Activation of AMPK/mTORC1-mediated autophagy by metformin reverses Clk1 deficiency - sensitized dopaminergic neuronal death

Qiuting Yan, Chaojun Han, Guanghui Wang, John L. Waddington, Longtai Zheng, Xuechu Zhen

Jiangsu Key Laboratory of Translational Research and Therapy for Neuropsychiatric Diseases and College of Pharmaceutical Sciences, Soochow University, Suzhou, Jiangsu 215021, China (QY, CH, GW, JLW, LZ, XZ); College of Pharmaceutical Sciences and the Collaborative Innovation Center for Brain Science, Soochow University, Suzhou, Jiangsu 215021, China (QY, CH, GW, LZ and XZ); Molecular and Cellular Therapeutics, Royal College of Surgeons in Ireland, Dublin 2, Ireland (JLW).
Running title: Clk1 deficiency inhibits autophagy in Parkinson’s disease

Corresponding author:
Xuechu Zhen, Jiangsu Key Laboratory of Translational Research and Therapy for Neuropsychiatric Diseases and College of Pharmaceutical Sciences, Soochow University, Suzhou, Jiangsu 215021, China, Tel: +86 512 6588 0369, fax: +86 512 6588 2089, e-mail: zhenxuechu@suda.edu.cn

Manuscript Info
Text Pages: 45
Figure 1-8
References: 47
Number of words in the –
Abstract: 207
Introduction: 641
Discussion: 818

Abbreviations:
PD, Parkinson’s disease; ALP, autophagy-lysosome pathway; AMPK, AMP-activated protein kinase; mTORC1, rapamycin complex 1; TFEB, transcription factor EB; SNc, substantia nigra pars compacta; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; WB, Western blotting; SDS-PAGE, sulfate polyacrylamide gel electrophoresis; q-PCR, real-time quantitative PCR.
Abstract

The autophagy-lysosome pathway (ALP) plays a critical role in the pathology of Parkinson’s disease (PD). Clk1 (coq7) is a mitochondrial hydroxylase that is essential for Coenzyme Q (ubiquinone) biosynthesis. We have reported previously that Clk1 regulates microglia activation via modulating microglial metabolic reprogramming, which contributes to dopaminergic neuronal survival. This study explores the direct effect of Clk1 on dopaminergic neuronal survival. We demonstrate that Clk1 deficiency inhibited dopaminergic neuronal autophagy in cultured MN9D dopaminergic neurons and in SNc of Clk1+/− mutant mice and consequently sensitized dopaminergic neuron damage and behavioral defects. These mechanistic studies impair that Clk1 regulates the AMPK/mTORC1 pathway, which in turn impairs the autophagy-lysosome pathway and TFEB nuclear translocation. As a result, Clk1 deficiency promotes dopaminergic neuronal damage in vivo and in vitro that ultimately contributes to sensitize MPTP-induced dopaminergic neuronal death and behavioral impairments in Clk1-deficient mice. Moreover, we found that activation of autophagy by the AMPK activator metformin increases dopaminergic neuronal survival in vitro and in the MPTP-induced PD model in Clk1 mutant mice. These results reveal that Clk1 plays a direct role in dopaminergic neuronal survival via regulating autophagy-lysosome pathways that may contribute to the pathological development of PD. Modulation of Clk1 activity may represent a potential therapeutic target for PD.
Introduction

Parkinson’s disease (PD) is a common aging-related neurodegenerative disease with progressive loss of dopaminergic neuron in the substantia nigra pars compacta (SNc) (Savitt et al., 2006; Ye et al., 2013; Kalia and Lang, 2015). Although neuroinflammation, oxidative stress and mitochondrial dysfunction are believed to contribute to the damage of dopaminergic neurons, the precise pathological mechanism remain unknown (Dias et al., 2013; Camilleri and Vassallo, 2014; Menzies et al., 2015; Moon and Paek, 2015; Morris and Berk, 2015; Segura-Aguilar and Kostrzewa, 2015). As a mitochondrial hydroxylase responsible for Coenzyme Q (ubiquinone) biosynthesis, Clk1 (coq7) plays an important role in electron transference in the mitochondrial respiratory chain (Nakai et al., 2001; Lapointe and Hekimi, 2008). Clk1+/− mutant mice exhibit a series of changes in mitochondrial metabolism such as reduced mitochondrial oxygen consumption, reduced electron transport and mitochondrial ATP synthesis (Hekimi, 2013). Moreover, Clk1 deficiency induces apoptosis associated with mitochondrial dysfunction, which may lead to embryonic lethality in mice around E10.5 (Takahashi et al., 2008). We reported recently that Clk1 deficiency sensitizes microglia-mediated neuroinflammation by altering metabolic reprogramming in microglial cells and subsequently increasing dopaminergic neuronal death induced by MPTP treatment, suggesting that Clk1 plays an important role in the survival of dopaminergic neurons via modulating microglia activation (Gu et al., 2017). However, the direct functional role of Clk1 in dopaminergic neurons remains unknown.
The autophagy-lysosome pathway (ALP) is a critical cellular quality control system that involves degradation of dysfunctional cellular components and organelles. Altered ALP is known to be associated with the pathological development of various neurodegenerative diseases including PD (Maiuri et al., 2007). Enhancement of autophagy with over-expression of beclin1 effectively reduced the accumulation of α-synuclein and protected neurons in an animal model of PD (Spencer et al., 2009). Furthermore, deletion of genes essential for autophagy, such as ATG7, resulted in PD-like neurodegeneration in mice (Komatsu et al., 2006). Moreover, MPTP treatment leads to defective autophagosomal clearance due to impairment of lysosomal function in a model of PD (Dehay et al., 2010). Therefore, ALP is essential for the survival of neuronal cells in response to an increased burden of misfolded protein or neurotoxicity (Decressac et al., 2013).

An *in vivo* study with CCI-779, a derivative of rapamycin, reduced the accumulation of α-synuclein by activation of autophagy, indicating that mTOR pathway plays a important role in the pathological development of neurodegenerative diseases (Ravikumar et al., 2004; Malagelada et al., 2006; Santini et al., 2009; Tain et al., 2009; Bar-Peled and Sabatini, 2014). AMP-activated protein kinase (AMPK) is a upstream of target of rapamycin complex 1 (mTORC1), and activation of AMPK inhibits mTORC1 activity, thereby promoting autophagy (Choi et al., 2010). Furthermore, the AMPK activator metformin protects dopaminergic neurons in SNC in a mouse PD model via enhancement of AMPK-mediated autophagy, suggesting that the AMPK signaling pathway may constitute a potential target for PD therapy.
(Sardiello et al., 2009; Wu et al., 2011). Recently, it has been shown that mTORC1 is a key regulator of the cellular localization and activity of transcription factor EB (TFEB) (Wong and Cuervo, 2010; Martina et al., 2012; Roczniak-Ferguson et al., 2012; Settembre et al., 2012; Martina and Puertollano, 2013). TFEB is a major transcriptional regulator of the ALP pathway by regulating the expression of autophagic gene products such as ATG5, Beclin-1 and ATG9B, lysosomal gene products such as LAMP1, and cathepsins (Pena-Llopis et al., 2011; Settembre et al., 2011). Altered TFEB function is involved in neurodegenerative diseases through regulating cargo recognition, autophagosome-lysosome fusion, and TFEB nuclear localization in ALP (Decressac et al., 2013; Chua et al., 2014; Cortes et al., 2014).

The present study is designed to investigate the direct functional role of Clk1 in the regulation of dopaminergic neurons. We found that loss of Clk1 strongly increased MPTP neurotoxicity and inhibited autophagy through the AMPK/mTORC1 pathway. Our data reveal a novel mechanism of Clk1-regulated dopaminergic neuronal survival.
Materials and Methods

Materials. Cell culture reagents were purchased from Hyclone (Thermo, MA, USA). MPP⁺, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), bafilomycin A1, puromycin, rapamycin, and MTT assay reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Metformin was purchased from Beyotime (Shanghai, China). Quantitative reverse-transcription polymerase chain reaction (q-PCR) primers were synthesized by Sangon Biotech (Shanghai, China). Antibodies to P62, phosphor-AMPK (T172), AMPK, phosphor-mTOR (S2448), mTOR, phosphor-p70s6k (T389) and p70s6k were from Cell Signaling Technology (Danvers, MA, USA). Antibodies for LC3 and LAMP1 were purchased from Abcam (Cambridge, MA, UK). Antibodies for Actin were purchased from Sigma (Sigma-Aldrich, USA). The antibody for TH was from Millipore (MA, USA) and the antibody for Clk1 was from Proteintech Technology (MO, USA). All drugs were freshly prepared before each experiment. MPTP was dissolved in saline. MPP⁺ was dissolved in PBS and added to cells for a final concentration of 250 µmol/L. Metformin was dissolved in saline for injection into mice at 50 mg/kg. For cell culture, metformin was prepared in PBS at 1 mol/L stock solution and kept at -20°C; the stock solution was diluted with cell culture medium to a designated concentration of 2 mmol/L. Rapamycin was dissolved using DMSO to a 100 mmol/L stock solution and kept at -20°C; the stock solution was diluted with cell culture medium to a designated concentration of 200 nmol/L. The final concentration of DMSO was no more than 0.1%.
Animals. Wild type and Clk1\(^{+/-}\) mutant mice were obtained from Rugen Therapeutics (Suzhou, China). Mice were maintained in plastic cages with free access to food and water in SPF conditions (temperature: 21±1°C; air exchange per 20 min; 12 h/12 h light-dark cycle). Animals were allowed free access to a standard laboratory diet and water. All animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee of Soochow University and were in compliance with the Guidelines for the Care and Use of Laboratory Animals (Chinese National Research Council, 2006).

Cell culture. Murine dopaminergic MN9D cells (Choi et al., 1992) were cultured in Dulbecco’s modified Eagle’s medium (Gibco, 12430-054) containing 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, 15140-122) at 37°C under 5% CO\(_2\) air environment. MN9D cells were grown until 70-80% confluent before further treatment in all experiments.

Plasmid and lentivirus (LV). EGFP-N3-tagged TFEB plasmid was provided by Dr. Guanghui Wang. LV gene transfer vectors encoding shClk1 (LV2-shClk1, 5’-TGCCTTTGTTGAAGAGGATTAT-3’) and scrambled shRNA used as a negative control (LV2-NC, 5’-TTCTCCGAACGTGTCACGTTTC-3’) were synthesized by GenePharma (Shanghai, China). The titer of lentivirus used was \(\geq 3\times10^8\) units. Polybrene was employed to promote the transduction of lentivirus and puromycin was applied to select successfully infected cells. After LV2-shClk1 was stably expressed, total RNA and proteins were extracted from MN9D cells to confirm the knockdown efficiency. For Clk1 over-expression assays and EGFP-TFEB assays, cells were
transfected with plasmids using Lipofectamine 2000 (Invitrogen, USA) according to manufacturer’s instructions; total RNA and proteins were isolated from MN9D cells.

**Cell viability assays.** Cell viability was determined by the MTT assay. Briefly, MN9D cells were seeded in 96-well plates to a final density of $2 \times 10^4$ cells per well. After treatment with indicated drugs for 24 h, 30 μL of MTT solution (0.5 mg/mL) was added to each well. The plates were incubated at 37°C for 2 h in the dark. Then, after addition of 100 μL DMSO, the absorbance was read at 570 nm using a spectrophotometer.

**Immunofluorescence.** MN9D cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Then, the cells were incubated with blocking solution containing 3% BSA, 0.3% Triton-X 100 in PBS for 1 h, followed by incubation overnight at 4°C with primary antibodies anti-LC3 (1:500). Subsequently, cells were washed 3 times with PBS and subjected to incubation with secondary fluorescent antibodies (1:400) for 2 h at room temperature. After 3 washes with PBS, samples were dyed with DAPI for 30 min at 37°C, followed by 3 more washes. Confocal microscopy (Carl Zeiss, Germany) was applied to the resultant images.

**Western blotting (WB).** Cells or tissues were lysed in RIPA buffer (Cell Signaling Technology, USA) on ice and incubated at 95°C for 10 min. Nuclear protein extraction was performed according to manufacturer’s instructions (Thermo, MA, USA). Protein was quantified using a bicinchoninic acid protein assay kit (Thermo,
USA). Denatured protein was loaded into sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride membrane (Millipore, USA). After blocking with 5% milk for 2 h, the membranes were incubated overnight at 4°C with primary antibodies against, respectively: Clk1 (1:1000), TH (1:1000), LC3 (1:3000), P62 (1:1000), LAMP1 (1:1000), p-AMPK (1:1000), AMPK (1:1000), p-mTOR (1:1000), mTOR (1:1000), p-p70s6k (1:1000), p70s6k (1:1000) or internal reference antibodies Actin (1:10000). Then, the membranes were washed 3 times in TBST (TBS containing 0.1% Tween 20) and subjected to incubation with anti-rabbit or anti-mouse IgG polyclonal secondary antibodies (Sigma-Aldrich, USA). Following incubation with enhanced chemiluminescence (Millipore, USA), the band was determined with a ChemiScope 3300 Mini (CLINX, Shanghai, China).

**RNA isolation and real-time quantitative PCR (q-PCR).** Total RNA was isolated by TRIZOL reagent (Takara, Shiga, Japan) according to manufacturer’s instructions. After assessment with NanoDrop (ND-2000C, Thermo Scientific, Waltham, MA, USA), cDNA was reverse-synthesized from 1000 ng RNA using oligo (dT), RNase free water, 10Mm dNTP, M-MLV buffer, M-MLV and recombinant RNase inhibitor (Takara, Shiga, Japan). Quantitative assay of mRNA was performed using SYBR Green PCR master mix (Takara, Shiga, Japan) in a 7500 real-time PCR system (Applied Biosystems). The cycling parameters were as follows: 50°C, 2min; 95°C, 10min; 95°C, 10min; 95°C, 15 s; 40 cycles; 60°C, 1 min. The primer sequences for each gene are listed as follows: GAPDH, forward primer: 5'-
GACAAGCTTCCGTTTCAG-3', reverse primer: 5'-
GACTCAACGGATTTGGTCTG-3'; Clk1, forward primer:
5'-GATTGCATTCAAGGGTGCGAC-3', reverse primer:
5'-TTCCCATCAGCATGCCAGATC-3', ATP6v1h, forward primer: 5'-
CATTGCAAGGTGCTGTGATG-3', reverse primer: 5'-
TGCTTGTCTCCTGAAACTTCT-3'; CTSA, forward primer: 5'-
TCCAGATGAACCTTCAAG-3', reverse primer: 5'-
AGTAGGCAAAGTAGACCGGG-3'; CTSD, forward primer: 5'-
TGCTCAAGAATACATGGACGC-3', reverse primer: 5'-
CGAAGACGACTGTGAAGACT-3'; CTSF, forward primer: 5'-
AGAGAGGCCCAATCTCCGT-3', reverse primer: 5'-
GCATGGTCATGAGGCAAAGG-3'; HEXA, forward primer: 5'-
ACGTCCCTTTACCCGAACACT-3', reverse primer: 5'-
CGAAAAGGCAGGTCAAGCGATAG-3'; LAMP1, forward primer: 5'-
CAGATGTTTAGTGCAACCCA-3', reverse primer: 5'-
TTGAAAAGGTACGGCTTGATG-3'; ATG5, forward primer: 5'-
TGGATGGGACTGCGAATGA-3', reverse primer: 5'-
GATCTCAAGTGTGTCAGGC-3'; ATG9B, forward primer: 5'-
TGGCATCACATCCAGA-3', reverse primer: 5'-
CATTGTAATCCACGCAGCGA-3. Gene expression was normalized to GAPDH in each sample.

**ADP/ATP Ratio Assay.** ADP/ATP ratio was measured using an ADP/ATP Assay
Kit (Sigma-Aldrich, USA) according user instructions. Cells were seeded in a 96-well plate to a final density of $10^3$-$10^4$ cells per well. ATP reagent was prepared with assay buffer 95 μL, substrate 1 μL, co-substrate 1 μL and ATP enzyme 1 μL; 90 μL of ATP reagent was added to each well and the plate tapped briefly to mix. The plate was incubated for 1 min at room temperature. Luminescence activities were read and determined using a luminescence Reporter Assay System (Promega, USA) for the ATP assay (RLU$_A$). ADP reagent was prepared with 5 mL water and 1 μL ADP enzyme. After incubation for 10 min, luminescence was read as for ATP (RLU$_B$). Immediately following the reading of RLU$_B$, 5 mL ADP Reagent was added to each well and mixed by tapping the plate or pipetting. After 1 min, luminescence was read as above (RLU$_C$). ADP/ATP ratio was calculated using the formula ADP/ATP ratio = RLU$_C$ – RLU$_B$ / RLU$_A$.

**MPTP administration.** MPTP-induced mouse model of PD was prepared according to our previously described procedures (Ren et al., 2016; Gu et al., 2017), in which mice received intraperitoneal (i.p.) injections of 25 mg/kg free base MPTP or saline for 7 consecutive days. Briefly, wild type and Clk1 +/- mutant mice were divided into three treatment groups: (1) saline; (2) MPTP (25 mg/kg); (3) metformin (50 mg/kg) + MPTP (25 mg/kg). For the metformin-treated group, metformin (50 mg/kg, i.p.) was given 3 days prior to and during each of the subsequent 7 daily MPTP injections. On day 11, behavioral tests were conducted. After the behavioral tests, mice were sacrificed and brain tissue prepared for immunohistochemistry or western blotting.
Rotarod test. One day before testing, mice were trained until they could remain on the rotarod (UGO Basile, Italy) for 120 s without falling. During testing, mice were placed on the rotarod and the rotation speed increased from 5 to 40 rev/min during 5 min. Latency (time) to fall from the rotarod was automatically recorded. Each mouse underwent three independent trials with a 20 min inter-trial interval, with latency to fall calculated as the average of the three trials (Ren et al., 2016).

Pole test. Mice were placed head down on the top of a vertical wooden pole (60 cm in length, 2 cm in diameter) with a rough surface. Latency for the mice to climb down from the top of the pole to the base was measured. Trials were considered a failure if the mouse jumped or slid down the pole. Each mouse underwent three trials, with latency to climb down calculated as the average of the three trials (Ren et al., 2016).

Immunohistochemistry. Following behavioral tests, animals were anesthetized with chloral hydrate (10%, i.p.) and brain tissue prepared as described previously. A freezing microtome was used to cut the midbrain into 20 μm serial sections containing the SNc for subsequent immunofluorescence staining. For staining, the midbrain sections were rinsed three times with 0.01 mol/L PBS and incubated in PBS containing 3% BSA and 0.3% Triton for 2 h at room temperature. The sections were washed in PBS and then incubated with anti-TH antibody (1:400) at 4°C for 24 h. After washing with PBST (PBS containing 0.3% Triton), secondary fluorescent antibodies (1:400) were added for 2 h. The slides were then observed using a confocal microscope (Carl Zeiss, Germany).
**Statistical analysis.** Data were expressed as means ± SEM and analyzed using GraphPad Prism software (version 6.0). One-way ANOVAs or two-way ANOVAs followed by Bonferroni post hoc tests were utilized for multiple-group comparisons. Student’s t-test was used for comparisons between two groups. $P<0.05$ was considered statistically significant. If two or three measurements were conducted from same sample, $p<0.025$, $p<0.0167$, respectively, was considered significant. In some experiments, the multiple measurements were actually from different sets of cells or tissues (i.e., not from same samples), in this case, $p<0.05$ was considered statistically significance.
Results

Clk1 deficiency promotes MPP⁺-induced dopaminergic neuronal death in MN9D cells. Recently, we reported that Clk deficiency in microglia cells sensitized dopaminergic neuron toxicity in response to MPP⁺ treatment in a neuroglia co-culture system (Gu et al., 2017). To explore the direct effect of Clk1 on dopaminergic neuronal survival, MN9D cells were cultured with the indicated concentrations of MPP⁺ for 24 h. As expected, MPP⁺ produced neurotoxicity in MN9D cells (Fig. 1A). Interestingly, we detected a decrease in expression of Clk1 in MN9D cells treated with MPP⁺, suggesting that altered Clk1 expression may be involved in dopaminergic neuronal survival (Fig. 1B&C). Knockdown of Clk1 with lentivirus expressing Clk1 (LV2-shClk1) enhanced neuronal death induced by MPP⁺ relative to LV2-NC-transfected MN9D cells (Fig. 1D&E). This indicated that deficient Clk1 in dopaminergic neurons enhanced MPP⁺-induced cell death.

Clk1 regulates autophagy in MN9D cells. We next explored if Clk1 is involved in the regulation of autophagy. Clk1 deficiency resulted in decreased expression of LC3-II and lysosomal protein LAMP1 in Clk1-deficient MN9D cells. Decreased expression of LC3-II is known to result from either a decrease in the formation of autophagosomes or an increase in the flux of autophagosomes to autolysosomes. Effective autophagic flux will lead to a decrease in its substrates, such as P62. However, we found that expression of P62 was increased (Fig. 2A). This clearly indicated impairment in autophagy and lysosomal biogenesis in Clk1-deficient MN9D
cells. To further confirm the effect of Clk1 on autophagy, we measured autophagic activity by immunofluorescence of LC3 and found decrease in LC3 in Clk1-deficient MN9D cells (Fig. 2B). In contrast, over-expression of Clk1 increased expression of LC3-II and LAMP1, whereas expression of P62 was decreased (Fig. 2C&D). To further evaluate the autophagic flux (Klionsky et al., 2016), cells were treated for 12 h with 100 nM bafilomycin A1, which is known to inhibit vacuolar-type ATPases and blocks the fusion of autophagosome and lysosome and ultimately leads to the inhibition of autophagosome degradation. The level of LC3-II was also decreased in Clk1-deficient MN9D cells compared with LV2-NC-transfected MN9D cells in response to bafilomycin A1 treatment (Fig. 2E), indicating that loss of Clk1 decreased autophagosome synthesis. In the contrast, over-expression of Clk1 increased expression of LC3-II with bafilomycin A1 treatment (Fig. 2F). In addition, MPP⁺ treatment enhanced expression of LC3-II without altering P62 expression (Fig. 2G). These results indicate that MPP⁺ impairs autophagic flux in MN9D cells. Furthermore, knockdown of Clk1 by transfecting MN9D cells with LV2-shClk1 inhibited MPP⁺-enhanced expression of LC3-II (Fig. 2H). Taken together, Clk1 deficiency regulated dopaminergic autophagy by altering autophagy and lysosomal biogenesis under either basal conditions or in MPP⁺-treated neurons.

**Clk1 regulates the AMPK/mTORC1 pathway.** We reported previously that Clk1 regulated glycolytic metabolism in microglia cells (Gu et al., 2017). As the ADP/ATP ratio is an important index of cellular energy status that directly affects activity of AMPK (Hardie, 2007), we examined the effect of Clk1 on the ADP/ATP
ratio and activity of AMPK in dopaminergic MN9D cells. ADP/ATP ratio and phosphorylation of AMPK on Threonine 172 (T172) were decreased with Clk1 knockdown (Fig. 3A). It is known that mTORC1 activation plays an important role in the regulation of autophagy, while AMPK phosphorylation negatively regulates mTOR activation (Puente et al., 2016). We found that Clk1 knockdown enhanced phosphorylation of mTOR on Serine 2448 (S2448) and the mTOR substrate p70s6k on Threonine 389 (T389) in MN9D cells (Fig. 3B). These results indicated that Clk1 is involved in the regulation of the AMPK/mTORC1 signaling pathway in MN9D cells.

**Clk1 mediates autophagy through the AMPK/mTORC1 pathway.** To identify the roles of the AMPK/ mTORC1 pathway in Clk1-mediated autophagy in MN9D cells, Clk1-deficient MN9D cells were incubated with metformin, an AMPK activator, and rapamycin, an mTORC1 inhibitor, with cells then subjected to western blotting assays. As shown in Fig. 4A&B, metformin and rapamycin enhanced expression of LC3-II, whereas expression of P62 was decreased in Clk1-deficient MN9D cells. Accordingly, we detected increased activation of AMPK and inhibition of mTOR and p70s6k in Clk1-deficient MN9D cells in response to metformin and rapamycin. These results suggest that activation of AMPK or inhibition of mTORC1 reversed the inhibition of autophagy induced by Clk1-deficiency in MN9D cells. Furthermore, metformin prevented the effect of Clk deficiency to sensitize MN9D cell death in response to MPP⁺ (Fig. 4C). Collectively, these results clearly showed that Clk1 inhibited autophagy through the AMPK/mTORC1 signaling pathway and that
activation of autophagy by metformin protected dopaminergic neurons from MPP+ insult in Clk1-deficient MN9D cells.

**Clk1 regulates the nuclear translocation of TFEB.** TFEB, which is regulated by mTORC1 (Wong & Cuervo, 2010; Pena-Llopis et al., 2011; Settembre et al., 2011; Decressac et al., 2013; Chua et al., 2014), is a transcription factor involved in autophagy and is functionally associated with neurodegenerative disease (Settembre et al., 2012; Martina and Puertollano, 2013). We therefore considered whether Clk1 is able to regulate TFEB. Nuclear translocation of TFEB was decreased by Clk1 knockdown after starvation in MN9D cells (Fig. 5A), while over-expression of Clk1 led to increased nuclear translocation of TFEB (Fig. 5B). Moreover, metformin increased nuclear translocation of TFEB in Clk1-deficient MN9D cells (Fig. 5C). These findings indicate that Clk1 regulates the nuclear translocation of TFEB. To further confirm this observation, we next assessed the expression levels of lysosomal and autophagic genes in Clk1-deficient cells. Clk1-deficient cells indeed resulted in a global decrease in mRNA expression levels of a set of TFEB target genes, including genes involved in lysosomal biogenesis and function, such as LAMP1, genes encoding subunits of vacuolar ATPases and cathepsins, and genes involved in autophagy (ATG5 and ATG9B; Fig. 5D).

**Clk1 mutant mice exhibit the impaired autophagy in striatum and SNc.** In order to detect autophagy in vivo, we employed Clk+/- mutant mice to explore the expression of proteins associated with autophagy. Consistent with observations in MN9D cells, LC3-II and LAMP1 expression were decreased, while P62 expression
was increased in striatal and SNC tissue in Clk\textsuperscript{+/−} mice (Fig. 6A&B). Moreover, 
AMPK phosphorylation was decreased and phosphorylation of mTOR and p70s6k 
was increased in tissue from Clk\textsuperscript{+/−} mice (Fig. 7A&B). These data further 
demonstrated that Clk1 deficiency results in impaired autophagy both \textit{in vitro and in vivo}

**Metformin promotes autophagy and ameliorates MPTP neurotoxicity on**
**dopaminergic neurons in Clk\textsuperscript{+/−} mice.** To determine if inhibition of autophagy by 
Clk1 deficiency is associated with enhanced dopaminergic neuronal death in Clk\textsuperscript{+/−} 
mice (Gu et al, 2017), we investigated if activation of AMPK and autophagy by 
metformin administration to Clk\textsuperscript{+/−} mice could reverse sensitization to dopaminergic 
neuronal toxicity induced by MPTP. A schematic diagram of drug administration is 
given in Fig. 8A. As expected, MPTP-treated Clk\textsuperscript{+/−} mice showed enhanced behavioral 
impairment as compared to wild type mice. Pretreatment with metformin attenuated 
the behavioral impairments induced by MPTP treatment (Fig. 8B&C). Furthermore, 
metformin pretreatment attenuated the decrease in number of TH-positive neurons in 
SNC, as indicated by immunostaining or TH protein levels by western blot, in mice 
treated with MPTP (Fig. 8D). In agreement with the data observed in MN9D cells, 
LC3-II expression was up-regulated and P62 expression was down-regulated in SNC 
of Clk-deficient MPTP-treated mice pretreated with metformin (Fig. 8E). Taken 
together, the present data indicate that metformin enhanced autophagy and 
ameliorated MPTP neurotoxicity on dopaminergic neurons in Clk\textsuperscript{+/−} mice.
Discussion

In this study, we provide the first evidence that Clk1 is an important regulator in the autophagy pathway and that maintaining adequate Clk1 activity may be critical for dopaminergic neuronal survival. We demonstrated that Clk1 deficiency inhibited dopaminergic neuronal autophagy in cultured MN9D dopaminergic neurons and in SNc of Clk^{+/−} mutant mice. We found that Clk1 regulated the AMPK/mTORC1 pathway, which in turn impaired the autophagy-lysosome pathway and TFEB nuclear translocation, and consequentially promoted dopaminergic neuronal damage in vivo and in vitro; this ultimately contributed to sensitization to MPTP-induced dopaminergic neuronal death and behavioral impairments in Clk1-deficient mice. Moreover, we found that activation of autophagy by metformin treatment increased dopaminergic neuronal survival in vitro and in the MPTP model of PD. These results reveal that Clk1 plays an important role in dopaminergic neuronal survival via regulation of ALP pathways that may contribute to the pathological development of PD. Thus, modulation of Clk1 activity may represent a potential therapeutic target for PD.

Clk1 (coq7) is essential for electron transference in the mitochondrial respiratory chain (Nakai et al., 2001; Lapointe and Hekimi, 2008). Deficiency in Clk1 function is known to alter mitochondrial metabolism, including reduced mitochondrial oxygen consumption, impaired electron transport and mitochondrial ATP synthesis (Hekimi, 2013). Our previous study found that loss of Clk1 in microglia promotes neuroinflammation through regulation of microglial metabolic reprogramming and,
consequently, enhances dopaminergic neuronal death (Gu et al., 2017). However, a direct role of Clk1 in the regulation of dopaminergic neuronal survival has not been explored. In the present study, we found that silencing Clk1 sensitized MPP\(^+\)-induced neurotoxicity toward dopaminergic neurons in cultured MN9D dopaminergic cells. These findings indicate that Clk1 is indeed involved in the regulation of dopaminergic neuronal survival. To explore the underlying mechanism, we found that silencing Clk1 inhibited autophagy in MN9D cells, as evidenced by down-regulation of LC3-II and LAMP1 in MN9D cells and in SNc of Clk\(^+/-\) mice (Fig. 6A&B). Importantly, activation of autophagy by metformin treatment protected dopaminergic neurons from the neurotoxic effects of MPTP administration in both Clk1-deficient and in MPTP-treated wild type animal (Fig. 8B&C). Although it has been reported that inhibition of autophagy favors cell survival (Feng et al., 2014), here we showed that Clk1 deficiency resulted in impairment of autophagy that sensitized cells to MPTP-induced neurotoxicity. In agreement with other reports (Lee et al., 2015; Hwang et al., 2016), our data indicate that induction of autophagy is protective on cell survival in our system. Although a role for the ALP pathway in the development of PD may appear complex, it may be explained by following points: (1) In the early stage of PD, when the lysosome is not severely impaired, enhancement of autophagy may be a complementary response to sustain normal functions by degrading dysfunctional cellular components and organelles, hence, the ALP pathway could play differing roles according to the stage of PD development; (2) We found that Clk1 deficiency inhibited autophagy under basal conditions and in MPP\(^+\)-treated neurons.
The serine/threonine kinase mTOR regulates cell survival and proliferation and is also known to be a major negative regulator of autophagy. Upstream of mTORC1, AMPK is a metabolic sensor, such that activation of AMPK protects neuronal survival through regulating mitochondrial biogenesis and maintaining energy balance (Egan et al., 2011). Activation of AMPK inhibits mTOR and induces autophagy (Li et al., 2013; Guo et al., 2014). As we have previously shown that Clk1 modulates AMPK activity and energy metabolism in microglia cells, we therefore investigated the role of the AMPK/mTORC1 signaling pathway in inhibition of autophagy by Clk1 deficiency in dopaminergic neurons. We found knockdown of Clk1 in MN9D dopaminergic neurons resulted in suppression of AMPK activation and elevation of phosphorylation of mTOR and the mTOR substrate p70s6k (Fig. 3B). Furthermore, we found that Clk1 regulated transcription factor TFEB nuclear translocation and TFEB-targeted genes, which was dependent on the AMPK/mTORC1 signaling pathway. Mechanistic studies demonstrated that Clk1 regulated TFEB nuclear translocation through the AMPK/mTORC1 signaling pathway.

Our previous study has demonstrated that increased dopaminergic neuronal loss may be associated with enhanced microglia activation in Clk1+/− mice treated with MPTP, (Gu et al., 2017). The present study provides evidences that Clk1 is directly involved in the regulation of dopaminergic neuronal survival. This appears to be mediated via changes in autophagy consequent to alterations in the AMPK/mTORC1 pathway. Indeed, pretreatment with metformin ameliorated MPTP-induced behavioral
impairment and dopaminergic neuronal survival in Clk<sup>+/−</sup> mice (Fig. 8B-D). The underlying mechanism may be attributed to enhanced autophagy by AMPK/mTORC1 in dopaminergic neurons. Our data thus indicates that, in addition to modulating microglia-mediated inflammation, Clk1 exerts neuroprotection by directly regulating autophagic activity of dopaminergic cells.

In conclusion, we found that Clk1 is directly involved in the regulation of dopaminergic neuronal survival by mediating autophagy via the AMPK/mTORC1 pathway. Modulation of Clk1 activity may represent a potential novel therapeutic approach for PD.
Authorship Contributions

Participated in research design: Yan and Zhen.

Conducted experiments: Yan.

Performed data analysis: Yan, Han, Wang and Zheng.

Wrote or contributed to the writing of the manuscript: Yan, Zhen and Waddington.
References


Neurosci 13, 805-811.


Footnotes:

This study was supported by grants from the National Science Foundation of China [No. 81372688 and 81373382] and the National Basic Research Plan (973) of the Ministry of Science and Technology of China [2011CB504403]. Additionally, support from the Specialized Research Fund for the Doctoral Program of Higher Education of China [20133201110017] and the Priority Academic Program Development of the Jiangsu Higher Education Institutes (PAPD) is also appreciated.
Figure Legends

**Fig. 1 Clk1 deficiency promotes MPP⁺-induced dopaminergic neuronal death in MN9D cells.** (A) MN9D cells were cultured with indicated concentrations of MPP⁺ for 24 h before MTT assay. *P<0.05 and **P<0.001 vs control group. (B) MN9D cells were treated with 125 μM, 250 μM, 500 μM and 1000 μM MPP⁺ for 24 h. Cells were then harvested and protein levels of Clk1 and TH determined by Western blot analysis. *P<0.025 and **P<0.001 vs control group. (C) MN9D cells were treated with 250 μM MPP⁺ for 12, 24 and 36 h. Cells were then harvested and protein levels of Clk1 and TH were determined by Western blot analysis. *P<0.025, **P<0.001 vs control group. (D) MN9D cells were transfected with LV2-shClk1 or LV2-NC for 48 h. Cells were then collected for RNA extraction or lysis preparations, and used for q-PCR or Western blot assay, respectively. *P<0.05 vs LV2-NC. (E) MN9D cells were transfected as in (D); cells were treated with 250 μM MPP⁺ for a further 24 h before MTT assay. *P<0.05 vs LV2-NC+MPP⁺. Data are presented as means ± SEM from at least 3 independent experiments.

**Fig. 2 Clk1 regulates autophagy in MN9D cells.** (A) MN9D cells were transfected with LV2-shClk1 for 48 h. Cells were then harvested and protein levels of LC3, P62, LAMP1 and Clk1 determined by Western blot analysis. *P<0.001 vs LV2-NC. (B) MN9D cells were transfected as in (A), followed by immunofluorescence assay using antibody against LC3 (green). DAPI (blue) was used for nuclear staining. Cells were visualized using confocal microscopy (scale bar 5 μm), with quantification of LC3
punctuation. *P<0.001 vs LV2-NC. (C) MN9D cells were transfected with mClk plasmid for 48 h. Cells were then harvested and protein levels of LC3, P62, LAMP1 and Clk1 determined by Western blot analysis. *P<0.0167 and **P<0.01 vs control group. (D) MN9D cells were transfected as in (C), followed by immunofluorescence assay using antibody against LC3 (green). DAPI (blue) was used for nuclear staining. Cells were visualized using confocal microscopy (scale bar 5 μm), with quantification of LC3 punctation. *P<0.05 vs control group. (E) MN9D cells were transfected as in (A) and incubated with bafilomycin A1 (Bafı) (100 nM) for 12 h. Then, cells were then harvested and protein levels of LC3 determined by Western blot analysis. **P<0.001 vs LV2-NC+Bafi. (F) MN9D cells were transfected as in (C), the cells were incubated with bafilomycin A1 (Bafi) (100 nM) for 12 h. Then, cells were then harvested and protein levels of LC3 determined by Western blot analysis. *P<0.05 vs Con+Bafi. (G) MN9D cells were treated with 125 μM, 250 μM, 500 μM and 1000 μM MPP⁺ for 24 h. Cells were then harvested and protein levels of LC3 and P62 determined by Western blot analysis. *P<0.025 and **P<0.001 vs control group. (H) MN9D cells with LV2-shClk1 transfection were treated with MPP⁺ for 24 h and protein levels of LC3 and P62 determined by Western blot analysis. *P<0.01 vs LV2-NC+MPP⁺. Data are presented as means ± SEM from at least 3 independent experiments.

Fig. 3 Clk1 regulates the AMPK/mTORC1 pathway. (A) MN9D cells were transfected with LV2-shClk1 for 48 h. ADP/ATP ratio was then measured as described
in Methods. *P<0.01 vs LV2-NC. (B) MN9D cells were transfected as in (A). After 48 h, cells were harvested and protein levels of p-AMPK, p-mTOR, p-p70s6k and Clk1 determined by Western blot analysis. *P<0.0167 and **P<0.01 vs LV2-NC. Data are presented as means ± SEM from at least 3 independent experiments.

Fig. 4 Clk1 mediates autophagy through the AMPK/mTORC1 pathway. (A) MN9D cells with LV2-shClk1 transfection were treated with metformin for 24 h and protein levels of LC3, P62, p-AMPK, p-mTOR and p-p70s6k then analyzed with Western blot analysis. *P<0.025 vs LV2-shClk1. (B) MN9D cells with LV2-shClk1 transfection were treated with rapamycin for 4 h and protein levels of LC3, P62, p-AMPK, p-mTOR and p-p70s6k determined by Western blot analysis. **P<0.01 and ***P<0.001 vs LV2-shClk1. (C) MN9D cells with LV2-shClk1 transfection were treated with MPP⁺ or metformin before MPP⁺ treatment. Four groups of cell viabilities were determined by MTT assay. *P<0.05 vs LV2-shClk1+MPP⁺. Data are presented as means ± SEM from at least 3 independent experiments.

Fig. 5 Clk1 regulates the nuclear translocation of TFEB. (A) MN9D cells were transfected with LV2-shClk1 for 48 h and then re-transfected with TFEB-EGFP 24 h after transfection, the cells were fixed. DAPI (blue) was used for nuclear staining. Cells were visualized using confocal microscopy (scale bar 5 μm). *P<0.05 vs LV2-NC. (B) MN9D cells were transfected with mClk plasmid for 48 h and then re-transfected with TFEB-EGFP 24 h after transfection, the cells were fixed. DAPI
(blue) was used for nuclear staining. Cells were visualized using confocal microscopy (scale bar 5 μm). **P<0.01 vs control group. (C) Clk1-deficient MN9D cells were transfected with TFEB-EGFP and treated with metformin for 24 h. The cells were then fixed. DAPI (blue) was used for nuclear staining (scale bar 5 μm). **P<0.01 vs LV2-shClk1. (D) Similar transfection as in (A) was performed, and transfected cells were processed for q-PCR analysis. The mRNA levels of lysosomal and autophagic genes were quantified and normalized relative to GAPDH. *P<0.05 and **P<0.01 vs LV2-NC. Data are presented as means ± SEM from at least 3 independent experiments.

Fig. 6 Striatum and SNc of Clk1 mutant mice exhibit impaired autophagy. (A, B) Wild type (WT) and Clk1⁺/⁻ mouse striatum and SNc were dissected and processed as described in Methods. Protein levels of LC3, P62, LAMP1, TH and Clk1 were determined by Western blot analysis. n=4 mice per group. *P<0.0167, **P<0.01, ***P<0.001 vs wild type.

Fig. 7 Striatum and SNc of Clk1 mutant mice exhibit impaired the expression of AMPK/mTORC1 signal pathway protein. (A, B) Wild type (WT) and Clk1⁺/⁻ mouse striatum and SNc were dissected and processed as described in Methods. Protein levels of p-AMPK, p- mTOR, p-p70s6k and Clk1 were determined by Western blot analysis. n=4 mice per group. *P<0.0167 and ** P<0.01 vs wild type.
Fig. 8 Metformin promotes autophagy and ameliorates MPTP-neurotoxicity on dopaminergic neurons in Clk<sup> +/-</sup> mice. (A-C) Schematic diagram of drug administration is in (A). WT and Clk1<sup> +/-</sup> mice were treated with saline, metformin and MPTP as described in Methods. The pole-climbing test and rotarod test for bradykinesia were performed on day 8, after which mice were sacrificed and brains collected, n=10 mice per group. *P<0.05 and **P<0.01 vs control group; ## P<0.01 vs Clk1<sup> +/-</sup> treated with MPTP. (D) Immunostaining for TH in SNc, n=6 mice per group (scale bar 200 µm). WT and Clk1<sup> +/-</sup> mouse SNc were dissected and processed as described in Methods. *P<0.05 and **P<0.01 vs control group; # P<0.05 vs Clk1<sup> +/-</sup> treated with MPTP. (E) Protein levels of LC3, P62, TH, p-AMPK and Clk1 were determined by Western blot analysis. n=4 mice per group, **P<0.01 vs control group, # P<0.05 vs Clk1<sup> +/-</sup> treated with MPTP.
Figure 2

A. LV2-NC  LV2-shCkI
LC3  P62  LAMP1  CkI  Actin

B. Anti-LC3  Merge
LV2-NC  LV2-shCkI

C. Control  mCkI
LC3  P62  LAMP1  CkI  Actin

D. Anti-LC3  Merge
Control  mCkI

E. LV2-NC  LV2-shCkI
Control  Bafl
LC3  Actin

F. Control  Bafl
mCkI

G. MPP⁺ (µM)
LC3  P62  Actin

H. LV2-NC  LV2-shCkI
Control  MPP⁺
LC3  P62  Actin
Figure 3

A

![Graph showing ADP/ATP ratio for LV2-NC and LV2-shClk1](image)

B

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Phospho-AMPK/total-AMPK</th>
<th>Phospho-mTOR/total-mTOR</th>
<th>Phospho-p70s6k/total-p70s6k</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV2-NC</td>
<td><img src="image" alt="Phospho-AMPK/total-AMPK" /></td>
<td><img src="image" alt="Phospho-mTOR/total-mTOR" /></td>
<td><img src="image" alt="Phospho-p70s6k/total-p70s6k" /></td>
</tr>
<tr>
<td>LV2-shClk1</td>
<td><img src="image" alt="Phospho-AMPK/total-AMPK" /></td>
<td><img src="image" alt="Phospho-mTOR/total-mTOR" /></td>
<td><img src="image" alt="Phospho-p70s6k/total-p70s6k" /></td>
</tr>
</tbody>
</table>

**Legend:**
- p-AMPK
- AMPK
- p-mTOR
- mTOR
- p-p70s6k
- p70s6k
- Clk1
- Actin

* indicates p < 0.05, ** indicates p < 0.01.
Figure 4

A

<table>
<thead>
<tr>
<th>Condition</th>
<th>LC3</th>
<th>P62</th>
<th>p-AMPK</th>
<th>AMPK</th>
<th>p-mTOR</th>
<th>mTOR</th>
<th>p-p70S6k</th>
<th>p70S6k</th>
<th>Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV2-NC</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LV2-shClk1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Met</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Condition</th>
<th>LC3</th>
<th>P62</th>
<th>p-mTOR</th>
<th>mTOR</th>
<th>p-p70S6k</th>
<th>p70S6k</th>
<th>Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV2-NC</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>LV2-shClk1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Rapa</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

C

Cell Viability (% of control) vs. MPP⁺(μM)

- LV2-NC+MPP⁺+metformin
- LV2-NC+MPP⁺
- LV2-Clk1+MPP⁺+metformin
- LV2-Clk1+MPP⁺

* * *
Figure 5

A

Starved
LV2-NC
LV2-shClik1

% cells with TFEB in cytoplasm

LV2-NC
LV2-shClik1

% cells with TFEB in nucleus

B

Control
mClik1

% cells with TFEB in cytoplasm

Con mClik1

% cells with TFEB in nucleus

C

LV2-shClik1
LV2-shClik1+Met

% cells with TFEB in cytoplasm

LV2-shClik1 LV2-shClik1+Met

% cells with TFEB in nucleus

D

LV2-NC
LV2-shClik1

Relative mRNA expression

ATP6V1B CTSB CTSD CTSE HSPA5 LAMP1 NAV15 NYO1
Figure 7

A. Striatum

- p-AMPK
- AMPK
- p-mTOR
- mTOR
- p-p70s6k
- p70s6k
- Clk1
- Actin

B. SNC

- p-AMPK
- AMPK
- p-mTOR
- mTOR
- p-p70s6k
- p70s6k
- Clk1
- Actin

<table>
<thead>
<tr>
<th>Striatum</th>
<th>WT</th>
<th>Clk1&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho-AMPK/total-AMPK</td>
<td>1.5</td>
<td>0.5**</td>
</tr>
<tr>
<td>Phospho-mTOR/total-mTOR</td>
<td>2.5</td>
<td>0.5**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SNC</th>
<th>WT</th>
<th>Clk1&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho-AMPK/total-AMPK</td>
<td>1.5</td>
<td>0.5**</td>
</tr>
<tr>
<td>Phospho-mTOR/total-mTOR</td>
<td>2.5</td>
<td>0.5**</td>
</tr>
</tbody>
</table>

**p < 0.01
Figure 8

A

B

C

D

E

This article has not been copyedited and formatted. The final version may differ from this version.