LOW-FREQUENCY VIBRATORY SOUND INDUCES NEURITE OUTGROWTH IN PC12M3 CELLS IN WHICH NERVE GROWTH FACTOR-INDUCED NEURITE OUTGROWTH IS IMPAIRED

Yoshihisa KOIKE1*, Soutarou IWAMOTO2, Yuko KIMATA3, Tsutomu NOHNO4, Fukumi HIRAGAMI5, Kenji KAWAMURA5, Keizo NUMATA1, Hiroyasu MURAI1, Kyota OKISIMA1, Miyuki IWATA1 and Yoshio KANO1

1 Department of Occupational Therapy, School of Health Science, Kibi International University, 8 Iga-machi Takahashi City, Okayama 716-8508, Japan
2 Department of Occupational Therapy, School of Health Science, Kyushu University of Health and Welfare, 1714–1 Yosino-cho Nobeoka City, Miyazaki 882–8508, Japan
3 Department of Occupational Therapy, Faculty of Comprehensive Rehabilitation, Osaka Prefecture College of Nursing, 3–7–30 Habikino Habikino City, Osaka 583–8555, Japan
4 Department of Molecular Biology, Kawasaki Medical School, 577 Matusima, Kurashiki City, Okayama 701–0192, Japan
5 Department of Physical Therapy, School of Health Science, Kibi International University, 8 Iga-machi Takahashi City, Okayama 716–8508, Japan

Abstract: A drug-hypersensitive PC12 mutant cell line (PC12m3) was obtained during continuous culturing of neural PC12 cells. In this study, PC12m3 cells were exposed to vibratory sound stimuli of frequencies ranging from 10 to 200 Hz for 30 minutes at intensity 5 under the condition of NGF treatment. The results showed that low-frequency vibratory sounds of 10–100 Hz induced enhancement of neurite outgrowth, whereas vibratory sounds 150 Hz and 200 Hz had little effect on neurite outgrowth. The frequency of neurite outgrowth induced by 40-Hz low-frequency vibratory sound stimuli was approximately 3-fold greater than that induced by NGF alone. The activation of p38 MAPK has been shown to play an important role in neuronal differentiation in PC12m3 cells. Therefore, we examined whether the ability of low-frequency vibratory sound stimulus to induce neurite outgrowth of PC12m3 cells is a reflection of its effect on p38 MAPK activity. The results demonstrated that 40-Hz low-frequency vibratory sound stimuli had an enhancing p38 MAPK activity and indicated that vibratory sound induces neurite outgrowth via a p38 MAPK signaling pathway in PC12m3 cells.

Key words: Low-frequency vibratory sound, p38 MAPK, PC12m3 cell, Alzheimer’s disease

Introduction

Dementias, such as Alzheimer’s disease (AD), involve a progressive deterioration of memory functioning (malignant amnesia) and mood disorders. Although the mechanisms of neuronal degeneration in AD are not clear, several investigators have reported an increase in AD patients’ self-expression following music therapy1,2. Music therapy might provide a new form of rehabilitative intervention, being particularly effective in reducing behavioral symptoms2. Although these effects appear to be mediated by the release of neurotransmitters and neurohormones, the specific neurohormonal systems involved have not been fully investigated3,4.

The purpose of our study was to clarify the
effect of music therapy for the treatment of AD. The effect of low-frequency vibratory sound on neurite outgrowth of drug-hypersensitive PC12 mutant cells (PC12m3 cells) was investigated. PC12 cells are para-neuronal culture cells of rat adrenal pheochromocytoma origin. The PC12 cell is useful as an undifferentiated cell model that is sensitive to the activity of nerve growth factor (NGF) for induction of differentiation to a nerve cell and extension of neurites (5, 6). Sustained activation of mitogen-activated protein kinase (MAPK) plays an important role in neurite outgrowth of PC12 cells (7). Due to the widespread use of PC12 cells in various culture conditions, spontaneous variants are often encountered (8, 9). We developed a drug-hypersensitive cell line (PC12m3 cells) in which neurite outgrowth is stimulated by various drugs, such as FK506, cAMP, and calcimycin (10).

PC12m3 cells displayed neurite outgrowth when treated with Kampo medicines, such as Tokishakuyakusan, that are known to be for treatment of AD (11). However, Tokishakuyakusan did not induce activation of MAPK (ERK). It appeared that Tokishakuyakusan induces neurite outgrowth in PC12m3 cells via a mechanism independent of the MAPK (ERK) signaling pathway (12).

Mammalian cells contain at least three MAPK pathways, which regulate the activities of extracellular signal-regulated kinase (ERK; also known as p42 and p44 MAPK), c-Jun N-terminal kinase (JNK), and the p38 mitogen-activated protein kinase (p38 MAPK). The ERK pathway is important for regulation of cell differentiation. JNK pathways have been implicated in the regulation of neuronal apoptosis. However, the p38 MAPK signaling pathway’s specific system has not been elucidated (13).

In this study, we found that p38 MAPK activity, which appears to be enhanced in AD, is involved in the mechanism by which neurite outgrowth on PC12m3 cells is induced by low-frequency vibratory sound stimulus.

Materials and Methods

Reagent and cell culture

NGF (2.5s) was purchased from Takara (Osaka, Japan). PC12m3 cells were cultured in Dulbecco’s modified eagle’s medium (DMEM) supplemented with 0.35% glucose, 10% horse serum, 5% fetal bovine serum (FBS), and 100 unit/ml kanamycin. All cells were grown at 37°C with 5% CO².

Determination of neurite outgrowth

A single-cell suspension of PC12m3 cells was obtained by trituration in DMEM. For culture experiments, cells were plated at a density of 2–5 x 10⁵ cells per flask onto 25cm² flasks with serum-containing DMEM, and then the cells were immediately treated with two kinds of sounds (vibratory sound or sound waves) in the presence of NGF. An oscillator (KENWOOD 204D, KENWOOD Co. Ltd., Tokyo, Japan) was used to produce the low-frequency sounds. The frequency of sound was measured with a multi-function counter (AD-5182, A and D Co. Ltd., Tokyo, Japan). Oscillation of vibratory sound and that of sound waves were done as follows. A culture flask containing PC12m3 cells was placed on a speaker, and the cells were oscillated for 30 minutes at various frequencies to detect the effect of vibratory sound. Another culture flask containing PC12m3 cells was suspended at a distance of 12 centimeters from the speaker and PC12m3 cells were oscillated for 30 minutes at various frequencies to detect the effect of sound waves.

After 5–7 days of incubation, the frequency of neuritogenesis was determined by measuring the neurite length and neurite numbers. In other experiment, cells were allowed to attach and proliferate for three days before exposure to sound, and then NGF was added to the culture medium and incubated for 7 days for measurement of neurite outgrowth. Cells with one or more neurites of a length 1.5 times greater than the diameter of the cell body were measured as previously described (13, 14). Each value is the mean ± S.E.M. for 100–200 cells sampled from three independent experiments.

Detection of activated p38 MAPK

p38 MAPK activity was determined by the modified method previously described (15). Briefly, PC12m3 cells were plated at a density of...
Low-frequency vibratory sound induces neurite outgrowth

$1 \times 10^6$ cells/25 cm$^2$ in a flask of serum-containing medium and cultured for 3 days. Then, cultures were replaced by 0.5% FBS-containing medium for 48 hours. Before vibratory sounds treatment, cells were incubated for 2 hours in serum-free medium. Cells were stimulated for 30 minutes by low-frequency vibratory sounds. p38 MAPK activity was then assayed in the cell lysates. The cells were lysed in lysing buffer. Aliquots of the lysates (10–15 µg) from each sample were fractionated on SDS-10% polyacrylamide gel and transferred to polyvinylidene difluoride membranes (0.45 µm pore size Immobilon-P, Millipore). The blots were probed with the phospho-p38 MAPK antibody (New England BioLabs) at a dilution of 1:1000 in a blocking buffer (5% nonfat dry milk) for 12 hours at 4°C. The blots were then probed with secondary antibody, horseradish peroxidase-linked anti-rabbit IgG, at a dilution of 1:2000 in the blocking buffer for 60 minutes at room temperature. The blots were stained for 1 minute using the nucleic acid chemiluminescence reagent (LumiGLO chemiluminescent reagent, Kirkegaard and Perry laboratories) and exposed to x-ray film.

Fig. 1. Stimulation of neurite outgrowth in PC12m3 cells by low-frequency vibratory sound. The cells were exposed to sound immediately after (A, B, C) or three days after (D, E, F) cultivation under the condition of NGF treatment. (A, D) PC12m3 cells treated with NGF. (B, E) PC12m3 cells stimulated with 40-Hz low-frequency vibratory sound under the condition of NGF treatment, and (C, F) PC12m3 cells stimulated with 200-Hz vibratory sound under the condition of NGF treatment. Phase-contrast photomicrographs of PC12m3 cells were taken 7 days after treatment (x 200).
Data analysis
Results were analyzed by one-way analysis of variance (ANOVA) and Dunnett's test to identify significant differences from the control. Correlational analyses were performed using the Student-Newman-Keuls (SNK) test.

Results
Establishment of PC12m3 cells
During continuous culturing of neural PC12 cells, a drug-hypersensitive PC12 mutant cell line (PC12m3) was obtained. PC12 cells are subject to spontaneous mutations that lead to the generation of PC12 variants. We obtained a variant cell line in which NGF-induced neurite outgrowth was impaired. When these culture cells were cultivated for 2 weeks under acidic conditions of Cl⁻, several surviving clones appeared. By using the ring isolation procedure, ten colonies (named PC12m1, PC12m2, PC12m3, and so on) were selected and propagated in a mass culture. PC12m3 cells exhibited poor neurite outgrowth in response to NGF. PC12m3 cells treated with NGF showed enhancement of neurite outgrowth in response to various stimulants, such as FK506, c-AMP, and calcimycin. Furthermore, the cells exhibited sustained activation of ERK induced by various stimulants^{10}.

Induction of neurite outgrowth of PC12m3 cells by sounds
PC12m3 cells were tested for their sensitivity to physical stimulations, such as stimulation by vibratory sounds. Fig. 1 shows photomicrographs of PC12m3 cells treated with NGF alone and those treated with low-frequency vibratory sounds for 30 min at 40 Hz or 200 Hz in the presence of NGF. Forty-Hz vibratory sound combined with NGF resulted in much greater enhancement of neurite outgrowth than did treatment with NGF alone. However, 200-Hz vibratory sound had no effect on neuritogenesis in PC12m3 cells (Fig. 1. A,B,C). Furthermore, neurite outgrowth was measured in PC12m3 cells that were allowed to attach, spread and proliferate for three days before exposure to vibratory sound as same condition as p38 MAPK assay (Fig. 1. D,E,F).

![Fig. 2. Change in the frequency of vibratory sound-induced neurite outgrowth by time and intensity of amplitude in PC12m3 cells.](image)

(A) The percentages of PC12m3 cells with neurites were determined 7 days after exposure to 40-Hz low-frequency vibratory sound stimuli for 15 min, 30 min and 60 min. Values represent means ± S.E.M. (n=3). In post-hoc Dunnett analysis, percentages of cells with neurites after stimulation for all three time periods were significantly different from the percentage of control cells with neurites. *P<0.01. In post-hoc SNK analysis, the percentage of cells with neurites after 15-min stimulation was significantly different from that after 30-min stimulation at **P<0.01. (B) The percentages of PC12m3 cells with neurites were determined 7 days after 40-Hz low-frequency vibratory sound stimuli at intensities of 2, 5, and 8. Values represent means ± S.E.M. (n=3). In post-hoc Dunnett analysis, the percentages of cells with neurites after stimulation at all three intensities were significantly different from the control at *P<0.01. In post-hoc SNK analysis, the percentage of cells with neurites after stimulation at intensity of 2 was not significantly different from that after stimulation at intensities of 5 and 8.

84
Fig. 3. Frequencies of neurite outgrowth induced by vibratory sound ranging from 10 to 200 Hz for 30 minutes and at an intensity of 5 in PC12m3 cells. Values represent means ± S.E.M. (n=3). In post-hoc Dunnett analysis, the frequencies of neurite outgrowth induced by 20-100 Hz low-frequency vibratory sounds were significantly different from the control at **P<0.01, and the frequency of neurite outgrowth induced by 10-Hz low-frequency vibratory sound was significantly different from the control at *P<0.05.

Fig. 4. Frequency of neurite outgrowth induced by vibratory sounds or sound waves in PC12m3 cells. Values represent means ± S.E.M. (n=3). In post-hoc Dunnett analysis, frequencies of neurite outgrowth induced by 40-Hz low-frequency vibratory sound and by 200-Hz sound waves were significantly different from the frequency of neurite outgrowth in control cells and the frequencies of neurite outgrowth induced by 40-Hz low-frequency sound waves and by 200-Hz vibratory sound at **P<0.01.
The period of sound stimulation required for neurite outgrowth was 30 minutes (Fig. 2A). The intensity of sound required for neurite outgrowth was level 2 (Fig. 2B). In this study, PC12m3 cells were exposed to vibratory sounds of various frequencies ranging for 10 to 200 Hz for 30 minutes at level 5 under the condition of NGF treatment (Fig. 3). It was found that low-frequency vibratory sounds of 10–100 Hz induced enhancement of neurite outgrowth, whereas vibratory sounds of 150 Hz and 200 Hz had little effect on neurite outgrowth. The frequency of neurite outgrowth induced by 40-Hz low-frequency vibratory sound was approximately 3-fold greater than that of neurite outgrowth induced by NGF alone. Fig. 4 shows that the effects of two kinds of sounds on neurite outgrowth in PC12m3 cells have two patterns. One is vibratory sound and the other is the sound wave. The frequency of neurite outgrowth induced by vibratory sound was greater than that of neurite outgrowth induced by the sound wave.

![Pospho p38](image)

**Fig. 5.** Activation of p38 MAPK by 40-Hz low-frequency vibratory sound in PC12m3 cells. PC12m3 cells were serum-starved and stimulated or not for 30 minutes with vibratory sounds. After treatment, cells were lysed and protein extracts were analyzed on Western blot with an anti-phospo-p38 MAPK antibody. Arrows labeled phospho p38 indicate the position of the phosphorylated forms of p38 MAPK protein.

![Images of neurite outgrowth](images)

**Fig. 6.** Inhibition of neurite outgrowth induced by low-frequency vibratory sound under the condition of NGF treatment. (A) PC12m3 cells treated with NGF, (B) PC12m3 cells stimulated with 40-Hz low-frequency vibratory sound under the condition of NGF treatment, and (C) PC12m3 cells stimulated with 40-Hz low-frequency vibratory sound were treated with a specific p38 MAPK inhibitor, SB203580, under the condition of NGF treatment. Phase-contrast photomicrographs of PC12m3 cells were taken 7 days after treatment (× 200).
Low-frequency vibratory sound induces neurite outgrowth

Activation of p38 MAPK by 40-Hz low-frequency vibratory sound in PC12m3 cells

Since activation of p38 MAPK has been shown to play an important role in neuronal differentiation in PC12 cells\(^1\), we examined whether the ability of low-frequency vibratory sound stimulus to induce neurite outgrowth of PC12m3 cells is a reflection of its effect on p38 MAPK activity. PC12m3 cells were stimulated with vibratory sounds of 40 Hz and 200 Hz for 30 minutes or were not stimulated (as controls), and then p38 MAPK activity was detected by immunoblotting (Fig. 5). The results showed that 40-Hz vibratory sound stimuli enhanced p38 MAPK activity. However, neurite outgrowth induced by 40-Hz vibratory sound was inhibited by a specific p38 MAPK inhibitor, SB203580 (Fig. 6). Furthermore, p38 MAPK activation in cells stimulated with 200-Hz vibratory sound and in control cells were not enhanced. Thus, it appeared that low-frequency vibratory sound induce neurite outgrowth via the p38 MAPK signaling pathway in PC12m3 cells.

Discussion

During the early stages of AD, the levels of various growth factors and mitogenic compounds are elevated\(^2\). Most of these factors elicit their effects on cells through activation of a ras-dependent MAPK cascade, a pathway that is also involved in the regulation of expression and post-translational modification of the beta-amyloid precursor protein (APP) and consists largely of the microtubule-associated protein tau\(^3\). A previous study demonstrated differential expression of MAPK in association with tau deposits in AD, and in particular p38 MAPK activation, especially during the early stage of the disease\(^4\). p38 MAPK participates in cascade-controlling cellular responses to cytokines and stress\(^5\). It is activated by a variety of cellular stresses, including stresses caused by osmotic shock, pro-inflammatory cytokines, UV radiation and growth factors\(^6\). Strong phosphorylated-p38 immunoreactivity has been found in about 50–70% of neurons with neurofibrillary tangles, consisting largely of the tau protein, and in dystrophic neurites of senile plaques, which made up most of the APP, in AD patients\(^7\).

However, another recent study has demonstrated that p38 MAPK participates in the preservation of the neurite growth cone, in neurite outgrowth, and in the regulation of cellular processes of differentiation and survival\(^8\). In the present study, p38 MAPK was activated by low-frequency vibratory sound stimuli and it promoted neurite outgrowth approximately 3-fold greater than that induced by NGF alone in PC12m3 cells. Furthermore, induction of neurite outgrowth by low-frequency vibratory sound was inhibited by a specific p38 MAPK inhibitor, SB203580, in the presence of NGF. These results suggest that p38 MAPK does work for the formation of neurite outgrowth stimulated by vibratory sound. These findings support the speculation that p38 MAPK preserves neurons and promotes neuronal regeneration in the early stage of AD. In fact, some investigators have reported an increase in AD patients’ self-expression following music therapy\(^9\).

Recently, we detected activity of the cyclic-AMP responsive element (CRE)-binding protein (CREB) stimulated by sound waves in PC12 mutant cells\(^10\). CREB is a transcription factor that is the target of a variety of signaling pathways mediating cell responses to extracellular stimuli, including proliferation, differentiation, and adaptive responses of the cell process. p38 MAPK could activate CREB binding to CRE\(^11\). These studies have suggested that p38 MAPK might induce neurite outgrowth via a CREB signaling pathway in PC12m3 cells. CREB is also activated by phosphorylation in response to depolarization-induced Ca\(^{2+}\) influx\(^12\).

Neuronal degeneration in AD appears to involve increased oxidative stress and disruption of cellular calcium homeostasis\(^13\). A recent study demonstrated that a Kampo medicine called Chotosan was effective in treating vascular dementia (AD)\(^14\). Its pharmacological function has been investigated, and it has been demonstrated that its extract has a Ca\(^{2+}\) channel-interference effect\(^15\). It has also been reported that Tokishakuyakusan reduced the cognitive disruption caused by central cholinergic dysfunction\(^16\). These findings suggest that Tokishakuyakusan...
may be a useful medicine for treatment of various types of senile dementia such as AD. Several studies have suggested that AD is associated with neurodegeneration of the brain. It has been demonstrated by using PC12 cells that the Kampo medicine Inyokaku may facilitate recovery from brain degradation through stimulating neural regeneration. Inyokaku appears to enhance the activity of voltage-dependent Ca\(^{2+}\) channels in neuronal cells.

Calcium influx through voltage-dependent calcium channels also seems to cause extensive neurite outgrowth in PC12 cells. The calcium-induced neurite outgrowth is mediated through the initial actions of tyrosin kinases. Calcium signals regulate the transcription factor CREB via a Rap1-ERK pathway. Kampo medicines such as Sokeikakketsuto and Tokishakuyakusan have no effect on ERK activity, despite the fact that they enhance neurite outgrowth in PC12m3 cells. However, the p38 MAPK signaling pathway was activated by Kampo medicine in PC12m3 cells. These findings suggest that low-frequency vibratory sound induces neurite outgrowth via a p38 MAPK signaling pathway in PC12m3 cells.

Skille reported that VibroAcoustic Therapy might prove to be a professional challenge for music therapy. Most of the effects of VibroAcoustic Therapy were found in the octave between 40 and 80 Hz, the range in the very center of the VibroAcoustical area. VibroAcoustic Therapy is a process in which vibrations are applied directly to the body in the form of low-frequency sinusoid tones in combination with selected music. The impulses given are perceived both by acoustical receptors and vibrotactile receptors in humans. Our results showed that both vibratory sound and sound waves induce neurite outgrowth. The most effective frequencies of vibratory sound and sound wave were 40 Hz and 200 Hz, respectively. The reason of the discrepancy in frequency is not clear. However, the effect of 40-Hz of vibratory sound is consistent with the effect of the VibroAcoustic Therapy which use mainly vibratory sound at between 40-Hz and 80-Hz.

Various drugs enhanced neurite outgrowth in PC12m3 cells under the condition of NGF treatment. This is the first analysis of enhanced formation of neurite outgrowth by low-frequency vibratory sound in PC12m3 cells, and we could not detect such effects in other PC12 mutant and parental cells. Furthermore, the effect of other mechanical stimulation has not yet been clarified.

All of these findings indicate that the effect of music therapy is depend on the recovery of damaged neurons through the p38 MAPK pathway and that music therapy is useful for the enhancement of self-expression in Alzheimer’s disease patients.

Reference

9) Teng, K. K., Georgieff, I. S., Aletta, J. M., Nunez, J., Shelanski, M. L. and Greene, L. A.: Characterization of a PC12 cell sub-clone (PC12-C41) with enhanced


28) Chan, S.L., Mayne, M., Holden, C.P., Geiger, J.D. and Mattoson, M.P.: Presenilin-1 mutations increase levels of ryanodine receptors and calcium release in


(Accepted 23 June 2004)