Full Paper

Effects of Acute Repetitive Transcranial Magnetic Stimulation on Extracellular Serotonin Concentration in the Rat Prefrontal Cortex

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Abstract. Repetitive transcranial magnetic stimulation (rTMS) changes the function of the cortex. This study clarified the effects of acute rTMS treatment on extracellular serotonin (5-HT) concentrations in the rat prefrontal cortex (PFC) by using in vivo microdialysis methods. Each rat received acute rTMS treatment of the frontal brain at 500 stimuli from twenty trains applied at 25 Hz for 1 s at 1-min intervals between trains. Sham-treated rats received the same handling procedure and sound of the stimulator. Sham treatment increased the extracellular 5-HT levels compared with the non-treated group. However, rTMS treatment using the stimulation intensity of 110% motor threshold eliminated the increase in 5-HT levels induced by the sham treatment. Acute rTMS treatment of the frontal brain is related to the serotonergic neuronal system in the rat PFC, and it may have therapeutic implications for emotional disorders.

Keywords: transcranial magnetic stimulation, in vivo microdialysis, prefrontal cortex, serotonin, dopamine

Introduction

Transcranial magnetic stimulation (TMS) that uses a pulsed magnetic field generated by a conducting coil influences regional electrical activity in the brain (1). The rapidly changing magnetic field produces electrical currents that activate neurons (1). TMS is used to cause muscle contraction by trans-synaptic stimulation of the pyramidal tract (2, 3). Treatment with trains of repetitive TMS (rTMS) can change the cortical function (4, 5) and is thought to have therapeutic effects such as antidepressive effects (6, 7). Acute application of rTMS to the dorsolateral prefrontal cortex (PFC) induces reductions in anxiety associated with a contralateral increase in theta activity (8).

The elevated plus-maze (plus-maze) test is one of the most widely used non-conditioned animal models of anxiety (9). It is well characterized and has been extensively validated pharmacologically as well as ethologically (10). Some studies have provided evidence for a possible relationship between the serotonergic neuronal system and anxiety (11–14). The amount of

released brain serotonin (5-HT) in guinea pig frontal cortex (11) and rat hippocampus (12) increases by placing rats on the plus-maze according to in vivo microdialysis. Rex and Marsden (11) reported that the extracellular concentration of 5-HT increases in the frontal cortex during the plus-maze test and that when diazepam, an established anxiolytic drug, is administered, the time spent in the open arms and the number of entries into open arms increase in the plus-maze test and the 5-HT increase is inhibited in the frontal cortex. A three-day series of rTMS treatment increases the time spent in the open arms and the number of entries into open arms during the plus-maze test and suppresses the increases in the extracellular 5-HT levels induced by the plus-maze test (15).

Effects of acute and chronic rTMS on neurotransmitters, such as 5-HT, in the rat brain have been described previously. Acute rTMS treatment selectively increases 5-HT_{1A} receptor numbers in the frontal cortex (16), a finding that suggests that rTMS may affect the 5-HT_{1A} system through the 5-HT_{1A} receptors. However, acute rTMS treatment causes no significant change in the amount of 5-HT in both the frontal cortex and striatum, calculated by using brain homogenates (17). Chronic rTMS reduces the sensitivity of somatodendritic

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5-HT_{1A} autoreceptors and presynaptic 5-HT_{1B} autoreceptors (18). Thus, 5-HT levels at the synaptic cleft may increase by rTMS treatment. However, according to an *in vivo* microdialysis method, intrahippocampal release of 5-HT does not change significantly after acute rTMS (19), and chronic rTMS treatment shows no changes in tissue monoamine levels in the frontal cortex (20).

Compared with the growing number of clinical trials using rTMS, several animal studies have examined the basic mechanisms of rTMS action (6, 7, 21). Most of these studies have been performed using rodents and are limited in their applicability to the rTMS actions in humans. For example, since rTMS can not be administered focally in rodents due to limitation of coil size, the entire brain receives stimulation; therefore, the rTMS application is considerably more focal in humans (7). Weissman et al. (22) suggested that the efficacy of magnetic stimulation is reduced in a small rodent brain. Therefore, the validity and usefulness of studies using rodents is questionable (18). Stimulation patterns have not been tested for their analogy to similar patterns used under clinical conditions (7, 21, 23). When a figure-of-eight-shaped coil is applied tangentially to the volume conductor, the electrical activity greatly increases focally and a sharp central peak parallel to the long axis is bounded on each side by smaller peaks (22, 24). In addition, rTMS treatment in rats is a rather stressful experience, due to the handling procedure, the sound of the magnetic stimulator, and the direct effects of rTMS on the muscles. Thus, a sham controlled study is required. Extracellular dopamine (DA) release in the rat PFC has been shown to be increased strongly by stress (25), exposure to a novel environment, and the handling procedure (26, 27). The responses of extracellular DA release in the PFC are graded according to the intensity of stimulus (26, 27).

In the present study, we used a small, figure-eight-shaped stimulation coil (24, 28) and evaluated the motor threshold (MT) intensity as an analogy to the clinical conditions (29). This study aimed to clarify the neurochemical effects of acute rTMS treatment on 5-HT and in the frontal brain of rats. An *in vivo* microdialysis method was used to measure the extracellular concentrations of 5-HT and DA in the PFC of freely moving rats.

Materials and Methods

Animals

Male Wistar rats weighing 270–390 g (10–13-week-old) were purchased from Shizuoka Laboratory Animal Center (Hamamatsu). The animals were housed individually in a temperature-controlled ($21 \pm 2^\circ\text{C}$) and

light-controlled room (12:12 h light: dark cycle with lights on at 19:00), with food and water available. All rats were handled in accordance with the guidelines for the Care and Use of Laboratory Animals of the Animal Research Committee of Hokkaido University School of Medicine.

rTMS procedure

A magnetic stimulator (AAA-10723; Nihon Kohden, Tokyo) was used for the rTMS. The stimulus waveform was biphasic and had a pulse width of 0.3 ms and a rise time of 0.08 ms. A figure-eight-shaped stimulation coil (inner diameter: 35 mm; outer diameter: 70 mm; 7 windings) (AAA-15062, Nihon Kohden) was used with the handle pointing upward. The coil angle was 130 degrees at the intersection of the two wings. The initial current direction was clockwise in the right wing and counterclockwise in the left wing. The fundamental performances of the magnetic stimulator and the stimulation coil were determined according to the previously described methods of Cohen et al. (30). The maximum induction intensity at a stimulator power of 100% machine output at 2 cm below the intersection of the two wings was 1.0 Tesla. Freely moving rats were handled with the head slightly fixed between two fingers. The center of the intersection was fixed above the surface of the head so that the intersection was vertical (24) and was tangential to the sagittal axis on the midline surface of the frontal brain (23). To mimic clinical conditions (8), the center of the intersection was set at the site of the PFC anterodorsally, as determined according to the atlas of Paxinos and Watson (31). The distance between the head surface, the intersection and the coordination of the coil were kept at 2 cm by using rulers made of complete nonconducting materials.

An acute rTMS treatment consisted of 500 stimuli using 60% machine output that resulted from twenty trains at 25 Hz for 1 s with 1-min intervals between trains for cooling to prevent overheating of the coil (32). Sham-treated rats received the same handling procedure and exposure to the sound of the stimulator at the stimulation intensity of 60% machine output by placing the stimulation coil perpendicular to the head surface. Non-treated rats received neither rTMS nor sham treatment.

Fundamental TMS procedure

The MT intensities when using rats were measured preliminarily (15, 29). Rats were anesthetized with 100 mg/kg ketamine intraperitoneally and were transferred to a stereotactic frame. The stimulating coil was stereotactically placed at 2 cm above the head surface. The procedure used to measure the MT intensity was

previously described (15). Briefly, electromyograph (EMG) (MEB-5508, Nihon Kohden) electrodes were implanted into a subcutaneous pocket over the left calf muscle. The optimal stimulation point (hot spot) was assessed by applying a single TMS stimulation to the presumed motor area in the right hemisphere to produce motor-evoked potential (MEP) (high-pass: 1 Hz, low-pass: 5 kHz) responses from the contralateral calf muscle. The threshold intensity was defined as the stimulation to the hot spot that produced 50% suprathereshold responses at inter-stimulus intervals of 7 s (MEP peak-to-peak amplitude $\geq 15 \mu\text{V}$). The onset latencies (point of initial deflection from baseline), first negative peak (N1) latencies, and MEP peak-to-peak amplitudes were measured using MEP data (MEP peak-to-peak amplitude $\geq 15 \mu\text{V}$) at each rat's MT intensity. The MEP latency and MEP peak-to-peak amplitude were expressed as the average of each rat's data.

The mean of the MT intensities ($n = 5$) of rats was $54.4 \pm 5.9\%$ machine output (mean $\pm$ S.E.M.). The MEP obtained at the MT intensity consisted of an initial negative deflection (N1) followed by one or two positive peaks. The mean of the onset latencies ($n = 5$), N1 latencies ($n = 5$), and peak-to-peak amplitudes ($n = 5$) of rats were 6.8 ± 0.4 ms, 9.1 ± 0.6 ms, and $58.7 \pm 13.5 \mu\text{V}$, respectively (mean $\pm$ S.E.M.). The MT measurement of each rat was completed within 60 min (range 20–40 min) after administration of the anesthetics.

In vivo microdialysis

Rats were anesthetized intraperitoneally with 100 mg/kg ketamine, and a guide cannula (CG-4FS; Eicom, Kyoto) was stereotactically implanted into the right PFC (rostral, 3.2 mm; lateral, 0.7 mm; and ventral, 4.0 mm) according to the atlas of Paxinos and Watson (31). More than two days after surgery, a dialysis probe with a membrane length of 3 mm (C-I-4-03-FEP, Eicom) was inserted through the guide cannula into the PFC. The guide cannula and the probe were made of metal-free material, fluorinated ethylene propylene, to avoid heat injury. Once an experiment procedure was completed with a rat, the animal was sacrificed and its brain examined histologically to determine the precise insertion site of the dialysis probe according to the previously described method (33).

The probe was continuously perfused with Ringer's solution (147 mM NaCl, 4 mM KCl, 2.3 mM CaCl_2) at a flow rate of $1 \mu\text{l}/\text{min}$. Sampling was started 3 h after implantation of the probe. Successive samples were collected at 20-min intervals in vials, each containing $10 \mu\text{l}$ of 0.05 N acetic acid. A $20\text{-}\mu\text{l}$ sample from each vial was injected directly into a high-performance

liquid chromatography (HPLC) column and was assayed by using an HPLC-electrochemical detection system. Extracellular 5-HT and DA concentrations were measured by using a previously described method (15, 34, 35). Briefly, the working electrode was maintained at 400 mV against an Ag-AgCl reference electrode, and the mobile phase consisted of 0.1 M sodium dihydrogen phosphate-0.1 M disodium hydrogen phosphate buffer (pH 6.0) with 2.31 mM-octanesulfonate and 0.13 mM ethylenediaminetetraacetate-2Na. The probe's recovery was expressed as the average of data calculated before and after the *in vivo* dialysis experiment by using a standard solution (15, 35).

Statistics

The results are expressed as the mean $\pm$ S.E.M. For the studies using *in vivo* microdialysis, extracellular monoamine concentrations were expressed as percentages of the baseline levels calculated just before the treatment (0 min). Maximum responses were the highest extracellular monoamine levels after the treatment, which were expressed as percentages of the baseline levels (0 min). Statistical analysis (*in vivo* microdialysis data at individual time points and maximum responses) was performed by a one-factor analysis of variance (ANOVA) followed by a post hoc Scheffé's *F* test to calculate differences between the treatment groups. Analysis of the time course was performed by repeated measure ANOVA followed by a post hoc Scheffé's *F* test. Values of *P* less than 5% were considered significant.

Results

Effects of acute rTMS treatment on extracellular DA levels in rat PFC

Significant increases in the extracellular DA concentrations induced by the rTMS ($n = 6$) and sham ($n = 6$) treatments in the rat PFC were observed, when compared with that of the non-treated group ($n = 6$). However, these increases in the extracellular DA concentrations were transient, which returned to the original levels after the treatments (almost 1 h).

The maximum responses in the extracellular DA levels induced by the rTMS and sham treatments in the rat PFC were significant, when compared with that of the non-treated group (Fig. 1). No significant difference was detected between the rTMS-treated and sham-treated groups ($P = 0.8838$).

Effects of acute rTMS treatment on extracellular 5-HT levels in rat PFC

Sham treatment ($n = 6$) showed significant increases

Fig. 1. Effects of acute rTMS treatment on extracellular DA levels in rat PFC. Maximum response of extracellular DA concentration in the rat PFC. Extracellular DA concentrations are expressed as percentages of the baseline levels calculated just before acute rTMS treatment (0 min). Each column is the mean $\pm$ S.E.M. (% of basal values). * $P < 0.05$ vs non-treated group, according to the Scheffé's F test.

in extracellular 5-HT concentrations in rat PFC, compared with that of the non-treated ($n = 6$) ($P < 0.05$) and rTMS-treated groups ($n = 6$) ($P < 0.05$) (Fig. 2A). The non-treated and rTMS-treated groups showed no significant difference ($P = 0.2783$). The maximum responses in 5-HT levels in the sham-treated group were significantly increased, when compared with that of the non-

treated ($P < 0.05$) and rTMS-treated groups ($P < 0.05$) (Fig. 2B).

The mean of the basal values of extracellular 5-HT concentrations corrected by the recovery of each probe was 23.1 ± 4.1 fmol/sample ($n = 18$). The groups showed no significant difference ($F_{[2,17]} = 0.2164$, $P = 0.8079$).

Discussion

Significant increases in the extracellular 5-HT levels in the rat PFC were revealed in the sham-treated group, when compared with that of the non-treated and rTMS-treated groups. The rTMS-treated and non-treated groups showed no significant difference. On the other hand, in the rTMS-treated and sham-treated group, significant increases in the extracellular DA levels in the rat PFC were revealed, when compared with that of the non-treated group. No significant difference in the maximum responses was detected between the rTMS-treated and sham-treated groups.

Feenstra and Botterblom (27), using in vivo microdialysis methods, reported the effects of physiological stimuli on the extracellular DA levels in the rat PFC. The extracellular DA levels in the rat PFC react very rapidly to external stimuli. Holding the rat in the hand for 5.5 min, food presentation and exposure to novelty result in differentially increased extracellular DA levels in the dialysates, with maximum responses of 190%,

Fig. 2. Effects of acute rTMS treatment on extracellular 5-HT levels in rat PFC. A: Time course of extracellular 5-HT concentration in the rat PFC. Extracellular 5-HT concentrations are expressed as percentages of the baseline levels calculated just before acute rTMS treatment (0 min). Each point is the mean $\pm$ S.E.M. B: Maximum response of extracellular 5-HT concentration in the rat PFC. Each column is the mean $\pm$ S.E.M. (% of basal values). * $P < 0.05$ vs non-treated group, # $P < 0.05$ vs sham-treated group, according to the Scheffé's F test.

150%, and 135%, respectively (27). After the maximum responses, the extracellular DA levels return to the original levels again (almost 1 h) (27). The effects of graded stressful conditions on the extracellular DA levels in the rat PFC were also reported by using an *in vivo* microdialysis method (26). Just picking up the rat twice with a 20-min interval increases the extracellular DA levels to 120%; exposure to a novel environment by placement in a clean cage for 20 min, to 150%; and holding the rat in the hands for 20 min, to over 200% (26). Diazepam decreases the extracellular DA levels to about 75% and attenuates the novelty- and handling-induced increases (26). Preliminarily, we found that acute rTMS treatment did not change the extracellular DA and 5-HT levels in the rat PFC under urethane anesthesia (1 mg/kg, *i.p.*) (repeated measure ANOVA, data not shown). In addition, rTMS treatment does not change the increases in the extracellular DA levels in the rat PFC induced by the plus-maze test (15). Therefore, the changes in the extracellular DA levels in the rat PFC induced by the rTMS treatment and by sham treatment in the present study may result from interference stress, which includes the handling procedure or the sound of the magnetic stimulator. That is, a similar interferential stress is assumed to be exerted on the rats by the rTMS and sham treatments (25–27). Taken together, we concluded that the rTMS treatment with 60% machine output in rats eliminated the increase in the extracellular 5-HT levels induced by the sham treatment.

To compare the effects of TMS in the rat brain with TMS effects under clinical conditions in humans, we measured the MT intensity (15, 29). For an accurate evaluation (29, 36) and ethical reasons to avoid pain and restriction, animal sedation might be required. Most sedatives, however, reduce neuronal excitability and synaptic transmission that reduce or eliminate muscle responses to TMS (29). Under ketamine anesthesia, although MEP is initially present, it becomes suppressed after a long period of administration (almost 3 h), which may be explained by accumulation of metabolites (29). Taking these facts together, we chose to sedate the animal by ketamine and completed the MT measurement quickly. Luft et al. (29) reported the rat MEP to TMS (MEP_{TMS}) is consistent with responses evoked by electrical stimulation of the cervical spinal cord (MEP_{CES}). MEP_{TMS} has an onset latency of 6.7 ± 1.3 ms (mean $\pm$ S.D.). MEP_{CES} shows a significantly shorter latency (onset latency: 5.3 ± 0.2 ms, mean $\pm$ S.D.). Latency differences between CES and TMS suggest a supraspinal origin of the MEP_{TMS} (29). Also, two morphologies, $MEP_{TMS, 1}$ and $MEP_{TMS, 2}$ are distinguished by the N1 latency and peak-to-peak amplitude

(29). $MEP_{TMS, 1}$ consists of an N1 followed by one or two peaks of a lower amplitude (onset latency: 6.7 ± 1.3 ms, N1 latency: 8.4 ± 1.3 ms, peak-to-peak amplitude: 61 ± 68 μ V, mean $\pm$ S.D.). $MEP_{TMS, 2}$ has a larger amplitude and higher N1 latency than $MEP_{TMS, 1}$ and lacks the P2 peak (onset latency: 6.5 ± 0.5 ms, N1 latency: 9.5 ± 1.3 ms, peak-to-peak amplitude: 507 ± 626 μ V, mean $\pm$ S.D.). The two morphologies likely reflect different cortical or subcortical origins of MEP_{TMS} . The lesser penetration depth of the magnetic stimulus at low intensities makes the cortex or cortical descending fibers a likely origin of $MEP_{TMS, 1}$. Higher intensities excite deeper structures which may account for $MEP_{TMS, 2}$. In the present study, the configuration of MEP obtained at the MT intensity was predominantly $MEP_{TMS, 1}$ and showed an onset latency of 6.8 ± 0.4 ms, N1 latency of 9.1 ± 0.6 ms, and peak-to-peak amplitude of 58.7 ± 13.5 μ V (mean $\pm$ S.E.M.). Taken together, these results suggest that 60% rTMS treatment (110% MT intensity) on the rat brain in the present study activated the frontal brain primarily.

Recently, Kanno et al. (15) reported that a 3-day series of rTMS treatment using 74% or 110% MT intensity has anxiolytic effects, which increase the time in the open arms and the number of entries into the open arms during the plus-maze test. Three-day series of rTMS treatment using 110% MT intensity suppresses the increases in the extracellular 5-HT levels induced by the plus-maze test (15). However, it is also reported that a 3-day series of rTMS treatment using 147% MT intensity does not change the behavior during the plus-maze test and that a 3-day series of rTMS treatment using 110% MT does not change the basal values of extracellular 5-HT concentrations in the rat PFC (15). Isogawa et al. (37) reported that chronic rTMS treatment using almost 150% MT intensity has anxiogenic effects, which decrease the time in the open arms and the number of entries into open arms during the plus-maze test. These facts suggest that chronic rTMS treatment can be anxiolytic or anxiogenic, depending on the experimental conditions. Either effect is thought to appear by experimental conditions such as stimulation intensity. That is, sufficient attention to the planning of experimental conditions and evaluation is necessary.

Agents acting on the 5-HT receptor attract attention as anxiolytic agents. However, evaluation of these agents based on the elevated plus-maze test is not fixed (38). With respect to buspirone, a 5-HT_{1A} agonist and a typical anxiolytic agent, actions which are different from the anxiolytic effect are reported (13), no-action (39) or an anxiogenic effect (40) in the plus-maze test. This fact indicates that this agent shows

both an anxiolytic effect and an anxiogenic effect, and either effect is thought to appear according to experimental condition (41). Thus, it is reasonable to consider that the changes in the extracellular 5-HT levels in the rat PFC induced by the 3-day series of rTMS treatment under the condition showing anxiolytic effects are related to the anxiolytic mechanism of rTMS, when a proper condition of stimulation is set up (15). Recently, it is suggested that selective 5-HT reuptake inhibitors (SSRIs) have an anxiolytic action (42, 43). The basic amount of 5-HT release is increased in the rat PFC by administration of SSRIs, and enhancement of transmission of serotonergic neurons forms the basis of the anxiolytic effect (42). At the least, there has been no report that rTMS treatment shows anxiolytic effects under these conditions, which lead to increase in the extracellular 5-HT levels in the rat PFC (6, 7). Therefore, it is suggested that there is the existence of a mechanism different from SSRIs in the expression of rTMS-induced anxiolytic effects. Taken together, we speculated that the inhibitory effects of rTMS treatment on the serotonergic nervous system in the PFC of this study were related to therapeutic effects of rTMS treatment, such as anxiolytic effects (8, 15).

In conclusion, this study showed that acute rTMS treatment with 60% machine output (110% MT intensity) of the frontal brain eliminated the increase in 5-HT levels induced by the sham treatment in the rat PFC. Thus, rTMS treatment is related to the serotonergic neuronal system in the PFC.

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