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ORIGINAL ARTICLE

Nyap1 Regulates Multipolar–Bipolar Transition and Morphology of Migrating Neurons by Fyn Phosphorylation during Corticogenesis

Shuzhong Wang[1], Xuzhao Li[1], Qianru Zhang[1], Xuejun Chai[2], Yi Wang[3], Eckart Förster[4], Xiaoyan Zhu[1,*] and Shanting Zhao[1,*]

[1]College of Veterinary Medicine, Northwest A & F University, Yangling, Shaanxi 712100, PR China, [2]College of Basic Medicine, Xi'An Medical University, Xi'An, 710021, PR China, [3]State Key Laboratory of Chinese Medicine and Molecular Pharmacology (Incubation) and Shenzhen Key Laboratory of Food Biological Safety Control, Shenzhen Research Institute of Hong Kong Polytechnic University, Shenzhen 518057, PR China, [4]Department of Neuroanatomy and Molecular Brain Research, Ruhr University Bochum, Bochum 44801, Germany

Address correspondence to Shanting Zhao and Xiaoyan Zhu, College of Veterinary Medicine, Northwest A&F University, Yangling 712100, Shaanxi, PR China. Email: zhaoshanting@nwsuaf.edu.cn; xyzhu0922@163.com.

Abstract

The coordination of cytoskeletal regulation is a prerequisite for proper neuronal migration during mammalian corticogenesis. Neuronal tyrosine-phosphorylated adaptor for the phosphoinositide 3-kinase 1 (*Nyap1*) is a member of the *Nyap* family of phosphoproteins, which has been studied in neuronal morphogenesis and is involved in remodeling of the actin cytoskeleton. However, the precise role of *Nyap1* in neuronal migration remains unknown. Here, overexpression and knockdown of *Nyap1* in the embryonic neocortex of mouse by in utero electroporation-induced abnormal morphologies and multipolar–bipolar transitions of migrating neurons. The level of phosphorylated *Nyap1* was crucial for neuronal migration and morphogenesis in neurons. Furthermore, *Nyap1* regulated neuronal migration as a downstream target of Fyn, a nonreceptor protein-tyrosine kinase that is a member of the Src family of kinases. Importantly, *Nyap1* mediated the role of Fyn in the multipolar–bipolar transition of migrating neurons. Taken together, these results suggest that cortical radial migration is regulated by a molecular hierarchy of Fyn via *Nyap1*.

Key words: Fyn, in utero electroporation, neuronal migration, *Nyap1*, tyrosine phosphorylation

Introduction

The development of the mammalian neocortex is a complex process that tightly links the cosmos of proliferation, migration, differentiation, and apoptosis of multiple cell types. Newborn neurons originating from the differentiation of neural progenitor cells undergo cell-fate commitment and migrate in a precise temporal pattern to form the six layers of the cerebral neocortex (Berry and Rogers 1965; Takahashi et al. 1996; Geschwind and Rakic 2013; Tan and Shi 2013; Shepherd and Rowe 2017). Projection neurons are generated in the ventricular zone (VZ)/subventricular zone (SVZ), which then migrate through the intermediate zone (IZ) toward regions in the cortical plate (CP) (Bystron et al. 2008). Migrating neurons become multipolar with multiple neurites when they are mostly located above the VZ or in the multipolar accumulation zone.

Subsequently, neurons migrate through the IZ into the CP via radial glial cell (RGC)-guided locomotion, where they finally reach the outermost region of the CP and extend axonal and dendritic branches (Bystron et al. 2008). Neuronal migration and morphogenesis require a series of complex coordinated processes, the impairment of which may contribute to various neurological diseases, such as lissencephaly, epilepsy, and mental retardation (Heng et al. 2010).

The phosphoinositide 3-kinase (PI3K) signaling pathway is a canonical intracellular pathway in neurons. Substantial evidence suggests that the PI3K signaling pathway participates in various cellular events, including proliferation, survival, apoptosis, and morphogenesis (Rodgers and Theibert 2002; Shi et al. 2003; Jaworski et al. 2005; Kumar et al. 2005; Cheng et al. 2018b). By screening for tyrosine kinase substrates of PI3K in the brain, a new family of proteins named the neuronal tyrosine-phosphorylated adaptor for the PI3-kinase (NYAP) family was identified (Yokoyama et al. 2011).

NYAPs, the major phosphoproteins in the brain, occupy at least three-fourths of PI3K-associated tyrosine phosphorylation, which is involved in the activation of PI3K by binding to PI3K p85. The family members of NYAPs include *Nyap1*, *Nyap2*, and *Myosin16/Nyap3*. NYAPs are specifically expressed in neurons but not in astrocytes or oligodendrocytes, and all three members show expressions in the CP rather than VZ and IZ during embryonic stages (Yokoyama et al. 2011). It has been shown that NYAP-mediated remodeling of the actin cytoskeleton affects neuronal morphogenesis. Neurite outgrowth is regulated by the Contactin-Fyn-NYAP-PI3K pathway, which is sustainably present for a clear reduction in the NYAPs-KO brain size, on account of neuronal hypotrophy (Yokoyama et al. 2011). Currently, only basic features of NYAPs have been reported. However, the role of NYAPs in neuronal migration remains unclear.

The nonreceptor protein-tyrosine kinase Fyn plays important roles in neuronal morphogenesis and movements (Umemori et al. 1992; Sasaki et al. 2002; Morita et al. 2006; Kotani et al. 2007; Huang et al. 2017). Kuo et al. (2005) reported that Fyn knockout mice showed abnormalities of layer II-III neurons, and the layering of the CP in Fyn and Src (a tyrosine kinase) mutant mice displayed a reeler-like phenotype caused by a mutation of reelin. Existing evidence suggests that Fyn is one of the major tyrosine kinases in the brain (Umemori et al. 1992). Moreover, Fyn knockdown inhibits the radial migration of late-born neurons by regulating cytoskeletal dynamics and the multipolar–bipolar transition of newly generated neurons (Huang et al. 2017). NYAPs are tyrosine phosphorylated by Fyn upon contactin stimulation, but the detailed associated functions of Fyn and NYAPs in neurons remain unknown.

In the present study, the contribution of *Nyap1* for corticogenesis in the mouse brain was examined. We found that the instability of *Nyap1* expression disrupted radial migration of projection neurons by affecting neuronal morphology. The tyrosine phosphorylation of *Nyap1* is involved in the radial migration and morphology. Moreover, *Nyap1*-mediated radial migration during cortical development was regulated by Fyn phosphorylation.

Materials and Methods

Animal and Tissue

KunMing mice were obtained from the experimental animal center of Xi'an Jiaotong University, China. The mice were housed under standard temperature and humidity conditions and were provided with food and water. The time of vaginal-plug appearance was defined as embryonic day 0.5 (E0.5). All of the care and handling of animals and the experiment activities were conducted according to the Guide for Care and Experimental of Laboratory Animals of Northwest A&F University, and were carried out in accordance with the Animal Care Commission of the College of Veterinary Medicine, Northewest A&F University (certificate no.: SCXK [SHAAN] 2017-003).

Plasmid Construction

The Eukaryotic expression vector of pCAG-MCS-EGFP was used in this study, which contained a modified chicken β-actin promoter with the CMV-IE enhancer and Rabbit β-globin splice acceptor (CAG promoter). The full CDS length *Nyap1* (GenBank accession no. NM_ 001347505.1) was amplified by reverse transcription-polymerase chain reaction (PCR) from the neocortex complementary DNA library of an embryonic mouse with the following primers: F-Nyap-1: 5′-CGGAATTCCGATGAACCTTCTC TACCGAAAAACC-3′; R-Nyap-1: 5′-CCAAGCTTGGGATGGCAGTGT CCCAGAGGA-3′ (Sangon Biotech, China). The expression vector for Nyap1 fusion protein was constructed by Nyap1 CDS joined.

Fyn RNA interference (RNAi) vector has been described previously. *Nyap1* RNAi vectors were purchased from GenePharma Co., Ltd (Shanghai, China). RNAi target sequences were selected from the mouse *Nyap1* sequence (GenBank/accession no. 001347505.1). To construct the small interfering RNA expression vector to silence *Nyap1*, the following hairpin sequences (shNyap1–1: 5′-GGAATGCTGTGTGCCAGTTCG-3′; shNyap1–2: 5′-GCAAACGCTGCGTGTGCAAGG-3′) were cloned into a pGPH1 vector, for which RNAi was performed by using an RNAi expression vector with an H1 promoter. Synonymous mutations were performed for the third nucleotide of each codon in the target sequence to create Nyap1-R. Overlap-extension PCR was performed to create the mutants of *Nyap1*. Mutations of Tyr212 and Tyr257 to phenylalanine were used to construct $Nyap1^{Y212F}$, $Nyap1^{Y257F}$, and $Nyap1^{Y212/257F}$, respectively.

Cell Culture and Transfection

Human embryonic kidney (HEK) 293 T cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Gibco®, Life Technologies, USA) and 1% penicillin-streptomycin (10 000 U/mL, Sigma, Shanghai, China) at 37°C and 5% CO_2. Cells were transfected by using Lipo2000 (Sigma, Shanghai, China), and they were cultured for 2 days.

Western Blot Analysis

Proteins harvested from HEK-293 T cells were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes using an electroblotting apparatus (Bio-Rad, USA). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with Tween-20 (TBST) (pH 7.4, with 0.05% Tween 20) for 2 h at room temperature (RT), and subsequently probed overnight at 4°C with primary antibodies. The filters were washed three times in TBST and then incubated for 2 h at 20–25°C with secondary antibodies. The Enhanced Chemiluminescence Substrate kit (Solarbio, China) was used to detect the bands. For quantification of the western blots, band intensities were measured relative to the control bands. In each group, three western blots were analyzed.

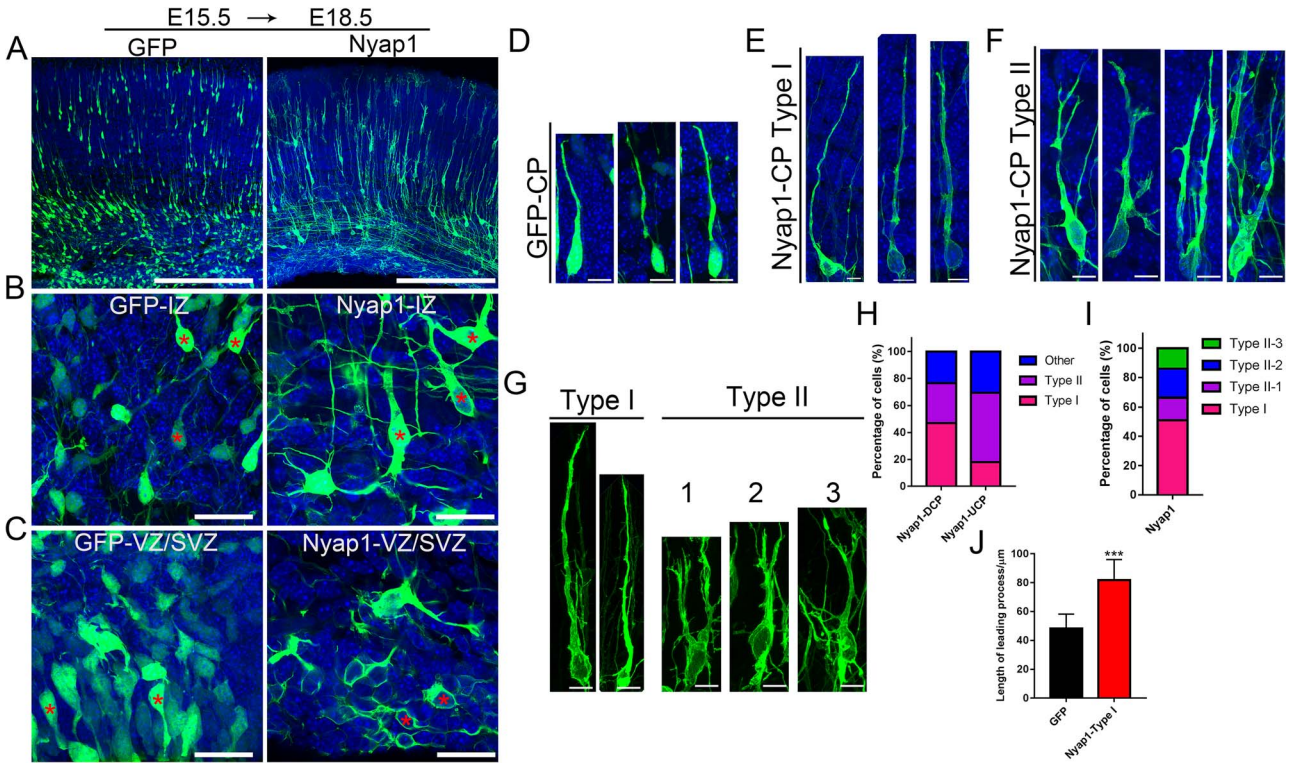

Figure 1. *Nyap1* overexpression alters the morphology of migrating neurons. (*A*) Overview of neocortex transfected with GFP (left) and *Nyap1* (right). The cell nuclei were counterstained with DAPI (blue). (*B*) Higher magnification in the IZ. Neurons showed multipolar morphology. The neurons transfected with *Nyap1* showed thick branching processes compared with those in the GFP control group. (*C*) Higher magnification in the VZ/SVZ. (*D–F*) Higher magnification of migrating neurons in the CP of E18.5 of brains transfected with GFP (*D*) and *Nyap1* (*E*, *F*). The labeled neurons in the GFP control group showed the typical bipolar morphology with long, thick leading processes and short, thin trailing processes. (*E*) Some neurons overexpressing *Nyap1* showed a longer leading process. (*F*) Other neurons overexpressing *Nyap1* gave rise to many branches from the leading process. (*G*) Classification of neurons in the *Nyap1* group in the CP. (*H*) Percentage of different neurons in the CP. (*I*) The distribution of different types of neurons in the CP. (*J*) The length of the leading process compared with GFP and Nyap1-Type 1. Data are collected from more than 100 cells in three brains. Scale bars, 250 μm (*A*), 25 μm (*B*, *C*) and 10 μm (*D–G*); E: embryo; Histograms show mean ± SEM (40 neurons in each group). ***$P < 0.001$ by Student's *t*-test.

In utero Electroporation

Plasmids were purified using the Endo-free Plasmid Maxi Kit (Omega Bio-Tek). All of the pregnant mice with embryos at E15.5 were deeply anesthetized with sodium pentobarbital (Sigma, Shanghai, China) and their intrauterine embryos were surgically manipulated, as described previously (Tabata and Nakajima 2001). Next, 1–2 μL plasmid DNA solutions (3–5 μg/μL) mixed with 0.1% Fast Green solution were injected into the lateral ventricles and in utero electroporations were performed with five pulses at 30 V separated by 950 ms (ECM 830; BTX). The uterine horns were then placed back until the embryos developed to the appropriate stages.

Immunohistochemistry

Mouse embryos at E17.5 to postnatal day 1 (P1) were sacrificed, and all of the brains were removed and immersion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, PH = 7.4) at 4°C for 3 days. All of the brains were embedded in 4% agar and sliced into 50-μm coronal sections using a vibratome (VT 1000S; Leica Microsystems, Wetzlar, Germany). The coronal brain sections were treated with Triton X-100 solution (0.1% Triton X-100 and 0.1 M PB) for 1 h at RT, and they were then blocked with blocking solution (0.1 M PB, 5% normal goat serum and 1% bovine serum albumin) for 2 h at RT. The primary antibody was incubated overnight at 4°C; secondary antibodies and propidium iodide or diaminophenylindole (DAPI) were incubated at RT for 4 h after the slices were rinsed three times with 0.1 M PB. The slices were mounted with fluorescent Mounting Medium (Dako, Carpentaria) on clean glass slides.

Photomicrographs and Analysis

All of the slices were photographed using a structured illumination microscope (Zeiss observer Z1) or a confocal laser-scanning microscope (Leica TCS SP8). Every group contained six slices from three brains in separate experiments. We used Image J analysis software to quantitatively analyze the labeling-cell distribution in vivo and morphology in each layer by electroporation of the expression vector. For analysis of variance, all of the differences for statistical significance were tested by using the Tukey–Kramer test, and then the mean ± SEM of the distances and quantities were calculated.

Results

Nyap1 Overexpression Promotes Neurite Branching and Elongation

In a previous study of the morphology of NYAPs-KO neurons in the cerebral cortex, the total neurite length of neurons was

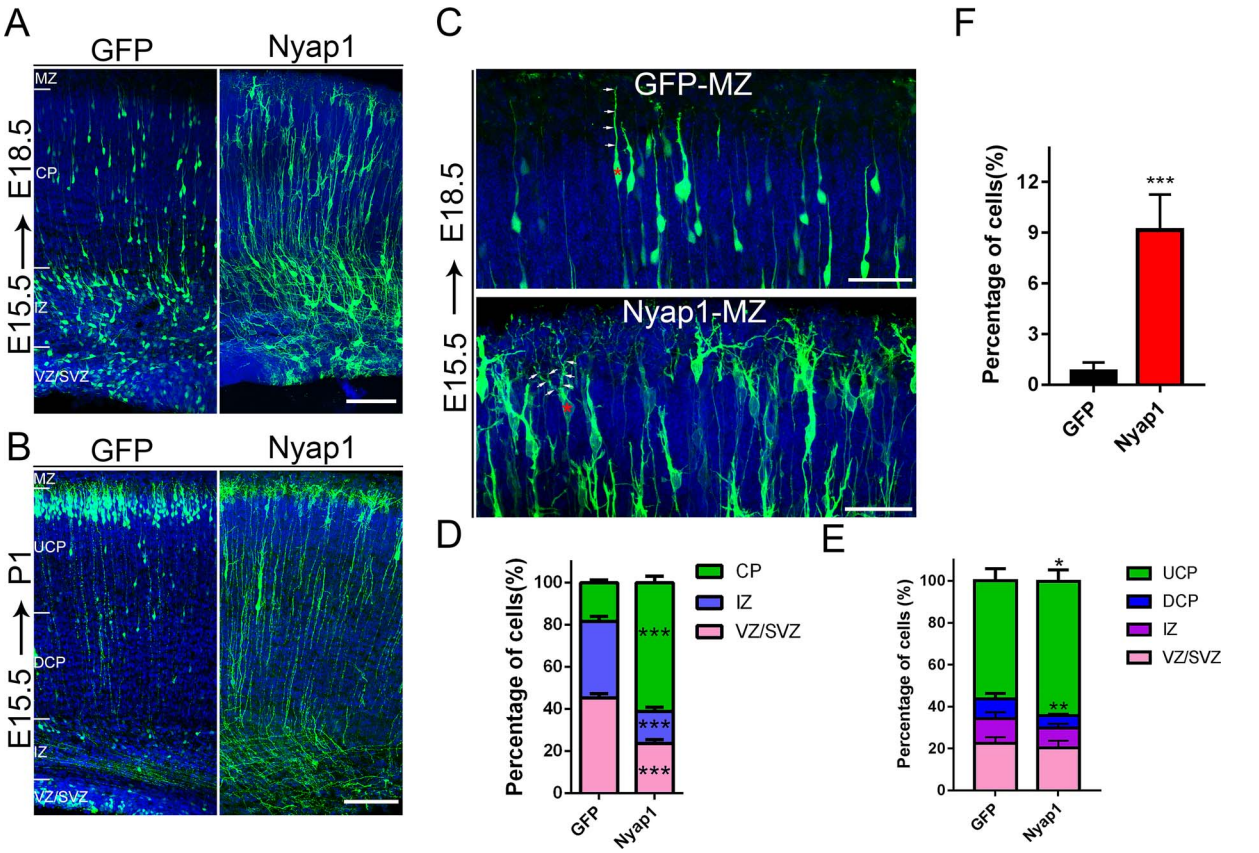

Figure 2. *Nyap1* overexpression results in neuronal migration completeness in advance. Comparison of E15.5–E18.5 (*A*, *D*) and E15.5–P1 (*B*, *E*) between GFP control and *Nyap1* was performed for the distribution of transfected neurons/zone (*D*, *E*). (*A*) More transfected neurons were found in the CP of *Nyap1* group than that of control slices. (*B*) Representative images of mouse cortices fixed at P1. Although almost all transfected neurons reached their final destinations near the marginal zone (MZ) in both of the GFP and the *Nyap1* group, fewer neurons appeared in the IZ in the *Nyap1* group. (*C*) Higher magnification of neurons transfected with GFP or *Nyap1* near the MZ. Arrows pointed representative cells in the MZ. (*D*, *E*) Quantification of the distribution of transfected cells in different layers for each condition at E18.5 or P1. (*F*) Quantification of the percentage of branching leading process of transfected neurons contacting MZ at E18.5. Histograms show mean ± SEM. Scale bars,100 μm (*A*, *B*). 50 μm (*C*). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's *t*-test. E: embryo; P: postnatal.

significantly shorter than that of control neurons when E16 mouse cortical neurons were cultured for 2 days in vitro (Yokoyama et al. 2011). To investigate the effects of *Nyap1* on migrating neurons in the cerebral cortex, we used the in utero electroporation system to introduce expression vectors for *Nyap1* with fusion green fluorescent protein (GFP) into newborn neurons at E15.5. The brains were collected at E18.5 and GFP was successfully detected in many neurons (Fig. 1A). The morphological characteristics of GFP-labeled migrating neurons were considered in the investigation.

High-magnification microscopy showed that Nyap1-positive neurons had thicker branching processes than GFP-positive neurons in the IZ (Fig. 1B). Moreover, Nyap1 protein was interspersed on the cytomembrane in the VZ/SVZ (Fig. 1C). Remarkably, we observed that GFP-positive neurons displayed typical bipolar morphology in the CP (Fig. 1D). However, Nyap1-positive neurons showed more branching (Type II) and elongation (Type I) of neurons (Fig. 1E,F). Next, we analyzed different types of neurons in the *Nyap1*-overexpression group. Type-I neurons showed one longer leading process (Fig. 1G). Type-II-1 neurons showed two leading processes, Type II-2 neurons showed branches with one leading process, and Type II-3 neurons showed branches with multiple leading processes (Fig. 1G). We found that the somata of neurons with a longer leading process were mainly located in the deep cortical plate (DCP), close to the upper intermediate zone (UIZ) (Fig. 1. H). Nearly 50% of neurons in the CP were Type I, while the other 50% of neurons in the CP were branched (Fig. 1I). The average length of the leading process of Nyap1-Type I neurons was 80 μm and were significantly longer than that of the GFP control group, which was 50 μm (Fig. 1J).

Collectively, these results suggest that overexpression of *Nyap1* affects neuronal morphogenesis by promoting branching and elongation of the neuronal leading process.

Overexpression of Nyap1 Accelerates Neuronal Migration

To study the role of *Nyap1* in neuronal migration, we analyzed the distribution of transfected neurons at E18.5 and P1. Overexpression of *Nyap1* resulted in a significant increase in the fraction of electroporated neurons entering in the CP, and a significant decrease in neurons remaining in the IZ at E18.5 (Fig. 2A,D). At P1, more neurons had finished migration and reached the MZ in the *Nyap1* group, and fewer retention neurons appeared in the IZ (Fig. 2B,E). Besides, some labeled cells located in the VZ/SVZ with the basal processes were detected in both of GFP and Nyap1 group (Fig. 2B). Based on the morphology of the cells and our previous study, the labeled cells were identified as RGCs (Chai et al. 2015; Lu et al. 2015).

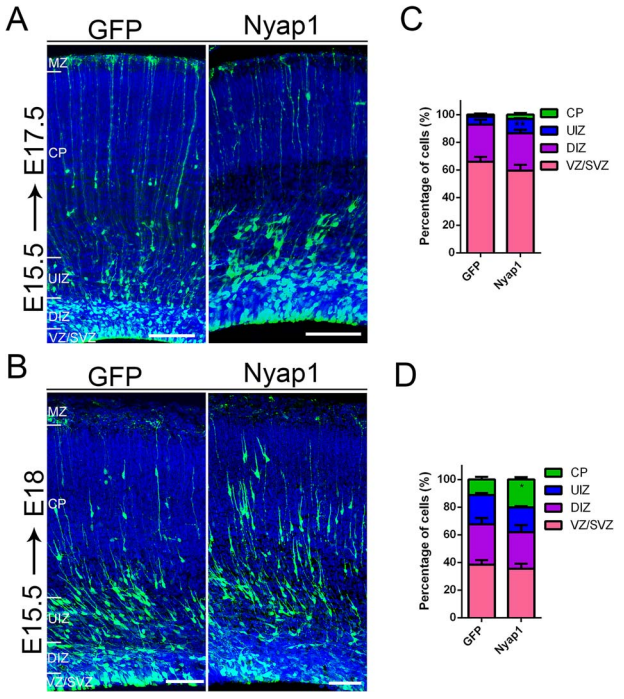

Figure 3. Overexpression of *Nyap1* promotes neurons migrating into CP. (*A*) Brains were electroporated at E15.5 with GFP and *Nyap1* and fixed at E17.5 (48 h). In GFP slices, the vast majority of transfected neurons just reached the deep intermediate zone (DIZ), whereas a considerable amount of neurons were found the UIZ. (*B*) Sections fixed at E18 (60 h). Compared with the GFP groups, an appreciable amount of transfected neurons reached the CP in the *Nyap1* group. (*C*, *D*) Statistical analysis of transfected neurons in four different cortical zones at E17.5 or E18. Histograms show mean ± SEM. Scale bars,100 μm (*A*, *B*). $^{*}P < 0.05$, $^{**}P < 0.01$ by Student's *t*-test. E: embryo.

Once the normal migrating neurons arrived at their final destinations, their leading processes would touch the MZ, and then they would terminate migration after finishing terminal translocation. Finally, these immature neurons would mature by extending their axons and dendrites (Coles and Bradke 2015). Remarkably, at E18.5, in the GFP control group, most neurons had just started to migrate from the IZ and few reached the MZ. Interestingly, an appreciable percentage of neurons reached the MZ in the *Nyap1* group, and most neurons extended their axons and dendrites (Fig. 2C,F). This result indicated that more neurons in the *Nyap1* group arrived at their final destinations.

All of the above abnormal phenomena, induced by *Nyap1* overexpression, demonstrate a function of *Nyap1* in radial neuronal migration. Combined with previous results, this suggests that overexpression of *Nyap1* promotes the completion of neuronal migration.

Overexpression of Nyap1 Induces Earlier Neuronal Migration Entry into the CP

To confirm that *Nyap1* promoted neuronal migration, we refined the time of data collection. Brains were collected at E17.5 (Fig. 3A). In the GFP control group, no transfected neurons appeared in the CP, while only some GFP-labeled neurons were found in the DIZ. The majority of transfected cells were still located in the VZ and SVZ. Similar to the GFP control group, no Nyap1-transfected cell existed in the CP. However, a considerable number of Nyap1-positive cells were found in the IZ. The percentage of Nyap1-positive cells located in the UIZ was higher than that in the GFP control group (Fig. 3C). Next, we examined the position of transfected neurons at E18 that were electroporated at E15.5 (Fig. 3B). Interestingly, a significant fraction of Nyap1-positive neurons was detected in the CP (Fig. 3D). These results indicate that overexpression of *Nyap1* promotes neurons to migrate from IZ to CP.

Nyap1 Knockdown Impairs Neuronal Migration

To further investigate the contribution of *Nyap1* in radial migration, we used an acute loss-of-function approach by RNAi. Two kinds of short-hairpin RNA (shRNA) were used to specifically knock down *Nyap1* messenger RNA. The western blotting analysis showed that the expression of Nyap1 was reduced by 70% in HEK293T cells transfected with shRNA for *Nyap1* (Fig. 4A,B).

Next, we coelectroporated the VZ cells of the cerebral cortex with *Nyap1* shRNA and a plasmid expressing GFP to visualize electroporated cells at E15.5. At E18.5, some neurons transfected with shNC (a control plasmid for RNAi) were found in the CP. A striking difference was observed in the CP and IZ when neurons were transfected with shNyap1–1 plasmids (Fig. 4C). More than 60% of transfected cells clustered in the IZ (Fig. 4E). At P1, *Nyap1* silencing resulted in a significant increase in the fraction of electroporated cells remaining in the IZ, and a significant decrease of cells reaching the CP (Fig. 4D,F). Another shRNA sequence targeting different regions of *Nyap1* called shNyap1-2 also resulted in a similar phenotype (Fig. 4C,D). This migration defect could be rescued by cotransfection of Nyap1-R, indicating that the shNyap1 was targeted specifically and that the resulting phenotype was caused by the loss of *Nyap1* (Fig. 4C,D).

As neurons migrate into the CP from the IZ, they transform from multipolar to bipolar morphology, stared from the cell body through the IZ-CP border, and are oriented toward the pial surface (Hatanaka et al. 2004). Together, these results demonstrate that *Nyap1* is required for the correct positioning of a large fraction of neurons from the IZ to the CP.

Loss of Nyap1 Alters the Morphology of Migrating Neurons

With the dynamic extension and retraction of neurites, migrating neurons first acquire a transient multipolar morphology in the IZ. Subsequently, they acquire a bipolar morphology with a leading process extending toward the pial surface and a thin trailing process toward the ventricle (Parnavelas and Nadarajah 2001; Tabata and Nakajima 2003; Noctor et al. 2004). To investigate whether *Nyap1* knockdown affects neuronal morphology, we analyzed the morphology of transfected neurons with different constructs on the multipolar-to-bipolar transition at P1 (Fig. 5A). We found that *Nyap1* silencing significantly increased the number of multipolar neurons in the IZ, and these multipolar neurons even approached the IZ–CP border (Fig. 5B,C). At high-magnification Nyap1-depleted neurons presented more and thinner neurites; approximately 50% of neurons exhibited this phenotype, demonstrating that *Nyap1* affects multipolar neurons (Fig. 5G,I).

To investigate whether *Nyap1* knockdown also affects neurons in the CP, we analyzed the morphology of CP-located migrating neurons. In the shNC group, most neurons showed the typical bipolar morphology (Fig. 5D,I). In contrast, Nyap1-silenced neurons showed thinner leading processes (Fig. 5E,I). Moreover, the leading processes of neurons transfected with shNyap1-1 gave rise to less branching than that in the shNC group when they reached the MZ (Fig. 5F). Together, these results

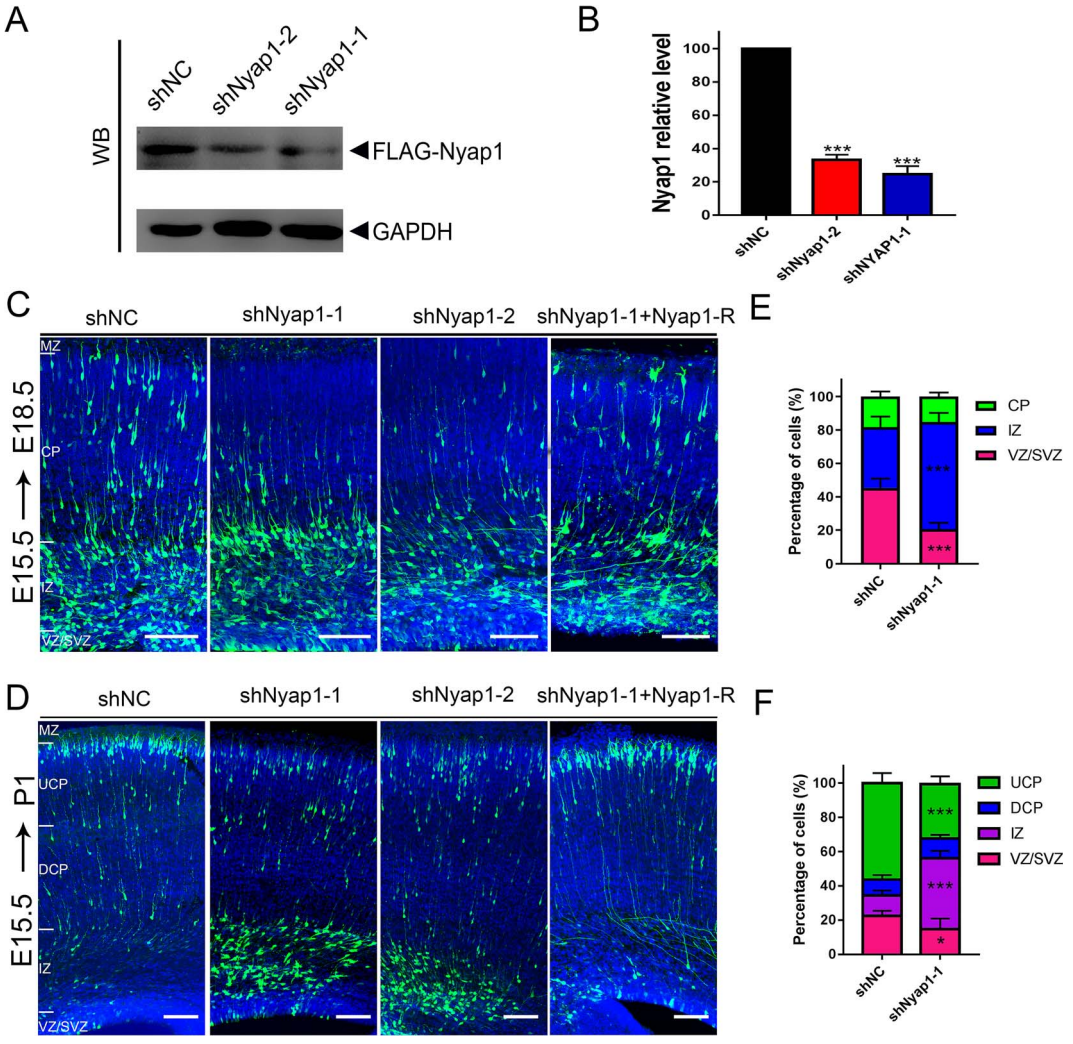

Figure 4. Knockdown of *Nyap1* causes ectopic accumulation of cortical neurons in the IZ. (*A*) Western blotting of Nyap1 in HEK293T cells 72 h after co-transfected with pCAG–FLAG-Nyap1 along with different shRNA constructs. (*B*) The quantification of Nyap1 in each group. (*C*, *D*) shNC, shNyap1-1, shNyap1-2 and shNyap1 plus Nyap1-R were in utero electroporated into the cerebral cortices of E15.5 mouse embryos, respectively. Brains were examined at E18.5 and P1 using coronal sections of cortices stained with GFP. An excessive number of Nyap1-KD cells positioned in the IZ and coexpression with Nyap1-R restored the positioning defects of Nyap1-KD neurons. (*E*, *F*) Quantification of the relative distribution of transfected neurons in different layers for each condition at E18.5 and P1. Histograms show mean ± SEM. Scale bars, 100 μm (*C*, *D*). $^{*}P < 0.05$, $^{***}P < 0.001$ by Student's *t*-test. E: embryo; P: postnatal.

indicate that *Nyap1* is crucial for the morphological dynamics of migrating neurons, which is essential for the multipolar-bipolar transition.

Tyrosine Phosphorylation Is Essential for Nyap1-Mediated Radial Migration

The NYAP Homology Motif (NHM) has previously been shown to contain conserved tyrosine residues and a proline-rich stretch (Fig. 6A). This domain has also been shown to be involved in cellular signal transduction (Yokoyama et al. 2011). To determine whether tyrosine phosphorylation contributes to *Nyap1* in neuronal migration, we investigated the capacity of a mutant version of *Nyap1* with mutated conserved tyrosine residues to phenylalanine (YF mutants) to affect radial migration. At E18.5, the percentage of neurons transfected with $Nyap1^{Y212F}$ or $Nyap1^{Y257F}$ was significantly increased in the CP compared with that transfected with GFP (Fig. 6B,C). There was no significant difference between the distributions of the two mutant groups. (Fig. 6C) However, compared with the *Nyap1* group, the transfected neurons located in the CP were significantly decreased in the $Nyap1^{Y212F}$ and $Nyap1^{Y257F}$ groups (Fig. 6C). This fact suggests that the mutation of *Nyap1* affects radial migration. Furthermore, we examined the morphology of transfected neurons expressing $Nyap1^{Y212F}$ and $Nyap1^{Y257F}$ in the CP and found that most transfected neurons recovered the typical bipolar morphology with one leading process (Fig. 6D,E). The percentage of transfected neurons with a branched leading process was significantly decreased in both mutants (Fig. 6F).

To further investigate the role of tyrosine phosphorylation of *Nyap1* in neuronal migration, both of the two conserved tyrosine residues in the NHM were mutated at the same time. Interestingly, fewer neurons entered the CP at E18.5 in the $Nyap1^{Y212/257F}$ group (Fig. 7A,F). However, most transfected neurons finally reached the normal position at P1, illustrating that the inhibiting effect of $Nyap1^{Y212/257F}$ was temporary

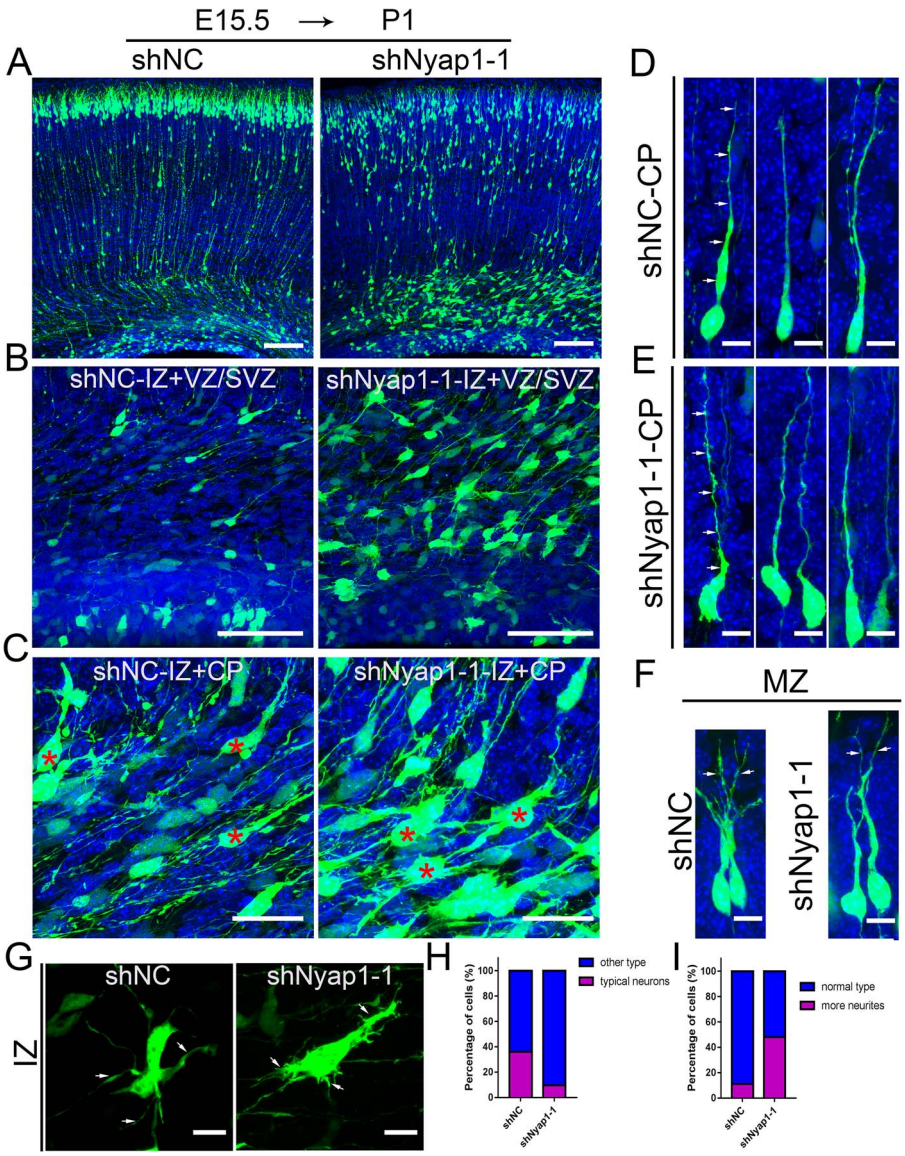

Figure 5. *Nyap1*-KD alters the morphology of migrating neurons. (*A*) Representative images of P1 cortices after in utero electroporation at E15.5 with plasmids encoding shNC or shNyap1-1. (*B*) Representative images of labeled neurons remaining in the IZ after in utero electroporation with plasmids encoding shNyap1-1 compare with the shNC-transfected group. (*C*) Morphological abnormalities of migrating neurons in the IZ-CP border after knockdown of *Nyap1*. Most neurons in the shNC control group showed bipolar morphology, whereas the percentage of neurons with more neurite outgrowth strikingly increased in the shNyap1-transfected group. (*D*, *E*) Higher magnification of neurons transfected with shNC or shNyap1-1 in the CP. Compared with the neurons with a typical thick leading process (white arrows) in the shNC group, the neurons in the shNyap1-1-transfected group showed a thinner leading process (white arrows). (*F*) Higher magnification revealed that neurons contacting the MZ decrease the branching number of leading process in the shNyap1-1-transfected group compared with the neurons transfected with shNC. (*G*) Higher magnification of transfected neurons in the IZ after in utero electroporation with plasmids encoding shNC or shNyap1-1 fixed at P1. (*H*) Quantitative analysis of the number of neurons with the typical leading process from neurons transfected with shNC or shNyap1-1. (*I*) Quantitative analysis of neurons transfected with shNyap1-1 showing increased neurite outgrowth. At least 100 cells from three brains were counted. Scale bars, (*A*), 75 μm (*B*), 25 μm (*C*), 10 μm (*D*–*G*). E: embryo; P: postnatal.

(Fig. 7B,G). Since few neurons migrated into the CP in the *Nyap1*$^{Y212/257F}$ group at E18.5, phosphorylation of *Nyap1* may contribute significantly to the multipolar-bipolar transition at the IZ-CP border. To address this possibility, we compared the morphology of the *Nyap1*$^{Y212/257F}$ group in each region with those in the control group. No significant difference was found between *Nyap1*$^{Y212/257F}$ transfected neurons and GFP-positive neurons in the CP; this finding is consistent with the results of the two single point mutations above (Fig. 7C). Remarkably, a significant difference was detected in neurons located at the IZ–CP border between the GFP control group and the *Nyap1*$^{Y212/257F}$ group (Fig. 7D). In the *Nyap1*$^{Y212/257F}$ group, the percentage of transfected neurons extending two leading processes at the IZ-CP border was much higher than that in the GFP control group (Fig. 7E,H). This indicates that expression of *Nyap1*$^{Y212/257F}$ affects neuronal morphology at the IZ–CP border where the multipolar–bipolar transition occurs and accounts for the inhibition of radial migration. Together, these results suggest that inhibition

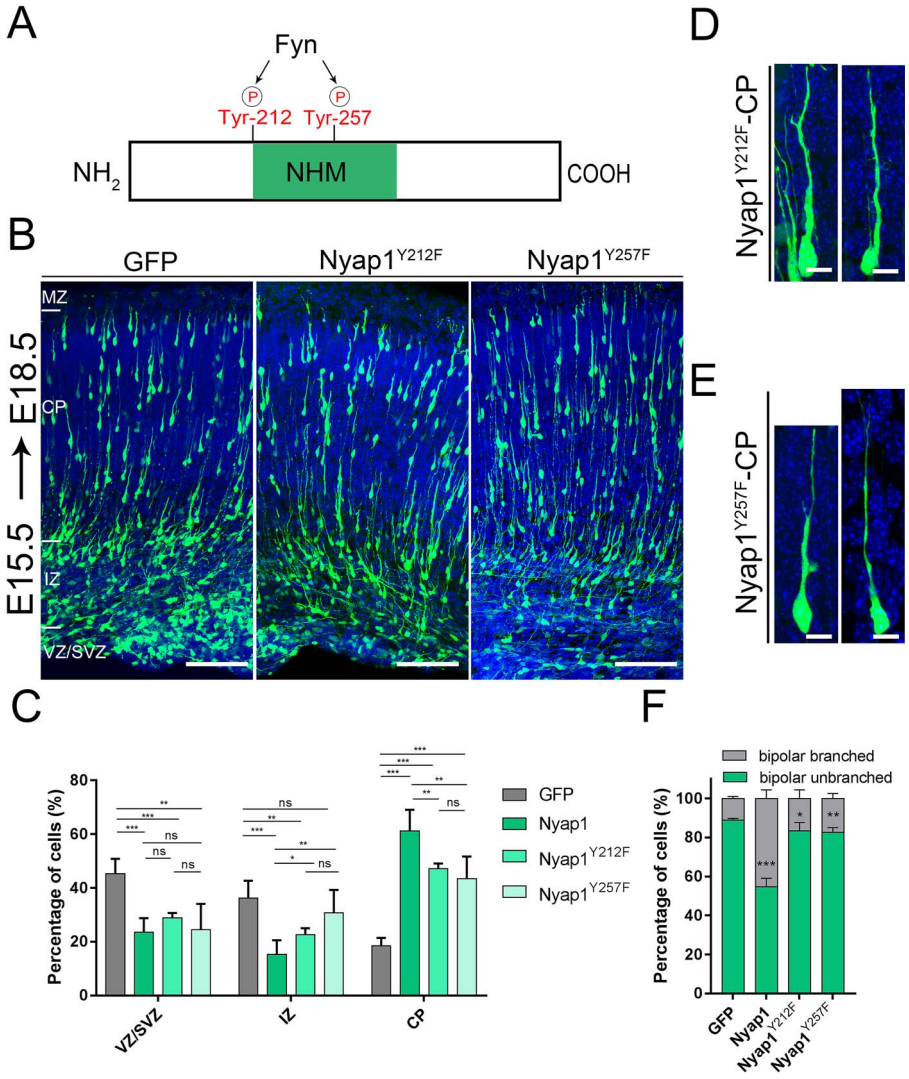

Figure 6. *Nyap1* affects radial migration relying on tyrosine phosphorylation. (*A*) Schematic representation of phosphorylation sites on the *Nyap1*. (*B*) Coronal sections fixed at E18.5 mice after in utero electroporation at E15.5 with plasmids encoding GFP or Nyap1^{Y212F} or Nyap1^{Y257F}. (*C*) Quantitation of distribution patterns in the GFP, *Nyap1*^{Y212F}, *Nyap1*^{Y257F} and *Nyap1* groups at E18.5. (*D*, *E*) Representative images of transfected neurons in the CP with plasmids encoding Nyap1^{Y212F}, Nyap1^{Y257F} at E18.5. (*F*) Quantitation of branched neurons in the CP at E18.5. Data were collected from more than 100 cells in three brains. Histograms show mean ± SEM. Scale bars, 100 μm (*B*), 10 μm (*D*, *E*). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's *t*-test. E: embryo.

of tyrosine phosphorylation of *Nyap1* impairs normal radial migration by affecting the multipolar–bipolar transition.

Nyap1 is Essential for Fyn-Mediated Radial Migration

In a previous study of the genes regulated by Fyn, most phosphorylation associated with Fyn occurred at the tyrosine residues in the NHM motifs of *Nyap1* (Yokoyama et al. 2011) (Fig. 6A). In addition, our previous studies indicated that Fyn affects the neuronal multipolar-bipolar transition and neuronal morphology, and that loss of Fyn inhibits radial migration (Huang et al. 2017). We speculated that *Nyap1* might contribute significantly to Fyn functionality in cortical development. To address this possibility, we investigated the capacity of *Nyap1* to rescue the Fyn knockdown phenotype when coexpressed with Fyn shRNA. As expected, expression of *Nyap1* rescued the defective migration, and more Fyn-silenced neurons migrated into the CP at E18.5 (Fig. 8A). At P1, no significant differences were found between the control group and the rescue group in the CP, suggesting that coelectroporation of *Nyap1* with the Fyn shRNA corrected the abnormal accumulation of Fyn-silenced cells in the IZ (Fig. 8B,D).

To investigate whether *Nyap1* requires Fyn-mediated tyrosine phosphorylation, we performed in utero coelectroporation of shFyn+*Nyap1*$^{Y212/257F}$. In this case, neurons rarely migrated into the CP at E18.5, which is consistent with the result of the shFyn group (Fig. 8A). At P1, numerous neurons still remained in the IZ, and about 30% of neurons cotransfected with shFyn and *Nyap1*$^{Y212/257F}$ were located in the IZ. The rescuing effect of *Nyap1*$^{Y212/257F}$ to shFyn indicates that the adaptor functions of *Nyap1* require Fyn-mediated tyrosine phosphorylation for neuronal migration (Fig. 8B,D).

To further examine the phosphorylation of *Nyap1* by Fyn, a reverse rescue was performed. We investigated whether phosphorylation activation of Fyn (Fyn-CA) disturbed normal

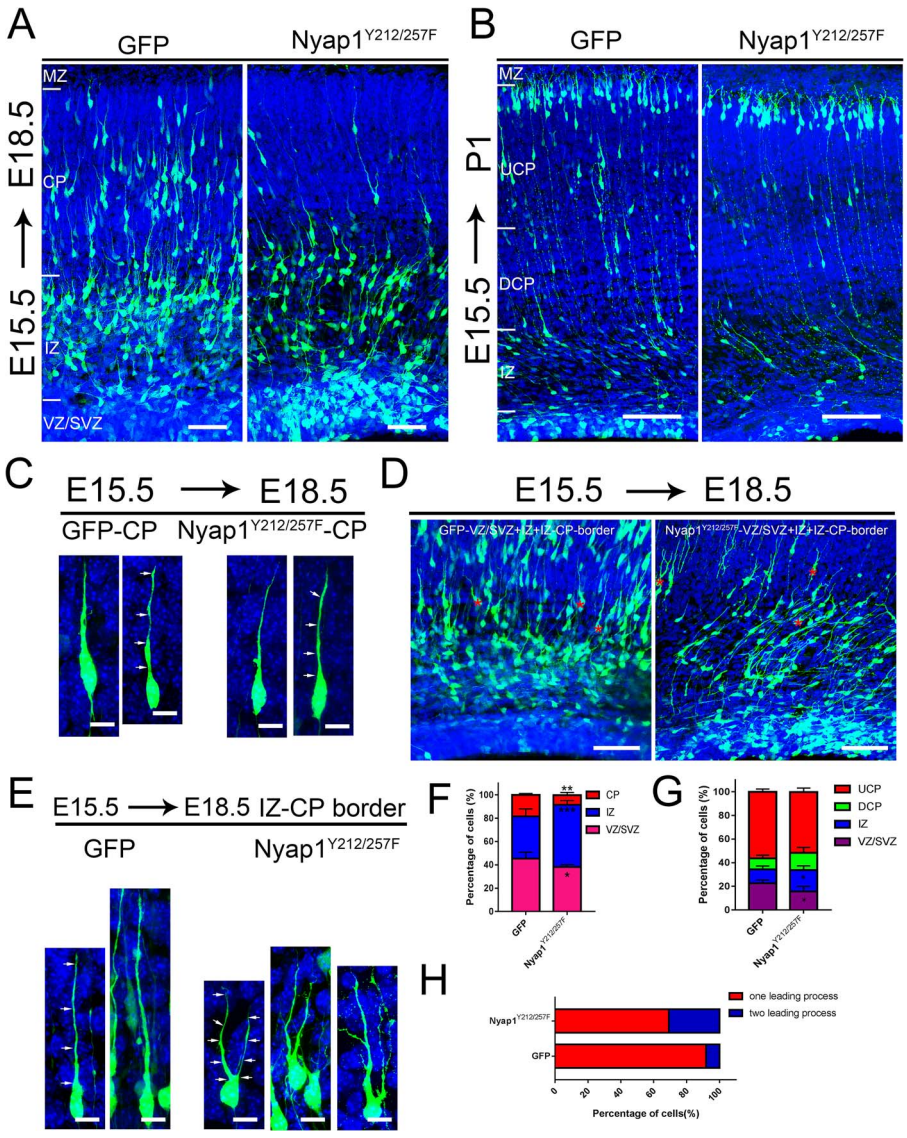

Figure 7. Absence of the tyrosine phosphorylation of *Nyap1* inhibits radial migration. Expression plasmids of GFP or Nyap1$^{Y212/257F}$ were injected into the lateral ventricle of E15.5 embryo brains and electroporated into the neocortex. (*A*) The brains were harvested and analyzed at E18.5. (*B*) Brains were fixed at P1. (*C*) Higher magnifications of the transfected neurons in the CP. (*D*) The representative image in the IZ, VZ/SVZ, and IZ-CP border. (*E*) Higher magnifications of neurons in the IZ-CP border. Compared with the GFP group, more neurons extend two leading processes in the *Nyap1*$^{Y212/257F}$ group. Quantify of transfected neurons in each of regions as a percentage of total GFP-positive cells, (*F*) at E18.5 (*G*) at P1. (*H*) Quantitation of two leading processes neurons in the IZ-CP border at E18.5. Data were collected from more than 100 cells in three brains. Histograms show mean ± SEM. Scale bars, 100 μm (*A*, *B*), 75 μm (*D*) 10 μm (*C*, *E*). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's *t*-test. E: embryo; P: postnatal.

radial migration when combined with the loss of *Nyap1*. Neuronal migration was severely impaired. When neurons were transfected with Fyn-CA, Fyn-CA-positive neurons showed a shape of drastic conglobate edelweiss (agglomeration and unobvious neurite) at P1. However, the effective rescue was not displayed when co-electroporated with Fyn-CA and shNyap1–1 (Fig. 8C,D), implying that there are other main effectors of phosphorylation activation of Fyn other than *Nyap1* during neuronal migration.

Altogether, these results demonstrate that *Nyap1* mediates the phosphorylation signal of Fyn, and that *Nyap1* regulates the crucial step of the multipolar-bipolar transition in the cerebral cortex downstream of Fyn during neuronal migration.

Discussion

In the present study, we demonstrated that *Nyap1* is an important molecule for radial migration by regulating the morphology of migrating neurons. The level of expression of *Nyap1* regulated multipolar-bipolar transition and affected the morphology of migrating neurons. The tyrosine phosphorylation of Nyap1 played a leading role in the corticogenesis. Moreover, *Nyap1* was regulated by Fyn to control radial migration, mainly by phosphorylating *Nyap1* to regulate the morphology of migrating neurons and the multipolar–bipolar transition.

The growth and migration of neurons require continuous cytoskeletal remodeling to provide a versatile cellular framework for force generation and guided movement, in addition

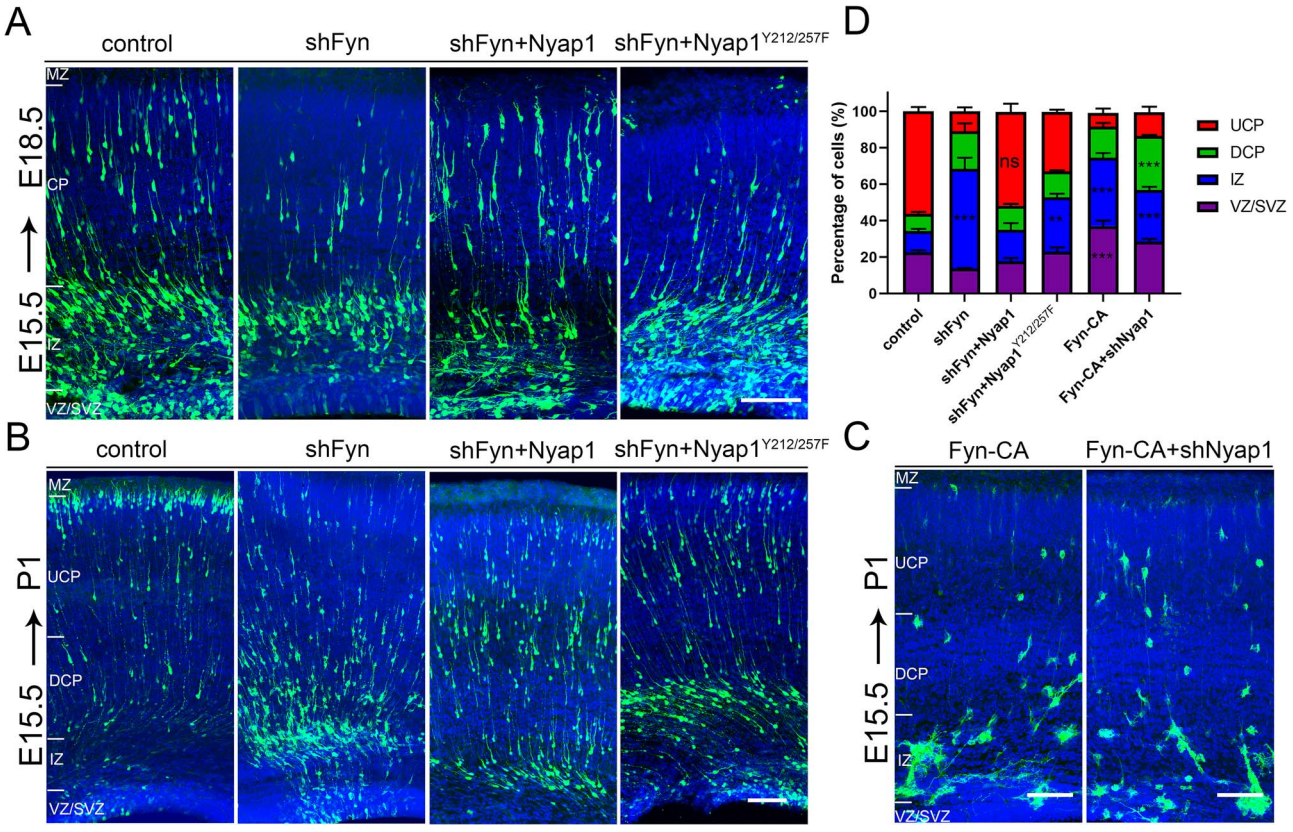

Figure 8. Fyn regulates radial migration of projection neurons via *Nyap1*. Migration defects associated with Fyn knockdown were restored by in utero co-electroporation with shFyn and *Nyap1* overexpression constructs into the cerebral cortices of E15.5 mouse embryos. (*A*) Coronal sections fixed at E18.5 after electroporation at E15.5 with plasmids. (*B*) Brains fixed at P1. Compared with control and shFyn alone, *Nyap1* co-expression rescued neuronal migration defects induced by shFyn. More transfected neurons migrated into the CP. *Nyap1*$^{Y212/257F}$ overexpression weakly rescued neuronal migration defects induced by shFyn, a large number of neurons still stagnated in the IZ. (*C*) Co-electroporated Fyn-CA with shNyap1-1 at E15.5 and visualized at P1. shNyap1-1 weakly counteracts the migration defects with existing of Fyn-CA. (*D*) Percentage distribution of GFP-positive neurons in each cortical layer was quantified and compared between the different groups at E18.5 or P1. Histograms show mean ± SEM. Scale bars, 100 μm (*A*, *B*, *C*). $^{*}P < 0.05$, $^{***}P < 0.001$ by Student's *t*-test. E: embryo; P: postnatal.

to structural support (Coles and Bradke 2015). Cytoskeletal components include microtubules and actin filaments. Remodeling of actin filaments plays critical roles in diverse physiological processes. This remodeling alters cellular morphology, such as neuronal outgrowth or extension. Mutations in actin result in malformations of cortical development (Suetsugu and Takenawa 2003). NYAPs have been shown to be important in the remodeling of the actin cytoskeleton in HeLa cells; actin stress fibers were observed to disappear and collapsed actin filaments were accumulated in the cytoplasm by transfection with various deletion mutants of NYAPs (Yokoyama et al. 2011). PI3K and WAVE complexes are associated with NYAPs, which contribute to actin cytoskeletal remodeling. The PI3K signaling pathway participates in neuronal morphogenesis by PI3K generating phosphatidylinositol 3,4,5-trisphosphate (PIP3) to mediate the recruitment and subsequent activation of PH domain-containing effector proteins (Cantrell 2001; Jaworski et al. 2005; Kumar et al. 2005). In addition, the phosphorylated tyrosine residues of NYAPs bind to the SH2 domains of the PI3K p85 subunit (Zhou et al. 1993; Yokoyama et al. 2011). Here, we demonstrated that gain-of-function *Nyap1* induced neurite outgrowth and extension, which is in accordance with previous reports. When there was loss-of-function of *Nyap1* in neurons, the neuronal leading process became thinner in the CP and the branching of neurons decreased in the MZ, which is similar to neuronal hypotrophy in triple-KO NYAPs mice. However, the total neurite length of NYAP-KO neurons was significantly shorter than that of normal neurons after being cultured for 2 days in vitro. In consideration of the decreased level of *Nyap1* by in utero electroporation, it is reasonable to accept the neuronal phenotype by knockdown *Nyap1*. Studies have shown that small GTPases of the Rho family are powerful initiators and modulators of structural changes in neurons (Zeug et al. 2018). The processes of promoting distinct forms of actin remodeling such as stress fibers, filopodia, and lamellipodia are mediated by Rho, Cdc42 (Cell Division Cycle 42), and Rac (Ras-related C3 botulinum toxin substrate) (Hall 1998). Thus, the relationship between *Nyap1* and the correlational factors mentioned above remains to be elucidated.

Neuronal migration is a coordinated movement associated with rearrangements of the cytoskeleton (Pollard and Borisy 2003; Jovceva et al. 2007). Stability of the leading process is crucial for nuclear translocation (Nadarajah et al. 2001; Nadarajah and Pamavelas 2002; Nishizawa et al. 2007). Meanwhile, actin polymerization is critical in leading processes to help guide migration (Lysko et al. 2011, 2014). The actin-binding protein, cofilin, participates in the dynamic reorganization of the actin cytoskeleton and redefines the direction of cell

migration (Ghosh et al. 2004). Our previous study has shown that the overexpression of cofilin induces the formation of supernumerary processes, which most likely contributes to concomitant migration defects (Chai et al. 2016). On account of this relationship, we assess the contribution of *Nyap1* on neuronal migration. The following two primary modes of neuronal migration have been identified: 1) Early somal translocation occurs before E14.5 and 2) later glial-guided locomotion occurs after E14.5 (Nadarajah et al. 2001). In addition, multipolar migration has been recently recognized as the third mode of neuronal migration in the developing cerebral cortex (Tabata and Nakajima 2003; LoTurco and Bai 2006). Multipolar neurons in the IZ or SVZ extend multiple thin processes in various directions independently of the radial glial fibers. It is an essential process of multipolar to bipolar transitioning when neurons migrate from the IZ into the CP (Sakakibara et al. 2014; Sakakibara and Hatanaka 2015).

Neurons entered the CP earlier when *Nyap1* was overexpressed and included the following: 1) an increase in the length of neurons in the DCP, 2) branching of neurons in the IZ, in which a multipolar shape can be more likely to transform into a bipolar type, and 3) a reduction of the waiting period in the IZ based on the phenotype at E17.5. To further explain the role of *Nyap1* in neuronal migration, knockdown of *Nyap1* was performed. A neuronal migration defect was observed after in utero electroporation at E15.5. Knockdown of *Nyap1* neurons displayed a maintained multipolar shape and increased neurites outgrowth in the IZ at P1, at which the continued movement of neurons depends on the multipolar-bipolar transition. The processes of regulating multipolar migration and the multipolar–bipolar transition remain complicated, although key molecules such as cyclin-dependent kinase 5, doublecortin (DCX), and Fyn have been proposed (Bai et al. 2003; Ohshima et al. 2007; Ye et al. 2014). NYAPs may play similar roles with Fyn partly, as the downstream factor of Fyn. In addition, at P1, knockdown of *Nyap1* resulted in nearly no neurons remaining in the DCP, but a number of neurons finally reached the upper cortical plate. This phenotype may contribute to *Nyap1* having a minimal effect on the formation of mammalian neocortical layers, which is in line with similar characteristics between wild-type and NYAP-KO mice in terms of cortical structure. Additionally, the hypothesis that the factors binding with NYAPs may compensate for the loss of NYAPs is consistent with the compensation for knockout DCX (Corbo et al. 2002; Bai et al. 2003).

In reference to the introduction of *Nyap1* single mutants, a nearly normal phenomenon was observed compared to the overexpression group. This result is consistent with the observation that the phosphorylation was almost abrogated in HEK293T cells when cells were transfected with *Nyap1*-mutations (Yokoyama et al. 2011). In addition, more neurons with two leading processes existed in the IZ–CP border with the expression of double mutants of tyrosine residues of *Nyap1*. Together, these data illustrate that phosphorylation of *Nyap1* is of critical importance in neurons.

To comprehensively understand the molecular mechanism of *Nyap1* in neuronal migration, we investigated the interactions of Fyn and *Nyap1*. The solid-phase phosphorylation screen showed that Fyn directly phosphorylated *Nyap1* in HEK293T cells. Moreover, the decreased phosphorylation of NYAPs was observed in Fyn-KO brains (Yokoyama et al. 2011). Most phosphorylation of *Nyap1* occurred at tyrosine residues in the NHM motifs via Fyn activation. The cortical thickness of NYAP-KO mice was reduced in accord with the attenuation of the cortical

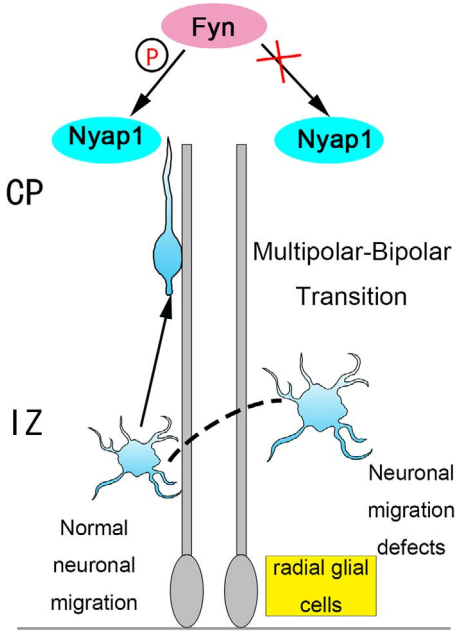

Figure 9. Summary of the role of *Nyap1* in neuronal migration by Fyn phosphorylation in the developing neocortex. The multipolar neurons migrate through the IZ into the CP need undergo multipolar–bipolar transition. Precise expression of Fyn and *Nyap1* could ensure normal neuronal migration. The transparent multipolar–bipolar transition in neurons relies on the phosphate group transmitting from Fyn to *Nyap1*. The multipolar neurons tend to accumulate in the IZ, when Nyap1 exists without Fyn phosphorylation.

thickness of Fyn-KO brains (Yokoyama et al. 2011). A previous study showed that the migration defect in the neocortex is limited to the upper layer in Fyn-deficient mice (Yuasa et al. 2004). Our recent study showed that the knockdown of Fyn causes the accumulation of neurons at the IZ–CP border in which the multipolar–bipolar transition appears (Huang et al. 2017). The above data are consistent with the results of the present study showing that loss of *Nyap1* impairs neuronal migration by affecting the neuronal multipolar–bipolar transition. In addition, a high concentration of Fyn induces the formation of filopodia and lamellipodia in CHO Cells via rearrangement of F-Actin, which is consistent with the finding that NYAPs were involved in the rearrangement of the actin cytoskeleton in HeLa cells (Yokoyama et al. 2011; An et al. 2014). These results demonstrate that Fyn may mediate its effects on neuronal migration via Nyap1 signaling. Furthermore, Fyn participates in the regulation of multiple pathways and targets, such as the Ras/PI3K/Akt signaling pathway and Reelin-Dab1 signaling pathway, as well as in phosphorylating collapsin response mediator proteins and focal adhesion kinase (FAK) (Arnaud et al. 2003; Bock and Herz 2003; Buel et al. 2010; Yadav and Denning 2011; Yeo et al. 2011; Zhang et al. 2018). Therefore, we hypothesize that *Nyap1* is a potential downstream effector of Fyn. Our present study suggests that the precise level of phosphorylation of *Nyap1* is crucial for normal radial migration. Overexpression of *Nyap1* could rescue the neuronal migration defect mediated by knockdown of Fyn, suggesting that these genes act in the same signaling pathway. In addition, we found that Nyap1 with lossing function of phosphorylation hardly rescued the defect of neuronal migration by introducing shFyn, which is consistent that the phosphorylation of *Nyap1* was almost abrogated in HEK 293 T cells when cotransfection of Fyn and *Nyap1*$^{Y212/257F}$ occurred (Yokoyama et al. 2011). All of

the above results indicate that *Nyap1* is a downstream factor of Fyn in neuronal migration. However, the therapeutic action for the migration defects by increasing phosphorylated Fyn is barely satisfactory by decreasing the expression level of *Nyap1*. This demonstrates that phosphorylated Fyn could perturb neuronal migration via downstream signaling targets other than *Nyap1* and that Fyn tyrosine kinase was important for neuronal migration. Together, these observations support the conclusion that *Nyap1* is regulated by Fyn-mediated phosphorylation to regulate the migration of neurons (Fig. 9).

Moreover, knockdown of Fyn in cortical neurons extended a few short neurites after being cultured for 3 days (Huang et al. 2017). Neuronal hypotrophy was observed in NYAP-KO mice after primary culture, whereas neurites did not extend in the presence of Contactin proteins (Yokoyama et al. 2011). Based on the Contactin-Fyn-NYAP pathway, we hypothesize that Fyn-deficient neurons have a Contactin-insensitive phenotype.

In addition, our previous study showed that overexpression of P85 affected neuronal migration and neuronal morphology (Cheng et al. 2018a). Based on the active phosphorylation of NYAPs to PI3K, the studies of *Nyap1* also demonstrate the important functions of PI3K in cortical development (Inaguma et al. 2016). This finding confirms the fact that the Fyn-NYAP-PI3K pathway plays an indispensable role in corticogenesis.

Although we have reported that *Nyap1* regulated neuronal migration, all three members of the NYAP family mediate remodeling of the actin cytoskeleton (Yokoyama et al. 2011). Based on this fact, we hypothesize that *Nyap2* and *Nyap3* affect neuronal morphology and migration as well. However, in situ hybridization analysis of NYAPs has shown that the spatial and temporal expression patterns of *Nyap2* and *Nyap3* are different from those of *Nyap1*. *Nyap1* is expressed in almost all of the CP zones, whereas *Nyap2* is mainly expressed in lower neocortex and *Nyap3* is mainly expressed in the upper CP. In addition, the expression of *Nyap2* begins at E18 and decreases thereafter (Yokoyama et al. 2011). Therefore, *Nyap2* and *Nyap3* might affect neuronal migration at different stages. However, the detailed mechanisms remain to be elucidated.

In summary, all of these data contribute to the understanding of the dynamics of cell movement and its underlying regulatory interactions.

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Notes

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