Biochemical and Biophysical Research Communications 436 (2013) 121–127

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Multiple effects of repetitive transcranial magnetic stimulation on neuropsychiatric disorders

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ARTICLE INFO

Article history:
Received 22 February 2013
Available online 21 March 2013

Keywords:
rTMS
Period 2
HSP70
Dopamine receptor 2

ABSTRACT

Repetitive transcranial magnetic stimulation (rTMS) is a new tool that has been used for the treatment of patients with neuropsychiatric disorders. However, the mechanisms underlying the effects of rTMS are still unclear. We analyzed the changes in mRNA expression in mouse brain that occurred after rTMS with an Affymetrix GeneChip. Following 20 days of rTMS, many genes were differentially expressed in the mouse brain. Downregulation of Period 2 and 3 mRNA expression levels and a subsequent decrease in food and water intake were observed. HSP70 mRNA expression levels were upregulated after transient and chronic rTMS. In N2A 150Q cells, an upregulation of HSP70 mRNA and protein levels and subsequent cell-protective effects were observed after chronic rTMS. In addition, dopamine receptor 2 mRNA expression levels were downregulated, and a subsequent decrease in the binding of [^{3}H]raclopride was observed. These results indicated that the modulation of several genes may be involved in the therapeutic mechanisms of chronic rTMS for patients with neuropsychiatric disorders.

1. Introduction

Repetitive transcranial magnetic stimulation (rTMS) is a novel tool that is considered to induce electric currents in the brain through a coil [1–4]. Initially, Merton and Morton [5] showed that it is possible to stimulate the motor areas of the human brain electrically through the intact scalp (transcranial electrical stimulation). Barker and his group later showed that it was possible to stimulate both nerves and the brain with external magnetic stimulation [1], which is a technique known as TMS. Recently, TMS has been used as a diagnostic tool in neurology for measuring central motor conduction because it is painless and noninvasive [6]. In contrast, electroconvulsive therapy provides highly reliable relief from depressive symptoms, but intense electrical stimulation is required because the skull resists electric currents [7]. The intensity of the stimulation that is usually used to treat patients induces a sustained after-discharge in cortical neurons, which causes convulsive seizures. After TMS was introduced, it was shown to have several therapeutic benefits in patients with neuropsychiatric disorders, such as depression, Parkinson's disease, and schizophrenia [8–10]. These neuropsychiatric disorders are considered to be associated with monoamine systems. Because of its safety and relative painlessness, TMS has many possible applications as a therapeutic device, and it may be beneficial in the treatment of some psychiatric disorders that have not yet been explored. If it ultimately proves useful for the treatment of neuropsychiatric disorders, TMS may well become a standard medical tool. The precise molecular mechanisms underlying the effects of TMS are unknown. Recent studies have measured monoamine release with microdialysis and imaging with raclopride after acute rTMS [11,12]. Recently, we described modulations in monoamine transporters following either acute or chronic rTMS [13]. However, there have been few reports of expression profiles following either acute or chronic rTMS. This prompted us to investigate the molecular effects of TMS in rodents by evaluating the expression of mouse genes after treatment with TMS. We found that the expression levels of dopamine receptor 2, HSP70, and several circadian rhythm-related genes were altered. And, a subsequent decrease in food and water intake were observed. In addition, our study showed that the binding of [^{3}H]raclopride was decreased after rTMS, suggesting that rTMS modulates D2 receptor gene expression and function, and it may be useful in the treatment of several neuropsychiatric disorders.

2. Materials and methods

2.1. Mice and conditions of rTMS

Male C57Black R6 mice (8 weeks old, 20–25 g) were chronically treated for 20 days (n = 42) or acutely for 1 day (n = 24) with rTMS. Mice were housed in a light-controlled room (8:00 a.m. on, 8:00 p.m. off). Stimulation was performed using a round-coil (7.5 cm

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0006-291X/$ - see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.bbrc.2013.03.017

outer diameter) and a Nihon Kohden Rapid Rate Stimulator (Nihon Kohden, Japan). Stimulation conditions were as follows: 20 Hz, 2 s; 20 times/day; inter-stimulus interval 1 min (30% machine output, representing about 0.75 T). The coil was placed over the head without touching the skull. Sham control mice were stimulated from a distance of more than 10 cm from the head. rTMS did not produce either notable seizures or changes in behavior, such as excessive struggling. Twenty-four hours after the last stimulation, the animals were sacrificed and their brains were processed for further analysis.

2.2. RNA extraction

Whole mouse brain was divided at the midbrain into cerebrum and cerebellum with brain stem (CBS). Total RNA was isolated from cerebrum and CBS by acid–phenol extraction [14]. Poly(A)+ RNA was isolated from the samples using an mRNA purification kit (TaKaRa Bio, Japan). Expression analysis by TaqMan real-time RT-PCR. We synthesized the TaqMan primer and probe sets with Primer Express Software (Applied Biosystems, Foster City, CA). The nucleotide sequence of the primers is shown in Supplementary data 3. Contaminating genomic DNA was removed with RNase-free DNase I (TaKaRa Bio, Japan). Complementary DNAs were synthesized using MMLV Reverse Transcription Reagents (Invitrogen, Carlsbad, CA). We used 1 μg of mRNA for the 100 μl reaction. The TaqMan PCR was performed as follows. We used 15 μl of TaqMan Universal PCR Master Mix (Applied Biosystems) in a 30 μl reaction. Primers and probes in optimal concentrations were added. We used 1 μl of RT mix for each PCR. Each sample was amplified in duplicate and the experiment was repeated at least three times. PCR conditions were standard for the 7700 Sequence Detector System (Applied Biosystems): 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. The mRNA quantity for the gene of interest was normalized by the quantity of GAPDH in each sample.

2.3. Ligand binding assay for dopamine receptor 2

Synaptosomes were prepared as previously described [15], using ice-cold buffer (50 mMTris–HCl, 120 mM NaCl, and 5 mM KCl) and incubated for 4 h at 4 °C with [3H]raclopride (3063.6 Gbq/mmol Perkin–Elmer), in binding buffer (50 mM Tris–HCl, 300 mM NaCl, and 5 mM KCl) containing 10 μM 7-OH-DPAT (Sigma) and 10 μM PD168077 (Sigma). Synaptosomes were washed four times rapidly with ice-cold buffer using filters (Whatman GF/B) for removal of excess radioligands, and any radioactivity remaining in the filters was measured by liquid scintillation spectrometry. Nonspecific uptake was determined in the presence of 10 μM raclopride (Sigma).

2.4. Cell culture

Neuro2a cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) at 37 °C under 5% CO2/95% air. Cells at subconfluence were harvested, diluted in culture medium, and plated in a 24-well culture plate for the uptake assay or in a 25 cm^2 culture flask for total RNA extraction. The cells were cultured with or without daily stimulation of chronic rTMS. And then, in the Neuro2a cellular model of Huntington disease [16], in which tNhtt-EGFP expression is induced and cells are

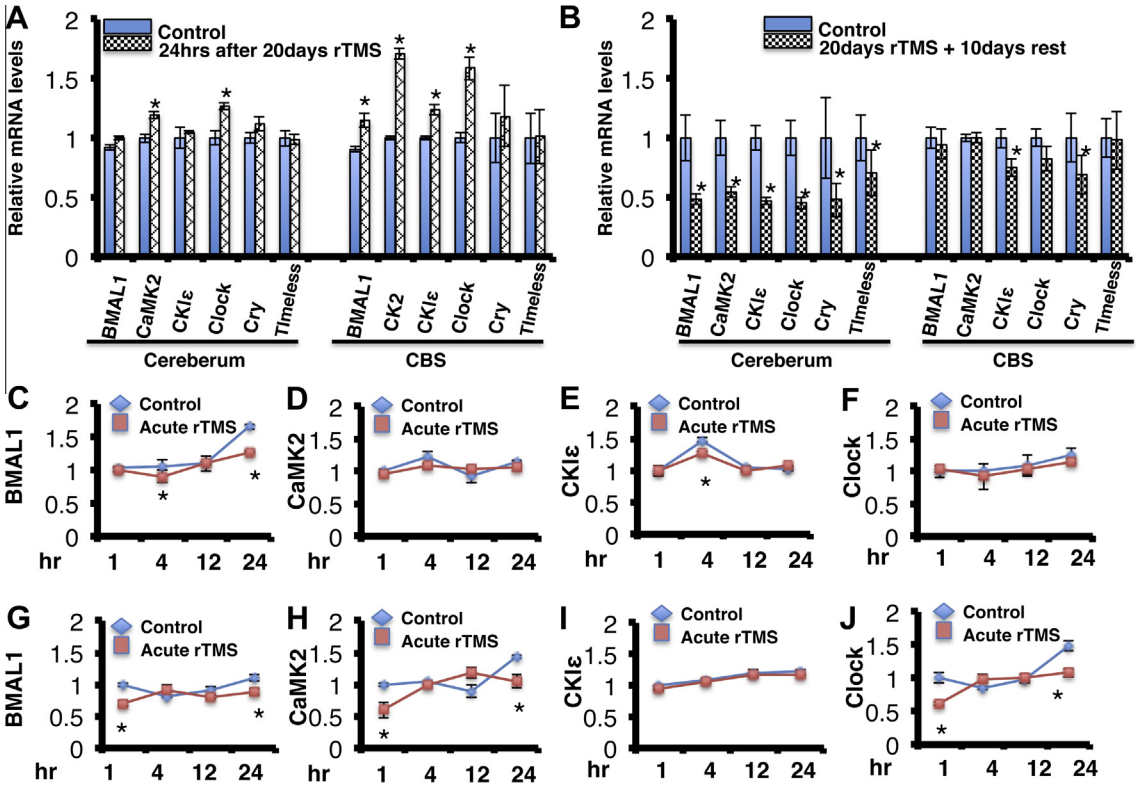

Fig. 1. Effects of rTMS on circadian rhythm related genes expression. (A, B) Effects of rTMS on circadian rhythm related genes mRNA levels. Mouse brains were treated by rTMS. The mRNA level was determined at 24 h and 10 days after 20 days rTMS stimulation. (C–F) Effects of acute rTMS on circadian rhythm related genes mRNA levels. Measurement were done at 1, 4, 12, 24 h after acute stimulation on cereberum. (G–J) Effects of acute rTMS on circadian rhythm related genes mRNA levels. Measurement were done at 1, 4, 12, 24 h after acute stimulation on CBS. mRNA level was compensated by GAPDH. Values represent means ± SEM of five experiments each performed in triplicate. *Significantly different from control at $P < 0.05$.

differentiated. Viability of tNhtt-150Q–EGFP cells was evaluated by MTT assay [16].

2.5. DNA chip profiling

We carried out comprehensive analysis of the altered gene expression in the cerebrum and CBS stimulated by chronic rTMS using a high-density oligonucleotide array (GeneChip; Affymetrix, Santa Clara, CA, USA), which has been described elsewhere [17]. Using the Affymetrix algorithm [18] and multiple analysis comparison software for assessing gene expression differences, mRNAs that were increased or decreased in the mouse brain with chronic rTMS stimulation relative to levels in control mouse brain were identified.

2.6. Western blotting and immunocytochemistry

Cells were washed with phosphate-buffered saline and treated with Laemmli sample buffer. Western blotting and immunocytochemistry were performed as described previously [16]. Mouse monoclonal anti-Hsp70 (SPA-810) were purchased from StressGen Biotechnologies (Victoria, British Columbia).

2.7. Data analysis

The data were represented as means ± SE of at least three independent experiments, each performed in triplicate or duplicate. Statistical analysis was performed using ANOVA (Fig. 1) and Student's *t*-test as appropriate.

3. Results

3.1. Changes in the expression of genes in the mouse brain after rTMS

We stimulated the brains of 8-week-old C57 Black mice for 20 days with rTMS and analyzed the changes in the expression of genes in mice brains that were stimulated by acute and chronic rTMS with an Affymetrix GeneChip microarray, and we found that the mRNA of many genes, including HSP70, dopamine receptor 2, and circadian-related genes, were altered in the cerebrum and CBS (Supplementary data 1 and 2). In order to evaluate the effects of rTMS on these genes, we measured the mRNA levels of several genes with RT-PCR (Tables 1–3).

3.2. Effects of acute and chronic rTMS on circadian rhythm-related genes

The GeneChip data showed that Period 2 mRNA levels were decreased after rTMS. Thus, we examined the mRNA levels of circadian-related genes at 24 h after 20 days of rTMS and after 10 days of rest after 20 days of rTMS. Twenty-four hours after 20 days of rTMS, Period 3, BMAL1, Calmodulin kinase II (CaMK2), casein kinase 1ε (CK-1ε), and Clock mRNA levels were increased in CBS, and CaMK2 and Clock mRNA levels were increased in the cerebrum (Table 2, Fig. 1A). However, Period 2 mRNA levels were

Table 1
Summary of gene expression profile by rTMS stimulation.

			20 days + 24 days	20 days + 10 days	1 h	4 h	12 h	24 h
RhoG	Cereberum	Mg+	0.705 ± 0.057[a]	1.094 ± 0.044	1.053 ± 0.47	0.721 ± 0.183[a]	0.903 ± 0.309	1.2 ± 0.129[a]
		Control	1.05 ± 0.059	1.137 ± 0.056	0.938 ± 0.418	0.89 ± 0.225	1.005 ± 0.139	1.0 ± 0.16
	CBS	Mg+	0.929 ± 0.015	0.824 ± 0.087[a]	0.913 ± 0.185	0.859 ± 0.198[a]	1.159 ± 0.285	0.83 ± 0.096
		Control	1.0 ± 0.08	1.0 ± 0.033	1.005 ± 0.231	1.3 ± 0.321	1.26 ± 0.309	0.87 ± 0.23
Periodl	Cereberum	Mg+	0.979 ± 0.014	0.848 ± 0.029[a]	1.927 ± 0.071[a]	1.429 ± 0.065[a]	1.581 ± 0.015	1.972 ± 0.141
		Control	1.002 ± 0.05	1.00 ± 0.013	0.992 ± 0.011	2.081 ± 0.113	1.511 ± 0.026	2.128 ± 0.051
	CBS	Mg+	1.254 ± 0.052	0.712 ± 0.027[a]	1.761 ± 0.036[a]	1.356 ± 0.011	2.432 ± 0.078	2.552 ± 0.134[a]
		Control	1.00 ± 0.018	1.00 ± 0.012	1.0 ± 0.024	1.465 ± 0.022	2.184 ± 0.041	1.875 ± 0.046
Period2	Cereberum	Mg+	0.601 ± 0.022[a]	0.764 ± 0.162[a]	1.176 ± 0.84	0.429 ± 0.159[a]	0.748 ± 0.325	0.796 ± 0.268
		Control	1.0 ± 0.056	1.0 ± 0.238	1.0 ± 0.937	0.688 ± 0.231	0.791 ± 0.374	0.756 ± 0.062
	CBS	Mg+	0.411 ± 0.128[a]	1.107 ± 0.27	1.08 ± 0.103	0.635 ± 0.074	1.457 ± 0.084	0.919 ± 0.055
		Control	1.0 ± 0.124	1.0 ± 0.062	1.08 ± 0.077	0.61 ± 0.094	1 457 ± 0.084	0.955 ± 0.05
Period3	Cereberum	Mg+	0.943 ± 0.012	0.929 ± 0.05	0.968 ± 0.071	1.295 ± 0.141	1.082 ± 0.035	1.347 ±0.12[a]
		Control	1.0 ± 0.047	1.0 ± 0.058	1.0 ± 0.071	1.494 ± 0.068	1.263 ± 0.127	0.985 ± 0.047
	CBS	Mg+	1.404 ± 0.026[a]	0.887 ± 0.096	0.875 ± 0.06	1.438 ± 0.047	1.396 ± 0.065	1.417 ± 0.032
		Control	1.0 ± 0.047	1.0 ± 0.0556	1.0 ± 0.062	1.300 ± 0.073	1.301 ± 0.089	1.429 ± 0.024
D-E-A-D	Cereberum	Mg+	0.913 ± 0.044	0.983 ± 0.137	1.545 ± 0.948	1.279 ± 0.228	1.457 ± 0.669	1.227 ± 0.176
		Control	1.0 ± 0.07	1.0 ± 0.203	1.0 ± 0.305	0.869 ± 0.258	1.112 ± 0.529	1.211 ± 0.28
	CBS	Mg+	1.519 ± 0.101[a]	1.096 ± 0.184	1.061 ± 0.125	1.300 ± 0.171[a]	1.1182 ± 0.136[a]	1.086 ± 0.061[a]
		Control	1.0 ± 0.04	1.0 ± 0.238	1.0 ± 0.255	2.255 ± 0.438	1.607 ± 0.223	1.244 ± 0.045
Dr-1	Cereberum	Mg+	0.689 ± 0.046[a]	1.066 ± 0.196	0.627 ± 0.241[a]	0.542 ± 0.061	0.693 ± 0.046	0.661 ± 0.171
		Control	1.0 ± 0.054	1.0 ± 0.144	1.0 ± 0.573	0.497 ± 0.054	0.594 ± 0.091	0.542 ± 0.187
	CBS	Mg+	0.992 ± 0.057	1.012 ± 0.116	1.023 ± 0.437	0.865 ± 0.159	0.991 ± 0.267	0.703 ± 0.192
		Control	1.0 ± 0.061	1.0 ± 0.148	1.0 ± 0.213	1.126 ± 0.664	0.964 ± 0.423	0.771 ± 0.237
IGF1 receptor	Cereberum	Mg+	0.819 ± 0.044	0.598 ± 0.069[a]	0.997 ± 0.023	1.028 ± 0.106	1.202 ± 0.075	1.143 ± 0.069
		Control	1.0 ± 0.121	1.0 ± 0.131	1.0 ± 0.041	1.191 ± 0.076	1.271 ± 0.092	1.198 ± 0.07
	CBS	Mg+	1.122 ± 0.216	0.744 ± 0.065	1.039 ± 0.007	0.927 ± 0.068	1.616 ± 0.122	2.050 ± 0.08
		Control	1.0 ± 0.145	1.0 ± 0.095	1.0 ± 0.05	1.046 ± 0.026	1.721 ± 0.097	2.186± 0.15
Cry1	Cereberum	Mg+			1.145 ± 0.08[a]	1.439 ± 0.166	1.182 ± 0.05 [a]	1.340 ± 0.141
		Control			1.0 ± 0.051	1.621 ± 0.11	1.367 ± 0.15	1.425 ± 0.244
	CBS	Mg+			1.103 ± 0.176	1.528 ± 0.532	1.56 ± 0.606*	2.029 ± 0.403 *
		Control			1.0 ± 0.293	1.293 ± 0.172	2.058 ± 0.094	1.663 ± 0.226
Timeless	Cereberum	Mg+			0.845 ± 0.04[a]	0.947 ± 0.104	1.007 ± 0.096	1.122 ± 0.134
		Control			1.0 ± 0.077	1.035 ± 0.006	1.093 ± 0.094	1.039 ± 0.134
	CBS	Mg+			0.613 ± 0.194[a]	0.859 ± 0.18 [a]	1.170 ± 0.273	1.151 ± 0.208
		Control			1.0 ± 0.274	1.039 ± 0.059	1.218 ± 0.252	1.310 ± 0.417

Values represent means ± SEM of three experiments each performed in triplicate.
[a] Significantly different from control at $P < 0.05$.

Table 2
Summary of gene expression profile by rTMS stimulation.

			20 days + 24 days	20 days + 10 days	1 h	4 h	12 h	24 h
AMPA	Cereberum	Mg+	0.926 + 0.128	1.028 + 0.244	0.546 + 0.081[a]	0.683 + 0.098[a]	0.982 + 0.156	0.653 + 0.149
		Control	1.0 + 0.051	1.0 + 0.162	1.0 + 0.4	1.634 + 0.523	0.987 + 0.21	0.656 + 0.23
	CBS	Mg+	1.459 + 0.083[a]	1.151 + 0.104[a]	0.071 + 0.06[a]	0.198 + 0.391[a]	0.571 + 1.574[a]	0.210 + 0.388[a]
		Control	1.0 + 0.089	1.0 + 0.08	1.0 + 2.024	0.963 + 1.653	0.295 + 0.733	0.135 + 0.264
NMDA1	Cereberum	Mg+	0.892 + 0.109	0.911 + 0.286	1.176 + 0.84	0.429 + 0.159[a]	0.748 + 0.325	0.796 + 0.268
		Control	1.008 + 0.038	1.0 + 0.115	1.0 + 0.937	0.689 + 0.231	0.791 + 0.374	0.756 + 0.062
	CBS	Mg+	0.816 + 0.119	1.4 + 0.259[a]	1.063 + 0.017[a]	0.954 + 0.122	1.075 + 0.027	0.834 + 0.02 [a]
		Control	1.0 + 0.016	1.095 + 0.152	1.0 + 0.036	1.134 + 0.183	1.119 + 0.058	0.995 + 0.057
5HT 1b	Cereberum	Mg+	1.337 + 0.008[a]	1.39 + 0.071[a]	1.150 + 0.047	1.408 + 0.197	1.203 + 0.048	1.136 + 0.013
		Control	1.0 + 0.016	1.057 + 0.057	1.0 + 0.008	1.667 + 0.117	1.135 + 0.084	1.173 + 0.043
	CBS	Mg+	1.836 + 0.051[a]	0.861 + 0.123	0.552 + 0.055	0.933 + 0.173	1.116 + 0.082	1.110 + 0.176
		Control	1.0 + 0.016	1.0 + 0.076	1.0 + 0.33	0.734 + 0.068	0.992 + 0.267	1.124 + 0.072
5HT4	Cereberum	Mg+	1.19 + 0.014[a]	1.240 + 0.101[a]	0.969 + 0.023	1.272 + 0.112	1.074 + 0.037	1.380 + 0.055[a]
		Control	1.0 + 0.025	1.0 + 0.141	1.0 + 0.032	1.345 + 0.093	1.116 + 0.04	1.169 + 0.025
	CBS	Mg+	1.886 + 0.01[a]	0.923 + 0.044	0.841 + 0.068	0.94 + 0.069[a]	0.69 + 0.087	0.824 + 0.172
		Control	1.0 + 0.005	1.0 + 0.007	1.006 + 0.214	0.614 + 0.022	0.714 + 0.11	0.694 + 0.05
5HT7	Cereberum	Mg+	1.240 + 0.03	1.136 + 0.035[a]	0.905 + 0.024[a]	1.204 + 0.058	0.911 + 0.015	1.0 + 0.009
		Control	1.0 + 0.016	1.0 + 0.017	1.0 + 0.014	1.334 + 0.075	1.036 + 0.042	1.039 + 0.043
	CBS	Mg+	1.682 + 0.01[a]	0.986 + 0.029	0.972 + 0.047	0.947 + 0.032[a]	1.036 + 0.013	1.054 + 0.066
		Control	1.0 + 0.022	1.0 + 0.051	1.0 + 0.036	1.049 + 0.017	1.082 + 0.067	1.042 + 0.041
Ab3	Cereberum	Mg+	0.936 + 0.039	0.937 + 0.065	0.817 + 0.028[a]	1.159 + 0.042	0.777 + 0.023	0.884 + 0.032[a]
		Control	1.0 + 0.018	1.0 + 0.05	1.0 + 0.038	1.201 + 0.061	0.883 + 0.036	0.587 + 0.028
	CBS	Mg+	0.978 + 0.004	0.877 + 0.033	1.129 + 0.043	0.965 + 0.004[a]	0.948 + 0.025[a]	0.597 + 0.025
		Control	1.0 + 0.005	1.0 + 0.046	1.0 + 0.031	0.908 + 0.004	0.658 + 0.017	0.591 + 0.031
Ca channel	Cereberum	Mg+	1.032 + 0.007	1.025 + 0.014	1.006 + 0.03	1.298 + 0.066[a]	1.068 + 0.008[a]	1.232 + 0.028
		Control	1.0 + 0.029	1.0 + 0.023	1 + 0.015	1.636 + 0.02	1.183 + 0.014	1.133 + 0.028
	CBS	Mg+	0.689 + 0.01[a]	0.990 + 0.047	0.891 + 0.003[a]	1.015 + 0.019[a]	1.386 + 0.022[a]	1.448 + 0.02
		Control	1.0 + 0.027	1.0 + 0.019	1.0 + 0.011	1.242 + 0.03	1.173 + 0.013	1.386 + 0.039
Cl channel	Cereberum	Mg+	987 + 0.023	1.184 + 0.147	0.803 + 0.03	0.960 + 0.048	0.799 + 0.005[a]	1.041 + 0.003
		Control	1.0 + 0.018	1.0 + 0.14	1.0 + 0.039	0.927 + 0.036	0.91 + 0.013	1.031 + 0.04
	CBS	Mg+	1.238 + 0.014[a]	0.936 + 0.14	0.387 + 0.155[a]	0.628 + 0.087	0.697 + 0.157	0.705 + 0.022[a]
		Control	1.0 + 0.031	1.0 + 0.058	1.0 + 0.521	0.570 + 0.066	0.890 + 0.344	0.843 + 0.027
D2	Cereberum	Mg+	0.565 + 0.01	1.273 + 0.066	1.011 + 0.016	1.276 + 0.029	1.087 + 0.008	1.188 + 0.013
		Control	1.0 + 0.009	1.0 + 0.071	1.0 + 0.003	1.423 + 0.041	1.151 + 0.014	1.236 + 0.031
	CBS	Mg+	0.772 + 0.01[a]	0.763 + 0.016[a]	0.688 + 0.012	0.728 + 0.01	1.010 + 0.027	0.915 + 0.012
		Control	1.0 + 0.002	1.0 + 0.009	1.0 + 0.066	0.640 + 0.007	0.816 + 0.028	0.783 + 0.018
D4	Cereberum	Mg+	1.198 + 0.031	1.278 + 0.057	0.651 + 0.089[a]	0.963 + 0.023[a]	0.454 + 0.015[a]	0.644 + 0.02
		Control	1.0 + 0.023	1.0 + 0.071	1.0 + 0.05	0.687 + 0.037	0.648 + 0.058	0.706 + 0.061
	CBS	Mg+	0.924 + 0.036	0.684 + 0.206[a]	0.845 + 0.09	0.914 + 0.129[a]	1.190 + 0.168	0.895 + 0.202[a]
		Control	1.0 + 0.005	1.0 + 0.165	1.0 + 0.376	0.579 + 0.09	1.254 + 0.316	0.468 + 0.08
GABA A3	Cereberum	Mg+	1.11 + 0.012	3.22 + 0.235[a]	0.901 + 0.037	1.240 + 0.226	0.972 + 0.055	1.20 + 0.079[a]
		Control	1.047 + 0.064	1.0 + 0.016	1.0 + 0.081	1.2 + 0.108	0.972 + 0.045	0.998 + 0.124
	CBS	Mg+	1.314 + 0.015[a]	1.456 + 0.278	0697 + 0.017	0.955 + 0.053[a]	1.098 + 0.026	0.896 + 0.049
		Control	1.0 + 0.009	1.0 + 0.266	1.0 + 0.116	0.684 + 0.008	0.992 + 0.069	0.857 + 0.022

Values represent means ± SEM of three experiments each performed in triplicate.
[a] Significantly different from control at $P < 0.05$.

decreased in the cerebrum and CBS (Table 2). After 10 days of rest after 20 days of rTMS treatment, Period 1, Period 2, BMAL1, CaMK2, CKIε, Clock, Cry1, and Timeless mRNA levels were decreased in the cerebrum, and Period 1, CK1ε, and Cry mRNA levels were decreased in CBS. There were no changes in Period 3 mRNA levels (Table 2, Fig. 1B). Next, we examined the acute effects of rTMS at 1, 4, 12, and 24 h. After acute rTMS, BMAL1 mRNA levels were decreased in the cerebrum at 4 and 24 h after acute rTMS (Fig. 1C) and in CBS at 1 and 24 h after acute rTMS (Fig. 1D). After acute rTMS, CaMK2 mRNA levels did not change in the cerebrum (Fig. 1D), but they were decreased after 1 and 24 h in CBS (Fig. 1H). CKIε mRNA levels were decreased in the cerebrum at 4 h after acute rTMS, (Fig. 1E) but they were not changed in CBS (Fig. 1I). Clock mRNA levels were not changed after acute rTMS in the cerebrum (Fig. 1F), but they were decreased in CBS at 1 and 24 h (Fig. 1J). Cry1 mRNA levels were upregulated at 1 h and decreased at 12 h in the cerebrum (Table 2); however, in CBS (Table 2), Cry1 mRNA levels were decreased at 12 h and upregulated at 24 h. Timeless mRNA levels were decreased 1 h after acute rTMS in the cerebrum (Table 2), and they were decreased at 1 and 4 h after acute rTMS in CBS (Table 2).

These findings suggested that the changes induced by rTMS in the mRNA expression patterns of the circadian rhythm-related genes may have some effects on neuropsychiatric disorders.

3.3. Effects of rTMS on water and food intake

Chronic rTMS changed the mRNA levels of the circadian rhythm-related genes. Thus, we examined whether chronic rTMS altered food and water intake. There were no changes in water and food intake at night (8:00 PM–8:00 AM) (Fig. 2A and B). However, in the daytime (8:00 AM–8:00 PM), rTMS inhibited water and food intake on days 14–20 and 15–20 (Fig. 2C and D).

3.4. Effects of acute and chronic rTMS on HSP70 induction

From the GeneChip data, we found that Heat Shock protein-related genes were altered (Supplementary data 1 and 2). Thus, we examined the effects of acute and chronic rTMS on HSP70 induction. The effects of rTMS on HSP70 induction were examined with tNhtt-150Q-EGFP cells and in the cerebrum and CBS. HSP70 mRNA and protein levels were increased after 5, 10, and 20 days of rTMS

Table 3
Summary of gene expression profile by rTMS stimulation.

			20 davs + 24 davs	20 davs + 10 davs	1 h	4 h	12 h	24 h
GABAb2	Cereberum	Mg+	1.203 + 0.036	1.20 + 0.064[a]	0.980 + 0.046	1.079 + 0.256	0.779 + 0.05[a]	1.082 + 0.085
		Control	1.0 + 0.107	1.0 + 0.037	1.0 + 0.17	0.891 + 0.088	0.864 + 0.046	1.044 + 0.232
	CBS	Mg+	1.618 + 0.034	1.167 + 0.108	0.452 + 0.017[a]	1.077 + 0.066	1.206 + 0.086	1.336 + 0.191[a]
		Control	1.0 + 0.051	1.0 + 0.057	1.0 + 0.176	1.075 + 0.092	1.210 + 0.014	1.921 + 0.163
NA channelbl	Cereberum	Mg+	0.951 + 0.019	1.074 + 0.032	0.870 + 0.014[a]	1.070 + 0.052	0.906 + 0.028	1.10 + 0.02[a]
		Control	1.0 + 0.026	1.0 + 0.043	1.0 + 0.008	1.230 + 0.028	1.004 + 0.035	0.864 + 0.002
	CBS	Mg+	1.022 + 0.03	1.025 + 0.065	0.816 + 0.05[a]	0.852 + 0.011[a]	1.077 + 0.019	1.043 + 0.085[a]
		Control	1.0 + 0.034	1.0 + 0.035	1.0 + 0.07	1.011 + 0.073	1.090 + 0.099	1.217 + 0.083
Opioid kappa	Cereberum	Mg+	0.961 + 0.008	1.044 + 0.047	0.936 + 0.012	1.141 + 0.04	0.923 + 0.008	1.014 + 0.013
		Control	1.0 + 0.031	1.0 + 0.021	1.0 + 0.008	1.048 + 0.017	1.002 + 0.018	1.021 + 0.034
	CBS	Mg+	1.5 + 0.005 [a]	0.969 + 0.014	0.633 + 0.007	0.792 + 0.022	0.873 + 0.025	0.708 + 0.022[a]
		Control	1.0 + 0.002	1.0 + 0.009	1.0 + 0.055	0.845 + 0.011	0.863 + 0.02	1.091 + 0.042
TSH	Cereberum	Mg+	0.816 + 0.015	1.191 + 0.221	0.943 + 0.067	1.240 + 0.046	0.690 + 0.021[a]	1.01 + 0.053
		Control	1.0 + 0.068	1.0 + 0.369	1.0 + 0.055	1.272 + 0.167	0.953 + 0.024	1.125 + 0.063
	CBS	Mg+	1.093 + 0.017	0.753 + 0.32	0.491 + 0.284[a]	0.938 + 0.449	0.518 + 0.171	0.637 + 0.443
		Control	1.0 + 0.021	1.0 + 0.245	1.0 + 0.192	0.631 + 0.157	0.535 + 0.389	0.518 + 0.332
HSP70	Cereberum	Mg+	0.852 + 0.076	0.987 + 0.041	1.23 + 0.248[a]	0.468 + 0.074	0.876 + 0.641	0.524 + 0.061
		Control	1.053 + 0.101	1.0 + 0.06	1.0 + 0 .201	0.656 + 0.322	0.676 + 0.073	0.484 + 0.091
	CBS	Mg+	0.765 + 0.037	1.099 + 0.076	1.951 + 0.322[a]	0.692 + 0.114[a]	1.027 + 0.069	0.585 + 0.062
		Control	1.0 + 0.136	1.0 + 0.052	1.0 + 0.106	1.295 + 0.592	1.124 + 0.223	0.691 + 0.146
HAP1	Cereberum	Mg+	0.940 + 0.055	0.950 + 0.08	1.047 + 0.408	0.680 + 0.09	0.819 + 0.218	0.92 + 0.056[a]
		Control	1.0 + 0.039	1.0 + 0.121	1.0 + 0.638	0.679 + 0.189	0.869 + 0.117	0.713 + 0.192
	CBS	Mg+	0.813 + 0.085	1.421 + 0.329	0.979 + 0.064	1.105 + 0.101[a]	1.097 + 0.127	1.023 + 0.003[a]
		Control	1.0 + 0.055	1.0 + 0.041	1.0 + 0.095	1.6 + 0.21	1.323 + 0.1	1.164 + 0.013
Tau	Cereberum	Mg+	0.759 + 0.083	1.029 + 0.147	0.91 + 0.637	0.574 + 0.208	0.764 + 0.47	0.694 + 0.146
		Control	1.0 + 0.026	1.0 + 0.113	1.0 + 0.995	0.850 + 0.3	0.832 + 0.138	0.589 + 0.139
	CBS	Mg+	1 330 + 0 087[a]	0.914 + 0.17	0.975 + 0.261	0.324 + 0.09[a]	0.408 + 0.289	0.291 + 0.056[a]
		Control	1.0 + 0.037	1.0 + 0.196	1.0 + 0.115	0.679 + 0.492	0.501 + 0.265	0.337 + 0.101
CUGBP	Cereberum	Mg+	0.861 + 0.061	0.906 + 0.078	1.062 + 0.74	0.641 + 0.185	0.637 + 0.154[a]	0.848 + 0.134[a]
		Control	1.0 + 0.022	1.0 + 0.134	1.0 + 0.971	0.697 + 0.229	0.819 + 0.07	0.631 + 0.184
	CBS	Mg+	1.258 + 0.08[a]	0.985 + 0.078	0.678 + 0.142[a]	0.837 + 0.169[a]	1.021 + 0.182	0.824 + 0.115
		Control	1.0 + 0.089	1.0 + 0.15	1.0 + 0.124	1.633 + 0.469	1.123 + 0.349	0.723 + 0.182
Caspase3	Cereberum	Mg+	0.731 + 0.037[a]	0.898 + 0.027	1.032 + 0.552	0.677 + 0.163	0.889 + 0.294	0.937 + 0.129[a]
		Control	1.0 + 0.088	1.0 + 0.06	1.0 + 0.649	0.696 + 0.064	0.832 + 0.099	0.721 + 0.179
	CBS	Mg+	1.022 + 0.055	0.985 + 0.028	0.889 + 0.213	0.997 + 0.177[a]	1.009 + 0.219[a]	0.989 + 0.181
		Control	1.0 + 0.063	1.0 + 0.057	1.0 + 0.209	1.285 + 0.39	1.299 + 0.326	1.055 + 0.137
SKD3	Cereberum	Mg+	0.938 + 0.019	1.081 + 0.063[a]	0.839 + 0.035[a]	1.126 + 0.08[a]	1.173 + 0.044	1.351 + 0.094
		Control	1.0 + 0.074	1.0 + 0.048	1.0 + 0.041	1.337 + 0.022	1.181 + 0.084	1.318 + 0.079
	CBS	Mg+	1.271 + 0.046[a]	1.126 + 0.058	0.665 + 0.028[a]	1.038 + 0.02	1.328 + 0.061	1.125 + 0.002[a]
		Control	1.0 + 0.044	1.0 + 0.024	1.0 + 0.057	1.195 + 0.115	1.217 + 0.05	1.503 + 0.117
TransG	Cereberum	Mg+	0.970 + 0.009	1.040 + 0.037	1.274 + 0.064[a]	0.950 + 0.009	0.862 + 0.064	0.924 + 0.04[a]
		Control	1.0 + 0.018	1.0 + 0.021	1.0 + 0.041	0.933 + 0.059	0.765 + 0.011	1.195 + 0.055
	CBS	Mg+	1.033 + 0.19	0.831 + 0.63	1.047 + 0.51	0.861 + 0.47	1.311 + 0.107	0.882 + 0.007
		Control	1.0 + 0.29	1.0 + 0.37	1.0 + 0.17	1.064 + 0.34	1.023 + 0.34	1.199 + 0.062
ATM	Cereberum	Mg+	0.772 + 0.026[a]	0.746 + 0.292[a]	0.888 + 0.114	0.880 + 0.199[a]	0.992 + 0.049[a]	0.992 + 0.065[a]
		Control	1.0 + 0.079	1.0 + 0.152	1.0 + 0.024	1.138 + 0.098	1.202 + 0.035	1.146 + 0.058
	CBS	Mg+	1.491 + 0.087[a]	1.088 + 0.217	0.947 + 0.271	0.883 + 0.084	1.114 + 0.226	0.907 + 0.082
		Control	1.0 + 0.097	1.0 + 0.194	1.0 + 0.212	1.548 + 0.493	1.615 + 1.195	0.960 + 0.194

Values represent means ± SEM of three experiments each performed in triplicate.
[a] Significantly different from control at $P < 0.05$.

in the tNhtt-150Q–EGFP cells (Fig. 3A, B and C). Because HSP70 is known to cure the misfoldings of the 150Q aggregate, we investigated whether 20 days of rTMS increased the cell viability of the tNhtt-150Q-EGFP cells. Twenty days of rTMS increased cell viability compared with controls (Fig. 3). In the mouse brain, acute rTMS increased HSP70 mRNA levels at 1 h and thereafter. However, no HSP70 mRNA changes were observed after 20 days of rTMS (Table 3).

3.5. Effects of rTMS on dopamine receptor 2

From the GeneChip data, we found that dopamine receptor 2 mRNA levels were changed. Therefore, we investigated dopamine receptor 2 mRNA levels and protein function after rTMS. We found that chronic rTMS decreased the mRNA levels of the D2 receptor, but no changes were found with acute rTMS (Table 3). In order to confirm these effects, we used a [^{3}H]ligand-binding assay of synaptosomes of the whole brain that were collected 24 h after mice were treated with rTMS for 20 days. We verified that [^{3}H]raclopride binding was decreased. A decrease in the B_{max} and no change in the K_D of [^{3}H]raclopride binding was observed in the binding assay (K_D: rTMS-treated, 14.74 ± 4.684 nM; control, 13.39 ± 5.219 nM; B_{max}: rTMS-treated, 4026 ± 305 fmol/μg of protein/min; control, 6296 ± 417 fmol/μg of protein/min) (Fig. 4).

4. Discussion

rTMS has been widely used to treat neuropsychiatric disorders. However, the underlying mechanisms of this treatment are not clear. In order to study the effects of chronic rTMS, we treated mice brains with a chronic rTMS protocol for 20 days. We have previously shown that acute and chronic rTMS alters monoamine transporter (MAT) mRNA, protein levels, and function [13]. In order to investigate the changes in gene expression that were induced by rTMS, we performed a GeneChip analysis. From these data (Supplementary data 1 and 2), we confirmed changes in some of the MAT-related genes.

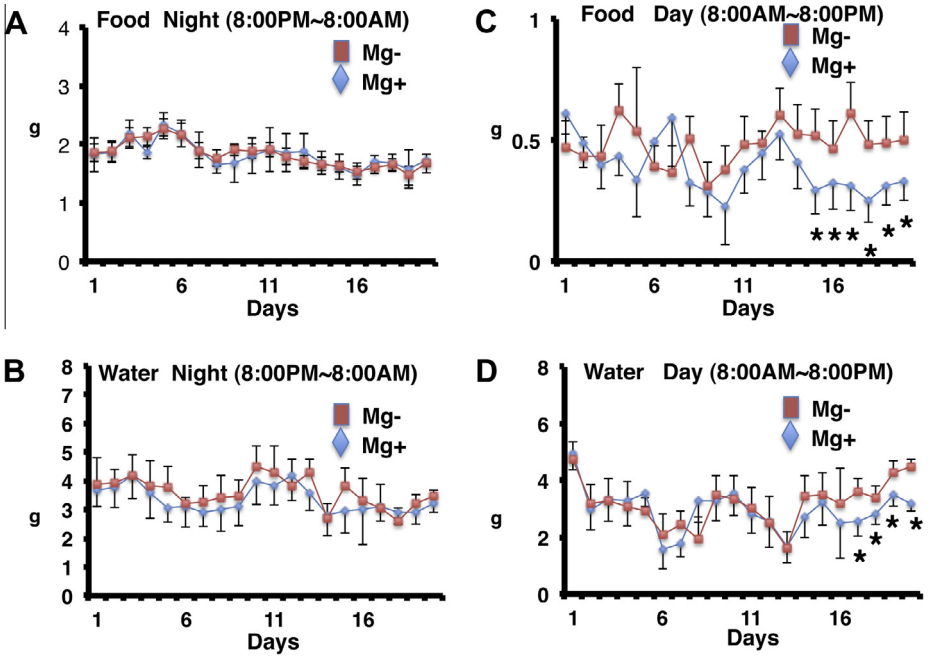

Fig. 2. Effects of rTMS on food and water intake. (A, C) Effects of rTMS on food intake. The weight of food intake was determined at 8:00 AM (A) and 8:00 PM (C). (B, D) Effects of rTMS on water uptake. The weight of water intake was determined at 8:00 AM (B) and 8:00 PM (D). Values represent means ± SEM of five experiments each performed in triplicate. *Significantly different from control at $P < 0.05$.

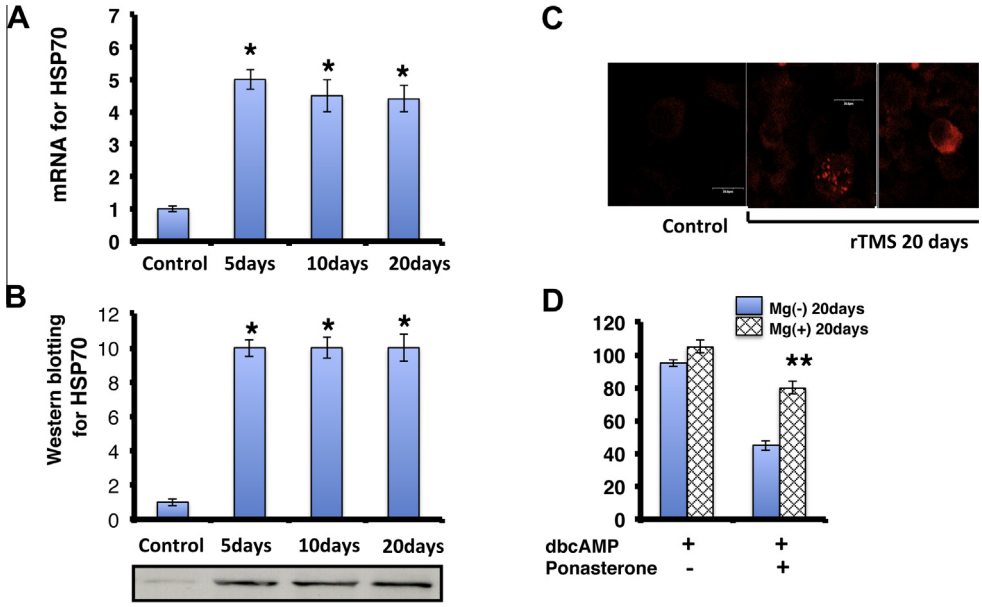

Fig. 3. Effects of rTMS on HSP70 mRNA and protein levels. (A–C) Effects of rTMS on HSP70 mRNA and protein levels. tNhtt-150Q–EGFP cells were treated by rTMS. The mRNA and protein level was determined at 24 h after stimulation. (D) Viability of tNhtt-150Q–EGFP cells stimulated by rTMS. Values represent means ± SEM of three experiments each performed in triplicate. *Significantly different from control at $P < 0.05$. **$P < 0.01$ versus control treatment with ponasterone A and dbcAMP.

We found that HSP70 mRNA and protein levels were increased in tNhtt-150Q-EGFP cells by chronic rTMS and in mouse cerebrum by acute rTMS. In addition, 20 days of rTMS increased the cell viability of tNhtt-150Q-EGFP cells. These results indicated that rTMS may result in improvement in patients with Huntington's diseases through the induction of HSP70. Further investigations are needed regarding the effects of chronic rTMS because we observed that acute rTMS increased HSP70 mRNA levels, whereas there were no changes observed 24 h after 20 days of rTMS.

In addition, we found that dopamine receptor 2 and dopamine receptor 4 mRNA levels were decreased in mouse brain after chronic rTMS. Thus, we examined the D2 receptor function with a ligand-binding assay. Chronic rTMS decreased [^{3}H]raclopride binding (Fig. 4). Because D2 receptor agonists are now widely used to treat schizophrenia, chronic rTMS may result in improvement in patients with psychiatric disorders through the effects on the D2 receptor.

We also observed that chronic rTMS inhibited food and water intake during the daytime (Fig. 2). Recently, circadian rhythm-related transcription factors have been found. Thus, we investigated whether chronic rTMS altered the mRNA levels of circadian rhythm-related genes. After 20 days of rTMS, Period 2 mRNA levels

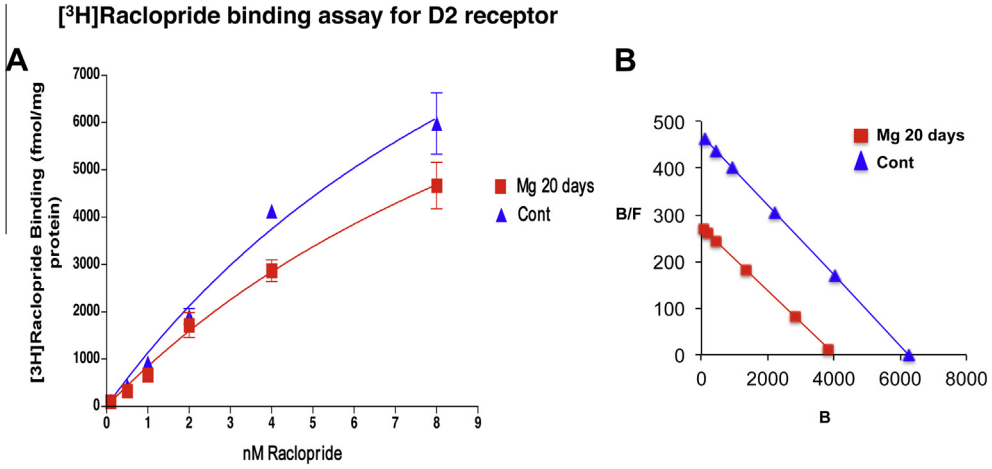

Fig. 4. Effects of chronic rTMS (20 days) on the binding of [^{3}H]raclopride in mouse brain. (A) raclopride binding assay-specific ligands for D2 receptor were evaluated using radiolabeled Raclopride. The Left panel shows the saturation analysis of [^{3}H]raclopride. Points and bars represent means ± SEM, $N = 3$. Right panel shows the Eadie–Hofstee plot of [^{3}H]raclopride binding. Each point represents the mean of three independent experiments obtained in (A, left panel). Values represent means ± SEM of three experiments each performed in duplicate.

were decreased in the cerebrum and CBS, whereas BMLA1, CaMK2, CKIε, and Clock mRNA levels were increased in CBS (CaMK2 and Clock were increased in the cerebrum too). These results indicated that circadian rhythm-related genes may be involved in the changes in the food and water intake that were induced by chronic rTMS.

Our results suggested the involvement of HSP70, the D2 receptor, and circadian rhythm-related gene expression changes in the therapeutic effects of chronic rTMS. Further research on the regulation of HSP70, the D2 receptor, and circadian rhythm-related gene expression by chronic rTMS will be needed in order to develop more effective rTMS therapies for the treatment of patients with neuropsychiatric disorders in which HSP70 and dopamine systems are involved. Moreover, it will be important to fine-tune the method or the apparatus to stimulate certain brain areas more specifically and reduce unnecessary side effects.

Acknowledgments

This work was supported partly by a grant from the JSPS (Japan Society for the Promotion of Science). This work was supported by Global COE Program "Center of Education and Research for the Advanced Genome-Based Medicine-For personalized medicine and the control of worldwide infectious disease-", MEXT Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.017.

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