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HIGHLIGHTS

- A single session of repetitive transcranial magnetic stimulation (rTMS) increases immediate early gene expression.
- The cumulative effect of rTMS includes increase of structural gene expression.
- MicroRNA might play role in rTMS-induced plasticity.

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Gene Expression Profile Changes in the Stimulated Rat Brain Cortex After Repetitive Transcranial Magnetic Stimulation

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ABSTRACT

Repetitive transcranial magnetic stimulation (rTMS) is gaining popularity as a research tool in neuroscience; however, little is known about its molecular mechanisms of action. The present study aimed to investigate the rTMS-induced transcriptomic changes; we performed microarray messenger RNA, micro RNA, and integrated analyses to explore these molecular events. Eight adult male Sprague-Dawley rats were subjected to a single session of unilateral rTMS at 1 Hz (n = 4) or sham (n = 4). The left hemisphere was stimulated for 20 minutes. To evaluate the cumulative effect of rTMS, eight additional rats were assigned to the 1-Hz (n = 4) or sham (n = 4) rTMS groups. The left hemisphere was stimulated for 5 consecutive days using the same protocol. Microarray analysis revealed differentially expressed genes in the rat cortex after rTMS treatment. The overrepresented gene ontology categories included the positive regulation of axon extension, axonogenesis, intracellular transport, and synaptic plasticity after repeated sessions of rTMS. A single session of rTMS primarily induced changes in the early genes, and several miRNAs were significantly related to the mRNAs. Future studies are required to validate the functional significance of selected genes and refine the therapeutic use of rTMS.

Keywords: Transcranial Magnetic Stimulation; Recovery of Function; Neuronal Plasticity

INTRODUCTION

Neuromodulation, which includes repetitive transcranial magnetic stimulation (rTMS) and transcranial direct current stimulation, has gained popularity in recent years. There has been a remarkable number of publications exploring the therapeutic potential of rTMS for various conditions, including central pain [1,2], depression [3], migraine [4], and stroke [5-7]. There is evidence of the after-effects of rTMS on cortical excitability, but the rationale for the use of rTMS under specific conditions is still not very strong [8]. One reason for this is that the molecular mechanisms of rTMS remain unclear.

Funding

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Conflict of Interest

The corresponding author of this manuscript is an editor of *Brain & NeuroRehabilitation*. The corresponding author did not engage in any part of the review and decision-making process for this manuscript. The other authors have no potential conflicts of interest to disclose.

The mechanisms suggested to underlie the after-effects of rTMS include long-term potentiation and long-term depression-like effects [9], transient shift in ionic balance [8], metabolic changes [10], and enhancement of neuroprotection [11]. Several studies have examined rTMS-induced molecular changes. High- and low-frequency rTMS have been reported to differentially activate immediate early genes, c-Foc and zif268, in different brain areas [12]. Regarding rTMS and the inhibitory interneuronal system, GAD65 and GAD67 showed different expression patterns with the stimulation protocols [13]. For glutamatergic, gamma-aminobutyric acid (GABA), and glycinergic systems, chronic rTMS induced the expressions of glutamate, GABA, and glycine transporters.[14] However, a genome-wide study of genes influenced by rTMS has not yet been performed.

MicroRNAs (miRNAs) are non-protein coding, 19–24 nucleotide, single-stranded RNAs that regulate gene expression by binding to specific target messenger RNAs (mRNAs) and disrupting their stability and translation.[15] miRNAs are thought to play a regulatory role in numerous biological processes.

In a preliminary study, we reported that the expression of the immediate-early genes Arc, Junb, and Egr2 increased after a single session of rTMS, and brain-derived neurotrophic factor (BDNF) increased after five sessions of rTMS.[16] In this study, we aimed to evaluate the effects of rTMS on mRNA and miRNA expressions in rat brains using microarrays. The immediate after-effect and cumulative effects were evaluated.

MATERIALS AND METHODS

Experimental design

In the first experiment, the rat brain was harvested 5 minutes after completion of a single session of rTMS to evaluate the immediate effect of rTMS (**Fig. 1**). The second experiment was performed to determine whether multiple sessions of rTMS resulted in changes in gene

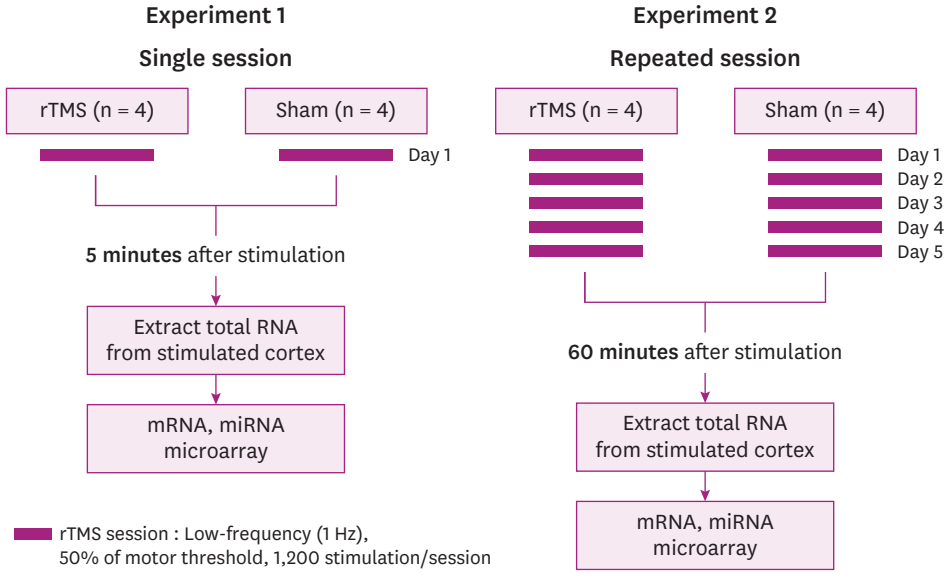

Fig. 1. Experimental design for assessing the immediate (A) and cumulative (B) effects after rTMS. rTMS, repetitive transcranial magnetic stimulation; miRNA, microRNA; mRNA, messenger RNA.

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expression profiles. Magnetic stimulation was applied for 20 min/day for 5 consecutive days. The rat brain was harvested 60 minutes after the completion of the last rTMS session (**Fig. 1**).

rTMS

Stimulation was applied using a repetitive stimulator (Magstim Rapid; Magstim Company Ltd., Wales, UK) delivering biphasic stimuli via a 25-mm figure-of-8 coil (1165-00; Magstim Company Ltd.). The coil size was selected in order to stimulate the unilateral cerebral cortex of rat. The maximum magnetic field strength was 4.0T for the coil. The magnetic coil was mounted firmly on a built-in holder. The motor threshold was defined as the minimum stimulus intensity evoking 5 or more palpable contractions in the contralateral forepaw in 10 stimulations. It was determined with the center of the coil positioned 0.5 cm lateral to the midline and the surface flat onto the calvarium. Motor thresholds were obtainable in all rats, and was measured as 74.4% (mean; range, 65%–80%) of maximal stimulator output.

The animals were subjected to a single session of unilateral rTMS of the left hemisphere. The left side was selected arbitrarily, since this study aimed to stimulate a unilateral cerebral hemisphere of rat. The stimulation intensity was set at 50% of the motor threshold. The 1-Hz stimulation was performed without rest, for 20 minutes. The center of the coil was angulated 45° to the ground to minimize the direct effect of rTMS on the contralateral cortex. For the sham stimulation, the coil was placed 2 cm apart and tilted perpendicular to the calvaria. We used a hydraulic cooling system to enable repeated stimulation for > 20 minutes, as in a previous study [16].

Animal preparation

Adult male Sprague-Dawley rats were used in this study. Seven-week-old rats were allowed to have 1 week to adapt to the new environment. Eight animals were used for the microarray analysis of each experiment. For each experiment, the animals were randomly allocated to the rTMS (1 Hz, n = 4) or sham stimulation (n = 4) groups. The animals were housed in cages (2 per cage) in a controlled environment under a 12 hours light/dark cycle with lights on at 7:00 A.M. Food and water were provided ad libitum. All the experimental procedures were performed between 8:30 A.M. and 4:00 P.M. The Institutional Animal Care and Use Committee of Seoul National University Hospital approved the experimental procedures.

To minimize the potential suppression of cortical excitability by the inhalation agent, intravenous anesthesia was maintained throughout the experiment. Initially, anesthesia was induced and maintained with 5% and 2% isoflurane dissolved in 40%/60% and 25%/75% oxygen/nitrogen applied via the chamber and nose cone, respectively. The anesthesia depth was adjusted to the level of abolishing abdominal contractions to the tail pinch. Body temperature was monitored and maintained using a homeothermic blanket equipped with a rectal probe (507222F; Harvard Apparatus, Holliston, MA, USA). A lateral tail vein was catheterized with a 24G venous catheter for transition to intravenous anesthesia. The animals were administered intravenous propofol (10 mg/kg over 10 minutes). Isoflurane was discontinued 5 minutes after initiating propofol loading. Propofol sedation was maintained with an infusion rate of 500–700 μg/kg/min. Oxygen was supplemented at 0.8 L/min through a nose cone. Magnetic stimulation was applied 10 minutes after the complete transition to intravenous anesthesia, as described in a previous study [16].

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mRNA microarray analysis

The rats were deeply anesthetized and decapitated after rTMS. The brains were carefully dissected, frozen on dry ice, and stored at −80°C until analysis. Brain tissue was harvested from the left cortex.

RNA preparation

Total RNA was extracted using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA) and purified using RNeasy columns (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. After processing with DNase digestion and clean-up procedures, the RNA samples were quantified, aliquoted, and stored at -80°C until use. For quality control, RNA purity and integrity were evaluated using denaturing gel electrophoresis (optical density, 260/280 ratio) and analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

Labeling and purification

Total RNA was amplified and purified using the Ambion Illumina RNA amplification kit (Ambion, Austin, TX, USA) to yield biotinylated complementary RNA (cRNA) according to the manufacturer's instructions. Briefly, 550 ng of total RNA was reverse-transcribed into complementary DNA (cDNA) using T7 oligo(dT) primers. Second-strand cDNA was synthesized, transcribed in vitro, and labeled with biotin-nucleoside triphosphate (NTP). After purification, cRNA was quantified using an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA).

Hybridization and data export

The Illumina RatRef-12 Expression BeadChip (Illumina, Inc., San Diego, CA, USA) was used for mRNA expression analysis. The labeled cRNA samples were hybridized to each rat-12 expression bead array for 16–18 hours at 58°C according to the manufacturer's instructions (Illumina, Inc.). The detection of the array signal was carried out using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK), following the bead array manual. The arrays were scanned with an Illumina bead array reader confocal scanner according to the manufacturer's instructions. Array data export processing and analysis were performed using Illumina BeadStudio v3.1.3 (Gene Expression Module v3.3.8).

Raw data preparation and statistical analysis

The quality of hybridization and overall chip performance were monitored by visual inspection of both internal quality control checks and raw scanned data. Raw data were extracted using software provided by the manufacturer (Illumina GenomeStudio v2009.2, Gene Expression Module v1.5.4). The array data were filtered using a detection p value of < 0.05 (similar to signal to noise) for at least 50% of the samples (we applied a filtering criterion for data analysis; a higher signal value was required to obtain a detection p value of < 0.05). The selected gene signal value was logarithmically transformed and normalized using the quantile method. Statistical significance of the expression data was determined using the local-pooled-errortest, independent t-test, and fold change; the null hypothesis was that no difference existed between the rTMS and sham groups.

The false discovery rate was controlled by adjusting the p-value using the Benjamini-Hochberg algorithm. After normalization and filtering, mRNAs showing significant differential expression ($|\text{fold-change}| \geq 1.2$, $p < 0.05$) were selected. Gene ontology (GO) analysis for the significant probe list was performed using PANTHER (http://www.pantherdb.org/panther/ontologies.jsp) and text files containing the Gene ID list and accession number

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of the Illumina probe ID. Gene Set Enrichment Analysis (GSEA) was performed to determine whether an a priori defined set of genes showed differential patterns for biological processes and molecular function states. For GSEA, genes with |fold changes of |≥ 1.2 were all included regardless of their p values. A one-tail Fisher's exact test was used to assess gene enrichment in annotation terms.

Hierarchical and K-means clustering were performed using complete linkage with a Euclidean metric. GenomeStudio v2009.2 was used for quantification and image analysis of the mRNA data. R scripts were used for all other analytical processes.

microRNA microarray analysis

Agilent rat miRNA microarrays (mirBASE 15.0) (Agilent Technologies, Inc., Santa Clara, CA, USA) were used. Raw data were extracted using the software provided by Agilent Feature Extraction Software (v. 10.7.1.1). The raw data for the same gene were summarized automatically using the Agilent feature extraction protocol to generate a gene view file, providing expression data for each gene probed on the array. The array data were filtered using gIsGeneDetected = 1 for all samples (1:detected). The selected miRNA gtotalGeneSignal values were logarithmically transformed and normalized using the quantile method.

The t-test and fold changes were performed. Statistical significance was adjusted using the Benjamini-Hochberg multiple-testing correction with the false discovery rate. Hierarchical cluster analysis was performed using complete linkage and Euclidean distance as similarity measures. All data analyses and visualization of differentially expressed genes were conducted using the R statistical language v. 2.4.1 (R Project for Statistical Computing, Vienna, Austria).

mRNA-miRNA integrative analysis

We first selected significantly up- or downregulated (1.2-fold) miRNAs and mRNAs from the rTMS and sham groups. We used miRNA target prediction information and miRBase Targets (Wellcome Trust Sanger Institute, http://microrna.sanger.ac.uk/targets/v5/). With this information, we extracted a list of putative miRNA-mRNA target pairs. From these target pairs, we extracted miRNA-mRNA regulatory association lists with negative correlations between miRNA and mRNA expression profiles. All data analyses and visualization were conducted using R 2.4.1 (R Project for Statistical Computing; www.r-project.org).

RESULTS

Microarray analysis reveals differentially expressed genes in the rat cortex after real or sham rTMS

To study rTMS-induced changes in mRNA expression in the stimulated cerebral cortex, mRNA expression profiling was performed using microarray analysis. The levels of expression were determined at two different time points and with different stimulation session numbers, namely 5 minutes after one session and 1 hour after 5 sessions. Significant differences in gene expression were observed at both time points after rTMS; 14 genes were upregulated and 11 were downregulated 5 minutes after 1 session (**Table 1**) and 67 genes were upregulated and 14 were downregulated 1 hour after 5 sessions (**Supplementary Table 1**). Cluster analysis showed a clear distinction between the rTMS and sham groups only in Experiment II (**Fig. 2**).

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Table 1. List of differentially expressed genes identified by microarray 5 minutes after 1 session of rTMS, ordered by FC (largest to smallest)

Genes	rTMS/Sham FC		p value
Early growth response 2	1.67	Up	< 0.001
Jun-B oncogene	1.53	Up	< 0.001
Activity regulated cytoskeletal-associated protein	1.53	Up	< 0.001
Proteolipid protein (myelin) 1	1.34	Down	< 0.001
Collagen, type I, alpha 2	1.34	Up	< 0.001
Purkinje cell protein 4	1.34	Down	< 0.001
Transferrin	1.34	Down	< 0.001
Myelin oligodendrocyte glycoprotein	1.40	Down	< 0.001
Osteoglycin, transcript variant 3*	1.29	Up	< 0.001
Breast carcinoma amplified sequence 1	1.34	Down	< 0.001
Myelin and lymphocyte protein, T-cell differentiation protein	1.37	Down	< 0.001
Dual specificity phosphatase 1	1.20	Up	< 0.001
Prostaglandin-endoperoxide synthase 2	1.26	Up	< 0.001
Zinc finger, CCHC domain containing 12	1.25	Up	0.002
2',3'-cyclic nucleotide 3' phosphodiesterase	1.21	Down	0.002
Insulin-like growth factor 2*	1.24	Up	0.002
Zgc:56193 (RGD1562162)*	1.29	Up	0.004
Heparan sulfate (glucosamine) 3-O-sulfotransferase 2	1.42	Down	0.005
Septin 4*	1.22	Down	0.007
TCDD-inducible poly(ADP-ribose) polymerase*	1.42	Up	0.017
Death effector domain-containing (Dedd)	1.41	Down	0.018
Coagulation factor C homolog (Limulus polyphemus)*	1.27	Up	0.020
Matrix Gla protein	1.25	Up	0.031
UDP galactosyltransferase 8	1.30	Down	0.042
Retinoblastoma binding protein 4 (RGD1563620)*	1.24	Up	0.046

rTMS, repetitive transcranial magnetic stimulation; FC, fold change.
*Predicted.

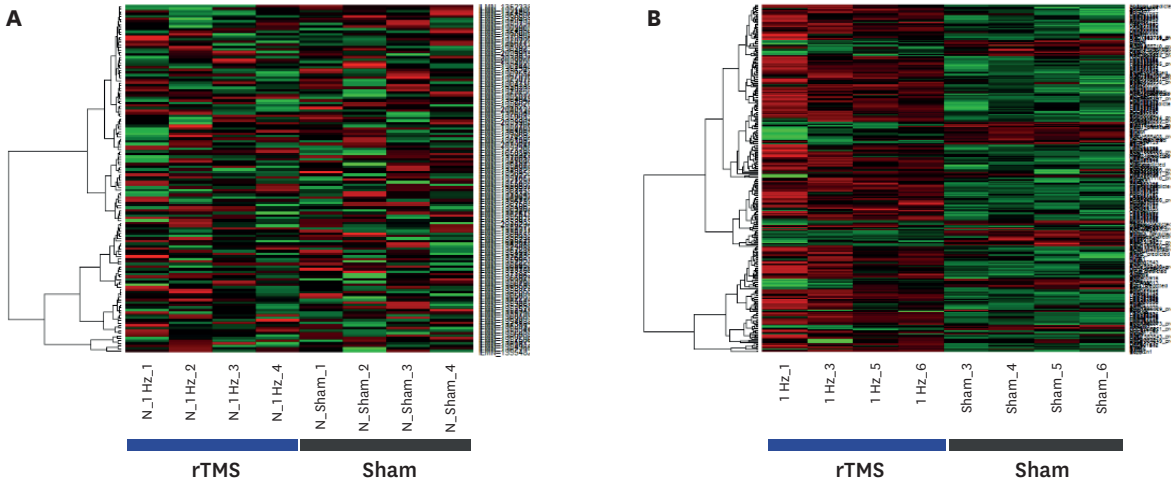

Fig. 2. Results of cluster analysis. (A) Experiment I did not show a clear distinction between groups. (B) Experiment II showed obvious differences in the gene expressions in the groups.
rTMS, repetitive transcranial magnetic stimulation.

Overrepresented GO categories of differentially expressed genes

To classify the observed rTMS-induced regulation of gene expression in the cerebral cortex, we performed GO enrichment analysis using the hyperGTest function in the GOstats R package to test whether selected functional categories were overrepresented (enriched) in our dataset.

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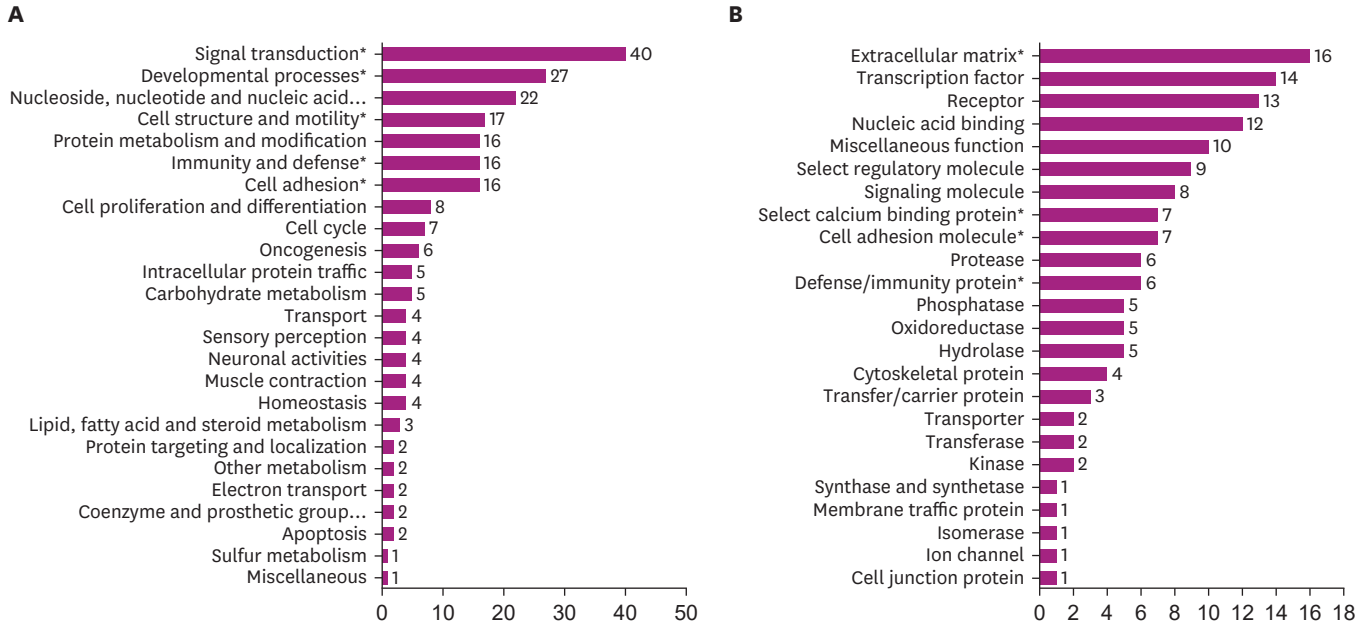

Fig. 3. The results of gene set enrichment analysis of biological processes (A) and molecular functions (B) based on all the genes with |fold change| of ≥ 1.2. The asterisk designates significant enrichment.

For the changes observed 5 minutes after a single session of rTMS, GSEA of the biological process revealed a significant enrichment of genes related to signal transduction ($p < 0.01$), developmental processes ($p < 0.01$), cell structure and mobility ($p < 0.01$), immunity and defense ($p = 0.03$), and cell adhesion ($p < 0.01$; **Fig. 3A**). Analysis of molecular function showed enrichment of genes responsible for the extracellular matrix ($p < 0.01$), calcium-binding protein ($p = 0.02$), cell adhesion molecule ($p=0.02$), and defense/immunity protein ($p = 0.02$; **Fig. 3B**).

From the list of differentially expressed genes 1 h after 5 rTMS sessions, as many as 78 significantly overrepresented GO terms were identified with p values of < 0.001 (**Supplementary Table 2**). The overrepresented gene ontology categories included positive regulation of axon extension, axonogenesis, intracellular transport, and synaptic plasticity.

Microarray analysis reveals differentially expressed miRNAs in the rat cortex after real or sham rTMS

To study rTMS-induced changes in miRNA expression in the stimulated cerebral cortex, miRNA expression profiling was performed using microarrays. Both experiments I and II showed no significant difference in miRNA expression after rTMS. The 25 and 44 miRNAs with |fold change| of > 1.2 in experiments I and II are summarized in **Tables 2** and **3**, respectively.

Integrative analysis of mRNA-miRNA pairs

Among the a priori selected putative microRNA-mRNA target pairs, six and nine miRNAs showed significant hypergeometric test results in experiments I (**Table 4**) and II (**Table 5**), respectively.

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Table 2. A list of miRNAs with |FC| > 1.2 in the experiment I, ordered by fold change (largest to smallest)

Systematic name	p value	1 Hz/Sham FC	
rno-miR-672	0.28	2.57	Down
rno-miR-206	0.28	2.35	Down
rno-miR-133b	0.83	2.19	Down
rno-miR-539	0.28	2.17	Down
rno-miR-483	0.79	2.07	Up
rno-miR-674-5p	0.28	2.04	Down
rno-miR-674-3p	0.28	2.02	Down
rno-miR-466b	0.28	1.98	Down
rno-miR-484	0.79	1.93	Up
rno-miR-465	0.28	1.85	Down
rno-miR-188	0.66	1.75	Up
rno-miR-541	0.28	1.71	Down
rno-miR-490	0.79	1.56	Down
rno-miR-409-3p	0.28	1.35	Down
rno-miR-139-3p	0.61	1.32	Up
rno-miR-181d	0.82	1.30	Up
rno-miR-500	0.52	1.28	Down
rno-miR-409-5p	0.28	1.26	Down
rno-miR-873	0.61	1.25	Up
rno-miR-425	0.76	1.25	Up
rno-miR-30b-5p	0.79	1.24	Up
rno-miR-223	0.61	1.22	Down
rno-miR-148b-3p	0.79	1.22	Up
rno-miR-186	0.86	1.22	Up
rno-miR-874	0.61	1.20	Up

miRNA, microRNA; FC, fold change.

DISCUSSION

Using microarray mRNA expression profiling, we provided a comprehensive view of the genes induced in the adult rat brain by single or repetitive sessions of low-frequency rTMS. Differentially expressed genes after repeated sessions of low-frequency rTMS are involved in synaptic plasticity, positive regulation of axon extension, axonogenesis, and intracellular transport. We demonstrated that low-frequency rTMS induced significant molecular changes in the stimulated cortex of rat brain.

Several studies have explored the expression of selected genes after magnetic stimulation. An earlier study reported that low- and high-frequency rTMS induced the differential expression of immediate-early genes (c-fos and zif-268) [12]. GAD65 and GAD67 also showed different expression patterns according to the stimulation protocol [13]. Chronic rTMS induces the expressions of glutamate, GABA, and glycine transporters [14]. Our previous report showed increased expression of immediate early genes after a single session of rTMS and increased expression of BDNF after 5 days of rTMS.

In a microarray study of multiple rTMS protocols for 2 weeks using the middle cerebral artery occlusion model, angiogenetic genes (Bai1 and Vegfa), immediate-early genes (Fos, Jun, and JunB), modulatory genes for stress response (GADD45), and G protein-coupled receptor signaling genes (Arrb1, Adcy8, and BDNF) were mostly induced in the intermittent theta-burst protocol than the 1-Hz protocol [17]. This study found significant expressions of similar genes despite the 1-Hz protocol, and the differences may be attributed to the disease model animals, differences in total stimulation numbers (cited research vs. current study, 24,000 vs. 6,000), or the duration of the entire study (10 vs. 5 days).

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Table 3. A list of miRNAs with |FC| > 1.2 in the experiment II, ordered by fold change (largest to smallest)

Systematic name	p value	1 HZ/Sham FC	
rno-miR-2985	0.57	1.89	Up
rno-miR-139-3p	0.57	1.76	Down
rno-miR-466b-1*	0.57	1.67	Up
rno-miR-206	0.60	1.64	Down
rno-miR-878	0.57	1.61	Down
rno-miR-196c	0.57	1.60	Up
rno-miR-1949	0.57	1.50	Up
rno-miR-125a-3p	0.57	1.48	Down
rno-miR-409-3p	0.57	1.47	Up
rno-miR-222	0.57	1.41	Up
rno-miR-203	0.57	1.41	Down
rno-miR-191*	0.65	1.39	Down
rno-miR-342-5p	0.57	1.37	Down
rno-miR-145	0.57	1.36	Up
rno-miR-664	0.57	1.35	Up
rno-miR-290	0.57	1.35	Up
rno-miR-483	0.68	1.33	Up
rno-miR-652*	0.68	1.33	Up
rno-miR-743a	0.71	1.33	Down
rno-miR-144	0.58	1.32	Up
rno-miR-7a-2*	0.68	1.31	Up
rno-miR-135a	0.60	1.30	Up
rno-miR-344b-5p	0.60	1.29	Up
rno-miR-138	0.57	1.29	Up
rno-miR-138-2*	0.57	1.28	Up
rno-miR-34a*	0.57	1.28	Down
rno-miR-346	0.57	1.27	Up
rno-miR-208*	0.60	1.25	Down
rno-miR-3593-3p	0.60	1.25	Up
rno-miR-139-5p	0.57	1.25	Up
rno-miR-28	0.60	1.24	Down
rno-miR-344b-2-3p	0.57	1.24	Up
rno-miR-3085	0.60	1.24	Up
rno-miR-3562	0.68	1.24	Down
rno-miR-665	0.72	1.23	Up
rno-miR-92b	0.57	1.23	Up
rno-miR-219-2-3p	0.66	1.22	Down
rno-miR-330	0.57	1.22	Down
rno-miR-3584-5p	0.60	1.21	Down
rno-miR-3573-3p	0.57	1.21	Up
rno-miR-542-3p	0.57	1.21	Down
rno-miR-361*	0.57	1.21	Up
rno-miR-1188-3p	0.70	1.20	Up
rno-miR-18a	0.65	1.20	Down

miRNA, microRNA; FC, fold change.
*Star strand of the microRNA.

Table 4. A list of miRNAs showing significant negative relationship with putative mRNA targets in experiment I, ordered by p value (smallest to largest)

miRNA	Target size	Detected target size	NR	p value
rno-miR-409-5p	858	380	7	0.001
rno-miR-409-3p	814	335	5	0.009
rno-miR-541	608	248	4	0.014
rno-miR-206	645	273	4	0.019
rno-miR-674-5p	775	332	4	0.034
rno-miR-873	781	344	4	0.037

miRNA, microRNA; mRNA, messenger RNA; NR, number of negative relationship pairs between miRNAs and mRNAs.

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Table 5. A list of miRNAs showing significant negative relationship with putative mRNA targets in experiment II, ordered by p value (smallest to largest)

miRNA	Target size	Detected target size	NR	p value
rno-miR-139-3p	771	543	7	< 0.001
rno-miR-743a	753	481	6	< 0.001
rno-miR-203	649	406	5	0.002
rno-miR-290	850	552	5	0.008
rno-miR-878	890	582	5	0.009
rno-miR-206	645	396	4	0.012
rno-miR-28	659	446	4	0.017
rno-miR-18a	837	574	4	0.035
rno-miR-125a-3p	957	630	4	0.045

miRNA, microRNA; mRNA, messenger RNA; NR, number of negative relationship pairs between miRNAs and mRNAs.

There was a striking difference between the expressions of genes after the single and repeated sessions of rTMS (**Tables 1** and **2**). The immediate early genes (e.g., *Egr2*, *Jun-B*, and *Arc*) increased five minutes after a single session of rTMS. The immediate early genes are activated transiently and rapidly in response to various stimuli, and mediate diverse signaling pathways [18]. The mRNA level of immediate early genes typically rise within minutes after stimulation and reach its maximum at 30 minutes after challenge, and normalizes over several hours [19]. The time between the start of stimulation and the harvest in this study was 25 minutes, which was in accordance with the previously known optimal range of time. The result of single session low-frequency rTMS indicates that the rTMS could sufficiently stimulate the cortex of rat brain to induce neuronal activation. However, repetitive rTMS sessions primarily induced changes in the structural genes (*Metrn* and *Map1b*), which were associated with neurogenesis and brain development. This finding suggests that repeated sessions of rTMS can upregulate structural genes and have long-term effects on local neuronal circuits. The low-frequency rTMS is well-known to decrease cortical excitability in may human studies. The results of this study may indicate that the resultant increase of structural gene expression may be linked to the reinforcement of the local inhibitory neuronal network. Further studies are required to elucidate the therapeutic potential of rTMS in axonogenesis.

The early growth response 2 gene (*Egr2*), *krox-20*, produces a transcription factor with tandem C2H2-type zinc fingers. Defects in this gene are associated with severe peripheral nervous system disorders, such as Charcot-Marie-Tooth disease type 1D, Charcot-Marie-Tooth disease type 4E, and Dejerine-Sottas syndrome,[20] and it is believed to be important for the production and maintenance of myelin and neuronal protection. However, an association between acute induction of *Egr2* and long-term changes after rTMS has not been established.

The decreased concentrations of miR-206 may be significantly involved in the molecular mechanism of rTMS. The predicted targets of miR-206 in humans include more than 1,000 genes. BDNF is a target of miR-206 of particular interest. BDNF has various functions such as modulating dendritic branching and dendritic spine morphology, synaptic plasticity, and long-term potentiation. Increased concentrations of miR-206 have been reported to play roles in Alzheimer's disease [21] and depression [22] via the BDNF regulation. miR-206 may also regulate the expression of the Otx2 gene. Otx2 is the earliest homeobox-containing gene that is expressed in the neuroectoderm, plays a role in brain development, and downregulates apoptosis. MiR-206 downregulates Otx2 expression and contributes to apoptosis [23]. As the interaction of miR-206 with BDNF or Otx2 has a detrimental effect on the neural system, the decrease in the concentrations of miR-206 by rTMS may contribute to the beneficial effect of rTMS on the brain.

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The concentrations of miR-125a-3p also showed a negative correlation with mRNA concentrations. miR-125a-3p inhibits oligodendrocyte differentiation and is upregulated in patients with multiple sclerosis and Parkinson's disease [24]. The decrease in the miR-125a-3p concentrations in our study also explains the beneficial effect of rTMS.

Unfortunately, there is a paucity of studies on the function of miRNAs in *Rattus norvegicus*. rno-miR-206 is known to be abundant in the muscle and has also been reported to be present in the rat hippocampus and striatum. miR-206 delays the progression of amyotrophic lateral sclerosis in mice [25]. Future studies on the function of miRNAs in humans and other species are needed.

Several rTMS protocols are commonly used in clinical practice. Low-frequency (≤ 1 Hz) and high-frequency (> 5 Hz) rTMS have different effects on cortical excitability. Low-frequency stimulation leads to depression in the stimulated cerebral cortex via a long-term depression-like mechanism. The present study investigated molecular events induced by low-frequency rTMS. Low- and high-frequency rTMS may have different cellular effects; however, future studies are required to confirm this.

Positive regulation of axonal extension, axonogenesis, intracellular transport, and synaptic plasticity were among the overrepresented gene ontology categories following repeated sessions of low-frequency rTMS. Axonal spouting, regrowth and neurogenesis are known as major recovery mechanisms after stroke [26]. The use of rTMS in the early subacute phase after stroke may provide a favorable cellular environment for neural repair. Also, the up- or down- regulated genes indicate that structural changes can be induced by repeated low-frequency rTMS.

The present study had several limitations. First, we established a small animal model of rTMS that primarily affected the unilateral hemisphere but the stimulation was not as focal as rTMS in human research. The figure-of-eight coil with a 25 mm diameter is still too large for focused stimulation of the rat brain. Thus, any attempt to extrapolate the present results to clinical settings seems premature at present. However, the model presented here can provide insights into the molecular mechanisms of rTMS. Understanding how rTMS affects innate mechanisms for recovery after ischemic stroke can provide an important clue to uncover how and why rTMS is used for various conditions. Second limitation of the present study was the short duration after stimulation. The time between stimulation and harvest was only a hour, shorter than the general clinical situations. Third, there were no further validation of the microarray analysis, such as western blot assay or ELISA, to quantify the protein that is related to up-down regulation genes, although it could give more robust information. Finally, the stimulation intensity—50% of motor threshold—was lower than that of clinical studies, because the smaller magnetic coil adopted in our study was particularly vulnerable to overheating. This may have caused mechanistic differences between the present study and other clinical rTMS studies.

In conclusion, experiment I demonstrated that rTMS stimulates the underlying cortex. Experiment II suggested that genes linked to various biological processes (including axonogenesis) were enriched by repetitive sessions of low-frequency rTMS. The mRNA-miRNA integrative analysis showed that miR-206 and miR-125a-3p may play a mediating role in rTMS-induced plasticity. The putative roles of various miRNAs in rTMS-induced changes in cortical excitability should be validated by further research.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1

List of differentially expressed genes identified by microarray 1 hour after 5 sessions of rTMS, ordered by FC (largest to smallest)

Click here to view

Supplementary Table 2

The enriched gene ontology categories, 1 hour after 5 sessio7ns of rTMS, ordered by p value (smallest to largest)

Click here to view

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