The effect of Repetitive Magnetic stimulation on Gene Expression in Neuronal Cells

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The effect of Repetitive Magnetic stimulation on Gene Expression in Neuronal Cells

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Those who sow in tears will reap with songs of joy [Psalms 126:5]. It is the words always lived in my heart. Finally, a moment of joy came to me.

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Table of Contents

List of Figures

List of Tables

Abstract

The Effect of Repetitive Magnetic Stimulation on Gene Expression in Neuronal Cells

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Repetitive transcranial magnetic stimulation (rTMS) is a non-invasive and painless tool that can be used to various neuropychiatric and neurorehabilitation disorders. Although rTMS is widely used in clinical field, precise biological mechanism and effectiveness is not well known.

Therefore, the current study examined the temporal changes in global gene expression pattern depending on differential effects of repetitive magnetic stimulation (rMS) in neuronal cell to generate a comprehensive view of the mechanisms.

Neuro 2a (mouse neuroblastoma cell line) cells were used as the cell model in this study. Dishes of cultured cells were randomly divided into three groups : sham, low-frequency (0.5 Hz) and high-frequency (10 Hz) and were stimulated for 3 days. Three groups were systematically characterized the transcriptome analysis. Differentially expressed genes (DEG), the result of transcriptome analysis, were then analyzed by a

program Database of Annotation Visualization and Integrated Discovery (DAVID), which yielded Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. DEG was validated by qRT-PCR & western blot.

Comparing between low-frequency and high-frequency of rMS, long-term potentiation, neurotrophin signaling pathway, cholinergic synapse, dopaminergic synapse were shown as the enriched significant pathways. Grin1, Camk2d and Camk2a were increased, and Camk2g was decreased. These genes can activate the Ca^{2+}-CaMKII-CREB pathway. Furthermore, high frequency of rMS increased phosphorylation of cAMP-response element binding protein (p-CREB), BDNF transcription and synaptic marker protein expression via activation of CaMKII-CREB pathway in neuro 2a cells.

Taken together, these data established the mechanisms of Ca^{2+}-CaMKII-CREB signaling pathway, which can be regulated by rMS in neuro 2a cells depending on different frequencies. These findings might help clarify further therapeutic mechanisms of rTMS.

Key words : Repetitive transcranial magnetic stimulation, Repetitive magnetic stimulation, low-frequency, high-frequency, Ca^{2+}-CaMKII-CREB signaling pathway, BDNF

Introduction

Transcranial magnetic stimulation (TMS) is a noninvasive tool that allows electrical stimulation of the nervous system and could be an ideal treatment tool due to its ability to modify brain plasticity. [1] TMS can produce a strong electric current of up to several kA is discharged briefly (1< ms duration) through a TMS coil [2] and also can produce a short 100μs biphasic electromagnetic pulse, which generates an electric current in the central nerve system (CNS) [3]. When given at regular frequencies, it is termed 'repetitive transcranial magnetic stimulation' or 'rTMS' [3].

rTMS is safe and painless noninvasive brain stimulation technique that has recently received increasing interest as a therapeutic neurorehabilitative tool [4]. rTMS has been used in clinical field such as movement disorders, stroke and schizophrenia [5]. Also, different rTMS techniques can produce different modulatory effects [6]. For example, high-frequency rTMS (> 5 Hz) stimulates cortical excitability and generates long-term potentiation (LTP)-like effects [6]. In contrast, low-frequency rTMS (<1 Hz) reduces cortical excitability and produces long-term depression (LTD) [6]. It is known that the functional effects of rTMS on cortical excitability depend on stimulation intensity, frequency and the overall stimulation pattern [7]. However, low and high frequency rTMS-induced neurobiological mechanisms remain not well understood.

Several experiments have shown that rTMS has the ability to mediate neuroplasticity by enhancing the expression of glutamate neurotransmitters and brain-

derived neurotrophic factor (BDNF) in rat brains [8]. BDNF is a critical protein that supports the development, differentiation, maintenance and plasticity of brain function [9]. Also, different forms of BDNF are implicated in the mechanisms of LTP and LTD [10]. BDNF promotes survival through inactivation of components of the cell death machinery and also through activation of the transcription factor cAMP-response element binding protein (CREB). [11]

CREB is a key regulator of gene expression [12]. CREB-regulated gene transcription has been shown to increase growth factor activity and to promote regenerative processes such as dendritic sprouting and neurogenesis [13]. Recently, it is revealed that novel signaling pathway of Ca^{2+}-CaMKII-CREB pathway plays crucial role in electrical stimulation (ES)-induced BDNF transcription and neurite outgrowth in cultured rat postnatal dorsal root ganglion neurons (DRGNs) [14].

In the present study, we aimed to profile neurobiological mechanisms of rTMS underlying effect. We investigated the temporal changes in global gene expression pattern depending on low and high frequency effects of repetitive magnetic stimulation (rMS) *in* neuronal cell to generate a comprehensive view of the mechanisms *in vitro*.

Materials and Methods

1. Cell cultures

The mouse neuroblastoma cell line Neuro-2a (CCL-131™) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Neuro-2a cells were maintained in Dulbecco's Modified Eagle medium-low glucose (DMEM-LG; Hyclone**,** Logan, UT**,** USA) containging 10% fetal bovine serum (FBS; Serum Source International, Charlotte, NC, USA) and 1% Penicillin-Streptomycin solution **(**Gibco, Rockville, MD, USA) in a humidified 5% CO_2 atmosphere at 37℃

At 80% confluence cells were harvested using 0.25% trypsin-EDTA (Gibco). Cells were washed, centrifuged, resuspended and seeded in new plates. The medium was replaced every 2 to 3 days.

2. Repetitive magnetic stimulation

In each experiment, cells were rendered quiescent for 6 hours by the addition of DMEM-LG without serum in a humidified 5% CO_2 atmosphere at 37℃, then stimulated rMS. Dishes of cultured cells were divided into 3 groups such as sham group (exposed to the rMS and did not receive stimulation), low-frequency group (0.5 Hz) and high-frequency group (10 Hz) and were stimulated for 3 days. Briefly, the coil was placed above a single dish (one dish per treatment group), and its center was aligned with the center of the dish; the distance between the dimensional center of the

coil and the culture dish was approximately 1.0 cm. The repetitive magnetic stimulation was performed with 0.5 Hz and 10 Hz frequencies (on-off interval, 3 s), with a 100% machine output stimulation intensity at a stimulation duration of 10 min per day. The treatments were performed daily for 3 days. The sham group was handled in a similar manner as treatment groups, exposed to the magnetic stimulation, but did not receive stimulation. After 3 days stimulation, cells were harvested using 0.25% trypsin-EDTA (Gibco) as described above. The experimental scheme is presented in figure 1.

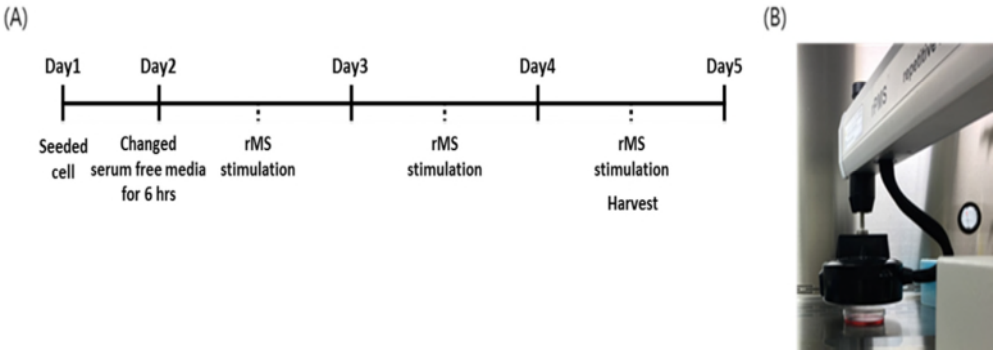

Figure 1. Experimental design.

(A) Schematic timeline of the experimental procedures. (B) The figure of rMS treatment in the neuro-2a cells. Dishes of cultured cells were divided into sham, low frequency and high frequency and were stimulated for 3 days.

3. RNA preparation

Total RNA was prepared from the cell pellets using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturers' protocols. For quality control, RNA quality and quantity were evaluated by 1% agarose gel electrophoresis and the 260/280 ratio was confirmed with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

4. Transcriptome array and analysis

RNA sequencing was performed by Macrogen Inc (Seoul, Korea). The procedures were explained previous studies [15, 16]. Briefly explained that mRNA was converted to a library of template molecules suitable for subsequent cluster generation using the reagents provided in the Illumina ® TruSeq™ RNA Sample Preparation Kit. The first step in the workflow involves purification of the poly-A containing mRNA molecules using poly-T oligo-attached magnetic beads. Following purification, the mRNA is fragmented into small pieces using divalent cations at elevated temperature. The cleaved RNA fragments are reverse transcribed into first strand cDNA using reverse transcriptase and random primers. This is followed by second strand cDNA synthesis using DNA polymerase I and RNase H. These cDNA fragments then go through an end repair process, the addition of a single "A" base, and ligation of the adapters. The products are then purified and enriched with PCR to create the final cDNA library.

Illumina utilizes a unique "bridged" amplification reaction that occurs on the surface of the flow cell. A flow cell containing millions of unique clusters is loaded into the HiSeq 2000 for automated cycles of extension and imaging. Solexa's Sequencing-by-Synthesis utilizes four proprietary nucleotides possessing reversible fluorophore and termination properties. Each sequencing cycle occurs in the presence of all four nucleotides, leading to higher accuracy than that of methods where only one nucleotide is present in the reaction mix at a particular time point.

5. Transcriptome data analysis

The lists of significant differentially expressed genes (DEGs) (fold change $\geq |1.5|$) were submitted to the database for annotation, visualization, and integrated discovery (DAVID v6.8) (http://david.abcc.ncifcrf.gov/) for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis.

6. Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

DEGs of interest were selected for the validation of transcriptome analysis results by qRT-PCR. Total RNA was reverse-transcribed into cDNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. The mRNA expression for genes of interest was profiled using qPCRBIO SyGreen Mix Hi-ROX (PCR BIOSYSTEMS, London, UK) in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

Data analysis was performed using the $2^{-\Delta\Delta^{CT}}$ method. Primers used for qRT-PCR were described in Table1.

Table 1. Primers used for qRT-PCR

Gene symbol	Forward primer (5'→3')	Reverse primer (5'→3')
Grin1	CAG GAT CGT CAG GCA AGA CA	CCA AGC AAC TGA GGG TCC TT
Camk2δ	TGC ACC TGG TAG GGG ACG AT	GAA TAC AGG GTG GCT TGA TGG GT
Camk2α	TGC TGC TCT TTC TCA CGC TG	TCA ATG GTG GTG TTG GTG CT
Camk2γ	TTG TGC GTC TCC ATG ACA GT	TGT CAT GCT GGT GGA TGT GG
BDNF	GGG TCA CAG CGG CAG ATA AA	ATT GCG AGT TCC AGT GCC TT

Grin 1, glutamate receptor ionotropic NMDA1 ; Camk2*d*, calcium/calmodulin-dependent protein kinase type II subunit delta; Camk2*a*, calcium/calmodulin-dependent protein kinase II alpha; Camk2*g*, calcium/calmodulin-dependent protein kinase II gamma; BDNF, Brain-derived neurotrophic factor

7. Western blot analysis

To assess Calmodulin-Dependent Protein Kinase II (CAMKII), phospho-cAMP response element binding (p-CREB) and BDNF from the cell pellets were homogenized and dissolved in RIPA buffer (Thermo Scientific, Rockford**,** IL**,** USA**)** with protease inhibitors (Abcam**,** Cambridge, MA, USA). Total proteins were quantified using BCA™ Protein Assay Kit (Thermo Scientific). The samples were denatured and separated by 4–12% Bis-Tris gels in 1X NuPage MES SDS running buffer **(**Invitrogen, Eugene, OR, USA). Bands were transferred onto a polyvinylidene difluoride (PVDF) membrane (Invitrogen) in 20% (vol/vol) methanol in NuPage Transfer Buffer (Invitrogen) at 4 °C. Membranes were blocked and then incubated overnight at 4 °C with following antibodies: CAMKII (1:1000, Abcam), p-CREB (1:1000, Santa Cruz Biotechnology), and BDNF (1:1000, Abcam). The next day, the blots were washed 3 times with TBS plus Tween 20 and incubated for 1 hour with horse-radish peroxidase–conjugated secondary antibodies (1:4000; Santa Cruz) at room temperature. After being washed 3 times with TBS plus Tween 20, the protein was visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Little Chalfont, UK).

8. Statistical Analysis

All results were expressed as means ± standard error of the mean. Statistical analyses were conducted using the premier vendor for Statistical Package for Social Sciences (SPSS) version 23.0. Non-parametric statistical analysis such as Mann-

Whitney *U* test was used for the comparison of two groups. Student *t* test was used to confirm statistical results. Results with *P*-value < 0.05 was considered statistically significant.

Results

1. Gene expression profile by transcriptome analysis

To identify DEGs depending on different frequencies, we performed transcriptome analysis by RNA sequencing. Total 21,567 genes were differentially expressed at 3 different pairs—low-frequency group (indicated as N2A-0.5 Hz) compared to sham group (indicated as N2A-Sham), high-frequency group (indicated as N2A-10 Hz) compared to sham group (indicated as N2A-Sham), high-frequency group (indicated as N2A-10 Hz) compared to the low-frequency group (indicated as N2A-0.5 Hz). Among total genes in the low-frequency group (indicated as N2A-0.5 Hz), 407 transcripts were 1.5-fold higher and 429 transcripts were 1.5-fold lower than the sham group (indicated as N2A-Sham) in figure 2(A). 362 transcripts were 1.5-fold higher and 388 transcripts were 1.5-fold lower in the high-frequency group (indicated as N2A-10 Hz) compared to the sham group (indicated as N2A-Sham) in figure 2(B). Also in the high-frequency group (indicated as N2A-10 Hz), 383 transcripts were 1.5-fold higher and 415 transcripts were 1.5-fold lower, compared to the low-frequency group (indicated as N2A-0.5 Hz) in figure 2(C).

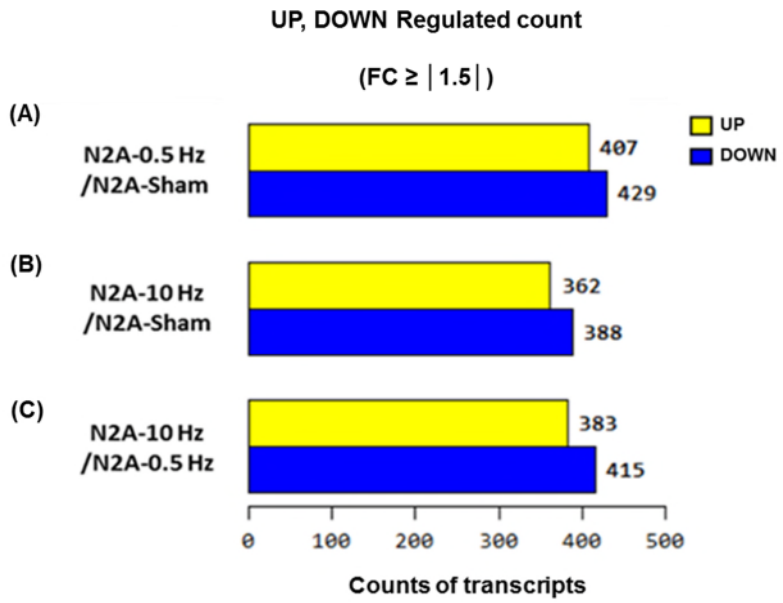

Figure 2. Gene expression profile by transcriptome analysis.

Bar graphs show the number of DEGs with fold change ≥ |1.5| at different phases. (A) The number of DEGs in the low-frequency group (indicated as N2A-0.5 Hz) compared to the sham group (indicated as N2A-Sham). (B) The number of DEGs in the high-frequency group (indicated as N2A-10 Hz) compared to the sham group (indicated as N2A-Sham). (C) The number of DEGs in the high-frequency group (indicated as N2A-10 Hz) compared to the low-frequency group (indicated as N2A-0.5 Hz).

2. Enriched KEGG pathway analysis

DEGs of 3 different pairs were classified based on KEGG pathways using DAVID Gene Functional Classification Tool. Several pathways were identified in low-frequency group and high-frequency group compared to the sham group, though these pathways were not related to the properties of neurons. Enriched KEGG pathways were identified from the high-frequency group compared to low-frequency group and listed in table 2. Among these pathways, mmu04720: Long-term potentiation (LTP) pathway is statistically significant ($p < 0.05$). Previous studies indicated that rMS is capable of inducing LTP. [4] Significantly up-regulated genes were as follows: Grin1, Camk2δ, ribosomal protein S6 kinase polypeptide 1 (Rps6ka1) and Camk2α. Also significantly down-regulated genes were Camk2γ, protein phosphatase 3 catalytic subunit alpha (Ppp3cα), protein kinase cAMP dependent catalytic alpha (Prkacα) and voltage-dependent L-type calcium channel subunit alpha-1C (Cacnαa1c).

Table 2. The enriched KEGG pathways compared between low-and high-frequency of rMS.

Term	Count	%	P-Value	Genes
mmu05031:Amphetamine addiction	9	0.0092	0.0009	FOS, CAMK2G, GRIN1, CAMK2D, PRKACA, FOSB, PPP3CA, CACNA1C, CAMK2A
mmu04713:Circadian entrainment	10	0.0102	0.0027	FOS, GNGT2, CAMK2G, GRIN1, CAMK2D, PER1, PRKACA, PER3, CACNA1C, CAMK2A
mmu04720:Long-term potentiation	8	0.0082	0.0036	RPS6KA1, CAMK2G, GRIN1, CAMK2D, PRKACA, PPP3CA, CACNA1C, CAMK2A
mmu04722:Neurotrophin signaling pathway	11	0.0113	0.0037	PDPK1, RPS6KA1, MAPK14, CAMK2G, PIK3CD, CAMK2D, SH2B2, SH2B1, MAPK7, MAP2K7, CAMK2A
mmu04917:Prolactin signaling pathway	8	0.0082	0.0063	FOS, SOCS2, MAPK14, SOCS1, PIK3CD, JAK2, STAT1, STAT3
mmu04725:Cholinergic synapse	10	0.0102	0.0070	FOS, ACHE, GNGT2, CAMK2G, PIK3CD, CAMK2D, PRKACA, JAK2, CACNA1C, CAMK2A
mmu05231:Choline metabolism in cancer	9	0.0092	0.0111	FOS, PDPK1, TSC1, CHKB, TSC2, PIK3CD, PIP5K1C, GPCPD1, RALGDS
mmu04261:Adrenergic signaling in cardiomyocytes	11	0.0113	0.0152	TNNT2, ATP2B4, MAPK14, CAMK2G, PIK3CD, CAMK2D, CACNB1, PRKACA, CACNA1C, CAMK2A, TPM1
mmu04912:GnRH signaling pathway	8	0.0082	0.0167	MAPK14, CAMK2G, CAMK2D, PRKACA, MAPK7, CACNA1C, MAP2K7, CAMK2A
mmu04728:Dopaminergic synapse	10	0.0102	0.0198	FOS, GNGT2, MAPK14, CAMK2G, CAMK2D, PRKACA, COMT, PPP3CA, CACNA1C, CAMK2A
mmu04919:Thyroid hormone signaling pathway	9	0.0092	0.0207	PDPK1, SIN3A, NCOA2, TSC2, PIK3CD, PRKACA, RCAN1, STAT1, MED1
mmu04010:MAPK signaling pathway	15	0.0154	0.0207	FGFR1, FOS, RPS6KA1, MAPT, MAPK14, TAOK3, CACNB1, MAPK8IP3, PRKACA, PPP3CA, MAPK7, ECSIT, CACNA1C, MAP2K7, MAP3K12
mmu04921:Oxytocin signaling pathway	11	0.0113	0.0211	FOS, CAMK2G, PIK3CD, CAMK2D, CACNB1, PRKACA, RCAN1, PPP3CA, MAPK7, CACNA1C, CAMK2A
mmu04910:Insulin signaling pathway	10	0.0102	0.0255	PHKA2, PDPK1, SOCS2, TSC1, FLOT2, TSC2, SOCS1, PIK3CD, PRKACA, SH2B2
mmu04150:mTOR signaling pathway	6	0.0061	0.0321	PDPK1, RPS6KA1, TSC1, TSC2, PIK3CD, STRADA
mmu04380:Osteoclast differentiation	9	0.0092	0.0365	FOS, MAPK14, SOCS1, MITF, PIK3CD, FOSB, PPP3CA, STAT1, MAP2K7
mmu05152:Tuberculosis	11	0.0113	0.0402	LSP1, MAPK14, CAMK2G, CAMK2D, TIRAP, NFYC, JAK2, APAF1, PPP3CA, STAT1, CAMK2A
mmu05205:Proteoglycans in cancer	12	0.0123	0.0433	FGFR1, PDPK1, ARHGEF1, TIAM1, MAPK14, CAMK2G, PIK3CD, CAMK2D, PRKACA, RDX, CAMK2A, STAT3
mmu04114:Oocyte meiosis	8	0.0082	0.0483	RPS6KA1, CPEB3, CAMK2G, CAMK2D, PRKACA, PPP3CA, CDC27, CAMK2A

These pathways are statistically significant (p-value < 0.05).

3. Validation of qRT-PCR and western blot for transcriptome

We conducted qRT-PCR and western blot to validate the altered gene expression related in LTP pathway for results of RNA-seq. We performed qRT-PCR for more accurate quantitative analysis. There were not any significantly changed in the low-frequency group compared to the sham group in Fig. 3(A) and the expression values were as follows: Grin1 (0.8253-fold, f= 9.810), Camk2δ (0.9199-fold, f= 1.117), Camk2α (0.8573-fold, f= 45.24), and Camk2γ (1.030 -fold, f= 1.407). The expression of Grin1, Camk2δ and Camk2α were also significantly increased and Camk2γ expression was decreased in the high-frequency group compared to the sham group and the expression values were as follows : Grin1 (1.263-fold, f= 1.477), Camk2δ (1.258-fold, f= 5.155), Camk2α (1.250-fold, f= 7.649), and Camk2γ (0.8665-fold, f= 2.901) (Fig. 3(A)). Also in the high-frequency group compared to low-frequency group, Grin1, Camk2δ and Camk2α were also significantly increased and Camk2γ expression was decreased in Fig.3(B) The expression values were as follow : Grin1 (1.530-fold, f= 14.49), Camk2δ (1.368-fold, f= 4.614), Camk2α (1.244-fold, f= 6.530), and Camk2γ (0.8409-fold, f= 4.083). These genes, Grin1, Camk2δ, Camk2α and Camk2γ, were associated with the Ca^{2+}-CaMKII-CREB pathway.

Next, to confirm further validation, CAMKII and p-CREB were determined by western blot. The expression of CAMKII and p-CREB were significantly increased in the high-frequency group compared to the control group and low-frequency group in Fig. 3(C)and (D). The expression values in the high-frequency group compared to the

sham control group were as follows : CAMKII (1.210-fold, f= 34.06) and p-CREB (1.258-fold, f= 5.155). Also in the high-frequency group compared to the low-frequency group were as follows : CAMKII (1.270-fold, f= 4.512) and p-CREB (9.414-fold, f= 124.5). In the low-frequency group compared to the sham group, the expression values were as follows : CAMKII (0.9123-fold, f= 40.33) and p-CREB (0.1664-fold, f= 2.938).

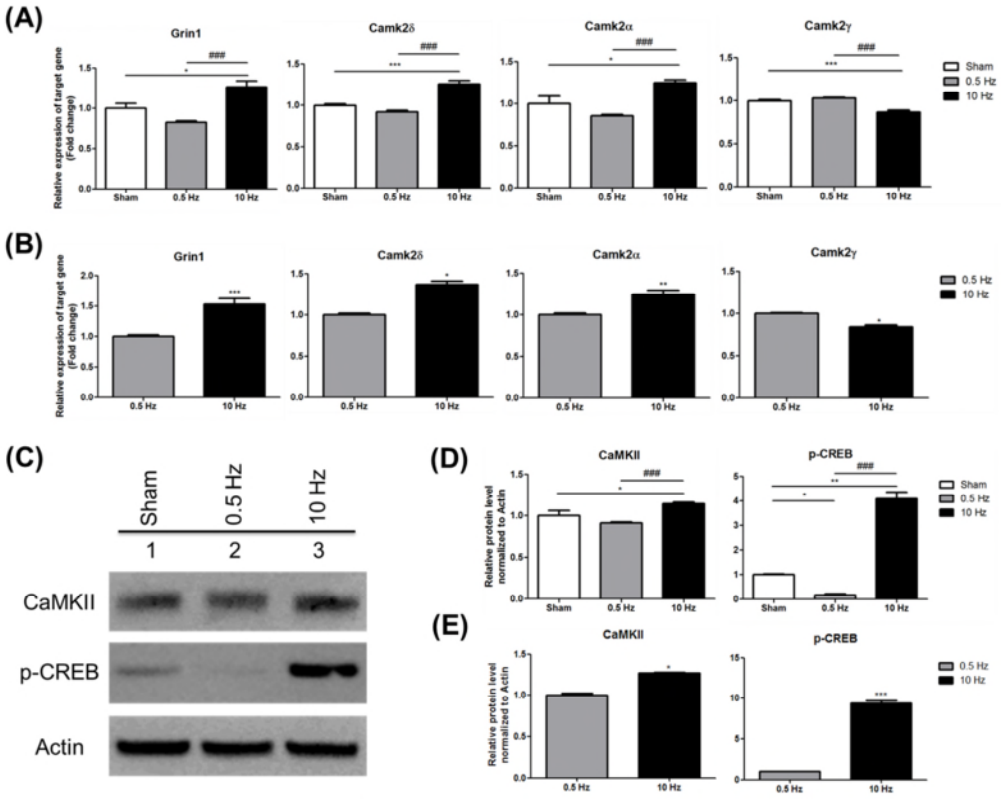

Figure 3. Validation of mRNA and protein quantifications using qRT-PCR and western blot.

Grin1, Camk2δ, Camk2α and, Camk2γ expressions were validated. (A) The relative expression of target genes was normalized by sham and were calculated using $2^{-\Delta\Delta Ct}$ method by qRT-PCR. (B) The relative expression of target genes was normalized by 0.5 Hz and were calculated using $2^{-\Delta\Delta Ct}$ method by qRT-PCR. (C) Western blot analysis was performed using antibodies against CaMKII, p-CREB and actin (as control). (D) Comparison of relative protein expression from the rMS treated groups versus the sham control group. (E) Comparison of relative protein expression from

the high-frequency group versus the low-frequency group. All results were expressed as means ± standard error of the mean (SEM).

4. rMS in neuro 2a cells facilitates BDNF expression

Recently, it is reported that Ca^{2+}-CaMKII-CREB pathway plays crucial role in BDNF transcription [14], we confirmed BDNF expression by qRT-PCR and western blot. In the low-frequency group compared to the sham control group, mRNA (0.9309-fold, f= 2.451) (Fig. 4(A)) and protein (0.8327-fold, f= 26.93) (Fig. 4(B), (C)) expressions were not significantly increased. As well as the high-frequency group compared to the sham control group, mRNA (1.200-fold, f= 1.017) (Fig. 4(A)) and protein (1.430-fold, f= 9.476) (Fig. 4(B), (C)) expressions were significantly increased. Furthermore in the high-frequency group compared to the low-frequency group, mRNA and protein expression of BDNF were significantly increased and expression values were as follows : (1.289-fold, f = 7.731, Fig. 4(A)), (3.352-fold, f = 31.49, Fig. 4(B),(C)).

Overall the study, ‘Long term potentiation pathway’ was confirmed to enriched KEGG pathway in the rMS treated neuro 2a cells depending on different frequencies. Also 10 Hz of rMS can activated the Ca^{2+}-CaMKII-CREB signaling pathway. Furthermore BDNF expression was increased via activation of the Ca^{2+}-CaMKII-CREB signaling pathway (Fig. 5).

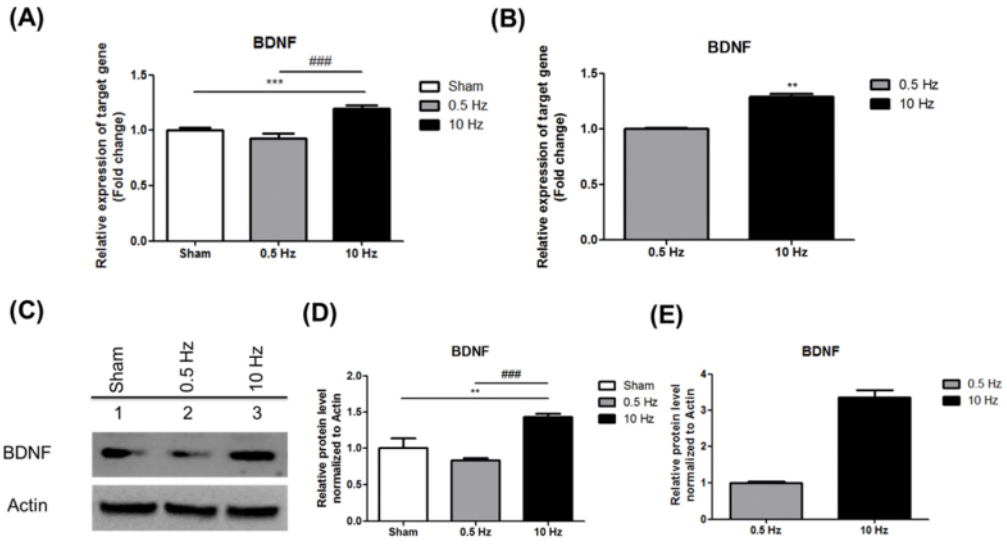

Figure 4. rMS treatment increase BDNF expression in neuro 2a cell.

BDNF expression was validated. (A) The relative expression of BDNF was normalized by sham and were calculated using $2^{-\Delta\Delta Ct}$ method by qRT-PCR. (B) The relative expression of BDNF was normalized by 0.5 Hz and were calculated using $2^{-\Delta\Delta Ct}$ method by qRT-PCR. (C) Western blot analysis was performed using antibodies against BDNF and actin (as control). (D) Comparison of relative protein expression from the rMS treated groups versus the sham control group. (E) Comparison of relative protein expression from the high-frequency group versus the low-frequency group. All results were expressed as means ± standard error of the mean (SEM).

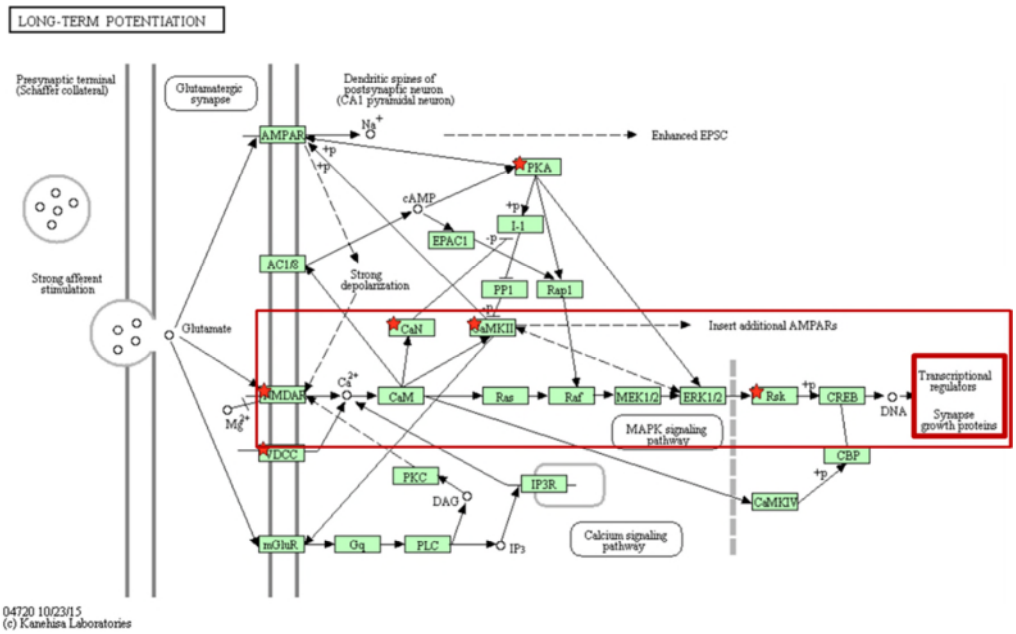

Figure 5. Illustration of the validated KEGG pathways

Long-term potentiation was shown as the enriched significant pathway comparing between 0.5 Hz and 10 Hz. Especially, high-frequency of rMS can activate the Ca^{2+}-CaMKII-CREB pathway.

Discussion

It has been established that different rTMS techniques can produce different modulatory effects [6]. It is unclear neurobiological mechanism of low-frequency and high-frequency rTMS underlying effect. The current study, we focused on global gene expression pattern depending on different frequency rMS in neuronal cells. We demonstrated that high-frequency (10 Hz) rMS can activate the p-CREB, BDNF and synaptic marker protein expression via Ca^{2+}-CaMKII-CREB pathway.

In recent years, rTMS has been generally acknowledged to affect attention, memory, and other brain functions in degenerative brain disease, but the effects of the various stimulation parameters (e.g. frequency, intensity, and total stimulation period) and the neural mechanisms remain unclear [17]. Several studies reported that changes in TMS frequency and stimulation patterns can result in varying long-term effects [10]. High-frequency stimulation (>3 Hz) generally results in facilitation-an effect that shares similarities with long-term potentiation (LTP), while low-frequency rTMS (≤1 Hz) induces reduction of synaptic efficiency-an effect that shares similarities with long-term depression (LTD) [6, 10]. In current study, comparing between 0.5 Hz and 10 Hz, long-term potentiation was shown as the enriched significant pathway.

Long-term potentiation (LTP), the enduring functional enhancement of synaptic connections, or structural modification of neuronal connectivity, is an important neurophysiological underlying mechanism of learning and memory processes [18]. It

was found that LTP was significantly increased in rTMS which compared 5 Hz rTMS group and Sharm group [19]. LTP is a long-lasting increase in synaptic efficacy resulting from the high-frequency stimulation of afferent fibers [20].

Another finding showed that Grin1, Camk2d and Camk2a were increased, and Camk2g was decreased. These genes can activate the Ca^{2+}-CaMKII-CREB pathway. Grin1, one of the subunits of the NMDA-R [21] is up regulated on 10Hz rMS in neuronal cells.

N-methyl-D-aspartate receptors(NMDARs) play important roles in normal brain development and function, such as synaptic plasticity, neural development, learning and memory [22]. NMDAR activity mediates CaMKII translocation to the postsynaptic density (PSD) where it is maintained through a direct interaction with the c-terminal tail of the NMDAR [23]. In mammals, four CaMKII genes denoted Camk2a, Camk2b, Camk2d, and Camk2g [24]. Studies show that Camk2d and Camk2a were increased, and Camk2g was decreased. CaMKII is a Ca^{2+}–activated enzyme, which plays an important role in learning and memory [9].

Seminal studies in the early 1990s showed that a stimulation paradigm that induces LTP in the CA1 region of the hippocampus also upregulates the BDNF mRNA. [25] BDNF and its cognate receptor tyrosine receptor kinase B (TrkB), a member of the neurotrophin receptor tyrosine kinase family, have emerged as important upstream regulators of LTP in brain region, including hippocampus and neocortex [26]. It is reported hippocampal BDNF levels were increased by rTMS treatment and accompanied by an increased level of NMDAR subunits and enhanced LTP. [27]

Studies show that 10 Hz rMS can activates BDNF transcription via Ca^{2+}-CaMKII-CREB pathway.

CREB has been strongly implicated in memory formation in a wide range of species [12]. The richness of CREB signaling is greatly increased by its responsiveness to multiple intracellular signal transduction cascades [12]. CREB can be activated by various kinases in response to electrical activity, neurotransmitter, hormones, and neurotrophins, among other, promoting expression of many genes that contain cAMP response elements (CREs) [28]. Multiple signaling cascades converge onto CREB phosphorylation, including Ca^{2+}/ Calmodulin kinase (CaMK) [28]. CREB may activate it by phosphorylation of ser-133 or, alternatively, may inhibit CREB binding in some cell lines by phosphorylating it on ser-142 [29].

The results of the present experiments indicate that high frequency rMS induces gene expression of Grin1, Camk2d, Camk2a and Camk2g. And these genes can activate the Ca^{2+}-CaMKII-CREB pathway which involved in long-term potentiation. We proposed that different effect of rMS depending on different frequencies and it is proved in this study. Ca^{2+}-CaMKII-CREB pathway plays a critical role in high frequency (10 Hz) rMS-indeced BDNF transcription and expression of synaptic protein marker. These findings provide better understand the neurobiological mechanisms of neuroplasticity on different frequency rTMS and basic information of clinical application

Summary and Conclusion

Comparing between 0.5 Hz and 10 Hz, long-term potentiation was shown as the enriched significant pathway. 10 Hz of rMS can activate the Ca^{2+}-CaMKII-CREB pathway. Furthermore, p-CREB, BDNF and synaptic marker protein expression were increased via activation of Ca^{2+}-CaMKII-CREB pathway. Taken together, Ca^{2+}-CaMKII-CREB signaling pathway was modulated by rMS depending on different frequencies. These findings might help clarify further therapeutic mechanisms of rTMS

References

[1] A. Kapoor, Repetitive transcranial magnetic stimulation therapy for post-stroke non-fluent aphasia: A critical review, Topics in Stroke Rehabilitation, DOI 10.1080/10749357.2017.1331417(2017) 1-7.
[2] F. Muller-Dahlhaus, A. Vlachos, Unraveling the cellular and molecular mechanisms of repetitive magnetic stimulation, Frontiers in molecular neuroscience, 6 (2013) 50.
[3] J.Y. Lee, H.J. Park, J.H. Kim, B.P. Cho, S.R. Cho, S.H. Kim, Effects of low- and high-frequency repetitive magnetic stimulation on neuronal cell proliferation and growth factor expression: A preliminary report, Neuroscience letters, 604 (2015) 167-172.
[4] A. Vlachos, F. Muller-Dahlhaus, J. Rosskopp, M. Lenz, U. Ziemann, T. Deller, Repetitive magnetic stimulation induces functional and structural plasticity of excitatory postsynapses in mouse organotypic hippocampal slice cultures, The Journal of neuroscience : the official journal of the Society for Neuroscience, 32 (2012) 17514-17523.
[5] J.P. Lefaucheur, N. Andre-Obadia, A. Antal, S.S. Ayache, C. Baeken, D.H. Benninger, R.M. Cantello, M. Cincotta, M. de Carvalho, D. De Ridder, H. Devanne, V. Di Lazzaro, S.R. Filipovic, F.C. Hummel, S.K. Jaaskelainen, V.K. Kimiskidis, G. Koch, B. Langguth, T. Nyffeler, A. Oliviero, F. Padberg, E. Poulet, S. Rossi, P.M. Rossini, J.C. Rothwell, C. Schonfeldt-Lecuona, H.R. Siebner, C.W. Slotema, C.J. Stagg, J. Valls-Sole, U. Ziemann, W. Paulus, L. Garcia-Larrea, Evidence-based guidelines on the therapeutic use of repetitive transcranial magnetic stimulation (rTMS), Clinical neurophysiology : official journal of the International Federation of Clinical Neurophysiology, 125 (2014) 2150-2206.
[6] J. Luo, H. Zheng, L. Zhang, Q. Zhang, L. Li, Z. Pei, X. Hu, High-Frequency Repetitive Transcranial Magnetic Stimulation (rTMS) Improves Functional Recovery by Enhancing Neurogenesis and Activating BDNF/TrkB Signaling in Ischemic Rats, International journal of molecular sciences, 18 (2017).
[7] M.R. Ljubisavljevic, A. Javid, J. Oommen, K. Parekh, N. Nagelkerke, S. Shehab, T.E. Adrian, The Effects of Different Repetitive Transcranial Magnetic Stimulation (rTMS) Protocols on Cortical Gene Expression in a Rat Model of Cerebral Ischemic-Reperfusion Injury, PloS one, 10 (2015) e0139892.
[8] J.Y. Lee, S.H. Kim, A.R. Ko, J.S. Lee, J.H. Yu, J.H. Seo, B.P. Cho, S.R. Cho, Therapeutic effects of repetitive transcranial magnetic stimulation in an animal model of Parkinson's disease, Brain research, 1537 (2013) 290-302.
[9] D. Min, F. Guo, S. Zhu, X. Xu, X. Mao, Y. Cao, X. Lv, Q. Gao, L. Wang, T. Chen, C. Shaw, L. Hao, J. Cai, The alterations of Ca2+/calmodulin/CaMKII/CaV1.2 signaling in experimental models of Alzheimer's disease and vascular dementia, Neuroscience letters, 538 (2013) 60-65.
[10] R. Gersner, E. Kravetz, J. Feil, G. Pell, A. Zangen, Long-term effects of

repetitive transcranial magnetic stimulation on markers for neuroplasticity: differential outcomes in anesthetized and awake animals, The Journal of neuroscience : the official journal of the Society for Neuroscience, 31 (2011) 7521-7526.
[11] A.E. West, W.G. Chen, M.B. Dalva, R.E. Dolmetsch, J.M. Kornhauser, A.J. Shaywitz, M.A. Takasu, X. Tao, M.E. Greenberg, Calcium regulation of neuronal gene expression, Proceedings of the National Academy of Sciences of the United States of America, 98 (2001) 11024-11031.
[12] G.Y. Wu, K. Deisseroth, R.W. Tsien, Activity-dependent CREB phosphorylation: convergence of a fast, sensitive calmodulin kinase pathway and a slow, less sensitive mitogen-activated protein kinase pathway, Proceedings of the National Academy of Sciences of the United States of America, 98 (2001) 2808-2813.
[13] J. Hellmann, R. Juttner, C. Roth, M. Bajbouj, I. Kirste, I. Heuser, K. Gertz, M. Endres, G. Kronenberg, Repetitive magnetic stimulation of human-derived neuron-like cells activates cAMP-CREB pathway, Eur Arch Psychiatry Clin Neurosci, 262 (2012) 87-91.
[14] X. Yan, J. Liu, Z. Ye, J. Huang, F. He, W. Xiao, X. Hu, Z. Luo, CaMKII-Mediated CREB Phosphorylation Is Involved in Ca2+-Induced BDNF mRNA Transcription and Neurite Outgrowth Promoted by Electrical Stimulation, PloS one, 11 (2016) e0162784.
[15] M.S. Kim, J.H. Yu, M.Y. Lee, A.L. Kim, M.H. Jo, M. Kim, S.R. Cho, Y.H. Kim, Differential Expression of Extracellular Matrix and Adhesion Molecules in Fetal-Origin Amniotic Epithelial Cells of Preeclamptic Pregnancy, PLoS One, 11 (2016) e0156038.
[16] Y.H. Won, M.Y. Lee, Y.C. Choi, Y. Ha, H. Kim, D.Y. Kim, M.S. Kim, J.H. Yu, J.H. Seo, M. Kim, S.R. Cho, S.W. Kang, Elucidation of Relevant Neuroinflammation Mechanisms Using Gene Expression Profiling in Patients with Amyotrophic Lateral Sclerosis, PloS one, 11 (2016) e0165290.
[17] J. Ma, Z. Zhang, L. Kang, D. Geng, Y. Wang, M. Wang, H. Cui, Repetitive transcranial magnetic stimulation (rTMS) influences spatial cognition and modulates hippocampal structural synaptic plasticity in aging mice, Experimental gerontology, 58 (2014) 256-268.
[18] K. Monte-Silva, M.F. Kuo, S. Hessenthaler, S. Fresnoza, D. Liebetanz, W. Paulus, M.A. Nitsche, Induction of late LTP-like plasticity in the human motor cortex by repeated non-invasive brain stimulation, Brain stimulation, 6 (2013) 424-432.
[19] Y. Shang, X. Wang, X. Shang, H. Zhang, Z. Liu, T. Yin, T. Zhang, Repetitive transcranial magnetic stimulation effectively facilitates spatial cognition and synaptic plasticity associated with increasing the levels of BDNF and synaptic proteins in Wistar rats, Neurobiology of Learning and Memory, 134 Pt B (2016) 369-378.
[20] M. Ogiue-Ikeda, S. Kawato, S. Ueno, The effect of repetitive transcranial magnetic stimulation on long-term potentiation in rat hippocampus depends on stimulus intensity, Brain research, 993 (2003) 222-226.
[21] M.C. Diana, M.L. Santoro, G. Xavier, C.M. Santos, L.N. Spindola, P.N. Moretti,

V.K. Ota, R.A. Bressan, V.C. Abilio, S.I. Belangero, Low expression of Gria1 and Grin1 glutamate receptors in the nucleus accumbens of Spontaneously Hypertensive Rats (SHR), Psychiatry research, 229 (2015) 690-694.
[22] W. Chen, C. Shieh, S.A. Swanger, A. Tankovic, M. Au, M. McGuire, M. Tagliati, J.M. Graham, S. Madan-Khetarpal, S.F. Traynelis, H. Yuan, T.M. Pierson, GRIN1 mutation associated with intellectual disability alters NMDA receptor trafficking and function, Journal of human genetics, 62 (2017) 589-597.
[23] F.J. Bustos, N. Jury, P. Martinez, E. Ampuero, M. Campos, S. Abarzua, K. Jaramillo, S. Ibing, M.D. Mardones, H. Haensgen, J. Kzhyshkowska, M.F. Tevy, R. Neve, M. Sanhueza, L. Varela-Nallar, M. Montecino, B. van Zundert, NMDA receptor subunit composition controls dendritogenesis of hippocampal neurons through CAMKII, CREB-P, and H3K27ac, J Cell Physiol, DOI 10.1002/jcp.25843(2017).
[24] F.Z. Saddouk, R. Ginnan, H.A. Singer, Ca2+/Calmodulin-Dependent Protein Kinase II in Vascular Smooth Muscle, Advances in pharmacology, 78 (2017) 171-202.
[25] G. Leal, C.R. Bramham, C.B. Duarte, BDNF and Hippocampal Synaptic Plasticity, vitamins and hormones, 104 (2017) 153-195.
[26] H.Y. Wang, D. Crupi, J. Liu, A. Stucky, G. Cruciata, A. Di Rocco, E. Friedman, A. Quartarone, M.F. Ghilardi, Repetitive transcranial magnetic stimulation enhances BDNF-TrkB signaling in both brain and lymphocyte, The Journal of neuroscience : the official journal of the Society for Neuroscience, 31 (2011) 11044-11054.
[27] N. Zhang, M. Xing, Y. Wang, H. Tao, Y. Cheng, Repetitive transcranial magnetic stimulation enhances spatial learning and synaptic plasticity via the VEGF and BDNF-NMDAR pathways in a rat model of vascular dementia, Neuroscience, 311 (2015) 284-291.
[28] M. Muller, C. Cardenas, L. Mei, K.H. Cheung, J.K. Foskett, Constitutive cAMP response element binding protein (CREB) activation by Alzheimer's disease presenilin-driven inositol trisphosphate receptor (InsP3R) Ca2+ signaling, Proceedings of the National Academy of Sciences of the United States of America, 108 (2011) 13293-13298.
[29] H. Zeng, Y. Liu, D.M. Templeton, Ca(2+)/calmodulin-dependent and cAMP-dependent kinases in induction of c-fos in human mesangial cells, Am J Physiol Renal Physiol, 283 (2002) F888-894.

국문 요약

반복 자기 자극이 신경세포의 유전자 발현에 미치는 영향

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박 은 지

반복경두개자기자극은 비침습적이며, 통증이 없는 도구로써 신경정신학적 질환과 신경재활에서 다양하게 사용되어왔다. 비록 반복경두개자기자극이 임상 영역에서 널리 쓰여왔다고는 하지만 정밀한 생물학적 기전과 그 효과는 여전히 알려지지 않았다. 그러므로 본 연구의 목적은 신경세포에 서로 다른 반복자기자극을 가하여 시간 변화에 따른 유전자의 전반적인 발현 양상을 조사하고, 포괄적인 기전을 밝히는 것이다. 본 연구에서는 Neuro 2a cells (mouse neuroblastoma cell line)이 본연구의 cell model로 사용되었으며, 배양된 세포는 무작위로 Sharm, 저빈도(0.5 Hz), 고빈도(10 Hz) 군으로 나뉘었으며 3일간 자극하였다. 세 그룹은 transcriptome analysis를 통해 체계적으로 분석하였다. Transcriptome analysis 결과는 Database of Annotation Visualization and Integrated Discovery (DAVID) 의 프로그램인 Differentially expressed genes (DEG) 로 분석하여 Kyoto Encycolpedia of Genes and Genomes (KEGG) pathway 로 나타내었으며, DEG 결과는 qRT-PCR 과 Western blot analysis로 검증하였다. 저빈도와 고빈도의 반복자기자극을 비교한 결과 long-term potentiation, neurotrophin signaling pathway, cholinergic synapse, dopaminergic synapse 가 주요 전달로로 강화되었으며, Grin1, Camk2d, Camk2a 유전자는 증가하였으며, Camk2g 는 감소하였다. 이 유전자들은 Ca^{2+}-CaMKII-CREB pathway를 활성화 시키며 또한, 고빈도의 반복자기자극은 cAMP반응요소결합단백 cAMP-response element binding protein (CREB)의 인산화(Phosphorylation)를 증가시키며, CaMKII-CREB pathway를 통해 신경영양인자 Brain-derived neurotrophic factor(BDNF) 전사(Transcription)와 시냅스표지단백질 (synaptic marker protein)의 발현을 증가시킨다. 종합적으로, Neuro 2a cell 에 가한 서로 다른 빈도의 반복자기자극에 따른 기전을 Ca^{2+}-CaMKII-CREB signaling pathway를 통해 입증하였다. 이러한 발견은 추후 반복경두개자기자극의 치료 기전에 도움이 될 것이다.

핵심되는말 : 반복경두개자기자극, 반복자기자극, 저빈도, 고빈도, Ca^{2+}-CaMKII-CREB pathway, 신경영양인자