Chinese herbal formula *Tongluo Jiunao* injection protects against cerebral ischemia by activating neurotrophin 3/tropomyosin-related kinase C pathway

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Abstract

The Chinese herbal formula *Tongluo Jiunao*, containing the active components *Panax notoginseng* and *Gardenia jasminoides*, has recently been patented and is in use clinically. It is known to be neuroprotective in cerebral ischemia, but the underlying pathway remains poorly understood. In the present study, we established a rat model of cerebral ischemia by occlusion of the middle cerebral artery, and administered *Tongluo Jiunao*, a positive control (*Xuesai Tong*, containing *Panax notoginseng*) or saline intraperitoneally to investigate the pathway involved in the action of *Tongluo Jiunao* injection. 2,3,5-Triphenyltetrazolium chloride (TTC) staining showed that the cerebral infarct area was significantly smaller in model rats that received *Tongluo Jiunao* than in those that received saline. Enzyme-linked immunosorbent assay revealed significantly greater expression of neurotrophin 3 and growth-associated protein 43 in ischemic cerebral tissue, and serum levels of neurotrophin 3, in the *Tongluo Jiunao* group than in the saline group. The reverse transcription polymerase chain reaction and immunohistochemical staining showed that after treatment with *Tongluo Jiunao* or *Xuesai Tong*, tropomyosin-related kinase C gene expression and immunoreactivity were significantly elevated compared with saline, with the greatest expression observed after *Tongluo Jiunao* treatment. These findings suggest that *Tongluo Jiunao* injection exerts a neuroprotective effect in rats with cerebral ischemia by activating the neurotrophin 3/tropomyosin-related kinase C pathway.

Key Words: neural regeneration; cerebral ischemia; Chinese herbal formula; Tneurotrophic factor; ongluo Jiunao injection; nerve growth factor receptor; Xuesai Tong; neuroprotection; NSFC grant; neuroprotection; neural regeneration

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Introduction

After central nervous system injury, activation of certain intracellular pathways can reduce infarct size and damage to the brain. The neurotrophin-tropomyosin-related kinase (Trk) signaling pathway is important in this process (Bachis et al., 2002; Barnabe-Heider and Miller, 2003). Neurotrophin 3 (NT3), one of the most recently discovered members of the neurotrophin family, acts on several intracellular pathways *via* the TrkC receptor (Kahn et al., 1999; Yamauchi et al., 2003). The NT3/TrkC pathway is fundamental to neuroregeneration as it stimulates the growth and activity of glial cells (Postigo et al., 2002; Hess et al., 2007). Furthermore, NT3 is not only a mediator for TrkC, but also upregulates the expression of TrkA and TrkB on neuronal membranes (Lewis et al., 2006).

In the present study, we used *Xuesai Ton*g (XST) as a positive control drug. XST promotes blood circulation, making it useful for the treatment of unstable angina pectoris (Liu

et al., 2008). In addition, an *in vivo* study showed that XST decreases thrombosis (Chen et al., 2007). This is achieved by the inhibition of platelet activation *via* a reduction in arachidonic acid release (Wang et al., 2004). A clinical trial revealed that continuous infusion of XST can improve nerve conductivity in patients with diabetic peripheral neuropathy (Han, 2003). In stroke, XST has several therapeutic effects; it promotes blood circulation during ischemic stroke and reduces tissue inflammation following ischemia (Ai et al., 2004; He and Xu, 2006). XST therapy improves learning and memory, and increases neurotrophin expression in a rat model of cerebral ischemia/reperfusion injury (He et al., 2004, 2005; Chuang et al., 2008; Son et al., 2009; Li et al., 2009).

The Chinese herbal formula *Tongluo Jiunao* (TLJN) has recently been patented (Li et al., 2007; Qing et al., 2007; Si et al., 2009; Wang et al., 2009). TLJN protects endothelial cells in cerebral blood vessels against ischemic attacks (Li et al.,

2007; Qing et al., 2007), suggesting that it may be useful in protecting the blood-brain barrier. TLJN injection promotes the proliferation and differentiation of neural stem cells in the rat cortex following devascularization, by upregulating endogenous nerve growth factors (Si et al., 2009). TLJN also exhibits protective effects following ischemic injury *in vivo* (Wang et al., 2009). The compound is a combination of two active components, *Panax notoginseng* and *Gardenia jasminoides*. In Chinese medicine, stroke is represented by a toxic heat in the head and brain collaterals; thus, any herb that can clear this toxic heat and promote blood flow is regarded as an effective therapy. *Panax notoginseng* and *Gardenia jasminoides* are known herbs with this function.

The purpose of the present study was to investigate the effects of TLJN injection on the NT3/TrkC pathway in a rat model of cerebral ischemia induced by middle cerebral artery occlusion (MCAO).

Materials and Methods

Animals

A total of 312 male, specific pathogen-free Sprague-Dawley rats, 4 months old and weighing 283 ± 36 g, were purchased from the Experimental Animal Center of Beijing Vital River, China (License No. SCXK (Jing) 2007-0001). The study was approved by the Experimental Animal Ethics Committee of Dongzhimen Hospital Affiliated to Beijing University of Chinese Medicine in China. The rats were randomly and equally divided into four groups: normal (naïve control), model (MCAO only), TLJN (MCAO + TLJN), and XST (MCAO + XST). Four time points were used for observation: 0.5 days ($n = 15$), 1 day ($n = 21$), 3 days ($n = 21$), and 7 days ($n = 21$) following the onset of ischemia.

Establishment of rat MCAO models

Focal cerebral ischemia was induced using the reversible filament occlusion model as previously described (Longa et al., 1989). In brief, animals were anesthetized with 10% chloral hydrate (0.4 g/kg intraperitoneally (i.p.)). The left external carotid artery was carefully dissected through a median incision in the neck. A piece of nylon suture, with a diameter of 0.25 mm and one end rounded by heat into a ball with a diameter of < 0.3 mm, was introduced into the transected lumen of the external carotid artery and gently advanced into the internal carotid artery to block the origin of the left middle cerebral artery. The retracted soft tissues were then replaced, the incision sutured, and the rats were returned to their home cages. Body temperature was maintained at 37°C with a heat lamp and heating pad during and after surgery, until the rats regained consciousness.

Neurological evaluation was performed before and 2 hours after surgery according to a previously described method (Bederson et al., 1986). Neurological scores prior to surgery were 0 for all rats. Following surgery, rats with a neurological score of ≥ 2 were selected for the study, and rats with a score of < 2 were considered to be unsuccessful MCAO candidates.

Drug treatment

Concentrated TLJN solution (approval No. 2004L01620)

was provided by Tianjin Hongri Pharmaceutical Co. (Tianjin, China) and contained 4.95 mg/mL geniposide, 1.02 mg/mL ginsenoside Rg1, and 1.73 mg/mL geniposidic acid. TLJN was administered daily at a dose of 3 mL/kg i.p., equivalent to the clinical dose (Si et al., 2009). Concentrated XST solution (approval No. Z53020662) was provided by Kunming Pharmaceutical Corp. (Kunming, China) and contained 25 mg/mL ginsenoside Rg1. XST was administered daily at a dose of 1.4 mL/kg (i.p.), equivalent to the clinical dose. In the model group, an equivalent volume of saline solution was injected instead of drugs. The first injection of each solution was given 2 hours after MCAO.

Infarct volume percentage

Rats were anesthetized and decapitated 1, 3, and 7 days after surgery ($n = 6$ in each group per time point). Brains were rapidly removed and cut into coronal sections (2-mm thick). Slices were stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO, USA) in 0.1 M PBS (pH 7.4) for 30 minutes at room temperature, and fixed in 10% buffered formalin overnight. The sections were photographed with an HPIAS-1000 image acquirement system (Beijing Kong Hai, China). Infarct volumes were quantified using the Cavalieri principle (Gundersen, 1986; Yang, 1990). Quantification was carried out directly by summing the infarct volumes of all sections, or indirectly, correcting for brain edema, by subtracting the volume of undamaged ipsilateral hemisphere from contralateral hemisphere. The infarct volume percentage was calculated by dividing the infarct volume by the total tissue volume and multiplying by 100%. All quantification was carried out by an investigator blind to experimental grouping.

Brain tissue and serum enzyme-linked immunosorbent assay (ELISA)

At 0.5, 1, 3, and 7 days after MCAO, rats ($n = 5$–6 per group per time point) were deeply anesthetized with 10% chloral hydrate, and blood samples were collected from the abdominal aorta. The brains were removed and immediately dissected on ice. The cerebral hemisphere ipsilateral to the MCAO was dissected out and stored at −80°C. Subsequently, brain samples were homogenized in PBS buffer, the homogenate was centrifuged for 45 minutes at $9,727 \times g$ at 4°C, and the supernatant was collected. Concentrations of brain lysate and serum NT3, as well as brain levels of growth-associated protein 43 (GAP-43), were quantified using a commercially available NT3 and GAP-43 ELISA kit (Ever Systems Biology Laboratory, Sacramento, CA, USA). Protein concentrations were determined using the bicinchoninic acid method (Ye et al., 2007).

RT-PCR

RT-PCR was used to measure TrkC gene expression in the cerebral tissue ipsilateral to the MCAO ($n = 3$ rats per group per time point). TrkC mRNA primer sequences were designed using Primer Premier 5.0 software (Premier, Vancouver, Canada) as follows: rat TrkC019248 251 bp: upstream, 5′-CAA GCC CAC CCA CTA CAA C-3′; downstream, 5′-AGA GGA CCA CCA GAA GGA C-3′; rat β-actin NM_031144.2 150 bp: upstream, 5′-CCC ATC TAT GAG GGT TAC GC-3′;

downstream, 5′-TTT AAT GTC ACG CAC GAT TTC-3′. Band density was determined using Gel-Pro 32-bit image analysis software (Media Cybernetics, Silver Spring, MD, USA), and TrkC densities were divided by corresponding β-actin values, resulting in relative expression levels of TrkC mRNA.

Immunohistochemical staining

Rats in each group were anesthetized and decapitated at 0.5, 1, 3, and 7 days after MCAO ($n = 5$ rats per time point). The brains were immersed in silicone gel and 8-μm thick coronal sections were cut using a freezing microtome (Leica Microsystems, Wetzlar, Germany) for immunohistochemistry. Sections were dehydrated through an increasing ethanol gradient and stored at −20°C until use. The sections were rinsed in 20 mM PBS and incubated in 3% H_2O_2/10% ethanol for 20 minutes at room temperature to block endogenous peroxidase. The sections were then boiled in 0.01 M citrate buffer for 10 minutes for antigen retrieval, and incubated overnight in rabbit anti-TrkC polyclonal antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by biotinylated goat anti-rabbit IgG (Zhongshan Golden Bridge Biotechnology, Beijing, China) at 1:1,000 dilution in 2% bovine serum albumin, for 1 hour. Finally, diaminobenzidine was used for coloration. PBS was used instead of the primary antibody as a negative control. TrkC-stained sections were digitized using a mounted camera on an Eclipse 50i microscope (Nikon, Tokyo, Japan) and an image analysis system (Nikon). To quantify TrkC expression, five high-magnification (× 100) visual fields were selected in the peri-infarct zone, and the area of positive expression was assessed compared with the total area of each field. Immunohistochemical analysis was performed blind to experimental grouping.

Statistical analysis

Data are expressed as the mean ± SD. One-way analysis of variance, followed by *post-hoc* analysis using the Student-Newman-Keuls multiple comparison test, was performed using SPSS 11.0 software (SPSS, Chicago, IL, USA). $P < 0.05$ was regarded as statistically significant.

Results

XST and TLJN reduced ischemic infarct size in rats with MCAO

TTC staining showed that the infarct volumes in the TLJN and XST groups were smaller than in the model group (**Figure 1**). One day after MCAO, total infarct volume in the TLJN group was smaller than that in the model group ($P < 0.05$). On days 3 ($P < 0.01$) and 7 ($P < 0.05$), infarct volume was significantly smaller in both treatment groups than in the model group, with no significant difference between the XST and TLJN groups.

TLJN treatment increased GAP-43 in the ipsilateral cerebral hemisphere in rats with MCAO

A markedly lower level of GAP-43 was observed at all time points in the model group than in the normal (naïve control) group ($P < 0.01$). One day after MCAO, the level of GAP-43 in the TLJN group was significantly higher than that

in the model and XST groups ($P < 0.05$). At 0.5 ($P < 0.05$), 3 ($P < 0.01$) and 7 ($P < 0.01$) days after surgery, GAP-43 levels in the XST group were significantly greater than those in the model group (**Figure 2**).

TLJN treatment increased NT3 in the serum and ipsilateral cerebral hemisphere of rats with MCAO

ELISA revealed that serum NT3 peaked 1 day after surgery in MCAO model rats, but no significant differences were observed between the model and normal groups throughout the experiment (**Figure 3A**). In the brain, NT3 level was significantly lower in the model group than in the normal group 7 days after surgery ($P < 0.05$) (**Figure 3B**). Rats that had received TLJN showed significantly higher serum NT3 levels than rats in the model group 7 days after MCAO ($P < 0.05$) (**Figure 3A**). Rats that received XST had significantly lower brain NT3 levels than those in the model group at 0.5 days ($P < 0.01$) and 1 day ($P < 0.05$). At 3 days after MCAO, brain NT3 level in the TLJN group was significantly greater than that in the model and XST groups ($P < 0.01$) (**Figure 3B**).

TLJN treatment increased TrkC gene expression in the ipsilateral cerebral hemisphere of rats with MCAO

RT-PCR showed that at 0.5 ($P < 0.01$), 1 ($P < 0.05$), and 7 ($P < 0.01$) days after MCAO, TrkC gene expression was significantly lower in the model group than in the normal group (**Figure 4**). In the XST group, TrkC gene expression peaked 0.5 days after MCAO and was significantly greater than that in the normal and model groups ($P < 0.01$) (**Figure 4**). At 1 day after MCAO, TrkC gene expression was significantly greater in the TLJN group than in the model and XST groups ($P < 0.01$ and $P < 0.05$ respectively).

TLJN treatment did not alter TrkC immunoreactivity in the ipsilateral cerebral hemisphere of rats with MCAO

Compared with the normal group, rats with MCAO showed greater TrkC immunoreactivity at 0.5 days and 1 day after surgery ($P < 0.05$). Brain TrkC immunoreactivity was significantly higher in the XST group than in the model group from 1 to 7 days after MCAO ($P < 0.01$). TrkC immunoreactivity was not significantly different between the TLJN and model groups (**Figure 5**).

Discussion

Nerve growth factor (NGF)/TrkA, brain-derived neurotrophic factor (BDNF)/TrkB, and NT-3/TrkC pathways are known to be important in healing and regeneration after central nervous system injuries. In the present study, we evaluated the effects of TLJN injection on the NT3/TrkC pathway. Our results suggest that TLJN promotes recovery after ischemic injury by increasing NT3 levels, reducing the necrotic area, and upregulating brain GAP-43.

Hypoxia can increase NT3 and TrkC gene expression in central nervous system pericytes, which increases the tolerance of neurons to hypoxia (Arimura et al., 2012). There is evidence that injection of exogenous NT3 into the brain after MCAO in rats can alleviate brain injury by decreasing TrkC expression (Wyatt et al., 1999; Zhang et al., 1999). In the
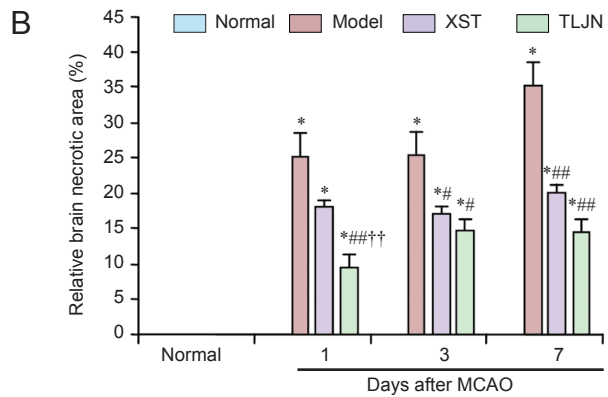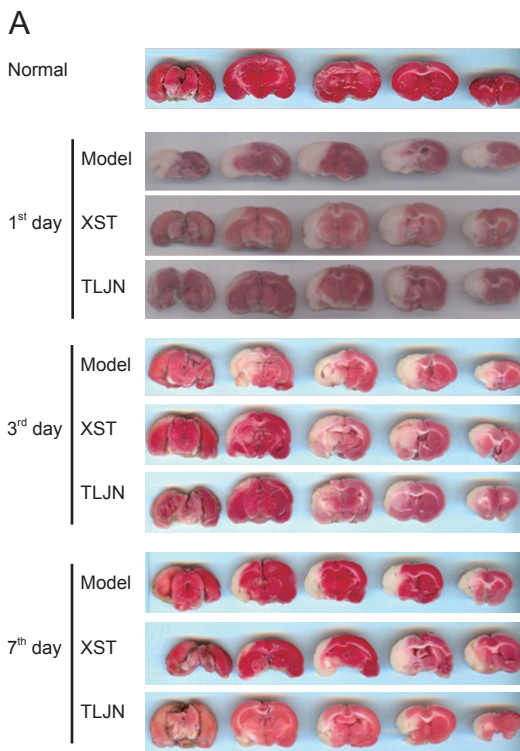
Figure 1 *Tongluo Jiunao* (TLJN) injection treatment reduced the ischemic infarct size detected by 2,3,5-triphenyltetrazolium chloride staining.

(A) The white area represents ischemic brain tissue where cellular metabolic activity had ceased, whereas the red area represents non-ischemic brain tissue where metabolic activity has been maintained. In the model group, infarct volume gradually increased from normal level (zero) to 7 days after surgery. In the TLJN and *Xuesai Tong* (XST) groups, less necrosis could be seen compared to the model group at all intervals. (B) The infarct volume percentage (%) in rats subjected to middle cerebral artery occlusion (MCAO) after TLJN treatment. Data are expressed as the mean ± SD ($n = 6$ rats for each time point). *$P < 0.05$, *vs.* normal group; #$P < 0.01$, ##$P < 0.05$, *vs.* model group; ††$P < 0.01$, *vs.* XST group (one-way analysis of variance, followed by *post-hoc* analysis, was used for significance with the Student-Newman-Keuls multiple comparison test).
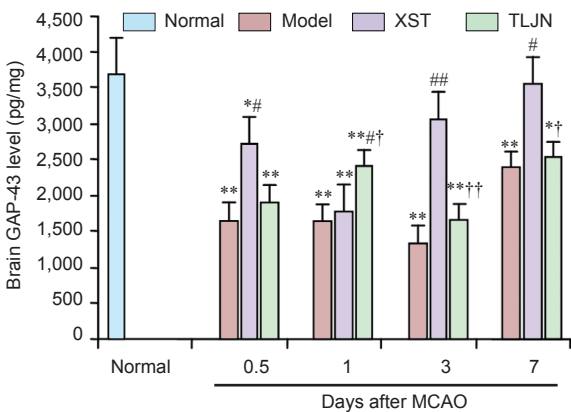
Figure 2 Growth-associated protein 43 (GAP-43) levels, detected by enzyme-linked immunosorbent assay, in rats with middle cerebral artery occlusion (MCAO) followed by daily injections of *Tongluo Jiunao* (TLJN), *Xuesai Tong* (XST) or saline (Model).

Data are expressed as the mean ± SD ($n = 6$ rats per group). *$P < 0.05$, **$P < 0.01$, *vs.* normal group; #$P < 0.05$, ##$P < 0.01$, *vs.* model group; †$P < 0.05$, ††$P < 0.01$, *vs.* XST group (one-way analysis of variance and Student-Newman-Keuls multiple comparison test).
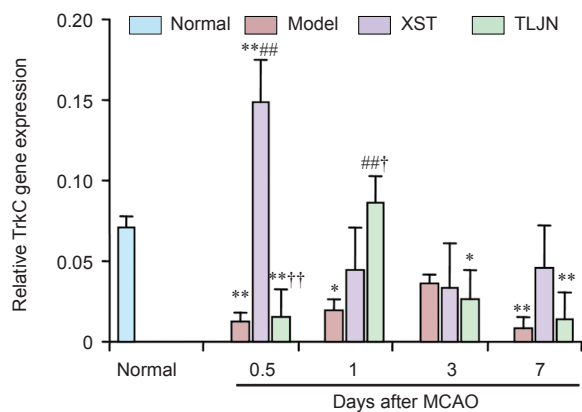
Figure 4 Relative TrkC gene expression levels, detected by reverse transcription polymerase chain reaction, in rats with cerebral ischemia induced by middle cerebral artery occlusion (MCAO) followed by daily injections of *Tongluo Jiunao* (TLJN group), *Xuesai Tong* (XST group) or saline (model group).
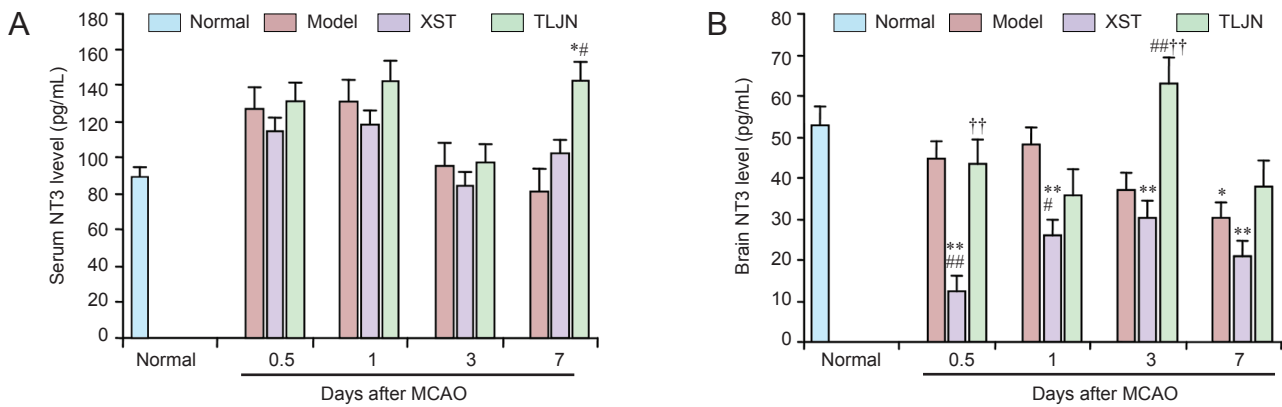
Data are expressed as the mean ± SD ($n = 3$ rats per group). *$P < 0.05$, **$P < 0.01$, *vs.* normal group; ##$P < 0.01$, *vs.* model group; †$P < 0.05$, ††$P < 0.01$, *vs.* XST group (one-way analysis of variance and Student-Newman-Keuls multiple comparison test).

present study, serum NT3 level did not change after MCAO. In addition, brain NT3 decreased on the 7th day after MCAO. Brain NT3 levels in the XST group remained low throughout the period of observation, indicating that the therapeutic effects attributed to XST are not related to an effect on NT3. However, in the TLJN group, NT3 levels were particularly high during the later stages of the observation period.

TTC staining is a nonspecific method used to evaluate tissue necrosis (Hua et al., 2008; Musiolik et al., 2010), whereas GAP-43 is a specific neuronal marker and plays a role in sprouting and branching (Yuan et al., 2009; Korshunova and

Mosevitsky, 2010). In the present study, infarct volume was smaller in model rats that received TLJN than in those that received saline, indicating that TLJN protects brain tissue against necrosis after an ischemic attack. In addition, GAP-43 expression was elevated after TLJN, suggesting that it may improve brain function following ischemic injury. However, expression of NT3, but not TrkC, was elevated after TLJN injections. This suggests that the TLJN strengthened the NT3/TrkC pathway by increasing NT3 expression, thereby resulting in better recovery from the ischemic attack.

NT3 release occurs in both the central and peripheral ner-

Figure 3 Serum (A) and brain (B) neurotrophin 3 (NT3) levels, detected by enzyme-linked immunosorbent assay, in rats with cerebral ischemia induced by middle cerebral artery occlusion (MCAO) followed by daily injections of *Tongluo Jiunao* (TLJN group), *Xuesai Tong* (XST group), or saline (model group).

$*P < 0.05$, $**P < 0.01$, *vs.* normal group; $\#P < 0.05$, $\#\#P < 0.01$, *vs.* model group; $\dagger\dagger P < 0.01$, *vs.* XST group (one-way analysis of variance and Student-Newman-Keuls multiple comparison test). Data are expressed as the mean $\pm$ SD (n = 5–6 rats per group).
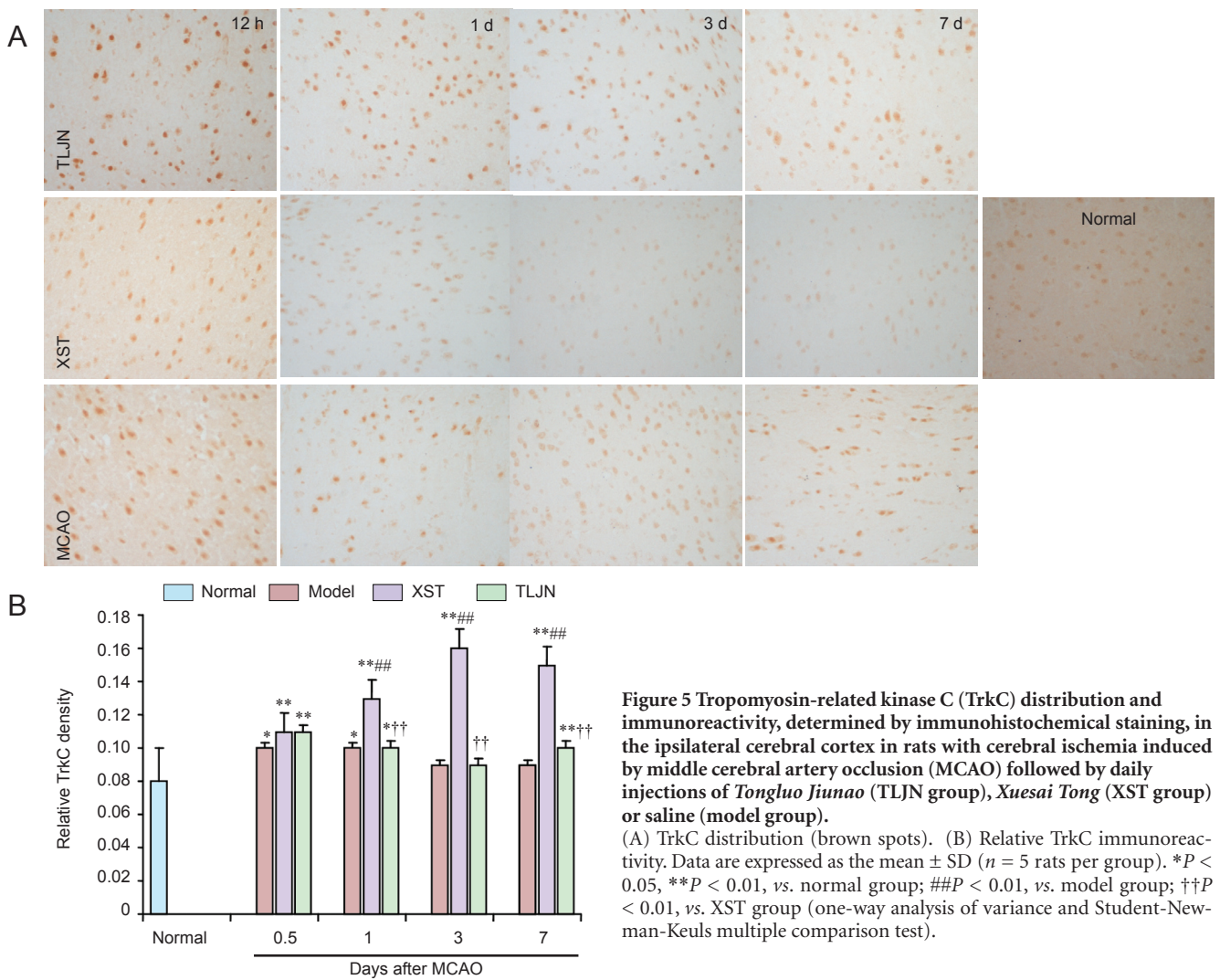
Figure 5 Tropomyosin-related kinase C (TrkC) distribution and immunoreactivity, determined by immunohistochemical staining, in the ipsilateral cerebral cortex in rats with cerebral ischemia induced by middle cerebral artery occlusion (MCAO) followed by daily injections of *Tongluo Jiunao* (TLJN group), *Xuesai Tong* (XST group) or saline (model group).

(A) TrkC distribution (brown spots). (B) Relative TrkC immunoreactivity. Data are expressed as the mean $\pm$ SD (n = 5 rats per group). $*P < 0.05$, $**P < 0.01$, *vs.* normal group; $\#\#P < 0.01$, *vs.* model group; $\dagger\dagger P < 0.01$, *vs.* XST group (one-way analysis of variance and Student-Newman-Keuls multiple comparison test).

vous systems, so we measured levels of NT3 in the brain and serum. In the TLJN group, brain NT3 levels peaked on day 3 in the brain but day 7 in serum, indicating that the systemic increase is secondary to central NT3 changes. We conclude, therefore, that TLJN increases brain NT3, and since XST can markedly lower NT3 in the brain, its mechanism of action is independent of the NT3/TrkC pathway.

Our findings confirm that TLJN and XST cause upregulation of TrkC gene expression. However, overexpression of TrkC after XST, particularly in the later stages of ischemic injury, may be counteracted by certain regulatory mechanisms, resulting in more extensive necrosis; this is avoided with TLJN. Further studies are needed to determine the effects of TLJN following brain ischemia in humans.

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