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Genes Dev. 2005 19: 1-49
Access the most recent version at doi:10.1101/gad.1256405

REVIEW

The role of the Rho GTPases in neuronal development

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Our brain serves as a center for cognitive function and neurons within the brain relay and store information about our surroundings and experiences. Modulation of this complex neuronal circuitry allows us to process that information and respond appropriately. Proper development of neurons is therefore vital to the mental health of an individual, and perturbations in their signaling or morphology are likely to result in cognitive impairment. The development of a neuron requires a series of steps that begins with migration from its birth place and initiation of process outgrowth, and ultimately leads to differentiation and the formation of connections that allow it to communicate with appropriate targets. Over the past several years, it has become clear that the Rho family of GTPases and related molecules play an important role in various aspects of neuronal development, including neurite outgrowth and differentiation, axon pathfinding, and dendritic spine formation and maintenance. Given the importance of these molecules in these processes, it is therefore not surprising that mutations in genes encoding a number of regulators and effectors of the Rho GTPases have been associated with human neurological diseases. This review will focus on the role of the Rho GTPases and their associated signaling molecules throughout neuronal development and discuss how perturbations in Rho GTPase signaling may lead to cognitive disorders.

The Rho GTPases

Rho GTPase family members include Rho (RhoA, RhoB, RhoC, RhoD, RhoT), Rac (Rac1, Rac2, and Rac3), Cdc42, TC10, TCL, Wrch1, Chp/Wrch2, RhoG, RhoH/TTF, and Rnd (Rnd1, Rnd2, and Rnd3/RhoE) (Van Aelst and D'Souza-Schorey 1997; Burridge and Wennerberg 2004). They are low-molecular-weight guanine nucleotide-binding proteins that function as binary molecular switches by cycling between an active GTP-bound state and an inactive GDP-bound state. Their activity is determined by the ratio of GTP to GDP in the cell and can be influenced by a number of different regulatory molecules. Guanine nucleotide exchange factors (GEFs) activate GTPases by enhancing the exchange of bound GDP for GTP (Schmidt and Hall 2002); GTPase activating proteins (GAPs) act as negative regulators of GTPases by enhancing the intrinsic rate of GTP hydrolysis of a GTPase (Bernards 2003; Bernards and Settleman 2004); and guanine nucleotide dissociation inhibitors (GDIs) prevent exchange of GDP for GTP and also inhibit the intrinsic GTPase activity of GTP-bound GTPases (Zalcman et al. 1999; Hoffman et al. 2000). The finding that constitutively active (CA) and dominant-negative (DN) mutants of the same Rho GTPase can cause aberrant phenotypes underscores the importance of tight regulation of Rho GTPase activity and the ability to cycle between an active GTP-bound form and an inactive GDP-bound form for normal cellular function (Luo et al. 1994). Activated, GTP-bound Rho GTPases interact with specific effector molecules to transduce upstream signals, and recent studies indicate that GEFs contribute to the signaling specificity of their downstream target GTPase via association with scaffolding molecules that link them and the GTPase to specific GTPase effectors (Buchsbaum et al. 2002, 2003; Jaffe et al. 2004). Spatial and temporal regulation of Rho GTPase GEFs and effectors further contribute to specificity, and future fluorescence resonance energy transfer (Fret) analyses should discriminate molecular interactions between Rho GTPases and particular regulators and effectors (Del Pozo et al. 2002; Yoshizaki et al. 2003; Aoki et al. 2004; Pertz and Hahn 2004).

Of the Rho GTPase family members, RhoA, Rac1, and Cdc42 have been characterized most extensively. These Rho GTPases are best known for their effects on the actin cytoskeleton. In classic fibroblast studies, activation of RhoA, Rac1, and Cdc42 leads to reorganization of the actin cytoskeleton into distinct structures: stress fibers and focal adhesions, veil-like lamellipodia, and filopodial microspikes, respectively. In addition to their regulation of the actin cytoskeleton, the Rho GTPases have been shown to play a role in transcriptional activation, membrane trafficking, and microtubule dynamics. These cellular processes contribute to the effects of the

[*Keywords*: Rho GTPases; neurite formation; axon formation, guidance, and regeneration; dendrite formation; spine morphogenesis; nervous system disorders]
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Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.1256405.

Rho GTPases on cell growth control, cytokinesis, cell motility, cell–cell and cell–extracellular matrix adhesion, cell transformation and invasion, and recently, neuronal development. Given the dependency of neuronal development on regulation of the actin and microtubule cytoskeletons by the Rho GTPases, a brief description of key effectors of Rac1, Cdc42 and RhoA, and their general mechanisms of action on the cytoskeleton is presented here and schematized in Figure 1. Their involvement in neuronal development is subsequently discussed in the appropriate sections below. For more comprehensive reviews on Rho effectors, the reader is referred to the following references: Van Aelst and D'Souza-Schorey (1997); Bishop and Hall (2000); Gundersen (2002); Luo (2002); Bokoch (2003); Miki and Takenawa (2003); Riento and Ridley (2003); Burridge and Wennerberg (2004); Millard et al. (2004).

Well-known effectors that can be activated by both Rac and Cdc42 are the p21-activated kinase (PAK) family of serine/threonine kinases. PAK proteins have been ascribed roles in regulating actin cytoskeleton dynamics and gene expression. A total of six members (PAK1–6) have been identified, of which PAK1–3 have been most extensively studied (Jaffer and Chernoff 2002; Bokoch 2003). These kinases exist in a dormant state in the cytoplasm as a result of the N-terminal autoinhibitory region, which assumes a conformation that prevents the activation of the C-terminal kinase domain (Lei et al. 2000; Buchwald et al. 2001; Buchsbaum et al. 2003). Upon binding to Rac-GTP or Cdc42-GTP, the autoinhibition is disrupted, resulting in PAK activation and its autophosphorylation. Cyclin-dependent kinase 5 (Cdk5) and its neuron-specific regulator p35, the latter of which has been shown to directly associate with Rac in a GTP-dependent manner, have been reported to influence the duration of PAK activation. When in a complex with Cdk5, p35 causes hyperphosphorylation of PAK1 in a Rac-dependent manner, resulting in down-regulation of PAK1 kinase activity (Nikolic et al. 1998). Thus, at the same time as activating PAK, Rac may activate the p35/Cdk5 complex to limit the duration of PAK activity. One mechanism by which PAKs affect the actin cytoskeleton involves phosphorylation and activation of the Lin-11, Isl-1, and Mec-3 (LIM) domain-containing kinases (Yang et al. 1998; Edwards et al. 1999; Dan et al. 2001). Once active, these kinases phosphorylate and inhibit cofilin, an actin filament depolymerizing/severing factor, and as such stabilize actin filaments and promote actin polymerization (Bamburg 1999; Stanyon and Bernard 1999). The regulation of myosins is likely to be another component of PAK-mediated cytoskeletal signaling. There is evidence that PAK1 can interfere with myosin light chain (MLC) function via direct phosphorylation and inhibition of myosin light chain kinase (MLCK) (Sanders et al. 1999; Bokoch 2003). This action of PAK may assist in the disassembly of actin stress fibers triggered by PAK. More recent studies have implicated PAK proteins as possible regulators of microtubule dynamics, likely through their ability to phosphorylate stathmin/Op18 at Ser 16. Phosphorylation of Op18 at this position prevents Op18 from binding to microtubules and therefore inhibits its microtubule destabilizing function, leading to stabilization of microtubules (Daub et al. 2001; Cassimeris 2002; Wittmann et al. 2003).

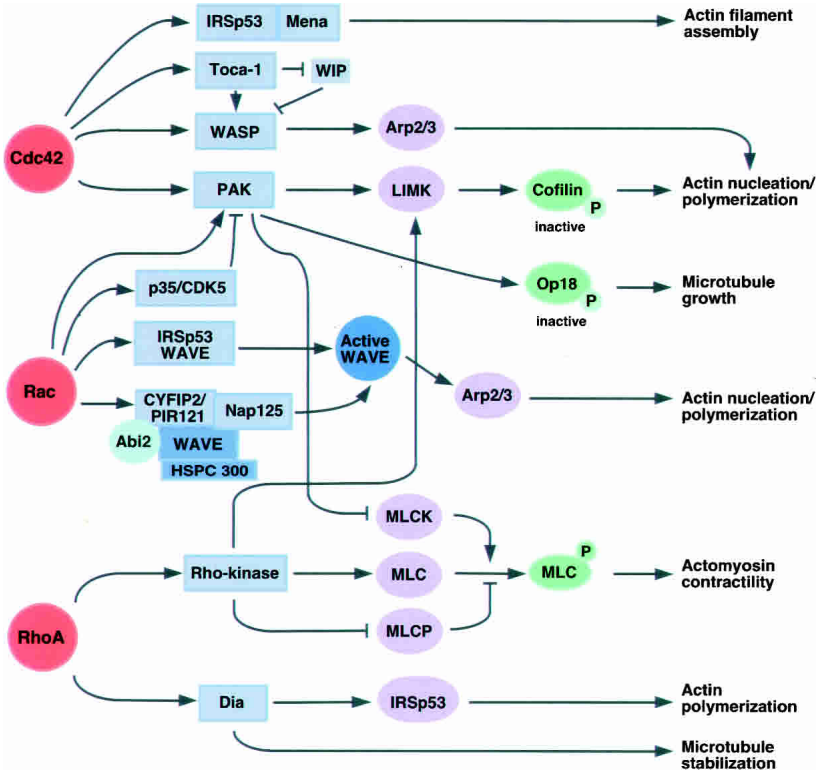

Figure 1. Rho GTPase effectors implicated in actin and microtubule dynamics. See main text for explanation. (Toca-1) Transducer of Cdc42-dependent actin assembly; (WIP) WASP-interacting protein; (WASP) Wiskott-Aldrich-syndrome protein; (Arp2/3) actin-related proteins 2 and 3; (PAK) p21-activated kinases; (LIMK) Lin-11, Isl-1, and Mec-3 kinase; (Cdk5) cyclin-dependent kinase 5; (IRSp53) insulin receptor substrate of 53 kDa; (Mena) mammalian Ena (Enabled); (WAVE) WASP family Verprolin-homologous protein; (CYFIP) cytoplasmic FMR1-interacting protein; (PIR121) a p53-inducible mRNA; (Nap125) Nck-associated protein; (Abi2) Abl interactor 2; (HSPC) heat-shock protein C; (MLCK) myosin light chain kinase; (MLC) myosin light chain; (MLCP) myosin light chain phosphatase; (Dia) Diaphanous-related formins.

Accumulating evidence indicates that another key mechanism by which Rac and Cdc42 relay signals to the actin cytoskeleton involves the Wiskott-Aldrich-syndrome family of scaffolding proteins (Machesky and Insall 1999; Suetsugu et al. 2002; Miki and Takenawa 2003; Snapper and Rosen 2003; Millard et al. 2004; Smith and Li 2004). The Wiskott-Aldrich-syndrome protein (WASP) and its closest cousin neuronal WASP (N-WASP) are regulated by Cdc42 (Rohatgi et al. 1999). Three other members of this family, WAVE1–3 (also known as Scar proteins) mediate actin-based processes triggered by Rac (Miki et al. 1998; Machesky et al. 1999; Suetsugu et al. 1999; Yamazaki et al. 2003; Yan et al. 2003). Both the WASP and WAVE family members are linked to the actin cytoskeleton through their interaction with the Arp2/3 complex (see reviews above). In the case of WASP/N-WASP, these proteins have been shown to directly bind to the activated form of Cdc42. This induces a conformational change that releases the WASP VCA domain from auto-inhibition, allowing it to activate the Arp2/3 complex to nucleate the formation of new actin filaments in vitro (Machesky et al. 1999; Rohatgi et al. 1999, 2000; Kim et al. 2000). Recent studies, however, have revealed that an additional component, Toca-1 (transducer of Cdc42-dependent actin assembly), is required to mediate Cdc42-induced actin polymerization in a physiological context. Toca-1 binds both N-WASP and Cdc42 and mediates actin nucleation by either directly activating N-WASP and/or inhibiting WIP, which is a negative regulator of N-WASP (Ho et al. 2004).

The WAVE proteins mediate actin cytoskeletal changes downstream of Rac without directly binding to Rac. In an effort to find a molecular connection between Rac and WAVE, a recent study succeeded in isolating an inhibitory WAVE complex that is responsive to Rac signaling (Eden et al. 2002). In its inactive state, this complex includes Nap125 (Nck-associated protein), PIR121 (a p53-inducible mRNA), Abi2 (Abl interactor 2) and the heat-shock protein, HSPC300. Notably, Nap125 (also called Nap1, NCKAP1, and Hem2) has been linked to Rac1 via p140 protein (Kitamura et al. 1997; Yamamoto et al. 2001). The p140 protein corresponds to Sra-1, which is an isogene of PIR121, and directly interacts with both Rac1 and Nap125 (Kobayashi et al. 1998). The PIR121 protein was previously identified as a profilin-interacting protein called POP (Witke et al. 1998), and interestingly both p140/Sra-1 and PIR121 proteins have recently been identified as fragile X mental retardation protein (FMRP)-interacting proteins and are referred to as CYFIP1 and CYFIP2, respectively (Schenck et al. 2001). When active Rac (Rac-GTP) is added, the complex dissociates, freeing WAVE and HSPC300, which then activates the Arp2/3 complex to induce actin polymerization. Subsequent studies in *Drosophila* and *Caenorhabditis elegans* have shown that the orthologs of Sra-1/PIR121 and Nap125 act in a common pathway linked to Rac-mediated actin-based protrusions and cell migration (Soto et al. 2002; Kunda et al. 2003; Schenck et al. 2003). Moreover, recent studies in mammalian cells also revealed that Abi1, Sra-1/PIR121, and Nap1 are essential intermediates of a signaling pathway from Rac activation to WAVE2-based nucleation of lamellipodial actin filaments. These studies, however, show that dissociation of the Abi1/Nap1/PIR121 complex from WAVE2 does not occur in vivo and that Rac activation recruits this complex to lamellipodia to cause site-directed nucleation of actin filaments (Innocenti et al. 2004; Steffen et al. 2004).

Another molecule, the insulin receptor substrate of 53 kDa (IRSp53), has been shown to bind both Rac and Cdc42, and links these Rho GTPases to WAVE2 and Mena (mammalian Ena), respectively (Miki et al. 2000; Krugmann et al. 2001; Miki and Takenawa 2002). The N-terminal portion of IRSp53 also binds a partial CRIB (Cdc42/Rac1 interactive binding) domain within itself, producing an intramolecular autoinhibitory interaction. This intramolecular interaction appears to be relieved by the binding of a Rho GTPase to the partial CRIB motif, or by the binding of another effector to the SH3 domain of IRSp53, which allows for the initiation of actin filament assembly (Krugmann et al. 2001). Overexpression of IRSp53 in fibroblasts causes the formation of filopodia, and IRSp53 synergizes with Mena to do so (Krugmann et al. 2001). Interestingly, a more recent study shows that IRSp53 localizes to both lamellipodia and filopodia, as does WAVE2, even in the absence of Ena/Vasp family members, including Mena (Nakagawa et al. 2003). In addition to the above interactions, IRSp53 binds the post-synaptic scaffolding molecules Shank1 (Bockmann et al. 2002; Soltau et al. 2002) and post-synaptic density (PSD)-95 (Soltau et al. 2004). Activation of IRSp53 by Cdc42 allows docking of a preassembled IRSp53/PSD-95 complex to the proline-rich region of Shank1 (Soltau et al. 2004). Given that Shank and PSD-95 organize a complex array of signaling molecules at the synapse (Ehlers 1999; Tu et al. 1999; Sheng 2001; Boeckers et al. 2002) and are important for the structure and function of dendritic spines (Ehlers 1999; Sala et al. 2001; Sheng 2001; Boeckers et al. 2002), IRSp53 via its association with these molecules is likely to be important for post-synaptic function as well. Interestingly, β-PIX, a GEF for Rac and Cdc42, also binds the PDZ domain of Shank (Park et al. 2003) and plays a role in spine morphogenesis (Zhang et al. 2003). This GEF/scaffolding molecule complex may help to confer specificity on Rho GTPase signaling by linking a particular Rho GTPase with a particular effector, such as IRSp53. Lastly, mDia binds IRSp53 in a Rho-dependent manner (Fujiwara et al. 2000), further implicating IRSp53 in cytoskeletal reorganization.

Two major downstream effectors of RhoA that mediate this GTPase's effects on the cytoskeleton are members of the Rho-kinase (also called ROK/ROCK) family and the Diaphanous formin subfamily (Dia). Rho-kinase was identified as ROKα (Leung et al. 1995) and ROCK2 (Matsui et al. 1996; Nakagawa et al. 1996), with ROKβ (Leung et al. 1996)/ROCK1 (Ishizaki et al. 1996) constituting an isoform of Rho-kinase. Rho-kinases are serine/threonine kinases that play several roles in RhoA-induced actin reorganization (Riento and Ridley 2003). They control actin filament bundling by directly phosphorylating and activating MLC, or by phosphorylating

and inactivating MLC phosphatase, thereby indirectly increasing MLC phosphorylation and activation (Amano et al. 1996; Kimura et al. 1996). Furthermore, Rho-kinases may promote F-actin accumulation by phosphorylating and activating LIMK, which in turn phosphorylates and inactivates the actin depolymerization factor (ADF) cofilin (Maekawa et al. 1999; Sumi et al. 1999, 2001; Ohashi et al. 2000a,b; Amano et al. 2001).

The Diaphanous-related formins are defined by their ability to interact with activated Rho GTPases, and murine members include mDia1 (which binds RhoA-C), mDia2 (which binds RhoA and Cdc42), and mDia3 (which binds Cdc42, RhoA, Rac1, and RhoD) (Olson 2003; Wallar and Alberts 2003; Yasuda et al. 2004). As seen for WASP and PAK proteins, binding of an activated Rho GTPase seems to relieve an autoinhibitory interaction between the NH_2- and COOH-terminal regions of Dia proteins, which have been shown to regulate the actin and microtubule cytoskeletons (Alberts 2001). Yeast formins and mDia1 bind the barbed ends of actin filaments while still allowing elongation (Pring et al. 2003; Zigmond et al. 2003), and they enhance actin-nucleation via their Formin homology FH1-FH2 or FH2 domains (Tominaga et al. 2000; Copeland and Treisman 2002; Pruyne et al. 2002; Sagot et al. 2002; Kovar et al. 2003; Li and Higgs 2003; Pring et al. 2003; Shimada et al. 2004). In mammalian cells, a large proportion of actin monomer is bound to Profilin, preventing spontaneous nucleation and the addition of actin monomer to the pointed end of the filament, but not to the barbed end (Pollard et al. 2000). The binding of Profilin to the FH1 domain of mDia1 may allow mDia1 to use Profilin-bound actin monomers for nucleation (Li and Higgs 2003; Higashida et al. 2004). Another study demonstrates that mDia cooperates with Rho-kinase to induce actin fiber formation (Maekawa et al. 1999). However, this action of mDia seems to be exerted through its effect on microtubule alignment (Ishizaki et al. 2001), and recent evidence shows that Dia proteins function as downstream effectors of RhoA to regulate the formation and orientation of stable microtubules (Ishizaki et al. 2001; Palazzo et al. 2001).

Taken together, these studies show that the Rho GTPases exert their effects on the cytoskeleton through a large number of effectors. The involvement and potential functions of these Rho effectors in neuritogenesis, axon formation and guidance, dendritic development, and dendritic spine formation are discussed below.

Neurite initiation and outgrowth

Immediately after neuronal commitment, extracellular cues activate membrane receptors to induce neuritogenesis, the formation of cylindrical extensions off of the neuronal cell body that serve as precursors to axons and dendrites. Each neuronal population forms neurites in a manner that is specific to its program of differentiation. However, regardless of the type of neuron, the original round shape of the cell must be broken to form a bud that develops into a neurite. Neurite initiation and outgrowth involves coordinated changes between the actin cytoskeleton, which provides a means of generating force within the cell, and the microtubule network, which stabilizes and maintains the neurites (da Silva and Dotti 2002). As a regulator of both actin cytoskeletal reorganization and microtubule orientation and stabilization, the Rho GTPases have a profound effect on neuritogenesis.

Rho GTPase studies in neuronal cell lines established their involvement in neurite formation and suggested that they act antagonistically toward each other to determine neuronal morphology. Treatment of rat pheochromocytoma PC12 cells with nerve growth factor (NGF), or serum starvation of mouse N1E-115 neuroblastoma cells, results in the formation of neurites, which depends on Rac and Cdc42 activity, since DN mutant forms of these GTPases inhibit neurite outgrowth (Sarner et al. 2000; Aoki et al. 2004). Interestingly, a recent study using FRET-based probes has shown that localized activation of Rac1 and Cdc42 is required for neurite outgrowth. Immediately after the addition of NGF to PC12 cells, Rac1 and Cdc42 are transiently activated in broad areas of the cell periphery, with a subsequent localized cycling of activity and inactivity of these GTPases at the mobile tips of protrusions. High Rac1 activity is observed in the distal half of neurite tips, while strong Cdc42 activity is concentrated in microspikes projecting from the tips (Aoki et al. 2004). Furthermore, NGF-induced recruitment of Rac1 to cell surface sites to form filamentous actin-rich protrusions is associated with a concomitant decrease in RhoA activity (Yamaguchi et al. 2001). Conversely, Rho activation is generally associated with inhibition of neurite initiation and retraction in PC12 and N1E-115 cells. RhoA signaling induces the formation of a thick ring-like structure of cortical actin filaments at the cell periphery and inhibits NGF-induced recruitment of Rac1 to protrusions (Yamaguchi et al. 2001). Furthermore, CA RhoA is sufficient to prevent neurite initiation and induce neurite retraction (Kranenburg et al. 1997; Amano et al. 1998; Hirose et al. 1998; Katoh et al. 1998a,b; Sebok et al. 1999), while inhibition of Rho using C3 exoenzyme from *Clostridium botulinum* or DN RhoA mimics actin-based lamellipodia and filopodia structures induced by Rac1 and Cdc42 and induces neurite formation (Tigyi et al. 1996a; Kozma et al. 1997; Kranenburg et al. 1997; Sebok et al. 1999; Brouns et al. 2001; Fujita et al. 2001). Taken together, these studies show that Rac and Cdc42 activation promote the formation of lamellipodia and filopodia, and play a role in neurite formation, while Rho activity prevents neurite initiation and induces neurite retraction.

The generalization that Rac and Cdc42 activation promote neurite formation, while Rho activation antagonizes it in neuronal cell lines, holds true in several primary cell systems. In agreement with the PC12 and N1E-115 experiments above, neurite outgrowth is stimulated by the chick-specific Rac1 subtype cRac1B in chick primary retinal neurons and inhibited by an inactive form of cRac1B (Albertinazzi et al. 1998), and CA Rac1 increases and DN Rac1 decreases neurite extension

in dissociated rat hippocampal neurons (Schwamborn and Puschel 2004). CA Rho prevents neurite outgrowth of cultured hippocampal neurons, including that promoted by neurotrophin 3 (NT-3), brain-derived neurotrophic factor (BDNF), NGF, and coating on a laminin substrate (Da Silva et al. 2003; Schwamborn and Puschel 2004), and neurite outgrowth is promoted by Rho inhibition using C3 exoenzyme in chick dorsal root ganglia (DRG) and cultured rat hippocampal neurons (Jin and Strittmatter 1997; Sarner et al. 2000; Da Silva et al. 2003; Fournier et al. 2003). C3 exoenzyme also rescues the arrest of neurite formation and elongation that results from the plating of cultured rat hippocampal neurons on a collagen substrate (Da Silva et al. 2003).

In contrast to the above findings, there are several instances in which CA and DN mutant forms of Rac and Cdc42 have been found to induce opposite phenotypes. Both CA Rac1 and CA Cdc42 expressing *Drosophila* giant fiber system neurons exhibit a lack of neurite outgrowth (Allen et al. 2000), CA Rac1 decreases the length of the longest neurite in cultured rat cortical neurons (Kubo et al. 2002), and DN Rac promotes neurite outgrowth in chick DRG (Fournier et al. 2003). These differential effects of the Rho GTPases on neurite formation may be due to the diversity of species, cell types, and age of cells or organisms used in primary cell studies, or reflect the need for the Rho GTPase to cycle between an active GTP-bound and inactive GDP-bound state in order to properly regulate neuritogenesis. This last possibility is emphasized in studies in which CA and DN mutants of a Rho GTPase produce the same effect. For example, both CA and DN Rac1 mutants retard growth cone advance, neurite outgrowth, and differentiation in primary chick embryo motor neurons (Kuhn et al. 1998). The important information gleaned from these studies is that the Rho GTPases play an important role in neurite formation in primary neurons, and that tight regulation and cycling of the Rho GTPases are required for normal neurite initiation and outgrowth.

Rac and Cdc42 signaling pathways that promote neurite formation

Given the effects of the Rho GTPases on neurite formation discussed above, a recurrent theme is the requirement of concurrent Rac and Cdc42 activation and Rho inactivation to promote neurite formation. Signaling cues that positively affect Rac and Cdc42 activity, while negatively impacting Rho activity, include growth factors, receptors, and Rho GTPase regulatory molecules. NGF is well known for its ability to stimulate the formation of neurites in neuronal cell lines (Negishi and Katoh 2002), and the NGF-elicited signaling pathway that leads to neurite formation through modulation of Rho GTPase activity has been largely elucidated. The Ras-linked tyrosine kinase receptor TrkA mediates NGF-activation of Rac1 and inactivation of RhoA (Nusser et al. 2002), and PI 3-kinase is required for NGF activation of both Rac1 and Cdc42 (Nusser et al. 2002; Aoki et al. 2004), NGF-induced inactivation of RhoA (Nusser et al. 2002), and NGF and Ras-induced process formation (Kobayashi et al. 1997; Kita et al. 1998; Sarner et al. 2000). Furthermore, activated Ras and PI 3-kinase-induced neurite outgrowth requires both Rac1 and Cdc42 activity, while neurite outgrowth induced by activated Cdc42 is Rac1 dependent (Sarner et al. 2000). Conversely, CA RhoA is able to antagonize neurite outgrowth induced by activated Ras (Sarner et al. 2000), and NGF treatment of PC12 cells causes a decrease in active Rho and its effector Rho-kinase downstream of Rac activation (Nusser et al. 2002). Thus, the following signaling cascade has been proposed for NGF-induced neurite outgrowth: NGF, TrkA, Ras, PI 3-kinase, Cdc42, and Rac, with a concomitant decrease in Rho and Rho-kinase activities downstream of Rac. NGF-induced Rac and Cdc42 activation and neurite formation may be further modulated by a specific splice variant of the RhoGAP Nadrin, Nadrin-116. Nadrin-116 inhibits NGF-induced neurite outgrowth in PC12 cells, which is dependent on Nadrin's GAP activity, suggesting a Rac- or Cdc42-directed preference for this molecule in relation to neurite formation (Furuta et al. 2002).

In addition to acting downstream of Ras signaling to regulate neuritogenesis, Rac and Cdc42 have been found to mediate the neurite-promoting effects of another Ras-like small GTPase, Rin. Rin is expressed predominantly in adult neurons and binds calmodulin (CaM) (Lee et al. 1996). Rin expression in PC12 cells increases Rac and Cdc42 activity and induces neurite formation in a MAP-kinase pathway independent manner. Rin-induced neurite outgrowth can be suppressed by DN Rac, DN Cdc42, or CaM inhibitor, and a Rin mutant unable to associate with CaM fails to induce neurite outgrowth. Furthermore, interfering with Rin function inhibits potentiation of neurite formation induced by a combination of forskolin, an adenylyl cyclase activator, and KCl, which induces extracellular calcium entry through voltage-dependent calcium channels. Rin-induced neurite outgrowth therefore requires Rac and Cdc42 activation and Rin association with CaM, and may involve Rin/calcium/CaM-mediated neuronal signaling. It should be noted that Rin expression also increases Rho activity, and interfering with RhoA levels using RNA interference (RNAi) induces the formation of more neurite branch points in Rin-induced neurites (Hoshino and Nakamura 2003). However, given that Rin-induced Rho activation does not appear to antagonize neurite formation as expected, how Rin balances Rac, Cdc42, and Rho activity to promote neurite formation remains to be elucidated.

Several Rac-specific GEFs have also been shown to play a role in neurite formation, including Tiam1 (the invasion-inducing T-lymphoma and metastasis 1 protein), STEF (SIF and Tiam1-like exchange factor), and FIR (FERM domain including RhoGEF). Tiam1 is a Rac-specific GEF that is highly expressed in the developing nervous system and may play a role in the migration of granule cells to their final destination (Habets et al. 1994; Ehler et al. 1997). Overexpression of Tiam1 in N1E-115 cells promotes cell spreading and neurite out-

growth on the extracellular matrix protein laminin through the recruitment of the α6β1 integrin and activation of Rac1. Neurite formation induced by Tiam1 can be enhanced by inactivation of RhoA and overcome by coexpression of CA RhoA (Leeuwen et al. 1997), once again highlighting the importance of Rac activation and Rho inactivation for neurite formation. Tiam1 also plays a role in neurite outgrowth promoted by ephrin-B1/EphB2-mediated reverse signaling and ephrin-A1/EphA2-mediated forward signaling (Tanaka et al. 2004). Ephrins are transmembrane or glycosylphosphatidylinositol-anchored molecules that are ligands of Eph receptor tyrosine kinases (Wilkinson 2001; Cutforth and Harrison 2002; Guan and Rao 2003; Huber et al. 2003). Tiam1 interacts with both ephrin-B1 and EphA2, and Rac1 is activated by extracellular stimulation of clustered soluble EphB2 receptors in cells coexpressing Tiam1 and ephrin-B1 or by soluble ephrin-A1 in cells coexpressing Tiam1 and EphA2. Importantly, primary cortical neurons from mouse embryos and neuroblastoma cells significantly extend neurites on surfaces coated with the extracellular domain of EphB2 or ephrin-A1, an effect that is negated by expression of DN ephrin-B1, DN EphA2, or DN Tiam1 (Tanaka et al. 2004). Another Rac1-specific GEF, STEF, is predominantly expressed in the brain, and CA STEF induces neurite-like processes that can be inhibited by DN Rac1 in N1E-115 cells (Matsuo et al. 2002), suggesting that STEF, like Tiam, activates Rac to promote neurite formation. In contrast to these findings, the Rac-specific GEF FIR appears to hinder neurite growth in cortical neurons. Ectopic expression of FIR results in shortened neurites and excessive growth cones in a Rac-dependent manner, as does CA Rac1 in this system (Kubo et al. 2002).

An additional protein linked to Rac and Cdc42-promoted neurite outgrowth is CD47 (also known as integrin-associated protein), a transmembrane protein that belongs to the immunoglobulin (Ig) superfamily. CD47 has been shown to activate Rac and Cdc42 and induce the formation of neurites and filopodia through Rac and Cdc42, respectively, in N1E-115 neuroblastoma cells. CD47-induced neurite and filopodia formation are also likely triggered by the CD47 ligand SHPS-1 (BIT/SIRPα) and involve integrins that contain the β3 subunit (Miyashita et al. 2004).

Once activated, Rac and Cdc42 relay neurite-promoting signals by binding and activating downstream effector molecules. PAK kinases are prominent effectors of both Rac and Cdc42 shown to play a role in neurite formation. PAK1 induces neurite outgrowth independent of its kinase activity when targeted to membrane in PC12 cells (Daniels et al. 1998) and is also indirectly linked to neurite outgrowth in cortical neurons through its association with both Rac and p35 (CDK5 kinase) (Nikolic et al. 1998). p35 is a neuron-specific regulator for CDK5 that colocalizes with Rac in neuronal growth cones, associates directly with Rac in a GTP-dependent manner (Nikolic et al. 1998), and regulates neurite outgrowth in cortical neurons in culture (Nikolic et al. 1996). Interestingly, PAK1 is present in a Rac/p35/Cdk5 complex and active p35 causes hyperphosphorylation of PAK1 in a Rac-dependent manner, resulting in down-regulation of PAK1 activity. Thus Rac not only activates PAK1, but also appears to regulate the duration of its activity via a p35/CDK5 complex (Nikolic et al. 1998), perhaps to modulate neurite formation. PAK5 is another member of the PAK family that has been implicated in neurite formation. PAK5 is highly expressed in mammalian brain and induces the formation of filopodia and neurite-like processes in N1E-115 cells. However, unlike PAK1, the action of PAK5 on neurite outgrowth is dependent upon its kinase activity. Furthermore, an activated PAK5 inhibits Rho activity and its effect on neurite outgrowth can be abolished by an activated RhoA mutant (Dan et al. 2002), suggesting that PAK5 acts downstream of Rac/Cdc42 to antagonize Rho signaling.

Other Cdc42 effectors involved in neurite outgrowth include myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK)α, N-WASP, and Cdc42Hs-associated kinase-1 (ACK-1). Mutants of MRCKα, including a kinase-dead MRCKα, and N-WASP, including N-WASP mutants unable to bind Cdc42 or activate Arp2/3, are able to block NGF-induced neurite outgrowth in PC12 cells (Chen et al. 1999; Banzai et al. 2000), and the N-WASP mutant unable to activate Arp2/3 inhibits neurite formation in cultured rat hippocampal neurons (Banzai et al. 2000). Cdc42 and its effector ACK-1 also regulate neurite formation downstream of muscarinic cholinergic receptor (mAChR) activation in human SH-SY5Y neuroblastoma cells expressing M_3-type muscarinic receptors. The mechanism by which this occurs involves phosphorylation of ACK-1 via a Fyn tyrosine kinase signaling pathway, which is dependent upon Rho-kinase signaling (Linseman et al. 2001). In addition to the above Cdc42 effectors, an adaptor protein that binds Cdc42, the 58-kDa substrate of the insulin receptor tyrosine kinase (IRS-58) has been shown to colocalize with F-actin and induce the formation of filopodia in fibroblasts and promote highly complex neurite outgrowth in N1E-115 cells, while an IRS-58 mutant unable to bind Cdc42 antagonizes these effects (Govind et al. 2001). Notably, IRS-58 is identical to IRSp53, except for differences in post-translational modifications (Abbott et al. 1999). Taken together, these findings not only implicate these Cdc42 effectors in neurite formation, but also underscore the importance of actin cytoskeletal changes for this process.

The role of Rho signaling in neurite retraction

While Rac and Cdc42 activation, coupled with Rho inactivation, promote neurite formation, Rho activation antagonizes this effect and causes neurite retraction, a process that may play an important role in the remodeling of neuronal protrusions important for guidance and synaptic plasticity. Rho activation and neurite retraction have been linked to lysophosphatidic acid (LPA) activation of G-protein coupled receptors, thrombin receptor activating peptide (TRP) activation of thrombin receptor, activation of prostaglandin E receptor EP3 subtype, acti-

vation of sphingosine-1-phosphate receptor, and the addition of serum to cells (Jalink et al. 1994; Katoh et al. 1996, 1998a; Postma et al. 1996). It was originally noted that the addition of LPA or TRP causes rapid growth cone collapse, neurite retraction, and cell rounding, and that *C. botulinum* C3 exoenzyme is able to inhibit this (Jalink et al. 1994; Tigyi et al. 1996a,b). C3 exoenzyme ADP ribosylates and specifically inhibits Rho activity and is able to cause neurite outgrowth (Nishiki et al. 1990; Kamata et al. 1994; Tigyi et al. 1996a; Kozma et al. 1997; Kranenburg et al. 1997; Sebok et al. 1999; Brouns et al. 2001; Fujita et al. 2001). Later studies suggested that LPA activates Rho through the heterotrimeric G-proteins (Tigyi et al. 1996a), including Gα12, Gα13, and Gαq, though they are believed to induce Rho-dependent neurite retraction through different signaling pathways. Gαq-induced neurite retraction can be blocked by inhibiting protein kinase C or extracellular calcium, and both Gα13 and Gαq-elicited neurite retraction, as well as LPA-induced RhoA activation, can be blocked by tyrosine kinase inhibitors (Katoh et al. 1998b; Kranenburg et al. 1999). In nonneuronal cells, the Rho-specific GEF PDZ–RhoGEF mediates both Gα12 and Gα13-induced Rho activation (Fukuhara et al. 1999), and p115 RhoGEF stimulates Gα12 and Gα13 activity and mediates Gα13-induced Rho activation, which is negatively regulated by Gα12 (Hart et al. 1998; Kozasa et al. 1998).

Rho regulatory molecules implicated in neurite retraction include a more recently identified Rho-specific GEF, KIAA0380, and a number of Rho GAPs. The RhoGEF KIAA0380 is related to p115 RhoGEF, is highly expressed in brain, activates Rho-kinase, and induces stress fiber formation in nonneuronal cells. In Neuro2a cells, KIAA0380 is present in the tips of neurites, and expression of an N-terminal fragment inhibits LPA-induced neurite retraction, suggesting that KIAA0380 plays a role in neurite retraction through Rho signaling (Togashi et al. 2000). Rho GAPs involved in neurite retraction include p190 RhoGAP and Grit. p190 RhoGAP is a Rho-specific GAP (Billuart et al. 2001) which is the principal Src (Src/Fyn) substrate in developing and mature nervous system. Overexpression of p190 RhoGAP causes extensive neurite outgrowth on laminin in N1E-115 cells and extensive outgrowth of long and highly branched neurites in neuroblastoma N2A cells. This growth is dependent on the RhoGAP domain and can be blocked by RhoA (Brouns et al. 2001). Grit is a novel GAP for the Rho family of GTPases that is highly expressed in neuronal cells. Grit directly interacts with the TrkA NGF receptor, and the GAP domain alone of Grit exhibits GAP activity mainly toward RhoA and Cdc42, with only moderate activity toward Rac1. Overexpression of the TrkA-binding region of Grit inhibits NGF-induced neurite elongation in PC12 cells, while full-length Grit promotes neurite formation upon NGF stimulation, suggesting Rho-directed activity in these cells (Nakamura et al. 2002). Lastly, a novel RhoGAP family member, p200RhoGAP, acts preferentially toward RhoA and Rac1, and costains with cortical actin in naive N1E-115 cells and the actin-rich ends of neurites in differentiated cells. Expression of either full-length p200RhoGAP or the RhoGAP domain alone induces differentiation (Moon et al. 2003), a phenotype reminiscent of decreased Rho activity.

Additional Rho proteins implicated in neurite formation are Dvl, Wnt, and δ-catenin. Dvl mediates Wnt signaling in the β-catenin and planar cell polarity pathways and has recently been shown to play a role in Wnt-3a-dependent neurite retraction. Dvl-1 is capable of activating Rho and Rho-kinase in PC12 cells, and expression of Dvl-1 inhibits NGF-induced neurite outgrowth in PC12 cells and serum-starvation-dependent neurite outgrowth in N1E-115 cells, which can both be prevented by the Rho-kinase inhibitor Y-27632. Furthermore, a Dvl-1 mutant incapable of activating Rho-kinase fails to induce neurite retraction. With regard to Wnt signaling, NGF-induced neurite formation in PC12 cells is suppressed by Wnt-1 or Wnt-3, and neurite formation under these conditions is enhanced by Y-27632. Wnt-3a protein also stimulates β-catenin and Rho-kinase activity, and the Dvl-1 mutant unable to activate Rho-kinase prevents Wnt-3a-induced neurite retraction (Kishida et al. 2004). Together, these data indicate that Dvl acts downstream of Wnt to regulate Rho and Rho-kinase activities necessary for neurite retraction. Another potential Rho protein implicated in neurite formation is δ-catenin, which is a neuronal protein that binds to the juxtamembrane segment of classical cadherins, and interacts with cortactin. δ-Catenin is phosphorylated by Src family kinases, which inhibits binding of δ-catenin to cortactin and causes cells to extend unbranched primary processes upon NGF treatment in PC12 cells. Inhibition of Rho using C3 exoenzyme changes the effect of δ-catenin from primary process extension to branch formation, and both DN RhoA and Rho-kinase inhibitor inhibit δ-catenin process formation. An activated RhoA mutant also decreases the length of protrusions compared with δ-catenin alone. Interestingly, growth induced by δ-catenin is more robust and is restricted to dendrites in cultured hippocampal neurons (Martinez et al. 2003). Thus these findings suggest that δ-catenin regulates process extension through Rho signaling and plays a role in dendrite formation.

A major downstream target of RhoA, its effector Rho-kinase, has been shown to mediate Rho-driven neurite retraction. Both wild-type and CA Rho-kinase arrest cells in a round state or induce neurite retraction (Amano et al. 1998; Hirose et al. 1998; Katoh et al. 1998a; Da Silva et al. 2003), including that promoted by NT-3, BDNF, NGF, and a laminin-coated substrate in cultured rat hippocampal neurons (Da Silva et al. 2003). Conversely, DN Rho-kinase or the Rho-kinase-specific inhibitor Y-27632 promotes the formation of neurites (Hirose et al. 1998; Fujita et al. 2001; Da Silva et al. 2003), DN Rho-kinase mutants inhibit both LPA-elicited (Amano et al. 1998; Hirose et al. 1998) and EP3 prostaglandin E receptor-induced neurite retraction (Katoh et al. 1998a), and Rho-kinase inhibition via Y-27632 rescues collagen-induced arrest of neurite sprouting and elongation in cultured rat hippocampal neurons (Da Silva et al. 2003).

The effects of activated RhoA and Rho-kinase on neurite retraction are likely brought about by increased actomyosin contractility. LPA activation of RhoA causes rapid F-actin assembly, along with neurite retraction and cell rounding, suggesting that contraction of the cortical actin cytoskeleton is important for neurite retraction (Kranenburg et al. 1997). Rho-kinase has been shown to directly phosphorylate and activate MLC (Amano et al. 1996, 1998) and is required for LPA-induced MLC phosphorylation in N1E-115 cells (Hirose et al. 1998). The use of a mutant MLC with an activated myosin ATPase promotes neurite retraction (Amano et al. 1998), while a MLCK inhibitor blocks LPA and TRP-induced neurite retraction (Jalink et al. 1994). Conversely, NGF and C3-induced neurite outgrowth can be counteracted by a phosphatase inhibitor, and both NGF and C3 exoenzyme treatment of PC12 cells and of C3 exoenzyme have been shown to decrease MLC phosphorylation. These findings suggest that NGF induces neurite outgrowth in part through inhibition of the RhoA/Rho-kinase signaling pathway that results in MLCP activation, causing a transient decrease in phosphorylated MLC (Fujita et al. 2001). The finding that myosin IIA antisense oligonucleotides inhibit LPA or thrombin-induced neurite retraction in Neuro-2A neuroblastoma cells, as does Y-27632, shows that the myosin isoform IIA is the myosin motor that drives neurite retraction (Wylie and Chantler 2003).

Additional Rho-kinase substrates linked to regulation of the actin cytoskeleton are the LIM kinases. As mentioned in the introduction, LIM kinases are serine/threonine kinases that phosphorylate and thus inactivate the actin depolymerizing protein cofilin. LPA treatment of N1E-115 cells causes phosphorylation of cofilin in a Y-27632-sensitive manner. This increase in cofilin phosphorylation is achieved by Rho-kinase's direct phosphorylation and activation of LIM-kinase, which then phosphorylates cofilin (Maekawa et al. 1999). N-terminal LIM domains also inhibit PC12 cell differentiation after stimulation with NGF and Y-27632, while the PDZ domain of LIM-kinase only reduces neurite outgrowth induced by Y-27632 (Birkenfeld et al. 2001).

The actin monomer binding protein Profilin has also been suggested to regulate neuritogenesis downstream of Rho-kinase by modulating actin stability. Different Profilin isoforms include Profilin I (PI), which is ubiquitously expressed, Profilin II (PIIa) and Profilin IIb (PIIb), which are largely restricted to the brain (Witke et al. 1998), and a third Profilin that is restricted to kidney and testes (E. Hu et al. 2001). Rho-kinase has been shown to associate with and phosphorylate PIIa (Witke et al. 2001; Da Silva et al. 2003), though this phosphorylation is not necessary for the binding of these two proteins (Da Silva et al. 2003). Interestingly, PIIa deficiency or overexpression causes phenotypes similar to impaired RhoA/Rho-kinase function and RhoA/Rho-kinase activation, respectively, in cultured rat hippocampal neurons. Neurons from *PII*-deficient mice or those subjected to antisense directed against *PIIa* exhibit an increase in neurite number, length, and branching, and a decrease in F-actin content. Overexpression of PIIa has the opposite effect. PIIa overexpression is capable of rescuing the effects of Rho and Rho-kinase inhibition on neurite outgrowth and F-actin content, while a reduction in PIIa ameliorates the effects of CA RhoA and Rho-kinase. Similarly, PIIa expression antagonizes increased neurite number and length and decreased F-actin caused by the actin depolymerizing factor cytocholasin D, suggesting that PIIa increases actin stability by increasing F-actin content. PIIa expression also antagonizes the induction of neurite formation by NT-3, BDNF, NGF, and a laminin-coated substrate, as do CA RhoA and Rho-kinase, and reduction of PIIa, Rho, or Rho-kinase activity rescues collagen-induced arrest of neurite sprouting and elongation. Thus, PIIa acts downstream of RhoA/Rho-kinase signaling to regulate actin stability during neuritogenesis in mammalian hippocampal neurons (Da Silva et al. 2003).

A recently discovered Rho-kinase-interacting protein potentially involved in neurite outgrowth is $p21^{Cip1/WAF1}$, which binds to and inhibits both cyclin/Cdk kinases and proliferating cell nuclear antigen. It is induced in the cytoplasm during differentiation of chick retinal precursor cells and N1E-115 cells. Without its nuclear localization signal, it affects the formation of actin structures similar to inactivation of Rho and forms a complex with Rho-kinase that inhibits its activity in vitro and in vivo. Neurite outgrowth and branching from hippocampal neurons are promoted if $p21^{Cip1/WAF1}$ is expressed abundantly in the cytoplasm (Tanaka et al. 2002). Together, the studies above demonstrate an important link between Rho-kinase and proteins associated with regulation of the actin cytoskeleton, underscoring the importance of actin reorganization for neurite retraction.

Rho-kinase has also been implicated in the reorganization of microtubules and intermediate filaments necessary for neurite retraction. An early study showed that DN Rho-kinase interferes with cytoskeletal collapse of microtubules and the intermediate filament peripherin caused by the presence of serum in N1E-115 cells (Hirose et al. 1998). Interestingly, tubulin disappears in retracting neurites, whereas vimentin and actin remain colocalized. Vimentin is one of the intermediate filaments and a major cytoskeletal component in developing neurons, and is phosphorylated by Rho-kinase, which causes disassembly of these filaments. Interfering with Rho-kinase activity, using a DN mutant or inhibitor, abolishes Rho-kinase-induced phosphorylation of vimentin and results in irregular neurite outgrowth (Nakamura et al. 2000). Thus the Rho/Rho-kinase signaling pathway not only regulates actin organization necessary for growth cone collapse, but also the microtubule and intermediate filament cytoskeletons vital to cell structure.

The role of other Rho GTPases in neurite formation

Additional Rho GTPases that play a role in neurite formation and retraction include RhoG, Rnd1/2, TC10, and RhoT. RhoG has been implicated in several signaling pathways and complexes that contribute to its ability to promote neurite formation. Downstream of Ras, RhoG

activates Rac and Cdc42 to promote neurite outgrowth. Wild-type RhoG is capable of inducing neurite outgrowth in PC12 cells independent of NGF stimulation and enhances outgrowth in its presence in a Rac1- and Cdc42-dependent manner. In support of this, DN mutants of Rac1 and Cdc42 interfere with RhoG-induced neurite outgrowth, CA RhoG elevates endogenous Rac1 and Cdc42 activities, and DN RhoG experiments show that interfering with RhoG function suppresses outgrowth, including that induced by activated Ras (Katoh et al. 2000). RhoG activity is further regulated by the RhoGEF Trio, which contains two RhoGEF domains; GEFD1, which activates Rac through RhoG; and GEFD2, which activates RhoA. Human Trio induces neurite outgrowth in PC12 cells in a GEFD1-dependent manner through RhoG (Estrach et al. 2002), indicating that neurite outgrowth involves Trio/RhoG/Rac1-Cdc42 signaling. A recent study suggests that RhoG also interacts with Elmo in a GTP-dependent manner and forms a ternary complex with Dock180 to activate Rac1. Coexpression of CA RhoG with Elmo and Dock180 promotes relocalization of Elmo and Dock180 from the cytosol to the plasma membrane, and increases Dock180 and Elmo-mediated activation of Rac1. In addition, DN RhoG, and Dock180 and Elmo mutants, are all able to prevent NGF-induced neurite outgrowth in PC12 cells (Katoh and Negishi 2003). Another RhoG-interacting protein, kinectin, is a regulator of microtubule-dependent kinesin activity. Kinectin selectively binds GTP-bound RhoG, inhibition of RhoG activity leads to relocalization of both kinectin and RhoG from the cell periphery to a perinuclear distribution, and changes in the activity of both proteins influence microtubule transport of lysosomes in nonneuronal cells (Vignal et al. 2001). Thus kinectin's ability to link RhoG to microtubule-dependent transport in cells may prove important for neuronal differentiation. Together, these studies show that RhoG acts downstream of Ras and Trio, and may act in concert with Elmo and Dock180, to activate Rac and Cdc42 in order to promote neurite formation. Furthermore, RhoG could potentially regulate microtubule-dependent transport necessary for the growth of neuronal processes.

Rnd proteins, which include Rnd1, Rnd2, and Rnd3 (also known as RhoE), are relatively new members of the Rho family of GTPases that have low intrinsic GTPase activity and are thought to be CA (Foster et al. 1996; Guasch et al. 1998; Nobes et al. 1998). In general, Rnd1 and Rnd3 prevent stress fiber and focal adhesion formation (Guasch et al. 1998; Nobes et al. 1998), suggesting that they function in part by antagonizing the RhoA signaling pathway. In line with this, Rnd1 causes the formation of numerous neurites that contain microtubules but little filamentous actin and neurofilaments in PC12 cells, and the formation of these processes by Rnd1 is inhibited by DN Rac1, suggesting that Rnd1 induces Rac-dependent neurite formation by disrupting cortical actin filaments (Aoki et al. 2000). More recent studies provide direct evidence for Rnd inhibition of RhoA signaling in nonneuronal cells, where Rnd proteins regulate reorganization of the actin cytoskeleton by reducing cellular levels of GTP-bound RhoA through a p190 RhoGAP mechanism and by binding and sequestering Rho-kinase to prevent it from phosphorylating MLCP (Riento et al. 2003; Wennerberg et al. 2003). Rnd2 has also been implicated in neurite formation. Rnd2 interacts with the protein Rapostlin in a GTP-dependent manner, and Rapostlin binds directly to microtubules and regulates reorganization of both actin filaments and microtubules. Rapostlin induces neurite branching in response to Rnd2 in PC12 cells and primary hippocampal neurons, an effect that is dependent upon the microtubule-binding region of Rnd2 (Fujita et al. 2002). Together, the Rnd GTPases appear to promote neurite formation and branching by antagonizing RhoA signaling and coordinating actin and microtubule cytoskeletal changes.

TC10 and RhoT have also been implicated in neurite outgrowth. TC10 was identified as a gene whose expression is up-regulated in response to nerve injury, while RhoA, Rac1, and Cdc42 only show slight up-regulation (Tanabe et al. 2000). This GTPase can associate with some of the same downstream effectors as Cdc42 and Rac, including PAK, N-WASP, and MRCK (Neudauer et al. 1998; Abe et al. 2003). TC10 is highly expressed in muscular tissue and brain and is induced during differentiation of C2 skeletal muscle cells and neuronal differentiation in N1E-115 and PC12 cells. RhoT is expressed in heart and uterus and during neuronal differentiation of N1E-115 cells. Both TC10 and RhoT have been found to induce neurite outgrowth in PC12 and N1E-115 cells (Abe et al. 2003), and TC10 induces neurite extension in cultured rat DRG neurons (Tanabe et al. 2000). DN mutants of TC10 and RhoT are capable of preventing neuronal differentiation induced by dibutyryl cyclic AMP in PC12 cells and by serum starvation in N1E-115 cells (Abe et al. 2003), but DN TC10 is unable to inhibit NGF-induced neurite outgrowth in PC12 cells (Murphy et al. 2001). In addition, neurite formation induced by TC10 and RhoT is mediated by N-WASP, given that DN N-WASP mutants, including one deficient for GTPase binding and one deficient for ARP2/3 binding, can inhibit TC10 and RhoT-induced neurite outgrowth (Abe et al. 2003).

Neuronal polarization, axon growth, and regeneration

How does a neuron develop the unique and intricate architecture that allows it to receive and store information vital for cognitive function? Neurons possess a highly polarized structure that provides for the vectorial flow of information. They typically have several dendrites that receive inputs from presynaptic neurons and one axon that relays information to post-synaptic neurons. The development of these processes has been studied extensively in rat primary hippocampal cultures and consists of five stages (see Fig. 2). Upon plating, round neurons attach to the substratum and form a lamellipodium (Stage 1), which is breached by the sprouting of several minor neurites (Stage 2). These neurites are cylindrical

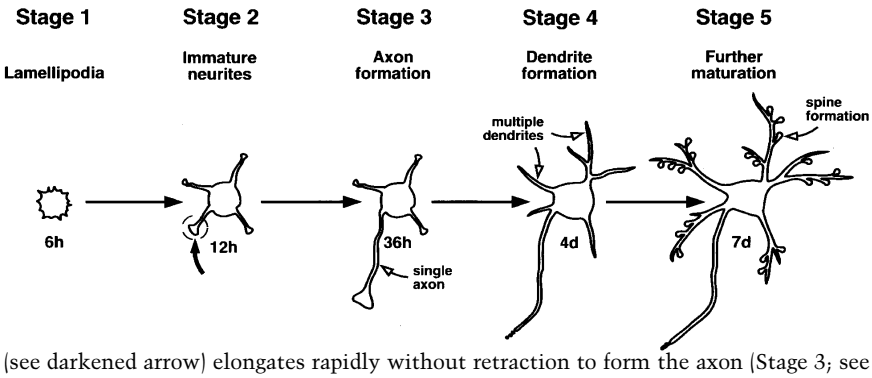

Figure 2. Establishment of polarity and stages of neuronal development in hippocampal neurons. Cultured hippocampal neurons form a lamellipodium when they attach to the substratum (Stage 1), and shortly after neurites begin to sprout (Stage 2). The neurites are cylindrical protrusions that contain a growth cone at their distal tip and they lack molecular and structural characteristics of mature axonal or dendritic processes. These neurites extend and retract without net elongation. The following day, one of the neurites with an enlarged growth cone at its tip (see darkened arrow) elongates rapidly without retraction to form the axon (Stage 3; see open arrow). Several days later, the remaining neurites continue to grow and branch to form the dendrites (Stage 4), and in a final step of maturation, the axon and dendrites develop further and dendritic protrusions, or spines, appear (Stage 5). See text for further explanation and references.

extensions with a growth cone at the distal tip, and while they continue to extend and retract, there is no net elongation of these processes during this time. Within 24 h, one of these neurites will elongate rapidly without retraction to form the axon (Stage 3), and several days later the remaining neurites will grow at a slower rate to form the dendrites (Stage 4). Lastly, the axon develops further, dendrites continue to branch, and dendritic spines are formed, allowing for the formation of synaptic contacts and spontaneous electrical activity throughout the neuronal network (Stage 5) (Dotti et al. 1988; Higgins et al. 1997; da Silva and Dotti 2002; Arimura et al. 2004).

Neuronal polarization is therefore an early step in the differentiation of a neuron and is generally accomplished when one neurite with an enlarged growth cone rapidly elongates to become the axon (between Stages 2 and 3). A prevalent question in neurobiology, though, is how neuronal polarization is actually established and maintained. What triggers or specifies the enlargement and elongation of that particular growth cone and neurite, respectively, thus marking it to become the axon? A model has been suggested in which Stage 2 neurites send a growth-promoting signal to themselves and a growth inhibitory signal to the other neurites, creating a balance between the two signals that prevents net elongation. Eventually, one fast-growing neurite manages to overcome this balance, while sending a stronger inhibitory signal to the other neurites, thus suppressing their growth (Andersen and Bi 2000; Bradke and Dotti 2000). Rapid neurite growth may therefore play a role in the specification of neuronal polarization. In addition, a prominent feature of early cellular polarization in general is the presence of a physical spatial landmark that marks the section of the plasma membrane that will initiate polarization, such as the bud scar in yeast or points of intercellular adhesion in epithelial cells (Chant and Herskowitz 1991; Yeaman et al. 1999). Cellular polarization also requires a segregation of protein and lipid components (Ledesma and Dotti 2003), and depends upon membrane protein diffusion barriers (Kobayashi et al. 1992; Winckler et al. 1999; Nakada et al. 2003), protein complex stabilization at the membrane by scaffolding molecules (Harris and Lim 2001), and protein targeting along the secretory or endosomal pathways (Jacob and Naim 2001; Keller et al. 2001; Kreitzer et al. 2003). Mechanisms such as these also contribute to neuronal polarization (Horton and Ehlers 2003), given that the axonal growth cone differs from those of neurites and dendrites in its membrane constitution and there is an accumulation of axon-specific proteins (Goslin et al. 1988; Fletcher et al. 1991; van Lookeren Campagne et al. 1992; Deitch and Banker 1993; Bradke and Dotti 1997; Silverman et al. 2001). In addition to these drastic changes in membrane composition and molecular sorting, a polarizing cell also requires coordinated changes between the actin and microtubule cytoskeletons to promote and support growth (Baas 2002; Ledesma and Dotti 2003; Rodriguez et al. 2003). The actin cytoskeleton of both the neurite that is to become the axon and a mature axon is more dynamic and unstable compared with that of other neurites or dendrites (Bradke and Dotti 1999), which presumably allows microtubules to elongate and push through the outer peripheral area of the growth cone to allow axon growth (Bradke and Dotti 1999; Baas 2002; Ledesma and Dotti 2003). Thus a fast growing neurite receives a polarized accumulation of materials and undergoes extensive cytoskeletal rearrangements to become the axon.

Role of Rho signaling in neuronal polarization

Given that the process of neuronal polarization has been studied most extensively in cultured hippocampal neurons, the best evidence for a role for the Rho GTPases in this neuronal process comes from studies in this system. The first evidence for the involvement of the Rho GTPases in the establishment of neuronal polarity comes from the treatment of Stage 2 hippocampal neurons with *Clostridium difficile* toxin B, an inhibitor of the Rho family of GTPases. Toxin B causes a loss of F-actin and the production of multiple axons, similar to treatment with the actin depolymerizing agents cytocholasin D and latrunculin B (Bradke and Dotti 1999). Since toxin B inhibits all Rho GTPase members, it is difficult to say from this study which Rho GTPase members are involved in axon formation. However, a recent study sug-

gests that Cdc42 plays a prominent role in the earliest stages of axon formation downstream of the Rap1B GTPase in cultured rat hippocampal neurons. Rap1B localizes to the tip of a single neurite just prior to axon formation, and both CA Rap1B and a fast cycling Cdc42 mutant are capable of inducing the formation of supernumerary axons. Furthermore, small hairpin RNAs for *Rap1B* and *Cdc42* prevent axon formation, and this loss of polarity upon Rap1B knock-down is rescued by the fast cycling Cdc42 mutant (Schwamborn and Puschel 2004). A potential Rac/Cdc42-interacting complex, the mPar3/mPar6/atypical protein kinase C (aPKC) protein complex, has also been found to play a role in the early stages of axon formation and may do so in collaboration with Cdc42. This protein complex has been shown to interact with activated forms of Rac1 and Cdc42 through mPar6 (Joberty et al. 2000; Johansson et al. 2000) and is required for the generation of anterior–posterior polarity in early *C. elegans* embryos, the apical–basal polarity of epithelial cells and neuroblasts in *Drosophila*, and the polarization of primary rat astrocytes during migration (Jan and Jan 2000; Kemphues 2000; Etienne-Manneville and Hall 2001; Ohno 2001). Before neuronal polarization, mPar3 is present in all neurites of Stage 2 rat hippocampal neurons, but becomes concentrated along with mPar6 in axonal growth cones after polarization (Stage 3). Importantly, neuronal polarization is prevented by overexpression and mislocalization of mPar3 or mPar6, by inhibition of aPKC or PI 3-kinase activity, or by overexpression of PTEN, a phosphatase that degrades the PI 3-kinase product PI(3,4,5)P3. In addition, inhibition of PI 3-kinase activity or overexpression of PTEN causes mislocalization of mPar3, which likely causes the disruption of polarity. These findings prompted the proposal of a model in which local PI 3-kinase activity recruits the Par complex to the growth cone to promote actin changes via Rac and Cdc42 and microtubule changes via aPKC (Shi et al. 2003). Given that Rac1 and Cdc42 enhance PI 3-kinase activity and stimulate Par complex formation (Tolias et al. 1995; Keely et al. 1997; Chan et al. 2002), it was suggested that a positive feedback loop may amplify growth cone signaling to break the symmetrical distribution of mPar proteins and promote neuronal polarization (Shi et al. 2003). However, it was not determined in this study whether PI 3-kinase activation and Par complex formation actually lie upstream of Rac and/or Cdc42 activation, or vice versa, to promote changes in the actin and microtubule cytoskeletons necessary for neuronal polarization. Evidence suggesting the involvement of Cdc42 in this signaling cascade comes from the study cited earlier in which Cdc42 lies downstream of Rap1B to promote neuronal polarization. This study suggests that Cdc42 activation lies downstream of PI 3-kinase and interacts with Par6 as well. Rap1B localization to the tip of the neurite that is to become the axon precedes axonal localization of Cdc42, phosphorylated Akt/PKB, and Par3, and the supernumerary axons induced by CA Rap1B or the fast cycling Cdc42 mutant contain Par3, phosphorylated aPKC, and phosphorylated Akt in the growth cone. In addition, unlike the fast cycling mutant of Cdc42, CA Rap1B is unable to reverse the loss of polarity resulting from uniform distribution of Par6c, suggesting that Par6 acts downstream of Rap1B and either acts upstream of, or in parallel with, Cdc42. Lastly, both CA Rap1B and the fast cycling Cdc42 mutant rescue axon loss caused by inhibition of PI3-kinase activity, suggesting that PI(3,4,5)P3 acts upstream of both Rap1B and Cdc42 (Schwamborn and Puschel 2004). Therefore the earliest molecular determinants of neuronal polarization thus far appear to be PI 3-kinase and Rap1B, which act upstream of the Rho GTPase Cdc42. The nature of the relationship between PI 3-kinase and Rap1B, including potential Rap1B GEFs involved, and between Cdc42, the Par complex, and aPKC to promote actin and microtubule changes necessary for axonal initiation and growth remain to be elucidated. An additional study implicates the plus-end-directed microtubule motor protein KIF3A in neuronal polarization via an interaction with Par3. Expression of DN Par3 and KIF3A constructs inhibits Par3 and aPKC accumulation at neurite tips and prevents polarization in cultured rat hippocampal neurons. This finding suggests that KIF3A actively transports Par3 to the distal axon tip in order to establish polarity (Nishimura et al. 2004). How KIF3A function is affected by PI 3-kinase, Rap1B, Cdc42, and Rac activities, or vice versa, also remains to be seen.

Additional potential regulators and effectors of the Rho GTPases that play a role in the polarization of cultured hippocampal neurons include those shown to associate with and influence the actin and microtubule cytoskeletons. The Rac-specific GEF Tiam1 has been implicated in the regulation of axon formation. Tiam1 localizes to the neurite that is to become the axon, and to its enlarged growth cone, in Stage 2–3 rat hippocampal neurons. Overexpression of Tiam1 promotes the formation of several long and thin axon-like processes, while suppression of Tiam1 using an antisense approach prevents axon formation. Treatment with cytochalasin D, an actin depolymerizing drug, reverses the antisense effect and causes the formation of multiple axons. These results suggest that Tiam1, like cytocholasin D, promotes growth cone actin reorganization and allows microtubules to extend into regions of the neurite tip devoid of actin filaments (Kunda et al. 2001).

In addition to Tiam1, a prominent substrate of Rho-kinase, collapsin response mediator protein-2 (CRMP-2)/TOAD-64/Ulip2/DRP-2, has been shown to promote axon formation and may do so by regulating the microtubule cytoskeleton. CRMP-2 is highly expressed in the developing axon of cultured hippocampal neurons. Overexpression of CRMP-2 causes the formation of multiple axons and even induces mature dendrites to become axon-like, while deletion mutants of CRMP-2 suppress axon formation (Inagaki et al. 2001). CRMP-2 is capable of binding tubulin heterodimers and promoting microtubule assembly, and, importantly, a CRMP-2 mutant unable to promote microtubule assembly inhibits axonal growth and branching (Fukata et al. 2002). With regard to Rho signaling, CRMP-2 antagonizes Rho/Rho-kinase signaling (Arimura et al. 2000, 2004; Hall et al. 2001; Leung

et al. 2002), and Rho-kinase phosphorylation of CRMP-2 prevents its association with tubulin dimer (Arimura et al. 2004), suggesting that Rho-kinase might inhibit axon formation in part by disrupting CRMP-2-promoted microtubule assembly. Finally, CRMP-2 also plays a role in endocytosis through its association with the α-adaptin-binding molecule Numb. Numb associates with the neuronal cell adhesion molecule L1, which is endocytosed and recycled at the growth cone, where CRMP-2 and Numb colocalize. Interfering with CRMP-2 function inhibits L1 endocytosis in the axonal growth cone and suppresses axon growth (Nishimura et al. 2003). Thus these studies imply that CRMP-2 modulates microtubule assembly and membrane trafficking necessary for axonal formation and growth, and that CRMP-2's activity may be modulated by Rho-kinase to regulate or limit these processes.

Studies in a number of systems other than cultured hippocampal neurons provide further support for the involvement of the Rho GTPases in axon formation. CA Rac1, DN Rac1, or CA Cdc42 inhibits axon formation in *Drosophila* sensory neurons (Luo et al. 1994) and *Xenopus* retinal ganglion cells (RGCs) (Ruchhoeft et al. 1999), as does CA Rac1 in *Drosophila* motor neurons (Kim et al. 2003). While these results seem contradictory to those above in dissociated hippocampal neurons where Cdc42 and Tiam1 activity promote axon formation, it should be noted that a CA Cdc42 mutant, as opposed to the fast cycling mutant, actually prevents neurite/axon formation in cultured hippocampal neurons as well (Schwamborn and Puschel 2004). The finding that DN Rac1 also inhibits axon formation in *Drosophila* sensory neurons (Luo et al. 1994) suggests that proper cycling of the GTPases between an active GTP-bound and an inactive GDP-bound form may be required for proper axon formation, but system-dependent effects of these GTPases on axon formation may play a part in the overall phenotype. Potential downstream effectors of Rac implicated in axon formation include the proposed Rac effector Cdk5 and the PAK family kinases. Inhibition of axon initiation by CA Rac1 can be partially rescued by DN Cdk5 in *Xenopus* RGCs (Ruchhoeft et al. 1999), and mice with a disrupted *PAK4* gene exhibit defects in neuronal development and axon formation in the hindbrain (Qu et al. 2003). In reference to the latter finding, another study has shown that non-CRIB domain containing effectors are also likely to play a role in axon formation, since axon formation defects persist in a subset of *Drosophila* motor neurons even when a Rac1 effector loop mutation disrupts its ability to bind PAK and other CRIB domain-containing effector proteins (Kim et al. 2003). Differences in a requirement for PAK in axon formation may be cell type or system dependent or may be due to the interference of different PAK family members.

In contrast to the positive effects of Cdc42 and Rac on axonogenesis mentioned above, Rho activation antagonizes axon formation. CA Rho prevents axon formation in *Xenopus* RGCs and cultured mouse cerebellar granule cells (Ruchhoeft et al. 1999; Bito et al. 2000), and strong activation of Rho using a high concentration of the neural chemokine SDF-1 (stromal cell-derived factor 1) inhibits axon formation in the latter system (Arakawa et al. 2003). Conversely, inhibition of Rho using C3 exoenzyme increases axon number in cultured mouse cerebellar granule neurons (Bito et al. 2000). The effects of the Rho effector Rho-kinase are consistent with those for Rho. Rho-kinase prevents axonogenesis in cultured mouse cerebellar granule neurons, while inhibition of Rho-kinase with Y-27632 or DN Rho-kinase mutants causes the immediate outgrowth of membrane ruffles and filopodia, followed by the generation of initial growth cone-like membrane domains from which axonal processes arise (Bito et al. 2000). In addition, Y-27632 treatment rescues the SDF-1-induced inhibition of axon formation in these cells (Arakawa et al. 2003).

RhoG has also been implicated in axon formation as a target of the dual Rho GEF Kalirin. Kalirin is similar to Trio and UNC-73 (Debant et al. 1996; Steven et al. 1998), and alternative splicing results in the formation of multiple isoforms (Johnson et al. 2000). Kalirin, however, is predominantly expressed in the nervous system, while Trio is broadly expressed throughout the body (May et al. 2002). Kalirin isoforms generally contain two Rho family GEF domains, with the first specific for Rac1 and the second specific for RhoA (Penzes et al. 2001a). Evidence suggests that the effects of Kalirin on axon formation are mediated by RhoG, since the first Kalirin GEF domain actually binds to RhoG more strongly than to Rac1 and is a potent activator of RhoG. Overexpression of Kalirin-9 or Kalirin-12, which both contain two Rho family GEF domains, initiates the formation of multiple prominent axonal outgrowths from the cell body in cultured rat superior cervical ganglion (SCG) neurons, similar to overexpression of the first GEF domain or CA RhoG. Axonal sprouting induced by Kalirin-12 or the first GEF domain is also blocked by RhoG inhibitors. The finding that the first GEF domain causes cellular and cortical actin adjacent to the plasma membrane to redistribute to newly formed lamellipodial fiber structures (May et al. 2002) suggests that Kalirin induces the formation of axons through RhoG-elicited cytoskeletal rearrangements.

Together, the studies above support a role for the Rho GTPases, their regulators, and effectors in multiple steps of neuronal polarization, which includes specification, maintenance, and elongation of the axon. In general, Cdc42 and Rac activation, and Rho inactivation, appear to promote axon formation. It is important to note, however, that separating the molecular components that actually specify whether a neurite is to become an axon from those that merely play a supporting role in the growth process is not always straightforward, since axon formation requires growth even at the earliest stages of initiation and growth itself may actually serve as a trigger for axon formation.

Role of Rho signaling in axonal extension and branching

Once the axon is formed and neuronal polarization has been established, the Rho GTPases continue to contrib-

ute to later stages of axon development, including axonal extension and branching. Several studies suggest that interfering with Rho GTPase function disturbs these aspects of axonal growth. Triple mutant *Drosophila* embryos lacking Rac1, Rac2, and Mtl exhibit growth defects in mushroom body (MB) neurons and at the CNS midline, with loss of these Rac GTPases preferentially causing axon stalling (Hakeda-Suzuki et al. 2002; Ng et al. 2002), suggesting that Rac activity positively promotes axon extension in these systems. Additional studies using CA and DN Rac mutants have further revealed that proper Rac function is required for axon growth, although the effects of dominant mutants on axon growth do not mimic those of the loss-of-function conditions. CA Rac1 in *Drosophila* peripheral neurons causes stalled axons (Luo et al. 1994), and Purkinje cells of transgenic mice expressing CA human Rac1 show a reduction of axon terminals in deep cerebellar nuclei (Luo et al. 1996b). DN Rac1 occasionally causes extension of the axon beyond the target muscle in *Drosophila* motor neurons (Kim et al. 2003), an increase in axonal branching in the *Drosophila* giant fiber system (Allen et al. 2000), and a decrease in axonal growth in cultured rat cortical cells (Threadgill et al. 1997). These phenotypic differences may be system dependent or reflect a need for Rac to cycle between an active GTP-bound and inactive GDP-bound form to properly regulate axon growth. In addition to Rac's involvement in axon extension, loss of Cdc42 function has been associated with increased branching of misguided *Drosophila* vertical system (VS) neurons (Scott et al. 2003), while DN Cdc42 decreases axonal growth in cultured rat cortical cells (Threadgill et al. 1997). Taken together, these studies clearly implicate Rac in axonal extension and branching, and suggest that Cdc42 may play a role in these processes as well.

The Rac regulator Trio has been shown to be important for axonal growth and likely confers specificity upon the Rac signaling pathway to promote axon growth. *Drosophila* embryos lacking Trio function exhibit severe stalling at the CNS midline, and overexpression of the first Trio GEF domain using an eye-specific promoter severely disrupts eye morphology and causes aberrant photoreceptor axon projections. The latter effect is suppressed in animals homozygous for loss-of-function mutations in *Rac1* or *Rac2*, and in *Rac1*/*Rac2*/*Mtl* triple mutants, suggesting that Trio regulates axonal growth through Rac. Furthermore, the effects of these experiments support the idea that Rac activity positively promotes axonal extension (Hakeda-Suzuki et al. 2002). In addition to potential specificity provided by upstream regulators of Rac, the effects of Rac on axonal growth have been suggested to depend upon the downstream effector involved, which is likely dependent upon the degree of Rac activation. The progressive loss of Rac1, Rac2, and Mtl in *Drosophila* MB neurons first causes defects in axonal branching, then guidance, and finally growth. A Rac effector binding loop mutant that fails to bind CRIB motif-containing effector proteins, including PAK, is capable of rescuing growth, only partly rescues guidance, and does not rescue branching defects caused by loss of the Rac GTPases (Ng et al. 2002). These findings suggest that CRIB motif-containing effector proteins are required for Rac's effect on axonal branching and are partly required for Rac's effect on axon guidance, but are not required for Rac's effect on growth. Axonal growth and guidance must therefore rely upon Rac signaling pathways other than, or in addition to, CRIB motif-containing effector pathways.

Interestingly, activation of Rho has been implicated in both axon retraction and extension. p190 RhoGAP positively regulates axonal outgrowth and stability through negative regulation of RhoA activity. Mice lacking functional p190 RhoGAP exhibit a specific reduction in subcortical axon number (Brouns et al. 2001), which may be due to inhibition of axon initiation and/or axon retraction due to elevated Rho activity. Studies in *Drosophila* MB neurons suggest that the latter may be the case. p190 RhoGAP overexpression causes axon overextension, and inactivation causes axon branch retraction in *Drosophila* MB neurons. The *p190 RhoGAP* inactivation phenotype is enhanced by RhoA expression and mimicked by CA Drok, *Drosophila* Rho-kinase. Additionally, the finding that the *p190 RhoGAP* inactivation phenotype is suppressed by loss of spaghetti squash (sqh), which encodes myosin regulatory light chain, suggests that Rho-induced axonal retraction is dependent upon actomyosin contractility (Billuart et al. 2001). This study therefore suggests that p190 RhoGAP regulates axonal extension by inactivating the Rho/Rho-kinase signaling pathway and shows that axon retraction is induced by Rho-kinase activation and requires actin cytoskeletal changes.

In contrast to the findings above, inhibition of Rho activity using DN RhoA reduces axonal branching and length in cultured mouse hippocampal neurons (Ahnert-Hilger et al. 2004), and another regulator of Rho, the dual Rho GEF Kalirin, activates Rho to enhance axonal extension. Overexpression of Kalirin-9 results in longer neurites with multiple lateral growth cones in cultured rat cortical neurons (Penzes et al. 2001a), and overexpression of Kalirin-9 or Kalirin-12 increases the branching of newly formed multiple axons in rat primary SCG cultures. Conversely, *Kalirin* antisense blocks the extension of pre-existing axons in SCG neurons (May et al. 2002). Overexpression of the first GEF domain of Kalirin drastically shortens axons and causes the formation of excessive growth cones in a Rac1-dependent manner in cortical neurons, while the second GEF domain induces overelongation of axons and abundant filopodial neurites through the RhoA/Rho-kinase signaling pathway in the same system (Penzes et al. 2001a). Thus the presence of two different GEF domains in Kalirin allows this protein to coordinate different incoming signals to regulate morphological changes, with activation of the Rho/Rho-kinase pathway promoting axonal extension.

A recent study suggests that different levels of GTP-bound Rho may trigger different downstream effector molecules to regulate axonal extension. SDF-1α is a neural chemokine shown to play a role in the migration of cerebellar granule cells (Klein et al. 2001; Lu et al. 2001; Tham et al. 2001; Zhu et al. 2002) and axon guidance

(Xiang et al. 2002; Chalasani et al. 2003). SDF-1α promotes axonal extension through a Rho/mDia signaling pathway at low concentrations, but inhibits axonal extension, and initiation as mentioned above, through a Rho/Rho-kinase signaling pathway at higher concentrations in cultured cerebellar granule neurons. However, even at a high concentration of SDF-1α, Rho/mDia-dependent axonal extension can be uncovered by inactivating Rho-kinase using the Rho-kinase inhibitor Y-27632. SDF-1α-induced axonal extension upon Y-27632 treatment is similar to that of CA mDia1 and can be counteracted by DN mDia or RNAi knock-down of mDia1, leading to a decrease in axon number and length. In addition, DN Rac represses axonal extension facilitated by CA mDia1 in the presence of Y-27632, and pull-down assays show that SDF-1α and Y-27632-treated cells exhibit an increase in GTP-bound Rac (Arakawa et al. 2003). Given that mDia controls actin polymerization and promotes the formation and orientation of stable microtubules (Palazzo et al. 2001), mDia likely promotes Rac-dependent axonal extension through spatial coordination of actin polymerization and microtubule stabilization and orientation. The finding that cells expressing CA mDia exhibit abnormal neuronal processes that are filled with excessive amounts of filamentous actin and β-tubulin lends further support to this supposition (Arakawa et al. 2003).

The studies cited above not only demonstrate a role for the Rho GTPases in axon growth, which is dependent upon actin and microtubule cytoskeletal changes, but also offer plausible explanations for the variety of phenotypes that can be elicited by a single Rho GTPase. Rho GTPase activity is not necessarily an all-or-none phenomenon. Activation of the Rho GTPases under normal conditions depends upon the presence of spatially and temporally regulated Rho GTPase regulators, such as Rho GEFs and Rho GAPs, and it is the fine balance between a number of these regulators that determines the local degree of Rho GTPase activity. Furthermore, it is the degree of Rho GTPase activation that at least partially determines which effector the Rho GTPase will interact with to elicit specific cellular responses. This scenario, in which a specific cellular response (axon growth) is determined by a combination of spatial and temporal regulation of the Rho GTPases by specific regulators and the activation of different effector pathways, is particularly illustrated by the SDF-1α, Kalirin, and Rac triple loss-of-function experiments described above.

Injury and axon regeneration

After injury, axonal regeneration in the CNS is prevented by a lack of growth-promoting molecules and the presence of inhibitory ones. A number of growth inhibitory molecules involved are derived from myelin and include myelin-associated glycoprotein (MAG), Nogo-A, chondroitin sulfate proteoglycans (CSPGs), and oligodendrocyte myelin glycoprotein (OMgp) (Niederost et al. 1999; McGee and Strittmatter 2003; Yiu and He 2003; Sandvig et al. 2004; Schwab 2004). A common finding in axonal regeneration studies is the involvement of the RhoA signaling pathway. Recent studies have demonstrated an increase in RhoA activity in situ after spinal cord injury using the Rho-binding domain (RBD) of the RhoA effector Rhotekin as a probe for active, GTP-bound RhoA. Incubating rat CNS tissue sections with RBD protein reveals active RhoA in both grey and white matter after spinal cord injury, an effect that is reversed by C3 exoenzyme and supported by biochemical analysis in both rats and mice (Dubreuil et al. 2003). RBD protein (made cell permeable by fusing it to the transduction domain of the human immunodeficiency virus protein PTD4) that is injected into the tail vein of rats with induced spinal cord injury also results in intense RBD protein staining of fibers in white matter compared with controls (Madura et al. 2004). Excitingly, axonal regeneration studies in vivo also provide hope that interfering with RhoA signaling may prove clinically relevant and aid recovery. It was first discovered that inactivation of Rho using C3 exoenzyme causes the regrowth of crushed adult rat optic nerve axons (Lehmann et al. 1999). Subsequent studies revealed that inactivation of Rho with C3 exoenzyme or Rho-kinase with Y-27632 promotes axonal regeneration and rapid recovery of locomotion and forelimb–hindlimb coordination after spinal cord injury in mice (Dergham et al. 2002). In adult rats, Y-27632 also enhances recovery after corticospinal tract lesions by increasing axonal sprouts and accelerating locomotor recovery (Fournier et al. 2003). Thus Rho signaling appears to mediate inhibitory effects on axonal growth after injury. Links between growth-inhibitory molecules and RhoA signaling are therefore discussed below.

MAG, Nogo-A, and OMgp are myelin-associated, glia-derived inhibitory factors, and Nogo-A is an integral membrane protein that contains at least two active domains, NiG and Nogo-66. The Nogo receptor (NgR) is an axonal glycosyl–phosphatidyl–inositol (GPI)-anchored protein that binds and mediates the inhibitory effects of Nogo-66, MAG, and OMgp (Domeniconi et al. 2002; Liu et al. 2002; Wang et al. 2002b; McGee and Strittmatter 2003; Yiu and He 2003; Schwab 2004). Mounting evidence suggests that CNS myelin and myelin-associated inhibitors cause growth cone collapse and limit axonal regeneration through the activation of Rho signaling. Active, GTP-bound RhoA is increased and neurite outgrowth is prevented in PC12 cells plated on myelin compared with cells plated on poly-L-lysine (Winton et al. 2002; Dubreuil et al. 2003). PC12 cells plated on MAG also exhibit a similar increase in active RhoA (Dubreuil et al. 2003), and both MAG and MAG receptors localize to lipid rafts, the latter of which also contain RhoA (Vinson et al. 2003). In addition, Nogo-66 has been shown to increase RhoA activity in PC12 cells and chick DRGs (Niederost et al. 2002), and NiG, Nogo-66, and the extracellular domain of MAG all activate RhoA, decrease Rac1 activity, and inhibit neurite outgrowth in post-natal rat cerebellar granule neurons (Fournier et al. 2003). Importantly, inhibition of the RhoA signaling pathway with C3 exoenzyme and Y-27632 abrogates the inhibitory effects of myelin, Nogo-A fragments, and MAG on

neurite outgrowth and growth cone collapse (Lehmann et al. 1999; Dergham et al. 2002; Niederost et al. 2002; Winton et al. 2002; Borisoff et al. 2003; Dubreuil et al. 2003; Fournier et al. 2003). In line with these findings, CA Rac1 protects against myelin-induced growth cone collapse and permits neurite outgrowth in chick motor neuron cultures. However, CA RhoA unexpectedly produces effects similar to those of CA Rac1 in this last study (Kuhn et al. 1999).

Given that NgR is GPI-linked and lacks an intracellular signaling domain, how does NgR transduce an inhibitory signal elicited by the above myelin-associated factors? Recent evidence suggests that NgR signals through the coreceptor $p75^{NTR}$, a low-affinity receptor for neurotrophins. Two independent groups have shown that $p75^{NTR}$ associates with NgR and that this association is required for inhibition induced by known NgR-ligands (Wang et al. 2002a; S.T. Wong et al. 2002). Primary neurons from *$p75^{NTR}$* knockout mice are no longer responsive to CNS myelin, Nogo-66, OMgp, and MAG (Wang et al. 2002a; Yamashita et al. 2002), and an antibody against $p75^{NTR}$ abolishes MAG-induced repulsive turning of *Xenopus* axonal growth cones and calcium elevation (S.T. Wong et al. 2002). With regard to Rho signaling, $p75^{NTR}$ has been shown to associate with and activate RhoA, which is abolished upon receptor binding of a number of neurotrophins, including NGF, BDNF and NT-3. In cultured chick ciliary neurons, inactivation of Rho by C3 exoenzyme mimics the effect of neurotrophins by increasing neurite elongation, while CA RhoA inhibits NGF-induced neurite outgrowth (Yamashita et al. 1999). A recent study shows that RhoA, probed with RBD protein, colocalizes with $p75^{NTR}$ in cerebellar granule neurons treated with Nogo peptide, and the RBD signal is more robust when the cerebellar granule neurons are plated on myelin as opposed to poly-L-lysine (Madura et al. 2004). Furthermore, $p75^{NTR}$ staining is markedly up-regulated in grey and white matter after induced spinal cord injury in rats, this $p75^{NTR}$ staining colocalizes with intense RBD staining (Dubreuil et al. 2003; Madura et al. 2004), and up-regulation of $p75^{NTR}$ in both neurons and glia after spinal cord injury can be blocked by C3 exoenzyme (Dubreuil et al. 2003). Additional evidence in support of a role for Rho downstream of $p75^{NTR}$ is provided by the finding that Rho activation is not observed immediately after spinal cord injury in mice lacking functional $p75^{NTR}$ (Dubreuil et al. 2003).

How does $p75^{NTR}$ link myelin-induced inhibitory signals to RhoA activation? An early study reported that $p75^{NTR}$ bound RhoA in a yeast two-hybrid screen, that the two proteins coimmunoprecipitate, and that $p75^{NTR}$ activates RhoA in a manner that is antagonized by neurotrophin binding to the receptor in a heterologous system (Yamashita et al. 1999). Subsequent studies showed that $p75^{NTR}$ colocalizes with MAG binding in mouse DRG neurons and that MAG inhibits neurite outgrowth in a Rho-dependent manner in post-natal cerebellar neurons, given that C3 exoenzyme counteracts this effect (Yamashita et al. 2002). In addition, as mentioned above, RhoA colocalizes with $p75^{NTR}$ in cerebellar granule neurons treated with Nogo peptide (Madura et al. 2004). Despite these findings, direct binding of $p75^{NTR}$ to MAG has not been demonstrated and $p75^{NTR}$ is now believed to indirectly associate with RhoA through Rho-GDI. Rho-GDI binds RhoA and renders it inactive by sequestering it in the cytoplasm. It is believed that the binding of Rho-GDI to the $p75^{NTR}$ receptor results in the release of RhoA from Rho-GDI, whereupon RhoA can be activated by as yet unidentified RhoGEFs. This interaction between $p75^{NTR}$ and Rho-GDI is further strengthened by MAG or Nogo and is antagonized by the binding of NGF to the receptor. Furthermore, a peptide that prevents association between $p75^{NTR}$ and Rho-GDI prevents inhibition of neurite outgrowth and activation of RhoA by MAG or Nogo in mouse cerebellar granule neurons (Yamashita and Tohyama 2003). Thus myelin-derived inhibitors of axon outgrowth facilitate an interaction between $p75^{NTR}$ and Rho-GDI in order to increase Rho signaling and prevent axonal regeneration.

Additional myelin-associated inhibitory molecules are the CSPGs (Niederost et al. 1999), which are also present in CNS injury scars (McKeon et al. 1991; Dow et al. 1994; Gates et al. 1996; Fitch and Silver 1997; Davies et al. 1999; Lemons et al. 1999). The inhibitory action of CSPGs on axonal regeneration was demonstrated by a recent study in which chondroitinase ABC, which degrades chondroitin sulfate glycosaminoglycan, was infused into the CNS after spinal cord injury in adult rats. Importantly, the presence of chondroitinase ABC promoted axonal elongation and improved recovery of postsynaptic activity below the lesion site upon electrical stimulation of corticospinal neurons, as well as functional recovery of locomotor and proprioceptive behaviors (Bradbury et al. 2002). Additional studies have suggested that Rho signaling is responsible for the inhibitory effects of CSPGs on axonal regeneration. In chick RGCs and rat primary cortical neurons, inactivation of the RhoA signaling pathway with C3 exoenzyme or Y-27632 blocks CSPG-induced repulsion and increases neurite formation on CSPG-containing substrates, such as glial scar tissue (Dergham et al. 2002; Monnier et al. 2003). In addition, a member of the CSPG family of neurite growth inhibitors, aggrecan, stimulates Rho activity and inhibits axonal growth in chick DRG, while inhibition of Rho-kinase using Y-27632 results in the formation of smaller growth cones with longer filopodia and smaller lamellipodia and increases axonal outgrowth on this substrate (Borisoff et al. 2003). In accordance with the findings above for other myelin-associated molecules, Rho signaling has been shown to play an inhibitory role downstream of CSPGs. However, the mechanism by which Rho activity is regulated by CSPGs, including potential receptors that transmit the CSPG signal, remain to be defined.

Despite the presence of a variety of axon inhibitory molecules in the CNS, it is evident from the studies above that Rho activation by these inhibitors is a common denominator in the prevention of axonal regeneration after spinal cord injury. As a result, the Rho signaling pathway provides a potential and promising target for

therapeutic intervention in order to aid and speed recovery after injury. However, it is likely that some degree of specificity by individual inhibitory factors is conferred by the activation of other signaling pathways in addition to that of RhoA. Therefore, further elucidation of these pathways, as well as of additional Rho regulators and effectors involved, will not only shed light on the basic molecular mechanisms involved in axon outgrowth, but will also provide a better sense of how to treat the debilitating effects of spinal cord injury and diseases that affect neuronal function.

Axon pathfinding and guidance

Axon pathfinding often involves the extension of axons over long distances to reach their targets and form connections as part of a complex neuronal network. The axonal growth cone, which contains receptors that sense environmental guidance cues, or molecules, helps navigate the axon en route to its final destination. Guidance cues can either cause attraction or repulsion, depending upon the type of receptor bound and the resulting cytoskeletal changes. Structural changes resulting from cytoskeletal reorganization within the growth cone are responsible for the axon's ability to extend and retract, with attractive cues promoting actin polymerization and extension, and repulsive cues decreasing actin polymerization, causing lamellipodial and filopodial collapse and axon retraction. Attractive and repulsive guidance cues are therefore read by the growth cone and translated into morphological changes that allow the axon to turn in the appropriate direction (K. Wong et al. 2002; Gordon-Weeks 2004; Zhou and Cohan 2004). In this section, we first discuss evidence for the involvement of the Rho GTPases and some of their regulators and effectors in axon guidance. This will be followed by a brief description of the role of the Rho GTPases in growth cone morphology as it pertains to axon guidance, given the general dependence of axon guidance on growth cone signaling and cytoskeletal changes influenced by the Rho GTPases. Lastly, we link guidance cues and their receptors with Rho GTPase signaling and describe their effects on axon repulsion and attraction, which majorly determines whether an axon will reach its appropriate target.

The role of Rho signaling in axon guidance

A growing body of evidence suggests that Rho GTPase function is essential for axons to reach and recognize their post-synaptic targets. Loss of function and overexpression experiments show that Rac plays a particularly important role in axon pathfinding. The progressive loss of three Rac GTPases, Rac1, Rac2, and Mtl in *Drosophila* MB neurons leads to an increasing percentage of neurons with misguided axons (Ng et al. 2002), as does loss of Mtl and Rac1 or Rac2 at the *Drosophila* CNS midline (Hakeda-Suzuki et al. 2002). In general, the loss of Rac function results in a "bypass" phenotype in which axons extend beyond their normal synaptic partners. This is seen for loss of *Rac1/Mtl* double mutants, and even more so for *Rac1/Rac2/Mtl* triple mutants in the *Drosophila* visual system, with local disruptions in topographic mapping and frequent misrouting of photoreceptor axons around and beyond the medulla (Hakeda-Suzuki et al. 2002). In agreement with these findings, interfering with Rac function by expressing DN Rac1 results in a bypass phenotype in the *Drosophila* giant fiber system (Allen et al. 2000), CNS (Fritz and VanBerkum 2002), and motor axons (Kaufmann et al. 1998; H. Hu et al. 2001). Conversely, expression of wild-type or CA Rac1 can cause the premature termination or stalling of axons in the *Drosophila* giant fiber system (Allen et al. 2000) and in Purkinje cells of CA Rac1 transgenic mice (Luo et al. 1996a). Expression of wild-type or DN Rac1 in the *Drosophila* giant fiber system (Allen et al. 2000), and CA Rac1 in the *Drosophila* CNS (Fritz and VanBerkum 2002), also causes inappropriate midline crossing. All in all, Rac activity appears to be required for the proper growth and guidance of axons, with loss of Rac function resulting in an overshoot of axons and increased Rac activity causing axons to stall.

Proper regulation of Cdc42 and Rho activities is also required for axon pathfinding. In contrast to Rac, however, activation of Cdc42 tends to cause inappropriate axon routing, which may result from an inability of the axon to recognize its target. Expression of CA Cdc42, and occasionally DN Cdc42, causes axon misrouting or stalling before reaching target muscles in *Drosophila* motor neurons (Kim et al. 2002, 2003), CA Cdc42 causes axon fasciculation and midline crossing errors in *Drosophila* CNS interneurons (Kim et al. 2002), and CA Cdc42 and DN RhoA cause midline crossing errors in the *Drosophila* CNS (Fritz and VanBerkum 2002). Wild-type Cdc42 and DN RhoA also cause axon targeting errors in *Xenopus* RGCs (Ruchhoeft et al. 1999). Taken together, these findings suggest that moderate levels of active Rac and Cdc42 are required for an axon to reach and recognize its appropriate target, and that Rho activity is required to prevent an axon from extending into an inappropriate area. One should keep in mind, however, that it is difficult to determine whether some of the effects of the Rho GTPases on axon pathfinding are caused or influenced by their effects on growth as well as path and target recognition, particularly in cases of axon stalling or overextension.

What signaling molecules integrate, or pathways mediate, the effects of the Rho GTPases on axon pathfinding? Numerous studies implicate the Rac1 activator Trio in this developmental process. Loss-of-function *trio* mutations in *Drosophila* result in the misdirection or stalling of axons in embryos and cause a malformation of the MB due to altered neurites (Awasaki et al. 2000). Another study shows that in addition to CNS axon pathfinding defects, axons of motor neurons deficient in *trio* activity are defective in their ability to reach their target muscles (Bateman et al. 2000). These results are similar to those mentioned above for *Rac1*, and genetic interaction experiments show that *trio* interacts with *Rac.* Removal of a single copy of *trio* increases the penetrance of

a DN Rac1 bypass phenotype, while expression of wild-type Trio suppresses the DN Rac1 bypass phenotype (Bateman et al. 2000). Another potential link between Trio and Rac1 involves Trio's association with the receptor phosphatase LAR. Strong zygotic *Drosophila Lar*, *Dlar*, mutants also exhibit a bypass phenotype, and partial reduction of *trio* activity or expression of DN Rac1 can enhance this phenotype (Kaufmann et al. 1998; Bateman et al. 2000). Conversely, the bypass phenotype caused by *Dlar* mutations can be suppressed by a partial reduction in *Abelson tyrosine kinase* (*Abl*), suggesting that this phosphatase and kinase act antagonistically to regulate axon pathfinding (Wills et al. 1999). Interestingly, while *trio* cooperates with *Dlar* to regulate motor axon pathfinding, in the CNS *trio* cooperates with *Abl* to control axon guidance at the midline (Liebl et al. 2000).

Several Rho GTPase and Rho GTPase-related genes in *C. elegans* play a role in axon pathfinding and guidance and provide further support for the involvement of a Trio/Rac signaling pathway in this process. Both activated and null alleles of a widely expressed Rho family member closely related to *Mtl*, *mig-2*, inhibit cell migration in vivo, and a small percentage of activated *mig-2* mutant hermaphrodite-specific neurons either fail to grow axons of normal length or extend long axons that wander aimlessly (Zipkin et al. 1997). Mutations in *unc-73*, which is highly related to the Trio and Kalirin family of proteins, cause a variety of axon pathfinding/guidance phenotypes similar to those for *mig-2* (Steven et al. 1998; Kubiseski et al. 2003). Axon pathfinding/guidance defects caused by these inactivating *unc-73* mutations can be rescued by a short UNC-73 isoform, UNC-73B. This isoform contains the first GEF domain, which exhibits guanine nucleotide exchange activity toward Rac and Mig-2 (Steven et al. 1998; Kubiseski et al. 2003).

The Rac and Cdc42 effector PAK is believed to contribute to Rac-dependent axon pathfinding, and genetic interactions implicate PAK in a Trio/Rac/PAK signaling pathway important for this developmental process. An effector loop mutation in Rac1 that disrupts its ability to bind to PAK and other CRIB motif-effector proteins is capable of partially rescuing CA Rac-induced axon pathfinding/guidance defects in *Drosophila* MB neurons (Ng et al. 2002), suggesting that PAK activity contributes to Rac-dependent axon guidance. It has been proposed that *Drosophila* photoreceptor axons require recruitment of PAK to the membrane by the adaptor protein Dock, where its activity can be regulated by Trio/Rac signaling. Loss of Trio function causes photoreceptor axonal projection errors, including misrouting of axon bundles beyond the medulla and into deeper regions of the brain, called a "medulla bypass" phenotype, similar to that seen in *PAK* or *dock* loss of function mutants (Newsome et al. 2000). Catalytic activity of the first Trio GEF domain, which acts on Rac, is vital for Trio function in photoreceptor axon guidance, and projection defects resulting from overexpression of the first Trio GEF domain are enhanced by a *Rac1* transgene, and to a lesser extent by a *Mtl* transgene. Additionally, heterozygosity for *trio* increases the frequency of photoreceptor axon pathfinding errors due to partial loss of *dock*, while heterozygosity for *PAK* increases the frequency of errors due to partial loss of *trio*, suggesting that these proteins act in a common pathway to control photoreceptor guidance (Newsome et al. 2000).

Another Rac effector, cytoplasmic FMR1-interacting protein (CYFIP), the fly ortholog of vertebrate FMRP interactors CYFIP1 and CYFIP2, is also associated with axon pathfinding abnormalities (see also nervous system disorders section below). Fragile X syndrome (FRAXA) is one of the most common monogenic forms of mental retardation (MR). The *FMR1* gene encodes the RNA-binding protein FMRP, which acts as a translational repressor (Zalfa and Bagni 2004) and is absent in FRAXA patients due to transcriptional silencing (Pieretti et al. 1991). CYFIP interacts biochemically and genetically with both FMRP and CA Rac1. Null *CYFIP* mutants exhibit a range of axon abnormalities, including midline crossing defects and defects in motor axons classified as stalling and abnormal branching. Arrest of CA Rac1 motor neuron axons can be ameliorated by expression of CYFIP and enhanced by loss of CYFIP. Furthermore, overexpression of dFMR1 causes synapse undergrowth resulting in reduced synaptic length, and coexpression of CYFIP rescues synapse length, while decreasing *CYFIP* dosage further reduces synaptic length. Thus CYFIP effects are opposite to those of Rac1 and FMR1, suggesting that they act in a common pathway and that Rac1 positively regulates FMR1 function (Schenck et al. 2003). Additional interactions and functions of these proteins suggest that they regulate axon guidance through modulation of the actin and microtubule cytoskeletons. CYFIP1 interacts with active Rac1 (Kobayashi et al. 1998) and is part of a complex that includes the Rac effector WAVE1, which directly interacts with ARP2/3 to regulate actin polymerization and induce actin cytoskeleton remodeling. In addition, FMRP inversely regulates mRNA translation of Futsch, the *Drosophila* homolog of MAP1B, shown to be required for dendritic, axonal, and synaptic development (Zhang et al. 2001). Thus, a model has been proposed in which CYFIP binds to GTP-bound Rac, releasing FMRP and WAVE1, which can then go on to regulate the microtubule and actin cytoskeletons, respectively (Billuart and Chelly 2003).

Regulation of Rho by Rho-specific GAPs and GEFs has also been shown to influence the navigation ability of axons. Mice lacking functional p190 RhoGAP exhibit defects in axon guidance and fasciculation (Brouns et al. 2001), and overexpression of GEF64C causes *Drosophila* CNS axons to project abnormally across the midline in a RhoA-dependent manner. Notably, GEF64C is capable of binding RhoA, Rac1, and Cdc42 and exhibits exchange activity for Rho and Rac in vitro, but appears to act specifically for RhoA in an in vivo context (Bashaw et al. 2001). The GEF64C finding is surprising given that Rho is generally believed to mediate axon repulsion at the midline, and suggests that Rho may also play a role in attractive guidance. Downstream of Rho signaling, Rho-

kinase has been implicated in repulsive growth cone turning required for axon guidance, with inhibition of Rho-kinase antagonizing repulsive guidance in cultured *Xenopus* spinal neurons and rat cerebellar granule cells (Yuan et al. 2003).

The studies outlined above provide evidence for the involvement of the Rho GTPases in axon pathfinding. In particular, attention has focused on the Trio/Rac/Pak signaling pathway in this developmental process. The findings above also provide a plausible link between Rac activation and coordination of actin polymerization with control of mRNA translation through a CYFIP/Rac1/dFMR1/WAVE1 complex. With regard to the latter finding, it is interesting to note that the study of mRNA localization and local protein translation in axonal growth cones is becoming increasingly popular and is believed to be important for autonomous regulation of structure and function (Martin 2004). It will be interesting to see whether Rac truly regulates mRNA translation through FMRP and how this affects the axonal cytoskeleton. Additionally, proper regulation of Cdc42 and Rho, which also regulate the actin and microtubule cytoskeletons, has been shown to be important for axonal pathfinding and target recognition, and a balance between all three Rho GTPases likely determines whether an axon will arrive at its appropriate destination.

Rho GTPase-regulated growth cone morphology pertinent to axon guidance

The Rho GTPases have profound effects on growth cone morphology and function generally thought to be indispensable for axon pathfinding and guidance. Rac activity is important for the formation of actin-based lamellipodia, which provide substrate adhesion and tension necessary for movement and extension. Endogenous Rac1 is present in growth cone bodies of primary chick embryo motor neurons, is present in the tips and shafts of filopodia, and colocalizes with actin filament structures (Kuhn et al. 1998). Introduction of CA or DN Rac1 causes an increase or decrease in rhodamine phalloidin staining, respectively, demonstrating Rac1-dependent changes in actin filament accumulation in the growth cone, and both mutants retard growth cone advance on laminin and fibronectin in a β1 integrin-dependent manner in this system (Kuhn et al. 1998). Similarly, dysregulation of Rac activity upon expression of CA Rac1 or DN Rac1 in *Drosophila* motor neurons also causes growth cone arrest (Kaufmann et al. 1998). It was shown in primary chick embryo motor neurons that CA or DN Rac1 mutants retard growth cone advance by affecting rates of extension, but not retraction (Kuhn et al. 1998). Thus proper regulation of Rac1 activity, and actin filament formation and dynamics, is required for the forward movement of growth cones.

Cdc42 activity is particularly important for growth cone morphology and function and is responsible for the formation of filopodia that serve as environmental sensors and thus orient growth. Expression of CA Cdc42 causes an increase in growth cone area and filopodia in chick spinal neurons (Brown et al. 2000), wild-type Cdc42 results in larger and more complex growth cones in *Xenopus* RGCs (Ruchhoeft et al. 1999), and DN Cdc42 produces the opposite effect in *Xenopus* RGCs (Ruchhoeft et al. 1999; Yuan et al. 2003). CA Cdc42 also causes arrest of *Drosophila* motor neuron growth cones, suggesting that hyperactivation of Cdc42 disrupts leading edge motility (Kaufmann et al. 1998). While it is generally accepted that Cdc42 activation regulates growth cone navigation by promoting filopodial activity, it is important to note that Cdc42 may be able to independently control filopodia activity in growth cones and pathfinding (Kim et al. 2002). As mentioned above, *Drosophila* motor neurons expressing CA Cdc42 exhibit axon pathfinding defects, including axon misrouting or stalling before reaching their target muscles (Kim et al. 2002, 2003), and some CA Cdc42 neurons that do not exhibit targeting defects exhibit an increase in growth cone filopodial activity (Kim et al. 2002). Interestingly, Cdc42 has been shown to mediate both attractive and repulsive growth cone turning. Expression of CA or DN Cdc42 has been shown to abolish chemoattractive growth cone turning induced by a BDNF gradient in cultured *Xenopus* spinal neurons and rat cerebellar granule cells. BDNF in the presence of a PKA inhibitor actually inhibits Cdc42 activity and induces repulsive growth cone turning, which is also diminished by CA or DN Cdc42 in rat cerebellar granule cells (Yuan et al. 2003).

Rho activity also affects growth cone morphology and induces growth cone collapse to mediate axon repulsion. *Xenopus* RGC growth cones expressing wild-type RhoA exhibit a decrease in growth cone area, while inhibition of RhoA using DN RhoA results in growth cones with abnormal, thickened filopodia with a balled appearance (Ruchhoeft et al. 1999). DN RhoA also causes a slight increase in growth cone filopodia in cultured *Xenopus* spinal neurons (Yuan et al. 2003). Inhibition of RhoA or Rho-kinase using DN RhoA or the Rho-kinase inhibitor Y-27632, respectively, revealed that RhoA and Rho-kinase mediate LPA-induced and BDNF (in the presence of a PKA inhibitor)-induced chemorepulsion in cultured *Xenopus* spinal neurons and rat cerebellar granule cells (Yuan et al. 2003). Inhibition of Rho using C3 exoenzyme, or Rho-kinase using Y-27632, also inhibits the collapse and retraction of chick temporal RGC axons (Thies and Davenport 2003).

Not unexpectedly, additional studies reveal an actin cytoskeletal contribution to growth cone collapse, which is likely to occur downstream of Rho GTPase signaling, and provide evidence for cross-talk between the Rho GTPases to regulate growth cone turning. Blocking MLCK activity with high concentrations of an MLCK inhibitory pseudosubstrate peptide causes growth cone collapse, but low concentrations of peptide result in fewer filopodia and faster growth cone advance, both of which are associated with a reduction in F-actin. These data show that varying MLCK activity inversely affects the rate of growth cone advance by regulating the amount of F-actin (Schmidt et al. 2002). Furthermore, filopodial asymmetry across the growth cone caused by

inhibition of actin polymerization or myosin contractile activity is sufficient to trigger a growth cone turning response, and the use of a myosin ATPase or MLCK inhibitor shows that turning is mediated by myosin activity. Myosin ATPase or MLCK inhibitor is able to convert repulsion induced by LPA or BDNF in the presence of a PKA inhibitor to attraction. Given that BDNF-triggered repulsion (in the presence of PKA inhibitor) is abolished by DN Cdc42, DN RhoA, Y-27632, myosin ATPase, and MLCK, there seems to be cross-talk between Cdc42 and RhoA signaling pathways that converges on myosin activity to regulate growth cone guidance (Yuan et al. 2003).

Guidance cues, receptors, and Rho GTPases

Essentially four families of guidance cues provide directional information to growing axons: the semaphorins, ephrins, slits, and netrins. These guidance cues are read by growth cone receptors, and signal transduction pathways downstream of these receptors converge onto the Rho GTPases to elicit changes in cytoskeletal organization that determine which way the growth cone will turn (Guan and Rao 2003; Huber et al. 2003). The signaling pathways linking axon guidance cues to Rho GTPases are discussed below and outlined in Figure 3.

Semaphorins, also known as collapsins, are secreted and membrane associated proteins that can either promote repulsive or attractive axon guidance, but generally function as chemorepellents that direct axons away from tissues marked by their expression. Their effects are mediated by a variety of receptor complexes, including members of the neuropilin and plexin protein families, as well as other membrane proteins belonging to the Ig superfamily (Raper 2000; Guan and Rao 2003; Huber et al. 2003). Semaphorin3A (Sema3A), semaphorin 3D (Sema3D), and semaphorin 4D (Sema4D) cause growth cone collapse and inhibit axonal outgrowth (Jin and Strittmatter 1997; Vastrik et al. 1999; Swiercz et al. 2002). In chick DRG, Sema3D-induced growth cone collapse is mediated by Rac1, since CA Rac1 enhances collapse, and DN Rac1 prevents collapse and restores neurite outgrowth (Jin and Strittmatter 1997). Peptides that compete with activated Rac1 for target binding can also inhibit Sema3A-induced growth cone collapse (Vastrik et al. 1999), in addition to Sema3A-induced endocytosis (Jurney et al. 2002). In chick motor neurons, DN mutants of both Rac1 and Cdc42 reduce Sema3D-induced growth cone collapse and neurite inhibition (Kuhn et al. 1999). Interfering with RhoA activity and the activity of a Plexin-B1-interacting Rho-specific GEF, PDZ–RhoGEF, can also rescue Sema4D-induced growth cone collapse in rat primary hippocampal neurons (Swiercz et al. 2002), while inhibition of Rho-kinase activity may or may not interfere with Sema3A-induced growth cone collapse in chick DRG (Arimura et al. 2000; Dontchev and Letourneau 2002). Thus, Rac, Cdc42, and Rho play a part in semaphorin-mediated repulsion.

Several studies in nonneuronal cells indicate that the semaphorin receptor Plexin-B1 interacts directly with activated Rac, but causes actin cytoskeletal changes indicative of Rho activity that are actually dependent upon

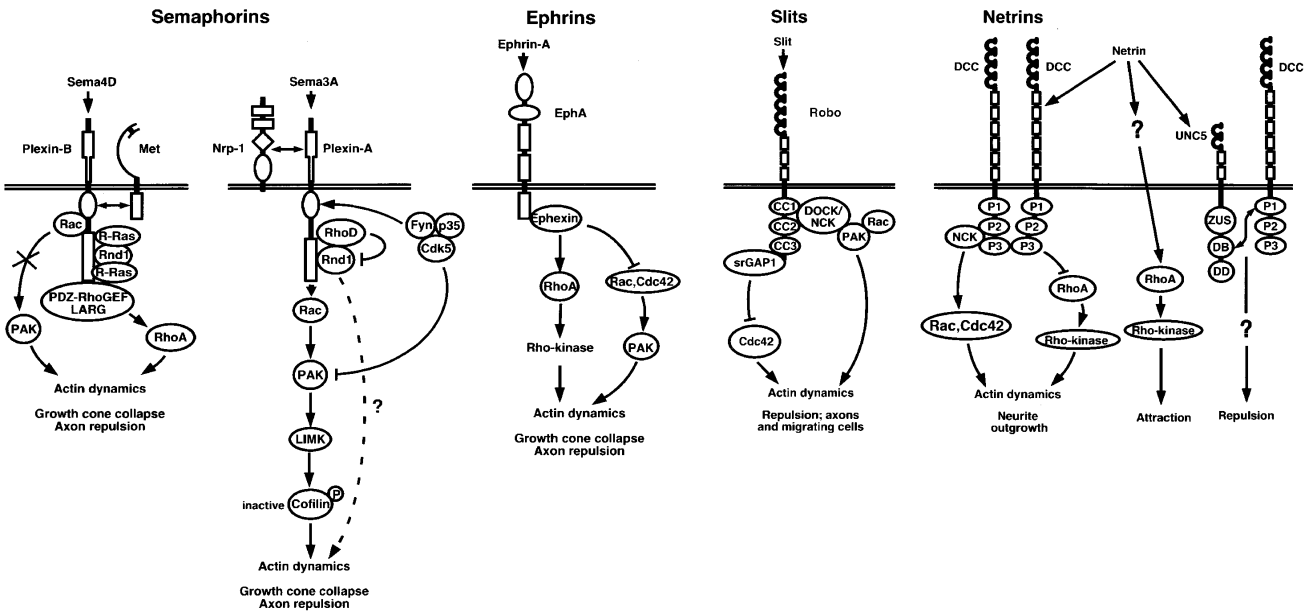

Figure 3. Rho GTPase signaling downstream of four axon guidance cue families, the semaphorins, ephrins, netrins, and slit proteins. These guidance cues are read by growth cone receptors, and signal transduction pathways downstream of these receptors converge onto the Rho GTPases to elicit changes in cytoskeletal organization that determine which way the growth cone will turn. See text for explanations. (Sema) Semaphorin; (Met) hepatocyte growth factor receptor; (LARG) leukemia-associated Rho GEF; (PAK) p21-activated kinases; (Nrp-1) neuropilin-1; (Fyn) Src family kinase; (Cdk5) cyclin-dependent kinase 5; (LIMK) Lin-11, Isl-1, and Mec-3 kinase; (Robo) Roundabout; Dock Dreadlocks; (srGAP1) Slit–Robo GAP 1; (DCC) deleted in colorectal cancer; (UNC5) uncoordinated 5.

both GTPases (Rohm et al. 2000; Vikis et al. 2000; Driessens et al. 2001). Expression of Rac also enhances Sema4D binding to Plexin-B1, and Plexin-B1 and PAK compete for Rac1 binding, with Plexin-B1 inhibiting Rac-induced *PAK* activation (Vikis et al. 2002). *Drosophila* studies further show that Plexin-B associates with active Rac, inhibits Rac by sequestering it away from PAK, and enhances RhoA activity to regulate axon guidance. When Plexin-B is overexpressed in motor neurons, axons fail to defasciculate and reach their distalmost target muscles. Combined with Plexin-B overexpression, reduced *Rac* gene dosage results in increased penetrance of these Plexin-B phenotypes, while increased *Rac* dosage causes suppression of these phenotypes, suggesting that Rac antagonizes Plexin-B signaling. Coexpression of Plexin-B with DN Rac1 also enhances a DN Rac1 axon bypass phenotype, while loss of Plexin-B reduces this phenotype, suggesting that the presence of Plexin-B further inhibits the remaining endogenous Rac1 signaling in these cells. Interestingly, the Plexin-B stall phenotype is similar to a *trio* loss-of-function phenotype, and reducing *trio*, an activator of Rac, also enhances the Plexin-B phenotypes. Plexin-B was found to compete with the Rac downstream effector PAK for binding to Rac using an in vitro pull-down competition assay, and overexpressing PAK with Plexin-B cancels the Plexin-B phenotypes, demonstrating that Plexin-B signaling can be antagonized by the Rac effector PAK in vivo. Finally, reducing *RhoA* gene dosage suppresses the Plexin-B phenotype, suggesting that RhoA acts antagonistically to Rac and that RhoA partially mediates Plexin-B signaling (H. Hu et al. 2001). It is noteworthy that *Drosophila* Plexin-B interacts directly with RhoA (H. Hu et al. 2001), while the Rho-specific GEFs PDZ–RhoGEF and LARG link mammalian Plexin-B to RhoA activation (Aurandt et al. 2002; Chikumi et al. 2002; Hirotani et al. 2002; Perrot et al. 2002; Swiercz et al. 2002). The mechanism by which Plexin-B1 activates RhoA through these Rho GEFs has recently been shown to involve the transmembrane tyrosine kinase ErbB-2. Plexin-B family members associate with ErbB-2, and the binding of Sema4D to Plexin-B1 stimulates the intrinsic kinase activity of ErbB-2, resulting in phosphorylation of both Plexin-B1 and ErbB-2. Furthermore, DN ErbB-2 blocks Sema4D-induced RhoA activation and axonal growth cone collapse in rat primary hippocampal neurons. In addition, CA Rac enhances Sema4D-induced Plexin-B1 phosphorylation in cells coexpressing Plexin-B1 and PDZ–RhoGEF, providing further evidence that active Rac modulates Plexin-B1 signaling (Swiercz et al. 2004). Taken together, these data suggest that semaphorin activation of plexin receptors coordinately regulates Rac and RhoA activity to regulate repulsive guidance.

There is evidence that LIM-kinase phosphorylation, and thus inactivation, of the actin depolymerizing factor cofilin is required for Sema3A-induced growth cone collapse. Sema3A increases cofilin phosphorylation with subsequent dephosphorylation in mouse DRG growth cones. A peptide containing a cofilin phosphorylation site and DN LIM-kinase (unable to be activated by Rho-kinase or PAK) are both able to inhibit cofilin phosphorylation and Sema3A-induced growth cone collapse in DRG, while CA LIM-kinase increases cofilin phosphorylation and induces morphological growth cone changes (Aizawa et al. 2001). Furthermore, LPA and Sema3D-induced neurite retraction are both dependent upon MLC activity, since retraction can be inhibited by a MLCK inhibitor (Jalink et al. 1994; Jin and Strittmatter 1997). Thus regulation of actin dynamics and actomyosin contraction is important for repulsive guidance.

The Rho GTPases Rnd1 and RhoD have also been implicated in semaphorin-induced growth cone collapse and axon guidance. Rnd1 and RhoD bind to the cytoplasmic domain of Plexin-A1, with RhoD counteracting Rnd1-mediated cytoskeletal collapse in Cos-7 cells expressing Plexin-A1 and Neuropilin-1 (Nrp-1). CA RhoD also blocks Sema3A-induced repulsion of chick sympathetic axons (Zanata et al. 2002). It should be noted that while Rnd1 binds well to Plexin-A1, it is also capable of binding Plexin-B1 to a lesser extent (Rohm et al. 2000). In light of this, Rnd1 has been shown to interact directly with the cytoplasmic domain of Plexin-B1 (Oinuma et al. 2003), enhance Sema4D-induced Plexin-B1 phosphorylation (Swiercz et al. 2004), and promote interaction between Plexin-B1 and PDZ–RhoGEF to potentiate Plexin-B1-induced RhoA activation. Coexpression of Rnd1 and Plexin-B1 in nonneuronal cells causes cell contraction in response to Sema4D, which can be suppressed by DN RhoA, Rho-kinase inhibitor, or DN PDZ–RhoGEF, suggesting the involvement of a PDZ–RhoGEF/RhoA/Rho-kinase pathway (Oinuma et al. 2003). In addition to its ability to activate Rho signaling in order to bring about cell contraction, Rnd1 has recently been found to induce Plexin-B1-mediated GAP activity toward R-Ras via the receptor's RasGAP homologous domains in a heterologous Cos-7 cell system, similar to effects of Sema4D. Stimulation of NGF-differentiated PC12 cells with Sema4D also decreases GTP-bound R-Ras and induces neurite retraction, which can be mimicked by knock-down of R-Ras levels or activity and blocked by CA R-Ras or Plexin-B1 mutants unable to interact with Rnd1 or R-Ras. Sema4D-induced growth cone collapse in rat hippocampal neurons is also dependent upon Rnd1, mimicked by R-Ras knock-down, and blocked by CA R-Ras. Furthermore, CA R-Ras is also able to suppress Sema3A-induced growth cone collapse, suggesting that down-regulation of R-Ras activity may be a general phenomenon downstream of semaphorin/Plexin signaling (Oinuma et al. 2004).

Taken together with the studies above, semaphorin-mediated repulsive guidance involves a balance between the activity of multiple Rho GTPases to bring about actin cytoskeletal changes that mediate growth cone collapse and axon repulsion.

Ephrins are transmembrane or glycosylphosphatidylinositol-anchored molecules that are ligands of Eph receptor tyrosine kinases, which induce growth cone collapse and play key roles in axon guidance (Wilkinson 2001; Cutforth and Harrison 2002; Guan and Rao 2003;

Huber et al. 2003). One mechanism by which they induce growth cone collapse is through regulation of Rho GTPase activity. Their effects on growth cone collapse are modulated by ephexin, an Eph receptor-interacting protein that exhibits GEF activity toward Rho and Cdc42, and to a small extent Rac1 (Shamah et al. 2001). Ephrin-A5-treatment of chick RGC cultures stimulates RhoA activity while decreasing Rac activity, and inhibition of the RhoA signaling pathway with C3 exoenzyme or Y-27632 reduces the collapse rate of retinal growth cones (Wahl et al. 2000). Ephrin-A1 stimulation of EphA receptors also elicits growth cone collapse in purified rat RGCs and inhibits ephexin-induced Rac1-dependent PAK phosphorylation in cultured embryonic cortical cells. Furthermore, ephrin-A1-induced growth cone collapse is enhanced by wild-type ephexin expression and inhibited by an ineffective ephexin GEF mutant. Therefore, EphA receptor activation likely inhibits ephexin activity toward the Rac1/Cdc42/Pak signaling pathway, while enhancing the ability of ephexin to activate RhoA, resulting in a shift in balance of Rho GTPase activities to regulate axon growth and guidance (Shamah et al. 2001).

In contrast to the Ephrin/Eph studies above, another study suggests that Ephrin-A2 actually requires Rac1 activity to induce growth cone collapse and promote endocytosis in chick retinal cells and DRG. After treatment with ephrin-A2, Rac1 is transiently inactivated, but then its activity resumes and is temporally correlated with growth cone collapse. Ephrin-A2-induced depolymerization and reorganization of F-actin in growth cones correlates with this resumption of Rac1 activity. Furthermore, use of an inhibitory Rac1 peptide shows that Rac1 activity is necessary for ephrin-A2-induced growth cone collapse, endocytosis of growth cone plasma membrane, and reorganization of F-actin, but not for depolymerization of F-actin during collapse. DN Rac1 gives similar growth cone collapse and endocytosis results. Further support for a role for Rac1 downstream of Ephrin/Eph signaling is provided by the finding that *EphA3* or *Rac1* antisense injected into embryonic chick eye in vivo both cause the retinotectal projection to develop without normal topography (Jurney et al. 2002). Given these findings, it remains to be determined whether an increase in Rac activity occurs after an initial decrease in Rac activity in the Wahl et al. (2000) and Shamah et al. (2001) studies discussed above. The Jurney et al. (2002) study suggests that ephrin may coordinate activation of the Rho/Rho-kinase signaling pathway to induce actin depolymerization with activation of Rac to regulate actin reorganization and membrane endocytosis during growth cone collapse. The nature of the transient inactivation of Rac after ephrin treatment remains to be determined.

Members of the Slit family are important repulsive guidance factors present at the midline of the nervous system that act through the Roundabout (Robo) receptors (K. Wong et al. 2002; Guan and Rao 2003; Huber et al. 2003). The role of the Rho GTPases in axon repulsion at the midline has been studied in the *Drosophila* CNS by examining the interaction of CA and DN mutants of the Rho GTPases with Robo. CA mutants of Rac1 and Cdc42 cause axons to cross the midline inappropriately, as does a reduction in Robo repulsion. Heterozygous loss of *robo* enhances the CA Rac1 phenotype, but suppresses midline crossing caused by CA or DN Cdc42. Both CA and DN Rho enhance the heterozygous *robo* mutant phenotype. Thus tight regulation of Rho GTPase activity is required for axons to respond appropriately to Robo activation (Fritz and VanBerkum 2002).

In contrast to the study cited above, which suggests that a decrease in Rac activity is required for Robo-mediated axon repulsion at the *Drosophila* CNS midline, another study shows that Slit stimulation recruits the adaptor protein Dreadlocks (*Drosophila* Dock/vertebrate Nck) and PAK to Robo and increases Rac activity to regulate axon repulsion. In this study, pan-neural expression of DN Rac had no significant effect on midline crossing. However, DN Rac1 strongly enhanced midline crossing defects resulting from a decrease in Slit, or Slit and Robo, expression. Homozygous loss of *Rac1* in a heterozygous *slit* background also significantly affected midline crossing. These results support a role for Rac in repulsive Robo signaling (Fan et al. 2003).

Since Rac is generally viewed as a positive regulator of axon outgrowth that facilitates attractive guidance, how might Rac serve to mediate repulsive guidance as well? The effects of Rac on axon guidance may depend upon the molecular context in which it is activated and the overall level of Rac activity. As an adaptor protein, Dock can bind the Rac/Cdc42 effector PAK (Hing et al. 1999) and Robo's cytoplasmic domain, with the latter association enhanced by stimulation with Slit. PAK only interacts with Robo in the presence of Dock in a Slit-dependent manner, and Slit stimulation of cells expressing Robo and Rac causes a modest increase in Rac1 activity and smaller increases in Rac2 and Mtl activities. Importantly, a Robo mutant unable to bind Dock, and therefore unable to recruit PAK, fails to mediate a Slit-dependent increase in Rac activity. These findings suggest that the binding of Slit to Robo recruits Dock and PAK and potentially Rac1 into a multiprotein complex important for repulsive guidance (Fan et al. 2003).

This scenario is likely to occur in vivo since genetic interaction experiments show that limiting *dock* and *PAK* partially disrupts Slit/Robo repulsion, as interfering with Rac activity does. Removal of both maternal and zygotic *dock* components results in phenotypes reminiscent of loss of *robo* function, including thickening of commisural axon bundles, a commensurate reduction in the thickness of longitudinal axon bundles, and ectopic midline crossing. In addition, removal of one copy of *dock* against a heterozygous *slit/robo* background enhances a mild *slit/robo* phenotype, while removal of maternal *dock* causes an even greater enhancement of midline crossing defects. Removing one copy of *dock* also enhances defects associated with pan-neural expression of a truncated DN Robo receptor. PAK is also likely to play a role in Robo repulsion. Removal of one copy of *PAK* enhances the *slit/robo* phenotype, as does a *PAK* allele containing a mutated Dock-binding site. A CA ver-

sion of PAK is also capable of partially suppressing Rac-dependent effects in a heterozygous *slit* background. However, wild-type and CA PAK unexpectedly enhance midline crossing in a wild-type *Rac* background with heterozygous loss of *slit* or *slit* and *robo*, suggesting that tight regulation of PAK activity is required for Robo repulsion. Taken together, these genetic interaction experiments indicate that Dock, PAK, Rac, and Robo proteins function in a common process, and may interact in the manner indicated above. In support of this, Robo containing a mutated Dock-binding site fails to rescue *robo* loss of function phenotypes. However, it is important to note that this mutation may also disrupt the binding of other proteins to Robo, such as Enabled (Ena), leaving the exact mechanism by which these proteins interact in a context of axon guidance to be definitively determined. In addition, the vertebrate homolog of Dock, Nck, also interacts with the attractive netrin receptor vertebrate deleted in colorectal cancer (DCC) (X. Li et al. 2002a), leaving open the possibility that Dock, like Rac, may play an additional role in attraction (Fan et al. 2003).

The role of Rho in Robo signaling is likely one of repulsion. While pan-neural expression of DN Rho has no effect on axon guidance in a wild-type background, it has a modest but significant ability to enhance a heterozygous *slit*, or *slit* and *robo*, midline crossing phenotype. Interestingly, restricted expression of DN Rho in a subset of neurons in embryos heterozygous for *slit*, or *slit* and *robo*, produces strong enhancement of midline defects (Fan et al. 2003), and CA and DN Rho enhance a heterozygous *robo* mutant phenotype as well (Fritz and VanBerkum 2002). In support of a role for Rho in repulsive midline guidance, DN Rho enhances and CA Rho suppresses midline crossing errors caused by homozygous loss of the Ras GEF *Son-of-sevenless* (*Sos*) in the *Drosophila* CNS (Fritz and VanBerkum 2002). Robo-dependent repulsive guidance at the *Drosophila* CNS midline is also influenced by the RhoGEF GEF64C (Bashaw et al. 2001). Overexpression of GEF64C causes *Drosophila* CNS axons to project abnormally across the midline in a manner similar to that of loss of *robo*, and GEF64C overexpression is able to overcome Robo repulsion. The ability of this RhoGEF to block axon repulsion, however, is unexpected given that its effects are RhoA, and not Rac1 or Cdc42, dependent (Bashaw et al. 2001).

Cdc42 has also been implicated in Robo-mediated axon guidance, and plays a role in Slit/Robo repulsion of migrating cells. While pan-neural expression of DN Cdc42 does not modulate heterozygous loss of *slit*, or *slit* and *robo*, midline crossing errors (Fan et al. 2003), restricted expression of both CA and DN Cdc42 suppresses heterozygous loss of Robo midline crossing in the *Drosophila* CNS (Fritz and VanBerkum 2002). From the latter findings, it appears that tight regulation of Cdc42 activity is required for *robo* repulsion, and the finding that CA Cdc42 enhances a homozygous loss of *Sos* midline crossing phenotype (Fritz and VanBerkum 2002) suggests that axon repulsion may require down-regulation of Cdc42 activity. The necessity of down-regulating Cdc42 activity for Slit/Robo repulsion is further demonstrated by the involvement of a Cdc42-specific GAP in the repulsion of migrating cells. The binding of Slit to Robo induces association of the receptor with a novel family of Cdc42 and Rac-specific GAPs, Slit–Robo GAPs (srGAPs) 1–3, and down-regulation of Cdc42 activity by Slit through srGAP1 contributes to repulsive guidance. The ability of Slit to inactivate Cdc42 is counteracted by DN srGAP1, and Slit repulsion of migratory cells from the anterior subventricular zone (SVZa) of the forebrain can be blocked by DN srGAP1 or CA Cdc42 (Wong et al. 2001). Thus limiting Cdc42 activity/function appears to be required for Slit/Robo repulsion.

Netrins are a family of conserved, secreted proteins that can act as either chemoattractants or repellents for axon guidance. Receptors for netrins include DCC family members, such as *C. elegans* Unc40, *Drosophila* Frazzled, and DCC and Neogenin. The composition of netrin receptor complexes dictates whether they promote attraction or repulsion. For example, the binding of netrin to DCC homodimers leads to growth cone attraction, while the binding of netrin to UNC-5/DCC heterodimers leads to growth cone repulsion (Chisholm and Tessier-Lavigne 1999; Guan and Rao 2003; Huber et al. 2003). Evidence in nonneuronal or neuroblastoma cell lines suggests that netrin-1/DCC signaling induces cytoskeletal rearrangements through the Rac and Cdc42 GTPases. Netrin-1 increases Cdc42 and Rac1 activities in DCC-expressing HEK 293T cells, and DN mutants of Cdc42 and Rac1 block netrin-1/DCC-induced increases in filopodia number and cell surface area, respectively, in HEK293T cells and NG108–15 neuroblastoma cells (Shekarabi and Kennedy 2002). Rac1 and Cdc42 are further shown to be required for neurite outgrowth induced by the DCC receptor, while down-regulation of RhoA and its effector Rho-kinase enhances the ability of DCC to induce neurite outgrowth in N1E-115 neuroblastoma cells. In Swiss 3T3 fibroblasts, however, netrin-1/DCC-induced actin reorganization is Rac1, and not Cdc42, dependent (X. Li et al. 2002b). The findings that DCC interacts constitutively and directly with the adaptor protein Nck in rat commissural neurons, and that DN Nck-1 inhibits netrin-1/DCC-induced neurite outgrowth in N1E-115 cells and Rac1 activation in Swiss 3T3 fibroblasts, suggests that Nck may couple DCC signaling to the Rho GTPases (X. Li et al. 2002a). Importantly, mutations in the *Drosophila* homolog of *Nck*, *dreadlocks* (*dock*), disrupts photoreceptor cell axon guidance and targeting (Garrity et al. 1996), and Dock has been implicated in Rac1-mediated Slit/Robo repulsion (Fan et al. 2003).

In support of a role for Cdc42 and Rac in netrin-1-induced process formation, Rac1 and Cdc42 are required for the axonal outgrowth of mouse precerebellar neurons (PCNs) in the presence of a netrin-1 source, since pharmacological inhibition of Rac and Cdc42 using lethal toxin LT-9048 inhibits this process. However, it is interesting that RhoA, and not Rac or Cdc42, mediates netrin-induced attractive migration and axon guidance in these neurons. During migration, all PCNs first emit a

leading process that leads the way and then the nuclei translocate inside the leading process. Inhibition of Rho using C3 exoenzyme causes a significant reduction in the nuclear migration of PCNs combined with a potentiation of axon outgrowth. C3-exoenzyme-treated cells exhibit neurites that are less fasciculated and lack direction, and axons are "tortuous" in morphology compared with straight, control axons. PCN migration and axon outgrowth and guidance are also dependent upon the Rho effector Rho-kinase, since the Rho-kinase inhibitor Y-27632 produces effects similar to those of C3 exoenzyme (Causeret et al. 2004). Thus Rho/Rho-kinase signaling unexpectedly plays a role in attractive, netrin-induced axon guidance in PCNs. However, the netrin-1 receptor that mediates this effect remains to be determined.

In conclusion, the Rho GTPases play important roles in axon pathfinding and guidance, and their involvement in attraction or repulsion likely depends upon the regulators and degree of Rho GTPase activity involved. It is important to note that effects of Rho GTPase activity on axon pathfinding and guidance are not easily teased apart from potential effects on axon growth. Some axon pathfinding and guidance errors could be explained as secondary effects of defects in the rate of axon extension. However, the variety of effects elicited by the Rho GTPases and their regulators and effectors, depending in part upon the guidance cues, receptors, and other signaling molecules involved, suggests that the Rho GTPases likely play specific roles in axon pathfinding and growth cone guidance.

The Rho GTPases and dendrite formation

Dendrites typically extend from the cell body, branching repeatedly to form a complex tree-like structure that receives and processes inputs from other neurons, and the degree to which dendrites arborize tends to correlate with the number and distribution of inputs on the neuron (Jan and Jan 2001). How this intricate dendritic architecture develops has been revealed to some degree by in vivo imaging of fluorescently labeled individual neurons in relatively transparent Zebrafish and *Xenopus laevis* tadpoles (Cline 2001). Fine filopodial branches are continually added and retracted during the elaboration of dendritic arbors. Some of these branches stabilize and extend, serving as a substrate for further branch dynamics and eventual stabilization and extension. Thus dendritic growth occurs when the rate of branch addition is greater than that of retraction and the added branches stabilize and extend. The cytoskeleton of newly added dendritic branches is thought by some to be composed entirely of actin, with microtubules subsequently invading to provide stability, so that mature branches are composed mainly of microtubules with a cortex of actin in close proximity to the plasma membrane (Cline 2001; Van Aelst and Cline 2004). Due to the dependency of cytoskeletal reorganization on the Rho GTPases, these molecules have been shown to be required for the major structural changes that underlie dendrite formation and elaboration (see Fig. 4).

Studies in both vertebrate and invertebrate model systems revealed that Rho activity has a profound effect on dendrite formation, with its activation negatively impacting dendritic arbor growth. CA Rho drastically reduces dendritic arbor growth in *Xenopus* RGCs (Ruchhoeft et al. 1999), *Drosophila* MB neurons (T. Lee et al. 2000), pyramidal neurons in rat organotypic hippocampal slices (Nakayama et al. 2000), and cultured mouse and rat hippocampal neurons (Ahnert-Hilger et al. 2004; Pilpel and Segal 2004), while LPA activation of RhoA significantly reduces the dendritic arbor growth rate of *Xenopus* optic tectal neurons (Li et al. 2000). CA Rho also decreases the rate and extent of dendritic movements in chick RGCs and produces arbors that exhibit secondary and tertiary branching, but few small terminal processes (Wong et al. 2000). Conversely, loss of *RhoA* function causes dendrites to overextend in *Drosophila*

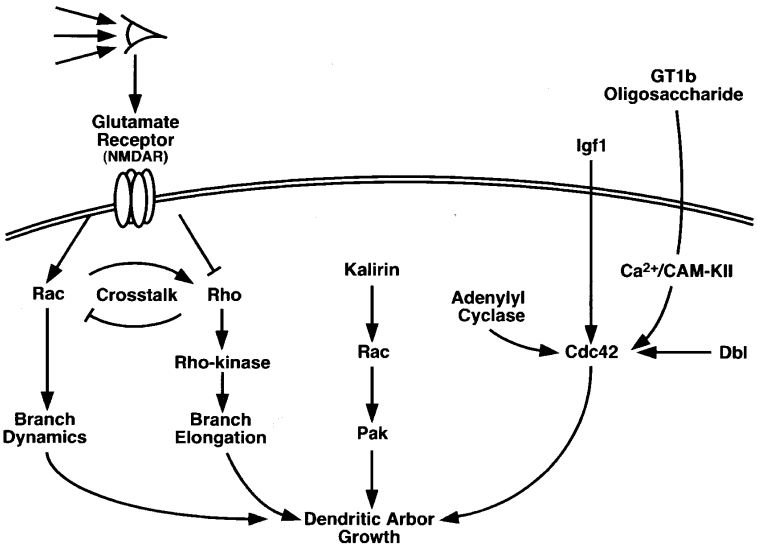

Figure 4. The Rho GTPases play a role in dendrite formation. Activation of Rac and Cdc42 promotes dendritic branch dynamics and generally positively regulates dendritic arbor growth. Inactivation of Rho/Rho-kinase signaling downstream of N-methyl-D-aspartate receptor (NMDAR) activation further contributes to dendritic arbor growth by allowing the extension of dendrites. See text for details. (GT1b) Neu5Acα3Galβ3GalNAcβ4 (Neu5Acα8 Neu5Acα3)Galβ4GlcCer; (CAM-KII) Ca^{2+}/calmodulin-dependent protein kinase II; (Igf1) insulin-like growth factor 1.

MB neurons (T. Lee et al. 2000), and DN RhoA significantly increases dendritic arbor growth rate and thus extension in *Xenopus* optic tectal neurons (Li et al. 2000), increases the dendritic length of cultured mouse hippocampal neurons (Ahnert-Hilger et al. 2004), and increases the rate and extent of dendritic movements in chick RGCs (Wong et al. 2000). Blocking Rho function using DN RhoA, however, fails to affect the dendritic morphology of pyramidal neurons in rat organotypic hippocampal slices (Nakayama et al. 2000). With regard to this finding, a model was suggested in which Rho signaling is relatively low in this system under physiological conditions in order to allow dendritic growth to occur and may be locally activated to limit dendritic growth when needed. Taken together, these studies show that Rho inactivation allows for the extension of dendrites, and activation reduces growth or causes retraction. Thus regulation of Rho activity may play an important role not only in dendrite formation and elaboration, but also in remodeling, a process thought to be vital for synaptic plasticity underlying learning and memory.

The RhoA effector Rho-kinase is also important for dendritic morphology, with Rho-kinase phenotypes being similar to those of RhoA. Exposure of *Xenopus* tadpoles to the Rho-kinase specific inhibitor Y-27632 increases the growth rate of tectal cell dendritic arbors similar to that of DN RhoA (Z. Li et al. 2002), and treatment of rat hippocampal slices with the inhibitor blocks CA RhoA-induced dendritic simplification in pyramidal cells (Nakayama et al. 2000). In addition, CA Rho-kinase reduces pyramidal cell dendritic complexity similar to CA RhoA in the rat hippocampal slice system, suggesting that Rho-kinase is necessary and sufficient to prune dendritic branches (Nakayama et al. 2000). Taken together, these studies demonstrate that activation of the Rho signaling pathway negatively affects dendrite growth, while inactivation positively affects this aspect of neuronal development.

Rac has emerged as a positive regulator of dendritic growth and a key regulator of dendritic branch dynamics. Loss of function of *Rac1*, *Rac2*, and *Mtl* genes in *Drosophila* MB neurons causes a significant reduction in dendritic length and branching (Ng et al. 2002), and DN Rac1 decreases dendrite number in mouse cortical pyramidal neurons (Hayashi et al. 2002), cultured rat primary cortical neurons, and *Xenopus* RGCs (Threadgill et al. 1997; Ruchhoeft et al. 1999), with CA Rac producing opposite phenotypes in the last two systems. DN Rac1 also causes a decrease in the length of the longest dendrite in *Xenopus* RGCs (Ruchhoeft et al. 1999). Time-lapse imaging experiments have revealed that Rac promotes dendritic arbor growth through its ability to regulate the formation and turnover of dendritic branches. Such experiments in *Xenopus* optic tectal neurons show that CA Rac selectively increases dendritic branch additions and retractions, while DN Rac increases branch retractions (Li et al. 2000). Similar experiments in chick RGCs show that CA Rac slightly increases the rate and extent of dendritic motility, and neurons exhibit fewer secondary and tertiary branches, but an increased number of short terminal processes, with DN Rac producing phenotypes opposite to those of CA Rac in this system (Wong et al. 2000). Thus Rac contributes to dendritic arbor growth through its effect on branch dynamics.

In contrast to the above findings, Rac has either no effect or a very mild effect on dendrite length and complexity in some systems, including CA and DN Rac1 in *Drosophila* peripheral nervous system (PNS) neurons (Luo et al. 1994), transgenic mice expressing CA Rac1 in Purkinje cells (Luo et al. 1996a), and CA and DN Rac1 in pyramidal cells in rat hippocampal slices (Nakayama et al. 2000). These results may be cell-type dependent or reflect the necessity for Rac GTPase to cycle in order to promote proper dendrite formation. This is particularly likely given that dendritic studies on regulators and effectors of Rac discussed below suggest that Rac activation positively affects dendritic growth.

The Rho family GEF Kalirin and the Rac/Cdc42 effector PAK are required for dendritic growth. As previously mentioned, Kalirin is predominantly expressed in the nervous system and alternative splicing results in the formation of multiple isoforms, which generally contain two Rho family GEF domains. The first Kalirin GEF domain is specific for RhoG and Rac1 and the second is specific for RhoA (Penzes et al. 2001a; May et al. 2002). Reduction of Kalirin levels leads to reduced dendritic complexity in dissociated rat hippocampal neurons and CA1 pyramidal neurons in rat hippocampal slices, as well as reduced dendritic length in the dissociated hippocampal neurons (Ma et al. 2003). Reduction of Kalirin levels also arrests dendritic development and results in the partial retraction of existing dendritic branches in rat primary sympathetic neurons (May et al. 2002). These studies suggest that Kalirin activity is necessary for dendritic development and/or maintenance, and that its activation of RhoG and Rac1, as opposed to RhoA, may play a role in this process, given that activation of RhoA is believed to antagonize dendrite formation. PAK1 is further thought to mediate the effects of Rac1 on dendrite formation. CA PAK1 increases dendrite number in mouse cortical pyramidal neurons, with DN PAK1 producing the opposite effect. While PAK is an effector for both Rac and Cdc42, the effect of DN PAK on dendrite number is similar to that of DN Rac1 in this system, suggesting that PAK acts downstream of Rac1 to regulate dendrite number (Hayashi et al. 2002). Thus two Rac-linked molecules, Kalirin and PAK, are required for dendrite formation, further providing support for the importance of Rac signaling in the promotion of dendritic growth.

Cdc42 has also been found to regulate dendritic branch dynamics and growth, though to a lesser degree than Rac. CA Cdc42 increases the percentage of transient branches in *Xenopus* optic tectal neurons imaged over time (Li et al. 2000), and stimulates dendritic outgrowth in chick primary spinal neurons (Kuhn et al. 2000). Conversely, DN Cdc42 reduces dendrite number in mouse cortical pyramidal neurons and causes a decrease in the number and length of dendrites in *Xenopus* RGCs (Ruchhoeft et al. 1999). While loss of *Cdc42* fails to affect den-

dritic growth in single cell clones of MB neurons, analyses of mutant *Cdc42* clones in VS neurons, which are more complex and stereotyped compared with MB neurons, demonstrate a requirement for Cdc42 in regulating dendritic morphology, branching, and guidance (Scott et al. 2003). Additional studies suggest that Cdc42 may negatively regulate dendrite formation in certain systems. Expression of CA Cdc42 in *Drosophila* PNS neurons results in abnormal or absent dendrites (Luo et al. 1994), including the presence of more than one dorsal primary branch, thickened primary branches that fail to fully extend, and a reduction in secondary branch number (Gao et al. 1999). CA Cdc42 also occasionally causes short dendrites in mouse hippocampal pyramidal neurons (Tashiro et al. 2000), and both CA and wild-type Cdc42 decrease dendrite number and length in *Xenopus* RGCs (Ruchhoeft et al. 1999). As mentioned above for Rac, these differential effects of Cdc42 on dendrite formation may be system specific and/or result from an inability of Cdc42 to cycle between an active GTP-bound state and an inactive GDP-bound state in order to properly regulate dendritic growth. Again, the finding that potential regulators of Cdc42 positively promote dendritic growth lends support to the idea that Cdc42 plays a positive role in this developmental process.

Though most of the potential Cdc42 regulators discussed below are only indirectly linked to Cdc42 activation in the context of dendritic growth, it is notable that they are all required for, or positively promote, dendrite formation. First, the Rho GEF Dbl, shown to be specific for Cdc42 and Rho, has been implicated in dendrite elongation, since *Dbl*-null cortical pyramidal neurons exhibit reduced dendrite length (Hirsch et al. 2002). Given that Cdc42 is capable of promoting dendrite formation, while Rho activation generally causes dendrite retraction, it is likely that Dbl acts preferentially toward Cdc42 in this case. Second, another study has shown that GT1b oligosaccharide stimulates Cdc42 activation in a Ca^{2+}/calmodulin-dependent protein kinase II (CAM-KII)-dependent manner, and facilitates CAM-KII-dependent dendritic outgrowth and branch formation in rat cerebellar Purkinje neurons, likely through Cdc42-mediated actin reorganization (Chen et al. 2003). Third, activation of Cdc42, using a combination of forskolin, an adenylyl cyclase activator, and Rolaprim, a phosphodiesterase 4 inhibitor, is likely responsible for a reduced rate of dendritic branch retraction so that more branched extensions persisted in cultured rat hippocampal neurons (Leemhuis et al. 2004). Lastly, a reduction in Cdc42 protein in *insulin-like growth factor 1* $(Igf1)^{-/-}$ brains has been associated with a decrease in dendritic length and complexity of cortical pyramidal neurons (Cheng et al. 2003). Together, these studies suggest that Cdc42 acts as a positive regulator of dendritic growth.

The studies outlined above show that each of the Rho GTPases plays a prominent role in dendritic arbor development. Furthermore, the converse dendritic phenotypes elicited by Rac/Cdc42 and RhoA activation in the studies described above, that is, promotion and prevention of dendritic arbor growth, respectively, suggest that interplay between these Rho GTPases must ultimately determine the degree of dendritic arbor complexity. Support for coordination between the Rho GTPases to regulate dendritic arbor development comes from a demonstration of cross-talk between the Rho GTPases in vivo by the same group that performed the *Xenopus* time-lapse imaging experiments (Li et al. 2000). Using an in situ version of the pull-down GTPase activity assay in tadpole optic tectum tissue sections, PAK, WASP, and Rhotekin GTPase-binding domains were used as probes for active, endogenous Rac, Cdc42, and RhoA, respectively. Under these conditions, the expression of CA Rac causes an increase in endogenous RhoA activity, and CA RhoA decreases endogenous Rac activity, suggesting the possibility of a regulatory feedback loop among the Rho GTPases (Z. Li et al. 2002; Van Aelst and Cline 2004). In another study, inhibition of the Rho effector Rho-kinase using Y-27632 slightly enhances the number of actin-containing dendritic branches in cultured rat hippocampal neurons through an increase in Rac activity (Leemhuis et al. 2004). These findings provide support for the idea that a balance between the activities of Rac, Cdc42, and RhoA determines dendritic morphology, and it does so in a manner reminiscent of that for neuritogenesis (see neurite initiation and outgrowth section above).

Extracellular cues, dendrite regulation, and the Rho GTPases

Extracellular cues, such as neurotrophins and well-characterized axon guidance factors, including semaphorins, ephrins, and slit, have been shown to affect the dendritic morphology of neurons (Miller and Kaplan 2003; Van Aelst and Cline 2004). However, the molecular mechanism by which these molecules influence dendrite formation has not been well defined, and the involvement of the Rho GTPases, though likely given their role in dendritic arbor development and axon guidance downstream of these cues, remains to be determined. Two rare examples of a link between extracellular cues, Cdc42 signaling and dendrite formation, are mentioned above for GT1b oligosaccharide and Igf1 (Chen et al. 2003; Cheng et al. 2003). Interestingly, many extracellular molecules or their signaling pathways are regulated by neuronal activity (Wong and Ghosh 2002; Miller and Kaplan 2003), and neuronal activity has been well documented to affect dendritic morphology as well (Cline 2001; Jan and Jan 2001; Wong and Ghosh 2002; Miller and Kaplan 2003). While a direct link between neurotrophins or guidance cues and the Rho GTPases is lacking, recent studies have revealed that neuronal activity induced by glutamatergic neurotransmission promotes dendritic motility and normal dendritic arbor development through the modulation of Rho GTPase activity (Li et al. 2000, Z. Li et al. 2002; Sin et al. 2002; Van Aelst and Cline 2004).

Initial experiments in *Xenopus* optic tectal neurons showed that inhibition of the N-methyl-D-aspartate (NMDA) receptor decreases dendritic arbor growth, which can be counteracted by DN RhoA, suggesting a

model in which NMDA receptor activation decreases RhoA activity in order to promote growth (Li et al. 2000). Further experiments solidified this presumption by showing that optic nerve stimulation decreases RhoA activity and increases Rac1 activity in the optic tectum, which can be blocked by a combination of NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor antagonists (Z. Li et al. 2002). These data were particularly exciting as they were the first direct evidence linking synaptic input to the regulation of endogenous Rho GTPase activity, and additional evidence for the role of Rho GTPases downstream of glutamate-receptor-mediated synaptic transmission came shortly after from the same lab. Dendritic arbor growth resulting from enhanced visual activity driven by a light stimulus was shown to be dependent upon glutamate-receptor-mediated synaptic transmission, low RhoA and Rho-kinase activities, and high Rac and Cdc42 activities. In this study, DN Rac, DN Cdc42, CA RhoA, and CA Rho-kinase all blocked light-induced dendritic arbor development (Sin et al. 2002). From these experiments, it appears that glutamate receptor activation decreases RhoA activity while increasing Rac activity, with these relative levels of Rho and Rac activity reinforced by cross-talk between the Rho GTPases themselves. This increase in Rac activity promotes dendritic branch additions and retractions (Li et al. 2000), which likely play a role in the sampling of axonal connections (Witte et al. 1996; Ruthazer et al. 2003). These dendritic connections with axons may be further stabilized by increased adhesion and strengthened synaptic transmission (Van Aelst and Cline 2004), upon which the decrease in RhoA activity allows for dendritic extension, resulting in an overall increase in dendritic growth (Li et al. 2000; Van Aelst and Cline 2004). It should be noted that in contrast to the finding that glutamate receptor activation decreases Rho activity in *Xenopus* optic tectal neurons, Rho is activated by synaptic transmission in the CA1 region of the hippocampus in rat organotypic slices (O'Kane et al. 2003).

A recent study further provides a potential link between actin cytoskeletal regulation by cadherin/catenin complexes, Wnt (Wingless) signaling, neural activity, and RhoA activity. β-Catenin is a component of the cadherin/catenin complex that helps to stabilize the actin cytoskeleton and mediate cell adhesion (Nelson and Nusse 2004). In addition, it is part of the Wnt signaling pathway important for embryogenesis and it regulates gene transcription (Cadigan and Nusse 1997; Bienz and Clevers 2000; Polakis 2000). Increasing the levels of β-catenin or other members of the cadherin/catenin complex, N-cadherin and αN-catenin, enhances dendritic arborization in a manner that is independent of Wnt/β-catenin transcription. Furthermore, overexpressing the intracellular domain of N-cadherin, which sequesters β-catenin and prevents its binding to functional partners, decreases dendritic branch tip number and total dendritic branch length, and prevents the enhancement of dendritic arborization resulting from neural activity induced by high K^+. Enhancement of dendritic growth by neural activity is also prevented by antagonists of ion channels such as NMDA receptors and L-type voltage sensitive calcium channels, and it requires increased Wnt release. These results suggest that Wnt/β-catenin signaling mediates dendritic development. Given that an important intracellular function of the catenins is the tethering of the actin cytoskeleton to the cell membrane, the role of RhoA in β-catenin-induced dendritic growth was also examined. CA RhoA drastically reduced dendritic arborization, and this phenotype could not be rescued by expression of β-catenin, suggesting that an intact actin cytoskeleton is required for β-catenin to promote dendritic branching. Thus extreme RhoA activation appears to regulate the actin cytoskeleton in such a way that growth-promoting signals are antagonized or overridden (Yu and Malenka 2003).

While the studies above focus on the consequences NMDA receptor regulation of Rho activity, another study has examined the contribution of AMPA receptor signaling to the phosphorylation of an actin-cytoskeletal protein implicated downstream of Rho signaling. Moesin is an ERM family protein that has been proposed to link membrane to the actin cytoskeleton (Bretscher et al. 2002), and Rho-kinase has been reported to phosphorylate and thus activate moesin (Jeon et al. 2002). Interestingly, glutamate induces the phosphorylation of moesin in rat hippocampal neurons (Jeon et al. 2002) and H19-7/IGF-IR cells (Kim et al. 2004) and translocates RhoA to membrane in H19-7/IGF-IR cells. Furthermore, the kinetics of glutamate translocation of RhoA to membrane parallels that of AMPA-induced moesin phosphorylation, and Y-27632 blocks AMPA-induced phosphorylation of moesin (Kim et al. 2004).

Together, these studies reveal a link between neuronal activity and regulation of Rho GTPase activity important for dendritic morphology and plasticity. Future experiments, however, are needed to further define the signaling cascades involved and to tie together signaling pathways elicited by extracellular cues, such as neurotrophins and axon guidance factors, with those elicited by neuronal activity to collectively modulate Rho GTPase activity and thus dendritic development.

The Rho GTPases and spine morphogenesis

Some neurons called spiny neurons have small protrusions emanating from their dendrites, which serve as the main sites of excitatory synapses in the brain. These protrusions are highly motile and heterogeneous in size and shape. Immature dendritic protrusions, classified as filopodia, tend to be long and thin, while mature protrusions, or spines, tend to have a well-defined head and neck structure (Harris 1999; Hering and Sheng 2001). The cytoskeleton of spines and filopodia are largely actin based, and the importance of actin for spine shape and function (Bonhoeffer and Yuste 2002; Lisman 2003) naturally suggested that the Rho GTPases play crucial roles in their formation, maintenance, and physiology. The Rho GTPase signaling pathways important for spine for-

mation and/or maintenance are outlined below and illustrated in Figure 5.

The introduction of Rho GTPase mutants into neurons produces characteristic spine phenotypes. CA RhoA decreases spine density and length in pyramidal neurons in rat and mouse hippocampal slices, mouse cortical slices, and cultured rat hippocampal neurons (Nakayama et al. 2000; Tashiro et al. 2000; Pilpel and Segal 2004). With regard to inhibition of Rho, different effects have been reported. Inhibition of Rho using C3 exoenzyme causes elongation of spine necks, unexpectedly reduces spine density, and increases filopodia density in cultured rat hippocampal neurons (Pilpel and Segal 2004). It also increases the density and length of spines in some mouse cortical and hippocampal pyramidal neurons in organotypic slices (Tashiro et al. 2000), although DN RhoA does not affect pyramidal spine density in rat hippocampal slices (Nakayama et al. 2000). The reasons for these discrepancies remain to be defined but may involve differences in the local levels of RhoA activity, the age and type of neurons examined, and the definition of "spine" used in these studies.

The effects of CA RhoA on hippocampal spines has been shown to be mediated by the Rho effector Rho-kinase, given that the Rho-kinase inhibitor Y-27632 restores the spine density of CA RhoA-expressing pyramidal neurons in rat organotypic slices close to control levels (Nakayama et al. 2000). In this study, however, Y-27632 alone did not affect spine density (Nakayama et al. 2000), nor did it affect the spine length of CA1 pyramidal neurons cotransfected with a GFP expression plasmid and a nonspecific siRNA in rat hippocampal slices (Govek et al. 2004). In contrast to these findings, a recent study in younger mouse hippocampal slices has shown that Y-27632 treatment causes a decrease in spine density and an increase in spine length, resulting from the formation of new, longer protrusions, with a subset of spines exhibiting an increased rate of spine length change (Tashiro and Yuste 2004). Again, the discrepancies between these studies remain to be defined, but may include varying levels of local RhoA activity, organismal and developmental stage differences, or different "spine" definitions. All in all, these findings show that RhoA/Rho-kinase signaling antagonizes spine formation and maintenance.

The loss of a negative regulator of Rho GTPase signaling, the RhoGAP oligophrenin-1, has also been shown to compromise spine morphogenesis. In particular, a reduction of oligophrenin-1 levels in CA1 neurons of rat hippocampal slices results in a significant decrease in dendritic spine length, an effect that is Rho-kinase dependent, suggesting that loss of oligophrenin-1 results in an increase in RhoA/Rho-kinase signaling that disturbs dendritic spine morphology (Govek et al. 2004). This study therefore highlights the importance of tight regulation of RhoA/Rho-kinase signaling for proper dendritic spine morphogenesis.

Rac1 has been shown to play a role in both the formation and maintenance of dendritic spines. Neurons expressing CA Rac1 tend to form overlapping protrusions and veil or ruffle-like structures, which are actually composed of numerous "mini" spines. Rac activation therefore decreases spine size and increases their number. This phenotype has been seen in transgenic mice expressing CA human Rac1 in Purkinje cells (Luo et al. 1996a), mouse cortical and hippocampal pyramidal neurons in slices (Tashiro et al. 2000), rat hippocampal pyramidal neurons in slices (Nakayama et al. 2000), and cultured rat hippocampal neurons (Pilpel and Segal

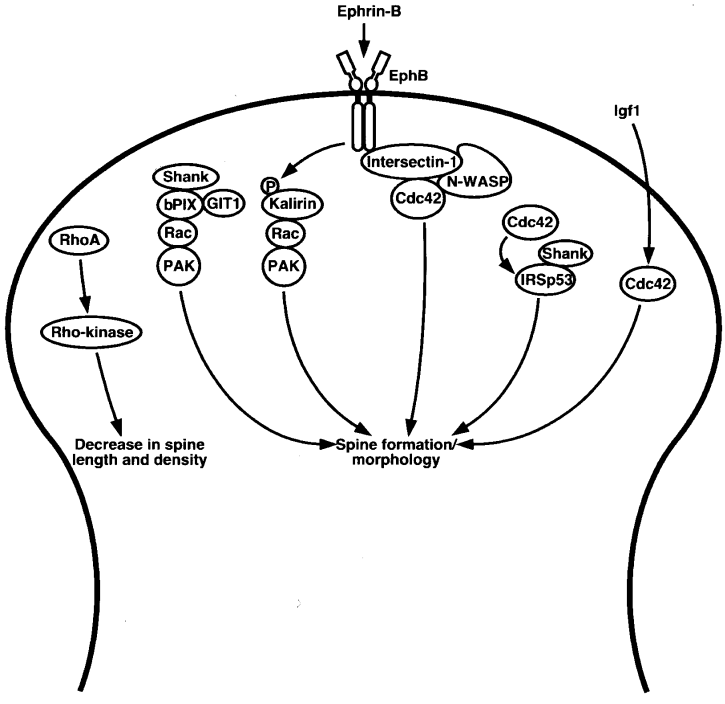

Figure 5. Rho GTPase signaling cascades that affect dendritic spine formation. In general, activation of Rac and Cdc42 signaling promotes dendritic spine formation, while activation of Rho/Rho-kinase causes spine loss. See text for details. (GIT1) G protein-coupled receptor kinase-interacting protein; (PAK) p21 activated kinase; (N-WASP) neuronal Wiskott-Aldrich-syndrome protein; (IRSp53) insulin receptor substrate of 53 kDa; (Igf1) insulin-like growth factor 1.

2004). Furthermore, these mini spines often form supernumerary synapses. Spines with double synapses, though rare in normal animals, are found frequently in CA Rac1 transgenic animals (Luo et al. 1996a). CA Rac also increases the spine head size of more mature spines at a later developmental stage (Tashiro and Yuste 2004). In contrast to these CA Rac1 findings, DN Rac causes a reduction in spine density in rat and mouse pyramidal neurons in hippocampal slices (Nakayama et al. 2000; Tashiro and Yuste 2004), and increases the length of a subset of pre-existing "stable" spines, while slightly decreasing the size of the spine head in the latter system. This increase in spine length and decrease in head size suggests the formation of filopodia-like protrusions. DN Rac also compromises spine stability and spine head motility (Tashiro and Yuste 2004).

Regulators of Rac examined in the context of dendritic spine morphogenesis include Kalirin and β-PIX. As previously mentioned, Kalirin consists of multiple isoforms, most of which are dual RhoGEFs, with a Rac1-specific GEF domain and a RhoA-specific GEF domain (Penzes et al. 2001a). However, Kalirin-7 is one of the most prevalent isoforms in the adult rat brain and contains the first Rac1 GEF domain, but lacks the second RhoA GEF domain, and as such has been shown to activate Rac1 (Penzes et al. 2000, 2001b). In primary cortical neurons, ectopically expressed Kalirin-7 is targeted to spines and increases the number and size of spine-like structures, which is dependent upon its ability to interact with PDZ domain-containing proteins and its GEF activity. Kalirin-7 is further found to interact with PSD-95 at synapses, which may reduce its ability to activate Rac (Penzes et al. 2001b). Conversely, reduced expression of Kalirin in CA1 neurons in hippocampal slices or dissociated rat hippocampal neurons results in reduced spine density, with dispersion of PSD-95 and elimination of presynaptic endings in the latter system (Ma et al. 2003). Furthermore, Kalirin-7 has been implicated in a signaling cascade whereby ephrin-B1 treatment of cultured neurons induces phosphorylation and activation of the EphB2 receptor, translocation of the Rho-GEF Kalirin to synapses, and activation of Rac1 and its effector PAK, leading to an increase in the number and size of dendritic protrusions with different morphologies. Notably, a DN EphB receptor, catalytically inactive Kalirin, DN Rac1, or inhibition of PAK interferes with ephrin-B1-induced spine development (Penzes et al. 2003). Thus it appears that Kalirin plays a role in regulating Rac activity at post-synaptic sites for proper dendritic spine development.

β-PIX is a Rac GEF implicated in dendritic spine morphogenesis involving G protein-coupled receptor kinase-interacting protein (GIT)1. In cultured hippocampal neurons, a DN GIT1 mutant results in a significant decrease in the number of synapses and normal mushroom-shaped spines, with a concomitant increase in the number of long, thin dendritic protrusions. This phenotype results from disruption of the synaptic localization of GIT1 and mislocalization of its binding partner β-PIX and Rac (Zhang et al. 2003). β-PIX and GIT1 have also been found in a complex with PAK and Shank/ProSAP (Park et al. 2003), a post-synaptic scaffolding protein shown to interact with glutamate receptors and actin cytoskeletal proteins (Ehlers 1999; Tu et al. 1999; Boeckers et al. 2002). Overexpression of Shank in rat cultured hippocampal neurons promotes synaptic accumulation of β-Pix and PAK (Park et al. 2003). Given that Shank plays a role in spine morphogenesis (Sala et al. 2001), Shank may recruit β-Pix and PAK to spines for the regulation of post-synaptic structure (Park et al. 2003). Interestingly, dPIX, the *Drosophila* homolog of mammalian PIX, regulates post-synaptic structure and protein localization at the *Drosophila* glutamatergic neuromuscular junction. *dpix* mutations decrease synaptic levels of the PDZ protein Dlg, the cell adhesion molecule Fas II, the glutamate receptor subunit GluRIIA, and PAK (Parnas et al. 2001). In addition, Dock-regulated PAK signaling has also been shown to be important for post-synaptic GluRIIA abundance at the *Drosophila* neuromuscular junction (Albin and Davis 2004). Taken together, these results indicate that PIX proteins are recruited to the synapse where they regulate post-synaptic structure and influence dendritic spine morphogenesis.

The study of transgenic mice in which the catalytic activity of PAK1–3 is inhibited by using the PAK autoinhibitory domain (AID-PAK) has provided further evidence that this Rac/Cdc42 effector is important for dendritic spine morphology, as well as memory consolidation. Cortical neurons in mice expressing DN PAK have fewer dendritic spines and larger synapses, which is correlated with enhanced mean synaptic strength and reduced bidirectional synaptic modifiability (enhanced long-term potentiation, LTP, and reduced long-term depression, LTD) in the cortex. They also exhibit deficits in the consolidation phase of hippocampus-dependent memory, including spatial memory and context-dependent fear memory (Hayashi et al. 2004). Thus this study nicely correlates changes in spine number and size with changes in synaptic plasticity underlying learning and memory, suggesting that aberrant Rho GTPase signaling can affect not only spine structure, but also function.

The role of Cdc42 in spine morphogenesis is less defined compared with Rho and Rac. While neither CA nor DN Cdc42 appear to have any significant effect on spine density or length in mouse cortical and hippocampal pyramidal cells in slices (Tashiro et al. 2000), Cdc42 may affect spine formation in other systems. Loss of function of *Cdc42* in VS neurons in the *Drosophila* visual system leads to a reduction in the density of spine-like structures (Scott et al. 2003), and reduced Cdc42 protein expression is associated with reduced cortical pyramidal neuron spine density and synapses in $Igf1^{-/-}$ brains (Cheng et al. 2003). These studies suggest that Cdc42 activity may be important for spine formation and/or maintenance in certain cell types.

Regulators and effectors of Cdc42 implicated in dendritic spine regulation include intersectin-1, N-WASP, and insulin receptor tyrosine kinase substrate (IRSp53). Activated EphB2 receptor physically associates with the Cdc42 GEF intersectin-1 and activates its GEF activity

in cooperation with neural N-WASP, a regulator of Arp2/3-mediated actin nucleation. This is turn activates Cdc42 and spine morphogenesis in dissociated mouse hippocampal neurons. Notably, DN mutants of intersectin-1, N-WASP, and Cdc42 are all capable of inhibiting spine formation in this system, leading to an increase in protrusion length and a decrease in width, suggesting the loss of mature spines and an increase in filopodia (Irie and Yamaguchi 2002). It will be interesting to see whether these DN mutants also interfere with Eprin-B-induced spine morphology. An additional link between Cdc42 and spine morphogenesis involves the binding of the Cdc42 effector IRSp53 to ProSAP2/Shank3. IRSp53's interaction with ProSAP2/Shank3 suggests that it can be recruited to the PSD, and may contribute to morphological reorganization of spines after insulin receptor and/or Cdc42 activation (Bockmann et al. 2002).

The Rho GTPase Rnd1 has also been found to affect spine formation. *Rnd1* mRNA is highly expressed in rat brain during the post-natal synaptogenic period and is present in neurons, including pyramidal neurons of the hippocampus. Furthermore, Rnd1 protein is present in synaptosomal membrane fractions, suggesting a role at the synapse. Importantly, expression of Rnd1 promotes the elongation of spines in dissociated rat hippocampal neurons, while knock-down using an antisense approach increases the percentage of headless protrusions, which also exhibit a reduction in length, and decreases spine number and width. *Rnd1* antisense spines immunostain less for PSD-95, suggesting that *Rnd1* antisense spines tend to be immature (Ishikawa et al. 2003). Given the ability of Rnd proteins to antagonize Rho signaling in a context of stress fiber and neurite formation in fibroblasts and PC12 cells, respectively (Guasch et al. 1998; Nobes et al. 1998; Aoki et al. 2000; Riento et al. 2003; Wennerberg et al. 2003), and Rnd1's ability to stimulate Rho signaling and down-regulate R-Ras activity in a context of semaphorin-induced growth cone collapse and axon guidance (Oinuma et al. 2003, 2004), it will be interesting to see if Rnd1 regulates Rho and/or R-Ras activity in order to influence dendritic spine morphology.

Taken together, these studies clearly demonstrate a role for the Rho GTPases in spine formation and maintenance, suggesting that they are capable of modulating synapse formation and function important for synaptic plasticity underlying learning and memory. Given the Rho GTPase-linked molecules involved in spine morphogenesis described above, and the importance of the actin cytoskeleton for spine structure and function (Bonhoeffer and Yuste 2002; Lisman 2003), it is likely that effects of the Rho GTPases on spine morphology are at least in part due to their effects on actin organization. However, while actin cytoskeletal rearrangements are ultimately likely to be responsible for the observed Rho GTPase effects on spine number and morphology, little is known about the external cues that might influence Rho GTPase activity in the context of spine morphogenesis. Exciting links, though, between neural activity and regulation of the actin cytoskeleton important for spine morphogenesis and motility have recently been reported, and the involvement of regulators of actin polymerization known to act downstream of the Rho GTPases, such as ADF/cofilin and Profilin, indirectly implicates the Rho GTPases in these signaling pathways in spines (Ackermann and Matus 2003; Fukazawa et al. 2003). Actin filaments within spines have been shown to turn over rapidly, and this process is modulated by synaptic transmission and calcium influx (Star et al. 2002; Portera-Cailliau et al. 2003). Interestingly, a recent study has shown that tetanic stimulation causes a shift of actin equilibrium toward F-actin with a concomitant increase in spine head size in rat hippocampal neurons, while prolonged low frequency stimulation causes a shift in actin equilibrium toward G-actin, resulting in a loss of post-synaptic actin and structure (Okamoto et al. 2004). Long-lasting increases in F-actin content within dendritic spines, and changes in spine number, size, and shape, which are associated with LTP, are dependent upon NMDA receptor activation and inactivation of ADF/cofilin, an actin depolymerizing protein (Fukazawa et al. 2003). NMDA receptor activation has also been shown to recruit a regulator of actin polymerization, Profilin, to spine heads to stabilize actin and block actin-based changes in spine shape (Ackermann and Matus 2003). Notably, ADF/cofilin and Profilin both act downstream of Rho GTPases to organize the actin cytoskeleton (Wear et al. 2000; Sarmiere and Bamburg 2004). Thus the possibility that Rho GTPase activity is regulated by synaptic activity to induce actin cytoskeletal changes in spines is tempting. In addition, given this link between neural activity and Rho-associated molecules important for actin polymerization, it will be interesting to see whether Rho GTPase signaling is influenced by or affects calcium dynamics and post-synaptic receptor signaling.

Rho signaling and nervous system disorders

As discussed above, the Rho family of GTPases and related regulatory molecules play critical roles in many aspects of neuronal development, particularly in determining neuronal morphology via their various actions on the actin cytoskeleton. Given their importance in neuronal processes, it is therefore not surprising that mutations in genes encoding a number of regulators and effectors of the Rho GTPases are associated with diseases affecting the nervous system. Here we will consider the involvement of Rho GTPase signaling in the etiology of MR and amyotrophic lateral sclerosis (ALS).

Rho GTPases and MR

MR affects ~2%–3% of children and young adults. It is characterized by reduced cognitive function, defined by an intelligence quotient lower than 70, together with associated functional deficits in adaptive behavior (Chelly and Mandel 2001). While the underlying causes of MR are extremely heterogeneous and include nongenetic factors such as infectious disease, very premature

birth, and fetal alcohol syndrome, a prominent cause is the result of single gene mutations. Positional cloning efforts in affected families have led to the identification of many disease genes associated with MR, including a number that are involved in Rho signaling. Rho-linked genes have been found to underlie various forms of MR, including syndromic and nonsyndromic X-linked forms (XLMR), as well as autosomal syndromic MR (see Table 1; Boettner and Van Aelst 2002; Ramakers 2002). Syndromic X-linked MR (MRXS) is clinically recognizable due to a specific and consistent pattern of symptoms combined with MR, whereas in the case of nonsyndromic or nonspecific X-linked MR (MRX), no other consistent functional or anatomical abnormalities are observed besides cognitive impairment. To date, 30 genes responsible for MRXS and 13 genes responsible for MRX have been cloned. In recent years it has become evident that the distinction between nonspecific and syndromic forms of MR is not always clear and in a number of instances, mutations in the same XLMR gene have been reported to result in nonsyndromic, as well as syndromic forms of this condition (Frints et al. 2002).

As discussed above, Rho GTPases modulate actin cytoskeletal dynamics that are critical for neuronal morphogenesis and dendritic plasticity, including changes in dendritic spine number and morphology, in the developing and mature nervous system. Such synaptic remodeling and dendritic plasticity are thought to underlie the anatomic basis for learning and memory formation (Matus 2000). The current view of how mutations in Rho-linked genes could result in MR is that they disrupt the normal development, structure, and/or plasticity of neuronal networks via perturbations in the regulation of the actin cytoskeleton (Negishi and Katoh 2002; Ramakers 2002; Chechlacz and Gleeson 2003). Evidence supporting such a hypothesis has come from MR patients, mouse models of MR, and RNAi studies in hippocampal slices (Purpura 1974; Comery et al. 1997; Irwin et al. 2001; Nimchinsky et al. 2001; Govek et al. 2004). MR is in many cases associated with abnormalities in dendritic

Table 1. *Mental retardation genes involved in Rho GTPase regulation and signaling*

Gene	Locus	Protein	Function	Mutations
Nonsyndromic X-linked MR (MRX)				
OPHN1	Xq12	Oligophrenin-1	Rho family GAP. Knockdown causes decreased spine length.	T(X;12) translocation, loss of function. MRX60 family: 1-bp deletion causing frameshift; mRNA dramatically reduced.
PAK3	Xq22	PAK3	Ser/Thr kinase. Rac1/Cdc42 effector.	MRX30 family: R419X truncation, abolishes kinase activity. MRX47 family: R67C missense mutation near CRIB domain A365E missense mutation.
ARHGEF6	Xq26	αPIX, Cool-2	GEF for Rac1/Cdc42. Interacts with PAK.	T(X;21) translocation, loss of function. MRX46 family: Mutation in intron 1 causes exon skipping of exon 2 resulting in 28 amino acid deletion.
FGD1	Xp11	FGD1	GEF for Cdc42.	P312L missense mutation.
Syndromic X-linked MR (MRXS)				
OCRL1 Lowe syndrome	Xq24	OCRL1	Rac GAP. Also encodes a PIP_2 5-phosphatase.	Numerous, mostly loss of function. Several mutations in Rho GAP domain.
FMR1 Fragile X syndrome	Xq27	FMRP	RNA binding protein that interacts with CYFIP, a Rac effector, to regulate protein translation and actin dynamics.	CGG repeat expansion in 5′ UTR of *FMR1* gene.
Autosomal Syndromic MR				
MEGAP 3p– syndrome	3p25	MEGAP, WRP, srGAP3	GAP for Rac and Cdc42. Interacts with WAVE and Robo.	T(X;3) translocation to identify gene; terminal deletions of several megabases causes $3p^-$ syndrome.
LIMK1 Williams syndrome	7q11	LIMK1	Ser/Thr kinase. Downstream of Rac and RhoA, phosphorylates and inactivates cofilin.	Large heterozygous deletion encompassing ~20 genes including elastin gene.

For references, see main text. (CYFIP) Cytoplasmic FMRP interacting protein; (Cool-2) cloned out of library 2; (FGD1) Faciogenital dysplasia protein 1; (FMRP) fragile X mental retardation protein; (GAP) GTPase activating protein; (GEF) guanine nucleotide exchange factor; (LIMK1) LIM kinase 1; (MEGAP) mental disorder-associated GAP; (OCRL) oculocerebrorenal syndrome of Lowe; (PAK3) p21 activated kinase 3; (PIX) PAK interacting exchange factor; (srGAP3) Slit-Robo GAP3; (WRP) WAVE-associated Rac GAP protein.

branching and spine morphology, and since spine shape and function are intricately linked (Engert and Bonhoeffer 1999; Maletic-Savatic et al. 1999; Toni et al. 1999; Matsuzaki et al. 2001; Yuste and Bonhoeffer 2001; Kasai et al. 2003), the observed changes are likely to impair neuronal connectivity and synaptic plasticity, leading to reduced cognitive function (Chechlacz and Gleeson 2003).

Nonsyndromic or nonspecific X-linked MR (MRX)

Of the 13 MRX genes identified to date, three encode regulators or effectors of the Rho GTPases (Chelly and Mandel 2001; Frints et al. 2002; Ramakers 2002). They are (1) *OPHN1*, a Rho-GAP (Billuart et al. 1998a); (2) *PAK3*, a serine/threonine kinase downstream of Rac and Cdc42 (Allen et al. 1998); and (3) *ARHGEF6*, a Rac GEF also known as α*PIX* or *Cool-2* (Kutsche et al. 2000). Other MRX genes, such as *IL1RAPL*, *TM4SF2*, and *RSK2*, have been indirectly linked to Rho signaling (Boettner and Van Aelst 2002), while a novel missense mutation in the Cdc42-specific exchange factor *FGD1*, the gene associated with faciogenital dysplasia (Aarskog-Scott syndrome), has been reported in three brothers with MRX that lack the distinct characteristics of this syndrome (Lebel et al. 2002).

OPHN1 was the first Rho-linked MRX gene to be identified and encodes the protein oligophrenin-1 that has a Rho-GAP domain shown to negatively regulate RhoA, Rac, and Cdc42 in vitro and in nonneuronal cells (Billuart et al. 1998a; Fauchereau et al. 2003). It was identified by the analysis of a balanced translocation t(X;12) in a female patient with mild MR (Bienvenu et al. 1997). Subsequently, its involvement in MRX was reported by the identification of a 1-bp deletion causing a frameshift at the end of the GAP domain in an MRX family (MRX 60) (Billuart et al. 1998a). In these two cases, the *OPHN1* mutations were associated with a loss of, or dramatic reduction in, mRNA product (Billuart et al. 1998a). More recently, *OPHN1* mutations have been found in families with MR associated with cerebellar hypoplasia and/or epilepsy (Tentler et al. 1999; Bergmann et al. 2003; Philip et al. 2003), and indeed, a new report indicates that members of the original MRX 60 family also suffer seizures and cerebellar dysgenesis (des Portes et al. 2004). This suggests that there may be a broader spectrum of phenotypes associated with *OPHN1* mutations than just cognitive impairment.

In addition to the Rho GAP domain, oligophrenin-1 contains two other identifiable functional domains: an N-terminal BAR domain and a pleckstrin homology (PH) domain (Billuart et al. 1998b; Peter et al. 2004). BAR domains generally serve as a dimerization, membrane-binding, and membrane curvature-sensitive module (Zimmerberg and McLaughlin 2004), while PH domains generally play a role in the localization of proteins to membranes (Cozier et al. 2004). Recent studies, however, have indicated that PH domains may play multiple roles in GTPase signaling, including acting as an effector-binding site for Rac (Snyder et al. 2003) and regulating GTPase activation (Rossman et al. 2003). Together, these two domains enable oligophrenin-1 to tubulate and bind liposomes (Peter et al. 2004), although the functional significance of these domains in vivo is unknown. Following this is the Rho GAP domain and there is a C-terminal region that directly binds actin, although it does not contain a conventional actin-binding domain (Billuart et al. 1998b; Fauchereau et al. 2003). Oligophrenin-1 is expressed in multiple tissues, with highest expression in brain. It is found in neurons in all major regions of the brain, including the hippocampus and cortex, and it is present in axons, dendrites, and dendritic spines (Fauchereau et al. 2003; Govek et al. 2004). It is also present in peripheral myelin (Xiao et al. 2004) and in neurons and varicose axons of the enteric nervous system (Xiao et al. 2003). A recent study provided the first insights into how mutations in the *OPHN1* gene may impact neuronal function. By using RNAi and antisense RNA approaches in organotypic hippocampal slices, knock-down of oligophrenin-1 levels in CA1 pyramidal neurons results in a significant decrease in dendritic spine length. This phenotype is recapitulated when using a CA form of RhoA and is rescued by inhibiting Rho-kinase, indicating that loss of oligophrenin-1 may cause changes in spine morphology during development due to effects on actomyosin contractility via RhoA and Rho-kinase (Govek et al. 2004). This finding further supports the notion that mutations in Rho-linked genes result in MR via their effects on neuronal morphology, most likely by involving the actin cytoskeleton, which in turn impairs synaptic structure and function. Oligophrenin-1 has also been found to bind the post-synaptic adapter protein Homer (Govek et al. 2004), a protein involved in organizing glutamate receptor complexes and in determining dendritic spine morphogenesis (Xiao et al. 2000; Fagni et al. 2002). Although the function of this interaction remains to be elucidated, it is tempting to speculate that oligophrenin-1 may act downstream of glutamatergic receptors via Homer to regulate RhoA activity and, in turn, influence spine shape. To date, spine phenotypes of patients carrying *OPHN1* mutations have not been examined in post-mortem material, but the generation of knockout mice will allow the examination of spine phenotypes both in developing and mature synapses.

The second Rho-linked MRX gene to be identified, *PAK3*, encodes a member of the larger family of PAKs, whose members are downstream effectors of Rac and Cdc42 (see introduction). PAK3 is highly expressed in brain and in post-mitotic neurons of the developing and post-natal cerebral cortex and hippocampus (Allen et al. 1998). Three different mutations in the *PAK3* gene have been isolated in different MRX-pedigrees. The first *PAK3* mutation, R419X, found in family MRX30, introduces a premature stop codon that abolishes the kinase activity of the truncated product (Allen et al. 1998). The second *PAK3* mutation, an R67C missense mutation in family MRX47, is located in a polybasic region upstream of the Cdc42 and Rac CRIB-binding domain, and likely affects GTPase binding and activation of PAK3, although this remains to be tested (Bienvenu et al. 2000). The conse-

quence of the third mutation (A365E), also a missense mutation, on PAK3 function is not yet defined, although it occurs in a highly conserved region of the protein that may affect catalytic kinase domain function (Gedeon et al. 2003).

The third Rho-linked MRX gene, *ARHGEF6* (also known as α*PIX* or *Cool-2*), encodes a ubiquitously expressed Cdc42/Rac exchange factor, which interestingly has been found to interact with and regulate PAK (Bagrodia et al. 1998; Manser et al. 1998). Cool-2 has recently been found to act as a Rac-specific GEF when it is a dimer, though as a monomer it can act as a GEF for Rac or Cdc42, but only when PAK or Cbl binds to its SH3 domain (Feng et al. 2004). The first mutation in *ARHGEF6* associated with MR was found in a male carrying a reciprocal X;21 translocation breakpoint located between exons 10 and 11 of the *ARHGEF6* gene (Kutsche et al. 2000). Subsequently, affected members of a large MRX family (MRX46) were found to possess a mutation in the first intron of this gene that resulted in preferential skipping of exon 2, predicted to produce a protein lacking 28 amino acids (Kutsche et al. 2000).

How mutations in *PAK3* and *ARHGEF6* affect neuronal morphology and/or function and contribute to MRX is currently unknown. ARHGEF6 has recently been shown in nonneuronal cells to interact with PARVB (β-parvin or affixin) and colocalize with PARVB and integrin-linked kinase (ILK), which are known to interact with each other. Interestingly, mutant ARHGEF6 proteins that mimic those produced in patients with MR are mislocalized and unable to bind to PARVB. Given that PARVB and ILK both localize to focal adhesions, which are essentially integrin clusters, it is likely that ARHGEF6 is involved in integrin-mediated signaling that leads to activation of Rho GTPases and changes in cell morphology (Rosenberger et al. 2003). How these findings relate to events occurring during normal neuronal development and function is unclear. Further hints as to the function of ARHGEF6/αPIX and PAK3 in neurons are provided by studies demonstrating a role for closely related family members in different aspects of neuronal development and morphology. Both PAK1 and β-PIX proteins have been implicated in neurite outgrowth in PC12 cells (Daniels et al. 1998; Shin et al. 2002, 2004). PAK1 has also been shown to regulate dendrite initiation of cortical neurons (Hayashi et al. 2002) and to mediate ephrin-B-induced spine development (Penzes et al. 2003). Recently, transgenic mice that express a DN *PAK* transgene in the post-natal forebrain were generated (Hayashi et al. 2004). The transgene consists of the AID-PAK, which binds to the catalytic domain of PAK1, PAK2, and PAK3 to block their activation. Cortical pyramidal neurons in these mice have fewer spines than control animals and show a shift in the overall spine population toward shorter spines with larger heads. This demonstrates that collectively, the PAK family members play a role in determining spine morphogenesis. Interestingly, these mice also show enhanced LTP and reduced LTD in cortex, as well as specific deficits in the consolidation phase of hippocampus-dependent memory, suggesting a role for PAK in memory retention (Hayashi et al. 2004). Meanwhile, β-PIX was found to interact with the postsynaptic protein Shank (Park et al. 2003), and GIT1 (Bagrodia et al. 1999). Both Shank and GIT1 target β-PIX to the synapse, where it may locally activate Rac and PAK (Park et al. 2003). This recruitment of β-PIX appears to be necessary for the formation and stabilization of synapse-bearing spines (Zhang et al. 2003). Consistent with these findings, the *Drosophila* homolog, dPIX, has been shown to play a major role in regulating post-synaptic structures and protein localization at the glutamatergic neuromuscular junction (Parnas et al. 2001). It will therefore be of interest to investigate whether mutations in *PAK3* and α*PIX* affect spine morphology and postsynaptic signaling and whether both molecules act in a common pathway underlying cognitive function.

Syndromic X-linked MR (MRXS)

Rho-related genes are also associated with syndromic forms ofMR, including Lowe syndrome and FRAXA. The oculocerebrorenal syndrome of Lowe (OCRL) is a rare X-linked disorder characterized by severe MR, bilateral congenital cataracts, and renal Fanconi syndrome. Lowe syndrome results from mutations in the *OCRL1* gene that encodes a phosphatidylinositol 4,5 bisphosphate 5-phosphatase (PIP_2 5-phosphatase) with a C-terminal Rho GAP domain (Attree et al. 1992). OCRL1 is found predominantly in the trans-Golgi network (TGN) and functions as an active phosphatase with PIP_2 as its preferred substrate (Suchy et al. 1995; Zhang et al. 1995; Dressman et al. 2000). Increased levels of PIP_2, as well as abnormalities in the actin cytoskeleton, were observed in cells from patients with Lowe syndrome, suggesting that increased levels of PIP_2 may contribute to the disease phenotype (Suchy and Nussbaum 2002). Whether the PIP_2 induced changes in actin polymerization involve Rho GTPases has not been investigated. Notably, several mutations of *OCRL1* in Lowe syndrome patients have been mapped to the RhoGAP domain (Lin et al. 1997, 1998; Satre et al. 1999). This domain has been shown to interact with Rac1 and to possess weak GAP activity toward Rac1 (Faucherre et al. 2003). The contribution of disrupting OCRL1's Rho GAP domain and associated functions to the disease phenotype remains unknown. However, given that the regulation of actin dynamics is important for the formation of epithelial cell adhesion sites critical for renal proximal tubule function in the kidney and lens differentiation in the eye (Cereijido et al. 1993; Stevenson and Begg 1994; Wittchen et al. 1999; A. Lee et al. 2000), as well as neuronal differentiation, it would not be surprising if disruption of the Rho GAP function contributed to clinical symptoms of Lowe syndrome.

The regulation of Rac also plays a role in FRAXA, the most frequent cause of hereditary MR. FRAXA is caused by a CGG repeat expansion and hypermethylation of CpG dinucleotides in the 5′ untranslated region of the *FMR1* gene (Pieretti et al. 1991). The *FMR1* gene encodes the RNA-binding protein FMRP, which is absent in

FRAXA patients due to transcriptional silencing. Fragile X patients exhibit a number of different clinical abnormalities in addition to MR, including evocative facial features, prominent jaw and large ears, post-pubescent macro-orchidism, hyperactivity, attention deficit, and autistic-like behaviors (Billuart and Chelly 2003). As described in the introduction, it is the association of FMRP with CYFIP1 and CYFIP2 that uncovered the relationship between FMRP and Rac signaling (Schenck et al. 2001, 2003). CYFIP1 (also known p140/Sra-1) binds activated, GTP-bound Rac1 (Kobayashi et al. 1998), while CYFIP2 (or PIR121 or POP) is part of an inactive protein complex that includes the Rac effector WAVE1 (Witke et al. 1998; Saller et al. 1999; Eden et al. 2002). From studies in *Drosophila*, a model was put forward that is analogous to the mechanism of WAVE activation. CYFIP was proposed to dissociate from FMRP upon interaction with activated Rac, releasing FMRP in an active complex that can then regulate local protein translation. Since CYFIP is in a complex with both FMRP and WAVE1, it is likely to coordinate the regulation of actin cytoskeletal reorganization, as well as the control of local protein translation, processes that are key to neuronal morphogenesis and connectivity (Billuart and Chelly 2003; Schenck et al. 2003). Indeed, abnormal dendritic spines have been observed in FRAXA patients, where spines are thin, elongated, and have small synaptic contacts typical of immature spines (Hinton et al. 1991; Irwin et al. 2001); in *FMR1* knockout mice (Comery et al. 1997; Nimchinsky et al. 2001); and transgenic Rac1 mice (Luo et al. 1996a), supporting the idea that loss of FMRP may negatively affect Rac signaling, resulting in aberrant neuronal network formation and accompanying cognitive dysfunction.

Autosomal syndromic MR

In addition to the identification of Rho-linked MR genes on the X chromosome, there are several examples of mutations in Rho-linked genes associated with MR on autosomes. These include the *MEGAP/WRP/srGAP3* gene, which encodes a Rho GAP, and also the *LIMK1* gene, encoding a downstream effector of both Rac1 and RhoA. Interruption of the *MEGAP* gene is associated with 3p$^-$ syndrome, while loss of *LIMK1*, along with a number of other genes, is associated with Williams syndrome (WS).

The 3p$^-$ syndrome is characterized by MR associated with microcephaly, hypotonia, growth failure, heart and renal defects, and facial abnormalities (Mowrey et al. 1993). A female patient exhibiting severe MR and hypotonia, but none of the other classical 3p$^-$ symptoms, was found to possess a balanced translocation, with one of the breakpoints mapping within the 3p$^-$ deleted region. This translocation breakpoint interrupted the mental disorder-associated GAP (*MEGAP*) gene, which is also deleted in 3p$^-$ patients that present with MR (Endris et al. 2002). The *MEGAP* gene encodes a RhoGAP that is highly expressed in fetal and adult brain, including the cortex and the hippocampus, and strongly activates intrinsic Rac GTPase activity and to a lesser extent intrinsic Cdc42 GTPase activity (Endris et al. 2002). The protein was previously identified as a WAVE-interacting protein called WRP and shown to act as a Rac-GAP (Soderling et al. 2002), as well as a Robo-interacting protein called srGAP3 (Wong et al. 2001). As discussed in the introduction, WAVE acts downstream of Rac to regulate actin polymerization by acting on the Arp2/3 complex. MEGAP/WRP/srGAP3 may therefore be part of a negative feedback loop to regulate Rac activity and modulate Rac-induced actin reorganization. Notably, loss of WAVE1, which is brain-specific and is present in the hippocampus and the cerebellum, causes symptoms in mice analogous to those of 3p$^-$ syndrome patients. The *WAVE1* null mice are runted and exhibit reduced viability, as well as reduced anxiety, poor balance, reduced coordination, and impaired hippocampus-dependent learning and memory (Soderling et al. 2003). In the case of srGAP3, this molecule is one of three srGAPs (srGAP1–3) shown to bind the repulsive guidance receptor Robo1 by yeast two-hybrid analysis. Interestingly srGAP1 has been shown to interfere with Slit-triggered repulsion of migratory cells from the SVZa of the forebrain (Wong et al. 2001), suggesting that MEGAP/WRP/srGAP3 may also have a role in neuronal migration and guidance. The concrete cellular effects brought about by mutations in *MEGAP/WRP/srGAP3* or *WAVE1* remain to be studied, although disrupting the function of these genes is likely to affect actin nucleation, which in turn may perturb synaptic connections and neuronal responses.

WS is a complex developmental disorder characterized by mild MR, cardiovascular problems, distinctive craniofacial features, poor visual-motor integration, attention deficits, and at times hyperactivity (Morris and Mervis 2000). The vast majority of WS patients share in common a heterozygous deletion on chromosome 7q11.23 that encompasses a region containing ~20 genes. One of the genes present in this WS critical region is *LIMK1*. Loss of *LIMK1* is believed to be responsible for some of the neurological aspects of WS, particularly impaired visuo-spatial cognition (Frangiskakis et al. 1996). The *LIMK1* gene encodes a serine/threonine protein kinase that is highly expressed in the developing brain. LIMK acts downstream of both Rac and RhoA to phosphorylate and inactivate ADF/cofilin, thus controlling actin dynamics (Arber et al. 1998; Yang et al. 1998). Recent studies in which *LIMK1* knockout mice were generated and analyzed provide the first insights as to how lack of *LIMK1* may contribute to certain symptoms of WS. *LIMK1* knockout mice show morphological changes in dendritic spines of pyramidal neurons: The spine head regions are reduced in size, while the spine necks are significantly thicker. In addition, these mice show enhanced hippocampal LTP following repeated high-frequency stimulation. Not surprisingly, these knockout mice show altered ADF/cofilin phosphorylation as well as actin cytoskeleton abnormalities, and it is likely that both the altered spine morphology and synaptic function are at least partly due to perturbations in actin filament organization. Importantly, the *LIMK* knockout mice also show abnormal behavioral responses consistent with

those found in human WS patients, including heightened locomotor activity, impaired spatial learning, and altered fear responses (Meng et al. 2002). Based on these findings in mice, it would be of interest to see whether similar abnormalities in spine morphology and actin dynamics are present in WS patients and whether the spine pathology accounts for some of the cognitive changes, as has been suggested for other disorders that include MR.

The above findings clearly document the importance of Rho signaling in the regulation of normal cognitive function and support the notion that MR results from the dysfunction of genes required for processes such as the remodeling, establishment, and stabilization of synaptic connections. Tying together the physiological and morphological aspects of MR with the molecular and cellular details of Rho GTPase signaling will be a major challenge for future research. The use of RNAi approaches and animal models carrying targeted disruptions in the Rho-linked MR genes will be of great benefit in further understanding the relationships between Rho signaling, neuronal morphology, and cognitive function.

Rho GTPases and motor neuron disease

It has become apparent that mutations in Rho-associated genes can also result in disorders of the PNS. ALS is a heterogeneous neurological disorder characterized by the progressive degeneration of motor neurons, normally resulting in death due to respiratory paralysis (Rowland and Shneider 2001). Although only 5%–10% of cases are inherited, positional cloning efforts in affected families have led to the identification of two genes that when mutated, result in ALS. Mutations in *SOD1*, which encodes for Cu-Zn superoxide dismutase 1, cause the classical form of ALS as a result of an unknown, but toxic gain-of-function mechanism (Rosen et al. 1993) The second gene, *ALS2,* encodes the protein alsin that contains three potential guanine nucleotide exchange (GEF) domains, including the tandem DH-PH domain module characteristic of a Rho GTPase exchange factor (Hadano et al. 2001; Yang et al. 2001). Mutations in *ALS2* give rise to a rare recessive juvenile form of ALS2 that presents early in life and progresses much more slowly than the classical form. In addition, mutations in *ALS2* are associated with two other progressive neurodegenerative disorders, a recessive juvenile form of primary lateral sclerosis and infantile-onset ascending hereditary spastic paralysis (Yang et al. 2001; Eymard-Pierre et al. 2002; Devon et al. 2003; Gros-Louis et al. 2003). The mutations in *ALS2* that give rise to these disorders are predicted to generate truncated forms of alsin, although disease-causing alsin mutants have been shown to be unstable and rapidly degraded when expressed in cultured cells, suggesting that alsin loss of function generates the disease phenotypes (Yamanaka et al. 2003).

By sequence prediction analysis, alsin contains multiple protein domains involved in signal transduction, membrane localization, and protein trafficking. As mentioned above, there are three predicted GEF domains: an N-terminal region that shares homology with RCC1 (regulator of chromatin condensation), a GEF for the Ran family of small GTPases; a central tandem arrangement of a DH and PH domain, which is the hallmark of Rho family exchange factors; and finally a C-terminal vacuolar protein sorting 9 (VPS9) domain that is the hallmark of GEFs for the Rab5 small GTPase. Ran family members are involved in nuclear transfer as well as chromatin condensation (Yamanaka et al. 2003), while Rab5 is essential for protein trafficking through the endocytic pathway (Zerial and McBride 2001).

Recent experimental analyses of alsin have shed some light on its function. Alsin is most abundant in brain, with lower levels found in the spinal cord and other tissues (Otomo et al. 2003; Yamanaka et al. 2003). With respect to alsin's role as a Rho family GEF, recent experiments have shown that alsin is an exchange factor for Rac1 (Topp et al. 2004). Alsin specifically interacts with Rac1 and shows little, if any, association with Rac3, RhoA, or Cdc42. Alsin is able to stimulate Rac1 nucleotide exchange in a GTPase pull-down assay and colocalizes with Rac1 in membrane ruffles and lamellipodia in NIH3T3 cells, although overexpression of alsin does not induce these structures in cells. It is also clear that alsin has exchange activity for Rab5 and has been implicated in controlling endosomal function (Otomo et al. 2003; Yamanaka et al. 2003; Topp et al. 2004). Whether alsin also has Ran GTPase exchange activity is unclear at present, although it has been suggested that the N-terminal region containing the predicted Ran GEF region may actually be of structural, rather than enzymatic, importance (Otomo et al. 2003; Topp et al. 2004).

At present it is unknown how the Rac1 and Rab5 exchange activities of alsin influence motor neuron maintenance and survival. One possibility is that alsin utilizes both GEF activities to regulate actin remodeling events required for endocytic events in motor neurons. It is now appreciated that endocytic trafficking and actin dynamics are intimately linked (Lanzetti et al. 2001; McPherson 2002; Schafer 2002) and alsin is poised to act at the interface between these processes by its actions on Rac1 and Rab5. There are a number of neuronal processes that could utilize alsin in this way. For example, alsin may be involved in regulating the clathrin-mediated endocytosis of glutamate receptors at the post-synaptic membrane (Topp et al. 2004), a process that is thought to require actin reorganization, as well as the endocytic machinery (Zhou et al. 2001). Alternatively, alsin may have a role at the presynaptic nerve terminal during synaptic vesicle trafficking. Indeed, synaptic vesicles are associated with both Rab5 and actin filaments (Gotow et al. 1991; de Hoop et al. 1994; Fischer von Mollard et al. 1994). Interestingly, Rac1 has been shown to associate with synaptojanin 2, a phosphatidylinositol lipid phosphatase that is related to synaptojanin 1, which in turn has been implicated in multiple steps of clathrin-mediated endocytosis of synaptic vesicles at the nerve terminal (Malecz et al. 2000). Immunolocalization studies suggest that alsin is found in punctuate structures in both dendrites and axons of hippocampal neurons (Topp et al. 2004), so further studies will be required

to understand the role of alsin in normal motor neuron function as well as in motor neuron disease. The generation of knockout animals and RNAi approaches will be of great interest to determine whether loss of alsin impairs receptor and/or vesicle trafficking in motor neurons, which may ultimately underlie their death. Thus, it may be that Rho signaling impacts a range of critical neuronal processes by influencing cytoskeletal and trafficking pathways, which when perturbed, result in disease.

Concluding remarks

The elaborate architecture of neurons serves as a basis for the formation of a vast network within the brain, which allows us to learn, remember, and reason. While each compartment of the neuron serves a particular purpose designed for the vectorial flow of information, it has become evident that the Rho GTPases play a prominent role in the formation and function of each part of these highly differentiated cells, largely through their regulation of the cellular cytoskeleton. In addition, they are indispensable for the formation of interneuronal connections and signaling, and play a role in dendritic and synaptic plasticity thought to underlie learning and memory. The importance of the Rho GTPases in neuronal development and function is underscored by the association of numerous nervous system disorders with perturbation of Rho GTPase signaling, and inappropriate Rho signaling appears to be responsible for the inability of CNS neurons to regenerate after injury. As a result, therapeutic intervention that targets Rho GTPase activity or that of their associated molecules exists as a possibility for the diseases discussed in this review. Further elucidation of the molecular mechanisms by which the Rho GTPases contribute to these disorders will shed light not only on the epidemiology of these diseases, but also on the basic mechanisms of neuronal development and function. To date, many of the developmental programs of mammalian neurons that we have come to accept remain to be confirmed in vivo. However, recent advances in live-cell imaging in intact animals, in both a wild-type and disease context, will surely enhance our knowledge and understanding of mental function.

Acknowledgments

L.V.A. is supported by the National Institutes of Health and the Dana Foundation and S.E.N. is a Wellcome Trust Traveling Fellow.

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