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FYN REGULATES MULTIPOLAR–BIPOLAR TRANSITION AND NEURITE MORPHOGENESIS OF MIGRATING NEURONS IN THE DEVELOPING NEOCORTEX

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Abstract—Fyn is a non-receptor protein tyrosine kinase that belongs to Src family kinases. Fyn plays a critical role in neuronal migration, but the mechanism remains unclear. Here, we reported that suppression of Fyn expression in mouse cerebral cortex led to migration defects of both early-born and late-born neurons. Morphological analysis showed that loss of Fyn function impaired multipolar–bipolar transition of newly generated neurons and neurite formation in the early phase of migration. Moreover, Fyn inhibition increased the length of leading process and decreased the branching number of the migrating cortical neurons. Together, these results indicate that Fyn controls neuronal migration by regulating the cytoskeletal dynamics and multipolar–bipolar transition of newly generated neurons during cortical development.

Key words: Fyn, RNAi, *in utero* electroporation, neuronal migration.

INTRODUCTION

The mammalian cerebral cortex is a well-organized six-layer structure, which is formed by precisely orchestrated processes. There are two modes of neuronal migration, ‘‘radial” and ‘‘tangential” (Nadarajah et al., 2001; Kriegstein and Noctor, 2004), and radial migration plays a key role in the establishment of the layered structures of the cerebral cortex (Rakic, 1972; Edmondson and Hatten, 1987). Neurons are generated in the ventricular zone and subventricular zone (VZ/SVZ). They migrate through the intermediate zone (IZ) to their destinations in the cortical plate (CP) in an ‘‘inside-out” manner (Cooper, 2008; Kubo et al., 2010). Early-born neurons migrate by radial glia-independent somal translocation and stay in the deep layers (Nadarajah et al., 2001; Hatanaka et al., 2004), whereas later-born neurons pass beyond their predecessors by radial glia-guided locomotion and form the more superficial layers (Rakic, 1972; Tabata and Nakajima, 2003; Noctor et al., 2004). Migrating neurons undergo a series of morphological changes along with their changes in location. After the final division, neurons become multipolar with multiple neurites above the VZ or in the multipolar accumulation zone (MAZ) (Tabata et al., 2009). Neurons transform from multipolar to bipolar and attach to radial glia cells in the upper IZ, and migrate by radial glia-guided locomotion through the IZ and CP (Rakic, 1972; Tabata and Nakajima, 2003; Noctor et al., 2004). When neurons migrate beneath the outermost region of the CP, they switch to terminal translocation and their leading processes attach to the MZ; by shortening their leading processes, neurons move their cell bodies to their final destinations in a radial glia-independent manner (Nadarajah et al., 2001; Sekine et al., 2011). The migration and morphogenesis of cortical neurons are complex and well-coordinated processes, the impairments of which lead to severe brain malformations and disorders, such as lissencephaly, schizophrenia and epilepsy (Dobyns et al., 1997; Kalus et al., 1997; Morris et al., 1998; Gleeson and Walsh, 2000; Sheen and Walsh, 2003; Stouffer et al., 2016).

Fyn, a non-receptor protein tyrosine kinase belonging to Src family kinases (SFKs), plays important roles in the nervous system. Fyn deficiency decreases dendritic spine density (Babus et al., 2011) and impairs long-term potentiation and spatial learning (Grant et al., 1992; Minami et al., 2012). In *Fyn* knockout mice, the development of the CP shows obvious defects: late-born neurons dislocate in deeper layers (Yuasa et al., 2004) and pyramidal neurons in layer V display inverted orientation (Sasaki et al., 2002). The double knockout of *fyn* and *src* leads to inverted layering of the CP, which is a reeler-like phenotype caused by the mutant of Reelin (Kuo et al., 2005). Reelin is an extracellular molecule that activates signaling cascades eventually leading to ‘‘inside-out” layering of neurons in the cerebral cortex. Reelin binds to its two receptors, apolipoprotein E receptor 2 (ApoER2) and very low-density lipoprotein receptor (VLDLR), leading to

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Abbreviations: Cdk5, cyclin-dependent kinase 5; CP, cortical plate; EGFP, enhanced green fluorescent protein; FAK, focal adhesion kinase; IZ, intermediate zone; MZ, marginal zone; PB, phosphate buffer; PFA, paraformaldehyde; RNAi, RNA interference; SFKs, Src family kinases; SVZ, subventricular zone; VZ, ventricular zone.

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the phosphorylation of the intracellular adaptor protein Dab1 by Fyn and initiating signal transduction cascades critical for neuronal migration (Bock and Herz, 2003; Arnaud et al., 2003). Our previous studies have shown that mutations in different domains of Fyn impair neuronal migration (An et al., 2014; Lu et al., 2015). However, the mechanism behind Fyn's control of neuronal migration remains poorly understood.

In the present study, we knocked down Fyn expression in the developing mouse cerebral cortex using RNA interference (RNAi) combined with *in utero* electroporation to explore the role and mechanism of Fyn in neuronal migration. We found that inhibition of Fyn expression impaired neuronal migration and affected morphology for both early-born and late-born neurons. Absence of Fyn led to defects in the morphological transition from multipolar to bipolar and the development of neurites. These results suggest that Fyn is critical for regulating neuronal migration and morphogenesis during cortical development.

MATERIALS AND METHODS

Animals

C57BL/6 mice used *in utero* electroporation were purchased from Xi'an Jiaotong University, China. Mice were kept in standard temperature and humidity conditions and provided with food and water freely. The time of vaginal plug appearance was defined as embryonic day 0.5 (E0.5). All animal experiments were conducted according to the guidelines established by Northwest A&F University.

DNA construction

Enhanced green fluorescent protein (EGFP) sequences cloned from pEGFP-N1 (Clontech, Mountain View, CA, USA) or a constructed myc sequence were inserted into a pCAG-MCS vector after the cloning site to produce pCAG-EGFP or pCAG-myc. *Fyn* mouse cDNA was inserted into the cloning site to generate pCAG-Fyn-GFP, and *Fyn* chicken (cFyn) cDNA was inserted to generate pCAG-cFyn-myc. RNAi vectors were purchased from GenePharma Co., Ltd (Shanghai, China). To generate mouse Fyn shRNA, the following hairpin sequences were cloned into a pGPH1/GFP/Neo vector (GenePharma Co., Ltd.): 5′-CACCGCGATCAG CAAACATTCTAGTTTCAAGAGAACTAGAATGTTGCTG ATCGCTTTTTTG-3′, 5′-CACCGCTTCCTGGAGGAC TACTTTATTCAAGAGATAAAGTAGTCCTCCAGGAAAG CTTTTTTG-3′, named as shFyn1 and shFyn2, respectively. A scrambled sequence 5′-CACCGTTCTCC GAACGTGTCACGTCAAGAGATTACGTGACACGTTCG GAGAATTTTTTG-3′ was cloned into the same vector as a control (shNC).

In utero electroporation

In utero electroporation was performed as previously described (Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001). In brief, pregnant C57BL/6 mice were deeply anesthetized with sodium pentobarbitone (50 mg/g), and their uterine horns were exposed. Plasmids were prepared using the Endo Free plasmid purification kit (Qiagen, Shanghai, China). Plasmid DNA solution (3–5 μg/μl) mixed with 0.1% Fast Green solution was injected into one of the embryonic brain lateral ventricles using a glass micropipette. After plasmid injection, electroporations with five pulses at 30 V separated by 950 ms were applied (ECM 830; BTX). The uterine horns were then placed back into the abdominal cavity, allowing the embryos to continue normal development.

Cell culture and transfection

Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah, USA) and 1% penicillin–streptomycin (10,000 U/ml, Sigma, Shanghai, China) at 37 °C and 5% CO_2. Cells were cotransfected with the pCAG-Fyn-EGFP and RNAi vector using the X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland) according to the manufacturer's guidelines.

Antibodies and agents

Primary antibodies used for immunohistochemistry study and western blot analysis were mouse anti-Fyn (1:250; sc-434; Santa Cruz, Shanghai, China), rabbit anti-GFP (1:1000; AB3080P, Millipore, Massachusetts, USA), goat anti-Brn2 (1:1000; sc-31983, Santa Cruz), and mouse anti-GAPDH (1:10,000; G8795; Sigma, Shanghai, China). Bovine serum albumin fraction V, Triton X-100, DAPI, and normal goat serum were purchased from Sigma.

Immunohistochemistry

The mouse embryos were sacrificed and their brains were removed and immersion-fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4) at 4 °C overnight. The postnatal mice were perfused transcardially with saline followed by 4% PFA in 0.1 M PB for 15 min, and brains were removed and postfixed with 4% PFA for 48 h at 4 °C. Brains were embedded in 4% agar and cut into 50 μm sections using a Leica VT 1000S vibratome. Brain sections were incubated with specific primary antibodies diluted in 0.1 M PB with 0.1% Triton at 4 °C overnight. After rinsing in 0.1 M PB, sections were incubated with the corresponding Alexa Fluor-conjugated secondary antibodies donkey-anti-rabbit 488 (1:300, Millipore) and donkey-anti-goat 568 (1:300, Millipore) at room temperature in the dark for 3 h. Sections were counterstained with DAPI (1:1000) and mounted in Dako.

Immunoblot analysis

HEK-293T cells were harvested 72 h after transfection and lyzed in cold RIPA buffer. Lysates were centrifuged at 4 °C and 15,000×*g* for 20 min; supernatant was collected and mixed with 5× concentrated gel-loading buffer (50 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 5%