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Research paper

Effects of low- and high-frequency repetitive magnetic stimulation on neuronal cell proliferation and growth factor expression: A preliminary report

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HIGHLIGHTS

- We studied the differential effects of repetitive magnetic stimulation.
- We used low and high-frequency repetitive magnetic stimulation.
- We examined neurotrophic/growth factors and proliferation in neuroblasts.
- High-frequency rMS induced upregulation of neurotrophic/growth factors.
- Upregulation of neurotrophic/growth factors caused cell proliferation.

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ABSTRACT

Repetitive magnetic stimulation is a neuropsychiatric and neurorehabilitation tool that can be used to investigate the neurobiology of sensory and motor functions. Few studies have examined the effects of repetitive magnetic stimulation on the modulation of neurotrophic/growth factors and neuronal cells *in vitro*. Therefore, the current study examined the differential effects of repetitive magnetic stimulation on neuronal cell proliferation as well as various growth factor expression. Immortalized mouse neuroblastoma cells were used as the cell model in this study. Dishes of cultured cells were randomly divided into control, sham, low-frequency (0.5 Hz, 1 Tesla) and high-frequency (10 Hz, 1 Tesla) groups ($n = 4$ dishes/group) and were stimulated for 3 days. Expression of neurotrophic/growth factors, Akt and Erk was investigated by Western blotting analysis 3 days after repetitive magnetic stimulation. Neuroblastoma cell proliferation was determined with a cell counting assay. There were differences in cell proliferation based on stimulus frequency. Low-frequency stimulation did not alter proliferation relative to the control, while high-frequency stimulation elevated proliferation relative to the control group. The expression levels of brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), neurotrophin-3 (NT-3) and platelet-derived growth factor (PDGF) were elevated in the high-frequency magnetic stimulation group. Akt and Erk expression was also significantly elevated in the high-frequency stimulation group, while low-frequency stimulation decreased the expression of Akt and Erk compared to the control. In conclusion, we determined that different frequency magnetic stimulation had an influence on neuronal cell proliferation *via* regulation of Akt and ERK signaling pathways and the expression of growth factors such as BDNF, GDNF, NT-3 and PDGF. These findings represent a promising opportunity to gain insight into how different frequencies of repetitive magnetic stimulation may mediate cell proliferation.

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1. Introduction

Transcranial magnetic stimulation is based on electromagnetic induction, which was initially observed by Michael Faraday almost 200 years ago [1]. A transcranial magnetic stimulator with a coil can produce a short 100 ms biphasic electromagnetic pulse, which generates an electric current in the central nervous system (CNS) [2]. The resulting magnetic field induces electrical current in nervous tissue and, at sufficient strength, produces neuronal depolarization [3]. When administered at regular frequencies, this process is referred to as 'repetitive transcranial magnetic stimulation' or 'rTMS' [4]. rTMS has been used to treat CNS diseases because it non-invasively stimulates brain areas, and also has less pain and fewer side-effects than other treatments [3,5]. rTMS is a neuropsychiatric and neurorehabilitation tool [4,6,7] that can be used to investigate the neurobiology of sensory and motor functions [6,8,9]. High-frequency (>3 Hz) stimulation generally results in facilitation, while low-frequency (<1 Hz) rTMS induces reduction of synaptic efficiency [10]. Based on this mechanism, several clinical trials have revealed that rTMS provides kinetic function improvements and change neurotransmitter system [6,7,11,12]. Additionally, other studies have reported that rTMS affects neural stem cell proliferation in the subventricular zone [13]. However, few studies have examined the *in vitro* and *in vivo* effects of repetitive magnetic stimulation. The effects of stimulation in different frequencies also remain uncertain. Therefore, we examined changes in cell proliferation and expression of growth factors in mouse neuroblastoma cells.

2. Materials and methods

2.1. Cell cultures

The immortalized mouse neuroblastoma cell line N1E-115 (ATCC® CRL-2263™), which expresses normal neuronal phenotypes, was used as a cell model in this study. Mouse neuroblastoma cells were seeded at 2×10^5 cells/cm^2 in 100ϕ culture dish in Dulbecco's Modified Eagle Medium without pyruvate (DMEM; Invitrogen-Gibco, Rockville, MD, USA) but containing 10% fetal bovine serum (FBS; Invitrogen-Gibco) and 100 U/ml penicillin/streptomycin (Invitrogen-Gibco) in a humidified 5% CO_2 atmosphere at 37 °C. The media was replaced at three day intervals. Neuroblastoma cells were subcultured when they reached 80–90% confluence. The numbers of neuroblastoma cells were counted using the manual hemocytometer method.

2.2. Repetitive magnetic stimulation

Dishes of cultured cells were randomly allocated into the following three groups: untreated controls ($n = 4$), low-frequency (0.5 Hz) stimulation group ($n = 4$), and high-frequency (10 Hz) stimulation group ($n = 4$). 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 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 20 min per day. The treatments were performed daily for 3 days. Control neurons were handled in a similar manner to the treatment group exposed to the magnetic stimulation apparatus for the identical length of time but was shielded by Mu metal, therefore did not receive any stimulation.

2.3. Western blotting

To compare the expression levels of neurotrophic factors, neuroblastoma cells ($n = 4$ per group) were lysed in 300 μl of cold RIPA buffer (50 mM Tris–HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), and 1% sodium deoxycholate) with a protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, USA). Cells lysate was centrifuged at 13,000 × *g* for 15 min at 4 °C. The supernatant was harvested, and protein concentration was analyzed using a Qunt-iT protein assay kit (Molecular Probes, Eugene, Oregon, USA). For electrophoresis, 50 μg of protein was dissolved in a sample buffer (60 mM Tris–HCl, pH 6.8, 14.4 mM β-mercaptoethanol, 25% glycerol, 2% SDS, and 0.1% bromophenol blue), boiled for 10 min and separated on a 10% SDS reducing gel. Separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Invitrogen, Carlsbad, CA) using a *trans*-blot system. Blots were blocked for 1 h in Tris-buffered saline (TBS) (10 mM Tris–HCl, pH 7.5, 150 mM NaCl) containing 5% nonfat dry milk at room temperature, washed three times with TBS and incubated at 4 °C overnight with an anti-rabbit polyclonal brain-derived neurotrophic factor (BDNF, 1:1000, Abcam, Cambridge, MA, USA), anti-rabbit glial cell-derived neurotrophic factor (GDNF, 1:1000, Abcam), anti-rabbit neurotrophin-3 (NT-3, 1:1000; Abcam), anti-rabbit platelet-derived growth factor (PDGF, 1:1000; Abcam), and anti-β actin (1:3000, Cell signaling, Boston, MA, USA) antibody in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.02% Tween 20) containing 5% nonfat dry milk. On the next day, blots were washed three times with TBST and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (1:3000, Santa Cruz Biotech, Santa Cruz, CA, USA) in TBST containing 3% nonfat dry milk. After washing three times with TBST, proteins were visualized with an ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.4. Blockage of growth factors

To determine the relation between growth factors and cell proliferation, we used NT-3, GDNF and PDGF blocking peptide. Mouse neuroblastoma cells were seeded at a density of 1×10^6 cells/cm^2 in 100ϕ culture dish. After 24 h, cells were washed with Dulbecco's phosphate buffered saline (DPBS; Invitrogen-Gibco) five times, and media were replaced with serum-free media containing 2 μg/ml NT-3 blocking peptide (Santa Cruz Biotech), GDNF blocking peptide (Biovision, CA, USA) and PDGF blocking peptide (Santa Cruz Biotech). Cells were incubated with or without growth factor blocking peptides for 12 h. The cells were then treated rTMS stimulation with 0.5 Hz and 10 Hz daily for 3 days.

2.5. Statistical analysis

Results shown in the bar graphs are the mean ± SE of at least 4 independent experiments. Statistical analysis of cell proliferation was performed in untreated, 0.5 Hz and 10 Hz stimulation groups using *t*-test and analysis of variance (ANOVA) with post hoc Bonferroni comparison. A *P*-value <0.05 was considered statistically significant.

3. Results

3.1. Immunoblot analysis of neuronal growth factors

To identify the growth factors associated with the functional recovery repair process and the neuroprotective and/or neurorestorative effects induced by repetitive magnetic stimulation, the expressions of various neurotrophic/growth factors including BDNF, GDNF, NT-3, and PDGF were investigated using Western blot