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Rho Family GTPases as Key Regulators for Neuronal Network Formation

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Rho family GTPases act as transducers of signals from extracellular stimuli to the cytoskeleton and gene expression. Their actions are temporal and spatial determinants for cellular functions. The cellular functions of Rho family GTPases have been studied in fibroblasts and endothelial cells, and recent advances have revealed their roles in the regulation of neuronal network formation, including migration, neurite outgrowth, polarity, axon guidance, dendrite maturation and synapse formation. In addition, a significant number of X-linked mental retardation genes have been shown to encode components directly involved in signal transduction pathways of Rho family GTPases, underscoring the view that Rho family GTPases essentially participate in the neuronal network formation. In this review, we will overview current understanding of the functions of Rho family GTPases in neuronal network formation.

Key words: G protein, neuron, neuronal network, Rho.

Rho family GTPases as molecular switches

Small GTPases of the Rho family are key regulators of the actin cytoskeleton in eukaryotic cells from yeast to humans (*1, 2*). Like other small GTPases, the Rho family GTPases serve as molecular switches by cycling between an inactive GDP-bound state and an active GTP-bound state and, once activated, they can interact with their specific effectors, leading to a variety of biological functions. Activation of the Rho family proteins requires GDP-GTP exchange catalyzed by various guanine nucleotide exchange factors (GEFs) in response to a variety of extracellular stimuli, and their activation is turned off by GTPase-activating proteins (GAPs), which stimulate the intrinsic GTPase activities of the G proteins. Extracellular stimuli regulate activities of Rho family GTPases through control of either GEFs or GAPs. In addition, guanine nucleotide dissociation inhibitors inhibit the exchange of GDP for GTP and might also serve to regulate their localization (*3*).

At least 14 mammalian Rho family proteins have been identified: Rho (A, B, and C), Rac (1, 2, and 3), Cdc42, RhoD, RhoG, RhoH/TTF, TC10, and Rnd (1, 2, and 3). Among these, the functions of Rho, Rac, and Cdc42 have been extensively characterized. In fibroblasts, the activation of Rho leads to formation of actin stress fibers and focal adhesion, whereas the activation of Rac and Cdc42 induces formation of lamellipodia and filopodia, respectively. These proteins are also involved in other cellular activities such as gene transcription and cell polarity.

These cellular functions of Rho family GTPases are mediated by a variety of downstream effectors that specifically associate with GTP-bound active form of GTPases. A well-studied Rho effector, Rho-kinase, also known as ROKα and ROCK, has been reported to be involved in several functions of Rho: the regulation of myosin phosphorylation, the formation of stress fibers and focal adhesions, and the regulation of cytokinesis. Rac- and Cdc42-induced regulation of the actin cytoskeleton is mediated by a variety of effectors, including N-WASP and PAK. These effectors work as direct regulators of the actin cytoskeleton in response to Rho, Rac, and Cdc42.

Another type of Rho family GTPases, such as RhoG and RhoD, regulates the activities of Rho, Rac, and Cdc42, indicating that RhoG and RhoD are upstream regulators for the actin cytoskeleton. As summarized in Fig. 1, many of these Rho family GTPases mediate a variety of neuronal morphological changes.

Neurite retraction and growth cone collapse

Neurons extend axons over potentially long distances to reach their target tissues during the development of the nervous systems. Axon formation involves periods of retraction and elongation, both of which are crucial for proper navigation to the targets. One of the first observations of the functions of Rho family GTPases in neurons was of neurite retraction mediated by lysophosphatidic acid (LPA) and thrombin through Rho activation, the involvement of Rho being verified by using C3 exoenzyme, a specific inhibitor for Rho (*4*). These neuronal actions are induced by the binding of LPA and thrombin to their specific receptors, which are heterotrimeric G protein–coupled rhodopsin-type receptors.

In addition to LPA and thrombin receptors, prostaglandin EP3 receptor and sphingosine-1-phosphate receptor also induce neurite retraction *via* activation of Rho (*5, 6*). Constitutively active RhoA, $RhoA^{V14}$, itself induces neurite retraction, indicating that activation of Rho is strong enough to induce neurite retraction. Rho regulates a variety of cellular functions through its specific binding to downstream effectors, including Rho-kinase, mDia, and protein kinase N (*7*).

Among these Rho effectors, Rho-induced neurite retrac-

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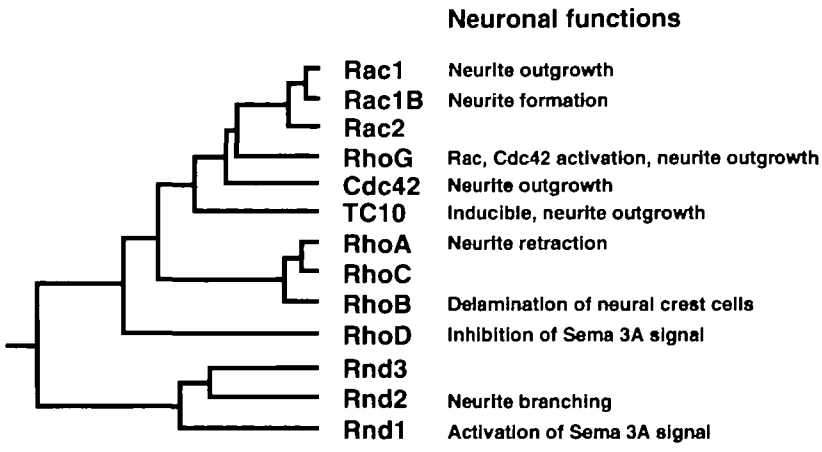

Fig. 1. **Summary of neuronal functions of Rho family GTPases.** Phylogenic analysis of Rho family GTPases is shown. Rho family GTPases consist of several subfamilies, including Rac, Rho and Rnd subfamilies. The group of Rac and Cdc42 promotes the neurite outgrowth, while the group of Rho inhibits the extension and induces retraction. These groups act antagonistically for neurite formation.

tion is mediated by Rho-kinase, and activation of Rho-kinase induces neurite retraction (*8, 9*). Rho-kinase is known to phosphorylate the myosin-binding subunit of myosin phosphatase and myosin light chain, causing smooth muscle contraction (*10*). Rho-kinase is located in the growth cones of neurites, which are rich in filamentous actin (F-actin) and myosin. Neurites extended from the cell bodies of neurons usually adhere to an extracellular matrix *via* growth cones, and cell bodies and growth cones are connected by stable microtubules.

At neurite extension state, actin polymerization is stimulated towards their leading edges, and the pulling action of myosin II activity induces retrograde flow of actin filaments coupled to the extracellular substrate towards the cell interior. These actions provide the driving force for extension of the growth cone, like the movement of a caterpillar. When the myosin II activity increases, the resultant promotion of the retrograde flow is thought to trigger neurite retraction. Therefore, myosin II activation through the phosphorylation of myosin light chain by Rho-kinase activation triggers contraction of actomyosin and eventually promotes neurite retraction (*11*).

Dynamic remodeling of actin filaments in growth cones participates in neurite retraction. Cofilin, which exhibits actin-depolymerizing activity, is a key regulator for the actin filament dynamics, and the cofilin activity is inhibited by LIM-kinase–mediated phosphorylation. Phosphorylation of cofilin occurs during Rho-mediated neurite retraction, and phosphorylation and activation of LIM-kinase by Rho-kinase increases the phosphorylation of cofilin, contributing to Rho-induced reorganization of the actin cytoskeleton (*12*).

Neurite retraction is a well-organized morphological change through regulation of three types of cytoskeleton, microfilaments, microtubules, and neurofilaments. Neurite retraction mediated by actomyosin contraction is concurrently accompanied by depolymerization of microtubules and neurofilaments. Microtubule depletion is known to cause axons to retract in a microfilament-dependent manner. Recently, axonal retraction induced by myosin-mediated forces was shown to be counterbalanced or attenuated by dynein-mediated microtubule forces (*13*). Therefore, the release of microtubule-associated forces is important for neurite retraction. Recenly, Rho has been shown to regulate formation and orientation of stable microtubules through another Rho effector, mDia (*14*). However, it is not well understood how Rho participates in regulation of microtubule dynamics during neurite retraction.

Concerning regulation of intermediate filaments, Rho induces translocation of Rho-kinase to the cell periphery, which is rich in vimentin, one of the intermediate filaments, and the activated Rho-kinase phosphorylates vimentin, causing the collapse of filamentous vimentin (*15*). The phosphorylation of the intermediate filaments by Rho-kinase may be involved in neurite retraction.

Signaling pathways from G protein–coupled receptors to Rho activation for neurite retraction have been extensively studied. Among various heterotrimeric G proteins, Gα12, Gα13, and Gαq induce Rho-dependent neurite retraction through different pathways, while Gαi2 fails to activate Rho (*16, 17*). Rho activation induced by Gα13, but not Gα12, involves a tyrosine kinase, while the Gαq-induced activation is mediated by protein kinase C. Receptors utilize a specific G protein pathway: muscarinic M1 receptor is coupled to Gq for Rho activation, thrombin receptor is coupled to G12, and LPA and prostaglandin EP3 receptors are coupled to G13 (*18, 19*). Gα13-induced Rho activation is mediated by p115 RhoGEF, which contains a DH-PH domain and regulators of G protein signaling (RGS) box (*20*). Gα13 directly binds to the RGS box of p115 and activates its Rho GEF activity. In contrast to Gα13, Gα12 can associate with the RGS box of p115 but has no ability to activate it. Since Gα12 actually stimulates Rho activity, it may activate Rho *via* a different system. In addition to p115, another RhoGEF, PDZ-RhoGEF, which contains an RGS box and a PDZ domain, has been identified to link G12 family G proteins to Rho (*21*). In contrast to p115, which is mainly expressed in thymus and spleen, PDZ-RhoGEF is highly expressed in brain, and induces the neurite retraction, indicating that PDZ-RhoGEF links G12 family G proteins to Rho activation for neurite retraction (*22*).

Neuronal growth cones navigate along specific pathways to establish the proper connections to target neurons and form neuronal networks. Various guidance factors serve as either attractants or repellents to influence the direction of growth cone extension (*23*). Axon guidance navigated by growth cones is regulated by a variety of extracellular guidance factors, which induce changes in the actin-based cytoskeleton of growth cones. Guidance factors bind to their specific receptors on the axonal surface of the growth cone

and trigger the cytoskeletal reorganization associated with oriented neurite extension or retraction.

Growth cone collapse is the prominent morphological change of growth cones induced by repulsive guidance factors. In contrast to signaling pathways of G12 family G proteins–Rho for neurite retraction, molecular mechanisms for guidance factor–induced collapse have not been well investigated, but the involvement of Rho family GTPases in the signaling pathways for some guidance factors has been revealed (Fig. 2). Semaphorin 3, a well-known axon guidance factor, induces growth cone collapse, and Rac1 is involved in this collapse, because semaphorin 3A–induced collapse is inhibited by dominant negative Rac1 or an inhibitory Rac1 peptide (*24–26*). Semaphorins function in neurons through binding to their receptor complexes composed of members of neuropilin and plexin families, and plexins are essential components for signal transduction to intracellular cascades (*27*). Recently, an active form of Rac1 has been shown to physically associate with plexin B1 in non-neuronal cells, and the activation of the plexin B1 exhibited Rho-activating phenotype in a Rac1-dependent manner (*28–30*). Furthermore, it has been reported in *Drosophila* that plexin B associates with active Rac and sequesters it from the downstream effector PAK and then enhances RhoA activity, guiding axon pathfinding (*31*). Activation of plexin B recruits activated Rac to the membrane and this may lead to activation of RhoA through an unknown mechanism.

Ephrins are another type of growth cone collapse factor that are transmembrane or glycosylphosphatidylinositol-anchored molecules, and Ephrin A plays a critical role in axonal patterning in retinotropic nervous system. Ephrin-A5 induces collapse of growth cones by activating Rho and Rho-kinase, indicating that the Rho-Rho-kinase pathway is involved in the growth cone collapse (*32*). The novel Rho family GEF ephexin has been isolated (*33*). Ephexin associates with the cytoplasmic domain of tyrosine kinase receptor–type EphA receptors, and Ephrin-A stimulation of EphA receptors modulates the activity of ephexin, leading to RhoA activation, Cdc42 and Rac1 inhibition, and cell morphology changes. This differential regulation of Rho family GTPases by Ephrin-A through ephexin may induce regional reorganization of actin cytoskeleton in the growth cones, leading to axon guidance.

Slit is an important axon guidance factor at the midline of the nervous system, functioning as a repulsive cue. Slit guides neurons through binding to its transmembrane receptor, Roundabout (Robo). The binding of Slit to Robo accelerates association of the intracellular domain of Robo with a novel family of RhoGAPs, the Slit-Robo GAPs (srGAPs), expressed in regions responsive to Slit (*34*). srGAPs display GAP activity for Cdc42, and a dominant negative srGAP or a constitutively active Cdc42 blocks the repulsive activity of Slit, indicating that srGAP mediates Slit repulsive activity by inhibiting Cdc42.

Collectively, these findings suggest that the guidance factors induce growth cone collapse though activation of the Rho-Rho-kinase pathway and inhibition of Rac and Cdc42. Actin bundle loss correlated with F-actin redistribution away from the leading edge of growth cone contributes to collapse (*35*). Phosphorylation of cofilin by LIM-kinase, which is phosphorylated and activated by Rho-kinase, is necessary for semaphorin 3A–induced growth cone collapse (*36*). Reorganization of actin cytoskeleton by the Rho-Rho-kinase-LIM-kinase pathway in growth cones may be linked to growth cone collapse.

Neurite formation and outgrowth

Neurons first initiate the formation of neurites and then extend them. Rat pheochromocytoma PC12 cells have been used as a model system for neuronal differentiation and neurite outgrowth. After stimulation with nerve growth factor (NGF), neurons stop growing and begin to extend neurites. In contrast, epidermal growth factor (EGF) does not induce neurite outgrowth but stimulates proliferation of PC12 cells (*37*). The receptors for NGF and EGF belong to a family of tyrosine kinase receptors, and they transduce signals *via* similar transduction pathways, including a Ras-dependent mitogen-activated protein kinase (MAPK) cascade. Studies using a dominant negative Rac1 and Cdc42 show that Rac1 and Cdc42 are involved in the NGF-induced neurite outgrowth (*38*). Both NGF and EGF induce

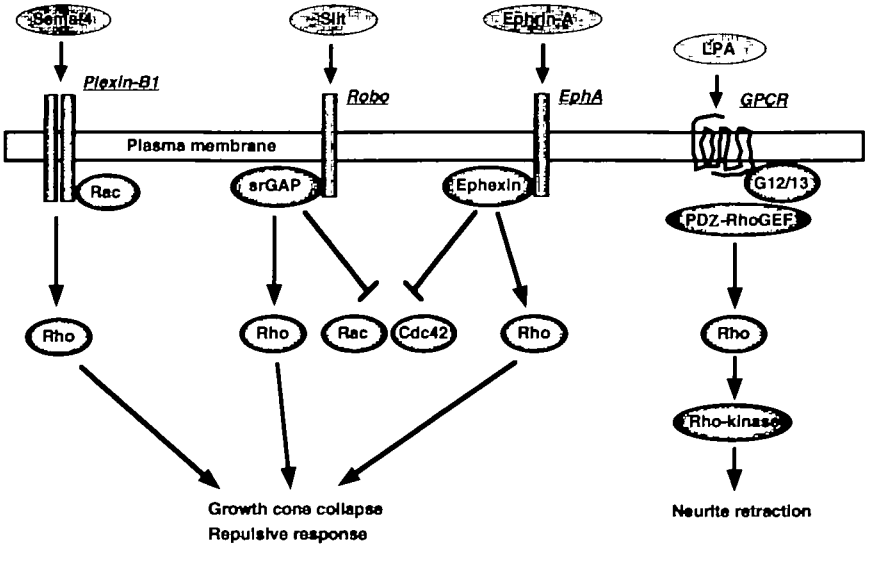

Fig. 2. **The role of Rho family GTPases in the signaling of axon guidance factors.** Three types of repulsive factors, Sema-4, Slit and Ephrin-A, bind to their specific receptors and stimulate Rho activity but suppress Rac and Cdc42 activities through distinct mechanisms, segregation of active Rac, GAP and GEF, respectively.

transient activation of Rac1, but their activation pathways are different: the former involves phosphoinositide 3-kinase, but the latter does not (*39*). In addition, NGF induces recruitment of active Rac1 to protrusion sites on the cell surface and subsequent accumulation of F-actin, triggering F-actin–rich protrusion and neurite formation, while EGF neither accumulates active Rac1 nor forms Rac1- and F-actin–rich protrusions.

NGF induces MAPK activation, and this activation has been thought to be required for neurite outgrowth in PC12 cells. The Rac1 activation and the Rac1-mediated protrusion by NGF are independent of the MAPK cascade, but the subsequent neurite extension requires the cascade. Thus, the initial neurite formation step and the subsequent extension step of the neurite outgrowth are regulated by different mechanisms. Strong Rac1 activation is essentially required for the initial neurite formation (*40*).

In contrast to Rac1, activation of Rho inhibits the neurite outgrowth (*41, 42*). Activation of RhoA inhibits the NGF-induced Rac1 activation, rapid Rac1 recruitment to the protrusion sites and neurite formation through Rho-kinase activation due to stabilization of cortical actin filaments at the cell periphery in PC12 cells (*43*). Activation of Rho-kinase by RhoA induces phosphorylation and activation of myosin, located at the cell periphery, and consequently elevates contractile activity of actomyosin, enhancing the stabilization of cortical actin filaments. This stabilization disturbs the Rac1-mediated reorganization of the actin cytoskeleton at the cell periphery and subsequent protrusion. Rac and Rho counteract each other's activity, and the balance between Rac and Rho activities is a crucial point for neuronal morphology. In PC12 cells, NGF rapidly activates Rac1 but simultaneously suppresses RhoA activity (*43*). Decrease in RhoA activity induces destabilization of cortical actin filaments at the cell periphery and offers the Rac1-mediated protrusion sites (Fig. 3). The opposite regulation of Rho family GTPases by NGF allows the cells to bring about neurite formation. NGF-induced Rac1 activation is mediated by TrkA receptor, a tyrosine kinase receptor, linking Ras activation pathway. On the other hand, NGF-induced RhoA inhibition is mediated by another NGF receptor, p75, which is a member of the tumor necrosis factor receptors (*44*). p75 associates with and activates RhoA, and NGF binding to p75 abolishes this RhoA activation. Therefore, NGF differentially regulates Rac1 and RhoA activities through different receptors. Decrease in Rho activity is critical for neurite formation. Actually, suppression of Rho-Rho-kinase activity has been shown to be critical for the initiation of axon outgrowth in cerebellar granule neurons (*45*). Rho activity is usually regulated by RhoGAP. Among various RhoGAP, p190 RhoGAP is co-enriched with F-actin in the distal tips of axons, and overexpression of p190 RhoGAP promotes extensive neurite outgrowth (*46*). p190 RhoGAP-knockout mice exhibit defects in axon guidance fasciculation. The essential role of p190 RhoGAP was also shown in axon stability in mushroom body neurons in *Drosophila* (*47*). Suppression of Rho activity by p190 RhoGAP plays a key role in axon outgrowth and its guidance.

The signaling pathway connecting NGF to Rac has been studied. NGF binding to TrkA receptor activates Ras *via* Sos, a Ras-GEF, leading to Rac activation. Another type of Rho family GTPase, RhoG, transduces the Ras signal to Rac1 and Cdc42 activation in PC12 cells (*48*). Thus, RhoG is an upstream regulator for Rac1 and Cdc42, and RhoG activation causes Rac1 and Cdc42 activation. The expression of RhoG by itself induces neurite outgrowth, while the active forms of Rac1 and Cdc42 together cannot produce neurites from cells, which instead form filopodia or lamellipodia, indicating that sustained activation of both Rac1 and Cdc42 is not sufficient to induce neurite outgrowth in PC12 cells. Neurite outgrowth requires not only GTPase cycle of Rac1 and Cdc42 activities but also their appropriate localization to the sites where neurites are formed and extend,

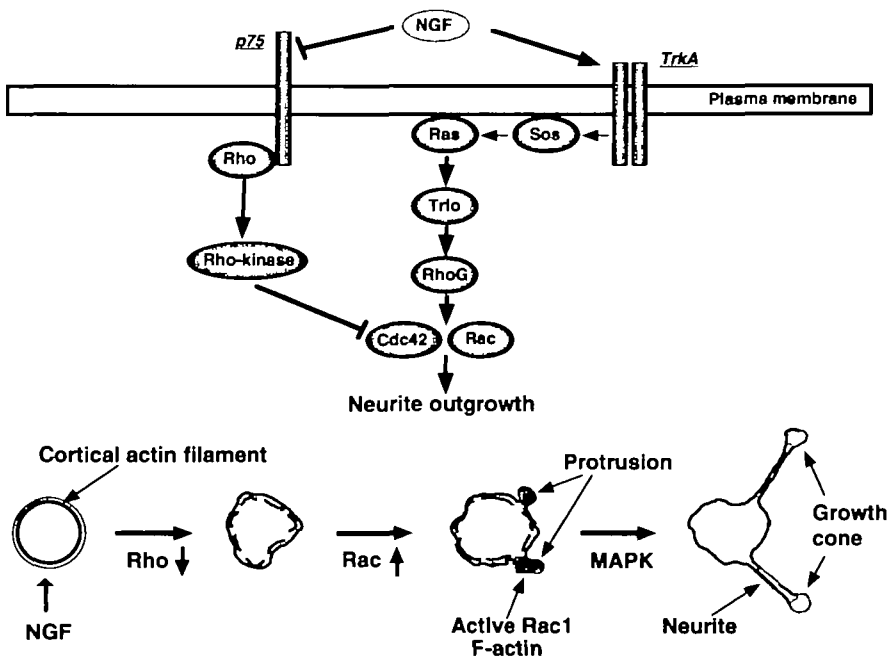

Fig. 3. **Signaling pathways for NGF-induced neurite outgrowth.** NGF binds to two different receptors, TrkA and p75, and differentially regulates Rac and Rho activities, respectively. NGF inhibits Rho activity but stimulates Rac activity and induces neurite formation.

and RhoG may function to appropriately promote the GTPase cycle of Rac1 and Cdc42 and localize them to the protrusion sites. Recently, kinectin, a regulator of microtubule-dependent kinesin activity, has been identified as a binding partner for RhoG and found to link RhoG to microtubule-dependent cellular activity (*49*). This microtubule-dependent RhoG activity may determine the spatial regulation of Rac1 and Cdc42 activities for neurite outgrowth.

Several molecules displaying *in vitro* GEF activity for RhoG have also been identified, including Vav and Trio. Trio is an upstream regulator of RhoG for the NGF-induced neurite outgrowth (*50*) and has been shown to play an essential role in patterning of axons by regulating their directional extension in *Drosophila* (*51, 52*). Trio-RhoG-Rac1/Cdc42 pathway is a central signaling route for neurite outgrowth. It is unknown how RhoG activates Rac1 and Cdc42. A variety of GEFs for Rac and Cdc42 have been so far isolated. Among them, Tiam1 and STEF promote neurite outgrowth in a Rac1-dependent manner (*53, 54*). These Tiam1 family GEFs may be involved in the signaling from RhoG to Rac activation.

Rac and Cdc42 reorganize the actin cytoskeleton through binding to their specific downstream effectors. PAK1, an effector for both of Rac and Cdc42, has been reported to induce neurite outgrowth, but this effect is independent of the kinase activity, while it may act upstream of Rac (*55, 56*). Another subtype, PAK5, highly expressed in brain, operates downstream of Cdc42 and Rac and antagonizes Rho activity, leading to neurite outgrowth in a kinase activity–dependent manner (*57*).

The Cdc42 effectors MRCKα and N-WASP have been shown to be involved in neurite extention (*58, 59*). N-WASP directly induces actin polymerization through activation of Arp2/3 complex, and thus the cytoskeletal reorganization at the growth cone induced by N-WASP may lead to extension of the growth cone. Another Cdc42-binding adaptor protein, IRS-58, comprising a Cdc42-binding domain, an SH3-binding site, an SH3 domain, and a WW-binding domain, induces neurite outgrowth with high complexity (*60*). This adaptor protein has the ability to reorganize the actin cytoskeleton, involving loss of stress fibers and filopodia formation, suggesting that Cdc42-bound IRS-58 facilitates neurite outgrowth through actin cytoskeleton reorganization.

Axon and dendrite development

Development of neuronal cells has been extensively studied with cultured hippocampal neurons. Development of hippocampal neurons is divided into five stages. Neurons form lamellipodia (stage 1) and extend several minor processes (stage 2) within the first 24 h, after which one of the processes rapidly extends to form an axon (stage 3). The residual processes then become dendrites (stage 4), which undergo dendrite branching and spine formation (stage 5). Rho family GTPases act as a key player in each step.

Axonal polarity is determined at stage 3 with the appearance in one of the multiple neurites of a large growth cone containing a labile actin network and abundant dynamic microtubules. Tiam1, a GEF for Rac1, is localized in the growth cone and involved in the axonal polarity, because overexpression of Tiam1 in neurons causes extension of several axon-like neurites, and suppression of Tiam1 prevents axon formation (*61*). Rac1-mediated actin reorganization in the growth cone appears to be critical for the axon polarity. Disruption of the actin network in an individual growth cone induces its neurite to become the axon, and local instability of the actin network restricted to a single growth cone is a physiological signal specifying neuronal polarization (*62*).

Recently, CRMP-2, collapsin response mediator protein-2, has been shown to be accumulated in growing axons and to be a critical determinant for axonal polarity in cultured hippocampal neurons (*63*). CRMP-2 is a dominant substrate for Rho-kinase *in vivo* and *in vitro*, raising the possibility that Rho-signaling pathways link extracellular signals and CRMP-2–mediated axonogenesis (*64*).

Dendritic patterning exerts a profound influence on neuronal connectivity. Dendritic development consists dendrite initiation, dendrite growth, dendrite branching, and spine formation, and RhoA, Rac1, and Cdc42 influence distinct aspects of dendritic patterning (*65*).

In dendrite initiation, dominant negative forms of Rac1 and Cdc42 lead to a significant reduction in the number of primary dendrites in cortical neurons, suggesting that Rac and Cdc42 are involved in the dendrite initiation (*66*). Among members of the Rac subfamily, cRac1B, which is mainly expressed in brain, induces an increment in the number of neurites per neuron with dramatic neurite branching in neural retinal cells, and the carboxy-terminal portion of cRac1B, which is different from those of other Rac subfamily, is essential for the action (*67*). Rac1B is responsible for the dendrite initiation, and putative downstream effector for Rac1B may recognize its carboxy-terminal portion.

In contrast to Rac and Cdc42, neurons lacking RhoA overextend their dendrites and expression of activated RhoA causes a reduction of dendrite growth, indicating that RhoA is a negative regulator for dendrite growth (*68*). RhoA-induced reduction in dendrite growth was also observed in *Xenopus* tectal neurons, while Rac1 and Cdc42 appeared not to have less or no effect (*69*).

The process of branching is a dynamic combination of branch addition and branch elimination as well as branch extension and retraction. In the beginning of the branch formation, F-actin–rich filopodia and lamellipodia are extended from the dendrites. Cortical protrusions enlarge from the filopodial and lamellipodial area, and then branches emerge. While branches are formed, the protrusion sites are invested with microtubules, indicating that branch formation is closely accompanied by reorganization of both the actin cytoskeleton and microtubules. Dendrite branching is controlled by Rac1 and Cdc42; dominant negative forms of Rac1 and Cdc42, but not RhoA, lead to a reduction in the dendrite branching of *Xenopus* retinal ganglion neurons (*70*). In contrast, RhoA suppresses branch formation, and this suppression is mediated by Rho-kinase in hippocampal pyramidal neurons (*71*). Rac1 and RhoA reciprocally regulate dendrite branching.

Dendrite spines are tiny protrusions that receive synaptic input and compartmentalize postsynaptic actions (*72*). F-actin, consisting of β- and γ-isoforms of actin, is highly enriched in dendritic spines, while microtubules are usually sparse or missing. Spine formation and stability are largely determined by the actin cytoskeleton. The first report of the effect of Rho family GTPases on spines is an increase in the number of Purkinje cell spines by constitu-

tively active Rac1, suggesting that Rac1 plays a role in spine formation (*73*). On the other hand, dominant negative Rac1 causes progressive reduction in spine number, indicating that Rac1 activity is crucial for maintenance of spine density (*71*). Expression of Kalirin-7, a GEF for Rac1, is targeted to spines and induces the number and size of spines (*74*). Kalirin-7 is enriched in postsynaptic density by binding to PSD95 or other PDZ domain–containing proteins. Targeting of Kalirin-7 to postsynaptic density and subsequent activation of Rac1 may regulate spine morphology. In contrast to Kalirin-7, SIF, another GEF for Rac, was shown to localize in the periactive zone of *Drosophila* neuromuscular synapses, suggesting that the SIF-Rac pathway controls synaptic development (*75*). On the other hand, active RhoA decreases spine density, while the inhibition by C3 exoenzyme increases it, suggesting that Rac and Rho signaling act antagonistically in spine formation (*76*). Compared with Rac and Rho, Cdc42 seems to have little effect on spine morphology and density (*76*).

Roles of other Rho family GTPases in neuronal morphology

As mentioned above, Rho family GTPases consist of at least 14 members, and Rho, Rac and Cdc42 have been extensively studied with regard to their roles in neuronal morphology. TC10 expression is lower in developing and mature motor neurons, but axotomy of motor neurons induces dramatic expression of TC10, suggesting that TC10 is a nerve injury–inducible Rho family GTPase and plays a crucial role in nerve regulation (*77*). The expression of TC10 exhibits drastic neurite extension. TC10 can associate with the same targets as Cdc42 and Rac, such as PAK, N-WASP, and MRCK, suggesting that the neuronal functions of TC10 are mediated by the same effectors as those of Rac and Cdc42 (*78*).

A new branch of Rho family GTPases, the Rnd subfamily, consisting of Rnd1, Rnd2, and Rnd3, has been identified (*79*). Rnd1 and Rnd2 are mainly expressed in brain, while Rnd3 is expressed ubiquitously. Unlike other Rho family GTPases, Rnd1 and Rnd3 possess very low intrinsic GTPase activity and are thought to be constitutively active due to point substitutions at the residues equivalent to valine and serine, respectively, in Ras G12. Expression of Rnd1 or Rnd3 in fibroblasts results in loss of actin stress fibers and focal adhesion, indicating the antagonistic effect on the Rho-regulated signaling pathway. Expression of Rnd1 induces the formation of many neuritic processes from the PC12 cell body, with disruption of the cortical actin filaments that is probably due to the inhibition of the Rho signaling pathway (*80*).

Xenopus Rnd1 is expressed in tissues undergoing extensive morphogenetic changes, such as marginal zone cells, somitogenic mesoderm, and neural crest cells, and overexpression of *Xenopus* Rnd1 induces the disruption of cell adhesion and is reversed by *Xenopus* RhoA expression (*81*). Therefore, Rnd1 and Rnd3 possess antagonistic effects on the action of RhoA. Rnd1 binds to the cytoplasmic domain of plexin-A1, a receptor for semaphorin 3A, and this binding triggers cytoskeletal collapse, suggesting possible involvement of Rnd1 in the semaphorin 3A–mediated axon guidance (*82, 83*) (Fig. 4).

In contrast to Rnd1, RhoD, originally identified as a regulator of endocytosis, has been shown to bind to the cytoplasmic domain of plexin-A1 but to block the Rnd1-induced plexin-A1 activation and repulsion of sympathetic axons by semaphorin 3A, indicating that Rnd1 and RhoD exert antagonistic actions in semaphorin 3A guidance signal (*83*). Recently, Socius, a novel Rnd GTPase–interacting protein, has been identified and found to be involved in Rnd1-induced loss of stress fibers, suggesting that Socius mediates in part the antagonistic effects of Rnd1 for Rho signaling (*84*).

In contrast to Rnd1 and Rnd3, the neural functions of Rnd2 remain to be elucidated, although Rnd2 is expressed in neurons in brain and spinal cord, suggesting that Rnd2 plays an important role in neural functions (*85*). We have revealed that Rnd2 specifically associates with its specific downstream effector, Rapostlin, showing high similarity to CIP4, a Cdc42 effector protein (*86*). Rapostlin has two functional domains, an FCH domain at the amino-terminus and an SH3 domain at the carboxy-terminus. Rapostlin directly binds to microtubules *via* the amino-terminal region including FCH domain and regulates the reorganization of both actin filaments and microtubules. Rapostlin induces neurite branching in response to Rnd2 in PC12 cells and primary hippocampal neurons, and the microtubule-binding amino-terminal region exerts a dominant negative effect on neurite branching, suggesting that Rnd2 and Rapostlin play an important role in branching formation through binding to microtubules.

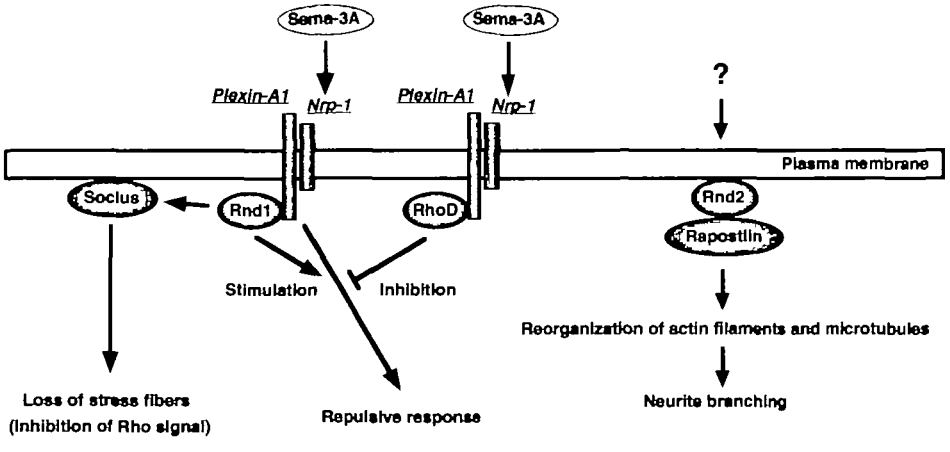

Fig. 4. **Neuronal functions of Rnd subfamily.** Rnd1 mediates the semaphorin 3A–induced repulsive response, while the guidance factor(s) regulating Rnd2 activity is unknown.

Human neurological diseases

Rho family GTPases are key players in neuronal morphology. Thus, a loss of function of Rho family GTPase signaling pathways has been speculated to cause disorganization of neuronal network formation and eventual neural diseases. Mental retardation is characterized by a deficit in cognitive functioning without observable phenotypic abnormalities or gross abnormalities in brain structure. Mental retardation is diagnosed during child development and is considered to be a developmental disorder. Mental retardation is associated with an immature morphology of synaptic spines, structures involved in neurotransmission and memory processes, suggesting that mental retardation is due to a deficiency in neuronal network formation. Recently, several genes involved in X-linked mental retardation (MRX) have been cloned, and a significant number of MRX genes have been found to be directly involved in signal transduction pathways of Rho family GTPases (*87*). Oligophrenin-1 encodes a RhoGAP stimulating GTPases activities of RhoA, Rac1, and Cdc42 *in vitro* (*88*). Oligophrenin-1 is expressed in both fetal and adult brain, and loss of function mutations result in mental retardation. A point mutation of PAK3, an effector for Rac and Cdc42, disrupts the kinase function and causes MRX (*89*). Mutations in ARHGEF6 (known as αPIX or Cool-2), encoding a GEF for Rac, have been found in patients with MRX (*90*). FGD1 is a RhoGEF specific for Cdc42 and a gene for the Aarskog-Scott syndrome that is associated with mental retardation (*91*). These genes mentioned above encode GEF, GAP, or an effector for Rho family GTPases, indicating that deficiency in the regulation of Rho family GTPase activity leads to MRX. Another type of gene, the fragile X mental retardation protein (FMRP), is responsible for MRX. FMRP is an RNA-binding protein associated with polysomes as part of an mRNP complex, suggesting a role in local protein translation at neuronal dendrites and in dendritic spine maturation. Recently, FMRP has been shown to interact with CYFIP1/2, a binding partner of Rac1, suggesting that these protein complexes are implicated in development of dendritic spine structures (*92*). In mentally retarded fragile X patients, dendritic spines are thin, elongated and have small synaptic contacts, typical of immature spines. The immature spine morphology supports the notion that mental retardation is due to abnormalities in morphology of actin cytoskeleton structures of spines regulated by Rho family GTPases. MRX strongly brings forward the physiological significance of Rho family GTPase signaling pathways in the neuronal network formation.

In addition of MRX, William's syndrome is a neurological condition characterized by mild mental retardation and defects in visuo-spatial cognition, and this syndrome results from deletions of the gene for LIM-kinase 1. LIM-kinase acts downstream of both Rac-PAK and Rho-Rho-kinase to phosphorylate cofilin, regulating reorganization of the actin cytoskeleton (*12, 93*). This syndrome presents an importance of the cytoskeletal dynamics regulated by Rho family GTPases through LIM-kinase.

Conclusions and perspectives

Here, we have summarized recent advances in our understanding of the molecular mechanisms for Rho family GTPase-regulated neuronal network formation. Rho family GTPases are key regulators for neuronal development and especially neuronal network formation, requiring organized morphological changes. They are also signal transducers connecting the extracellular guidance signals to cytoskeletal elements. Rho family GTPases act as an on-off switch temporally and spatially regulated in response to extracellular stimuli, and this guidance factor–induced temporal and spatial activation allows neurons to extend neurites in defined directions and to form connections with target cells. Guidance factors activate or inhibit the activities of Rho family GTPases through regulation of GEF or GAP activity. GEFs and GAPs are multifunctional molecules having a variety of functional modules in addition to catalytic domains. These diverse modules of GEFs and GAPs realize fine and complicated formation of neuron networks. Molecular biological and genetic approaches to the regulation of Rho family GTPase activity will provide further mechanistic insight into the molecular control of neuronal network formation. Emerging areas of interest in the signal transduction of Rho family GTPases include cross-talk among various Rho family GTPases and between Rho family GTPases and other small GTPases such as Ras and ARF families. These approaches are expected to reveal the entire picture of the signaling cascades of Rho family GTPases. As mentioned above, several human genetic neural diseases have been shown to result from mutations in factors involved in Rho signaling pathways. Many more disease-causing mutations in the human genes for molecules in Rho signaling pathways will be unveiled in the future, providing an important concept of how Rho family GTPases function in a variety of neural diseases. Research along these lines will also contribute to the design of strategies for therapeutic intervention and diagnostic purposes.

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