Specific effects of c-Jun NH2-terminal kinase-interacting protein 1 in neuronal axons

Shu Tang\(^1,2\), Qiang Wen\(^2\), Xiao-jian Zhang\(^1\), Quan-cheng Kan\(^1\)

1 Department of Pharmacy, the First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan Province, China
2 Department of Clinical Pharmacology, Basic Medical College, Zhengzhou University, Zhengzhou, Henan Province, China


Funding: This study was supported by the Henan Province Education Department Key Project of Science and Technology Research in China, No. 12A35006.

Graphical Abstract

Abstract
c-Jun NH2-terminal kinase (JNK)-interacting protein 1 plays an important role in brain-derived neurotrophic factor/tropomyosin-related kinase B (TrkB) anterograde axonal transport. It remains unclear whether JNK-interacting protein 1 mediates similar effects, or whether JNK-interacting protein 1 affects the regulation of TrkB anterograde axonal transport. In this study, we isolated rat embryonic hippocampus and cultured hippocampal neurons in vitro. Coimmunoprecipitation results demonstrated that JNK-interacting protein 1 formed TrkB complexes in vitro and in vivo. Immunocytochemistry results showed that when JNK-interacting protein 1 was highly expressed, the distribution of TrkB gradually increased in axon terminals. However, the distribution of TrkB reduced in axon terminals after knocking out JNK-interacting protein 1. In addition, there were differences in distribution of TrkB after JNK-interacting protein 1 was knocked out compared with not. However, knockout of JNK-interacting protein 1 did not affect the distribution of TrkB in dendrites. These findings confirm that JNK-interacting protein 1 can interact with TrkB in neuronal cells, and can regulate the transport of TrkB in axons, but not in dendrites.

Key Words: nerve regeneration; c-Jun NH2-terminal kinase-interacting protein; neurons; brain-derived neurotrophic factor; tropomyosin-related kinase B; axons; hippocampus; dendrites; regulation; neural regeneration

Introduction
Proteins can be synthesized in neuronal cell bodies, and such proteins play important roles in dendrites and axons (Tan et al., 2010; Plazas et al., 2013). Accuracy of protein transport from the cell body to synaptic terminals directly affects the integrity of neuronal function. Brain-derived neurotrophic factor (BDNF) plays an important role in synaptic function. BDNF binds to tropomyosin-related kinase B (TrkB), and activated TrkB causes a series of neuron responses affecting the targets of such neurons (Nakaji-Hirabayashi et al., 2009; Sánchez-Migallón et al., 2011; Parkhurst et al., 2013a). Theoretically, after TrkB is synthesized, it is immediately transported to axon terminals, where it plays an important role in BDNF signal transmission. c-Jun NH2-terminal kinase (JNK)-interacting proteins (JIPs) are a series of JNK isoforms. Huang et al. (2011) suggested that JIP3 plays an important role in TrkB anterograde axonal transport. However, whether JIP1 mediates similar effects or precisely regulates TrkB anterograde axonal transport remains poorly understood. This study sought to analyze the regulatory effect of JIP1 on TrkB anterograde axonal transport in hippocampal neurons, and aimed to provide new data on the function of JIP1.
Materials and Methods

Ethics statement
This study was approved by the Experimental Animal Ethics Committee of the Experimental Animal Center, Zhengzhou University of China. Animal studies were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Precautions were taken to minimize suffering and the number of animals used in each experiment.

Animals
Sprague-Dawley rats aged 8–10 weeks and weighing 220–290 g (mean 273.7 ± 10.4 g) at gestational day 18 were purchased from the Experimental Animal Center of Zhengzhou University of China (animal license No. SCXK(Yu)2010-0002). All rats were allowed free access to food and water, and housed at 20–22°C in a 12-hour light/dark cycle. The rats were allowed to acclimate for 1 week.

Hippocampal neuron culture
After rats were sacrificed, the brains of embryos at embryonic day 18 were obtained. The hippocampi were isolated under an inverted microscope (TS100; Nikon, Tokyo, Japan) (Paxinos and Watson, 2005). Hippocampi were cut into blocks, and digested with 0.05% trypsin-ethylenediamine tetraacetic acid (EDTA) solution at 37°C for 15–20 minutes. The digestive waste was discarded. All samples were then incubated with Dulbecco’s modified Eagle’s medium (DMEM)/F12 media (Shanghai BioSun Sci&Tech Co., Ltd., Shanghai, China), trituated and deposited for 2 minutes. After removal of supernatant, samples were triturated into a single cell suspension, quantified using a hemocytometer (Shanghai Huake Experimental Equipment Co., Ltd., Shanghai, China), and diluted according to the density of the single cell suspension (5 × 10^6/mL). All samples were seeded onto a polyllysine-coated culture dish at 37°C, 5% CO₂, and saturated humidity.

Passagging and culture of HEK293 cells
HEK293 cells (ATCC, Manassas, VA, USA) were rapidly defrosted, incubated with DMEM/F12 media, trituated, and mixed. Cell suspensions were incubated in the culture dish at 37°C, 5% CO₂, and saturated humidity. At 2 days after culture, cells reached 80% confluency and began to subculture. The medium was discarded. After washing with PBS, cells were digested with EDTA-PBS solution (0.2 mM) for 5 minutes. After the digestive waste was discarded, cells were incubated with HEK293 media, triturated into a single cell suspension, and centrifuged at 1,000 r/min for 2 minutes. After the supernatant was discarded, samples were incubated with cell media and triturated into a single cell suspension. The passage ratio was 1:3. Cells were then incubated in three culture dishes at 37°C, 5% CO₂, and saturated humidity.

Plasmid construction
pcDNA3.1 (Life Technologies, Carlsbad, CA, USA) was used as a vector. Flag-labeled TrkB and HA-labeled JIPA (Shanghai Sangon Biological Technology Co., Ltd., Shanghai, China) were made, and sequencing was conducted. Interfering RNA expression was initiated using the H1 promoter. The JIP1 sequence was 5′-GCG TCA GCG TCG AGT TTA A-3′.

Transfection
HEK293 cell suspension (5 × 10^6) was centrifuged in a centrifuge tube at 1,000 r/min for 5 minutes. The supernatant was discarded. 100 µL of electrotransfer buffer (2.5 µg/µL) was added to the cell suspension, triturated, and mixed. The mixture was placed in an electric rotor (nerve cells O-003 and HEK293 cells A-023). After electroporation, 500 µL of media was added and placed in a centrifuge tube in a 37°C water bath for 10 minutes. These cells were incubated in media at 37°C, 5% CO₂, and saturated humidity for 6 hours. The plasmids were then transfected.

Immunoprecipitation of proteins
Cultured cells were removed from the media, washed twice with PBS, and lysed with TNE buffer containing phosphatase and protease inhibitors. Lysed cells were scraped with a cell scraper, placed into a centrifuge tube, and lysed with a silent mixer (Kylin-Bell Lab Instruments Co., Ltd., Haimen, Jiangsu Province, China) for 30 minutes, and centrifuged at 25,000 × g for 10 minutes. Part of the cell lysate was uniformly mixed with an equal volume of 4× loading buffer. The mixture was heated at 98°C for 5 minutes. Mouse anti-rat HA monoclonal antibody, rabbit anti-rat Flag monoclonal antibody, rabbit anti-rat IgG monoclonal antibody, rabbit anti-rat TrkB monoclonal antibody, and mouse anti-rat JIP1 monoclonal antibody (1:1,000; GenScript Co., Ltd., Nanjing, China) were added to the remaining cell lysate in the silent mixer at 37°C for 2 hours. According to the primary antibody source, Protein A- or Protein G-coupled beads (secondary antibody; Beyotime Biotechnology, Shanghai, China) were added to the silent mixer at 4°C overnight. The mixture was centrifuged at 25,000 × g for 3 minutes, and the supernatant was discarded. TNE buffer was added to suspend the precipitate beads in triplicate. An equal volume of 4× loading buffer was added. Proteins were boiled at 98°C for 5 minutes, and experimental results were observed by electrophoresis.

Western blot assay
A premade plastic frame was placed into an electrophoresis tank, and an appropriate amount of buffer was added. Immunoprecipitation protein and cell lysate were added in the plastic frame in order. All samples were electrophoresed at a constant current for 1 hour, and transferred at 110 V for 2 hours. Polyvinylidene fluoride membrane was incubated with mouse anti-rat HA monoclonal antibody, rabbit anti-rat Flag monoclonal antibody, rabbit anti-rat IgG monoclonal antibody, rabbit anti-rat TrkB monoclonal antibody, and mouse anti-rat JIP1 monoclonal antibody (1:10,000; GenScript) at 4°C overnight. The membrane was washed three times with Tris-Buffered Saline and Tween 20 (TBST), and then treated with TBST-diluted horseradish peroxidase-antibody/mouse IgG (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 2 hours, followed by three washes with TBST.

Immunocytochemical staining
In accordance with a previously published staining method (Tang et al., 2011), samples were observed under the inverted fluorescence microscope (Nikon). Images were obtained by cold light sources (XD-301; RWD Life Science, Shenzhen, China), and analyzed using MetaMorph (Universal Imaging Corp, Downingtown, PA, USA). Dendrites were distinguished.
JIP1 expression was significantly higher in the distal end and processes. In 4–9 days, JIP1 gradually congregated in the longest processes. At this time, processes already displayed the rudimentary features of axons. In 7–9 days, JIP1 expression was significantly higher in the distal end compared with the proximal end (P < 0.05; Figure 2). This result suggested that 2–4 days of culture was a key stage in axon growth.

Distribution of JIP1 in the hippocampus

Immunocytochemical staining demonstrated that in 1–3 days of development, JIP1 could be detected in cell bodies and processes. In 4–9 days, JIP1 gradually congregated in the longest processes (Figure 2). At this time, processes already displayed the rudimentary features of axons. In 7–9 days, JIP1 expression was significantly higher in the distal end compared with the proximal end (P < 0.05; Figure 2).

Interaction of JIP1 and TrkB

Immunoprecipitation was performed in plasmids encoding JIP1-HA and TrkB-Flag using HA and Flag antibodies. Results revealed that JIP1-labeled HA and TrkB-labeled Flag could be co-precipitated by corresponding molecules, indicating that highly expressed JIP1 could form a complex with TrkB. Flag: Labeled plasmid; HA: rabbit monoclonal antibody. JIP1: c-Jun NH2-terminal kinase-interacting protein 1; TrkB: tropomyosin-related kinase B.

**Figure 1 JIP1 expression in hippocampal neurons (western blot assay).**

Data are expressed as the mean ± SD. Experiments were performed in triplicate. Intergroup comparisons were conducted using paired t-tests. JIP1: c-Jun NH2-terminal kinase-interacting protein 1; TrkB: tropomyosin-related kinase B.

**Figure 2** Lamin B + Tang S, et al. / Neural Regeneration Research. 2016;11(1):114-118.

**Figure 3 Interaction of JIP1 and TrkB in axons.**
TrkB was precipitated by endogenous JIP1 antibody, suggesting that JIP1 could form a complex in vivo. FL: TrkB receptor subtype 1; T1: TrkB receptor subtype 2; Ab: endogenous JIP1 antibody; JIP1: c-Jun NH2-terminal kinase-interacting protein 1; TrkB: tropomyosin-related kinase B.

**Figure 4 Interaction of JIP1 and TrkB in axons.**
TrkB was precipitated by endogenous JIP1 antibody, suggesting that JIP1 could form a complex in vivo. FL: TrkB receptor subtype 1; T1: TrkB receptor subtype 2; Ab: endogenous JIP1 antibody; JIP1: c-Jun NH2-terminal kinase-interacting protein 1; TrkB: tropomyosin-related kinase B.

**Figure 5** The effects of JIP1 on TrkB distribution in axons and dendrites (immunocytochemistry).

The ratio of gray values of TrkB in axons/dendrites to values in cell bodies represents the relative distribution of TrkB. Data are expressed as the mean ± SD. Experiments were performed in triplicate. Intergroup comparisons were conducted using paired t-tests. †P < 0.05, vs JIP1-HA group; JIP1: c-Jun NH2-terminal kinase-interacting protein 1; TrkB: tropomyosin-related kinase B.
demonstrated that TrkB could be precipitated, indicating that JIP1 could form a complex in vivo (Figure 4).

The effects of JIP1 on the distribution of TrkB in axon terminals

Immunocytochemistry showed that the distribution of TrkB was significantly increased in axon terminals when JIP1 was highly expressed. After JIP1 knockout, the distribution of TrkB was significantly decreased in axon terminals. The difference in the distribution of TrkB remained significant whether JIP1 was knocked out or not (P < 0.05). However, in dendrites, JIP1 knockout did not affect the distribution of TrkB (P > 0.05; Figure 5).

Discussion

TrkB is a high affinity receptor for BDNF. TrkB is mainly distributed over neuronal cell bodies, dendrites, and axon terminals. Zhai et al. (2011) verified that the precise location of TrkB is important for normal nerve function in highly differentiated neurons. Postsynaptic TrkB receptors can promote the release of glutamate and increase the activity of the Na+/H+ antiporter on target tissues (Numakawa et al., 2009). Postsynaptic TrkB receptors can enhance N-methyl-D-aspartic acid receptor function in central neurons. Thus, the precise location and correct transport of TrkB receptors is important in propagating the correct response to BDNF release. The CRMP-2/S1p1/Rab27B complex has been shown to mediate TrkB and affect kinesin-1 to regulate TrkB transport in axons (Lähteinen et al., 2004; Butowt and von Bartheld, 2007; Arimura et al., 2009; Poopalasundaram et al., 2011). When the CRMP-2/S1p1/Rab27B complex was knocked out, TrkB transport to synapses could not be completely blocked. This finding confirmed that other adapter proteins are likely involved in axon TrkB transport. Gatto et al. (2013) confirmed that TrkB transport from cell bodies to dendrites or axons occurs via a complex and precise regulatory mechanism. JIP3 can mediate the interaction of kinesin-1 light chain and TrkB, and regulate anterograde axonal transport of TrkB (Kanato et al., 2009; Arimoto et al., 2011; Fu and Holzbaur, 2013; Parkhurst et al., 2013b; Sun et al., 2013; Kim et al., 2014). However, the mechanism regulating TrkB is complex in neuronal cells. Additionally, other subtypes of the JNK family are possibly involved.

JIP1 was the first subtype of the JNK family to be discovered. Exogenous application of JIP1 can restore neurological function in mice with disconnection of the telencephalon induced by JIP3 knockout (Hayashi et al., 2007; Fujieda and Sasaki, 2008; Yamanaka et al., 2008; Nakaji-Hirabayashi et al., 2009). These results suggest that JIP1 and JIP3 have common features. Whether JIP1 has the same effect on mediating anterograde axonal transport of TrkB as JIP3 remains poorly understood. The present study first verified that JIP1 can form a complex with TrkB in vivo and in vitro, indicating that JIP1 potentially plays an important role in anterograde axonal transport of TrkB. The JIP3 and CRMP-2/S1p1/Rab27B complex pathways involved in regulating anterograde axonal transport of TrkB are completely different (Kim et al., 2004; Roberts et al., 2006; Bogush et al., 2007; Burkhalter et al., 2007; Caldeira et al., 2007; Zhang et al., 2007; Yamanaka et al., 2008). Thus, JIP1 may mediate anterograde axonal transport of TrkB, and in-turn, TrkB is a receptor involved in multiple regulatory pathways. Whether there is a link between these mechanisms is unconfirmed.

To further verify whether JIP1 has a similar role in dendrites and axons, we overexpressed JIP1-HA and JIP1 knockout in cultured neuronal cells in vitro. Immunocytochemistry showed that when JIP1 was highly expressed, the distribution of TrkB apparently increased in axon terminals. After endogenous JIP1 knockout, the distribution of TrkB was clearly reduced in axon terminals. Nevertheless, the distribution of TrkB was not associated with JIP1 expression in the ends of dendrites. This finding indicates that JIP1 only affected TrkB transport in axon terminals, minimally affected the distribution of TrkB in dendrites, and further verified that JIP1 exerted an effect on anterograde axonal transport of TrkB.

In conclusion, JIP1 may interact with TrkB in neuronal cells and regulate anterograde axonal transport of TrkB. Interestingly, the regulatory effect of JIP1 was exerted only in axons. This study demonstrated the effects of JIP1 on anterograde axonal transport of TrkB, but the precise mechanism and whether other proteins or molecules are involved during transport requires further investigation.

Author contributions: All authors designed and evaluated the study, performed the experiments and approved the final version of the paper. ST wrote the paper.

Conflicts of interest: None declared.

Plagiarism check: This paper was screened twice using CrossCheck to verify originality before publication.

Peer review: This paper was double-blinded and stringently reviewed by international expert reviewers.

References


Figure 2 Relative expression of JIP1 in the hippocampus.

(A) JIP1 expression in different axonal developmental stages (immunofluorescence staining). × 400. Stage 1: 1–3 days of culture; stage 2: 4–6 days of culture; stage 3: 7–9 days of culture. Arrows show axons. Pane: Localization of JIP1. JIP1 is the red color, and α-tubulin is the green color. (B) JIP1 expression in different locations in stage 3. Data are expressed as the mean ± SD. Experiments were performed in triplicate. Intergroup comparisons were conducted using paired t-tests. *P < 0.05, vs the proximal end; #P < 0.05, vs the center. JIP1: c-Jun NH2-terminal kinase-interacting protein 1.