Loss of PINK1 inhibits apoptosis by upregulating α -synuclein in inflammation-sensitized hypoxic-ischemic injury in the immature brains

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Abbreviations: PINK1, PTEN-induced putative kinase 1; α -Syn, α -synuclein; LPS, lipopolysaccharide; CP, cerebral palsy; HI, hypoxia-ischemia; TTC, 2,3,5-triphenyltetrazolium chloride; TUNEL, TdT-mediated dUTP nick end-labeling; CC3, cleaved caspase-3; siRNA, small interfering RNA.

Abstract

The incidence of preterm birth is rising worldwide. Among preterm infants, many face a lifetime of neurologic impairments. Recent studies have revealed that systemic inflammation can sensitize the immature brain to hypoxic-ischemic (HI) injury. Therefore, it is important to identify the mechanisms involved in inflammation-sensitized HI injury in immature brains. PTEN-induced putative kinase 1 (PINK1) is a regulatory protein that is highly expressed in the brain. We have previously found that PINK1 gene knockout can protect matured brains from HI injury in postnatal day 10 mice. However, the mechanisms are unknown. In this study, we employed an inflammation-sensitized HI injury model using postnatal day 3 mice to study the roles and mechanisms that PINK1 plays in the immature brains. Lipopolysaccharide (LPS) was injected intraperitoneally into the mice before HI treatment to set up the model. We found that PINK1-knockout mice had fewer brain infarcts and less cell apoptosis than did the wild-type mice. Furthermore, we found that α -synuclein was markedly higher in the PINK1-knockout mice than in the wild-type mice, and inhibition of α -synuclein through small interfering RNA (siRNA) reversed the protective effect in the PINK1-knockout mice. Collectively, these findings indicate that loss of PINK1 plays a novel role in the protection of inflammation-sensitized HI brain damage.

Key words: PINK1; α -Synuclein; Lipopolysaccharide; Hypoxia-ischemia; Apoptosis; Immature brain.

1. Introduction

Preterm birth, defined as birth before 37 weeks of gestation, is currently a major cause of neonatal morbidity worldwide. The incidence of preterm birth is rising in most countries, representing more than one-tenth of all births worldwide (Blencowe et al., 2012). Progress in neonatal medical care has decreased the preterm mortality rate. However, among preterm infants who survive, many face a lifetime of adverse developmental outcomes, including cerebral palsy (CP), mental retardation, reduced cognitive performance, delayed language skills, behavioral difficulties, and learning difficulties (Allen et al., 2011; Jarjour, 2015).

Preterm birth is strongly associated with infection (Goldenberg et al., 2008), and it is estimated based on microbiological studies that 25-40% of preterm births are caused by genital tract infection (Nadeau et al., 2016). Because of the deficient amount of pulmonary surfactant and the immature respiratory center/musculature, premature infants tend to suffer from respiratory distress and respiratory failure, resulting in cyanosis and hypoxia. Recent studies revealed that systemic inflammation can sensitize the immature brain to hypoxic-ischemic (HI) injury (Wang et al., 2014a; Wang et al., 2014b), but the mechanisms responsible for this sensitization have not been fully elucidated. Thus, the mechanisms by which inflammation sensitizes the immature brain to HI injury is under investigation, and the exploration of therapeutic methods minimizing HI injury has gained great attention in modern perinatal care for preterm babies.

PTEN-induced putative kinase 1 (PINK1), a mitochondrial serine/threonine kinase, is a regulatory protein that is highly expressed in the brain. Mutations in this gene cause autosomal recessive early-onset Parkinson's disease (Valente et al., 2004). PINK1 participates in mitochondrial quality control by identifying damaged mitochondria and targeting specific mitochondria for degradation via autophagy (Youle and van der Bliek, 2012). Many studies have indicated that PINK1 is a protective regulatory protein that is critical for neuronal survival following HI insult

and that knockout of PINK1 results in exacerbated HI-induced damage (Chen et al., 2015; Li and Hu, 2015; Lin et al., 2014; Zhao et al., 2013). Nevertheless, a few recent studies have reported that the suppression of PINK1 function *in vivo* under normal physiological conditions does not affect mitochondrial turnover events as had been predicted (Devireddy et al., 2015) but rather sustains cell proliferation by reprogramming glucose metabolism (Requejo-Aguilar et al., 2014). Furthermore, loss of PINK1 function was reported to be protective in neurons in a murine model (Qi et al., 2011). We have previously found that PINK1 gene knockout can protect neonatal mice (at postnatal day 10, which corresponds to full term infants) from HI brain damage (Huang et al., 2016). However, the roles and mechanisms of PINK1 in the pathogenesis of damage to the immature brain remain unclear.

The downstream effectors of PINK1 are complicated. Among them, α -synuclein (α -Syn) is important. α -Syn was found to be upregulated in PINK1-knockout mice, and the misfolding and aggregation of α -Syn clearly causes Parkinson's disease (Minami et al., 2015). By contrast, many studies have indicated that α -Syn performs neuroprotective activities through suppressing cell apoptosis (Bornhorst et al., 2014; Jin et al., 2011).

In the present study, we generated a mouse model of inflammation-sensitized HI injury in the immature brain by exposing postnatal day 3 (P3) mice to low oxygen levels after left carotid artery ligation following intraperitoneal lipopolysaccharide (LPS) injection, and we investigated the effect and underlying mechanisms of PINK1 in this animal model.

2. Results

2.1. Genotyping of wild-type and PINK1-knockout C57BL/6 mice

PCR products amplified from genomic DNA of wild-type and PINK1-knockout mice confirmed that the samples from the wild-type mice displayed a 355-bp band but that samples from PINK1-knockout mice lacked this band (figure 1).

2.2. The effect of PINK1-knockout on brain infarcts in LPS-sensitized HI brain injury in neonatal mice

TTC staining was employed to detect brain infarcts 24 h and 72 h after LPS and/or HI insult. First, we tested whether LPS injection or HI insult alone induced brain damage. TTC revealed that neither 0.05 mg/kg LPS injection nor HI (20 min duration) insult alone caused any significant brain infarct in the wild-type and PINK1-knockout neonatal mice (figure 2A). However, in the WT LPS+HI group, there were significant infarcts (figure 2B, C), which indicated that LPS exposure could sensitize neonatal mice to HI brain injury. Nevertheless, we did not detect significant brain infarcts in the KO LPS+HI group (figure 2B, C), which meant that loss of PINK1 can dramatically protect the immature brain from LPS-sensitized HI injury. Naturally, in both the WT Sham and KO Sham groups, there were no brain infarcts.

2.3. The effect of PINK1-knockout on cell apoptosis in LPS-sensitized HI brain injury in neonatal mice

The TUNEL method was used to detect cell apoptosis 24 h after LPS+HI insult. In the cortical area of the left hemisphere (also referred to as the ipsilateral cortex area), both the WT Sham group ($1.37 \pm 0.77\%$) and the KO Sham group ($1.33 \pm 0.89\%$) had hardly any cell apoptosis. This result indicates that there is little cell apoptosis under physiological conditions in the brains of wild-type and PINK1-knockout mice. After the LPS+HI insult, the KO LPS+HI group did not show any significant cell apoptosis ($3.08 \pm 1.39\%$) compared with the sham groups, whereas the WT LPS+HI group displayed dramatic cell apoptosis ($55.67 \pm 7.81\%$) based on confocal laser scanning microscopy ($P < 0.01$) (figure 3).

2.4. The effect of PINK1-knockout on the expression of CC3 in LPS-sensitized HI brain injury in neonatal mice

Western blotting was used to measure the expression of the cell apoptosis-related protein CC3 in brain tissues of the ipsilateral cortex and hippocampus at 4 h, 8 h, 12 h and 24 h after the LPS+HI insult. The expression level of CC3 began to be elevated at 8 h in both the WT LPS+HI and KO LPS+HI groups compared with their respective sham groups ($P<0.05$). However, in accordance with the TUNEL staining results, the expression level of CC3 was much lower in the KO LPS+HI group than in the WT LPS+HI group. At 8 h after the LPS+HI insult, the CC3 expression level was significantly higher in the WT LPS+HI group than in the KO LPS+HI group ($P<0.05$). Furthermore, at 12 h and 24 h after the LPS+HI insult, the CC3 expression level was dramatically higher in the WT LPS+HI group than in the KO LPS+HI group ($P<0.01$) (figure 4).

2.5. The effect of α -Syn on cell apoptosis in LPS-sensitized HI brain injury in PINK1-knockout neonatal mice

First, we measured the expression of α -Syn in the brain tissue of neonatal PINK1-knockout and wild-type mice. Western blotting revealed that the expression level of α -Syn was markedly higher in the neonatal PINK1-knockout mice than in the neonatal wild-type mice ($P<0.05$) (figure 5A).

Next, we inhibited the expression of α -Syn in neonatal PINK1-knockout mice via an intracerebroventricular injection of the siRNA targeting α -Syn at P1. The results shown in figure 5B demonstrate that α -Syn expression in the neonatal PINK1-knockout mice was successfully attenuated at P3, 48 h after the injection of siRNA at P1 ($P<0.01$).

At P3, the neonatal PINK1-knockout mice treated with siRNA to inhibit the expression of α -Syn were exposed to LPS+HI as described above. Then, after 24 h of the insult, TTC staining was used to detect brain infarcts, TUNEL staining was employed to measure cell apoptosis, and Western blotting was performed to evaluate the expression of the cell apoptosis-related protein CC3 in brain tissues. TTC staining

revealed notable infarcts in neonatal PINK1-knockout mice deficient in α -Syn expression (the KO siRNA group); however, there was no significant infarct in the control group of neonatal PINK1-knockout mice intracerebroventricularly injected with phosphate-buffered saline (PBS) (figure 6A). TUNEL staining revealed a considerable percentage of cell apoptosis in the KO siRNA group ($49.01 \pm 2.92\%$) but no significant cell apoptosis in the KO PBS group in the ipsilateral cortex area ($3.92 \pm 0.62\%$) (figure 6B). The expression of CC3 was maintained at a very low level in the KO PBS group. In contrast, the expression level of CC3 was significantly increased in the KO siRNA group ($P < 0.01$) (figure 6C).

3. Discussion

The incidence of preterm birth is rising worldwide, at more than 10% of all births. Moreover, preterm birth is estimated to account for approximately 50% of all long-term neurologic impairments observed in children (Subramaniam et al., 2012). CP is a common major disability in premature infants (Hirvonen et al., 2014; Jarjour, 2015). Inflammation and HI are two major mechanisms of CP in preterm infants (Beaino et al., 2010; O'Shea, 2002). Perinatal pre-exposure to systemic inflammation may worsen HI damage to the immature brain (Wang et al., 2014a; Wang et al., 2014b). In our study, TTC staining revealed dramatic infarction in P3 mice subjected to the LPS+HI insult. By contrast, there was no significant infarction in P3 mice insulted with LPS or HI alone. This finding indicates that LPS exposure can sensitize mice to HI brain injury at P3, when the brain maturation status is equivalent to that of very preterm infants.

Cell apoptosis is an important form of cell death following neonatal HI (Northington et al., 2011). Caspase-3 is cleaved and activated in the brain after HI in neonatal rodents, and the extent of caspase-3 cleavage and activation is more evident in developing rodents than in adults (Zhu et al., 2005). In this study, we found dramatic cell apoptosis and increased expression of CC3 following LPS+HI insult in

P3 mouse pups. Thus, we consider apoptosis an important form of cellular degeneration in LPS-sensitized HI injury in the immature brain. Loss of PINK1 can protect the immature brain against cell apoptosis induced by LPS-sensitized HI injury.

PINK1, a serine/threonine kinase that is highly expressed in the brain, contains an N-terminal mitochondrial localization sequence, a putative transmembrane sequence, a serine/threonine kinase domain, and a C-terminal regulatory sequence (Valente et al., 2004). PINK1 present throughout the cytoplasm serves as a “scout” to probe for damaged mitochondria (Narendra et al., 2012; Youle and van der Bliek, 2012), and relevant studies have shown that knockout of the PINK1 gene exacerbates injury induced by hypoxic stress (Chen et al., 2015; Lin et al., 2014). Mutations in the PINK1 gene, resulting in a failure to protect against stress-induced mitochondrial dysfunction, can cause hereditary early-onset Parkinson’s disease (Valente et al., 2004). Other studies indicate that healthy mitochondria and cell proliferation in the intact nervous system can be sustained despite PINK1 deficiency (Devireddy et al., 2015; Requejo-Aguilar et al., 2014). Moreover, Qi et al. demonstrated a protective effect of PINK1 silencing in mice, primarily in the early phase of PINK1 deficiency (Qi et al., 2011). To our surprise, there was no significant infarction in PINK1-knockout P3 mice insulted with LPS+HI, and this protective effect primarily resulted from inhibition of the activity of caspase-3 for suppressing cell apoptosis. These results indicate that loss of PINK1 can protect neonatal mice against brain damage caused by LPS-sensitized HI.

However, the effector molecules regulating apoptosis in neonatal PINK1-knockout mice are unknown. There was evidence showing that loss of PINK1 leads to enhanced α -Syn expression even in fibroblasts prior to an objective diagnosis of Parkinson’s disease (Hoepken et al., 2008). In the present study, we examined the expression of α -Syn in the brains of neonatal mice and found that α -Syn expression is significantly higher in neonatal PINK1-knockout mice than in their wild-type counterparts. However, the function of α -Syn is largely unknown, as it shows both neurotoxic and

neuroprotective properties. Misfolding and aggregation of α -Syn, the major component of Lewy bodies, are associated with Parkinson's disease (Guella et al., 2016). However, the neuroprotective role of α -Syn has been demonstrated during the early stage of manganese exposure in a model of Parkinson's disease (Harischandra et al., 2015). Bornhorst et al. revealed a neuroprotective role of α -Syn in attenuating oxidative stress (Bornhorst et al., 2014). Jin et al. reported that α -Syn can suppress apoptosis in neurons by downregulating the proapoptotic kinase PKC δ (Jin et al., 2011). In our study, we inhibited the expression of α -Syn in PINK1-knockout mice using siRNA and found that α -Syn-silenced PINK1-knockout mice did not exhibit the protective effect of PINK1 loss against LPS-sensitized HI injury.

In summary, the present study showed that apoptosis is an important form of cellular degeneration in LPS-sensitized HI injury in the immature brain. Loss of PINK1 can protect the immature brain against cell apoptosis induced by LPS-sensitized HI injury. Moreover, α -Syn plays a neuroprotective role in LPS-sensitized HI brain damage in PINK1-knockout neonatal mice. These findings indicate that loss of PINK1 plays a novel role in the protection of inflammation-sensitized HI injury in the immature brain.

4. Experimental procedures

4.1. Establishing a mouse model of inflammation-sensitized HI injury in the immature brain

The animal protocol was approved by the Animal Care and Use Committee of Sichuan University. All mice in the C57BL/6 genetic background were housed with free access to food and water in an environment of 22-25°C, 55-60% relative humidity and a 12 h/12 h light/dark cycle. Female and male neonatal mice were assigned to each group without sex difference. Six animals were used for each group. At least 3 independent experiments were performed in each experimental time point.

Here, we focused on the inflammation-sensitized HI injury in the immature brain. The brain maturity of P3 mice developmentally corresponds to that of preterm infants at ~28-30 weeks of age. The mouse model was adapted from Wang's method (Wang et al., 2014a). Briefly, an LPS concentration of 0.05 mg/ml was diluted in PBS. The volume of 1 μ l LPS per gram body weight of mice was intraperitoneally injected using a Hamilton (USA) syringe with 33G needle to minimize the injury by the injection. The mice were returned to their dams following LPS injection for 2 h until HI. Next, the mice were anesthetized by cooling on ice to the point of unresponsiveness to noxious stimulation (Shen et al., 2010). Following disinfection of the skin with alcohol, a midline ventral incision was made in the anterior neck. Under a dissecting microscope (Leica, Germany), the left common carotid artery was ligated following isolation from the nearby vagus nerve and cut off from the middle of the two ligations on the artery. After occluding the artery, the skin incision was sutured. The average length of surgery was 3-4 min. After surgery, the mice were recovered by warming on a heating pad (36°C) for 1 hour. The mice were then placed in a chamber on the heating pad (36°C) and supplied with 8% oxygen for 20 min. Finally, the mice were returned to their dams.

The control group of C57BL/6 mice on P3 was intraperitoneally injected with 1 μ l PBS/g body weight. After 2 h, the mice were treated as described above, except that the left common carotid artery was isolated only from the nearby vagus nerve without occlusion or the subsequent hypoxic insult.

4.2. PINK1-knockout mice and experimental grouping

PINK1-knockout mice in the C57BL/6 genetic background were kindly provided by Dr. Changan Jiang (Sichuan University, China). Genotyping of PINK1-knockout mice was performed via PCR. First, tail tissue was boiled in a 100°C water bath in 50 mmol/L NaOH for 30-40 min. Then, 1 mol/L Tris-HCl (pH 8.3) was added, and the solution was mixed. After centrifugation at 14,000 rpm for 5 min, the supernatant was

collected as genomic DNA. The PCR reaction mixture contained primers, master mix (Thermo Fisher Scientific, USA), and the extracted template RNA as described above. The PINK1 primer 1 sequence was 5'-GAG CCT GAA GTG CAA ACT CC-3'; the PINK1 primer 2 sequence was 5'-GCT CTG GCT TCT GAG GAA GA-3'. The amplification conditions included an initial denaturation step at 95°C for 2 min followed by 3 cycles of denaturation at 95°C for 15 sec, annealing at 64°C for 15 sec, and amplification at 72°C for 75 sec; 3 cycles of denaturation at 95°C for 15 sec, annealing at 61°C for 15 sec, and amplification at 72°C for 75 sec; 3 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 15 sec, and amplification at 72°C for 75 sec; a final 33 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 15 sec, and amplification at 72°C for 75 sec; and a final extension step at 72°C for 3 min. After the reaction, the sample containing amplified products was held at 4°C. PCR products were examined via horizontal electrophoresis in a 1.8% agarose gel containing goldenvue dye at 60 V for 1.5 h in 1× TAE buffer (40 mmol/L Tris base, 20 mmol/L sodium acetate, 1.8 mmol/L EDTA, and pH adjusted to 7.8 with glacial acetic acid) and photographed under ultraviolet light. Samples from the wild-type mice showed a 355-bp band, and samples from PINK1-knockout mice lacked this band.

Wild-type C57BL/6 mice were randomly assigned to two groups: wild-type control (**WT Sham**) group: injected with PBS and no exposure to HI; and the wild-type experimental (**WT LPS+HI**) group: intraperitoneally injected with 0.05 mg/kg LPS and exposed to HI. The PINK1-knockout C57BL/6 mice were also randomly assigned to two groups: the PINK1-knockout control (**KO Sham**) group: injected with PBS and no exposure to HI; and the PINK1-knockout experimental (**KO LPS+HI**) group: intraperitoneally injected with 0.05 mg/kg LPS and exposed to HI. Around 15% of the mice in the LPS+HI group were sacrificed, and no mice were sacrificed in the sham group.

4.3. 2,3,5-triphenyltetrazolium chloride (TTC) staining

TTC staining was employed to detect brain infarcts. At 24 h and 72 h after the LPS+HI insult, the mice were deeply anesthetized by cooling on ice. Their brains were harvested and sectioned into 2 mm coronal slices. The brain slices were then submerged in 2% TTC in the dark at 37°C for 20 min and then fixed in 4% paraformaldehyde for approximately 24 h for photography. The infarct area of each coronal slice was analyzed using the Image-Pro Plus software. An indirect method for assessing the infarct area was used because of potential swelling of the infarct (Lin et al., 1993). In brief, $LI=RT-LN$, where LI=infarct area in the left hemisphere measured by the indirect method, RT=total cortex area in the right hemisphere of the same brain, and LN=noninfarcted cortex area in the left hemisphere of the same brain. The ratio of infarct volume was calculated using the following formula (Jin et al., 2013): $\text{Infarct volume ratio} = (\sum \text{Infarct area} \times \text{thickness}) / (\sum \text{the whole left brain area} \times \text{thickness}) \times 100\%$. Here, for easy and convenient operation, we also harvested the whole brains for TTC staining without sectioning into slices at 24 h after the LPS+HI insult.

4.4. Western blotting

The cortex and hippocampus of the left hemisphere (also referred to as the ipsilateral cortex and hippocampus) were homogenized in cold RIPA buffer, and the protein concentrations were determined using the BCA method (Bio-Rad, USA). Samples (50 μg) were separated via 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes. After blocking with 5% skim milk in TBST (Tris-HCl 20 mmol/L, NaCl 150 mmol/L, and Tween-20 0.1%, pH 7.4), the membranes were incubated with primary antibodies. Immunoreactivity was detected using horseradish-conjugated secondary antibodies and visualized via enhanced chemiluminescence. The primary antibodies used were anti-cleaved caspase-3 (anti-CC3, rabbit source, 1:1000, Cell Signaling Technology, USA), anti- α -Syn (mouse source, 1:250, R&D Systems, USA) and anti- β -actin (mouse source, 1:5000,

Zen BioScience, China). The horseradish-conjugated secondary antibodies used were anti-rabbit IgG (1:5000, Zen BioScience, China) and anti-mouse IgG (1:5000, Zen BioScience, China). The signals from immunoreactive bands were quantified using QuantityOne imaging software by a person who was blinded to the treatments.

4.5. TdT-mediated dUTP nick end-labeling (TUNEL) staining

At 24 h after the LPS+HI insult, the mice were deeply anesthetized by cooling on ice and transcardially perfused with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (PB, pH 7.3). The brains were harvested and fixed in 4% paraformaldehyde for 24-48 h. Following embedment in 3% agarose, the brains were coronally sectioned (50 μ m in thickness) from the genu of the corpus callosum to the end of the dorsal hippocampus using an oscillating tissue slicer. Two sections per brain, one at the level of the striatum (0.5 mm anterior to Bregma) and the other at the level of the dorsal hippocampus (1.0 mm posterior to Bregma) according to a mouse brain atlas, were selected for TUNEL staining according to the manufacturer's instructions (DeadEndTM fluorometric TUNEL system, Promega). The sections were washed in PBS and permeabilized with 0.2% Triton X-100 in PBS for 30 min. After washing 3 times $\times$ 5 min in PBS, the sections were equilibrated in Equilibration Buffer for 5-10 min. Next, the sections were incubated with 50 μ l of TdT reaction mixture for 60 min at 37°C in a humidified chamber (away from light from this step forward). Then, 2 $\times$ SSC (300 mmol/L NaCl, 30 mmol/L sodium citrate, pH 7.0) was added for 15 min to stop the TdT reaction. Afterwards, the sections were washed 3 times $\times$ 5 min in PBS. Then, nuclear staining was performed by applying 1 μ g/ml 4,6-diamidino-2-phenylindole (DAPI, Roche) in PBS for 15 min at room temperature. After washing another 3 times $\times$ 5 min in PBS, the sections were mounted onto glass slides and cover-slipped with anti-fade mounting medium (Solarbio, China). The sections were observed using the appropriate laser beams and filter settings for detection of green-emitting DyLight 488 (excitation peak, 493 nm; emission peak,

518 nm) under a confocal laser scanning microscope (Olympus, Japan). Digital images were captured with FV10-ASW-3.1 software (Olympus, Japan).

4.6. Silencing of α -Syn expression via intracerebroventricular injection of small interfering RNA (siRNA)

PINK1-knockout C57BL/6 neonatal mice were anesthetized by cooling on ice to the point of unresponsiveness to noxious stimulation. After disinfecting the scalp with alcohol, suspensions of 2 nmol siRNA mixture (target sequences: GGAAGACATGCCTGTGGAT, GGAAGGCTACCAAGACTAT, and GGAGTCCTCTATGTAGGTT, RiboBio, China) targeting α -Syn mRNA in a volume of 1 μ l were injected (0.2 μ l/min) into the left lateral ventricle using a Neuros syringe (Hamilton, USA). The stereotaxic coordinates for injection into the left lateral ventricle were 1.8 mm anterior to lambda, 1.8 mm lateral to lambda, and a 1.8 mm depth from the skin. At the end of the injection, the needle was held in place for 5 min and then slowly removed. For the control groups, the same volume of PBS (1 μ l) was injected into the left lateral ventricle using the same procedure as described above.

In this experiment, the PINK1-knockout C57BL/6 mice were randomly assigned to two groups. For the “**KO siRNA**” group, at P1, α -Syn expression was silenced using siRNA, and after 48 h (at P3), the mice were exposed to LPS+HI using the same procedure as described above. For the “**KO PBS**” group, at P1, the same volume of PBS was intracerebroventricularly injected, and after 48 h (P3), the mice were exposed to LPS+HI using the same procedure as described above. Around 15% of the mice in the two groups were sacrificed.

4.7. Statistical analysis

All statistical analyses were performed using SPSS 23.0 software. For comparisons between values for two groups, the independent-samples *t*-test was adopted for statistical analysis. One-way ANOVA was performed for comparisons of multiple

groups. One-way ANOVA was followed by the Student-Newman-Keuls test if homogeneity of variance was assumed or by Dunnett's test if homogeneity of variance was not assumed. $P < 0.05$ was considered to indicate statistical significance: * and #, $P < 0.05$; **, $P < 0.01$.

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Author contributions

JZ, YQ and DM conceived and designed the experiments; JZ, YQ, ZL, FZ, LZ, YH and CJ performed the experiments; JZ, YQ and DM analyzed the data and wrote the paper. All authors read and approved the final version of the manuscript.

Ethics statement

All procedures on animals were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). This study was approved by the Animal Care and Use Committee of Sichuan University. All efforts were made to minimize the number of animals used and their suffering.

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Figure legends

Figure 1. Genotyping of the wild-type and PINK1-knockout C57BL/6 mice.

Horizontal electrophoresis of the PCR products showed a 355 bp band in wild-type mice (WT) but no 355 bp band in PINK1-knockout mice (KO).

Figure 2. PINK1-knockout reduced the brain infarct volume in LPS-sensitized HI brain injury in neonatal mice. TTC staining was used to detect brain infarcts. (A)

There was no significant brain infarct 24 h after LPS injection alone or HI insult alone in both wild-type and PINK1-knockout neonatal mice. (B) Arrowheads denote brain infarcts in the WT LPS+HI group 24 h after the insult; however, there was no significant infarct in the KO LPS+HI group. In both the WT Sham and KO Sham groups, there were no brain infarcts. (C) The ratio of infarct volume was significantly increased in the WT LPS+HI group at both 24 h and 72 h after the insult (** $P<0.01$), whereas there was no significant infarct in the WT Sham, KO Sham and KO LPS+HI groups.

Figure 3. PINK1-knockout inhibited the apoptosis in LPS-sensitized HI brain injury in neonatal mice (magnification, 100×; scale bar=100μm). TUNEL staining revealed cell apoptosis in brain slices from neonatal wild-type and PINK1-knockout mice after the LPS+HI insult. In the ipsilateral cortex area, the WT Sham, KO Sham and KO LPS+HI groups rarely displayed apoptotic cells, as demonstrated by green nuclear staining (cell nuclei were stained blue with DAPI). In contrast, compared with these groups, the WT LPS+HI group showed many green apoptotic cells. ** $P<0.01$.

Figure 4. PINK1-knockout downregulated the CC3 in LPS-sensitized HI brain injury in neonatal mice. Western blotting was used to measure the expression of CC3 in the brain tissues of neonatal wild-type and PINK1-knockout mice at different time points after the LPS+HI insult. “#” indicates that the expression level of CC3

was significantly higher than in the corresponding sham group ($P<0.05$), “*” indicates that the expression level of CC3 was significantly higher in the WT LPS+HI group than in the KO LPS+HI group ($P<0.05$), and “**” indicates that the expression level of CC3 was significantly higher in the WT LPS+HI group than in the KO LPS+HI group ($P<0.01$).

Figure 5. The expression level of α -Syn in brain tissue. (A) Western blotting showed a much higher expression level of α -Syn in PINK1-knockout mice than in wild-type mice (* $P<0.05$). (B) The expression of α -Syn was significantly downregulated by the injection of siRNA targeting α -Syn in PINK1-knockout neonatal mice, as demonstrated by Western blotting (** $P<0.01$).

Figure 6. Inhibition of the expression of α -Syn reversed the protective effect in LPS-sensitized HI brain injury in PINK1-knockout neonatal mice. (A) TTC staining showed the brain infarcts in the whole brain tissue of PINK1-knockout mice after the LPS+HI insult. Arrowheads show the brain infarct in the KO siRNA group, in which α -Syn expression was inhibited. However, there was no significant infarct in the KO PBS group. (B) TUNEL staining indicated cell apoptosis in brain slices of the neonatal PINK1-knockout mice after the LPS+HI insult (magnification, 100 $\times$; scale bar=100 μ m). In the ipsilateral cortex area, the KO siRNA group showed many apoptotic cells, but the KO PBS group exhibited few apoptotic cells. There was a significant difference in the percentage of apoptotic cells between the groups (** $P<0.01$). (C) Western blotting revealed the expression of CC3 in brain tissues of PINK1-knockout mice after the LPS+HI insult. Compared with the KO PBS group, the KO siRNA group displayed significantly increased CC3 expression at 24 h after the LPS+HI insult (** $P<0.01$).

Figure

A **B** **C**

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