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Short Communication

Chronic Repetitive Transcranial Magnetic Stimulation (rTMS) Does Not Affect Tyrosine Hydroxylase (TH) and Dopamine-β-Hydroxylase (DBH) Expression in Rats In Vivo

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Repetitive transcranial magnetic stimulation (rTMS) is a new tool in psychiatry in which a pulsed electric current is applied to the scalp via a coil (Post and Keck, 2001). The electric current generates a magnetic field which passes the skull to depolarize the subjacent neuronal tissue (Roth et al., 1991). rTMS has been proposed to exert therapeutic effects in diseases associated with an altered dopaminergic and/or noradrenergic neurotransmission, namely, depression (George et al., 2000) and Parkinson's disease (Siebner et al., 1999). This is in line with Keck et al. (2000), who described enhanced hippocampal release of serotonin and dopamine in rats following rTMS.

The catecholamines noradrenaline (NA) and dopamine (DA) serve as neurotransmitters both in the peripheral and central nervous system. Mesencephalic tyrosine hydroxylase (TH) is the key enzyme for DA synthesis, while TH and dopamine-β-hydroxylase (DBH) are expressed in the noradrenergic locus coeruleus. There is evidence that the upregulation of the catecholamine-synthesizing enzymes and a subsequent enhancement of monoamine-mediated neurotransmission accompanies the therapeutic effect of most antidepressant agents in the rat (Blier and de Montigny, 1994). The aim of this study was to investigate if chronic rTMS is able to affect late gene and protein expression of TH and DBH in vivo in the rat.

Six adult 250 g male Sprague Dawley rats were chronically treated for 14 days with rTMS. Stimulation was performed using a small-sized figure-8-coil of 2.3 cm outer diameter manufactured by Magstim Co. (Whitland, UK). A small coil was chosen because Weissman et al. (1992) showed that the efficacy of magnetic stimulation is smaller the smaller the stimulated brain is compared to the size of the coil. In order to easily evaluate the induced electric field they established a rule of thumb distilled from a mathematical model in which they compared peak electric fields induced by magnetic coils of different sizes. This model allows prediction of peak electric field above a butterfly coil calculated for spherical targets of 0–6 cm radius. In our model the coil used (2.3 cm) theoretically produces an electric field of 0.56 V/cm (Volt/cm) covering an area with a radius of 3.6 cm. Thus, the rat brain as a whole is stimulated. Rats were gently restrained by hand and the coil was held over the head without touching the skull. The middle of the central segment of the figure-8-coil, which is known to induce the largest electric field (Maccabee et al., 1990), was positioned at the center of the rat's skull. The handle of the coil was placed parallel to the vertebral column of the animal.

rTMS treatment was performed with a Magstim rapid stimulator as described previously (Hausmann et al., 2000). Stimulation consisted of one train (20 Hz) administered over 10 sec (with 75% machine output, representing approximately 1.0 T). Six control animals were handled similarly but were only exposed to the acoustic artifact of stimulation (1 m distance). All rTMS applications were performed once per day between 6 and 7 AM. The rTMS treatment did not produce seizures nor behavioral changes. Twelve hours after the last stimulation the animals were processed for in situ hybridization or immunohistochemistry.

In situ hybridization was performed as described previously by Hutter et al. (1996). Briefly, animals were decapitated, the brains frozen in a CO_2 stream,

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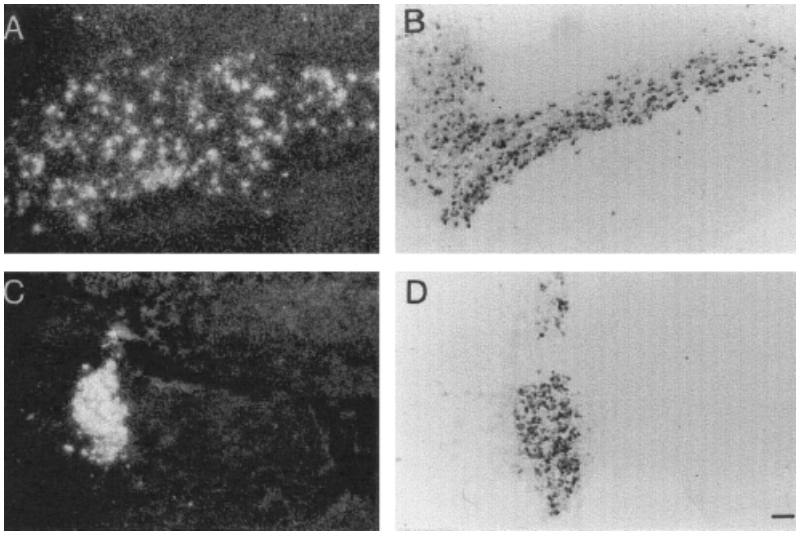

Fig. 1. Rats were treated for 14 days with rTMS (one train per day 10 sec, 20 Hz, and 75% machine output) or sham-treated and analyzed 12 h after the last stimulation. In situ hybridization (**A,C**) shows expression of TH-mRNA in the ventral mesencephalon (**A**) and DBH-mRNA in locus coeruleus (**C**) of rat brains. Immunohistochemistry (**B,D**) shows TH-like immunoreactivity in the ventral mesencephalon (**B**) and in the locus coeruleus (**D**) of chronically stimulated rats as analyzed 12 h after the last stimulation. **A,C:** Darkfield microscopic pictures; **B,D:** brightfield pictures. Scale bar = 85 μm (**A,D**); 165 μm (**B**); 45 μm (**C**).

sectioned in a cryostat (14 μm), and hybridized with 35-S-labeled oligonucleotides. Sections were dipped in Kodak NTB-3 photoemulsion, exposed for 6 weeks at 4°C, developed, fixed, and analyzed. Immunohistochemistry using the avidin-biotin technique was performed on fresh frozen sections as described in detail by Hausmann et al. (2000). Briefly, fresh-frozen 14 μm sections were fixed, washed, blocked, and incubated with a primary polyclonal TH antibody (1:100, Chemicon, Temecula, CA) overnight at room temperature. Since the DBH-specific antibody did not work on fresh-frozen tissue, the well-established TH-antibody was used for mesencephalic as well as for locus coeruleus (LC) neurons. Sections were washed and incubated with secondary biotinylated anti-rabbit antibody (1:100, Vectastain), washed again, and incubated in Vectastain reagent. The signal was detected by using 3,3′-diaminobenzidine (DAB) as a substrate. A computer-assisted image analysis system (Image pro Plus Software, connected to an Olympus BX60 microscope via a Sony video camera) was used to analyze brain sections. Multistatistical analysis was obtained by one-way ANOVA, followed by a subsequent Fisher post-hoc test by comparing controls against the respective treatments, where $P < 0.05$ is significant.

In situ hybridization revealed a strong signal for TH mRNA in the ventral mesencephalon, including the substantia nigra and ventral tegmental area (Fig. 1A). The staining was localized over neurons and silver grains were found exclusively associated with neurons (not shown). In situ hybridization for DBH mRNA showed a very strong positive signal in the LC (Fig. 1C). Immunohistochemistry was performed for TH on sections of the ventral mesencephalon (Fig. 1B) and LC (Fig. 1D). This antibody revealed a strong and specific staining of fresh-frozen sections and the pattern was identical to the mRNA expression. Immunohistochemistry using the DBH antibody showed high background staining on unfixed fresh-frozen sections (not shown).

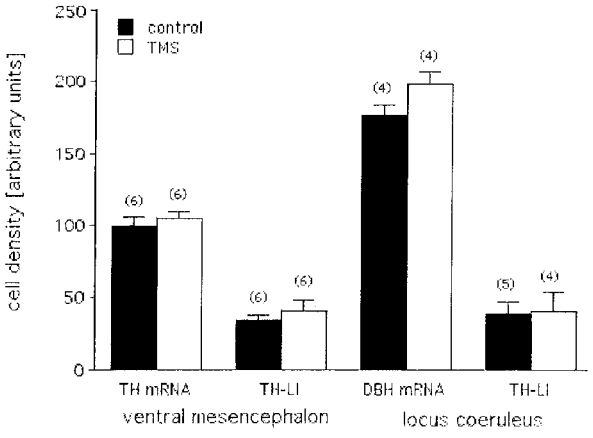

Fig. 2. Quantitative analysis of gene and protein expression of TH and DBH in the ventral mesencephalon or locus coeruleus. Animals were treated for 14 days with rTMS (open bars) and analyzed 12 h after the last treatment compared to sham-treated rats (filled bars). The density of mRNA expression and protein-like immunoreactivity was measured using a computer-assisted image analysis system. Values are given as mean ± SEM arbitrary units (AU). Values in parentheses give the number of analyzed animals. Statistical analysis was performed by one-way ANOVA with subsequent Fisher PLSD post-hoc test.

Quantitative analysis (Fig. 2) of sham-treated animals revealed 99 ± 6 arbitrary units (n = 6) for TH-mRNA, 176 ± 8 AU (n = 6) for DBH-mRNA, 34 ± 4 AU (n = 6) for TH like protein (TH-LI) in the mesencephalon and 38 ± 9 AU (n = 6) for TH-LI in the LC. Comparing sham-treated animals with rTMS-treated animals (statistical analysis using ANOVA) did not show a statistical difference for all measured parameters (Fig. 2).

We have recently shown that magnetic stimulation of the brain markedly induces c-fos mRNA in the brain (Hausmann et al., 2000). Thus, it was the rationale of this study to investigate if late gene and protein expression of TH and DBH may be altered after magnetic stimulation, which could be possibly linked to c-fos

activation. However, our present study shows that magnetic stimulation of the brain does not affect either the expression of DA- or NA-synthesizing enzymes. Our data were obtained using in situ hybridization and immunohistochemistry at the cellular level and quantitative analysis was performed by computer-assisted image analysis. The publication of negative results is often very difficult to interpret, especially if there are no positive controls included. However, both techniques (in situ hybridization and immunohistochemistry) are well established in our laboratory and we have shown, e.g., that DBH mRNA expression in the LC was significantly decreased after an axotomy lesion using an even smaller number of animals (Hutter et al., 1996). We also have measured TH-positive DA neurons and nerve fibers in the mesostriatal system using computer-assisted image analysis (Schatz et al., 1999). We are fully aware of the problem that semiquantitative analysis alone may not be sufficient to quantify small changes in gene or protein expression. While additional quantitative methods, like Northern blots or radioimmunoassays or DA/NA analysis using HPLC/EC detection may overcome this problem, the observed data did not justify use of additional animals for in vivo experiments with a large number of rats.

Although the present data do not show changes in the expression of the DA- and NA-synthesizing enzymes after magnetic stimulation, these data may help to add additional information about the effects after magnetic stimulation. First, these data may suggest that other neurotransmitter systems are primarily involved or activated after magnetic stimulation in the brain. In fact, glutamate is the predominant excitatory neurotransmitter and all corticofugal pathways are believed to use glutamate and activation of cortical c-fos could be linked to glutamate activation. This is strengthened by a study showing that activation of NMDA receptors generate long-term potentiation (LTP), an effect of cerebral plasticity previously described as an rTMS-induced phenomenon (Wang et al., 1996). Alternatively, magnetic stimulation may also stimulate the serotonin system, which plays a predominant role in depression. In fact, Keck et al. (2000) have shown that TMS mediates the serotonin content in the hippocampus. Second, other mechanisms may play a more important role after magnetic stimulation, like modulation at the receptor level, by, e.g., changing the receptor expression in the brain or altering the receptor affinities.

In conclusion, our data show that chronic rTMS does not directly affect gene and protein expression of dopaminergic and noradrenergic neurons in rats. It seems promising to study whether dopaminergic or noradrenergic receptors are affected by chronic rTMS in vivo and may mediate the antidepressant effect in humans.

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