SUPPLEMENTARY MATERIALS

Neuronal and vascular protection by the prostaglandin E_2 EP4 receptor in a mouse model of cerebral ischemia

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I. Supplementary Methods

Characterization of anti-mouse EP4 IgY

pActin-HA-PTGER4-IRES-EGFP constructs were generated by cloning the mouse PTGER4 gene (Thermo Scientific, Huntsville, AL) into the pActin-IRES-GFP construct (1). HEK293T cells were transfected with 0.05μg or 0.2μg of plasmid using FuGENE HD (Roche, Indianapolis, IN) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were fixed with 4% PFA and stained with rabbit anti-human EP4 (1/200; Cayman Chemical, Ann Arbor, MI) or chicken anti-mouse EP4 (1/200; GenScript Corp., Piscataway, NJ). Secondary antibodies were Cy3-conjugated donkey-anti-rabbit (1/1500) and donkey-anti-chicken (1/1500; Jackson Immunoresearch Laboratories Inc., West Grove, PA). Nuclei were visualized using Hoechst 33258 dye (MP Biomedicals, Solon, OH). Images were acquired using a Nikon Eclipse E600 microscope (Nikon Instruments, Melville, NY) and a Hamamatsu Orca-ER digital camera (Hamamatsu Photonics, Bridgewater, NJ).

Transient focal ischemia model

MCAo-RP was carried out as previously described (2, 3). All MCAo-RP experiments were performed by an experimenter who was blinded to genotype and/or pharmacological agent. Mice were anesthetized and maintained with 2% isoflurane in 70% nitrous oxide/30% oxygen. Rectal temperature was maintained at 37°C ± 0.5°C with a thermostatically controlled heating pad (DC Temperature Controller, Dowdoin, ME, USA). Briefly, left MCA occlusion was performed by passing a 7.0 nylon silicon rubber-coated monofilament (Doccol, Redlands, CA USA) into the internal carotid artery about 10mm from the internal carotid/pterygopalatine artery bifurcation via an external carotid artery stump. The suture was placed intraluminally for 30 or 60 minutes and then withdrawn, and the mice were allowed to recover for 24h or 7 days. Sham controls underwent a similar procedure without insertion of the suture into the MCA. In pharmacologic studies, the selective EP4 agonist AE1-329 or vehicle (0.01% ethanol) was administered after MCAo-RP either at 2 and 8 hours, or in subsequent experiments 3 hours after MCAo. Control experiments examining effect of vehicle vs normal saline did not show significant differences in hemispheric infarct size (47.24 ± 5.578 N=11 normal saline, 39.09 ± 4.751 N=12 vehicle; p=0.276).

Quantification of infarct volume

After 23 hours of reperfusion, mice were lethally anesthetized and brain tissue was harvested for infarct quantification using either 1% 2,3,5-triphenyl-tetrazolium chloride (TTC) staining for pharmacological experiments or cresyl violet for genetic experiments, as previously described (2). For TTC, brains were sectioned coronally into five 2 mm sections and incubated with TTC in saline for 15 min at 37°C. The area of infarct, identified by the lack of TTC staining, was measured on the rostral and caudal surfaces of each slice and numerically integrated across the thickness of the slice to obtain an estimate of infarct volume in each slice (OpenLab software; Improvision, Waltham, MA). Infarct volume was measured separately in the cerebral cortex, striatum, and hemisphere. For cresyl violet measurements, anesthetized animals were perfused with 10 U/mL heparin and subsequently with 4% paraformaldehyde in phosphate buffered saline (PBS) pH 7.4. Brains were removed, postfixed in 4% paraformaldehyde for 24 hours, cryoprotected in 30% sucrose, and sectioned coronally at 30 μm thickness. Sections at 720 μm intervals were selected for cresyl violet staining. For both TTC and cresyl violet quantification, infarct area was measured using digital imaging and image analysis software (Image J, National Institutes of Health). Infarct volume was corrected for swelling as previously described (4).

Measurement of relative cerebral blood flow

Laser Doppler Flowmetry (LDF) was performed in a blinded fashion as previously described (2, 3). A small incision was made in the skin over the left skull, and a laser Doppler probe (PeriFlux System 5000, Perimed, Sweden) was positioned 2 mm posterior and 3 mm lateral to the bregma over the parietal skull surface. After placement of the probe, the MCA was occluded, and LDF was monitored before and during ischemia and for 60 min after the beginning of reperfusion.

Behavior

In the first experiment (Fig 1b), behavior was measured at 24h using the Bederson rating scale, as previously described (2). Motor coordination and balance were assessed with the Rotarod apparatus (San Diego Instruments, San Diego, CA). Training on the Rotarod began with a constant low-speed rotation (5 rpm) for 5 minutes on the first day of training. On all subsequent days, each mouse was placed on the rod with rotation speed increasing from 5 to 10 rpm over 5 minutes. The time the mouse was able to stay on the rotating rod before falling was determined up to a maximum duration of 300 seconds. Each mouse was trained 3 times a day, 3 days/wk for 3 weeks before MCAo surgery. Mice that failed to learn to remain on the rotarod for 300 seconds for the last two sessions were excluded from further study, and mice were randomized to vehicle and treatment MCAo-RP groups. Behavior was assessed by an examiner blinded to treatment group at 48h and 7 days after MCAo-RP. Mice were scored at 48h and 7 days after MCAo according to the maximum time (up to 300 sec) they were able to remain on the rotarod.

Cultured primary neurons and organotypic hippocampal slices

Primary neuronal cell cultures were established from dissociated cerebral cortical cells from day 17 C57B6 mouse embryos as described previously (2, 5). Dissociated cells were plated at a density $2.5x10^5$ cells/cm^2 and maintained in Neurobasal Medium (NB), B-27, 0.5mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin for 7-10 days. For oxygen glucose deprivation studies (OGD) cells were washed twice with 37ºC Balanced Salt Solution (BSS: 125mM NaCl, 5mM KCl, 1.2 mM NaH_2PO_4, 26mM $NaHCO_3$, 1.8mM $CaCl_2$, 0.9mM $MgCl_2 \cdot 6H_2O$, 10mM HEPES, 10mM D- glucose) and once with glucose-free BSS. Plates were then moved to a Modular Incubator Chamber (MIC-101, Billups-Rothenberg, Inc., Del Mar, CA) flushed with 2% O_2 /5% CO_2/93% N_2 for 3h at 37ºC. OGD was terminated by returning the cultures to NB medium and normoxic conditions at 37ºC. BSS was added to control cultures for 3 hours at 37ºC under normoxic conditions. For glutamate toxicity assays, cells were incubated for 24h with 250µM glutamate, which induces a 50% increase in LDH levels after 24h (data not shown). Assessment of cell injury was made by measurement of lactate dehydrogenase (Roche, Indianapolis, IN) or by

quantification of propidium iodide (PI) fluorescence as previously described (2, 5). For PI quantification of neuronal death, cells were washed with PBS, stained with 0.01mg/ml PI to measure dead cells and 0.01mg/ml Hoechst 33342 to measure total cell number in 2.5mM glucose solution for 10min at 37ºC. Cells were then fixed with PFA (4%) for 15min, washed 3 times with PBS, and counted by an experimenter blinded to the experimental condition. For quantification of caspase 3, cell lysates were prepared in 1x Cell lysis buffer (Cell Signaling Technology, Danvers, MA). Caspase 3 activity was measured using the Caspase 3/7 Glo® assay (Promega, Madison, WI) according to the manufacturer's instructions. 1mM PMSF and 0.1% Protease Inhibitor Cocktail were added directly before use. 0.04µg/µl protein samples (~ 30,000 cells) were assayed using a luminometer (Spectra Max M5, Molecular Devices, Sunnyvale, CA). All samples were assayed in duplicates. AE1-329 (dissolved in 0.05% ethanol) or vehicle (0.5% ethanol) was added to cultures at the same time as glutamate and left for 24h. AE1-329 or vehicle were added to cultures at the initiation of OGD; after OGD, AE1-329 was added to fresh medium for 24h until PI quantification. H89 was dissolved in 0.05% DMSO.

Organotypic hippocampal cultures were prepared from post-natal day 7 rat pups and subjected to oxygen glucose deprivation (OGD) for 75 minutes or N-methyl D-aspartate (NMDA) for 60 minutes as described previously (2, 6). Neuronal death was assayed over three days by quantification of mean PI fluorescence in the CA1 subregion of each hippocampal slice (OpenLab software) at t_b, representing PI fluorescence measurements before treatment, t_{24h} or 24 hours after either NMDA or OGD, and at t_{max} following overnight incubation with 10µM NMDA to elicit maximal cell death. The percent neuronal death was normalized for each individual slice and was calculated as follows: $(t_{24h} - t_b) / (t_{max} - t_b)$. Experiments were repeated 3-6 times, with n=15 slices per condition).

Immunohistochemistry

The following primary antibodies were used: anti-human EP4 (1/1000; Cayman Chemicals, Ann Arbor, MI), NeuN monoclonal antibody (1/1000; Chemicon, Temacula, CA), anti-MAP (1/3000; Sigma, St Louis, MO), and anti-ß dystroglycan (1/1000; Novocastra Laboratories, Benton Lane, U.K.). Secondary antibodies and detection reagents included goat anti-mouse Alexa 555, and anti-rabbit Alexa 486 (Molecular Probes, Eugene, OR). Specific staining of the EP4 antibody was confirmed using blocking peptide and no primary antibody in control experiments. An affinity purified peptide polyclonal chicken anti-mouse EP4 antibody was generated using a 15-residue peptide at the carboxy-terminal sequence of mouse EP4 (GenScript Corp., Piscataway, NJ;

Supplementary Figure 2). This antibody was generated as a more specific antibody to mouse EP4 for in vivo immunostaining.

Quantitative Western blot analysis

Protein (15 ug per lane) was fractionated using 4-12% SDS-PAGE and electrophoretically transferred to PVDF membranes (Bio-Rad, Hercules, CA). Primary antibodies used included anti–phospho-Akt (Ser473; 1:1000; Cell Signaling Technology), anti-Akt (1:2000; Cell Signaling Technology), anti-phospho-CREB (Ser133) (1:1000; Cell Signaling Technology), anti-CREB (1:2000; Cell Signaling Technology), anti-phospho eNOS Ser^{1177} (Cell Signaling, Danvers, MA), anti-eNOS (Millipore, Billerica, MA), anti-actin (Sigma, St. Louis, MO) and anti-GAPDH (1:5000; Abcam, Cambridge, MA). Secondary antibodies used were sheep anti-rabbit or sheep anti-mouse HRP-conjugated secondary Ab (Amersham Biosciences, Arlington Heights, IL). Immunoreactivity was detected by ECL or Supersignal West Pico Substrate (Thermo Scientific, Rockford, IL). Experiments were repeated in duplicate.

II. Supplementary Figure Legends

Supplementary Figure 1. Immunostaining of primary cortical neurons with anti-mouse EP4 IgY. Primary cortical neurons (DIV 10) derived from E16 C57B6 embryos are stained for EP4 receptor using a novel affinity purified polyclonal chicken anti-mouse EP4 antibody (mEP4IgY) that was generated using a 15-residue peptide at the carboxy-terminal sequence of mouse EP4 (see **Supplementary Figure 2 and Supplementary Methods**). Note colocalization of anti-mouse EP4 IgY (mEP4IgY) and anti-human EP4 rabbit IgG (hEP4 IgG) in MAP-2 positive neurons (scale bar=10μm).

Supplementary Figure 2. Validation of anti-mouse EP4 IgY antibody. This antibody was developed to achieve more specific immunostaining *in vivo* than that afforded by the commercially available rabbit anti-human EP4 rabbit polyclonal antibody that yields high background staining *in vivo* in brain tissue (Cayman Chemicals, Ann Arbor, MI). Both chicken IgY **(A)** and rabbit IgG **(B)** anti-EP4 antibodies detect mouse EP4 receptor in HEK293T cells transfected with plasmid containing GFP and mouse EP4 ORFs. EP4 signal is only detected in GFP-positive cells, and endogenous HEK EP4 receptor is not detected (vector). Scale bar = 50μm.

Supplementary Figure 3: Representative slices from organotypic hippocampal slice cultures (OHC) are shown for OGD and NMDA treatment experiments. Please see Supplementary Methods for detailed OHC protocol. Note lower PI staining at T24h in the AE1-329 treated slices in both OGD and NMDA slices. All experiments were repeated 3-4 times, and representative dose responses are shown in Figures 2g and 2h.

Supplementary Figure 4: EP4 receptor expression in cortical neurons and cerebral cortex after ischemia. (A) Primary mouse cortical neurons were subjected to 3h of OGD and harvested immediately after OGD (0h), 3h, and 24h after OGD. qPCR of EP4 receptor was performed as previously described (7) (ANOVA $p<0.0001$; post hoc $p<0.001$ at 3h; n=6 per group). (B) Confocal examination of colocalization of EP4 receptor and NeuN in sham control and MCAo-RP at 24h demonstrates expression of EP4 in NeuN+ cells in cerebral cortical peri-infarct area (scale bar=15μm).

Supplementary Figure 5. Validation of Thy-1 Cre recombinase activity. **(A)** To analyze Cre recombinase activity, Thy-1 Cre transgenic females were crossed to ROSA26 male reporter mice. X-gal staining of ROSA26:Thy1-Cre+ mouse brain sectioned rostral to caudal demonstrates Cre recombinase activity in cerebral cortex and striatum (scale bar =2 mm). **(B)** Immunofluorescent staining of brain sections from ROSA26: Thy1-Cre+ mouse demonstrates co-localization of ß-galactosidase with the neuronal marker NeuN (arrowheads, top row), but not with the astrocytic marker GFAP (arrows, bottom row) in cerebral cortex (scale bar= 50 μm).

Supplementary Figure 6. Representative serial sections from Thy-1 Cre;EP4 mice subjected to 60 min of MCAo followed by 24h reperfusion**.** As described in the Methods, fixed and cryoprotected brains were sectioned coronally at 30 μm thickness, and sections at 720 μm intervals were selected for cresyl violet staining. Shown here are three representative serial sections per genotype through striatum and rostral hippocampus stained with cresyl violet; loss of cresyl violet in the left hemisphere (ipsilateral stroked hemisphere) indicates infarcted area.

Supplementary Figure 7. Quantification of edema in Thy-1 Cre;EP4 and VECad-Cre-ER^{T2};EP4 MCAo cohorts. Edema was quantified as volume of (ipsilateral hemisphere-contralateral hemisphere)/volume of ipsilateral hemisphere. **(A)** Edema from F4 Thy-1 Cre;EP4 mice. **(B)** Edema from F6 Thy-1 Cre;EP4 mice +/- AE1-329. **(C)** Edema from VE CadCre-ER^{T2};EP4 mice (ANOVA $p=0.085$).

Supplementary Figure 8. Immunostaining and confocal immunolocalization of EP4 receptor in cerebral microvasculature. **(A)** EP4 receptor colocalizes by confocal microscopy with the endothelial marker Factor VIII (FVIII) in cerebral microvasculature of VECad-Cre-ER^{T2}:EP4+/+ mice (scale bar 15μm) 4 hours after MCAo. **(B)** Confocal microscopy demonstrates low to undetectable levels of EP4 receptor in sham VECad-Cre-ER^{T2};EP4$^{+/+}$ and VECad-Cre-ER^{T2};EP4$^{lox/lox}$ cerebral cortical vasculature. At 4 hours after MCAo however, EP4 expression is upregulated in endothelium and colocalizes with ß-dystroglycan in VECad-Cre-ER^{T2};EP4$^{+/+}$ but not VECad-Cre-ER^{T2};EP4$^{lox/lox}$ mice (anti-mouse EP4 IgY used here; scale bar=20μ). **(C)** At 24 hours after MCAo, EP4 expression remains upregulated in endothelium, but is absent in VECad-Cre-ER^{T2};EP4$^{lox/lox}$ mice (anti-mouse EP4 IgY used here; scale bar=15μ).

Supplementary Figure 9. Body temperature graphs for Thy-1 Cre;EP4 and VECad-Cre-ER^{T2};EP4 mice during MCAo. **(A)** Thy-1 Cre;EP4 mice and **(B)** VECad-Cre-ER^{T2};EP4 mice.

Supplementary Figure 10. Expression of EP4 receptor in astrocytes and microglia in cortical peri-infarct region. Colocalization of EP4 receptor with astrocytic marker GFAP does not show colocalization either in sham or at 24h after MCAo in cortical peri-infarct area. Colocalization with Iba1 shows low level of microglial EP4 signal in both sham and 24h after MCAo sections. Scale bar 20μm.

Supplementary Table 1. Physiological measurements in VECad-Cre-ER^{T2}:EP4 mice do not show significant differences between genotypes at time points before, during, and after ischemia.

III. References

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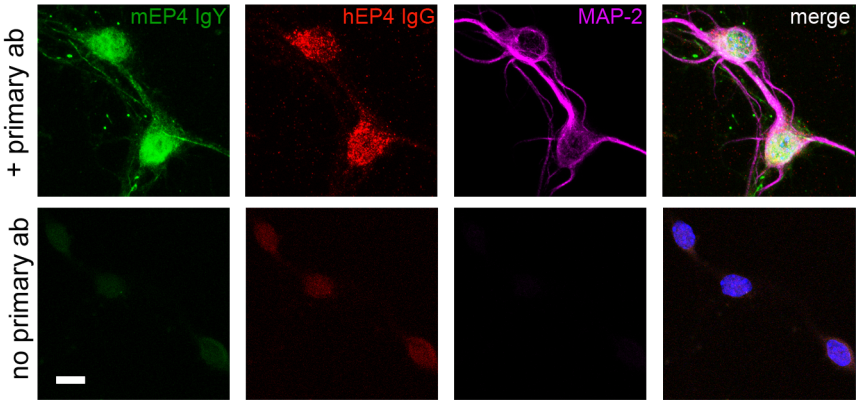
Supplementary Figure 1



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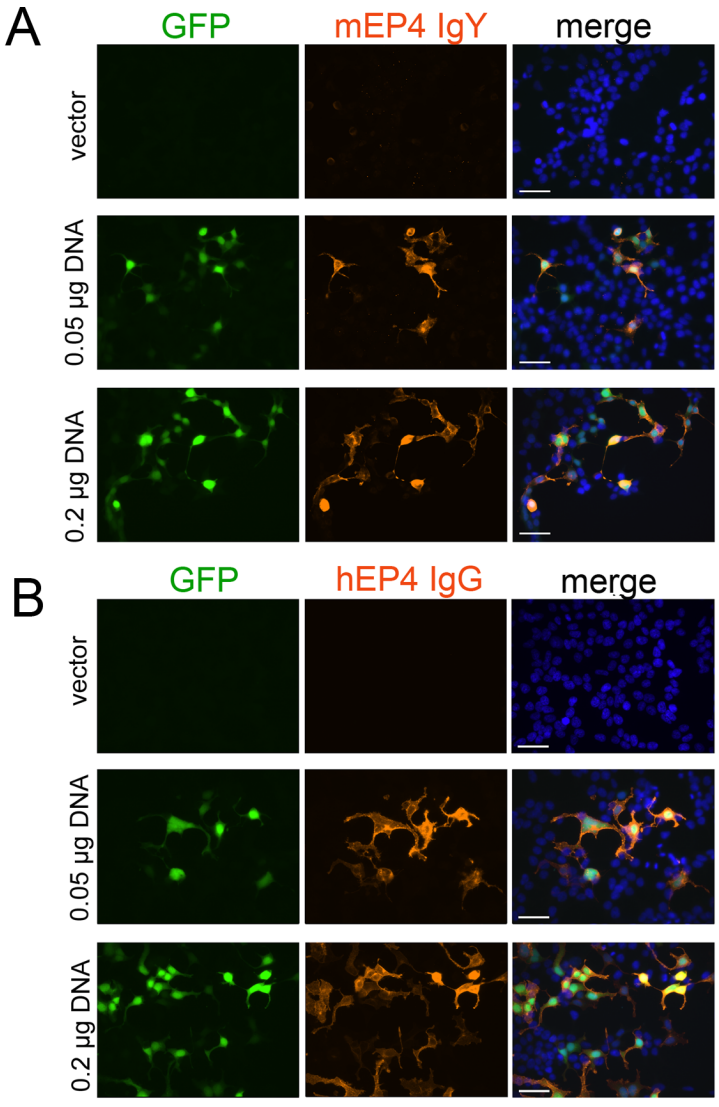

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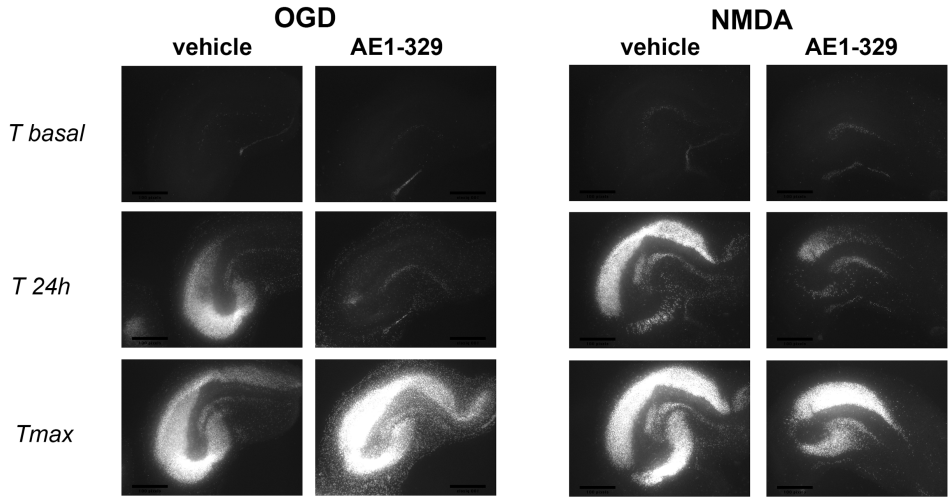
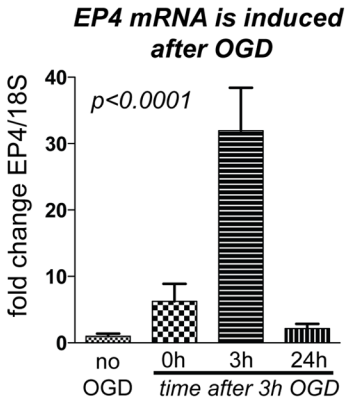


Supplementary Figure 4

A



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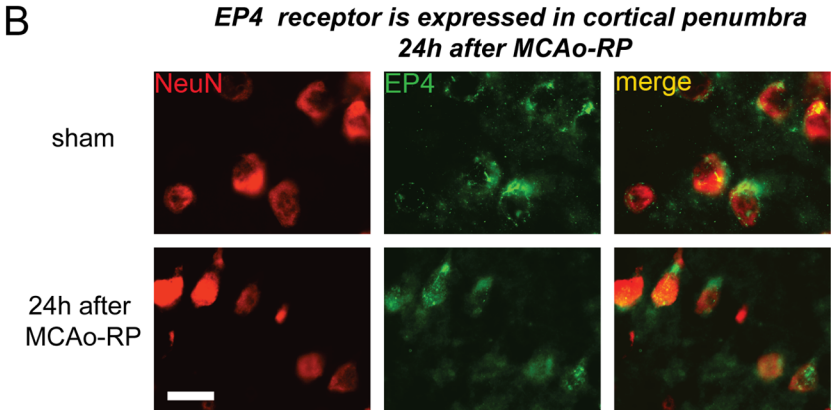

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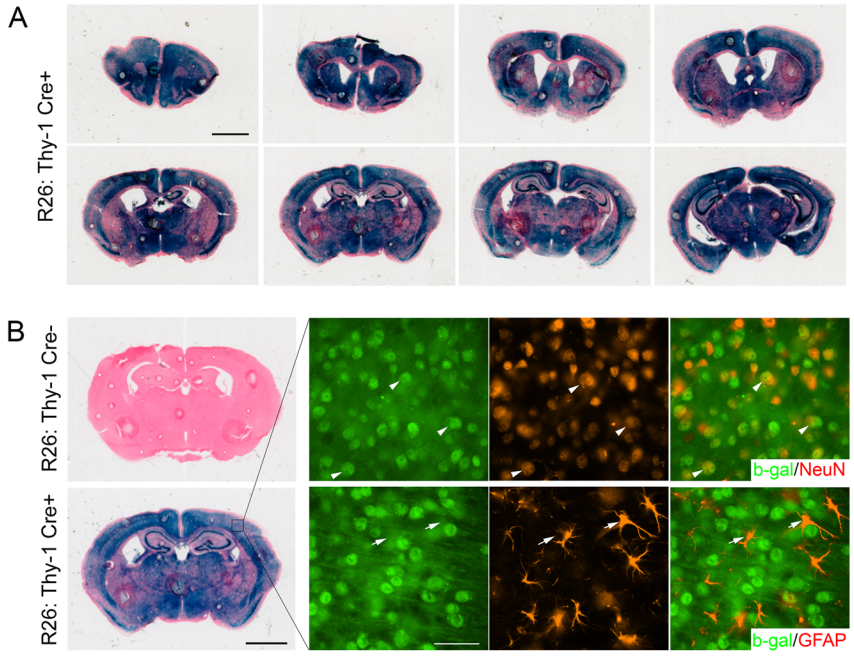

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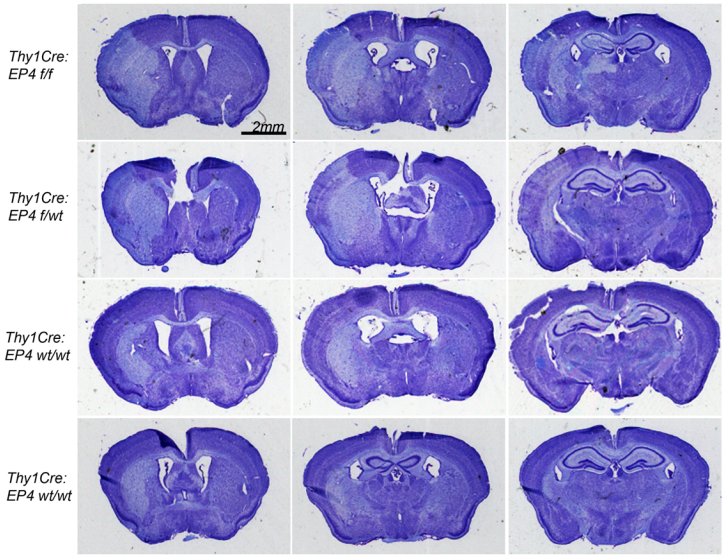

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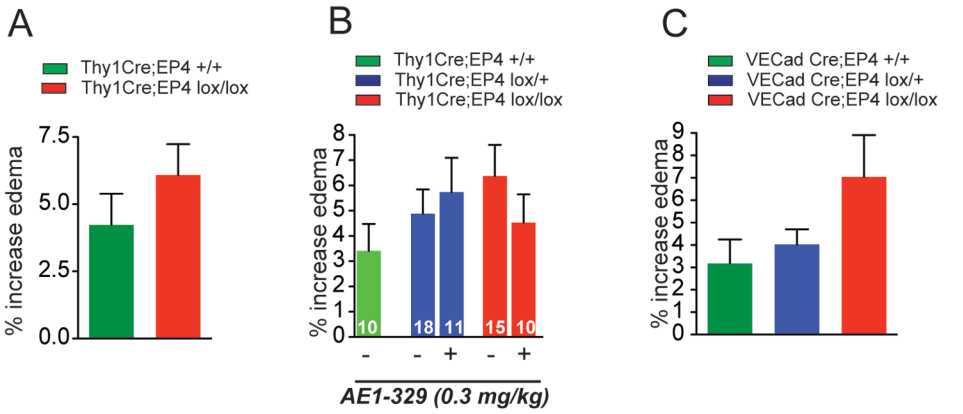

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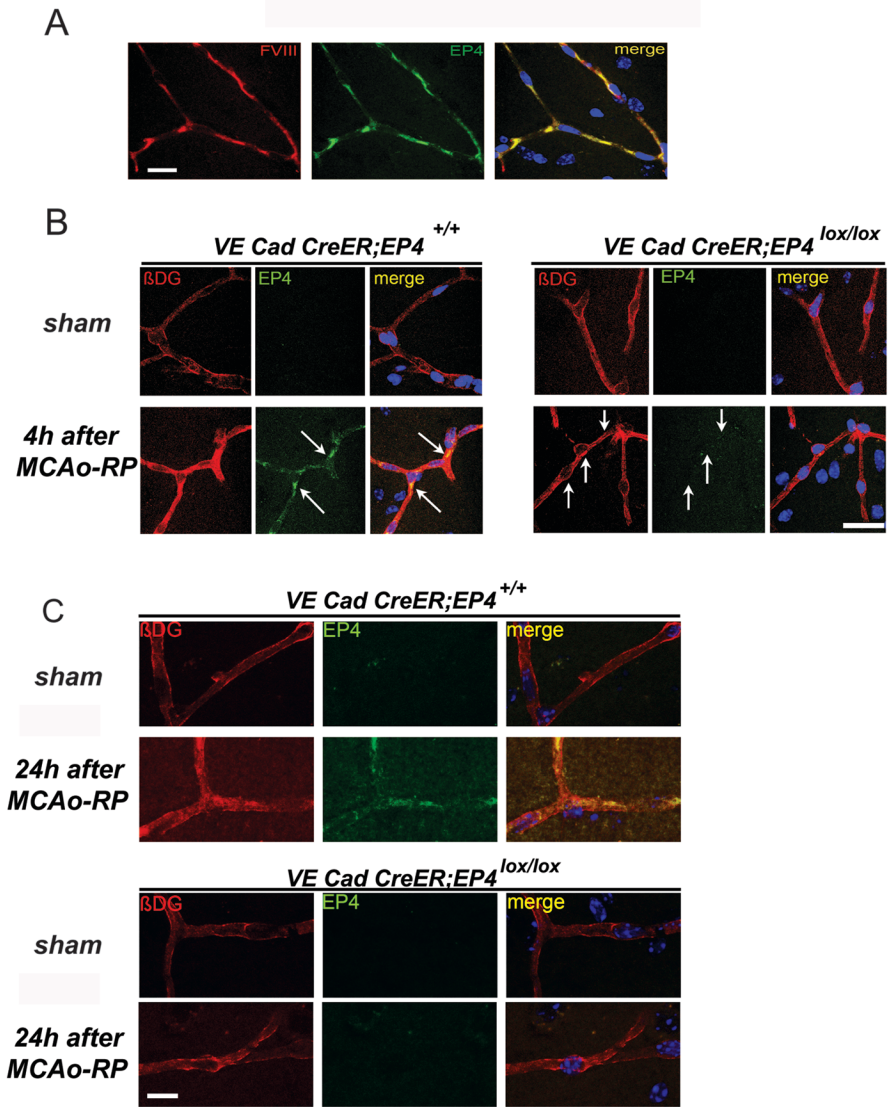

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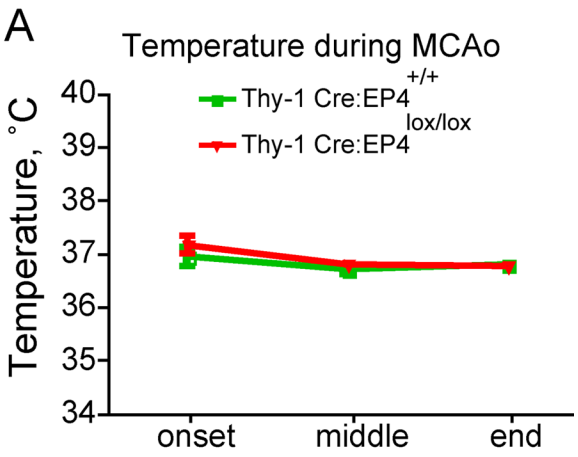
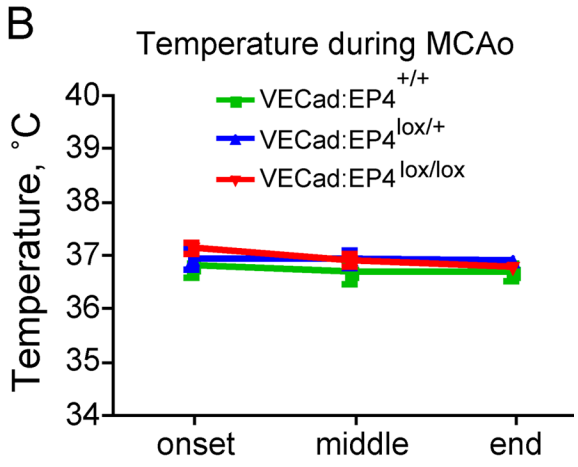
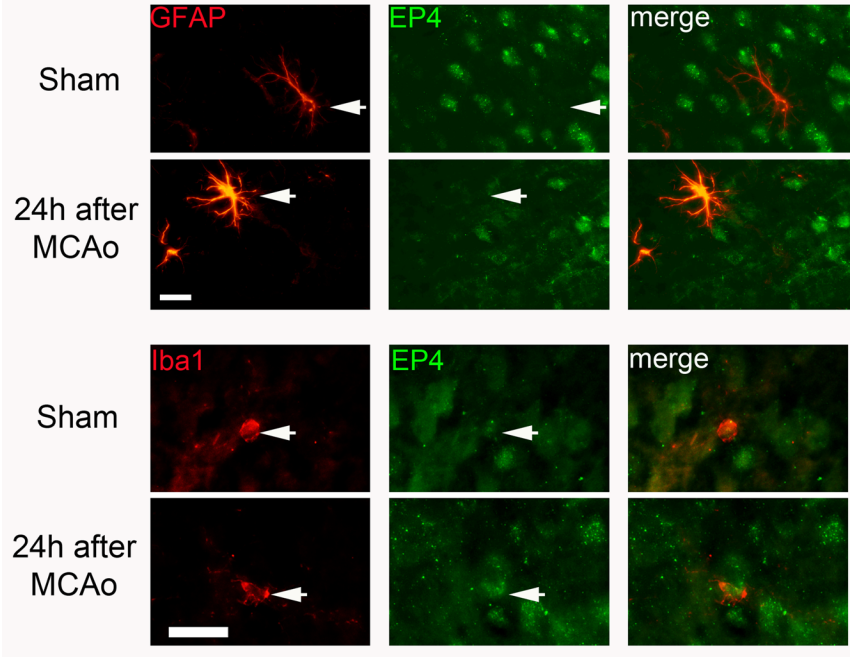




Supplementary Figure 10



Supplementary Table 1

		VECad-Cre-ER^{T2}:EP4$^{+/+}$ (n=3)	VECad-Cre-ER^{T2}:EP4$^{lox/lox}$ (n=4)
pH	before MCAO during MCAO after MCAO	7.42±0.05 7.38±0.09 7.27±0.05	7.44±0.03 7.37±0.07 7.31±0.06
pCO_2 (mmHg)	before MCAO during MCAO after MCAO	24.23±2.95 28.4±7.72 30.3±8.3	22.03±2.51 27.93±10.13 25.97±2.11
pO_2 (mmHg)	before MCAO during MCAO after MCAO	161.33±8.81 169.33±8.76 166±13.87	175.33±10.02 143.67±18.77 142.0±42.67
Na^+ (mmol/L)	before MCAO during MCAO after MCAO	148±2.06 144.67±3.92 144.33±2.38	148±2.65 143.33±2.08 146±2.65
K^+ (mmol/L)	before MCAO during MCAO after MCAO	3.73±0.7 4.6±0.26 4.77±0.4	4.43±1.12 5.43±1.37 5.6±0.85
Ca^{2+} (mmol/L)	before MCAO during MCAO after MCAO	1.14±0.06 1.16±0.07 1.09±0.08	1.13±0.05 1.12±0.1 1.08±0.1
Glucose (mg/dL)	before MCAO during MCAO after MCAO	174.33 ±31.56 202.67±80.31 173.67±62.61	178.75±19.81 224±46.73 212.75±30.02
Hct% (PCV)	before MCAO during MCAO after MCAO	37±3 40.67±2.52 34±1	37±3.92 37±4.76 32±4.24