ONLINE SUPPLEMENT

Supplemental Materials and Methods

Materials

The following materials were obtained from the source indicated: L-glutamic acid monosodium salt, and lactate dehydrogenase (LDH) cytotoxicity assay kit (Sigma-Aldrich, St. Louis, MO); α-tocotrienol (Carotech Inc, Malaysia); DharmaFECT™1 transfection reagent (Dharmacon RNA Technologies, Lafayette, CO); Absolutely RNA® Miniprep kit (Stratagene, La Jolla, CA); *mir*Vana™ miRNA isolation kit (Ambion, Austin, TX); PicoPure RNA Isolation kit (Arcturus, Sunnyvale, CA); dual-luciferase reporter assay system (Promega Corporation, Madison, WI); calcein acetoxymethyl ester, Lipofectamine™ LTX and PLUS™ reagent (Invitrogen Corporation, Carlsbad, CA); 4-hydroxynonenal (HNE) and monoclonal antibody to MRP1 (Enzo Life Sciences, Plymouth Meeting, PA); rabbit polyclonal MRP1 antibody (Abbiotec, San Diego, CA); Fluoro-Jade® C (Millipore, Billerica, MA).

For cell culture, Dulbecco's modified Eagle medium, fetal calf serum, and penicillin and streptomycin were purchased from Invitrogen Corporation, Carlsbad, CA. Culture dishes were obtained from Nunc, Denmark.

Cell culture

Mouse hippocampal HT4 neural cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in humidified atmosphere of 95% air and 5% CO_2 as described previously[1-3]. *Glutamate treatment*. Immediately before experiments, the culture medium was replaced with fresh medium supplemented with serum and antibiotics. Glutamate (5 mmol/L for primary neurons and 10 mmol/L for HT4 neural cells) was added to the cell culture medium as an aqueous solution. No change in the medium pH was observed in response to the addition of glutamate[2,4]. *α-Tocotrienol (TCT) treatment*. A stock solution of α-TCT (Carotech Inc, Malaysia) was prepared in ethanol. Before experiments, culture medium was replaced with fresh medium supplemented with serum and antibiotic, α-TCT was then added to the culture medium as described in the corresponding legends.

Primary cortical neurons

Cells were isolated from the cerebral cortex of rat feti (Sprague-Dawley, day 17 of gestation; Harlan, Indianapolis, IN) as described previously[1,4]. After isolation from the brain, cells were grown in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 40 μmol/L cystine, and antibiotics (100 μg/ml streptomycin, 100 units/ml penicillin, and 0.25 μg/ml amphotericin). Cultures were maintained at 37°C in 5% CO_2 and 95% air in a humidified incubator. All experiments were carried out 24 hours after plating.

siRNA delivery and analysis of genes

Primary cortical neurons (1.5-2.5 $\times$ 10^6 cells/ well in 12-well plate) or HT4 neural cells (0.1 $\times$ 10^6 cells / well in 12-well plate) were seeded in antibiotic free medium for 24h

prior to transfection. DharmaFECT™1 transfection reagent was used to transfect cells with 100 nmol/L siRNA pool (Dharmacon RNA Technologies, Lafayette, CO) for 72h as described previously[5-7]. For controls, siControl non-targeting siRNA pool (mixture of 4 siRNA, designed to have ≥ 4 mismatches with the corresponding gene) was used. HT4 cells were harvested and re-seeded for treatment with glutamate or α-TCT as indicated in the respective figure legends. For the primary neurons, media were changed after 24h of seeding. Neurons were treated with glutamate or α-TCT as indicated after 72h of transfection. For determination of mRNA expression after siRNA transfection, total RNA was isolated from cells using the Absolutely RNA® Miniprep kit (Stratagene, La Jolla, CA). The abundance of mRNA for MRP1 was quantified using real time PCR using SYBR green-I (Applied Biosystems, Forster City, CA). The following primer sets were used: m_MRP1_F, 5'-GGT CCT GTT TCC CCC TCT ACT TCT T-3'; m_MRP1_R, 5'-GCA GTG TTG GGC TGA CCA GTA A-3'; m_GAPDH_F, 5'-ATG ACC ACA GTC CAT GCC ATC ACT-3'; m_GAPDH_R, 5'-TGT TGA AGT CGC AGG AGA CAA CCT-3'; r_MRP1_F, 5'-TGA ACC ATG AGT GTG CAG AAG GT-3'; r_MRP1_R, 5'-TCA CAC CAA GCC AGC ATC CTT-3'; r_GAPDH_F, 5'- TAT GAC TCT ACC CAC GGC AAG TTC A-3'; r_GAPDH_R, 5'- CAG TGG ATG CAG GGA TGA TGT TCT-3'.

miRIDIAN microRNA (miR) mimic/ inhibitor delivery

Primary cortical neurons (1.5-2.5 $\times$ 10^6 cells/ well in 12-well plate) or HT4 neural cells (0.1 $\times$ 10^6 cells / well in 12-well plate) were seeded in antibiotic free medium for 24h prior to transfection. DharmaFECT™ 1 transfection reagent was used to transfect cells with miRIDIAN rno-miR-199a-5p mimic / rno-miR-199a-5p inhibitor or mmu-miR-199a-5p mimic / mmu-miR-199a-5p hairpin inhibitor (Dharmacon RNA Technologies, Lafayette, CO) as per the manufacturer's instructions. miRIDIAN miR mimic or inhibitor negative controls (Dharmacon RNA Technologies, Lafayette, CO) were used for control transfections. Samples were collected after 72h of miR mimic/inhibitor delivery for quantification of miR, mRNA and protein expression as described[8].

pGL3-MRP1-3'UTR luciferase reporter assay

miRIDIAN mmu-miR-199a-5p mimic or mmu-miR-199a-5p hairpin inhibitor was delivered to HT4 neural cells followed by transfection with pGL3-MRP1-3'UTR firefly luciferase expression construct (Signosis, Sunnyvale, CA) together with renilla luciferase pRL-cmv expression construct using Lipofectamine™ LTX PLUS™ reagent. Luciferase assay were performed using the dual-luciferase reporter assay system (Promega, Madison, WI). Firefly luciferase activity was normalized to renilla luciferase expression for each sample as described[8].

Quantification of microRNA expression

Total RNA including miR fraction was isolated using miRVana™ miRNA isolation kit (Ambion, Austin, TX), according to the manufacturer's protocol. miR-199a-5p levels were quantified using Taqman Universal Master Mix (Applied Biosystems, Forster City, CA). miR levels were quantified with the $2^{(-\Delta\Delta CT)}$ relative quantification method using miR-16 as the house keeping miR[7-10].

Western blot

After protein extraction, the protein concentration was determined using BCA protein reagents. The samples (40-50 μg of protein / lane) were separated on a 4-12% SDS-polyacrylamide gel electrophoresis as described[2,11-12] and probed with anti-MRP1 (1:20 dilution, Enzo Life Sciences, Plymouth Meeting, PA). To evaluate the loading efficiency, membranes were probed with anti-GAPDH antibody (Sigma-Aldrich, St. Louis, MO).

Cell viability

The viability of primary cortical neurons or HT4 neural cells in culture was assessed by measuring leakage of lactate dehydrogenase (LDH) from cells into media using an *in vitro* toxicology assay kit from Sigma-Aldrich (St. Louis, MO, USA). The protocol has been described in detail in a previous report[5-6,11,13]. In brief, LDH leakage was determined using the following equation: % total LDH leaked = (LDH activity in the cell culture medium / total LDH activity) $\times$ 100. Total LDH activity represents the sum of LDH activities in the cell monolayer, detached cells, and the cell culture medium.

Immunocytochemistry

HT4 neural cells (0.5×10^6 cells / well) were seeded in 35mm plates for 24h then treated with or without 10 mmol/L glutamate. Cells were washed with PBS three times and fixed in 10% buffered formalin for 20 min, then underwent permeabilization using 0.1% Triton X-100/PBS for 15 min. The cells were washed and incubated with 10% goat serum (Vector Laboratories, Burlingame, CA) for 1h at room temperature, and incubated with MRP1 antibody (1:50, Abcam, Cambridge, MA) overnight at 4°C. After incubation with primary antibody, cells were washed with PBS three times and incubated with an Alexa-flour 488 (1:200 dilution) for 1h at room temperature. After three washes and incubation with 4',6'-diamino-2-phenylindole (1:10,000 dilution) for 2 min, cells were mounted in gelmount (aqueous mount, Vector Laboratories, Burlingame, CA) for microscopic imaging as described previously[5,11].

Calcein clearance assay

Calcein clearance assay was used to measure MRP1 activity in both primary cortical neurons and HT4 neural cells against glutamate insult. To improve poor specificity of pharmacological inhibitors, RNA interference approach was applied to measure specific MRP1 activity. After 72h of MRP1 siRNA transfection as described above, HT4 cells were re-seeded and treated with glutamate as described in relevant legends. After glutamate challenge, calcein-AM (25 nmol/L) was loaded to the cells for 15 min at 37°C. Cells were washed with PBS, collected and analyzed using the Accuri C6™ (Accuri Cytometers, Ann Arbor, MI) flowcytometer. MRP1 activity was measured on the basis of intracellular calcein retention[14]. For primary neurons, media were changed after 72h of transfection, and the neurons were treated with 5 mmol/L glutamate for 1h. After glutamate challenge, calcein-AM (5 μmol/L) was loaded to the cells for 30 min at 37°C. Then, fluorescence was measured by using the Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT) with the excitation wavelength of 485nm and the emission wavelength of 528nm.

Mouse stroke model

Transient focal cerebral ischemia was induced in 8 weeks old MRP1 deficient (n=20, male, Taconic, Hudson, NY) or background FVB mice (n=15, male, Taconic, Hudson, NY) by middle cerebral artery occlusion (MCAO) as previously described[1,11,15-16]. Occlusion of the right middle cerebral artery was achieved by using the intraluminal filament insertion technique. Briefly, mice were anesthetized by inhaling halothan, and 6-0 nylon monofilament was inserted into the internal carotid artery, *via* the external carotid artery. Then the filament tip was positioned for occlusion at a distance of 6mm beyond the internal carotid artery-pterygopalatine artery bifurcation. We observed that this approach results in a 70 ± 10% drop in cerebral blood flow as measured by laser Doppler (DRT4, Moor Instruments). Once the filament was secured, the incision was sutured and the animal was allowed to recover from anesthesia in its home cage. After 90min of occlusion, the animal was briefly re-anesthetized, and reperfusion was initiated via withdrawal of the filament from MCA. This surgical protocol typically results in a core infarct limited to the parietal cerebral cortex and caudate putamen of the right hemisphere. After 48h of reperfusion, T2-weighted image was taken to measure infarct volume. Mice suffering from surgical complications (*e.g.* hemorrhage or death) during MCAO were excluded. Immediately after imaging, tissues from control and stroke-affected hemispheres of MRP1 (n=12) and FVB (n=8) were harvested.

MRI

T2-weighted imaging was performed on stroke-affected mice. Imaging experiments were carried out using a 11.7T (500 MHz) MR system comprised of a vertical bore magnet (Bruker Biospin, Ettlingen, Germany) as described previously by our group[11,17-18]. Briefly, animals were placed inside a 30 mm radio frequency coil (resonator) and finally the whole arrangement was placed inside the vertical magnet. Shimming was performed to adjust field inhomogeneities on the subject. After several localizer scans were completed for three different orientations (saggital, coronal and axial), a T2-weighted spin echo rapid acquisition with relaxation enhancement (RARE) sequence was optimized for some key parameters such as: field of view (FOV) = 30×30 mm, acquisition matrix 256×256, repetition time (TR) = 3000 ms, echo time (TE) = 30 ms, flip angle (FA) = 180 degrees, images in acquisition = 15, resolution = 8.533 pixels/mm, and number of averages 4 was used to acquire T2-weighted MR images from the mouse head on the 11.7-T MRI system to generate 15 images corresponding to 15 short axis slices. For stroke-volume calculations, raw MRI images were converted to digital imaging and communications in medicine (DICOM) format and read into ImageJ software (NIH).

α-Tocotrienol supplementation

C57BL/6 (5 weeks, male, Harlan, Indianapolis, IN) mice were randomly divided into two groups, control (n=18) and supplemented (n=23) group. The control group was orally gavaged with vitamin E stripped corn oil with volume matching the mean volume of the supplement in the test group. Stock solution of α-TCT supplement solution was prepared in vitamin E-stripped corn oil. The test group was orally gavaged with α-TCT (Carotech Inc, Malaysia) in oil (same as placebo) at a dosage of 50 mg/kg body weight for 13 weeks. Incorporation of orally supplemented vitamin E to the brain is a slow process. Longer supplementation period improves bioavailability of vitamin E to the

brain. Stroke was performed at 20 to 24h after the last supplementation of α-TCT or corn oil. After 48h of MCAO, T2-weighted image was taken to measure infarct volume. Mice suffering from surgical complications (*e.g.* hemorrhage or death) during MCAO were excluded. Immediately after imaging, tissues from control and stroke-affected hemispheres of control (n=9) and α-TCT fed (n=10) mice were harvested. Mice were maintained under standard conditions at 22±2°C with 12:12 dark: light cycles. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Ohio State University, Columbus, Ohio.

HPLC-electrochemical detection

Vitamin E analyses of TCT or corn oil fed mice brains were performed using a HPLC-coulometric electrode array detector (Coularray Detector - model 5600 with 12 channels; ESA Inc., Chelmsford, MA). This system enables the simultaneous detection of TCTs and TCPs in the same run as described by us previously[2,19]. Glutathione measurement was performed using an HPLC system coupled with a electrochemical coulometric detector as described[1,4,20]. The CoulArray detector employs multiple channels set at specific redox potentials. Data were collected using channels set at 600, 700, and 800mV. The samples were snap-frozen and stored in liquid nitrogen until HPLC assay. Sample preparation, composition of the mobile phase, and specification of the column used were as described previously[2,20].

mRNA expression assay from laser-captured microdissected somatosensory cortex of brain tissue

Laser microdissection and pressure catapulting (LMPC) was performed using the microlaser system from PALM Microlaser Technologies AG (Bernreid, Germany) as described[8,21-23]. Briefly, mice were euthanized immediately after MRI imaging and coronal slices of brain tissue were collected using a mouse brain matrix. OCT-embedded in slices were subsequently cut in 12μm thick sections on a Leica CM 3050 S cryostat (Leica Microsystems, Wetzlar, Germany). Settings used for laser cutting were UV-Energy of 70-80 and UV-Focus of 70. Matched area (2×10^6 μm^2) of contralateral or stroke-affected somatosensory cortex was captured into 25μl of RNA extraction buffer. The total RNA was isolated using PicoPure RNA Isolation kit (Arcturus, Sunnyvale, CA) to measure MRP1 mRNA expression. To determine miR-199a-5p expression in MCAO-induced mouse brain, miR fraction was isolated using *miR*Vana™ miR isolation kit (Ambion, Austin, TX) as described above.

Histology

OCT-embedded frozen brain was sectioned (12μm) and mounted onto slides. brain sections were stained with rabbit polyclonal antibody to MRP1 (1: 200, Abbiotec, San Diego, CA), 0.0001% Fluoro-Jade® C (Millipore, Billerica, MA) or 4-HNE (1:1000, Enzo Life Sciences, Plymouth Meeting, PA). Coronal slices of cortical sections were analyzed by fluorescence microscopy (Axiovert 200M, Zeiss, Göttingen, Germany) and images were captured using Axiovert v4.8 software (Zeiss)[6,24].

Statistics

Data are reported as mean $\pm$ SD of at least three independent experiments. Difference in means was tested using Student's *t*-test or one-way ANOVA with Tukey's test. $P<0.05$ was considered statistically significant.

Supplemental Figures and Figure Legends

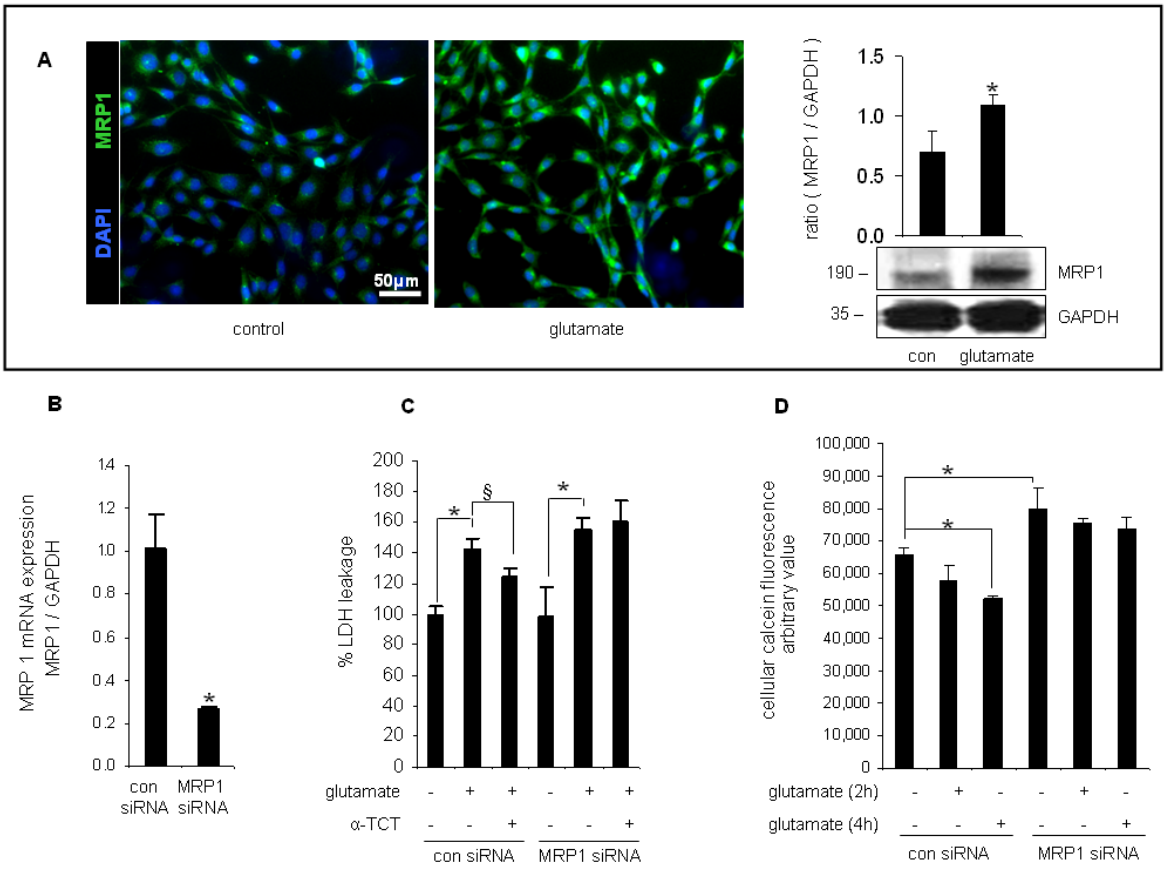

Figure S1. MRP1 knockdown attenuated the neuroprotection of α-tocotrienol. **A**, Glutamate (10mmol/L, 12h) challenge induced the expression of MRP1 in HT4 neural cells (blue-DAPI stained nuclei; green-MRP1 protein). After MRP1 siRNA transfection, MRP1 mRNA expression was significantly down-regulated (**B**). **C**, Cells were re-split after transfection. α-TCT (1μmol/L) was added into cell culture medium 6h before glutamate treatment. After glutamate (10mmol/L, 12h) challenge, LDH leakage was measured. Neuroprotection of α-TCT was compromised under conditions of MRP1 knockdown. **D**, Glutamate-challenged cells with MRP1 knockdown exhibited loss of functional MRP1 by retaining more calcein compared to corresponding control cells. Bar=50μm. n=3, $^{*}P<0.05$ compared with control; $^{\S}P<0.05$ compared with control siRNA-transfected, glutamate-treated HT4 cells.

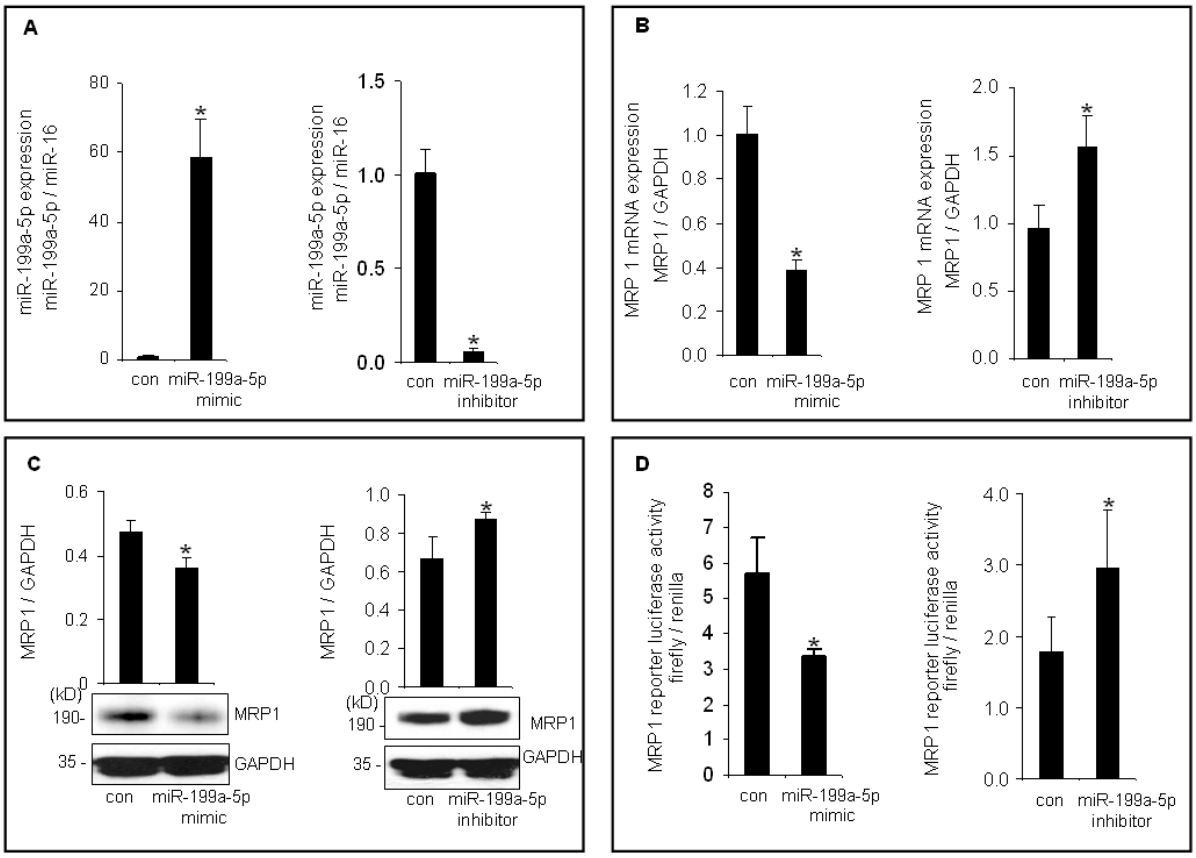

Figure S2. miR-199a-5p targets MRP1 expression. After 72h of miR-199a-5p mimic delivery, miR-199a-5p expression was significantly upregulated while miR-199a-5p hairpin inhibitor significantly downregulated miR-199a-5p in HT4 neural cells (**A**). miR-199a-5p mimic/inhibitor negatively regulated mRNA (**B**), and protein (**C**) of MRP1. To test whether MRP1 is a direct target of miR-199a-5p, HT4 cells were transfected with a pGL3-MRP1-3'UTR firefly luciferase expression construct and co-transfected with control renilla luciferase reporter construct along with miR-199a-5p mimic or inhibitor. miR-199a-5p mimic delivered cells showed lower luciferase activity while miR-199a-5p hairpin inhibitor delivered cells showed higher luciferase activity (**D**). n=3, $^{*}P<0.05$ compared with control.

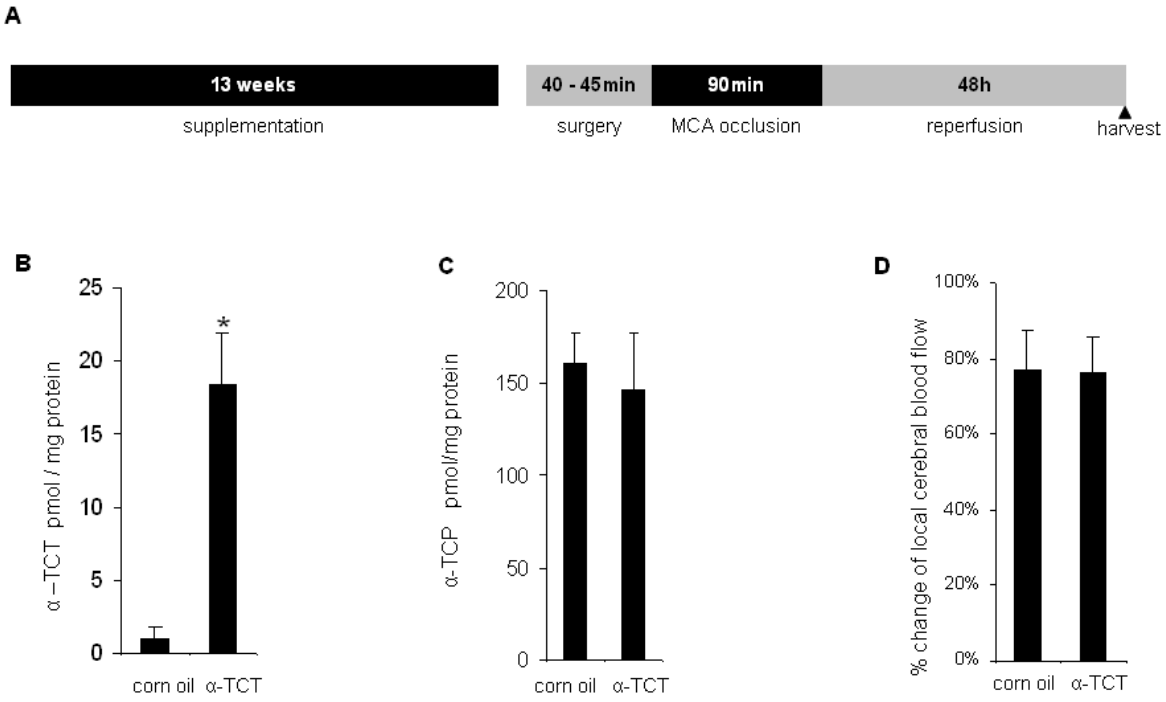

Figure S3. Orally supplemented α-tocotrienol did not change brain α-tocopherol levels or cerebral blood flow. **A**, C57BL/6 mice were randomly divided into two groups, and orally gavaged with vitamin E-stripped corn oil (n=18) or 50mg α-TCT per kg body weight (n=23) for 13 weeks. After 24h of last supplementation, MCAO was performed for 90min. T2 weighted MRI imaging was performed 48h after reperfusion, then mice were sacrificed and brain were harvested. **B**-**C**, Oral α-TCT supplementation significantly increased α-TCT level in the brain without affecting brain tissue α-TCP level (n=5). **D**, Reduction in the MCA-area blood flow in corn oil fed and α-TCT fed mice during occlusion was found to be comparable (corn oil, n=9; α-TCT, n=10). $^{*}P<0.05$ compared with corresponding control.

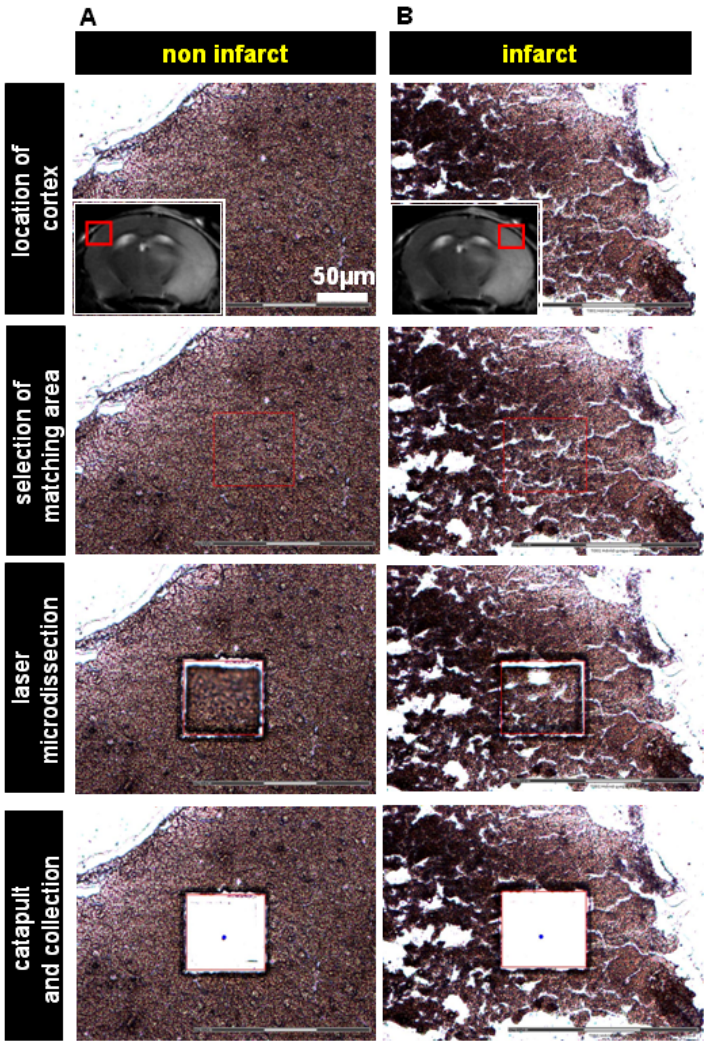

Figure S4. Collection of somatosensory cortical tissue elements using a laser microdissection and pressure catapulting (LMPC) system. MCAO-challenged brains were embedded in OCT. OCT-embedded slices were cut in 12μm thick coronal section. Matched area (2×10^6 μm^2) of contralateral or stroke-affected cortex was captured using a LMPC system (***A***, control hemisphere; ***B***, infarct hemisphere). Bar=50μm.

Reference for Supplementary Material

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