Histol Histopathol (2014) 29: 871-879
DOI: 10.14670/HH-29.871

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Review

Roles of Rho small GTPases in the tangentially migrating neurons

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Summary. Rho small GTPases are members of the Ras superfamily of monomeric 20~30 kDa GTP-binding proteins. These proteins function as molecular switches that regulate various cellular processes such as migration, adhesion and proliferation. Cycling between GDP-bound inactive and GTP-bound active forms is regulated by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and GDP-dissociation inhibitors (GDIs). Among 20 different mammalian Rho GTPases identified to date, RhoA, Rac1 and Cdc42 have been most extensively investigated; regulation of migration, adhesion and proliferation by these proteins have been well documented in a variety of cell types, including neurons. In neurons, RhoA, Rac1 and Cdc42 are crucial for axon guidance, dendrite formation and spine morphogenesis, where molecular machineries required for cell migration and adhesion play essential roles. Recently, accumulating experimental data indicate the participation of Rho GTPases in neuronal cell migration. To establish the cortical lamination and synapse network formation, highly specialized modes of neuron migration are essential, which include 1) radial migration of excitatory pyramidal neurons along radial glial fibers, 2) tangential migration of GABAergic cortical (inhibitory) interneurons along emerging axon tracts and 3) chain migration of interneurons ensheathed in a glial network, which is observed only in olfactory bulb-directed adult neurogenesis. While roles of Rho GTPases in the radial migration have been well reviewed, knowledge of their functions in tangential migration and chain migration are fragmentary to date. In this review, we focus on the roles of Rho small GTPases and their related molecules in tangential migration, together with the possible application of the electroporation method to analyses for this mode of migration in embryonic and postnatal mouse brain.

Key words: Brain development, Rac, Rho A, Cdc42, Neurodevelopmental disorders

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Introduction

The Rho family small GTPases are known to play important roles in various cellular processes, such as actin cytoskeletal reorganization, transcriptional activation, tumor cell invasion, cell morphology, cell motility and cytokinesis (Bishop and Hall, 2000; Etienne-Manneville and Hall, 2002). Like other GTPases, they act as a molecular switch by cycling between an active GTP-bound state and an inactive GDP-bound one. The balance of these two states is regulated by 3 groups of regulatory factors (Fig. 1). Guanine nucleotide-exchange factors (GEFs) catalyze exchange of GDP for GTP, leading to activation of small GTPases in response to various signals. GTPase-activating proteins (GAPs) increase the intrinsic GTP-hydrolysis activity, resulting in inactivation of the proteins. Guanine nucleotide dissociation inhibitors (GDIs) have the ability to block the cycling between the

GDP- and GTP-bound forms by preventing the exchange of GDP for GTP (Bernards and Settleman, 2004).

For cell migration, polarization in the direction of movement is the first crucial step, followed by formation of cell peripheral actin-based structures, filopodia and lamellipodia, and organized vesicle trafficking toward the leading edge. In the regulation of filopodia and lamellipodia, actomyosin motors play pivotal roles in concert with cell adhesion molecules to provide the mechanical force required for forward movement. In the classic studies with fibroblasts, activation of RhoA, Rac1 and Cdc42 were shown to induce actin cytoskeleton reorganization and formation of stress fiber, lamellipodia and filopodia, respectively (Bishop and Hall, 2000). On the other hand, in neuronal cells, Rho family proteins have been reported to regulate a variety of functions, including axon guidance, dendrite formation and spine morphogenesis (Govek et al., 2005). Rac and Cdc42 signaling pathways promote neurite formation while Rho signaling plays a crucial role in neurite retraction in cultured neuronal cells. About 30 potential effector proteins have been identified that interact with GTP-bound active forms of the Rho family proteins. Although it is still unclear which effector molecules are responsible for the diverse biological effects of Rho GTPases *in vivo*, some have been shown to play pivotal roles in the Rho family-dependent neuronal cellular events.

Experimental data indicating the importance of the Rho family of small GTPases in radial migration of excitatory neuron precursors have been accumulated and comprehensively reviewed (Govek et al., 2011). In contrast, involvement of Rho proteins in the migration of interneuron precursors is still enigmatic. In this review, we first describe the origins and features of interneurons generated in embryonic and postnatal stages. In the second part, we summarize the roles of Rho small GTPases and their related molecules in the tangential migration of interneurons. Finally, involvement of Rho signaling molecules in developmental disorders is summarized.

Interneurons generated during corticogenesis and after birth

Neuronal migration is an essential step for architectural and functional brain development. During the developmental process, there are 2 general types of migration in the cerebral cortex on the basis of its orientation: radial migration, in which postmitotic immature neurons migrate radially toward the pial surface, and tangential migration, in which cells migrate into the developing cortex in a trajectory tangential to the radial plane (Marin and Rubenstein, 2003; Métin et al., 2008). In the developing cerebral cortices, excitatory projection neurons generated in the dorsal ventricular zone (VZ) show radial migration, while interneurons, originated from the lateral or medial ganglionic eminence (LGE or MGE) of the basal ganglia, exhibit tangential migration where neurons migrate orthogonally to the direction of radial migration. It is notabale that neuronal precursors born in LGE differentiate to striatum interneurons (medium spiny neurons) and olfactory interneurons, while MGE-derived precursors become interneurons in the cerebral cortex (Wichterle et al., 2001) (Fig. 2A). Most of these central nervous system interneurons use the neurotransmitter γ-Aminobutyric acid (GABA), and orchestrate the cortical network through synaptic inhibition of excitatory pyramidal neurons. The classification of the interneurons is highly complicated since their unequivocal identification requires a combination of morphological, neurochemical and electrophysiological properties (Ascoli et al., 2008; DeFelipe et al., 2013). Indeed, based on these properties, more than 20 different classes of interneurons have so far been identified in the hippocampus and neocortex, each of them with distinctive spatial and temporal capabilities to influence cortical circuits (Bartolini et al., 2013). In a recent review, neocortical interneurons have been intelligibly classified into 5 broad categories as follows (Bartolini et al., 2013). Based on the classification, the most abundant group includes two main classes of interneurons, basket cells and chandelier cells, which generally express a Ca-binding protein parvalbumin (PV). A second group is characterized by the expression of a neuropeptide somatostatin (SST), including Martinotti cells with a characteristic long axon. The large majority of PV- and SST-positive cells derive from the MGE. The third group of neocortical interneurons includes double-bouquet cells typically positive for vasointestinal peptide (VIP) and a Ca-binding protein calretinin (CR). Neurogliaform cells constitute a fourth group of neocortical interneurons, which generally express reelin and ionotropic serotonin receptor 3a, and possess highly branched short dendrites

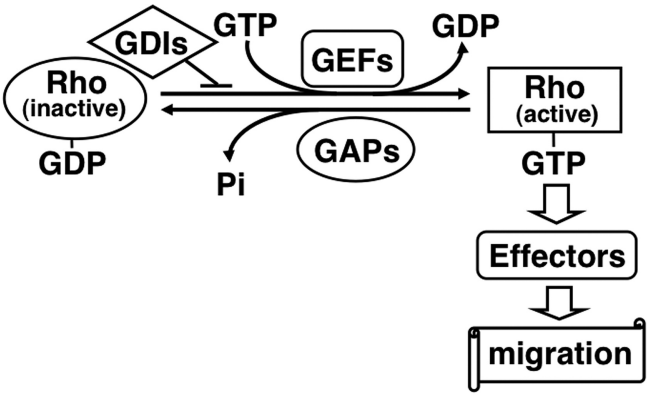

Fig. 1. Model of activity regulation of Rho family small GTPases. Cycling between the active, GTP-bound, and the inactive, GDP-bound forms is regulated by GEFs, GAPs and GDIs. GTP-bound Rho family protein is an activated form and interacts with a number of downstream effectors, leading to a variety of biological responses, including cytoskeletal reorganization and integrin complex assembly which are crucial for cell migration.

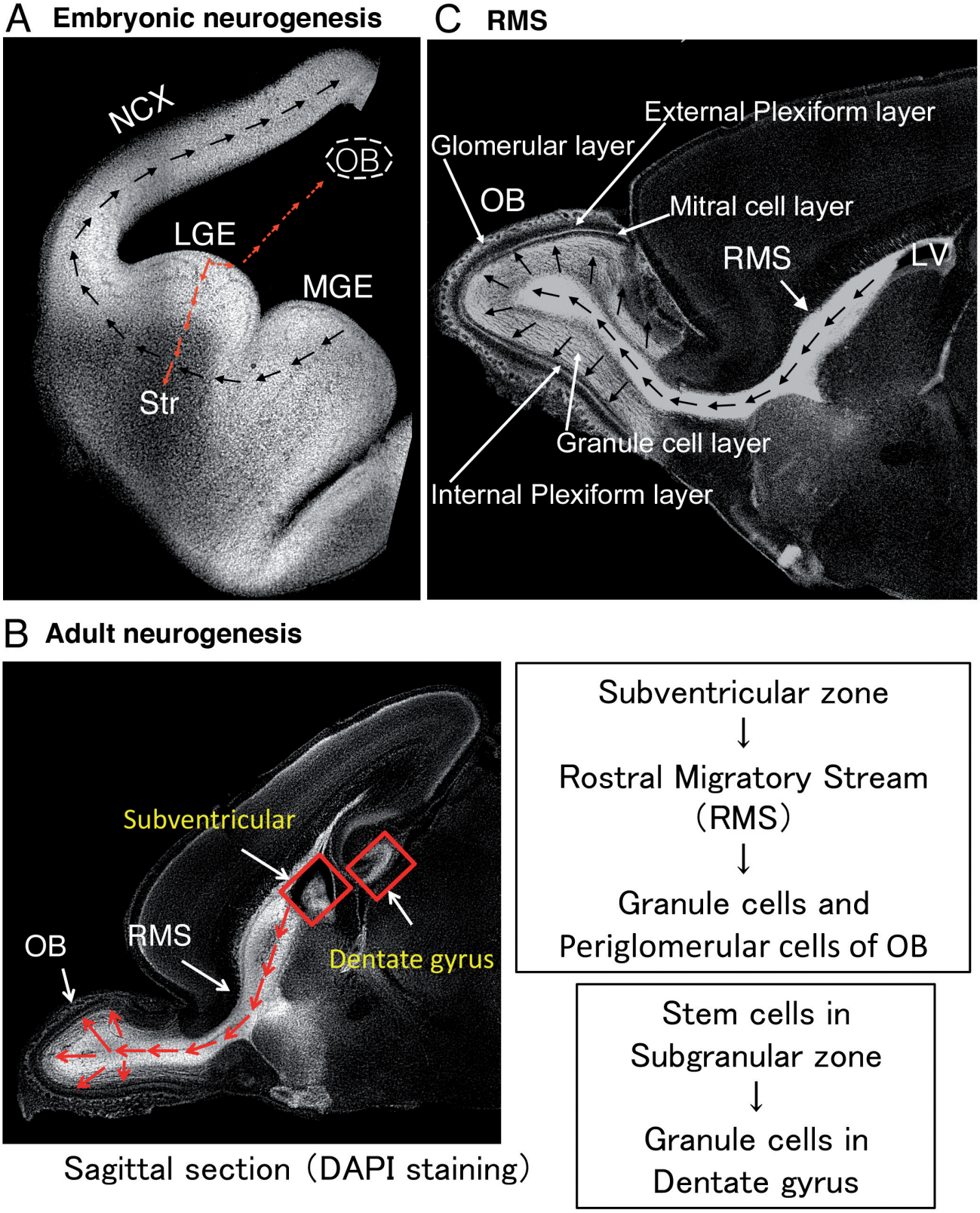

Fig. 2. Pathways of the tangentially migrating neurons in the developing brain. **A.** Embryonic neurogenesis. Coronal section of E14.5 rat brain was stained with DAPI. Arrows indicate the migratory pathways of interneuron precursors. During corticogenesis, most of the precursor cells born in LGE give rise to interneurons in striatum and OB, while MGE-derived precursors migrate into NCX and become interneurons in the cerebral cortex. **B.** Postnatal and adult neurogenesis. Sagittal section of postnatal days 7 (P7) mouse brain was stained with DAPI and arrows indicate the migratory pathways of interneurons generated after birth. Interneuron precursors generated in SVZ migrate into OB while cells generated in the subgranular zone of hippocampus move to dentate gyrus. **C.** RMS. Sagittal section as in **(B)** was stained with DAPI and arrows indicate the migratory pathways of cells. Neural precursors born in SVZ of LV migrate tangentially along the RMS and become interneurons in OB. LGE, lateral ganglionic eminence; LV, lateral ventricle; MGE, medial ganglionic eminence; NCX, neocortex; OB, olfactory bulb; RMS, rostral migratory stream; Str, striatum.

and a defining dense local axonal plexus. Finally, the fifth group of interneurons consists of multipolar cells that often contain neuropeptideY (NPY). Different interneruon subtypes tend to exhibit different membrane firing properties and to have axons with different arborization patterns and synaptic targets.

On the other hand, in postnatal and adult brain, precursors of olfactory interneurons are generated in the subventricular zone (SVZ) of the lateral ventricle and migrate tangentially along the rostral migratory stream (RMS) to the olfactory bulb (OB) (Alvarez-Buylla and Garcia-Verdugo, 2002) (Fig. 2B,C). Since olfactory bulb interneurons have not been as extensively characterized as cortical interneurons, they were classified largely based on marker expression into 3 main populations: granule cells, external plexiform layer interneurons, and periglomerular cells (Batista-Brito et al., 2008).

To clarify the mechanism of the interneuron generation *in vivo*, targeted viral-mediated gene transfer, knockout or transgenic mice have been commonly employed. During the last decade, *in utero* electroporation of embryonic mouse brain and *in vivo* electroporation of postnatal mouse brain have been applied for the investigation of interneuron development *in vivo*. *In utero* electroporation system has been mainly used for the analysis of embryonic radial migration of neural progenitor/precursor cells (Fukuchi-Shimogori and Grove, 2001; Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001). At a later time, the electroporation of expression plasmids into the ganglionic eminences was exploited and this system allowed the selective labeling of migratory interneuron progenitor cells in embryonic stage (Borrell et al., 2005). More recently, *in vivo* electroporation has been applied to investigate postnatal olfactory neurogenesis (Boutin et al., 2008; Chesler et al., 2008; Fernandez et al., 2011). When we injected GFP-expressing plasmids into neuronal stem cells or precursors at the lateral ventricle of early postnatal mouse (P0-P2), applied electric pulses and continued to raise the pup, GFP-positive cells were observed in the ventricular wall, RMS and OB (Fig. 3). This method needs neither surgery nor stereotaxic apparatus but results in the robust expression of exogenous genes in specific cell populations. This technique is now considered a powerful method to investigate the mechanism of postnatal olfactory neurogenesis.

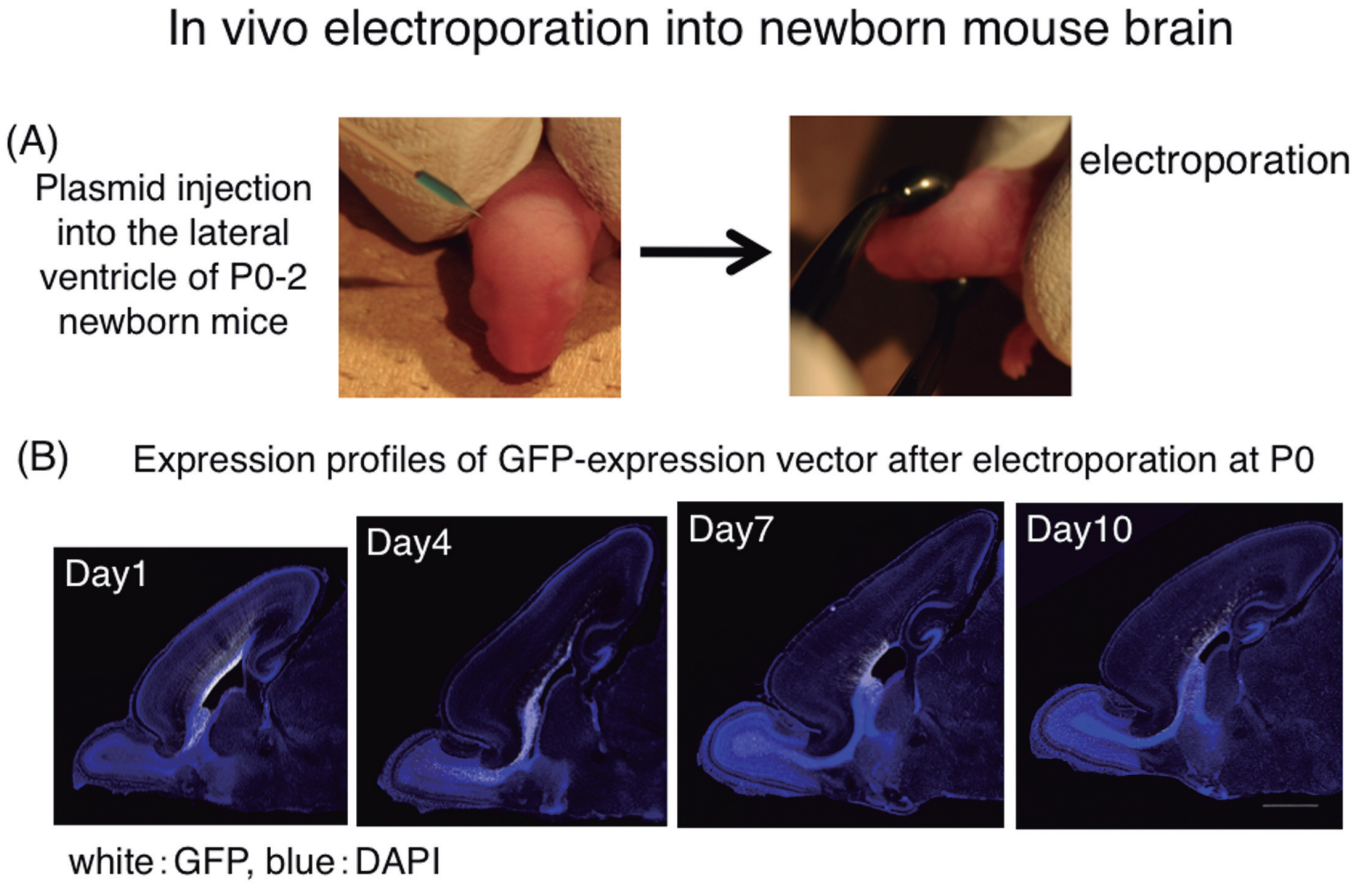

Fig. 3. *In vivo* electroporation. **A.** DNA injection into a P0 mouse brain. Electroporation by holding the mouse head with forceps-type electrodes. **B.** RMS Neuronal migration pathway was highlighted with GFP at various days after electroporation.

Role of Rho family proteins in the tangential migration

There are several reports indicating the participation of Rho family proteins in embryonic and postnatal interneuron migration (Table 1). We will introduce roles of Rac, RhoA and Cdc42 signaling molecules in the tangential migration of interneuron progenitors and precursors in detail.

Rac and its related molecules

The Rac subfamily consists of 3 members, Rac1 ~ 3 (Didsbury et al., 1989; Haataja et al., 1997). The importance of Rac1 in radial migration has been demonstrated using an *in utero* electroporation system (Kawauchi et al., 2003; Konno et al., 2005). Then, involvement of Rac in tangential migration has been studied using conditional knockout mice. Rac1/Foxg1-Cre mice embryos, which lack Rac1 expression in the VZ progenitor/stem cells, had disproportionately smaller OB compared with control embryos; interneurons were absent in the glomerular and granular cell layers in OB at E18.5 whereas GABAergic interneurons were normally formed and present within in the ventral telencephalon (Chen et al., 2007). In contrast, radial migration of cortical pyramidal neurons was preserved in this knockout mouse, although migration delay was observed. The contrasting severity of defects in radial and tangential migration raised 2 possibilities; one is that Rac1 has different roles in these 2 migration modes, and the other is that Rac1 may have a unique role during progenitor differentiation in the ventral telencephalon (LGE and MGE) and endow the nascent neurons with the migration competency. In this context, discernible morphological abnormalities have not been found in E18.5 Rac1/Dlx5/6-CIE(Cre-IRES-EGFP) mice, where Rac1 was deleted in cells emerging from VZ in LGE and MGE, and the granule cell and glomerular layers were formed properly in the olfactory bulb (Chen et al., 2007). These results may indicate that Rac1 is not essential for the cell motility per se but is required for the differentiation of VZ progenitors in LGE and MGE to establish the competency of migration in subsequent nascent neurons.

When Rac1 expression was selectively ablated in MGE-derived progenitors during corticogenesis, migration impairment was observed; Rac1-depleted interneurons were found to form aggregates in the ventral telencephalon and show morphological defects in their growing processes (Vidaki et al., 2012). In proliferating MGE-derived progenitors of cortical interneurons, Rac1 appeared to be necessary for their transition from G1 to S phase, at least in part by regulating cyclin D1 expression and retinoblastoma protein phosphorylation (Vidaki et al., 2012). It should be noted, however, that development of Rac1-deficient postmitotic interneurons was almost normal; any morphological differences, such as the length and number of leading process and the number of primary branches, were not observed in the interneurons, suggesting the developmental stage-specific requirement of Rac1 activity (Vidaki et al., 2012).

Rac3 is specifically expressed in neurons in peripheral and central nervous systems (Corbetta et al., 2005) and the expression has a peak at late

Table 1. Participation of Rho GTPase signaling molecules in the development of tangentially migrating interneurons.

Molecules	Materials	Results	References
Rac1	Rac1/Foxg1-Cre KO mice	Migratory defects of LGE- and MGE-derived neural cells.	Chen et al., 2007
	Rac1/Nkx2.1-Cre KO mice	Perturbation of cell cycle exit and aggregation of MGE-derived neural cells in ventral telencepharon.	Vidaki et al. 2012
	Rac1 KD by virus injection	Reduction of relative number of cells that migrate to OB.	Khodosevich et al., 2009
Rac1/Rac3	Rac1/Rac3 dKO mice	Selective reduction of PV-positive cells in cortex and hippocampus. Migratory defects in cortical interneuron.	Vaghi et al., 2014
WAVE1	WAVE1 KD in SVZ	Reduction of relative number of cells that migrate to OB.	Khodosevich et al., 2009
PAK3	PAK3 KD in Dlx1/Dlx2 dKO mice	PAK3 KD rescues the migratory defect on tangentially migrating neuron.	Cobos et al., 2007
IQGAP1	IQGAP1 KO mice	Delayed differentiation of neural progenitors into neural precursor cells in RMS.	Balenci et al. 2007
Vav3	Vav3 KD in SVZ	Reduction of newborn neuron in OB.	Khodosevich et al., 2009
RhoA	Expression of RhoA CA or DN in SVZ explants	RhoA CA decreases the migration. RhoA DN increases the migration.	Wong et al., 2001
ROCK	ROCK KD in NPCs prepared from SVZ	Decrease of chain formation and increase of migration.	Leong et al., 2011
	Y-27632 treatment with SVZ explants	Reduced number of migrating cells.	Shinohara et al., 2012
mDia	mDia1/mDia3 dKO mice	Inhibition of tangential migration of cortical interneurons and SVZ neuroblasts.	Shinohara et al., 2012
Cdc42	Expression of Cdc42 DN in SVZ explants	Reduced number of migrating cells.	Wong et al., 2001

CA, constitutively active; dKO, double knockout; DN, dominant negative; KD, knockdown; KO, knockout; LGE, latelal ganglionic eminence; MGE, medial ganglionic eminence; NPC, neural precursor cell; OB, olfactory bulb; PV, parvalbumin; RMS, rostral migratory stream; SVZ, subventricular zone.

embryonic/early postnatal developmental stage (Bolis et al., 2003). Selective reduction of PV-positive interneurons was found in the cerebral cortex of Rac1/Rac3 double knockout (Rac1/Rac3 dKO) mice whereas such cells were normally generated and distributed in Rac3 single knockout mice (Vaghi et al., 2014). Length of leading processes and number of interneurons were also affected in the cortex of Rac1/Rac3 dKO mice (Vaghi et al., 2014). Taken together, Rac1 and Rac3 may synergistically affect the development of cortical interneurons. Involvement of Rac pathway in the postnatal olfactory neurogenesis has also been shown; Rac1 inhibitor reduced the migration of SVZ-derived neuronal precursors in RMS, and knockdown of Rac1 or its effector, WAVE1, in neonatal mouse brain caused marked reduction of newborn neuron in OB (Khodosevich et al., 2009).

Rac-related signaling molecules have also been shown to participate in interneuron migration. Involvement of PAK3 kinase, a downstream effector of Rac and Cdc42 (Bokoch, 2003), in the tangential migration has been reported in the analysis of Dlx1/2 homeobox transcription factors knockout mice (Cobos et al., 2007). Dlx1/2 are essential for the tangential migration of interneurons to neocortex, as well as neurite maturation in the cerebral cortex. In Dlx1/2-double knockout mice, interneuron precursors failed to migrate tangentially and showed increased neurite length, perhaps due to the premature expression of cytoskeleton-regulating proteins, including PAK3. While PAK3 expression is nearly undetectable in MGE of wild type mice embryo, the kinase comes to be upregulated once interneuron precursors arrive at the cortex. In this context, overexpression of PAK3 in the MGE blocked the tangential migration of interneurons. Consistently, siRNA-mediated reduction of PAK3 in the MGE of Dlx1/2$^{-/-}$ mutants, which robustly express the kinase, partially rescued the tangential migration defects. Taken together, repression of PAK3 by Dlx1/2 appears to be critical for the promotion of tangential migration of interneuron precursors from MGE during corticogenesis.

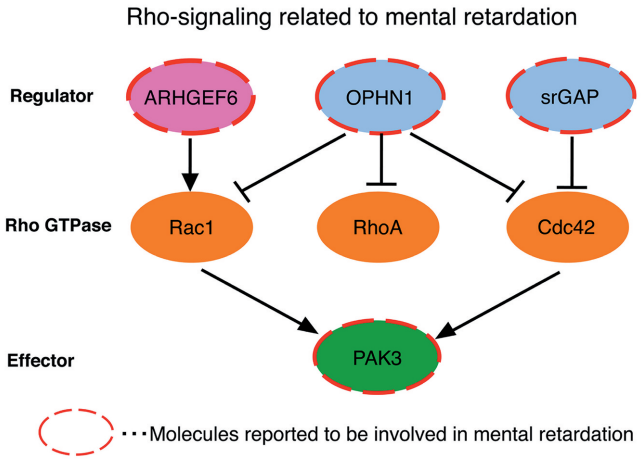

Fig. 4. Rho-signal molecules involved in mental retardation.

IQGAPs are effectors for Rac1 and Cdc42 that comprise 3 proteins, IQGAP1 - 3, in mammals (Kuroda et al., 1996; White et al., 2009). IQGAP1 is best characterized among the isoforms and is known to interact with various molecules such as actin (Fukata et al., 1997), ß-catenin and E-cadherin (Kuroda et al., 1998). This molecule is expressed both in multipotent neural progenitors in the SVZ (also called type C cells) and more differentiated neural precursor cells migrating in the RMS (type A cells), in the adult mouse brain (Balenci et al., 2007). In the IQGAP1-null mouse brain, differentiation of type C cells into type A cells was delayed although no difference was observed in their proliferation profiles (Balenci et al., 2007).

Vascular endothelial growth factor (VEGF), a signal protein that stimulates vasculogenesis and angiogenesis (Neufeld et al., 1999), was reported to play crucial roles in neuronal migration and differentiation during the brain development (Mackenzie and Ruhrberg, 2012). VEGF was also shown to regulate the interaction of Rac1 and Cdc42 with IQGAP1 in neurospheres prepared from adult wild type mouse (Balenci et al., 2007). VEGF-induced migration and differentiation of neuronal progenitor cells were impaired in neurospheres isolated from the ventricular wall of IQGAP1-null mice. VEGF is supposed to be secreted from astrocytes in RMS and SVZ, and stimulate the association of IQGAP1 and Rac1/Cdc42 in neural precursors, facilitating their chain migration to OB and differentiation there.

Vav family proteins that consist of Vav1-3 are common GEFs for Rho, Rac and Cdc42 (Movilla and Bustelo, 1999; Sachdev et al., 2002; Hornstein et al., 2004). Knockdown of this molecule in neuronal progenitor cells from postnatal mouse brain resulted in the reduction of the newborn neuron number in OB (Khodosevich et al., 2009). In addition, Vav3-suppressed neuronal precursors migrating in RMS have a lesser number of large growth cones, which may lead to impaired migration. Although the actual Vav3 target is yet unidentified *in vivo*, some Rho family member(s) are considered to play essential roles in RMS precursor proliferation and tangential migration.

RhoA and its effector molecules

RhoA and its downstream effectors have been reported to be involved in interneuron migration. Expression of a dominant negative version of RhoA in SVZ cells increased the number of cells migrating from SVZ explants, whereas expression of constitutively active Rho reduced the number (Wong et al., 2001).

Adult mouse neuronal precursors in SVZ express two RhoA effectors; Rho-dependent kinases (ROCK1 and ROCK2), which has been reported to regulate their migration (Leong et al., 2011). Application of a ROCK inhibitor, Y-27632, has been shown to induce precursor

cell morphological change into an elongated and bipolar shape, and facilitate migration. Silencing of ROCK1 and ROCK2 in SVZ-derived neural precursor cells also caused morphological changes and decrease of chain formation which abrogates cell-cell adhesion and leads to scattered cell migration (Leong et al., 2011). Although another group reported that Y-27632 inhibits the migration of neuronal precursors from SVZ explants (Shinohara et al., 2012), a difference in experimental conditions may explain the apparent discrepancy.

Recently, a role for mDia, another RhoA effector, has been reported in the tangential migration of interneuron precursors (Shinohara et al., 2012). Among the 3 isoforms (mDia1 – 3), mDia1 and mDia3 are present in developing and adult brain tissues. While mice lacking both mDia1 and mDia3 (mDia double knockout mice; mDia dKO) showed normal radial migration of excitatory cortical neurons, tangential migration of cortical interneurons during corticogenesis and of SVZ precursors in RMS during adult neurogenesis were interrupted. Further analyses revealed that SVZ neuronal precursors in mDia dKO mice showed reduced movement of the centrosome and cell body during migration, perhaps due to the functional defects of actin cytoskeleton rather than microtubule, leading to impaired migration.

Cdc42 and its related molecules

While Slit is a secreted protein expressed in midline glial cells in *Drosophila* (Rothberg et al., 1990), Roundabout (Robo) receptors that belong to the immunoglobulin (Ig) superfamily cell adhesion molecules (CAM) regulate midline crossing of commissural axons in the fly (Seeger et al., 1993). Homologues of both proteins have been identified in many species, including mammals. Interaction of Slit leucine-rich repeat (LRR) regions with Robo receptors Ig domain is known to regulate essential events for cortical development such as axon guidance and neuronal migration (Ypsilanti et al., 2010). In addition, Slit-Robo has been shown to regulate the tangential migration of interneuron precursors in RMS from SVZ to OB (Hu, 1999; Wu et al., 1999; Nguyen-Ba-Charvet et al., 2004; Sawamoto et al., 2006).

Slit-Robo GAPs (srGAPs), specific for RhoA and Cdc42 but not Rac1, interact with the intracellular domain of Robo (Wong et al., 2001). Since Slit increases interaction of srGAP1 with Cdc42 while decreasing the interaction with RhoA, srGAP1 is considered to function as a Cdc42-specific GAP when the Slit-Robo signal is activated. When SVZ explants were co-cultured with an aggregate of HEK293 cells secreting Slit, neuronal precursor cells migrating from SVZ explants were repelled by Slit, whereas overexpression of a constitutively active Cdc42 in explants diminished the repulsive effect (Wong et al., 2001). On the other hand, expression of dominant negative Cdc42 reduced the number of migrating cells from SVZ explants under the above assay conditions. These results suggest that srGAPs regulate Slit-Robo-mediated Cdc42 activity which is important for proper migration of SVZ precursors (Wong et al., 2001).

Rho-signaling and neurodevelopmental diseases

Neuronal migration following neurogenesis is essential for establishing the architecture of the cerebral cortex, and disruption of the ordered migration leads to morphological and functional abnormalities of brain architectures. Thus, involvement of Rho GTPases-mediated signaling in neuronal migration strongly suggests their pathophysiological relevance in neuronal and psychiatric disorders. Indeed, mutations in Rho family GTPase-related genes, including oligophrenin-1 (OPHN1), p21-activating kinase 3 (PAK3), FGD1, ARHGEF9 and ARHGEF6, have been identified in patients with X-linked intellectual disability (Fig. 4) (Nadif Kasri and Van Aelst, 2008). Oligophrenin-1 is a GAP for RhoA, Rac1 and Cdc42 (Billuart et al., 1998). While PAK3 is an effector for Rac1 and Cdc42 (Allen et al., 1998), ARHGEF6 is a GEF for these GTPases (Manser et al., 1998). FGD1, a causative gene for faciogenital dysplasia (Aarskog-Scott syndrome) (German Pasteris et al., 1994), and ARHGEF9 are GEFs specific for Cdc42 (Olson et al., 1996; Reid et al., 1999). In X-linked intellectual disability, functional defects of Rho family-related molecules are most likely to hamper actin-reorganization machinery and cause abnormal neuronal morphology and movement, leading to the defects in corticogenesis and consequent mental retardation.

Conclusion and perspective

Our knowledge concerning molecular mechanisms of neurogenesis and radial migration of excitatory neural precursors has been accumulating rapidly for the last decade. On the other hand, recent studies have just begun to reveal the precise molecular mechanisms of tangential migration of interneuron precursors. Rho GTPases and their regulatory and effector molecules play pivotal roles in the migration of both excitatory and inhibitory neuronal precursors. Further study of Rho signaling molecules in migration of interneruon progenitors/precursors may contribute to the understanding of neuropsychiatric disease etiology, and *in vivo* electroporation methods should be a useful tool for future analyses.

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Accepted February 13, 2014