WIN55,212-2 protects oligodendrocyte precursor cells in stroke penumbra following permanent focal cerebral ischemia in rats

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Aim: To explore whether the synthetic cannabinoid receptor agonist WIN55,212-2 could protect oligodendrocyte precursor cells (OPCs) in stroke penumbra, thereby providing neuroprotection following permanent focal cerebral ischemia in rats.

Methods: Adult male SD rats were subjected to permanent middle cerebral artery occlusion (p-MCAO). The animals were administered WIN55,212-2 at 2 h, and sacrificed at 24 h after the ischemic insult. The expression of cannabinoid receptor type 1 (CB1) in the stroke penumbra was examined using Western blot assay. The pathological changes and proliferation of neural glial antigen 2-positive OPCs (NG2+ cells) in the stroke penumbra were studied using immunohistochemistry staining.

Results: p-MCAO significantly increased the expression of CB1 within the stroke penumbra with the highest level appearing at 2 h following the ischemic insult. Administration of WIN55,212-2 (9 mg/kg, iv) significantly attenuated the brain swelling, and reduced the infarct volume as well as the number of tau-immunoreactive NG2+ cells (tau-1+/NG2+ cells) in the stroke penumbra. Moreover, WIN55,212-2 significantly promoted the proliferation of NG2+ cells in the stroke penumbra and in the ipsilateral subventricular zone at 24 h following the ischemic insult. Administration of the selective CB1 antagonist rimonabant (1 mg/kg, iv) partially blocked the effects caused by WIN55,212-2.

Conclusion: Tau-1 is expressed in NG2+ cells following permanent focal cerebral ischemic injury. Treatment with WIN55,212-2 reduces the number of tau-1+/NG2+ cells and promotes NG2+ cell proliferation in the stroke penumbra, which are mediated partially via CB1 and may contribute to its neuroprotective effects.

Keywords: stroke; permanent focal cerebral ischemia; penumbra; oligodendrocyte precursor cells; neural glial antigen 2 (NG2); tau-1; cannabinoid receptor type 1 (CB1); WIN55,212-2; rimonabant

Introduction

Considerable evidence demonstrates that the preservation of only gray matter neurons following stroke may be insufficient for recovery of the neurological function, suggesting the importance of protecting glial cells in the white matter as well[1]. Following occlusion of the middle cerebral artery for 30 min, rats exhibit a swelling of oligodendrocytes[2], and in neonatal hypoxic-ischemic rats, oligodendrocyte precursor cells (OPCs) die rapidly[3]. Remyelination is performed by OPCs, and the promotion of OPC survival may contribute to the development of reparative strategies for demyelinating diseases[4]. Therefore, preclinical studies of drugs with therapeutic potential in acute stroke should include an assessment of oligodendrocyte and OPC survival.

The quantification of oligodendrocytes that are immunoreactive for the microtubule-associated protein tau can be used to assess potential therapeutic interventions for pathologies in which oligodendrocytes have been exposed to ischemia[5, 6]. As OPCs contain tau[7], a remaining question is whether tau-immunoreactivity is increased in OPCs following cerebral ischemia. Notably, OPC membranes contain the chondroitin sulfate proteoglycan neural glial antigen 2 (NG2)[8], and NG2-
positive cells are present in the rat brain during development and in adulthood\[9\]. To determine whether OPCs express tau-1 following permanent middle cerebral artery occlusion (p-MCAO), we performed immunohistochemistry to co-label tau-1 and the OPC marker NG2 following 24 h p-MCAO injury.

Cannabinoid receptor type 1 (CB1) immunoreactivity is up-regulated following transient MCAO injury\[10\], and numerous studies have suggested that endocannabinoids, acting through CB1, may be promising neuroprotective agents in several degenerative brain conditions. Whether a p-MCAO injury has effects on the expression of CB1 remains controversial. In this study, we identified the expression of CB1 in penumbral areas following p-MCAO injury and explored the hypothesis that treatment with the synthetic cannabinoid agonist WIN55,212-2, at a time when CB1 is highly expressed, could reduce the number of tau-immunoreactive NG2-positive cells and promote their proliferation, thereby providing neuroprotection.

Materials and methods

Chemicals and reagents

We purchased the non-selective cannabinoid receptor agonist R(+)-WIN55,212-2 mesylate (C22H21Cl3N4O2·HCl, purity: 99%; Axon Medchem, Groningen, Netherlands) and the selective CB1 antagonist, rimonabant (1 mg/kg) at 1.5 h following p-MCAO, and WIN55,212-2 (9 mg/kg) combined with rimonabant (1 mg/kg). After model assessment\[13\], 30 male SD rats were used in the experiment (n=6). The rats in the first two groups were intravenously administered a vehicle solution at 2 h following p-MCAO. The rats in the WIN55,212-2 (1 mg/kg) and WIN55,212-2 (9 mg/kg) groups received an intravenous administration of WIN55,212-2 at 2 h following p-MCAO. The rats in the last group were intravenously administered rimonabant (1 mg/kg) at 1.5 h following p-MCAO, and WIN55,212-2 (9 mg/kg) was injected 30 min later. All of the animals were humanely sacrificed 24 h following p-MCAO, and the brain samples were isolated to determine cerebral infarct volume and swelling. Finally, to investigate the pathology and proliferation of NG2-positive cells, immunofluorescence staining was performed. A total of 30 male SD rats were randomly assigned to five groups. The grouping and methods of drug administration were identical to the second experiment. After model assessment\[13\], 20 male SD rats were used in the experiment (n=4). The S-phase marker 5-bromo-2′-deoxyuridine (BrdU) (Sigma) was dissolved in saline and administered to SD rats at a dose of 100 mg/kg ip at 10 and 20 h following p-MCAO to label dividing cells\[14\].

Animals

One hundred and twelve adult male Sprague-Dawley (SD) rats weighing 250-300 g were purchased from Zhejiang Laboratory Animals Center (Hangzhou, China) and kept under standard housing conditions at a temperature between 20 °C and 23 °C with a 12 h light-dark cycle at a relative humidity of 50%. All animal tests and experimental procedures were approved by the Administration Committee of Experimental Animals in Jiangsu Province and the Ethics Committee of China Pharmaceutical University.

Induction of permanent focal cerebral ischemia

Permanent focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) according to the method of Koizumi et al\[11\]. To block the origin of the middle cerebral artery, a monofilament nylon suture (approximate diameter: 0.26 mm) was prepared by rounding the tip by heating and coating with poly-l-lysine (Sigma). The nylon suture was coating with poly-l-lysine (Sigma). The nylon suture was advanced approximately 18–20 mm intracranially from the common carotid artery bifurcation. Body temperature was maintained at 37.0 °C with a temperature control system. As recommended by Longa et al\[12\], the neurological evaluation was carried out as follows: score 0, no apparent neurological deficits; score 1, contralateral forelimb flexion; score 2, decreased resistance to lateral push; score 3, spontaneous movement in all directions and contralateral circling when pulled by the tail; score 4, no spontaneous locomotion and depressed levels of consciousness.

Animal treatment

First, we observed the time course of CB1 protein expression following p-MCAO in male SD rats. A total of 42 SD rats were randomly assigned to 7 experimental groups (n=6 per group). After assessing the model\[13\], 28 rats were used for the experiment (n=4 per group). Protein expression was evaluated at 1, 2, 3, 4, 5, and 6 h following p-MCAO insult, by using Western blot. Next, we examined whether post-treatment with the synthetic cannabinoid agonist WIN55,212-2 has neuroprotective roles, such as decreasing the infarct volume, brain swelling and neurological deficits, while CB1 is highly expressed. A total of 40 male SD rats were randomly assigned to five groups receiving one of the following treatments: sham; vehicle; WIN55,212-2 (1 mg/kg); WIN55,212-2 (9 mg/kg); WIN55,212-2 (9 mg/kg) combined with rimonabant (1 mg/kg). After model assessment\[13\], 30 male SD rats were used in the experiment (n=6). The rats in the first two groups were intravenously administered a vehicle solution at 2 h following p-MCAO. The rats in the WIN55,212-2 (1 mg/kg) and WIN55,212-2 (9 mg/kg) groups received an intravenous administration of WIN55,212-2 at 2 h following p-MCAO. The rats in the last group were intravenously administered rimonabant (1 mg/kg) at 1.5 h following p-MCAO, and WIN55,212-2 (9 mg/kg) was injected 30 min later. All of the animals were humanely sacrificed 24 h following p-MCAO, and the brain samples were isolated to determine cerebral infarct volume and swelling. Finally, to investigate the pathology and proliferation of NG2-positive cells, immunofluorescence staining was performed. A total of 30 male SD rats were randomly assigned to five groups. The grouping and methods of drug administration were identical to the second experiment. After model assessment\[13\], 20 male SD rats were used in the experiment (n=4). The S-phase marker 5-bromo-2′-deoxyuridine (BrdU) (Sigma) was dissolved in saline and administered to SD rats at a dose of 100 mg/kg ip at 10 and 20 h following p-MCAO to label dividing cells\[14\].

Assessment of cerebral infarct volume and brain swelling

To analyze the degree of cerebral infarction, 2,3,5-triphenyltetrazolium chloride (TTC) staining was assessed using methods previously published by Leker et al\[15\]. Infarct volumes are expressed as a percentage of the contralateral hemisphere as measured by image analysis software\[15\]. Brain swelling was calculated according to the following formula: (infarct volume+undamaged ipsilateral–contralateral volume)×100/ contralateral volume (%)\[16\].

Western blots

The protocol used to isolate brain samples from the penumbra...
and normal contralateral areas was published previously by Kramer et al[17]. Thirty-microgram protein samples were size-fractionated by 10% SDS-PAGE and immunoblotted with a polyclonal rabbit anti-CB1 antibody (1:500, GeneTex) and an HRP-conjugated secondary antibody (1:1000, Chemicon). β-Actin, detected by a mouse monoclonal anti-β-actin antibody (1:1000, Sigma), served as a loading control.

Immunohistochemistry staining
The method used for immunofluorescence staining has been published previously by Wojtowicz et al[14]. The following primary antibodies were used: polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) (1:200, Chemicon), polyclonal rabbit anti-NG2 (1:300, Chemicon), monoclonal mouse anti-tau-1 (1:600, Chemicon), monoclonal mouse anti-Ki67 (1:300, Novocastra Laboratories), and monoclonal mouse anti-BrdU (1:300, Chemicon). Following incubation with the primary antibody, sections were incubated for 90 min with Cy2- and Cy3-conjugated goat anti-rabbit and anti-mouse secondary antibodies (1:300, Invitrogen) in blocking solution at room temperature. Finally, to detect nuclei, sections were incubated with 4',6-diamidino-2-phenylindole (DAPI) in potassium phosphate buffered saline for 10 min at room temperature. The GFAP+ region was used to delineate the penumbra surrounding the core of the infarct (Supplementary Figure 1)[18]. The number of cells in the penumbra and subventricular zone (SVZ) that were positive for various markers was quantified using digitalized confocal images, captured from a minimum of five serial coronal sections spaced 250 μm apart, corresponding to the Bregma coordinates -0.8 mm through 2.5 mm[19]. The data are presented as the total number of positive cells within the specific region analyzed.

Statistical analysis
Except for the neurological deficit scores, all values are expressed as the mean±SD, and significant differences between the groups were determined by a two-way analysis of variance (ANOVA). The neurological deficit scores were expressed as the medians, and significant differences between the groups were determined by a non-parametric Mann-Whitney test. P-values<0.05 were considered an indication of statistical significance.

Results
Determination of CB1 expression in p-MCAO rats
Following 2 h of p-MCAO, the level of CB1 protein expressed within the penumbra, standardized to β-actin protein levels, was significantly increased compared to 1 h following p-MCAO (Figure 1B, 1C, n=4, P<0.01). No significant changes in CB1 protein expression in the contralateral control area were observed in any group (Figure 1A, 1C, n=4, P>0.05).

WIN55,212-2 protects against p-MCAO injury partially through CB1
We next explored whether treatment with WIN55,212-2 2 h following p-MCAO, during which time CB1 was highly expressed, has neuroprotective roles, decreasing the infarct volume, brain swelling and neurological deficits in the p-MCAO model. There are not only two doses including 3 mg/kg, 1 mg/kg used previously in the pre-treatment of cerebral ischemic reperfusion[20], but the dose of 9 mg/kg also have been designed in order to study the dose-dependent effect of p-MCAO model in the preliminary experiments. The infarct volume (% of the contralateral hemisphere) in the vehicle animals was 32.1%±1.8%. The WIN55,212-2 (9 mg/kg) and WIN55,212-2 (3 mg/kg) groups both had significantly smaller infarct volumes than the vehicle group (Supplementary Figure 2A, n=4, P<0.05). However, there was no significant difference in the infarct volume between the WIN55,212-2 (1 mg/kg) and vehicle groups (Supplementary Figure 2A, n=4, P>0.05). Consequently, we chose 9 and 1 mg/kg of WIN55,212-2 as the drug treatment doses in the formal experiment.

In the formal experiment, the ischemic infarctions were white following 24 h p-MCAO, and TTC staining revealed regular inclusion of the neocortex and basal ganglia. The results showed that the infarct volume (% of the contralateral hemisphere) in vehicle animals was 31.2%±3.1% and that the volume in the WIN55,212-2 (9 mg/kg) group was significantly lower (reduced by 61.6% compared with the vehicle animals; 12.5%±2.3% infarct volume) (Figure 2A, n=6, P<0.01). The percentage of brain swelling was significantly different between rats in the vehicle group (11.3%±1.2%) and the WIN55,212-2
mg/kg) combined with rimonabant (2 mg/kg). The administration of rimonabant alone led to infarct volumes of 29.1%±5.6% and 30.2%±3.1% (% of the contralateral hemisphere) for the doses of 1 and 2 mg/kg, respectively, which did not have any influence on infarct volumes compared with the vehicle group (Supplementary Figure 2A, n=4, P>0.05). Compared with the WIN55,212-2 (9 mg/kg) group, the neuroprotective effects were partially antagonized by the combined treatment with rimonabant (1 or 2 mg/kg) (Supplementary Figure 2A, n=4, P<0.01). There was no significant difference in infarct volume between the rimonabant 1 mg/kg and 2 mg/kg alone/combined treatment (Supplementary Figure 2A, n=4, P>0.05). Therefore, we chose 1 mg/kg rimonabant for the combined treatment with WIN55,212-2 (9 mg/kg) in the formal experiment. We found in the formal experiment that compared to the WIN55,212-2 (9 mg/kg) group, all neuroprotective effects were partially antagonized by the combined treatment with rimonabant (1 mg/kg) (Figure 2, n=6, P<0.01).

NG2-positive cells are immunoreactive for tau-1 following p-MCAO, and WIN55,212-2 reduces the number of NG2+/tau-1+ cells by acting partially through CB1

Twenty four hours following p-MCAO insult, NG2, and tau-1 co-expressing cells were detectable in the penumbral area. Compared to the sham group, we found that the numbers of tau-1+/DAPI+ and tau-1+/NG2+ cells (% of DAPI+ cells) in the penumbra were significantly higher in the vehicle group 24 h following p-MCAO (Figure 3Af, 3Ag, 3Ba, 3Bb, n=4, P<0.01). Compared to the vehicle group, WIN55,212-2 (9 mg/kg) treatment was able to decrease the numbers of tau-1+/DAPI+, tau-1+/NG2+, and tau-1+/NG2- cells (% of DAPI+ cells) (Figure 3Ag, 3Ai, 3Ba, 3Bb, n=4, P<0.01); however, the low dosage of WIN55,212-2 (1 mg/kg) did not have these effects (Figure 3Ag, 3Ah, 3Ba, 3Bb, n=4, P>0.05). There was no significant difference in the number of NG2+/tau-1+ (% of DAPI+ cells) between the rimonabant (1 mg/kg) and vehicle groups (Supplementary Figure 2Ba, 2Bd, 2C, n=4, P>0.05). Following the combination treatment with rimonabant, the numbers of both NG2+/tau-1+ and NG2+/tau-1− cells (% of DAPI+ cells) were significantly higher than in the group treated with WIN55,212-2 (9 mg/kg) alone (Figure 3Ai, 3Aj, 3Bb, n=4, P<0.01). In the contralateral control area, there was no significant alteration in the number of tau-1+/DAPI+ cells (% of DAPI+ cells) compared to the sham group (Figure 3Aa–3Ae, 3Ba, 3Bb, n=4, P>0.05).

WIN55,212-2 selectively promotes the proliferation of NG2-positive cells in the penumbra partially through CB1

One concern when using BrdU immunohistochemistry is that cells undergoing DNA repair are detected in addition to cells undergoing cell division[21]. In this study, we first used another proliferation marker, Ki67, which labels cells in all of the phases of the cell cycle except for G0[14]. Compared to the vehicle group, the numbers of Ki67+/DAPI+ and Ki67+/NG2+ cells (% of DAPI+ cells) were significantly higher than in the group treated with WIN55,212-2 (9 mg/kg) alone (Figure 4Ag, 4Ai, 4Ba, 4Bb, n=4, P<0.01). In the contralateral control area, there was no significant alteration in the number of Ki67+/DAPI+ cells (% of DAPI+ cells) compared to the sham group (Figure 4Aa–4Ac, 3Ba, 3Bb, n=4, P>0.05).
were observed in the WIN55,212-2 (1 mg/kg) group (Figure 4Ag, 4Ah, 4Ba, 4Bb, n=4, P>0.05). There was no significant change in the numbers of Ki67+/NG2+ cells between the rimonabant (1 mg/kg) and vehicle groups (Supplementary Figure 2Bb, 2Bc, 2Bd, 2Be, 2Bf, 2C, n=4, P>0.05). Following rimonabant treatment, the number of NG2+/Ki67+ cells (% of DAPI+ cells) was significantly lower than that of the group treated with WIN55,212-2 (9 mg/kg) alone, whereas the number of NG2+/Ki67+ cells (% of DAPI+ cells) was significantly higher (Figure 4Ai, 4Aj; 4Bb, n=4, P<0.01, P<0.05). In the contralateral control area, the number of DAPI+/Ki67+ cells (% of DAPI+ cells) was not significantly different between the sham and WIN55,212-2 (9 mg/kg) groups (Figure 4Aa, 4Ad, 4Ba, n=4, P>0.05). To identify the
source of the new NG2-positive cells generated following WIN55,212-2 treatment, we analyzed the population of rapidly proliferating cells by BrdU labeling. Only a few NG2⁺ cells (0.52±0.1%) were BrdU⁺ in the vehicle group 24 h following p-MCAO (Figure 5Ab, 5B). Interestingly, compared to the vehicle group, the numbers of BrdU⁺/NG2⁺ cells (% of DAPI⁺ cells) was significantly higher (Figure 5Ab–5Ad, n=4, P<0.01). However, following rimonabant treatment, the number of BrdU⁺/NG2⁺ cells (% of DAPI⁺ cells) was significantly lower than in the group treated with WIN55,212-2 (9 mg/kg) alone (Figure 5Ad, 5Ae, 5B, n=4, P<0.01).

WIN55,212-2 increases the proliferation of ipsilateral SVZ NG2-positive progenitor cells partially through CB1

In addition to the penumbral areas, the SVZ is a source of post-
natal glial precursors that can migrate to nearby areas affected by infarction and then differentiate into oligodendrocytes [22]. Quantitative data analysis showed that, in the ipsilateral SVZ, the number of Ki67+/DAPI+ cells (% of DAPI+ cells) was significantly higher in the WIN55,212-2 (9 mg/kg) group than in the vehicle and sham group (Supplementary Figure 3Aa, 3Ab, 3Ad, 3Ba, n=4, P<0.01). Compared to the vehicle group, the number of NG2+/Ki67+ cells (% of DAPI+ cells) was significantly higher in the WIN55,212-2 (9 mg/kg) and WIN55,212-2 (1 mg/kg) groups (Supplementary Figure 3Ab1, 3Ac1, 3Ad1, 3Bb, n=4, P<0.01). Following rimonabant co-treatment, the numbers of Ki67+/DAPI+ and NG2+/Ki67+ cells (% of DAPI+ cells) were significantly lower than the group treated with WIN55,212-2 (9 mg/kg) alone (Supplementary Figure 3Ad1, 3Ae1, 3Ba, 3Bb, n=4, P<0.01). However, following rimonabant co-treatment, the number of BrdU+/NG2+ cells (% of DAPI+ cells) was significantly lower than that in the group treated with WIN55,212-2 (9 mg/kg) alone (Supplementary Figure 4Ad, 4Ae, 4B, n=4, P<0.01).

Discussion
In this study, we used a permanent focal cerebral ischemia model in adult rats to investigate the neuroprotective effects of WIN55,212-2 on NG2-positive cells, and our results highlighted the following: (1) tau-1 is expressed in NG2-positive cells in the penumbra following 24 h p-MCAO insult; (2) post-treatment with WIN55,212-2 (9 mg/kg) when CB1 is highly expressed significantly decreases the cerebral swelling, cerebral infarction volume and the number of tau-immunoreactive NG2-positive cells within the stroke penumbra; and (3) WIN55,212-2 selectively increases the proliferation of NG2-positive cells following 24 h p-MCAO insult. In addition, by using the selective CB1 antagonist rimonabant, we identified a partial role of CB1 in mediating the effects mentioned above.

Previous studies have reported that the activation of CB1 is a neuroprotective strategy in models of cerebral ischemia [10]. However, whether a p-MCAO injury affects the expression of CB1 remains controversial. In this study, we identified high levels of CB1 expression in the penumbral area following 2 h of p-MCAO. Of note, the infarct volume reached its maximum at 2 h after p-MCAO insult [23]. Based on this information, we speculated that the exogenous cannabinoid agonist WIN55,212-2 should be administered prior to 2 h post-p-MCAO, while the ischemic injury is still progressing and the level of CB1 protein is increasing. Following preliminary dose-response experiments, we chose 9 and 1 mg/kg of WIN55,212-2 as the formal experimental doses. We found that treatment with WIN55,212-2 (9 mg/kg) 2 h following insult significantly decreased the cerebral swelling and cerebral infarction observed in p-MCAO rats. We also used the selective CB1 antagonist rimonabant to verify the above results. Rimonabant is an extensively studied CB1 inverse agonist that antagonized the effects of WIN 55,212-2 in a dose-dependent manner. McMahon reported that intravenous administration of 0.32 and 1.0 mg/kg SR 141716A (rimonabant) in rhesus monkeys increased the ED50 of subsequent WIN55,212-2 doses to 2.9- and 4.3-fold, respectively [24]. Based on these data, we
that oxidative stress promotes tau dephosphorylation at increased in the penumbra. Previous studies have reported reasons why the number of tau-1 positive cells significantly decreased the number of tau-1/NG2 cells. This could indicate an initial cellular response against oxidative insults. However, the high levels of tau dephosphorylated at the tau-1 epitope were associated with greater vulnerability to apoptosis induced by hydrogen peroxide, with mechanisms involving a failed dephosphorylation/activation of Bcl-2.

Therefore, the phosphorylation and dephosphorylation levels of tau proteins could not be used to accurately identify the survival of neurons. WIN55,212-2 treatment decreased the number of tau-1/NG2 cells, which might have some relationship with the survival of neurons. However, further study is required to determine the effects of WIN55,212-2 on neurons.

To study whether WIN55,212-2 could promote NG2-positive proliferation, we used both Ki67 and BrdU staining in the penumbra and ipsilateral SVZ. We found that WIN55,212-2 treatment (9 mg/kg) significantly increased the numbers of Ki67+/NG2- and BrdU+/NG2- cells in the penumbra. Interestingly, the vehicle-treated rat brains showed spontaneous proliferation; however, the majority of these BrdU cells did not express NG2. Following rimonabant co-treatment, the number of NG2+/Ki67- and NG2+/BrdU- cells were significantly decreased, while the percentage of NG2/Ki67+ cells was significantly increased. These results indicate that WIN55,212-2 may selectively increase the proliferation of NG2-positive cells partially via CB1. One previous study suggested that the levels of CB1 mRNA and protein in OPCs appear to be increased relative to other types of glial cells, and this is one likely mechanism to explain the selective effects observed here. In this study, WIN55,212-2 was administered 2 h after p-MCAO, when low levels of CB1 protein were observed in the contralateral cerebral hemisphere, and the number of NG2+/Ki67+ cells in the WIN55,212-2 (9 mg/kg) group was significantly increased. It is therefore conceivable that the capacity of WIN55,212-2 to promote proliferation in NG2-positive cells is related to the level of CB1 expression.

In addition to the classic cannabinoid receptors, novel receptors capable of binding cannabinoids, such as the transient receptor potential vanilloid 1 (TRPV1), have recently been identified. In particular, TRPV1 is expressed in the sensory neurons of the dorsal root ganglion and has been demonstrated to play a critical role in the induction of thermal hyperalgesia in inflammatory pain models. WIN55,212-2 can evoke anti-hyperalgesia by promoting dephosphorylation of TRPV1 at Thr144 and Thr750 in sensory neurons. Although there were no reports of WIN55,212-2 directly protecting OPCs or increasing oligodendrogligenesis through TRPV1, we cannot ignore the possible involvement of TRPV1 in the mediation of the indirectly neuroprotective effects of cannabinoids in stroke. Muzzi et al reported that rimonvan, a TRPV1 agonist, could induce mild hypothermia in promising candidates for...
hypothermic treatment of stroke\[38\]. To prevent the potential hypothermic effects of WIN55,212-2 in this study, we used a heat lamp to maintain consistent temperatures for each rat. It is of great interest and value to investigate whether the hypothermic effects of WIN55,212-2 are mediated through TRPV1 receptors, which might be further studied by our subsequent research.

In conclusion, we determined that tau-1 is expressed in NG2-positive cells following p-MCAO injury and that WIN55,212-2 protects the NG2-positive cells in the penumbra by reducing the co-expression of tau-1 and promoting proliferation. By using the selective CB1 antagonist rimonabant, we demonstrated that the neuroprotective mechanism of WIN55,212-2 on NG2-positive cells is, in part, mediated through CB1.

Acknowledgements
This research was supported in part by the National Natural Science Foundation of China (No 81070967) and the Natural Science Foundation of Jiangsu Province (No BK2009296). We wish to thank Su-juan YUAN in Yancheng City No 1 People’s Hospital for technological assistance.

Author contribution
Jing SUN, Hong LIAO, Lu-yong ZHANG, and Shu SONG designed the research plan; Jing SUN, Yin-quan FANG, Tao CHEN, Hong REN, Jing-jing GUO, and Jun YAN performed the research; Yin-quan FANG and Tao CHEN analyzed the data; and Jing SUN and Hong LIAO wrote the paper.

Supplementary information
Supplementary figure is available at the Acta Pharmacologica Sinica website.

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