RHO GTPASES IN NEURONAL MORPHOGENESIS

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The Rho family of small GTPases act as intracellular molecular switches that transduce signals from extracellular stimuli to the actin cytoskeleton and the nucleus. Recent evidence implicates Rho GTPases in the regulation of neuronal morphogenesis, including migration, polarity, axon growth and guidance, dendrite elaboration and plasticity, and synapse formation. Signalling pathways from membrane receptors to Rho GTPases and from Rho GTPases to the actin cytoskeleton are beginning to be discovered. Mutations in these signalling pathways have been reported in human neurological diseases, which underscores their importance in the development and function of the nervous system.

CONSTITUTIVELY ACTIVE
Mutant proteins that remain active in the absence of upstream signals. In the case of Rho GTPases, common constitutively active mutants act by inhibiting the GTPase activity, thereby preventing them from being 'switched off'.

DOMINANT-NEGATIVE
Non-functional mutant proteins that interfere with the functions of the endogenous wild-type proteins. In the case of Rho GTPases, common dominant-negative mutants act by titrating guanine nucleotide exchange factors. The specificity of a dominant-negative mutant therefore relies on targeting the factor(s) that are specific for the GTPase of interest.

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The organization of the nervous system relies on the exquisite morphological complexity of neurons. The development of functional neural circuitry involves several discrete morphological steps. Newborn neurons must migrate to their characteristic locations, extend axons and dendrites into proper target regions, and form synapses with appropriate partners. These seemingly different processes all depend on the regulation of the cytoskeleton in response to extra- or intracellular cues that orchestrate the morphological development of neurons.

The small GTPases of the Rho subfamily are critical regulators of the actin cytoskeleton in eukaryotic cells from yeast to humans. This article reviews recent evidence indicating that these Rho GTPases may mediate the many morphological changes that can be observed during neuronal development, focusing on the roles of Rho GTPases in axon growth and guidance, and in dendrite elaboration. I will also summarize recent advances in our understanding of the signal-transduction pathways from extracellular ligands to Rho GTPases, and from Rho GTPases to the actin cytoskeleton. Several excellent reviews provide general information on growth cone guidance[1,2] or Rho GTPase signalling in general[3–5].

Rho GTPases as molecular switches

Rho GTPases constitute a subfamily of the Ras superfamily of small GTPases (~200 amino acids long). The number of Rho GTPases varies from about six in worms and flies to over a dozen in mammals. The best-studied Rho GTPases are RhoA (Ras homologous member A), Rac1 (Ras-related C3 botulinum toxin substrate 1) and Cdc42 (cell division cycle 42). For simplicity, I refer to the specific proteins as Rho, Rac and Cdc42, and use Rho GTPases to refer to the entire subfamily.

Rho GTPases act as molecular switches (FIG. 1). They exist in two states: a GDP-bound inactive state and a GTP-bound active state. Two classes of protein facilitate the switch between these two states. Guanine nucleotide exchange factors (GEFs) facilitate the exchange of GDP for GTP, thereby switching Rho GTPases on. When bound to GTP, activated Rho GTPases can bind to various effectors and elicit different biological activities. By contrast, GTPase activating proteins (GAPs) increase the endogenous GTPase activity of Rho GTPases, thereby helping to switch them off (FIG. 1).

From fibroblasts to neurons

The first clues about the cellular functions of Rho GTPases came from studies in mammalian fibroblasts (FIG. 2a). Microinjecting CONSTITUTIVELY ACTIVE Rho GTPases into fibroblasts resulted in clear and characteristic changes to the actin cytoskeleton that depended on the specific Rho GTPases used. By contrast, microinjecting DOMINANT-NEGATIVE Rho GTPases inhibited the formation of specific actin structures in response to appropriate extracellular stimuli. In this way, different Rho family members were found to have different cellular functions.

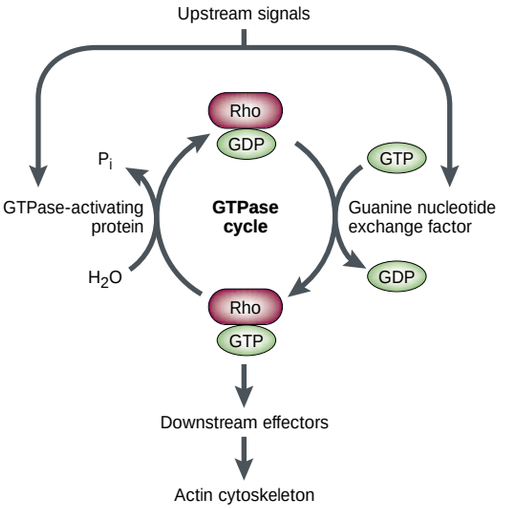

Figure 1 | **Rho GTPases as molecular switches.** Upstream signals transduce signals to Rho GTPases through regulation of the activities of guanine nucleotide exchange factors or GTPase-activating proteins, which facilitate switching on or switching off Rho GTPases. In their GTP-bound state, Rho GTPases bind to and activate their effectors to transduce the signal downstream.

Rho seems to be important for regulating the formation of STRESS FIBRES and focal adhesions, whereas Rac and Cdc42 regulate the formation of LAMELLIPODIA and FILOPODIA, respectively[6–9]. In addition to their downstream effects on the actin cytoskeleton, Rho family members affected each other's activity, and a signal-transduction pathway from Cdc42 to Rac to Rho was also proposed[8]. Rho GTPases also cause the formation of characteristic cytoplasmic protrusions in yeast cells and, indeed, Cdc42 was originally identified in budding yeast as a key regulator of cell polarity during bud formation[10,11].

Do these observations of fibroblasts and budding yeast have any relevance to neuronal development? Neuronal growth cones, the structures that lead and direct the growth of neuronal processes, have filopodia and lamellipodia that are structurally analogous to those seen in fibroblasts (FIG. 2b). Filopodia and lamellipodia in neuronal growth cones or at the leading edge of migrating neurons sense environmental cues. Extensions of filopodia and lamellipodia in the right direction are subsequently stabilized, possibly by increases in adhesion and cytoskeletal stability[12], while the growth cone calculates its next move. The similarities of yeast budding and outgrowth of neuronal process[13], and the fact that filopodia and lamellipodia are important structures in neuronal growth cones, prompted researchers to investigate the function of Rho GTPases in different aspects of neuronal development.

Regulation of neuronal morphogenesis

Rho GTPases play an essential role in the generation of neurons by regulating CYTOKINESIS[14,15] but here I will focus on postmitotic events. Once a neuron is born, it might migrate a long way before it finds its destination and begins to differentiate[16]. It then sends out two classes of process: a single axon to carry its output and several dendrites to collect input[17,18]. Once this neuronal polarity is established, the axon navigates through a complex environment to find its target[1,2], and dendrites can undergo extensive growth and branching. The last step in constructing a rough scaffold of the nervous system is the establishment of synaptic connections between different neurons[19]. Accumulating evidence now indicates that Rho GTPases are involved in all of the above developmental processes and that, in certain situations, they might be key regulators. I will first introduce the Rho GTPases in the context of axon and dendrite growth and guidance, and then move on to examine how migration, polarity and synapse formation are regulated by Rho GTPases.

Axon growth and guidance. The first investigations of Rho GTPases in neurons identified their function in regulating outgrowth and retraction of axons or neurites[20,21]. In *Drosophila melanogaster* embryonic sensory neurons, expression of either constitutively active or dominant-negative Rac results in selective defects in axonal outgrowth (both initiation and elongation) without notably affecting dendrite growth. *Cdc42* mutations affected both axons and dendrites[20]. Expression of constitutively active *Rac* had a similar selective effect on the growth of axons but not dendrites in cerebellar Purkinje cells in transgenic mice[22]. Rho is thought to mediate neurite retraction, because activation of *Rho* in neuronal cell lines led to neurite retraction, whereas expression of dominant-negative *Rho* prevented neurite retraction in response to extracellular stimuli[21].

Subsequent studies in systems from neuron-like cell lines and primary neurons to *in vivo* systems support the following general scheme. Rac and Cdc42 seem to be positive regulators[20,23–30], whereas Rho seems to be a negative regulator[21,23,25,28,31,32] of process outgrowth, although there are exceptions[31,33]. These distinct effects of different Rho GTPases, as well as the differential effects of each Rho GTPase in different neuronal compartments, indicate that the functions of Rho GTPases may be more complex in neurons than in fibroblasts. Often, the expression of constitutively active and dominant-negative GTPase mutants produce similar, rather than opposite, phenotypes[20,27,29,34]. This might mean that the Rho GTPase signalling pathway has a cyclic mode of action. For example, if filopodia are required to cycle between extension and retraction during axonal growth for the growth cone to advance, then blocking filopodia in either state (with either constitutively active or dominant-negative GTPase mutants) would have the same net outcome of decreased neurite outgrowth.

Rho GTPases are also involved in guiding growing axons. As axons grow, their growth cones encounter many cues that guide them towards or away from specific cells or pathways. Guidance cues act by causing the selective stabilization or destabilization of actin-based filopodia and lamellipodia. Accumulating evidence indicates that Rho GTPases participate in mediating these actions. In *Caenorhabditis elegans*, mutations in a

STRESS FIBRES
Axial bundles of F-actin underlying the cell bodies.

LAMELLIPODIA
Structures at the edge of cells composed of a crosslinked F-actin meshwork.

FILOPODIA
Long, thin protrusions at the periphery of cells and growth cones. They are composed of F-actin bundles.

CYTOKINESIS
The division of cytoplasm of a parent cell after nuclear division.

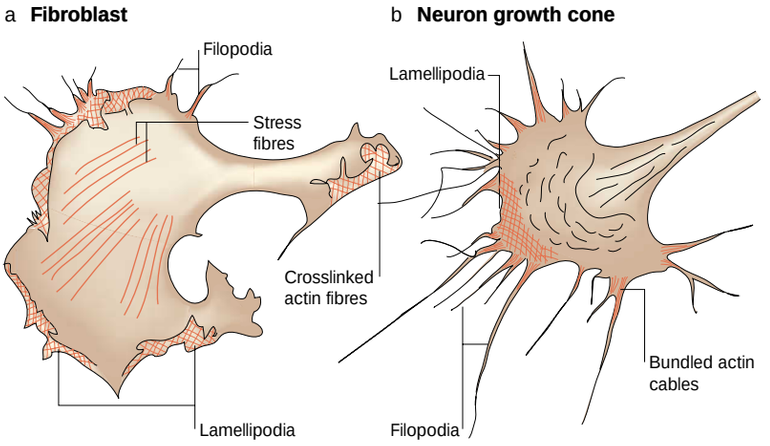

Figure 2 | **Actin-based structures in a fibroblast and a neuronal growth cone.** Prominent F-actin based structures in (**a**) a migrating fibroblast and (**b**) a neuronal growth cone. Red indicates F-actin-based structures, including filopodia and lamellipodia in both the fibroblast and the neuronal growth cone, and the stress fibres in the fibroblast.

Rho-like GTPase *mig-2* (*migration 2*) caused both outgrowth defects and occasional guidance defects[34]. Similarly, in *Drosophila*, expression of dominant-negative *Rac* caused motor axon guidance errors at specific choice points[35]. In addition, overexpression of dominant-negative *Rho* or wild-type *Cdc42* caused axon guidance defects in *Xenopus laevis* retinal ganglion neurons *in vivo*[29]. Genetic loss-of-function mutants in GEF (FIG. 1) for Rho GTPases or downstream effectors (see below) have been shown to result in axon guidance defects[36–41], further supporting the roles of Rho GTPases in axon guidance.

What is the relationship between axon growth and guidance, and how are they both affected by perturbation of Rho GTPases? Previous experiments have shown that cytochalasin treatment inhibits actin filament assembly, eliminates filopodia and results in defects in axon guidance. However, the extension of axons is affected to different extents, depending on the dose of cytochalasin and the adhesive nature of the substrates[42–44]. The effect of GTPase perturbation might be influenced by interactions between the growth cone and the substrate[27], and by the degree to which GTPases are perturbed[20,35]. Another outstanding issue is whether these GTPases have an instructive or permissive function in axon guidance. For instance, is Rac essential for the formation of lamellipodia, which are necessary structures for the growth cone to be guided (permissive), or does Rac interpret the guidance signal directly (instructive)? Understanding the signalling pathways of the Rho GTPases in the growth cone (see below) might help us to resolve these issues.

Dendrite development and plasticity. Dendrites are inherently difficult to study because of their morphological complexity. As a result, our general understanding of the molecular mechanisms that regulate dendrite growth and branching notably lags behind analogous studies on axon growth and guidance. However, technological advances in imaging and genetic manipulation have allowed the routine examination of the function of candidate genes in dendrite development in physiological settings[14,29,45–47] and even the development of forward-genetic screens for the discovery of novel genes that regulate dendrite development[48] (L. Luo, unpublished observations).

By expressing dominant-negative and constitutively active mutants, the functions of Rho, Rac and Cdc42 in dendrite development have been examined in Purkinje cells in transgenic mice[22], dissociated rat cortical neurons[49], *Xenopus* retinal ganglion cells in retinal explants[47] or *in vivo*[29], *Xenopus* tectal neurons *in vivo*[46], and pyramidal neurons in hippocampus slices[45]. The effects of expressing different mutant GTPases vary quantitatively and sometimes qualitatively in different studies, and this might be due to variations in expression of transgenes, to the assays used or to the developmental state, which might determine the basal level of GTPase activity. However, given the caveat that constitutively active and dominant-negative mutants sometimes result in similar, rather than opposite, phenotypes (see above), there is a general trend (similar to their regulation of axon outgrowth) that Rac and Cdc42 promote dendrite growth and dynamics, whereas Rho inhibits dendrite growth. The role of Rho was further supported by genetic analysis of RhoA using null mutations in mosaic *Drosophila* brains, in which *RhoA* mutant neurons overextended their dendrites[14].

Dendrite branches are highly dynamic and might contribute to the structural basis of neural plasticity. Indeed, several live imaging studies have shown that Rho GTPases might regulate the rate of dendrite extension and retraction, with Rac promoting and Rho inhibiting dendrite remodelling[46,47]. Another special feature of dendrites that might contribute to neural plasticity is dendritic spines, which are special protrusions that are the primary sites of excitatory synapses and might be the basic unit of synaptic integration[50]. Perturbation of Rac activity in cerebellar Purkinje cells or hippocampal pyramidal neurons resulted in a selective effect on the formation and maintenance of dendritic spines with minimal effect on dendrite growth and branching[22,45], indicating that regulation of Rac activity might be crucial for the morphological development and plasticity of dendritic spines.

Neuronal migration

Rho GTPases have been implicated in the regulation of cell migration in many cell types, from mammalian fibroblasts during wound healing[51] to somatic follicle cells during *Drosophila* oogenesis[52]. Neuronal migration involving a Rho GTPase was first reported for *C. elegans mig-2* (REF. 34), which encodes a novel subclass of Rho GTPase. In *mig-2* activating mutants, the migration of many cell types was arrested, including neurons, indicating that hyperactivation might have disrupted proper signalling. In *mig-2* null mutants, however, the migration of only a small subset of cells was arrested, indicating that its function may be compensated by other Rho GTPases[34].

Box 1 | Rho GTPases and human neurological diseases

Many human genetic diseases result from mutations in components of Rho signalling pathways. Two that primarily affect nervous system development and/or function are nonsyndromic X-linked mental retardation and William's syndrome.

Nonsyndromic X-linked mental retardation (MRX)
There are about 65 loci on the X chromosome that, when mutated in hemizygous males, cause mental retardation without other obvious symptoms. Recent advances in positional cloning have allowed some of them to be identified[99]. The first identified locus encodes Oligophrenin 1, which has a GTPase-activating protein (GAP) domain for Rho GTPases and a GTPase-activating activity for Rho (Ras homologue), Rac (Ras-related C3 botulinum toxin substrate) and Cdc42 (cell division cyle 42) *in vitro*[100]. Another identified locus encodes PAK3 (p21-activated kinase 3), a Rac/Cdc42 downstream effector[101]. A third MRX locus, *ARHGEF6*, encodes a guanine nucleotide exchange factor (GEF) for Rho GTPases[102]. So, both an increase (owing to *Oligophrenin 1* mutation) and a decrease (owing to *Pak3* or *ARHGEF6* mutation) in Rho GTPase signalling can lead to abnormal mental function.

William's syndrome
This is a contiguous gene deletion disorder that affects many developmental processes. Loss of one copy of the gene for the LIM-domain-containing protein kinase (LIM-kinase) is believed to be responsible for one particular symptom: impaired visuospatial constructive cognition (the ability to visualize an object or a picture as a set of parts and to construct a replica from those parts)[103]. LIM-kinase acts downstream of both Rac and Rho to phosphorylate the actin depolymerization factor cofilin.

Extensive neuronal migration is also integral to the development of the mammalian cortex. Neurons that are produced later must migrate past the earlier neurons to establish the well-characterized 'inside-out' pattern of the neocortex[16]. In mice with a mutant Cdk5 (cyclin-dependent protein kinase 5) and its essential subunit p35, the 'inside-out' migration pattern of cortical neurons is converted to an 'inside-in' pattern[53,54]. The p35 protein was recently identified as a downstream effector for Rac[55] (see below), further implicating the Rho GTPases, and Rac signalling in particular, in the regulation of neuronal migration.

Establishing neuronal polarity. Hippocampal pyramidal neurons in primary culture have long been a model system of the initial events leading to the establishment of neuronal polarity[17,18]. Shortly before axon formation, the actin cytoskeleton in the growth cone of future axons becomes selectively dynamic and unstable compared with growth cones of future dendrites[56]. Treatment of cultured hippocampal neurons with the Rho GTPase inhibitor toxin B could mimic the actin destabilization in growth cones to generate several axon-like processes[56]. However, because toxin B inactivates all the Rho GTPases, it is not clear whether one GTPase in particular regulates the dynamics and stability of the singular growth cone destined to differentiate into the axon. Some evidence indicates that Rac might be involved in establishing polarity, because perturbation of Rac preferentially affects the outgrowth of axons but not dendrites *in vivo*[20,22].

Synapse development. The formation of highly specialized presynaptic terminals and postsynaptic specializations is another example of the highly specialized morphology of neurons. One important step in the formation of the vertebrate neuromuscular synapse is the clustering of acetylcholine receptors in the muscle fibre, which is induced by neuronal expression of agrin (REF. 19). How does agrin control receptor clustering? Recent evidence shows that it induces the expression of Rac and Cdc42 in myotubes, and that Rac and Cdc42 are necessary and sufficient to cause acetylcholine-receptor clustering in response to agrin[57]. Other evidence pointing to the involvement of Rho GTPases in synapse development includes the concentration of RhoGEFs in pre- and postsynaptic terminals[37,58], and reduction of the postsynaptic density marker post-synaptic density 95 (PSD-95) in dominant-negative *Rac*-expressing hippocampal pyramidal neurons[45]. Although it has not yet been proved, it is likely that Rho GTPases regulate the formation of both pre- and postsynaptic specialized structures.

Rho GTPase signal transduction pathways

How do Rho GTPases regulate such a wide variety of neuronal developmental processes? Do these different processes share a common mechanism? A common theme in all of the above processes is the regulation of cytoskeletal reorganization in response to extracellular cues (as in neuronal migration, axon guidance or dendrite elaboration) or possible intrinsic cues (as in the establishment of neuronal polarity). Understanding the signalling pathways that regulate the activity of these Rho GTPases and the pathway by which they regulate the actin cytoskeleton would offer insights into the mechanisms shared by these different processes, as well as the unique conditions that set them apart.

Below, I discuss the known signalling mechanisms upstream and downstream of Rho GTPases, focusing on those already observed or likely to be used in the developing nervous system. Mutations in some of these signalling pathways have been reported in human neurological diseases (BOX 1). Although most of the examples discussed are drawn from studies of axon growth and guidance, it is already apparent that analogous mechanisms are often used in other developmental processes. For instance, it has recently been shown that axon guidance and cell migration share the same extracellular ligand[59], as does the guidance of axons and dendrites[60].

Linking upstream signals to Rho GTPases. The common view is that Rho GTPases are regulated by upstream signals through the regulation of GEFs and GAPs (FIG. 1). These enzymes far outnumber the Rho GTPases themselves in a given organism. For instance, there are six Rho GTPases in the *Drosophila* genome but there are around 20 RhoGAPs and 20 RhoGEFs, and there are eight predicted RhoGAPs on human chromosome 22 alone[61]. In addition to their GEF and GAP domains, RhoGEFs and RhoGAPs also have other signalling motifs, including SH2, SH3, PLEKSTRIN-HOMOLOGY, PDZ and phorbol-ester-binding domains, which probably allow the GEFs and GAPs to be regulated by various upstream signals.

The best-studied RhoGEF in the nervous system is the triple functional domain protein (TRIO). Originally identified in mammals as a binding partner of the

SH2 DOMAIN
(Src-homology region 2). Protein sequence of about 100 amino acids found in many proteins involved in signal transduction.

SH3 DOMAIN
(Src-homology region 3). Protein sequence of about 50 amino acids that recognizes and binds to sequences rich in proline.

PLECKSTRIN-HOMOLOGY DOMAIN
A sequences of about 100 amino acids present in many signalling molecules. Pleckstrin is a protein of unknown function originally identified in platelets. It is a principal substrate of protein kinase C.

PDZ DOMAIN
(Postsynaptic density-95, Discs-large, Zona occludens-1). Protein–protein interaction domain.

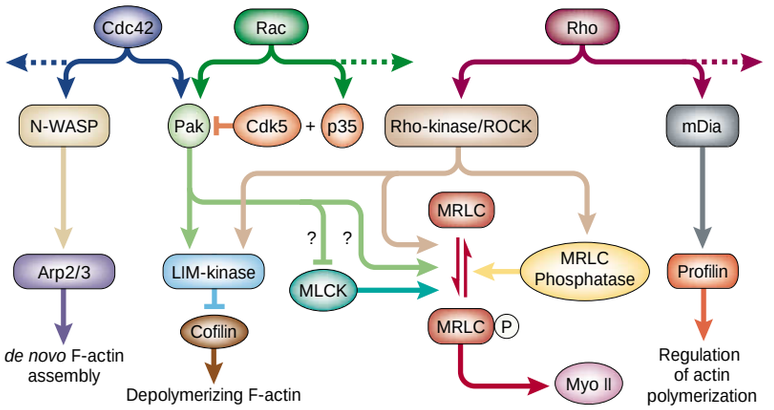

Figure 3 | **Signalling pathways from Rho GTPases to the actin cytoskeleton that are likely to be used in neurons.** Pointed arrows represent activation, flat arrows represent inhibition. Dashed arrows from Rho GTPases indicate that additional downstream effectors are implicated for each of the GTPases in regulating neuronal morphology. The question mark indicates that the direction of modulation of MRLC phosphorylation by Pak is still controversial. (Arp2/3, actin-related proteins 2 and 3; Cdc42, cell division cycle 42; Cdk5, cyclin-dependent protein kinase 5; LIM-kinase, LIM-domain-containing protein kinase; mDia, mammalian diaphanous; MLCK, myosin light chain kinase; MRLC, myosin II regulatory light chain; Myo II, myosin II; N-WASP, neuronal Wiskott–Aldrich syndrome protein; p35, the essential subunit of Cdk5; Pak, p21-activated protein kinase; Rac, Ras-related C3 botulinum toxin substrate; Rho, Ras homology gene family, member A; ROCK, Rho-associated, coiled-coil-containing protein kinase.)

receptor tyrosine phosphatase LAR[62], TRIO contains two GEF domains that probably act on Rac and Rho. Recent evidence indicates that the *Drosophila* TRIO homologue is genetically required for the growth and guidance of sensory, motor and central nervous system neurons[37–40]. The *C. elegans* homologue, uncoordinated-73 (UNC-73), is also required for cell migration and axon guidance[36]. Although the physical interaction with LAR might not be conserved in worms or flies[38], the amino-terminal SH3 domain and ankyrin repeat domains are conserved in mammals, flies and worms, and might allow TRIO to be linked to conserved axon guidance receptors. So far, the only RhoGAP studied extensively in the context of neuronal morphogenesis is n-chimaerin. This was shown to co-operate with Rac and Cdc42 to induce the formation of lamellipodia and filopodia in neuronal cell lines[63]. Given the number of GAPs encoded in the genome and the other signalling motifs that they possess, it is tantalizing to speculate that GAPs, like GEFs, might transduce signals from receptors to the Rho GTPases.

How are RhoGEFs, and possibly RhoGAPs, regulated by extracellular cues? There will be rapid progress in the context of axon guidance because many guidance cues and receptors are being identified, and more studies are focusing on the downstream signalling pathways of known axon guidance cues and their receptors. Indeed, Rho GTPases have already been suggested to mediate the effects of semaphorins (Rac)[31,33] or ephrins (Rho)[64] on axon guidance, and the effects of NMDA (*N*-methyl-D-aspartate) activity on dendrite elaboration (Rho)[46]. In the case of ephrin-A5, biochemical assays indicated that application of the ligand resulted in an increase in the concentration of Rho–GTP without changing the total level of Rho, showing that Rho is downstream of the ephrin receptor rather than acting in a parallel pathway[64].

Why are there so many GEFs and GAPs? The tissue distribution and subcellular location of different GEFs and GAPs might well be responsible for fine regulation of the Rho GTPases in different neurons at different developmental stages and in specific cellular compartments. It is also possible that different GEFs and GAPs in the same growth cone integrate signals from different receptors and converge on the regulation of Rho GTPases. These possibilities might, in turn, explain why Rho GTPases are so versatile, regulating seemingly different processes in neuronal development in different developmental contexts.

Although GEFs and GAPs are important regulators of GTPases, not all upstream signals must proceed through GEFs and GAPs. The low-affinity neurotrophin receptor p75 has been shown to bind directly to Rho and to activate its activity. This binding and activation is abolished when neurotrophin binds to the receptor, inactivating Rho and so permitting process outgrowth[32].

Linking Rho to the actin cytoskeleton. In their GTP-bound states, Rho GTPases bind to downstream effectors to elicit their biological functions (FIG. 3). At least half a dozen EFFECTORS have been identified for each of the three GTPases Rho, Rac and Cdc42. One important common effector for Rac and Cdc42 is the p21-activated kinase[65] (Pak), which might explain some of the common phenotypes seen when Rac or Cdc42 activity is perturbed. LOSS-OF-FUNCTION MUTANTS of *Drosophila* Pak resulted in photoreceptor axon guidance defects[40,41]. Pak is likely to be regulated by both Rac[40] and DOCK, which is an adaptor protein containing SH3 and SH2 domains[40,41,66].

Identified substrates for the Pak kinase include: first, Pak itself, which leads to its activation[65]; second, LIM-domain-containing protein kinase (LIM-kinase)[67], which can subsequently phosphorylate and inhibit cofilin, an actin depolymerization factor[68,69]; and last, myosin light chain kinase, which inhibits Pak kinase and causes decreased myosin-regulatory light chain (MRLC) phosphorylation[70] and a presumed decrease in myosin motor activity. However, other studies have suggested that Pak activation leads to increased MRLC phosphorylation[71,72], possibly through a direct phosphorylation of MRLC[72] (FIG. 3). Whether any of these Pak substrates are important in the context of neuronal morphogenesis remains to be determined. Overexpression of active (non-phosphorylated) cofilin results in increase of neurite outgrowth[73], which is consistent with Rac providing a positive signal for neurite outgrowth.

The essential cofactor for Cdk5, p35, is also a downstream effector for Rac[55]. The p35–Cdk5 complex is essential for cortical neuronal migration *in vivo*[53,54]. Perturbation of p35–Cdk5 activity also results in defects of neurite outgrowth in neuronal culture[74] and of axon patterning in *Drosophila* embryos[75]. In addition, expression of dominant-negative Cdk5 has also been reported to suppress an axonal initiation phenotype

EFFECTORS
Proteins that bind to Rho GTPases only when they are in an active GTP-bound state and are therefore likely to transduce signals downstream of the Rho GTPases.

LOSS-OF-FUNCTION MUTATION
Mutation that causes a decrease or the total loss of the activity of the encoded protein. Although genetic loss-of-function mutants provide the most rigorous test for the function of genes, mutations in Rho GTPases are likely to cause pleiotropic defects. Conditional knockouts in small populations of neurons might provide more useful information.

caused by expression of constitutively active *Rac*[29]. Biochemically, p35–Cdk5 can inhibit Pak activity[55]. So, at the same time as activating Pak, Rac might activate p35–Cdk5, which acts as a built-in timer to limit the duration of Pak activation.

Among the many Cdc42 effectors, neuronal Wiskott–Aldrich syndrome protein (N-WASP) has received a lot of recent attention because of its potential role in regulating the actin cytoskeleton. N-WASP is a ubiquitous protein but is enriched in the nervous system[76] and is essential for Cdc42-mediated filopodium formation in fibroblasts[77]. N-WASP binds to the actin-related proteins 2 and 3 complex (Arp2/3) and stimulates nucleation of actin polymerization[78]. Other effectors for Cdc42 include *Drosophila* Gek (Genghis Khan)[79] and its mammalian homologue MRCK (myotonic dystrophy kinase-related Cdc42-binding kinase)[80] — a multidomain serine/threonine kinase similar to myotonic dystrophy kinase and Rho-associated, coiled-coil-containing protein kinase (ROCK), the non-receptor tyrosine kinase Ack (activated cdc42Hs-associated kinase)[81] and calmodulin-binding protein IQGAP (IQ motif-containing GTPase-activating protein 1)[82]. The functions of these molecules in the nervous system have not been reported.

Of the half dozen or so Rho effectors, the best-studied is Rho-kinase/ROCK. ROCK is a serine/threonine kinase with a large coiled-coil domain and a few other signalling motifs[83–85]. ROCK mediates the effect of RhoA on neurite retraction in cultured neuroblastoma cells[86], on preventing axonal initiation in cultured cerebellar granule cells[87] and on dendrite retraction in hippocampal pyramidal neurons in cultured brain slices[45]. More than half a dozen substrates of ROCK have been identified so far. ROCK phosphorylates the regulatory light chain of myosin II and the regulatory subunit of myosin light chain phosphatase. Both lead to increased phosphorylation of a critical residue in the myosin light chain, which then promotes the ATPase and motor activity of myosin II (REFS 88–90). Phosphorylation of myosin light chain has been shown to accompany neurite retraction in neuroblastoma cells in response to Rho activation, suggesting the relevance of regulating myosin activity in neuronal morphogenesis[86]. Another RhoA effector that might function during neuronal morphogenesis is a formin-related protein, mammalian diaphanous (mDia)[91]. This cooperates with ROCK in regulating stress-fibre formation in fibroblasts[92] and also binds to the actin-binding protein profilin (REF. 91), which is genetically required for axon growth in *Drosophila*[93]. However, the neuronal function of mDia remains to be examined.

Studies investigating the downstream signalling pathways of Rho GTPases have been crucial in helping us to understand the varied and sometimes conflicting results seen when the activities of different Rho family members are perturbed. For example, ROCK can phosphorylate LIM-kinase and increase its kinase activity[94,95], as can Pak[67]. Therefore, activation of Rac or Rho could, in theory, lead to similar phenotypes. However, although Rho activation leads to increased myosin light chain phosphorylation (through ROCK), Rac activation might lead to a decrease in myosin light chain phosphorylation (through inhibition of myosin light chain kinase by Pak). This might explain the opposing effect of Rho and Rac observed on many occasions. The crosstalk between different signal-transduction pathways downstream of the Rho GTPases, coupled with shared upstream regulators such as GEFs and GAPs, add more complexity to the task of dissecting the signal-transduction pathways through which these GTPases regulate biological processes.

Rho GTPase signalling pathways do not act alone in transducing extracellular signals to the cytoskeleton in the neuronal growth cone. For instance, regulation of tyrosine phosphorylation has long been implicated in transducing signals from axon guidance receptors to the cytoskeleton[1,2]. Interestingly, several recent studies indicate a possible close relationship between tyrosine phosphorylation and Rho GTPase signalling. For example, the Rac effector Pak is also regulated by dreadlocks (DOCK)[41], which binds to the tyrosine-phosphorylated axon guidance receptor DSCAM (Down's syndrome cell adhesion molecule) through its SH2 domain[96]. In addition, strong genetic interactions have been reported between the Rac GEF TRIO and the receptor tyrosine phosphatase Dlar, the non-receptor tyrosine kinase Abelson (Abl) and their common target Enabled[38,39], all of which are required for axon guidance[97]. Investigating the mechanisms that bring together Rho GTPases and tyrosine-phosphorylation signalling holds the promise of a unified understanding of how extracellular signals regulate the actin cytoskeleton in neuronal growth cones.

Summary and future perspectives

Rho GTPases are important regulators of the actin cytoskeleton and are involved in many neuronal development processes that require morphological changes. Extracellular signals converge on GTPases through a large numbers of GEFs and GAPs. In turn, each of the GTPases regulates a diverse range of downstream pathways by activating distinct effectors. The position of these GTPases at the bottleneck of signal-transduction pathways might explain the myriad defects seen when these GTPases are misregulated. To help disentangle these functions, future experiments will use more specific tools to examine Rho GTPase functions in more physiological settings. For example, genetic loss-of-function mutants in these GTPases and in specific upstream regulators and downstream effectors can be generated in specific subpopulations of neurons, and are likely to result in more specific defects.

It will also be essential to extend the investigation of signal-transduction pathways from extracellular stimuli to the cytoskeleton elements that include each of the Rho GTPases, and to delineate more clearly the cross-talk between these pathways. These investigations will provide more insight into the question of how activation of these Rho GTPases in different contexts brings about the different steps of neuronal morphogenesis. Another interesting and important direction will be to develop imaging assays to follow the activities of Rho GTPases and their signalling partners in live cells, tissues and *in vivo*. Indeed, while this

manuscript was in review, the first imaging assay of Rac activity using fluorescence resonance energy transfer was reported[98]. With these assays in hand, more roles for Rho GTPases will surely be uncovered. Ideally, we will gain a comprehensive understanding of how these molecular switches work to modulate the seemingly diverse processes of neuronal development, plasticity and function.

Links

DATABASE LINKS Rho GTPases | RhoA | Rac1 | Cdc42 | GEFs | GAPs | Cdk5 | p35 | TRIO | LAR | n-chimaerin | ephrin-A5 | p75 | Pak | DOCK | LIM-kinase | cofilin | N-WASP | ROCK | Ack | IQGAP | DSCAM

FURTHER INFORMATION Luo lab homepage

ENCYCLOPEDIA OF LIFE SCIENCES Dendrites | Axon guidance

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Acknowledgements

I thank all members of my lab, in particular P. Billuart and A. Y. N. Goldstein, as well as many colleagues in the field, for their contributions to the idea and work described here. I also thank many lab members and J. Goldberg for helpful comments on the manuscript. Work in the lab was supported by grants from the NIH, the MDA, and the McKnight, Klingenstein and Sloan foundations.