Eur Arch Psychiatry Clin Neurosci (2012) 262:87–91
DOI 10.1007/s00406-011-0217-3

SHORT COMMUNICATION

Repetitive magnetic stimulation of human-derived neuron-like cells activates cAMP-CREB pathway

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Received: 2 December 2010 / Accepted: 3 May 2011 / Published online: 12 May 2011

Abstract Repetitive transcranial magnetic stimulation (rTMS) is a non-invasive neurostimulatory technique widely used in research, diagnostics, and neuro-psychiatric therapy. Despite its growing popularity, basic molecular mechanisms underlying the clinical effects of rTMS have remained largely under-researched. Here, we present a human-derived neuronal cell culture system responsive to rTMS effects. SH-SY5Y neuroblastoma cells were differentiated by retinoic acid treatment for 10 days, resulting in a neuronal phenotype characterized by upregulation of neuronal marker proteins and generation of an action potential in response to depolarizing current step injection. Repetitive magnetic stimulation of these cells resulted in increased intracellular cAMP levels and increased phosphorylation of transcription factor CREB. Pretreatment with ketamine (1 μM) potentiated, while pretreatment with lithium (2 mM) attenuated this cellular response to repetitive magnetic stimulation. In conclusion, we introduce here a novel in vitro system responding to rTMS at the level of second messenger signaling. The use of human-derived cells with neuron-like properties will prove useful for further studies on the cellular effects of rTMS.

Keywords rTMS · cAMP · CREB · SH-SY5Y neuroblastoma cells

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Introduction

Repetitive transcranial magnetic stimulation (rTMS) represents a relatively novel and versatile tool with a number of diagnostic, research, and therapeutic implications [15, 17]. rTMS is a non-invasive technique for brain stimulation, which makes use of the principle of electromagnetic induction as first described by Michael Faraday. The direct effects of rTMS are therefore largely confined to cortical neurons in the proximity of an externally placed magnetic coil [3].

Despite the widespread use of rTMS in the clinical setting, very little is known about the molecular mechanisms behind it. Importantly, there is currently no model system available in basic neuro-psychiatric research to study neurobiological mechanisms of rTMS in vitro. In antidepressant pharmacotherapy, it is widely accepted that key biological mechanisms underlying therapeutic effects involve plasticity-associated signal transduction pathways converging on CREB, leading to modulation of the transcription of cAMP responsive genes [4, 12, 14, 21]. Especially in hippocampus, CREB-regulated gene transcription has been shown to increase growth factor activity and to promote regenerative processes such as dendritic sprouting and neurogenesis [2].

So far, only few studies have investigated the biological effects of repetitive magnetic stimulation (rTMS) on

cultured cells. Notably, these studies generally failed to demonstrate effects on second messenger production or cell signaling, which were expected from studies in animals [9]. Moreover, previous investigations in vitro used either non-neuronal, undifferentiated neuronal or non-human cell systems [18–20]. The paucity of observed effects in these earlier reports may be due in large part to the use of undifferentiated or non-neuronal cells. Here, we hypothesized that to become fully useful for the study of rTMS, cells have to develop neuronal characteristics first. Therefore, in this study, we employed SH-SY5Y neuroblastoma cells after retinoic acid (RA)-induced differentiation as a neuronal, human-derived culture system. After providing data on neuronal differentiation, we show that rTMS induces the formation of cAMP and subsequent phosphorylation of CREB. Furthermore, we used the anesthetic ketamine and mood stabilizer lithium as two examples to demonstrate that our system is also suitable to study interactions between drug treatment and rTMS.

Materials and methods

Cell culture

Human SH-SY5Y neuroblastoma cells (Geweberesourcenzentrum Braunschweig, Germany) were seeded at an initial density of 4×10^5 cells/cm^2 for cAMP and pCREB experiments and 1-5 $\times$ 10^5 cells/cm^2 for electrophysiological and immunofluorescence studies. Cells were cultured as described in detail previously [7]. Briefly, cells were grown in minimum essential medium containing Earle's salts, 100 units/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (Biochrom, Berlin, Germany). Retinoic acid (RA; Sigma–Aldrich, Taufkirchen, Germany) was applied at a final concentration of 10 μM. Lithium aspartate (Sigma) was added to a final concentration of 2 mM, and ketamine (CuraMED, Karlsruhe, Germany) was added to a final concentration of 1 μM.

Repetitive magnetic stimulation (rTMS)

Cells were placed on the magnetic coil of a conventional Magstim Pro^{TM} device. Stimulation parameters were set to either 60 or 180 series at maximum intensity, resulting in a total of 600 or 1,800 stimuli, respectively, applied in series of 10 pulses at 5 Hz with a 10-s interval between series. In the sham-stimulation condition, cells were removed from the incubator and exposed to the same environment as the stimulated cells for the same period of time. Serum was removed from culture medium 24 h before stimulation. Drugs were applied in serum-free medium 1 h prior to stimulation.

Analysis of intracellular cAMP levels

Cells from four wells of a 96-well plate were pooled as one sample ($\sim 5 \times 10^5$ cells). Intracellular cAMP levels were determined by a non-acetylation method using a cAMP-specific enzyme-linked immunosorbent assay (RPN 225, GE Healthcare, Buckinghamshire, UK).

Western blot analysis

SDS–PAGE and Western blotting were performed as described in detail elsewhere [7, 8]. In brief, cells were lysed with ice-cold mammalian protein extraction reagent (Pierce Biotechnology, Rockford, Ill., U.S.A.) and cellular debris was precipitated by centrifugation at 25,000*g* and 4°C for 25 min. Protein concentration was determined using BCA assay (Pierce Biotechnology). Equal amounts of protein were loaded on sodium dodecyl sulfate polyacrylamide gels (10–20%) and blotted onto PVDF membranes (Millipore, Schwalbach, Germany). Blots were probed with the following antibodies: anti-GFAP (1:500; Sigma), polyclonal anti-neurofilament (1:1,000; Sigma), anti-MAP2 (1:500; Sigma), anti-β-actin (HRP-labeled, 1:10,000; Sigma), anti-CREB, and anti-phospho-CREB (both 1:1,000; Cell Signaling Technologies, Danvers, MA, USA). Densitometric quantification was performed using the LAS 3000 imaging system and Aida image analysis software, version 4.1 (Raytest, Straubenhardt, Germany).

Immunofluorescence

Cells were cultured on poly-L-lysine-coated glass coverslips. Fixation was with 4% paraformaldehyde (PFA) in TBS buffered to pH 7.4. Cells were stained with anti-MAP2 antibody (1:500; Sigma) and RhodX-conjugated secondary antibody (anti-mouse; 1:250; JacksonImmunoResearch, West Grove, PA). Nuclear dye Sytox Green (Invitrogen, Karlsruhe, Germany) was used at a concentration of 1:5,000. All confocal microscopy was performed using a spectral confocal microscope (TCS SP2; Leica, Nussloch, Germany).

Electrophysiology

RA-treated cells were analyzed by whole-cell patch-clamp recordings for passive membrane properties and voltage-gated currents in voltage-clamp mode as well as for generation of action potentials in current-clamp mode using a patch-clamp amplifier (EPC-9, HEKA Elektronik, Lambrecht, Germany) as described in detail previously [22]. The recording pipette solution contained (in mM): 120 KCl, 4 NaCl, 5 ethylene glycol-bis(β-aminoethyl ether)

N,N,N′,N′-tretraacetic acid (EGTA), 5 N-2-hydroxyethylpiperzine-N′-2-ethanesulfonic acid (HEPES), 5 glucose, 0.5 $CaCl_2$ and 4 $MgCl_2$ buffered to pH 7.3. During the experiment, cells were bathed in a solution containing (in mM): 145 NaCl, 3 KCl, 20 N-2-hydroxyethylpiperzine-N′-2-ethanesulfonic acid (HEPES), 20 glucose, 2 $CaCl_2$, 1 $MgCl_2$ adjusted to pH 7.3. Signals were sampled at a rate of 10 kHz using WinTida software (HEKA Elektronik, Lambrecht, Germany) and Bessel filtered at 3 kHz.

Statistical analyses

Values are presented as mean ± SEM. All numerical analyses were performed using the Graph Pad Prism® program. Differences between means were analyzed by analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Results

SH-SY5Y cells adopt neuronal characteristics upon differentiation with retinoic acid

The SH-SY5Y neuroblastoma cell line is widely used as an in vitro model to study biochemical and functional properties of neurons. Importantly, it is a human-derived cell line [1]. SH-SY5Y cells were differentiated for 10 days in the presence of RA. Subsequent histological analysis consistently yielded strong MAP2-immunoreactivity in all cells throughout the cultures, indicating neuronal differentiation (Fig. 1a). Similarly, immunoblot analysis of undifferentiated (0 DIV) and differentiated (10 DIV) SH-SY5Y cells revealed increasing levels of neuronal marker proteins neurofilament and MAP2 with differentiation whereas expression of glial marker protein GFAP receded (Fig. 1b).

Differentiated SH-SY5Y cells are able to produce action potentials

The physiological properties of SH-SY5Y cells treated for 10 days with RA were characterized using whole-cell patch-clamp recordings. Analysis of passive membrane properties revealed a whole-cell membrane capacitance of 13.0 ± 1.2 pF and an input resistance of 1270.1 ± 113.6 MΩ. Furthermore, activation of voltage-gated channels by depolarizing pulses induced voltage-gated Na^+-currents of 499.1 ± 85.2 pA and voltage-gated K^+-currents of 725.1 ± 85.9 pA. Importantly, cells generated an action potential (AP) upon current injection of at least 30 pA (Fig. 1c).

Repetitive magnetic stimulation (rTMS) increases intracellular cAMP levels and induces CREB phosphorylation in differentiated SH-SY5Y cells

SH-SY5Y cells were differentiated in the presence of RA for 10 days. Cells were subjected to 180 consecutive series of rTMS or sham stimulation (sham rTMS) as described above. rTMS resulted in a significant increase in intracellular cAMP levels both in vehicle-treated cells and in cells that had been pretreated with ketamine. By contrast, lithium treatment attenuated this effect of rTMS on intracellular cAMP levels (Fig. 1d).

The transcription factor CREB (cAMP response element-binding protein) is activated by phosphorylation. Chronic antidepressant treatment has previously been shown to increase CREB phosphorylation in mice [21]. Here, we studied the effects of either 60 or 180 consecutive series of rTMS on the levels of phosphorylated CREB (pCREB); 180 consecutive series of rTMS significantly increased pCREB levels. Ketamine pretreatment potentiated, while lithium treatment largely inhibited the effects of rTMS on the amount of pCREB (Fig. 1e, f). Total levels of CREB protein were not affected by either 1-h pretreatment with drugs or the stimulation procedure (not shown).

Discussion

To study the molecular effects of rTMS, it would be beneficial to have a cell culture system that responds to magnetic stimulation in a parallel fashion to in vivo responses. While many animal models exist, an in vitro model, although more removed from the in vivo system, carries some additional advantages. In vitro models allow for a higher throughput at a greatly reduced cost. Also, human cells such as SH-SY5Y could then be studied rather than rodent neurons, and the different responses between the two evaluated. Lastly, stimulating neurons in vitro strips away the additional complications of penetration through skin and bone and allows the pure study of rTMS directly on the tissue of interest.

In a hallmark study in rats, Ji and co-workers demonstrated effects of rTMS on immediate early gene expression and CREB phosphorylation in several brain areas [9]. Since activation of CREB may promote neuroplastic responses such as synaptogenesis and neurogenesis, these in vivo findings link rTMS to a host of neurobiological mechanisms, which may be therapeutically harnessed [11, 23]. Importantly, long-term rTMS has also been shown to increase the expression of CREB target gene BDNF in rat hippocampus [13].

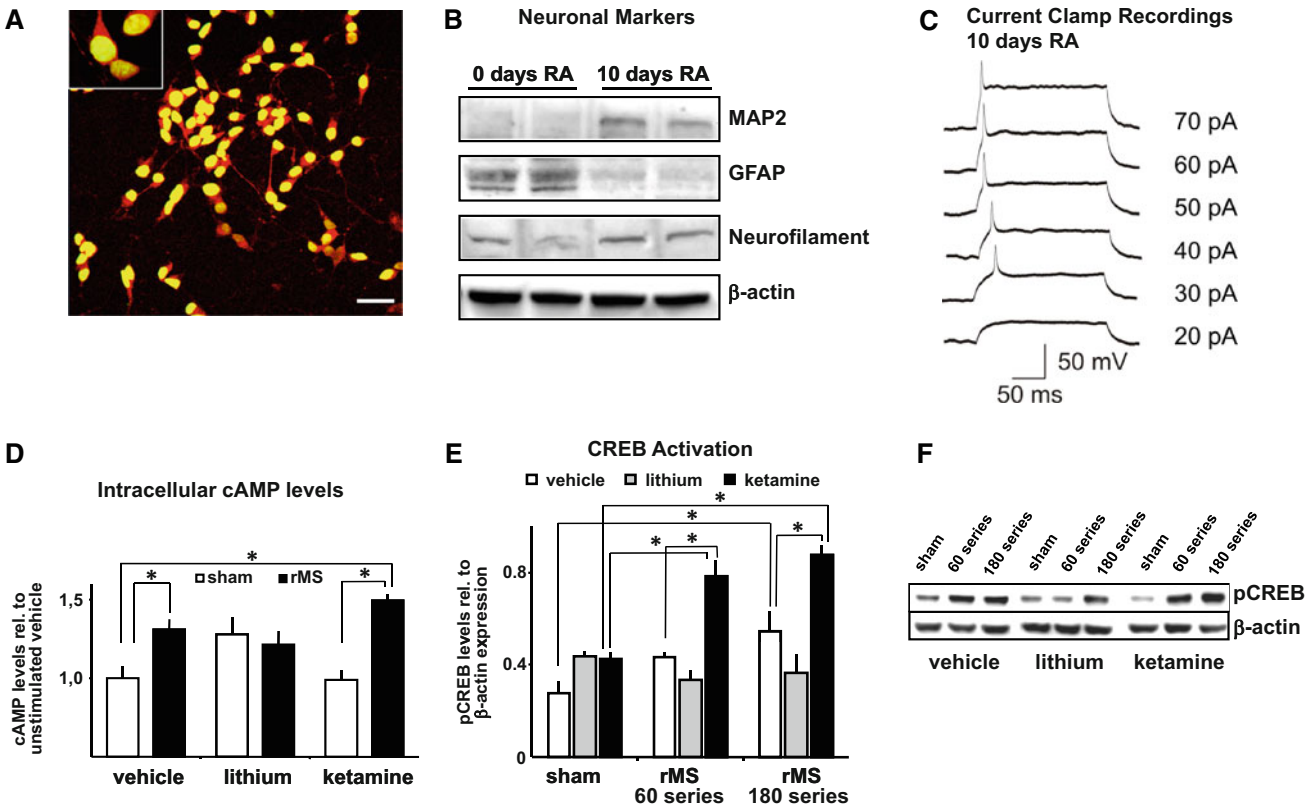

Fig. 1 Human-derived neuronal cell culture system for the study of the effects of repetitive magnetic stimulation. SH-SY5Y human neuroblastoma cells were differentiated for 10 days in the presence of RA (10 μM). **a** Confocal analysis of cells stained with nuclear dye Sytox Green (*green*) consistently demonstrated co-staining with neuronal marker protein MAP2 (*red*). Scale bar 35 μm. **b** Representative Western blots of undifferentiated (0 DIV) and differentiated (10 DIV) SH-SY5Y cells demonstrating increase in neuronal marker proteins MAP2 and neurofilament and decreased expression of glial marker protein GFAP with differentiation. **c** Current-clamp recording from an RA-treated neuron-like differentiated cell. Sample traces for membrane responses to depolarizing current step injections (20–70 pA) for 200 ms show that cells were able to generate a single action potential in response to current injection of at least 30 pA. The reference potential was set to −70 mV. **d**, **e**, **f** Differentiated SH-SY5Y neuroblastoma cells were investigated as a model system for studying potential molecular effects of repetitive magnetic stimulation in vitro. **d**. Intracellular cAMP levels. **e** Densitometric quantification of pCREB bands, presented as ratios of pCREB optical density (O.D.) over *β*-actin O.D. Note that ketamine pretreatment potentiated, while lithium treatment inhibited the inducing effect of rTMS on pCREB. **f** Representative blots of pCREB. All experiments were performed at least in triplicate. $^{*}P < 0.05$, one-way ANOVA, Tukey's post hoc test

Previous studies in vitro yielded partly conflicting results concerning rTMS effects on cell culture systems. Sontag & Kalka did not find significant effects of either pulsed magnetic fields or of rTMS on cAMP content and neurotransmitter release using undifferentiated rat pheochromocytoma cells [19, 20]. While Shaul and co-workers did not investigate second messenger signaling, they did observe stimulation frequency-dependent alterations in neurotransmitter metabolism in undifferentiated SH-SY5Y cells [18]. Obviously, results from different studies are hard to compare, not least due to the use of varying cell culture systems and stimulation parameters. We hypothesized that the use of non-neuronal and undifferentiated cells, respectively, in previous studies may also have contributed to the lack of any strong effects on second messenger signaling. Therefore, we first induced differentiation of SH-SY5Y neuroblastoma cells into a more mature neuron-like phenotype by application of RA. We outlined a method of cell culture preparation that induces neuronal differentiation as observed by the appearance of neuronal markers, growth of connecting processes and the potential for AP signaling, and used this method of preparation to study the molecular effects of rTMS. We observed increased levels of cAMP following rTMS, an effect which was intensified by pretreatment with ketamine and attenuated by pretreatment with lithium. This pattern of regulation was corroborated by the levels of downstream pCREB. CREB becomes transcriptionally active following phosphorylation [5]. Taken together, the data presented here suggest that activation of the cAMP/CREB pathway may also underlie some of the clinical actions of rTMS.

So far, studies on the effects of concomitant medications with a neurostimulatory technique have primarily focused on electroconvulsive therapy (ECT). Synergistic effects of

ketamine and ECT have been described [10]. Interestingly, ketamine has also been reported to increase human motor cortex excitability (Di Lazzaro et al., 2003). Furthermore, combined treatment with ketamine and a tricyclic antidepressant has recently been shown to produce increases of CREB and BDNF protein levels in prefrontal cortex, hippocampus and amygdala [16]. By contrast, use of lithium during ECT remains controversial, because it may lead to serious central nervous system side effects. At this point, our results concerning lithium are hard to interpret. To our knowledge, the combined effects of lithium and rTMS have not yet been studied in the clinical setting. It should also be noted that the effects of lithium on the cAMP/CREB pathway may depend crucially on the duration of treatment [6].

In summary, while much work remains to be done to further refine the model system introduced here, our data can be regarded as an encouraging step toward the study of rTMS effects in vitro. Further advancement on this model may help us to understand the effects of rTMS on a molecular level and how these effects are influenced by specific drugs.

Acknowledgments This study was supported by grants from the VolkswagenFoundation (Lichtenberg program), Bundesministerium für Bildung und Forschung (Center for Stroke Research Berlin), Deutsche Forschungsgemeinschaft (DFG RA 424/5-1 to R.J.) and the Hermann and Lilly Schilling Foundation.

Conflicts of interest None.

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