MLKL inhibition attenuates hypoxia-ischemia induced neuronal damage in developing brain

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Running headline: MLKL mediates HI induced neuronal damage

Abbreviations

ANOVA, analysis of Variance; BHA, butylated hydroxyanisole; CCA, common

1 carotid artery; Drp1, dynamin-related protein-1; HI, hypoxia-ischemia; MLKL, mixed
2 lineage kinase domain-like protein; NAC, N-acetylcysteine; Nec-1, necrostatin 1;
3 NSS, neurological severity score; OGD, oxygen-glucose deprivation; PGAM5,
4 phosphoglycerate mutase family member 5; RIP1, receptor interacting protein kinase
5 1; RIP3, receptor interacting protein kinase 3; ROS, reactive oxygen species; SD,
6 sprague-dawley; TRPM7, transient receptor potential melastatin related 7; TTC,
7 triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl
8 transferase-mediated dUTP nick end-labeling.

9

Abstract

Mixed lineage kinase domain-like protein (MLKL) is a critical molecule mediating cell necroptosis. However, its role in brain injury remains obscure. We first investigated the functions and mechanisms of MLKL in mediating neuronal damage in developing brain after hypoxia-ischemia. Neuronal necroptosis was induced by oxygen-glucose deprivation (OGD) plus caspase inhibitor zVAD treatment (OGD/zVAD). We found that two important necroptosis related proteins, receptor-interacting protein 1 and 3 (RIP1, RIP3) were upregulated. Furthermore, the interaction of RIP1-RIP3 with MLKL increased. Inhibition of MLKL through siRNA diminished RIP1-RIP3-MLKL interaction and attenuated neuronal death induced by OGD/zVAD. The translocation of oligomerized MLKL to the neuronal membrane leading to the injury of cellular membrane is the possible new mechanism of neuronal necroptosis. Animal experiment with neonatal rats further proved that MLKL inhibition attenuated brain damage induced by hypoxia-ischemia. These findings suggest that MLKL is a target to attenuate brain damage in developing brain.

Keywords: Brain damage; Hypoxia-ischemia; MLKL; Neonate; Neuron

1 **Introduction**

2 Apoptosis has been thought to be the predominant mechanism of regulated cell
3 death. In recent years, another important cell death type, necroptosis, also termed
4 programmed necrosis, has been promoted as an alternative cell death pathway
5 (Degterev et al., 2005). The increasing attention on necroptosis is largely due to recent
6 findings of its physiological and pathological importance, especially in ischemic brain
7 injury (Degterev et al., 2005; Northington et al., 2011) Receptor-interacting protein 1
8 (RIP1) and receptor-interacting protein 3 (RIP3) have been implicated in necroptosis
9 (Cho et al., 2009; Zhang et al., 2009), and together they form the backbone of the
10 large necrosome as a crucial platform for recruiting other components and stimulating
11 the downstream execution mechanisms of necroptosis (Li et al., 2012). Among these
12 components, mixed lineage kinase domain-like protein (MLKL) has been reported to
13 be the most important one. The formation of RIP1-RIP3-MLKL interaction leads to
14 the phosphorylation of MLKL, which has been proposed to be the marker of
15 necrosome activation (Sun et al., 2012). Although there are studies examining the
16 roles of RIP1 or RIP3 in ischemic brain injury, the role of MLKL has remained
17 unclear. In the present study, we investigate the possible functions and related
18 mechanisms of MLKL in mediating hypoxia-ischemia (HI) induced neuronal damage
19 in neonatal rats.

20 **Materials and methods**

21 **Primary culture of cortical neurons**

22 All animal protocols were approved by the Sichuan University Committee on
23 Animal Research and complied with the ARRIVE guidelines. Cortical neurons were
24 prepared from brains of 18-day-old Sprague–Dawley rat embryos. Brains were
25 cleaned of meninges and blood vessels. The whole cerebral cortex was isolated and
26 cells were dissociated in 0.25% trypsin solution for 10 min at 37 °C. Fetal bovine
27 serum was added and the dissociated cells were forced through a mesh. After
28 centrifuging, the cortex cells were resuspended and plated in poly-D-lysine (Sigma, St
29 Louis, MO, USA) coated six-well plates, and grown in Neurobasal (NB) (Life, New
30 York, NY, USA) with 500μM glutamine (Life) and 2% B27 supplement (Life) in a

1 humidified incubator with 5% CO₂ at 37°C, and used for subsequent experiments
2 after 10 days in vitro (DIV). The purity of neurons was measured by staining with
3 anti-microtubule associated protein 2 (MAP-2) antibody (1:100; Santa Cruz
4 Biotechnology, Santa Cruz, CA, USA) and counterstaining with DAPI using standard
5 protocols. The purity quotient was calculated as MAP-2 positive cells /DAPI positive
6 cells.

7 **Oxygen-glucose deprivation (OGD) insult**

8 To test the effects of Nec-1 (20 µM, Sigma), BHA (100-200 µM, Sigma),
9 N-acetylcysteine (NAC, 0.05-10mM, Sigma), nifedipine (100 µM, Sigma), NiCl₂
10 (1 mM, Sigma), LaCl₃ (100 µM, Sigma), 2-APB (50 µM, Sigma) and bupivacaine
11 (300 µM, Sigma), cortical neurons at DIV 11 were pretreated with each of the
12 reagents at the mentioned concentrations together with z-VAD (20 µM, Sigma)
13 for 1 h, then followed by OGD insult. Control groups were treated with vehicle
14 (DMSO, Sigma) only together with z-VAD for 1 h, followed by OGD insult for the
15 indicated time periods. This dose schedule of drugs was selected based on the results
16 of previous reports (Cai et al., 2014; Lee et al., 2003; Liu et al., 2014; Miao et al.,
17 2013; Vieira et al., 2014).

18 To initiate OGD, neurons were exposed to Dulbecco's modified Eagle's medium
19 (DMEM, Sigma) without serum or glucose but with above reagents in a humidified
20 atmosphere and flushed with a gas mixture of 93% N₂/5% CO₂/2% O₂ for the
21 indicated time periods. Sham control was cultured in normal DMEM with glucose,
22 serum and above reagents maintained in 95% O₂/ 5% CO₂ at 37 °C.

23 Calcium-free or sodium-free minimum essential medium (MEM) was produced
24 according to the MEM formula without adding calcium or sodium. For cation-free
25 medium treatment, neurons were pre-incubated with cation-free medium together with
26 zVAD for 1 h, then exposed to cation-free medium without serum or glucose in a
27 humidified atmosphere and flushed with a gas mixture of 93% N₂/5% CO₂/2% O₂ for
28 the indicated time periods.

29 **RNA interference**

30 Small interfering RNA duplexes (si-RNA) targeting the RIP3 (ID246240)

(si-RIP3, Ribobio, Guangzhou, China), MLKL (ID690743) (si-MLKL, Ribobio), or nonspecific sequences (Scrambled) (si-Scr, Ribobio) without modification were synthesized for cell treatment by Ribobio. Ten nmol of siRNA was transfected into cells. The same concentration of nonspecific sequences was used as scrambled control. Cells were cultured for 24 h and subjected to OGD.

Transmission electron microscopy

Cells were fixed in PBS containing 2% paraformaldehyde/2% glutaraldehyde for 60 min. After washing in the same buffer, cells were gently scraped off, centrifuged, and postfixed with 1% OsO₄, 0.8% potassium ferricyanide, and 5mM CaCl₂ in 0.1M cacodylate buffer. Cells were washed twice in the same buffer, dehydrated in acetone and embedded in PolyBed 812 overnight at 60°C. Ultrathin sections (90 nm) were stained with uranyl acetate and lead citrate and observed under a H-600IV transmission electron microscope (Hitachi, Japan).

Detection of cell death using propidium iodide (PI) staining

PI (1 mg/ml; Sigma) and Hoechst 33258 (10 mg/ml; Sigma) were added to media and incubated with cells for 5 min. For quantification of PI positive cells, photographs were randomly taken from three individual ×200 fields per well. There were 6 wells per experimental condition. PI positive cells were expressed as a percentage of Hoechst positive cells.

Cell membrane leakage assay

Cell membrane leakage assay was performed using the CytoTox-Glo™ Cytotoxicity Assay Kit according to the manufacturer's instructions (Promega, Fitchburg, Wisconsin, USA). Luminescence was recorded with a microplate reader (Thermo Varioskan Flash).

Immunofluorescent staining

Neurons on cover slip were blocked (1× PBS, 2% normal goat serum and 0.1% Triton X-100) for 1 h, then were incubated overnight at 4 °C with the following primary antibodies: anti-RIP1 antibody (1:400; Abcam, Cambridge, MA, USA) , anti-RIP3 antibody (1:400; Abcam) and anti-MLKL antibody (1:400; Abcam). The neurons were washed three times with 0.1 M PBS and then incubated with Alexa

1 Fluor 488 anti-rabbit IgG (1:400; Life) for 1 h at room temperature, then were
2 photographed for green fluorescence with a fluorescent microscope (Leica, Germany).

3 **Crude cell membrane fraction**

4 Cells (2.5×10^6 cells) were detached from the culture plates with a non-enzymatic
5 solution consisting of HBSS (Sigma) with 1mM EDTA (Sigma) and washed twice
6 with PBS (Sigma). Cells were re-suspended in 1ml of the fractionation buffer (250
7 mM sucrose, 20 mM HEPES at pH7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA
8 and 1 mM EGTA) and placed on ice for 10 min. Cells were disrupted by freezing with
9 liquid nitrogen for 5 min and then were thawed on ice, repeated three times. The
10 lysates were passed through a 25G needle (BD Biosciences, New Jersey, USA) for 10
11 times. Nuclei and unbroken cells were removed by centrifugation at 750g for 5 min.
12 The supernatant was collected and centrifuged again at 10,000g for 5 min. The
13 supernatant was then centrifuged at 100,000g in an Optima TLX Ultracentrifuge
14 (Beckman Coulter, California, USA) for 1 h at 4 °C. The supernatant containing
15 cytosolic proteins were concentrated using an acetone precipitation method. The
16 pellets containing membrane proteins were washed with the fractionation buffer and
17 were re-centrifuged at 100,000g for 45 min. The pellets were collected and lysed in
18 M2 buffer (20 mM Tris, pH7, 0.5% NP40, 250 mM NaCl, 3 mM EDTA, 3 mM
19 EGTA, 2 mM DTT, 0.5 mM phenylmethylsulphonyl fluoride, 20 mM glycerol
20 phosphate, 1 mM sodium vanadate and 1 µg/ml leupeptin). For reducing gel analysis,
21 normal SDS-PAGE was performed as described below. For non-reducing gel analysis,
22 cells were lysed in M2 buffer without DTT and separated by SDS-PAGE without
23 mercaptoethanol.

24 **Western blotting**

25 Cells were lysed in ice-cold lysis buffer (10 mM Tris-HCl, pH 7.8, 100 mM
26 NaCl, 10 mM EDTA, 0.5% Nonidet P-40, and 0.5% sodium deoxycholate)
27 supplemented with protease and phosphatase inhibitors. Homogenates were
28 maintained in ice for 30 min and then centrifuged at 15,000 g for 10 min at 4°C, the
29 supernatant was recovered. For fragmentation, cytosolic and mitochondrial extracts
30 were prepared using the ProteoExtract Cytosol Mitochondria Fraction Kit

(Calbiochem, San. Diego, CA, USA) according to the manufacturer's protocol. Protein concentration was determined by BCA protein assay kit (Life). Proteins were resolved in SDS-PAGE (10% polyacrylamide), transferred to PVDF membrane and incubated with primary antibodies. The reactions were followed by incubation with secondary antibodies peroxidase labelled (Life). Primary antibodies used were: DRP1 (D8H5) Rabbit mAb (1:1000, CST, Danvers, MA, USA), Phospho-DRP1 (Ser616) (D9A1) Rabbit mAb (1:1000, CST), Phospho-DRP1 (Ser637) (D3A4) Rabbit mAb (1:1000, CST), anti- β -actin (1:5000, Abcam), anti-COX4 (1:1000, Abcam), anti- Na^+ - K^+ -ATPase (1:400, Santa Cruz).

Immunoprecipitation

Cells were homogenized in cold lysis buffer, lysis was precleared by adding resuspended Protein A/G PLUS-Agarose (Santa Cruz), incubate at 4°C for 30 min, centrifuged for 8 min at 2,500g, the pellet was discarded. Protein (250ug) was combined with anti-MLKL antibody (2 μ g, Abcam) and incubated overnight, gently mixing at 4°C. Immobilized protein A (Life) 100 μ L was added to the antigen-antibody complex and gently mixed at room temperature for 2 h. To remove any unbound protein, the samples were washed 4 times with 0.5mL of immunoprecipitation buffer (Life) and centrifuged for 3 min at 2,500g. The supernatant was discarded after each wash. The pellet was washed with 0.5mL distilled H_2O , centrifuged for 3 min at 2,500g, and the supernatant discarded. The beads were resuspended in 50 μ L of 2 $\times$ treatment buffer and boiled for 5 min, then centrifuged at 14,000g for 5 seconds. The supernatant (20 μ L per lane) was loaded onto a gel for SDS-PAGE. Blots were incubated overnight at 4°C with anti-RIP1, anti-RIP3, or anti-MKLR, then exposed to corresponding secondary antibody and developed with enhanced chemiluminescence. The same amount of irrelevant mouse IgG (Santa Cruz) was used as the nonspecific binding control.

Animal model of HI

Postnatal day 10 Sprague-Dawley rats were anesthetized and then subjected to hypoxia and ischemia using previously described methods (Qu et al., 2009). Briefly,

1 the right common carotid artery (CCA) was exposed and ligated with a 7-0 silk suture
2 through a midline cervical incision. Surgery lasted less than 5 min. Rats with
3 excessive bleeding were excluded. After ligation of the CCA, the rats were returned to
4 the cage for 1 h to recover from anesthesia. Rats were then exposed to hypoxia (8%
5 O₂/92% N₂) for 2.5 h, and returned to their cage. Sham control rats were subjected to
6 isolation and stringing of vessels without occlusion and subsequent ischemia. Body
7 temperature of the rats was maintained at about 37°C using an infrared heating lamp
8 and monitored with a thermal probe. Rats were sacrificed at the indicated time points
9 after HI and their brains were removed for analysis.

10 For in vivo gene delivery, rat pups immediately after right common carotid
11 artery were given a single intracerebroventricular (ICV) injection of methyl and
12 cholesterol modified small interfering RNA duplexes (si-RNA) targeting the MLKL
13 (ID690743) (si-MLKL, Ribobio), or nonspecific sequences (Scrambled) (si-Scr,
14 Ribobio), followed by hypoxia treatment as described above. For each rat, 1nmol
15 si-RNA plus 0.5 nmol transfection control (Ribobio) were complexed, and injected
16 into the lateral ventricle using a Hamilton syringe with a 26-gauge needle. Cy3 was
17 used to monitor success of transfection. Protein level was detected through western
18 blotting and immunoprecipitation as described above used in neurons.

19 For in vivo PI detection, PI (120 ng, Sigma) was administered via tail vein 5 h
20 prior to sacrifice. Following tissue sectioning, brain sections were imaged by
21 fluorescent microscope.

22 **Triphenyltetrazolium chloride (TTC) staining**

23 Brain infarct volume was measured through TTC staining using the method
24 described by Liu et al (Liu et al., 2009). Briefly, brains were chilled at -80°C for 4
25 min. Then five of 2 mm coronal sections were made from the olfactory bulb to the
26 cerebellum and then stained with 1.5% TTC (Sigma) at 37°C for 15 min. After
27 staining, the sections were scanned and measured using an image analysis software
28 (NIH image, USA) to determine the ischemic infarct volume in a blinded manner.
29 Total infarct area was multiplied by the thickness of the brain sections to obtain

infarct volume as described previously (Khan et al., 2005). In order to minimize the error introduced by edema, an indirect method for calculating infarct volume was used (Swanson et al., 1990). The non-infarcted area in the ipsilateral hemisphere was subtracted from that in the contralateral hemisphere, and infarct volume was calculated using the following formula: corrected percentage of infarct volume = (contralateral hemispheric volume – ipsilateral non-infarcted volume) / contralateral hemispheric volume.

Neurological severity score (NSS)

The severity of neurological damage was evaluated using Chen's NSS system (Chen et al., 2001). Neurologic function was graded on a scale of 0-18 (0, normal score; 18, maximal deficit score). The NSS is a composite of motoric, sensory, reflex, and balance assessments. In the severity score of injury, one point was awarded for the inability to perform a task or for the lack of an assessed reflex; thus, the more severe the injury, the higher the score. The scores of all rats were recorded 21 days after HI.

Statistical analysis

Data are presented as mean $\pm$ S.E.M from 3 independent experiments. Student's t-test was used when comparing between two groups. Analysis of Variance (ANOVA) with Fisher's post hoc test was used when comparing more than two groups. The value of p less than 0.05 was defined as the threshold for significance.

Results

Oxygen-glucose deprivation plus caspase inhibitor zVAD treatment (OGD/zVAD) induced a component of necroptotic neuronal death

MAP2 and DAPI double fluorescent staining showed that the neurons were successfully cultured in vitro with high purity (96%) (Fig.1A). Then we investigated whether necroptotic neuronal death occurs when neurons are submitted to OGD, an in vitro model of global ischemia. Meanwhile, zVAD, a broad-spectrum caspase inhibitor, was used to facilitate cell death from apoptosis to necrosis. As the result, OGD/zVAD insult induced membrane permeability that is seen as PI positive staining (Fig.1B). On electron microscopy, a morphological pattern between classical

1 apoptosis and classical necrosis was shown in cell death, with accumulation of
2 cytoplasmic vacuoles as seen in classical necrosis, whereas the nuclei underwent
3 progressive compaction of chromatin into few, discrete, large clumps as seen in
4 classical apoptosis (Fig.1C). When we used the CytoTox-Glo kit to detect the protease
5 activity in cell culture media, we also found membrane leakage of neurons induced by
6 OGD or OGD/zVAD. After 4 h of insult, the cell death reached 60% in OGD neurons
7 and 70% in OGD/zVAD neurons (Fig.1D).

8 **OGD/zVAD upregulated the expressions of RIP1, RIP3 and the interaction of** 9 **RIP1-RIP3-MLKL**

10 Since RIP1-RIP3-MLKL interaction is regarded as the critical step in the
11 formation of the necrosome leading to necroptosis (Wang et al., 2012), we
12 investigated RIP1-RIP3-MLKL interaction in neurons subjected to OGD/zVAD
13 treatment. Immunofluorescent staining demonstrated increased RIP1 and RIP3
14 expression after OGD/zVAD compared to the control (Fig.2A). MLKL expression
15 was not obviously changed after OGD/zVAD (Fig.2A). Immunoprecipitation with
16 anti-MLKL showed an increase in RIP1-RIP3-MLKL interaction after OGD/zVAD
17 insult (Fig.2B).

18 **Down-regulation of RIP1-RIP3-MLKL interaction attenuated neuronal death** 19 **after OGD/zVAD**

20 Since RIP1-RIP3-MLKL interaction was significantly upregulated in neurons
21 subjected to OGD/zVAD insult, we next examined the possible roles of
22 RIP1-RIP3-MLKL interaction in regulating neuronal death after OGD/zVAD insult.
23 We first determined whether the RIP1 inhibitor, necrostatin-1 (Nec-1), could affect
24 neuronal death after OGD/zVAD insult. Immunoprecipitation revealed Nec-1
25 significantly attenuated RIP1-RIP3-MLKL interaction, and the neuronal death was
26 decreased. (Fig.3A). We then examined the possible functions of RIP3 and MLKL in
27 this model. Western blotting analyses and immunoprecipitation showed that RIP3
28 siRNA or MLKL siRNA treatment significantly reduced RIP3 or MLKL expression,
29 RIP1-RIP3-MLKL interaction, as well as the neuronal death after OGD/zVAD insult
30 (Fig.3B, 3C).

OGD/zVAD induced neuronal death was independent of reactive oxygen species (ROS) accumulation, or calcium and sodium influx

Previous work have shown that ROS accumulation is required for RIP3-mediated necrosis in certain cell lines such as mouse embryonic fibroblast (He et al., 2011; Zhang et al., 2009), so we evaluated the role of ROS in OGD/zVAD induced neuronal death by using two widely used ROS scavengers, butylated hydroxyanisole (BHA) and N-acetylcysteine (NAC). In the presence of BHA or NAC, neurons underwent cell death in response to OGD/zVAD, while BHA and NAC had no inhibitory effect on OGD/zVAD induced cell death of neurons (Fig.4A).

Recently, calcium or sodium influx into cells has been reported as a downstream executor of MLKL to induce necroptosis (Cai et al., 2014; Chen et al., 2014). We tested whether calcium or sodium influx is involved in OGD/zVAD induced neuronal death. It showed that neurons cultured in either calcium or sodium free media were still able to undergo cell death (Fig.4B); the L-type voltage-dependent calcium channel blocker nifedipine, the T-type voltage-dependent calcium channel blocker NiCl_2 , the non-voltage-sensitive channel blockers LaCl_3 and 2-APB, or sodium channel blocker bupivacaine did not protect neurons from OGD/zVAD induced cell death (Fig.4C).

MLKL inhibition did not affect dynamic-related protein 1 (Drp1) translocation to mitochondria

Drp1 has been regarded as an important down-stream effector of the RIP1-RIP3-MLKL necrosome; its phosphorylation states have been reported to be critical to determine its translocation to mitochondria and execution of necroptosis (Zhang et al., 2013). Therefore, we next investigated whether MLKL inhibition could affect Drp1 translocation to mitochondria in neurons after OGD/zVAD insult. Western blotting showed that total Drp1 in cytoplasm increased 4 h after OGD/zVAD, but total Drp1 in mitochondria was not changed after OGD/zVAD insult. The phosphorylation of Ser 616 and Ser 637 in Drp1 was not affected by OGD/zVAD insult and inhibition of MLKL (Fig.5).

MLKL inhibition attenuated oligomerization of MLKL in neuronal membrane

fraction after OGD/zVAD

MLKL oligomerization and membrane translocation have been reported to mediate necroptosis in human embryonic kidney 293 cells (Cai et al., 2014). Therefore we analyzed MLKL protein in membrane or non-membrane fractions of neuronal lysis by SDS-PAGE under nonreducing conditions. As shown in Fig.6, MLKL was largely in non-membrane fraction as monomers (about 50 kDa) under normal culture conditions, but formed an oligomer above 250 kDa in membrane fractions when neurons were subjected to OGD/zVAD. Moreover, we also found that MLKL inhibition through siRNA attenuated oligomerization of MLKL in neuronal membrane fraction after OGD/zVAD (Fig.6).

MLKL inhibition attenuated brain damage and promoted neurological recovery in rat HI models

We next examined the function of MLKL in vivo. siRNA technology was applied to inhibit MLKL. Western blotting and immunoprecipitation showed that MLKL siRNA notably inhibited MLKL expression and RIP1-RIP 3-MLKL interaction in rat cortex and hippocampus subjected to HI, whereas scrambled control did not show these effects (Fig.7A). Representative PI staining of cortex and hippocampus were recorded using a fluorescence microscope, it showed that PI staining was obviously weaker in the MLKL siRNA group than in scrambled control 48 h after HI (Fig.7B). TTC staining showed that HI caused severe infarcts in rat brains, with more damage in males than in females. MLKL siRNA decreased the infarct volume from about 50% to 25% in females and from about 75% to 22% in males 7 days after HI (Fig.7C).

Since MLKL inhibition attenuated brain damage after HI, we then determined the long term effects of MLKL inhibition on neurological outcome. NSS evaluation 21 days after HI showed that the NSS was significantly higher in HI group compared with sham group, without obvious difference in females and males. MLKL inhibition lowered the NSS. There was no obvious difference of NSS after MLKL inhibition in males and females, showing equivalent long term effects of MLKL inhibition without a gender difference (Fig.7D).

Discussion

1 In the present study, we found that necroptotic neuronal death occurred when
2 neurons were submitted to OGD, an in vitro model of global ischemia. OGD/zVAD
3 insult upregulated the expressions of RIP1 and RIP3, and the interaction of
4 RIP1-RIP3-MLKL. Inhibition of MLKL diminished RIP1-RIP3-MLKL interaction
5 and attenuated neuronal death induced by OGD/zVAD insult. The mechanism
6 involved the translocation of oligomerized MLKL to the neuronal membrane after
7 OGD/zVAD, but was independent of ROS accumulation, calcium or sodium influx,
8 and Drp1 translocation to mitochondria. Neonatal rats subjected to HI further showed
9 that MLKL inhibition reduced brain damage. This is the first study that suggests
10 MLKL plays a key role in mediating hypoxia-ischemia induced brain damage in
11 neonatal rats.

12 The interaction of RIP1-RIP3-MLKL constitutes the core compartment of
13 necrosis signaling complex, the necrosome. The execution mechanism downstream of
14 MLKL in necroptosis is very complicated, with tissue and cell type specificity. Drp1
15 has been reported to be one of the crucial downstream executors of MLKL. The
16 formation of RIP1-RIP3-MLKL interaction recruits phosphoglycerate mutase family
17 member 5 (PGAM5) long form to the necrosome and forms a complex, then the
18 complex transfers and binds PGAM5 short form in the mitochondria, where PGAM5
19 may activate Drp1. Drp1 undergoes a number of steps to mediate mitochondrial
20 fission, including translocation from the cytoplasm to the mitochondrial outer
21 membrane, higher-order assembly into spirals, GTP hydrolysis associated with a
22 conformational change and membrane deformation, and ultimately disassembly (Cho
23 et al., 2013). Dephosphorylation of Drp1 in Ser 637 has been shown to promote
24 translocation of the cytosolic Drp1 to mitochondria and concurrent fission events
25 (Cribbs and Strack, 2007). Another experiment showed that mitochondrial dynamics
26 proceed with a fission process, produced by Drp1 phosphorylation in Ser 616 (Godoy
27 et al., 2014). Following mitochondrial dysfunction, ROS production might increase
28 and enhance necroptosis. (Ye et al., 2012). In the present study, we found that
29 although OGD/zVAD enhanced Drp1 expression, it did not increase the translocation

1 of Drp1 to mitochondria, nor were the phosphorylation states at both sites (Ser 616
2 and 637) affected by the OGD/zVAD insult, or MLKL inhibition, suggesting that
3 Drp1 is not the critical executor of cell death downstream of MLKL in
4 hypoxia-ischemia induced neuronal damage. Meanwhile, we found that ROS
5 scavengers BHA and NAC had no inhibitory effect on OGD/zVAD induced neuronal
6 death, suggesting that OGD/zVAD induced neuronal death is independent of ROS
7 accumulation.

8 Recent reports reveal that plasma membrane translocation of oligomerized MLKL
9 protein is another critical event mediating necroptosis (Cai et al., 2014; Chen et al.,
10 2014). Cai et al (2014) found that MLKL forms a homotrimer through its
11 amino-terminal coiled-coil domain and locates to the cell plasma membrane during
12 necroptosis, where it activates transient receptor potential melastatin related 7
13 (TRPM7), which mediates Ca^{2+} influx and induces necroptosis. Chen et al found that
14 upon phosphorylation by RIP3, MLKL forms tetramers and translocates from
15 cytoplasm to lipid rafts of the plasma membrane, and the plasma membrane MLKL
16 complex acts either by itself or via other proteins to increase the sodium influx, which
17 increases osmotic pressure, eventually leading to membrane rupture (Chen et al.,
18 2014). In the present study, we found that neurons cultured in either calcium or
19 sodium free media were still able to undergo death, and the channel blockers of
20 calcium and sodium did not protect neurons from death, suggesting that OGD/zVAD
21 induced neuronal death is independent of calcium or sodium influx. However, we did
22 find that MLKL formed tetramers and translocated from cytoplasm to the neuronal
23 membrane after OGD/zVAD insult. Recently, Wang et al reported that
24 phosphorylated MLKL can form an oligomer and move from the cytosol to the
25 plasma and intracellular membranes where it binds to phosphatidylinositol lipids and
26 cardiolipin, inserts deeply into the bilayers of membrane, and directly disrupts
27 membrane integrity, finally resulting in cell death (Wang et al., 2014). Therefore, we
28 suggest that although the oligomerization and translocation of MLKL in neurons
29 subjected to OGD/zVAD insult does not change ion channels, it might induce
30 neuronal death through disrupting membrane integrity directly.

1 Gender differences have been found in neonatal rodent models of HI brain injury
2 (Zhu et al., 2006). We analyzed cohorts of male and female mice in the animal
3 experiments. We found that HI caused more severe infarcts in males than in females,
4 and the infarct volume decreased to almost the same after MLKL inhibition,
5 suggesting MLKL inhibition provided significant efficacy both in males and females,
6 providing MLKL as a potential target to attenuate hypoxia-ischemia induced brain
7 damage in neonates.

8

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Author contribution

DM, YQ designed the study; YQ, JS, YT, FZ, SL and MJ performed cell culture, oxygen-glucose deprivation, drug administration, gene delivery, and western blotting; SL performed and analyzed immunoprecipitation; XL and XP performed immunostaining and electron microscopy; JS, XL and FZ performed triphenyltetrazolium chloride staining and neurological severity score evaluation. We thank Donna M. Ferriero for her critical evaluation of this manuscript.

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Figure legends

Figure 1. OGD/zVAD induces a component of necroptotic neuronal death.

A. MAP2 and DAPI double fluorescent staining showed that the neurons were successfully cultured in vitro with high purity. Most of the DAPI positive cells are also MAP2 positive (Scale bar = 40 μ m).

B. Representative images and quantification of PI-positive neurons treated with OGD/zVAD (20 μ M) for 4 h. Photographs were randomly taken from three individual $\times 200$ fields per well. There were 6 wells per experimental condition. PI positive cells were expressed as a percentage of Hoechst positive cells. Data are presented as mean $\pm$ S.E.M from 3 independent experiments (Scale bar = 20 μ m) (* $p < 0.05$, comparing the OGD or OGD/zVAD group and the sham group).

C. Detection of the neurons under EM demonstrated a morphological pattern between classical apoptosis and classical necrosis where an accumulation of cytoplasmic vacuoles occurred as seen in classical necrosis, whereas nuclei underwent progressive compaction of chromatin into few, discrete, large clumps as similar as classical apoptosis, in contrast with the formation of numerous, smaller, irregular chromatin clumps observed in classical necrosis (Scale bar=4 μ m).

D. Cell death was determined by measuring released protease activity in culture medium. The data are represented as the mean $\pm$ S.M.E from 3 independent experiments, $n=6$ for each group in each experiment.

Figure 2. OGD/zVAD upregulates the expressions of RIP1 and RIP3, and the interaction of RIP1-RIP3-MLKL.

A. Immunofluorescence staining showed increased RIP1 and RIP3 expression after OGD/zVAD insult compared to the sham, whereas MLKL expression was not obviously changed (Scale bar=40 μ m). S: sham; O/z: OGD/zVAD.

B. MLKL was immunoprecipitated from neuronal lysates to detect the interaction with RIP1 or RIP3. The interaction of RIP1-RIP3-MLKL was increased after OGD/zVAD insult. Data are presented as mean $\pm$ S.E.M from 3 independent experiments, $n=6$ for each group in each experiment ($p < 0.05$, compared to the sham

1 control). S: sham; O/z: OGD/zVAD.

2 **Figure 3. Down-regulation of RIP1-RIP3-MLKL interaction attenuated**
3 **neuronal death after OGD/zVAD.**

4 **A.** Immunoprecipitation showed that RIP1-RIP3-MLKL interaction was decreased
5 after Nec-1 treatment. Meanwhile, neuronal death determined by measuring released
6 protease activity in culture medium was significantly decreased after Nec-1 treatment.
7 Data are presented as mean $\pm$ S.E.M from 3 independent experiments, n=6 for each
8 group in each experiment ($p<0.05$, comparing the Nec-1 group and the OGD/zVAD
9 group). S:sham; O/z: OGD/zVAD; N: Nec-1; Ni: Nec-1 inhibitor.

10 **B.** Western blotting showed that RIP3 siRNA treatment significantly reduced RIP3
11 expression. Immunoprecipitation assay showed that RIP1-RIP3-MLKL interaction
12 was decreased after the inhibition of RIP3 through RIP3 siRNA treatment. Meanwhile,
13 neuronal death determined by measuring released protease activity in culture medium
14 was significantly decreased after RIP3 inhibition. Data are presented as mean $\pm$ S.E.M
15 from 3 independent experiments, n=6 for each group in each experiment ($p<0.05$,
16 comparing the siRNA group and the OGD/zVAD group). S: sham; O/z: OGD/zVAD;
17 Si: RIP3 siRNA; Sc: RIP3 siRNA scramble.

18 **C.** Western blotting showed that MLKL siRNA treatment significantly reduced
19 MLKL expression. Immunoprecipitation assay showed that RIP1-RIP3-MLKL
20 interaction was decreased after the inhibition of MLKL through MLKL siRNA
21 treatment. Meanwhile, neuronal death determined by measuring released protease
22 activity in culture medium was significantly decreased after MLKL inhibition. Data
23 are presented as mean $\pm$ S.E.M from 3 independent experiments, n=6 for each group in
24 each experiment ($p<0.05$, comparing the siRNA group and the OGD/zVAD group). S:
25 sham; O/z: OGD/zVAD; Si: MLKL siRNA; Sc: MLKL siRNA scramble.

26 **Figure 4. OGD/zVAD induced neuronal death is independent of ROS**
27 **accumulation, or calcium and sodium influx.**

28 **A.** Neurons were treated with vehicle, BHA or NAC at the indicated concentration
29 together with zVAD 1 h before OGD insult, cell death was determined by measuring
30 released protease activity in culture medium. Data are presented as mean $\pm$ S.E.M

from 3 independent experiments, n=6 for each group in each experiment. S: sham; O/z: OGD/zVAD; V: vehicle.

B. Neurons were cultured in MEM with or without calcium or sodium and subjected to OGD/zVAD insult. Neuronal death was determined by measuring released protease activity in culture media. Data are presented as mean $\pm$ S.E.M from 3 independent experiments, n=6 for each group in each experiment.

C. Neurons were treated with vehicle, nifedipine (100 μ M), NiCl₂ (1 mM), LaCl₃ (100 μ M), 2-APB (50 μ M) or bupivacaine (300 μ M) together with zVAD 1 h before OGD insult, cell death was determined by measuring released protease activity in culture medium. Data are presented as mean $\pm$ S.E.M from 3 independent experiments, n=6 for each group in each experiment. S: sham; O/z: OGD/zVAD; V: vehicle.

Figure 5. MLKL inhibition did not affect Drp1 translocation to mitochondria.

Western blotting showed that OGD/zVAD insult increased total Drp1 in cytoplasm but not in mitochondria 4 h after OGD/zVAD; MLKL inhibition did not decrease total Drp1 both in cytoplasm and mitochondria. The level of phosphorylated Drp1 (p-Ser 616 and p-Ser 637) was not changed by OGD/zVAD insult both in cytoplasm and mitochondria, and MLKL inhibition did not affect the level of phosphorylated Drp1 in either cytoplasm or mitochondria. The absence of COX4 in the cytoplasm indicated lack of contamination between cytoplasmic and mitochondrial fractions. S: sham; O/z: OGD/zVAD; Si: MLKL siRNA; Sc: MLKL siRNA scramble.

Figure 6. MLKL inhibition attenuated oligomerization of MLKL in neuronal membrane fraction after OGD/zVAD.

Neurons were subjected to OGD/zVAD, then the total, non-membrane and membrane fractions were resolved either on reducing or non-reducing gel and analysed by western blotting. It showed that MLKL siRNA treatment significantly attenuated oligomerization of MLKL in neuronal membrane fraction after OGD/zVAD. The absence of Na⁺-K⁺-ATPase in the non-membrane fraction indicated that there's no contamination between non-membrane and membrane fractions. T, total cell lysate; NM, non-membrane fraction; M, membrane fraction; S: sham; O/z: OGD/zVAD; Si:

1 MLKL siRNA; Sc: MLKL siRNA scramble.

2 **Figure 7. MLKL inhibition attenuated brain damage in a rat HI model.**

3 **A.** Western blotting showed that MLKL siRNA treatment significantly reduced
4 MLKL expression in both cortex and hippocampus. Immunoprecipitation assay
5 showed that RIP1-RIP3-MLKL interaction was decreased after the inhibition of
6 MLKL through MLKL siRNA treatment. Data are presented as mean $\pm$ S.E.M from 3
7 independent experiments, n=6 for each group in each experiment ($p < 0.05$, comparing
8 the siRNA group and the HI group). S: sham; HI: hypoxia-ischemia; Si: MLKL
9 siRNA; Sc: MLKL siRNA scramble.

10 **B.** Representative PI staining images of cortex and hippocampus were recorded using
11 a fluorescence microscope (Scale bar=60 μ m). It showed that PI staining was
12 obviously weaker in the MLKL siRNA group than in scrambled control 48 h after HI.
13 S: sham; HI: hypoxia-ischemia; Si: MLKL siRNA; Sc: MLKL siRNA scramble.

14 **C.** Representative TTC staining images of brain sections and infarct volume by
15 gender were shown. HI caused severe infarcts in rat brains, with more damage in
16 males than in females. MLKL siRNA decreased the infarct volume from about 50% to
17 25% in females and from about 75% to 22% in males 7 days after HI. The data shown
18 were the means $\pm$ S.E.M. from 3 independent experiments, n=6 for each group in each
19 experiment (* $p < 0.05$, comparing the siRNA group and the HI or scrambled control
20 groups). S: sham; HI: hypoxia-ischemia; Si: MLKL siRNA; Sc: MLKL siRNA
21 scramble.

22 **D.** NSS evaluation 21 days after HI showed that the NSS was significantly higher in
23 HI group compared with sham group (* $p < 0.05$), without obvious difference in
24 females and males. MLKL inhibition significantly lowered NSS (* $p < 0.05$, comparing
25 the siRNA group and the HI or scrambled control groups). There was no obvious
26 difference of NSS after MLKL inhibition in males and females. Data are presented as
27 mean $\pm$ S.E.M from 3 independent experiments, n=8 for each group in each
28 experiment. M: male; F: female; S: sham; HI: hypoxia-ischemia; Si: MLKL siRNA;
29 Sc: MLKL siRNA scramble.

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