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ORIGINAL RESEARCH

Repetitive Transcranial Magnetic Stimulation Alleviates MPTP-Induced Parkinson's Disease Symptoms by Regulating CaMKII-CREB-BMAL1 Pathway in Mice Model

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Background: Repetitive transcranial magnetic stimulation (rTMS) is a noninvasive neuromodulation technique that shows promise for the treatment of Parkinson's disease (PD). However, there is still limited understanding of the optimal stimulation frequencies and whether rTMS can alleviate PD symptoms by regulating the CaMKII-CREB-BMAL1 pathway.

Methods: A PD mouse model was induced intraperitoneally with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and treated with 1 Hz, 5 Hz, and 10 Hz rTMS. The neurological function, survival of dopaminergic neurons, and protein levels of Tyrosine hydroxylase (TH), α-synuclein(α-syn), and brain-derived neurotrophic factor (BDNF) in the striatum were measured to determine the optimal stimulation frequencies of rTMS treatment in PD mice. The levels of melatonin, cortisol, and the circadian rhythm of Brain and muscle ARNT-like 1 (BMAL1) in PD model mice were detected after optimal frequency rTMS treatment. Additionally, KN-93 and Bmal1siRNA interventions were used to verify that rTMS could alleviate PD symptoms by regulating the CaMKII-CREB-BMAL1 pathway.

Results: Administration of 10 Hz rTMS significantly improved neurological function, increased the protein levels of TH and BDNF, and inhibited abnormal aggregation of a-syn. Furthermore, administration of 10 Hz rTMS regulated the secretion profile of cortisol and melatonin and reversed the circadian arrhythmia of BMAL1 expression. After the KN-93 intervention, the MPTP+rTMS+KN-93 group exhibited decreased levels of P- Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)/CaMKII, P-cAMP-response-element-binding protein (CREB)/CREB, BMALI, and TH. After Bmal1siRNA intervention, the protein levels of BMAL1 and TH were significantly reduced in the MPTP+10 Hz+ Bmal1siRNA group. At the same time, there were no significant changes in the proportions of P-CaMKIIα/CaMKIIα and P-CREB/CREB expression levels. Finally, immunohistochemical analysis showed that the number of TH-positive neurons was high in the MPTP+10 Hz group, but decreased significantly after KN-93 and Bmal1siRNA interventions.

Conclusion: Treatment with 10 Hz rTMS alleviated MPTP-induced PD symptoms by regulating the CaMKII-CREB-BMAL1 pathway. This study provides a comprehensive perspective of the therapeutic mechanisms of rTMS in PD.

Keywords: Parkinson's disease, transcranial magnetic stimulation, brain and muscle ARNT-like 1, Ca^{2+}/calmodulin-dependent protein kinase II, cAMP-response-element-binding protein

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and abnormal aggregation of α-synuclein (α-syn).[1,2] The

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clinical manifestations of PD include both motor and non-motor symptoms. In addition, non-motor symptoms present early in disease development and emerge with disease progression,[3] such as sleep disorders and circadian rhythm.[4] Currently, PD treatment mainly involves pharmacological therapies and deep brain stimulation (DBS). However, these treatments also have several disadvantages. Long-term treatment with drugs often causes motor complications such as dyskinesia and motor fluctuations.[5] DBS surgery is an invasive therapy with limitations in effectiveness and battery consumption.[6] Additionally, these treatments mainly improve the motor symptoms of PD, but do not alleviate non-motor symptoms and DA neuron degeneration.[7] Therefore, it is necessary to explore non-invasive and effective therapeutic measures to improve motor and non-motor symptoms, as well as to delay neurodegenerative processes.

Repetitive transcranial magnetic stimulation (rTMS) is a noninvasive neuromodulation technique that is promising for the diagnosis and treatment of neurological and psychiatric disorders.[8,9] rTMS has been widely used as an experimental nonpharmacological therapy for PD, which has a variable influence on motor and non-motor symptoms in PD patients.[10,11] In addition, rTMS induced dopamine release in the caudate nucleus.[12,13] It is well known that different stimulation frequency (1 Hz, 5 Hz and 10 Hz) can produce different biochemical, molecular, and cellular effects.[14] At present, it remains unclear which stimulation frequency has a better therapeutic effect in PD patients.[11,15,16] Moreover, the mechanisms by which rTMS improves motor and non-motor symptoms are not fully understood.

Circadian arrhythmia, a common non-motor symptom in patients with PD, has also been implicated in the pathogenesis of PD.[17,18] Brain and muscle ARNT-like 1 (BMAL1) is a positive regulator of the circadian clock feedback loops.[19] The abnormal expression of BMAL1 has been confirmed in animal models of PD and PD patient.[4,20] Abnormal BMAL1 expression is closely associated with PD pathogenesis.[21] Therefore, the regulation of BMAL1 may be a potential target for the treatment of PD. Previous studies[22,23] have suggested that the circadian clock gene can be influenced by the cytoplasmic Ca^{2+} circadian rhythm and is involved in the signaling pathway of the resultant phosphorylation of the cAMP-response-element-binding protein (CREB) by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which has been reported that rTMS can activate the CaMKII-CREB signaling pathway and subsequently enhance the downstream target genes and related protein expression in Neuro-2a cells.[24] However, it remains unclear whether rTMS can regulate BMAL1 expression by modulating CaMKII-CREB signaling pathways in PD.

Therefore, we designed this study to investigate (I) the effects of 1 Hz, 5 Hz, and 10 Hz rTMS interventions on DA neurons, neurological functions, and circadian rhythm in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD mice, and (II) elucidate the molecular mechanism by which rTMS could alleviate PD symptoms by regulating CaMKII-CREB-BMAL1 pathways.

Material and Methods

Experimental Groups and Research Design

The mice were allocated in a randomized manner using a random number table and distributed by a technician blinded to the group assignments. Prior to establishing the MPTP-induced PD model, all mice were randomly divided into the Control and MPTP groups. Following the construction of the MPTP-induced PD model, the MPTP mice were randomly assigned to different subgroups based on a random number table by the same technician. Further details are provided below and illustrated in Figure 1.

Experiment 1: The Effects of Different rTMS Frequencies on the Neurobehavior and DA Neurons, α-Syn and BDNF Expression in MPTP-Induced PD Mice

The mice were randomly allocated to the control and MPTP groups. The MPTP group was further divided into four subgroups (sham, 1 Hz, 5 Hz, and 10 Hz rTMS) based on the different stimulation frequencies for subsequent analysis. Neurobehavioral assessments, including rotarod and Morris Water Maze (MWM) tests, were conducted on all experimental mice. SNpc and striatum tissues from all experimental mice were collected simultaneously for immunohistochemical and Western blot analyses (Figure 1A).

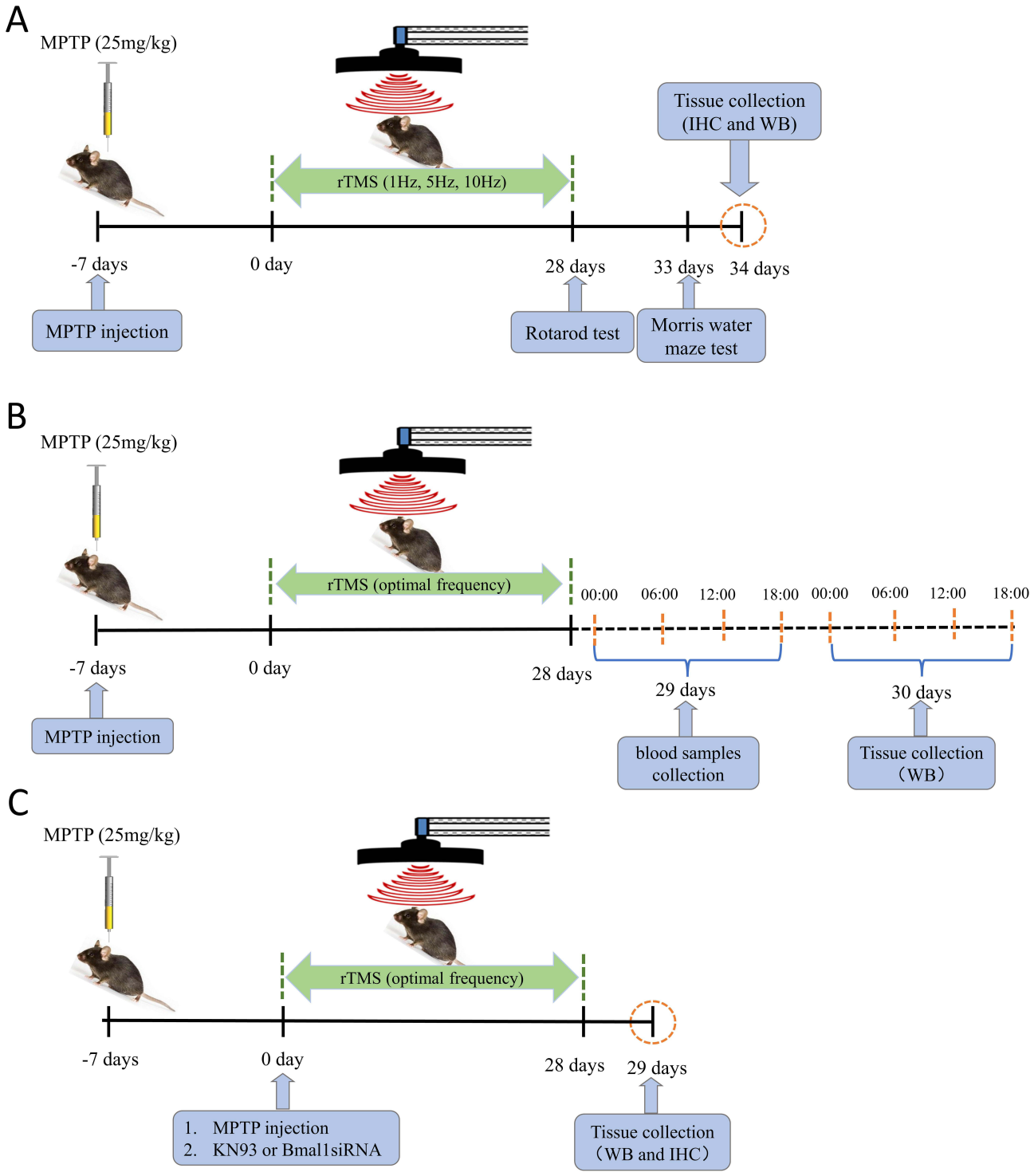

Figure 1 Experimental design.
Notes:(A) Experiment 1: The effects of different rTMS frequencies on the neurobehavior and DA neurons, α-syn and BDNF expression in MPTP-induced PD mice. (**B**) Experiment 2: The effects of optimal rTMS frequencies on secretion levels of cortisol and melatonin and BMAL1 expression in MPTP-induced PD mice. (**C**) Experiment 3: The effects of optimal rTMS frequencies on the CaMKII-CREB-BMAL1 pathway in MPTP-induced PD mice.
Abbreviations: rTMS, repetitive transcranial magnetic stimulation; DA, Dopamine; a-syn, α-synuclein; BDNF, brain-derived neurotrophic factor; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; CAMKII, Ca2+/calmodulin-dependent protein kinase II; CREB, cAMP-response-element-Binding protein; BMAL1, Brain and muscle ARNT-like 1.

Experiment 2: The Effects of Optimal rTMS Frequencies on Secretion Levels of Cortisol and Melatonin and BMAL1 Expression in MPTP-Induced PD Mice

The mice were randomly assigned to different groups based on the optimal frequency determined in Experiment 1. The four research groups were designated as the control, MPTP, MPTP+Sham rTMS, and MPTP+rTMS groups. After establishing the MPTP-induced PD model and administering rTMS, 30 mice (30 mice were used, but 24 mice survived after intervention) were randomly allocated to four groups for blood sample collection. The serum melatonin and cortisol levels were measured using specific ELISA kits. Additionally, brain tissues from each group were collected at various time points throughout a 24-hour period to analyze circadian rhythm alterations of BMAL1 protein via Western blot analysis. Brain tissues from all four groups were collected simultaneously at specific time points (06:00, 12:00, 18:00, and 24:00) for Western blot analysis to compare the expression levels of the BMAL1 protein among the groups (Figure 1B).

Experiment 3: The Effects of Optimal rTMS Frequencies on the CaMKII-CREB-BMAL1 Pathway in MPTP-Induced PD Mice

This study aimed to investigate the potential involvement of rTMS in modulating the CaMKII-CREB-BMAL1 pathway. We assessed the expression levels of p-CaMKIIα, CaMKIIα, p-CREB, CREB, BMAL1, and TH in the striatum using KN-93 and Bmal1siRNA. First, we established an MPTP-induced PD model and performed KN-93 intervention. The research groups were divided into MPTP+rTMS and MPTP+rTMS+KN-93 groups. After a 4-week rTMS treatment, immunohistochemistry (IHC) and Western blot analyses were conducted on SNpc and striatum tissues from both groups. Similarly, we established an MPTP-induced PD model with Bmal1siRNA intervention in another set of research groups: MPTP+rTMS and MPTP+rTMS+Bmal1siRNA. After a 4-week rTMS treatment period, IHC and Western blot analyses were performed on SNpc and striatum tissues (Figure 1C).

Animals and MPTP Treatment

All male mice (C57/BL6) (25–30g) were housed in a room with controlled temperature (24°C ± 2°C) and humidity (50% ± 5%), maintained on a half day light/dark cycle, and fed standard rodent chow and water. MPTP-induced PD mice were established as described previous studies.[25] Briefly, after anesthetization with isoflurane, mice were treated intraperitoneally with MPTP (25 mg/kg) once a day for seven days. Mice in the control group were injected with the same volume of physiological saline. The experimental protocol was approved by the Animal Subject Review Committee of Soochow University (No. 2020147) and conducted according to the recommendations of the National Institutes of Health Guidelines for the Care and Use of Experimental Animals. Efforts have been made to reduce the number of animals used and their suffering.

Reagents

MPTP (Sigma, M0896, USA) was used for intraperitoneal injection. The si-RNAs (sense: 5′-CCU CAA UUA UAG CCA GAA UTT-3′, antisense: 5′-AUU CUG GCU AUA AUU GAG GTT-3) targeting the Bmal1siRNA were purchased from RiboBio Corporation (Guangzhou, China). As Bmal1iRNA can be delivered into the cerebrospinal fluid, an intracerebroventricular injection was selected to conduct Bmal1SiRNA or control siRNA interventions. Based on previous studies,[26] Bmal1SiRNA or control siRNA was injected intracerebroventricularly (AP= 0.46, L=1.0, DV=2.5) 0 days before rTMS treatment in this study (Figure 1C). KN-93 (HY-15465) was purchased from the MedChemExpress (MCE) Corporation (USA). According to previous methods,[27] KN-93 (10 mg/kg) or the same volume of DMSO was administered by intraperitoneal injection at 0 day before rTMS treatment in this study (Figure 1C).

rTMS Intervention

The MPTP-induced PD mice were randomly divided into five groups: MPTP, Sham, 1 Hz, 5 Hz, and 10 Hz. The rTMS apparatus (CCY-IA) was provided by the Yiruide Medical Equipment Company (Hubei, China). The rTMS was implemented using a circular coil for rodent use (2.5 cm internal diameter, 5 cm external diameter). Over the course of four weeks, the center of the coil was positioned in the center of the interocular line with the handle pointing forward. Mice in the rTMS

group received the corresponding real rTMS intervention five days per week for four consecutive weeks in a suitable awake state. Sham stimulation was applied by using a coil perpendicular to the target area of the scalp.[28] In accordance with previous studies,[28–30] the magnetic stimulation intensity was set at 20% maximum output, and the main parameters for 1 Hz were the 30 seconds(s) train length with a 4 s intertrial interval and repetition of 1050 pulses per day. The main parameters for 5 Hz were the 1s train length with a 3 s intertrial interval and a repetition of 1500 pulses per day. The parameters for 10 Hz were a 5 s train length with a 10s intertrain interval and repetition of 2000 pulses per day. During stimulation, the movement of the mice was restrained. To rule out the putative effects of nonspecific stress, each mouse was allowed to adapt to the rTMS intervention and subjected to a 10-min Sham stimulation before commencing the 4-week rTMS treatment.

Animal Neurobehavioral Evaluation

Rotarod Test

Motor function was assessed using a rotarod system (ZH-300; China).[31] The mice in each group received consecutive training (three times per day) and were ready to undergo the formal test. When the rotarod test speed reached 5 rpm and 20 rpm, the mice were tested three times at 30-minute intervals. Average time was used as the final result.

Morris Water Maze Test

The Morris water maze test was used to assess the learning and memory functions of mice in each group. Based on previously published methods,[32,33] all mice were trained for five consecutive days before the test. When the mice found the submerged platform, a computer tracking system (Noldus Ethovision, Tacoma, WA) recorded their swimming paths and escape latency times to assess their learning function. After the platform was removed, a probe trial was conducted to observe the time spent in the target quadrant and evaluate the memory function of the mouse.

Western Blotting

Fresh striatal and suprachiasmatic nucleus (SCN) tissues were collected from the brains of mice. The Ice-cold RIPA lysis buffer (Beyotime, China) was used to homogenize the tissues, and the homogenates were centrifuged for 10 min (4°C, 12,000 g). The supernatant was collected, and protein concentrations were measured using an enhanced BCA protein assay kit (Beyotime). Protein extracts from each group were loaded into SDS sulfate-polyacrylamide gels, separated, and electrically transferred to nitrocellulose membranes (Billerica, MA, USA). Subsequently, the membrane was blocked with 5% bovine serum albumin (BSA, BioSharp, China) for 1 h at 25°C. Afterward, The membranes was incubated with primary antibodies overnight at 4 °C. The following primary antibodies were used: TH (1:5000, Cat# T1299, Sigma, USA), α-syn (1:500, Cat#sc-53226, Santa Cruz Biotechnology, USA), BDNF (1:1000, Cat#T55577, Abmart, China), BMAL1 (1:500, Cat#MA5-25133, Invitrogen, USA), phospho-CAMKIIα^{Thr286} (1:500, Cat#12716, Cell Signaling Technology, USA), CaMKIIα (1:1000, Cat#3357, Cell Signaling Technology, USA), phospho-CREB^{Ser133} (1:1000, Cat#9198S, Cell Signaling Technology, USA), CREB (1:1000, Cat#9197S, Cell Signaling Technology, USA), β-actin (1:5000, Cat#A3854, Sigma, USA), GAPDH (1:5000, Cat#M20006, Abmart, China) and β-tubulin (1:2000, Cat#2146, Cell Signaling Technology, USA). Then, we incubated the membrane with an horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at 25 °C. Finally, band signals were detected using an enhanced chemiluminescence kit (Beyotime, China), and relative protein levels were analyzed using ImageJ software (NIH, Bethesda, MD, USA). Two technicians, who were blinded to the experimental groups, performed the analyses.

Immunohistochemistry (IHC)

According to previous experimental methods,[34] the mice were intracardially perfused with saline and the brain tissues were fixed with 4% paraformaldehyde after anesthetization with isoflurane. The fixed brain tissues were sectioned and embedded in paraffin. Midbrain sections containing SNpc were deparaffinized before staining. After antigen retrieval, we incubated the sections from were incubated with primary antibodies against TH (1:1000, T1299, Sigma, USA) at 4 °C overnight. After washing thrice, the sections were incubated with HRP-conjugated secondary antibodies, and a DAB kit (A10027, Abmart, China) was used to stain the sections. Finally, the sections were observed and photographed using

a microscope (Nikon, Japan). The number of TH-positive cells in the SNpc was counted in every six sections by two researchers who were blinded to the experimental conditions.

Elisa

After establishing PD mice and rTMS administration, blood samples were collected from each mouse by puncturing the heart under anesthesia. The Blood samples were centrifuged at 1000 g and 4 °C for 5 min. The supernatants were collected to detect melatonin and cortisol levels using specific ELISA kits (AB-2963 and AB-3301, Abmart, China) according to the manufacturer's instructions.

Statistical Analysis

Data are expressed as the mean ± SEM, and data analysis was performed using GraphPad Prism 8 software. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to analyze multiple groups. Cosinor analysis was performed using R (version 4.2.1; R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org/) using the CircaCompare and Cosinor2 packages. A *P*-value less significance was set at $P < 0.05$.

Results

Treatment with 10Hz rTMS Improves Neurological Function in Mice with MPTP-Induced PD

To compare the therapeutic efficacy of different frequencies of rTMS on neurobehavioral outcomes in MPTP-induced PD mice, we conducted Rotarod and Morris water maze tests to evaluate motor and cognitive functions after rTMS intervention. MPTP-treated mice (25 mg/kg) exhibited a significantly diminished rotarod retention time compared to the control group, indicating impaired muscle coordination ($P<0.01$). Among the different frequencies of rTMS, only 10 Hz stimulation significantly enhanced grip strength in MPTP mice ($P<0.01$), while no differences were observed in motor coordination or balance between the control group and those treated with 1 Hz or 5 Hz rTMS (Figure 2A and B). Furthermore, the MPTP+10 Hz rTMS group spent less time searching for the platform during the spatial learning trials, suggesting that 10 Hz stimulation significantly improved their spatial learning abilities (Figure 2C and D). In the memory tests conducted after removing the platform, mice in the MPTP+10 Hz rTMS group spent more time in the target platform quadrant than those receiving rTMS at other frequencies (Figure 2C and E). Based on these findings, it can be concluded that treatment with 10 Hz rTMS effectively improved both motor and cognitive function in MPTP-induced PD mice.

Treatment with 10Hz rTMS Protected DA Neurons Against MPTP Lesion and Increases the BDNF Level in MPTP-Induced PD Mice

The therapeutic effects of different rTMS frequencies on the survival of DA neurons in the SNpc and the protein expression of TH, α-syn, and BDNF in the striatum were analyzed in this study. Seven days after MPTP injection, the number of TH-positive DA neurons in the SNpc significantly decreased in the sham rTMS group. However, administration of 10 Hz rTMS reduced the loss of TH-positive neurons induced by MPTP (Figure 3A and B). The tissue lysates dissected from the striatum of different groups were analyzed by Western blotting. Significant restoration of TH expression in the striatum was detected after 10 Hz rTMS for 4 weeks (Figure 3C and D). Additionally, compared to the MPTP group, downregulated a-syn expression and increased BDNF protein levels in the striatum were observed after 10 Hz rTMS for 4 weeks (Figure 3C and E). However, there were no significant differences in the survival of DA neurons and the expression levels of TH, a-syn, and BDNF among the sham, 1 Hz, and 5 Hz rTMS groups after 4 weeks of rTMS (Figure 3C–F). These data indicate that 10 Hz rTMS for 4 weeks exhibited significant beneficial effects on the survival of DA neurons against MPTP lesions, whereas other frequencies exhibited only modest beneficial effects.

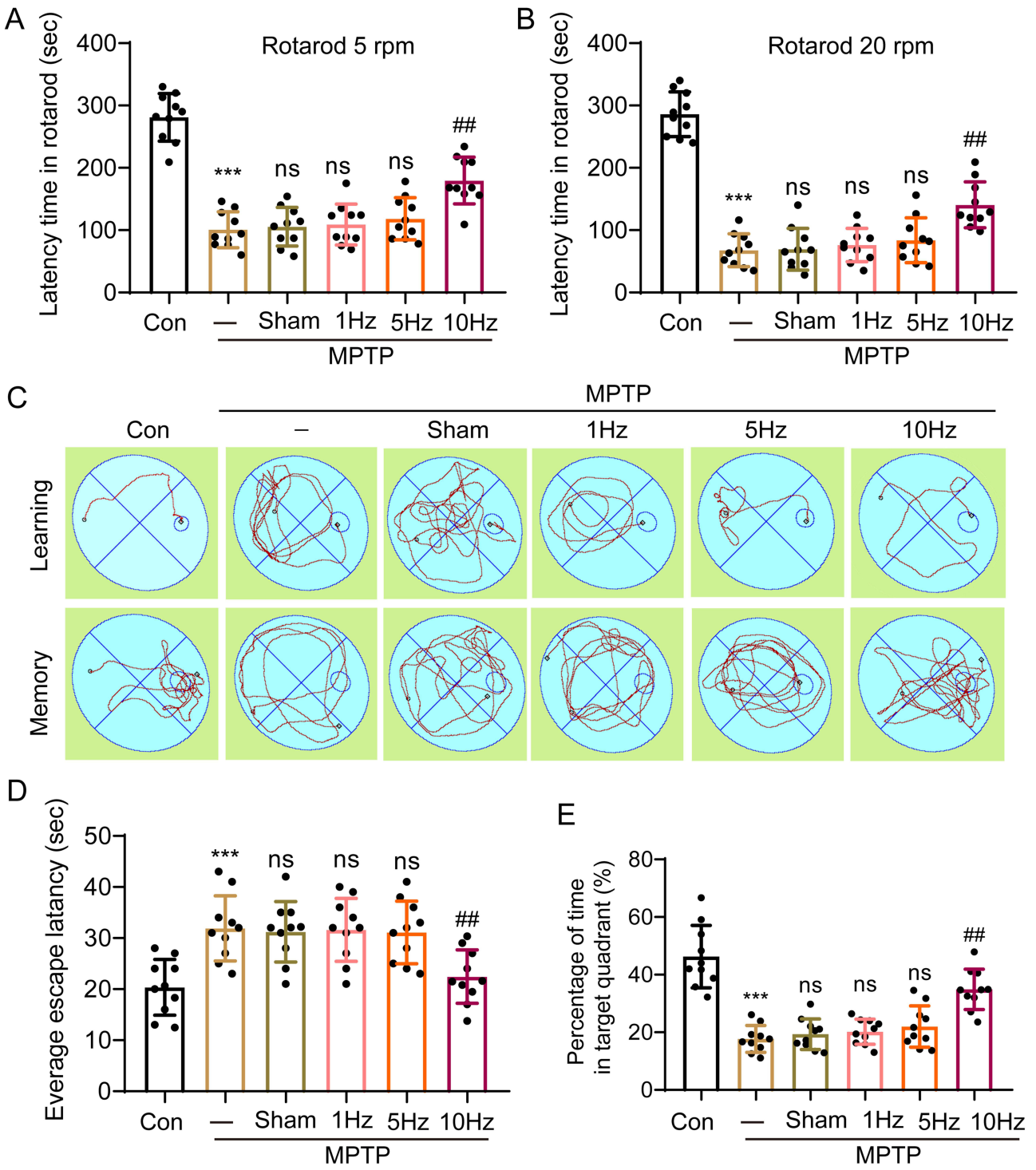

Figure 2 Treatment with 10Hz rTMS improves neurological function of mice with MPTP-induced PD.

Notes: (**A, B**) Rotarod performance after different frequencies rTMS treatment and MPTP at 5rpm and 20rpm rotarod speeds. (**C**) The learning and memory of representative images illustrate swimming trajectories in the Morris water maze test in each group. (**D, E**) Everage escape latency and time spent in the Morris water maze test in various groups. Values are given as mean ± SD (n = 6). ***$P<0.001$ vs control group; $^{\#\#}P<0.01$ vs MPTP group; ns, no significant difference vs MPTP group.

Abbreviations: rTMS, repetitive transcranial magnetic stimulation; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; rpm, Revolutions per minute; SD, Standard deviation.

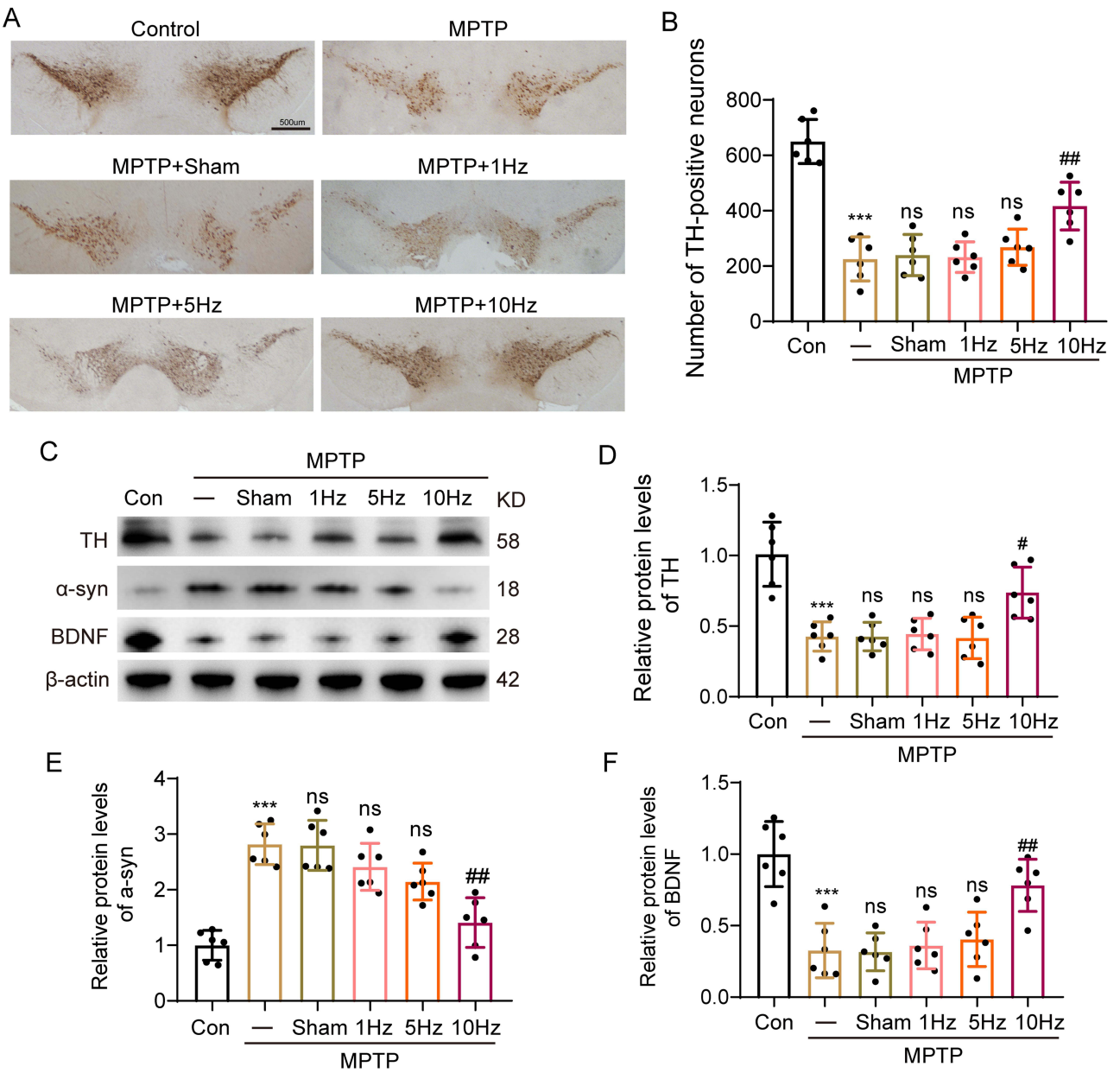

Figure 3 Treatment with 10Hz rTMS protected DA neurons against MPTP lesion and increases the BDNF Level in MPTP-induced PD mice.

Notes: (**A, B**) Representative immunohistochemistry showing TH-positive neurons in the SNpc of each group. (**C, D**) Representative Western blot and quantification of TH protein expression in the striatum (**C, E**) Representative Western blot and quantification of α-syn protein expression in in the striatum. (**C, F**) Representative Western blot and quantification of BDNF protein expression in the striatum. All data are represented as mean ± SD (n=6); ***P<0.001 vs control group; $^{\#\#}P$<0.01 vs MPTP group; $^{\#}P$<0.05 vs MPTP group; ns, no significant difference vs MPTP group.

Abbreviations: rTMS, repetitive transcranial magnetic stimulation; DA, Dopamine; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; TH, Tyrosine hydroxylase; a-syn, α-synuclein; BDNF, brain-derived neurotrophic factor; SNpc, Substantia nigra pars compacta; SD, Standard deviation.

Treatment with 10Hz rTMS Regulates the Secretion Profile of Cortisol and Melatonin in MPTP-Induced PD Mice

Melatonin and cortisol are key biomarkers of the circadian rhythm. First, compared to the MPTP group, there was a significant time-dependent effect on the secretion profile of cortisol in the control group (P<0.05). Following the 10 Hz rTMS treatment, a similar pattern was observed in the MPTP+10 Hz group. Moreover, there was a noticeable disparity in cortisol secretion levels between the MPTP+S + ham and MPTP+10 Hz groups (P<0.001). Specifically, when compared to the MPTP+Sham group, cortisol levels were significantly decreased at 12:00 (P<0.05) in the MPTP+10 Hz group, resembling those seen in the control group (Figure 4A).

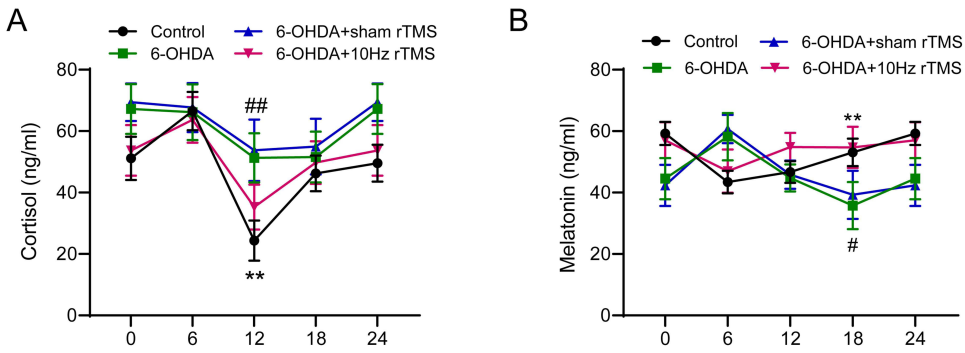

Figure 4 Treatment with 10Hz rTMS regulates the secretion profile of cortisol and melatonin in MPTP-induced PD mice.
Notes: The concentrations of cortisol (**A**) and Melatonin (**B**) were detected by ELISA. Values are given as mean ± SD, n =6 for each time point, equals to n = 12 in the Control, MPTP, MPTP+Sham rTMS, and MPTP+10Hz rTMS groups. $^{**}P<0.01$ for MPTP group versus control group; $^{\#\#}P<0.01$ and $^{\#}P<0.05$ for MPTP+10Hz rTMS group versus MPTP+Sham rTMS group.
Abbreviations: rTMS, repetitive transcranial magnetic stimulation; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; ELISA, enzyme-linked immunosorbent assay; SD, Standard deviation.

However, no obvious time-dependent variation in melatonin secretion patterns was observed among the four groups ($P>0.05$). However, the analysis of the area under the curve (AUC) for plasma melatonin indicated a decrease in the MPTP group (Figure 4B). The peak level of melatonin occurred at 24:00 in the control group but shifted to 6:00 in the MPTP group. Notably, treatment with 10 Hz rTMS appeared to reverse this phase shift to normalcy. Furthermore, similar to that seen in the control group, melatonin levels were significantly higher at 18:00 in both the MTP+10 Hz and control groups than in the MTP + Sham group ($P<0.05$).

Treatment with 10Hz rTMS Reverses Circadian Arrhythmia of BMAL1 in MPTP-Induced PD Mice

Cosinor analysis was employed to examine circadian rhythm alterations in BMAL1 protein expression in the suprachiasmatic nucleus (SCN) over a 24-hour period (Figure 5A–C). The acrophase values for the control, MPTP, and MPTP+10 Hz group were 0.394±0.058, 0.201±0.086, and 0.271±0.065, respectively. The corresponding peak times of the acrophase occurred at 5.3 hours in the control group, 10 h in the MPTP-lesioned group, and at a time point approximately two hours after treatment with 10 Hz rTMS stimulation. Notably, significant shifts in circadian phases were observed following administration of 10 Hz rTMS ($P<0.01$). Subsequently, BMAL1 protein levels were assessed at identical time points across all four groups, revealing significantly lower levels of BMAL1 protein in the MPTP-induced PD mice compared to controls at both 06:00 and12:00 (Figure 5D–G). However, upon application of high-frequency rTMS (10 Hz), BMAL1 protein levels at time points: 06:00,18:00, and 24:00. Conversely, a sharp decrease was observed at 12:00 h (Figure 5D–G). These findings demonstrate that intervention with 10 Hz rTMS can effectively reverse circadian arrhythmia associated with altered expression patterns of BMAL1 in MPTP-induced PD mice.

Protective Effects of 10Hz rTMS Against PD Symptoms are Dependent on the CaMKII-CREB-BMAL1 Pathway

To determine the involvement of CaMKII-CREB-BMAL1 pathway modulation by 10 Hz rTMS, we initially assessed the expression levels of P-CaMKIIα, CaMKIIα, P-CREB, CREB, and TH in the striatum and BMAL1 protein expression in the SCN using KN-93 intervention. As shown in Figure 6A–C, there was a significant decrease in the ratio of P-CaMKII/CaMKII and P-CREB/CREB expression levels after KN-93 intervention in the MPTP+10 Hz rTMS group. Concurrently, BMAL1 expression levels were also noticeably reduced in SCN following KN-93 treatment (Figure 6A and D). Moreover, decreased TH protein levels were detected in the MPTP+10 Hz rTMS+KN-93 group (Figure 6A and E). Additionally, immunohistochemical analysis revealed a significant reduction in the number of TH-positive DA neurons within the SNpc after KN-93 treatment (Figure 6F–G).

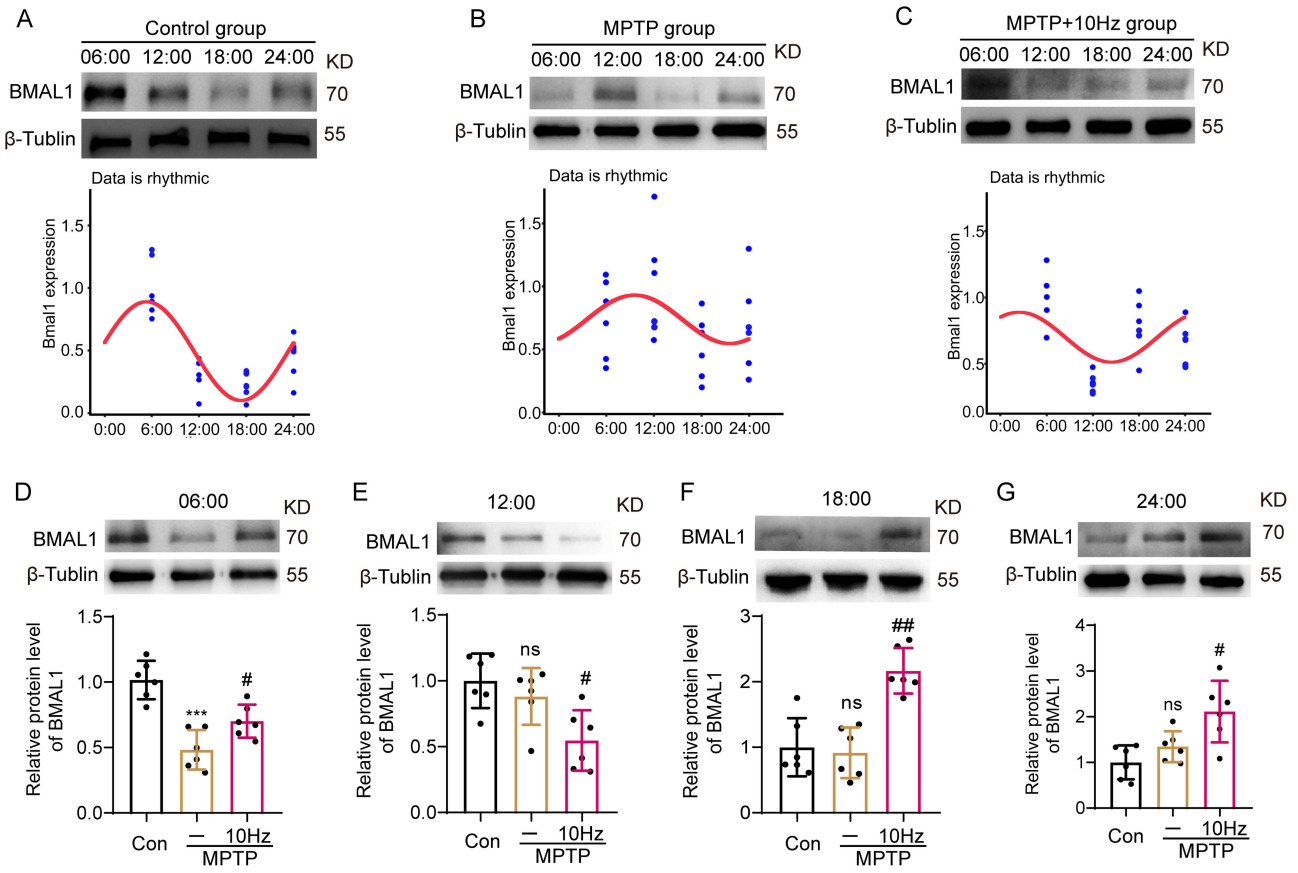

Figure 5 Treatment with 10Hz rTMS reverses circadian arrhythmia of BMAL1 in MPTP-induced PD mice.

Notes: (**A-C**) Representative Western blot and cosinor analysis of BMAL1 protein circadian expression levels in the SCN among control, MPTP and MPTP+10Hz rTMS groups at different time points during a day (06:00,12:00,18:00, 24:00). (**D-G**) Representative Western blot and quantification of BMAL1 level in various groups at the same time point (06:00, 12:00, 18:00, 24:00). Values are given as mean ± SD (n = 6). ***P<0.01 vs control group; $^{\#\#}P$<0.01 and $^{\#}P$<0.05 vs MPTP group; ns: no significant difference vs control group.

Abbreviations: rTMS, repetitive transcranial magnetic stimulation; SCN, suprachiasmatic nucleus; BMAL1, Brain and muscle ARNT-like 1; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; SD, Standard deviation.

On the other hand, we further used Bmal1siRNA to determine BMAL1 expression via regulating CaMKII-CREB pathway after 10 Hz rTMS treatment. Figure 7A–D, there were no significant differences in the proportion of P-CaMKIIα/CaMKIIα and P-CREB/CREB expression levels in the striatum between the MPTP+10 Hz group and MPTP+10 Hz+Bamal1siRNA group. To explore the effects on DA neuron survival in MPTP mice induced by BMAL1 depletion via Bmal1siRNA intervention, BMAL1 protein expression within the SCN and TH protein expression within the striatum were evaluated. The results indicated an evident decrease in both BMAL1 and TH expressions following Bmal1siRNA intervention (Figure 7A–E). Immunohistochemical analysis performed on midbrain sections containing the SNpc further revealed a substantial reduction in TH-positive neurons within the SNpc subsequent to Bmal1siRNA intervention (Figure 7F–G). Therefore, the notable modifications induced by KN-93 or Bmal1siRNA interventions during 10 Hz rTMS treatment indicated that the effects of rTMS on PD symptoms were dependent on the CaMKII-CREB-BMAL1 pathway.

Discussion

In this study, we conducted a comparative analysis of the therapeutic effects of rTMS at frequencies of 1 Hz, 5 Hz, and 10 Hz in MPTP-induced PD mice. Our findings demonstrate that 10 Hz rTMS exhibits beneficial effects on the protection of DA neurons, abnormal aggregation of α-synuclein, neurological functions, and circadian rhythm regulation. Interestingly, we observed that 10 Hz rTMS modulated the expression level and circadian rhythm of BMAL1 protein in MPTP-induced PD mice. Further application of a CaMKIIα antagonist and Bmal1siRNA confirmed that the

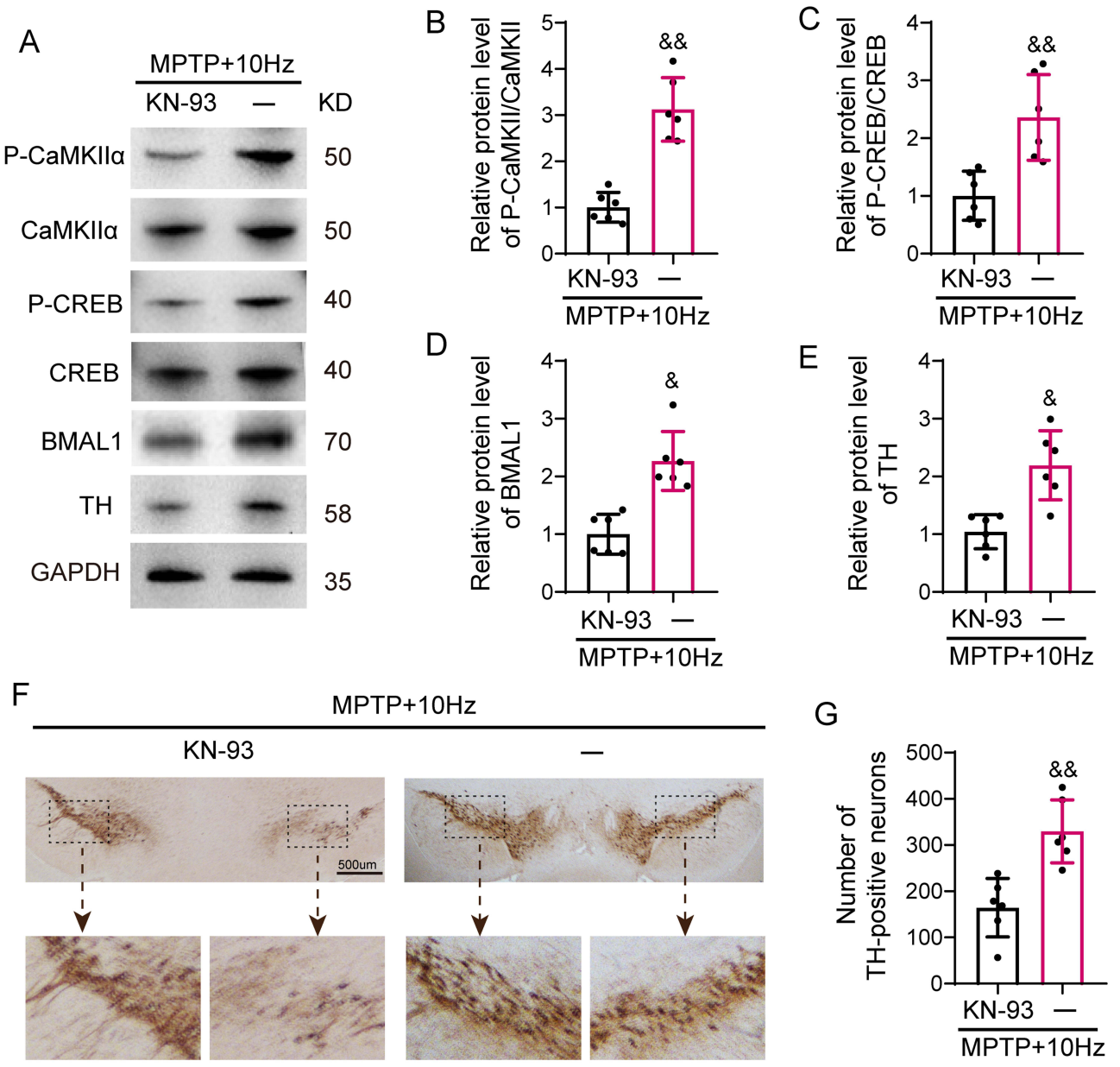

Figure 6 Effects of 10Hz rTMS treatment on the CaMKII-CREB-BMAL1 pathway in in MPTP-induced PD mice via using KN-93 intervention.

Notes: (**A, B**) Western blot analysis and quantification of P-CaMKIIα and CaMKIIα protein expression in the striatum between MPTP+10HzrTMS+KN-93 and MPTP+10HzrTMS groups. (**A, C**) Western blot analysis and quantification of P-CREB and CREB protein expression in the striatum. (**A, D**) Western blot analysis and quantification of BMAL1 protein expression in the SCN at the same time point (06:00). (**A, E**) Western blot analysis and quantification of TH protein expression in the striatum. (**F**) Representative immunohistochemistry showing TH-positive neurons in the SNpc. (**G**) Quantification of the numbers of TH-positive neurons in the SNpc. All data are displayed as means ± SD. $^{\&\&}P<0.01$, $^{\&}P<0.05$.

Abbreviations: rTMS, repetitive transcranial magnetic stimulation; a-syn, α-synuclein; BDNF, brain-derived neurotrophic factor; CAMKII, Ca2+/calmodulin-dependent protein kinase II; CREB, cAMP-response-element-Binding protein; BMAL1, Brain and muscle ARNT-like 1; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; SCN, suprachiasmatic nucleus; SNpc, Substantia nigra pars compacta; TH, Tyrosine hydroxylase, SD, Standard deviation.

neuroprotective effects of 10 Hz rTMS on surviving DA neurons by regulating the CaMKII-CREB-BMAL1 signaling pathway. Therefore, from a biological rhythm perspective, our study provides novel insights into the molecular mechanisms underlying rTMS therapy in PD.

In clinical practice, we make the standard rTMS treatment protocols according to the types of diseases and dysfunction, which mainly includes the setting of stimulation frequency, stimulation site and intensity.[8] However, conventional rTMS protocols may not be effective for all patients. Therefore, optimization strategies of traditional rTMS, such as deep, priming,

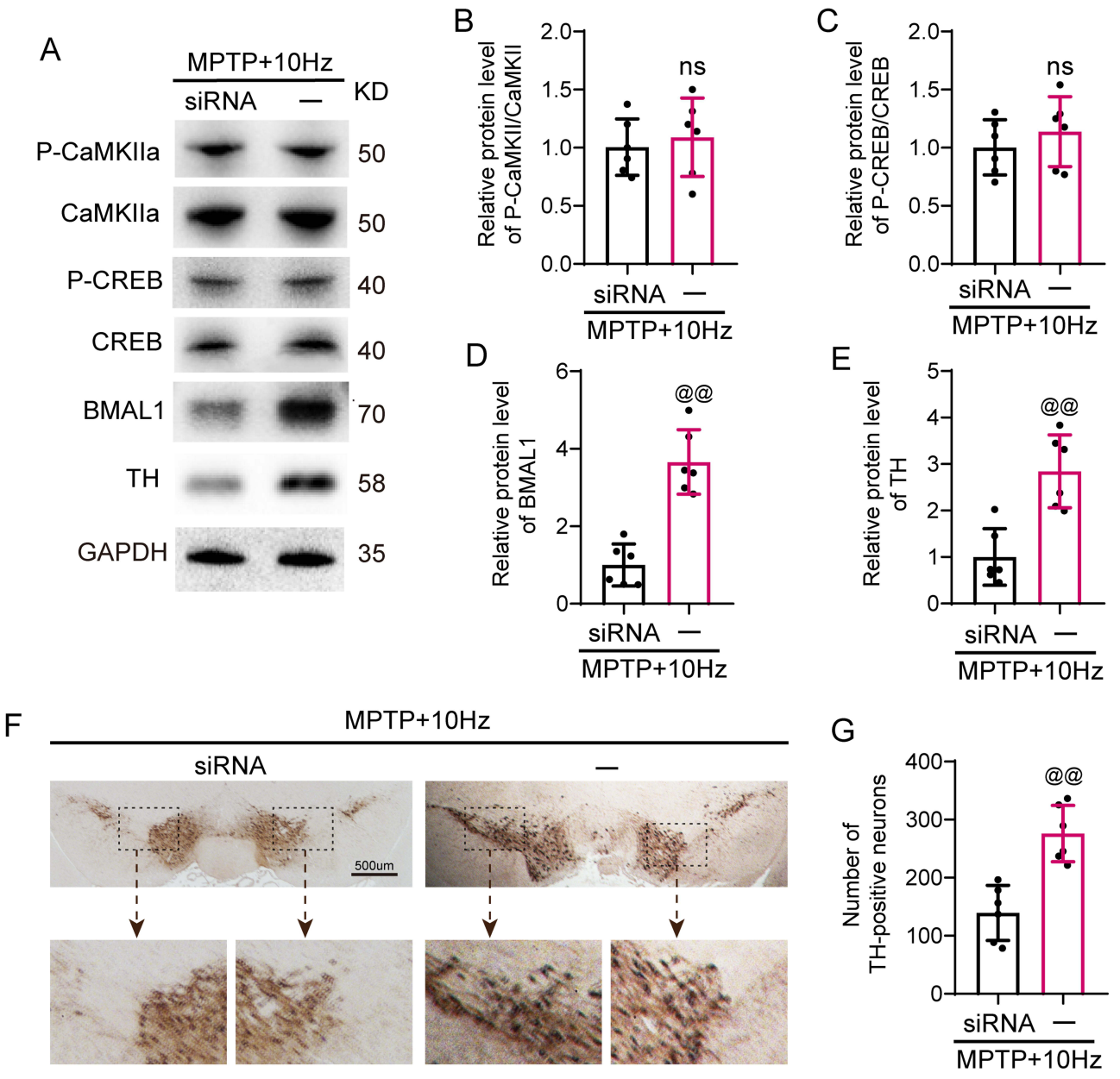

Figure 7 Effects of 10Hz rTMS treatment on the CaMKII-CREB-BMAL1 pathway in MPTP-induced PD mice via using Bmal1siRNA intervention.

Notes: (**A, B**) Western blot analysis and quantification of P-CaMKIIα and CaMKIIα protein expression in the striatum between MPTP+10HzrTMS+SiRNA and MPTP+10HzrTMS groups. (**A, C**) Western blot analysis and quantification of P-CREB and CREB protein expression in the striatum. (**A, D**) Western blot analysis and quantification of BMAL1 protein expression in the SCN at the same time point (06:00). (**A, E**) Western blot analysis and quantification of TH protein expression in the striatum. (**F**) Representative immunohistochemistry showing TH-positive neurons in the SNpc. (**G**) Quantification of the numbers of TH-positive neurons in the SNpc. All data are displayed as means ± SD. $^{@@}P<0.01$.

Abbreviations: rTMS, repetitive transcranial magnetic stimulation; siRNA, small interfering RNA; a-syn, α-synuclein; BDNF, brain-derived neurotrophic factor; CAMKII, Ca2+/calmodulin-dependent protein kinase II; CREB, cAMP-response-element-Binding protein; BMAL1, Brain and muscle ARNT-like 1; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; SCN, suprachiasmatic nucleus; SNpc, Substantia nigra pars compacta; TH, Tyrosine hydroxylase; SD, Standard deviation.

accelerated, or synchronized TMS or theta burst stimulation (TBS), and Stanford neuromodulation therapy (SNT), have been developed to enhance the treatment effects.[35,36] SNT is a precision-targeted TMS therapy developed by researchers at Stanford University School of Medicine, which is mainly used in the treatment of treatment-resistant depression (TRD).[37] Although there is no evidence-based evidence about STN treatment of PD, the basic principles of SNT have shown potential for treating PD. PD is characterized by the degeneration of dopamine-producing neurons in the substantia nigra region of the brain, leading to an imbalance between excitatory (D1 neurons) and inhibitory (D2 neurons) neural circuits.[38] SNT, through its

precise targeting capabilities, aims to restore this balance by modulating the activity of specific neural circuits involved in motor control.[36] Nevertheless, to fully assess the efficacy and safety of SNT in PD, rigorous clinical trials are necessary. These trials should evaluate the long-term effects of SNT, its ability to improve motor function and quality of life, and its potential as a stand-alone or adjunctive therapy to traditional medications. Additionally, the individualized rTMS stimulation has also been proposed to optimize the effectiveness of rTMS, including targeting specific cortical regions based on individualized neuroimaging data, adjusting stimulation parameters such as stimulation target and frequency.[39,40] Hence, in order to optimize the effectiveness of rTMS treatment strategies, stimulation frequency is an important factor that must be considered.[8] However, the optimal stimulation frequency for treating Parkinson's disease (PD) remains a subject of debate.

According to previous studies,[11,15,41–43] the stimulation frequencies of rTMS used to treat PD are mainly classified into three types: low frequency (1 Hz), low- and high-frequency (5 Hz), and high-frequency (10 Hz). However, a clinical study involving late-stage PD patients found that sequential application of 1 Hz rTMS over the left and right primary motor cortices did not result in any changes in motor or executive functions.[44] Similarly, another clinical trial showed that low-frequency rTMS over the vertex had no significant therapeutic effects on sleep in patients with PD when compared with the appropriate sham stimulation control.[15] Several previous studies have suggested that 5 Hz rTMS treatment may improve motor function and sleep parameters.[11,45,46] However, one study failed to find consistent or potential therapeutic effects on motor performance in patients with PD after 5 Hz rTMS treatment.[47] Our study also yielded negative results under 5 Hz rTMS intervention, as we did not observe significant improvements in neurological function or the survival of dopamine neurons in MPTP-induced PD mice. Recent randomized controlled trials and meta-analyses suggest that high-frequency (mainly 10 Hz) rTMS treatment is effective in improving both motor and non-motor symptoms of PD[40,48,49] Consistent with recent guidelines[8] and previous studies,[29] our study comparing the therapeutic effects of different frequencies of rTMS in MPTP mice demonstrated that high-frequency 10 Hz rTMS provided protection for dopamine neurons and significantly improved motor behavior and circadian rhythm.

Circadian rhythms are biological processes that regulate most physiological functions, and their disruption can lead to a multitude of health risks, including an increased susceptibility to developing multiple diseases such as PD.[18,50] Recently, it has been demonstrated that PD patients often manifest dysfunction in circadian systems such as temporal patterns of body temperature, blood pressure, cortisol and melatonin production, and sleep disorders.[4,51] Therefore, it is plausible that the circadian regulatory system is affected in individuals with PD. An increasing number of studies have supported an interaction between the dopamine (DA) system and the circadian rhythm.[52] The loss of DA neurons may play a role in circadian disruption observed in mice with PD. In MPTP-induced PD mice, primates manifested an immediate disruption of the sleep/wake cycle,[53] as well as changes in REM sleep and increased daytime sleepiness.[54,55] Furthermore, MPTP treatment significantly reduced motor activity during active periods in mice while promoting arrhythmia in core body temperature patterns and inhibiting clock gene expression.[56] In this study, melatonin and cortisol were used as indicators of biological rhythms to assess the effects of 10 Hz rTMS on the circadian rhythm of MPTP-induced PD mice, suggest that 10 Hz rTMS could potentially exert neuroprotective effects on DA neurons by restoring the disrupted circadian rhythm system.

As previously mentioned, circadian dysfunction may be a novel mechanism involved in PD pathology[57] and perhaps in the development of PD symptoms, including sleep disorders.[4,55] Increasing evidence suggests that rTMS has potential as a complementary therapy for impaired sleep-wake cycles in the PD population.[8,11] Several studies have reported that cortical rTMS exerts remote effects by modulating the subcortical areas involved in sleep regulation, such as the hypothalamus.[12,58] However, the molecular mechanisms underlying the therapeutic effects of rTMS on sleep disorders remain unclear. BMAL1 serves as a central regulator of the clock machinery, driving transcriptional-translational feedback loops for itself and other genes.[59] Studies investigating alterations in the molecular clock mechanism have revealed disrupted BMAL1 expression in patients with PD.[4,60] Compared with healthy individuals, decreased expression levels of BMAL1 were observed in total leukocytes from patients with PD and correlated positively with disease severity.[60] In this study, we observed reduced levels of BMAL1 and disrupted circadian rhythms associated with its expression; these changes were partly reversed by 10 Hz rTMS treatment in MPTP-induced PD mice. Therefore, our findings suggest that 10 Hz rTMS may regulate BMAL1 expression and subsequently alleviate symptoms associated with PD.

CaMKII, a calmodulin-dependent protein kinase, plays crucial roles in synapse formation, cytoskeleton modification, neurotransmitter synthesis and secretion, gene expression, and neuroplasticity regulation.[61,62] Four CaMKII isoforms, CaMKIIα, CaMKIIβ, CaMKIIδ, and CaMKII,[63] which regulate calcium channel activity and gene expression. CaMKIIα is

a major component necessary for the induction of long-term potentiation (LTP).[64] Under physiological conditions, most CaMKIIα exists in a non-phosphorylated state, which is dependent on intracellular calcium.[65] According to previous studies, small changes in intracellular Ca^{2+} concentrations can cause a change in the conformation of the calc-binding protein (C), which binds to CaMKIIα and phosphorylates the 286 Threonine site, thereby increasing the phosphorylation level of CaMKIIα itself.[66] Thus, phosphorylation of CaMKIIα can further induce biochemical reactions such as phosphorylation of the transcription factor CREB.[67] According to previous studies, CaMKIIα is abundant in striatal medium spiny neurons,[68] and dynamically regulated by changes in DA signaling.[69] In addition, CaMKIIα inhibition decreased DA release and downregulated CaMKIIα levels.[70] Therefore, modulation of the CaMKII-CREB signaling pathway may represent a molecular mechanism underlying rTMS therapy in PD. In this study, KN-93 intervention was employed to confirm whether rTMS modulates the CaMKII-CREB signaling pathway and exerts neuroprotective effects on dopaminergic neurons in MPTP-induced PD mice. We found that the phosphorylation levels of CaMKIIα and CREB were significantly reduced after KN-93 intervention in PD mice treated with 10 Hz rTMS, whereas there were no changes in the total levels of CaMKIIα and CREB.

Additionally, CaMKIIα serves as a component of the cell-autonomous clock and as a synchronizer that integrates circadian behavioral activities.[23,71] Our study also found that the protein levels of BMAL1 and TH were significantly decreased in PD mice treated with 10 Hz rTMS and KN-93. This finding is consistent with the previously reported role of CaMKII signalling in light entrainment of the circadian clock.[71] We further aimed to determine whether 10 Hz rTMS could modulate the CaMKII-CREB signaling pathway and promote BMAL1 expression using Bmal1siRNA intervention. Moreover, we found that the protein levels of BMAL1 and TH were significantly decreased, while Bmal1siRNA did not change the levels of CaMKIIα, P-CaMKIIα, CREB, and p-CREB. Hence, our study suggests that 10 Hz rTMS could regulate BMAL1 expression by modulating the CREB-BMAL1 signaling pathway and alleviating PD symptoms (Figure 8).

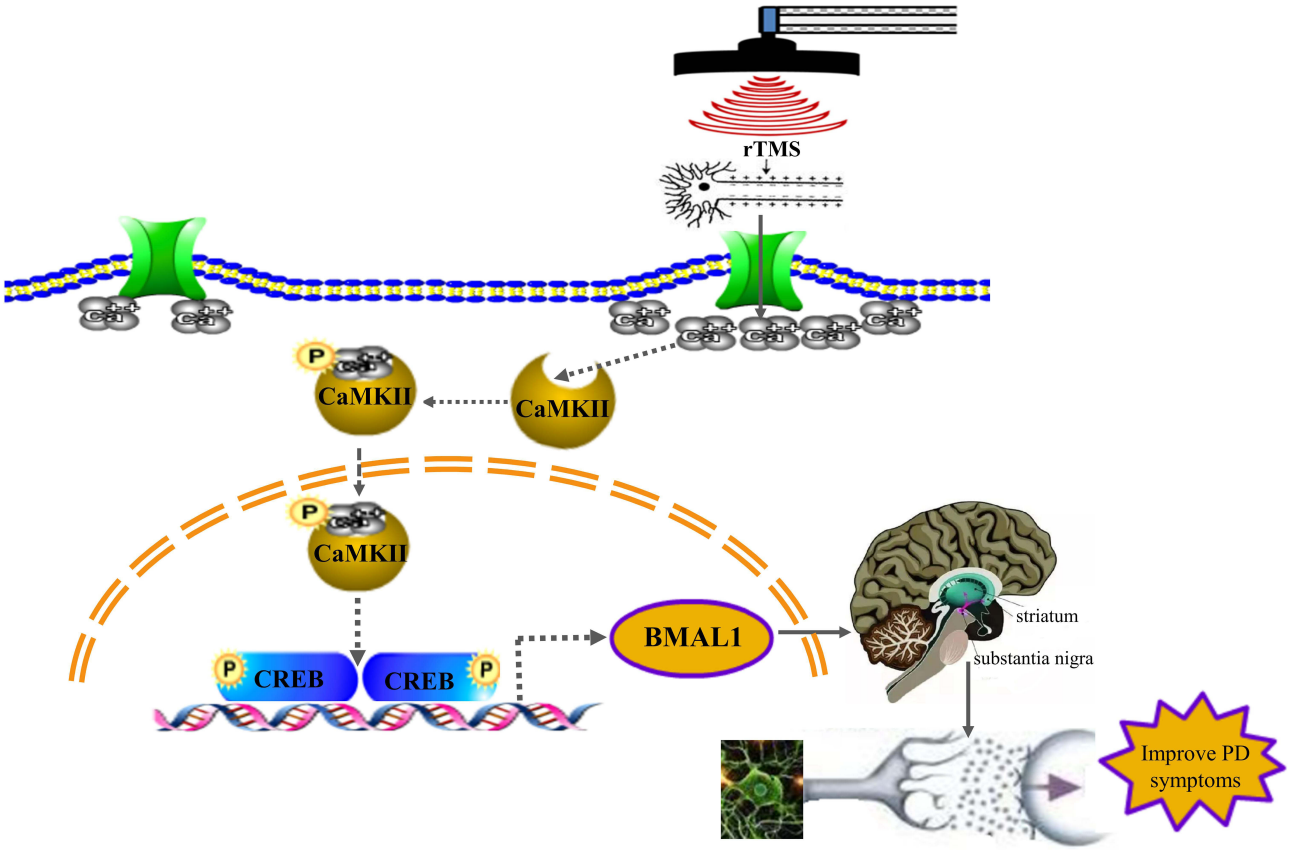

Figure 8 Schematic representation of the role and related mechanism of rTMS for treating PD.
Abbreviations: rTMS, repetitive transcranial magnetic stimulation; PD, Parkinson's disease.

This study aimed to explore the anti-PD effects and potential mechanisms of action of rTMS in mice with MPTP-induced PD. However, this study had some limitations that should be acknowledged. First, only adult male mice were used to establish the PD model, whereas most patients in clinical settings were older females. Therefore, future studies should include animals of different sexes and ages for testing purposes. Second, achieving spatial targeting and specificity using rTMS coils has long been a challenge in animal experiments. Although a commercially available circular coil designed for rodents was utilized in this study, it is worth noting that its size is still significantly larger than that of a mouse brain. Thus, smaller rodent-specific TMS coils should be developed in future studies. Thirdly, during the stimulation, manual restriction and visual tracking were employed for mouse head movements, which may have affected the therapeutic outcomes of rTMS. Finally, we established MPTP-induced PD models in mice that are different from those in PD patients. The conclusions of this study need to be confirmed by large-scale and rigorous clinical trial studies in PD patients.

In conclusion, the current study demonstrates that 10 Hz rTMS over 1 Hz and 5 Hz rTMS can alleviate dopaminergic neuron damage and restore circadian rhythm disturbances in MPTP-induced PD mice. Furthermore, this study elucidates the molecular mechanism by which rTMS may alleviate MPTP-induced Parkinson's disease symptoms by regulating the CaMKII-CREB-BMAL1 pathway. These findings provide a new comprehensive perspective on the therapeutic mechanisms of rTMS in PD.

Data Sharing Statement

Data will be made available on request from the corresponding author.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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