Supplemental Material

Materials and Methods

Trypan blue assay

Trypan blue assay was used to analyze the mortality of NPCs in response to H_2O_2 induced ROS stress. In brief, neurospheres were incubated in maintenance medium with 50 μmol/L H_2O_2 for 24 h. Then, neurospheres were collected and dissociated into single cells, incubated in 0.04% trypan blue staining solution (Solabio) for 5 min, and analyzed using a Cell Counter (JIMBIO-FIL) detect trypan blue positive cells. Cell mortality was calculated as the percentage of trypan blue positive cells to total cells.

Cell proliferation assay

The proliferation of NPCs was tested using a 5-ethynyl-2´-deoxyuridine (EdU) assay kit (Beyotime). NPCs cultured as neurospheres were incubated in maintenance medium with EdU (diluted to recommended concentration by manufacturer's instruction) for 2 h to label proliferating cells. Then, neurospheres were dissociated, planted on a poly-L-ornithine and laminin coated glass slide at 5×10^4 cells/cm^2, and fixed by 4% paraformaldehyde (PFA) (Beyotime). The incorporation of EdU into nucleus was detected as described in the manufacturer's instructions. Fluorescence images were obtained using a Nikon A1 confocal microscope system and randomly selected scopes were captured using a 20 x objective lens. Results were analyzed by ImageJ software (National Institutes Health).

Glucose uptake assay

^{18}F-FDG, a radioactive glucose analog, was used to test glucose uptake of NPCs or neurons [1]. In brief, NPCs or neurons cultured in 6 well plates were incubated in culture medium supplemented with ^{18}F-FDG (provided by Department of Nuclear Medicine, the Second Affiliated Hospital of Zhejiang University School of Medicine) at the concentration of 200 kBq/ml under humidified atmosphere with 5% CO_2 at 37°C for 40min. After rapid washing with phosphate buffer saline (PBS) (Hyclone), NPCs or neurons were dissociated from the culture plates and collected into a centrifuge tube. The radioactivity of ^{18}F-FDG was assessed by wizard 2 γ counter (Perkin Elmer).

Measurement of glycolysis and mitochondrial respiration

Glycolysis and mitochondrial metabolism were measured by extracellular acidification rate (ECAR) and oxygen consumption rate (OCR), respectively, using a Seahorse XF96 analyzer (Seahorse Bioscience) [2, 3]. Briefly, NPCs or neurons were cultured on XF96 well plates coated by L-ornithine and laminin at a density of 10000 cells/well, and then incubated in XF base medium (Seahorse Bioscience) containing Glutamax (1x, Gibco), D-Glucose (10 mmol/L, Sigma), and pyruvate sodium (1 mmol/L, Sigma) at 37℃ in the absence of CO_2 for 1 h before seahorse analysis. During seahorse analysis, basal OCR or ECAR and their changes in response to the sequential injection of 1 μmol/L oligomycin (Sigma), 1 μmol/L FCCP (MCE) and a mixture of 5/1 μmol/L rotenone/antimycin A (MCE) was tested by 3 repeated measurements with a 3 minutes interval, respectively. The basal respiration and the basal glycolysis were

determined as the mean value of three measurements before the injection of oligomycin.

Quantitative real-time PCR (qRT-PCR)

To analyze the mRNA level of PGC-1, differently treated cells (Control/hcNPC/hcNPC + Res) were harvested using TRIzol™ Reagent (Invitrogen) in the light of the manufacturer's protocols at Week 2 after neuronal differentiation. RNA was quantified with NANO drop 2000 (Thermo), and 1μg RNA was reverse-transcribed into cDNA using the PrimeScript™ RT Master Mix reverse transcription System (Takara). The relative levels of PGC-1α and PGC-1β expression were analyzed by qRT-PCR with TB Green™ Premix Ex Taq™ II (Takara) using the iCycler iQ5 (Bio-Rad). The gene GAPDH was used for internal normalization and the relative quantification of gene expression was calculated according to the △CT method. The qRT-PCR primers were as follows:

PGC-1α sense, 5'-CCAAAGGATGCGCTCTCGTTCA-3';

antisense, 5'-CGGTGTCTGTAGTGGCTTGACT-3';

PGC-1β sense, 5'-TGAGCAGACCTTGACAGTGGAG-3';

antisense, 5'-GACTATGCTTGATGTCTGGTTTGA-3'.

Assessment of mitochondrial membrane potential (Δψm)

Δψm dependent dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbo-cyanineiodid (JC-1) and

tetramethylrhodamine methyl ester (TMRM) were used in this study to assess Δψm in NPCs and neurons, respectively [4]. Briefly, JC-1 (Beyotime) and TMRM (AAT Bioquest) were diluted in Tyrode's solution (in mmol/L: 129 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 HEPES, 30 Glucose, pH 7.4) to a final concentration of 15 μmol/L and 100 nmol/L, respectively. Then, NPCs or neurons were incubated in dye solution for 20 min under a humidified atmosphere of 5% CO_2 at 37°C, followed by 3 times rinse by Tyrode's solution. Nucleus were stained by Hochest 33342 (Beyotime) diluted in Tyrode's solution at 1 μg/ml for 20 min.

Fluorescence image of JC-1 and TMRM staining were captured under Zeiss LSM800 confocal system and Nikon A1 confocal system, respectively. Δψm was assessed by the red/green fluorescence (aggregates/monomer) ratio of JC-1, and by the red fluorescence intensity of TMRM.

Calcium imaging

The time-lapse fluorescence imaging of Fluo-4, a Ca^{2+} indicator, was used to analyze calcium homeostasis of neurons [5]. Neurons cultured in glass bottom dish were incubated in 2 μmol/L Fluo-4 AM (Beyotime) diluted in Tyrode's solution supplemented with 0.02% Pluronic F-127 for 40 min under a humidified atmosphere of 5% CO_2 at 37°C. After a 20 min interval, fluorescence imaging was performed by an Olympus IX81-FV1000 confocal microscope system. Firstly, the basal Fluo-4 signal of neurons was recorded. Then, neurons were exposed to high KCL Tyrode's solution (in mmol/L: 44 NaCl, 90 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 HEPES, 30 Glucose, pH

7.4) to induce neuron depolarization. Fluo-4 fluorescence change in response to depolarization stimuli was recorded every 2 s using time-lapse imaging, and was quantified by Image J software.

Immunocytofluorescence assay

Immunocytofluorescence assay was performed to determine neuronal fate commitment of NPCs and synaptic development of neurons. Briefly, NPCs or neurons cultured in poly-ornithine and laminin coated glass bottom dishes were fixed by 4% PFA (Sigma) at 4ºC for 20 min, and then were rinsed 3 times by PBS for 5 min. Fixed cells were permeabilized with 0.2% Triton X-100 (Thermo) in PBS for 10 min, and subsequently incubated in 5% bull serum albumin (BSA) (HaoXin) for 1 h. Then, cells were incubated overnight in primary antibody against TUJ1 (Abcam) or PSD95 (Proteintech) at 4 ºC. After 3 times rinsing in PBS, cells were stained with fluorescence dye labeled secondary antibodies (Abbkine) at room temperature for 1 h. Nucleus were stained by 5 μg/ml DAPI (Fermentas) diluted in PBS. Fluorescence imaging was performed by an Olympus IX81-FV1000 confocal microscope system, and then analyzed by Image J software.

Tissue immunofluorescence staining

Tissue immunofluorescence staining was used to determine the migration and neuronal differentiation of transplanted NPCs in host brain. Rats were transcardially perfused with cold PBS and subsequently with 4% PFA. Fixed rat brain was

dehydrated, embedded in OCT compound (Sakura), and then cut into 20 μm thickness coronal slices at two defined positions (< 100 μm and > 500 μm adjacent to the transplantation site). Brain slices were permeabilized in 0.2% Triton X-100 in PBS for 10 min and blocked with 5% BSA for 60 min. Then brain slices were incubated in primary antibody against TUJ1 (Proteintech) overnight at 4ºC. After 3 times rinses by PBS, brain slices were incubated with fluorescence dye labeled secondary antibodies (Abbkine) for 1 h. DAPI was applied to stain nucleus. Immunofluorescence images of brain section were performed by a Nikon A1 confocal microscope system, and were analyzed by Image J software.

Western blot assay

Western blot was used to analyze the expression change of translocase of mitochondrial outermembrane 20 (TOMM20) during neuronal differentiation of NPCs, and postsynaptic density 95KD (PSD95) during synaptogenesis of neurons. Cultures were lysed by RIPA lysis buffer (Beyotime), and then were tested by an enhanced BCA protein assay kit (Beyotime) to determine the protein concentration of cell lysate. Extracted protein was separated on SDS-PAGE gels (Solarbio) and transferred to polyvinylidene fluoride membranes (Millipore). Membranes were incubated in 3% BSA blocking solution for 60 min, and then incubated overnight at 4°C in primary antibodies against TOMM20 (Proteintech), PSD95 (Proteintech) and β-actin (Proteintech), followed by 60 min incubation with HRP-conjugated secondary antibody (Abbkine). The immunoreactive bands were visualized by ECL detection

reagents (Millipore).

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

To identify the ischemia injury induced by photothrombosis, TTC staining was performed as described previously [6]. Briefly, rats were anesthetized and decapitated. Rat brain was harvested and sliced into 2 mm thickness slices using a Brain Matrix (RWD Life Science Corporation). Brain slices were stained with 2% TTC (Sigma) for 20 min at 37°C and then fixed with 4% PFA solution (Beyotime). Color images of these slices were obtained with a digital camera (Panasonic).

Luminescence imaging

Bioluminescent imaging was performed to trace transplanted NPCs using IVIS Spectrum Imaging System (PerkinElmer) as described previously [7]. Briefly, rats transplanted with NPCs were intraperitoneally administrated with D-luciferin (150 mg/kg body weight, Synchem). From 15 min to 45 min after administration, serial images were acquired with a fixed interval of 3 min. Image with maximum bioluminescent intensity was selected and analyzed with IVIS Live Image System (PerkinElmer). Bioluminescent intensity acquired at Week 2 and Week 4 was expressed as relative change to initial bioluminescent intensity acquired at Day 1.

PMOD analysis

PMOD analysis was used to assess the accurate site of ischemia area after photothrombosis. PET images were reconstructed using a maximum a posteriori 2

(MAP2) algorithm. Reconstructed PET image was registered with T2 magnetic resonance image (MRI) template and aligned with Schiffer rat brain MRI atlas using PMOD fusion tool (version 3.9, PMOD Technologies). Sensory cortex and the adjacent motor cortex were automatically defined on the lesion and contra-lateral normal hemispheres.

Results:

After hypoxia conditioning for 24h, EdU incorporated nucleus was decreased in hcNPCs compared with the control NPCs (Fig. S1), indicating reduced proliferation of hcNPCs.

One day after photothrombosis (Day -6), TTC staining showed the infarcted area on rat brain cortex (Fig. S2A). PMOD analysis of ^{18}F-FDG PET image indicated that photothrombosis induced infarction in the sensory cortex of rat brain, and didn't injure the adjacent area (motor cortex) (Figure S2B). The infarcted area showed reduced ^{18}F-FDG uptake in the region of interest (ROI) on injured hemisphere of rat brain compared with that on the contralateral hemisphere ($P < 0.0001$, Fig. S2C-D). These results confirmed the ischemia injury and metabolic deficiency induced by photothrombosis.

Figures

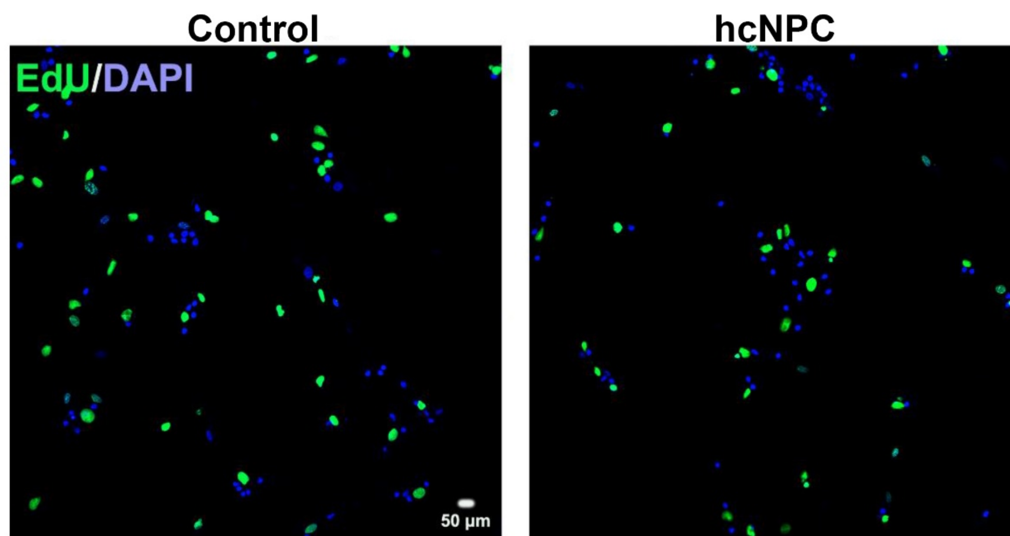

Fig. S1 Representative fluorescence images of EdU immunostaining of the control and hcNPC groups. Nucleus was stained by DAPI and shown in blue. EdU was shown in green.

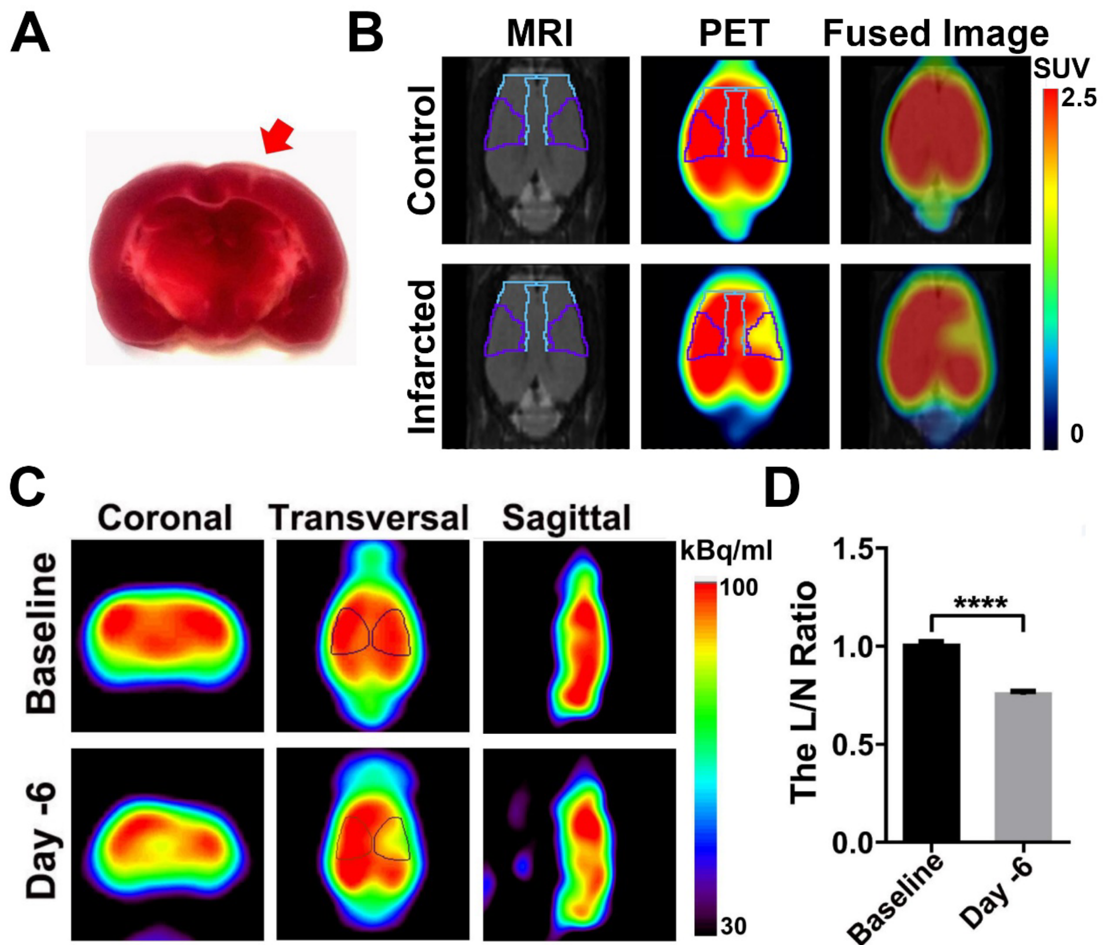

Fig. S2 Ischemia injury and metabolic deficiency induced by photothrombosis on rat brain cortex. A: Representative image of 2, 3, 5-triphenyltetrazolium chloride (TTC) staining on coronal brain slices of stroke rat at Day -6, red arrow indicated the ischemia injury on cortex induced by photothrombosis. B: PMOD analysis proved the infarction on the sensory cortex of rat brain after photothrombosis. The purple line indicated the sensory cortex, the blue line indicated the motor cortex. The whole brain SUV (standard uptake value) was normalized to the SUV of pons. C: Representative PET imaging of the health rat brain and stroke brain at Day -6 after photothrombosis. D: Semi-quantification of the lesion to normal (L/N) ratio of somatosensory cortex on stroke rat brain, n = 5. (mean ± SEM, unpaired *t* test, **** $P < 0.0001$.)

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