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Time-course of changes in neuronal activity markers following iTBS-TMS of the rat neocortex

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HIGHLIGHTS

- We studied the expression of neuronal activity markers after iTBS rTMS in a rat model.
- Increase in c-Fos and GAD65 for 40 min signals excitatory and inhibitory activity.
- Activity markers of inhibitory neurons decrease after about 40 min.
- Late decrease in c-Fos (>120 min) reflects general cortical hypoactivity.
- iTBS rTMS drives excitatory and inhibitory neurons but leads to late hypoactivity.

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ABSTRACT

In a rat model of transcranial magnetic stimulation we could recently show that intermittent theta-burst stimulation (iTBS) affects the neocortical expression of the immediate early gene products c-Fos and zif268 as well as that of the two glutamic acid decarboxylase isoforms GAD65 and GAD67 and that of the calcium-binding proteins calbindin (CB) and parvalbumin (PV), known as markers of excitatory and inhibitory activity. We now analyzed in more detail the time course of changes in the expression of these proteins at 10, 20, 40, 80 and 160 min following a single block of iTBS consisting of 600 stimuli. Initial increase in c-Fos, zif268 and GAD65 (20 min) signals transient activation of excitatory and inhibitory neurons, thereafter first followed by a decrease in markers of activity of inhibitory neurons (GAD67, PV, CB: 20–80 min) and then by a late decrease in c-Fos and GAD65 expression (160 min). The results demonstrate that one iTBS block may have an after-effect of at least two different phases, an early phase with increased neuronal activity (c-Fos, zif268) but also the likelihood of increased GABA-release (GAD65), followed by a late phase (>40 min) of reduced neuronal activity in excitatory and inhibitory systems which may indicate a state of reduced excitability.

1. Introduction

Repetitive transcranial magnetic stimulation (rTMS) of the human brain is able to modify cortical excitability in a comfortable and non-invasive fashion, thereby being a promising therapeutic tool for neuronal diseases [20,24]. Intermittent and continuous theta-burst stimulation (iTBS, cTBS) are two efficient protocols by which changes in human cortical excitability lasting for up to 1 h can be induced within minutes, with iTBS enhancing and cTBS decreasing cortical excitability [11,18]. We recently established a rat model of rTMS showing that the expression of distinct cortical proteins known as activity markers of excitatory and inhibitory neurons is specifically affected in a manner related to the stimulation protocol used [2,4,5,16,25]. Especially the theta-burst protocols iTBS and cTBS appeared to be powerful modulators of the activity of inhibitory neurons since they strongly decreased the expression of the cytosolic isoform of the gamma-aminobutyric acid (GABA) synthesizing enzyme glutamic acid decarboxylase (GAD67) [27] and the calcium-binding proteins calbindin (CB) and parvalbumin (PV) expressed in two different classes of GABAergic interneurons [12,14]. These changes could last several days if animals were housed in a standard cage [2] but could quickly reverse in a cortical area activated during a sensory discrimination task [16]. By contrast, the increase in c-Fos and GAD65 appeared to be short-lived, signalling the direct activation of excitatory neurons but also GABAergic synaptic terminals, respectively [5,16,25]. So far however, we had little knowledge about the exact (early) time course of changes in these activity markers and, therefore, we analyzed these markers at 10, 20, 40 80 and 160 following one block of iTBS. If the early increase in GAD65 and c-Fos is directly related to increased neuronal and synaptic activity during stimulation but does not reflect lasting changes in neuronal excitability, the

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expression of these markers should quickly return to control levels. Changes in the expression of GAD67, CB and PV seem to reflect such lasting changes but so far we do not know how early these effects set in. This is of particular interest with regard to the combination of rTMS with other interventions like specific training procedures or the repeated application of rTMS or other stimulation procedures at distinct intervals. Since rTMS is believed to drive homeostatic processes of synaptic function [21,22,28], repeated cortical stimulation can invert the effect of distinct stimulation protocols [6–8].

2. Materials and methods

2.1. Animals and experimental procedures

As done in a previous study [16], adult male Sprague–Dawley rats ($n = 42$, 580–620 g, 15 weeks old) were trained to tolerate the manual restrain and the noise and sensations related to TMS over a period of 2 weeks using food rewards as positive feedback. One block of iTBS [11] lasting 192 s was applied at an intensity of 23% of the maximal machine output (MagStim rapid, The MagStim Company, Whitland, Dyfed, UK) via a conventional 70 mm figure-of-eight coil to the conscious animal. The handle of the coil was oriented at right angle to the head-body axis of the rat to induce a mediolaterally oriented electric field within the brain, suitable to activate axons of the corpus callosum and thereby inducing synaptic activity primarily in layers 2/3 of most cortical areas [5]. An intensity of 21–23% has previously been determined as being just subthreshold to evoke motor activity in the conscious rat [16]. A fine-tuning was performed by manually varying the mean distance between head and coil (~10 mm) during stimulation to avoid muscle twitches. For sham-treatment, coil-to-head distance was increased to 100 mm. The following deep anaesthesia (sodium-pentobarbital, 300 mg/kg body weight, Narcoren©, Rhone Merieux GmbH, Laubheim, Germany) was appropriately timed to perfuse rats 10, 20, 40, 80 or 160 min after stimulation with physiological saline containing Heparin (5.000 I.E., Liquemin©, Roche, Switzerland), followed by 4% Paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). In case of the 10 min interval, procedures were timed as follows: 0 min: onset of iTBS, 3.5 min: injection of Narcoren, ~6.5 min: start of preparation, ~8.0 min: start of saline perfusion, 10 min: start of Paraformaldehyd perfusion (terminating metabolic processes). All procedures were conforming to the guidelines of the animal welfare laws in Germany, UK and the United States and were performed with the permission of the government (AZ. 87-51.04.2010.A097) and the local animal welfare committee.

2.2. Immunohistochemistry

Immunohistochemical processing of the brain tissue was done as previously described [2,16]. After postfixation (1 day) and cryoprotection in 30% sucrose brains were frozen and cut coronally to sections of 30-μm. Every tenth section was stained with cresyl violet for cytoarchitectural control. After blocking endogenous peroxidase activity (0.3% H_2O_2 for 20 min) and blocking unspecific epitopes (10% normal serum, 20% avidin in PBS containing 0.2% Triton X-100) free-floating slices were exposed overnight to the following primary antibodies diluted in phosphate buffered saline (PBS) containing 20% biotin, 1% normal serum and 0.2% Triton X-100: 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) and c-Fos (polyclonal, 1:1000, Santa Cruz Biotech.). NeuN labelling was used to normalize marker labelling with regard to the total number of neurons within the region of interest (ROI). After processing the sections with matching secondary antibodies (1:500 in PBS, 90 min, room temperature, Vectastain, Vector Laboratories, Burlingame, CA, USA) and a standard avidin-biotinylated peroxidase complex (ABC) kit (1:000, 90 min, room temperature Vectastain, Vector Laboratories), visualization of specific labelling was done via 3,3′-diaminobenzidine as chromogen (0.05% diaminobenzidine and 0.01% H_2O_2 in PBS). Finally sections were rinsed one last time in PBS, dehydrated, cleared in xylene and coverslipped with Depex (Serva, Heidelberg, Germany).

2.3. Statistics

As in previous studies [2,16,25], cells labelled for c-Fos, zif268, GAD67, CB and PV were manually counted at a final magnification of 100× in sectors dorsoventrally extending from the pia mater to the white matter and with a mediolateral width of about 720 μm for the following regions of interest (ROI): frontal cortex (3.7 mm anterior [+] to bregma according to Paxinos and Watson [17]), motor cortex (+1.2 mm), somatosensory cortex (hind paw representation, 1.8 mm posterior [−] to bregma) and visual cortex (−4.3 mm), all about 1.5 mm lateral to midline. Since corpus callosum stimulation obviously activates both hemispheres equally, cell counts of both hemispheres were pooled for each cortical area as done in the previous studies. NeuN-labelled cells were counted automatically as previously described [2] and number of cells labelled for the markers c-Fos, zif268, GAD67, CB and PV were normalized with regard to the total number of NeuN-positive cells within each ROI by expressing marker-labelled cells as percent of the total number of NeuN-labelled cells within each ROI. This way, differences in cell density or variation in the absolute size of the counted area due to tissue shrinkage, which would lead to a different absolute number of neurons within the ROI, were taken into account. Quantification of GAD65 labelling was done by measuring the optical density (OD, at magnification of 50× [19]) of neuropil staining, since it is mainly expressed in the synaptic terminals. First, data of the different cortical regions were separately analyzed but finally pooled because no principal differences were evident. All ROIs showed the same direction of change in marker expression and only a few of the 30 samples given by the six markers and the five time points quantitatively differed (ANOVA with factor ROI and Tukey post hoc tests, see results). Statistical analysis on the pooled data was performed using uni-factorial ANOVA with factor TIME and Mann–Whitney *U*-test for pair-wise comparison of sham and verum iTBS groups. A p-value <0.05 was taken as reference for significant differences in all cases (no Bonferroni correction because of independent samples of sham and verum iTBS treatment for each time point). For reasons of better readability, verum data are shown as mean percent change (±SEM) of the paired sham-group data, because the latter exhibit a more or less pronounced increase in the expression of all marker proteins over time, which may be due to the handling of the animals. On average, 1909 NeuN+ cells, 1454 zif268+ cells, 333 c-Fos+ cells, 98 GAD67+ cells, 43 CB+ cells and 92 PV+ cells were counted per ROI at time point 10 min for the sham groups. We first started with three rats per time point (both, for verum and sham stimulation) to keep number of experimental animals as small as possible, but added further three to the first (10 min) and latest (160 min) time point for statistical reasons (to verify initially weak significant differences especially in case of zif268 and c-Fos, verum & sham group), ending up with a total of 42 rats.

3. Results

Figs. 1 and 2 demonstrate that three different phases of changes in marker protein expression are evident when comparing verum