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Dose-Dependence of Changes in Cortical Protein Expression Induced with Repeated Transcranial Magnetic Theta-Burst Stimulation in the Rat

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ABSTRACT

Background: Theta Burst stimulation (TBS) applied via transcranial magnetic stimulation (TMS) effectively modulates human neocortical excitability but repeated applications of the same TBS protocol at short intervals may be not simply accumulative.
Objective: Our aim was to investigate the impact of multiple blocks of either intermittent (iTBS) or continuous TBS (cTBS) on the expression of neuronal activity marker proteins in rat cortex.
Methods: Up to four iTBS- or cTBS-blocks of 600 stimuli were applied to urethane-anesthetized rats followed by immunohistochemical and Western blot analyses.
Results: The effects of iTBS and cTBS were similar but slightly differed with regard to the number of stimuli applied. The expression of the 65-kD isoform of glutamic acid decarboxylase (GAD65) increased with each stimulation block, while that of the 67-kD isoform (GAD67), and that of the calcium-binding proteins (CaBP) Parvalbumin (PV) and Calbindin (CB) and that of the immediate early gene c-Fos progressively decreased. Both TBS protocols increased the expression of the vesicular glutamate transporter 1 (VGLUT1) with 1200–1800 stimuli but then decreased them after the 4th block.
Conclusion: Our findings indicate that repeated TBS elicits no simple accumulative dose-dependent effect for all activity-markers but distinct profiles with threshold characteristics and a waxing-and-waning effect especially for the markers of inhibitory activity CB and GAD67. Interestingly, somatic activity markers, such as c-Fos for mainly excitatory and GAD67, CB and PV for inhibitory neurons, decreased with repeated stimulation while synaptic activity markers mainly increased which could be a result of the artificial stimulation of axons.

Introduction

Repetitive transcranial magnetic stimulation (rTMS) is a technique able to modify human cortical excitability in a frequency-dependent manner, with low-frequency rTMS (0.5–1 Hz) reducing cortical excitability and high-frequency rTMS (>2 Hz) increasing it [1,2]. Since 2005 more effective theta-burst stimulation (TBS) protocols have been applied [3]. Mainly two types of TBS protocols are currently in use, which also allow to up- and down-regulate human cortical excitability: continuous and intermittent TBS (cTBS and iTBS). In the case of conventional cTBS, using 600 stimuli in total, two hundred 50 Hz-bursts of 3 pulses each are applied at 5 Hz in a single 40-s train. In iTBS, the train is split into 20 trains of 2 s each, repeated at 10 s intervals. Interestingly, this rather small difference in the stimulation protocol leads to opposite effects: iTBS usually enhances cortical excitability while cTBS decreases it [3], but see Ref. [4]. While the number of possible applications of rTMS is consistently increasing, relatively little is known about the physiological mechanisms underlying the evolution of rTMS effects and what the concrete stimulus conditions are which might lead to facilitation or depression of cortical activity.

Involvement of some forms of synaptic plasticity, analogous to long-term depression and long-term potentiation (LTP) known from in vitro studies [5], is generally accepted as a key concept for understanding rTMS effects [6,7]. Sometimes, however, interpretation of rTMS effects on this basis is difficult, as in the case of cTBS. It is

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suppressing cortical activity although composed of a high-frequency stimulation pattern (5 Hz repetition of 50 Hz bursts). Besides changes in synaptic transmission characteristics, different types of neurons or cortical subsystems, like the excitatory or inhibitory system, may be more or less sensitive to different stimulation protocols, as shown by experimental approaches, like electrophysiological recordings in humans, magnetic resonance spectroscopy and animal studies [8–10]. Moreover, the history of cortical activation is also an important factor in terms of metaplasticity of synaptic transmission and has been shown for combinations with different primer stimulation protocols, motor activity or learning procedures [11–13]. In the case of TBS, changing the number of stimuli within one series [14,15] or repeating a protocol at different intervals [16] can lead to a different outcome regarding modulation of cortical excitability.

In a rat TMS model, we have already demonstrated that cortical excitatory and inhibitory processes are differently affected depending on the rTMS protocol applied, as reflected by changes in the expression of proteins related to cortical activity in general (c-Fos and zif268) and those signaling activity of inhibitory interneurons, like Parvalbumin (PV), Calbindin (CB) and the glutamate decarboxylase isoforms GAD65 and GAD67 [10,17,18]. In the current study, we tested a kind of "dose-dependence" of TBS by applying up to 4 blocks of 600 stimuli each at intervals of 15 min, both for the iTBS and the cTBS protocol.

Materials and methods

Analogous to previous studies [10,17,18], all TBS applications were performed in the lightly sedated rat (adult male Sprague–Dawley, 460–540 g, Elevage Janvier) to minimize stress effects [19]. For immunohistochemical analysis 9 experimental groups were treated with different TBS-protocols. The control groups received sham stimulation (5 animals, 600 pulses), while for verum cTBS and iTBS, 4 groups (3 rats per group each hemisphere analyzed separately) were treated with a different number of stimuli (600, 1200, 1800 and 2400), applied as blocks of conventional cTBS or iTBS (consisting of 600 pulses each [3]), separated by an interval of 15 min (see below). Another 7 animals were used for Western blotting, 3 receiving cTBS (600, 1800 and 2400 pulses; one animal each), 3 receiving iTBS respectively, and one sham-treated control. In this study, we applied only 600 sham stimuli to the control group while 3000 sham-stimuli were applied in previous studies [10,18]. No significant differences were observed between these groups and in relation to naïve control animals [10,18] indicating no effect of sham-stimulation on the cortical expression of the analyzed proteins. Anesthesia (intra-peritoneal injections of urethane: 1.0 g/kg body weight, lowest amount to prevent body motion) was maintained until the rats were transcardially perfused under deep anesthesia (300 mg/kg body weight pento-barbital-sodium i.p., Narcoren©, Rhone Merieux GmbH, Laubheim, Germany) 60 min after the end of the last rTMS block. During this period, scalp EEG recordings were performed above frontal cortical region (0.1–300 Hz, CyberAmp 380, Axon Instruments, Molecular Devices, LLC, Sunnyvale, CA) to check for pathological activity. No significant changes in EEG pattern were observed following TMS. All experiments were performed with permission of the government (AZ. 87-51.04.2010.A097) and the local welfare committee. All procedures were conforming to the guidelines of the animal welfare laws in the EU and United States.

TMS

TMS was applied using a MagStim Rapid device and a 70 mm figure-of-eight coil (The MagStim Company, Whitland, Dyfed, UK). As in previous experiments [10,18], the coil was centered 10 mm above the interhemispheric cleft of the occipital forebrain with the handle pointing to the left to primarily activate supragranular cortical layers of both hemispheres via stimulation of the callosal axons and their collaterals [20,21]. Thereby, all cortical areas connected by the corpus callosum are assumed to be stimulated directly. Stimulus strength was just subthreshold for inducing peripheral motor activity (27–30% of maximal machine-output), corresponding to 37–50 V/m of the mediolateral oriented induced electric field. Due to their length, callosal axons need lower electric field strength to be activated than intracortical axons. For sham stimulation, coil-to-brain distance was increased to 100 mm.

Theta-burst stimulation protocols used followed the description by Huang and colleagues [3], with one iTBS block consisting of 20 trains of ten 50 Hz bursts (3 pulses) repeated at 5 Hz (lasting 192 s with 10 s intervals between trains) and one cTBS block being a single 40 s train of bursts repeated at 5 Hz. In both cases, each block contained 600 pulses. Application of a higher number of stimuli (1200, 1800 and 2400) was done by repeating blocks of cTBS (or iTBS respectively) at intervals of 15 min, resulting in total stimulation period between 192 s and 48.2 min for iTBS (600 and 2400 pulses, resp.) and 40 s to 45.7 min for cTBS (600 and 2400 pulses, resp.).

Immunohistochemistry

Immunohistochemical analysis was done according to earlier experiments (extensively described elsewhere [10,22]. Regions of interest (ROI) were chosen according to [10,22]: frontal cortex (FC, 3.7 mm anterior to bregma), motor cortex (MC, 1.2 mm anterior to bregma), somatosensory cortex (SC, 1.8 mm posterior to bregma) and visual cortex (VC, 4.3 mm posterior to bregma). The following primary antibodies were used: GAD67 (monoclonal, 1:2000, clone 1G10.2; Millipore), GAD65 (polyclonal, 1:2000, Millipore, Billerica, MD, USA), NeuN (Neuron-specific Nuclear antigen, monoclonal, 1:1000, clone A60, Millipore), PV (monoclonal, 1:1000, clone 234, Swant, Bellinzona, Switzerland), CB (monoclonal, 1:1000, clone 300, Swant), c-Fos (polyclonal, 1:1000, Santa Cruz Biotech., CA), VGLUT1 (polyclonal, 1:8000, Millipore) and VGLUT2 (polyclonal, 1:750, Synaptic Systems, Goettingen, Germany). The neuronal marker NeuN served as a control to rule out neuronal cell loss as a possible reason for changes in the number of cells labeled for one of the other markers, and to normalize cell numbers with regard to the total number of neurons within one ROI during the evaluation of TBS-effects. Visualization of specific labeling was done via 3,3′-diaminobenzidine as chromogen.

Western blotting

Samples from all ROIs were prepared and equal amounts of protein from all animals were separated by SDS-PAGE and processed for conventional immunoblotting, using antibodies against PV, CB, GAD65, GAD67 and β-tubulin (Sigma–Aldrich). Immunodetection was performed with horseradish peroxidase-coupled anti-rabbit or anti-mouse IgGs. Subsequent visualization was done with an enhanced chemiluminescence system (GE healthcare). Finally, optical density of protein-labeling was quantified and normalized by the optical density of β-Tubulin (set as 100%).

Data collection and analysis

Brain slices were photographed using a Leitz Wetzlar Dialux 20 microscope (Leica, Germany) equipped with a color video camera (CCD KH 609, Heimann; Metamorph, Universal Imaging). All cell counting procedures (for PV, CB, GAD67 and NeuN) were performed according to earlier studies (for further details see Refs. [10,22,23]). Cell counting was done in a defined cortical area of