minimum amino acids of P_{30} peptide in ProTα is responsible for neuroprotection. In the present study, we evaluated the neuroprotective effect of 9-amino acid peptide derived from ProTα against the ischemic stress.

2. Materials and methods

2.1. Animals

Male C57/BLJ mice weighing 20–25 g were purchased from Tagawa Experimental Animals (Nagasaki, Japan) and used for all the experiments. Mice were kept in a room maintained at constant temperature (21 ± 2 °C) and relative humidity (55 ± 5%) with an automatic 12 h light/dark cycle with free access to standard laboratory diet and tap water. Animal care and all experimental procedures were formally approved by Nagasaki University Animal Care and Use Committee (Animal Experiments Approval Number: 1104190914).

2.2. Determination of short peptide by alanine scanning

The procedure for the identification of neuroprotective peptide sequence P_{30} (P_{30}: amino acids 49–78) in ProTα has been described previously [17]. To design ProTα-derived shorter neuroprotective peptide, alanine scanning of P_{30} was performed to determine the contribution of specific amino acid residues that retain the original function of P_{30} peptide. Neuroprotective activity of primary cultured cortical neurons was measured at 12 h after the start of serum-free culture in the presence or absence of various peptides. Cultures and methods of the measurement of survival activity were previously described [17,45].

2.3. Peptide administration

Peptide P_9 was dissolved in 0.05% dimethyl sulfoxide (DMSO), which was diluted with 0.1 M potassium (K^+)-free phosphate buffered saline (PBS). Following the protocol of injection in the eye as described previously [17], P_9 was administered intravitreously (i.vt.) with doses of 1, 3 and 10 pmol/μl at 24 h after retinal ischemia ($n = 5$, $n = 7$ and $n = 7$, respectively). On the other hand, P_9 was injected intravenously (i.v.) with doses of 0.1, 0.3 and 1 mg/kg ($n = 5$, $n = 6$, and $n = 7$, respectively) 1 h after the cerebral ischemia (tMCAO). Vehicles were treated with equal volume of 0.05% DMSO in similar manners.

2.4. Ischemic models

Two types of *in vivo* ischemic models were used throughout the experiments. Retinal ischemia was performed following the method as described previously [13,17]. Briefly, mice were anesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg) and pupils were fully dilated with 1% atropine sulfate drops (Nitten, Nagoya, Japan). The anterior chamber of the eye was cannulated with a 33-gauge needle attached to an infusion container of sterile intraocular irrigating solution (BSS PLUS dilution buffer, Alcon, Fort Worth, TX, USA). Retinal ischemia was induced by elevating the IOP to generate a hydrostatic pressure of 130 mm Hg for 45 min by lifting the container. Following 45 min after retinal ischemic stress, the needle was withdrawn and 0.3% ofloxacin (Santen Pharmaceutical Co. Ltd., Osaka, Japan) was applied topically into the eye to avoid infection.

Another ischemic model is a transient middle cerebral artery occlusion (tMCAO) model, which was induced following the method as described previously [16]. Briefly, mice were anesthetized by 2% isoflurane (Mylan, Tokyo, Japan), and body temperature was monitored and maintained at 37 °C during surgery. After a midline neck incision, the middle cerebral artery was occluded transiently using 8-0 in size monofilament nylon surgical suture (Natsume Co. Ltd., Tokyo, Japan) coated with silicon (Xantopren, Bayer dental, Osaka, Japan) that was inserted through the left common carotid artery and advanced into the left internal carotid artery. Following 1 h tMCAO, the animals were briefly re-anesthetized with isoflurane and the monofilament was withdrawn for reperfusion studies. As the silicon-coated nylon suture also plugs the branch from middle cerebral artery to supply blood to hippocampus in mice, due to small brain size, the ischemia-induced brain damages are also observed in the hippocampus. Cerebral blood flow was monitored by laser Doppler flowmeter (ALF21, Advance Co., Tokyo, Japan) using a probe (diameter 0.5 mm) of a laser Doppler flowmeter (ALF2100, Advance Co., Tokyo, Japan) inserted into the left striatum (anterior: −0.5 mm, lateral: 1.8 mm from Bregma; depth: 4.2 mm from the skull surface) through a guide cannula.

2.5. Electroretinogram

Electroretinogram (ERG) study was performed following the protocol as previously described [13,17]. Briefly, mice were dark-adapted for 3–4 h, then anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg) and pupils were dilated with 1% atropine. A contact electrode (KE-S, Kyoto contact lenses, Kyoto, Japan) was placed topically on the corneal apex and reference electrode was placed near the ipsilateral eye. The ground was a subdermal platinum needle electrode near the abdominal area. ERGs were produced by 20 J flash intensities. The flash stimulus source (SLS-3100, Nihon Kohden, Tokyo, Japan) illuminated the eye by diffuse reflection off the interior surface of the ganzfeld. Maximum flash luminance was measured with detector (MEB-9104, Nihon Kohden, Tokyo, Japan). After the intensity series, an incandescent background light sufficient to desensitize the rod system was turned on, and ERGs produced by the standard stimulus were recorded every 2 min for 20 min. The background was then turned off, and ERGs were produced by the standard stimulus every 2 min for the first 30 min of dark adaptation. The a- and b-wave amplitudes were measured online (Neuropack m, QP-903B, Nihon Kohden, Tokyo, Japan). ERG was performed at day 7 after retinal ischemia.

2.6. Tissue processing

All *in vivo* experiments were performed using retinal and brain tissues. For retinal tissue preparation, mice were deeply anaesthetized with sodium pentobarbital (50 mg/kg, i.p.). Eye was quickly isolated, washed with saline and 4% paraformaldehyde (PFA). Eye was then nicked through pupil, post-fixed in 4% PFA for 24 h and finally transferred to 25% sucrose solution (in 0.1 M K^+-free PBS) overnight for cryoprotection. Following freeze in cryoembedding compound, retinal sections were prepared at 10 μm thickness for staining. For brain tissue preparation, mice were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with 0.1 M K^+-free PBS, followed by 4% PFA. Brain was then quickly removed, post-fixed in 4% PFA and transferred immediately to 25% sucrose solution overnight. Brain was frozen in cryoembedding compound and coronal sections were cut at 30 μm thickness for staining.

2.7. Morphological assessment of retinal damages

For hematoxylin and eosin (H&E) staining, on the other hand, frozen retinal sections were washed with 0.1 M K^+-free PBS, immerged in Mayer's hematoxylin solution (WAKO, Osaka, Japan) for 5 min at room temperature (25 °C) and then washed with tap

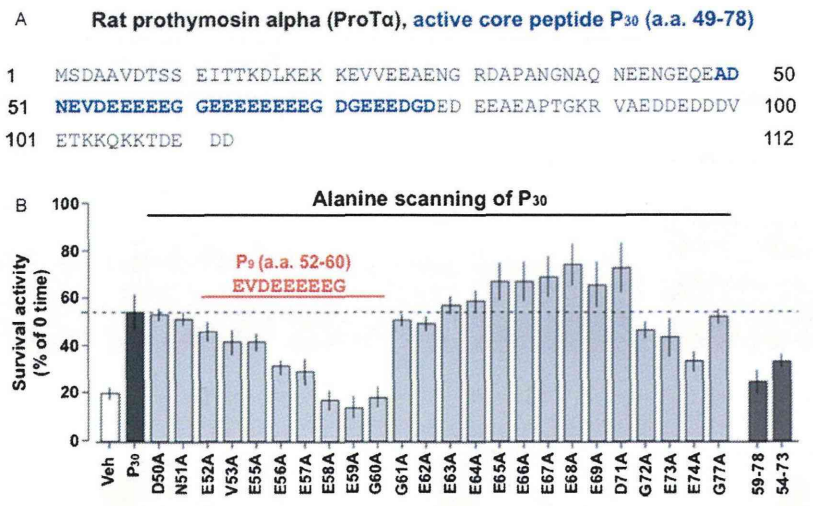

Fig. 1. Central 9-amino acid peptide in ProTα is an essential domain for its survival activity. (A) Amino acid (a.a.) sequence of rat ProTα and active core peptide P_{30}. Blue colored sequence indicates P_{30} (a.a 49–78). (B) Screening of essential amino acid residue of P_{30} in survival activity using alanine scanning of P_{30} and deletion mutant analysis. Survival activity of primary cultured cortical neurons was measured at 12 h after serum-free ischemic stress. Survival activity of P_{30} was significantly abolished by replacement of amino acids between E56 and G60 by alanine, whereas E52A, V53A, and E55A showed partial survival activity. However, the 54–73 amino acid peptide and the 59–78 amino acid peptide, C-terminus of P_{30}, have no significant survival activity. Red colored line and sequence indicate an essential 9-amino acid peptide P_9 and its survival activity. Dashed line indicates the level of the survival activity of P_{30}. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

water for 20 min. Following brief treatment with 95% ethanol, sections were immerged in eosin–alcohol solution (WAKO) for 4 min at 25 °C. Sections were dehydrated through a series of ethanol solutions, xylene, and over-slipped with Permount (Fisher Scientific, Waltham, MA, USA). Sections were then analyzed using a BZ-8000 microscope with BZ Image Measurement Software (KEYENCE, Osaka, Japan).

2.8. TTC staining

For 2,3,5-triphenyltetrazolium chloride (TTC) staining, brain was quickly removed at 24 h after cerebral ischemia (1 h tMCAO) followed by P_9 administration ($n = 6$ for each group), sectioned coronally with 1-mm thickness and washed with K^+-free PBS. Brain slices were incubated in 2% TTC (Sigma–Aldrich, St. Louis, MO, USA) in 0.9% NaCl in dark place for 15–20 min at room temperature (25 °C) and transferred in 4% PFA overnight. Images of brain slices were then collected by scanner, and infarct volume was calculated by Image J software (NIH, Bethesda, MD, USA).

2.9. Evaluation of damaged blood vessels

To perform fluorescence staining of blood vessels, biotinylated *Lycopersicon esculentum* (tomato) lectin (Vector Laboratories, Burlingame, CA, USA) was diluted with PBS and injected (1 mg/ml, 100 μl, i.v.) at 24 h after cerebral ischemia (1 h tMCAO). Mice were perfused 5 min after tomato lectin injection. Following tissue preparation as described in Section 2, coronal brain sections were washed with 0.1 M K^+-free PBS and blocked with 2% BSA in 0.1% PBST for 2 h at 25 °C. Sections were then incubated with Alexa Fluor 488 streptavidin conjugates (Molecular Probes, Eugene, OR, USA) for 2 h at room temperature (25 °C). Sections were thoroughly washed with PBS and cover-slipped with Perma Fluor. Images were collected using an LSM 710 confocal microscope with ZEN Software (Carl Zeiss, Oberkochen, Germany).

2.10. Behavioral assessments

Following P_9 administration with doses of 0.1, 0.3 and 1 mg/kg (i.v., $n = 7$, $n = 6$ and $n = 7$, respectively) 1 h after cerebral ischemia (1 h tMCAO), clinical scores were assessed at 24 h after the ischemic stress. Clinical score was evaluated in the following way: 0, no observable deficits; 1, failure to extend the forepaw fully; 2, circling; 3, falling to one side; 4, no spontaneous movement; 5, death. In this study, 0.5 point was added to each score when the motor dysfunction was severe for scores between 1 and 4.

2.11. Statistical analysis

All results are shown as means ± S.E.M. Two independent groups were compared using the Student's *t*-test. Multiple groups were compared using Dunnett's multiple comparison tests after a one-factor ANOVA or a Repeated Measures ANOVA. Survival rate was compared using Logrank test after Kaplan–Meyer method. $P < 0.05$ was considered significant.

3. Results

3.1. Identification of functionally active short 9-amino acid peptide

More recently, we succeeded in identifying 30-amino acid peptide (P_{30}: amino acids 49–78) from the sequence of ProTα, bearing neuroprotective activity *in vitro* and *in vivo* [17]. To design shorter amino acid peptide from P_{30} peptide in ProTα (Fig. 1A), we screened essential amino acid residue of P_{30} by use of alanine scanning technique (Fig. 1B). Following replacement of amino acids D50 and N51 by alanine (D50A and N51A), no change in survival activity of P_{30} in cultured cortical neurons against ischemic stress was observed at 12 h after the start of culture, whereas E52A, V53A, and E55A showed partial survival activity (Fig. 1B). Interestingly, the survival activity of P_{30} was markedly abolished by replacement of amino acids between E56 and G60 by alanine (Fig. 1B). On the other hand, the survival activity was absent in peptide comprised of 54–73 amino acid residues as well as in C-terminus of P_{30} (amino acids 59–78) against ischemic stress (Fig. 1B). These results indicated that 9-amino acid peptide (E52–G60) is core domain of P_{30} for its full neuroprotective action.

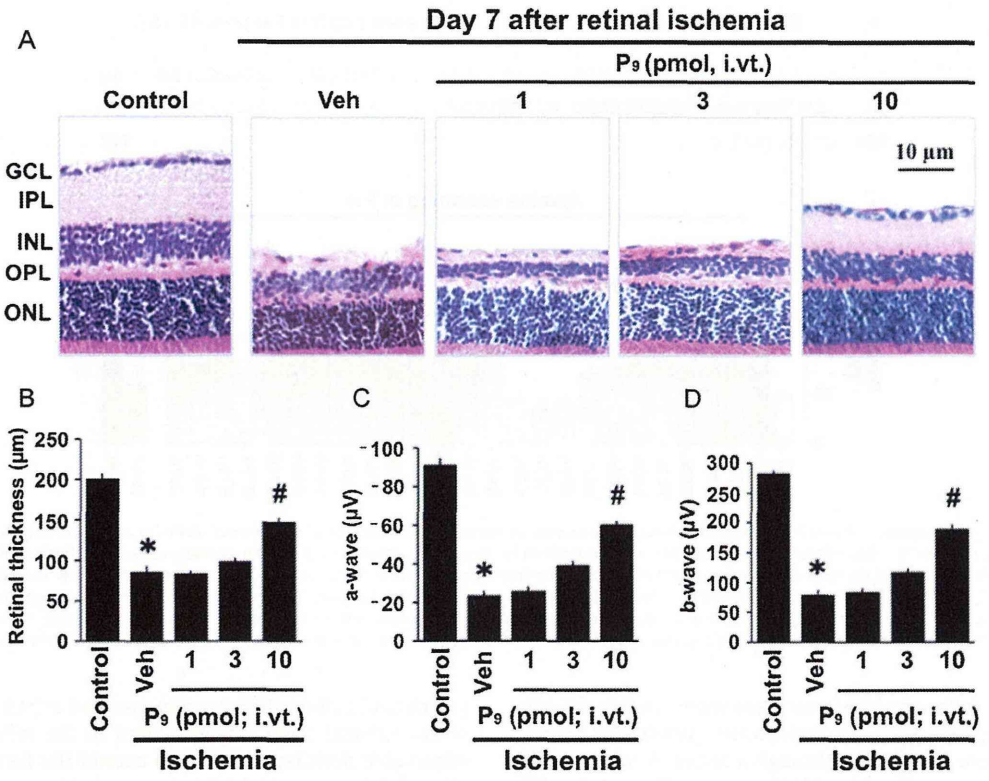

Fig. 2. P_9 peptide inhibits retinal ischemic damages. P_9 is injected intravitreously (i.vt.) with doses of 1, 3 and 10 pmol/μl in the ipsilateral eye at 24 h after retinal ischemia. Vehicle is treated with 0.05% DMSO in a similar manner. (A–D) Dose-dependent protective activity of P_9 against ischemic damages. (A) H&E staining of retinal section is performed in control (left panel), vehicle (middle panel) and P_9 post-treated mice (right panel) at day 7 after retinal ischemia. (B–D) Measurement of retinal thickness (B) as well as the a-wave (C) and b-wave (D) amplitudes of ERG analysis are performed at day 7 after retinal ischemia in control, vehicle and P_9 post-treated mice. The median effective dose of P_9 is 8 pmol/eye for a-wave ($n = 10$), and 4 pmol/eye for b-wave ($n = 10$) of ERG analysis. Data are mean ± S.E.M. (*$P < 0.05$, *vs.* control, #$P < 0.05$, *vs.* Veh) from experiments using 5–7 mice for each group.

3.2. P_9-induced recovery of retinal ischemic damages

To examine whether P_9 has *in vivo* protective effect against ischemic damages, P_9 was injected intravitreously (i.vt.) in the ipsilateral eye with doses of 1, 3 and 10 pmol/μl at 24 h after retinal ischemia. The hematoxylin and eosin (H&E) staining data revealed that number of cells in different retinal layers and retinal thickness are significantly decreased in the vehicle-treated mice at day 7 after ischemia, whereas 10 pmol P_9, but not 1 and 3 pmol maximally and significantly blocked the cellular loss and decrease in retinal thickness at day 7, compared to vehicle (Fig. 2A and B). The a- and b-wave amplitudes in electroretinogram (ERG) study represent the functional activity of photoreceptor cells in the outer nuclear layer (ONL), and mixture of cells in different retinal layers including bipolar, amacrine, Muller and ganglion cells, respectively [13,17]. Experiments using ERG analysis showed that a- and b-wave amplitudes are significantly decreased in the vehicle-treated mice at day 7 after retinal ischemia, compared to control (Fig. 2C and D). Following P_9 injection, amplitudes of a- and b-waves were gradually increased in a dose-dependent manner and 10 pmol P_9 induced the maximum protective effect against ischemic damages at day 7 after ischemia, compared to vehicle (Fig. 2C and D). The a-wave value for naive (non-ischemia) was 94 ± 6 μV ($n = 7$), while 22 ± 3 μV ($n = 7$) for vehicle (ischemia alone). Thus, the median effective dose for P_9 to give 50 μV was 8 pmol/eye. On the other hand, b-wave values for naive (non-ischemia) and vehicle (ischemia alone) were 292 ± 8 μV ($n = 7$) and 90 ± 4 μV ($n = 7$), respectively. In the case of b-wave, the median effective dose for P_9 to give 150 μV was 4 pmol/eye.

3.3. P_9 ameliorates the cerebral ischemic brain

To evaluate the P_9-induced protection against the ischemic damages in brain, P_9 was intravenously (i.v.) administered with doses of 0.1, 0.3 and 1 mg/kg at 1 h after cerebral ischemia (1 h tMCAO), and subsequent 2,3,5-triphenyl tetrazolium chloride (TTC) staining and behavioral tests were performed at 24 h after the ischemic stress. The infarct volume was calculated as the percentage of damaged areas in the ischemic brain on the ipsilateral side to the contralateral side. TTC staining data showed that cerebral ischemia causes a significant increase in ipsilateral infarct volume in the vehicle-treated ischemic mice to 51 ± 3% ($n = 7$) of control. Following systemic injection of P_9 (1 mg/kg, i.v.), infarct volume was maximally and significantly decreased at 24 h in the ischemic brain treated with 0.3 or 1 mg/kg of P_9, which had been given at 1 h after the ischemic stress (33 ± 3%, $n = 7$, and 30 ± 2%, $n = 7$, respectively), compared to vehicle (Fig. 3A and B). On the other hand, the behavioral study showed that clinical scores are significantly increased at 24 h after ischemia in vehicle-treated mice (score: 3.5 ± 0.5, $n = 6$) (Fig. 3C). The systemic injection (i.v.) with 0.3 or 1 mg of P_9 at 1 h after tMCAO caused the significant decline in clinical scores (scores: 2.5 ± 0.5, $n = 6$, and 2.0 ± 0.5, $n = 7$, respectively), as shown in Fig. 3C.

3.4. Blockade of cerebral ischemia-induced blood vessel damages by P_9

To investigate whether P_9 inhibits the ischemia-induced blood vessel damages, P_9 was injected (1 mg/kg, i.v.) at 1 h after cerebral

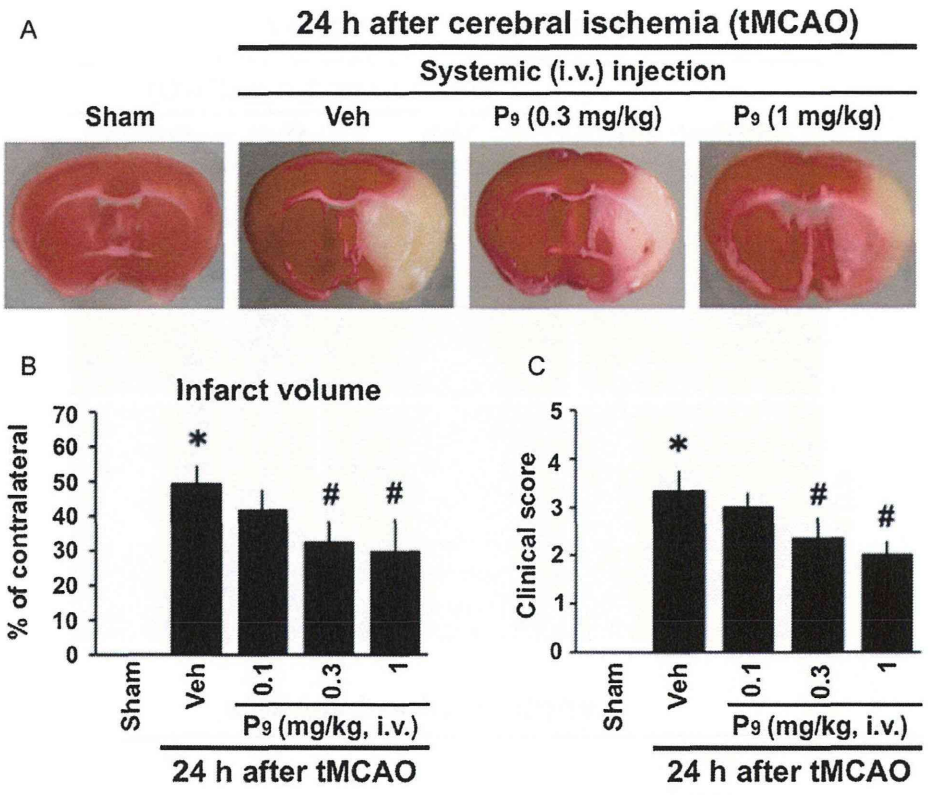

Fig. 3. P_9 improves cerebral ischemic brain. P_9 is injected intravenously (i.v.) with doses of 0.1, 0.3 and 1 mg/kg at 1 h after cerebral ischemia (1 h tMCAO). Vehicles are treated with equal volume of K^+-free PBS in a similar manner. (A–C) TTC staining (A), measurement of infarct volume (B) and clinical scores (C) are performed in sham-operated, vehicle-treated and P_9-treated mice at 24 h after tMCAO. Data represent the means ± S.E.M. (*$P < 0.05$, *vs.* Sham, #$P < 0.05$, *vs.* Veh). Experiments are performed using 5–7 mice for each group.

ischemia (1 h tMCAO). Following *in vivo* binding with biotinylated tomato lectin, the damages of blood vessels after fixation were evaluated by staining with Alexa Fluor 488 streptavidin at day 1 after tMCAO. When the damage was calculated as the ratio of averaged length of blood vessels in the region of somatosensory cortex on the ipsilateral side to the contralateral side, the cerebral ischemia caused a significant decrease to 45 ± 5% ($n = 6$) of control. The systemic injection of P_9 (1 mg/kg, i.v.) completely prevented the ischemia-induced damages of blood vessels (108 ± 6%, $n = 7$), as shown in Fig. 4A and B. Similar significant prevention by P_9 was observed in the striatum (control: 100%, $n = 6$; ischemia alone: 47 ± 4%, $n = 6$; ischemia + P_9: 104 ± 3%, $n = 7$) and hippocampus (control: 100%, $n = 6$; ischemia alone: 34 ± 5%, $n = 6$; ischemia + P_9: 102 ± 4%, $n = 7$).

4. Discussion

The present study includes the following important findings: (1) the 9-amino acid peptide P_9 (amino acids 52–60) derived from ProTα shows the full survival activity in cultured neuronal cells against ischemic stress, (2) P_9 significantly ameliorates the ischemic damages in retina and brain, and (3) P_9 prevents the cerebral ischemia-induced disruption of blood vessels.

The previous *in vitro* investigations suggested that the action of ProTα is related to cell survival [6,23,24,30,45,47], though the sequence-specific diverse functions of ProTα also have been reported [5,14,15,36,42,43]. Most recently, we demonstrated using various deletion mutants of GST-ProTα that the active core peptide P_{30} (amino acids 49–78), but not N-terminal (amino acids 2–29) including the sequence (amino acids 1–48) or C-terminal sequence (amino acids 79–112 and 102–112) in ProTα induces original survival effect in cultured cortical neurons against the ischemic stress [17]. In the present study, we firstly applied alanine scanning technique for the determination of minimum peptide sequence of P_{30} that exerts full neuroprotective effect in cultured cortical neurons under ischemic (serum-free) condition. The findings revealed that the replacement of amino acids D50 and N51 with alanine (D50A and N51A) retains the original survival activity of P_{30} in cortical neurons against the ischemic stress, whereas alanine replacement of E52, V53, and E55 (E52A, V53A, and E55A) exerted partial survival action. Interestingly, the neuroprotective effect of P_{30} was significantly abolished when replaced amino acids between E56 and G60 by alanine. However, the peptide comprised of 54–73 amino acid residues as well as the C-terminal sequence of P_{30} (amino acids 59–78) induced no neuroprotective action against ischemic stress. Therefore, our *in vitro* study clarified that this 9-amino acid peptide P_9 (amino acids 52–60) represents the short active core sequence in P_{30}, which is required for full-length neuroprotection.

Next, we performed *in vivo* experiments to examine the neuroprotective effect of P_9 against ischemic stress in retina and brain. Retinal ischemia is associated with injury-related mechanism-induced destruction of cellular and functional response in the different cell layers of retina, leading to visual disorders and blindness [31,32]. Since retinal ischemic model provides higher reproducibility to comprehend the pathophysiological alternation and signaling cascades [37], this ischemic stress was used as a simple model for screening the neuroprotective action of P_9 by intravitreous administration. It is evident that ProTα improves the ischemic damages in retina [13,47,50]. Most recently, our *in vivo* study demonstrated that P_{30} peptide in ProTα inhibits

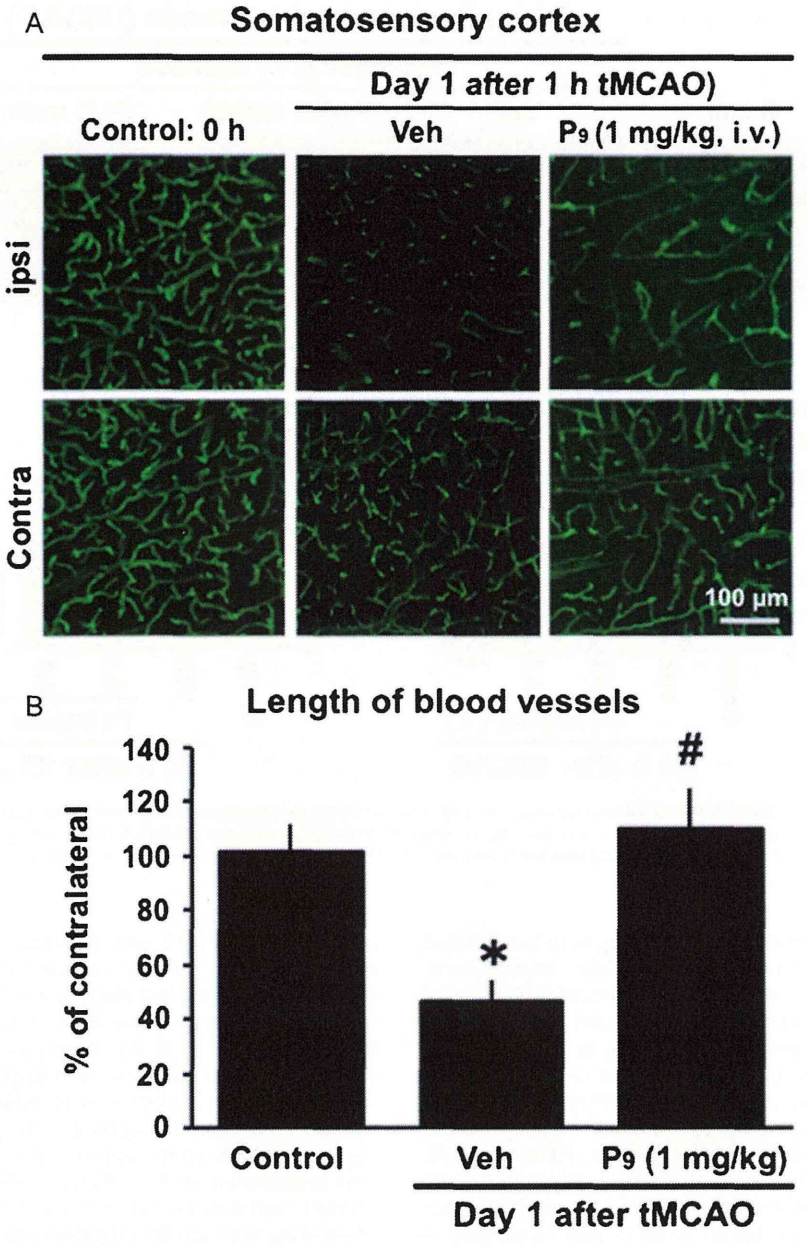

Fig. 4. P_9 prevents cerebral ischemia-induced blood vessel disruption. P_9 is administered (1 mg/kg, i.v.) at 1 h after cerebral ischemia (1 h tMCAO). (A and B) Following biotinylated tomato lectin injection (1 mg/ml, 100 μl, i.v.) at 24 h after tMCAO, and subsequent perfusion of mice 5 min after tomato lectin delivery, the staining of blood vessels using Alexa Fluor 488 streptavidin (A) as well as measurement of the length of blood vessels (B) is performed at day 1 after the ischemic stress. Data represent the means ± S.E.M. ($^*P < 0.05$, *vs.* control, $^\#P < 0.05$, *vs.* Veh) from experiments using 5–7 mice for each group.

ischemia-induced retinal damages [17]. The present findings using H&E staining and ERG analysis revealed that intravitreous treatment with 10 pmol of P_9 at 24 h after retinal ischemia markedly rescues the ischemia-induced decrease in retinal thickness and cell number in different layers in the retina.

Brain ischemia is one of the major clinical issue that is associated with irreversible neurological damages, along with dysfunction of motor, sensory and cognitive systems [10,20,39,40]. There are several reports about ProTα-induced blockade of ischemic brain damages [12,17,48]. Following cerebral ischemia (tMCAO), in the present study, the experiments using TTC staining and behavioral assessment in terms of clinical score suggested that systemic administration of P_9 (1 mg/kg, i.v.) at 1 h after tMCAO significantly improves the ischemic brain. Ischemic stress in the brain causes adequate breakdown of blood vessels [2,19], whereas this disruption of blood vessels is protected by P_{30} peptide [17]. The experiment of fluorescence staining by *in vivo* tomato lectin administration showed the P_9-induced (1 mg/kg, i.v.) significant prevention of ischemia-induced cerebral blood vessel damages at day 1.

It is interesting to examine the potency ratio of P_9-induced protection to P_{30}, which has recently been reported. In the present retinal ischemia model, the median effective dose of P_9 to give 50 μV was approximately 8 pmol/eye for a-wave, and to give 150 μV was 4 pmol/eye for b-wave of ERG analysis, respectively, while these values for P_{30} were 2 and 1 pmol/eye, respectively [17],

indicating that P_9 is 4 times less potent than P_{30}. In the cerebral ischemia model, on the other hand, systemic treatment (i.v.) with 0.3 or 1 mg/kg of P_{30} causes markedly decrease in infarct volume at 24 h after the ischemic stress ($33 \pm 3\%$ and $30 \pm 2\%$, respectively), compared to ischemia alone ($52 \pm 3\%$), suggesting that the value with 0.3 mg/kg (approximately 0.3 μmol/kg) of P_9 is equivalent to that with 0.3 mg/kg (~0.1 μmol/kg) of P_{30}. Regarding the clinical score, the value (score: 2.0 ± 0.5) with 1 mg/kg (~1.0 μmol/kg) of P_9 is equivalent to that (score: 2.0 ± 0.5) with 1 mg/kg (~0.3 μmol/kg) of P_{30} [17] indicating that P_9 is 3 times less potent than P_{30} in cerebral ischemia-induced infarction and clinical score. All together, it is evident that the relative potencies of P_9 to P_{30} in prevention of retinal and brain damages seem to be similar, though the administration routes are different (local/intravitreous and systemic/intravenous, respectively). Cerebral ischemic stress disrupts the blood–brain barrier (BBB) [26,35], but we previously found that ProTα through intraperitoneal (i.p.) route is penetrated into the damaged regions in cerebral and retinal ischemia models [12,13]. It is considered that smaller peptides are generally preferable in BBB-penetration, while at the same time they are more susceptible to enzymatic degradation [22,38]. Thus, similar relative potency of P_9 and P_{30} in the present study may be a result of these opposite factors. The present study suggests that P_9 would be a promising prototype of small peptides to prevent the stroke. However, much more detailed studies should be performed prior to the future studies of peptide modification. They include (1) the quantitative beneficial actions in cultured neuronal cell death, and its mechanisms, (2) different administration schedules *in vivo*, (3) assessment studies of BBB-penetration and metabolic stability. All these studies are in progress in our group.

In conclusion, ProTα-derived 9-amino acid peptide P_9 induced potent actions against the cerebral and retinal ischemic damages. Thus, the present study would be a key demonstration to develop a new type of peptidic medicines against stroke.

Conflict of interest

Authors have no conflict interest to report.

Acknowledgments

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ORIGINAL ARTICLE

Novel neuroprotective action of prothymosin alpha-derived peptide against retinal and brain ischemic damages

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Abstract

Prothymosin alpha (ProTα), a nuclear protein, is implicated in the inhibition of ischemia-induced necrosis as well as apoptosis in the brain and retina. Although ProTα has multiple biological functions through distinct regions in its sequence, it has remained which region is involved in this neuroprotection. This study reported that the active core peptide sequence P_{30} (amino acids 49–78) of ProTα exerts its full survival effect in cultured cortical neurons against ischemic stress. Our *in vivo* study revealed that intravitreous administration of P_{30} at 24 h after retinal ischemia significantly blocks the ischemia-induced functional damages of retina at day 7. In addition, P_{30} completely rescued the retinal ischemia-induced ganglion cell damages at day 7 after the ischemic stress, along with partial blockade of the loss of bipolar, amacrine, and photoreceptor cells. On the other hand, intracerebroventricular (3 nmol) or systemic (1 mg/kg; i.v.) injection of P_{30} at 1 h after cerebral ischemia (1 h tMCAO) significantly blocked the ischemia-induced brain damages and disruption of blood vessels. Systemic P_{30} delivery (1 mg/kg; i.v.) also significantly ameliorated the ischemic brain caused by photochemically induced thrombosis. Taken together, this study confers a precise demonstration about the novel protective activity of ProTα-derived small peptide P_{30} against the ischemic damages *in vitro* and *in vivo*.

Keywords: blood vessel, brain ischemia, neuroprotective peptide, prothymosin alpha, retinal ischemia.

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Ischemic damages in the central nervous system including brain and retina are associated with the rapid and severe loss of functional and cellular responses through the mechanisms of necrosis as well as apoptosis by several types of cytotoxic mediators (White *et al.* 2000; Paolucci *et al.* 2003; Ueda and Fujita 2004; Feigin 2005; Flynn *et al.* 2008; Fornage 2009; Dvoriantchikova *et al.* 2010; Neroev *et al.* 2010; Sims and Muyderman 2010; Yin *et al.* 2010; Iadecola and Anrather 2011; Witmer *et al.* 2011). At the same time, several neuroprotective molecules such as brain-derived neurotrophic factor (BDNF), fibroblast growth factor (FGF), and erythropoietin (EPO) are produced upon ischemia to play limited attenuation of ischemic damages through the anti-apoptosis mechanisms, without exerting protective activity against necrosis (Siren *et al.* 2001; Korada *et al.* 2002; Maiese *et al.* 2004; Blanco *et al.* 2008; Fujita *et al.* 2009; Madinier *et al.* 2009; Ueda *et al.* 2010; Bejot *et al.* 2011).

Prothymosin alpha (ProTα) has been identified in the conditioned medium of serum-free primary culture of cortical neurons, as an anti-necrosis factor (Ueda *et al.* 2007). In addition, ProTα potently inhibits the ischemia-induced damages in brain and retina (Fujita and Ueda 2007; Fujita *et al.* 2009; Ueda *et al.* 2010). It is interesting that ProTα

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Abbreviations used: a.a., amino acid; BDNF, brain-derived neurotrophic factor; Chx10, ceh-10 homeodomain-containing homolog; ERG, electroretinogram; GCL, ganglion cell layer; H&E, hematoxylin and eosin; i.c.v., intracerebroventricularly; i.v., intravenously; i.vt., intravitreously; INL, inner nuclear layer; IPL, inner plexiform layer; NeuN, neuronal nuclei; ONL, outer nuclear layer; P_{30}, peptide sequence comprised of 30 amino acids; PIT, photochemically induced thrombosis; ProTα, prothymosin alpha; tMCAO, transient middle cerebral artery occlusion; TTC, 2,3,5-triphenyltetrazolium chloride.

has distinct actions, which are all related to the cell survival (Jiang *et al.* 2003; Ueda 2008; Mosoian *et al.* 2010; Ueda *et al.* 2012). Some studies revealed that different peptide sequences in ProTα are implicated with these survival actions. The peptide sequence in the central domain of ProTα (amino acids; a.a. 32–52) is related to the interaction with Kelch-like ECH-associated protein 1 (Keap1), which play roles in the induction of oxidative stress-protecting genes expression by liberating Nrf2 from the Nrf2-Keap1 inhibitory complex (Karapetian *et al.* 2005). The N-terminal sequence in ProTα (a.a. 2–29), corresponding to thymosin alpha 1, which has an ability to induce anti-cancer effects (Garaci *et al.* 2007; Danielli *et al.* 2012). In addition, thymosin alpha 1 has been approved in 35 countries for the treatment of hepatitis B and C, and as an immune stimulant and adjuvant (Goldstein and Goldstein 2009; Pierluigi *et al.* 2010). Previous reports suggested that C-terminal region (a.a. 89–109, 99–109 and 100–109) of human ProTα exerts immunoenhancing effects including pro-inflammatory activity through the stimulation of monocytes via toll-like receptor (TLR) signaling, induces dendritic cell maturation and adopts β-sheet conformation (Skopeliti *et al.* 2009). Most recently, there is a report about the survival activity of the middle part (a.a. 41–83) of human ProTα against mutant huntington-caused cytotoxicity in the cultured cells (Dong *et al.* 2012). However, it remains to be elucidated which region is responsible for the neuroprotection against ischemia-induced neuronal damages. In this study, we have attempted to see the neuroprotective activity of ProTα-derived small peptide against ischemic damages *in vitro* and *in vivo*.

Materials and methods

Animals

Male C57/BLJ mice weighing 20–25 g were purchased from Tagawa Experimental Animals (Nagasaki, Japan) and used for all the experiments. Mice were kept in a room maintained at constant temperature (21 ± 2°C) and relative humidity (55 ± 5%) with an automatic 12 h light/dark cycle with free access to standard laboratory diet and tap water. Animal care and all experimental procedures were formally approved by Nagasaki University Animal Care and Use Committee (Animal Experiments Approval Number: 1104190914).

Expression constructs and purification procedures for GST-fusion rat ProTα deletion mutants

The rat ProTα gene was amplified from cDNA derived from rat embryonic brain. The gene constructions for expression of recombinant GST-ProTα deletion mutants (Full-length, Δ1-29, Δ1-48, Δ1-68, Δ1-86, Δ30-112, Δ58-112, Δ79-112, and Δ102-112) were previously described (Ueda *et al.* 2007; Matsunaga and Ueda 2010). Here, we newly made a GST-ProTα-49-78. The amplified genes blunted at their 5′-ends and cloned in-frame into the BamHI (blunted)-EcoRI sites of pGEX-5X-1 (GE Healthcare Bio-Science Corp, Piscataway, NJ, USA). The PCR primers used were as follows: Δ1-48-F, 5′-AGGGATCCAATGGCTGACAATGAGGT AGATG-3′ and Δ79-112-R, 5′-TTGAATTCCTAATCTCCATC TTCTTCCTC-3′. F-primer contains a BamHI site, while all R-primer contains a stop codon and an EcoRI site. The recombinant proteins were purified using Glutathione-Sepharose™ (GE Healthcare Bio-Science Corp).

Identification of functional active core domain in ProTα

To determine the active core domain in ProTα, we measured the survival activity in primary cultured rat cortical neurons. The preparation and culture of cortical neurons were previously described (Ueda *et al.* 2007). The culture of neurons was started at low density (1×10^5 cells/cm^2) under the serum-free conditions in the presence or absence of GST and GST-ProTα deletion mutants (100 nM). After 12 h from the start of culture, survival activity was evaluated by WST-8 reduction activity (Cell Counting Kit-8; DOJINDO, Kumamoto, Japan). Finally, we successfully obtained the functional active core domain comprised of 30 amino acids in ProTα (a.a. 49–78) and referred as P_{30} according to number of amino acids.

Peptide administration

Intravitreal injection was performed using a 33-gauge needle connected to a microsyringe and the needle was inserted approximately 1 mm behind the corneal limbus, guided under a stereoscopic microscope to avoid lens and retinal injury. Peptide P_{30} was dissolved in 0.05% dimethyl sulfoxide (DMSO), which was diluted with 0.1 M potassium-free phosphate buffered saline (K^+-free PBS). P_{30} was injected intravitreously (i.vt.) in the eye with doses of 1, 3 and 10 pmol/μL at 24 h after retinal ischemia ($n = 5$, $n = 6$ and $n = 7$, respectively). Vehicle was treated with equal volume of 0.05% DMSO in a similar manner. On the other hand, P_{30} was injected intracerebroventricularly (0.03 and 3 nmol/5 μL, i.c.v.; $n = 6$ and $n = 7$, respectively) in the brain at 1 h after cerebral ischemia (1 h tMCAO). P_{30} (0.3 and 1 mg/kg) was administered intravenously (i.v.) at 1 h after cerebral ischemia. In addition, P_{30} was delivered (1 mg/kg, i.v.) at 3 and 6 h after the cerebral ischemic stress.

Retinal ischemia

Retinal ischemia was performed following the method as described previously (Fujita *et al.* 2009). Briefly, mice were anesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg) and pupils were fully dilated with 1% atropine sulfate drops (Nitten, Nagoya, Japan). The anterior chamber of the eye was cannulated with a 33-gauge needle attached to an infusion container of sterile intraocular irrigating solution (BSS PLUS dilution buffer; Alcon, Fort Worth, TX, USA). Retinal ischemia was induced by elevating the IOP to generate a hydrostatic pressure of 130 mm Hg for 45 min by lifting the container. Following 45 min after retinal ischemic stress, the needle was withdrawn and 0.3% ofloxacin (Santen Pharmaceutical Co. Ltd., Osaka, Japan) was applied topically into the eye to avoid infection.

Electroretinogram

Electroretinogram (ERG) study was performed following the protocol as previously described (Fujita *et al.* 2009). Briefly, mice were dark-adapted for 3–4 h, then anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg) and pupils were

dilated with 1% atropine. A contact electrode (KE–S; Kyoto contact lenses, Kyoto, Japan) was placed topically on the corneal apex and reference electrode was placed near the ipsilateral eye. The ground was a subdermal platinum needle electrode near the abdominal area. ERGs were produced by 20 J flash intensities. The flash stimulus source (SLS-3100; Nihon Kohden, Tokyo, Japan) illuminated the eye by diffuse reflection off the interior surface of the ganzfeld. Maximum flash luminance was measured with detector (MEB–9104; Nihon Kohden). After the intensity series, an incandescent background light sufficient to desensitize the rod system was turned on, and ERGs produced by the standard stimulus were recorded every 2 min for 20 min. The background was then turned off, and ERGs were produced by the standard stimulus every 2 min for the first 30 min of dark adaptation. The a- and b-wave amplitudes were measured online (Neuropack m, QP-903B; Nihon Kohden). ERG was performed at day 7 after retinal ischemia.

Middle cerebral artery occlusion model

The transient middle cerebral artery occlusion (tMCAO) model was induced following the method as described previously (Halder *et al.* 2012). Briefly, mice were anesthetized by 2% isoflurane (Mylan, Tokyo, Japan), and body temperature was monitored and maintained at 37°C during surgery. After a midline neck incision, the middle cerebral artery was occluded transiently using 8-0 in size monofilament nylon surgical suture (Natsume Co. Ltd., Tokyo, Japan) coated with silicon (Xantopren, Bayer dental, Osaka, Japan) that was inserted through the left common carotid artery and advanced into the left internal carotid artery. Following 1 h tMCAO, the animals were briefly re-anesthetized with isoflurane and the monofilament was withdrawn for reperfusion studies. Cerebral blood flow was monitored by laser Doppler flowmeter (ALF21; Advance Co., Tokyo, Japan) using a probe (diameter 0.5 mm) of a laser Doppler flowmeter (ALF2100; Advance Co.) inserted into the left striatum (anterior: −0.5 mm, lateral: 1.8 mm from bregma; depth: 4.2 mm from the skull surface) through a guide cannula.

Photochemically induced middle cerebral artery thrombosis

Photochemically induced thrombosis (PIT) was produced following the protocol as described previously (Nagai *et al.* 2007). Briefly, anesthesia was induced with 3% isoflurane, and the rectal temperature was maintained at 37°C. The temporal muscle was dissected, the skull was exposed, and a 1.5-mm opening was made over the middle cerebral artery (MCA). Photo-illumination of green light (wavelength: 540 nm) was achieved with a xenon lamp (model L-4887, Hamamatsu Photonics, Hamamatsu, Japan) with heat-absorbing and green filters, via an optic fiber with a focus of 1 mm, placed on the opening in the skull. Rose Bengal (Wako, Osaka, Japan) was injected (3 mg/kg, i.v.) in mice, and photo-illumination (5000 lx) was applied for 10 min, after which the temporal muscle and skin were replaced. The MCA occlusion time (from the start of light exposure until the flow in the MCA is stopped) was monitored by observation in real time under the microscope.

Retinal and brain tissue preparation

For retinal tissue preparation, mice were deeply anaesthetized with sodium pentobarbital (50 mg/kg, i.p.). Eye was quickly isolated, washed with saline and 4% paraformaldehyde (PFA). Eye was then nicked through pupil, post-fixed in 4% PFA for 24 h and finally transferred to 25% sucrose solution (in 0.1 M K^+-free PBS) overnight for cryoprotection. Following frozen in cryoembedding compound, retinal sections were prepared at 10 μm thickness. For brain tissue preparation, mice were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with 0.1 M PBS, followed by 4% PFA. Brain was then quickly removed, post-fixed in 4% PFA and transferred immediately to 25% sucrose solution overnight. Brain was frozen in cryoembedding compound and coronal sections were cut at 30 μm thickness for immunohistochemical analysis.

Hematoxylin and eosin staining

For hematoxylin and eosin (H&E) staining, frozen retinal sections were washed with 0.1 M K^+-free PBS, immerged in Mayer`s hematoxylin solution (Wako) for 5 min at 25°C and then washed with tap water for 20 min. Following brief treatment with 95% ethanol, sections were immerged in eosin-alcohol solution (Wako) for 4 min at 25°C. Sections were dehydrated through a series of ethanol solutions, xylene, and over-slipped with Permount (Fisher Scientific, Waltham, MA, USA). Sections were then analyzed using a BZ-8000 microscope with BZ Image Measurement Software (KEYENCE, Osaka, Japan).

Immunohistochemical analysis

To perform fluorescence immunohistochemistry, retinal sections were washed with 0.1 M K^+-free PBS and incubated with 50% methanol followed by 100% methanol for 10 min. Following treatment with blocking buffers [bovine serum albumin (BSA) as well as 10% goat serum with 0.1% Triton X-100 in phosphate buffered saline (PBST)], retinal sections were incubated overnight at 4°C with following primary antibodies: anti-NeuN (1 : 100; mouse monoclonal IgG_1, clone A60; Chemicon, Temecula, CA, USA); anti-syntaxin-1 (1 : 500; mouse monoclonal; Sigma-Aldrich, St. Louis, MO, USA); and anti-Chx10 (1 : 300; sheep polyclonal; Exalpha Biologicals Inc., MA, USA). Sections were then incubated with Alexa Fluor 488-conjugated anti-mouse IgG and Alexa Fluor 488-conjugated anti-sheep IgG secondary antibodies (1 : 300; Molecular Probes, Eugene, OR, USA). The nuclei were visualized with Hoechst 33342 (1 : 10 000; Molecular Probes). Samples were then washed thoroughly with PBS and cover-slipped with Perma Fluor (Thermo Shandon, Pittsburgh, PA, USA). Images were collected using a BZ-8000 microscope with BZ Image Measurement Software.

For blood vessels staining, biotinylated *Lycopersicon esculentum* (tomato) lectin (Vector Laboratories, Burlingame, CA, USA) is diluted with PBS. Biotinylated tomato lectin was injected (1 mg/mL, 100 μL, i.v.) at 24 h after cerebral ischemia (1 h tMCAO). Mice were perfused 5 min after tomato lectin injection. Following tissue preparation as described in the method section, coronal brain section was blocked with 2% BSA in 0.1% PBST for 2 h, and then incubated with Alexa Fluor 488 streptavidin conjugates for 2 h at 25°C. Sections were washed with PBS and cover-slipped with Perma Fluor. Images were collected using an LSM 710 confocal microscope with ZEN Software (Carl Zeiss, Oberkochen, Germany).

TTC staining

For 2,3,5–triphenyltetrazolium chloride (TTC) staining, brain was quickly removed at 24 h after cerebral ischemia (1 h tMCAO)