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Research report

Chronic repetitive transcranial magnetic stimulation enhances c-fos in the parietal cortex and hippocampus

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Abstract

Repetitive transcranial magnetic stimulation (rTMS) is a novel non-invasive method with anti-depressant properties. However, the mechanism of activation on the cellular level is unknown. Twelve hours after the last chronic rTMS treatment (14 days, once per day, 20 Hz, 10 s, 75% machine output, the transcription factor c-fos was markedly increased in neurons in layers I–IV and VI of the parietal cortex and in few scattered neurons in the hippocampus of Sprague–Dawley rats. The cortical activation was not blocked by the NMDA antagonist MK-801. The increase of c-fos was not paralleled by an increased glial response and activation of cortical growth factors. Thus, it is concluded that chronic rTMS differentially activates parietal cortical layers and this might be involved in mediating anti-depressant activity in other brain areas.

Keywords: Transcription factor; Brain; Depression; Cortex

1. Introduction

Repetitive transcranial magnetic stimulation (rTMS) has anti-depressant effects in humans [13,14,16,22,27] and may eventually replace electroconvulsive therapy [30,36]. TMS is a new non-invasive therapeutic approach in psychiatry [2] in which electromagnetic fields are applied via an electrode to the scalp. These electromagnetic waves in turn induce action potentials in the subjacent neuronal tissue. The great advantages of TMS for the patients are, that no anaesthesia is needed and it is painless and not associated with significant side effects. Some data suggest that TMS and electroconvulsive shock (ECS) share common neurobiological effects, although acute TMS was by far not as efficient in activating c-fos in definite brain regions compared to ECS [20]. Acute TMS down-regulated cortical beta-adrenergic receptors and decreased cortical cAMP generation [11]. The acute effect of TMS might seem to be mediated by an initial increased release of serotonin because TMS caused an acute reduction in serotonin content in the hippocampus [3]. A recent report showed that acute rTMS increased the number of serotonin 5-HT_{1A} and glutamate NMDA binding sites in some brain areas [23]. Post et al. [29] demonstrated that rTMS had a neuroprotective effect against oxidative stressors such as amyloid-beta and glutamate.

Immediate early genes, including those of the fos–jun family, are induced by different stimuli [7,25]. The expression of c-fos is proposed to be a rapid indicator for neuronal activation and to be a suitable marker to study activation after different stimuli. Strong central stressors, such as seizures induced by kainic acid [28], kindling [9], electrical stimulation [32] or ischemia [28], activate the c-fos gene. Mild physiological stressors such as changes in the environment [15] or light [1,31] also may induce c-fos expression in different brain areas.

The aims of this study were (1) to observe the effects of chronic rTMS treatment on the expression of the immediate early gene c-fos in the rat brain, (2) to determine if glutamate is involved in a possible induction of c-fos mRNA, and (3) to investigate whether this new anti-depressant therapeutic strategy leads to glial activation.

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2. Materials and methods

2.1. Chronic rTMS treatment

Adult 250-g male Sprague–Dawley rats ($n = 5$, stimulation group) were chronically treated for 14 days with rTMS. The rats were fixed by hand and the figure-8-coil (2.3 cm) was held over the head of the rats without touching the skull. The handle of the coil was placed parallel to the vertebral column of the rat. The rTMS treatment was performed with a Magstim 200 rapid stimulator (Magstim, Whitland, UK). The stimulation consisted of one train (20 Hz) administered over 10 s (with 75% machine output). Control animals ($n = 5$, sham group) were handled in the same way, but were only exposed to the noise of the coil (in 1 m distance). All rTMS applications were performed once per day between 06.00 and 07.00 h. The rTMS treatment did not produce either notable seizures or changes in behavior. Another group of animals was treated with 0.2 mg/kg MK-801 about 15 min before stimulation with rTMS ($n = 4$, stimulation-MK-801 group) or before sham-treatment ($n = 4$, sham-MK-801 group). Twelve hours after the last stimulation, the animals were processed for in situ hybridization or immunohistochemistry.

2.2. In situ hybridization

Radioactive in situ hybridization using oligonucleotides was performed as described recently by us [10,21]. Antisense oligonucleotides against c-fos (5′-GAC GGA CAG ATC TGC GCA AAA GTC CTG TGT-3′ [4,7], brain-derived neurotrophic factor (BDNF) (5′-CTC CAG AGT CCC ATG GGT CCG CAC AGC TGG GTA GGC CAA GTT GCC TTG-3′ [19], fibroblast growth factor-2 (FGF-2) (5′-GCC GCC GTC CTC CGG CAG TGC GGG AAG CGA AGT GAT GCT GCC GGC AGC-3′ [18], and glial fibrillary acidic protein (GFAP) (5′-GAT CTC CTC CTC CAG CGA CTC AAC CTT CCT CTC CAG ATC-3′ and 5′-CTC TGG CTA GAG ATT CTT TGC CTC GGG ATG TTT TCC TTT-3′, GeneBank Accession L27219) were commercially synthesized by MWG Biotech (Germany). Briefly, animals were decapitated, the brains frozen in a CO_2 stream, sectioned in a cryostat (14 μm) and stored at −20°C until used. Oligonucleotides were labeled at the 3′ end with [α-^{35}S]dATP using terminal deoxyribonucleotidyl transferase (NEN, DuPont, Austria) and purified using the Qiagen nucleotide removal kit. Thawed sections were hybridized at 42°C overnight in a humidified chamber with 0.1 ml per slide of the hybridization solution (50% formamide, 4× SSC, 0.02% polyvinyl-pyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin, 10% dextrane sulfate, 0.5 mg/ml sheared salmon sperm DNA, 1% sarcosyl (*N*-lauroyl sarcosine), 0.02 M phosphate buffer (pH 7.0), 50 mM dithiothreitol) containing 1×10^7 cpm/ml probe. Sections were subsequently rinsed, washed four times (15 min each) at 54°C in 1× SSC, cooled to room temperature, dehydrated through 70%, 90% and 99% ethanol and air-dried. Sections were exposed on X-ray film for 5 weeks. Other sections were dipped in Kodak NTB-3 photo emulsion (diluted 1:1 in water), exposed for 6 weeks at +4°C, developed, fixed, and lightly counterstained with Cresyl violet. Sections were analyzed using light and dark-field microscopy (Olympus BH-2) and photographed on Kodak Technical-pan film.

2.3. Immunohistochemistry

Immunohistochemistry, using the avidin–biotin technique, was performed as described previously [34]. Animals were anesthetized and transcardially perfused with 50 ml cooled isotonic saline, followed by 200 ml of cooled fixative (4% paraformaldehyde (w/v) in 0.1 M PBS). The brains were immersed in the fixative at 4°C for 90 min and rinsed in 10% sucrose in 0.1 M PBS during 48 h. Coronal brain sections (20 μm) were taken throughout the dorsal hippocampus using a cryostat. All sections were rinsed three times in 50 mM Tris-buffered saline (TBS), blocked in 10% horse serum and incubated with a primary polyclonal c-fos antibody (1:1000, Santa Cruz Biotechnology) in a humidity chamber overnight at room temperature. Antisera were diluted in TBS containing 0.1% Triton X-100. The sections were washed and incubated with secondary biotinylated anti-rabbit antibody (1:100, Vectastain) for 1 h at room temperature. After washing, sections were incubated in Vectastain reagent for 30 min, washed in TBS and the signal detected by using 0.5 mg/ml 3,3′-diaminobenzidine (DAB) including 0.003% H_2O_2 as a substrate. The sections were dehydrated and embedded in Entellan. Unspecific staining was defined by omitting the primary antibody.

2.4. Evaluation of sections and statistics

The expression of c-fos mRNA on X-ray films was analyzed by using a computer-assisted image analysis system (Image pro Plus Software, connected to an Olympus BX60 microscope via a Sony video camera). Representative X-ray sections were quantified on a grey scale between 0 and 255, where 0 represents black and 255 white. To obtain specific values, brain areas with unspecific background levels were subtracted from brain areas with specific signal. The number of c-fos positive nuclei was counted in a calibrated 0.29 mm^2 square in all cortical layers under the microscope. Multistatistical analysis was obtained by one-way ANOVA, followed by a subsequent Fisher post-hoc test by comparing controls against the respective treatments, where $p < 0.05$ represents significance.

3. Results

3.1. In situ hybridization for c-fos mRNA

In control brains, c-fos mRNA was detectable in a low number of scattered neurons in the parietal cortex (Fig.

1A, C, E) and in the hippocampus (Fig. 4A). Twelve hours after chronic rTMS (14 days), neuronal c-fos mRNA expression was markedly enhanced in layers I–IV and VI of the parietal cortex (Fig. 1B, D, F; Fig. 3A). Only scattered neurons in the hippocampus showed enhanced c-fos expression (Fig. 4B). The c-fos mRNA was not increased in

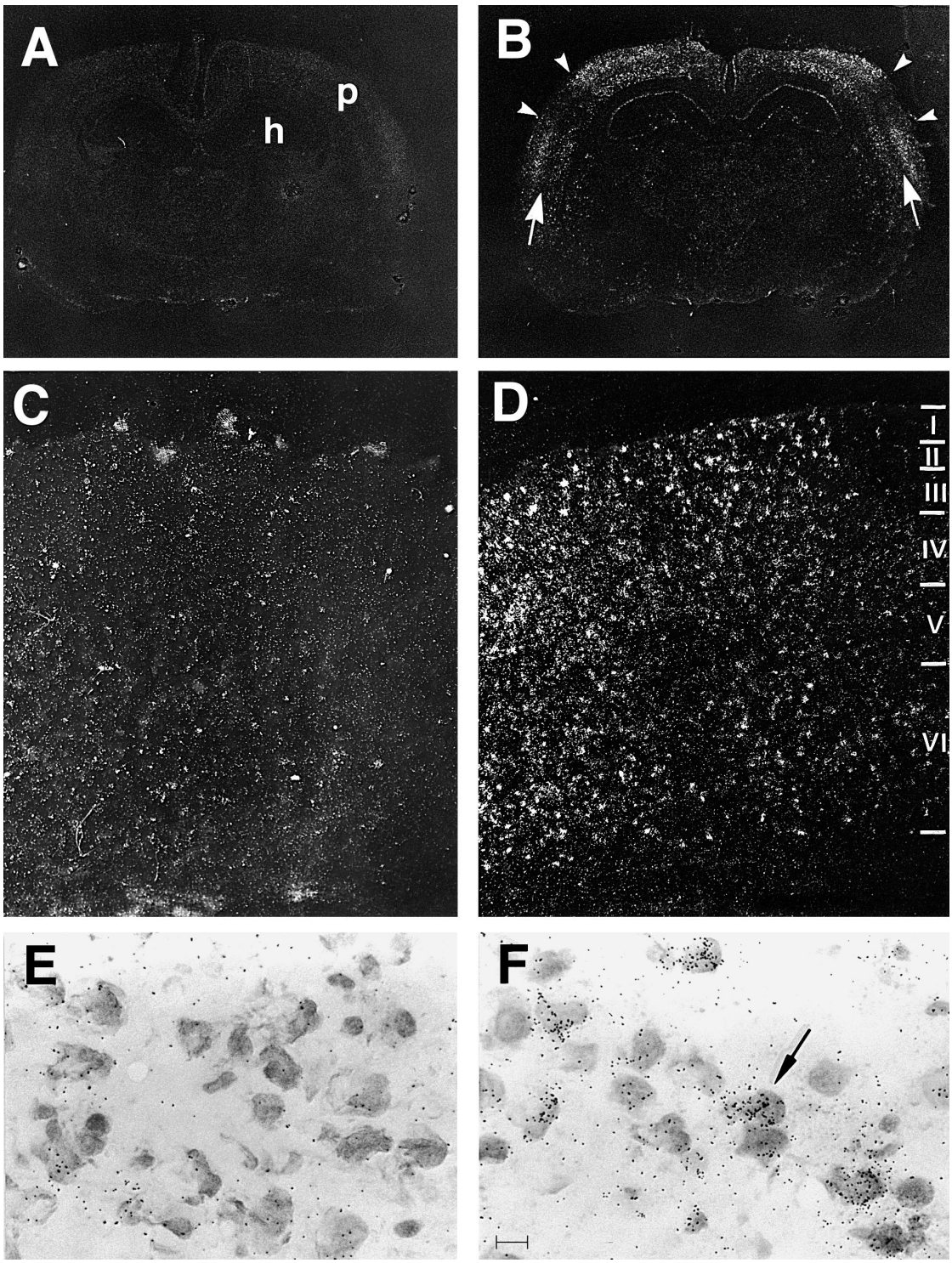

Fig. 1. In situ hybridization shows expression of c-fos mRNA in controls (A, C, E) or chronic rTMS-treated (B, D, F) rats. Rats were treated for 14 days with or without rTMS, and analyzed 12 h after the last stimulation. When rats were stimulated with rTMS, a marked increase in c-fos mRNA in the parietal cortex (p) and hippocampus (h) compared to control rats was seen. Note the specific layering (arrows in B) and distinct cortical profiles (arrowheads in B). The increase was found predominantly in layers I–IV and VI (B, D). Bright field microscopy presents the specific silver grains located over Cresyl violet counterstained cells of rTMS-treated rats (F, arrow) compared to controls (E). Scale bar = 1400 μm (A, B), 150 μm (C, D), 20 μm (E, F).

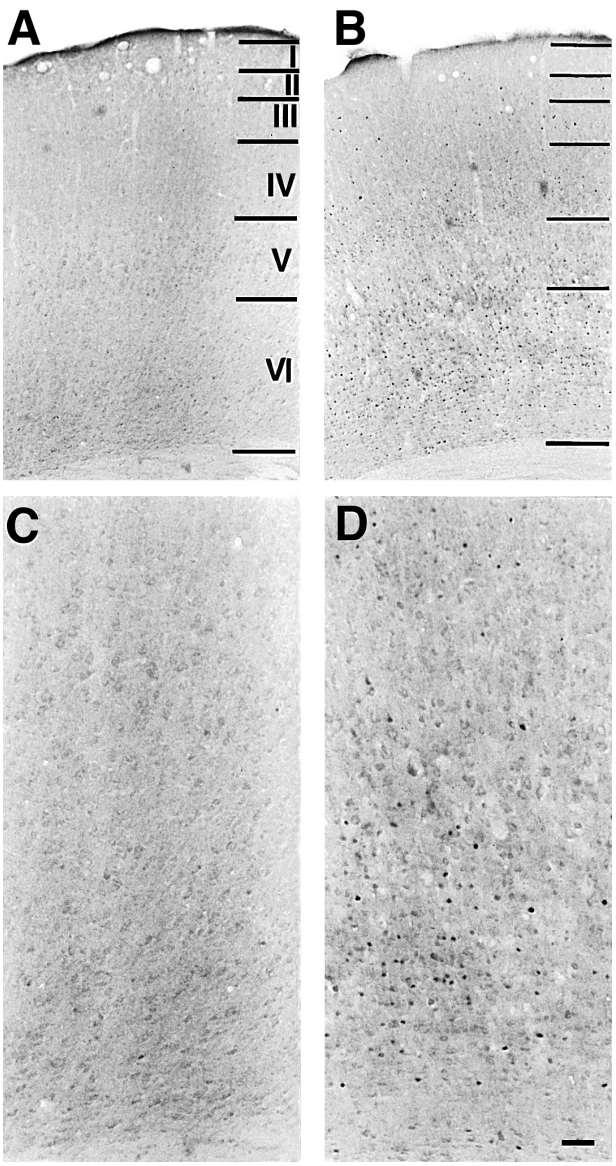

Fig. 2. Immunohistochemistry shows c-fos-like immunoreactive positive neuronal nuclei in the parietal cortex of controls (A, C) or chronic rTMS-treated (B, D) rats, as analyzed 12 h after the last stimulation. Controls (A, C) display only few scattered single c-fos positive nuclei in the cortical layers (A). In rTMS-treated rats, an increase in c-fos positive nuclei is found predominantly in the layer VI of the parietal cortex (D). Scale bar = 150 μm (A, B), 50 μm (C, D).

layer V (Fig. 1B, arrows; Fig. 3A). A distinct region of parietal cortex on both sides of the brain was not activated and did not reveal enhanced c-fos mRNA expression (Fig. 1B, arrowheads). No effect of MK-801 pre-treatment on c-fos induction was observed (Fig. 3B). Pre-treatment of animals with MK-801 prior to rTMS stimulation did not alter the c-fos expression pattern (Fig. 3B). The c-fos mRNA expression was not significantly different when comparing animals treated or not treated with MK-801 (Fig. 3A, B).

3.2. Immunohistochemistry for c-fos-LI

In control animals, the number of c-fos-positive neuronal nuclei was low in all layers of the parietal cortex (Fig. 2A, C). Chronic rTMS treatment significantly increased c-fos-like immunostaining exclusively in layer VI of the parietal cortex (Fig. 2B, D). In the parietal cortex,

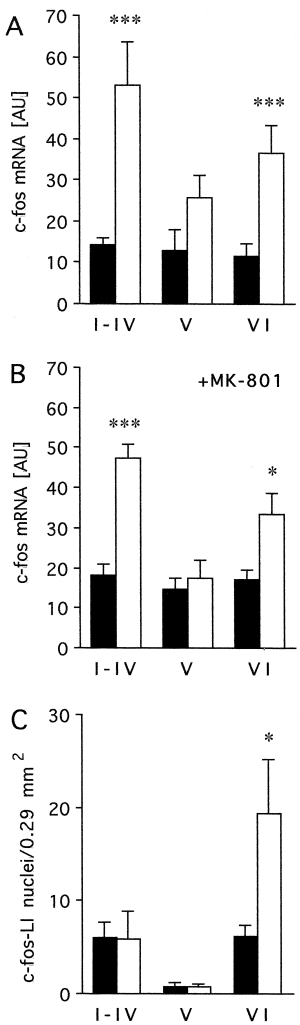

Fig. 3. Quantitative analysis of c-fos mRNA and c-fos-like nuclei in parietal cortical layers I–IV, V, and VI after 14 days of chronic rTMS, as measured 12 h after the last treatment. The c-fos mRNA density was measured in controls (filled bars) and rTMS-treated (open bars) rats using a computer-assisted image analysis system (Panel A). Panel B shows c-fos mRNA density in controls pre-treated with 0.2 mg/kg MK-801 alone (filled bars) and rats pre-treated with 0.2 mg/kg MK-801 and then exposed to rTMS (open bars). Values are given as arbitrary units [AU]. Areas were measured in samples at both sides of the brain, and then averaged ($n = 5$). Panel C presents the quantitative analysis of c-fos-like positive nuclei in controls (filled bars) and rTMS-treated rats (open bars). Positive c-fos-like nuclei were counted under the microscope using a 20× magnification in an area of 0.29 mm^2 on both sides of the cortex. Values are given as mean ± S.E.M. ($n = 4–6$). Statistical analysis was performed by one-way ANOVA with subsequent Fisher PLSD post-hoc test ($^{*}p < 0.05$; $^{***}p < 0.001$).

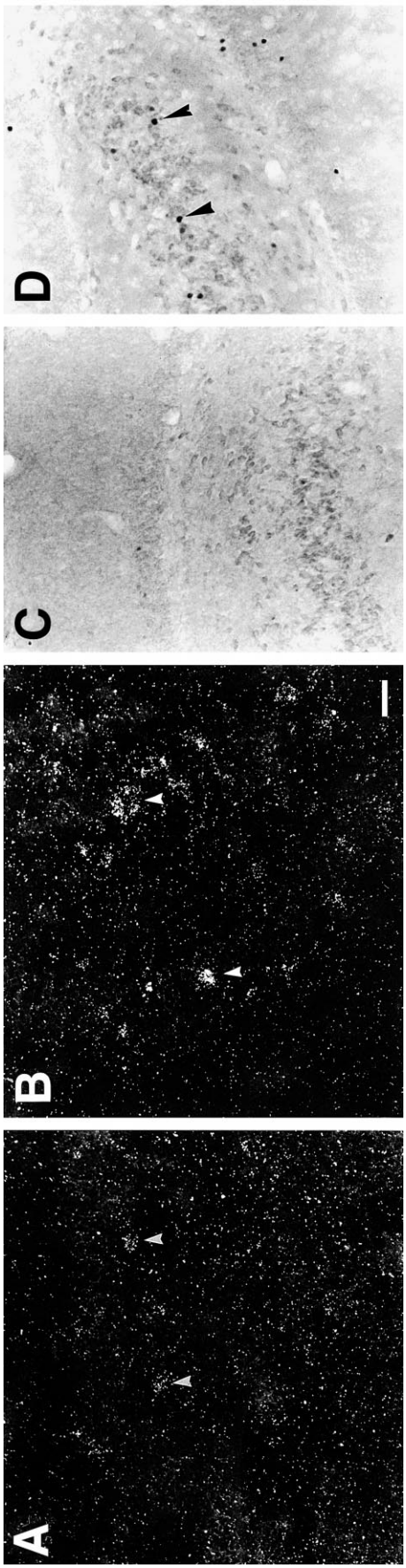

Fig. 4. C-fos mRNA and protein in the hippocampus of sham (A, C) or chronic rTMS-treated rats (B, D) as determined by in situ hybridization (A, B) and immunohistochemistry (C, D), respectively. In rTMS-treated rats only scattered cells (arrowheads) in the hippocampus (hilar region of dentate gyrus) showed increased c-fos mRNA expression compared to controls. Note the nuclear staining of c-fos-LI. Scale bar = 50 μm.

the number of c-fos positive nuclei was about three times increased compared to controls (Fig. 3C). In the hilus of the hippocampus, very few c-fos positive nuclei were seen (Fig. 4C), but chronic rTMS treatment increased the number of c-fos positive nuclei (Fig. 4D).

3.3. In situ hybridization for GFAP, BDNF and FGF-2

GFAP mRNA was expressed at a low level in the parietal cortex and hippocampus in controls (data not shown). BDNF mRNA expression was high in the dentate gyrus and in pyramidal neurons of the hippocampus and weak in the parietal cortex in controls (data not shown). FGF-2 mRNA expression was strongly expressed in the CA2 and medial CA1 region of the hippocampus in controls (data not shown). Chronic rTMS treatment did not significantly enhance cortical GFAP ($110 \pm 16\%$ of control), BDNF ($94 \pm 9\%$ of control) or FGF-2 ($92 \pm 8\%$ of control) mRNA expression 12 h after the last stimulation.

4. Discussion

The present study shows a significant induction of c-fos mRNA and protein after chronic rTMS stimulation in the rat cortex and hippocampus. Our data point to a specific activation of a definite pattern in the parietal cortex, by increasing c-fos mRNA in layers I–IV and VI but not layer V and c-fos-like protein in layer VI. In addition, a small part of the parietal cortex was not activated by rTMS, suggesting that only the activated cortical areas might be involved in mediating the effects of rTMS. In fact, there is strong evidence that rTMS treatment affects cortical brain areas. Kole et al. [23] reported substantial increases in receptor binding in selected layers of the parietal cortex after acute rTMS treatment. In Parkinsonian patients, rTMS improved symptoms, possibly by an increased cortical inhibition in the motor cortex [24]. In the auditory cortex of gerbils, rTMS treatment induced both long term potentiation and long term depression [35]. Our data indicate that a constant chronic high frequency rTMS treatment stimulates cortical neurons and yet unknown cortico-fugal pathways. Such an activation might further induce neurotransmitter systems related to depression, such as, e.g., the raphe nuclei or the locus coeruleus, which are the site of synthesis of the neurotransmitter serotonin and noradrenaline, respectively. Such a hypothesis is in line with Ben-Shachar [3] showing altered serotonin release due to acute TMS treatment. It seems possible that, e.g., cortico–thalamo-raphe pathways may mediate the anti-depressant activity of rTMS.

Strong stimuli such as epilepsy or ischemia normally increase c-fos mRNA rapidly within 30 min and c-fos-like protein within 3 h, which transiently declines to baseline levels within 3–6 h [33]. It is interesting to note that chronic rTMS treatment increased c-fos mRNA 12 h after the last stimulation. This indicates that the c-fos increase is not only a stress-mediated effect due to handling of the animals but a selective effect due to the rTMS stimulation. Recently, Onodera et al. [26] demonstrated that the c-fos mRNA expression in the rat neocortex is dependent on the circadian rhythm and transiently increased at the beginning of the light phase (between 08.00 and 09.00 h). In our study, the rTMS treatments were performed between 06.00 and 07.00 h and the brains analysed 12 h later (between 18.00 and 19.00 h), thus any changes found after rTMS are not related to circadian rhythms. Our data also indicate, that the c-fos mRNA expression is chronically upregulated and not transient after daily rTMS stimulation. Such a chronic upregulation might indicate permanent activation of cortical neurons giving rise to cortico-fugal pathways. However, the increase of c-fos mRNA was not paralled by an increase of c-fos protein in layers I–IV. It seems possible that the enhancement of c-fos protein in these layers is delayed and may occur at earlier or later time points, while the increase in layer VI is clearly time-associated with a chronic expression of cortical c-fos mRNA.

Only one recent study has evaluated the effects of rTMS application on c-fos expression in rat brain, and this showed that an acute single rTMS train markedly enhanced c-fos mRNA in the cingulate and frontal but not parietal cortex [20]. The most pronounced increase in c-fos after acute rTMS was found in the paraventricular nucleus of the thalamus and in regions involved in circadian rhythms [20]. Repeated acute rTMS (three trains) enhanced the effects slightly, being comparable to ECS at least in the cingulate and frontal cortex [20]. Our study shows for the first time that chronic rTMS markedly increased neuronal activity in the parietal cortex with a less intense effect in few neurons in the hippocampus. Taken together, the data suggest that acute ($1–3 \times$ /day) rTMS markedly differs from chronic ($1 \times$ /day for 14 days) rTMS. Thus, while a single acute rTMS was not able to provoke any activation in the parietal cortex, chronic rTMS did. In fact, such a chronic treatment more likely resembles the in vivo conditions used for anti-depressant therapy in patients. Our regimen is comparable to the regimen used in the anti-depressant treatment in humans where 10 trains per day are applied over 12 days with 20 Hz at 80% machine output [14] or 10 Hz at 90–100% machine output [27]. A repeated stimulation with rTMS seems to be an essential paradigm for activating neurons in the parietal cortex.

The increase of c-fos by different stimuli, such as epilepsy or ischemia, is normally mediated by glutamate. In both the cerebral cortex and the hippocampus, glutamate-induced increases in c-fos mRNA are inhibited by the glutamate NMDA antagonist MK-801 [5]. However, MK-801 treatment alone increases c-fos mRNA in several cortical areas and in the midline thalamic nucleus [8]. Castren et al. [6] showed that 1 mg/kg MK-801 enhanced BDNF mRNA in the rat cortex, indicating neurotoxic effects. In our experiments a dose of 0.2 mg/kg MK-801

was used to avoid such unspecific stimulation. Pre-treatment with MK-801 did not block the rTMS-enhanced c-fos expression in the parietal cortex. This indicates that the chronic rTMS effects are not mediated by the neurotransmitter glutamate acting on NMDA receptors, but rather that rTMS treatment directly activates a specific pattern of intrinsic or cortico-fugal neurons. While the anti-depressant effects of ECS seem to be mediated by an induction of seizures and subsequent release of glutamate, rTMS likely exhibits its anti-depressant activity via an alternative mechanism. Thus, the induction of seizures seems not to be essential for the anti-depressant effects of rTMS.

A recent study reported that acute rTMS (30 trains at 25 Hz) markedly enhanced the glial marker GFAP in the hippocampus after 24 h [12]. Such a finding indicates glial reactions, which are similar to those seen after brain injury [12]. In our experiments, chronic rTMS treatment did not enhance GFAP levels in the parietal cortex, indicating that no glial responses occurred. The c-fos stimulation in the parietal cortex after chronic rTMS treatment was also not paralleled by an increase of two sensitive trophic factors; neither BDNF nor FGF-2 (also bFGF) was enhanced in the parietal cortex after chronic rTMS treatment. In fact both trophic factors are highly sensitive markers activated after brain injury. FGF-2 especially is expressed in astrocytes and is increased after reactive gliosis or cell death [17,18]. Our data suggest that chronic rTMS does not evoke any reactive glial responses in the brain, indicating the non-damaging nature of chronic rTMS.

In conclusion, our data show that chronic rTMS has a layer specific effect on neurons in the parietal cortex, without inducing glial reactions. Such a cortical activation might further activate related associated brain regions which play a role in the anti-depressant effect of rTMS. This study helps to clarify the neuronal basis of rTMS as a new therapeutic tool.

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