Neurochemistry International 128 (2019) 143–153

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Bis(propyl)-cognitin potentiates rehabilitation of treadmill exercise after a transient focal cerebral ischemia, possibly via inhibiting NMDA receptor and regulating VEGF expression

Zheng Ke[a,1], Shengquan Hu[b,1], Wei Cui[b], Jing Sun[a], Shaojie Zhang[a], Shinghung Mak[b], Jiajun Wang[b], Jing Tang[c], Yuanping Pang[c], Yifan Han[b,*], Kaiyu Tong[a,d,**]

[a] Interdisciplinary Division of Biomedical Engineering, The Hong Kong Polytechnic University, Hong Kong
[b] Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hong Kong
[c] Mayo Cancer Center, Department of Pharmacology, Mayo Clinic, Rochester, MN, USA
[d] Department of Biomedical Engineering, The Chinese University of Hong Kong, Hong Kong

Keywords:
bis(propyl)-cognitin
Exercise
Ischemia
Motor recovery
Stroke
VEGF

A B S T R A C T

Combination therapies may have greater efficacy compared with monotherapy in treating stroke. We investigated the molecular mechanisms by which the combination of bis(propyl)-cognitin, an uncompetitive antagonist of NMDA receptor, and treadmill exercise promote rehabilitation after ischemic stroke. Rats were distributed into 3 treatment groups: infarct/bis(propyl)-cognitin(drug only group, DO); infarct/treadmill exercise(exercise only group, EO); infarct/bis(propyl)-cognitin + treadmill exercise (drug + exercise group, DE). The DE group had further separated to 3 sub-groups to investigate the effects achieved by different time for drug administration (60 min before stroke (DE-60 m), 15 min (DE + 15 m) and 60 min (DE + 60 m) after stroke). Although all infarct groups improved over time, the combination of bis(propyl)-cognitin and treadmill exercise effectively enhanced motor recovery during 14-day intervention. Early drug intervention has a best recovery result, the DE + 15 m group with drug intervention at 15-min after stroke had better motor recovery than DE + 60 m, DO, EO and control groups. Both bis(propyl)-cognitin and treadmill exercise significantly elevated brain VEGF expression and decreased brain infarct volume at 14 day post-ischemia. Our study reveals that bis(propyl)-cognitin potentiated rehabilitation of treadmill exercise after ischemic stroke, possibly via regulating brain VEGF expression, indicating that the combination of NMDA receptor antagonists and exercise might be useful for stroke rehabilitation.

1. Introduction

Stroke is the second leading cause of death among elderly in the world, leaving 50% of the stroke survivors permanently disabled (Shi et al., 2018; Stoop et al., 2017). Extensive studies have been carried out to develop effective interventions for treating stroke (Diener et al., 2013; Hasegawa et al., 2017). However, no single effective therapy for this devastating disease is available due to its multifaceted manifestations and complicated pathogenesis. Combination therapy might increase the efficacy above levels achievable with monotherapy (O'Collins et al., 2012), suggesting that combination therapy is an appealing and important strategy for the treatment of stroke.

As suggested by earlier studies (Burnett et al., 2006; DeBow et al., 2003), exercise is able to enhance memory and cognitive functions, and improve functional recovery after stroke. Among various exercise experimental paradigms, treadmill exercise is one of the most commonly applied rehabilitation scheme in both animal models and clinical studies (Ke et al., 2011; Munari et al., 2018; Yang et al., 2003). For example, treadmill exercise greatly reduced brain infarct volume and improve neurological functions in rats after cerebral artery occlusion/

Abbreviations: BSA, bovine serum albumin; CV, cresyl violet; GFAP, glial fibrillary acidic protein; MCA, middle cerebral artery; MCAo/r, middle cerebral artery occlusion/reperfusion; NMDA, N-methyl-D-aspartate; OD, optical density; SDS, sodium dodecyl sulfate; VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor-2

* Corresponding author. Rm Y842, Lee Shau Kee Building, 11 Yuk Choi Road, Hung Hom, Kowloon, Hong Kong.
** Corresponding author. Rm 429, Ho Sin Hang Engineering Building, The Chinese University of Hong Kong, Shatin, N. T., Hong Kong.
E-mail addresses: bcyfhan@polyu.edu.hk (Y. Han), kytong@cuhk.edu.hk (K. Tong).

[1] The first two authors contributed equally to this work.

https://doi.org/10.1016/j.neuint.2019.04.016
Received 3 January 2019; Received in revised form 24 April 2019; Accepted 25 April 2019
Available online 26 April 2019
0197-0186/

reperfusion (MCAo/r) (Ke et al., 2011; Yang et al., 2003). Additionally, high-intensity treadmill training markedly improved gait ability and cost of walking in stroke survivors (Mehrholz et al., 2014; Munari et al., 2018). Several important mechanisms, particularly including the increase of vascular endothelial growth factor (VEGF) expression in the striatum and motor cortex (Ma et al., 2013; Xie et al., 2018; Zhang et al., 2013). Have been proposed to be responsible for the long-term repair and restoration process of the brain. Moreover, to a great extent, exercise training improves recovery after stroke by increasing collateral blood flow (Zhang et al., 2013).

Recent studies have highlighted the involvement of glutamate-induced excitotoxicity in the pathogenesis of stroke (Chamorro et al., 2016; Ikonomidou and Turski, 2002; Lai et al., 2014), suggesting that intervening this mechanistic steps that lead to excitotoxicity may prevent stroke damage. Glutamate induces neurotoxicity mainly through the over-activation of N-methyl-D-aspartate (NMDA) receptors in the post-synaptic membranes of neurons. Consequently, NMDA receptor antagonists were proved to rescue the dying neurons after stroke (Chen et al., 2017). Bis(propyl)-cognitin (B3C) is an effective dimer that has been demonstrated to possess multiple pharmacological activities. For instance, B3C is an uncompetitive NMDA receptor antagonist (Luo et al., 2010), indicating that B3C preferentially blocked glutamate-induced neuronal death while sparing physiological neurotransmission mediated by the NMDA receptor. Additionally, B3C effectively decreased brain infarct volume in rats after MCAo/r. Moreover, B3C greatly activated VEGF/vascular endothelial growth factor receptor-2 (VEGFR-2) system to produce neuroprotective activities in primary neurons (Hu et al., 2013), highlighting that B3C might also increase VEGF expression in the brain.

The combination of pharmacological agents and exercises, which brings about better recovery, has been emerged as one of the most potent therapy for stroke (Sakakima et al., 2012). .Although B3C treatment and treadmill exercise were reported to protect against brain injury and improve recovery after stroke individually, the effects of their combination have not been studied before. In this study, the effects and underlying molecular mechanisms by which the combination of B3C and treadmill exercise promote rehabilitation were investigated in the rat model of MCAo/r.

2. Material and methods

2.1. Subjects and group assignment

All experiment procedures were approved by the Animal Ethics Review Committee of the Hong Kong Polytechnic University and conformed to international guidelines on the ethical use of animals. A total of 155 young male Sprague-Dawley rats (280–320 g) were enrolled in the experiment. Animals were allowed to access to food and drink *ad libitum*. Rats were first trained to run on a treadmill (KN-73, Natsume Ltd., Tokyo, Japan) as the accommodation phase for 3 days. The minimum running distance on treadmill (600 m/d) was based on our previous study (Ke et al., 2011). 14 rats that did not fulfill the running requirements were excluded.

2.2. Surgical procedures

The MCAo/r surgery was performed as we previously described (Ke et al., 2011). Briefly, after anesthetization, the neck region of the rat was opened and the right common carotid artery and the right external carotid artery were ligated, whereas the right internal carotid artery was isolated. A 3–0 uncoated monofilament nylon suture with a rounded tip was inserted from the internal carotid artery to the right middle cerebral artery (MCA) and thus blocked the blood flow to the MCA and caused occlusion. After 90 min, reperfusion was allowed by withdrawing the suture. Rats in the Sham group were subjected to the same surgical procedures except that the monofilament nylon suture was not inserted into the internal carotid artery.

2.3. Drug administration and treadmill exercise

Drug preparation and administration protocol was carried out as we previously reported (Carlier et al., 1999; Zhao et al., 2011). In the drug administration, rats were separated to 3 groups to investigate the effects of the time to administrate the drug (60 min before stroke (DE-60 m), 15 min (DE + 15 m) and 60 min (DE + 60 m) after stroke). Bis(propyl)-cognitin (0.3 mg/kg, the Drug group) and saline (the Non-drug group) were *i.p.* administered. DE-60 m group: 1 h before occlusion at the concentration of 0.3 ml/kg body weight; DE + 15 m and DE + 60 m groups were 15 min or 60 min after the reperfusion of the MCAO surgery at the concentration of 0.3 ml/kg body weight respectively. Our previous study suggested that *i.p.* injection of bis(propyl)-cognitin reached the brain within 1 h (Luo et al., 2010). Therefore, animals were received the first injection of bis(propyl)-cognitin 1 h before or immediately (e.g. 15 or 60 min) after the MCAo/r to prevent ischemia-induced impairments. One day after the surgery, rats were further assigned to a 14-day intervention period. Briefly, rats in the Non-drug group were randomly divided into two groups: the control group without treatment (Con) and the treadmill exercise group (exercise only, EO). Similarly, rats in the Drug group were also randomly divided into two groups: the bis(propyl)-cognitin group (drug only, DO) and the bis(propyl)-cognitin + treadmill exercise group (drug & exercise, DE). Rats in the DO group daily received *i.p.* injection of 0.3 mg/kg bis (propyl)-cognitin. Rats in the DE group daily received *i.p.* injection of 0.3 mg/kg bis(propyl)-cognitin 1 h before the treadmill exercise. Treadmill exercise was carried out in the EO and the DE group rats and the running protocol was described in our previous study (Ke et al., 2011). Rats in the EO and the DE groups were trained to run on the treadmill. The speed of the treadmill was set at 20 m/min and the slope was set at 0°. The daily training duration was 30 min which was separated into three 10-min sessions. Between each session, rats were allowed for a 10-min rest. During the rehabilitation process, the settings were not changed for the rats. Rats in the Con group were put in standard cages without any intervention and allowed spontaneous recovery after ischemia.

2.4. Neurological test

The Longa's neurological test was used to measure the neurological deficits at 24 h after the MCAo/r surgery (Longa et al., 1989). Briefly, rats were scored as follows: 0 - no obvious neurological impairment; 1 – can not fully stretch contralateral forelimbs; 2 - contralateral circling while walking; 3 - contralateral fall over while walking; 4 – can not walk and decrease of consciousness. Rats with more neurological deficits, and presumably larger areas of infarctions, would obtain higher scores in this test.

2.5. Behavioral tests

De Ryck's behavioral and the beam walking tests are relatively simple and sensitive measurements to evaluate motor deficits in rats (De Ryck et al., 1989; Goldstein and Davis, 1990). Therefore, these behavioral tests were applied in this study to investigate the motor recovery.

De Ryck's behavioral test is a sensitive test which has been widely applied in functional evaluation in stroke rat models. It is a 16-point scale with eight sub-tests, each of which had a score from 0 (maximum deficit) to 2 (no deficit). Among the 8 tests, 6 tests were designed to examine the forelimb's functions, while the other 2 evaluated the hindpaw's placing.

The beam walking test involves scoring the foot slips while a rat transverses of a 2 cm-wide and 130 cm-long beam. The beam was elevated 110 cm above the ground and placed horizontal to the home cage

at the target end. The rats were placed at one end of the beam, and their foot slips were counted during their walk to their home cage. Beam-walking test was a six-point scoring system as previously described: 0 - unable to put the affected hindpaw on the horizontal surface; 1 - place the hindpaw on the horizontal surface and maintain balance for at least 5 s; 2 - traverse the beam with dragging the affected hindpaw; 3 - traverse the beam and at least once put the affected hindpaw on the horizontal surface; 4 - crosse the beam with more than half steps slipped; 5 - use the affected hindpaw more than half its steps; 6 - traverse the beam with less than three footslips.

The behavioral tests were conducted from the 6th hour after the MCAo/r surgery to monitor the immediate influence of experimental drug on any behavioral outcome. And according to our experience and literature (Hüske et al., 2016), the rats generally waken from chloral hydrate anesthesia in about 3–4 h after operation, which means that most rats were conscious and able to respond to the behavioral tests.

2.6. Western blot analysis

Western blot analysis was performed as we previously described (Hu et al., 2015, 2018a, 2018b). Briefly, the rat's brain was obtained from the skull immediately after sacrifice. Brain tissue samples were homogenized at 4 °C for 1 min in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 5 μg/ml leupeptin, 1 μg/ml aprotinin and 5 μg/ml pepstatin). After centrifugation at 16,000 g for 10 min, protein concentration in the supernatant was determined by Bradford assay. Samples (40 μg) were separated by SDS-PAGE, and transferred to polyvinylidene fluoride membrane for 2 h at 100 V. The membranes were further blocked with 5% non-fat milk in PBST (0.1% Tween 20 in phosphate buffered saline) for 2 h. The blots were incubated overnight at 4 °C with various primary antibodies, including VEGF (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin (1:1000, Santa Cruz Biotechnology). After three times washes with PBST, the membranes were further incubated with secondary antibody. Blots were developed by using an enhanced chemiluminescence plus kit (Amersham Bioscience, Aylesbury, UK), and then exposed to Kodak films. All data were resulted from three independent experiments. Data were expressed as a ratio to optical density (OD) values of controls for statistical analyses.

2.7. Tissue preparation

After the 14-day experiment, rats were deeply anesthetized and perfused transcardially with 4% para-formaldehyde in 0.1 M phosphate buffer. The brains were then carefully removed and immersed in 30% sucrose solution in distilled water at 4 °C until they sank to the bottom of the sucrose solution. Brain tissue was cut into series of 20-μm coronal sections on a freezing microtome and stored in cytoprotectant at −20 °C.

2.8. Immunofluorescence staining

Immunofluorescence staining was performed as described previously (Lu et al., 2012). Briefly, brain sections were blocked with 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 h at room temperature, and incubated at 4 °C overnight with a rabbit polyclonal antibody against VEGF (1:200, Santa Cruz Biotechnology) together with a mouse monoclonal antibody against glial fibrillary acidic protein (GFAP, 1:300, Cell Signaling Technology Inc, Beverly, MA, USA). After three washes with PBS, brain sections were incubated with Alexafluor-568 anti-rabbit secondary antibodies (1:300, Life Technologies, USA) and Alexafluor-488 anti-mouse (1:300, Life Technologies, Carlsbad, CA, USA) for 1 h at room temperature in the dark. Brain sections were then washed three times with PBS. Images were acquired using a fluorescence microscope (Nikon Instruments Inc. Melville, NY, USA).

2.9. Cresyl violet staining

Cresyl violet (CV) staining was performed as described previously (Lafargue et al., 2012). Briefly, brain sections were stained by using 1% cresyl violet acetate (Sigma Chemicals) at room temperature for 10 min, and then dehydrated in 70, 90, and 100% ethanol. To evaluate the lesion, an image analysis software (ImageJ, National Institutes of Health, USA) was used (Rousselet et al., 2012). The injury volume was calculated in pixels. To normalize the brain slice, we used the following formula: corrected infarct volume percentage = (contralateral hemispheric volume - ipsilateral non-infarcted volume)/contralateral hemispheric volume (Valtysson et al., 1994).

2.10. Statistical analysis

For analysis of neurological tests, Western blot on VEGF and CV staining on brain infarct volumes among groups, we used one-way analysis of variance (ANOVA) with Bonferroni post-hoc test. For comparison of the De Ryck's behavioral test and beam walking test, the multivariate analysis of variance (MANOVA) with Wilk's λ test, and the Kruskal-wallis with Bonferroni post-hoc test were applied. All the analyses were performed using SPSS (version 20.0). Data were presented as mean ± SD. The level of statistical significance was set at 0.05.

3. Results

3.1. CONSORT statement flow diagram

After accommodation, a total of 141 rats were randomly divided into three groups: 12 rats of the Sham group (no infarct/no treatment), 49 rats of the Non-drug group, and 80 rats of the Drug group. Rats of the Non-drug group and the Drug group were enrolled in the MCAo/r surgery (Fig. 1). The Drug group had separated to 3 sub-groups to investigate the effects of the time to administrate the drug (60 min before stroke, 15 min and 60 min after stroke). Within 24 h after MCAo/r surgery, 9 rats of the Non-drug group and 6 rats of the Drug group died. Moreover, 7 rats of the Non-drug group and 8 rats of the Drug group did not exhibit motor deficits. Therefore, ischemic stroke was successfully induced with motor deficits in 33 rats of the Non-drug group and 66 rats of the Drug group. At 24 h after MCAo/r surgery, 6 rats of the Sham group, and 6 rats each of the Non-Drug and the Drug groups with motor deficits were sacrificed for the evaluation of VEGF expression. Remaining rats of the Non-drug group with motor deficits (n = 27) were further divided into the Control (infarct/no treatment, n = 13), the EO (infarct/treadmill exercise, n = 14) groups. Similarly, remaining rats of the Drug groups with motor deficits (n = 60) were divided into the DO (infarct/bis(propyl)-cognitin, n = 13), and the DE (infarct/bis(propyl)-cognitin + treadmill exercise, n = 47) groups. 60 of these rats (10 rats of the Control group, 10 rats of the EO group, 10 rats of the DO group, and 10 rats of each DE group [DE-60 m, DE+15 m, DE+60 m]) completed the 14-day intervention, and were scarified for neurochemical and immunofluorescence analysis at 14 day post-ischemia.

3.2. Bis(propyl)-cognitin effectively attenuates neurological and motor deficits at 24 h post-ischemia

A five-point Longa's neurological score was employed to measure the neurological deficits at 24-h post-ischemia. The neurological scores of the Sham group, the Non-drug group and the Drug group were 0.0 ± 0.0, 2.27 ± 0.62 and,1.83 ± 0.58, respectively (Fig. 2A). Neurological scores in both infarct groups were significantly higher than those in the Sham group (ANOVA and Bonferroni test, $p < 0.001$). Moreover, rats of the Drug group have significantly less neurological

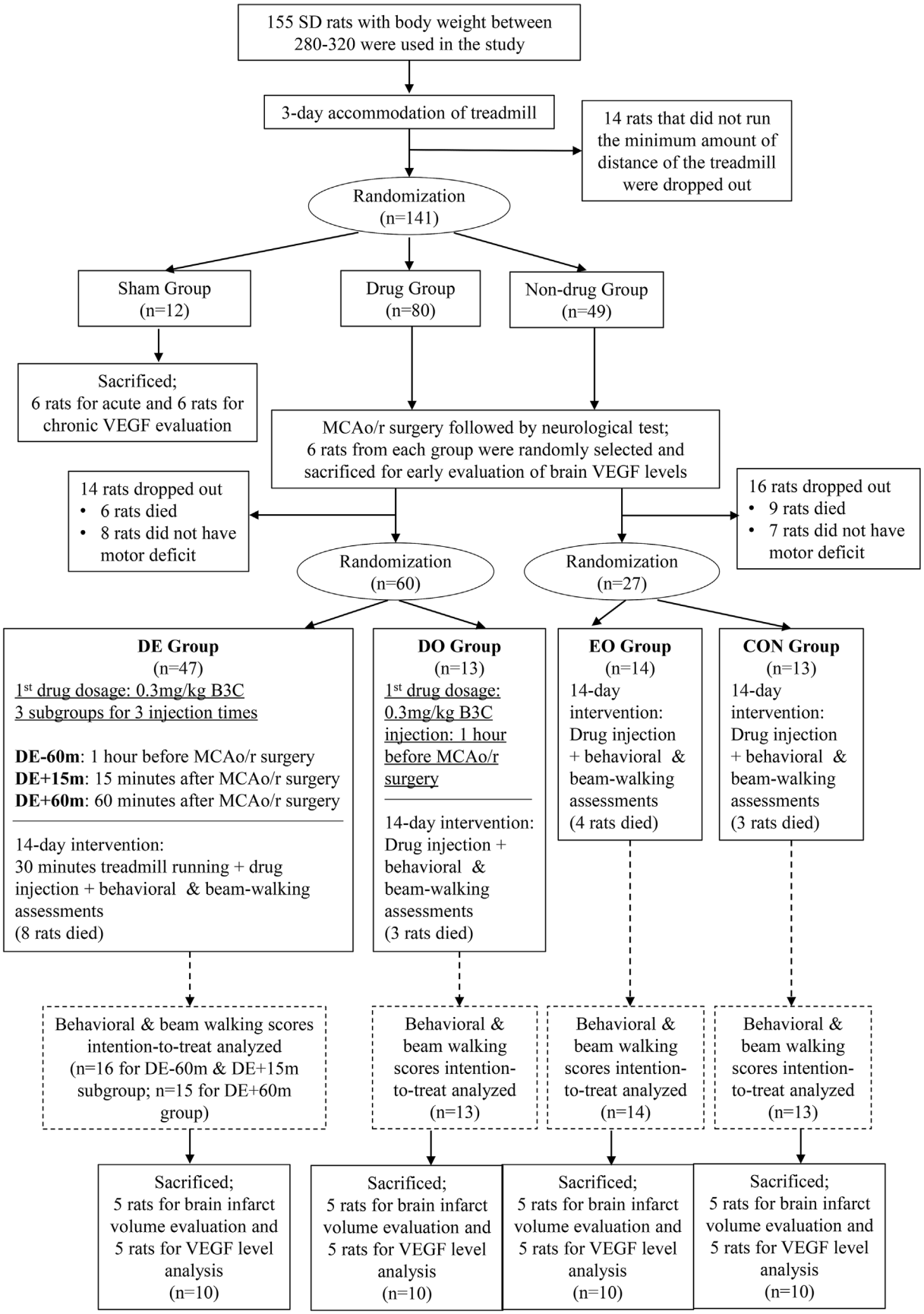

Fig. 1. CONSORT flowchart of the experimental procedure. All rats were firstly accommodated to treadmill exercise. Rats in the Drug group were administered bis(propyl)-cognitin (B3C). Rats in the DE and the DO groups received daily B3C injection while rats in the DE and the EO rats daily exercised on treadmill during the 14-day intervention post-ischemia. De Ryck's behavioral test and the beam walking test were conducted daily during the 14-day intervention. Brain infarct volume and VEGF expression were analyzed after sacrificed at 14 day post-ischemia. (DE: drug & exercise; DO: drug only; EO: exercise only).

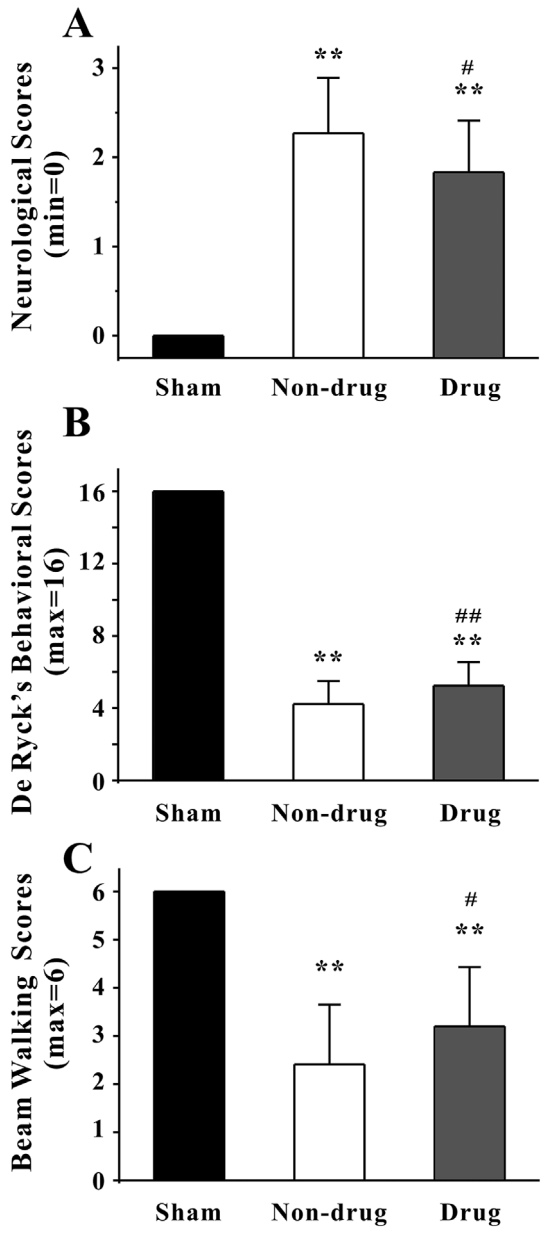

Fig. 2. Bis(propyl)-cognitin significantly attenuates neurological and motor deficits at 24 h post-ischemia. (A) Bis(propyl)-cognitin significantly attenuated neurological deficits at 24 h post-ischemia. Animals in the Drug group received 0.3 mg/kg B3C 1 h before the MCAo/r. A five-point Longa's neurological test (max = 4) was employed to measure the neurological deficits of rats in the Sham group (N = 12), the Non-drug group (N = 27) and the Drug group (N = 25) at 24 h post-ischemia. (B) Bis(propyl)-cognitin significantly increased De Ryck's behavioral scores at 24 h post-ischemia. Animals in the Drug group received 0.3 mg/kg B3C 1 h before the MCAo/r. De Ryck's behavioral test (max = 16) was applied to measure the motor deficits of rats in the Sham group, the Non-drug group and the Drug group at 24 h post-ischemia. (C) Bis(propyl)-cognitin significantly increased beam walking scores at 24 h post-ischemia. Animals in the Drug group received 0.3 mg/kg B3C 1 h before the MCAo/r. Beam walking test (max = 6) was applied to measure the motor deficits of rats in the Sham group, the Non-drug group and the Drug group at 24 h post-ischemia. Data were the mean ± SD; *p < 0.001 *versus* the Sham group, #p < 0.05 and p < 0.01 *versus* the Non-drug group (ANOVA and Bonferroni test).

deficits than those of the Non-drug group (ANOVA and Bonferroni test, $p < 0.05$).

The motor recovery outcomes were presented as changes in De Ryck's behavioral scores (max = 16) and the beam walking scores (max = 6). Rats of the Sham group did not show significant motor deficits evidenced by both De Ryck's behavioral scores and beam walking scores. At 24-h post-ischemia, De Ryck's behavioral scores of the Sham group, the Non-drug group and the Drug group were 16.0 ± 0.0, 4.23 ± 1.27 and 5.25 ± 1.30, respectively (Fig. 2B), and the Kruskal-wallis detected a significant difference among groups ($p < 0.001$). De Ryck's behavioral scores in both infarct groups were significantly lower than that in the Sham group ($p < 0.001$). Moreover, the Drug group have significantly higher De Ryck's behavioral scores than those of the Non-drug group ($p < 0.01$). Similarly, at 24-h post-ischemia, beam walking scores of the Sham group (n = 12), the Non-drug group (n = 27) and the Drug group (n = 25) were 6.0 ± 0.0, 2.41 ± 1.24 and 3.20 ± 1.23, respectively (Fig. 2C), and the Kruskal-wallis detected a significant difference among groups ($p < 0.001$). Beam walking scores in both infarct groups were significantly lower than that in the Sham group ($p < 0.001$). Additionally, the Drug group have significantly higher beam walking scores than those of the Non-drug group ($p < 0.05$).

3.3. Bis(propyl)-cognitin synergistically potentiates motor recovery of treadmill exercise during 14-day intervention post-ischemia

During 14-day intervention, significant differences of De Ryck's behavioral scores among four infarct groups (the Con, EO, DO and DE groups) were found from the second day post-ischemia and lasted until the end of the 14-day intervention (Fig. 3A). MANOVA analysis further demonstrated that both bis(propyl)-cognitin (Wilk's $\lambda = 0.076$, $p < 0.001$) and treadmill exercise (Wilk's $\lambda = 0.183$, $p < 0.001$) interventions had significant effects on the increase of De Ryck's behavioral scores. Importantly, the interaction between bis(propyl)-cognitin and treadmill exercise also showed significant effects on the increase of De Ryck's behavioral scores (MANOVA, Wilk's $\lambda = 0.376$, $p < 0.05$). At the end of the 14-day intervention, De Ryck's behavioral scores in all three treatment groups were significantly higher than that in the Con group (Kruskal-wallis and Bonferroni test, $p < 0.001$). Moreover, the DE-60 m group had significantly higher De Ryck's behavioral score than the DO (Kruskal-wallis and Bonferroni test, $p = 0.001$) and the EO groups (Kruskal-wallis and Bonferroni test, $p < 0.001$). No significant difference was found between DO and EO groups (Kruskal-wallis and Bonferroni test, $p = 0.363$).

Similarly, significant differences of beam walking scores among four infarct groups (the Con, EO, DO and DE groups) were found from the second day and lasted until the end of the 14-day intervention (Fig. 3B). MANOVA analysis further showed that both bis(propyl)-cognitin (Wilk's $\lambda = 0.120$, $p < 0.001$) and treadmill exercise (Wilk's $\lambda = 0.163$, $p < 0.001$) interventions had significant effects on the increase of beam walking scores. Importantly, the interaction between drug treatment and treadmill exercise also showed significant effects on the increase of beam walking scores (MANOVA, Wilk's $\lambda = 0.294$, $p < 0.01$). At the end of the 14-day intervention, beam walking scores in all three treatment groups were significantly higher than that in the Con group (Kruskal-wallis and Bonferroni test, $p < 0.01$). And the DE-60 m group had significantly higher beam walking score than the DO (Kruskal-wallis and Bonferroni test, $p < 0.05$) and the EO (Kruskal-wallis, and Bonferroni test $p < 0.01$) groups.

3.4. Bis(propyl)-cognitin and treadmill exercise significantly decreased brain infarct volume at 14 day post-ischemia

The brain infarct volume was measured by CV staining at 14 day post-ischemia. The striatum and the frontoparietal cortex were mainly affected after ischemia (Fig. 4). Rats of the Sham group did not show significant brain infarct. One-way ANOVA and Bonferroni test revealed significant differences among four infract groups in the brain infarct volume. After the 14-day intervention, the infarct volume in the Con

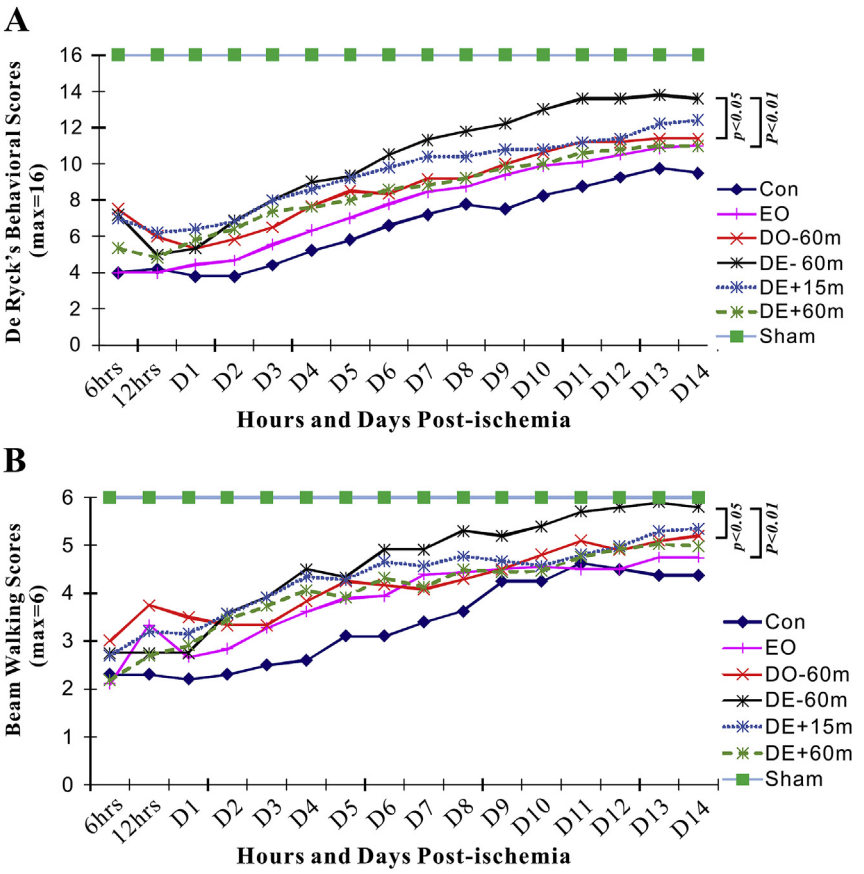

Fig. 3. Bis(propyl)-cognitin synergistically potentiates motor recovery of treadmill exercise during 14-day intervention post-ischemia. (A) Bis(propyl)-cognitin and treadmill exercise synergistically increase De Ryck's behavioral scores during 14-day intervention post-ischemia. De Ryck's behavioral test (max = 16) was applied to measure the daily motor recovery of rats in Sham group (N = 6), Con group (N = 10), DO group (N = 10), DE-60 m, DE + 15 m, DE + 60 m groups (N = 10 for each DE sub-group) during 14-day intervention post-ischemia. (B) Bis(propyl)-cognitin and treadmill exercise synergistically increase beam walking scores during 14-day intervention post-ischemia. Beam walking test (max = 6) was applied to measure the daily motor recovery of rats in Sham group (N = 6), Con group (N = 10), DO group (N = 10), DE-60 m, DE + 15 m, DE + 60 m groups (N = 10 for each DE sub-group) during 14-day intervention post-ischemia. Data were the mean ± SD (ANOVA and Bonferroni test).

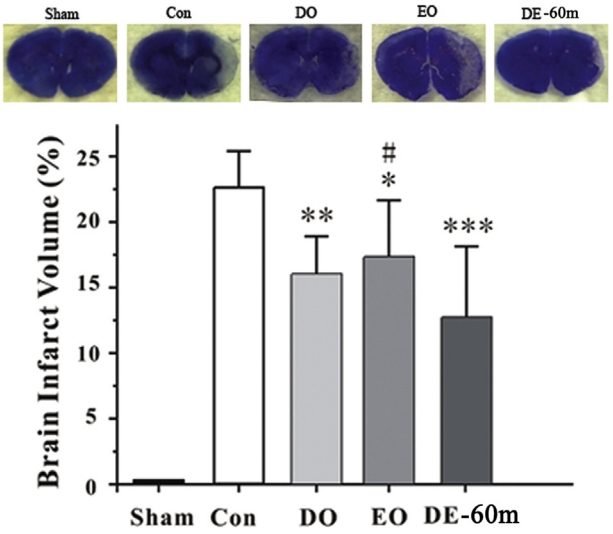

Fig. 4. Bis(propyl)-cognitin and treadmill exercise significantly decreased brain infarct volume at 14 day post-ischemia. CV staining was applied to measure the brain infarct volume of rats in the Sham, the Con, the DO and the DE-60 m groups (N = 4 per group) at 14 day post-ischemia. Data were the mean ± SD; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *versus* the Con group, $^{\#}p < 0.05$ *versus* the DE-60 m group (ANOVA and Bonferroni test).

group (22.7 ± 2.7%) was significantly larger than that in the groups of EO ($p < 0.05$, 17.4 ± 4.3%), the DO ($p < 0.01$, 16.1 ± 2.8%) and the DE-60 m ($p < 0.001$, 12.3 ± 4.1%) (Fig. 4). Moreover, the infarct volume in the DE-60 m group was significantly smaller than that in the EO (ANOVA and Bonferroni test, $p < 0.05$) group. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, compared to Con group; #, $p < 0.05$, compared to EO group.

3.5. Bis(propyl)-cognitin significantly decreased ischemia-increased VEGF expression in the cortex and the striatum at 24 h post-ischemia

VEGF levels in the brain of rats were determined by Western blot analysis. At 24 h post-ischemia, VEGF level in the hippocampus did not show a significant difference among the Sham, the Non-drug and the Drug groups (Fig. 5A). However, VEGF levels of the Non-drug group in the cortex (ANOVA and Bonferroni test, $p < 0.01$) and the striatum (ANOVA and Bonferroni test, $p < 0.01$) were significantly higher than those of the Sham group (Fig. 5B and C). Moreover, the Drug group had significantly lower VEGF level in the cortex (ANOVA and Bonferroni test, $p < 0.01$) and the striatum (ANOVA and Bonferroni test, $p < 0.01$) than the Non-drug group.

3.6. Bis(propyl)-cognitin and treadmill exercise markedly increase VEGF expression in the hippocampus, cortex and the striatum at 14 day post-ischemia

At 14 day post-ischemia, VEGF levels of the Con group was significantly lower than those of the Sham group in all brain regions (Fig. 6, ANOVA and Bonferroni test, $p < 0.01$). Moreover, all three treatment (DO, EO and DE-60 m) groups had significantly higher VEGF levels in the cortex, the striatum and the hippocampus than the Control group (ANOVA and Bonferroni test, $p < 0.05$). Fig. 7 further showed DE + 60 m group also had significantly higher VEGF levels in the cortex, the striatum and the hippocampus than the Control group (ANOVA and Bonferroni test, $p < 0.05$).

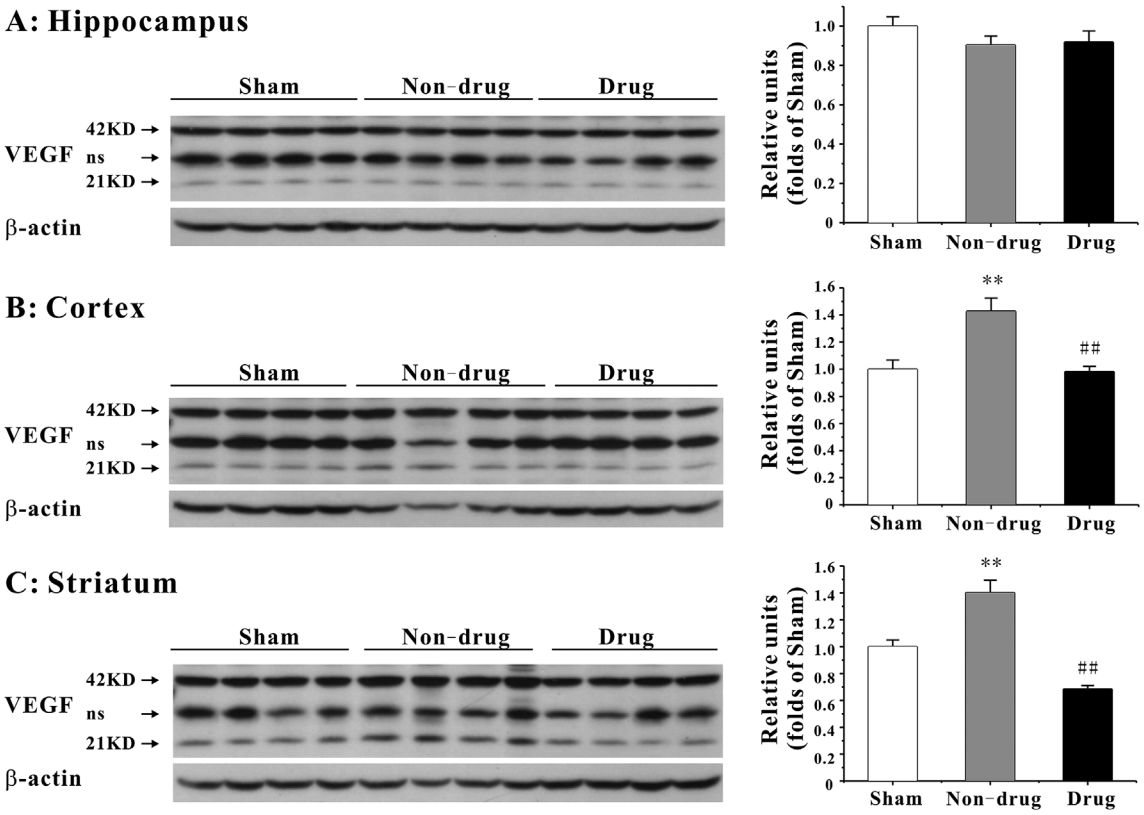

Fig. 5. Bis(propyl)-cognitin significantly decreased ischemia-increased VEGF expression in the cortex and the striatum at 24 h post-ischemia. Animals in the Drug group received 0.3 mg/kg B3C 1 h before the MCAo/r. VEGF expression in (A) the hippocampus, (B) the cortex and (C) the striatum of rats in the Sham (N = 12), the Non-drug (N = 27) and the Drug (N = 25) groups were detected by Western blotting at 24 h post-ischemia (n = 4 per group). Data, expressed as percentage of the Sham group, were the mean ± SD; $^{**}p < 0.01$ *versus* the Sham group, $^{\#\#}p < 0.01$ *versus* the Non-drug group (ANOVA and Bonferroni test).

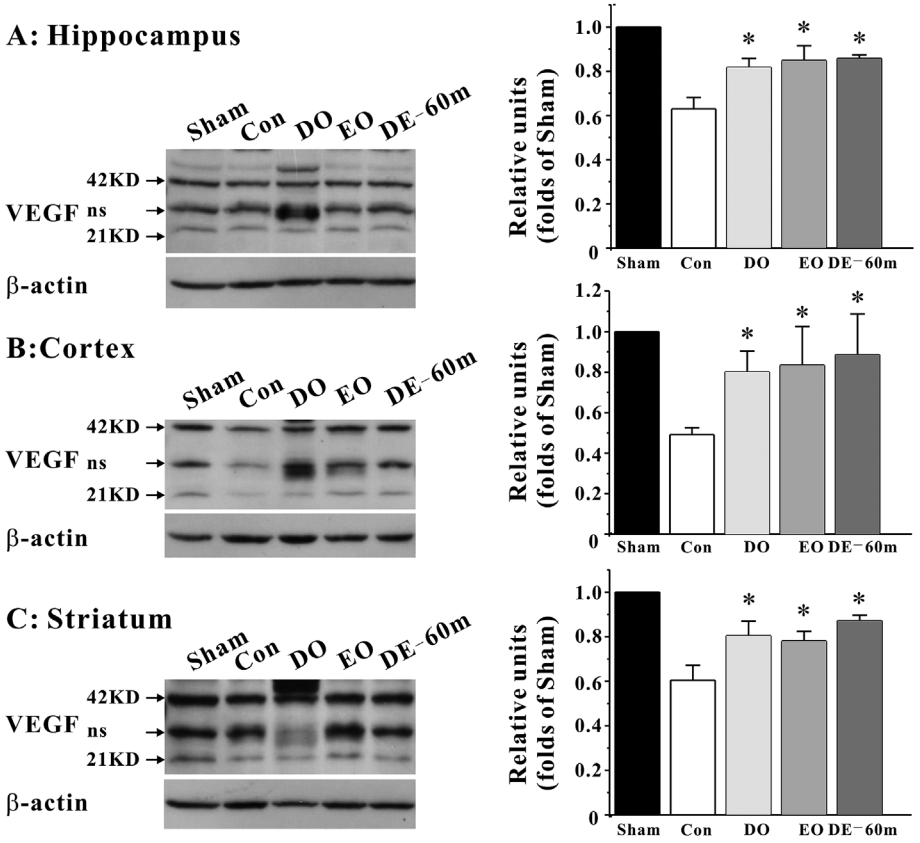

Fig. 6. Group DE-60m, first injection of Bis (propyl)-cognitin was 60 min before stroke and treadmill exercise significantly increased VEGF expression at 14 day post-ischemia. VEGF expression in (A) the hippocampus, (B) the cortex and (C) the striatum of rats in the Sham (N = 12), the Con (N = 13), the DO (N = 13), the EO, (N = 14) and the DE-60 m groups (N = 13) were detected by Western blotting at 14 day post-ischemia (n = 3 per group). Data, expressed as percentage of Sham group, were the mean ± SD; $^{*}p < 0.05$ *versus* the Con group (ANOVA and Bonferroni test).

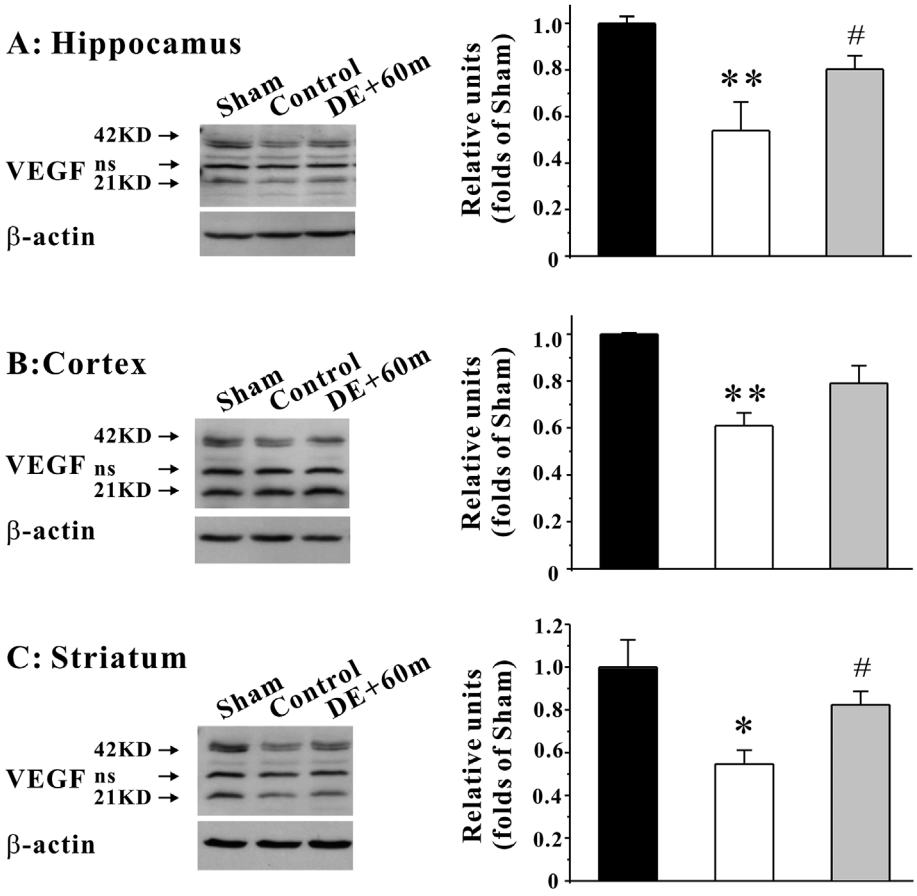

Fig. 7. Group DE + 60m, first injection of Bis (propyl)-cognitin was 60 min after stroke and treadmill exercise significantly increased VEGF expression at 14 day post-ischemia. VEGF expression in (A) the hippocampus, (B) the cortex and (C) the striatum of rats in the Sham (N = 12), the Con (N = 13), and the DE+60 m (N = 13) groups were detected by Western blotting at 14 day post-ischemia (n = 3 per group). Data, expressed as percentage of Sham group, were the mean ± SD; $^{\#}p < 0.05$ *versus* the Con group (ANOVA and Bonferroni test).

3.7. Bis(propyl)-cognitin and treadmill exercise increase astrocytes expressed-VEGF in the striatum

Our previous study suggests that the striatum is the main brain region affected by ischemic stroke using the current MCAo/r protocol (Ke et al., 2011). Moreover, VEGF is mainly expressed by GFAP-positive astrocytes around or in the lesion after brain injury (Skold et al., 2005). Therefore, we performed immunofluorescence straining to confirm that treatment groups had higher VEGF levels in the striatum than the Control group as evidenced by Western blotting analysis and to investigate the expression of VEGF by GFAP-positive astrocytes induced by various treatments. At 14 day post-ischemia, VEGF could be detected in the striatum of all four infarct groups (Fig. 8). Strong immunoreactivity of VEGF was found in all three treatment groups (DO, EO, DE). Moreover, in these treatment groups, double immunolabeling with GFAP and VEGF showed that VEGF immunoreaction mainly co-localized with GFAP-positive astrocytes, suggesting that bis(propyl)-cognitin and treadmill exercise might increase astrocytes expressed-VEGF in the striatum.

4. Discussion

In this study, we supported the notion that either drug treatment of bis(propyl)-cognitin (DO) or treadmill exercise rehabilitation (EO) alone could effectively decrease neurological impairments and improve motor function after ischemic stroke. The results further demonstrated that early bis(propyl)-cognitin drug intervention has a best recovery result after 14-day intervention. By taking drug after 15 min and then with exercise, rats had better motor recovery than by taking drug after 60 min with exercise, drug only, exercise only and control groups.

The middle cerebral artery is the most common place for the occurrence of ischemic stroke (Hsu et al., 2010). Taken MCAo/r, a well-established and widely accepted ischemic stroke model, as an example, occlusion of middle cerebral artery causes a loss of blood supply to the infarct core, leading to the ischemic cascade. The deprivation of oxygen or glucose in the infarct core results in the failure of energy-dependent process which is necessary for the survival of neurons (Ikonomidou and Turski, 2002). Therefore, the primary lesion of ischemic stroke is the loss of neurons in the infarct core. Subsequently, the loss of neurons in the infarct core cause the secondary damage, leading to the excessive release of glutamate, which could over-activate NMDA receptors and result in neuronal death in the penumbra (Im et al., 2012). Thus, NMDA receptor antagonists, which protect against glutamate-induced NMDA receptor over-activation, are proposed to be good drug candidates because they could protect against the secondary damage of ischemic stroke (Lipton, 2007). In this study, bis(propyl)-cognitin, an uncompetitive NMDA receptor antagonist, effectively protected against ischemic stoke-induced neuronal and motor impairments, suggesting that bis(propyl)-cognitin has the potential for the treatment of stroke.

Exercises was reported to be one of the most common physical interventions for stroke rehabilitation (DeBow et al., 2003). We previously demonstrated that treadmill exercise could facilitate motor recovery after ischemic stroke in animal models (Ke et al., 2011). Moreover, our results that the interaction between bis(propyl)-cognitin and treadmill exercise significantly increased the behavioral scores suggested that the combination of NMDA receptor antagonists and exercises may have greater efficacy compared with monotherapy in stroke rehabilitation, possibly by decreasing the infarct size, increasing collateral blood flow and inhibiting the secondary lesion simultaneously and synergistically, which resulted in a better behavioral outcomes. Nevertheless, the increased scores of behavioral tests along the experimental period in all stroke rats may also partially due to that rats could learn to compensate during the repeated daily behavioral tests.

Why bis(propyl)-cognitin was able to potentiate rehabilitation of

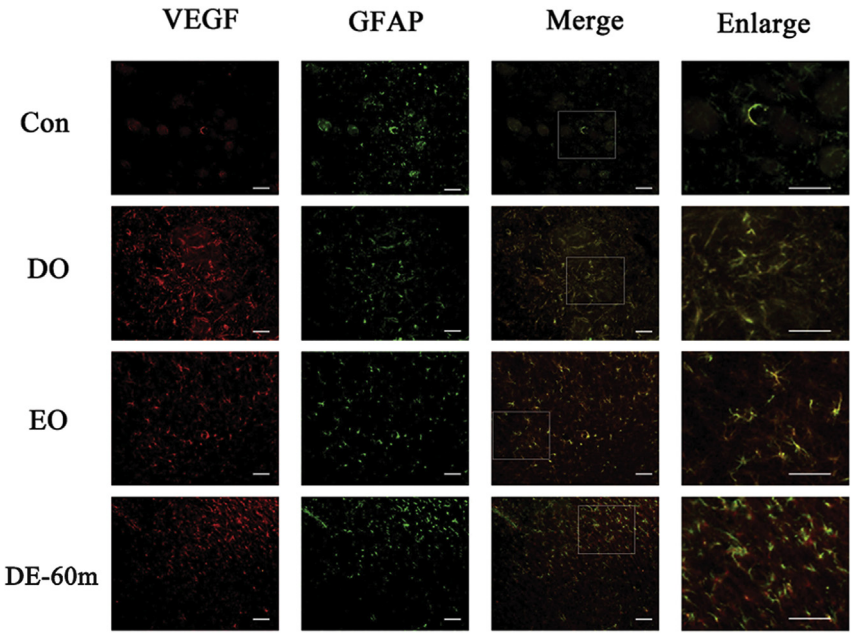

Fig. 8. Bis(propyl)-cognitin and treadmill exercise increase astrocytes expressed-VEGF in the striatum. Striatal VEGF expression of rats in the Con, DO, EO and DE-60 m groups was analyzed by co-staining of VEGF and GFAP at 14 day post-ischemia. Scare bar = 50 μM.

treadmill exercise after ischemic stroke? We speculated that bis(propyl)-cognitin protected against neuronal death via inhibiting the over-activation of the NMDA receptor. On the other hand, as demonstrated in previous studies and herein, treadmill exercise increased the collateral blood supply to the ischemic penumbra and promotes angiogenesis and other rehabilitated activities simultaneously. By restricting the brain infarct size, protecting against the secondary damage, promoting angiogenesis and providing other rehabilitated activities, bis(propyl)-cognitin and treadmill exercise may jointly enhance motor recovery after ischemic stroke.

VEGF, an inducer of angiogenesis, plays an important role in ischemic stroke (Jean LeBlanc et al., 2018; Slevin et al., 2000). Therefore, we investigated the effects of bis(propyl)-cognitin and treadmill exercise on brain VEGF expression after ischemic stroke. At the acute stage of ischemic stroke, VEGF expression is increased not only in the infarct area but also in the remote cortical regions (Stowe et al., 2008). Moreover, there is a correlation between infarction and VEGF level at the acute phase where higher VEGF level was related to a larger vessel infarction, suggesting that early VEGF expression may exacerbate brain damage (Plate et al., 1999). In accordance with these studies, cortical and striatal VEGF levels of the Non-drug group were significantly higher than those of the Sham group at 24 h post-ischemia. Moreover, cortical and striatal VEGF levels of the Drug group were significantly lower than those of the Non-drug group, suggesting that bis(propyl)-cognitin could reverse the increase of VEGF expression caused by ischemic stroke, and may consequently attenuate VEGF-mediated brain damage at the acute phase of the stroke.

However, at the chronic stage of ischemic stroke, VEGF was able to promote angiogenesis and functional recovery (Chen et al., 2005; Yang et al., 2010). Intranasal administration of VEGF starting from 3 days post MCAo/r induced angiogenesis in ischemic boundary and improved behavioral recovery in rats (Yang et al., 2010). Similarly, increased VEGF level caused by stem cell transplantation at 7 days post MCAo/r could suppress inflammation, increase neovascularization and promote functional recovery (Horie et al., 2011). In this study, VEGF levels in the intervention groups were significantly higher than that in the Con group at 14 day post-ischemia at the chronic stage of ischemic stroke, indicating that bis(propyl)-cognitin and treadmill exercises might enhance motor recovery via increasing VEGF expression. Results from immunofluorescence staining further revealed that bis(propyl)-cognitin and treadmill exercises increased astrocytes expressed-VEGF in the striatum at the chronic stage of ischemic stroke. The protein expression of VEGF in the control was lower compared to those in Sham and all the intervention groups, suggesting that VEGF expression might be responsible for functional recovery. However, we could not rule out any other possible contributing factors, including decreased brain infarct volume resulted from drug and/or exercise, exercise-induced neural plasticity, in affecting the functional recovery among the intervention groups.

Based on the fact that bis(propyl)-cognitin could decrease VEGF levels at the acute stage, while increase VEGF levels at the chronic stage of ischemic stroke, and that bis(propyl)-cognitin could activate VEGFR-2/VEGF system (Hu et al., 2013), we speculated that long-term treatment of bis(propyl)-cognitin might increase astrocytes expressed-VEGF at the chronic stage of ischemic stroke. Previous studies suggested that NMDA receptor over-activation was involved in hypoxia-induced VEGF expression, leading to the increase of VEGF expression at the early stage of stroke. Bis(propyl)-cognitin inhibits NMDA receptors and activates the VEGFR-2/VEGF system simultaneously. At the acute phase of ischemic stroke, NMDA receptors are over-activated by the excessive glutamate in the brain. Over-activated NMDA receptors could substantially increase the expression of VEGF. Although bis(propyl)-cognitin might active VEGFR-2/VEGF system at the same condition, it is exhibited that bis(propyl)-cognitin has inhibited NMDA receptor and decrease VEGF levels at the acute phase of ischemic stroke.

NMDA receptors play important roles not only in ischemia stroke-induced impairments, but also in various physiological activities, including post-stroke functional recovery. Therefore, NMDA receptor antagonists could reduce functional recovery. However, bis(propyl)-cognitin acts as an uncompetitive NMDA receptor antagonist with fast off-rate property. Uncompetitive antagonists are defined as chemicals whose inhibitory effects are contingent upon prior activation of the channel by agonist, and a fixed concentration of agonist rise to a greater extent than the response to low concentrations of agonist. Such kind of antagonists could preferentially block overactivated channels while sparing physiological functions. Therefore, bis(propyl)-cognitin could preferentially reduce ischemia stroke-induced peri-infarct region while not reducing functional recovery. Recently, memantine, an

uncompetitive antagonist of NMDA receptor, was reported to improve motor and sensory function recovery when administrated after photothrombotic stroke, which further indicated that such uncompetitive NMDA receptor antagonists might be suitable for the treatment of stroke (Yang et al., 2010). More encouragingly, bis(propyl)-cognitin might also activate VEGFR-2/VEGF system, to facilitate the functional recovery after stroke.

5. Conclusions

We herein demonstrated that bis(propyl)-cognitin effectively potentiated rehabilitation of treadmill exercise after focal cerebral ischemia. We further suggested that the enhanced rehabilitation is possibly through the inhibition of the NMDA receptor and the regulation of VEGF expression. Our results indicated that the combination of NMDA receptor antagonists and exercise might be useful for stroke rehabilitation. Future studies could consider to use quantitative analysis and objective measurement method to further evaluation the clinical efficacy.

Acknowledgment

This work was supported by the research grants from General Research Fund (14204315), PolyU (G-YBGQ, G-YZ95), the Research Grant Council of Hong Kong (15101014), ITSP-Guangdong–Hong Kong Technology Cooperation Funding Scheme (GHP/012/16GD), Shenzhen Basic Research Program (JCYJ20160331141459373) and the National Natural Science Foundation of China (No. 21576199). We sincerely thank Ms. Josephine Leung for proofreading our manuscript.

References

Burnett, M.G., Shimazu, T., Szabados, T., Muramatsu, H., Detre, J.A., Greenberg, J.H., 2006. Electrical forepaw stimulation during reversible forebrain ischemia decreases infarct volume. Stroke 37, 1327–1331.
Carlier, P.R., Han, Y.F., Chow, E.S., Li, C.P., Wang, H., Lieu, T.X., Wong, H.S., Pang, Y.P., 1999. Evaluation of short-tether bis-THA AChE inhibitors. A further test of the dual binding site hypothesis. Bioorg. Med. Chem. 7, 351–357.
Chamorro, A., Dirnagl, U., Urra, X., Planas, A.M., 2016. Neuroprotection in acute stroke: targeting excitotoxicity, oxidative and nitrosative stress, and inflammation. Lancet Neurol. 15, 869–881.
Chen, B., Wang, G., Li, W., Liu, W., Lin, R., Tao, J., Jiang, M., Chen, L., Wang, Y., 2017. Memantine attenuates cell apoptosis by suppressing the calpain-caspase-3 pathway in an experimental model of ischemic stroke. Exp. Cell Res. 351, 163–172.
Chen, J., Zhang, C., Jiang, H., Li, Y., Zhang, L., Robin, A., Katakowski, M., Lu, M., Chopp, M., 2005. Atorvastatin induction of VEGF and BDNF promotes brain plasticity after stroke in mice. J. Cereb. Blood Flow Metab. 25, 281–290.
De Ryck, M., Van Reempts, J., Borgers, M., Wauquier, A., Janssen, P.A., 1989. Photochemical stroke model: flunarizine prevents sensorimotor deficits after neocortical infarcts in rats. Stroke 20, 1383–1390.
DeBow, S.B., Davies, M.L., Clarke, H.L., Colbourne, F., 2003. Constraint-induced movement therapy and rehabilitation exercises lessen motor deficits and volume of brain injury after striatal hemorrhagic stroke in rats. Stroke 34, 1021–1026.
Diener, H.C., Foerch, C., Riess, H., Rother, J., Schroth, G., Weber, R., 2013. Treatment of acute ischaemic stroke with thrombolysis or thrombectomy in patients receiving antithrombotic treatment. Lancet Neurol. 12, 677–688.
Goldstein, L.B., Davis, J.N., 1990. Beam-walking in rats: studies towards developing an animal model of functional recovery after brain injury. J. Neurosci. Methods 31, 101–107.
Hasegawa, Y., Nakagawa, T., Matsui, K., Kim-Mitsuyama, S., 2017. Renal denervation in the acute phase of ischemic stroke provides brain protection in hypertensive rats. Stroke 48, 1104–1107.
Horie, N., Pereira, M.P., Niizuma, K., Sun, G., Keren-Gill, H., Encarnacion, A., Shamloo, M., Hamilton, S.A., Jiang, K., Huhn, S., Palmer, T.D., Bliss, T.M., Steinberg, G.K., 2011. Transplanted stem cell-secreted vascular endothelial growth factor effects poststroke recovery, inflammation, and vascular repair. Stem Cell. 29, 274–285.
Hsu, J.H., Wu, J.R., Chen, H.M., Huang, W.T., 2010. Stroke, infective endocarditis and a blood filled cyst. Lancet 376, 1338.
Hu, S., Cui, W., Mak, S., Xu, D., Hu, Y., Tang, J., Choi, C., Lee, M., Pang, Y., Han, Y., 2015. Substantial neuroprotective and neurite outgrowth-promoting activities by bis(propyl)-cognitin via the activation of alpha7-nAChR, a promising anti-alzheimer's dimer. ACS Chem. Neurosci. 6, 1536–1545.
Hu, S., Hu, H., Mak, S., Cui, G., Lee, M., Shan, L., Wang, Y., Lin, H., Zhang, Z., Han, Y., 2018a. A novel tetramethylpyrazine derivative prophylactically protects against glutamate-induced excitotoxicity in primary neurons through the blockage of N-Methyl-D-aspartate receptor. Front. Pharmacol. 9, 73.
Hu, S., Mak, S., Zuo, X., Li, H., Wang, Y., Han, Y., 2018b. Neuroprotection against MPP(+)-Induced cytotoxicity through the activation of PI3-K/Akt/GSK3beta/MEF2D signaling pathway by rhynchophylline, the major tetracyclic oxindole alkaloid isolated from uncaria rhynchophylla. Front. Pharmacol. 9, 768.
Hu, S.Q., Cui, W., Xu, D.P., Mak, S.H., Tang, J., Choi, C.L., Pang, Y.P., Han, Y.F., 2013. Substantial neuroprotection against K+ deprivation-induced apoptosis in primary cerebellar granule neurons by novel dimer bis(propyl)-cognitin via the activation of VEGFR-2 signaling pathway. CNS Neurosci. Ther. 19, 764–772.
Hüske, C., Sander, S.E., Hamann, M., Kershaw, O., Richter, F., Richter, A., 2016. Towards optimized anesthesia protocols for stereotactic surgery in rats: analgesic, stress and general health effects of injectable anesthetics. A comparison of a recommended complete reversal anesthesia with traditional chloral hydrate monoanesthesia. Brain Res. 1642, 364–375.
Ikonomidou, C., Turski, L., 2002. Why did NMDA receptor antagonists fail clinical trials for stroke and traumatic brain injury? Lancet Neurol. 1, 383–386.
Im, D.S., Jeon, J.W., Lee, J.S., Won, S.J., Cho, S.I., Lee, Y.B., Gwag, B.J., 2012. Role of the NMDA receptor and iron on free radical production and brain damage following transient middle cerebral artery occlusion. Brain Res. 1455, 114–123.
Jean LeBlanc, N., Guruswamy, R., ElAli, A., 2018. Vascular endothelial growth factor isoform-B stimulates neurovascular repair after ischemic stroke by promoting the function of pericytes via vascular endothelial growth factor receptor-1. Mol. Neurobiol. 55, 1104–1107.
Ke, Z., Yip, S.P., Li, L., Zheng, X.X., Tong, K.Y., 2011. The effects of voluntary, involuntary, and forced exercises on brain-derived neurotrophic factor and motor function recovery: a rat brain ischemia model. PLoS One 6, e16643.
Lafargue, M., Xu, L., Carles, M., Serve, E., Anjum, N., Iles, K.E., Xiong, X., Giffard, R., Pittet, J.F., 2012. Stroke-induced activation of the alpha7 nicotinic receptor increases Pseudomonas aeruginosa lung injury. FASEB J. 26, 2919–2929.
Lai, T.W., Zhang, S., Wang, Y.T., 2014. Excitotoxicity and stroke: identifying novel targets for neuroprotection. Prog. Neurobiol. 115, 157–188.
Lipton, S.A., 2007. Pathologically-activated therapeutics for neuroprotection: mechanism of NMDA receptor block by memantine and S-nitrosylation. Curr. Drug Targets 8, 621–632.
Longa, E.Z., Weinstein, P.R., Carlson, S., Cummins, R., 1989. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 20, 84–91.
Lu, H., Wang, Y., He, X., Yuan, F., Lin, X., Xie, B., Tang, G., Huang, J., Tang, Y., Jin, K., Chen, S., Yang, G.Y., 2012. Netrin-1 hyperexpression in mouse brain promotes angiogenesis and long-term neurological recovery after transient focal ischemia. Stroke 43, 838–843.
Luo, J., Li, W., Zhao, Y., Fu, H., Ma, D.L., Tang, J., Li, C., Peoples, R.W., Li, F., Wang, Q., Huang, P., Xia, J., Pang, Y., Han, Y., 2010. Pathologically activated neuroprotection via uncompetitive blockade of N-methyl-D-aspartate receptors with fast off-rate by novel multifunctional dimer bis(propyl)-cognitin. J. Biol. Chem. 285, 19947–19958.
Ma, Y., Qiang, L., He, M., 2013. Exercise therapy augments the ischemia-induced proangiogenic state and results in sustained improvement after stroke. Int. J. Mol. Sci. 14, 8570–8584.
Mehrholz, J., Pohl, M., Elsner, B., 2014. Treadmill training and body weight support for walking after stroke. Cochrane Database Syst Rev, pp. CD002840.
Munari, D., Pedrinolla, A., Smania, N., Picelli, A., Gandolfi, M., Saltuari, L., Schena, F., 2018. High-intensity treadmill training improves gait ability, VO2peak and cost of walking in stroke survivors: preliminary results of a pilot randomized controlled trial. Eur. J. Phys. Rehabil. Med. 54, 408–418.
O'Collins, V.E., Macleod, M.R., Donnan, G.A., Howells, D.W., 2012. Evaluation of combination therapy in animal models of cerebral ischemia. J. Cereb. Blood Flow Metab. 32, 585–597.
Plate, K.H., Beck, H., Danner, S., Allegrini, P.R., Wiessner, C., 1999. Cell type specific upregulation of vascular endothelial growth factor in an MCA-occlusion model of cerebral infarct. J. Neuropathol. Exp. Neurol. 58, 654–666.
Rousselet, E., Kriz, J., Seidah, N.G., 2012. Mouse model of intraluminal MCAO: cerebral infarct evaluation by cresyl violet staining. JoVE 69, e4038.
Sakakima, H., Khan, M., Dhammu, T.S., Shunmugavel, A., Yoshida, Y., Singh, I., Singh, A.K., 2012. Stimulation of functional recovery via the mechanisms of neurorepair by S-nitrosoglutathione and motor exercise in a rat model of transient cerebral ischemia and reperfusion. Restor. Neurol. Neurosci. 30, 383–396.
Shi, L., Rocha, M., Leak, R.K., Zhao, J., Bhatia, T.N., Mu, H., Wei, Z., Yu, F., Weiner, S.L., Ma, F., Jovin, T.G., Chen, J., 2018. A new era for stroke therapy: integrating neurovascular protection with optimal reperfusion. J. Cereb. Blood Flow Metab. 38, 2073–2091.
Skold, M.K., von Gertten, C., Sandberg-Nordqvist, A.C., Mathiesen, T., Holmin, S., 2005. VEGF and VEGF receptor expression after experimental brain contusion in rat. J. Neurotrauma 22, 353–367.
Slevin, M., Krupinski, J., Slowik, A., Kumar, P., Szczudlik, A., Gaffney, J., 2000. Serial measurement of vascular endothelial growth factor and transforming growth factor-beta1 in serum of patients with acute ischemic stroke. Stroke 31, 1863–1870.
Stoop, W., Geyter, D., Verachtert, S., Brouwers, S., Verdood, P., Keyser, J., Kooijman, R., 2017. Post-stroke treatment with 17beta-estradiol exerts neuroprotective effects in both normotensive and hypertensive rats. Neuroscience 348, 335–345.
Stowe, A.M., Plautz, E.J., Nguyen, P., Frost, S.B., Eisner-Janowicz, I., Barbay, S., Dancause, N., Sensarma, A., Taylor, M.D., Zoubina, E.V., Nudo, R.J., 2008. Neuronal HIF-1 alpha protein and VEGFR-2 immunoreactivity in functionally related motor areas following a focal M1 infarct. J. Cereb. Blood Flow Metab. 28, 612–620.
Valtysson, J., Hillered, L., Andine, P., Hagberg, H., Persson, L., 1994. Neuropathological endpoints in experimental stroke pharmacotherapy: the importance of both early and late evaluation. Acta Neurochir. 129, 58–63.
Xie, Q., Cheng, J., Pan, G., Wu, S., Hu, Q., Jiang, H., Wang, Y., Xiong, J., Pang, Q., Chen, X., 2018. Treadmill exercise ameliorates focal cerebral ischemia/reperfusion-induced

neurological deficit by promoting dendritic modification and synaptic plasticity via upregulating caveolin-1/VEGF signaling pathways. Exp. Neurol. 313, 60–78.
Yang, J.P., Liu, H.J., Liu, X.F., 2010. VEGF promotes angiogenesis and functional recovery in stroke rats. J. Investig. Surg. 23, 149–155.
Yang, Y.R., Wang, R.Y., Wang, P.S., Yu, S.M., 2003. Treadmill training effects on neurological outcome after middle cerebral artery occlusion in rats. Can. J. Neurol. Sci. 30, 252–258.
Zhang, P., Yu, H., Zhou, N., Zhang, J., Wu, Y., Zhang, Y., Bai, Y., Jia, J., Zhang, Q., Tian, S., Wu, J., Hu, Y., 2013. Early exercise improves cerebral blood flow through increased angiogenesis in experimental stroke rat model. J. NeuroEng. Rehabil. 10, 43.
Zhao, Y., Dou, J., Luo, J., Li, W., Chan, H.H., Cui, W., Zhang, H., Han, R., Carlier, P.R., Zhang, X., Han, Y., 2011. Neuroprotection against excitotoxic and ischemic insults by bis(12)-hupyridone, a novel anti-acetylcholinesterase dimer, possibly via acting on multiple targets. Brain Res. 1421, 100–109.