Development and characterization of an inducible Dicer conditional knockout mouse model of Parkinson’s disease: validation of the antiparkinsonian effects of a sigma-1 receptor agonist and dihydromyricetin

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Parkinson’s disease (PD) is a common neurodegenerative disease characterized by motor impairment and progressive loss of dopamine (DA) neurons. At present, the acute application of neurotoxic drugs such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) are commonly used to simulate the pathology of PD; however, it is difficult to induce the progressive pathogenesis of PD with these models. In this study, we employed DAT promoter-mediated Cre transgenic mice to establish tamoxifen-inducible Dicer conditional knockout (cKO) mice in an effort to mimic the progressive loss of DA neurons and the development of PD-like behavioral phenotypes. The results showed that Dicer cKO mice exhibited progressive loss of DA neurons in the substantia nigra (SN) following tamoxifen administration. Significant DA loss was observed 6 weeks after tamoxifen administration; accordingly, progressive motor function impairment was also observed. We also found that a significant neuroinflammatory response, as evidenced by microglial proliferation, another hallmark of PD pathogenesis, accompanied the loss of DA neurons. The acute application of levo-DOPA (L-DOPA) relieved the PD-like motor impairments in Dicer cKO mice to exert its antiparkinsonian action, indicating that the model can be used to evaluate the antiparkinsonian efficacy of PD drugs. To further elucidate the potential application of this novel PD animal model for PD drug development, we employed the powerful neuroprotective agent dihydromyricetin (DHM) (10 mg/kg) and the selective sigma-1 receptor agonist PRE-084 (1 mg/kg), both of which were previously shown to produce antiparkinsonian effects. The results indicated that the chronic administration of either DHM or PRE-084 attenuated the Dicer cKO-induced loss of DA neurons and motor impairments, although the two drugs acted through different mechanisms. These data indicate that the Dicer cKO mouse model may be a useful model for investigating the pathological development of PD and intervention-mediated changes. In conclusion, this transgenic mouse model appears to simulate the progressive pathogenesis of PD and may be a potentially useful model for PD drug discovery.

Key words: Parkinson’s disease; Dicer; gene knockout; dihydromyricetin; PRE-084

INTRODUCTION

Parkinson’s disease (PD) is characterized by progressive damage to dopamine (DA) neurons in the midbrain substantia nigra (SN) [1, 2]. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine (6-OHDA), and other neurotoxic compounds are commonly used in the laboratory to establish PD models. These neurotoxins are selectively taken up by DA neurons and are mainly targeted to the mitochondrial complex, eventually causing the death of DA neurons, and they have been widely used for PD drug evaluation and pathological studies of diseases [3, 4]. However, these neurotoxin-induced animal models cause acute death of DA neurons and therefore do not simulate the progressive pathogenesis of PD. Thus, there is an unmet need to develop novel animal models that resemble the chronic pathological development of PD.

MicroRNAs (miRNAs) are single-stranded noncoding RNAs composed of 22 nucleotides that play an important role in neuronal development and function in both physiological and disease conditions [5–7]. Dicer is a key enzyme in the progression of miRNA synthesis. Dicer-specific knockout of hippocampal neurons using the calmodulin kinase (Camk II) promoter induces hippocampal neuronal death and neuroinflammation [8]; Dicer-specific knockout in the cerebellum also causes Purkinje cell death and behavioral ataxia in mice [9]. Moreover, Kim and colleagues constructed a mouse line with Dicer conditional knockout (cKO) driven by the DAT promoter using Cre recombinase, revealing the key role of miRNA in DA neuronal survival for the first time. They found that the loss of DA neurons began 3 weeks after Dicer deletion and that 90% DA cell death was reached after 8 weeks [10]. In addition, Pang et al. employed a different approach, using
Dicer conditional knockout Parkinson’s disease mouse model

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Materials and Methods

Inducible Dicer cKO mouse preparation

Floxed Dicer mice (Dicer<sup>f/f</sup>) and DAT-creER transgene mice were donated by Dr. Xinyuan Lv. (Medical College of Georgia, USA) and bred under SPF conditions (temperature: 22 ± 1 °C, humidity: 55% ± 5%, 12-h light–dark cycle) with free access to food and water. All animal experiments were approved by the Institutional Animal Care and Use Committee of Soochow University and were in accordance with the Guidelines for the Care and Use of Laboratory Animals. Dicer<sup>f/f</sup> mice were crossed with DAT-icreER mice to obtain Dicer<sup>f/f</sup>:DAT-creER mice; a 300-bp band indicated the Cre transgene, a 200-bp band indicating the positive control, a 120-bp band indicated Dicer<sup>f/f</sup>, and a 420-bp band indicated Dicer<sup>f/f</sup>. The PCR products were used to determine the genotypes of the mice. Total protein isolated from mouse SN tissues were lysed in RIPA buffer and denatured at 95 °C for 5 min. Protein concentrations were determined using a BCA Protein Assay Kit. Proteins (20 μg) were loaded on SDS-PAGE gels and transferred to PVDF membranes for 2 h at 250 mA. The membranes were incubated with 5% nonfat milk for 2 h at room temperature before incubation with the respective antibodies, including anti-TH (1:1000; Millipore, USA), anti-α-tubulin (1:10,000; Sigma-Aldrich, USA), and anti-β-tubulin (1:10,000; Sigma-Aldrich, USA), at 4 °C overnight. The membranes were washed three times with TBST. The membranes were incubated with respective mouse IgG (1:10,000; Sigma-Aldrich, USA) or rabbit IgG (1:10,000; Sigma-Aldrich, USA) secondary antibodies for 2 h at room temperature. The results were analyzed using ImageJ software.

Western blotting

After the behavioral tests, the mice were sacrificed to collect brain tissues for biological tests. Total protein isolated from mouse SN tissues were lysed in RIPA buffer and denatured at 95 °C for 5 min. Protein concentrations were determined using a BCA Protein Assay Kit. Proteins (20 μg) were loaded on SDS-PAGE gels and transferred to PVDF membranes for 2 h at 250 mA. The membranes were incubated with 5% nonfat milk for 2 h at room temperature before incubation with the respective antibodies, including anti-TH (1:1000; Millipore, USA), anti-α-tubulin (1:10,000; Sigma-Aldrich, USA), and anti-β-tubulin (1:10,000; Sigma-Aldrich, USA), at 4 °C overnight. The membranes were washed three times with TBST. The membranes were incubated with respective mouse IgG (1:10,000; Sigma-Aldrich, USA) or rabbit IgG (1:10,000; Sigma-Aldrich, USA) secondary antibodies for 2 h at room temperature. The results were analyzed using ImageJ software.

Immunofluorescence staining

After the behavioral tests, some mice were anesthetized with 4% chloral hydrate and then perfused through the left ventricle with PBS (pH 7.4) followed by 4% paraformaldehyde in PBS. Mouse brains were collected and postfixed in paraformaldehyde overnight at 4 °C and then dehydrated in 30% sucrose at 4 °C for 3 days. The brain tissues were serially cut into 20-μm coronal sections using a freezing microtome. For immunofluorescence staining, the sections were washed three times with PBS and incubated in PBS containing 3% BSA and 0.3% Triton for 2 h at room temperature. The sections were then incubated with an anti-TH (1:400, Millipore, USA) or anti-α-tubulin (1:400; Wako, Japan) antibody for 24 h at 4 °C. Then, the sections were washed in PBS (PBS containing 0.3% Triton) and incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:400; Thermo Fisher Scientific, USA) or Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:400; Thermo Fisher Scientific, USA) for 2 h at room temperature in the dark. The sections were washed and then mounted using mounting medium. Images were captured using a laser confocal fluorescence microscope.

Drug treatment

Dicer deletion was induced in 8- to 10-week-old mice. Briefly, 1 mg of tamoxifen (Sigma-Aldrich, USA, T5648) was diluted in sunflower oil at a final concentration of 10 mg/mL and injected intraperitoneally (i.p.) twice a day over five consecutive days. The animals then received different treatments: (1) tamoxifen administration alone: the mice were used to observe pathological and behavioral changes at various times following Dicer cKO; (2) the L-DOPA treatment group: the mice were injected with 8 mg/kg L-DOPA (Sigma-Aldrich, USA, D9628) combined with 15 mg/kg benserazide (Sigma-Aldrich, USA, B7283) 8 weeks after tamoxifen administration to validate the antiparkinsonian effect of L-DOPA through behavioral measurements; (3) DHM treatment: the mice were administered tamoxifen followed by daily injection of 10 mg/kg DHM (Nature Standard, China, ST03840120MG) for 8 weeks to evaluate the neuroprotection and antiparkinsonian effects of the drug; and (4) Sigma-1 receptor agonist treatment: PRE-084 (MCE, USA, HY-18100A) was injected daily together with tamoxifen for consecutive 8 weeks, and the antiparkinsonian effects were evaluated weekly after 6 weeks of tamoxifen administration.

Behavioral experiments

Pole test and rotarod test: The experiments were performed as previously reported by our group [12, 13]. In brief, before the experiments, the mice were trained to crawl downward. During the experiment, the mice were placed head down at the top of a rough wooden pole (60 cm length, 2 cm in diameter), and the time required for the mice to descend from the top of the pole to the ground was recorded. The experiment was repeated three times for each mouse. For the rotarod test, the mice were first trained until they were able to remain on the rotarod for more than 120 s at a speed of 20 revolutions per minute (r/min). During the experiment, the speed of the rotarod was increased from 20 to 40 r/min within 5 min. The mice were allowed to walk freely on the rotarod, and the latency time to fall from the rotarod was recorded. The measurement was repeated three times for each mouse.

After the behavioral tests, the mice were sacrificed to collect brain tissues for biological tests. Total protein isolated from mouse SN tissues were lysed in RIPA buffer and denatured at 95 °C for 5 min. Protein concentrations were determined using a BCA Protein Assay Kit. Proteins (20 μg) were loaded on SDS-PAGE gels and transferred to PVDF membranes for 2 h at 250 mA. The membranes were incubated with 5% nonfat milk for 2 h at room temperature before incubation with the respective antibodies, including anti-TH (1:1000; Millipore, USA), anti-α-tubulin (1:10,000; Sigma-Aldrich, USA), and anti-β-tubulin (1:10,000; Sigma-Aldrich, USA), at 4 °C overnight. The membranes were washed three times with TBST. The membranes were incubated with respective mouse IgG (1:10,000; Sigma-Aldrich, USA) or rabbit IgG (1:10,000; Sigma-Aldrich, USA) secondary antibodies for 2 h at room temperature. The results were analyzed using ImageJ software.

Immunofluorescence staining

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**RESULTS**

Dicer cKO mice showed a progressive decline in motor ability and damage to DA neurons

We first examined changes in the motor ability of the mice after tamoxifen administration to induce Dicer cKO in DA neurons at different time points. As shown in Fig. 1, impairments in motor activity were observed at week 6 and worsened progressively with time. There was no difference in motor activity between male and female animals. In association with this observation, we also detected the progressive loss of DA neurons in the SN, as measured by tyrosine hydroxylase (TH) expression using Western blotting. This was further confirmed by immunostaining with an anti-TH antibody (Fig. 2). These data confirmed that the induction of Dicer cKO in DA neurons in adult mice resulted in progressive DA neuronal loss and that the mice progressively developed a PD-like phenotype. These results indicated that these transgenic animals may represent a novel animal model for the study of PD, particularly the progressive development of PD-like behavioral and pathological phenotypes, that may be a useful tool for evaluating the protection of DA neurons or for studying the mechanism of the effects of interventions or drugs.

Progressive neuroinflammation during PD-like pathological development in Dicer cKO mice

Neuroinflammation is believed to contribute to the development of PD. The modulation of neuroinflammation has become an important drug target for neurodegenerative diseases, including PD [14–16]. To elucidate the inflammatory response during the development of PD-like phenotypes in the Dicer cKO mice, the animals were sacrificed and their brains were collected 4, 6, and 8 weeks after tamoxifen administration. Immunofluorescence staining for the microglial marker Iba1 was performed (Fig. 3). The results revealed a progressive increase in the number of Iba1 immunopositive cells from week 6, which coincided with the onset of DA neuronal death. The above results revealed that Dicer knockout can cause progressive neuroinflammation in the SN, which is one of the hallmarks of pathology in PD.

Acute application of L-DOPA to Dicer cKO mice relieved PD-like motor impairments

We characterized the PD-like behavioral and pathological phenotypes in inducible Dicer cKO mice. We next tested the potential application of this novel model to study the efficacy of PD drugs. Dicer cKO mice, which steadily developed PD-like behaviors in response to tamoxifen induction, were administered L-DOPA, and the results indicated that L-DOPA treatment significantly relieved motor impairments, as evidenced by performance on the pole test and rotarod test (Fig. 4), indicating that the Dicer cKO mouse line can be used as an alternative animal model for evaluating the efficacy of PD drugs.

Chronic application of the neuroprotective agent DHM or the sigma-1 receptor agonist PRE-084 attenuated DA neuronal damage and behavioral abnormalities in Dicer cKO mice

To further evaluate the potential application of Dicer cKO mice in PD research, we investigated whether this animal model can be used to study the preventive effect of neuroprotective agents or antiparkinsonian agents on PD pathological development given the progressive nature of PD-like pathology in Dicer cKO mice. To this end, we employed DHM, a powerful neuroprotective agent that was previously shown to protect DA neuron [13], and PRE-084, a selective sigma-1 receptor agonist, since the modulation of the sigma-1 receptor was recently identified an important target for PD drug discovery and treatment [17]. Dicer cKO mice were administered DHM, PRE-084, or vehicle for 8 consecutive weeks, and the drug treatments were started after tamoxifen induction. Behavioral tests were performed every other week starting from week 6 following tamoxifen administration. After the behavioral tests, the mice were sacrificed for biological tests (Fig. 5a). The results showed that, in comparison with tamoxifen alone, DHM treatment significantly attenuated Dicer cKO-induced impairments in motor functions (Fig. 5b, c). Similarly, the chronic administration of the selective sigma-1 receptor agonist PRE-084 also dramatically protected against impairments in motor functions (Fig. 5d, e).

In support of these findings, the Western blot results indicated that after DHM administration, the expression of TH was significantly higher than that in the control (no drug treatment group) (Fig. 6a, b). Meanwhile, we found that chronic DHM treatment significantly reduced the number of microglial cells, suggesting a decrease in neuroinflammation (Fig. 6c–e). In addition, treatment with the sigma-1 receptor agonist PRE-084 also significantly restored TH expression (Fig. 7a, b) and...
suppressed reactive microglia proliferation, as evidenced by the decreased number of Iba1-positive cells (Fig. 7c–e). Taken together, these results suggest that chronic DHM or PRE-084 treatment can protect DA neurons and reduce neuroinflammation in response to Dicer cKO.

DISCUSSION
In this study, we established an inducible DA neuron-specific Dicer cKO mouse line by crossing floxed Dicer mice (Dicer<sup>f/f</sup>) with DAT-iCreER mice to obtain DAT-iCreER;Dicer<sup>f/f</sup> mice, the latter were bred with Dicer<sup>fl</sup> mice to produce DAT-iCreER;Dicer<sup>f/fl</sup> mice, which we called Dicer cKO mice. Following tamoxifen induction, DA neurons experienced progressive death, and a significant loss of DA neurons was observed 6 weeks after the depletion of Dicer in the SN. Accordingly, the behavioral tests revealed progressive PD-like impairment of motor functions. In addition, we also observed time-dependent inflammation in the SN during the development of PD-like phenotypes, and microglia-mediated neuroinflammation was regarded as the hallmark of PD pathogenesis. These data revealed that Dicer cKO mice develop PD-like pathological and behavioral phenotypes, specifically in a progressive manner. The acute administration of L-DOPA to Dicer cKO mice significantly relieved the impairment of motor functions, indicating the potential of the Dicer cKO mouse model in evaluating PD drug efficacy. We further found that the chronic administration of DHM or a selective sigma-1 receptor agonist significantly attenuated the development of PD-like pathological and behavioral changes in Dicer cKO mice. Although the mechanisms of the respective drugs are different, this model can also be used to investigate the pathological mechanism of PD and to validate the effects of various antiparkinsonian drugs.
miRNAs are single-stranded noncoding RNAs that bind to targeted gene sequences to inhibit target gene translation. Recently, many studies have revealed the important functional roles of miRNAs in neuronal differentiation, development, and function [18, 19]. Moreover, alterations in miRNAs have been widely reported to be associated with neuropsychiatric diseases [20, 21]. For example, we recently reported that Let-7c and miRNA-3473b are involved in microglial activation and contribute to neuronal survival and functional recovery in stroke [22, 23], indicating the importance of miRNAs in neuroprotection and cognitive recovery.

**Fig. 3** Progressive neuroinflammation development in Dicer cKO mice. Animals were administrated with tamoxifen to induce the deletion of Dicer in DA neurons as depicted in Fig. 1. **a** Double immunofluorescence staining with respective anti-TH and anti-Iba1 antibodies were performed in brain sections at various time points. Iba1-positive-cell numbers in SN were increased with time in response to the Dicer cKO. Representative photomicrographs were shown at ×20 magnification (scale bars, 100 μm). **b** Quantitative analyses of TH-positive neurons and c Ibal-positive neurons. Data were presented as mean ± SEM. Statistical analyses were performed by one-way ANOVA. n = 3 per group (*P < 0.05, **P < 0.01; tamoxifen vs vehicle).

**Fig. 4** L-DOPA alleviated the motor disorder in Dicer cKO mice. Eight weeks after tamoxifen or vehicle administration, mice were subjected to behavioral tests to monitor the motor impairments. Mice were then injected with 8 mg/kg L-DOPA with 15 mg/kg benserazide before the behavioral tests. **a** Pole test and **b** Rotarod were tested 1 h later. Data were presented as mean ± SEM. Statistical analyses were performed by two-way ANOVA. n = 10 per group (**P < 0.01; No L-DOPA injection, vehicle vs tamoxifen. **P < 0.01; Dicer cKO mice, no L-DOPA vs L-DOPA).
neuroinflammation. In relation to the current study, alterations in miRNAs in PD pathological development have been widely studied, and circulating miRNAs have also been suggested to be potential novel biomarkers for PD. Moreover, it has been suggested that targeting specific miRNAs could be a potential therapeutic approach for PD. For instance, treatment with miR-124 agomir was shown to reduce the loss of DA neurons in an MPTP-treated mouse model, inhibiting Bim expression and thus suppressing Bax translocation to mitochondria [24]. Dicer is a type III RNase and is responsible for the processing of miRNA precursors into functional miRNAs, thus playing a crucial role in miRNA regulation [25]. Dicer expression is essential not only for midbrain development in mice and DA neuron maintenance and survival during early postnatal development, but also for DA neuron survival in adult mice [11]. Recently, a reduction in Dicer in the ventral midbrain and alterations in miRNA expression profiles in laser-microdissected DA neurons of aged mice have been demonstrated [26]. Moreover, miR-133b was reported to be specifically deficient in midbrain DA neurons from PD patient samples. It was further found that miR-133b alters the survival of DA neurons through negative feedback regulation with pitx3 [10]. Additionally, it was shown that the stimulation of miRNA biosynthesis promotes the survival of cultured DA neurons and reduces their vulnerability to thapsigargin-induced endoplasmic reticulum stress [26]. In agreement with these observations, our adult Dicer cKO mice show progressive loss of DA neurons in the SN in response to tamoxifen induction and consequently the presence of PD-like impairments in motor functions. We have characterized the detailed time-dependent changes and degeneration of DA neurons in the SN in response to the specific deletion of Dicer driven by DAT-Cre. A slightly different time course of PD-like phenotypes than that reported by a previous study that employed a different approach to delete Dicer from DA neurons, in which 90% DA loss was observed at week 8. We found dramatic DA loss at week 10 following tamoxifen induction [10]. The specific mechanism by which Dicer cKO induces DA neuron loss remains to be further studied. In addition to the direct effect of Dicer depletion on DA neurons, we found that the number of Iba1-positive cells in the SN increased significantly 6 weeks after tamoxifen injection, which was consistent with the time at which neurons began to die. The depletion of Dicer induced the activation of microglia-mediated neuroinflammation. Given that...
neuroinflammation is another hallmark of PD pathology and that miRNAs have been widely reported to be involved in the regulation of neuroinflammation [21], it is conceivable that Dicer depletion-mediated microglial activation may also contribute to DA neuron death.

In addition to pathological development, the mice also exhibited progressive impairments in motor functions that resembled the PD-like phenotypes. It was also noted that we did not detect any difference between males and females. Therefore, Dicer cKO mice may be an alternative PD animal model. Indeed, the acute application of L-DOPA significantly relieved motor impairments in response to Dicer cKO 8 weeks after tamoxifen induction. Furthermore, the chronic administration of neuroprotective agent DHM and the selective sigma-1 receptor agonist PRE-084 significantly attenuated the development of PD-like behaviors and pathological phenotypes. All data indicate that Dice cKO mice may serve as a useful PD model.

The chronic application of the neuroprotective agent DHM has been shown to produce powerful neuroprotective effects in models of different diseases, including PD and stroke [13, 27]. As a traditional Chinese medicine, it was initially used to treat cough and fever and later found to have many pharmacological effects, such as protective effects on the heart, diabetes, liver, and nervous system, possibly through reducing oxidative stress or anti-inflammation [28, 29]. The neuroprotective effects of DHM were further suggested to be associated with its modulation of the AKT-GSK3β signaling pathway [13]. In this study, we confirmed that DHM administration for 8 weeks reduced motor dysfunction, attenuated DA neuron death, and reduced microglial activation in mice DA neurons with Dicer cKO. The Sigma-1 receptor is an important target for a few CNS diseases [30, 31]. The sigma-1 receptor agonist PRE-084 can alleviate high spontaneous activity in mice and reduce DA outflow and release induced by acute methamphetamine administration [32]. In a chronic unpredicted mild stress model (CUMS), SOMCL-668, a recently discovered sigma-1 allosteric modulator, was reported to elicit an antidepressant effect through the BDNF-GSK3β pathway [31]. In addition, the administration of the low-dose sigma-1 receptor selective agonist PRE-084 for 5 consecutive weeks produced a good therapeutic effect in a 6-OHDA-induced PD model [17]. In agreement with these observations, we also found that...
long-term administration of PRE-084 improved motor ability, protected DA neurons, and suppressed neuroinflammation, further confirming that our transgenic mouse model is valuable for evaluating the efficacy of PD drugs.

In summary, the present study demonstrated that adult DAT-icre;Dicer<sup>fl/fl</sup> transgenic mice develop progressive DA neuron loss and PD-like behaviors accompanied by progressive neuroinflammation following tamoxifen induction. The acute application of L-DOPA can improve the impaired motor ability of Dicer cKO mice. Therefore, we believe that this model could be used to simulate the progressive and irreversible pathogenesis of PD and to study the pathogenesis of PD. In addition, we evaluated DHM and PRE-084, two compounds that have been shown to elicit antiparkinsonian effects through different mechanisms, and found that the chronic application of either DHM or PRE-084 attenuated the development of PD-like behavioral and pathological phenotypes, further implying that the use of this model is an alternative approach for PD drug discovery.

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AUTHOR CONTRIBUTIONS
CHG, XCZ, and JLW designed the research and wrote the paper; CHG and TC performed the experiments; CHG and LTZ analyzed the data.

ADDITIONAL INFORMATION
Competing interests: The authors declare no competing interests.

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