



# The Critical Role of Equilibrative Nucleoside Transporter-2 in Modulating Cerebral Damage and Vascular Dysfunction in Mice with Brain Ischemia-Reperfusion

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## Abstract

**Background** Cerebral vascular protection is critical for stroke treatment. Adenosine modulates vascular flow and exhibits neuroprotective effects, in which brain extracellular concentration of adenosine is dramatically increased during ischemic events and ischemia-reperfusion. Since the equilibrative nucleoside transporter-2 (Ent2) is important in regulating brain adenosine homeostasis, the present study aimed to investigate the role of Ent2 in mice with cerebral ischemia-reperfusion.

**Methods** Cerebral ischemia-reperfusion injury was examined in mice with transient middle cerebral artery occlusion (tMCAO) for 90 minutes, followed by 24-hour reperfusion. Infarct volume, brain edema, neuroinflammation, microvascular structure, regional cerebral blood flow (rCBF), cerebral metabolic rate of oxygen (CMRO<sub>2</sub>), and the production of reactive oxygen species (ROS) were examined following the reperfusion.

**Results** Ent2 deletion reduced the infarct volume, brain edema, and neuroinflammation in mice with cerebral ischemia-reperfusion. tMCAO-induced disruption of brain microvessels was ameliorated in Ent2<sup>-/-</sup> mice, with a reduced expression of matrix metalloproteinases-9 and aquaporin-4 proteins. Following the reperfusion, the rCBF of the wild-type (WT) mice was quickly restored to the baseline, whereas, in Ent2<sup>-/-</sup> mice, rCBF was slowly recovered initially, but was then higher than that in the WT mice at the later phase of reperfusion. The improved CMRO<sub>2</sub> and reduced ROS level support the beneficial effects caused by the changes in the rCBF of Ent2<sup>-/-</sup> mice. Further studies showed that the protective effects of Ent2 deletion in mice with tMCAO involve adenosine receptor A<sub>2A</sub>R.

**Conclusions** Ent2 plays a critical role in modulating cerebral collateral circulation and ameliorating pathological events of brain ischemia and reperfusion injury.

**Keywords** adenosine · blood-brain barrier · equilibrative nucleoside transporters · ischemia-reperfusion injury · neuroinflammation

## Background

Stroke is a leading cause of death and long-term disability, in which ischemic stroke is responsible for over 80% of the cases worldwide [1]. Without sufficient cerebral blood flow, oxygen and glucose deprivation causes ionic imbalance, cerebral infarcts, and edema. Along with these events, neuroinflammation and microvascular disruption are developed following the onset of stroke [2]. The primary goal of therapy for ischemic stroke is to restore cerebral blood flow. To date, intravenous injection of tissue plasminogen activator (t-PA) is the only approved medication for ischemic stroke, while mechanical thrombectomy can also be applied for the occlusion of large vessels. However, only a small portion of

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patients with ischemic stroke may receive the reperfusion therapy because of the limited treatment window. It is also known that the restoration of blood supply may generate reactive oxygen species (ROS), increase vascular permeability, and exacerbate neuroinflammation and neurological damage, leading to hemorrhagic transformation [2, 3]. Accordingly, following the cerebral reperfusion, the protection against secondary injuries, especially on the cerebral vascular system, is important to benefit the treatment of stroke.

The neurovascular unit that consists of brain microvessels, neighboring neurons, and glial cells plays an important role in stroke progression and recovery [4]. The interaction among these components controls neural activity, cerebral blood flow, and the permeability of the blood-brain barrier (BBB) [5]. Upon the onset of stroke, the affected vessels showed endothelial edema, preceding structural damage, eventually leading to endothelial loss and unsalvageable vascular damage [6]. On the other hand, astrocytic functions include BBB maintenance and the regulation of perivascular flow. The expression of astrocytic aquaporin-4 (Aqp4) controls water homeostasis and is involved in edema formation.

Adenosine is an endogenous purine nucleoside that modulates a variety of physiological functions, including inflammatory responses and vascular blood flow, through interactions with adenosine receptors. Upon the onset of stroke, ATP can be released from the injured cells and rapidly converted to adenosine by a series of ecto-nucleotidases. This process leads to a dramatical increase in adenosine concentration in the extracellular space (from nM to  $\mu$ M levels), which continuously elevates during reperfusion [7]. The neuroprotective effects of adenosine during ischemia have been demonstrated by several studies [8, 9]. The equilibrative nucleoside transporter-1 (ENT1) and ENT2 are the major transporters responsible for the transfer of adenosine across the plasma membrane in a concentration-dependent manner [10]. In particular, the mRNA level of Ent2 is significantly higher than that of Ent1 in the brain of mice [11] and was proposed to predominantly contribute to the transport of nucleosides (e.g., adenosine and inosine) in neurons and astrocytes [12]. Also, genetic deletion of Ent2 was shown to increase extracellular levels of adenosine in the brain and provide a protective effect on animals with lipopolysaccharide-induced neuroinflammation and neuronal injury [11]. In this regard, the present study aimed at investigating the role of Ent2 in the pathogenesis and treatment of mice with ischemia-reperfusion.

## Methods

### Animals

Ent2<sup>-/-</sup> (C57BL/6-Slc29a2<sup>em1</sup>) mice were generated using the CRISPR-Cas9 technique by Transgenic Mouse Models

Core at National Taiwan University as described previously [11, 13]. Adult Ent2<sup>+/+</sup> and Ent2<sup>-/-</sup> mice of both male and female were used and randomly allocated for outcome investigation (Supplementary Table 1). For examining the impact of the stroke on Ent2 expression, male C57BL/6 J mice (8–12 weeks old) purchased from Laboratory Animal Center (an AAALAC-accredited experimental animal facility) at National Taiwan University were used. All mice were maintained on a 12-hour light/dark cycle and given food and water ad libitum in the Laboratory Animal Center at National Taiwan University. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the National Taiwan University College of Medicine (IACUC: 20201107).

### Transient Middle Cerebral Artery Occlusion (tMCAO)

Unilateral transient focal cerebral ischemia was induced by occluding the middle cerebral artery as described in literature [14] with modifications. In brief, the animals were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). A craniotomy was performed and the distal middle cerebral artery (MCA) was ligated. After 90 minutes, the suture was removed to restore the disrupted blood flow.

### Treatments with A<sub>1</sub>R and A<sub>2A</sub>R Antagonists

Mice were treated with 2 mg/kg of an A<sub>1</sub>R antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (Tocris Bioscience, Bristol, UK), or 1 mg/kg of an A<sub>2A</sub>R antagonist, 2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]-triazolo[1,5-c]pyrimidin-5-amine (SCH58261) (Tocris Bioscience), intraperitoneally twice daily for 3 consecutive days, starting from the day before the induction of tMCAO. The dose was determined based on previous reports [15, 16].

### Histology

Infarct volume and edema were determined on coronal sections of brains by 2,3,5-triphenyltetraolium chloride (TTC) staining and Nissl staining. For TTC staining, the brain was coronally sliced into four sections, each 2 mm thick and separated by 2 mm from the anterior tip of the frontal lobe with an acrylic brain matrix. Brain sections were immersed with a 2% TTC (Sigma-Aldrich, St. Louis, MO, USA) solution at 37°C. The images were captured from the anterior and posterior sides of the tissue. The infarct volume and brain swelling were determined by summing the infarcted (pale) volume of the four sections and expressed as a percentage of the contralateral cortex volume as described previously [14]. Brain edema was estimated by the differences between

ipsilateral and contralateral cortex volume and expressed as a percentage of the contralateral cortex volume [17]. In terms of Nissl staining, the brain was fixed with 4% paraformaldehyde, immersed in 15% sucrose, and then in 30% sucrose solution before being sliced into 20  $\mu\text{m}$ -thick sections on a cryostat. Ten slices were picked with an interval distance of 200  $\mu\text{m}$  and brain sections were stained with 1% cresyl violet solution. The infarct volume was determined by the following formula:  $[(\text{contralateral cortex area} - \text{ipsilateral uninjured area}) \times (\text{thickness of sections} + \text{distance between sections}) / (\text{contralateral cortex area}) \times (\text{thickness of sections} + \text{distance between sections})] \times 100\%$  as described previously [18]. Edema volume was estimated as described above.

### Quantitative Real-Time PCR Analysis

The cortical tissues of the selected slices from the ischemic and non-ischemic hemispheres were collected for RNA isolation. Total RNA isolation, quality check, cDNA synthesis, and SYBR green-based quantitative real-time PCR (qRT-PCR) assay were performed as described previously [10]. The primer sequences were listed in Supplementary Table 2. The relative expression of target genes normalized to that of Gapdh was calculated by the comparative Ct ( $\Delta\text{Ct}$ ) and was expressed as  $2^{-\Delta\text{Ct}}$ . The relative quantity was determined by the formula:  $2^{-\Delta\Delta\text{Ct}}$ , in which  $\Delta\Delta\text{Ct}$  values were obtained by subtracting the  $\Delta\text{Ct}$  values of the wild-type (WT) controls.

### Immunofluorescence Staining

The brain sections fixed with 4% paraformaldehyde were immersed in 15% sucrose and then in 30% sucrose. The tissues were embedded in the optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA). Brain coronal sections (20  $\mu\text{m}$ -thick each) were prepared using a cryostat. Antigen retrieval was conducted by heating the slices in a retrieval buffer (pH 9; Dako, Carpinteria, CA, USA) at 80°C. The slices were immersed in 0.2% Triton X-100, blocked with 3% bovine serum albumin (BSA), and then incubated with primary antibodies listed in Supplementary Table 3. The immunolabeling was visualized using fluorescence-conjugated secondary antibodies with Rhodamine or Alexa Fluor 488 (1:400; Jackson Immuno-Research Laboratories, West Grove, PA, USA) diluted in the blocking solution. For the assessment of BBB integrity, brain sections were incubated with anti-mouse IgG conjugated to Rhodamine (1:400; Jackson Immuno-Research Laboratories) for 48 hours at 4°C. The slices were mounted with a mounting gel (DAPI Fluoromount G; Southern Biotech Birmingham, Alabama, USA). Images were acquired by a Zeiss AXIO Imager M1 microscope (Carl Zeiss, Göttingen, Germany)

and the quantification was performed using ImageJ (NIH, Bethesda, MD, USA).

### Immunoblotting

The ipsilateral and contralateral cortex tissues were dissected and homogenized in radioimmunoprecipitation assay buffer (RIPA; 50 mM NaCl, 50 mM Tris base, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 8) containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and a phosphatase inhibitor cocktail (Roche Diagnostics). The homogenate was then centrifuged at 14,000 g for 15 minutes and the supernatant was collected and stored at  $-80^\circ\text{C}$  until analysis. The protein concentrations of the samples were determined by the Bio-Rad DC Protein Assay Kit (Bio-rad, Hercules, CA, USA). Protein samples (20  $\mu\text{g}$  each) were diluted with a loading buffer (200 mM Tris-HCl, 1.43% 2-mercaptoethanol, 0.4% bromophenol blue, and 40% glycerol), heated at 98°C, and then separated by 10% SDS-PAGE electrophoresis. After electrophoresis, the proteins were transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA) in transfer buffer (0.3% Tris base, 1.88% glycine, and 20% methanol; pH 8.3). Nonspecific binding was blocked with 5% BSA in TBST buffer (10 mM Tris-HCl, 150 mM NaCl, and 0.2% Tween-20, pH 7.4) and the nitrocellulose membranes were incubated with primary antibodies listed in Supplementary Table 4. Protein loading controls were probed with mouse anti-Gapdh antibodies (1:160,000; Biorad International, Saco, Maine, USA). The membrane was washed by TBST buffer and incubated with horseradish peroxidase-conjugated anti-mouse IgG antibodies (1:5000; Cell Signaling, Danvers, Massachusetts, USA) or anti-rabbit IgG antibodies (1:5000; Cell Signaling, Danvers, Massachusetts, USA) in TBST buffer. The bound antibodies were detected using Chemiluminescence reagent Plus (PerkinElmer Life Science, MA, USA) and Bio-rad ChemiDoc™ XRS+ Systems. The Image Lab™ Software was used to obtain images under appropriate exposure time.

### Measurement of Cerebral Collateral Circulation and Hemoglobin Oxygenation

The dual-wavelength optical imaging system was used to simultaneously measure spatiotemporal characteristics of the relative cerebral blood flow (rCBF) of collateral blood vessels and cerebral metabolic rate of oxygen ( $\text{CMRO}_2$ ) of the cortical tissue, which combined the laser speckle contrast imaging (LSCI) and optical intrinsic signal imaging techniques [19]. The measured rCBF and  $\text{CMRO}_2$  of regions of interest (ROIs) were averaged by a 60-second imaging period to reduce the noise. To measure the changes of hemodynamics and metabolic responses in the same animal before and after MCAO with multiple times, each animal received the implantation of

glass coverslip based cranial window over the cortical surface for repeated *in vivo* imaging. The preparation and implantation procedure of cranial window were described previously [19].

### Measurement of Adenine Nucleotides

Tissues from the ipsilateral and contralateral cortex of mice with tMCAO were rapidly dissected and snap-frozen in liquid nitrogen to stop enzymatic activity. Brain tissues were then extracted in an ice-cold buffer (methanol/acetonitrile/water, 40/40/20; v/v/v) with DL-4-chlorophenylalanine (ACROS organics, Lancashire, UK) as an internal standard. After being centrifugated at 13,000 rpm for 15 min at 4°C, the supernatant was collected for analysis on the same day. The assay was conducted by an Agilent 1290 Infinity II ultra-performance liquid chromatography (UPLC) system (Agilent Technologies, Palo Alto, CA, USA), coupled to the Dual AJS electrospray ionization (ESI) source of an Agilent 6545 XT quadrupole time-of-flight (Q-TOF) mass spectrometer (Agilent Technologies, USA). The samples were eluted over an ACQUITY UPLC BEH amide column (1.7 μm, 2.1 × 100 mm, Waters Corp., Milford, MA, USA) by mobile phases composed of double-distilled water (eluent A) and 10% double-distilled water in acetonitrile (eluent B). Both eluents contained 15 mM ammonium acetate and 0.3% ammonium hydroxide solution. The column temperature was kept at 40°C at a flow rate of 300 μL/min. The instrument was operated in positive and negative full-scan mode, collected from an *m/z* of 60–1500. The chromatogram acquisition, detection of mass spectral peaks, and their waveform processing were performed using Agilent Qualitative Analysis 10.0 (Agilent, USA) and Agilent Profinder B.10.00 software (Agilent, USA).

### Measurement of ROS

ROS was detected using dihydroethidium (DHE, Invitrogen, Carlsbad, CA, USA) as described previously [20] with modifications. Thirty minutes following the reperfusion, mice were injected with DHE (20 mg/kg) intraperitoneally. Twenty-four hours after the injection, mice were sacrificed and the whole brains were collected. Brain ROS levels were measured by an IVIS spectrum imaging system (IVIS 200, Xenogen, CA, USA) with a fluorescence filter (Excitation: 500 nm, Emission: 600 nm). Data were analyzed by the software Living Image (Xenogen, CA, USA).

### Statistical Analysis

The investigators were unaware of their genotypes during surgeries and outcome evaluations. The exclusion criteria were applied as following: (1) death during surgeries due

to surgical causes or anesthetic problems; (2) unsuccessful MCAO; (3) failure to achieve reperfusion; (4) death before sacrifice for sampling; (5) absence of observable ischemic core in TTC staining. Statistical differences were evaluated by the Student's *t* test or two-way analysis of variance (ANOVA), followed by pairwise comparisons using Tukey's method. A *P* value <0.05 was considered statistically significant. Post-hoc analysis was performed with G\*power software (version 3.1) to examine whether the values of statistical power were all greater than 0.8 for the significant differences between WT and Ent2<sup>-/-</sup> mice.

## Results

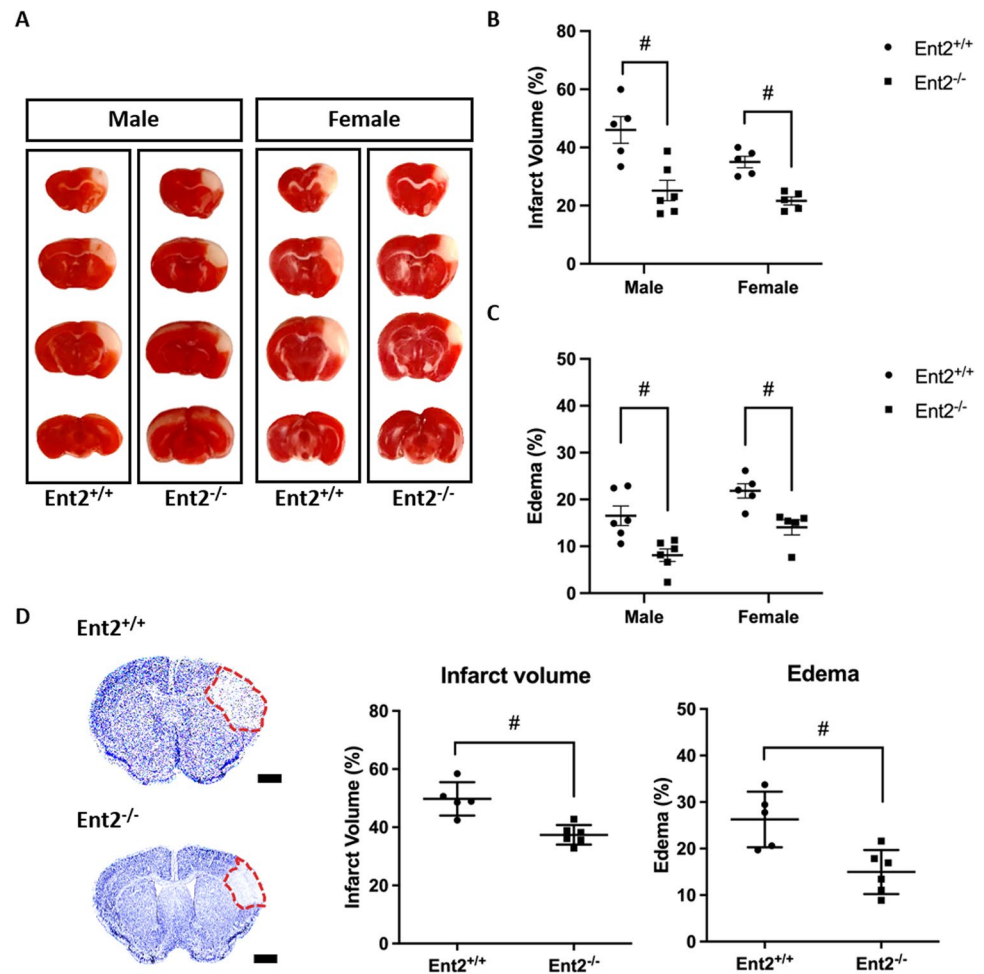
### Ent2 Deletion Reduces Infarct Volume and Brain Edema Caused by tMCAO in both Male and Female Mice

To investigate the impact of Ent2 deletion on brain injury caused by tMCAO, infarct volume and brain edema were examined in Ent2<sup>-/-</sup> mice and the WT controls. The TTC staining showed that the infarct volume and brain edema caused by tMCAO were significantly lower in both male and female Ent2<sup>-/-</sup> mice, compared with the WT controls (Fig. 1A to C). The results also showed that the protective effect of Ent2 deletion in ischemia-reperfusion injury was in a gender-independent manner. Further studies using the Nissl staining also showed that Ent2 deletion reduced both the infarct volume and brain edema of mice with tMCAO (Fig. 1D).

### Ent2 Deletion Ameliorates tMCAO-Induced Neuroinflammation

The neuroinflammatory response is an important event of brain ischemia, which may exacerbate the infarct volume and brain edema [21, 22]. Given that the proinflammatory cytokines, TNF-α, IL-1β, and IL-6, are considered to be critical in the progression of neuroinflammation and contribute to cerebral injury and BBB damage in stroke [22], the expression of these cytokines in the ipsilateral and contralateral cortex was examined. The qRT-PCR results showed that mRNA levels of TNF-α, IL-1β, and IL-6 were significantly increased in the ipsilateral cortex of Ent2<sup>+/+</sup> mice with tMCAO, whereas Ent2 deletion reduced the levels of these cytokines (Fig. 2A). On the other hand, the immunoblotting showed that Gfap protein was increased in the ipsilateral cortex of Ent2<sup>+/+</sup> mice, but not of Ent2<sup>-/-</sup> mice (Fig. 2B). The immunofluorescence analysis also indicated that Ent2 deletion can reduce the presence of reactive astrocytes in mice with tMCAO (Fig. 2C). However, different from the findings in the levels of Gfap protein, tMCAO did not cause

**Fig. 1** Ent2 deletion reduces infarct volume and brain edema in mice with ischemic-reperfusion. (A), TTC staining of brain slices of male and female Ent2<sup>-/-</sup> mice and the WT (Ent2<sup>+/+</sup>) controls 24 hours after tMCAO. (B-C), The ratio of cerebral infarction volume (B) and edema (C) in TTC-stained slices in males and in females. Data are presented by mean ± SEM (n = 5, 6, 5, and 5 for male Ent2<sup>+/+</sup>, male Ent2<sup>-/-</sup>, female Ent2<sup>+/+</sup>, and female Ent2<sup>-/-</sup> mice, respectively). (D), Nissl staining of brain slices and the quantification of the ratio of cerebral infarction volume and brain edema in Nissl-stained slices in Ent2<sup>-/-</sup> mice and the WT controls. The scale bar represents 1 mm. Data are presented by mean ± SEM (n = 5–6; mixed gender). #p < 0.05 compared with Ent2<sup>+/+</sup> group.



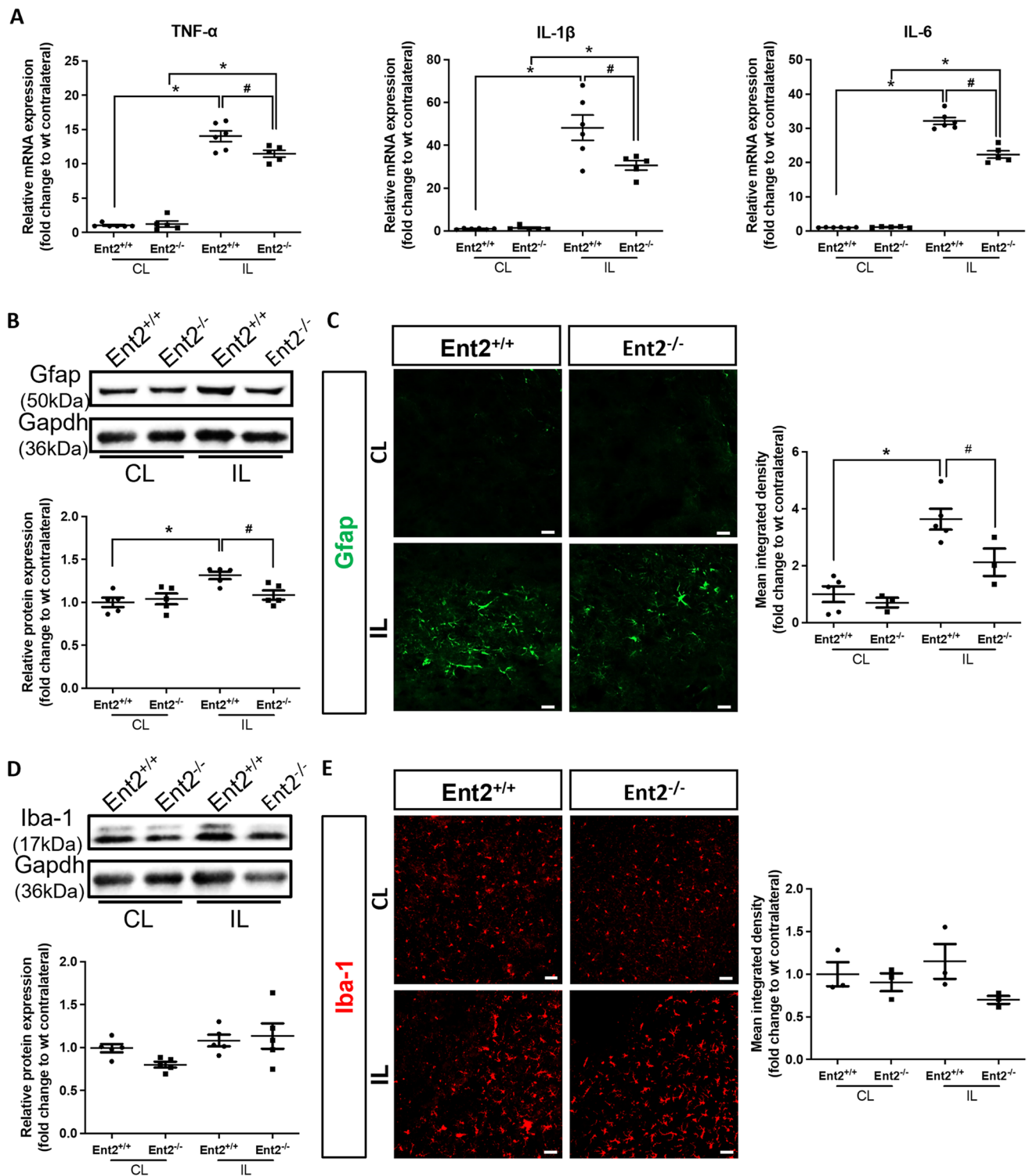
a significant change in the level of Iba-1 protein in mice with and without Ent2 (Fig. 2D and E), suggesting that Ent2 deletion reduces the number of Gfap-positive astrocytes, but did not alter the level of Iba-1-positive microglia/macrophages.

### Ent2 Deletion Attenuates tMCAO-Induced Impaired Microvascular Integrity and Function

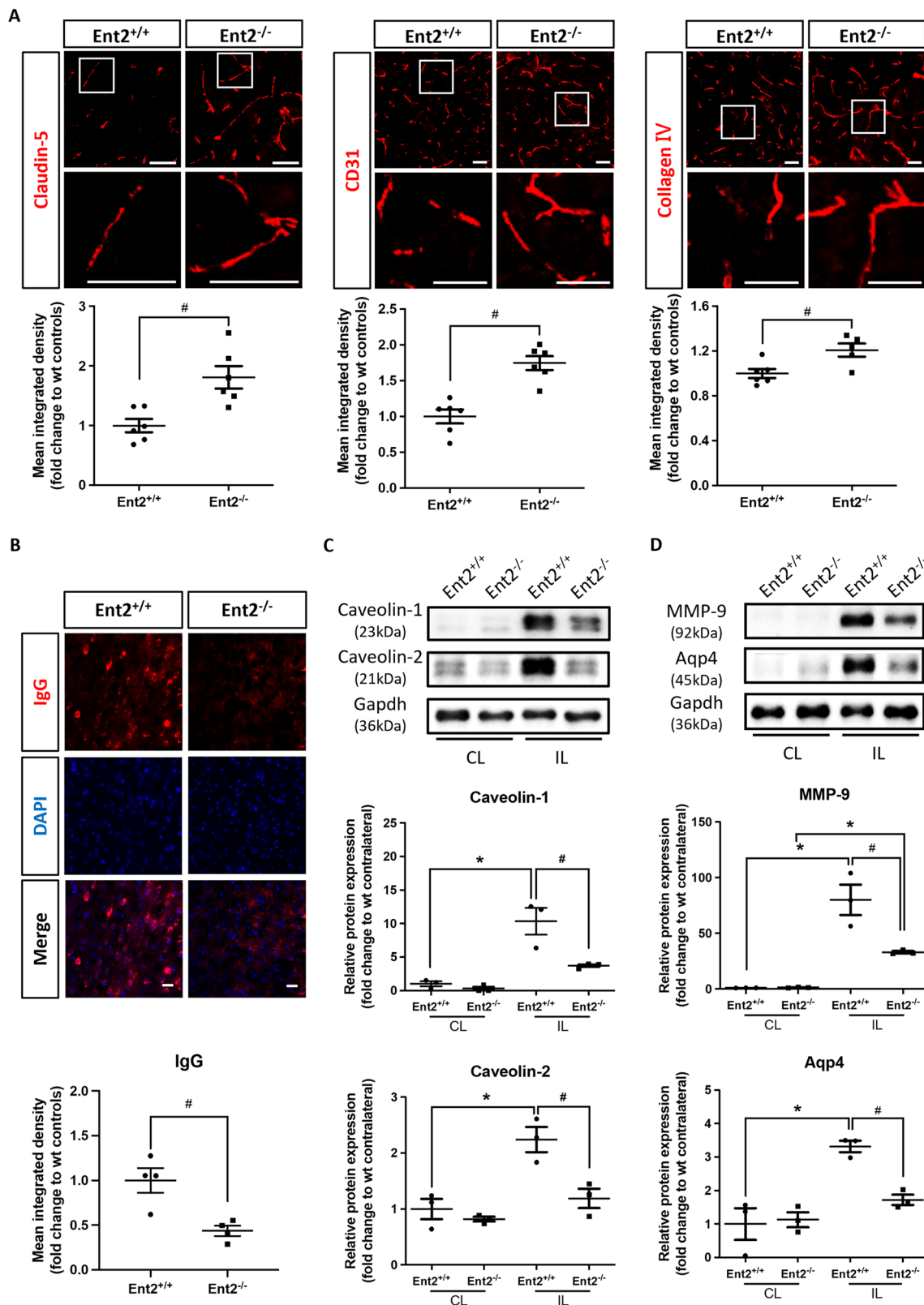
The disruption of brain blood vessels is a notable feature of ischemia and ischemia-reperfusion. Neuroinflammation can further disrupt the vessel component and increase the permeability of the BBB after MCAO [21, 23]. To investigate BBB integrity, claudin-5, CD31, and collagen-IV, marker proteins of the tight junction, endothelial cells, and basement membrane, respectively, were evaluated. In Ent2<sup>-/-</sup> mice, the expression of claudin-5, CD31, and collagen-IV in the penumbra area was significantly higher and more continuous than that in the WT controls (Fig. 3A). To evaluate BBB permeability, brain permeation of endogenous IgG was examined in the brain of WT and Ent2<sup>-/-</sup> mice after tMCAO. The results showed that the leakage of IgG was detected in the ipsilateral cortex of mice with tMCAO, but

was significantly ameliorated in mice with Ent2 deletion (Fig. 3B). In addition to the disruption of the paracellular barrier, enhanced transcellular transport has been reported to be an initial response of cerebral endothelium in stroke, in which caveolins are involved in this alternative mechanism for BBB opening [24]. Accordingly, the effect of Ent2 deletion on the transcellular pathway was examined in terms of caveolin-1 and caveolin-2 expression, markers for caveolae. As a result, tMCAO significantly increased the expression of both caveolin-1 and caveolin-2 in the cortex of the WT mice, whereas Ent2 deletion reduced elevated levels (Fig. 3C).

In terms of BBB integrity, MMP-9 is a key collagenase to cleave collagen-IV, leading to vascular hemorrhage. Following tMCAO, the protein level of MMP-9 in the ipsilateral cortex was significantly increased (Fig. 3D). However, tMCAO-induced overexpression of MMP-9 was attenuated in Ent2<sup>-/-</sup> mice (Fig. 3D), consistent with the reduced structural loss of collagen-IV in Ent2<sup>-/-</sup> mice (Fig. 3A). Except for MMP-9, Aqp4, the major water channel expressed in brain perivascular astrocyte processes, plays an important role not only in the formation of brain edema, but also in BBB integrity [25]. Our results showed that Aqp4



**Fig. 2** Ent2 deletion reduces neuroinflammation in the ipsilateral cortex of mice with ischemia-reperfusion. **(A)**, The mRNA levels of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in the contralateral and ipsilateral cortex of Ent2<sup>-/-</sup> mice and the WT controls. Data are presented by mean  $\pm$  SEM ( $n=5-6$ ; mixed gender). **(B-C)**, Protein levels of Gfap examined by immunoblotting **(B)** and immunostaining **(C)** in the ipsilateral and contralateral cortex and the quantitative results. **(D-E)**, Protein levels of Iba-1 examined by immunoblotting **(D)** and immunostaining **(E)** in the ipsilateral and contralateral cortex and the quantitative results. For immunostaining, the scale bar represents 50  $\mu$ m. IL=ipsilateral; CL=contralateral. Data are presented by mean  $\pm$  SEM ( $n=3-5$ ; mixed gender). \* $p < 0.05$  compared with the contralateral brain regions; # $p < 0.05$  compared with Ent2<sup>+/+</sup> group.



**Fig. 3** Ent2 deletion protects against BBB disruption in the cortex of mice with ischemia-reperfusion. **(A)**, Representative images and the quantitative results of claudin-5 (**left**), CD31 (**middle**), and collagen-IV (**right**) immunostaining in the penumbra area. **(B)**, Representative images and the quantitative results of endogenous IgG in the penumbra area. **(C-D)**, Immunoblots and the quantitative results of caveolin-1 and caveolin-2 (**C**), MMP-9 and Aqp4 proteins (**D**) in Ent2<sup>-/-</sup> mice and the WT controls. The scale bar represents 50 μm. IL=ipsilateral; CL=contralateral. Data are presented by mean ± SEM (n = 3–6; mixed gender). \*p < 0.05 compared with the contralateral brain regions; #p < 0.05 compared with Ent2<sup>+/+</sup> group.

expression was significantly increased in the ipsilateral cortex after ischemia-reperfusion, but can be reversed by Ent2 deletion (Fig. 3D). These findings demonstrated that Ent2 deletion protects against tMCAO-induced BBB breakdown and water flux.

### Ent2 Deletion Modulates rCBF Recovery and Reduces ROS Production in Mice with tMCAO

With more intact microvessels in Ent2<sup>-/-</sup> mice with tMCAO, therefore, we examined the changes in rCBF and hemoglobin oxygenation at pial collateral blood vessels. The rCBF decreased in both Ent2<sup>-/-</sup> and WT groups 90 minutes after MCAO. Following the reperfusion, the rCBF of the WT mice was quickly restored to the baseline. However, in mice with Ent2 deletion, rCBF remained at a low level at the initial phase (i.e., 4 minutes) of reperfusion, but was then higher at the later phase (i.e., 24 hours) of reperfusion (Fig. 4B).

Consistent with the findings of rCBF, the level of CMRO<sub>2</sub>, an indicator for tissue oxidative metabolism, was significantly decreased in both Ent2<sup>-/-</sup> and WT mice at all stages post-MCAO. Nonetheless, mice with Ent2 deletion showed a higher recovery of CMRO<sub>2</sub> 24 hours after reperfusion (Fig. 4C). Further study showed that tMCAO-induced cortical ATP depletion was restored in Ent2<sup>-/-</sup> mice (Fig. 4D), corroborating the findings in CMRO<sub>2</sub>. On the other hand, since ROS production is a critical contributor to ischemia-reperfusion injury, we then measured the ROS levels. The results showed that ROS production was significantly lower in Ent2<sup>-/-</sup> mice shortly (i.e., 30 minutes) after the reperfusion (Fig. 4E). These data demonstrated that Ent2 deletion can modulate the restoration of the blood flow, restore oxygen metabolism, and reduce ROS production after ischemia-reperfusion.

### The Activation of A<sub>2A</sub>R, but Not A<sub>1</sub>R, Is Involved in the Protective Effects of Ent2 Deletion in Mice with tMCAO

Since Ent2 deletion can result in an increase in extracellular adenosine levels [11], we then examine whether the protective effects of Ent2 deletion are mediated by adenosine receptors. Given that A<sub>1</sub>R and A<sub>2A</sub>R are high-affinity receptors, the A<sub>1</sub>R antagonist, DPCPX, and the A<sub>2A</sub>R antagonist, SCH58261, were administered for 3 consecutive days in mice with tMCAO. The results showed that treatment with SCH58261, but not DPCPX, abrogated the attenuation of infarct volume caused by Ent2 deletion (Fig. 5A, C). Additionally, SCH58261 treatment abolished the protective effects of Ent2 deletion on microvascular integrity, examined by claudin-5, CD31, and collagen-IV (Fig. 5B) and brain accumulation of IgG (Supplementary Fig. 1). In contrast, the

administration of DPCPX did not block the protective effects of Ent2 deletion on BBB structure (Fig. 5D). These data suggest that A<sub>2A</sub>R, but not A<sub>1</sub>R, is involved in the protective effects of Ent2 deletion in mice with ischemia-reperfusion.

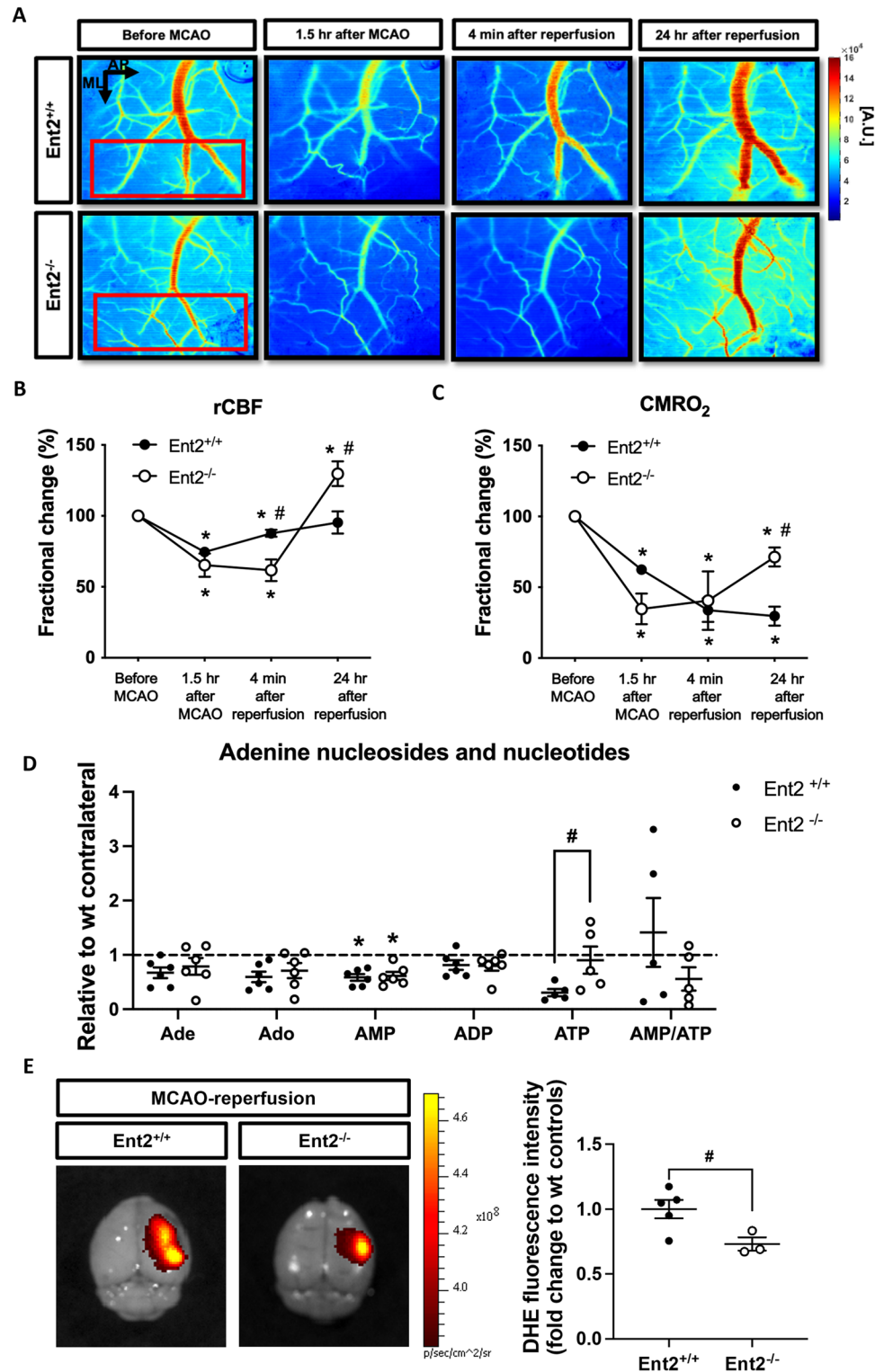
## Discussion

The blockage of cerebral blood flow in ischemic stroke leads to neuronal death, neuroinflammation, BBB breakdown, edema, and long-lasting neurological dysfunctions. While the restoration of cerebral blood flow is the treatment of choice, the reperfusion causes a significant increase in oxidative stress and may lead to hemorrhage transformation. Accordingly, vascular protection is important for the treatment of ischemic stroke. In response to the insult of ischemia-reperfusion, brain extracellular level of adenosine, an important neuromodulator, is dramatically increased. In the present study, we demonstrated that the deletion of Ent2, a major transporter for cellular transfer of adenosine in the brain, provides beneficial effects against tMCAO-induced infarct volume, edema, neuroinflammation, neurovascular-associated injury, and ROS production. These findings demonstrated the important role of Ent2 in the pathogenesis and treatment of ischemia-reperfusion in the brain.

Previous studies have shown that Ent1 inhibition protected animals from tMCAO-triggered neuronal apoptosis and neurological deficit [26]. Compared to Ent1, the expression of Ent2 is more abundant in the brain of mice [11]. After the induction of tMCAO, the mRNA level of Ent2 was significantly increased in the ipsilateral cortex, compared to the contralateral cortex in mice (Supplementary Fig. 2), suggesting a potential role of Ent2 in maintaining adenosine homeostasis under the ischemia/reperfusion condition. In addition to the amelioration of neuronal damage, the present findings further showed that Ent2 deletion preserved vascular integrity/function in mice with tMCAO (Figs. 3 and 4). In relation to vascular integrity, the dysregulation of MMP-9 plays a critical role in causing neurovascular disruption, leukocyte infiltration, cerebral edema, and even hemorrhage [27, 28]. MMP-9 level is associated with infarct size and poor neurological outcomes in animals with stroke [29], in which MMP-9 knockout mice had significantly smaller lesions compared to the WT mice after permanent and transient focal ischemia [30]. While MMP-9 can be activated by a number of factors, including cytokines (i.e., TNF- $\alpha$  and IL-1 $\beta$ ) [31], the administration of t-PA can also promote MMP-9 upregulation after focal cerebral ischemia [32]. Of note, intracranial hemorrhage caused by vascular disruption is the most feared complication of thrombolytic therapy. The reduced MMP-9 in Ent2<sup>-/-</sup> mice indicates the concomitant use of Ent2 inhibitors, if available, with thrombolytic agents is worth attention. In relation to this, the effects of

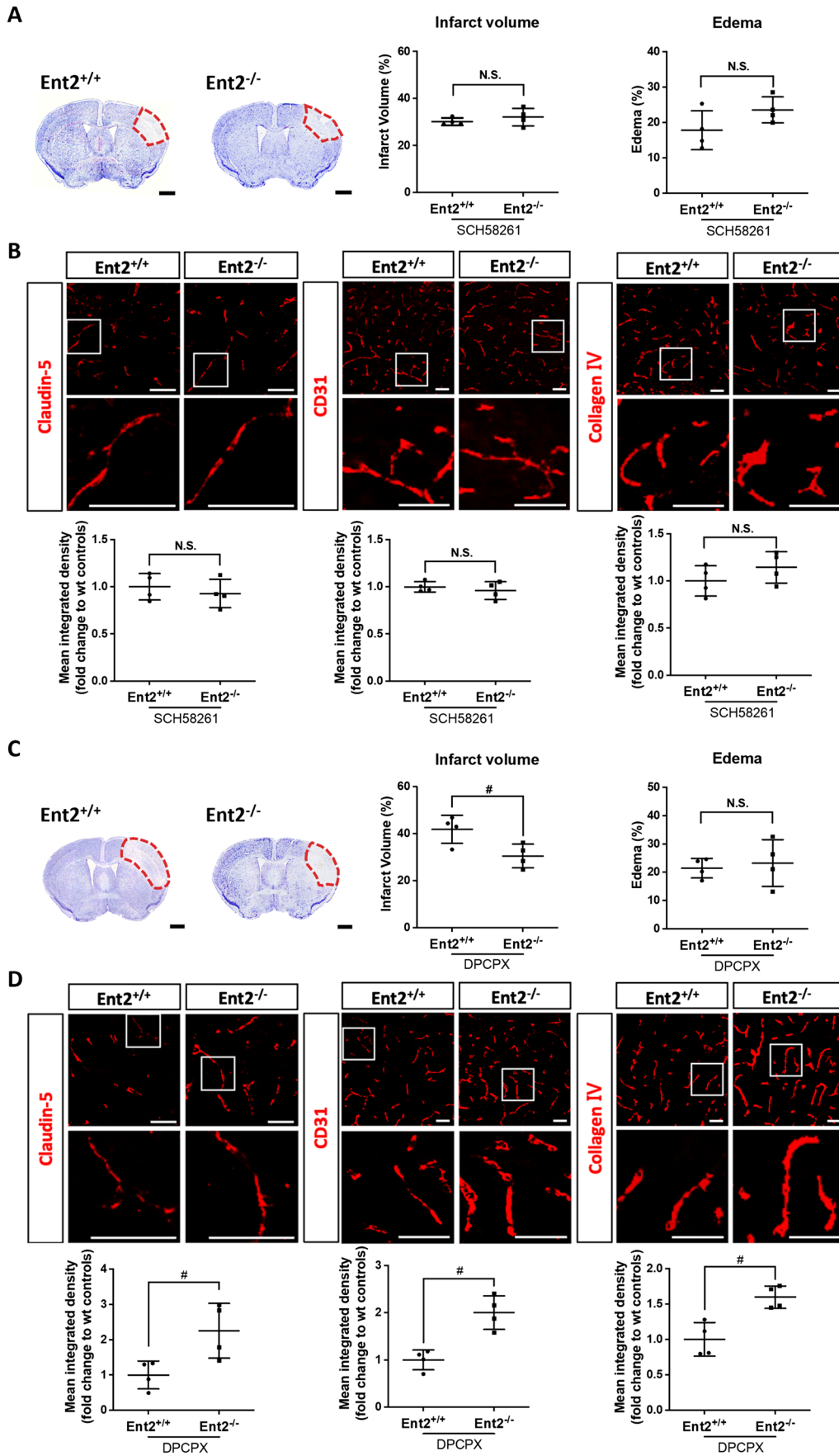


**Fig. 4** Ent2 deletion alters hemodynamics, metabolic responses, and ROS production after tMCAO. (A), Pseudo-color maps of rCBF measured with LSCI before MCAO (the baseline), 1.5 hours after ischemia, 4 minutes post-reperfusion, and 24 hours post-reperfusion in mice with tMCAO. ROIs placed in collateral vessels (red boxes) were used to measure the rCBF and CMRO<sub>2</sub> of collateral circulation. (B–C), The fractional changes of rCBF (B) and CMRO<sub>2</sub> (C) of pial collateral circulation before and after MCAO. Data are presented by mean ± SEM (n = 4–5; mixed gender). (D), The levels of adenine nucleosides and nucleotides in the ipsilateral cortex of Ent2<sup>-/-</sup> mice and the WT controls (relative to those in the contralateral cortex of WT mice). Data are presented by mean ± SEM (n = 6; mixed gender). (E), ROS production at 30 minutes following the reperfusion. Data are presented by mean ± SEM (n = 3–5; mixed gender). \*p < 0.05 compared with the values before MCAO in (B)–(C) and compared with the values in the contralateral cortex of WT mice in (D); #p < 0.05 compared with the WT group in (B)–(E).



dipyridamole, an inhibitor of both Ent1 and Ent2, on the treatment of ischemic stroke have been investigated by a number of clinical studies. A large randomized controlled trial showed that dipyridamole alone or in combination with aspirin significantly reduce recurrent ischemic stroke and

other vascular events [33]. However, others did not provide positive results, possibly due to a small sample size and low statistical power [34]. Nonetheless, it should also be noted that the transport of dipyridamole across the BBB is rather limited [35]. The development of a BBB-permeable Ent2



**Fig. 5** Adenosine receptor  $A_{2A}R$ , but not  $A_1R$ , is involved in the protective effects of Ent2 deletion against tMCAO-induced damages. (A), The infarct volume and edema estimated by Nissl staining in brain slices of Ent2<sup>-/-</sup> and the WT mice with the treatment of SCH58261 (an  $A_{2A}R$  inhibitor). (B), Immunostaining of claudin-5, CD31, and collagen-IV in the penumbra of Ent2<sup>-/-</sup> and the WT mice with the treatment of SCH58261. (C), The infarct volume and edema of brain slices of Ent2<sup>-/-</sup> and the WT mice with the treatment of DPCPX (an  $A_1R$  inhibitor). (D), Immunostaining of claudin-5, CD31, and collagen-IV in the penumbra of Ent2<sup>-/-</sup> and the WT mice with the treatment of DPCPX. The scale bar represents 1 mm in Nissl staining and 50  $\mu$ m in immunostaining, respectively. Data are presented by mean  $\pm$  SEM (n=4; mixed gender). N.S. represents not significant ( $p > 0.05$ ), # $p < 0.05$  compared with Ent2<sup>+/+</sup> group.

inhibitor would be helpful to further investigate the role of Ent2 in the pathogenesis and treatment of stroke.

Except for the activation of MMPs, edema is also considered as one of the cardinal signs of neuroinflammation in stroke. Ent2 deletion not only ameliorated neuroinflammation but also reduced brain edema (Figs. 1 and 2), supporting the connection between inflammatory responses and edema formation. On the other hand, edema formation is a critical issue associated with cerebrovascular dysfunction after ischemic stroke. Aqp4 controls water flux on the endfeet of perivascular astrocytes. Aqp4 upregulation may induce astrocyte swelling and exaggerate the ischemic brain injury. Acutely inhibiting Aqp4 decreased edema and astrogliosis in cerebral ischemia [36]. Aqp4 deletion reduced brain edema and improved neurological and BBB functions in ischemia [37, 38]. Accordingly, the attenuation of tMCAO-induced brain edema in Ent2<sup>-/-</sup> mice may involve the reduction in Aqp4 protein expression (Figs. 1 and 3D), which also suggests that Ent2 may play a role in regulating Aqp4-dependent perivascular flow.

Collateral circulation is an important predictor of the outcome of acute ischemic stroke. The promotion of collateral circulation may reduce ischemic brain injury [39]. Yet, ROS production caused by ischemia-reperfusion is involved in neuronal cell injury and edema formation in mice with tMCAO [40]. ROS production was significantly increased shortly (i.e., 10–30 minutes) after the reperfusion [41, 42]. It was suggested that ROS generated during the initial stages of cerebral ischemia contributes to brain injury [42]. In the present study, while the rCBF at the collateral blood vessels of the WT mice was restored quickly, ROS production in WT mice was also significantly increased (Fig. 4). Unlike the findings in WT mice, rCBF of Ent2<sup>-/-</sup> did not return to the baseline (i.e., the level before MCAO) promptly after the reperfusion. Instead, it resumed slowly at the initial phase and then reached a higher level later. The delayed recanalization in Ent2<sup>-/-</sup> mice was accompanied by a lower increase in ROS level, providing protection from a sudden increase in ROS attack. In terms of the recanalization after stroke, cerebrovascular dysregulation in the ischemic region leads to

the inability of the cerebral blood vessels to dilate and draw blood from neighboring vascular territories with normal blood supply [43]. Interventions such as increasing adenosine level on vasodilation can improve rCBF and result in the amelioration of tissue damage in ischemic stroke [44, 45]. Nonetheless, it was found that, in response to an intravenous injection of dipyridamole, an inhibitor of Ent1/Ent2, the increases in plasma adenosine levels appeared to be biphasic [46]. Therefore, the slower recovery rate of the rCBF in Ent2<sup>-/-</sup> mice may be due to the changes of adenosine level that leads to vasodilatory effect and  $A_{2A}R$  signaling. Although the detailed mechanism of the regulation of rCBF in Ent2<sup>-/-</sup> condition remains to be defined, the present study suggests that adenosine augmentation by targeting Ent2 is a promising approach to modulate collateral cerebral blood flow following reperfusion.

While extracellular adenosine level is increased in ischemia and ischemia-reperfusion [7], Ent2 deletion may further increase the level [11], which may in turn activate both  $A_1R$  and  $A_{2A}R$ . In the present study, the administration of the  $A_{2A}R$  antagonist, SCH58261, blocked the reparative effects of Ent2 deletion in tMCAO mice, whereas treatment with DPCPX (an  $A_1R$  antagonist) did not reverse the protection. Although acute activation of  $A_1R$  is commonly considered to be neuroprotective by inhibiting glutamate release and excitatory synaptic transmission, the neuroprotection of Ent2 deletion appeared not to be mediated by  $A_1R$  activation. One potential explanation for this finding is that  $A_{2A}R$  activation may cause  $A_1R$  desensitization, especially under conditions of high adenosine levels [47]. Targeting  $A_{2A}R$  has been proposed as a therapeutic strategy for stroke treatment. Yet, the beneficial effects caused by  $A_{2A}R$  antagonists/agonists seem to depend on the time window following stroke onset, in which  $A_{2A}R$  antagonists provide early protection by controlling excitotoxicity, whereas  $A_{2A}R$  agonists provide protracted protection by controlling inflammatory responses [48]. In addition,  $A_{2A}R$  activation can inhibit MMP-9 release [49] and exhibit vasodilated effects [50], both of which may preserve microvascular structure and function that are critical for stroke treatment. In addition to the effects of adenosine, intracerebroventricular infusion of inosine was shown to reduce cerebral infarction and glutamate responses [51]. As inosine can be formed by the deamination of adenosine extracellularly, the elevation of extracellular adenosine level may be accompanied by an increase of inosine level. However, it was noted that adenosine ( $EC_{50} \sim 6$  nM) has a significant higher affinity for  $A_{2A}R$  than that of inosine ( $EC_{50} \sim 300$   $\mu$ M) [52]. Since our findings suggest that  $A_{2A}R$  activation is involved in the effect of Ent2 deletion (Fig. 5), the contribution of inosine in this aspect may be relatively minor.

In terms of nucleoside transporters, the concentrative nucleoside transporters (Cnts) are also expressed in the

mouse brain. Cnts are unidirectional nucleoside transporters coupled with the sodium/proton gradient. Compared with Ents, Cnts have higher apparent affinity but a much lower turnover rate for nucleosides [53]. In this regard, Cnts are considered to be associated with nucleoside sensing and signal transduction [54]. While the expression level of Cnt1 and Cnt3 is reported to be low in the brain [55] and Cnt2 expression is downregulated in the infarcted tissue [56], we cannot exclude the implication of Cnts in ischemia/reperfusion. On the other hand, since the damaged region of the ligation MCAO model was only confined to the cerebral cortex, deficits of neurobehavioral functions were not obvious in this model, as observed in the present study (data not shown). Further study employing a stroke model induced by intraluminal filament MCAO, which may result in a more severe damage in the cerebral cortex, striatum, and hippocampus, is required to explore the role of Ent2 in long-term neurobehavioral outcomes.

## Conclusions

Ent2 deletion provides beneficial effects on mice with cerebral ischemia and reperfusion injury by reducing the infarct volume, brain edema, and neuroinflammation, with the restoration of brain microvascular function. These protective effects involve A<sub>2A</sub>R activation.

**Abbreviations** Aqp4: aquaporin-4; A<sub>2A</sub>R: adenosine 2A receptor; CD31: cluster of differentiation 31; CMRO<sub>2</sub>: cerebral metabolic rate of oxygen; Ent: equilibrative nucleoside transporter; MMP-9: matrix metalloproteinase-9; rCBF: regional cerebral blood flow; ROS: reactive oxygen species; tMCAO: transient middle cerebral artery occlusion.

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**Authors' Contributions** H-L C contributed to the conception and design, acquisition and analysis of data, and manuscript preparation; K-C W contributed to the acquisition and analysis of data and manuscript preparation; Y-Y C, C-J H, H-L W, Y-H F, and W-Y C contributed to the acquisition and analysis of data; C-J L contributed to the conception and design, advised, manuscript preparation, and funding acquisition. All authors read and approved the final manuscript.

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**Data Availability** The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

## Declarations

**Ethical Approval and Consent to Participate** All animal experiments were approved by the Institutional Animal Care and Use Committee of National Taiwan University College of Medicine (IACUC number: 20201107).

**Consent for Publication** Not applicable.

**Competing Interests** The authors declare no competing interests.

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