

# Melatonin attenuates MPTP-induced neurotoxicity via preventing CDK5-mediated autophagy and SNCA/ $\alpha$ -synuclein aggregation

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**Keywords:** autophagy, CDK5, melatonin, MPTP, SNCA, TH

**Abbreviations:** ATG7, autophagy-related 7; BAF1, bafilomycin A<sub>1</sub>; CDK5, cyclin-dependent kinase 5; CDK5R1/p35/p25, cyclin-dependent kinase 5, regulatory subunit 1 (p35); DA, dopamine; DAPI, 4',6-diamidino-2-phenylindole; i.p., intraperitoneally; s.c., subcutaneously; ICV, intracerebroventricular; MAP1LC3B/LC3B, microtubule-associated protein 1 light chain 3  $\beta$ ; MAP2, microtubule-associated protein 2; MPTP, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine; PD, Parkinson disease; PEBP1, phosphatidylethanolamine binding protein 1; PI, propidium iodide; SNc, substantia nigra pars compacta; SNCA/ $\alpha$ -synuclein, synuclein,  $\alpha$  (non A4 component of amyloid precursor); TH, tyrosine hydroxylase.

Autophagy is involved in the pathogenesis of neurodegenerative diseases including Parkinson disease (PD). However, little is known about the regulation of autophagy in neurodegenerative process. In this study, we characterized aberrant activation of autophagy induced by neurotoxin 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) and demonstrated that melatonin has a protective effect on neurotoxicity. We found an excessive activation of autophagy in monkey brain tissues and C6 cells, induced by MPTP, which is mediated by CDK5 (cyclin-dependent kinase 5). MPTP treatment significantly reduced total dendritic length and dendritic complexity of cultured primary cortical neurons and melatonin could reverse this effect. Decreased TH (tyrosine hydroxylase)-positive cells and dendrites of dopaminergic neurons in the substantia nigra pars compacta (SNc) were observed in MPTP-treated monkeys and mice. Along with decreased TH protein level, we observed an upregulation of CDK5 and enhanced autophagic activity in the striatum of mice with MPTP injection. These changes could be salvaged by melatonin treatment or knockdown of CDK5. Importantly, melatonin or knockdown of CDK5 reduced MPTP-induced SNCA/ $\alpha$ -synuclein aggregation in mice, which is widely thought to trigger the pathogenesis of PD. Finally, melatonin or knockdown of CDK5 counteracted the PD phenotype in mice induced by MPTP. Our findings uncover a potent role of CDK5-mediated autophagy in the pathogenesis of PD, and suggest that control of autophagic pathways may provide an important clue for exploring potential target for novel therapeutics of PD.

## Introduction

Parkinson disease (PD) is the second most common neurodegenerative disease characterized by bradykinesia, tremors, and rigidity.<sup>1,2</sup> It is caused by the degeneration of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNc), which results in a decreased dopamine level in the striatum<sup>3,4</sup> and a formation of Lewy bodies that are predominantly composed of aggregated SNCA/ $\alpha$ -synuclein.<sup>5</sup> However, molecular mechanisms underlying PD have not been fully understood. The neurotoxin 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine

(MPTP), an inhibitor of mitochondrial complex I, has been commonly used as an agent to develop PD animal models for inducing specific loss of DAergic neurons in the SNc.<sup>6–8</sup> The neurotoxicity of MPTP depends on its active metabolite 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>), which is specifically absorbed into the DAergic neurons through SLC6A3/DAT (solute carrier family 6 [neurotransmitter transporter], member 3).<sup>9</sup> Several studies have shown that CDK5 (cyclin-dependent kinase 5), a key regulator of cell-cycle progression, plays a key role in dopamine loss after MPTP treatment and in the pathogenesis of PD.<sup>10–12</sup> Activation of CDK5 requires its regulatory partner

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CDK5R1/p35/p25 (cyclin-dependent kinase 5, regulatory subunit 1 [p35]).<sup>13</sup> Accumulating evidence indicates that MPTP increases CDK5 activity<sup>11,12</sup> and triggers neuronal loss through phosphorylation and inhibition of PEBP1/RKIP (phosphatidylethanolamine-binding protein 1),<sup>14</sup> survival factor MEF2 (myocyte enhancer factor 2),<sup>15</sup> antioxidant enzyme PRDX2/Prx2 (peroxiredoxin 2),<sup>16</sup> and APEX1/Ape-1 (APEX nuclease [multi-functional DNA repair enzyme] 1).<sup>11</sup>

Autophagy is a lysosomal degradation pathway that controls the turnover of cytoplasmic contents and organelles through the engulfment of cargo into double-membrane autophagosomes. It is characterized by increased MAP1LC3B-II/LC3B-II (microtubule-associated protein 1 light chain 3  $\beta$ -II) and decreased SQSTM1/p62.<sup>17-19</sup> Autophagy is essential for survival, differentiation, development, and homeostasis.<sup>17,18,20</sup> Recent studies have reported that deregulation of autophagy was implicated in PD.<sup>21,22</sup> Autophagosome accumulation is evident in the brain tissues of PD patients<sup>23,24</sup> and in MPTP-induced PD animal models.<sup>25</sup> Moreover, PINK1 (PTEN induced putative kinase 1)- and PARK2/parkin (parkin RBR E3 ubiquitin protein ligase)-mediated autophagy is actively involved in selective clearance of damaged mitochondria and protection of the neurons from death.<sup>26</sup> In addition, basal levels of autophagy and chaperone-mediated autophagy are necessary for the clearance of abnormal SNCA.<sup>27,28</sup> Excessive autophagy has also been associated with neuronal loss.<sup>20,29,30</sup> The CDK5-mediated autophagy contributes to neuronal loss in PD animal models.<sup>14,31</sup>

Melatonin, the main secretory product of the pineal gland, can easily cross the blood-brain barrier and has plentiful neuroprotective properties such as regulating circadian rhythms and providing anti-inflammatory and neuronal protection.<sup>32-34</sup> As a naturally occurring compound, melatonin has potential effects on inhibiting autophagy through a redox-mediated scavange of free radicals and lipid peroxidation,<sup>35</sup> as well as its well-known antioxidant properties.<sup>36</sup> Emerging evidence suggests that melatonin can be used as a neuroprotective agent in the rodent models of PD<sup>32,37,38</sup> by ameliorating the dysfunction of mitochondria,<sup>39-42</sup> blocking CASP3 (caspase 3, apoptosis-related cysteine peptidase) activation and cellular apoptosis,<sup>43</sup> and protecting TH (tyrosine hydroxylase)-positive nerve terminals.<sup>44</sup> However, the molecular underpinning of protective effect of melatonin on the loss of DAergic neurons in PD mouse model is still unknown. Our recent study suggests that melatonin can restore the mitochondrial DNA copy number to normal level by inhibiting autophagy induced by morphine.<sup>45</sup>

Based on these lines of evidence, we hypothesized that aberrant activation of autophagy may contribute to MPTP-induced neuronal cell death, which will lead to dopamine depletion in the striatum and motor symptoms of the PD animal models. We asked whether melatonin can reduce autophagic neuronal cell death induced by MPTP and which factor is mediating the process of dopaminergic neuronal cell loss and the bradykinesia of the PD animal models. Our results showed that melatonin attenuates MPTP-induced neurotoxicity via preventing CDK5-mediated autophagy and SNCA aggregation.

## Results

### MPTP induced autophagy in monkey brain tissues

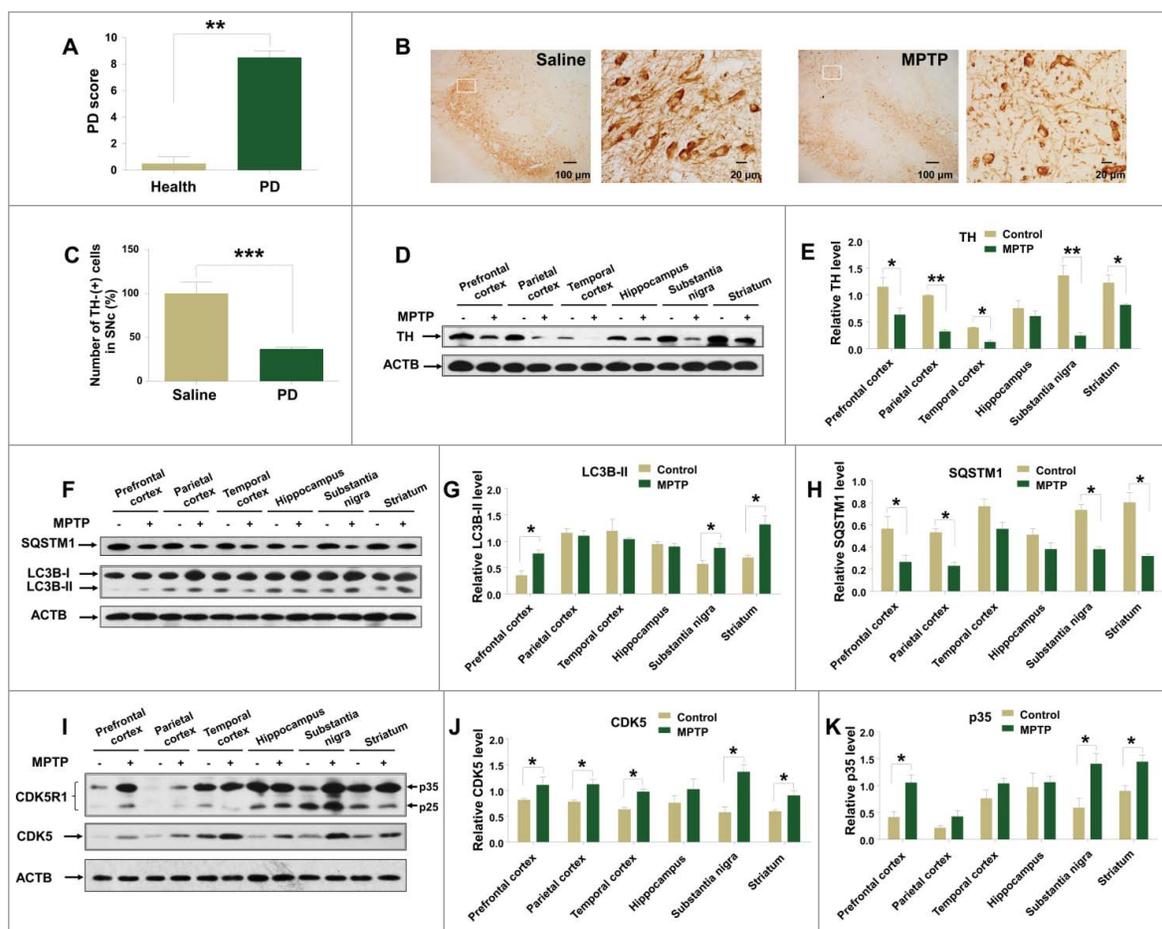
Male rhesus monkeys received MPTP injection (0.3 mg/kg, N = 2) and saline (N = 2) twice a wk for 3 mo and were then sacrificed for collection of brain tissues. We observed apparent parkinsonian symptoms in monkeys with MPTP treatment (Fig. 1A). Meanwhile, the number of TH-positive cells and dendrites of dopaminergic neurons were reduced in the SNc of monkeys with MPTP injection (Fig. 1B and C), along with the decreased TH level in different brain tissues (Fig. 1D and E), suggesting that the monkey PD model was successfully established. An increased protein level of LC3B-II (Fig. 1F and G) and a decreased protein level of SQSTM1 (Fig. 1F and H) were observed in different brain tissues from monkeys with MPTP injection, indicating induced autophagy by this agent.

Previous studies have demonstrated that CDK5 participates in the regulation of autophagy to mediate neurotoxicity and neuronal cell death.<sup>14,31,46</sup> We investigated whether CDK5 was changed during the development of the monkey PD model. We found that MPTP could induce higher CDK5 level in the monkey brain tissues, with the most striking effect in the substantia nigra region (Fig. 1I and J). In order to explore whether the increased CDK5 level was associated with the altered CDK5 kinase activity, the levels of CDK5R1 that contribute to CDK5 activity<sup>47</sup> were evaluated. We found that CDK5R1 levels were significantly upregulated in several brain regions after MPTP administration (Fig. 1I and K). These data showed that MPTP treatment could induce an increase of CDK5 protein level and its kinase activity in monkey brain tissues.

### MPTP induced CDK5-mediated autophagy, leading to death of C6 cells

To explore whether the elevated CDK5 level participates in MPTP-induced autophagy, we measured the protein levels of CDK5 and the autophagic marker, LC3B-II, as well as SQSTM1, in MPTP-treated C6 cells. An increased protein level of CDK5 was observed in MPTP-treated C6 cells, in a dose-dependent manner (Fig. 2A and B). Accordingly, we observed an increased protein level of LC3B-II and a decreased protein level of SQSTM1 (Fig. 2A and B). Along with the elevated LC3B-II and the decreased SQSTM1, we observed increased EGFP-LC3 puncta (Fig. 2C) upon MPTP treatment in C6 cells transfected with the N-terminally tagged pEGFP-C1-LC3 vector. Pretreatment with bafilomycin A<sub>1</sub> (BAFA1), an inhibitor of the vacuolar (V)-type ATPase that results in blockage of autophagosome-lysosome fusion, induced more LC3B-II accumulation compared to the control cells with or without MPTP treatment (Fig. 2D and E). However, the lysosomal function was unchanged in C6 cells treated with MPTP and/or melatonin (Fig. S1), suggesting that MPTP indeed increased autophagic flux. Treatment with MPP<sup>+</sup>, a metabolite of MPTP, similarly induced autophagy and upregulated CDK5 protein levels (Fig. S2).

To further identify the potential roles of CDK5 in regulation of MPTP-induced autophagy, we performed experiments using



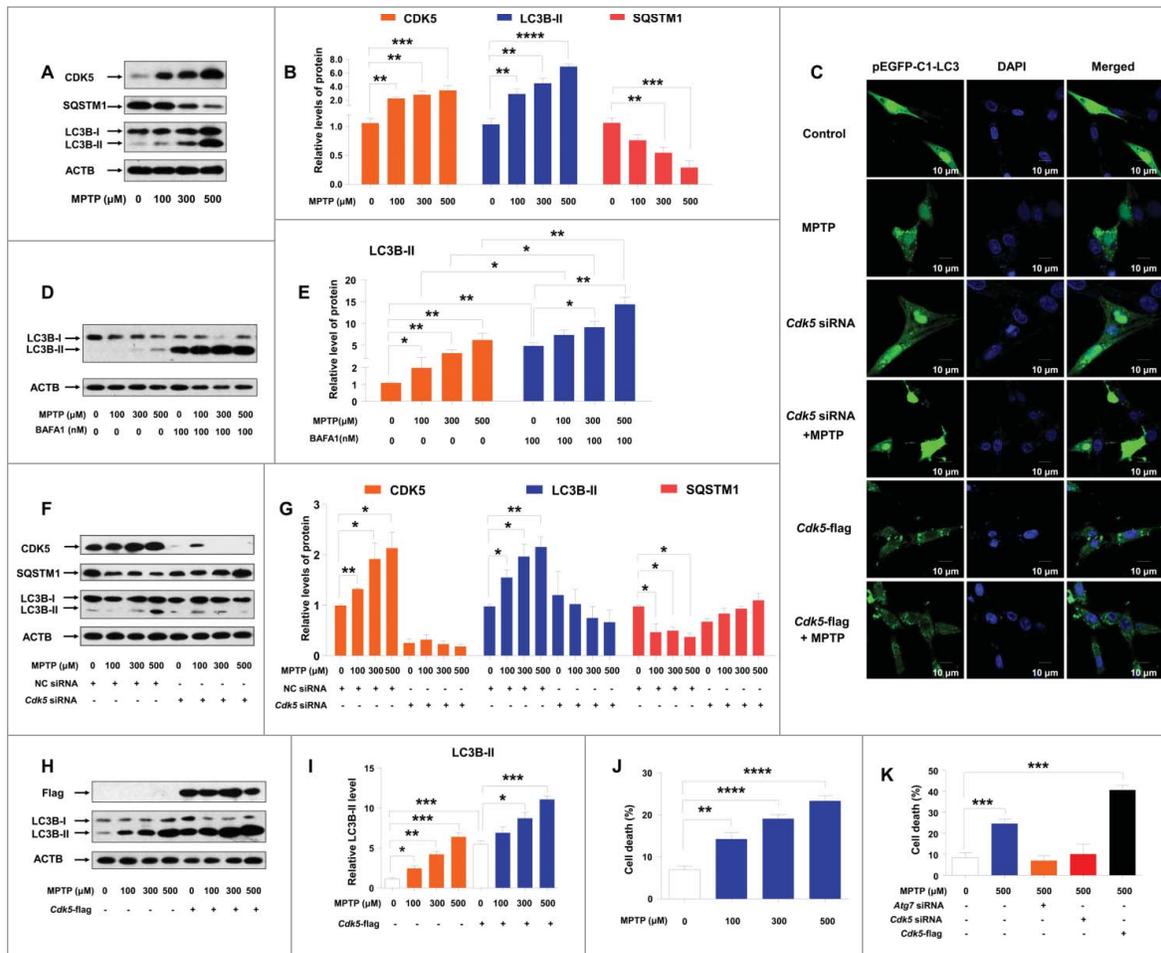
**Figure 1.** MPTP induces autophagy in the brain tissues of monkeys with MPTP treatment. Monkeys had a higher Parkinson disease (PD) score after MPTP injection compared to their status before the drug treatment (A). The number of TH-positive cells and dendrites of dopaminergic neurons was reduced in the SNc (B and C) and the protein levels of TH (D and E) were decreased in different brain tissues of monkeys treated with MPTP. There were increased protein levels of LC3B-II (F and G) and a decreased level of SQSTM1 (F, H). CDK5 levels were elevated (I and J), accompanied with increased levels of CDK5R1 (I, K), in different brain tissues of monkeys with MPTP treatment. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; Student *t* test. Bars represent mean  $\pm$  SEM.

siRNAs targeted to the *Cdk5* gene and also, overexpression of CDK5. Among the 3 siRNAs for the mRNA of the *Cdk5* gene, siRNA-1 had the best inhibitory effect (Fig. S3A and B) and was used in the following knockdown assays. Concordant with our expectation, knockdown of the mRNA of the *Cdk5* gene suppressed MPTP-induced formation of autophagosomes (Fig. 2C) and salvaged the increased level of LC3B-II and the decreased level of SQSTM1 (Fig. 2F and G). We got similar rescue results in a cell line stably expressing *Cdk5* shRNA (Fig. S3C and D and Fig. S4A and B). MPTP could induce autophagy in C6 cells with knockdown of CDK5 in the presence of BAF1 (Fig. S5A and B). Overexpression of CDK5 in C6 cells with or without MPTP resulted in increased autophagosome formation (Fig. 2C) and LC3B-II accumulation (Fig. 2H and I). These results provided direct evidence that the autophagy induced by MPTP was mediated by CDK5. Consistent with previous reports,<sup>14,46</sup> we found that MPTP induced cell death in C6 cells in a dose-dependent manner (Fig. 2J). Inhibition of autophagy by using *Atg7* siRNA prevented cell death progression induced by MPTP

(Fig. 2K). Knockdown of the *Cdk5* gene had a similar blocking effect on cell death triggered by MPTP (Fig. 2K). In contrast, overexpression of CDK5 had a synergetic effect and significantly increased cell death induced by MPTP (Fig. 2K). Taken together, the CDK5-mediated autophagy contributed to MPTP-induced cell death.

#### Melatonin restored CDK5-mediated autophagy and cell death in C6 cells, induced by MPTP

Melatonin is a well-known antioxidant molecule playing important roles against oxidative stress caused by MPTP.<sup>42</sup> It also has protective effects on methamphetamine-induced autophagic cell death in vitro.<sup>48</sup> We tested whether exogenous adding of melatonin could prevent MPTP-induced autophagy and cell death. As shown in Figure 3A and B, melatonin could restore the elevated CDK5 level induced by MPTP in C6 cells. In addition, melatonin reversed the increase of LC3B-II level, the decrease of SQSTM1 level (Fig. 3A and B), as well as formation of autophagosomes induced by MPTP (Fig. 3C). Pretreatment



**Figure 2.** MPTP treatment induces autophagy and cell death in cultured C6 cells and this effect is CDK5-mediated. An increased protein level of CDK5 and LC3B-II and a decreased level of SQSTM1 (**A and B**) were observed in C6 cells treated with MPTP. EGFP-LC3 puncta were induced by MPTP treatment (**C**). A treatment of 100 nM BFAF1 blocked the fusion of autophagosomes and lysosomes and led to accumulation of LC3B-II (**D and E**). Knockdown of the *Cdk5* gene by siRNA abolished the formation of EGFP-LC3 puncta (**C**) and the altered protein levels of LC3B-II and SQSTM1 (**F and G**) in C6 cells with MPTP treatment. Overexpression of CDK5 increased autophagosome formation (**C**), LC3B-II accumulation (**H and I**) and cell death (**K**) in C6 cells with and without MPTP treatment. MPTP induced cell death in C6 cells in a dose-dependent manner (**J**). Knockdown of the mRNA expression of the *Atg7* gene or the *Cdk5* gene blocked MPTP induced cell death (**K**). Relative protein abundance was normalized to ACTB. Data are representative of 3 independent experiments with similar results. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ ; one-way ANOVA with the Tukey post-hoc test. Bars represent mean  $\pm$  SEM.

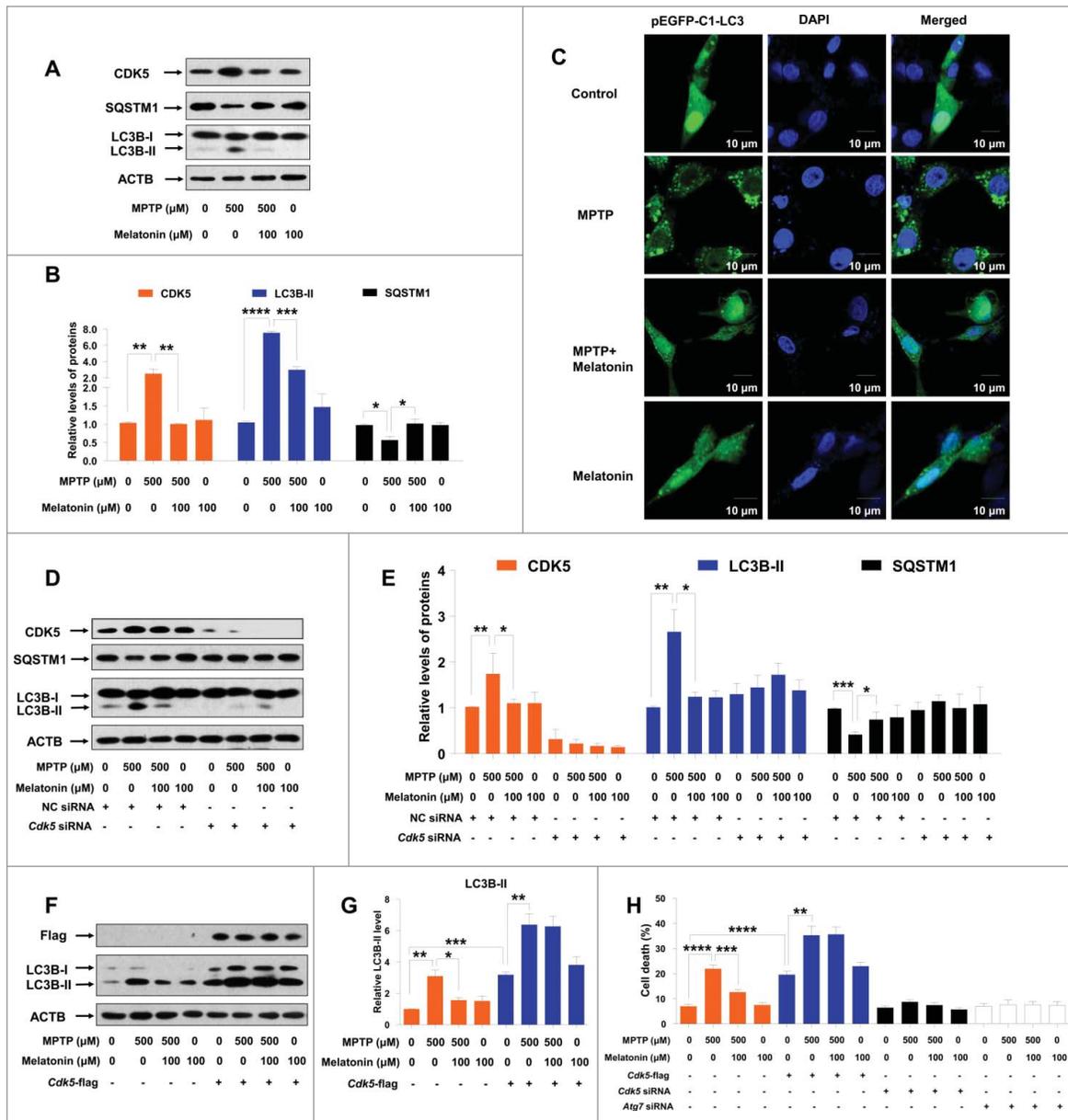
with melatonin could inhibit the autophagy induced by MPTP in C6 cells in the presence of BFAF1 (Fig. S5C and D). Melatonin alone had no effect on CDK5 protein level and autophagy (Fig. S6).

We further performed experiments using siRNA targeted to the *Cdk5* gene or overexpressing *Cdk5* to test whether the effect of melatonin on regulation of autophagy was CDK5-dependent. We found that melatonin had no effect on autophagy in cells with knockdown of the *Cdk5* gene by using siRNA (Fig. 3D and E), (partially) because CDK5 knockdown itself could inhibit the autophagy induced by MPTP. A similar result was observed in the stable cell line expressing *Cdk5* shRNA (Fig. S4C and D). Overexpression of CDK5 in C6 cells transfected with the *Cdk5*-flag vector abolished the protective effect of melatonin on MPTP-induced autophagy (Fig. 3F and G). Intriguingly, we found that melatonin

blocked cell death induced by MPTP (Fig. 3H and Fig. S7). Overexpression of CDK5 abolished the protective effect of melatonin on MPTP-induced cell death (Fig. 3H). Note that knockdown of the *Atg7* and *Cdk5* genes could also salvage the MPTP-induced cell death (Fig. 3H). These results suggested an active role of melatonin on protecting autophagy and cell death induced by MPTP and CDK5 was involved in this process.

### Melatonin reversed the dendritic morphological changes of neurons and autophagy induced by MPTP

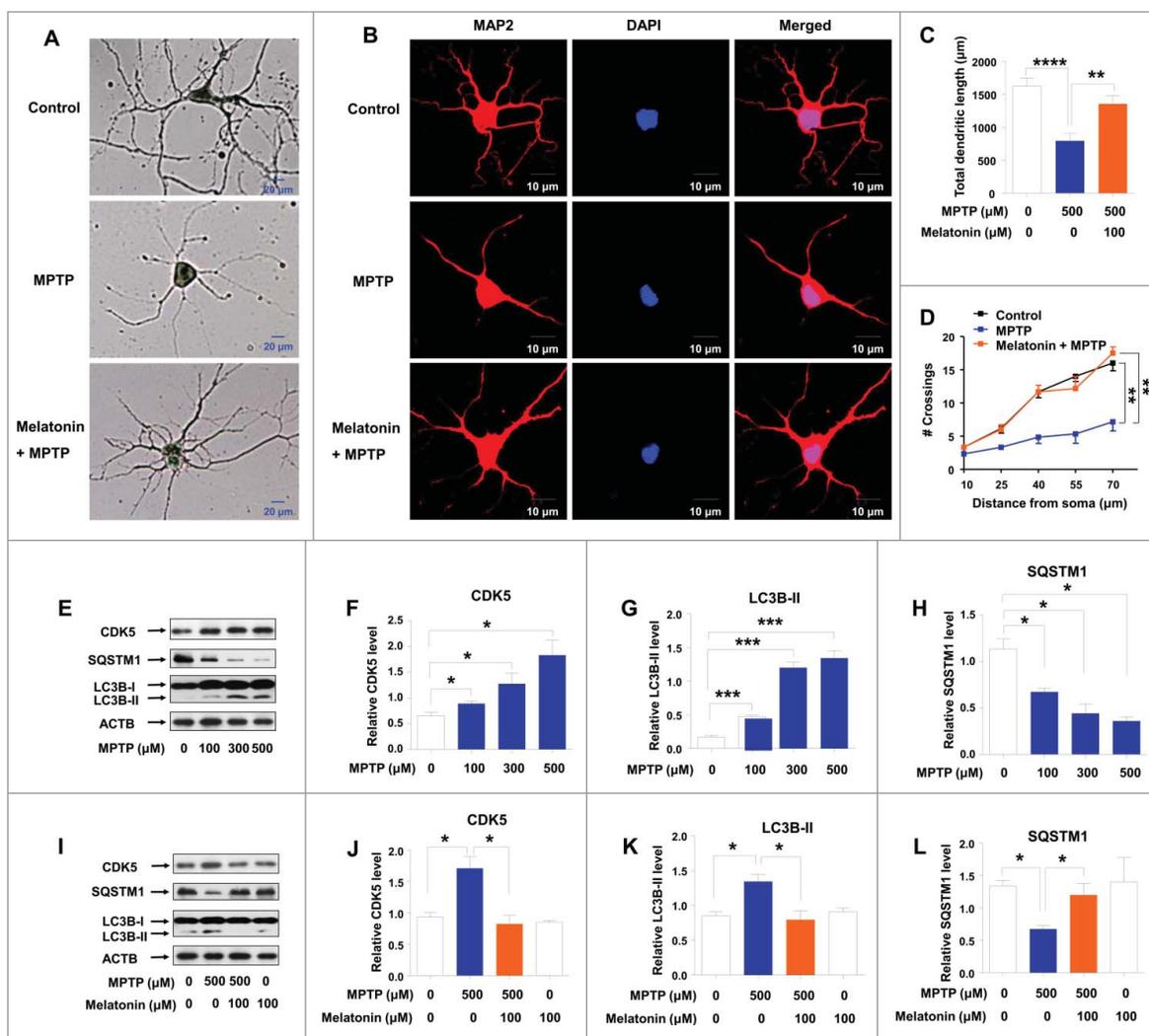
To confirm the importance of MPTP-induced autophagy in regulating neuronal cell survival, mouse primary cortical neurons were treated with 500  $\mu$ M MPTP for 24 h followed by staining with Golgi-Cox or TUBB3 (tubulin,  $\beta$  3 class III) to visualize neurons. MPTP treatment reduced the length of



**Figure 3.** Melatonin inhibits CDK5-mediated autophagy and cell death induced by MPTP in cultured C6 cells. The increased protein levels of CDK5 and LC3B-II and the decreased protein level of SQSTM1 (**A and B**) and formation of EGFP-LC3 puncta (**C**) in C6 cells induced by MPTP could be suppressed by melatonin treatment. Knockdown of the *Cdk5* gene abolished the effect of melatonin on the altered LC3B-II and SQSTM1 levels (**D and E**) in C6 cells with MPTP treatment. Melatonin failed to restore the altered protein level of LC3B-II (**D–G**) in C6 cells with knockdown or overexpression of the *Cdk5* gene. Knockdown of ATG7 and CDK5 or overexpression of CDK5 abrogated the protective effect of melatonin on cell death induced by MPTP (**H**). Data are representative of 3 independent experiments with similar results. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ ; one-way ANOVA with the Tukey post-hoc test. Bars represent mean  $\pm$  SEM.

Golgi-Cox or TUBB3-positive axons and dendrites compared to untreated neurons, and this effect could be reversed by melatonin pretreatment (Fig. 4A and Fig. S8). We next explored the impact of MPTP treatment on the structure of neuron by evaluating the change of total dendritic length and dendritic complexity, which was demonstrated by using immunostaining for the dendritic MAP2 (microtubule-associated protein 2) (Fig. 4B). We found that both total dendritic length (Fig. 4C)

and dendritic complexity (Fig. 4D) were significantly reduced in MPTP-treated neurons relative to untreated neurons. Pretreatment with melatonin reversed the neuronal morphological changes triggered by MPTP (Fig. 4A–D). These results indicated an active role of melatonin in regulating the neuro-morphological changes and counteracting the deleterious effect of MPTP, which would eventually prevent the dysfunction of the neurons. Similarly, MPTP treatment significantly elevated



**Figure 4.** Effect of melatonin on primary neuron dendritic morphogenesis and autophagy induced by MPTP. Mouse primary cortical neurons were cultured in Neurobasal<sup>®</sup> Medium containing 2% B27. Neurons were pretreated with melatonin (100 μM) for 1 h, followed by treatment with or without MPTP (500 μM) for 24 h. The morphological structure (A) and dendrites (B) of neurons were demonstrated by Golgi-Cox and MAP2 staining, respectively. Upon MPTP treatment, dendritic length (C) and dendritic complexity (D) were reduced. These changes could be reversed by a pretreatment with melatonin (A–D). MPTP treatment increased the protein levels of CDK5 (E and F), LC3B-II (E, G) and decreased the protein level of SQSTM1/p62 (E, H) in mouse cortical neurons. Pretreatment with melatonin restored the increased levels of CDK5 (I and J) and LC3B-II (I, K) and the decreased level of SQSTM1 (I, L) induced by MPTP. Data are representative of 2 independent experiments with similar results. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ ; one-way ANOVA with the Tukey post-hoc test. Bars represent mean  $\pm$  SEM.

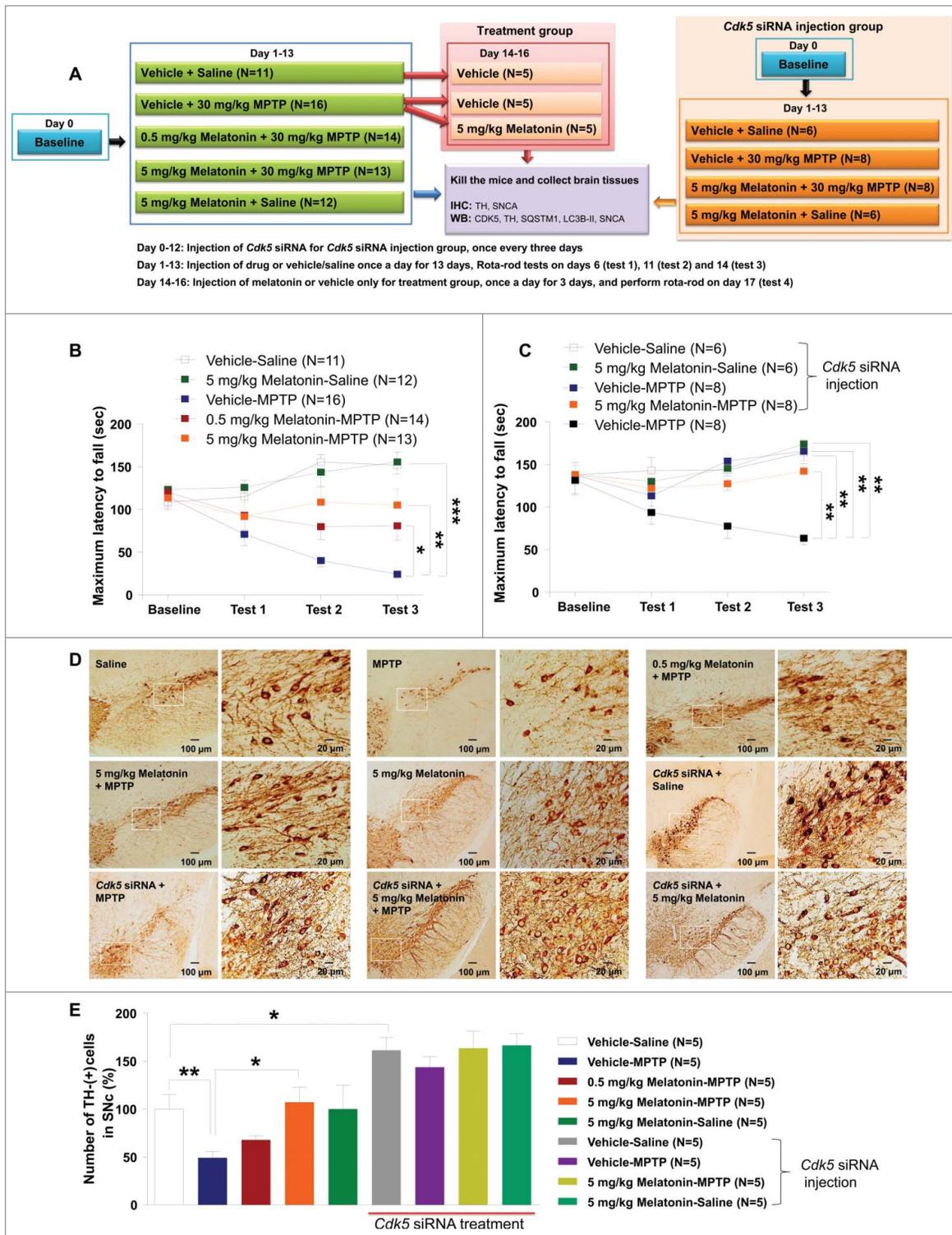
protein level of CDK5 with a dose-dependent manner in primary cortical neurons (Fig. 4E and F), accompanied with an increased protein level of LC3B-II (Fig. 4E and G) and a decreased level of SQSTM1 (Fig. 4E and H). Pretreatment with melatonin could restore the altered protein level of CDK5 (Fig. 4I and J), LC3B-II (Fig. 4I and K) and SQSTM1 (Fig. 4I and L) to the normal levels.

#### Melatonin ameliorated symptoms in the mouse PD model through preventing CDK5-mediated autophagy

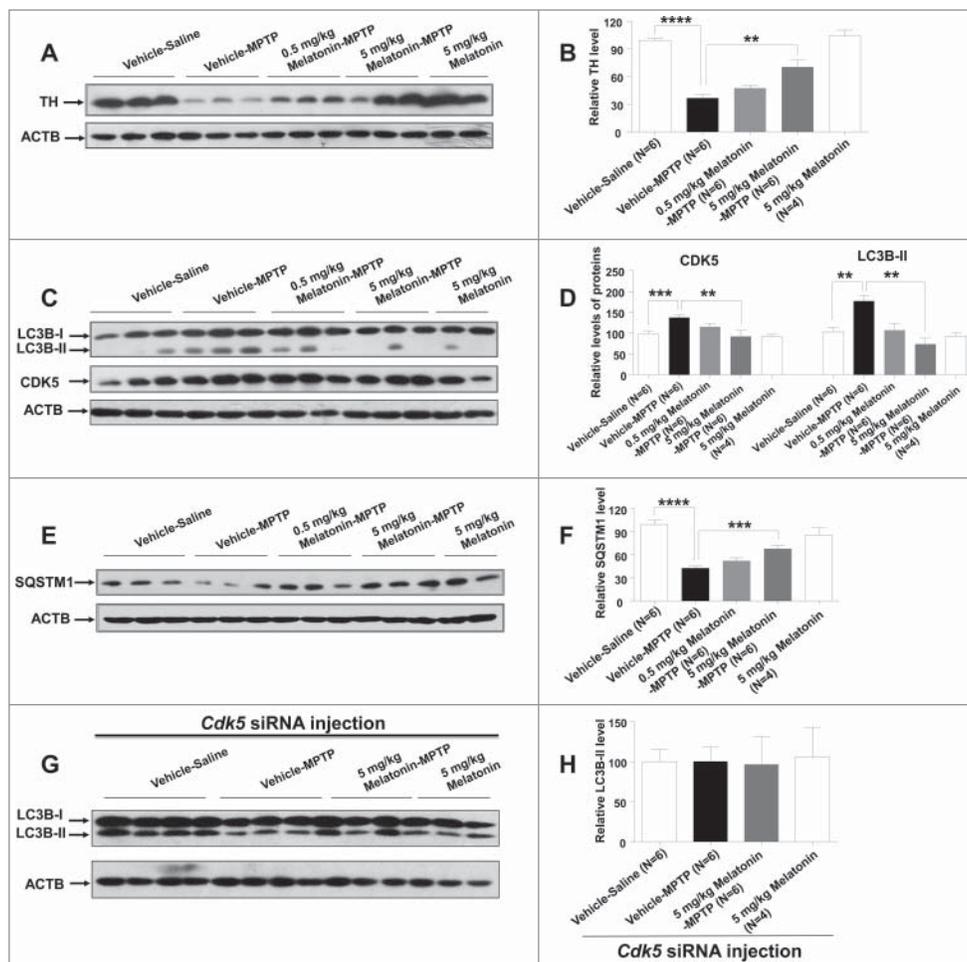
A mouse PD model was used to discern whether melatonin salvaged the MPTP toxic effect in vivo. The induced dyskinesia by chronic MPTP treatment was measured using a rota rod

apparatus after MPTP injection at 3 time points (Fig. 5A). The retention time on the rota rod was significantly decreased in mice subjected to 30 mg/kg MPTP treatment (subcutaneously [s.c.]) once per d for 2 wk compared to saline controls. Pretreatment with melatonin (intraperitoneally [i.p.]; 5 mg/kg and 0.5 mg/kg) before MPTP injection in mice significantly prevented dyskinesia induced by MPTP, with a dosage-dependent effect (Fig. 5B).

To test whether the CDK5 was involved in the pathogenesis of mouse PD model, we evaluated the effect of CDK5 inhibition by using intracerebroventricular (ICV) injection of siRNA oligonucleotide that targeted the *Cdk5* gene. Three d after ICV injection of *Cdk5* siRNA, we observed a significant decrease of CDK5 protein levels in different mouse brain regions, especially in the



**Figure 5.** Pretreatment with melatonin or knockdown of CDK5 counteracts the effect of MPTP on behavioral dyskinesias and dopaminergic neuron loss in mouse models. Mice were divided into different groups (A). MPTP-treated mice (Vehicle-MPTP group) exhibited a significantly decreased retention time on the rota rod apparatus compared to control mice without any treatments (Vehicle-Saline group). Pretreatment with melatonin (0.5 mg/kg [0.5 mg/kg Melatonin-MPTP group] and 5 mg/kg [5 mg/kg Melatonin-MPTP group]) significantly improved the retention time compared to the MPTP group (B). Knockdown of CDK5 increased the retention time on the rota rod of mice with and without MPTP injection (C). Shown are photomicrographs of TH immunoreactivity in the SNc central region of different mouse groups (D): TH-positive cell number and dendrites of DA neurons were reduced in the SNc of mice with MPTP injection, which could be rescued by injection of 5 mg/kg melatonin or knockdown of the *Cdk5* gene. The differences of TH-positive cell numbers between the control group and the MPTP group, between the MPTP group and the MPTP plus 5 mg/kg melatonin group (5 mg/kg Melatonin-MPTP group) were statistically significant (E). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; one-way ANOVA with the Tukey post-hoc test. Bars represent mean  $\pm$  SEM.



**Figure 6.** Protective effect of melatonin on MPTP-induced autophagy mediated by CDK5 in the mouse model of PD. TH protein level was significantly decreased in the MPTP group relative to the control group (Vehicle-Saline group) and this change could be reversed by pretreatment with 5 mg/kg melatonin (**A and B**). Pretreatment of 5 mg/kg melatonin restored the elevated protein levels of CDK5 and LC3B-II (**C and D**) and the decreased protein level of SQSTM1 (**E and F**) induced by MPTP in mouse striatum. The relative protein levels of CDK5, LC3B-II and SQSTM1 were statistically significant between the control group and the MPTP group, and between the MPTP group and the MPTP plus 5 mg/kg melatonin group (5 mg/kg Melatonin-MPTP group). MPTP failed to induce autophagy and melatonin had no salvaging effect on autophagy induced by MPTP in the striatum of mice after knockdown of the *Cdk5* gene (**G and H**). Data are expressed as mean  $\pm$  SEM. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ ; one-way ANOVA with the Tukey post-hoc test. Bars represent mean  $\pm$  SEM.

midbrain (Fig. S9). As expected, inhibition of CDK5 in brain tissues increased the retention time on the rota rod of mice with and without MPTP injection compared to those without *Cdk5* siRNA injection (Fig. 5C). These results indicated that CDK5 was involved in MPTP-induced dyskinesia of mice.

We detected the number and morphology of dopaminergic neurons in the midbrain of mice by using TH immunoreactivity. In control mice with saline injection, TH immunoreactivity was observed in cells with rounded somata and immunoreactive dendrites formed a dense lattice network within the SNc area (Fig. 5D). However, the number of TH-positive cells and dendrites of dopaminergic neurons was significantly reduced in the SNc area of mice with MPTP injection (Fig. 5D and E).

Pretreatment with 5 mg/kg melatonin (i.p.) or knockdown of CDK5 in mice significantly attenuated the dopaminergic deficit including the reduced number and altered morphology of dopaminergic neurons induced by MPTP (Fig. 5D and E). Pretreatment with a lower concentration of melatonin (0.5 mg/kg) also had a salvaging effect, albeit the effect was not significant. Treatment with melatonin alone had no effect (Fig. 5D and E). We observed an increased TH-positive cells and dendrites of dopaminergic neurons in the SNc of the mice in the presence of *Cdk5* siRNA (Fig. 5D and E). MPTP and melatonin had no significant effect on the TH-positive cells and dendrites of dopaminergic neurons in mice with *Cdk5* siRNA injection (Fig. 5D and E). In addition, MPTP treatment significantly decreased the protein level of TH, as compared to the control group (Fig. 6A and B). Pretreatment with 5 mg/kg melatonin (i.p.) in mice restored the decreased TH level compared to the MPTP-treated group (Fig. 6A and B). In the mice with *Cdk5* siRNA injection, MPTP treatment decreased the protein level of TH. However, pretreatment with melatonin had no significant effect on the level of TH in the striatum of these mice (Fig. S10).

To investigate whether melatonin exerted its neuroprotective role via inhibiting the CDK5-mediated autophagy, we analyzed the effect of melatonin on protein levels of

CDK5, LC3B-II, and SQSTM1 in the striatum of treated mice. In accordance with the results observed in vitro, we found increased levels of CDK5 and LC3B-II (Fig. 6C and D) and a decreased level of SQSTM1 (Fig. 6E and F) in the striatum of mice with MPTP treatment. Melatonin treatment (5 mg/kg) could restore the elevated CDK5 and LC3B-II levels and the decreased SQSTM1 level to normal levels in mouse striatum induced by chronic MPTP treatment (Fig. 6C–F). MPTP could not induce autophagy in the striatum in mice with *Cdk5* siRNA injection (Fig. 6G and H). Collectively, our results indicated that melatonin salvaged the dopaminergic neuronal loss and decreased neurite of neuron through inhibition of MPTP-induced autophagy mediated by CDK5.

### Melatonin treatment and CDK5 knockdown ameliorated SNCA aggregation

Previous studies suggest that SNCA aggregates are neurotoxic and that reducing SNCA aggregation can be used as a neuroprotective strategy against central nervous system neurodegeneration.<sup>49,50</sup> We investigated the involvement of SNCA aggregation induced by MPTP in the brain tissues of monkey and mouse. Our immunohistochemical staining results showed that MPTP increased SNCA aggregates in the SNc region of monkeys (Fig. 7A). Western blot assay further demonstrated an increased accumulation of SNCA (51 k<sub>D</sub>) in different brain tissues of monkeys injected with MPTP (Fig. 7B and C). However, MPTP did not significantly alter the SNCA monomer (17 k<sub>D</sub>) levels in these brain tissues (Fig. 7B and D).

We validated the observations in monkeys relative to MPTP treatment in mouse. An increased level of SNCA aggregates was found in the SNc region and striatum of mice injected with MPTP (Fig. 7E–G). Pretreatment with melatonin significantly reduced MPTP induced SNCA aggregation (Fig. 7E–G), but had no effect on the SNCA monomer level (Fig. 7F and H). Few SNCA aggregates were observed in the SNc region and striatum of mice in the presence of *Cdk5* siRNA (Fig. 7E and I). MPTP and melatonin had no effect on the aggregates of SNCA in these mice with *Cdk5* siRNA injection (Fig. 7E and I–K). It should be mentioned that melatonin enacted its protective effect only before the MPTP treatment. In the mice that were treated with MPTP and developed PD symptoms, melatonin treatment had no significant salvaging effect on behavioral change (Fig. S11A), deficiency of dopaminergic neurons (Fig. S11B and C), decrease of TH (Fig. S11D and E), upregulation of CDK5 (Fig. S12A and B), increased autophagic activity (Fig. S12A and B) and SNCA aggregation (Fig. S13). These results suggested that melatonin may not be used as a therapeutic drug, but its use as a food additive for people susceptible to PD is promising.

### Discussion

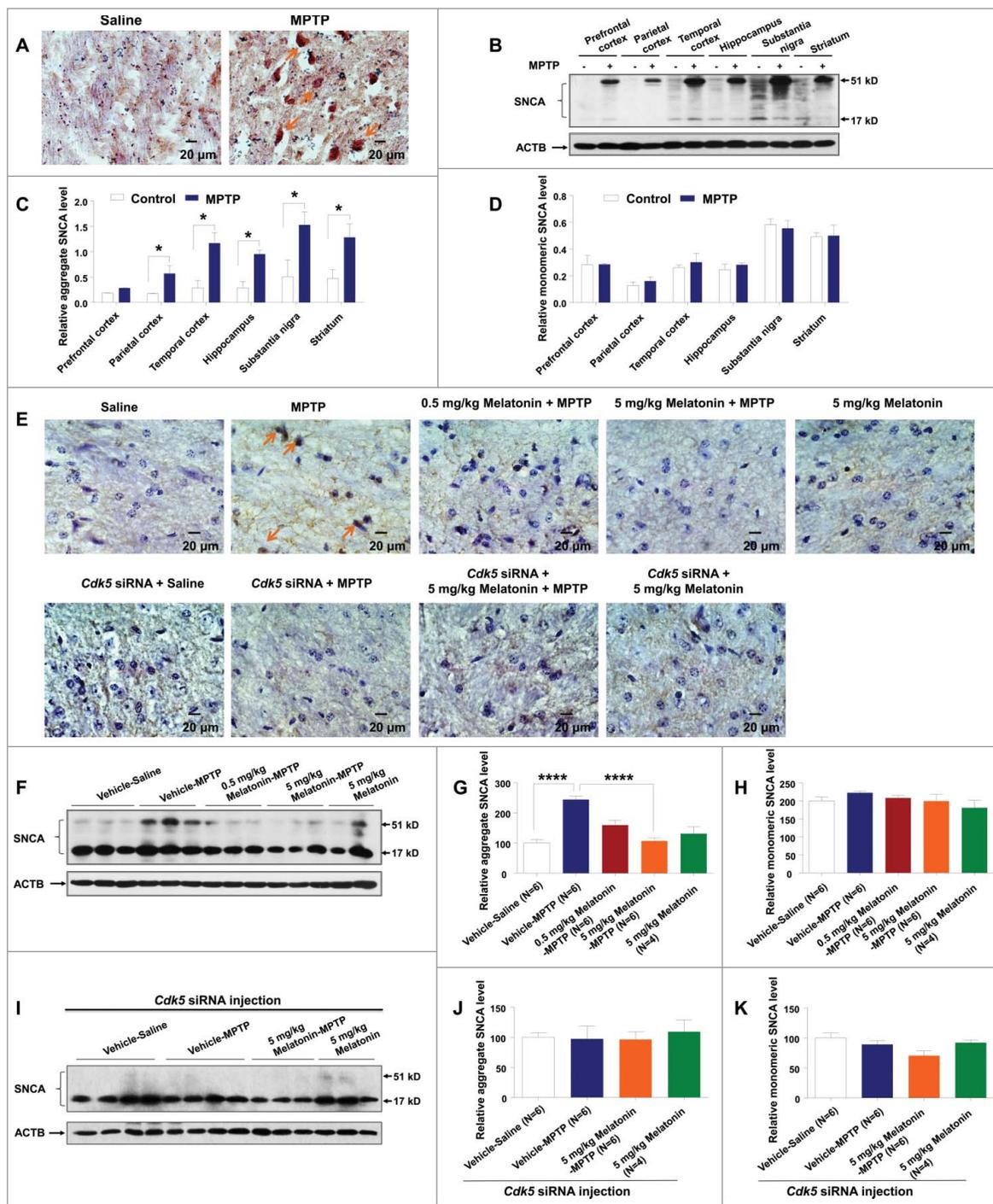
PD is a progressive, debilitating neurodegenerative disease characterized by Lewy body accumulation and dopaminergic neuron death in the SNc region.<sup>5,51</sup> The development of PD is affected by a growing number of genetic and environmental risk factors and synaptic dysfunction, which would be regulated by autophagy<sup>52</sup> and SNCA aggregates.<sup>50</sup> Autophagy-mediated self-digestion of cytoplasmic inclusions may be protective against the PD.<sup>53,54</sup> However, excessive autophagic activation evokes autophagic programmed cell death.<sup>14,55</sup> Recent studies report that CDK5-mediated phosphorylation and autophagy of PEBP1 regulates neuronal death in PD.<sup>14</sup> Overexpression of the leucine-rich repeat kinase 2 (LRRK2) mutant G2019S, which is associated with autosomal dominant and sporadic forms of PD in human neuroblastoma cells, causes activation of autophagy and significantly decreases dendritic length.<sup>56,57</sup> Similarly, overexpression of mutant SNCA increases cellular autophagosomes,<sup>58</sup> which actively influences and impacts synaptic functions. Previous studies also demonstrate that both autophagy inducers and

inhibitors serve as neuroprotectors against PD. For example, the autophagy inducer rapamycin (Rap) has neuroprotective effects on cellular and mouse models of PD.<sup>54</sup> The autophagy inhibitor 3-methyladenine can block 6-hydroxydopamine induced toxicity.<sup>59</sup> These findings suggest that maintaining balanced autophagy is critical for neuronal health and function.<sup>60</sup> In this study, we aimed to answer whether interference of autophagy could be of potential use in counteracting the progression of PD and whether melatonin, a readily available substance that is already widely in use in humans for other indications, could be potentially translated into human clinical trials for curing PD given melatonin's good safety profile and availability.

We presented direct evidence that MPTP could induce autophagic flux at the cellular level and this neurotoxicity effect was mediated by CDK5. These findings are in agreement with previous reports that the CDK5-mediated autophagy is actively involved in PD.<sup>14,31</sup> We next tested whether melatonin, with its antioxidant properties, could restore the abnormal autophagic activity induced by MPTP. Consistent with previous observations that melatonin can restore morphine-induced autophagy<sup>45</sup> and kainic acid-induced autophagy,<sup>61</sup> we found that melatonin inhibited MPTP-induced autophagy and cell death in vitro. This salvaging effect of melatonin was CDK5-dependent, and knock-down or overexpression of CDK5 abolished the protective effect of melatonin on MPTP-induced autophagy and cell death. Taken all these results together, it is evident that melatonin restored autophagy through inhibition of CDK5 upregulation at the cellular level.

Maintenance of axon and dendrite length is critical for neuron function and autophagy is actively involved in regulation of pathological remodeling.<sup>60</sup> Therefore, a direct test of melatonin's effect on neurons in vitro and in vivo, rather than on cell lines, is essential for further defining its role in the development of PD. Consistent with a previous finding that melatonin can save dopaminergic cells in MPTP-treated mice,<sup>62</sup> our study showed that MPTP treatment reduced the length of axons and dendrites in primary neurons and decreased the number and dendrites of dopaminergic neurons in the SNc region of monkeys and mice with MPTP injection. Furthermore, MPTP injection increased SNCA aggregation in the mouse PD model. Melatonin could reverse all these aberrant effects, including decreased number of dopaminergic neurons, increased SNCA aggregation and altered morphologic changes of neurons induced by MPTP. These results indicated an active role of melatonin in regulating the neuromorphological changes and counteracting the deleterious effect of MPTP, which might eventually prevent the dysfunction of the neurons.

Although previous studies show that melatonin ameliorates the symptomatic features of Maneb and Paraquat-induced PD in a mouse model,<sup>63</sup> the exact mechanism of the PD or PD-like symptoms induced by these drugs has not been fully understood. We were able to show that pretreatment with melatonin significantly prevented MPTP-induced dyskinesia, and salvaged the reduction of the number of dopaminergic neurons in mice with MPTP treatment. This neuroprotective role of melatonin against MPTP-induced neurotoxicity in vivo was mediated by CDK5.



**Figure 7.** MPTP induced SNCA aggregation in monkey and mouse brain. MPTP increased SNCA aggregation in the monkey brain tissues, assessed by immunohistochemical staining, followed by hematoxylin counterstaining (A) and western blot assay (B–D). Mice with chronic MPTP administration (30 mg/kg once daily) showed an increased aggregation of SNCA in the SNc (E) and striatum (F). Pretreatment with 5 mg/kg melatonin or knockdown of CDK5 inhibited the SNCA aggregation induced by MPTP in mouse SNc (E) and striatum (F–H) compared to the control group. MPTP and melatonin had no effect on the SNCA aggregation in the presence of *Cdk5* siRNA (E, I–K). \*,  $P < 0.05$ ; \*\*\*\*,  $P < 0.0001$ ; one-way ANOVA with the Tukey post-hoc test. Bars represent mean  $\pm$  SEM.

First, pretreatment of melatonin reversed the increased CDK5 protein level, SNCA aggregation, and autophagy induced by MPTP in the mouse PD model. Second, injection of *Cdk5*

siRNA ameliorated the behavioral dyskinesia of mice with MPTP injection and MPTP failed to induce autophagy in the striatum of treated mice. These lines of evidence suggested that

melatonin could prevent MPTP-induced CDK5-mediated autophagy and SNCA aggregation, and subsequently protected the substantia nigra dopaminergic neurons cells from autophagic cell death, and finally prevented the symptomatic features of PD (Fig. 8). Note that there are other more relevant PD models to study the impact of SNCA aggregation instead of the MPTP models,<sup>3</sup> and it might be rewarding to validate the above results in these PD models and to more thoroughly characterize the nature of the aggregates.

An interesting finding in this study is that injection with melatonin after MPTP treatment could not reverse PD symptoms and related deleterious effects such as dopaminergic neuronal loss, CDK5 upregulation, autophagy, as well as SNCA aggregation. These results suggested that melatonin has a protective effect only in the early stage of PD. Our study raises further questions for future investigations: What is the exact biological mechanism of CDK5 upregulation in MPTP-induced neurotoxicity and autophagy? Does it share the same pathway, i.e. CDK5-mediated phosphorylation of endophilin B1, during the induction of autophagy in PD models?<sup>31</sup> Mitochondrial dysfunction and excess reactive oxygen species have been strongly implicated in the pathogenesis of PD.<sup>64</sup> Consistent with previous studies,<sup>65</sup> we found that MPTP treatment increased the reactive oxygen species level (Fig. S14A) and decreased the mitochondrial Complex I activity (Fig. S14B and C), and these effects could be salvaged by melatonin (Fig. S14), suggesting that mitochondrial dysfunction might be actively involved in CDK5 upregulation. Conversely, deregulation of cellular Ca<sup>2+</sup> homeostasis would lead to synaptic dysfunction, which is a hallmark of several neurodegenerative diseases.<sup>66</sup> The Ca<sup>2+</sup>-dependent protease CAPN/calpain regulates CDK5R1-CDK5 in dopaminergic neuron death.<sup>67</sup> Is CDK5 upregulation during MPTP treatment caused by the Ca<sup>2+</sup>-dependent CAPN/calpain activation? Finally, might the increased activation of autophagy be compensatory, in response to MPTP, and perhaps even protective? In this case, cells induce autophagy as a coping response, and while it is correlated with cell stress and cell death, it is not itself a primary cause of cell death. Further studies are essential to answer these intriguing and important questions.

In summary, we found excessive activation of autophagy induced by MPTP, which is mediated by CDK5 in both cellular and animal models. The detrimental effects of MPTP-induced

autophagic activity could be counteracted by melatonin or CDK5 knockdown, which finally protected neuron function and prevented the behavioral abnormality (Fig. 8). It should be mentioned melatonin that itself could not affect autophagy under the dosage used in this study; this would exclude its potential toxicity associated with usage in other neurodegenerative disease models where autophagy induction is (apparently) beneficial. Importantly, the protective effect of melatonin occurred before the activation of CDK5. Taken together, our results suggested that pretreatment with melatonin salvaged the dopaminergic neuronal loss, decreased axon and dendritic length of neuron and dyskinesias induced by MPTP via inhibition of CDK5-mediated autophagy and SNCA aggregation. Control of autophagic pathways may provide important insights for exploring potential targets for PD therapy.

## Materials and Methods

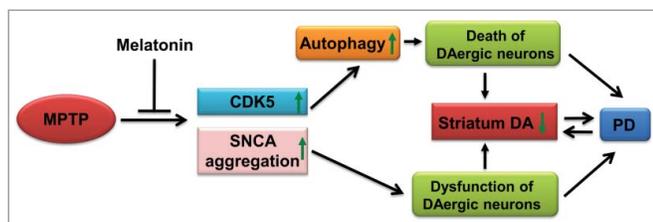
### Reagents, chemicals, and cells

The following antibodies and chemicals were used in this study: mouse monoclonal anti-CDK5 (Santa Cruz Biotechnology, sc-6247), rabbit monoclonal anti-LC3B (Cell Signaling Technology, 3868), mouse monoclonal anti-SQSTM1/p62 (Merck Millipore, MABC32), rabbit polyclonal anti-TH (Merck Millipore, AB152), rabbit polyclonal anti-SNCA/ $\alpha$ -synuclein (Merck Millipore, AB5038), rabbit polyclonal anti-MAP2 (Merck Millipore, AB5622), mouse monoclonal anti-ACTB/ $\beta$ -actin (Beijing Zhong Shan-Golden Bridge Biological Technology CO., LTD, TA-09), FITC-conjugated anti-rabbit antibody (KPL, 172-1506). MPTP (Sigma, M0896), melatonin (Sigma, M5250), poly-L-ornithine (Sigma, P4957) and DAPI (Roche, 10236276001), propidium iodide (PtdIns; Beyotime Institute of Biotechnology, C1502-2), pentobarbital (Sigma, P3761), procaine hydrochloride (Sigma, P9879), were purchased as indicated.

Rat C6 astroglial cells were introduced from Kunming Cell Bank, Kunming Institute of Zoology. Cells were maintained in DMEM medium (Gibco-BRL, 11965-092) with 10% fetal bovine serum (Gibco-BRL, 10099-141) at 37°C in a humidified atmosphere incubator with 5% CO<sub>2</sub>. Mouse cortical neurons were prepared and cultured using a previously described method.<sup>45</sup> Briefly, primary cortical neurons were isolated from mouse E15 embryos and were cultured in Neurobasal<sup>®</sup> Medium supplement with 2% B27 (Gibco-BRL, 17504).

### Establishment of a chronic PD model in monkeys

Four male rhesus monkeys (*Macaca mulatta*) were obtained from the Primate Research Center of Kunming Institute of Zoology, Chinese Academy of Sciences. Captive male rhesus monkeys (mean weight: 5.2 kg; mean age: 6.1 y) were housed individually in primate cages under a 12-h light/dark cycle with free access to water and food. Monkeys were divided into 2 groups: MPTP group (N = 2) and Control group (N = 2). MPTP was dissolved in 0.9% saline. Monkeys received intravenously (i.v.) injection of low doses of MPTP (0.3 mg/kg; Sigma, M0896) or saline twice weekly for 3 mo until obvious Parkinsonism was detected.<sup>68</sup> The behavioral changes were assessed as previously described.<sup>69,70</sup>



**Figure 8.** A schematic diagram of potential mechanism of MPTP-induced Parkinsonism and the reversing effect of melatonin. DA, dopamine; up arrow symbol, upregulation or activation; down arrow symbol, downregulation.

Briefly, the severity of PD was scored by using an established rating scale for (1) tremor, (2) posture, (3) gait, (4) bradykinesia, (5) balance, (6) gross motor skills, and (7) defense reaction. The minimal PD score was 0 and the maximum score was 20. All animals were euthanized by sodium pentobarbital overdose (150 mg/kg, i.v.), and the brains were removed quickly after death. Each brain was bisected along the midline. Different brain tissues were obtained and immediately frozen in liquid nitrogen and then stored at  $-80^{\circ}\text{C}$ .

#### Establishment of a chronic PD model in mice

Experiments were performed on 8-wk-old male C57BL/6 mice (body weight: 25 to 30 g) that were obtained from the animal core facility of the Kunming Medical University. Animals were group-housed under a 12-h light/dark cycle with free access to water and food. Mice were divided into the following groups: (1) Control group (N = 11), injected with vehicle and saline; (2) MPTP group (N = 16), injected with vehicle and MPTP; (3) MPTP + 5 mg/kg Melatonin group (N = 13), injected with melatonin and MPTP. (4) MPTP + 0.5 mg/kg Melatonin group (N = 14), injected with melatonin and MPTP. (5) Melatonin group (N = 12), injected with melatonin and saline (cf. Fig. 5). MPTP was dissolved in 0.9% saline; melatonin was dissolved in 0.5% ethanol saline; 0.5% ethanol saline (v/v) is regarded as vehicle. Mice were subcutaneously (s.c.) injected with MPTP (30 mg/kg) once a day for 2 wk following a previously described procedure.<sup>36</sup> Animals in the control group received an equivalent volume of 0.9% saline. Each mouse received an injection of MPTP or saline 30 min after intraperitoneal (i.p.) injections of melatonin (0.5 or 5 mg/kg) or vehicle (10 mL/kg, i.p.). For the treatment group (compare Fig. 5A in the main text), animals in the control group and the MPTP group received the same treatment as described above. Starting from d 14, 5 mice from the control group and 5 mice from the MPTP group were injected with vehicle only, another 5 mice from the MPTP group were injected with 5 mg/kg melatonin once a d for 3 consecutive d, followed by the behavioral test.

#### Behavioral analysis: rota-rod task

We created a mouse PD model and assessed it on a rota-rod apparatus (Panlab, LE8500) following a previously described procedure.<sup>71,72</sup> The instrument has a 30-mm diameter rotating rod and was divided into 5 compartments, thus permitting tests for 5 mice at a time. The rotation speed of the rod was fixed at 12 rpm and mice were tested for the total duration of 180 sec. Mice were trained to maintain themselves in a neutral position on a stationary rod. After the rota-rod was switched on for 3 sec, mice were timed until they fell off the rota-rod (or a maximum cutoff time of 180 sec). Mice that had a score of 180 sec were removed from the rota-rod and returned to their home cages. The animals had a total of 3 consecutive trials per d. Mice that could not hold on the stationary cylinder for 3 trials were scored as zero. The highest individual score of each animal every day was used in the analysis. In order to avoid potential data misinterpretation due to acute melatonin residual effects, we performed behavioral measurements at least 24 h after the last injection of melatonin. For all experiments in vivo, data acquisition and analysis were performed using a double-blind approach.

#### Surgery and intracerebroventricular (ICV) siRNA administration

The ICV cannulation was carried out as previously described.<sup>73</sup> In brief, mice were anesthetized with a pentobarbital (60 mg/kg, i.p.) and procaine hydrochloride (60 mg/kg, s.c.) combination. A guide cannula (RWD Life Science Co., 62003) was stereotaxically implanted with stereotaxic coordinates related to bregma (anterior-posterior  $-0.82$  mm; medial-lateral  $+1.5$  mm; dorsal-ventral  $+2.0$  mm) according to a previous study.<sup>74</sup> After surgery, animals were allowed to recover for a wk. A total of 5.0  $\mu\text{L}$  of *Cdk5* siRNA (20  $\mu\text{M}$ ) was injected over a period of 10 min into the right lateral ventricle with a microinjection pump (World Precision Instruments, Inc., UMP3-4) connected to an internal cannula via polyethylene tubing. The internal cannula was left in place for at least 2 min before being slowly withdrawn to avoid back flow. Drugs were administered 24 h later after siRNA injection.

All above animal study and procedures were approved by the institutional review board of Kunming Institute of Zoology.

#### RNA extraction, real-time quantitative PCR (RT-qPCR) assay, and western blot

Total RNA was isolated from tissues and cell lines using TRIzol (Invitrogen, 15596-018) and cDNA was synthesized by using the M-MLV Reverse Transcriptase (Promega, M170A) according to the manufacturer's instructions. The relative mRNA expression level of *Cdk5* was quantified by RT-qPCR, with normalization to the *Actb*/ $\beta$ -actin gene.

Western blot assays for the target proteins were performed using the common approach. Cell lysates of different monkey brain tissues (prefrontal cortex, parietal cortex, temporal cortex, hippocampus, substantia nigra and striatum), mouse striatum, and rat glioma C6 cells were prepared using the protein lysis buffer (Beyotime Institute of Biotechnology, P0013). Protein concentration was determined using BCA protein assay kit (Beyotime Institute of Biotechnology, P0012). A total of 25  $\mu\text{g}$  protein was separated by 15% SDS PAGE, and was transferred to a polyvinylidene difluoride membrane (Roche Diagnostics, IPVH00010). The membrane was soaked with 5% (w/v) skim milk for 2 h at room temperature. The membrane was incubated with primary antibodies against LC3B (Cell Signaling Technology, 3868; 1:1000), CDK5 (Santa Cruz Biotechnology, sc-6247; 1:1000), SQSTM1/p62 (Merck Millipore, MABC32; 1:1000), TH (Merck Millipore, AB152; 1:1000), SNCA (Merck Millipore, AB5038; 1:1000) and ACTB (Beijing Zhong Shan-Golden Bridge Biological Technology CO., LTD, TA-09; 1:1000) overnight at  $4^{\circ}\text{C}$ . The membranes were washed 3 times with TBST (Tris Buffered Saline [Cell Signaling Technology, #9997] with Tween 20 [0.1%; Sigma, P1379]), each time 5 min, followed by incubation with the peroxidase-conjugated anti-mouse (lot number 474-1806) or anti-rabbit (474-1506) IgG (1:5000; KPL) for 1 h at room temperature. The epitope was visualized using an ECL western blot detection kit (Millipore, WBKLS0500). ImageJ (National Institutes of Health, Bethesda, Maryland, USA) was used to evaluate the densitometry. Western blot for ACTB was used a loading control to measure the densitometry of CDK5, SQSTM1, TH and SNCA. The LC3B-II

densitometric signal is determined by the ratio of LC3B-II to LC3B-I before normalized to ACTB.

#### Measurement of cell death in C6 cells

C6 cells were cultured in DMEM supplemented with 5% fetal bovine serum in a humidified atmosphere incubator with 5% CO<sub>2</sub> at 37°C. After 24 h of drug (MPTP and/or melatonin) treatment, cells were harvested and washed once with PBS. Cell death was quantified by staining with propidium iodide (1 µg/ml). Briefly, cells were incubated with the dye at 37°C for 20 min, then washed twice with PBS, resuspended in PBS and kept on ice for an immediate detection on the FACScan (Becton Dickinson, USA).

#### Immunohistochemistry, confocal microscopy imaging, Golgi staining, and quantification

For immunohistochemical analysis, mice were anesthetized with pentobarbital immediately at the end of the cycle of drug treatment, and were intracardially perfused with saline, followed by 4% paraformaldehyde. The ventral midbrain was sectioned coronally at 10-µm thickness on a cryostat (Leica, CM1850UV-1-1, Amtzell, Germany). Sections were collected on slides and were incubated for 24 h with primary anti-mouse TH (1:1000; Merck Millipore, AB152) or anti-rabbit SNCA (1:1000). After 3 washes with PBST (Phosphate Buffer Saline [Beyotime Institute of Biotechnology, C0221A] with Triton<sup>TM</sup> X-100 [0.1%]), each time 5 min, sections were then incubated with a biotinylated secondary antibody (Fuzhou Maixin Biotech. Co., Ltd. SP KIT-C1) and a streptavidin horseradish peroxidase-conjugated tertiary antibody (Fuzhou Maixin Biotech. Co., Ltd., SP KIT-D1) and were visualized using a 3, 3'-diaminobenzidine/glucose oxidase reaction.<sup>75</sup> Loss of dopamine neurons was assessed by immunohistochemical analysis of TH-positive neurons in the anatomical region of the SNc of middle brain.

Cultured C6 cells were transfected with N-terminally tagged pEGFP-C1-LC3 (a kind gift from Dr. Quan Chen, Institute of Zoology, Chinese Academy of Sciences). Intact cells were imaged, using an Olympus FluoView<sup>TM</sup> 1000 confocal microscope (Olympus, Melville, NY, USA) after being treated with MPTP and/or melatonin. Primary neurons were fixed and stained with antibodies against MAP2 (Merck Millipore, AB5622; 1:500). Immunoreactivity was detected with a FITC-conjugated secondary antibody (KPL, 172-1506; 1:50) and nuclei were counterstained with DAPI and the slides were visualized under an Olympus FluoView<sup>TM</sup> 1000 confocal microscope (Olympus, America). Golgi-Cox staining

(FD NeuroTechnologies, PK401) was used to visualize neurons. Primary neurons were impregnated for 2 wk and processed according to the manufacturer's specifications. Dendrites of neurons were recognized by staining with the dendritic marker MAP2. ImageJ (National Institutes of Health, Bethesda, Maryland, USA) and Image-Pro Plus (IPP; Media Cybernetics, Inc., Washington, Maryland, USA) were used to evaluate the total dendritic length and dendritic complexity.

#### Statistical analysis

Comparisons of relative protein levels of LC3B-II, SQSTM1, CDK5, TH, and SNCA from animals of multiple groups and cells with different treatments were conducted by one-way analysis of variance (ANOVA) with the Tukey post-hoc test using the PRISM software (GraphPad Software, Inc., La Jolla, CA, USA). Differences in the animal rota rod task were determined by ANOVA, followed by LSD (Least Significant Difference) for post-hoc comparisons. Data were represented as mean ± SEM. A *P* value of <0.05 was considered to be significantly different.

#### Disclosure of Potential Conflicts of Interest

There were no potential conflicts of interest to be disclosed.

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#### Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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## Online Methods and Data Supplement

### Materials and Methods

#### Reagents, chemicals and cells

The following antibodies and chemicals were used in this study: rabbit monoclonal anti-ATG5 (Cell Signaling Technology, 8540), rabbit monoclonal anti-ATG7 (Cell Signaling Technology, 8558), Rabbit polyclonal anti-TIMM23/Tim23 (Proteintech Group, 11123-1-AP), rabbit polyclonal anti-LAMP1 (Abcam, ab24170), rabbit polyclonal anti-CTSD/cathepsin D (Proteintech, 21327-1-AP), rabbit polyclonal anti-CTSB/cathepsin B (ABZOOM, AM2528), mouse monoclonal anti-ACTB/ $\beta$ -actin (Beijing Zhong Shan-Golden Bridge Biological Technology CO., LTD , TA-09), mouse monoclonal anti-TUBB3/ $\beta$ -III tubulin (Abcam, ab7751), FITC-conjugated anti-rabbit antibody (KPL, 172-1506).

Human SH-SY5Y neuroblastoma cells were introduced from Kunming Cell Bank, Kunming Institute of Zoology. Cells were maintained in DMEM medium (Gibco-BRL, 11965-092) with 10% fetal bovine serum (Gibco-BRL, 10099-141) at 37°C in a humidified atmosphere incubator with 5% CO<sub>2</sub>.

#### Generation of *Cdk5* shRNA stable cell lines

For shRNA targeting of the rat *Cdk5* gene, the oligonucleotides were annealed and ligated into pTRIPZs according to the manufacturers' instructions (Open Biosystems, Dharmacon Research, RHS4750). The following oligonucleotides were used: *Cdk5* shRNA1:

TGCTGTTGACAGTGAGCGATTGGTGAATGTCGTGCCCAAATAGTGAAGCCA  
CAGATGTATTTGGGCACGACATTCACCAAGTGCCTACTGCCTCGGA; *Cdk5*

shRNA2:

TGCTGTTGACAGTGAGCGAAGCTACAACATCCTTGGTGAATAGTGAAGCCA  
CAGATGTATTCACCAAGGATGTTGTAGCTGTGCCTACTGCCTCGGA; *Cdk5*

shRNA3:

TGCTGTTGACAGTGAGCGACCTGAGATTGTGAAGTCACTCTAGTGAAGCC  
ACAGATGTAGAGTGAAGTTCACAATCTCAGGGTGCCTACTGCCTCGGA

(Generay Biotech Co, Ltd., Shanghai, China). All ligation plasmids were verified by DNA sequencing. The lentiviral vector carrying shRNA against rat *Cdk5* with packaging plasmid psPAX2 (Addgene, #35002) and envelop plasmid PMD2.G (Addgene, #12259) were cotransfected into a HEK293T producer cell line, and the culture supernatant was collected 48 h after transfection. The C6 cells were infected with the lentiviral supernatant in the presence of 1  $\mu$ g/ml polybrene (Sigma, H9268) for 24 h. Cells were then cultured in DMEM medium supplemented with 10% fetal bovine serum and 1  $\mu$ g/ml puromycin (Sigma, OGS541) at 37°C in a humidified atmosphere incubator with 5% CO<sub>2</sub>, and were selected for 2 weeks. The expanded cells were used for further experiments. Transduction efficiency of C6 cells in

response to doxycycline (1 µg/ml; Sigma, D9891) induction was determined by western blotting.

### **RNA extraction and real-time quantitative PCR (RT-qPCR) assay**

Total RNA was isolated from rat glioma C6 cells using TRIZOL (Invitrogen, 15596-018). 1 µg of total RNA was used to synthesize single-strand cDNA using the M-MLV Reverse Transcriptase (Promega, M170A) in a final volume of 25 µL reaction mixtures according to the manufacturer's instructions. The relative mRNA expression level of *Cdk5* was normalized by the housekeeping gene *Actb/β-actin*. The respective primer pairs of *Cdk5* and *Actb* were: *Cdk5* F: 5'-GCCAGACTATAAGCCCTAC-3'; *Cdk5* R: 5'-GGTTACACTTCAACAGGTTC-3'; *Actb* F: 5'- CAGGGTGTGATGGTGGTATG-3'; *Actb* R: 5'-GACAATGCCGTGTTCAATGG-3'. RT-qPCR was performed on the platform of iQ2 system (Bio-Rad Laboratories, Hercules, CA, USA) with SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II kit (TaKaRa, DRR081A).

### **RNA interference and transfection**

Three siRNAs targeting the mRNA expression of the *Cdk5* gene and the negative control siRNA (NC siRNA) were obtained from RiboBio (Guangzhou, China). C6 cells were cultured in DMEM medium with 10% fetal bovine serum in a humidified atmosphere incubator with 5% CO<sub>2</sub> at 37°C. In brief, cells (1×10<sup>5</sup> per well) were seeded in 12-well plates to grow to 50% confluence. Before transfection, the culture medium was removed and washed once with Opti-MEM medium (Gibco-BRL, 31985-070). *Cdk5* siRNA or NC siRNA was dissolved in Opti-MEM medium, and then mixed with 1 µL Lipofectamine<sup>™</sup> 2000 (Invitrogen, 11668-027) to achieve a final volume of 100 µL. The siRNA-Lipofectamine mixture was incubated at room temperature for 20 min before adding to each well, together with an additional 400 µL Opti-MEM medium. After a 6 h incubation in the CO<sub>2</sub> incubator, the medium was removed and 1 mL fresh medium was added to each well for growth. We optimized the siRNA concentration for transfection and the time for cell harvest by testing 5 different concentrations of siRNA (6.25 nM, 12.5 nM, 25 nM, 50 nM, and 100 nM) and an optimized 36-h time point. The efficiency of siRNA to downregulate the expression of the *Cdk5* mRNA was determined by RT-qPCR analysis.

### **Measurement of cellular reactive oxygen species in C6 cells**

C6 cells were cultured in DMEM supplemented with 5% fetal bovine serum in a humidified atmosphere incubator with 5 % CO<sub>2</sub> at 37°C. After 24 h of drug (MPTP and/or melatonin) treatment, cells were harvested and washed once with PBS. The intracellular reactive oxygen species level was measured by staining with DCFH-DA (2 µM). Briefly, cells were incubated with the dye at 37°C for 20 min, then washed twice with PBS, resuspended in PBS and kept on ice for an immediate detection on the FACScan (Becton Dickinson, USA).

### **Mitochondria isolation and measurement of mitochondrial complex I activity**

C6 cells were treated with MPTP (500  $\mu$ M) and/or melatonin (100  $\mu$ M) for 24 h. Mitochondria were isolated using the Mitochondria/cytosol Fractionation Kit (Beyotime Institute of Biotechnology, C3601) following the manufacturer's instructions. The protein concentration was determined using BCA protein assay kit (Beyotime Institute of Biotechnology, P0012). Bovine serum albumin (BSA; Beyotime Institute of Biotechnology, P0007) was used as a protein standard. The mitochondrial complex I activity was quantified in C6 cells treated with MPTP and/or melatonin by in-gel activity staining. In brief, mitochondrial proteins (400  $\mu$ g) were solubilized by Triton™ X-100 (Sigma, T8787) and protein complexes were resolved by 1D Blue Native-PAGE (Solarbio Life Science, PG20-0420) following a previously described method.<sup>1</sup>  $\beta$ -Nicotinamide adenine dinucleotide, reduced disodium salt hydrate (NADH, Sigma, N8129) and Nitrotetrazolium Blue chloride (NBT, Sigma, N6876) were used as the substrates for quantification of the complex I activity. Western blot for TIMM23 was used as a mitochondrial loading control.

### **Primary cortical neurons culture and immunofluorescence**

Mouse cortical neurons were prepared and cultured using a previously described method.<sup>2</sup> Briefly, primary cortical neurons were isolated from mouse E15 embryos and were cultured in Neurobasal® Medium supplement with 2% B27 (Gibco-BRL, 17504). Neurons were seeded in a 12-well plate with a density of  $1 \times 10^5$  on a coverslip coated with poly-L-ornithine (Sigma, P4957). After having allowed neurons to attach to the plate for 4 h, the medium was replaced with fresh medium. Neurons were cultured for 3 days, then were treated with MPTP (500  $\mu$ M) with and without melatonin (100  $\mu$ M) for 24 h followed by staining for TUBB3/ $\beta$ -III tubulin to visualize the neuronal outgrowth. After treatment, primary neurons were fixed with 4% paraformaldehyde for 30 min at room temperature, then were permeabilized with 0.4% Triton X-100 (Sigma, T8787) for 10 min. After washing with PBS, cells were blocked in 1% BSA for 1 h at 37°C, followed by incubation with an antibody against TUBB3/ $\beta$ -III tubulin (1:200; Abcam, ab7751) at 4°C overnight. Immunoreactivity was detected with FITC-conjugated secondary antibody (1:50; KPL, 172-1506) for 1 h at room temperature. Nuclei were counterstained with 1  $\mu$ g/mL DAPI (Roche Diagnostics, 10236276001) and the slides were visualized under an Olympus FluoView™ 1000 confocal microscope (Olympus, America).

### **Statistical analysis**

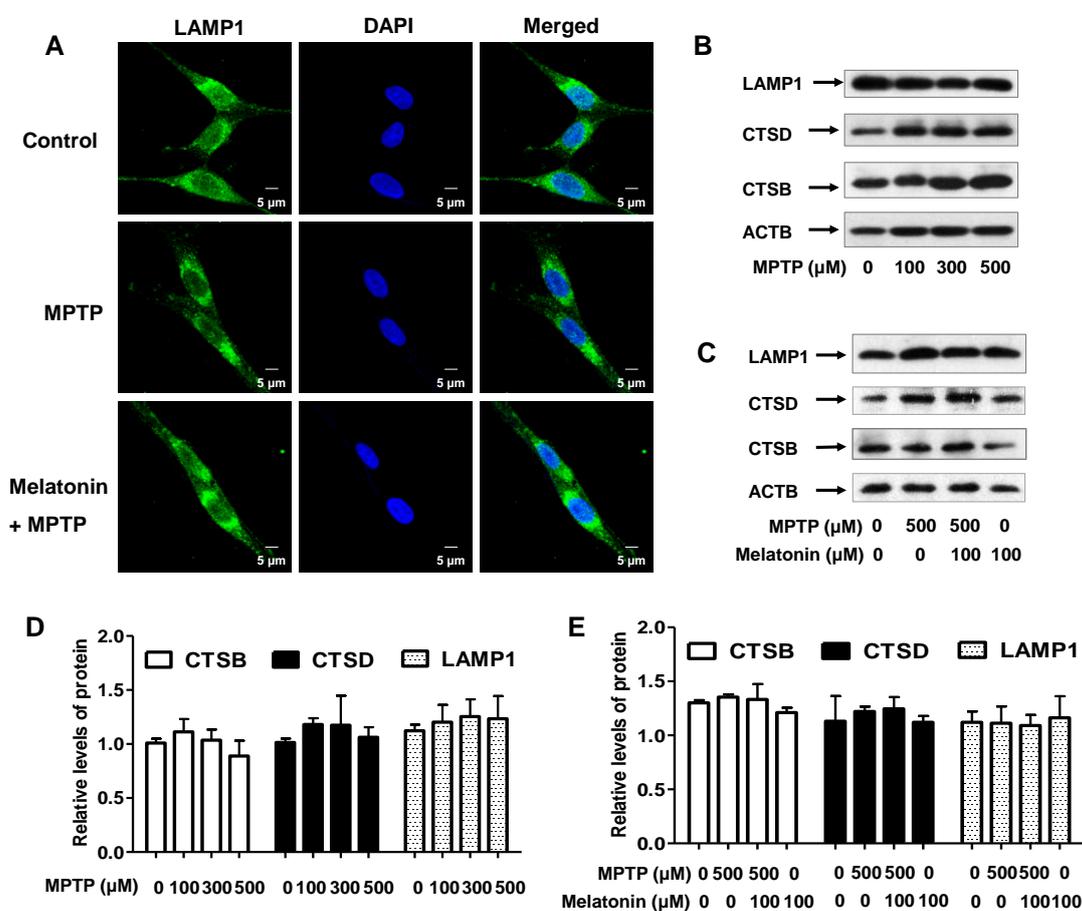
Statistical comparisons of relative protein expression levels of LC3B-II, SQSTM1, CDK5, TH and SNCA from animals of multiple groups and cells with different treatments were conducted by one-way analysis of variance (ANOVA) with the Tukey post-hoc test using the PRISM software (GraphPad Software, Inc., La Jolla, CA, USA). Data are represented as mean  $\pm$  SEM. In all cases, a *P* value of  $< 0.05$  was considered to be significantly different. All statistical analyses were two-tailed and with 95% confidence intervals (CI) using GraphPad PRISM software (GraphPad Software, Inc., La Jolla, CA, USA). Differences in animal rota-rod task were determined by ANOVA, followed by LSD (Least Significant Difference) for post-hoc

comparisons.

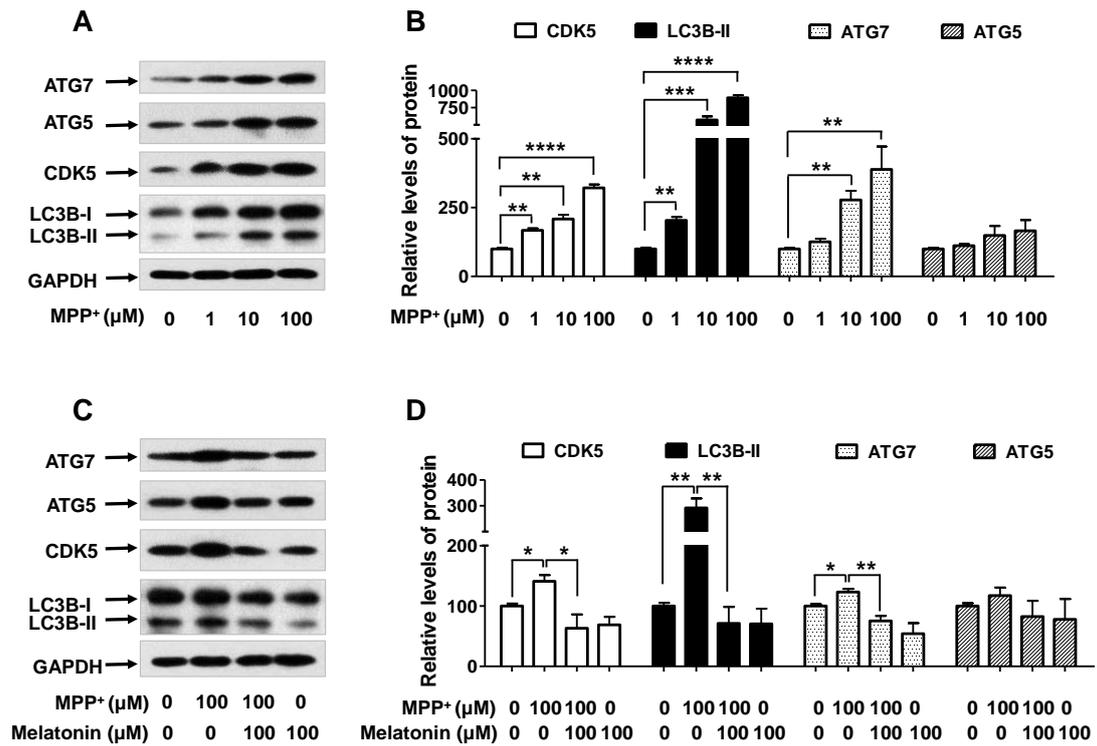
### **Supplementary References**

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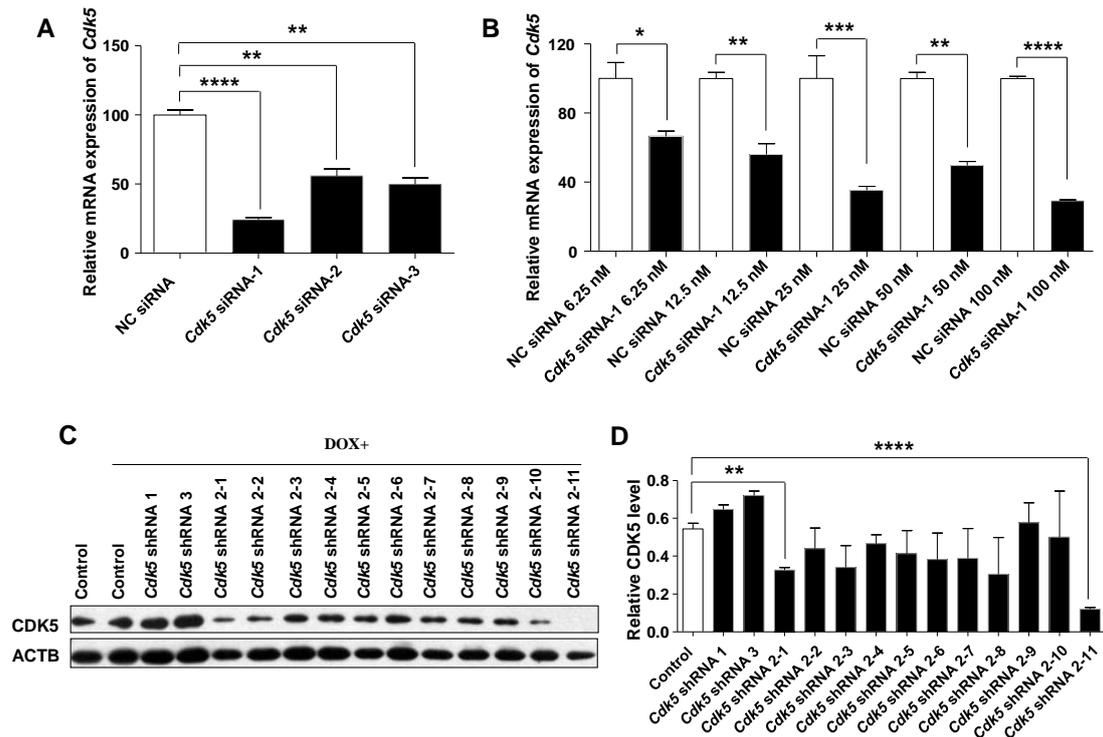
## Supplementary Figures and Figure Legends



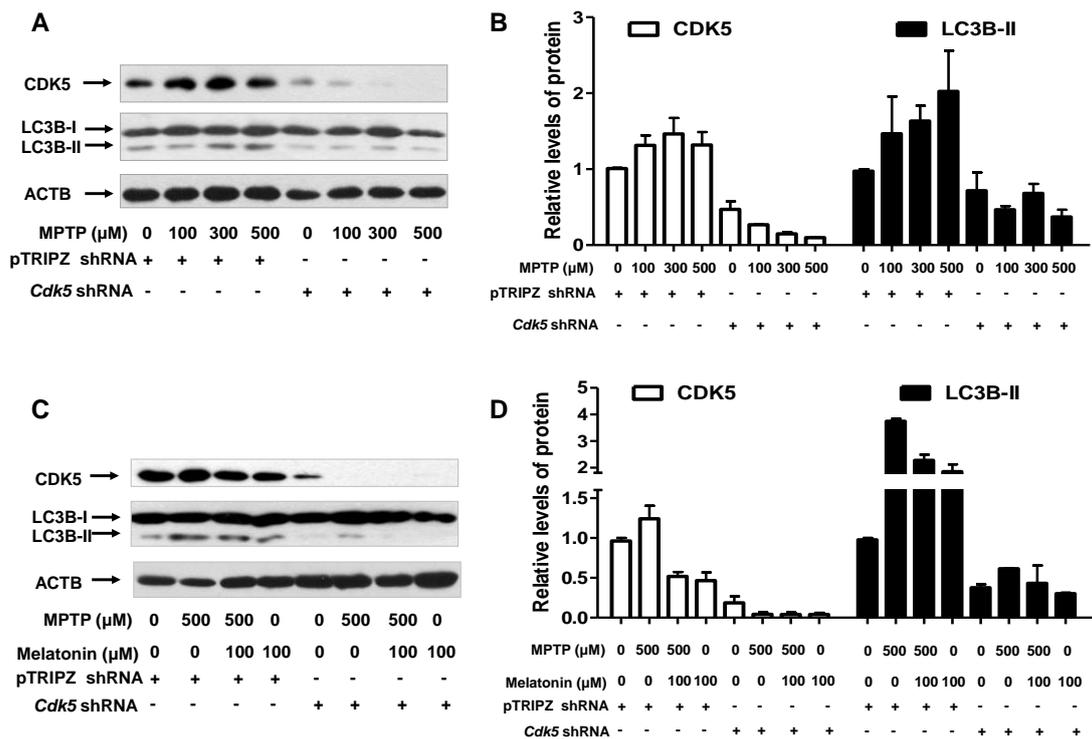
**Figure S1.** MPTP and melatonin had no effect on the lysosomal function in C6 cells. Cells were cultured in DMEM medium supplemented with 5% FBS and were treated with different concentrations of MPTP and/or melatonin for 24 h. There was no change in LAMP1 (a lysosome marker) in C6 cells treated with MPTP (500 μM) and/or melatonin (100 μM) (A). The protein levels of CTSB/cathepsin B, CTSD/cathepsin D and LAMP1 were consistent in C6 cells treated with different concentrations of MPTP (B). Melatonin had no effect on the protein levels of CTSB, CTSD and LAMP1 in C6 cells treated with and without MPTP (C). Quantifications of the protein levels are shown in (D) and (E)



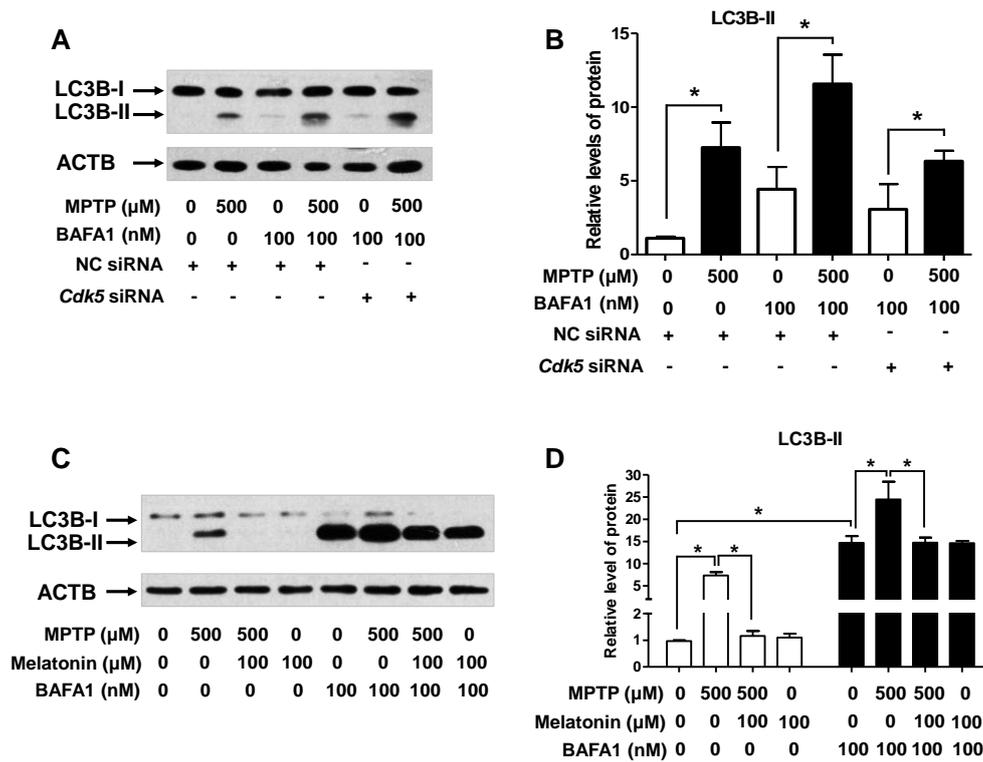
**Figure S2.** Melatonin salvages the elevated autophagy induced by MPP<sup>+</sup> in SH-SY5Y cells. Cells were cultured in DMEM-F12 medium containing 5% FBS and were treated with different concentrations of MPP<sup>+</sup> and/or melatonin for 36 h. Increased protein levels of CDK5, LC3B-II, ATG7 and ATG5 (**A and B**) were observed in SH-SY5Y cells treated with MPP<sup>+</sup>. The effect of MPP<sup>+</sup> could be prevented by melatonin (**C and D**). Relative protein abundance was normalized to GAPDH. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ ; one-way ANOVA with the Tukey post-hoc test. Bars represent mean  $\pm$  SEM. Data shown are representative of 3 experiments with similar results.



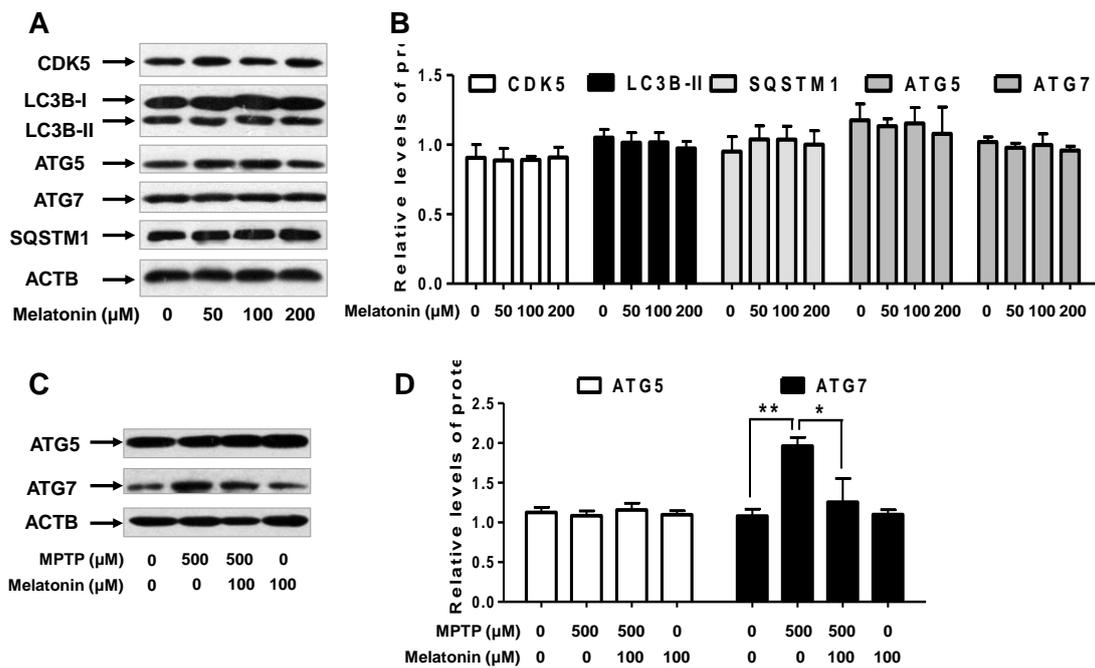
**Figure S3.** Knockdown of the *Cdk5* gene in C6 cells by using siRNA and shRNA. C6 cells were cultured in DMEM medium supplemented with 10% FBS in a 12-well plate, and were transfected with 3 siRNA oligonucleotides against the mRNA of the *Cdk5* gene for 36 hours. The *Cdk5* siRNA-1 had the most efficient inhibition effect (**A**) and was used in the following experiments, with the best-optimized concentration of 25 nM (**B**). Each treatment contains 3 repeats. For the stable expression, the oligonucleotides of the *Cdk5* gene were annealed and ligated into pTRIPZ empty lentiviral vector according to the manufacturers' instructions. The transduction efficiency of C6 cells with the doxycycline (DOX) induction was determined by western blot. C6 cells with overexpression of *Cdk5* shRNA 2-11 had the best inhibitory efficiency (**C and D**) and were used in the following experiments. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ , Student *t* test. Bars represent mean  $\pm$  SEM. A representative result of 3 independent experiments with similar results is presented.



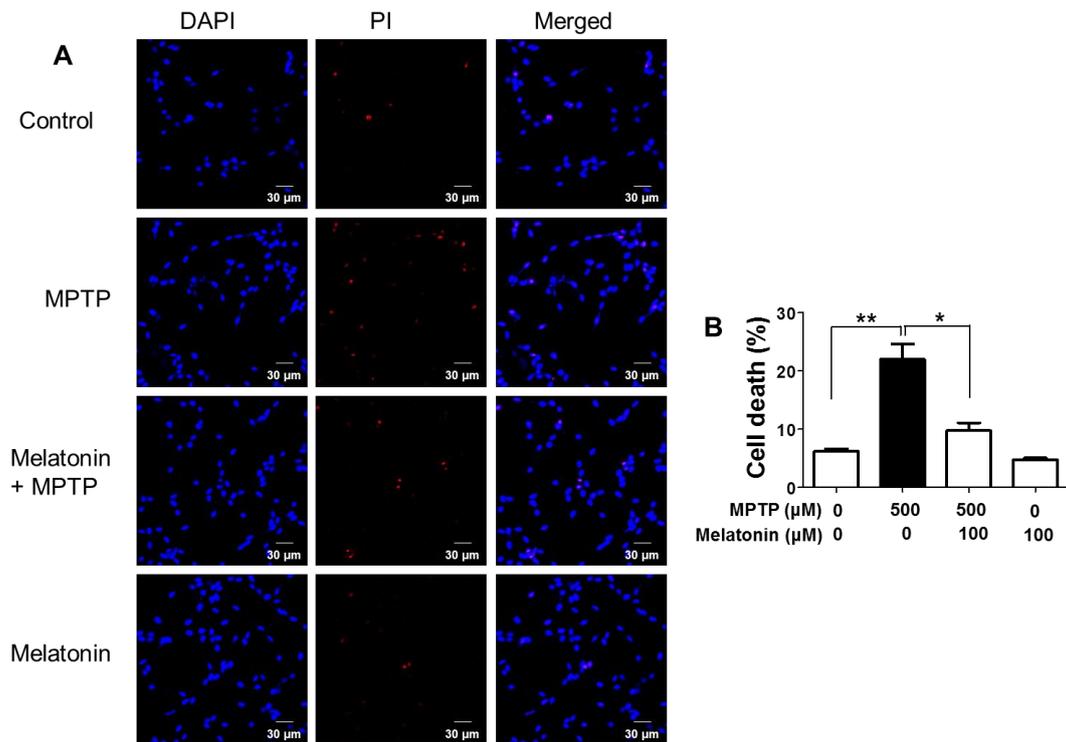
**Figure S4.** Knockdown of the *Cdk5* gene abrogated the autophagy induced by MPTP in C6 cells with overexpression of *Cdk5* shRNA 2-11. The C6 cells with stable expression of shRNA 2-11 were cultured in DMEM medium containing 10% FBS, and were treated by MPTP and/or melatonin for 24 hours. Knockdown of the *Cdk5* gene abolished the increased level of LC3B-II (**A and B**) induced by MPTP. Melatonin treatment had no effect (**C and D**) in the absence of CDK5 expression. Relative protein abundance was normalized to ACTB. Data are representative of 3 experiments with similar results.



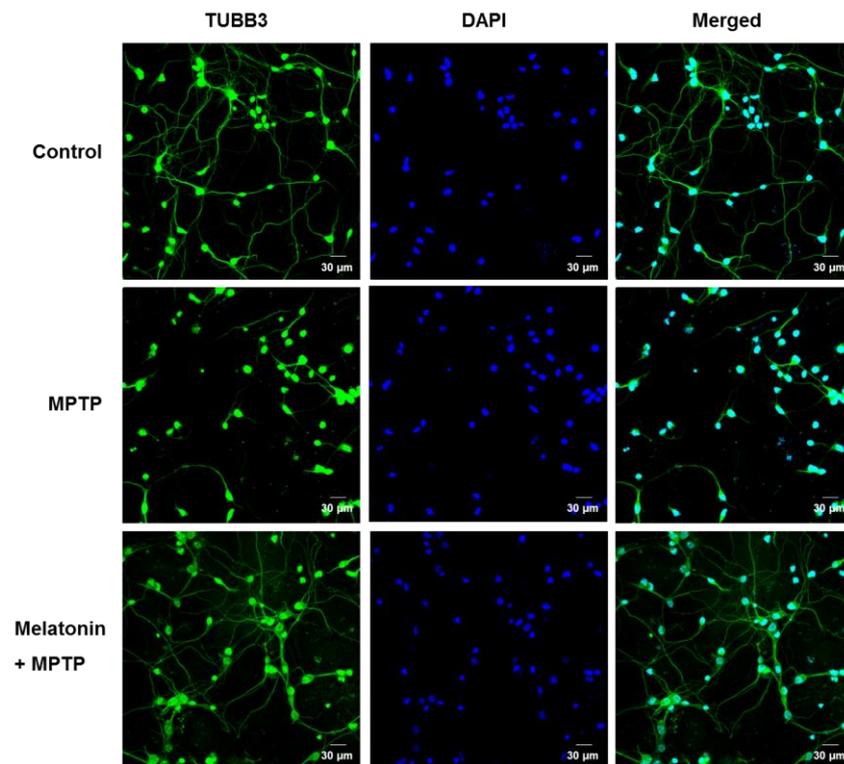
**Figure S5.** Effect of *Cdk5* siRNA and melatonin on autophagy in the presence of bafilomycin A<sub>1</sub>. MPTP induced autophagy in C6 cells in the presence of BAFA1, regardless of knockdown of CDK5 (**A and B**). Pretreatment with melatonin restored the autophagy induced by MPTP in C6 cells in the presence of BAFA1 (**C and D**). All cells received drug treatment for 24 hours before the harvest. \*  $P < 0.05$ , one-way ANOVA with the Tukey post-hoc test. Bars represent mean  $\pm$  SEM. Data are representative of 3 experiments with similar results.



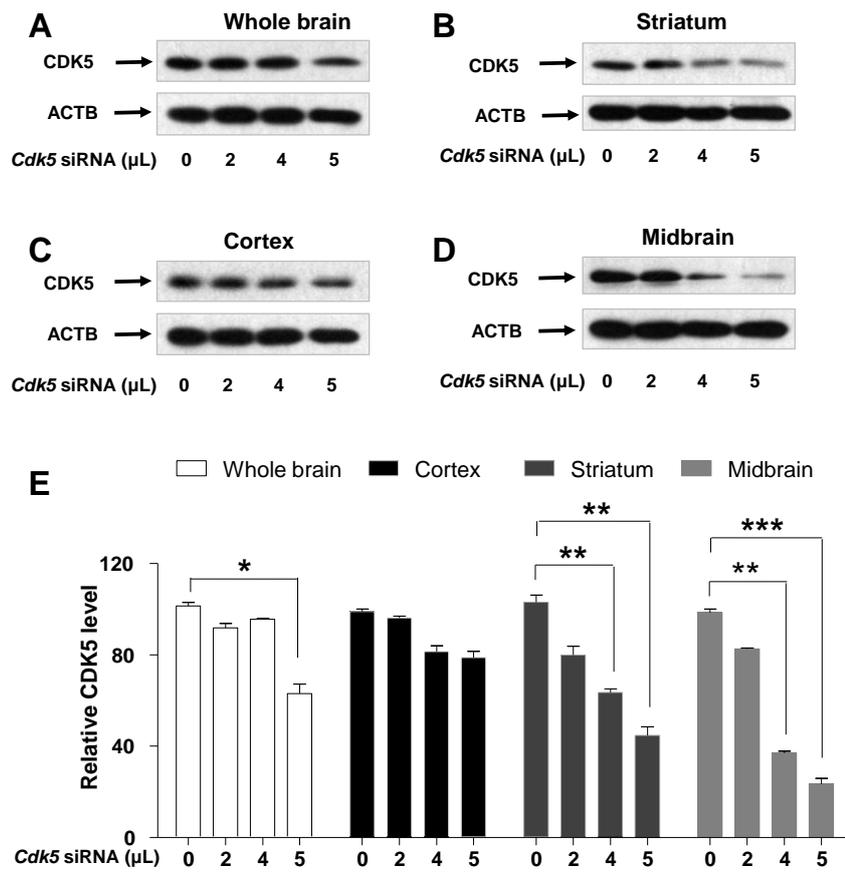
**Figure S6.** Effect of melatonin on autophagic activation and modulation of ATG7 protein levels in cultured C6 cells. The effect of melatonin on autophagic activation was demonstrated in cultured C6 cells. Melatonin had no obvious effect on regulating the levels of CDK5, LC3B-II, SQSTM1, ATG5 and ATG7 in C6 cells (**A**), as shown by the quantification of the respective protein level in C6 cells with or without melatonin treatment for 24 hours (**B**). Melatonin reversed the altered ATG7 protein level induced by MPTP. An increased protein level of ATG7 was observed in C6 cells treated with MPTP, whereas MPTP had no effect on ATG5 protein level (**C and D**). Pretreatment with melatonin restored the increased ATG7 level induced by MPTP (**C and D**). \*  $P < 0.05$ , \*\*  $P < 0.01$ , one-way ANOVA with the Tukey post-hoc test. Bars represent mean  $\pm$  SEM. Data are representative of 3 experiments with similar results.



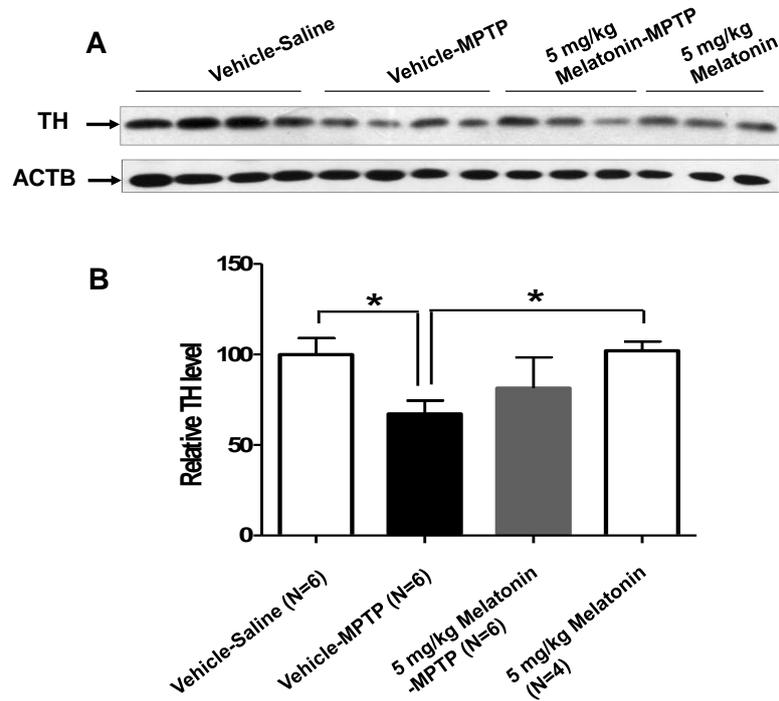
**Figure S7.** Melatonin inhibited cell death in C6 cells induced by MPTP. C6 Cells were cultured in DMEM medium containing 5% FBS and were treated with MPTP (500  $\mu$ M) and/or melatonin (100  $\mu$ M) for 24 hours. Confocal microscopy showed that MPTP induced cell death in C6 cells and this effect could be reversed by melatonin (**A and B**). \*  $P < 0.05$ , \*\*  $P < 0.01$ , one-way ANOVA with the Tukey post-hoc test. Bars represent mean  $\pm$  SEM. Data are representative of 3 experiments.



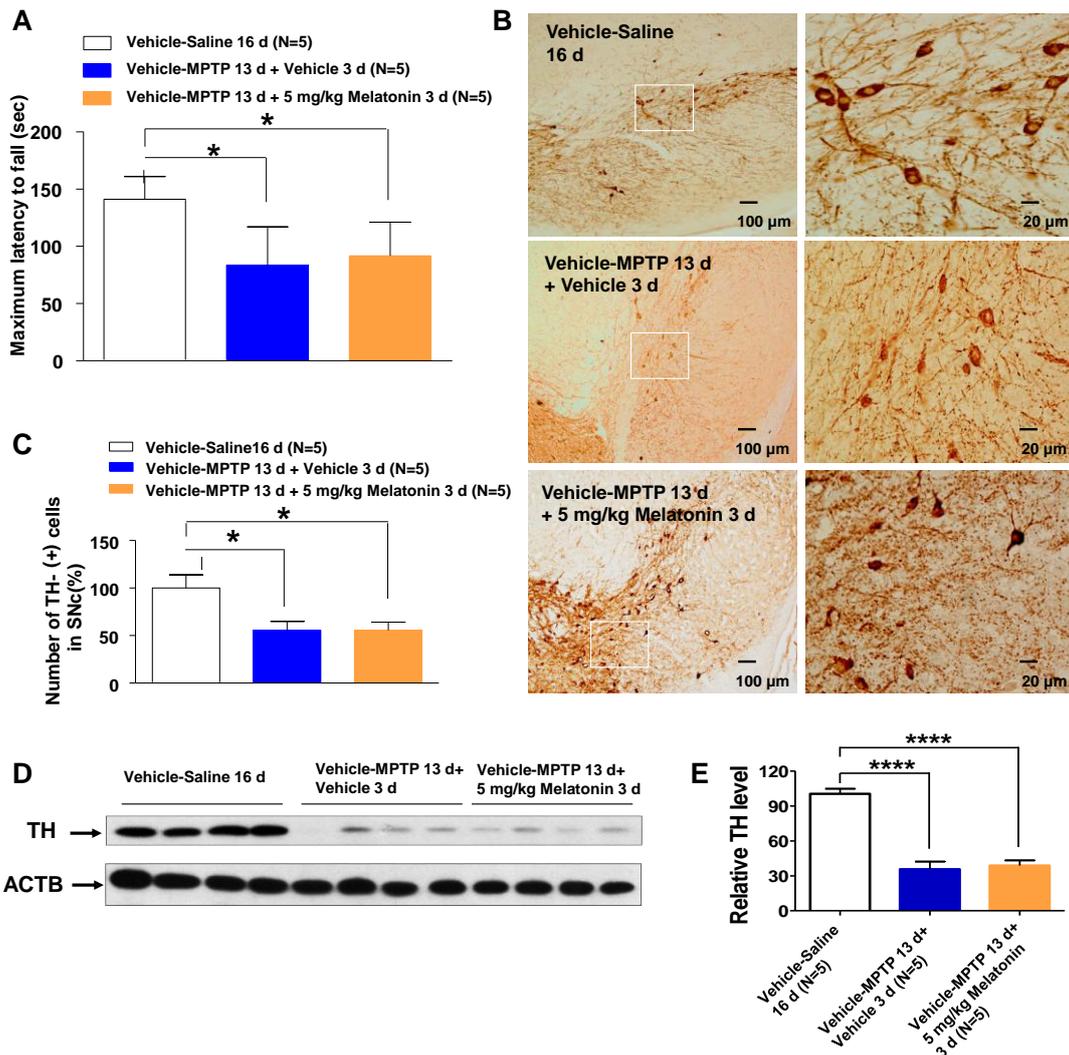
**Figure S8.** Effect of melatonin on primary neuron morphogenesis induced by MPTP. Mouse primary cortical neurons were cultured in Neurobasal® Medium containing 2% B27. Neurons were pretreated with melatonin (100  $\mu$ M) for 1 hour, followed by treatment with or without MPTP (500  $\mu$ M) for 24 hours. Upon MPTP treatment, axons and dendrites (as shown by TUBB3/ $\beta$ -III tubulin) were reduced and this change could be reversed by melatonin pretreatment.



**Figure S9.** Effect of the *Cdk5* siRNA knockdown in the brain tissues of C57BL/6 mice. After intracerebroventricular (ICV) injection of the *Cdk5* siRNA (20 μM) for 3 days, the silencing effect was tested in different brain regions, including whole brain (A, E), striatum (B, E), cortex (C, E) and midbrain (D, E). The results indicated that *Cdk5* siRNA had the most efficient inhibition effect in the midbrain (D, E). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; one-way ANOVA with the Tukey post-hoc test. Bars represent mean  $\pm$  SEM. Each concentration group had 2 animals.

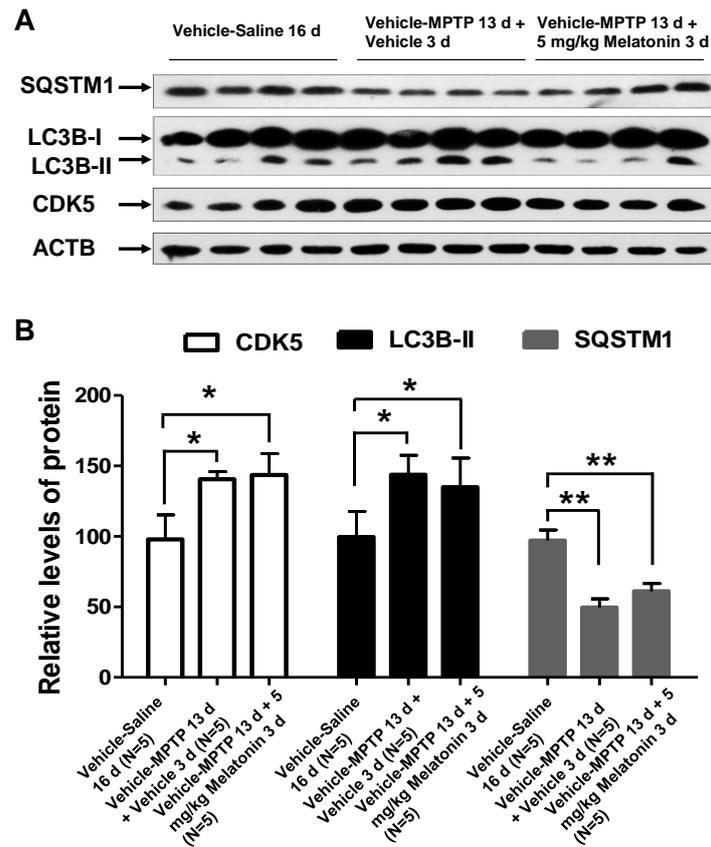


**Figure S10.** Knockdown of CDK5 abrogated the protective effect of melatonin on TH level in the striatum of mice with MPTP treatment. MPTP decreased the protein level of TH in the striatum of mice with *Cdk5* siRNA injection (Vehicle-MPTP group) compared to the control group (Vehicle-Saline group). Pretreatment with melatonin had no significant salvaging effect on the level of TH in these mice (5 mg/kg Melatonin-MPTP group) (A and B). For the procedures describing animal treatments, please refer to Fig. 5A. Mice with *Cdk5* siRNA injection were divided into 4 groups and received different drug treatments. \*,  $P < 0.05$ ; one-way ANOVA with the Tukey post-hoc test. Bars represent mean  $\pm$  SEM.

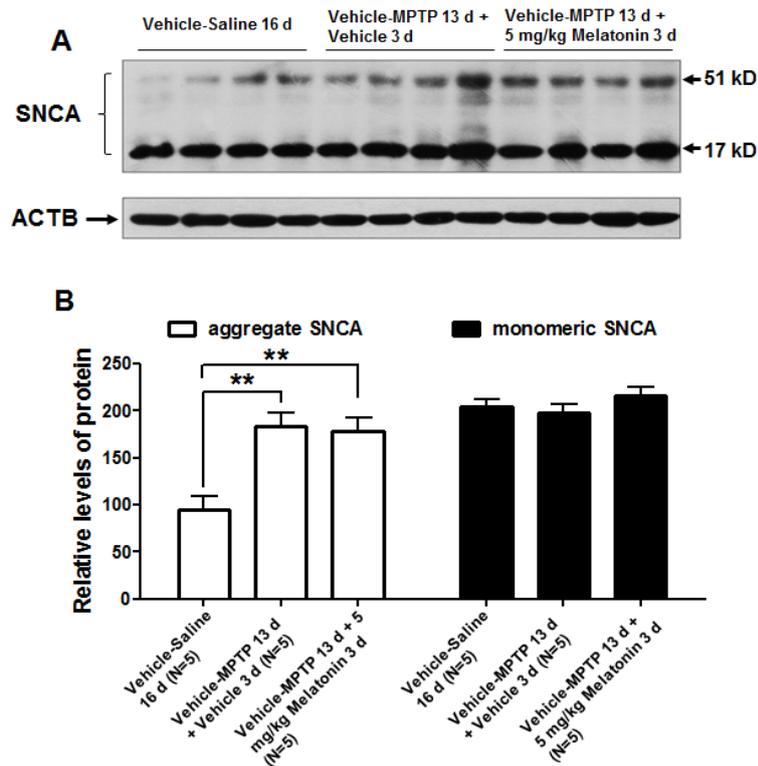


**Figure S11.** Melatonin had no salvaging effect on behavioral bradykinesia and dopaminergic neuronal loss in mouse model of PD after the animals presented PD symptoms. After treatment with MPTP (30 mg/kg once per day for 2 weeks; Vehicle-MPTP 13 d + Vehicle 3 d group), mice exhibited significantly decreased retention time in the rotarod test compared to the control mice (Vehicle-Saline 16 d group). Injection of melatonin (5 mg/kg once per day for 3 days from day 14 to day 16; Vehicle-MPTP 13 d + 5 mg/kg Melatonin 3 d group) could not improve the retention time in this mouse model of PD (A). Compared to control mice (Vehicle-Saline 16 d), the TH-positive cell number and dendrites of DA neurons were reduced in the SNc central region of mice receiving MPTP treatment (Vehicle-MPTP 13 d + Vehicle 3 d group and Vehicle-MPTP 13 d + 5 mg Melatonin 3 d group) (B and C). Melatonin treatment (5 mg/kg) could not salvage the reduction of TH-positive cells and dendrites of DA neurons in mouse model of PD (B). The differences of TH-positive cell numbers between the control group (Vehicle-Saline 16d) and the MPTP group (Vehicle-MPTP 13 d + Vehicle 3 d group), and between the

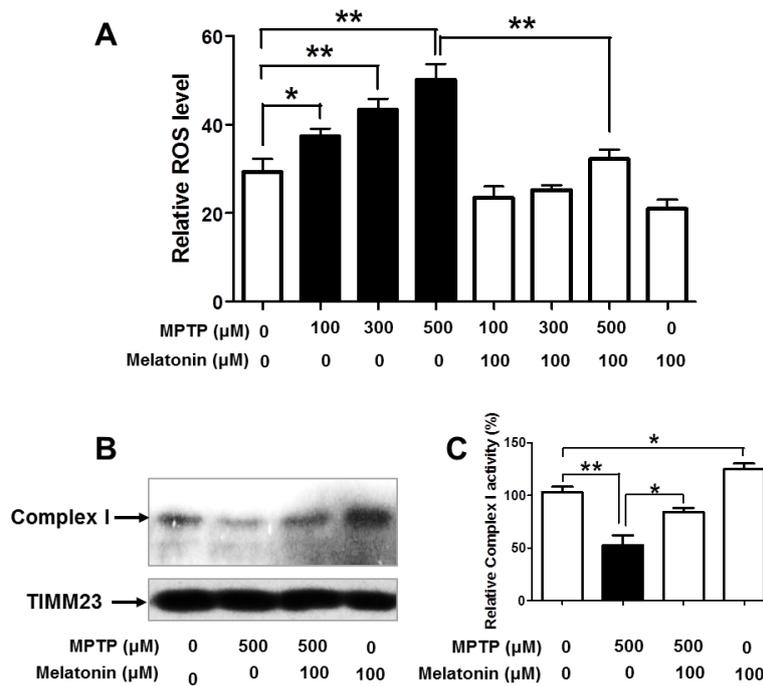
control group (Vehicle-Saline 16 d) and MPTP plus 5 mg/kg melatonin group (Vehicle-MPTP 13 d+5 mg/kg Melatonin 3 d group) were statistically significant (**C**). Melatonin treatment after MPTP withdrawal could not rescue the decreased level of TH (**D and E**). \*  $P < 0.05$ , \*\*\*\*  $P < 0.0001$ , one-way ANOVA with the Tukey post-hoc test. Bars represent mean  $\pm$  SEM.



**Figure S12.** Melatonin had no rescuing effect on the autophagy induced by MPTP in the mouse model of PD after the animals presented PD symptoms. Protein homogenates from mouse striatum were analyzed by western blot. Melatonin treatment after MPTP withdrawal (Vehicle-MPTP 13 d + 5 mg/kg Melatonin 3 d group) could not significantly restored the elevated protein level of CDK5, LC3B-II, and the decreased protein level of SQSTM1 (**A and B**) in mouse model of PD (Vehicle-MPTP 13d + Vehicle 3 d group). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; one-way ANOVA with the Tukey post-hoc test. Bars represent mean  $\pm$  SEM.



**Figure S13.** Melatonin had no inhibiting effect on SNCA aggregation induced by MPTP in the mouse model of PD after the animals presented PD symptoms. Protein homogenates from mouse striatum and brain tissues of monkey were analyzed by western blot. Melatonin treatment after MPTP withdrawal could not reverse SNCA aggregation (**A and B**) in the mouse model of PD. Treatment of MPTP or MPTP and melatonin had no effect on SNCA monomer levels in the striatum of mice (**A and B**). \*\*,  $P < 0.01$ ; one-way ANOVA with the Tukey post-hoc test. Bars represent mean  $\pm$  SEM.



**Figure S14.** Melatonin salvaged the elevated reactive oxygen species (ROS) and mitochondrial complex I activity induced by MPTP in C6 cells. Cells were cultured in DMEM medium supplemented with 5% FBS and were treated with different concentrations of MPTP and/or melatonin for 24 hours. MPTP treatment increased cellular ROS in a dose-dependent manner (**A**). The mitochondrial complex I activity in C6 cells treated with MPTP (and/or melatonin) was measured by in-gel activity staining. We used TIMM23 as a mitochondrial loading control (**B**). Pretreatment with melatonin restored the increased ROS level (**A**) and decreased mitochondrial complex I activity induced by MPTP. Melatonin alone could increase the complex I activity (**B to C**). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; one-way ANOVA with the Tukey post-hoc test. Bars represent mean  $\pm$  SEM.