Functional Dopaminergic Neurons in Substantia Nigra are Required for Transcranial Magnetic Stimulation-Induced Motor Plasticity

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Repetitive magnetic stimulation (rTMS), including theta burst stimulation (TBS), is capable of modulating motor cortical excitability through plasticity-like mechanisms and might have therapeutic potential for Parkinson’s disease (PD). An animal model would be helpful for elucidating the mechanism of rTMS that remain unclear and controversial. Here, we have established a TMS model in rat and applied this model to study the impact of substantia nigra dopamine neuron on TBS-induced motor plasticity in PD rats. In parallel with human results, continuous TBS (cTBS) successfully suppressed motor evoked potentials (MEPs), while MEPs increased after intermittent TBS (iTBS) in healthy rats. We then tested the effect of iTBS in early and advanced 6-hydroxydopamine (6-OHDA)-lesioned PD. Moreover, dopaminergic neurons in substantia nigra and rotation behavior were assessed to correlate with the amount of iTBS-induced plasticity. In results, iTBS-induced potentiation was reduced in early PD rats and was absent in advanced PD rats. Such reduction in plasticity strongly correlated with the dopaminergic cell loss and the count of rotation in PD rats. In conclusion, we have established a TMS PD rat model. With the help of this model, we confirmed the loss of dopaminergic neurons in substantia nigra resulting in reduced rTMS-induced motor plasticity in PD.

Keywords: motor evoked potential, Parkinson’s disease, rat, theta burst stimulation, 6-OHDA

Introduction

Repetitive transcranial magnetic stimulation (rTMS) is capable of producing long-lasting changes of cortical excitability beyond the short period of stimulation. Although the exact neurobiologic substrate for the mechanisms of rTMS remains unclear, several lines of evidence support that rTMS induces changes similar to long-term potentiation (LTP) or long-term depression (LTD) of synaptic strength (Huang et al. 2007). Hence, rTMS has been applied to study plasticity phenomena in healthy humans and patients. Moreover, the modulation effect of rTMS is recently considered having therapeutic potential in neurological or psychiatric disorders, for example, Parkinson’s disease (PD), dystonia and depression (Dragasavic et al. 2002; Koch et al. 2005; Huang et al. 2010, 2012; Benninger et al. 2011; Berlim et al. 2013).

rTMS has recently been used to investigate plasticity in PD patients with or without complications (Huang et al. 2011). Impaired motor plasticity was reported using rTMS in chronic and early PD patients with off medications and was restored by dopaminergic medications (Morgante et al. 2006; Ueki et al. 2006; Eggers et al. 2010; Huang et al. 2011; Suppa et al. 2011; Kishore et al. 2012). In contrast, others showed enhanced or normal motor plasticity in PD patients off medication (Bagnato et al. 2006; Zamir et al. 2012). Similar inconsistency was also found in the therapeutic effect of rTMS on PD. Although quite a few studies have shown clinical benefits of rTMS on PD (Leaucharer et al. 2004; Elahi and Chen 2009; Degardin et al. 2012), the results so far have been inconclusive (Benninger et al. 2011; Shirota et al. 2013). Such discrepancy between studies could be due to the clinical heterogeneity in patients, long-term pharmacological effects, different severity of the disease, and the variability of protocols (Muller-Dahlhaus et al. 2008; Bologna et al. 2012; Hamada et al. 2013).

Disease animal models may provide a more stable and controllable condition than patients to reduce the discrepancy (Picconi et al. 2003; Morgante et al. 2006; Calabresi et al. 2007). However, the studies in animals were mostly done with invasive electrical stimulation, which is not suitable for humans. For the translational purpose, between animals and humans, it would be valuable to study the effect of rTMS on animals. Recently, rTMS protocols have been translated to use in animal models for understanding their mechanistic insights (Aydin-Abidin et al. 2008; Benali et al. 2011; Funke and Benali 2011; Ghiglieri et al. 2012; Hoppenrath and Funke 2013; Volz et al. 2013). However, most of these studies focused on the mechanism at the cellular level. The physiological response to rTMS as measured in humans, for example, changes in motor evoked potentials (MEPs), has rarely been tested in the animal models. This lacking piece of information in animal models may restrict the usage of the knowledge obtained from the animal model. On the other hand, the literature is scant in the use of disease animal models for studying the plasticity phenomena of rTMS in disease conditions, which may provide insights into the underlying pathophysiology of the disease for future diagnostic purposes and therapeutic applications targeting synaptic plasticity.

We therefore first set up a plasticity rat model, which is comparable with that established in humans, to test the effects of theta burst form of rTMS (theta burst stimulation, TBS), which is adapted from theta burst paradigm commonly used in animal preparations (Larson and Lynch 1986; Hess et al. 1996), on MEPs in anesthetized normal rats. Then, the rat model served as
a platform to examine TBS-elicited plasticity responses in a 6-OHDA-lesioned rat model of PD. Two stages of PD rats reflecting different severity of dopamine depletion quantified by counting dopaminergic cell loss and the behavioral presentation tested by drug-induced rotation were utilized to understand the correlation between the disease severity and plasticity change.

Materials and Methods

Animal Preparation
Experiments were carried out on 45 male Wistar rats (350–400 g) obtained from the Animal Center of National Cheng Kung University Medical College. All experiments were approved beforehand by National Cheng Kung University Medical College Animal Use Committee. Among them, 27 rats were equally assigned to sham control, iTBS and cTBS groups (n = 9 for each group) for testing immediate effect of TBS. The other 18 rats were chosen for PD lesions and were separated into 2 equal groups to undergo iTBS at 1 week or 4 weeks postlesion (expressed as 1-wk PD and 4-wk PD, respectively) to identify motor plasticity during PD progression. The animals were housed in standard cages at a temperature of 25 ± 1 °C with a 12/12-h light/dark cycle and ad libitum water and food access before the experimental procedures.

Parkinsonian Rat Model
For the 6-OHDA lesion, the rat was deeply anesthetized by intraperitoneal injection of Tiletamine-Zolazepam (50 mg/kg, i.p.; Zoletil, Vibac, France) and xylazine (10 mg/kg, i.p.; Rompun, Bayer, Germany), then placed in a stereotactic apparatus (Stoelting, USA). A solution of 6-OHDA (8 µg, 4 µL, dissolved in 0.02% ascorbic saline, Sigma, USA) was injected intracranially at a rate of 0.5 µL/min into the left medial forebrain bundle (AP: +4.3 mm; lateral: 1.6 mm; DV: −8.2 mm) according to the stereotaxic brain atlas of Paxinos and Watson using a 10-µL Hamilton microsyringe (Paxinos and Watson 2005). The needle was left in the brain for 5 min before being slowly retracted (Truong et al. 2006; Hsieh et al. 2011).

Electrophysiological Recordings
The test rats were deeply anesthetized for ~4 h with Tiletamine-Zolazepam (65 mg/kg, i.p.; Zoletil, Vibac, France) plus xylazine (10 mg/kg, i.p.; Rompun, Bayer, Germany) and mounted in a stereotactic apparatus (Stoelting, USA). No anesthesia depth was adjusted to the absence of abdominal contractions during tail pinch. Recording of electromyographic (EMG) activity was obtained with monopolar uninverted EMG signal was amplified (1000×) (Cyberamp 380, Axon Instruments, USA) and filtered using 60-Hz notch and 10-Hz to 1-kHz bandpass filters prior to digitization at 4 kHz (DAQPad-6015, National Instruments, USA). All TMS and rTMS sessions were performed using a Rapid² magnetic stimulator and a figure-of-eight 25 mm coil (Magstim Co., Wales, UK). The coil was held in the stereotaxic frame and one of the rings (as a circular coil) was placed over the rat dorsal scalp, and was systematically adjusted to the best coordination for eliciting MEP in the right forelimb (see Supplementary Fig. 1). At the coil position, MEPS were reliably elicited from both forelimbs as previously reported (Magnuson et al. 1999; Nielsen et al. 2007; Gersner et al. 2011; Iglesias-Bregna et al. 2013). MEPS evoked by single-pulse TMS were recorded to evaluate the effect of TBS on cortical excitability. The resting motor threshold (RMT) was defined as the minimal intensity of stimulation required for eliciting MEPS from the right brachioradialis muscle with peak-to-peak amplitude of at least 20 µV in 5 of 10 consecutive trials performed during muscle relaxation due to anesthesia (Rotenberg et al. 2010; Vahabzadeh-Hagh et al. 2011). The coil was maintained at this location throughout the whole session. TMS intensity for RMT was documented as percent machine output (% MO) with 100% corresponding to the maximal strength of the generated magnetic field.

TBS Protocols
Two TBS protocols, including iTBS and cTBS, consisting of 3-pulse bursts at 50 Hz repeated at 5 Hz were used in the present study. For iTBS, a 2-s train of TBS was repeated every 10 s for 20 repetitions (total 600 pulses). In the cTBS paradigm, a 40-s train of uninterrupted TBS was given (600 pulses) (Huang et al. 2005). Because active motor threshold (AMT) could not be obtained in animals under anesthesia, 80% RMT, instead of AMT, was used for TBS. For sham intervention, the coil was placed 8 cm laterally and above the rat’s head (Aydin-Abidin et al. 2008) and the iTBS protocol was delivered.

MEP Assessment
For the assessment of MEP amplitudes, 15 single pulses were applied at 10- to 15-s random intervals at 120% RMT. The stimulus intensity remained unchanged throughout the whole session of experiment. Peak-to-peak amplitudes of MEP were analyzed offline.

Behavioral Tests
In addition, we measured the apomorphine-induced rotational behavior to correlate the severity of parkinsonian symptom with iTBS-induced changes in the MEP amplitude. Apomorphine (0.5 mg/kg in 0.1% ascorbic acid, s.c.; Sigma) was given to the PD rat (Yoon et al. 2007; Hsieh et al. 2011). Then the rat was placed in a 40-cm-diameter round bowl to assess the rotational behavior, which was recorded using a digital video camera to calculate the number of rotation at 10-min intervals offline, for 60 min. The number of rotations was calculated by subtracting the number of ipsilateral rotations from the number of contralateral turns with respect to the 6-OHDA injection side.

Histology Investigation
After electrophysiological and behavioral tests, the PD rat was sacrificed for tyrosine hydroxylase (TH) staining to quantify dopaminergic neuron loss in the brain. The brains were post-fixed 3 days and cryoprotected in 30% sucrose. The brains were frozen and sectioned at 30 µm “coronally” in the region spanning from −5.20 to −5.80 mm in the substantia nigra and from +1.70 to +2.30 mm in the striatum with respect to the bregma (Paxinos and Watson 2005). Free-floating sections were treated with 0.3% H2O2 in PBS for 10 min to remove endogenous peroxidase. After being washed, the sections were preincubated in PBS containing 0.3% Triton-X 100 (Merck, Germany), 3% horse serum (Vector Labs, USA), and 3% bovine serum albumin (BSA; Sigma, USA) for 30 min to block nonspecific antibody binding. Sections were then incubated in a 1:1000 dilution of rabbit primary anti-TH (AB152, Millipore, USA) containing 5% horse serum (Vector Labs) and 5% BSA for 15–18 h. Thereafter, consecutive sections were washed and then incubated for 1 h with an anti-rabbit horseradish peroxidase (1:200; ImmPRESS Anti-Rabbit Ig Kit # MP-7401, Vector Labs) containing 3% “normal horse serum” in PBS. Following rinsing with PBS, immunostaining was visualized with 3,3-diaminobenzidine (SK-4105, Vector Labs) for 3 min. The sections were then mounted on slides, dehydrated in a series of alcohols, cleared in xylene, and cover-slipped in DPX. After TH staining, the stained images were digitalized with a camera, connected to a microscope and a computer. The TH-positive cell bodies in the substantia nigra from both hemispheres were counted manually in each section (Truong et al. 2006; Hsieh et al. 2011). The percentage loss of TH-positive cells in the lesioned hemisphere was calculated and normalized with respect to the nonlesioned side. In addition, we measured the optical density of TH-positive fibers in the striatal sections using Image-Pro Plus 6.0 software (Media Cybernetics, USA) to calculate the dopaminergic fibers in the striatum. The optical density measurements were corrected for nonspecific background density measured at the corpus callosum. The percentage loss of dopaminergic

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fiber in the lesioned side was presented and normalized with respect to the nonlesioned side.

**Experimental Design**

The experimental design is illustrated in Figure 1. We first investigated the effect of different TBS protocols (i.e., iTBS, cTBS, and sham) on the MEP size in normal rats. To reach a stable condition of anesthesia, we waited for 60 min after anesthetics were injected to begin the investigation. MEPs were recorded every 5 min for 3 times before TBS as the baseline condition. Then TBS was given and MEPs were recorded again immediately and at 5, 10, 15, 20, 25, and 30 min after the end of TBS (Fig. 1A). For evaluating the effect of TBS in PD rats, we focused on the effect of iTBS. The same MEP assessment as above followed by apomorphine-induced rotational behavior test was performed. To prevent the possible interaction between MEP assessment and behavioral recording and to avoid any residual anesthesia, the behavioral test was done at 12 h after the MEP assessment. On the day after electrophysiological and behavioral tests, we sacrificed the rat for histological examination to correlate the TBS effect with dopamine depletion (Fig. 1B).

**Data Analysis**

Data were analyzed using SPSS for Windows version 17.0 with the significance level set at $P<0.05$. All data are presented as the average ± standard error of the mean (SEM). Statistics for the comparisons of TBS effects in healthy rats were performed on MEPs, which were normalized to the last 5-min pre-TBS baseline, using two-way repeated-measures analysis of variance (ANOVA) with PROTOCOL (iTBS, cTBS, sham) as between-subjects factor and TIME (0, 5, 10, 15, 20, 25, and 30 min after TBS) as within-subjects factor. When it was needed, a separate one-way ANOVA on absolute values to characterize the time-dependent change of each protocol. Post hoc Fisher’s LSD tests were used to compare between time points if needed.

Furthermore, to test whether the iTBS-induced plasticity was affected with the evolution of PD, the averaged iTBS-induced percentage change in the MEP size after iTBS were compared between 1-wk PD and 4-wk PD rats using t-tests. The percentage change of MEP size was calculated as the percentage of changes in averaged MEP size of all testing time points (i.e., 0, 5, 10, 15, 20, 25, and 30 min) after iTBS with respect to last 5-min pre-TBS MEP baseline.

We also used Pearson’s correlation test for evaluating the correlation between the iTBS-induced percentage changes of MEP size and dopaminergic cell loss and the count of apomorphine-induced rotation in the PD rats.

### Results

**Effects of TBS on MEPs in Healthy Rats**

RMT was 53.64 ± 1.93% (mean ± SEM) maximum machine output (MMO) in normal rats, 52.83 ± 2.04% MMO in 1-wk PD rats and 51.23 ± 1.33% MMO in 4-wk PD rats. No significant difference was found between groups. Two-way repeated-measures ANOVA performed on normalized MEPs showed a significant effect of PROTOCOL (F(2, 27) = 4.57, $P = 0.02$) and TIME (F(6, 162) = 27.34, $P = 0.00$) with a significant interaction between PROTOCOL and TIME (F(12, 162) = 3.34, $P = 0.00$). Post hoc Fisher’s LSD tests showed a significant increase in MEP size after iTBS compared with baseline (iTBS vs. sham: $P = 0.00$, cTBS vs. sham: $P = 0.00$, iTBS vs. cTBS: $P = 0.00$).

**Effects of TBS in PD Rats**

A two-way repeated-measures ANOVA performed on normalized MEPs showed a significant effect of GROUP (F(2, 27) = 4.57, $P = 0.02$) and TIME (F(6, 162) = 27.34, $P = 0.00$) with a significant interaction between GROUP and TIME (F(12, 162) = 3.34, $P = 0.00$). Post hoc Fisher’s LSD tests showed a significant increase in MEP size after iTBS compared with baseline (iTBS vs. sham: $P = 0.00$, cTBS vs. sham: $P = 0.00$, iTBS vs. cTBS: $P = 0.00$).

**Correlation Analysis**

Pearson’s correlation analysis showed a significant correlation between the iTBS-induced percentage change of MEP size and dopaminergic cell loss ($r = 0.73$, $P = 0.00$) and a significant correlation between the iTBS-induced percentage change of MEP size and apomorphine-induced rotation ($r = 0.67$, $P = 0.00$).

Figure 1. Experimental design. In the experiments for testing the effect of TBS on normal rats (A), 15 MEPs assessed every 10–15 s were recorded every 5 min for 3 times before TBS and every 5 min until 30 min after the end of TBS, including iTBS, cTBS, and sham stimulation. For the PD groups (B), the MEP assessment, behavioral, and histological investigations were performed on 1-wk and 4-wk PD rats.
measures ANOVA showed significant main effects of PROTOCOL ($F_{2,23} = 46.35, P < 0.001$ in left limb; $F_{2,23} = 26.78, P < 0.001$ in right limb) and TIME ($F_{6,144} = 2.64, P = 0.02$ in left limb; $F_{6,144} = 3.12, P = 0.007$ in right limb). Following one-way ANOVAs showed that MEPs were enhanced by iTBS ($F_{7,64} = 3.619, P = 0.002$ in left limb and $F_{7,64} = 2.60, P = 0.02$ in right limb), whereas they were suppressed by cTBS ($F_{7,64} = 2.51, P = 0.02$ in left limb and $F_{7,64} = 2.64, P = 0.02$ in right limb). Post hoc Fisher’s LSD analysis revealed that MEPs were significantly enhanced in 5 min ($P = 0.024$ in left limb; $P = 0.041$ in right limb) and remained enhanced up to 30 min ($P < 0.001$ in both limbs) after iTBS when compared with the baseline MEP. MEPs were suppressed immediately ($P = 0.002$ in left limb; $P = 0.015$ in right limb) and lasted for 30 min or more ($P = 0.049$ in left limb; $P = 0.009$ in right limb) after cTBS. No effect was noted on the MEP size in rats that had received sham stimulation ($F_{7,64} = 0.32, P = 0.85$ in right limb) (Fig. 2B-D).

**Effects of iTBS on MEPs in PD Rats**

Two-way repeated-measures ANOVA showed significant main effects of GROUP ($F_{2,23} = 5.783, P = 0.009$ in left limb; $F_{2,23} = 5.069, P = 0.015$ in right limb) and TIME ($F_{6,144} = 3.698, P = 0.002$ in left limb; $F_{6,144} = 4.789, P < 0.001$ in right limb). Further two-way ANOVA with factor GROUP confirmed significant differences between the 3 groups (normal vs. 1-wk PD: $F_{1,112} = 7.444, P = 0.007$ in left limb; $F_{1,112} = 4.821, P = 0.03$ in right limb; normal vs. 4-wk PD: $F_{1,112} = 88.422, P < 0.001$ in left limb; $F_{1,112} = 62.521, P < 0.001$ in right limb; 1-wk PD vs. 4-wk PD: $F_{1,112} = 15.313, P < 0.001$ in left limb; $F_{1,112} = 18.711, P < 0.001$ in right limb). Following one-way ANOVAs revealed that MEPs were enhanced by iTBS ($F_{7,64} = 3.619, P = 0.002$ in left limb and $F_{7,64} = 2.60, P = 0.02$ in right limb) in the normal group but not in either 1-wk PD group ($F_{7,64} = 0.699, P = 0.673$ in left limb and $F_{7,64} = 1.425, P = 0.211$ in right limb) or 4-wk PD group ($F_{7,64} = 0.338, P = 0.934$ in left limb and $F_{7,64} = 0.695, P = 0.676$ in right limb) (Fig. 3B-D). We further averaged the MEPs at all time points after iTBS to compare with the baseline MEP, and revealed that MEPs were enhanced by iTBS in 1-wk PD ($t = 3.071, P = 0.015$), but not in 4-wk PD ($t = 0.413, P = 0.691$). This result suggests that there is still some facilitatory effect of iTBS in 1-wk PD, but not in 4-wk PD.

**Dopaminergic Cell Depletion and Behavioral Deficits in PD Rats**

The results of TH-immunohistochemistry in the substantia nigra and striatum in 1-wk and 4-wk PD are shown in Figure 4A, B. A moderate reduction of TH-immunoreactive density was observed in the substantia nigra and striatum of the infusion side in rat at

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Figure 2. The effect of TBS on MEPs in healthy rats. Representative MEPs of a rat before and after sham stimulation, cTBS, and iTBS are shown at each measured time epoch (A). Averaged changes in the MEP amplitude after sham, cTBS, and iTBS interventions are presented for left (B), right (C), and average of both sides (D). Each point corresponds to the mean and standard error of MEP amplitude expressed as a ratio to the last block of baseline responses. *$P < 0.05$, **$P < 0.01$, significant post hoc Fisher’s LSD differences on the MEP size when compared with each time point after intervention to the last block of pre-TBS baseline.
Figure 3. The effect of iTBS on MEPs in PD rats. Representative MEPs before and after iTBS in normal, 1-wk and 4-wk PD rats are shown at each measured time epoch (A). The MEP amplitude increased in the normal rat but was less facilitated in PD rats after iTBS. Time course of averaged changes in the MEP amplitude in normal, 1-wk PD and 4-wk PD rats are presented for left (B: ipsilateral side), right (C: contralateral side), and average of both sides (D). These effects of iTBS in the 3 groups were significantly different from each other. *P < 0.05, **P < 0.01, significant post hoc Fisher’s LSD differences on MEP size compared between baseline and at each time point after iTBS.

Figure 4. The histological and behavioral changes correlate with the disease stage in PD rats. Representative TH-immunoreactive fibers at the substantia nigra (A) and striatum (B) from animals sacri­ficed at 1 week and 4 weeks post-6-OHDA lesion are shown in upper row, while the quantification of TH-positive neuron loss (C), TH-positive fiber loss (D), and the count of rotational behavior (E) are shown in the lower row. **P < 0.01, ***P < 0.001 (unpaired t-test).
The Correlation Between the Severity of PD and iTBS-Induced Plasticity

We then calculated the iTBS-induced percentage change in MEP size, which was significantly larger in 1-wk PD than in 4-wk PD rats (Fig. 5A: 1-wk PD: 22.83 ± 5.74%; 4-wk PD: −1.07 ± 3.60%; t = 3.524, P < 0.003), to correlate with the level of dopamine denervation and the number of apomorphine-induced rotation in PD rats. The Pearson’s correlation analysis showed a significant negative correlation between the percentage change of MEP size after iTBS and the loss level of dopaminergic neuron in substantia nigra (r = −0.87, P < 0.001) (Fig. 5B) and fibers in the striatum (r = −0.87, P < 0.001) (Fig. 5C). A similar significant negative correlation was also found between the rotational behavior and the percentage change of MEP size (r = −0.81, P < 0.001) (Fig. 5C). These results suggest that advanced PD rats have more evident deficit in plasticity in response to iTBS than early PD rats.

Discussion

In the present study, we successfully translated the human TMS protocols into an in vivo rat model. The study of motor plasticity in 6-OHDA-lesioned PD rats with this model showed that the amount of rTMS-induced plasticity decreased in PD rats and deteriorated as the disease progressed. Such impaired plasticity was strongly associated with the loss of dopaminergic nigral cells and striatal fibers proved histologically and the impairment of dopaminergic function tested by behavioral investigations.

rTMS-Induced Motor Plasticity in Vivo Rat Model

In the TMS rat model, we recorded consistent MEPs from anesthetized rats’ limbs as previous reports (Rotenberg et al. 2010; Vahabzadeh-Hagh et al. 2011). Analogous to those observed in humans (Huang et al. 2005), iTBS increased the size of MEPs for 30 min or more, while cTBS decreased the size of MEPs for at least 30 min. To avoid major stress effects, we had to anesthetize the rat. In contrast, most TMS-MEP measurements were studied in conscious humans. To our knowledge, the effect of rTMS on MEPS has never been reported in anesthetized humans. It is more difficult to induce plasticity in freely moving animals than in brain slices or anesthetized animals (Trepel and Racine 1998; Froc et al. 2000). This may explain why the plasticity like response in our rats seems to be larger and less variable than those reported in humans.

MEPs were recorded at several time points and sham controlled group was used to confirm no confounding effects of anesthesia. Eighty percent RMT instead of AMT was used for TBS because it is impossible to record AMT in anesthetized rats. Hence, TBS was given at a higher intensity when compared with that usually applied to human. In humans, it has been reported that TBS is sensitive to the given intensity. A subtle change in the intensity of TBS may selectively activate different circuit within the cortex and produce different effect on the corticospinal excitability (McAllister et al. 2009; Doeltgen and Ridding 2011). It has been demonstrated that cTBS at 90% AMT, which is close to 80% RMT, has stronger inhibition than cTBS at 80% AMT (Bruckner et al. 2013). This could be another reason why the TBS effects in the present study were larger than those in humans. However, the influence of the stimulus intensity on the effect of TBS deserves further careful studies. The current TMS rat model may be an ideal platform for this purpose.

MEPs were evoked by 120% RMT of the anesthetized rat. Thinking of the large size of the coil relative to the size of the rat brain and the relatively strong stimulus intensity, we do not propose that we are stimulating only the motor cortex. Nevertheless, the results were compatible with those in humans and showed pattern-dependent lasting effects on the size of MEP (Huang et al. 2005). Cellular studies of the similar protocols in rats showed that iTBS altered cortical neuronal activity along with significant changes in the expression of the synaptic plasticity-related proteins (Hoppenrath and Funke 2013). These lines of evidence suggest that plasticity-like effects were successfully activated by our magnetic stimulation protocol in the corticospinal pathway of rats in this model. Hence, this TMS model may serve as a bridge between animal and human plasticity studies, at least, in the motor pathway. On the other hand, the human protocols could be tested or manipulated in the rat model, for example, to clarify the underlying mechanisms, to improve protocols, and to test variability (Hamada et al. 2013) and the effect of stimulus intensity. If the disease models are available, it may also be used to match the findings between animal model and patients and clarify the pathophysiology or to test the therapeutic potential of rTMS protocols.

iTBS-Induced Plasticity in PD Rats

With the help of this model, we revealed that motor plasticity was reduced in PD rats. This result is in line with recent results showing reduced rTMS-induced plasticity in the motor cortex of PD patients and suggest that dopamine might play a key role for expression of plasticity (Morgante et al. 2006; Ueki et al. 2006; Huang et al. 2011; Suppa et al. 2011). Similar results have been shown in animal studies, in which plasticity was tested in vitro with brain slices and the corticostratial pathway was tested (Centonze et al. 1999; Picconi et al. 2003; Ghiglieri et al. 2012). However, the corticostratial pathway is difficult to test in humans, unless a deep brain stimulation electrode is implanted (Prescott et al. 2009). Hence, motor plasticity has commonly been studied instead.

The facilitatory effect of iTBS was absent in the later stage PD rats. In contrast, iTBS was still able to increase the MEPs amplitude in early-stage PD rats, although the amount of increase was less than that in normal rats. The result of TH staining suggests a lower level of functional dopaminergic cells in 4-wk than 1-wk PD. Previous studies have demonstrated that 6-OHDA induces an initial loss in dopaminergic phenotype

one week after 6-OHDA injection. Four weeks after the injection, TH immunoreactivity in the substantia nigra and striatum was much less detectable on the side of the infusion when compared with that at 1-week postlesion. Quantification of TH-positive cell loss in the substantia nigra and TH-positive fiber loss in striatum at 1 week and 4 weeks postlesion are presented in Figure 4C,D, respectively. The average TH-positive cell loss in the substantia nigra region when compared with the intact hemisphere was 61.56 ± 7.35 and 90.47 ± 1.43% at 1 week and 4 weeks postlesion, respectively (t = 3.93, P = 0.004). The average TH-positive fiber loss in the striatum was 76.16 ± 2.26 and 91.05 ± 1.10% at 1 week and 4 weeks postlesion, respectively (t = 5.90, P < 0.001). With regard to apomorphine-induced rotational behavior, apomorphine induced 516.4 ± 31.5 turns, which was significantly higher than 366.7 ± 40.1 turns that was recorded in 1-wk PD rats (t = 3.57; P = 0.003), in 60 min in 4-wk PD rats (Fig. 4E).
and then induces more neuronal loss in late stage (Jeon et al. 1995; Bowenkamp et al. 1996; Cohen et al. 2011). Similar phenomena were also observed in our PD rats (Fig. 4A), although we did not analyzed them quantitatively. Further analysis revealed a high correlation between the level of functional dopaminergic cell in the substantia nigra and iTBS-induced changes in MEP amplitudes. Moreover, there was the significant negative correlation between the level of dopaminergic fiber loss in stratum and versus the count of apomorphine-induced rotation (D). Asterisks indicate a significant difference (unpaired t-test, \( P < 0.01 \)).

Figure 5. The correlation between the percentage change of MEP and the disease severity. iTBS was able to enhance MEPs in 1-wk PD, but not in 4-wk PD (A). There was a significant negative correlation between the percentage change of MEP amplitude versus the level of dopaminergic neuron loss in substantia nigra (B), versus the level of dopaminergic fiber loss in striatum (C), and versus the count of apomorphine-induced rotation (D). Asterisks indicate a significant difference (unpaired t-test, \( P < 0.01 \)).

Although it has been reported that reduced plasticity could be restored by levodopa in PD patients (Huang et al. 2011; Kawashima et al. 2015), there has been no direct evidence of the correlation between iTBS-induced plasticity and the pathological change of the substantia nigra in PD. The present study for the first time provides such evidence and shows a strong correlation between the amount of plasticity tested by TMS and the functional dopaminergic neuron in the substantia nigra.

In the past few years, the therapeutic potential of rTMS has been shown on PD patients (Randhawa et al. 2013; Shirota et al. 2013). Few studies tried to answer the mechanism of the therapeutic effect of rTMS in 6-OHDA PD rats. Four weeks of daily rTMS could improve the motor deficits and have neuroprotective effect to dopamine neurons (Yang et al. 2010; Lee et al. 2013). A single session of cortical iTBS increased striatal excitability and restored corticostriatal LTD (Ghiglieri et al. 2012). So far, such issue has been mainly approached in the behavioral response or in histology or physiology analysis such that the sacrifice of animals is commonly required. Although we did not focus on this issue in the current study, we have demonstrated another option to assess the physiological condition in the PD rat model without sacrificing the animal.

Dopamine has long been known as a crucial chemical in the regulation of plasticity in the brain (Law-Tho et al. 1995; Calabresi et al. 2000). There is a growing evidence suggesting that the dopamine deficit alters N-methyl-D-aspartate (NMDA) receptors to impair striatal and hippocampal plasticity in PD rats (Costa et al. 2012; Picconi et al. 2012). Rebalancing the composition of NMDA receptors may restore plasticity and motor function (Paille et al. 2010). NMDA receptors are known to be...
crucial for rTMS to produce motor plasticity in humans (Huang et al. 2007, 2008) or in vitro (Vlachos et al. 2012). Hence, it is reasonable to speculate that the modulation of NMDA receptors by dopamine deficiency involves the impaired rTMS-induced plasticity in PD.

Our study shows that motor plasticity is strongly influenced by the degree of dopaminergic cell loss. However, it cannot be fully excluded that the impairment of motor plasticity in PD rats could reflect the secondary reorganization of motor cortex or the compensation of motor function with the progression of PD. This issue may be worth to be considered for further investigation. Also, for the translational application, in addition to test dopamine depletion in PD, it would be needed to understand the relationship between the changes of motor plasticity and other symptom-related indices such as the motor abilities when planning to test or modulate motor plasticity for therapeutic purposes.

Conclusions

In conclusion, we have set up an animal model for testing rTMS-induced motor plasticity in vivo in rats. Our results confirm that the TMS rat model mimics the situation in humans and might be used for translating TMS studies from humans to rats and vice versa. However, a slightly modified protocol in the stimulus intensity and the coil position and anesthesia were required for rat stimulation. Such technical limitations may cause some discrepancies between the model and human studies and should be aware in data interpretation and further applications. With the help of this model, we showed reduced rTMS-induced motor plasticity, which strongly correlated with the depletion of dopaminergic neuron in the substantia nigra and the severity of PD symptoms, in PD rats. To our knowledge, this is the first evidence that motor plasticity tested by TMS relies on the amount of dopamine in the substantia nigra. The newly developed TMS rat model would be useful for studying on the mechanism of rTMS-induced plasticity and for developing therapeutic strategies of rTMS for PD and other disorders.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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