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ORIGINAL ARTICLE

Calycosin Preserves BDNF/TrkB Signaling and Reduces Post-Stroke Neurological Injury after Cerebral Ischemia by Reducing Accumulation of Hypertrophic and TNF-α-Containing Microglia in Rats

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Abstract

Both brain-derived neurotrophic factor (BDNF) and microglia activation are involved in the pathogenesis of ischemic stroke. Herein, we attempt to ascertain whether Calycosin, an isoflavonoid, protects against ischemic stroke by modulating the endogenous production of BDNF and/or the microglia activation. This study was a prospective, randomized, blinded and placebo-controlled preclinical experiment. Sprague-Dawley adult rats, subjected to transient focal cerebral ischemia by middle cerebral artery occlusion (MCAO), were treated randomly with 0 (corn oil and/or saline as placebo), 30 mg/kg of Calycosin and/or 1 mg/kg of a tropomyosin-related kinase B (TrkB) receptor antagonist (ANA12) at 1 h after reperfusion and once daily for a total of 7 consecutive days. BDNF and its functional receptor, full-length TrkB (TrkB-FL) levels, the percentage of hypertrophic microglia, tumor necrosis factor-α (TNF-α)-containing microglia, and degenerative and apoptotic neurons in ischemic brain regions were determined 7 days after cerebral ischemia. A battery of functional sensorimotor test was performed over 7 days. Post-stroke Calycosin therapy increased the cerebral expression of BDNF/TrkB, ameliorated the neurological injury and switched the microglia from the activated amoeboid state to the resting ramified state in ischemic stroke rats. However, the beneficial effects of BDNF/ TrkB-mediated Calycosin could be reversed by ANA12. Our data indicate that BDNF/TrkB-mediated Calycosin ameliorates rat ischemic stroke injury by switching the microglia from the activated amoeboid state to the resting ramified state.

Keywords Stroke · Calycosin · Brain-derived neurotrophic factor · Microglia · Neuronal death

Abbreviations

BDNF	brain-derived neurotrophic factor
CBF	cerebral blood flow
CIV	corrected infarction volume
d	thickness of the brain slices
DAPI	4′,6-diamidino-2-phenylindole

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ECA	external carotid artery
ELISA	enzyme-linked immunosorbent assay
Iba-1	ionized calcium binding adaptor molecule 1
ICA	internal carotid artery
LT	left hemisphere
MCAO	middle cerebral artery occlusion
mNSS	modified neurological severity score
NeuN	neuronal nuclei
PBS	phosphate-buffered saline
RA	Radix Astragali
RI	right side infarct
RT	right hemisphere
SD	Sprague-Dawley
TNF-α	tumor necrosis factor-alpha
TrkB-FL	full-length tyrosine kinase receptor B
TTC	2,3,5-triphenyl tetrazolium chloride
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

Introduction

Radix Astragali (RA) taken from the medicinal plant Astragalus membranaceus has been prescribed for thousands of years to treat stroke (Fu et al. 2014). The major constituents of RA are polysaccharides, flavonoids, and saponins (Li et al. 2015). Calycosin, an isoflavonoid, exerts the principal function of the RA and ameliorates both the neurological deficit and infarct volume in experimental cerebral ischemia-reperfusion injury (Fu et al. 2014; Guo et al. 2012). However, the underlying mechanism of its neuroprotective effect remains unclear.

Activation of brain-derived neurotrophic factor (BDNF) with its tyrosine kinase receptors B (TrkB) mediates neuronal survival, differentiation, synaptic plasticity, and neurogenesis (Habtemariam 2018; Tejeda and Diaz-Guerra 2017; Wurzelmann et al. 2017; Chen et al. 2017). The post-stroke neurological injury is associated with reduced expression of BDNF/TrkB (Chen et al. 2015; Ferrer et al. 2001).

In addition, evidence has accumulated to indicate that microglia/macrophages play an important role in neuroinflammation and neurogenesis after stroke (Xiong et al. 2016). Cerebral ischemia transforms the morphology of microglia or macrophages from the resting ramified phenotype into the activated amoeboid (or hypertrophic) phenotype. It is generally believed that hypertrophic microglia/macrophages damage neurons, whereas resting ramified microglia/macrophages repair neurons (Lin et al. 2017; Jin et al. 2014; Mouihate 2014). These observations prompted us to think that Calycosin may exert its neuroprotective effects via modulating BDNF/ TrkB signaling pathway and/or microglial activation.

There are two folds of purpose for the present study. First, using a rat stroke model of middle cerebral artery occlusion (MCAO), we examined whether Calycosin improves outcomes of ischemic stroke injury by preserving BDNF/TrkB signaling in injured brain regions. Second, we ascertained whether Calycosin can improve neurological injury after MCAO by reducing the number of hypertrophic and TNF-α-containing microglia in ischemic brain regions.

Methods

Animals and Stroke Model

We purchased male Sprague-Dawley (SD) rats (weight 300–320 g) from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan) and followed their policies regarding the care and use of laboratory animals. We performed all experiments in accordance with the Institutional Animal Care and Use Committee of Chi Mei Medical Center (IACUC approved no. 105110328) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. We housed animals at a room temperature of 23 °C–24 °C with a 12 h light-dark cycle as well as a relative humidity of 65%. Pellet rat chow and tap water were available ad libitum. Since the majority (>70%) of ischemic stroke in human patients belongs to MCAO (Lee et al. 2014), we adopted a focal cerebral ischemic rat model to mimic human stroke (Durukan and Tatlisumak 2007). A reversible MCAO without craniotomy described previously (Bederson et al. 1986; Longa et al. 1989) was employed to block blood flow into the MCAO in rats under a mixture of Ketamine Hydrochloride (Ketalar® 50 mg/kg; Pfizer, New Taipei City, Taiwan), Atropine Sulfate (1 mg/kg; Tai Yu Chemical & Pharmaceutical Co. Ltd., Hsinchu, Taiwan), and Xylazine Hydrochloride (Rompun®, 5 mg/kg; Bayer, Leverkusen, Germany) anaesthesia with intramuscular injection. A laser Doppler monitor (Transonic Systems, Inc., Ithaca, NY, USA) was attached to the skull with dental cement and monitored cerebral blood flow (CBF) in each rat before, during, and after surgery. The rectal temperature of each rat was kept at 37 °C ± 0.5 °C controlled by a Homeothermic control unit (RightTemp® Temperature Monitor & Homeothermic Warming Control Module; Kent Scientific, Torrington, CT, USA). A 4–0 monofilament nylon suture (Doccol Co., Redlands, CA, USA) with silicone-rubber coated was inserted from the right external carotid artery (ECA) into the internal carotid artery (ICA) to occlude the right middle cerebral artery (MCA) at its origin. In the MCAO model to verify the occlusion, it is mandatory a CBF reduction higher than 80% in Laser Doppler monitor. Focal cerebral ischemia was maintained for 60 min, then the suture was removed for reperfusion, the ECA was ligated, and the wound was closed. Temperature, respiratory rate, oxygen saturation, and heart

rate were monitored during the surgery with a small animal Oximeter (PhysioSuite® Pulse Temperature & Homeothermic Warming, Oximeter, Heart Rate & Respiratory Rate Monitor Module; Kent Scientific, Torrington, CT, USA). After surgery, the rat was placed in a warm cage and allowed to awaken. Buprenorphine (0.05 mg/kg q12 hours for 2 days, subcutaneously; Sigma-Aldrich, St. Louis, MO, USA) was used for postoperative analgesia. The sham operative animals received the same surgical procedures and analgesia except MCAO and maintained their CBF within the normal range throughout the entire experiments. The awake animals were allowed to recover at room temperature and received a single dose of corn oil (i.g.), Calycosin (i.g.), normal saline (i.p.) or ANA12 (i.p.) at 1 h after reperfusion.

Experimental Groups

Two hundred and forty SD rats were randomly divided into the following 8 groups: a sham-operated group treated with vehicle (Sham+Vehicle; $n = 30$), a sham-operated group treated with ANA12 (Sham+ANA12; $n = 30$), a sham-operated group treated with Calycosin (Sham+Calycosin; $n = 30$), a sham-operated group treated with ANA12 plus Calycosin (Sham+ANA12 + Calycosin; $n = 30$), a stroke group treated with vehicle (MCAO+Vehicle; $n = 30$), a stroke group treated with ANA12 (MCAO+ANA12; $n = 30$), a stroke group treated with Calycosin (MCAO+Calycosin; $n = 30$), and a stroke group treated with ANA12 plus Calycosin (MCAO +ANA12 + Calycosin; $n = 30$). Calycosin (purity >98%, as verified by high-performance liquid chromatography; purchased from Fusol Material Co., Ltd., Tainan City 709, Taiwan) was dissolved in 2 ml of corn oil and administered intragastrically (i.g.) at a dose of 30 mg/kg at 1 h after reperfusion and once daily for seven consecutive days after MCAO. This particular concentration of Calycosin has been found to be neuroprotective in ischemic stroke rats (Guo et al. 2012). Each animal received another intraperitoneal (i.p.) injection of N-[2-[[(Hexahydro-2-oxo-1H-azepin-3-yl)amino]carbonyl]phenyl]benzo[b]thiophene-2-carboxamide (ANA12, 1 mg/kg; Tocris Bioscience, Bristol, UK) or vehicle (1% dimethylsulfoxide in physiological saline, 1 ml/kg; Sigma-Aldrich, St. Louis, MO, USA) (Cazorla et al. 2011), as appropriate, immediately after the intragastric irrigation of Calycosin and once daily for seven consecutive days after MCAO.

Evaluation of Neurobehavioral Functions

Behavioral testing was performed before MCAO and 3 and 7 days after MCAO by an observer who was unaware of the experimental treatments. We chose the time point of 7 days as the measuring point because ischemic damage on MRI occurred in rats 7 days following MCAO (Kurozumi et al. 2005).

Neurological function was evaluated by the mNSS described previously (Chen et al. 2001). The higher mNSS denotes the more neurological dysfunction.

The foot-fault placing test was used to examine forelimb function (Ding et al. 2004). The foot-placing apparatus consisted of an elevated (1 m) acrylic grid surface (12 cm × 110 cm, with a 9-cm × 9-cm square opening) whose ends (15 cm × 20 cm) were connected to platforms. The rats were placed on one platform during each trial and were encouraged to transverse the grid surface for 1 min. The rats sometimes placed their forelimbs inaccurately and fell through one of the openings in the grid. These mistakes were recorded as foot faults. The errors in the affected forelimb were calculated as faults per meter in 1 min (errors per meter). Fewer errors indicated better forelimb function.

The rats were placed in a glass cylinder scaled for their sizes; thus, the diameter of the cylinder was approximately 4 cm greater than the nose-to-hind quarter's length of each rat. The rats were observed for spontaneous rearings during a single 5-min observation session. The number of wall rearings for which each rat used both the left and right forelimbs, only the right forelimb, or only the left forelimb were recorded. Measurement were performed on the day before ischemia to control for pre-injury limb preference. The laterality score was computed as follows: (number of right-only+number of left-only)/(number of right-only+number of left-only+number of both together) (Schallert et al. 2000). The cylinder test aims to evaluate asymmetry in forelimb use during vertical exploratory activity (rearing).

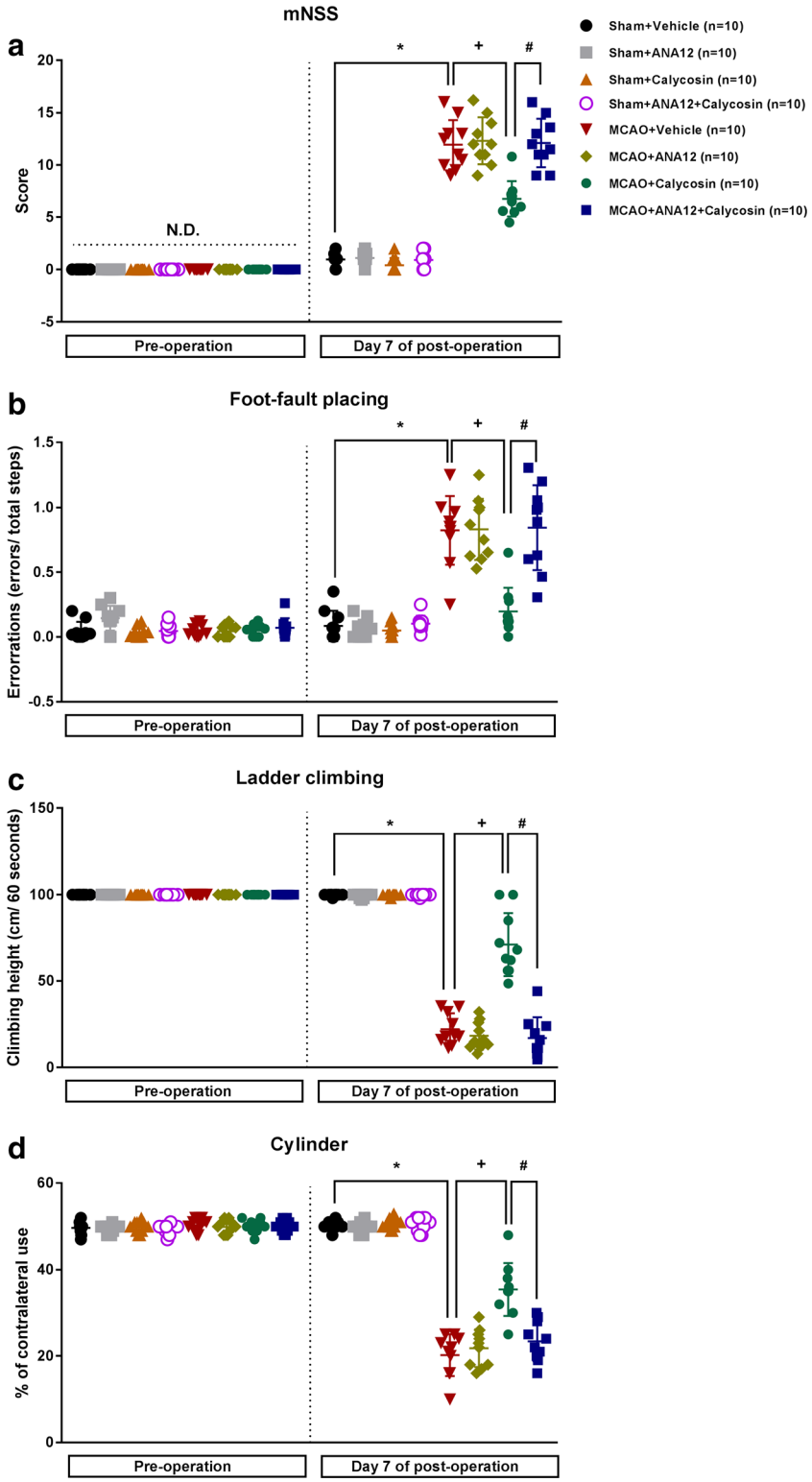
The ladder-climbing test, which was adapted from a previous study (Ding et al. 2004), was employed to examine the coordination of both forelimbs and both hind limbs. The animals were tested with a ladder-climbing task that required the coordination of both forelimbs and both hind limbs. A single wooden rod supported by cross bases at 3-cm intervals was suspended vertically from a platform 100 cm above the bases.

BDNF Detection Using ELISA

We used a BDNF ELISA Kit (Abcam Inc., Boston, MA, USA) to measure BDNF protein expression in different parts of the brain (including ipsilateral cortical and striatal tissues), according to the guidelines provided by the manufacturer (Liu et al. 2016).

TrkB Detection Using Western Blotting

We prepared total protein lysates by homogenizing cortical or striatal tissues ($n = 10$ in each group) in RIPA (radioimmunoprecipitation assay) lysis buffer with protease and phosphatase inhibitor (Thermo Fischer Scientific Inc.,



Waltham, MA, USA). The protein concentrations of the samples were subsequently determined with a protein assay kit (Bio-Rad, Hercules, CA, USA). Twenty micrograms of each protein sample were analysed by 10% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels (Zhong et al. 2016). The primary antibody used to detect rat TrkB protein was from R&D Systems, Inc., Minneapolis, MN, USA. β-actin (Chemicon International, Inc., Billerica, MA, USA) was probed as a protein loading control. For secondary antibody, donkey anti-goat IgG conjugated to horseradish peroxidase

◀ **Fig. 1** Calycosin attenuated the neurological motor deficits caused by MCAO; the beneficial effects of Calycosin could be reversed by ANA12. **a** mNSS-based assessments, **b** foot-fault placing tests, **c** cylinder tests, and **d** ladder-climbing tests were performed to evaluate behavioral performance. The data represent the mean ± S.D. of 10 rats in each group. $^{*}P < 0.05$, MCAO+Vehicle group vs. Sham+Vehicle group; $^{+}P < 0.05$, MCAO+Calycosin group vs. MCAO+Vehicle group; $^{\#}P < 0.05$, MCAO+ANA12+Calycosin vs. MCAO+Calycosin group. N.D.: no deficits. (●)Sham+Vehicle: sham rats treated with vehicle; ( )Sham+ANA12: sham rats treated with ANA12 (1 mg/kg); ( )Sham+Calycosin: sham rats treated with Calycosin (30 mg/kg); ( )Sham+ANA12 + Calycosin: sham rats treated with Calycosin (30 mg/kg) and ANA12 (1 mg/kg); ( )MCAO+Vehicle: MCAO rats treated with vehicle; ( )MCAO+ANA12: MCAO rats treated with ANA12 (1 mg/kg); ( )MCAO+Calycosin: MCAO rats treated with Calycosin (30 mg/kg); ( )MCAO+ANA12+ Calycosin: MCAO rats treated with Calycosin (30 mg/kg) plus ANA12 (1 mg/kg).

(HRP) (R&D Systems). Band densities were determined by scanning densitometry (GS-800, BIO-RAD, Hercules, CA, USA). The density of Western blot bands was quantified using an image analysis system (Image Pro-Plus; Media Cybernetics, USA). TrkB protein levels were determined after normalizing with β-actin.

2,3,5-Triphenyl Tetrazolium Chloride (TTC) Staining

The animals under general anesthesia were perfused intracardially with 0.05 mol/L phosphate-buffered saline (PBS) containing heparin (10 IU/mL, Sigma-Aldrich, St. Louis, MO, USA). The removed brain tissues were sectioned for TTC stain as described previously (Bederson et al. 1986). The infarct volume (mm^3) of each slice was measured and summed using computerized planimetry (Gartshore et al. 1997). The infarction volume by brain edema was corrected by the methods of Lin et al. (Lin et al. 1993). The following formula was utilized: CIV = (LT-(RT-RI)) x *d*, where the CIV was the corrected infarction volume in mm^3, LT was the area of the left hemisphere in

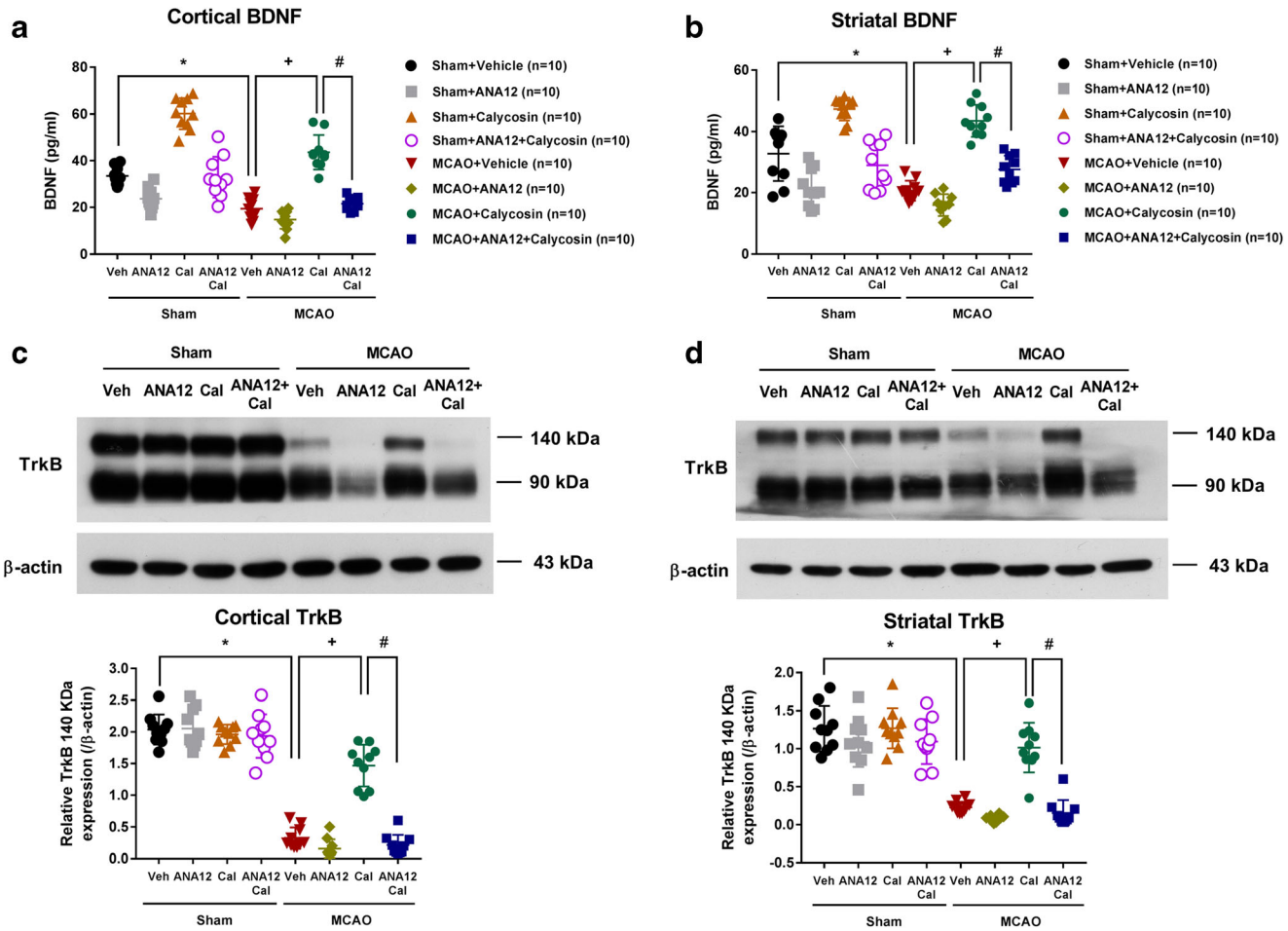


Fig. 2 Calycosin attenuated the reduced cortical and striatal expressions of BDNF/TrkB in caused by MCAO; the beneficial effect of Calycosin can be reversed by ANA12. Cortical (**a**) and striatal (**b**) BDNF concentrations were determined by ELISA whereas the cortical (**c**) and striatal (**d**) TrkB expression were determined by Western blotting. The data represent the mean ± S.D. of 10 rats in each group. $^{*}P < 0.05$, MCAO+Vehicle group vs. Sham+Vehicle group; $^{+}P < 0.05$, MCAO+Calycosin group vs. MCAO+Vehicle group; $^{\#}P < 0.05$, MCAO+ANA12+Calycosin vs MCAO+Calycosin group. Veh: vehicle; Cal: Calycosin. Please see the legend in Fig. 1 for an explanation of the group abbreviations or symbols

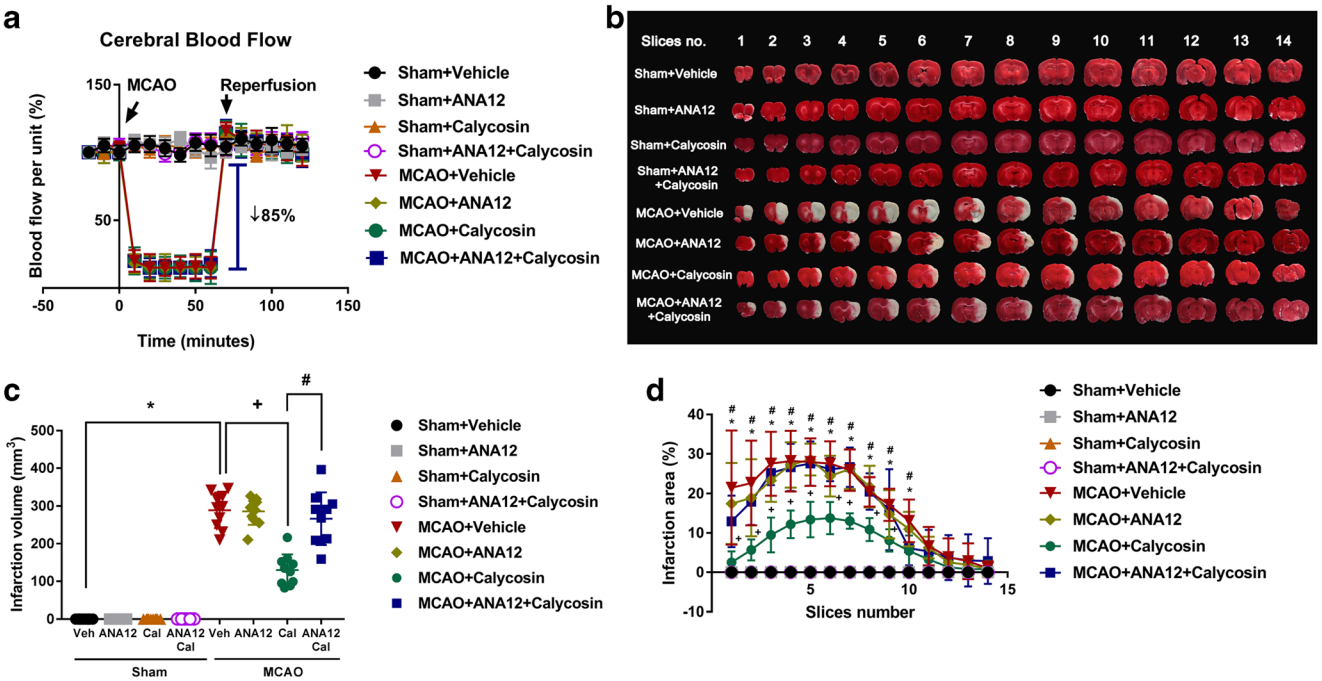


Fig. 3 Calycosin therapy attenuated cerebral infarct caused by MCAO, and ANA12 could reverse the beneficial effects of calycosin could be reversed by ANA12. **a** Relative blood flow before, during, and after 60 min of sham operation and MCAO in each group of rats. **b** Representative triphenyl tetrazolium chloride-stained brain sections. The bar graphs demonstrate brain infarction volume **c** and infarct area **d** in different groups of rats on day 7 after MCAO or sham operation. The data represent the mean ± S.D. of 10 rats in each group. $^{*}P < 0.01$, MCAO+Vehicle group vs. Sham+Vehicle group; $^{+}P < 0.05$, MCAO+Calycosin group vs. MCAO+Vehicle group; $^{\#}P < 0.05$, MCAO+ANA12+Calycosin. Please see the legend in Fig. 1 for an explanation of the group abbreviations or symbols

mm^2, RT was the area of the right hemisphere in mm^2, RI was the area of the infarct (non-stained) in the right hemisphere in mm^2, and *d* was the thickness of the slices (1 mm).

Immunofluorescence Staining

Brain sections were mounted onto silane-coated slides (MUTO PURE CHEMICALS Co.Ltd., Tokyo, Japan), as described in the Fluoro-Jade B staining section, and were allowed to air dry. After which they were blocked and then permeabilized in 0.1 M phosphate buffer with 0.3% Triton X-100 (Sigma-Aldrich) and 10% goat serum for 1 h. The sections were then incubated with the appropriate primary antibodies (mouse monoclonal anti-NeuN, mouse monoclonal anti-Iba-1, or mouse monoclonal anti-TNF-α antibody) before being incubated with the appropriate secondary antibodies (Alexa Fluor 568-conjugated goat anti-rabbit IgG or Alexa Fluor 568-conjugated goat anti-mouse IgG or Alexa Fluor 488-conjugated goat anti-mouse) for 1 h at room temperature. For quantification of neuron degeneration or apoptosis assay, the sections were stained with 0.001% Fluoro-Jade B (Merck Millipore, Billerica, MA, USA) in 0.1% acetic acid or were stained with a terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling (TUNEL) assay kit (Clontech, Palo Alto, CA, USA). The sections were subsequently washed with phosphate buffer, and the nuclei were co-stained with 4,6-diamidino-2-phenylindole (DAPI) using DAPI-staining mounting medium (Vectashield ®Vector Laboratories, Burlingame, CA, USA). The sections were analysed using a Carl Zeiss upright fluorescence microscope (Carl Zeiss) at excitation/emission wavelengths of 535/565 nm (rhodamine, red) and 470/505 nm (FITC, green), respectively. All the primary and secondary antibodies are described in detail in the supplementary section (Supplementary Table 1). Digital images were captured with a 40x objective (N.A. 0.75) and a 100x oil immersion objective (N.A. 1.4) by an upright fluorescence microscope system (Carl Zeiss Microscopy GmbH, Jena, Germany) with Zen Software (Carl Zeiss). The numbers of NeuN+Fluoro-Jade B + DAPI positive cells in cortical and striatal, hypothalamic and hippocampal parts of the ipsilateral brain were counted.

Immunohistochemical Staining and Quantification of Activated and Total Number of Microglia

Iba-1 immunostained brain slices were used to quantified activated microglia. At 7 days post-stroke, the rat brain slices were incubated with Iba-1 antibody at 1:200 dilution in 1× PBS, 0.3% Tween 20 and 1.0% bovine serum albumin at 4 °C overnight. After being washed three times in PBS with 0.3% Tween 20, slices were incubated with N-Histofine Simple Stain MAX PO (Nichirei Biosciences Inc., Tokyo, Japan) as a secondary antibody at room temperature for 60 min. Subsequently, sections were treated with 3, 3′-diaminobenzidine (DAB; K3468, Dako,

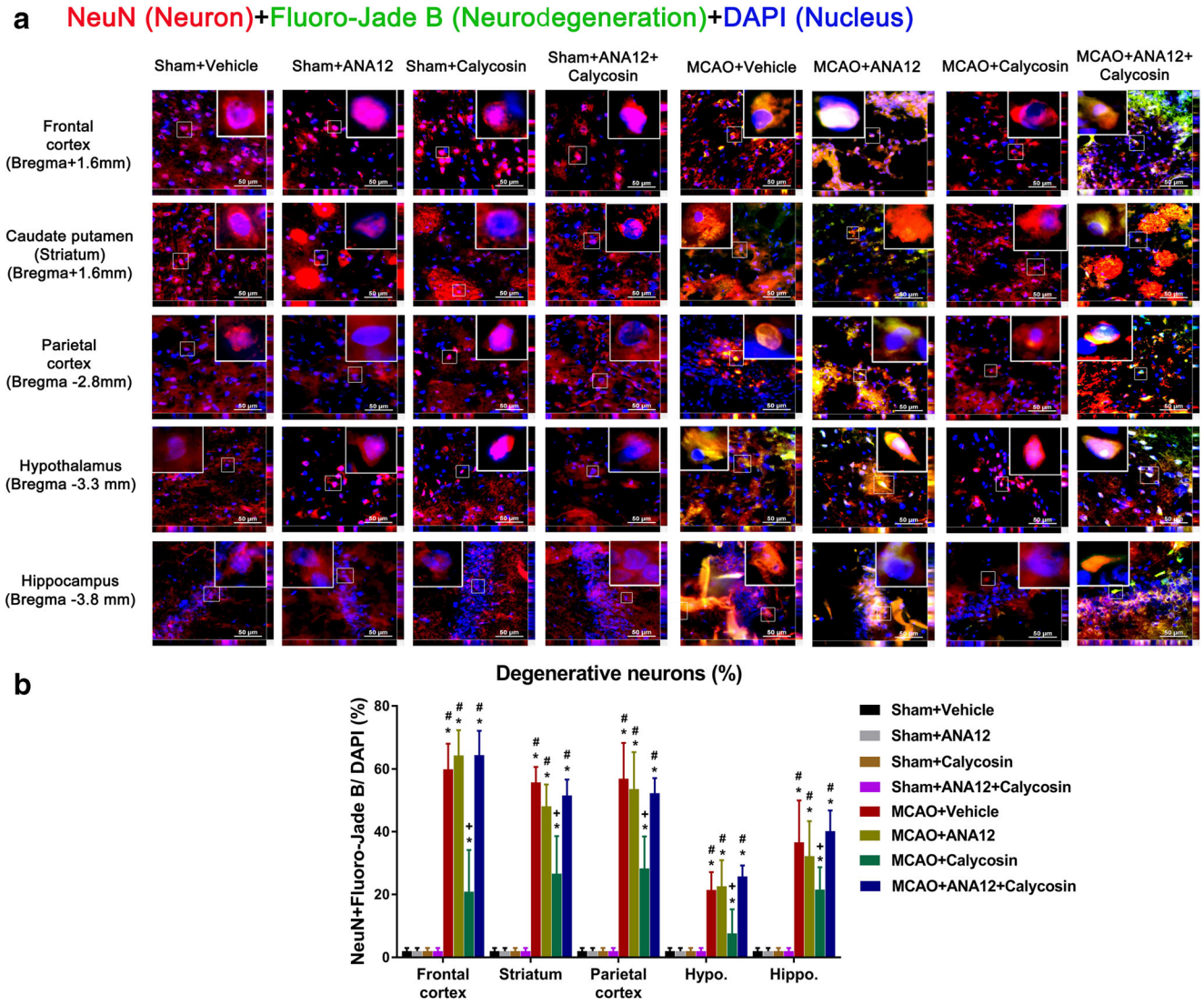


Fig. 4 Calycosin reduced the accumulation of degenerative neurons in the frontal cortex, striatum, parietal cortex, hippocampus (Hippo.), and hypothalamus (Hypo.) in rats after MCAO or sham operation. ANA12 could reverse the beneficial effects of calycosin. **a** Representative photographs of Fluoro-Jade B staining at 7 days after MCAO or sham operation. **b** The data represent the mean ± S.D. of 10 rats in each group. $^{*}P < 0.05$, MCAO+Vehicle group vs. Sham+Vehicle group: $^{+}P < 0.05$, MCAO+Calycosin group vs. MCAO+Vehicle group; $^{\#}P < 0.05$, MCAO+ANA12+Calycosin group vs. MCAO+Calycosin group

Denmark). After counterstained with Hematoxylin, sections were coverslipped with neutral balsam and observed under the microscope camera (Carl Zeiss) and 40x objective. Using acquired images and Image-Pro Plus analysis software, an observer blinded to experimental groups quantified the total number of activated amoeboid (or hypertrophic) phenotype of microglia, determined by their rounded appearance with few processes, as well as the total number of resting ramified microglia (Miller et al. 2015; Lin et al. 2017). Average microglial activation was determined in the frontal cortex, striatum, and parietal cortex. The counts obtained from five different images per sections per area for each rat were averaged. Results are expressed as percentage of activated microglia in the analyzed regions of interest, including the cortical, striatal, hypothalamic and hippocampal parts of the ipsilateral brain.

Statistical Analysis

The person charged with functional outcome measurements was the only one that was blinded to treatments among those working on animals (single blind). She used cage and animal codes to recognise individuals and to report repeated measurements on data collection forms. We performed all statistical analyses of the data with GraphPad Prism 7.01 (GraphPad Software Inc., CA, USA). The data are expressed as the mean ± standard deviation (S.D.). One-way analysis of variance (ANOVA) with Tukey-Kramer post hoc test was used by an investigator blinded to the treatment groups and the infarction volume, ELISA, and western blotting. We performed two-way ANOVA with Tukey's multiple comparisons tests to analyse behavioural performance. The immunostaining data were

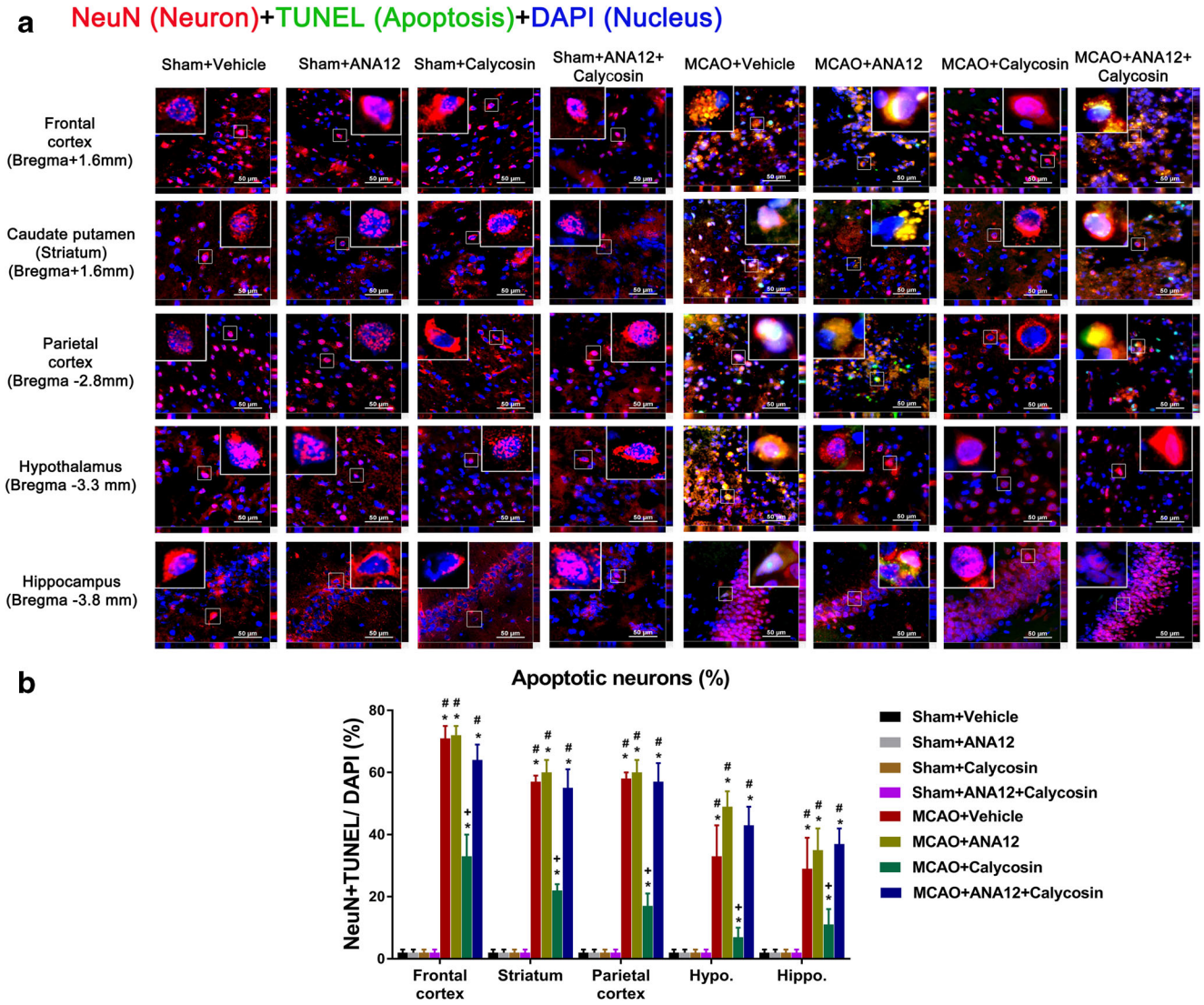


Fig. 5 Calycosin reduced the accumulation of apoptotic neurons in the frontal cortex, striatum, parietal cortex, hippocampus (Hippo.), and hypothalamus (Hypo.) in rats after MCAO or sham operation. **a** Representative photographs of TUNEL staining at 7 days after MCAO or sham operation operation. **b** The data represent the mean ± S.D. of 10 rats in each group. $^{*}P < 0.01$, MCAO+Vehicle group vs. Sham+Vehicle group; $^{+}P < 0.05$, MCAO+Calycosin group vs. MCAO+Vehicle group; $^{\#}P < 0.05$, MCAO+ANA12+Calycosin

analyzed by the Kruskal-Wallis test with Dunn's post hoc test. Values were considered significant when P was less than 0.05.

Results

Calycosin Reduced Neurological Injury in Ischemic Stroke Rats Evaluated by Neurological Motor Tests

To test neurological motor function in rats following ischemic stroke, we performed modified neurological severity score (mNSS)-based assessments (Fig. 1a), forelimb foot-fault placing tests (Fig. 1b), ladder-climbing tests (Fig. 1c) and cylinder tests (Fig. 1d) on different groups of rats. According to the data, the MCAO+Vehicle group achieved significantly higher scores in the mNSS, higher error rates in the forelimb foot-fault placing test, lower heights in the ladder-climbing test, and lower lateralities in the cylinder tests than the Sham+Vehicle group. However, Calycosin significantly attenuated the effects of neurological motor deficits after an MCAO in the MCAO+Calycosin group. ANA12 significantly reversed the beneficial effects of Calycosin in the MCAO +ANA12 + Calycosin group (Fig. 1). The difference in behavioral performance between the MCAO+Vehicle and MCAO+ANA12 group was insignificant (Fig. 1). These results revealed that ANA12 did not affect MCAO-induced neurological deficits but reversed the beneficial effects of Calycosin on MCAO-induced neurological deficits. The difference in behavioral performance between the Sham+Vehicle group and the Sham +ANA12 + Calycosin group was insignificant.

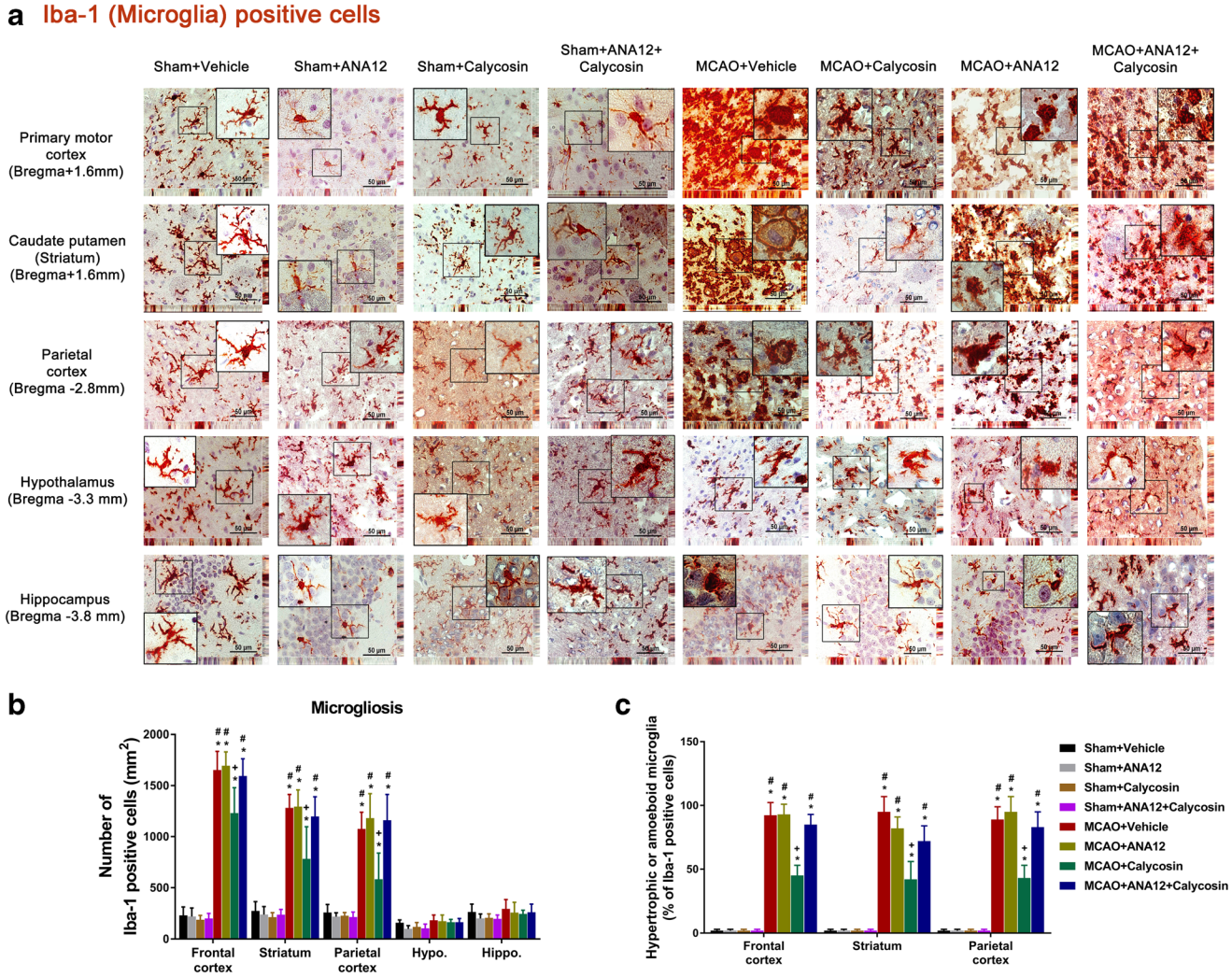


Fig. 6 Calycosin reduced microgliosis and shifted amoeboid-like or hypertrophic microglia/macrophages into ramified microglia/macrophages in the frontal cortex, striatum, parietal cortex, hippocampus (Hippo.), and hypothalamus (Hypo.) after MCAO or sham operation. The beneficial effects of Calycosin could be reversed by ANA12. **a** Photomicrographs of Iba-1 (microglia) staining in the ipsilateral brain regions at 7 days after MCAO or sham operation. The numbers of both Iba-1-positive cells (microgliosis) **b** and hypertrophic microglia **c** in different brain regions. The bars represent the mean ± S.D. of the numbers of Iba-1 positive cells and the % of hypertrophic or amoeboid microglia/macrophages in ipsilateral brain areas after MCAO or sham operation. $^{*}P < 0.05$, MCAO+Vehicle group vs. Sham+Vehicle group; $^{+}P < 0.05$, MCAO+Calycosin group vs. MCAO+Vehicle group; $^{\#}P < 0.05$, MCAO+ANA12 + Calycosin group vs. MCAO+Calycosin group

Calycosin Preserved BDNF/TrkB Levels in Ischemic Cortical and Striatal Tissues Evaluated by ELISA and Western Blot Analysis

We performed both ELISA and Western blot analysis on rat ipsilateral cortical and striatal tissue extracts 7 days after an ischemic stroke to ascertain whether Calycosin affects the decreased BDNF and truncated TrkB expression in the ischemic brain. Figure 2 shows that MCAO decreased BDNF (Fig. 2a and b) and TrkB (Fig. 2c and d, the original Westen blotting data were shown in Supplementary Fig. S1) expression in ischemic cortical and striatal tissues compared with control tissues. These changes are significantly ($P < 0.01$) attenuated by Calycosin. However, the beneficial effects of Calycosin on BDNF and TrkB expression in ischemic stroke rats were significantly ($P < 0.01$; Fig. 2) reversed by ANA12, as shown by the results for the MCAO +ANA12 + Calycosin group. BDNF and TrkB expression levels in the ipsilateral brain tissues of the Sham+ANA12 and MCAO+ANA12 group were insignificantly or slightly different from those of the Sham+Vehicle group, respectively (Fig. 2). These results revealed that ANA12 did not affect the basal levels of BDNF and TrkB in ipsilateral brain tissues but reverses the beneficial effects of Calycosin on BDNF and TrkB expression in MCAO rats.

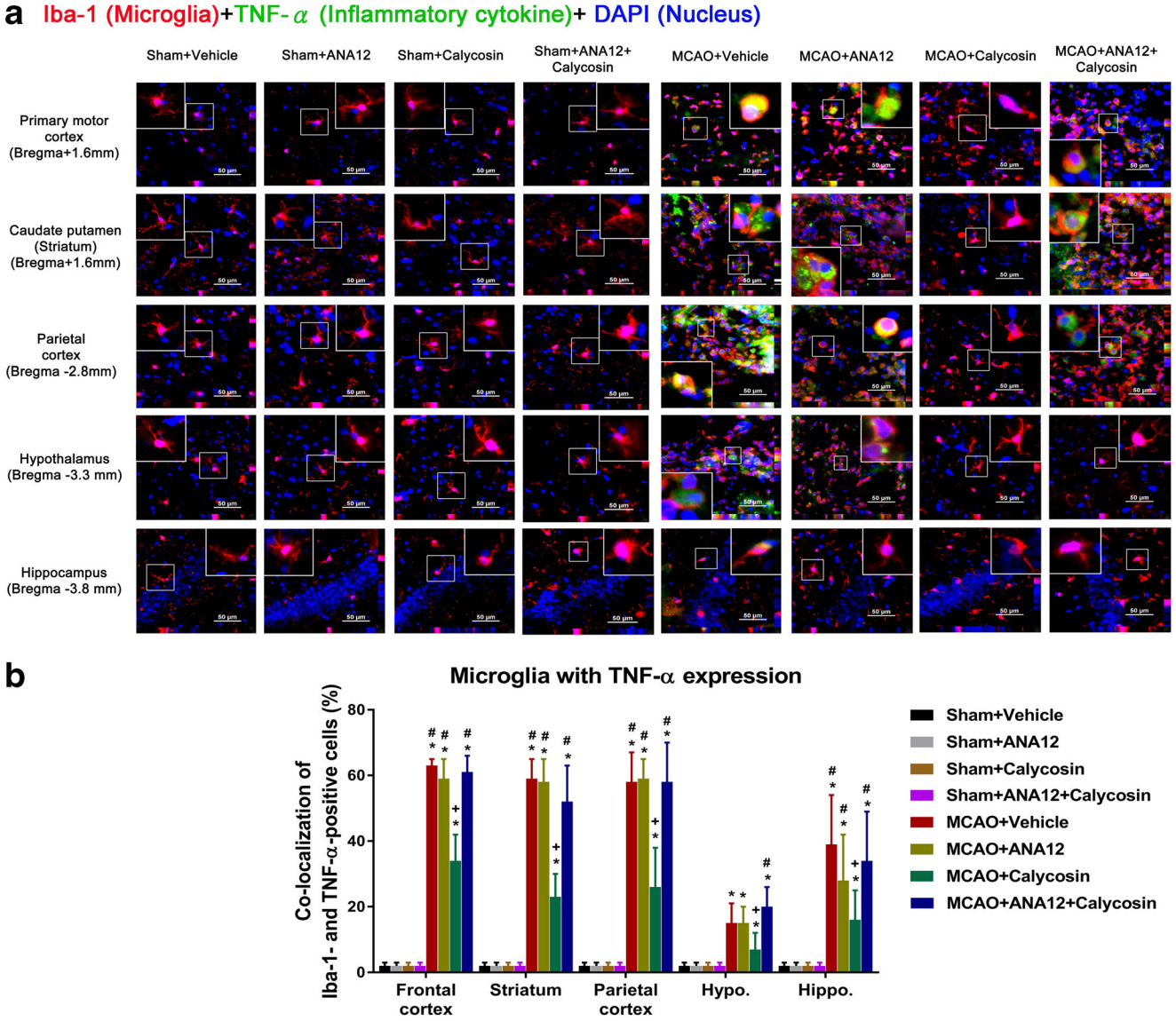


Fig. 7 Calycosin reduced the accumulation of TNF-α-containing microglia/macrophages in the frontal cortex, striatum, parietal cortex, hippocampus (Hippo.), and hypothalamus (Hypo.) after MCAO. ANA12 could reverse the beneficial effects of Calycosin. **a** Photomicrographs of Iba-1 (microglia) + TNF-α (an inflammatory cytokine)-positive cells in different brain regions at 7 days after MCAO. **b** The bars represent the mean ± S.D. of the percentage of TNF-α-containing microglia in different brain regions. $^{*}P < 0.05$, MCAO+Vehicle group vs. Sham+Vehicle group; $^{+}P < 0.05$, MCAO+Calycosin group vs. MCAO+Vehicle group; $^{\#}P < 0.02$, MCAO+ANA12 + Calycosin group vs. MCAO+Calycosin group

Calycosin Attenuated Brain Infarct in Ischemic Stroke Rats Evaluated by TTC Stain

Figure 3a demonstrates the relative blood flow before, during, and after 60 min of sham operation and MCAO in each group. We also evaluated the neuroprotective effects of Calycosin by measuring both infarct volumes (Fig. 3b and c) and infarct areas (Fig. 3d) at 7 days after MCAO. Both the infarct volume (Fig. 3c) and the infarct area (Fig. 3d) were higher in the MCAO+Vehicle group than in the Sham+Vehicle group ($P < 0.01$ vs. sham controls). However, Calycosin significantly reduced the infarct size compared with vehicle control ($P < 0.01$; Fig. 3). The beneficial effects of Calycosin on infarct size were significantly attenuated by ANA12 as shown in the MCAO +ANA12 + Calycosin group (Fig. 3). These results revealed that ANA12 did not affect brain infarct size but reversed the beneficial effects of Calycosin on MCAO-induced infarct size.

Calycosin Reduced the Percentage of both Degenerative and Apoptotic Neurons in Ischemic Stroke Rats Evaluated by Immunofluorescence Staining

We evaluated the neuroprotective effects of Calycosin further by counting the numbers of the degenerative neurons and

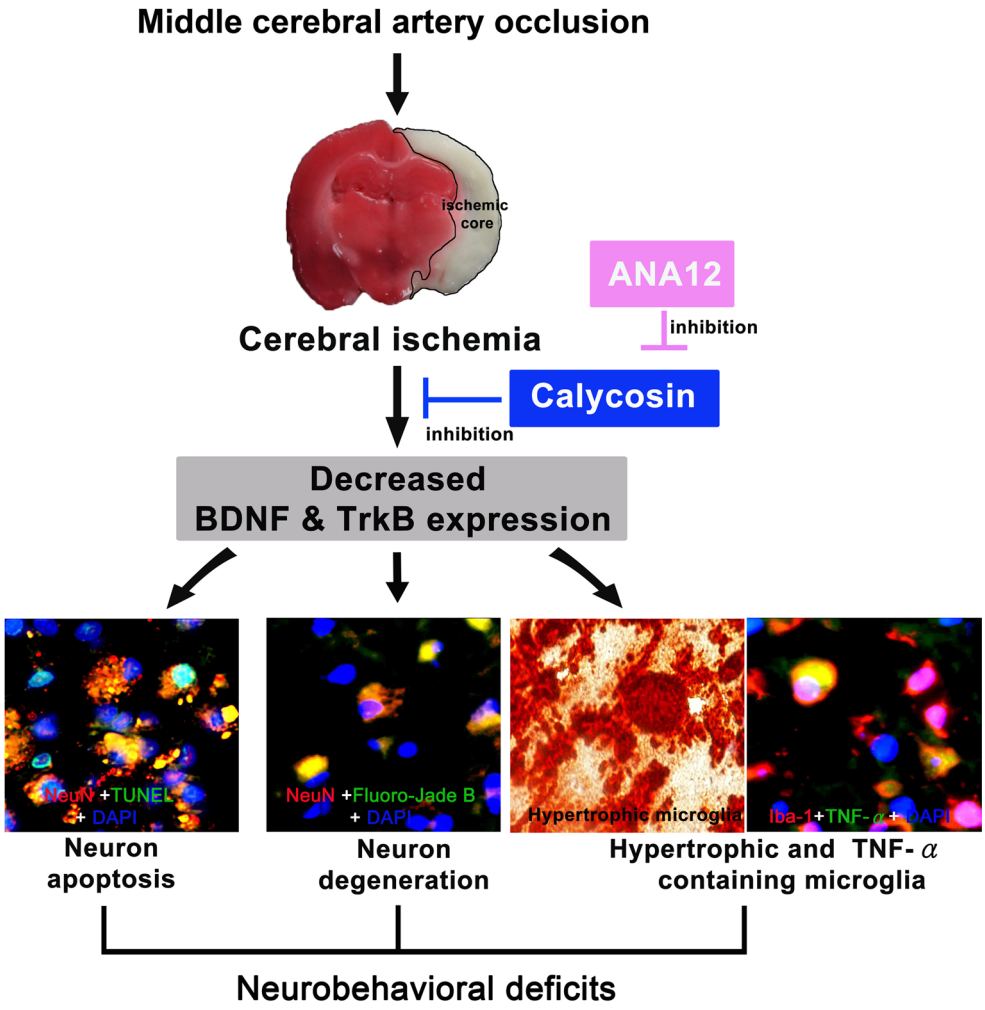


Fig. 8 A schematic diagram showing that Calycosin attenuates cerebral ischemia-induced BDNF/TrkB signaling, brain infarct, neuronal apoptosis and degeneration, accumulation of hypertrophic and TNF-α-containing microglia, and result in neurobehavioral deficits. The beneficial effects of Calycosin can be reversed by ANA12, a BDNF/TrkB antagonist.

apoptotic neurons by Fluoro-Jade-B (Fig. 4) and TUNEL (Fig. 5) staining, respectively (Butler et al. 2002). We found that MCAO significantly increased the % of both degenerative neurons and apoptotic neurons in the ischemic brain regions at 1 week after the procedure compared with sham surgery (Fig. 4). Moreover, Calycosin significantly attenuated the increases in the % of both degenerating and apoptotic neurons induced by MCAO (Figs. 4 and 5). The beneficial effects of Calycosin on neuronal apoptosis and degeneration were significantly attenuated by ANA12 treatment (Figs. 4 and 5). These results revealed that ANA12 did not affect MCAO-induced neurodegeneration and neuronal apoptosis but reverses the neuroprotective effects of Calycosin.

Calycosin Attenuated both Microgliosis and Accumulation of Hypertrophic or Amoeboid-like Microglia in Ipsilateral Brain Tissues Evaluated by Immunohistochemical Staining Following MCAO

Immunohistochemical staining performed 7 days after MCAO revealed that the total numbers of Iba-1-positive cells (or microglia) in the ischemic cortex and striatum of the MCAO+Vehicle group were significantly higher than those in the ischemic cortex and striatum of the Sham+Vehicle group (Fig. 6). The majority of these Iba-1-positive cells displays a hypertrophic or an amoeboid-like phenotype. Calycosin shifted the hypertrophic phenotypes of microglia into the resting ramified phenotype of microglia (Fig. 6). However, ANA12 significantly abolished the beneficial effects of Calycosin on Iba-1-positive cell numbers and morphological phenotypes after MCAO (Fig. 6). These results revealed that ANA12 did not affect MCAO-induced alterations in microglia counts and morphology but significantly reversed the beneficial effects of Calycosin on microglia counts and morphology.

Calycosin Attenuated Accumulation of TNF-α-Containing Microglia in Ischemic Brain Tissues Evaluated by Immunofluorescence Staining Following MCAO

Immunofluorescence staining performed 7 days after MCAO showed that MCAO rats treated with vehicle displayed greater

percentage of TNF-α-containing microglia in the ipsilateral brain regions than did the sham rats ($P < 0.01$; Fig. 7). Calycosin significantly reduced the accumulation of microglia and TNF-α-containing microglia in ipsilateral brain tissues (Fig. 7). However, the beneficial effects of Calycosin on the accumulation of TNF-α-containing microglia in ischemic brain tissues after MCAO were significantly attenuated by ANA12 (Fig. 7).

Discussion

As a neurotrophin, BDNF can regulate neuronal viability development and function (Habtemariam 2018). In addition to maintenance of synaptic plasticity, BDNF/ TrkB signaling pathways protect neurons from apoptosis also promote neurogenesis to recover neuronal deficit even in adulthood. Stroke is caused by decreased brain perfusion due to occlusion or hemorrhage of a cerebral blood vessel followed by oxygen and glucose deprivation in the deficiently irrigated brain tissue. This condition results in impairment of neurotransmission, neuronal survival and plasticity, and the processes of learning and memory (Tejeda and Diaz-Guerra 2017). Enhancement of BDNF production after ischemic stroke, mainly attributable to both neurons and microglia in the ischemic brain tissue, may act as a brain compensatory mechanism to prevent excessive neuronal death as well as neurological impairments (Bejot et al. 2011; Kokaia et al. 1995).

Post-stroke cognitive deficits (Chen et al. 2013) as well as depression-like behavior (Chen et al. 2015) in rodents can be alleviated by BDNF/ TrkB overexpression but exacerbated by ANA12, a TrkB antagonist. Probably, the most striking finding of the present study is that we provide first evidence to demonstrate that Calycosin might alleviate the outcomes of neuropsychiatric disorders in ischemic stroke rats by preserving the BDNF/ TrkB signaling and reducing the accumulation of hypertrophic and TNF-α-containing microglia in the ischemic brain tissues.

Recent evidence indicates that microglia-mediated neuroinflammation is one of the most striking hallmarks of pathology shared by several neurodegenerative diseases such as amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease and traumatic brain injury (Tang and Le 2016; Chio et al. 2015). M1 (hypertrophic or amoeboid-like) microglia produce various pro-inflammatory cytokines, such as TNF-α, interleukin-1β, and interleukin-6, and superoxide anions, reactive oxygen species and nitric oxide by activating the nuclear factor-kappa B and inducible nitric oxide pathways. M2 (resting or ramified) microglia facilitate the phagocytosis of cell debris and misfolded proteins, promote tissue repair, and support neuronal survival by secreting neurotrophic factors. M2 microglia antagonize M1 pro-inflammatory responses, resulting in immunosuppression and neuroprotection. M1 microglia generally predominate at injury sites at the end stage of diseases, at which time the immune resolution and repair processes facilitated by M2 microglia are dampened (Tang and Le 2016). During the pathogenesis of neurodegeneration, hypertrophic or amoeboid-like microglia contribute to post-ischemic inflammation by producing TNF-α, interleukin-1β, reactive oxygen species, and other pro-inflammatory mediators (Iadecola and Anrather 2011). The results of the present study show that microglia activation (signified by the accumulation of both hypertrophic Iba-1-positive cells and TNF-α-containing Iba-1-positive cells) occurs in the ischemic penumbra zone of the cortical and striatal regions of the brain in ischemic stroke rats. In vehicle-treated stroke rats, microglia activation is accompanied by neurological injury. Calycosin preserves BDNF/TrkB signaling but also suppresses cerebral neurological injury and promotes functional recovery post-stroke in rats. It is likely that Calycosin switches the microglia phenotypes from cytotoxic (hypertrophic or amoeboid) to neuroprotective (ramified) to alleviate the inflammation and neuron loss. This hypothesis is supported by the results of several previous investigations. For example, inflammation generated by stroke is a major factor that contributes to tissue damage (Moskowitz et al. 2010) and that damaged tissue favors an M1/hypertrophic phenotype of microglia (Hu et al. 2012; Denker et al. 2007; Schilling et al. 2005). Mice lacking an M2 (ramified) response, which downregulates inflammation and initiates repair, have worse outcomes after experimental cerebral ischemia than other mice (Perez-de Puig et al. 2013; Xiong et al. 2011). Additionally, a reduction in the levels of M2 microglia-associated cytokines (e.g., IGF-1) via the depletion of galectin-3 (a protein required for microglial activation) results in worse pathology after stroke (Lalancette-Hebert et al. 2012). Macrophages can also be classified into the following two groups: M1 (or hypertrophic) macrophages, which produce pro-inflammatory cytokines, chemokines, ROS, and NO; and M2 (ramified) macrophages, which produce anti-inflammatory cytokines (Mantovani et al. 2005). After cerebral ischemia, perivascular macrophages may drive inflammatory cell infiltration by releasing pro-inflammatory cytokines (Konsman et al. 2007). It is possible that BDNF/TrkB-mediated Calycosin may also induce the switching of microglia/macrophage phenotypes from M1 (hypertrophic or amoeboid) to M2 (ramified) to reduce inflammatory neurodegeneration and to promote neuroregeneration in stroke rats.

Conclusions

As summarized in Fig. 8, the BDNF/ TrkB-mediated Calycosin reverses ischemic stroke-induced reductions of BDNF/ TrkB expression in the injured brain tissues, neurological injury (e.g., brain infarct, neuronal degeneration and

apoptosis, and neurobehavioral deficits), and accumulation of hypertrophic and TNF-α-containing microglia in rats.

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Authors' Contributions C.C.H. and W.P.L. performed the MCAO surgeries and western blotting. T.W.K. performed the rat behavioral tests and immunohistochemical staining. C.C.H. and H.J.L. designed and coordinated the study. C.P.C. and H.J.L. conceived the study and wrote the manuscript. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Ethics Approval and Consent to Participate All animal research was ethically approved by the Institutional Animal Care and Use Committee of Chi Mei Medical Center (IACUC approved no. 105110328). Experiments adhered to guidelines from the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Consent for Publication Not applicable.

Availability of Data and Material The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing Interests The authors declare that there is no conflict of interest.

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