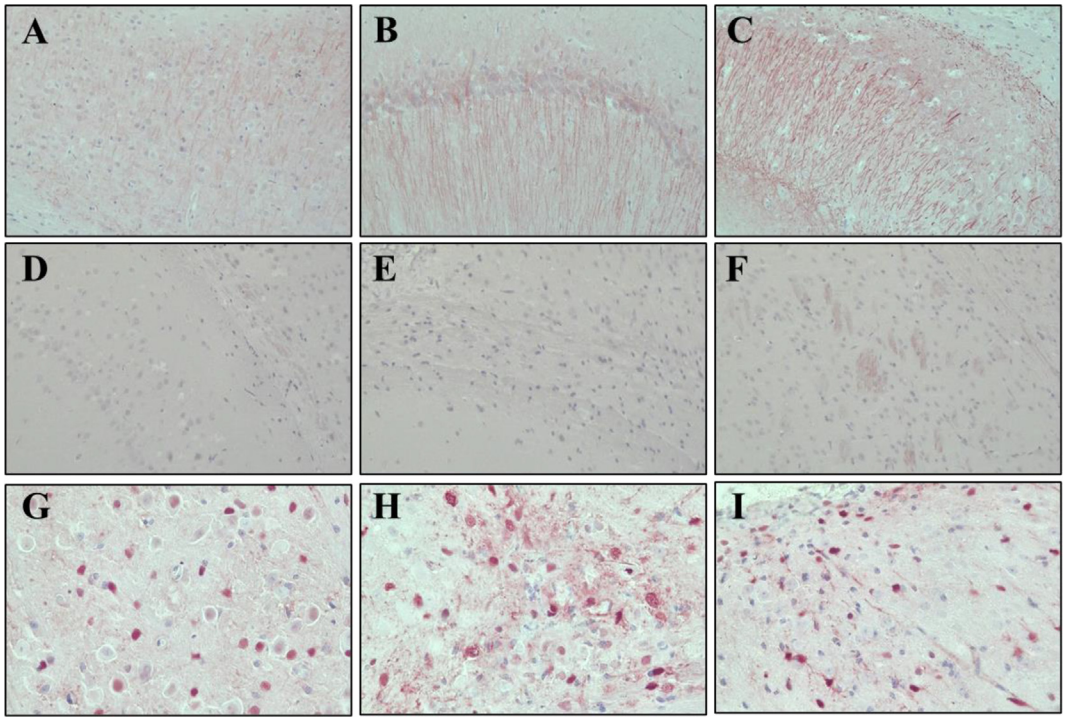

Fig. 11. Immunohistochemical staining of the ischemic injured lesion. (A–C) MAP2, (D–F) NF200, and (G–I) S100. A, D, G; stroke only, B, E, H; stroke with hMSC injection, C, F, I; stroke with both hMSC injection and sound exposure. Original magnification, A∼F: 200×, G∼I: 400×.

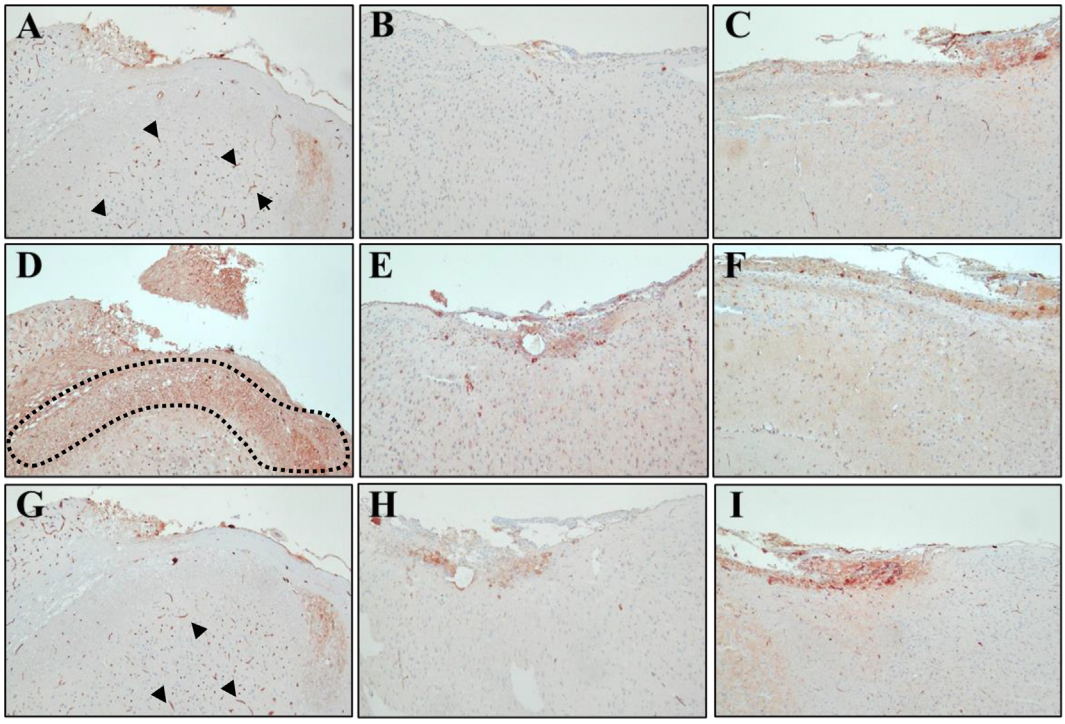

Fig. 12. Immunohistochemical staining of the ischemic injured lesion. (A–C) INFγ, (D–F) MMP9, and (G–I) TNFα. A, D, G; stroke only, B, E, H; stroke with hMSC injection, C, F, I; stroke with both hMSC injection and sound exposure. Original magnification, 100×.

mechanical stimulations such as tension [14,18,19]. Contrary to previous studies, little is known about the sound transmission of energy through air, water, or coupling gels.

Human SH-SY5Y neuroblastoma cells maintain their potential for neuronal differentiation and regression under culture conditions [26]. Differentiation was regulated by cell proliferation inhibition. Neurites, typical of neuronal morphology, were induced in SH-SY5Y cell lines by treatment with retinoic acid [27]. The toxicity of sound exposure to cells was evaluated by cell morphology and LDH assays *in vitro*. Our study showed that sound was not

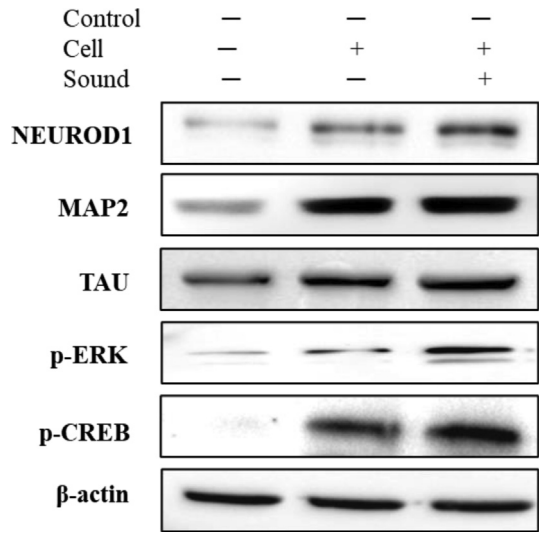

Fig. 13. Protein expression levels in stroke-induced mice. hMSCs were injected prior to sound exposure. After injection of hMSCs into the injured mouse brain, sound was exposed at the site of injury.

toxic in the neurite elongation process of SH-SY5Y cells and had a positive effect on the maturation of neuroblastoma cells in terms of regulating the expression of proteins involved in neural differentiation and maturation. To examine neuronal differentiation, we observed the expressions of neural related proteins induced by sound. MAP2, a neuron-specific cytoskeletal protein enriched in dendrites, belongs to the microtubule-associated protein family, and it stabilizes neuronal morphology during neuron development [28]. In this study, the expression of MAP2 protein was greatly increased in SH-SY5Y cells by sound. TAU, which interacts with TUBB3 provides stability to axon microtubules [29] and has a critical role in neuronal structure and function [30], and NFL forms a neuronal cytoskeleton. These proteins are involved in neuronal differentiation effects and synergize with sound as determined by immunocytochemical staining. This result suggests that sound affects neurite stabilization during neuronal differentiation. Neuronal migration is a process involved in the development of the nervous system and DCX is a microtubule-associated protein in neurogenesis and maturation [31,32]. We confirmed DCX expression through western blot when exposed to sound for 3 d, which shows that neuroblastoma cells are undergoing maturation by sound. However, expression of DCX was not detected in NeuN positive cells in FACS analysis. It is thought that expression of a sufficient amount of DCX is required until NeuN is expressed.

Synaptophysin is a synaptic vesicle protein, and in neurons it is used as a marker for presynaptic sites. It forms a kind of channel in the membrane of synaptic vesicle and acts as the major Ca^{2+}-binding protein [33]. The exact function of this protein is unknown. Sound upregulated synaptophysin indicating the neuronal network might be effectively restored after structural destruction. Interestingly, *CACNA1E* (human voltage-dependent, R-type calcium channel) was constituently expressed and not induced regardless of exposure to sound. However, *CACNA1G* and *CACNA1I* (human voltage-dependent, T-type calcium channels) were induced by sound. A total of five types of calcium channel subtypes exist in humans, with R- and T-type channels being found most often in neurons. T-type calcium channels are critical contributors to membrane excitability, exocytosis, and endocytosis of chromaffin cells in normal and stress-mimicking conditions [34,35,36]. These channels also regulate low-threshold spikes, oscillatory cell activity, cell growth, differentiation, and proliferation [36,37,38]. We could confirm that the presynaptic site was formed in SH-SY5Y neuroblastoma cells induced by the expression of synaptophysin. PSD95 is a kind of post-synaptic density protein expressed in post-synaptic sites, expressed by sound in SH-SY5Y cells. In the end, sound influences the differentiation and maturation of SH-SY5Y cells and induces the expression of synaptophysin and PSD95 expressed in presynaptic and postsynaptic sites.

In our previous study, mechanical stimulation affected the recovery of spinal cord injured rats [24]. We hypothesized that sound would restore damaged ischemic mouse brains. We used a PT cold laser to create the injury, and the mouse brain injury site was identified by H&E staining. To enhance the effect of sound on ischemic injury in mouse brain, we injected hMSCs through the penis prior to sound exposure. MNPs were incorporated into hMSCs prior to injection, and cells were detected at the injured site by Prussian blue staining. The size of the injury site was reduced by sound compared with the stroke only and stroke with hMSC injection and stroke with both hMSC injection and sound exposure. In this recovery process, the neuron-related proteins MAP2, NF200, and S100 were all expressed.

TNFα, an ischemia-induced proinflammatory cytokine, promotes the activation of MMP9 and induces blood–brain barrier breakdown leading to leukocyte extravasation into the injured brain [39,40,41,42]. During ischemia, MMP9 disrupts the connection between astrocyte endfeet and endothelial cells by degrading the basal lamina [43,44]. Because MMPs have beneficial roles in stroke, they might be potential therapeutic targets [45]. In this study, sound reduced the expressions of the inflammatory molecules, MMP9, IFNγ, and TNFα, in ischemic lesions. Fig. 13 shows the western blotting analysis of neuron-related proteins. NEUROD1, MAP2, and Tau proteins were increased by sound in the hMSC group, and in particular, MAP2 was increased almost 4-fold.

The exact mechanism of stimulation of neurogenesis by sound is unknown, but our previous studies reported the activation of ERK and CREB by ultrasound [46]. cAMP-response element binding protein (CREB) is the downstream signaling molecule of ERK and is activated by phosphorylated ERK. CREB is a key factor for cAMP-mediated reflection of growth regulation by myelin-associated inhibitors, suggesting it may play a role in axon growth in damaged tissues [47]. CREB-mediated gene expression is thought to be a protective response against ischemic injury [48] and it plays an important role in neural survival [49]. In this study, CREB activation was induced by sound in ischemic lesions, and consequently, sound was thought to have a protective role by promoting survival and neurogenesis.

The sound stimulus is transmitted from the outside through the medium to the membrane of SH-SY5Y cells. This signal is transmitted to the cytoplasm and begins with the activation of ERK and CREB proteins involved in the early stages of neuronal differentiation. Phosphorylation of ERK and CREB induced the expression of proteins such as MAP2, NEUROD1, and TAU, thereby promoting the neuron differentiation process of SH-SY5Y cells. Expression of DCX protein is thought to indicate that SH-SY5Y cells are in neuronal maturation. In *in vivo* studies, sound exposure to injured lesion for ischemic mouse brain recovery induces a decrease in inflammatory cytokines around the injured lesion, which is thought to create an environment for the recovery. At the same time, sound and hMSC stimulated the expression of neuronal differentiation-related proteins to induce recovery of the ischemic lesion of mouse brain.

Finally, the molecular mechanism triggered by sound is uncertain. However, some possible explanations exist. Stretch-activated ion channels are of special interest because they lead to cell membrane vibration and act as mechanotransducers, which mediate mechanical stimuli such as vibrations [50] These stretch-activated ion channels promote mechanical stimuli that play key roles in mechanosensory neurons and fibroblasts [50,51].

5. Conclusions

This study demonstrated that sound promoted the neural maturation of SH-SY5Y cells without any cellular damage. Therefore, sound can be a mechanical stimulus for the differentiation or maturation of nerve cells with advantages such as ease of application and non-invasiveness in tissue engineering and regenerative medicine.

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Conflict of interest

The authors declare no competing interests.

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