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JIP3 regulates neuronal radial migration by mediating TrkB axonal anterograde transport in the developing cerebral cortex

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Running title: JIP3 regulates neuronal radial migration

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Abstract

Radial migration is essential for the precise lamination and the coordinated function of the cerebral cortex. However, the molecular mechanisms for neuronal radial migration are not clear. Here, we report that c-Jun NH2-terminal kinase (JNK)-interacting protein-3 (JIP3) is highly expressed in the brain of embryonic mice and essential for radial migration. Knocking down JIP3 by in utero electroporation specifically perturbs the radial migration of cortical neurons but has no effect on neurogenesis and neuronal differentiation. Furthermore, we illustrate that JIP3 knockdown delays but does not block the migration of cortical neurons by investigating the distribution of neurons with JIP3 knocked down in the embryo and postnatal mouse. Finally, we find that JIP3 regulates cortical neuronal migration by mediating TrkB axonal anterograde transport during brain development. These findings deepen our understanding of the regulation of neuronal development by JIP3 and provide us a novel view on the regulating mechanisms of neuronal radial migration.

Keywords: JIP3, TrkB, radial migration, In utero electroporation
1. Introduction

The cerebral cortex is the largest structure of the brain. The precise functions of the cerebral cortex are directed by the highly patterned six distinct layers, which requires the distribution of cortical neurons to the proper positions [1-4]. These eminent developmental processes include neurogenesis, neuronal migration, differentiation and circuit formation [5, 6]. Radial migration, which is the process of newborn neurons migrating to their target destination, plays key roles in the formation of distinct lamina and the realization of the normal function of the cortex. For example, disruption of neuronal migration causes cortical malformation disorders and some neuropsychiatric diseases, such as epilepsy, schizophrenia, dysgnosia, autism, dyslexia, etc. [7-9]. However, the regulating mechanisms for neuronal radial migration remain unclear.

C-Jun NH2-terminal kinase (JNK)-interacting protein 3 (JIP3) is a member of the JNK-binding protein family and, as a scaffold protein for the JNK signaling pathway, facilitates JNK activation [10, 11]. In addition, JIP3 also acts as an adaptor protein to regulate the trafficking of cargo (APP, TrkB, etc.) by bridging them to kinesin [12, 13]. In addition to these functions of JIP3, many studies have focused on the influence of JIP3 on brain development. For example JIP3 knockout mice have been reported to die after birth with many brain morphology defects, such as the random distribution of telencephalic neurons and the disorganization of the telencephalic commissure [14, 15]. Furthermore, some other reports have proven that JIP3 regulates neuronal axon elongation and branching in cultured neurons [16, 17]. All of the reports suggest that JIP3 plays a major role in the development of brain and cultured neurons. However, the processes of brain development that are regulated by JIP3 are still not clear.

In this study, we showed that JIP3 participates in the migration of cortical neurons during cortical development by mediating TrkB axonal anterograde transport, providing a novel view on the regulating mechanisms of neuronal radial migration.
2. Materials and Methods

2.1 Animals

ICR mice used for in utero electroporation were obtained from Beijing Vital River Laboratory Animal Technology limited company (Beijing, China). JIP3 knockout mice were purchased from Model Animal Research Center of Nanjing University. All procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the institutional animal care and use committee of Shandong University.

2.2 Plasmid constructs

The Flag-TrkB plasmid was constructed as described previously [18]. For gene knockdown by RNA interference (RNAi), the pCAGGS-EGFP vector was used to transcribe functional small interfering RNA (siRNA) of JIP3 with a target sequence as follows: rat and mouse JIP3, CAG GCC GAG GAG AAA TTC A. EGFP-tagged JIP3-resistant plasmids and the mutants were created as follows: ΔJBD, ΔKBDALZ, ΔCC1 were subcloned into the pCAGGS expression plasmid. The pCAGGS-EGFP-JIP3-resistant plasmids were generated using the PCR site-directed mutagenesis, and the target sequence was as follows: CAA GCG GAA GAG AAG TTC A.

2.3 Cell culture and transfection

PC12 cells were maintained in DMEM (Invitrogen) supplemented with 5% fetal bovine serum (hyclone) and 10% horse bovine serum (hyclone) at 37°C in a 5% CO2 humidified atmosphere. PC12 cells were electroporated with plasmid in a Nucleofector device (Amaxa Bio-systems) according to the manufacturer’s instruction.

2.4 Immunoblotting

PC12 cells or brain cortical tissue samples were lysed in TNE buffer (10 mM Tris, pH 8.0; 1% NP-40; 1 mM EDTA) with protease inhibitors. The lysates were clarified by centrifugation at 1,4000 g for 15 min at 4°C and re-suspended in 4 × sample buffer. The samples were suspended in SDS-PAGE (Invitrogen) and transferred onto PVDF membranes.
Finally, they were subjected to immunoblotting for analysis with the indicated antibodies.

2.5 In utero electroporation (IUE)

The surgery on ICR pregnant mice and E15.5 embryo manipulation in the uterus used in IUE have been described previously [19]. Briefly, at E15.5, pregnant mice were anesthetized deeply with pentobarbital sodium, and the uterus was removed. Then, the plasmids at a high concentration (2-3 µg/µl) with Fast Green (0.1%) were injected into the lateral ventricle of embryos. The tweezer-type electrode was positioned flanking the ventricular zones of the embryo and pulsed five times at 40 V for 50 ms at intervals of 950 ms. Finally, the uterine horns were placed back into the abdominal cavity. For the rescue experiment, JIP3 siRNA plasmids were mixed with other expressing plasmids at a 1:3 ratio in molar concentration with a final concentration of 3 µg/µl.

2.6 Immunohistochemistry

At E18.5, P0 or P7, the brains were dissected out and post-fixed in 4% paraformaldehyde for 24 hours and moved to 30% sucrose in PBS for 24 hours at 4°C. The cortical sections were sliced coronally at 30 µm by using a freezing microtome (Leica, GER). The sections were coverslipped with 4’, 6-diamidino-2-phenylindole (DAPI) solution in anti-fluorescence quenching agent.

When BrdU staining was performed, sections were treated with 2 M HCl for 30 min and sodium tetraborate for 1 h after being washed 3 times in PBS. Then, the sections were blocked with PBS containing 30% normal donkey serum for 1 h at room temperature and incubated with anti-BrdU (Sheep) 1:500. After being washed three times in PBS, the brain sections were treated with Alexa Fluor 594-conjugated donkey anti-sheep IgG. All images were captured by Cell Observer SD (Microstructural Platform of Shandong University).

2.7 Statistical analysis

The statistical analyses were performed with IBM SPSS Statistics 19 software, and the statistical significance was analyzed by Student’s test or one-way ANOVA followed by post
hoc tests. The data are represented as the mean ± SEM, and the differences were considered significant at \( p < 0.05 \).
3. Results

3.1 JIP3 is necessary for the development of the cerebral cortex

To investigate the function of JIP3 on cortical development, we first examined the expression pattern of JIP3 in the cerebral cortex of mice at different developmental stages of the brain. By using western blot, the results showed that the expression levels of the JIP3 protein gradually increased from embryonic day 10 (E10) to postnatal day 10 (P10) (Fig. 1 A and B), which is consistent with previous studies and suggests that JIP3 plays a potential role in brain development [20]. Importantly, we found that the expression of JIP3 was obviously increased from E13 to E17, at a time of vigorous radial migration of cortical neurons [6, 20, 21]. This prompted us to hypothesize that JIP3 may participate in the migration of cortical neurons. Meanwhile, we found that the expression of JIP3 increased significantly in the P0-P10 period when the axons of cortical neurons were projecting to the contralateral cortex [22], which, along with our previous report, provided further evidence to suggest that JIP3 plays a role in the period in which cortical neurons extend into the contralateral cortex [16]. Above all, the results proved that the expression of JIP3 in the developing cerebral cortex is abundant and suggested that JIP3 may participate in cortical development.

To explore the necessity of JIP3 in cortical development, we further studied the structure of the cerebral cortex in the embryonic JIP3 knockout mouse. As is shown in Fig. 1 C, we found that the cortical cortex of knockout mice was a little thinner and the distribution of neurons was more disordered than that in the wild-type littermates (Fig. 1 C). These results suggested that JIP3 plays an important role in the formation of the lamellar structure and distribution of cortical neurons.

3.2 JIP3 regulates the radial migration of cortical neurons but not their proliferation and differentiation

Cortical formation undergoes four stages during cortical development: (1) proliferation of neural precursor cells (NPCs), (2) differentiation of multipolar neurons into bipolar neurons, (3) radial migration of neurons, and (4) circuit formation [23, 24]. To explore which developmental stages of the cortical cortex are regulated by JIP3, we investigated the
influence of JIP3 on these stages in turn. First, we analyzed the functions of JIP3 on the proliferation of NPCs. A PCAGGS-EGFP-siJIP3 plasmid, which has been used in our previous reports, was employed again, and its interfering efficiency on endogenous JIP3 was tested by western blot (Fig. 2 A) [12]. Then, the plasmids were transfected into E15.5 mice using in utero electroporation (IUE). The pregnant mouse was injected with BrdU to label the proliferating NPCs 24 hours after IUE treatment (Fig. 2 B). Then, 1 hour after BrdU injection, the mice were sacrificed, and the percentage of BrdU-positive cells co-localized with EGFP-positive cells compared to total EGFP-positive cells was analyzed. However, no significant difference was found between the scrambled and siJIP3 group (Fig. 2 C and D). The results suggested that JIP3 has no effect on NPCs proliferation.

Next, we tested whether JIP3 regulates neural differentiation. Similarly, we electroporated a scrambled or JIP3 siRNA construct into E15.5 embryos and examined their brain at E18.5. A previous study has mentioned that newborn neurons finish their multi-to-bipolar transition in the intermediate zone (IZ) during neuronal differentiation [23]. Therefore, we examined the ratio of the number of multipolar neurons and bipolar neurons in the IZ. The results showed that regardless of if the brain tissue was from the JIP3 knockdown group or the control group, approximately 90%± 0.99 EGFP-positive cells were multipolar neurons, and no significant difference was found between the two groups (Fig 2 E and F), which suggested that JIP3 does not participate in neuronal differentiation. Then, we further explored whether JIP3 participates in neuronal radial migration. The embryo brains were acquired at E18.5 after they were treated with scrambled or JIP3 siRNA via IUE at E15.5. The results showed that the proportion of neurons that had reached the cortical plate (CP) was significantly lower in the JIP3 knockdown group compared with that in the scrambled group (Fig 2 G and H), which suggested that JIP3 regulates the radial migration of cortical neurons. In conclusion, we proved that JIP3 regulates the radial migration of cortical neurons but not the proliferation of NPCs and the differentiation of neurons.

3.3 Knockdown of JIP3 delays but does not block the radial migration of cortical neurons

To verify whether knockdown of JIP3 inhibits the migration of the neurons to the CP, we
did not sacrifice the mice treated with IUE at E15.5 until P0 and P7, when the six-layer lamination structure of the cortex had been formed [16]. The results showed that although the percentage of neurons that migrated to II-IV at P7 was still lower in the brains from the siJIP3 group than those from the scrambled group, the percentage had increased from 9.031% ± 2.02 at P0 to 67.93% ± 1.14 at P7 (Fig. 3 A-D). This change suggests that knockdown of JIP3 only slows down but does not block the radial migration of the cortical neurons.

3.4 JIP3 regulates cortical neuronal migration by mediating TrkB anterograde axonal transport

Previous studies have reported that JIP3 has several biological functions dependent on different structural domains. To verify which structural domain in JIP3 is responsible for its regulation of neuronal radial migration, we performed an IUE rescue assay. In detail, we first constructed Res-JIP3 and several Res-JIP3 mutants without the respective JBD, KBD and LZ domains, whose target sequence of JIP3 siRNA was mutated based on the degenerate codon principle (Fig. 4A). Then, we co-transfected the JIP3 siRNA plasmid and some expressional plasmids into the ventricle of E15.5 embryos by IUE and investigated the migration of the cortical neurons at E18.5. The results showed that Res-JIP3 could rescue the migration defect caused by JIP3 siRNA, suggesting that our rescue system worked normally (Fig. 4B). More importantly, we found that Res-JIP3ΔJBD could rescue the defect in neuronal radial migration caused by JIP3 knockdown, but Res-JIP3ΔKBDΔLZ could not (Fig. 4B), which suggested that JIP3 may regulate neuronal migration by mediating cargo trafficking.

JIP3 has been reported to mediate TrkB axonal transport through its coiled-coil 1 (CC1) domain [12], and TrkB regulates the timing of cortical neuronal migration [25]. To precisely define the mechanism, we constructed the Res-JIP3ΔCC1 plasmid and co-transfected it with JIP3 siRNA into the embryo brain using IUE. The results showed that Res-JIP3ΔCC1 could not rescue the migration defect caused by JIP3 knockdown, which suggested that JIP3 regulates neuronal radial migration by mediating TrkB axonal transport in neurons (Fig. 4B and C). To prove that the expression of TrkB at the axonal tips was a sufficient condition for radial migration, we co-transfected JIP3 siRNA and Flag-TrkB plasmids into the embryo
using IUE, and the results showed that TrkB could rescue the migration defect caused by JIP3 knockdown (Fig. 4 B and C), suggesting that JIP3 regulates cortical neuronal migration by mediating TrkB axonal transport.
4. Discussion

The radial migration of newborn neurons is essential for cortical function. It is well known that the disruption of neuronal migration is associated with cortical malformation disorders and some neuropsychiatric diseases, such as epilepsy, schizophrenia, dysgnosia, autism, dyslexia, etc. [7-9].

Our study provides three novel insights. First, we obtained a time course of the expression levels of JIP3 in the cortical cortex during its developmental period for the first time. Previous studies have illustrated that JIP3 was expressed in the developing brain. However, these studies only performed qualitative research using immunocytochemical methods, and a sufficient quantitative study to reflect the relationship between JIP3 expression levels and brain development was still missing [20]. In our study, we found that JIP3 expression levels have two significantly elevated periods during cortical development. Interestingly, these two periods correspond to the elongation phase illustrated in our previous study and migration phase found in our current study [16]. These results illustrated that JIP3 regulates cortical development as a key control factor, and whether JIP3 plays important roles in formatting the neuronal cortex in later developmental stages is worthy of further investigation.

Second, we found that JIP3 knockdown delayed but did not block neuronal radial migration. The neuronal development requires sophisticated processes, including neurogenesis, differentiation, migration and cortical circuit formation. All of these processes are dependent on accurate time regulation. Starting at about E11.5, NPCs differentiate into neurons and start their radial migration [26]. If neurons miss their proper migrating time, the radial scaffold is dismantled, and the cortical circuit could not perform its normal functions [6]. In our study, although 58.9% neurons could accomplish their migration within P0 - P7 (Fig 3 A and B), we believe that they might have missed the correct time window, causing irretrievable functional loss. The reason these delayed neurons still continue to migrate to their destination even after missing the correct time window is a fascinating question.
Third, JIP3 regulates cortical neuron radial migration by mediating TrkB axonal transport. Neurons are highly polarized cells. Most proteins in neurons are synthesized in the soma, and their expression in the axons or dendrites requires transportation from the soma to their destination [27]. The transport process is based on a long-distance cargo trafficking system composed of the microtubule system and motor proteins [28]. Some studies have illustrated that motor proteins are important for neuronal radial migration. For example, a study in Caenorhabditis elegans found that the KIF5 ortholog UNC-116 could regulate neuronal cell body position in the PHB sensory neurons [29], and kinesin-5 has been reported to regulate neuronal migration [30]. The most important function of motor proteins is to mediate the precise trafficking of specific cargos to their destination; therefore, whether motor proteins regulate cortical neuronal migration by mediating the trafficking of specific cargoes is a fascinating question. Our study proved that JIP3 regulates neuronal migration by mediating TrkB axonal transport [12] and illustrated that kinesin regulates neuronal migration by the lateral trafficking of specific cargoes. Whether the trafficking of other cargo participates in the regulation of neuronal migration needs further study.

Meanwhile, TrkB, as a receptor for brain-derived neurotrophic factor (BDNF), plays important roles in brain development. For example, newborn neurons could not reach the outside of the cortex in TrkB conditional knockdown mice [25]. Furthermore, another study proved that BDNF and TrkB could regulate the migration of NPCs in the SVZ to the olfactory bulb [31]. In our study, we further found that TrkB in axon tips plays an important role in cortical neuron radial migration, which further defined the function of TrkB in the developing cortical neurons. However, whether TrkB in dendrites also participates in neuronal migration remains unclear.

In conclusion, we investigated the role of JIP3 in regulating cortical neuronal migration. In addition, we found that JIP3 knockdown only delays neuronal radial migration. Finally, we proved that JIP3 regulates cortical neuronal migration by enhancing TrkB axonal transport. The findings provide us a novel view on the regulating mechanisms of neuronal radial migration and help us understand more about how JIP3 regulates brain development.
Acknowledgments

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Figure legend

FIGURE 1. JIP3 is important for the development of the cerebral cortex. (A) Immunoblot showing JIP3 protein levels in the cerebral cortex at various developing stages. Actin was used for normalization. (B) Quantification of the expression levels of the JIP3 protein shown in A (**p <0.01, compared to E10; One-way ANOVA). (C) The cortex of JIP3 knockout mouse and wild-type littermate mice immunostained by DAPI at E19. The scale bar represents 100 µm.

FIGURE 2. Knockdown of JIP3 impairs neuronal migration. (A) Immunoblot showing the efficient knockdown of endogenous JIP3 expression in PC12 cells transfected with PCAGGS-EGFP-siJIP3 constructs or scrambled constructs. Tubulin was used for normalization. (B) The schematic shows the protocol for detecting cell proliferation. The scrambled or JIP3 siRNA plasmids were injected into embryos at E15.5. After 24 hours, the pregnant mouse was injected with BrdU and was then sacrificed after 25 hours. (C) Representative cortical sections were stained with antibodies and BrdU (Red) and DAPI (Blue). Arrows show the colocalization of BrdU and EGFP neurons. The scale bar represents 100 µm. (D) Quantification of the ratio of the number of newborn neurons to total positive neurons. (E) Magnified images of migrating neurons in the intermediate zone. Neuronal morphologies after the scrambled or JIP3 siRNA IUE are shown. Asterisks indicate representative neurons with unipolar/bipolar morphology. The scale bar represents 40 µm. (F) Quantification of the ratio of the number of unipolar/bipolar neurons to multipolar neurons in EGFP-positive neurons in the scrambled or JIP3 siRNA group. (G) E15.5 mouse embryos were electroporated with scrambled or siJIP3 and cortical sections were collected at E18.5. Representative coronal sections showed the migrating neurons of transfected-positive neurons. The sections were stained with DAPI (Blue). The scale bar represents 100 µm. (H) Quantification of the percentages of EGFP-positive neurons in each cortical zone (**p <0.01, ***p <0.001, JIP3 siJIP3 group compared to scrambled group; n.s., p>0.5; Student’s t-test).
**FIGURE 3.** Knockdown of JIP3 delays but does not block neuronal migration. (A-D) E15.5 embryos were electroporated with scrambled or siJIP3 by IUE and brains were collected at P0 or P7. (A and C) Representative cortical sections were stained with DAPI (Blue). The scale bar represents 100 µm (A) and 200 µm (C). (B and D) Quantification of the percentage of EGFP-positive neurons in different cortical sections ("p <0.01, ""p <0.001; Student’s t-test).

**FIGURE 4:** JIP3 regulates the radial migration of cortical neurons by mediating TrkB axonal anterograde transport. (A) Schematic representation of the Res-JIP3 mutants. The blank space corresponds to the appropriate domains within the JIP3 deletion constructs. (B) E15.5 embryos were electroporated with scrambled or siJIP3 only or overexpressed Res-JIP3 mutants in addition to siJIP3. The sections were stained with DAPI (Blue). The scale bar represents 100 µm. (C) Quantification of the percentage of migrating neurons in different cortical sections ("p <0.05, ""p <0.01, #p <0.1, n.s., p >0.05, compared to the control group; One-way ANOVA).
Figure 1

A

B

C

E19

Wide type

JIP3 KO mice
Figure 3

A

E15.5-P0

scramble  siJIP3

B

percentages of neurons in each zone (%)

II-IV  V  VI  WM

scramble  siJIP3

C

E15.5-P7

scramble  siJIP3

D

percentages of neurons in each zone (%)

II-IV  V  VI  WM

scramble  siJIP3
Figure 4

A

Res-JIP3

Res-JIP3ΔJBD

Res-JIP3ΔKBDΔLZ

B

E15.5- E18.5

scramble

siJIP3

siJIP3 + Res-JIP3

siJIP3 + Res-JIP3ΔJBD

siJIP3 + Res-JIP3ΔKBDΔLZ

siJIP3 + Res-JIP3ΔCC1

siJIP3 + Flag-TrkB

C

percentage of EGF+ cells

in each zone (%)

CP

IZ

V2/SVZ

con

siJIP3

siJIP3+Res-JIP3

siJIP3+Res-JIP3ΔJBD

siJIP3+Res-JIP3ΔKBDΔLZ

siJIP3+Res-JIP3ΔCC1

siJIP3+Flag-TrkB

n.s.
Highlights

A JIP3 expression-time curve during cortical cortex development was obtained.

JIP3 knockdown delayed but did not block neuronal radial migration.

JIP3 regulates cortical neuron radial migration by mediating TrkB axonal transport.
Conflict of interest:

The authors declare no competing financial interests.