Research Report

Therapeutic effects of repetitive transcranial magnetic stimulation in an animal model of Parkinson's disease

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\textbf{Article info}

Article history:
Accepted 26 August 2013
Available online 30 August 2013

Keywords:
Repetitive transcranial magnetic stimulation
Parkinson’s disease
Dopaminergic neuron
Neurotrophic/growth factor

\textbf{Abstract}

Repetitive transcranial magnetic stimulation (rTMS) is used to treat neurological diseases such as stroke and Parkinson's disease (PD). Although rTMS has been used clinically, its underlying therapeutic mechanism remains unclear. The objective of the present study was to clarify the neuroprotective effect and therapeutic mechanism of rTMS in an animal model of PD. Adult Sprague-Dawley rats were unilaterally injected with 6-hydroxydopamine (6-OHDA) into the right striatum. Rats with PD were then treated with rTMS (circular coil, 10 Hz, 20 min/day) daily for 4 weeks. Behavioral assessments such as amphetamine-induced rotational test and treadmill locomotion test were performed, and the dopaminergic (DA) neurons of substantia nigra pars compacta (SNc) and striatum were histologically examined. Expression of neurotrophic/growth factors was also investigated by multiplex ELISA, western blotting analysis and immunohistochemistry 4 weeks after rTMS application. Among the results, the number of amphetamine-induced rotations was significantly lower in the rTMS group than in the control group at 4 weeks post-treatment. Treadmill locomotion was also...
1. Introduction

Parkinson’s disease (PD) occurs as a result of degenerating cell death of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc). PD is characterized by certain motor disturbances such as resting tremor, bradykinesia, and gait disturbance. Dopamine replacement therapy is an effective medical treatment for the symptomatic improvement of PD. In spite of such effects, fluctuations in abnormal involuntary movement eventually occur in most patients after long-term dopaminergic treatment. Consequently, several studies have investigated various methods other than dopaminergic drugs for PD treatment (Wu et al., 2008).

Repetitive transcranial magnetic stimulation (rTMS) is a non-invasive therapeutic device that can alter the excitability of the cerebral cortex or neural network. rTMS has been used to treat neuropsychiatric diseases (Zwanzger et al., 2002; Bentwich et al., 2011; Fitzgerald and Daskalakis, 2011) and stroke (Theilig et al., 2011; Corti et al., 2012). Recently, several clinical trials have revealed that rTMS offers therapeutic benefit for functional recovery in PD (Siebner et al., 1999; Arias-Carrión, 2008; Wu et al., 2008; Yang et al., 2013). rTMS treatment has shown to improve motor function and cognition in patients with PD (Zamir et al., 2012). Additionally, increased DA levels in serum and subcortical areas have been observed following rTMS in both PD patients and experimental animals (Strafella et al., 2001, 2003; Khedr et al., 2007; Choand Strafella, 2009).

On the other hand, the underlying mechanism of rTMS still remains to be elucidated. Recently, several experiments have shown that rTMS has the ability to mediate neuroplasticity by enhancing the expressions of glutamate neurotransmitters and brain-derived neurotrophic factor (BDNF) in rat brains (Müller et al., 2000; Keck et al., 2000; Yue et al., 2009).

rTMS treatment did not only activate brain regions in terms of immediate early gene expression, but also increased the expression of BDNF-TrkB signaling in rats and humans (Ji et al., 1998; Hausmann et al., 2000; Doi et al., 2001; Wang et al., 2011). Additionally, rTMS modulated neurotrophic factors such as BDNF, cholecystokinin and neuropetide tyrosine in healthy humans and patients with depression and amyotrophic lateral sclerosis (Angelucci et al., 2004; Yukimasa et al., 2006; Gedge et al., 2012). However, there are few rTMS studies on the effects of various neurotrophic/growth factors in PD.

To clarify the neuroprotective effect of rTMS on SNc DA neurons and the therapeutic mechanism in overall brain after rTMS treatment, the present study investigated motor functions and tyrosine hydroxylase (TH)-immunoreactive DA neurons and DA nerve fibers in the SNc and striatum. In addition, the expression of neurotrophic/growth factors was examined after rTMS application in an animal model of PD.

2. Results

2.1. Effects of rTMS on amphetamine-induced rotation test

Adult Sprague-Dawley rats were unilaterally injected with 6-hydroxydopamine (6-OHDA) into the right striatum. Two weeks after 6-OHDA injection, rats with PD were treated with rTMS (circular coil, 10 Hz, 20 min/day) daily for 4 weeks (Fig. 1A–C). Functional benefit in rTMS-treated rats on amphetamine-induced rotation behavior was evaluated up to 4 weeks after treatment (n=12 each). Whereas untreated group showed continuous augmentation in rotational behavior, the increasing rate of amphetamine-induced rotations was significantly lowered in rTMS-treated rats at 4 weeks after treatment (Fig. 2A). The number of rotations was not significantly different between the rTMS group (387.3±43.4) and the untreated group (319.2±43.1) at 2 weeks post-treatment. However, the number of rotations in the rTMS group (386.9±43.8) was significantly lower than that in the untreated group (549.9±33.6) at 4 weeks post-treatment (F=5.52, p=0.03).

2.2. rTMS improved locomotor function in a PD animal model

Treadmill locomotion test was assessed to determine the effects of the DA lesion on motor functions (n=12 each). The rTMS-treated rats showed improvement in locomotor function 2 weeks after treatment (280±25.0 mm), compared with the untreated group (225.1±34.1 mm) (Fig. 2B). The running distance of rTMS-treated rats continuously increased to a final score of 320.2±23.1 mm throughout the treatment period, while the distance of untreated groups maintained to the score of 230±35.5 mm at 4 weeks post-treatment (F=3.56, p=0.04), implying that rTMS induced motor improvements in the PD rats.
2.3. Neuroprotective effect of rTMS on DA neurons in the SNc

The therapeutic effect of rTMS on DA neurons in SNc was assessed by immunohistochemistry \((n=5\) each). Following unilateral 6-OHDA injection, the number of TH\(^+\) neurons decreased compared to the corresponding contralateral (non-lesioned) SNc \(\text{(Table 1)}\). Four weeks after treatment of rTMS, the number of TH\(^+\) neurons \((210 \pm 10.0\) in the ipsilateral (lesioned) SNc was significantly higher than the number...
in untreated group (144 ± 7.7) (t = 5.34, p = 0.01) (Fig. 3A, B), whereas the number of TH⁺ neurons in the contralateral SNc was not different between the untreated group and rTMS-treated group (Table 1). In addition, western blotting analysis demonstrated that the expression of TH increased in the ipsilateral hemisphere 4 weeks after rTMS treatment (Fig. 3C). The results suggest that rTMS treatment might preserve or slow DA neuronal degeneration from 6-OHDA administration.

### Table 1 - TH⁺ dopaminergic neurons in the SNc at 4 weeks after rTMS treatment.

<table>
<thead>
<tr>
<th></th>
<th>Contralateral side</th>
<th>Ipsilateral side</th>
<th>Survival rate (%)</th>
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<tbody>
<tr>
<td>Untreated (n=5)</td>
<td>1163.4 ± 43.5</td>
<td>144.0 ± 7.5</td>
<td>12.38 ± 0.4</td>
</tr>
<tr>
<td>rTMS (n=5)</td>
<td>1205.6 ± 41.6</td>
<td>210.8 ± 9.9</td>
<td>17.44 ± 1.4</td>
</tr>
</tbody>
</table>

Note: Values are mean ± SEM. Survival rate (%) is the percentage of TH⁺ neurons in the ipsilateral SNc relative to those in the contralateral SNc. TH = tyrosine hydroxylase; SNc = substantia nigra pars compacta; Untreated = PD animals without rTMS treatments; rTMS = PD animals treated with rTMS.

* p < 0.05 compared with untreated group.

#### 2.4. Neuroprotective effect of rTMS on DA efferents in the striatum

The therapeutic effect of rTMS on the striatal DA efferent fibers was evaluated by striatal TH immunohistochemistry. Representative findings of striatal TH staining in the ipsilateral hemispheres were observed compared with those in the contralateral hemispheres (Fig. 3D). Expressing volume values as...
the percentage of TH⁺ fibers in the ipsilateral striatum relative to those in the contralateral striatum, the volume of dopaminergic TH⁺ fibers was significantly higher in the rTMS group (22±7.3%) than in the untreated group (2.0±5.5%) at 4 weeks post-treatment (t=4.73, p=0.003) (Fig. 3E). The results suggest that more striatal TH⁺ efferent fibers in concurrence with more TH⁺ neurons in the SNc, relative to the untreated group, could improve motor performance following rTMS treatment.

2.5. Effects of rTMS on the expression of neurotrophic/growth factors

To identify the growth factors associated with the repair process of functional recovery and the neuroprotective and/or neurorestorative effects induced by rTMS, the expressions of various neurotrophic/growth factors including nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), platelet-derived growth factor AA (PDGF-AA), and vascular endothelial growth factor (VEGF) were investigated in ipsilateral hemispheres by multiplex ELISA assay (n=3 each). Among the factors, NGF (t=3.79, p=0.03), PDGF (t=3.12, p=0.009) and VEGF (t=2.59, p=0.02) levels were significantly elevated in the 6-OHDA-injected hemispheres of rTMS group compared with those of the untreated groups at 4 weeks after treatment, although the change in CNTF expression was not statistically significant (Fig. 4A–D).

To confirm the expressions of neurotrophic/growth factors, brain derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), NGF, PDGF, and VEGF (n=5 each) were investigated in both hemispheres by western blotting analysis. Expressions of neurotrophic/growth factors BDNF (t=3.14, p=0.013), GDNF (t=2.28, p=0.02), NGF (t=3.79, p=0.04), PDGF (t=6.15, p=0.008) and VEGF (t=3.02, p=0.02) significantly increased in the rTMS-treated rats compared with those in untreated group (Fig. 5A–E). The expressions of neurotrophic/growth factors exhibited a similar pattern to the multiplex ELISA results in the ipsilateral hemispheres. On the other hand, the expressions of neurotrophic/growth factors in the contralateral hemispheres were not significantly increased at 4 weeks after treatment (Fig. 5A–E).

2.6. Effects of rTMS on the expression of neurotrophic/growth factors in the SNc

To identify the effect of rTMS on the expression of neurotrophic/growth factors in the SNc, the expression levels of BDNF, GDNF, NGF, PDGF and VEGF were performed using immunohistochemistry in the ipsilateral SNc at 4 weeks after treatment (n=4 each). Representative findings of BDNF, GDNF, PDGF, and VEGF staining in the ipsilateral SNc after rTMS treatment were shown in comparison with untreated group (Fig. 6A). According to the density of neurotrophic/growth factors’ cells (%), calculated as the immunoreactive cell area (μm²) divided by the SNc area (μm²), the expressions of BDNF (t=4.54, p=0.04), GDNF (t=3.03, p=0.03), PDGF (t=2.81, p=0.02), and VEGF (t=2.34, p=0.042, n=5) were

![Fig. 4 - Neurotrophic/growth factors by multiplex ELISA assay. (A–D) The expression levels of neurotrophic/growth factors were increased in the rTMS treatment group compared with the untreated group (n=3 each). The levels of NGF (A), PDGF-AA (B), and VEGF (C) were significantly higher in the rTMS group than in the untreated group at 4 weeks post-treatment. Change in CNTF expression (D) was not statistically significant (*p < 0.05, **p < 0.01). Untreated—PD animals without rTMS treatments, rTMS—PD animals treated with rTMS, NGF—nerve growth factor, PDGF—platelet-derived growth factor, VEGF—vascular endothelial growth factor, CNTF—ciliary neurotrophic factor.](image-url)
significantly increased in the rTMS group compared with the untreated group (Fig. 6B). On the other hand, NGF+ cell density was not increased after rTMS treatment (data not shown). Taken together, these results suggest that the upregulation of various neurotrophic/growth factors in rTMS-treated hemispheres and SNc might preserve DA neuronal damage, consequently inducing functional recovery in rTMS-treated PD rats.

3. Discussion

rTMS has been shown to modulate cortical network and corticomotor excitability, and to improve motor function in patients with PD (Fisher et al., 2008; Yang et al., 2013). However, this study focused on the alterations of neurotrophic/growth factors after rTMS in a PD animal model rather than modulation of the cortical network. This study showed the therapeutic potential of rTMS to improve motor functions and to preserve DA neuronal survival by upregulation of neurotrophic/growth factors in a 6-OHDA-lesioned PD condition. This might be the first report to demonstrate modulation of various trophic factors by rTMS treatment in PD rats.

We demonstrated that rTMS elicited neuroprotective effects on functional outcomes in the amphetamine rotational test and treadmill locomotion test. Namely, rTMS treatment alleviated unilateral 6-OHDA induced rotation 4 weeks after treatment. This result was consistent with a previous study that demonstrated that the number of rotations was reduced by low-frequency (0.5 Hz) rTMS treatment (Yang et al., 2010). Treadmill locomotion test also revealed that rTMS has the capability to improve reciprocal locomotion in unilateral DA-lesioned rats (Pellis et al., 1987; Johnson et al., 1999; Lee et al., 2012). Treadmill locomotion test nonspecifically reflects cortical dysfunction as well as dysfunction of basal ganglia loops including striatal dopamine depletion (Chang et al., 2003), and can immediately control not only basal ganglia loops but also cortical networks (Arias-Carrion et al., 2011). On the other hand, amphetamine-induced rotation results from a persistent and consistent one side preference, and is thought to be mediated by lateralized functioning of the dopaminergic nigrostriatal pathways in brain. Amphetamine rotation preferences of unilateral PD rats have been shown to be related to not only...
endogenous asymmetries in striatal dopamine content but also survived DA neurons on SNc or DA nerve terminals in striatum in a dopamine-dependent manner (Glick et al., 1986). The PD animal model of this study, which was made by the 6-OHDA injected into striatum, was characterized by slowly evolving and progressive nigrostriatal degeneration until 8 weeks after 6-OHDA administration and resembles the progressive nature of the neurodegenerative process of human PD (Sauer and Oertel, 1994; Blandini et al., 2007). We observed that improvement in treadmill locomotion was observed at 2 weeks after rTMS treatment (4 weeks after 6-OHDA administration), but amphetamine turning improvements was not shown at the time point. A possible explanation for the difference between the amphetamine-induced rotation and treadmill locomotion outcomes is that the discrepancy might be related to different mechanism of each test at the time when degeneration of DA neurons was ongoing in the SNc.

The histological results of this study revealed that rTMS-treated rats had more striatal TH⁺ efferent fibers as well as more TH⁺ neurons in the SNc than those of the untreated group, demonstrating that rTMS has a neuroprotective effects on survival of DA neurons and DA nerve terminals in the ipsilateral SNc and striatum of 6-OHDA-induced PD rats.

Our results showed that TH⁺ fibers were primarily increased in the ventromedial striatum rather than dorsolateral striatum which plays a key role in motor function. However, previous studies have shown that ventral striatum was also related with motor functions such as amphetamine or apomorphine rotation and forepaw motor functions (Grealish et al., 2010; Heuer et al., 2012; Nozaki et al., 2013). Taken together, our data suggested that rTMS treatment might preserve or slow neuronal degeneration by 6-OHDA administration, and consequently improve motor performances in an animal model of PD.

Finally, we evaluated endogenous neuroprotective and/or neurorestorative proteins from the 6-OHDA-lesioned brains after rTMS treatment. We demonstrated that rTMS promoted increases in expression levels of neurotrophic/growth factors such as BDNF, GDNF, NGF, PDGF, and VEGF in ipsilateral hemispheres and SNc. The present study confirmed that these factors increased in the rTMS group rather than in the untreated PD group at 4 weeks after treatment. rTMS can change signaling pathways and gene transcription, immediately early gene expression, and initiate the biosynthesis of new molecules which persist in the tissue beyond the period of stimulation, consequently increasing the synthesis of growth factors (Arias-Carrion et al., 2011).
Among these factors, BDNF is expressed by DA neurons in both the SN and the ventral tegmental area (Baquet et al., 2005; Peterson and Nutt, 2008). In postmortem samples of PD patients, BDNF as well as GDNF was lower in the SNC than in controls (Mogi et al., 1999; Howells et al., 2000; Peterson and Nutt, 2008). BDNF protected DA neurons in vitro from the neurotoxic effects of 1-methyl-4-phenylpyridinium ion and 6-OHDA. Intrastratal injection of BDNF in a rat model prior to lesioning reduced destruction of DA neurons in the SNC and decreased the apomorphine-induced rotation (Spina et al., 1992; Shults et al., 1995; Mogi et al., 1999). rTMS is well known for changing the expression of BDNF. Previous studies have reported that high-frequency rTMS increased the level of BDNF expression in vivo and in patients with neuropsychiatric disorders (Kole et al., 1999; Müller et al., 2000; Angelucci et al., 2004; Yukimasa et al., 2006; Yue et al., 2009). However, there are few reports that rTMS modulates other neurotrophic factors except BDNF.

GDNF has been shown to promote the survival of DA neurons and has powerful protective effects for neurotoxin-induced degenerated DA neurons (Lin et al., 1993; Höffer et al., 1994; Gill et al., 2003; Slevin et al., 2005; Lang et al., 2006). In the DA-depleted rat model, GDNF protects 6-OHDA, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and medial forebrain bundle axotomy-induced degenerated midbrain DA neurons (Kearns and Gash, 1995; Tomac et al., 1995; Cohen et al., 2003; Bradley et al., 2010). In addition, NGF supports sympathetic and sensory neurons, and functions in the development and maintenance of cholinergic neurons in the basal forebrain (Sofroniew et al., 2001). NGF level decreased in experimental models and patients with PD. DA neurons and NGF are strongly believed to be functionally related. When NGF was administrated in a 6-OHDA-lesioned rat model of PD, a significant increase of TH+ neurons was also observed in the SN, showing potential neuroprotective effects of NGF on nigrostriatal DA neurons (Lorigados Pedre et al., 2002; Chaturvedi et al., 2006). PDGF is a major mitogen for fibroblasts, smooth muscle cells and other cells (Heldin and Westermark, 1999). PDGF also has the neuroprotective and neurotrophic functions such as neuronal survival and neurite formation not only in cultures of rat DA neurons (Nikkhah et al., 1993; Othberg et al., 1995; Pietz et al., 1996), but also in DA neurons in the SN of 6-OHDA-lesioned rats (Funa et al., 1996). It has been suggested that PDGF agonists may be potential therapeutic agents for patients with PD (Othberg et al., 1995). VEGF is an established angiogenic factor with specificity for endothelial cells (Jin et al., 2000; Rosenstein and Krum, 2004). Recently, VEGF has been reported to have neuroprotective effects in cerebral ischemia and ischemic peripheral neuropathy, and neurogenesis effects in neurological disorders (Schatzberger et al., 2000; Yasuhara et al., 2004a). The increased survival of the DA neurons by VEGF might be mediated by neuroprotective mechanisms. Appropriate neovascularization could improve microcirculation around DA fibers in the striatum, and protect DA neurons (Jin et al., 2000; Yasuhara et al., 2004a, 2004b, 2005). Taken together, enhanced various neurotrophic/growth factors might protect neuronal populations against excitotoxic, oxidative, and metabolic insults as described in the previous reports (Mattson et al., 2002; Mattson and Lindvall, 1997).

In addition, there are evidences that a variety of neurochemical factors induced by physical exercise lead to motor functional improvement as well as neuroprotection in animal models of PD. (Tuon et al., 2012; Lau et al., 2011). Previous reports also showed that neurotrophic factors such as BDNF and NT-3 augment locomotor activity and rotational behavior, and striatal DA turnover, indicating that BDNF enhances the nigrostriatal DA system function (Shen et al., 1994; Martin-Iverson et al., 1994; Altar et al., 1994). Therefore, we considered that neurotrophic factors induced by rTMS might modulate nigrostriatal DA system, leading to functional recovery.

As a limitation of this study, we did not elucidate mechanisms of rTMS that involve modulation of the cortical network. Instead of functional alterations of the corticospinal pathway involving short interval intracortical inhibition and paired-association stimulation effects (Cantello et al., 2002), this study focused on the alterations of neurotrophic/growth factors after rTMS in a PD animal model. Because this study did not evaluate neurophysiologic function, such as motor evoked potentials, we could not interpret behavioral outcomes with respect to modulation of the cortical network or activation of the nigrostriatal dopaminergic pathway.

The other limitation of this study is that repeated amphetamine administration has been reported to induce sensitization and priming effects in hemiparkinson rats (Erhardt et al., 2004). Therefore, it is possible that rTMS can affect the priming process instead of behavioral improvement from preserved DA neuronal damage in the SNC and upregulation of neurotrophic/growth factors. However, amphetamine-induced rotation test is a method that has been widely used to evaluate motor function related to endogenous DA depletion in hemiparkinson rat models. In this study, amphetamine-induced rotational test revealed that both groups continuously increased in the number of rotations until 6 weeks after the 6-OHDA injection. A possible explanation for the increased rotations is that this experimental model was characterized by evolving progressive nigrostriatal degeneration, resembling the progressive nature of the neurodegenerative process of human PD (Sauer and Oertel, 1994; Blandini et al., 2007).

Another limitation of this study is that we did not provide evidence for neurorestorative effects, including the repopulation of neurons, increased endogenous neural stem/progenitor cells, synaptogenesis and angiogenesis. On the other hand, this study discerned the upregulation of neurotrophic/growth factors in rTMS-treated hemispheres and SNC. Recent literature demonstrated that GDNF involved neurorestorative processes (Airavaara et al., 2012). VEGF and PDGF were also shown to be related with neurorestoration by angiogenesis (Zhang and Chopp, 2009). Therefore, we need to validate the neurorestorative processes described above in future studies.

In conclusion, rTMS treatment improved motor functions and preserved DA neuronal damage by 6-OHDA administration in a rat model of PD. Additionally, rTMS promoted expression of various growth factors such as BDNF, GDNF, NGF, PDGF-AA, and VEGF. The neuroprotective effects might be induced by upregulation of neurotrophic/growth factors. Collectively, our results could support a therapeutic potential role of rTMS in the treatment of PD, extending its application to other neurological diseases.
4. Experimental procedure

4.1. Animals

Animal use and care protocols were approved by the Institutional Review Board, and the Institutional Animal Care and Use Committee. Thirty adult male Sprague-Dawley rats (body weight, 250–300 g; Charles River Lab, Wilmington, MA, USA) were used. All efforts were made to minimize the number of animals required and to ensure minimal suffering. Rats were housed with free access to food and water in a room maintained at constant room temperature (20–22 °C) with alternate 12-h light/dark cycles according to animal protection regulations.

4.2. 6-hydroxydopamine (6-OHDA) lesion

Animals were pretreated with norepinephrine transporter blocker desipramine hydrochloride (12.5 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) 30 min before surgery. For the surgery, rats were deeply anesthetized with an intraperitoneal injection of ketamine (70 mg/kg body weight) and xylazine (8 mg/kg body weight). Each animal was positioned within a stereotaxic apparatus (Stoelting Co., USA). The 20 μg/4 μl/site of 6-OHDA (Sigma-Aldrich, St. Louis, MO, USA) was injected into two sites (total of 40 μg) in the right striatum according to the brain atlas (Paxinos and Watson, 2009). The 6-OHDA was injected at the following coordinates relative to bregma and dura: anterior–posterior (AP) +0.5 mm, medial–lateral (ML) 2.5 mm, dorsal–ventral (DV) –5.0 mm and AP –0.5 mm, ML 4.2 mm, and DV –5.0 mm at a rate of 1 μl/min using a 26 G Hamilton syringe (Fig. 1B). The inserted needle was withdrawn from each location after 5 min, and the skin was sutured. Afterward, animals were kept on a heating pad at 37 °C until they completely recovered. Ten days after the injections, animals showing more than 100 rotations per 50 min were considered to be successfully altered and were used for further experiments (n=24). Animals were sacrificed at 6-week time intervals post-lesion. The contralateral side of the brain was used as the internal control.

4.3. Repetitive transcranial magnetic stimulation

The rats with PD were randomly allocated into the following two groups: rTMS group and untreated controls (n=12 each). The rats were immobilized with an acrylic holder, and the distance between the rat’s head and the coil was maintained at 1 cm. The coil was placed parallel to the skull of the rat. The rTMS was administered to awake animals by using a round coil (5-cm diameter) and the Bicon-100 (M-cube Tech, Korea) at a rate of 10 Hz for 1 s with 100% power that generates a field of approximately 1 T and the duration of stimulation was 20 min per day. The stimulation intensity was set to 100% machine output. The treatments were performed daily for 4 weeks (Fig. 1A).

4.4. Amphetamine-induced rotation test

Rotational behaviors were evaluated in response to intraperitoneal injection of d-amphetamine (5 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) on day 10 after the 6-OHDA injection. Animals were injected with the amphetamine and placed into an automated multichannel rotometer system (ROTORAT, MED Associates, Inc., St Albans, USA). Each animal was placed into a cylindrical test chamber for 50 min. Animals with more than 100 asymmetric turns per 50 min were used for experiments. Amphetamine-induced rotation test was performed at 2 and 4 weeks after treatment.

4.5. Treadmill locomotion test

The rats were placed on a treadmill which was covered with a see-through box with lines drawn from 0 to 40 cm. The treadmill belt moved at 72 rpm during the on cycles. After the rats adapted to running for 1 min, the lengths from the beginning of the box to the end of the rats’ noses were measured. One cycle was defined as 20 s of running and 20 s of rest. The treadmill locomotion test measurement was repeated five times, and the average result was used.

4.6. Immunohistochemistry

Six rats of each group were deeply anesthetized as described above, and the thorax was opened. The rats were perfused transcardially with 100 ml of 0.9% sodium chloride, then with 200 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brain was carefully removed from the skull, fixed for 12 h in the same fixative, and infiltrated with 30% sucrose solution at 4 °C until they sank. The brains were then placed on a brain blocker (David Kopf Instruments, St. Tujunga, USA) and sliced into a block containing the striatum or SNC, based on the atlas of Paxinos and Watson (2009). The block was then rapidly frozen in 2-methylbutane, chilled on dry ice, and mounted in Tissue-Tek OCT compound (Sakura Finetechical Co., Tokyo, Japan). Serial coronal sections 40 μm in thickness were obtained using a cryostat microtome (Leica Microsystems Inc., Wetzlar, Germany) and were distributed sequentially into a set of 12 tissue wells containing 0.1 M PB. After a brief wash with 0.1 M PB, the brain sections were transferred to another set of 12 tissue wells containing cryoprotection solution and then stored in a freezer at a temperature below –20 °C until use. The sections were washed three times with 0.1 M PBS and treated with 3% hydrogen peroxide for 15 min to suppress endogenous peroxidase activity. After washing with 0.1 M PBS, the sections were treated for 1 h with 5% normal serum obtained from the same host species to reduce non-specific binding and were then incubated with rabbit anti-TH monoclonal antibody (1:1000; Chemicon, Temecula, CA, USA), rabbit anti-BDNF (1:200; Abcam, Cambridge, UK), rabbit anti-GDNF (1:200; Abcam, Cambridge, UK), rabbit anti-NGF (1:200; Abcam, Cambridge, UK), rabbit anti-PDGF (1:200; Abcam, Cambridge, UK) and rabbit anti-VEGF(1:200; Abcam, Cambridge, UK) diluted in 0.1 M PBST for 12 h. Sections were washed in PBS and incubated for 1 h with biotinylated goat anti-rabbit IgG (1:200; Vector, Burlingame, CA, USA) for TH and goat-anti rabbit Alexa-488 for TH and growth factors. After rinsing, sections were exposed to avidin–biotin peroxidase complex (Vector) for 1 h prior to incubation. For peroxidase detection, chromogens including 3,3′-diaminobenzidine
tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, MO, USA) and DAB-nickel (Vector) chromogen were used for 3–5 min.

4.7. Image analysis

TH positive neurons on both sides of the SNc were counted with the analySIS image analyzer system (Olympus Soft Imaging System, Münster, Germany), and the percentage of TH⁺ neurons was calculated by dividing the average number of TH⁺ neurons in the ipsilateral SNc by the number of TH⁺ neurons in the contralateral SNc. Light photomicrographic images were acquired on a Nikon Optiphot microscope (Nikon Inc., Tokyo, Japan) fitted with a Nikon digital camera (DXM1200), using Nikon ACT-1 image capture software (ver. 2.2). TH positive neurons on both sides of the striatum were measured using the MetaMorph Imaging System (Molecular Device, Sunnyvale, CA), and the percentage of TH⁺ fibers was calculated by dividing the average volume of TH⁺ fibers in the ipsilateral striatum by the volume of fibers in the contralateral striatum. The area of the SNc was obtained using the MetaMorph Imaging System (Molecular Device, Sunnyvale, CA) and converted to volume by multiplying the area by the section thickness (40 μm). BDNF⁺, GDNF⁺, NGF⁺, PDGF⁺ and VEGF⁺ cells (%) in the SNc (mm²) were quantified using the MetaMorph Imaging System (Molecular Device, Sunnyvale, CA). The images were imported into Adobe Photoshop (ver. 7.0, Adobe Systems Inc., San Jose, CA, USA) and were adjusted for brightness and contrast to optimize photographic representation of the images obtained by the microscope.

4.8. Western blotting

To compare the expression levels of neurotrophic factors, ipsilateral hemispheres (n=5 per group) were lysed in 500 μl of cold RIPA buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), and 1% sodium deoxycholate) with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Tissue lysate was centrifuged at 13,000 × g for 15 min at 4 °C. The supernatant was harvested, and protein concentration was analyzed using a BCA assay kit (Thermo Scientific, H90014A, Sigma–Aldrich). To electrophorese, 50 μg of protein was dissolved in sample buffer (60 mM Tris–HCl, pH 6.8, 14.4 mM β-mercaptoethanol, 25% glycerol, 2% SDS, and 0.1% bromophenol blue), boiled for 10 min and separated on a 10% SDS reducing gel. The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Invitrogen, Carlsbad, CA) using a trans-blot system. Blots were blocked for 1 h in Tris-buffered saline (TBS) (10 mM Tris–HCl, pH 7.5, 150 mM NaCl) containing 5% nonfat dry milk at room temperature, washed three times with TBS and incubated at 4 °C overnight with an anti-rabbit polyclonal BDNF (1:1000, Abcam), anti-rabbit glial cell-derived neurotrophic factor (GDNF, 1:1000, Abcam), anti-rabbit NGF (1:1000; Abcam, Cambridge, UK), anti-rabbit PDGF (1:1000; Abcam, Cambridge, UK), anti-rabbit VEGF (1:1000; Abcam, Cambridge, UK) and anti-rabbit TH (1:1000; Abcam, Cambridge, UK) and anti-GAPDH (1:3000, Cell signaling, Boston, MA, USA) antibody in TBS (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.02% Tween 20) containing 5% nonfat dry milk. On the next day, blots were washed three times with TBS and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (1:3000, Santa Cruz Biotech, Santa Cruz, CA, USA) in TBST containing 3% nonfat dry milk at room temperature. After washing three times with TBST, proteins were visualized with an ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

4.9. Multiplex ELISA

To identify growth factors regulated by rTMS, a multiplex ELISA assay (Quantibody® array, RayBio-tech, Norcross, GA) was used to determine which of the following growth factors were detectable in the ipsilateral hemispheres (n=3 per group): NGF, CNTF, PDGF-AA and VEGF. Expression of growth factors was detected using an array scanner (Gene PIX™4000B, Axon instruments, USA).

4.10. Data analysis

Statistical analysis was performed using the t-test (Prism Graph Pad Software, San Diego, CA, USA) and two-way repeated ANOVA, followed by a Bonferroni post-hoc comparison (SAS version 9.2, SAS Institute Inc., Cary, NC, USA). The data were expressed as a mean ± standard error of the mean (SEM). The significance level was assumed at p<0.05, unless otherwise indicated.

Author contributions

Lee JY: Conception and design, collection and/or assembly of data, manuscript writing; Kim SH: Conception and design, data analysis and interpretation, manuscript writing; Ko AR, Lee JS,Yu JH, Seo JH: Collection and/or assembly of data; Cho BP: Administrative support, conception and design, data analysis and interpretation; Cho SR: Data analysis and interpretation, manuscript writing, final approval of manuscript.

Acknowledgments

This study was supported by grants from the National Research Foundation Research 2009-0077194; 2010-0020408; 2010-0024334 funded by the Ministry of Education, Science and Technology, Republic of Korea, and the Korea Health Technology R&D project, Ministry of Health & Welfare (A100054).


