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Cellular and Molecular Changes to Cortical Neurons Following Low Intensity Repetitive Magnetic Stimulation at Different Frequencies

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ABSTRACT

Background: Repetitive transcranial magnetic stimulation is increasingly used as a treatment for neurological dysfunction. Therapeutic effects have been reported for low intensity rTMS (LI-rTMS) although these remain poorly understood.
Objective: Our study describes for the first time a systematic comparison of the cellular and molecular changes in neurons *in vitro* induced by low intensity magnetic stimulation at different frequencies.
Methods: We applied 5 different low intensity repetitive magnetic stimulation (LI-rMS) protocols to neuron-enriched primary cortical cultures for 4 days and assessed survival, and morphological and biochemical change.
Results: We show pattern-specific effects of LI-rMS: simple frequency pulse trains (10 Hz and 100 Hz) impaired cell survival, while more complex stimulation patterns (theta-burst and a biomimetic frequency) did not. Moreover, only 1 Hz stimulation modified neuronal morphology, inhibiting neurite outgrowth. To understand mechanisms underlying these differential effects, we measured intracellular calcium concentration during LI-rMS and subsequent changes in gene expression. All LI-rMS frequencies increased intracellular calcium, but rather than influx from the extracellular milieu typical of depolarization, all frequencies induced calcium release from neuronal intracellular stores. Furthermore, we observed pattern-specific changes in expression of genes related to apoptosis and neurite outgrowth, consistent with our morphological data on cell survival and neurite branching.
Conclusions: Thus, in addition to the known effects on cortical excitability and synaptic plasticity, our data demonstrate that LI-rMS can change the survival and structural complexity of neurons. These findings provide a cellular and molecular framework for understanding what low intensity magnetic stimulation may contribute to human rTMS outcomes.

Introduction

Repetitive transcranial magnetic stimulation (rTMS) is used in clinical treatment to non-invasively stimulate the brain and promote long-term plastic change in neural circuit function [1,2], with benefits for a wide range of neurological disorders [3–5]. In addition, there is increasing evidence that low intensity magnetic stimulation (LI-rTMS) may also be therapeutic, particularly in mood regulation and analgesia [6–8]. Nonetheless, clinical outcomes of rTMS and LI-rTMS are variable [9] and greater knowledge of the mechanisms underlying different stimulation regimens is needed in order to optimize these treatments.

Investigating the mechanisms of both high and low intensity rTMS is important because most human rTMS protocols deliver a

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range of stimulation intensities across and within the brain. Human rTMS is most commonly delivered using butterfly figure-of-eight shaped coils [10,11] to produce focal high-intensity fields that depolarize neurons in a small region of the cortex underlying the intersection of the 2 loops [10,11], which in turn can modulate activity in downstream neural centers [12,13]. However, this stimulation focus is surrounded by a weaker magnetic field such that a large volume of adjacent cortical and sub-cortical tissue is also stimulated, albeit at a lower intensity that is below activation threshold [14,15]. While the functional importance of this parafocal low-intensity stimulation in the context of human rTMS is unclear, low-intensity magnetic stimulation on its own modifies cortical function [7,16] and brain oscillations [17]. Moreover, animal and *in vitro* studies demonstrate that low-intensity stimulation alters calcium signaling [18,19], gene expression [20], neuroprotection [21] and the structure and function of neural circuits [22,23]. However, the mechanisms underlying outcomes of low-intensity magnetic stimulation, particularly in conjunction with different stimulation frequencies, have not been investigated.

To address this, we undertook a systematic investigation of the fundamental morphological and molecular effects of five repetitive low intensity magnetic stimulation (LI-rMS) protocols in a simple *in vitro* system with defined magnetic field parameters. We show for the first time that LI-rMS induces calcium release from intracellular stores. Moreover, we show specific effects of different stimulation protocols on neuronal survival and morphology and associated changes in expression of genes mediating apoptosis and neurite outgrowth. Taken together, our data demonstrate that even low intensity magnetic stimulation induces long-term modifications to neuronal structure, which might have implications for understanding the effects of high-intensity human rTMS in the whole brain.

Methods

Animals

C57Bl/6j mice pups were sourced from the Animal Resources Centre (Canning Vale, WA, Australia). Experimental procedures were approved by the UWA Animal Ethics Committee (03/100/957).

Tissue culture

To investigate changes in neuron biology following LI-rMS stimulation, we used neuronal enriched cultures from postnatal day 1 mouse cortex. Pups were euthanased by pentobarbitone sodium (150 mg/kg i.p.), decapitated and both cortices removed. Pooled cortical tissue was dissociated and prepared following standard procedures [24]. Cells were suspended in NB media (Neurobasal-A, 2% B27 (Gibco®), 0.6 mg/ml creatine, 0.5 mM L-glutamine, 1% Penicillin/Streptomycin, and 5 mM Hepes) and plated on round poly-D-lysine coated coverslips at a density of 75,000 cells/well (day 0 *in vitro*; DIV 0). On DIV 3, half the culture medium was removed and replaced with fresh media containing cytosine arabinofuranoside (6 μM; Sigma) to inhibit glial proliferation. Cells were grown at 37 °C in an incubator (5% CO_2 + 95% air) for 10 days and half the medium was replaced on DIV 6 and 9. To ensure that any experimental effects were not due to either different litters or culture sessions, plated coverslips from each litter were randomly allocated to stimulation groups. The whole culture-stimulation procedure was repeated 3 times.

Repetitive magnetic stimulation

LI-rMS stimulation was delivered to cells in the incubator with a custom built round coil (8 mm inside diameter, 16.2 mm outside diameter, 10 mm thickness, 0.25 mm copper wire, 6.1 Ω resistance, 462 turns) placed 3 mm from the coverslip (Fig. 1A) and driven by a 12 V magnetic pulse generator: a simple resistor-inductor circuit under control of a programmable (C-based code) micro-controller card (CardLogix, USA). The non-sinusoidal monophasic pulse [25] had a measured 320 μs rise time and generated an intensity of 13 mT as measured at the target cells by hall effect (ss94a2d, Honeywell, USA) and assessed by computational modeling using Matlab (Mathworks, USA; Fig. 1B,C). Coil temperature did not rise above 37 °C, ruling out confounding effects of temperature change. Vibration from the bench surface (background) and the top surface of the coil were measured at 10 Hz stimulation using a single-point-vibrometer (Polytec, USA); coil vibration was within vibration amplitude of background (Fig. 1D).

Stimulation was delivered for 10 continuous minutes per day at 1 of 5 frequencies: 1 Hz, which reduces, or 10 Hz which increases, cortical excitability in human rTMS [26,27]; we also used 100 Hz, consistent with very low intensity pulsed magnetic field stimulation [8,28], continuous theta burst stimulation (cTBS: 3 pulses at 50 Hz repeated at 5 Hz) showing inhibitory effects on cortical excitability post-stimulation in human rTMS [29,30] or biomimetic high frequency stimulation (BHFS: 62.6 ms trains of 20 pulses, repeated at 9.75 Hz). The BHFS pattern was designed on electro-biomimetic principles [6], based on the main parameter from our previous studies [22,23] which was modeled on endogenous patterns of electrical fields around activated nerves during exercise (patent PCT/AU2007/000454, Global Energy Medicine). The total number of pulses delivered for each stimulation paradigm is shown in Table 1. We chose a standard duration of stimulation of 10 min (rather than a standard number of pulses) because studies of brain plasticity reveal that 10 min of physical training or LI-rTMS is sufficient to induce functional and structural plasticity [22,23,31]. For all experiments, controls were treated identically but the coils were not activated. An overview of experiments and summary of experimental design is shown in Fig. 1E.

Immunohistochemistry

To investigate the influence of different stimulation frequencies at the cellular level, we used immunohistochemistry to examine neuronal survival and the prevalence of different cell types. Cells plated on glass coverslips were grown in 12 spatially separated wells of a 24 well plate to ensure no overlap of magnetic field. Wells were stimulated for 10 min daily from DIV 6–9 and cells were fixed with 4% paraformaldehyde 24 h after the last stimulation. Mouse anti-active Caspase-3 (1:50, Abcam) and TUNEL (DeadEnd™ Fluorometric TUNEL System, Promega) double labeling were carried out to identify apoptosis. Glia and neurons were labeled with rabbit anti-GFAP (1:500, Dako) or mouse anti-βIII Tubulin (1:500, Covance). Subpopulations of neurons were identified, using rabbit anti-calbindin D-28K (inhibitory and small excitatory neurons; 1:500, Chemicon [32]) or mouse anti-SMI-32 (excitatory neurons; 1:2000, Covance [33]). Antibody binding was visualized using fluorescently labeled secondary antibodies (Alexa Fluor 546 and Alexa Fluor 488; Invitrogen). Cell nuclei were labeled with either Hoechst (1:1000, Sigma Aldrich) or Dapi (DeadEnd™). Coverslips were mounted with Fluoromount-G.

Histological analysis

For each experimental group, histological analyses were performed blind to stimulation paradigm on 12–18 images containing cultured cells from 2 to 3 different litters. Five semi-randomly distributed images per immunostained coverslip were taken from locations underneath the desired magnetic field (13 mT), in order to