Clk1 deficiency promotes neuroinflammation and subsequent dopaminergic cell death through regulation of microglial metabolic reprogramming

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Abstract

Clock (Clk)1/COQ7 is a mitochondrial hydroxylase that is necessary for the biosynthesis of ubiquinone (coenzyme Q or UQ). Here, we investigate the role of Clk1 in neuroinflammation and consequentially dopaminergic (DA) neuron survival. Reduced expression of Clk1 in microglia enhanced the LPS-induced proinflammatory response and promoted aerobic glycolysis. Inhibition of glycolysis abolished Clk1 deficiency-induced hypersensitivity to the inflammatory stimulation. Mechanistic studies demonstrated that mTOR/HIF-1 α and ROS/HIF-1 α signaling pathways were involved in Clk1 deficiency-induced aerobic glycolysis. The increase in neuronal cell death was observed following treatment with conditioned media from Clk1 deficient microglia. Increased DA neuron loss and microgliosis were observed in *Clk1*^{+/-} mice after treatment with MPTP, a rodent model of Parkinson's disease (PD). This increase in DA neuron loss was due to an exacerbated microglial inflammatory response, rather than direct susceptibility of *Clk1*^{+/-} DA cells to MPP⁺, the active species of MPTP. Exaggerated expressions of proinflammatory genes and loss of DA neurons were also observed in *Clk1*^{+/-} mice after stereotaxic injection of LPS. Our results suggest that Clk1 regulates microglial metabolic reprogramming that is, in turn, involved in the neuroinflammatory processes and PD.

Keywords: Clk1; microglia; glycolysis; MPTP; mTOR/HIF-1 α

1. Introduction

Neuroinflammation plays an important role in the pathological processes of neurodegenerative and neuropsychiatric disorders, including Parkinson's disease (PD) (Wang et al., 2014). Microglia, as the resident macrophages of brain, are recognized as the major innate immune cells in the central nervous system, and are key cellular mediators of neuroinflammatory processes (Gendelman, 2002; Walker et al., 2014). Like monocytes/macrophages, activated microglia produce and release proinflammatory cytokines and neurotoxic mediators after stimulation with lipopolysaccharide (LPS) or interferon (IFN)- γ *in vitro* (Dong and Zhen, 2015; Rice et al., 2015). Although inflammatory cytokines such as interleukin-6 (IL-6), interleukin-1 beta (IL-1 β) and tumor necrosis factor alpha (TNF- α) are endogenous mechanisms in response to various inflammatory insults, they are also toxic to neurons (Streit et al., 2004). In this regard, dopaminergic neurons are particularly vulnerable to proinflammatory toxic factors released by activated glial cells (Herrero et al., 2015). In both experimental PD animals and PD patients, the large number of reactive microglia and increases in proinflammatory cytokines in the substantia nigra pars compacta (SNc) have been repeatedly demonstrated to be associated with the pathological process of the diseases (Exner et al., 2012). Therefore, inhibition of the microglia-mediated inflammatory response has become a focal point in efforts to reduce tissue damage and ameliorate the neurodegenerative process.

Recent studies have indicated that glycolytic metabolism plays a critical role in inflammatory regulation (O'Neill and Hardie, 2013). The M1 type of activated

macrophages exhibit enhanced activation of the glycolysis and pentose phosphate pathway, whereas the M2 type undergoes a switch to enhanced oxidative phosphorylation (Krawczyk et al., 2010). When stimulated with LPS or interferon- γ (IFN- γ), macrophages undergo aerobic glycolysis that is essential to activate cellular defense mechanisms and to manage various micro-environments in inflamed tissue (Kelly and O'Neill, 2015). Several reports have demonstrated that inhibition of glycolysis can inhibit M1 polarization in macrophages, TH17 cells and dendritic cells, indicating that metabolic reprogramming could regulate inflammatory cell function (O'Neill, 2014). Similar to other immune cells, microglia also have the ability to switch from oxidative phosphorylation to aerobic glycolysis under inflammatory stimuli (Vazquez et al., 2010).

Clock (Clk)1 (also known as Coq7) encodes a mitochondrial hydroxylase that is involved in the biosynthesis of ubiquinone (UQ or coenzyme Q). Clk1 plays an essential role in electron transport in the mitochondrial respiratory chain and in antioxidant activity (Nakai et al., 2001). It was reported that inactivation of Clk1 or loss of one copy of Clk1 gene (*Clk1*^{+/-} mutant) results in prolonged life span in *Caenorhabditis elegans* and mice (Levavasseur et al., 2001), whereas complete loss of Clk1 is embryonic lethal in mice (Liu et al., 2005). In addition, *Clk1*^{+/-} mutant mice displayed altered mitochondrial metabolisms such as reduced mitochondrial oxygen consumption, reduced electron transport, reduced mitochondrial ATP synthesis, and an increase in the generation of mitochondrial ROS (mtROS) (Hekimi, 2013; Lapointe and Hekimi, 2008). It was also demonstrated that nuclear Clk1 was involved in

mitochondrial metabolism, the mitochondrial unfolded protein response and longevity through regulating gene expression which was likely independent of production of ubiquinone (Monaghan et al., 2015). Moreover, Wang *et al.* demonstrated that Clk1 deficient macrophage displayed increased proinflammatory cytokine and inflammatory responses to LPS through stabilization of HIF-1 α (Wang et al., 2010). In the later study, they also demonstrated that an enhanced immune response may contribute to the longevity of *Clk1*^{+/-} mutants (Wang et al., 2012). Although, the role of Clk1 in mitochondrial function and inflammatory responses has been demonstrated, the role for Clk1 in the pathogenesis of PD, particularly one that is closely associated with neuroinflammation and mitochondrial dysfunction, has not yet been explored. Given the importance of Clk1 in the inflammatory response and mitochondrial metabolism, the present study is designed to explore the role of Clk1 in regulation of the microglia-mediated inflammatory response and its potential significance in the pathological process of PD.

2. Material and methods

2.1 Experimental animals

Clk1^{+/+} (wild type) and *Clk1*^{+/-} mutant mice were obtained from Rugen Therapeutics Co., Ltd (Suzhou, China). Mice were kept on a 12 h light/dark cycle with free access to food and water. Animal experiments were conducted in accordance with NIH guidelines for the Care and Use of Laboratory Animals under a protocol that was approved by the Institute Animal Welfare Committee of Soochow University.

2.2 MPTP mouse model

This mouse model for PD was induced by administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Sigma, St. Louis, MO) as described previously (Park et al., 2015). Fourteen-week-old male mice of both genotypes were pretrained for the rotarod and pole climbing tests before MPTP injection. Wild type and *ClkI*^{+/-} mice were divided into two groups, each consisting of 10 mice that were injected with either normal saline (NS) or MPTP. After repeated intraperitoneal injection of MPTP (30mg/kg, once daily) or saline for 7 days, performance in the rotarod and pole climbing tests was then evaluated daily for 7 days. On day 15, mice were sacrificed, and brains harvested for immunohistochemistry or western blotting.

2.3 Behavioral testing

The rotarod test and pole-climbing test were performed to assess motor function as described previously (Goverdhan et al., 2012). For the rotarod test, mice were trained before MPTP treatment at a speed of 20 rpm to obtain a stable baseline. Then, at a constant speed of 30 rpm, the time each mouse remained on the rod was recorded during a 5 min trial; the trial ended while the mouse fell or jumped off the rod. This test was performed daily, in the morning, over 7 consecutive days after completion of MPTP treatment. On each day, mean time on the rod (in minutes) over three individual trials was recorded, with mice allowed to recover in their home cages for at least 1 h before starting a new trial. For the pole-climbing test, mice were trained before MPTP treatment to obtain a stable baseline. A 50 cm-long rough wooden pole

of 1 cm diameter, with a 2 cm diameter rough wooden ball at the top, was fixed vertically onto the base. Mice were placed on the wooden ball and allowed to climb down. The time from placement on the ball to the forelimbs reaching the base of the pole was recorded. This test was performed daily, in the afternoon, over 7 consecutive days after completion of MPTP treatment. On each day, mean time to climb down (in seconds) over three individual trials was recorded.

2.4 Immunohistochemistry

Immunohistochemistry was performed as described previously (Saijo et al., 2009). On the 15th day after initiation of MPTP treatment, wild type and *Clk1*^{+/-} mice were deeply anesthetized and perfused with 0.9% saline, followed by fixation with 4% paraformaldehyde. Brains were dissected and tissue processed as described previously (Ni et al., 2015). The brains were embedded for cryosection and cut into 15-μm-thick coronal sections using a microtome. Sections containing the SNc were blocked with phosphate buffer saline (PBS) containing 3% albumin from bovine serum (BSA) and 0.3% Triton-X100 for 2h at room temperature. The sections were incubated overnight at 4°C with the following primary antibodies: rabbit anti-tyrosine hydroxylase (TH) (1:500, Millipore, Billerica, MA, USA), and rabbit anti-ionized calcium-binding adapter molecule 1 (Iba-1) (1:500, Wako USA, Richmond, VA, USA). Expression of TH and Iba-1 was finally detected using secondary antibodies conjugated to Alex Fluor 488 and 594 (1:1000, Invitrogen, Carlsbad, CA, USA). Images were taken at 4×, 10× and 40× magnification, using a confocal microscope (Zeiss LSM510 META). The number of TH and Iba-1-positive cells was counted per image in order to

determine the number of surviving DA neurons, active astrocytes and microglia.

2.5 siRNA assay

Clk1, HIF-1 α and HK2 siRNAs were prepared by GenePharma (Shanghai, China) with the sequences in Table S2. All siRNA were performed with Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to manufacturer's instructions. BV-2 cells were transfected with 50nM siRNA for 24 h, followed by treatment. Protein knockdown was assessed by western blot analysis.

2.6 Stereotaxic injection of LPS into the mouse Substantia Nigra pars Compacta

Wild type and *Clk1*^{+/-} mice were divided into four groups (WT-NS, WT-LPS, *Clk1*^{+/-}-NS, *Clk1*^{+/-}-LPS). For stereotaxic injection, mice were anesthetized with 4% chloral hydrate (4g/kg, Sinopharm Chemical Reagent Co., Ltd) and immobilized in a stereotaxic apparatus. LPS or saline was infused into the right SNc using the following coordinates: AP -3.3 mm, ML -1.2 mm, DV -4.6 mm from bregma(Saijo et al., 2009; Thijs et al., 2015). 1 μ l LPS (5mg/ml) or saline was slowly injected over a 2-min period via a stainless steel cannula using a 5 μ l Hamilton microsyringe.

2.7 RNA isolation and quantitative 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), 1 μ g RNA was reverse transcribed using Oligod(T) primers (Takara, Shiga, Japan) according to manufacturer's protocol. qPCRs were performed in triplicate in a 20 μ l reaction with SYBR Premix II (Roche, Nutley, NJ, USA) using 50 ng cDNA on an ABI Prism 7500 sequence detection system (Thermo).

The cycling parameters were as follows: 50°C, 2min; 95°C, 10min; 95°C, 10min; 95°C, 15s, 40 cycles; 60°C, 1min. The primer sequences for each gene are shown in Table S1. We also assayed GAPDH RNA using 5ng total cDNA from each sample. Final results were normalized to GAPDH RNA and expressed as the ratios of the target genes to GAPDH.

2.8 NF-κB reporter assay

The establishment of a stable BV-2 cell line expressing the NF-κB reporter construct was performed as described previously (Xu et al., 2015). Briefly, the NF-κB construct BV-2 cells (Cignal™ Lenti Reporters, Qiagen) were plated on 24 well plates (1×10^5 cells/well). Luciferase activity was measured after 16 h of LPS treatment using a luciferase assay kit (Promega) according to manufacturer's instructions. Level of promoter activity was expressed as relative units (Xu et al., 2015).

2.9 Metabolic assays

Lactate levels in the culture medium were determined using a Lactate Assay Kit (BioAssay Systems) according to manufacturer's instructions. Glucose consumption was determined using a QuantiChrom™ Glucose Assay Kit (BioAssay Systems). Using whole-cell lysates, the ATP/ADP ratio was measured using an ATP/ADP Assay Kit (Sigma) and the NAD⁺/NADH ratio measured using a NAD⁺/NADH Assay Kit (BioAssay Systems). For analysis of extracellular acidification rate (ECAR), BV-2 cells were analyzed using an XF-24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA). ECAR was analyzed in response to 10mM glucose, 0.5μM oligomycin and 50mM 2DG (all from Sigma-Aldrich).

2.10 Statistical analysis

For multiple group comparisons, one-way ANOVA was performed, followed by application of the Bonferroni correction. For comparisons between two groups, Student's t-test was applied. A *p* value of less than 0.05 was considered statistically significant.

3. Results

3.1 Clk1 deficiency enhances the LPS-induced inflammatory response in microglia cells

To investigate the role of Clk1 in the neuroinflammatory response, we evaluated the consequences of reduced Clk1 expression in microglial responsivity to LPS. Knockdown of Clk1 in microglia cells was established by siRNA-mediated RNA interference (Fig. 1A). As shown in Fig. 1B-F, LPS-induced mRNA expressions of iNOS, COX-2, TNF- α , IL-1 β , and IL-6 were increased in Clk1 knockdown BV-2 microglia cells. LPS-induced production of NO, IL-1 β , IL-6, iNOS and COX-2 was also increased in Clk1 knockdown BV-2 microglia cells (Fig. 1G-J). We also tested the role of Clk1 in expression of M2 polarization marker genes including arginase (Arg)-1, chitinase 3-like-3 (YM-1) and IL-10. The results showed that knockdown of Clk1 did not change the expression of M2 polarization marker genes, however, it reduced IL-4 induced the expression of M2 polarization marker genes at transcriptional or protein levels (Fig.1L-O). We next confirmed this observation by employing primary microglia isolated from *Clk1*^{+/-} mice (Fig. 2A). As depicted in Fig.

2B-I, primary microglia from *Clk1*^{+/-} mice produced more NO and pro-inflammatory cytokines than BV-2 cells with Clk1 knockdown. Since NF-κB is a key transcriptional factor that induces proinflammatory cytokines and iNOS gene expression in glial cells, it was determined whether this hypersensitivity of Clk1 knockdown cells occurred through exaggeration of NF-κB activation in BV-2 microglia cells. The results revealed that Clk1 knockdown made microglia more sensitive to LPS-induced transcriptional activity of NF-κB (Fig. 1K). These results indicated that the increased sensitivity of Clk1-deficient microglial cells to LPS was dependent on the NF-κB pathway.

3.2 Clk1 deficiency results in increased production of reactive oxygen species in microglia cells

There is growing evidence that intracellular ROS or mitochondrial ROS is produced by activated microglia cells and plays an important role in regulating the inflammatory response (Chuang et al., 2015). Therefore, the effect of Clk1 on generation of intracellular ROS and mitochondrial ROS was determined using ROS-sensitive indicators DCFH-DA and MitoSOX Red, respectively. Knockdown of Clk1 in BV-2 microglia cells revealed increases in both intracellular ROS (Fig. 3A) and mtROS (Fig. 3B) for both basal and activated status.

3.3 Clk1 deficiency results in increased aerobic glycolysis

It is known that glycolytic reprogramming plays an important role in polarization of inflammatory cells such as macrophages and dendritic cells (Everts et al., 2014). Therefore, we tested the effect of Clk1 on glycolytic reprogramming in microglia

cells. Clk1 deficiency (siClk1) in BV-2 cells revealed that NAD^+/NADH ratios for both basal and activated status were markedly decreased (Fig. 4A) and extracellular lactate (Fig. 4B) and glucose consumption (Fig. 4C) were increased for both basal and activated status. In support, extracellular acidification rate (ECAR), an indirect indicator of lactate production and enhanced glycolytic metabolism, was elevated in siClk1 BV-2 cells following 6h (Fig. 4D) and 16h (Fig. 4E) of LPS treatment. Glucose transporter 1 (GLUT1), a key molecule in the cellular uptake and utilization of glucose, and hexokinase (HK), a vital rate-limiting enzyme for phosphorylating the glucose, were also upregulated in siClk1 BV-2 cells (Fig. 4F). These data clearly indicated that Clk1 deficiency promoted aerobic glycolysis in microglia cells.

3.4 Inhibition of glycolytic metabolism abolish the the enhanced inflammatory response in Clk1-deficient BV-2 cells

Aerobic glycolysis may control polarization and differentiation of immune cells (Pollizzi et al., 2015). Therefore, we next investigated whether enhanced glycolytic metabolism was related to the enhanced inflammatory response in Clk1-deficient microglia cells using siRNA to knockdown HK2 (Fig.5A). Increased expression of proinflammatory cytokines and NO production after LPS treatment in siClk1 cells were abolished on knockdown of HK2 (Fig. 5B-H). Increased NF- κ B activity was also rescued on knockdown of both HK2 and Clk1 cells (Fig. 5I). Similar results were observed following pretreatment with the glycolytic inhibitor 2DG in Clk1 deficient BV-2 microglia cells. As shown in Fig. 5J-O, LPS-induced production of NO, mRNA expression of proinflammatory cytokines, and NF- κ B activation were suppressed by

2DG in siClk1 BV-2 cells. These results indicated that the enhanced inflammatory response mediated by Clk1 deficiency was mediated by aerobic glycolysis in microglia cells.

3.5 Enhanced HIF-1 α expression mediates enhancement of aerobic glycolytic metabolism and the inflammatory response in Clk1-deficient microglia cells

Hypoxia-inducible factor 1 (HIF-1 α) plays an critical role in glucose metabolism by regulating the expression of glucose transporter 1 (GLUT1) and glycolytic enzymes such as HK and lactate dehydrogenase (Semenza, 2013). Therefore, we examined whether Clk1 deficiency-mediated aerobic glycolysis and enhanced inflammatory responses were dependent on HIF-1 α expression. As shown in Fig. 6A, mRNA and protein expression of HIF-1 α in siClk1 BV-2 cells were elevated both at basal level and in response to LPS stimulation, indicating that the observed increase in HIF-1 α expression was not only due to its enhanced stability in Clk1 deficient microglia cells.

In support, HIF-1 α expression was also elevated in *Clk1*^{+/-} microglia (Fig. 6B) and mouse SNc (Fig. 6C). To further explore linkage between elevated expression of HIF-1 α and increased aerobic glycolysis due to Clk1 deficiency, we assessed lactate level and glucose consumption in siClk1 BV-2 cells with or without HIF-1 α knockdown with siRNA (Fig. 6D). Knockdown of HIF-1 α decreased levels of lactate release and glucose consumption in Clk1-deficient microglia cells, indicating that aerobic glycolysis triggered by Clk1 deficiency was dependent on HIF-1 α expression (Fig. 6E,F). Next, we addressed whether enhanced expression of HIF-1 α contributed to the observed hypersensitive response to LPS stimulation in siClk1 cells.

Knockdown of HIF-1 α abolished enhanced expression of proinflammatory cytokines, NO production, and iNOS and COX-2 expression in response to LPS in siClk1 BV-2 cells (Fig. 6G-M), and NF- κ B activation (Fig. 6N). Taken together, these results indicated that Clk1 deficiency-induced aerobic glycolysis and increased inflammatory responses were dependent on HIF-1 α expression in microglia cells.

3.6 Expression of HIF-1 α is mediated by mTOR activation and ROS signaling

Since it has been reported that mTOR activation is involved in the regulation of transcription of HIF-1 α mRNA and protein stability (Hudson et al., 2002), we investigated activation of mTOR in siClk1 BV-2 cells. As expected, Clk1 deficiency resulted in enhanced activation of mTOR and S6 kinase (S6K) at basal level and in response to LPS stimulation (Fig. 7A). Activation of mTOR is regulated by at least two signaling pathways, including AMPK and Akt. AMPK phosphorylation negatively regulates mTOR activation, whereas Akt activation positively regulates mTOR (Kaper et al., 2006). We found inhibition in phosphorylation of AMPK in Clk1-knockdown BV-2 cells (Fig. 7A,B), while phosphorylation of Akt was unchanged (Fig. 7B). Likewise, the ADP/ATP ratio in siClk1 BV-2 cells was also lower than that of siCtrl cells at basal level and in LPS-treated cells (Fig. 7C). To further confirm the effect of AMPK-mediated mTOR signaling on HIF-1 α expression in Clk1-deficient microglia cells, siClk1 BV-2 cells were incubated with rapamycin (mTOR inhibitor), metformin (AMPK activator) and α -KG (PHD inhibitor, depleting HIF-1 α) for 2h. Increased expression of HIF-1 α and phosphorylation of S6K in siClk1 BV-2 cells were reduced after treatment with metformin and rapamycin (Fig. 7D),

indicating that AMPK signaling was upstream of mTOR activation and that the AMPK/mTOR pathway mediated expression of HIF-1 α in Clk1-deficient microglia cells.

There is growing evidence that increased ROS level drives HIF-1 α expression (Gao et al., 2007). Therefore, we tested the effect of the ROS scavenger NAC on HIF-1 α expression and HIF-1 α -mediated enhancement of the inflammatory response in Clk1-deficient BV-2 microglia cells. NAC inhibited the increased expression of proinflammatory cytokines (Fig.7E-J) and NO secretion (Fig.7K) in Clk1-knockdown BV-2 cells. This result showed that NAC partially abolished HIF-1 α expression in Clk1 deficient BV-2 microglia cells (Fig. 7E), indicating that ROS signaling was also involved in regulating HIF-1 α -mediated glycolytic reprogramming. Thus, Clk1 deficiency enhanced HIF-1 α expression to mediate glycolysis and inflammation through AMPK/mTOR signaling and ROS.

3.7 MPTP-induced loss of DA neurons is increased in *Clk1*^{+/-} mice

Overactivation of microglia is known to be closely related to neurotoxicity and is involved in the pathological development of neurodegenerative disorders, including PD (Pellerin et al., 2007; Kavanagh et al., 2015; Thome et al., 2015). Therefore, we employed *Clk1*^{+/-} mutant mice to explore the function of Clk1 in the MPTP-induced mouse PD model. The rotarod test and pole climbing test were conducted daily after MPTP injection from day 8 to day 14. As expected, MPTP treatment resulted in a shorter time in the rotarod test and a longer latency in the pole climbing test. These behavioral impairments were more severe in *Clk1*^{+/-} mice treated with MPTP (Fig.

8A-C). In agreement with this observation, the number of TH⁺ neurons in SNc of *Clk1*^{+/-} mice was lower than that of wild type mice at day 14 following the first MPTP injection (Fig. 8D-F). These data indicated that DA neurons in *Clk1*^{+/-} mice were more sensitive to MPTP-induced lesions.

To exclude the possibility that Clk1-deficient DA neurons were more sensitive to MPP⁺, the active species of MPTP, we first studied if Clk1 knockdown in DA neurons altered neuronal survival consequent to MPP⁺ lesions. As shown in Fig. 8H, there was no difference in MPP⁺-induced cell viability between siCtrl and siClk1 MES 23.5 cells, indicating that Clk1 deficiency in DA neuron did not enhance neuronal death induced by MPP⁺. We next explored any difference in the MPTP-induced inflammatory response between wild type and *Clk1*^{+/-} mice. Compared to wild type mice, Iba-1 positive cells and total protein level of Iba-1 in SNc were increased in *Clk1*^{+/-} mice after MPTP treatment (Fig. 8E, G). Clk1 deficiency also resulted in exaggerated expression of inflammatory mediators in SNc, including iNOS, COX-2, TNF- α , IL-6 and IL-1 β , at day 14 following the first MPTP injection (Fig. 8J). This suggested that Clk1 deficiency-sensitized microglial activation may be responsible for the enhancement of DA neuron death induced by MPTP.

3.8 Clk1 deficiency in microglia cells increases inflammation induced dopaminergic neuronal cell death

It is known that microglial overactivation facilitate neuronal cell death *in vitro* and *in vivo* (Hernandez-Romero et al., 2008). We next evaluated whether Clk1-deficient microglia influenced the survival of dopaminergic neuron *in vitro*. Compared to wild

type or siCtrl microglia cells, the conditioned media from *Clk1*^{+/-} primary microglia or siClk1 BV-2 cells, which had been treated with LPS for 24h, exacerbated the death of primary neurons and MES 23.5 dopaminergic cells, respectively (Fig. 9A-C). To further confirm this observation, we performed stereotaxic injections of LPS or NS into SNc of adult wild type and *Clk1*^{+/-} mice to establish the inflammatory PD model (Fig. 9D). Clk1 deficiency resulted in exaggerated expression of inflammatory mediators in SNc, including iNOS, COX-2, TNF- α , IL-6 and IL-1 β , in response to local LPS injection (Fig. 9E-I). Loss of TH⁺ neurons after local LPS injection normally takes 14-21 days (McGeer and McGeer, 2008). We detected greater loss of TH⁺ neurons in SNc of *Clk1*^{+/-} mice compared to wild types at 14 days after LPS treatment (Fig. 9J, K). These results indicated that Clk1 deficiency leads to increased DA cell death through microglial activation.

4. Discussion

In the present study we demonstrate that Clk1 deficiency resulted in enhanced aerobic glycolysis, which lead to an enhanced inflammatory response and contributes to increased DA neuron injuries in neuroinflammatory diseases. Clk1 deficiency-induced glycolysis was dependent on the AMPK/mTOR/HIF-1 α or ROS/HIF-1 α signaling pathway. Glycolytic reprogramming-mediated enhancement of NF- κ B activation likely contributed to the increased inflammatory response of Clk1-deficient microglia cells.

Clk1 (Coq7), a mitochondrial hydroxylase, is essential for ubiquinone

biosynthesis and plays an important role in mitochondrial metabolism and aging (Levavasseur et al., 2001). It has been shown that aged *Clk1*^{+/-} mutants show an enhanced immune reaction to infection (Wang et al., 2012) and that Clk1-deficient macrophage cells display an enhanced inflammatory response to LPS (Wang et al., 2010). In this study, we demonstrated that Clk1-deficient microglia cells also exhibited an enhanced inflammatory response to inflammatory stimuli, suggesting that Clk1 not only regulated activation of peripheral immune cells, but also regulated brain microglia cells. It is well known that NF-κB activation is a major event in proinflammatory gene expression in microglia cells. In the present study, it was observed that the transcriptional activity of NF-κB was more sensitive to activation by LPS in Clk1-deficient microglia cells. This elaborated previous reports that Clk1-deficient macrophages show increased expression of proinflammatory cytokines that is dependent on activation of NF-κB signaling (Wang et al., 2010).

There is increasing evidence that M1 polarization is often accompanied by a shift from oxidative phosphorylation towards aerobic glycolysis in innate immune cells (O'Neill and Hardie, 2013; Tannahill et al., 2013). It is also known that cellular metabolic reprogramming plays an important role in the regulation of the inflammatory response (Everts et al., 2014). Additionally, it has been shown that LPS induces macrophage glycolytic metabolism, while inhibition of glycolysis with 2DG suppresses LPS-induced expression of IL-1β (Tannahill et al., 2013). In TH17 cells, 2DG also inhibits the LPS-induced shift in glycolytic metabolism and expression of IL-17, and ultimately suppresses differentiation of TH17 cells (Shi et al., 2011). More

recently, the relationship between M1 polarization and increased aerobic glycolysis has been investigated in microglia (Rahman et al., 2016), and it is reported that LPS-induced aerobic glycolysis may contribute to the production of proinflammatory factors in BV-2 microglia cells. In the present study, we found that Clk1 deficiency promoted aerobic glycolysis in microglia cells, and inhibition of glycolysis by 2DG or knockdown of HK2 expression may abolish Clk1 deficiency-induced hypersensitivity to LPS in microglia cells, suggesting that Clk1 regulates microglial activation through metabolic reprogramming in microglia cells.

HIF-1 α is a key transcriptional factor that regulates glucose metabolism in immune cells (Courtney et al., 2015). Activation of HIF-1 α promotes aerobic glycolysis via regulation of transcriptional expression of GLUT1 and glycolytic enzymes such as HK and lactate dehydrogenase in macrophages and DC cells (Semenza, 2013). Studies document that Clk1-deficient RAW264.7 cells display increased expression of HIF-1 α , which may contribute to the enhanced immune response (Wang et al., 2010). We detected elevation in HIF-1 α expression in Clk1-knockdown BV-2 cells and in the brain of *Clk1*^{+/-} mice. Knockdown of HIF-1 α expression in microglia cells attenuated Clk1 deficiency-induced aerobic glycolysis and the inflammatory response, suggesting that regulating microglial metabolic reprogramming and subsequent inflammatory responses by Clk1 were dependent on the expression of HIF-1 α .

There is increasing evidence that expression of HIF-1 α is mediated by mTOR activation in immune cells. Induction of HIF-1 α in TH17 cell differentiation is

required for mTOR activation, and treating T cells with the mTOR inhibitor rapamycin inhibits glycolytic activity and production of IL-17 (Shi et al., 2011). The expression of HIF1- α is regulated by mTOR activation at both mRNA and protein level (Laughner et al., 2001; Zhong et al., 2000). Activation of mTOR can be regulated by AMPK and Akt signaling pathway (Puente et al., 2016). Akt signaling can stimulate mTOR activity, while AMPK inhibits such activity (Kaper et al., 2006). We found activation of mTOR and inhibition of AMPK phosphorylation in Clk1-deficient microglia cells, but no change in Akt activation. Inhibition of AMPK can abolish the activation of mTOR by Clk1 deficiency, suggesting that AMPK is an upstream signal molecule of mTOR in Clk1-deficient microglia cells. In addition to stimulation of protein synthesis, inhibition of its degradation also contributes to the expression of HIF-1 α (Shi et al., 2011). Studies document that increased ROS level drives HIF-1 α expression via inhibition of protein degradation (Desideri et al., 2014). In the current study, we found that the ROS scavenger NAC not only suppressed HIF-1 α expression but also abolished enhancement of the inflammatory response in Clk1-deficient microglia cells and found NAC could abolish a high level of inflammation. Thus, we can conclude that Clk1 depletion stimulates HIF-1 α expression through the ROS or AMPK/mTOR pathway.

In the present study we found that Clk1 deficiency increased the sensitivity of the LPS-induced inflammatory response, but did not change proinflammatory gene expression at basal status. Similarly, Clk1 deficiency increased the activity of NF- κ B transcriptional activity at activated status. These results suggest that Clk1 regulates

the sensitivity of microglia cells to the inflammatory response through NF- κ B pathway. However, the precise molecular mechanisms underlying this regulatory role of Clk1 are not known. It has been reported that the acetylation of the p65 subunit of NF- κ B, which is regulated by NAD⁺-dependent SIRT1 activity, is involved in sensitivity of macrophages to LPS (Yuk et al., 2015). Increased glycolysis leads to decreases in NAD⁺/NADH ratio and SIRT1 deacetylase activity (Ryall et al., 2015). We found a marked decrease in the NAD⁺/NADH ratio in Clk1-deficient BV-2 cells. It is possible that a drop of NAD⁺/NADH ratio by Clk1 deficiency inhibits SIRT1 deacetylase activity, which increases the acetylation of NF- κ B p65 and thus ultimately promotes transcriptional activity of NF- κ B. This should be investigated further in future studies.

Overactivation of microglia is closely related to neurotoxicity and is involved in the pathological development of neurodegenerative disorders, including PD (Block et al., 2007). In many cases of PD, neuroinflammation is an underlying process and could be an inducible mechanism of neuronal death (Lee et al., 2016). MPTP is a neurotoxic compound that is commonly used to create an animal model of PD. Inhibition of mitochondrial complex I activity is believed to be a mechanism underlying MPTP-induced neurotoxicity. In the present study, we found that Clk1 deficiency did not increase the sensitivity of neuronal cells to MPP⁺, suggesting that the effect of mitochondrial dysfunction on neuronal death is dependent on its magnitude and species. Indeed, some reports have demonstrated that mitochondrial dysfunction may contribute to resistance to apoptosis (Dawson and Dawson, 2003;

Yao and Wood, 2009). On the other hand, microglial activation is evident and contributes to DA neuron death in the MPTP-induced PD model (Pellerin et al., 2007). An inflammatory animal model, involving injection of the proinflammatory compound LPS into SNc, shows that the inflammatory process can induce dopaminergic neuronal death (Herrera et al., 2000). Compared to controls, the conditioned media from Clk1-deficient microglia cells treated with LPS exacerbated the death of DA neuronal cells. *In vivo*, local injection of LPS into SNc of *Clk1*^{+/-} mice resulted in exaggerated expression of inflammatory mediators and more severe loss of TH-positive DA neurons in SNc compared to wild types. Thus, it is reasonable to conclude that Clk1 deficiency promotes DA neuron death via enhanced microglia-mediated inflammation and hence contributes to the development of PD. However, as MPTP can be converted to MPP⁺ by astrocytic MAO-B, we cannot exclude the possibility of some change in MAO-B activity in *Clk1*^{+/-} astrocytes relative to wild type.

In conclusion, the present study demonstrates that 1) Clk1 modulates microglial activation via regulation of glycolytic reprogramming; 2) AMPK inactivation modulates the mTOR/HIF-1 α and ROS/HIF-1 α signaling pathway involved in Clk1-mediated glycolytic reprogramming; and 3) Clk1-mediated microglial activation contributes to DA neuron injury *in vitro* and *in vivo*. Therefore, modulation of Clk1 activity may constitute a potential therapeutic target for neuroinflammation-associated PD therapy.

Conflicts of interest

The authors declare no competing interest.

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Figure legends

Fig. 1. Clk1 deficiency-enhanced TLR4-induced inflammatory response in BV-2 cells. BV-2 cells were cultured as described in Methods. Knockdown of Clk1 was achieved using siRNA1 and 2 (50nM). (A) Validation of knockdown efficiency in BV-2 cells. Expression of Clk1 was determined by qPCR and western blotting. (B-F)

BV-2 cells with or without knockdown of Clk1 were stimulated with LPS (100ng/ml) for 6h and mRNA expression of iNOS, COX-2, TNF- α , IL-6 and IL-1 β was measured by qPCR. (G) Cells were incubated in the presence or absence of LPS for 24h and the amount of NO in the medium was measured by Griess reagent. (H, I) Cells were incubated in the presence or absence of LPS for 24h and secreted TNF- α and IL-6 in the medium was measured by ELISA. (J) Cells were incubated in the presence or absence of LPS for 12h and the expression of iNOS, COX-2 and Clk1 was determined by western blotting. (K) Cells were incubated with LPS for 16h and NF- κ B activity in BV-2 cells was determined by luciferase reporter assay. Cells were incubated in the presence or absence of IL-4 and the expression of Arg-1, YM-1 and IL-10 was determined by qPCR (L-M) or western blotting (O). *, $p<0.05$ compared to PBS. #, $p<0.05$ compared to siCtrl. Data are presented as mean $\pm$ SD for at least 3 independent experiments.

Fig. 2. Elevated classic activation in *Clk1*^{+/-} microglia. (A) Expression of Clk1 in primary microglia was determined by qPCR. **, $P<0.01$ compared to wild type (WT). Primary microglia were stimulated with LPS (200ng/ml). (B) After 24h, the amount of NO in the medium was measured by Griess reagent. (C-G) After 6h, mRNA levels of iNOS, COX-2, TNF- α , IL-1 β , and IL-6 were measured by qPCR. (H, I) After 24h, the secreted TNF- α and IL-6 in the medium was measured by ELISA. **, $P<0.01$ compared to PBS. #, $P<0.05$ compared to WT. All data are presented as mean $\pm$ SEM.

Fig. 3. Clk1 deficiency results in increased production of reactive oxygen species in microglia cells. BV-2 cells with or without knockdown of Clk1 were incubated with LPS (100ng/ml) for 2h and intracellular and mitochondrial ROS levels were measured by DCFH-DA (A) and MitoSOX (B).

Fig. 4. Clk1 deficiency results in increased aerobic glycolysis. BV-2 cells with or without knockdown of Clk1 by siRNA were cultured for indicated times. (A-C) BV-2 cells were treated with LPS for 6h. Knockdown of Clk1 (siClk1) in BV-2 cells altered the levels of NAD^+/NADH ratio, L-lactate production and glucose consumption. *, $p < 0.05$ compared to PBS. #, $p < 0.05$ compared to siCtrl. Rate of extracellular acidification (ECAR) was analyzed as an indicator of glucose oxidation. BV-2 cells were treated with LPS for 6h (D) and 16h (E). *, $p < 0.05$ compared to siCtrl. #, $p < 0.05$ compared to siCtrl+LPS. (F) Expression of GLUT1, HK2 and Clk1 in siCtrl and siClk1 BV-2 cells was determined by western blotting using respective antibodies. *, $p < 0.05$ compared to siCtrl. Data are presented as mean $\pm$ SD for at least 3 independent experiments.

Fig. 5. Inhibition of glycolytic metabolism abolish the enhanced inflammatory response in Clk1-deficient BV-2 cells. (A) Validation of knockdown efficiency of HK2 in BV-2 cells. Expression of HK2 was determined by qPCR and western blotting. **, $p < 0.01$ compared to siCtrl. siCtrl and siClk1 BV-2 cells with or without HK2 knockdown were treated with PBS or LPS. (B-F) mRNA expression of iNOS, COX-2,

TNF- α , IL-6 and IL-1 β was measured by qPCR after 6h LPS treatment. (G) Amount of NO in the medium was measured by Griess reagent. (H) Protein expression of iNOS and COX-2 was determined after 12h LPS treatment by western blotting using respective antibodies. (I) NF- κ B activity after 16h LPS treatment was determined by luciferase reporter assay system. *, $p < 0.05$ compared to LPS (+)/siHK2 (-). #, $p < 0.05$ compared to siCtrl. BV-2 cells were pretreated with 2DG (200 μ M) for 30min prior to and during LPS (100ng/ml) treatment. (J-N) mRNA levels of iNOS, COX-2, TNF- α , IL-6 and IL-1 β were measured by qPCR after 6h LPS treatment. (O) After 24h LPS treatment, amount of NO in the medium was measured by Griess reagent. *, $p < 0.05$ compared to LPS (+)/2DG (-). #, $p < 0.05$ compared to siCtrl. Data are presented as mean $\pm$ SD for at least 3 independent experiments.

Fig. 6. Enhanced HIF-1 α expression mediates enhancement of aerobic glycolytic metabolism and the inflammatory response in Clk1-deficient microglia cells.

BV-2 cells with or without knockdown of Clk1 were cultured in the presence or absence of LPS (100ng/ml) for indicated times. (A) Basal and stimulated (6h) expression of HIF-1 α in siCtrl and siClk1 BV-2 cells was determined by qPCR and western blotting. *, $p < 0.05$ compared to PBS. #, $p < 0.05$ compared to siCtrl. (B) Basal and stimulated (6h) expression of HIF-1 α in wild type (WT) and *Clk1*^{+/-} primary microglia was determined by qPCR. *, $p < 0.05$ compared to PBS. #, $p < 0.05$ compared to WT. (C) Expression of HIF-1 α in WT and *Clk1*^{+/-} mice SNc was determined by western blotting. (D) Expression of HIF-1 α was determined by qPCR. **, $p < 0.01$

compared to siCtrl. After 16h LPS stimulation, L-lactate level (E) and glucose consumption (F) in BV-2 cells were measured. *, $p<0.05$ compared to siHIF-1 α (-). #, $p<0.05$ compared to siCtrl. (G-K) After 6h, expression of iNOS, COX-2, TNF- α , IL-6 and IL-1 β was measured by qPCR. (L) After 24h, amount of NO in the medium was measured by Griess reagent. (M) After 12h, expression of iNOS and COX-2 was determined by western blotting. (N) After 16 h, NF- κ B activity in BV-2 cells was determined by luciferase reporter assay system. *, $p<0.05$ compared to LPS(+)/siHIF-1 α (-). #, $p<0.05$ compared to siCtrl. Data are presented as mean $\pm$ SD for at least 3 independent experiments.

Fig. 7. Clk1 deficiency enhances expression of HIF-1 α via AMPK/mTOR signaling pathway and ROS. BV-2 cells with or without Clk1 knockdown by siRNA were cultured as described. (A) Cells were incubated with LPS (100ng/ml) for the indicated times. The indicated phosphorylated (p-) and total proteins were analyzed by western blotting. (B) Phosphorylated (p-) and total proteins of Akt and AMPK were determined by western blotting in siCtrl and siClk1 BV-2 cells by respective antibodies. (C) Cells were incubated in the presence or absence of LPS (100ng/ml) for 2h in siCtrl and siClk1 BV-2 cells. ADP/ATP ratio was measured as described in Methods. *, $p<0.05$ compared to PBS. #, $p<0.05$ compared to siCtrl. (D) Cells were treated with rapamycin (rap, 200nM), metformin (Metf, 1mM), α -KG (1mM) or CoCl₂ (200 μ M) for 4h. Protein levels were determined by western blotting. Cells were pretreated with NAC for 30min and then incubated in the presence or absence of LPS

(100ng/ml) in siCtrl and siClk1 BV-2 cells. (E) After 12h LPS stimulation, expression of HIF-1 α and COX-2 was determined by western blotting. (F) After 24h LPS treatment, amount of NO in the medium was measured by Griess reagent. (G-K) After stimulating with LPS for 6h, expression of iNOS, COX-2, TNF- α , IL-1 β , and IL-6 was measured by qPCR. *, $p<0.05$ compared to LPS (+)/NAC (-). #, $p<0.05$ compared to siCtrl. Data are presented as mean $\pm$ SD for at least 3 independent experiments.

Fig. 8. MPTP-induced loss of DA neurons is increased in *Clk1*^{+/-} mice. (A-C) Wild type (WT) and *Clk1*^{+/-} mice received sequential intraperitoneal injections of MPTP (30mg/kg) or saline (NS) for 7 days as described in Methods. The pole-climbing test and rotarod test for bradykinesia were performed daily for 7 days. Mice were sacrificed and brains collected on day 15. Behavioral tests for MPTP-induced bradykinesia were conducted on the indicated days; n=10 mice per group. Data are presented as mean $\pm$ SEM. #, $p<0.05$, compared to respective NS. *, $p<0.05$. **, $p<0.01$ compared to WT+MPTP group. (D) Immunostaining for TH in SNc; n=6 mice per group. Bar: 200 μ M (E) Immunostaining for TH and Iba-1 in SNc; n=6 mice per group. Bar: 50 μ M (left); 25 μ M (right). Quantitative analysis of TH-positive (F) and Iba-1-positive (G) cells in SNc. **, $p<0.01$ compared to respective NS. # $p<0.01$, compared to WT+MPTP treatment. (H) MES 23.5 cells were cultured with indicated concentrations of MPP⁺ for 24h before MTT assay. Data are presented as mean $\pm$ SD for at least 3 independent experiments. Expression of Clk1 in MES 23.5 cells was

determined by western blotting. (I) Western blotting for TH and Iba-1 protein expression; n=4 mice per group. (J) Expression of iNOS, COX-2, TNF- α , IL-6 and IL-1 β in SNc was measured by qPCR. *, $p<0.05$ compared to NS; # $p<0.05$, compared to WT; n=3 per group.

Fig. 9. Clk1 deficiency in microglia cells increases inflammation induced dopaminergic neuronal cell death. Conditioned media (CM) treated with either PBS or LPS for 24h were harvested separately from primary microglia cultures in wild type (WT) and *Clk1*^{+/-} mice, or from siCtrl and siClk1 BV-2 cells. CMs were then transferred to MES 23.5 cells and primary neuron cultures to assess survival. (A-C) Viability of neurons was measured by MTT assay. *, $p<0.05$ compared to PBS. # $p<0.05$, compared to WT CM in response to LPS. (D) LPS or NS was injected into SNc of WT and *Clk1*^{+/-} mice as described in Methods. (E-I) After LPS or NS injection for 6h, expression of iNOS, COX-2, TNF- α , IL-6 and IL-1 β in SNc was measured by qPCR. *, $p<0.05$ compared to NS; n=4 per group. (J) Immunostaining for TH in SNc. Bar: 200 μ M; n=6 per group. (K) Quantitative analysis of TH-positive cell number in SNc. **, $p<0.01$ compared to NS. #, $p<0.05$, compared to WT injected with LPS. Data are presented as mean $\pm$ SEM. Each experiment repeated at least 3 times.

Figure1

Figure2

Figure3

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Figure 6

Figure 7

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Figure9