

Tanshinone IIA Promotes Axonal Regeneration in Rats with Focal Cerebral Ischemia Through the Inhibition of Nogo-A/NgR1/RhoA/ROCKII/MLC Signaling

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Purpose: The aim of this study was to evaluate the neuroprotective effect of tanshinone IIA (TSA) on focal cerebral ischemia in rats and to investigate whether it was associated with Nogo-A/NgR1/RhoA/Rho-associated protein kinase 2 (ROCKII)/myosin light chain (MLC) signaling.

Methods: In this study, focal cerebral ischemia animal model was used. Neurological deficit scores and infarction volume were investigated to evaluate the neuroprotection of TSA. Hematoxylin-eosin staining, Nissl staining, and immunofluorescence staining were conducted to detect ischemic changes in brain tissue and changes in neurofilament protein 200 (NF200) and growth-associated protein-43 (GAP-43) expression, respectively. Western blotting and qRT-PCR analyses were used to detect the expression levels of NF200, GAP-43 and Nogo-A/NgR1/RhoA/ROCKII/MLC pathway-related signaling molecules.

Results: TSA treatment can improve the survival rate of rats, reduce the neurological score and infarct volume, and reduce neuron damage. In addition, TSA also increased axon length and enhanced expression of NF200 and GAP-43. Importantly, TSA significantly attenuated the expression of Nogo-A, NgR1, RhoA, ROCKII, and p-MLC, and thus inhibiting the activation of this signaling pathway.

Conclusion: TSA promoted axonal regeneration by inhibiting the Nogo-A/NgR1/RhoA/ROCKII/MLC signaling pathway, thereby exerting neuroprotective effects in cerebral ischemia rats, which provided support for the clinical application of TSA in stroke treatment.

Keywords: tanshinone IIA, cerebral ischemia, axonal regeneration, neuroprotective effect, neurite outgrowth inhibitor-A, Nogo receptor, Rho-associated protein kinase

Introduction

Stroke is the leading cause of death and permanent disability in adults worldwide, especially in low-and middle-income countries, with important clinical and socio-economic implications.¹ Among them, ischemic stroke accounts for about 87% of stroke cases.² In the acute phase of the disease, neurons of the ischemic lesion die quickly, while other neuron groups in the ischemic penumbra are vulnerable to secondary injury.³ Unfortunately, despite advances in technology and pharmacology, there are still few drugs or treatments for stroke. To date, tissue plasminogen activator (t-PA) is the only FDA-approved treatment for stroke. However, due to narrow treatment window (<4.5h) and safety concerns, less than 5% of patients

benefit from the drug.⁴ Therefore, the development of safe and effective treatment methods with long treatment windows and effective drugs is imminent.

Danshen is a very important ingredient in traditional Chinese medicine and it is extracted from the dried root or rhizome of *salvia miltiorrhiza*. Its main ingredient is tanshinone IIA (TSA), which has traditionally been used to treat cardiovascular and cerebrovascular diseases, including ischemic stroke.^{5,6} It has been reported that TSA can penetrate the blood-brain barrier when brain content is limited to 30% of plasma concentration, possibly due to its role in reducing infarct volume and maintaining neuronal function.⁷ In addition, TSA has been shown to protect against focal cerebral ischemia/reperfusion (I/R) injury in animal models and has been used as a potential therapeutic agent in the treatment of heart, liver and cancer.⁸ However, there have been no reports on the neuroprotective effect after focal cerebral ischemia of TSA by promoting axonal regeneration.

Studies have shown that the central nervous system (CNS) in adults can repair itself after cerebral ischemia, but to a limited extent. The key factor is that it is difficult to regenerate axons due to the increased expression of axonal growth inhibitors after ischemic injury.⁹ Neurite outgrowth inhibitor-A (Nogo-A) is a well-known myelin-associated axon growth-inhibitory protein that has been shown to inhibit the migration and spread of nerve cells and play an important role in preventing axon regeneration and reconnection after stroke.^{10,11} It binds to the receptor Nogo receptor 1 (NgR1), triggering the downstream RhoA/ROCKII/MLC signaling pathway, inducing collapse of the growth cone and obstructing nerve repair and regeneration.¹² A previous study has shown anti-Nogo-A therapy improves neurological deficits and enhances neuronal plasticity, suggesting that Nogo-A-targeted therapies are expected to be regenerative after stroke.¹³ However, it is unclear whether the neuroprotective effect of TSA is related to the inhibition of Nogo-A signaling pathway. Herein, the purpose of this study was to evaluate the neuroprotective effects of TSA and to explore whether TSA promotes axonal regeneration and protects damaged nerves by inhibiting Nogo-A/NgR1/RhoA/ROCKII/MLC signal activation.

Methods and Materials

Animals and Groups

Ninety-six Sprague-Dawley (SD) rats, weighing 250–280 g, provided by Liaoning Changsheng biotechnology co., Ltd., were included in the study [SCXK (Liaoning) 2015-0001].

All rats were maintained in the house with a background of temperature $21 \pm 1^\circ\text{C}$ and humidity ($55 \pm 10\%$). The SD rats were randomly divided into four groups: Sham group (Sham, $n=24$), vehicle group (vehicle, $n=24$), Low TSA group (TSA-L, $n=24$) and High TSA group (TSA-H, $n=24$). The sham group received the same surgery and was injected with 10 mL/kg PBS including 1% DMSO; The MCAO model was established in the remaining three groups, the difference was that the vehicle group was injected intraperitoneally with 10 mL/kg PBS including 1% DMSO, while the TSA-L group was 10 mg/kg TSA, and the TSA-H group was 20 mg/kg TSA. TSA was administered to each rat by intraperitoneal (i. p.) injection 15 mins after surgery, and every 24 hrs for 7 consecutive days. The structural formula of TSA is shown in Figure 1. This study was approved by the ethics committee of Hebei University of Traditional Chinese Medicine. All procedures are in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The schedule of the experimental procedures is presented in Figure 2.

Focal Cerebral Ischemia Animal Model

Focal brain ischemia was induced by the permanent middle cerebral artery occlusion (pMCAO) method as previously described.¹⁴ Briefly, the rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (350 mg/kg). The rat's right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed, and then a 4-0 nylon suture was inserted into the ICA to occlude the middle cerebral artery (MCA).

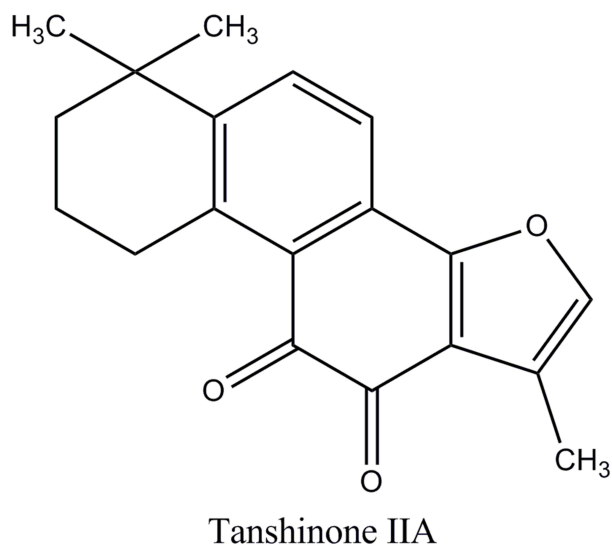


Figure 1 Chemical structures of tanshinone IIA (TSA).

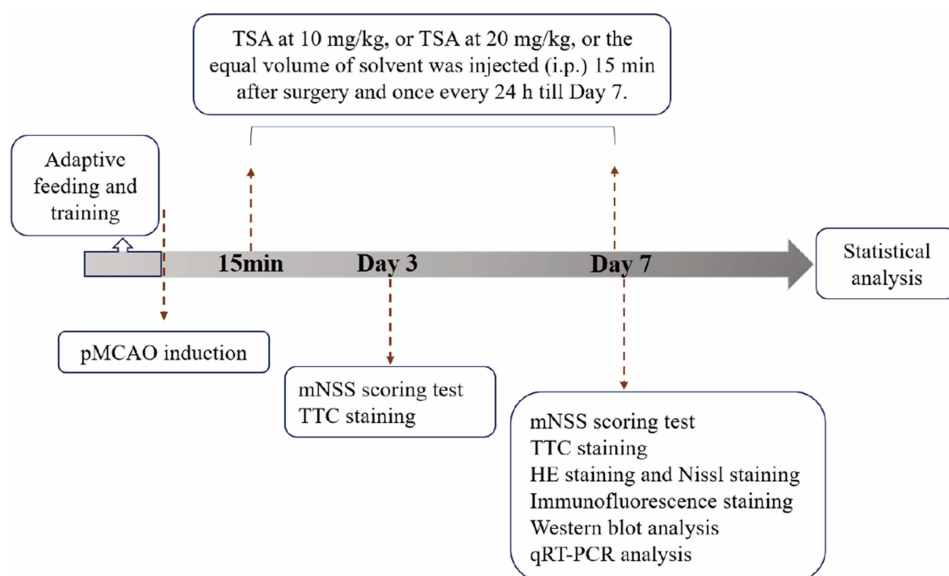


Figure 2 The schedule of the experimental procedures.

The temperature of the rats was adjusted by a thermostatic heating plate after surgery, and the rats were placed in the supine position until awakening and fed separately after resuscitation. The Zea-Longa scores test was used to evaluate whether the pMCAO was successful.¹⁵ More specifically, neurological deficits were scored with the following 5-point scale: 0, no neurological deficits; 1, failure to fully extend the left forelimb; 2, rotation to the left; 3, falling to the left during walking; and 4, no spontaneous walking. The rats of neurologic deficit score of 1–3 were considered to have a successful pMCAO and were included for subsequent evaluation.

Assessment of Neurological Deficits

Researchers who were blind to the experimental groups measured neurological scores of rats on the 3rd and 7th day after surgery to assess neurological deficits. The neurological impairment score system is based on the mNSS scoring system (0–18 points) developed by Chen et al.¹⁶ Score of 1 refers to an unfinished task or lack of response. Therefore, the higher the score, the more severe the injury.

Measurement of Cerebral Infarction Volume

To measure the infarct volume, rats were deeply anesthetized and sacrificed on the 3rd and 7th day after surgery, the brain was dissected and cut into 2 mm sections.

Subsequently, the sections were incubated in a 37°C 2% 2, 3, 5-triphenyltetrazolium chloride (TTC) solution for 20 mins and fixed with 4% paraformaldehyde. The staining images were recorded using a digital camera (Canon Oxus 950IS) and then quantified using an Image J (ver 1.51k, NIH).

Hematoxylin-Eosin Staining

On day 7 after modeling, the rats were decapitated immediately after deep anesthesia and heart perfusion with normal saline and 4% paraformaldehyde, and the brain was fixed in 4% paraformaldehyde for 24 hrs. Subsequently, the brain tissue was embedded in paraffin and cut into 5 μ m sections with a microtome. Dewaxing and rehydration were performed with a xylene and ethanol aqueous solution, followed by HE staining by a conventional method. Sections were stained with hematoxylin for 5 mins and eosin for 3 mins.

Nissl Staining

After 7 days of modeling, we conducted Nissl staining. Briefly, after dewaxing and rehydration, the brain sections were stained with 0.5% cresol violet at 37°C for 10 mins. Then, we use 0.25% glacial acetic acid ethanol to distinguish. The cell morphology of the cerebral cortex was observed under an optical microscope (Olympus BX53). Four high-power fields were randomly selected and Nissl positive cells were calculated using Image J, which was

Table 1 Primers Used for Real-Time PCR

Genes	Forward (5'-3')	Reverse (3'-5')
NF200	GGAGGCACTGAAAAGCACCA	GCCATCTCCCATTGGTGT
GAP-43	AGGGAGATGGCTCTGCTAC	CACATCGGCTTGTTAGGC
Nogo-A	AGTCTTGGGAAGGATAGTGA	GGTGCTTTCGGTTGC
NgR1	GAAAGAACCGCACCCGTAG	GGCCCAAGCACTGTCCAA
RhoA	TCGGAATGATGAGCACACAA	GCTTCACAAGATGAGGCAC
ROCK II	TCATAAGGCATCACAGAA	AACCACCCACGGACT
MLC	CTAAGGGACACGTTTGCT	GAAGGCGTTGAGAATGG
β -actin	GGAGATTACTGCCCTGGCTCCTAGC	GGCCGGACTCATCGTACTCCTGCTT

Abbreviations: NF200, neurofilament protein 200; GAP-43, growth associated protein-43; Nogo-A, neurite outgrowth inhibitor-A; NgR1, Nogo receptor 1; RhoA, Recombinant Ras Homolog Gene Family, Member A; ROCK II, Rho-associated protein kinase 2; MLC, myosin light chain; β -actin, β -non-muscle.

expressed as the number of complete neurons per 1 mm² length in the ischemic penumbra.

Immunofluorescence Staining

Immunofluorescence staining was performed 7 days after surgery. After the fixation with 4% paraformaldehyde, the brain sections were permeabilized with xylene, and blocked with goat serum for 20 mins. Then, the brain sections were immunolabeled with antibodies: NF200 (1:100; Proteintech) and GAP-43 (1:100; Proteintech). After incubation, the sections were washed with PBS followed by Cy3-conjugated secondary antibody. After the nuclei were counterstained with DAPI, the samples were observed under a fluorescence microscope (Olympus, Japan).

Western Blotting Analysis

On day 7 after surgery, the cortical tissue of the ischemic penumbra of the experimental rats was homogenized, and the protein concentration was measured using a BCA protein assay kit. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane. After blocking with TBST, 5% nonfat dry milk for 1 h, the membrane was incubated overnight at 4°C with antibodies against NF200 (1:1000; Proteintech), GAP-43 (1:5000; Proteintech), Nogo-A (1:500; Proteintech), NgR1 (1:1000; Bioss), RhoA (1:1000; Proteintech), ROCKII (1:500; Proteintech), MLC (1:5000; Bioss), p-MLC (1:5000; Proteintech) and β -actin (1:1000; Wanleibio) followed by incubation with a HRP-conjugated secondary antibody for 45 min at room temperature. The blot was observed with ECL reagent, and the optical density of the target band was measured using a gel image processing system (Gel-Pro-Analyzer software).

Real-Time PCR Analysis

On the 7th day after the surgery, total RNA was extracted from cortical tissue of ischemic penumbra of the experimental rats using TRIpure reagent (BioTeke, BEIJING) according to the standard instructions. After it was reverse transcribed to cDNA using a Sensiscript RT kit (ThermoFisher Scientific Inc., USA). Subsequently, RT-qPCR was performed. The thermocycling conditions were as follows: 94°C for 10 s, 60°C for 20 s; 40 cycles of 72°C for 30 s and extension at 72°C for 2.5 mins. The primer sequences are shown in Table 1.

Statistical Analysis

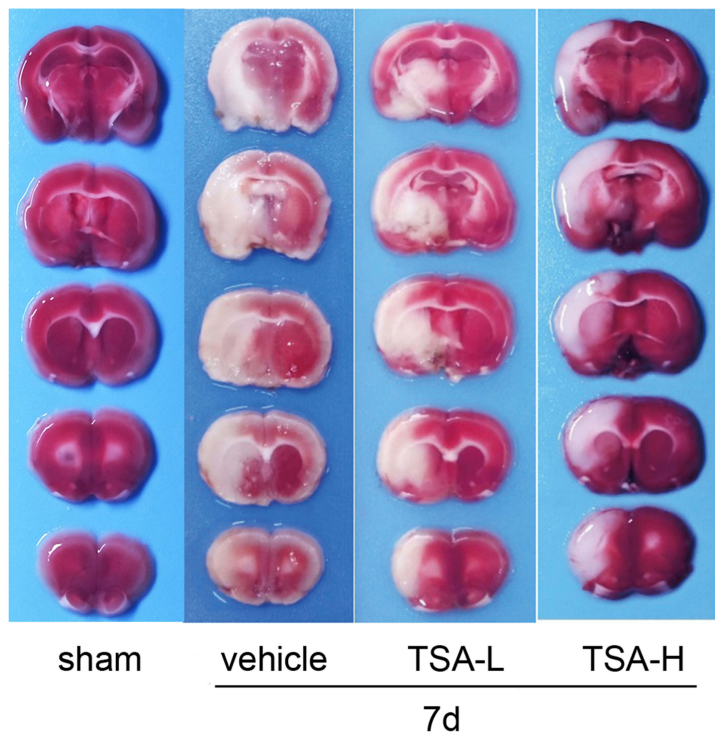
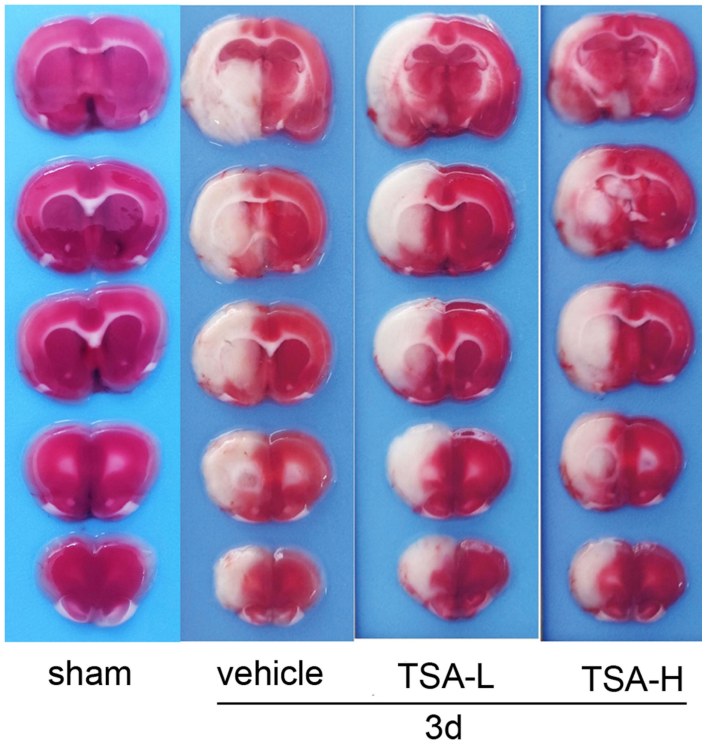
SPSS 20.0 (SPSS Inc, USA) was applied to analyze all data. The survival rates were analyzed by the Kaplan–Meier method, with comparisons performed using the Log-rank test. The neurological deficit scores were analyzed with a nonparametric Kruskal–Wallis test with pairwise comparisons and post hoc comparisons. Differences among multiple groups were statistically analysed using one-way ANOVA and post hoc comparisons (Bonferroni test). Values of $P < 0.05$ were considered statistically significant.

Results

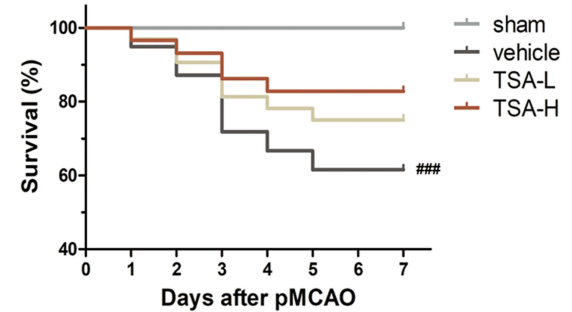
TSA Improves the Survival Rate of Rats After MCAO

As shown in Figure 3B, in the sham group, there was no death of rats during the test. Compared with sham group, the survival rate of rats in the vehicle group was 61.54%, lower than that in the sham group ($P < 0.001$). Further comparison found that there was no significant difference in survival between the vehicle group and the TSA-treated group (TSA-L group: 75.00%; TSA-H group: 82.76%),

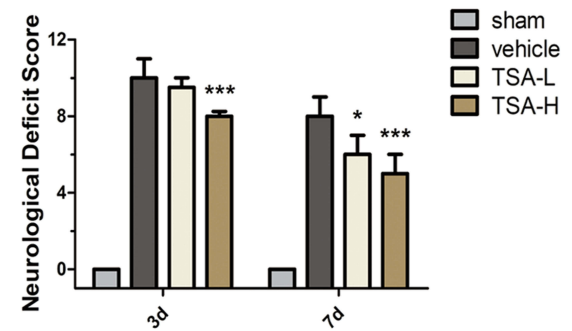
A



B



C



D

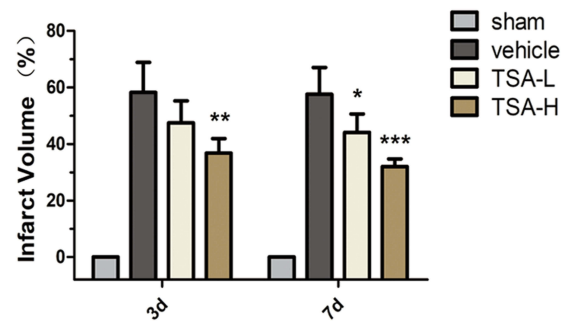


Figure 3 The effects of TSA on the survival rate, neurological deficit scores and infarct volume in pMCAO rats. **(A)** TTC staining of rats in different groups 3rd and 7th days. **(B)** The survival rate of rats in each group was expressed as the percentage. **(C)** Neurological deficit scores of rats in different groups 3rd and 7th days. **(D)** Infarct volume of rats in different groups 3rd and 7th days; ###P < 0.001 vs the sham group; *P < 0.05, **P < 0.01, ***P < 0.001 vs the vehicle group.

Abbreviation: pMCAO, permanent middle cerebral artery occlusion.

but the survival rate in the TSA-H group did increase by 21.22%.

TSA Reduces Neurological Deficit Score and Cerebral Infarction Volume

To test whether TSA has a neuroprotective effect, we measured the neurological score and cerebral infarct volume of rats after MCAO. In [Figure 3C](#), the sham group had a score of 0, showing no dysfunction, while the other groups all had neurological deficits of varying degrees. Compared with the vehicle group, the TSA-H group decreased the scores on both the 3rd and 7th days ($P<0.001$), while the TSA-L group reduced the scores only on the 7th day ($P<0.05$). 2,3,5-triphenyltetrazolium chloride (TTC) staining showed that each model had different degrees of ischemic changes. No infarction was observed in the sham group, but extensive lesions developed in the vehicle group ([Figure 3A](#)). More specifically, cerebral infarct volumes were both significantly reduced after treatment with 20 mg/kg TSA on the 3rd and 7th days, while only decreased on 7th day after treatment with 10 mg/kg TSA ([Figure 3D](#)).

TSA Alleviates Neuronal Injury After MCAO

The HE staining in [Figure 4A](#) shows the morphological changes of cerebral cortex. From the figure, we can clearly observe that the neurons in the sham group are neatly arranged without necrotic neurons. In the vehicle group, neurons were arranged disorderly and degenerate cells increased, the nucleus was deeply stained, and the cytoplasm shrank. Administration of TSA significantly improved neurons' morphology and reduced neurons' damage, especially group TSA-H. Further, we evaluated changes in the number of neurons with and without TSA. Nissl staining results indicated that compared with sham group, the neurons in the vehicle group showed lighter staining, looser cell arrangement and fewer neurons ($P<0.001$). When TSA was added, it significantly reduced the number of degenerated neurons and increased the number of intact neurons ([Figure 4B and C](#); $P<0.001$).

TSA Increases Axon Length and Promotes NF200 Expression

To detect the effect of TSA on axonal regeneration, we first observed changes in the expression of NF200 and the length of the axons labeled with NF200 by

immunofluorescence staining. In [Figure 5A and B](#), the axons in the sham group were intact, while the axon structure was broken and the axon length was reduced after MCAO ($P<0.001$). Compared with the vehicle group, the addition of TSA remarkably improved axon structure and increased axon length, especially group TSA-H ($P<0.001$). In addition, immunofluorescence staining showed a strong expression of NF200 in the sham group, while the fluorescence intensity in the carrier group decreased significantly in the vehicle group. TSA treatment enhanced NF200 immunofluorescence when compared with the vehicle group ([Figure 5C](#)).

To further verify the above results, we conducted Western blotting and qRT-PCR. The results showed that after MCAO, the protein expression of NF200 was significantly decreased when in comparison with the sham group ($P<0.001$). However, after treatment with 10 mg/kg or 20 mg/kg TSA, NF200 expression was significantly increased compared with the vehicle group ([Figure 5D](#); $P<0.05$ TSA-L group, $P<0.001$ in TSA-H group). Similarly, the transcription results of NF200 genes were consistent with the protein results, that is, MCAO decreased the mRNA expression of NF200, whereas TSA promoted the mRNA expression of NF200 ([Figure 5E](#)).

TSA Up-Regulates GAP-43 Expression

As presented in [Figure 6A](#), at 7th day after MCAO, GAP-43 immunofluorescence was evidently enhanced in comparison to the sham group ($P<0.001$). Interestingly, after TSA treatment, the protein expression of GAP-43 was up-regulated more significantly in comparison to vehicle group ([Figure 6B](#); $P<0.001$). Next, we performed Western blotting and qRT-PCR to detect the protein expression and mRNA expression of GAP-43, respectively. The Western blotting results showed that MCAO significantly increased GAP-43 protein expression of vehicle group, while the addition of TSA promoted the induction of MCAO to GAP-43 ([Figure 6C](#); $P<0.05$ in TSA-L group, $P<0.001$ in TSA-H group). qRT-PCR results also confirmed that TSA promoted the mRNA expression levels of GAP-43, which was consistent with previous protein results ([Figure 6D](#)).

TSA Inhibits Expression of Nogo-A/ NgR1/RhoA/ROCKII/MLC Signaling Pathways

To investigate the mechanism of axonal regeneration with TSA treatment, the expression of the vital target genes of the

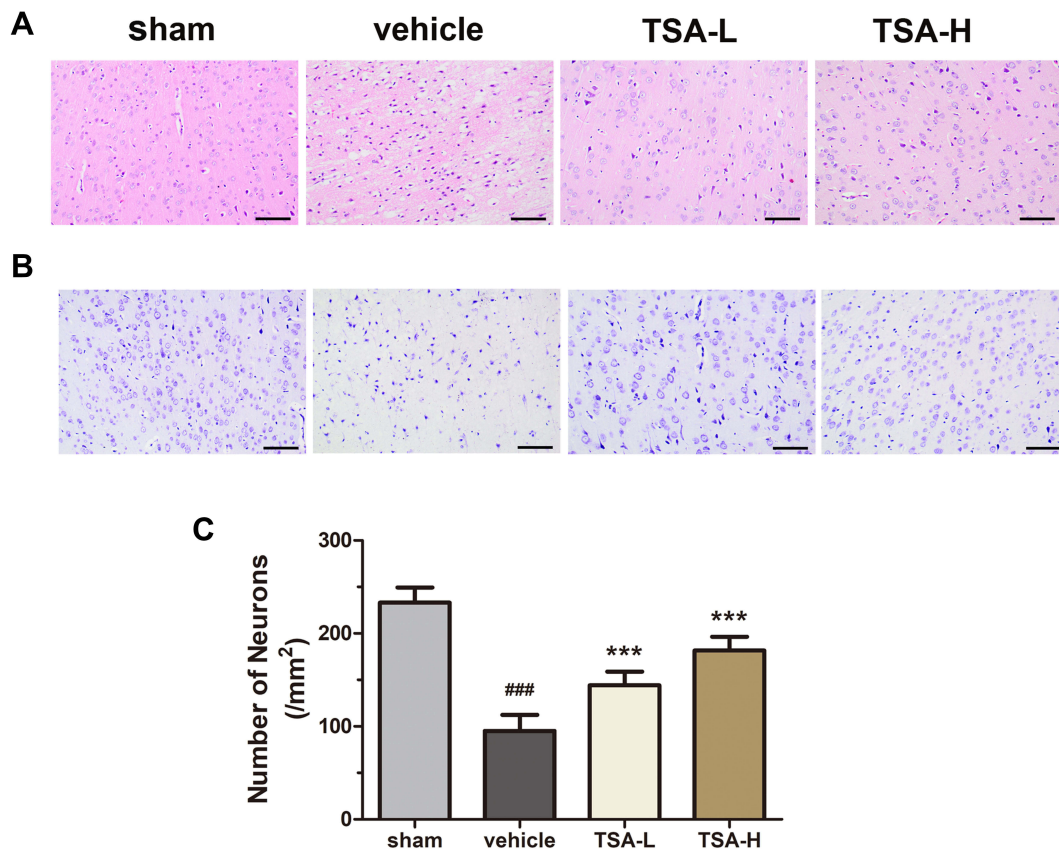


Figure 4 TSA alleviated neuronal damage. **(A)** The results of HE staining in different groups. **(B)** The results of Nissl staining in different groups. **(C)** The changes in the number of intact neurons in different groups. Scale bar= 100 μ m. ### P < 0.001 vs the sham group; *** P < 0.001 vs the vehicle group.

Nogo-A signaling pathway, Nogo-A, NgR1, RhoA, ROCKII and MLC, was investigated. As presented in Figure 7A, compared with sham group, the protein expression of Nogo-A, NgR1, RhoA, ROCKII and p-MLC were increased significantly after MCAO in vehicle group. However, after treatment with TSA, the expression of the above proteins was markedly down-regulated, especially in group TSA-H. Accordingly, the optical of Nogo-A, NgR1, RhoA, ROCKII and p-MLC increased significantly when compared with the sham group, respectively (P <0.001). However, they decreased significantly when TSA was added as compared with vehicle group (all P <0.001). Consistently, the qRT-PCR results also showed that MCAO induced the mRNA expression of Nogo-A, NgR1, RhoA, ROCKII and p-MLC, while TSA inhibited the induction of these proteins by MCAO (Figure 7B). In addition, there were no significant changes in MLC at the transcription and translation levels in each group.

Discussion

Ischemic stroke is one of the diseases with the highest mortality and disability rate in the world, and it often

leaves severe neurological deficit symptoms. Stroke treatment usually focuses on promoting nerve recovery to improve nerve function. Previous studies have shown that the mechanisms of nerve recovery are complex, including angiogenesis, neurogenesis and synaptic plasticity.¹⁷ Among these mechanisms, axon regeneration is an important link and basis for nerve recovery. Therefore, promoting axon regeneration is a key way to promote nerve recovery and improve nerve function recovery. One form of axon regeneration is the regeneration of axon buds in the injured area. However, due to severe self-injury, axons grow slowly, and the growth process is easily blocked by glial scars. Another form is the regeneration of axons around the injury. The regeneration of axon buds of neurons in the area around the infarction and even the contralateral area after cerebral infarction is enhanced.¹⁸ The axial protuberant buds in the area surrounding the infarct grow and extend to the injured area and establish connections to facilitate nerve recovery in the injured area. However, due to the presence of a large number of inhibitory factors on axonal

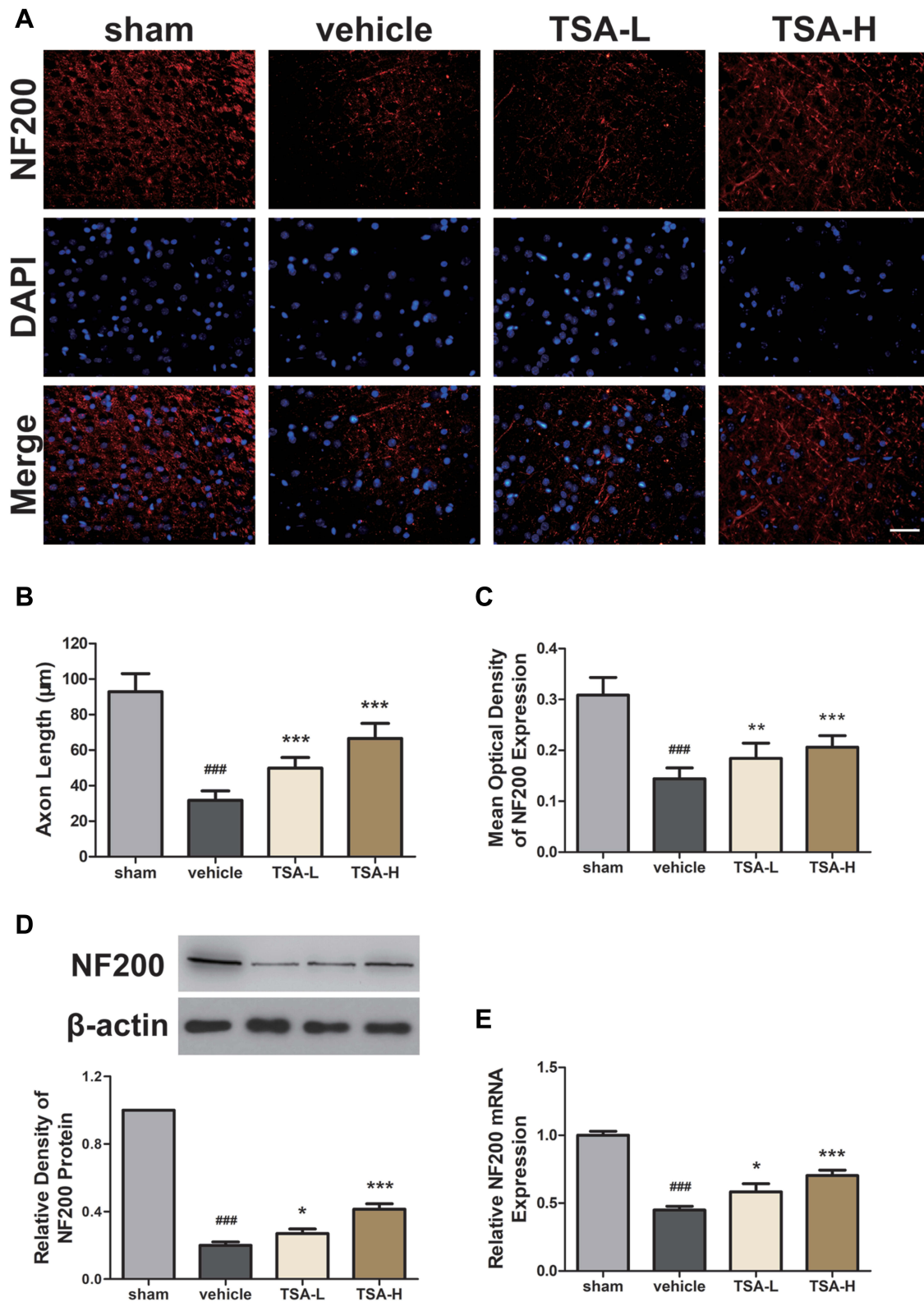


Figure 5 TSA increased axonal length and promoted NF200 expression. **(A)** Immunofluorescence staining for NF200, scale bar= 50µm. **(B)** The changes in the length of axons in different groups. **(C)** The mean optical density of NF200 in different groups. **(D)** The protein expression of NF200 in different groups. **(E)** The mRNA expression of NF200 in different groups; ####P < 0.001 vs the sham group; *P < 0.05, **P < 0.01, ***P < 0.001 vs the vehicle group. **Abbreviation:** NF200, neurofilament protein 200.

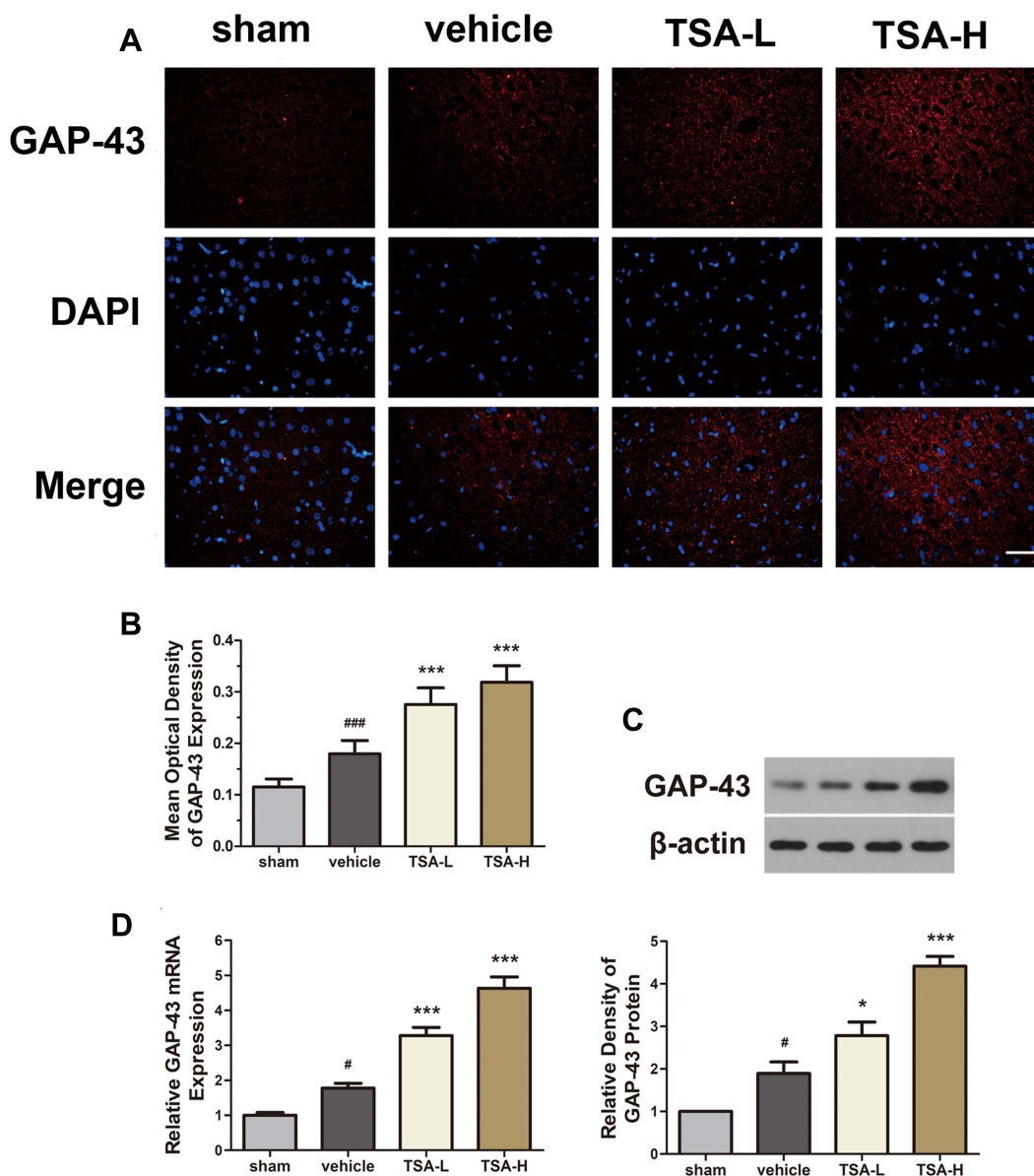


Figure 6 TSA up-regulated GAP-43 expression. **(A)** Immunofluorescence staining for GAP-43, scale bar= 50 μ m. **(B)** The mean optical density of GAP-43 in different groups. **(C)** The protein expression of GAP-43 in different groups. **(D)** The mRNA expression of GAP-43 in different groups; [#] $P < 0.05$, ^{###} $P < 0.001$ vs the sham group; ^{*} $P < 0.05$, ^{***} $P < 0.001$ vs the vehicle group.

Abbreviation: GAP-43, growth associated protein-43.

regeneration in the microenvironment, neural self-recovery is very limited, which cannot effectively promote the improvement of neurological dysfunction symptoms. Therefore, exogenous drug therapy is used to promote axonal regeneration, thereby promoting neurological recovery and improving neurological function recovery.

As a crude herbal medication isolated from the dried root or rhizome of *salvia miltiorrhiza bunge*, Danshen has been used in Asian countries for multiple therapeutic remedies including myocardial infarction, stroke and atherosclerosis.⁷ As one of the most pharmacologically

active components isolated from Danshen, TSA can easily cross the blood-brain barrier due to its lipophilic properties.¹⁹ Numerous experimental studies have shown that TSA can prevent damage from neurodegenerative diseases, including ischemic stroke.^{7,20} Consistent with previous studies, our study showed that TSA exerted neuroprotective effect, as indicated by the ameliorated neurological outcome, reduced neurological deficit score, cerebral infarct volume, and neuronal damage.

NF200 is a neuron-specific structural protein, which is mainly expressed on the axons of neurons and is important

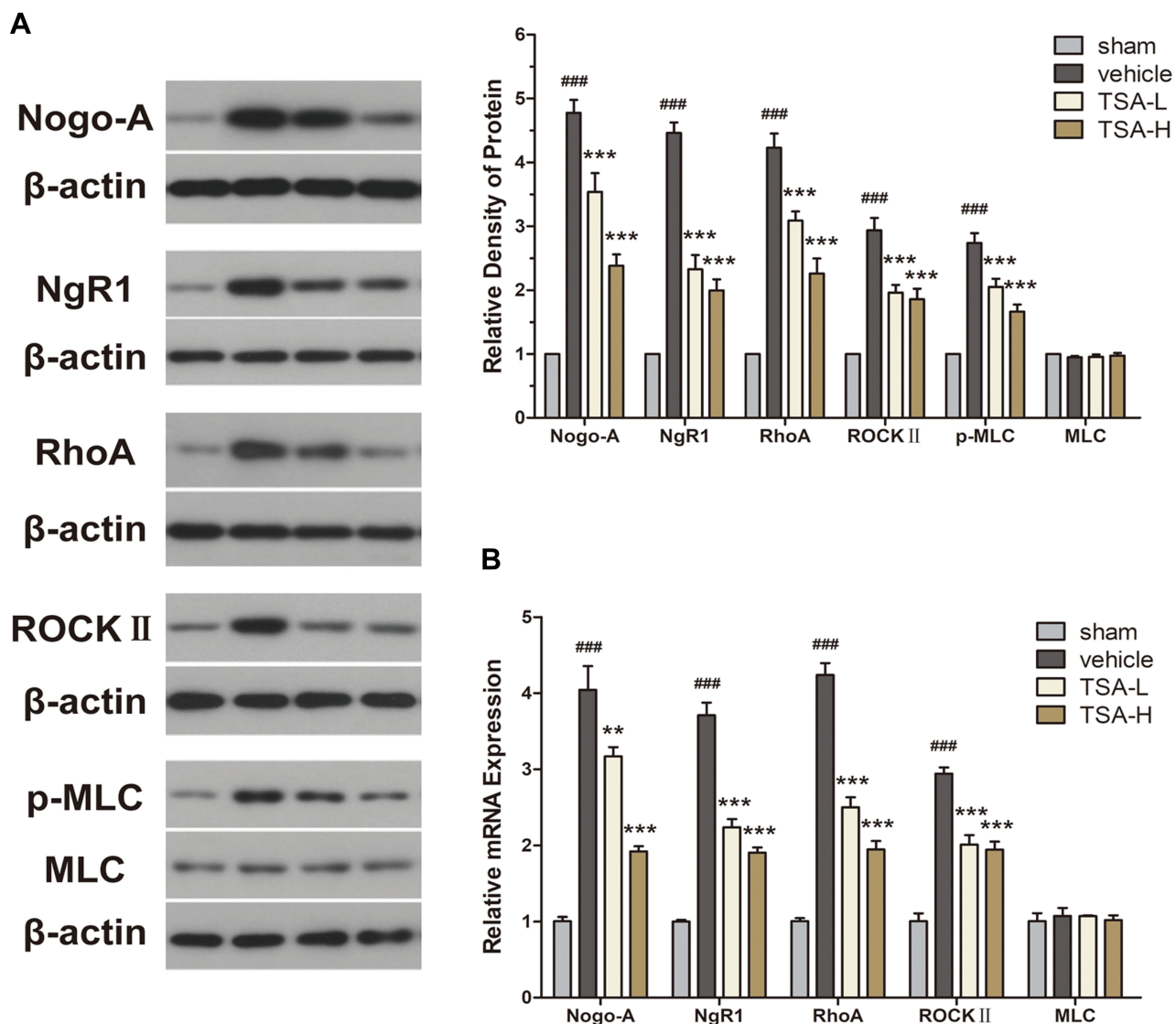


Figure 7 TSA inhibited the expression of the Nogo-A/NgR1/RhoA/ROCKII/MLC signaling pathway. **(A)** The protein levels of Nogo-A, NgR1, RhoA, ROCKII, MLC and p-MLC. **(B)** The mRNA levels of Nogo-A, NgR1, RhoA, ROCKII and MLC; ####P < 0.001 vs the sham group; **P < 0.01, ***P < 0.001 vs the vehicle group.

Abbreviations: Nogo-A, Neurite outgrowth inhibitor-A; NgR1, Nogo receptor I; RhoA, Recombinant Ras Homolog Gene Family, Member A; ROCK II, Rho-associated protein kinase 2; MLC, myosin light chain.

for the growth, maintenance and regeneration of axons. A previous study reported that NF200 reflects the morphological changes of neuron axons and the state of axon growth or repair.²¹ Therefore, NF-200 has been used as a marker for assessing axon regeneration in central neurons.²² In addition, GAP-43 is a membrane-associated phosphorylated protein, and the expression of GAP-43 is very low in the brain tissue of healthy adult mammals. However, after cerebral ischemic injury, GAP-43 becomes concentrated mainly at the growth cone terminus and the presynaptic terminal, and it is expressed at high levels during axonal growth and synapse formation.²³ Studies

have shown that axons exhibit regeneration and remodeling after cerebral ischemia, as reflected by the re-expression of GAP-43.^{24,25}

In the current study, our results indicated that TSA treatment significantly improved axon morphology in MCAO rats, increased the length of NF200-labeled axons, and up-regulated the expression of NF200 and GAP-43, suggesting that TSA promoted axon regeneration after ischemic stroke.

Membrane protein Nogo-A is a key factor that inhibits axon regeneration and nerve repair. In the adult central nervous system, Nogo-A acts as a negative regulator of

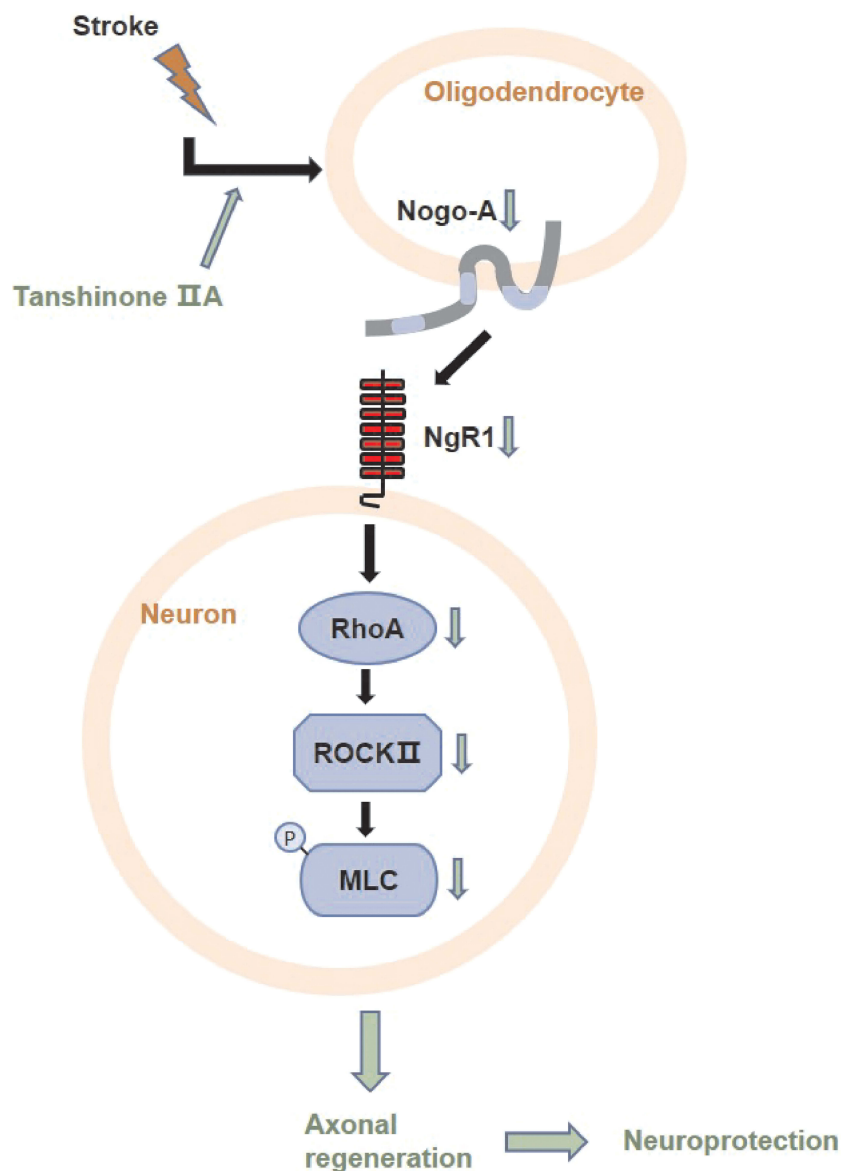


Figure 8 Schematic diagram of tanshinone IIA promoting axonal regeneration and neuroprotective effect on focal cerebral ischemia in rats by inhibiting the Nogo-A/NgR1/RhoA/ROCKII/MLC signaling pathway.

Abbreviations: Nogo-A, neurite outgrowth inhibitor-A; NgR1, Nogo receptor 1; RhoA, Recombinant Ras Homolog Gene Family, Member A; ROCKII, Rho-associated protein kinase 2; MLC, myosin light chain.

neuron growth, preventing abnormal fiber growth under physiological conditions to stabilize the central nervous system wiring. At the same time, axonal regeneration was inhibited under pathological conditions.²⁶ Studies have shown that ischemic stimulation can activate Nogo-A in central neurons, which binds to NgR1 and further activates the downstream protein RhoA to phosphorylate Rho kinase (ROCK). The activation of Rho protein leads to the formation of fibroblast actin stress fibers, which eventually leads to axon retraction and collapse of the growth cone.⁷ ROCKII is mainly distributed in the cortex

and hippocampus of the central nervous system. Phosphorylated ROCKII promotes the phosphorylation of the target protein myosin light chain (MLC), leading to rearrangement of the cytoskeleton, collapse of the growth cone, and ultimately inhibiting sprouting and growth of the axons.²⁷ Existing reports indicated that Rho/ROCK/MLC pathway-specific inhibitors such as Fausudil and Y27632 promote axonal regeneration and functional recovery after stroke by blocking this pathway.^{28,29} In the present study, we found that the expression of Nogo-A, NgR1, RhoA, ROCKII, and p-MLC increased in rats after the

establishment of a cerebral ischemia model, which is consistent with previous studies. After treatment TSA, the expressions of Nogo-A, NgR1, RhoA, ROCKII and p-MLC were significantly reduced, indicating that the neuroprotective effect of TSA and promotion of axon regeneration may be related to inhibiting Nogo-A/NgR1/RhoA/ROCKII/MLC pathway related (Figure 8).

There are some limitations in our manuscript. First, we did not conduct further studies on pathways, such as the use of pathway-specific inhibitors for interventions or gene knockout techniques. Second, the time window for TSA intervention is single, and no studies have been conducted on the delayed treatment of TSA (for example, starting administration at different time periods after modeling). In future studies, we will further explore the effects of TSA on Nogo-A/NgR1/RhoA/ROCKII/MLC signaling pathway of MCAO rats by using pathway-specific inhibitors or gene knockout techniques. At the same time, for the delayed treatment of TSA, we will explore the effective time window of TSA by administering drugs at different time periods after modeling, so as to make the experimental study more suitable for clinical application.

Conclusion

TSA plays an active role in promoting neurological recovery and axonal regeneration after ischemic stroke. The realization of this effect is related to the inhibition of Nogo-A/NgR1/RhoA/ROCKII/MLC signaling pathway, which may be one of the neuroprotective mechanisms induced by TSA.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Disclosure

The authors declare no conflicts of interest in this work.

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