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The Role of Small GTPases in Neuronal Morphogenesis and Polarity

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The highly dynamic remodeling and cross talk of the microtubule and actin cytoskeleton support neuronal morphogenesis. Small RhoGTPases family members have emerged as crucial regulators of cytoskeletal dynamics. In this review we will comprehensively analyze findings that support the participation of RhoA, Rac, Cdc42, and TC10 in different neuronal morphogenetic events ranging from migration to synaptic plasticity. We will specifically address the contribution of these GTPases to support neuronal polarity and axonal elongation. © 2012 Wiley Periodicals, Inc

Key Words: neuronal polarity, cytoskeleton dynamics, Rac1, Cdc42, RhoA, TC10

Introduction

The development and maintenance of the unique shape of most neuron types involves a complex series of events that start when spherical neuronal progenitors become asymmetric [Da Silva and Dotti, 2002]. They gradually adopt a polarized morphology by extending a single long axon and several much shorter dendrites that eventually connect with axons at synapses [Jan and Jan, 2003, 2010; Wiggin et al., 2005; Arimura and Kaibuchi, 2007; Barnes and Polleux, 2009; de la Torre-Ubieta and Boni, 2011]. There is considerable interest in learning about the mechanisms underlying neuronal shaping and polarization since they are central for understanding the complex connectivity and functioning of the nervous system, as well as for helping to solve many disorders involving nerve degeneration and failure to regenerate, as in spinal cord or brain injuries, cerebrovascular accidents, and neurodegenerative diseases [de la Torre-Ubieta and Boni, 2011]. This article focuses on the role of the small Rho GTPases family members RhoA, Rac, Cdc42 and Tc10 in regulating cytoskeletal organization and dynamics [Conde and Cáceres, 2009], as well as membrane addition [Pfenninger, 2009] during neuronal morphogenesis and polarization [Arimura and Kaibuchi, 2007; Tahirovic and Bradke, 2009; Hall and Lalli, 2010; de la Torre-Ubieta and Boni, 2011].

The initial and very well characterized observations on the functions of small Rho GTPases [Etienne-Manneville and Hall, 2002] established that they promote actin reorganization, with RhoA (Ras homology member A) regulating stress fiber formation and cell contraction [Ridley and Hall, 1992; Nobes and Hall, 1995], whilst Rac1 (Ras-related C3 Botulinum substrate 1) and Cdc42 (Cell division cycle 42) induce extensive protrusive activities that include the formation of lamellipodia and filopodia, respectively [Ridley et al., 1992; Hall et al., 1993a; Nobes and Hall, 1995; Hall, 1998; 2005]. More recent studies have revealed that small Rho GTPases in association with factors that control their expression, activity, lifespan, or subcellular localization, act as “spatiotemporal signaling modules” [Pertz, 2010] modulating microtubule organization, dynamics, plus-end capture and cross talk with actin-based structures, such as growth cone actin ribs [Paglini et al., 1998a), actin arcs [Zhang et al., 2003; Burnette et al., 2008], actin bundles and the subcortical cytoskeleton [Jaffe and Hall, 2005; Li and Gundersen, 2008; Lowery and Van Vactor, 2009].

The Rho Family of Small GTPases. Molecular and Structural Considerations

The Rho family of GTPases comprises a subgroup of the Ras superfamily of small (20–30 kDa) GTP-binding proteins. These proteins are ubiquitously expressed across many species, from yeast to human. The small GTPases act as *molecular switches*, cycling between an active GTP-

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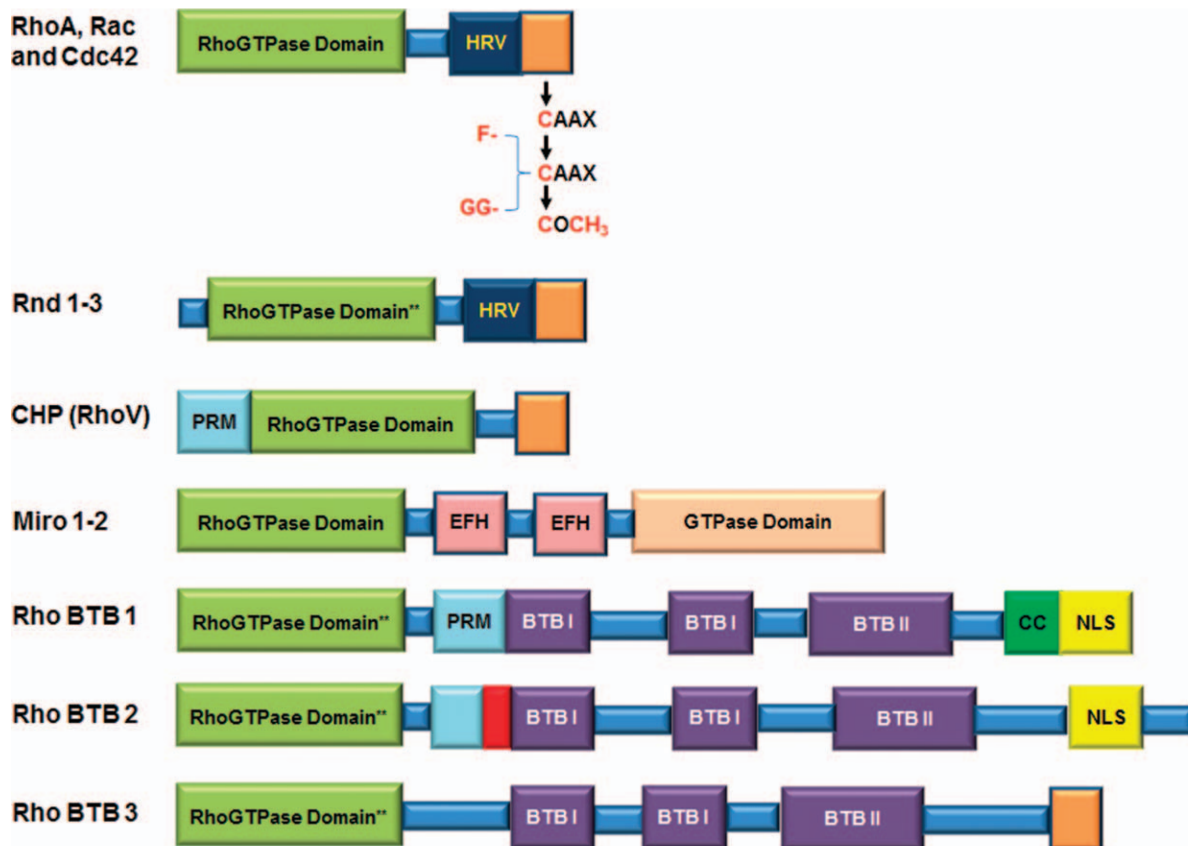

Fig. 1. Overall structure of canonical and atypical Rho GTPase domains and post translational modifications. Members of the RhoGTPase family share a functional GTPase domain (green boxes). However, not all of these domains are functional (** denotes deficient GTPase activity). The C-terminal region contains a hyper variable region (HVR, dark blue box) and adjacent to the HVR, a conserved CAAX region (orange box), which is post-translationally modified by the addition of farnesyl (F-) or genarylgenaryl (GG-) groups and subsequent methylation (CH3). The CAAX is isoprenylated in typical RhoGTPases (Rho, Rac and Cdc42) and members of the Rnd subfamily. CHP (RhoV) is not isoprenylated. CHP proteins display a proline rich motif (PRM, light blue box), which is also present in other atypical RhoGTPases (RhoBTB1 and RhoBTB2). Miro contains two EF-hand (EFH pink box) motifs and an additional GTPase domain located at the most C-terminal region of the protein (light orange box). RhoBTB proteins do not undergo any of the known post-translational modifications, and contain two broad complex/Tramtrack/Bric-a-brac domains (BTB). RhoBTB3 also lacks the C-terminal GTPase domain (light orange box). All RhoBTB family members differ in their C-terminal domain, where different domains critical for their functions, such as those involved in protein-protein interaction (CC, green box; PEST domain, red box) and nuclear localization signals (yellow box) can be found.

bound state and an inactive GDP-bound state [Etienne-Manneville and Hall, 2002; Pertz, 2010]. Rho GTPases are activated mainly through several cell-surface receptors via guanine-exchange factors, including cytokine, tyrosine kinase and adhesion receptors, as well as G-protein coupled receptors (GPCRs) [Kjoller and Hall, 1999; Schiller, 2006]. Rho GTPases interact with a myriad of effectors controlling many components of brain development and functioning such as neuronal migration [Hall, 2005], cell adhesion [Nobes and Hall, 1995] and morphogenesis [Hall and Nobes, 2000; Hall, 2005]. Thus, almost all aspects of neuronal polarization, one of the main issues of this review, are controlled by small Rho-GTPases [Luo et al., 1997; Luo, 2000; Ng et al., 2002; Jan and Jan, 2003, 2010; Ng and Luo, 2004; Govek et al., 2005; Arimura and Kaibuchi, 2007; de Curtis, 2008; Hall and Lalli, 2010].

To date, 22 mammalian members of the Rho GTPase family have been identified and subdivided into several groups on the basis of their amino acid composition. These members include Rho (A, B and C isoforms), Cdc42, Rac (1, 2 and 3 isoforms), Rnd (Rnd1, Rnd2 and Rnd3/RhoE), RhoD, Rif (RhoF), RhoG, TTF (RhoH), mitochondrial Rho/RhoT (Miro1 and Miro2), TC10/RhoQ, Chp/RhoV and atypical members such as RhoBTB.

The canonical structure of Rho GTPases consists of several motifs (Fig. 1). An effector domain becomes accessible to targets when GTP is loaded (active conformation). In contrast, when GDP is bound, the effector domain acquires an inactive conformation and is hidden for binding to downstream effectors. These changes in structure are restricted to two switch domains [Hakoshima et al., 2003]. In the C-terminal domain there is a Hyper Variable Region (HVR; Fig. 1) that differs not only between

the Rho GTPase subclasses but also within the same subclass in terms of the presence of either a polybasic region or a palmitoylation site [Ridley, 2006]. The polybasic region and palmitoylation site present in the HVR are involved in targeting of GTPases to plasma membrane or endomembrane compartments [Michaelson, 2001]. A C-terminal CAAX-box (C, cysteine; A, Aliphatic Amino acid; X, any amino acid) contains a cysteine residue, which is crucial for prenylation that adds a farnesyl or geranylgeranyl group, enhancing their interactions with membranes and very often defining their localization to specific membrane compartments. Then, the remaining aliphatic and variable amino acids undergo proteolysis and replacement by a methyl group [Adamson et al., 1992; Wennerberg and Der, 2004; Samuel and Hynds, 2010].

On the other hand, one striking feature of atypical Rho GTPases, like those in the RhoBTB group is a non-functional GTPase domain followed by a proline rich region, and a C-terminal Bric-a-brac, Tramtrack, and Broad-complex (BTB) domain, a module known to mediate protein-protein interactions. The RhoBTB subfamily consists of 3 isoforms: RhoBTB1, RhoBTB2, and RhoBTB3 (Fig. 1) [Ramos et al., 2002; Berthold et al., 2008]. Interestingly, RhoBTB3 behaves as an ATPase involved in Rab9-mediated late endosome to Golgi trafficking [Espinosa et al., 2009; Danglot and Galli, 2009].

The on/off cycling and target accessibility of Rho-GTPases are controlled by different groups of proteins. There are two primary classes of molecules that control the switching: 1) Guanine exchanging factors (GEFs), which catalyze the exchange of GDP for GTP [Hart et al., 1991; Schmidt and Hall, 2002]; and 2) GTPase-activating proteins (GAPs), which enhance the relatively slow intrinsic GTPase activity of Rho proteins. GEFs promote the activation of Rho-GTPase family members, whereas GAPs act as inhibitory factors. A third set of regulatory proteins are the guanine nucleotide-dissociation inhibitors (GDIs) that maintain a large pool of RhoGTPases in the cytosol in a GDP-bound state [Sasaki and Takai, 1998; Dovas and Couchman, 2005; Garcia Mata et al., 2011] (Fig. 2).

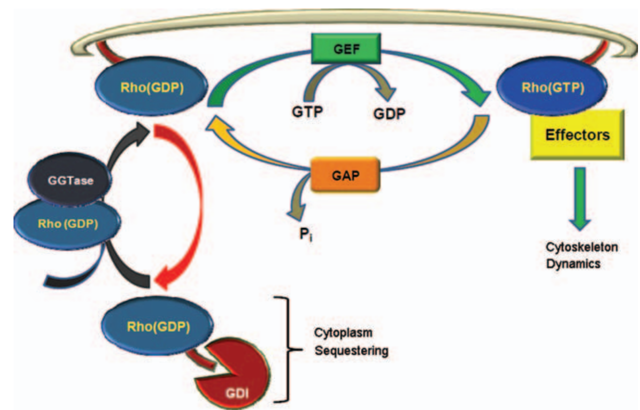

Fig. 2. Canonical RhoGTPases activation/inactivation mechanisms. RhoGTPases are firstly modulated by their destination to the plasma membrane which involves the activity of the genaryl-genaryl transferases (GGTase) shown in gray. The switch cycling between GDP- (inactive form) and GTP- (active form) bound states depends on the activity of GEF (green) and GAP (orange) proteins. The third general mechanism contributing to the regulation of RhoGTPases is shown in red, and is dependent on GDI proteins that recognize and bind the prenylated groups of RhoGTPase, inhibiting their translocation to the plasma membrane.

Regulators of Rho GTPases

Guanine Nucleotide Exchange Factor

The first mammalian GEF was originally identified as an oncogene from human diffuse B-lymphoma cells and designated as Dbl [Hart et al., 1991; Zheng et al., 1996; Zheng, 2001]. In humans, approximately 70 GEFs have been described; most of them contain a Dbl-homology (DH) domain and an adjacent Pleckstrin homology (PH) domain. In most cases, they provide the minimal structural unit required to catalyze the GDP-GTP exchange reaction [Chen et al., 1997; Baumeister et al., 2003]. A non-conventional Rho-GEF family, whose members lack DH domains, has also been identified [Meller and Merlot, 2005]. These proteins, designated as CZH (CDM and Zizimin homology) or 180 kDa protein downstream of CRK (DOCK180)-related proteins, contain two highly homologous regions [Dock homology regions (DHR1 and DHR2)] that mediate nucleotide exchange [Meller and Merlot, 2005].

DH domains interact extensively with the Switch regions of GTPases (Switch I, residues 28–44 and Switch II, residues 62–69 in human RhoA, respectively), which are domains for nucleotide binding [Hakoshima et al., 2003; Meller and Merlot, 2005]. The nucleotide exchange mechanism involves sequential steps in which the RhoGEF interacts with a rigid zone of the RhoGTPase and a portion of Switch II. This interaction (GTPase-GEF) promotes conformational changes of Switch I allowing dissociation of GDP and Mg^{2+} leaving the nucleotide binding pocket fully exposed for binding to GTP-Mg^{2+} [for details: Worthylake et al., 2000; Hakoshima et al., 2003; Rossman et al., 2005].

GTPase Activating Proteins

GAPs increase the rate of GTP hydrolysis by more than 100 times. The first GAP identified for Rho family GTPases was p50 RhoGAP [Garrett et al., 1989] and since then, more than 70 members have been characterized in eukaryotes. The human genome is predicted to encode between 59 and 70 proteins containing a RhoGAP domain, and to date more than half have been identified [Venter et al., 2001; Peck et al., 2002; Bernards, 2003]. The structure of p50 RhoGAP-RhoA has been well characterized [Rittinger et al., 1997a,b; Hakoshima et al., 2003].

Guanine Nucleotide Dissociation Inhibitors

There are only three well-defined RhoGDIs [Garcia Mata et al., 2011], including RhoGDI-1 (α), RhoGDI-2 (β) and RhoGDI-3 (γ). These proteins fold into an immunoglobulin-like β sandwich followed by a helix-turn-helix motif in the N-terminus that generates a hydrophobic pocket, which binds and encompasses the C-terminal regions of Rho GTPases containing isoprenylation of the conserved cysteine within the CAAX box. When associated with GTPases, the GDI-GTPase complex comprises a cytoplasmic pool of inactive prenylated proteins [Garcia Mata et al., 2011: for an excellent recent review on Rho-GDIs].

In the following sections, we will focus on specific GEFs, GAPs and GDIs for Rho, Rac and Cdc42, which have been implicated in neuronal morphogenesis.

The Rho Family (RhoA, RhoB, and RhoC)

This family is composed of three members, namely RhoA, B and C. RhoA and RhoC share a structural identity of 92% and 82% with RhoB, respectively, and all three members are post-translationally modified by prenylation at the C-terminal cysteine. Additionally, RhoA and RhoC can be geranylgeranylated [Wheeler and Ridley, 2004; Primeau and Lamarche-Vane, 2008] while RhoB can be geranylgeranylated, farnesylated and palmitoylated [Wherlock et al., 2004]. Differential post-translational modifications of Rho members are linked with differences in their subcellular localizations and functions [Robertson et al., 1995; Michaelson et al., 2001; Stamatakis et al., 2002; Perez-Sala et al., 2009; Samuel and Hynds, 2010]. While RhoA and RhoC are localized to the cytoplasm or the plasma membrane, RhoB is mainly localized to membranes and to an endo-lysosomal compartment for degradation [Perez-Sala et al., 2009].

Rho proteins are highly expressed in the nervous system, both in neuronal and glial cells [Suidan et al., 1997; Sepp and Auld, 2003]. All have been implicated in several aspects of neural morphogenesis, but RhoA has been the most investigated. Thus, a large body of evidence, mainly from studies in cultured cells, suggests that RhoA and one of its main downstream effectors, the Rho-associated coil-coiled containing-associated protein kinase [ROCK; Kiento and Ridley, 2003] act during a large part of the neuronal morphogenetic program, from neurogenesis and migration to dendritic formation and synaptic plasticity.

RhoA is expressed in the plasma membrane of neural crest progenitors and is down regulated upon delamination [Groysman et al., 2008]. More importantly, loss-of-function of RhoA or inhibition of overall Rho signaling with C3 transferase [Just et al., 2010] enhances premature delamination without affecting cell fate specification [Groysman et al., 2008]. In accordance with this, treatment of neural crest explants with Y27632, an inhibitor of ROCK, or cell permeable C3 toxin, accelerates migration without affecting proliferation [Groysman et al., 2008]. These treatments also reduce stress fiber formation, focal adhesions and membrane-bound N-cadherin. Conversely, activation of RhoA with LPA inhibits migration [Groysman et al., 2008]. A role for RhoA in regulating migration of cortical neurons has also been reported [Pacary et al., 2011]. These authors showed that two proneural transcription factors, Neurog2 and Ascl1, promote migration of newborn cortical neurons by inducing expression of the atypical Rho GTPases, Rnd2 and Rnd3, which in turn inhibit RhoA activity at different steps of the migratory process [Heng et al., 2008; Pacary et al., 2011; Lin and Anton, 2011].

Another set of studies showed that RhoA regulates apoptosis [Coleman et al., 2001; Coleman and Olson, 2002], proliferation and cell division in the developing cerebral cortex, rather than migration or layering. Programmed cell death plays a central role in shaping the organization of neuronal circuits and RhoA appears to be actively involved. Thus, a mouse line in which a dominant negative (DN) RhoA mutant (N19-RhoA) is expressed in all post-natal developing neurons led to no major changes in neocortical anatomy [Sanno et al., 2010]. However, the density and absolute number of neurons in the somatosensory cortex increased by 12–26% compared with wild-type littermates. This was not associated with a change in neuronal migration during formation of cortical layers, but rather with a large decrease in the amount of neuronal apoptosis at post-natal day 5, when a developmental peak of cortical apoptosis occurs. In addition, overexpression of RhoA in cortical neurons caused high levels of apoptosis. Moreover, inhibition of RhoA decreased the number of cleaved caspase-3-expressing neurons in the cortex, whereas overexpression of wild-type RhoA activated caspase-3, suggesting that RhoA acts upstream of this key executor of the apoptotic pathway [Sanno et al., 2010]. Proteins that promote RhoA activation such as GEF-H1 or Lfc (Lbc [lymphoid blast crisis]'s first cousin), have also been implicated in the generation of cortical neurons by regulating proliferative symmetrical versus neurogenic asymmetrical cell divisions [Gauthier-Fisher et al., 2009].

Neurite formation, a fundamental step for initiating migration and the extension of axon and dendrites, also involves RhoA. Both *in situ* and *in vitro* neuritogenesis begins with the breakage of the neuronal sphere, as budding neurites emerge from the cell body [Da Silva and Dotti, 2002; Da Silva et al., 2003]. These neurites extend progressively until one of them (the future axon) starts growing more rapidly; as neurites extend further and acquire their final axonal or dendritic identities, they establish functional synaptic contacts and consolidate polarization. Several lines of evidence suggest that RhoA is a negative regulator of neuritogenesis, including axon-dendrite formation [Da Silva et al., 2003; Conde et al., 2010].

Ectopic expression of constitutively active (CA) RhoA inhibits neurite sprouting in neuronal cell lines [Kozma et al., 1997] and minor process formation in cultured hippocampal pyramidal neurons [Threadgill et al., 1997; Chuang et al., 2005; Conde et al., 2010]. Likewise, factors that induce growth cone collapse and neurite retraction or arrest nerve regeneration, like LPA [Jalink et al., 1993, 1994] and Nogo [Fournier et al., 2000; Schwab, 2004; Kim et al., 2011; Schmandke and Strittmatter, 2007], or direct/stop axon guidance/advance, like netrin/slit [Murray et al., 2010] or Sema 3A [Aizawa et al., 2001] act through a RhoA-ROCK mediated signaling cascade. Disheveled (Dvl)-mediated activation of RhoA-ROCK is also involved in Wnt-3a-induced neurite retraction in rat PC-12 and mouse/N1E-115 cells [Kishida et al., 2004; Tsuji et al., 2010]. LIMK1 [Rosso et al., 2004], a kinase that phosphorylates and inactivates the actin depolymerizing factor (ADF) and cofilin [Bamburg, 1999; Bernstein and Bamburg, 2010], is phosphorylated and activated by ROCK, mediating several of the neurite retraction effects of RhoA [Aizawa et al., 2001; Ng and Luo, 2004].

Conversely, inactivation of RhoA/ROCK by pharmacological agents or ectopic expression of DN mutants enhances neurite formation. In hippocampal cultures, treatment of stage 2 neurons with toxin B [Bradke and Dotti, 1998], C3 toxin [Jalink et al., 1994; Da Silva et al., 2003; Arimura and Kaibuchi, 2007], Y27632 [Da Silva et al, 2003; Sanchez et al., 2008], or expression of DN RhoA [Chuang et al., 2005] or reduction of Rho A activity by suppression of Lfc [Conde et al., 2010] enhances axonal elongation and/or induces the formation of supernumerary axon-like neurites. Interestingly, neurons incubated with RhoA inhibitory C3 toxin lead to increased neuronal sprouting characterized by the presence of longer neurites. Moreover, these cells showed decreased actin filament polymerization [Da Silva et al., 2003], consistent with observations suggesting that increased actin dynamics is required for axon initiation [Bradke and Dotti, 1999; Kunda et al., 2001; Conde et al., 2010] and that down regulation of RhoA activity parallels the transformation of a minor neurite into an axon. Forster resonance energy transfer (FRET) experiments using a RhoA biosensor [Pertz, 2010] revealed high RhoA activity in growth cones of minor processes of stage 2 or 3 hippocampal neurons compared with axonal shafts or their growth cones (Fig. 3). Taken together, these observations argue for the existence of a RhoA-ROCK inhibitory tone that limits neurite sprouting, enhancing actin stability and preventing axon formation. LIMK1 and profilin IIa, as well as inhibition of a Rac-Cdc42 signaling pathway are likely to mediate the effects of RhoA-ROCK on actin dynamics, neuritogenesis and axon formation [Da Silva et al., 2003; Rosso et al., 2004; Conde and Caceres, 2009; Conde et al., 2010]. In other systems, the inhibitory role of RhoA on axon formation and polarization is less clear. For example, in rodent enteric neurons, Smurf, a SMAD-specific E-3 ubiquitin proteasome protein ligase 1 that causes RhoA degradation, promotes axon elongation without inducing multiple axon formation [Bhupinder et al., 2007].

RhoA has also been implicated in regulating the complex structure of dendritic arbors and their spines [Van Aelst and Cline, 2004; Jan and Jan, 2010]. Time-lapse imaging of developing dendrites within the brains of zebrafish or Xenopus tadpoles [Cline, 2001] has revealed that dendrogenesis follows a stereotyped sequence of events that involves the addition and retraction of many fine filopodial branches to the primary arbor; some branches are stabilized and become a substrate for further branching and extension. The iteration of this process (addition, stabilization and extension) leads to the generation of intricate dendritic trees [Van Aelst and Cline, 2004]. Expression of active forms of RhoA in Xenopus tectal and retinal ganglion cells [Ruchhoeft et al., 1999; Wong et al., 2000; Li et al., 2000], or "Drosophila" mushroom body (MB) neurons [Lee et al., 2000] or cultured hippocampal neurons [Nakayama et al., 2000] restrain dendrite growth. Conversely, suppression or inactivation of RhoA in MB neurons or Xenopus tectal neurons increased dendritic length [Li et al., 2000; Lee et al., 2000]. However, in other model systems, such as cultured hippocampal neurons, where overexpression of RhoA inhibits dendrogenesis, no effects were observed after ectopic expression of a DN mutant [Nakayama et al., 2000]. To explain this apparent discrepancy, it was proposed that inhibition of RhoA is a physiological phenomenon to allow dendrite growth; thus, no further inhibition can be achieved by expressing a DN mutant [Van Aelst and Cline, 2004]. Interestingly, Cux4, a transcription factor that limits dendritic growth in cortical pyramidal neurons acts through activation of RhoA [Li et al., 2010].

Dendritic growth is highly sensitive to afferent activity. Live imaging of dendritic arbors of tectal neurons expressing GFP fusion mutants of RhoGTPases, in the presence or absence of visual stimulation, revealed that branch addition and stabilization are Rac-mediated, whereas extension involves inhibition of RhoA activity [for further details: Sin et al., 2002; Niell et al., 2004; Van Aelst and Cline, 2004].

Dendritic spines are highly dynamic actin-rich protrusions that undergo remodeling triggered by neurotransmitters and neurotrophins. Not surprisingly, RhoA has emerged as an important regulator of spine formation and synaptic plasticity. Expression of a CA RhoA decreases spine length in slices of CA1 hippocampal pyramidal neurons; interestingly, this phenotype was mimicked by down regulation of oligophrenin-1 [Govek et al., 2004]. It was also shown that the effect of oligophrenin-1 on spine structure involves the RhoA-ROCK pathway [Van Aelst and Cline, 2004]. Furthermore, mutations in oligophrenin-1 are linked with mental retardation [Bienvenu et al.,

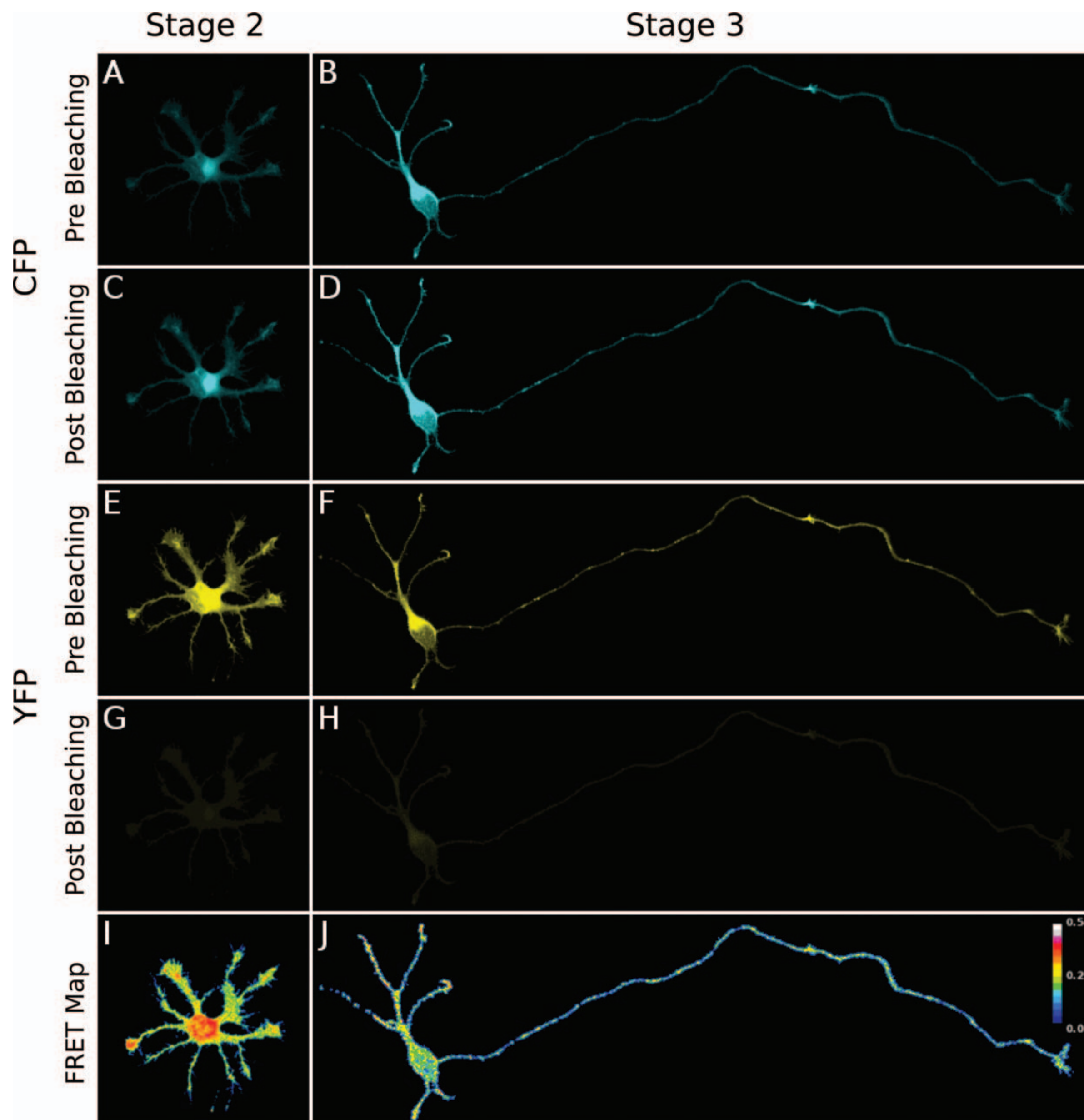

Fig. 3. Analysis of RhoA activity in cultured hippocampal pyramidal neurons by FRET using an Acceptor Photobleaching procedure. FRET map images reflecting Rho-A activity in developing neurons were calculated as described by Icaruso et al. [2011]. Briefly, prebleaching CFP (**A**, **B**) and YFP (**E**, **F**) images were sequentially acquired exciting the sample with 515 and 458 nm argon lasers, respectively. Photobleaching of the acceptor (YFP) was accomplished by repeatedly scanning the sample with the 458 nm laser at 100% power until YFP fluorescence was reduced at least to 80% of its initial value. Post-bleached CFP (**C**, **D**) and YFP (**G**, **H**) images were acquired with the exact same configuration used to acquire the prebleaching pair of images. FRET map images (Fig. **I**, **J**) were calculated using Image J software according to the following formula: FRET map = 1 – (prebleaching CFP / post bleaching CFP); Before FRET map calculation, images were background subtracted and carefully checked for correct alignment between pre and post images. Finally, a binary mask was created using the prebleaching YFP image to exclude non- neuronal signal from analysis. Note high RhoA FRET in minor neurites from stage II and stage III, compared with the axon shaft and growth cone. Transient transfection of a wild type Rho-A probe plasmid (Addgene cat #12150) was performed mixing 2.2 μg of plasmid with Lipofectamine 2000; the mixture was added into neurons 6 hr or 24 hr after plating. Cultures were fixed with 4% paraformaldehyde in 4% sucrose-containing PBS 16 hr after transfection, mounted and processed for FRET map calculation as described. Cells were visualized using either an Olympus FV300 or FV1000 confocal microscopes using a 60 × m 1.4 NA immersion objective.

1997; Bergmann et al., 2003]. Other studies have demonstrated that increases in spine density and length induced by NMDA receptor activity [Shepherd and Huganir, 2006] are negatively regulated by an Lfc-RhoA signaling cascade [Ryan et al., 2005; Kang et al., 2009]. The function of RhoA has also been tested in behavioral studies

where ROCK inhibition in the amygdala of intact animals blocks fear conditioning [Lamprecht et al., 2002].

Current evidence also suggests that RhoB has a role in regulating spine morphogenesis and synaptic plasticity [McNair et al., 2010]. For example, RhoB, but not RhoA or Rac, is specifically activated during LTP [O'Kane et al., 2003]. More recent studies have used RhoB-/- mice to show that the early phase of LTP is significantly reduced in the KO animals, whereas the later phase is unaffected [McNair et al., 2010]. In addition, the RhoB-/- animals have a decrease in phosphorylated LIMK1, increased dendritic branching, and fewer, but larger spines. The formin mDia2 interacts with RhoB in endosomes, and when down regulated with RNAi produces a spine phenotype similar to that of RhoB −/− neurons [Wallar et al., 2007; Hotulainen et al., 2009]. Membrane recycling is required for spine growth and maintenance [Park et al., 2006], so RhoB-mDia could be part of a signaling pathway controlling membrane trafficking from recycling endosomes to dendritic spine membranes during synaptic plasticity. Another unique aspect of RhoB is that it interacts with MAP1A [Lajoie-Mazenc et al., 2008], a microtubule-associated protein involved in activity-driven dendritic remodeling [Szebenyi et al., 2005]. In this regard, it is worth noting that MAP1B interacts with the Rac GEFs, Tiam1/Tiam2, and regulates spine formation by a mechanism involving increased RhoA activity [Tortosa et al., 2011]. These interactions are of great interest since they may serve to link dendritic spine microtubules [Hoogenraad and Bradke, 2009] with the actin cytoskeleton.

Rho Regulators and Effectors

Regulatory mechanisms are different for the three members of the Rho family (Fig. 4). For example, RhoA, B and C share high similarity except in a defined region around Switch 1, suggesting that they have different affinities for regulators or effector proteins [Wheeler and Ridley, 2004]. However, most work has focused on the modulation of RhoA activity. RhoGDIs inactivate Rho by inhibiting the dissociation of the GDP nucleotide [Adra et al., 1997; Dovas and Couchman, 2005] after removal from the plasma membrane [Dransart et al., 2005; Garcia Mata et al., 2011].

Current evidence suggests that RhoGDIs may have important neuronal functions. For example, Rho inhibition promotes axon growth on inhibitory substrates and regeneration in the injured CNS. Thus, several studies have shown that Nogo, MAG and other myelin-derived axon growth inhibitors activate RhoA [Pearse, 2004]. Interestingly, this activation involves sequestering RhoGDI from the membrane by $p75^{NTR}$; thus, inhibitors of RhoA are potential therapeutic targets for promoting recovery after spinal cord injury [Yamashita and Tohyama, 2003; Zurn and Bartlow, 2006]. Down regulation of RhoGDI leads to differentiation of the neural stem cell line C17.2, whereas suppression of RhoGDI was associated with decreased expression of RhoA, Cdc42, LIMK1 and WASP [Lu et al., 2008]. These intriguing results highlight the importance of further exploring the role of GDIs in neuronal development.

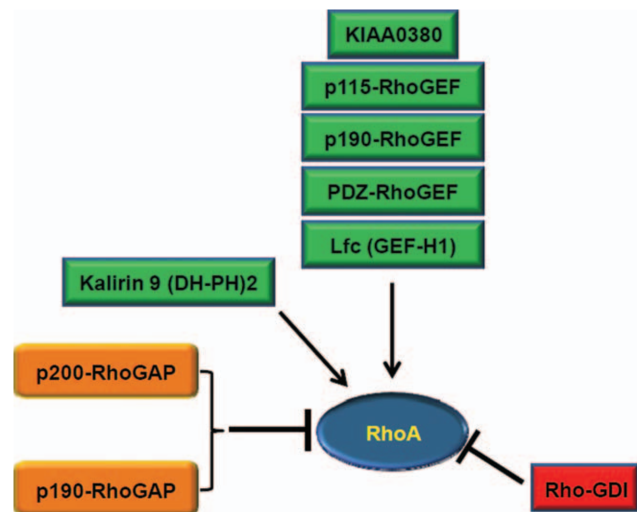

Fig. 4. RhoA regulators involved in the development of the axon. The participation of RhoA in the development of neuronal polarity is tightly regulated by several proteins acting as positive or negative regulators of RhoA activity. The figure shows the GEF protein that stimulate RhoA activity (in green), and the GAP proteins that lead to inactive RhoA (in orange). The role of Rho-GDI is also indicated (in red).

Phosphorylation can regulate the affinities between Rho and Rho-GDIs. A recent study has shown that cAMP induces phosphorylation of RhoA and increases binding with Rho-GDI. Moreover, a mutant RhoA (e.g., RhoA S188D) that mimics PKA-mediated phosphorylation induces greater neurite outgrowth than RhoA (S188A) mimicking the dephosphorylated form. Taken together these results suggest that inactivation of RhoA by PKA phosphorylation can participate in neuritogenesis affecting RhoA and the Rho-RhoGDI complex [Jeon et al., 2011]. Interestingly, PKA also phosphorylates and inactivates the Rho-GEF, Lfc [Meiri et al., 2009], which is implicated in axon formation [Conde et al., 2010]. Rho can also be modulated by heterotrimeric G proteins [Kranenburg et al., 1999; Sachdev et al., 2007].

The interaction of Rho-GEFs with microtubules is crucial to regulate cross talk with actin filaments and the plasma membrane. For example, the p190RhoGEF interacts with microtubules through its C-terminal domain adjacent to the DHPH domain. p190RhoGEF localizes and binds RhoA in the plasma membrane and when over expressed, inhibits neurite outgrowth [van Horck et al., 2001]. Another specific RhoA-GEF that binds to microtubules is the brain enriched PDZRhoGEF that is activated by Gα12/13 [Kuner et al., 2002] and interacts with LC2, a light chain of MAP1A or B. It has been proposed that this interaction is essential in the

maintenance of neuronal polarity [Longhurst et al., 2006]. Of note is that ubiquitin-mediated degradation of PDZRho-GEF in cultured hippocampal neurons promotes neurite outgrowth [Lin et al., 2011].

Another striking example of a microtubule-regulated Rho-GEF is GEF-H1, the human homolog of Lfc, which is activated upon microtubule depolymerization [Birkenfeld et al., 2008]. In primary cultured neurons, suppression of Lfc induces formation of multiple axon-like neurites and decreases RhoA activity; conversely, ectopic expression of Lfc prevents axon formation, inducing retraction of minor neurites [Conde et al., 2010]. Tctex-1, a dynein light chain implicated in axon outgrowth by modulating actin dynamics and Rac activity [Chuang et al., 2005], colocalizes and physically interacts with Lfc, inhibiting its GEF activity, decreasing Rho-GTP levels, and antagonizing Lfc during neurite formation [Conde et al., 2010].

Kalirin is a brain-specific GEF for RhoA, Rac and RhoG. Alternative splicing generates several Kalirin isoforms; one of them, Kalirin-7 is the most abundant in brain and is enriched in the post-synaptic density where it regulates spine morphology and synaptic plasticity. In fact, Kalirin-7 KO mice showed reduced dendritic branching, fewer spines, alterations in cortical layering, and cognitive deficits [Xie et al., 2007, 2008, 2010]. A Rac specific domain (e.g. GEF1 domain) is present in all isoforms, while a second domain (e.g. GEF2) specific for RhoA is present in Kalirin-9 and Kalirin-12 [Penzes et al., 2001]. Ectopic expression of the Kalirin GEF2 domain (DHPH2) enhances axonal elongation [Penzes et al., 2001]. In sympathetic neurons, ectopic expression of Kalirin induces prolific sprouting of new axonal fibers, a phenomenon dependent on the Kalirin GEF1 domain and mimicked by expression of CA RhoG [May et al., 2002].

A brain-specific GAP for RhoA and Rac, p200RhoGAP, was found in differentiated N1E-115 cells associated with the cortical actin cytoskeleton. It binds to SH3 domains, and is tyrosine-phosphorylated upon association with activated Src in cells. Transient expression of the RhoGAP domain or of the full-length molecule promotes N1E-115 differentiation [Moon et al., 2003]. p190 RhoGAP is a GAP with high specificity for RhoA, rather than Rac or Cdc42 [Kaibuchi et al., 1999]. Although not directly linked with neuronal polarization, some studies have shown its involvement in Nerve Growth Factor-(NGF) or Basic Fibroblast Growth Factor- (bFGF) induced PC12 differentiation and neurite extension, through a mechanism involving RhoA inactivation [Jeon et al., 2010, 2011].

Molecules that act downstream of Rho can be classified into three groups. Class I includes protein kinase N (PKN), rhophilin, and rhotekin; class II includes ROCK-I and ROCK-II9 [Kiento and Ridley, 2003]; and class III includes Citron [Fujisawa et al., 1998]. Class I proteins contain a Rho binding region of 70 amino acids near their N-terminus, known as the HR1 domain. In contrast, class II proteins contain the Rho-binding region towards the C-terminus. Finally, Citron contains a Rho-binding region, which is also localized at the C-terminus, but is unrelated to the Class II or the HR1 sequences [Wheeler and Ridley, 2004]. The Switch 1 region of Rho contains the sequence VFSKD, which is very important for binding to all classes of Rho effectors. This sequence is preserved in RhoA, B, and C and therefore most effector proteins could bind all three Rho family members [Fujisawa et al., 1998; Wheeler and Ridley, 2004].

RhoA, B and C activate ROCK, which in turn can phosphorylate several actin cytoskeleton regulatory proteins, such as myosin light chain (MLC) phosphatase and LIMK. The activation of LIMK by ROCK can then be associated with changes in the phosphorylation levels of ADF/cofilin. Regulation of LIMK1 and cofilin phosphorylation contributes to axon development [Rosso et al., 2004; Garvalov et al., 2007].

Other important downstream effectors of Rho family members are formins, a family of proteins which are essential for nucleation and polarization of straight, unbranched actin filaments [Pruyne et al., 2002]. To date, the most studied members of this family are the mammalian Diaphanous Formin (mDia) 1 and 2. Both formins possess a microtubule binding ability that is independent of their actin polymerization activity, and serves to coordinate actin cytoskeleton rearrangements to modulate microtubule dynamics in migrating cells [Spiering and Hodgson, 2011]. For example, the association of mDia1 with the microtubule plus end capping protein complex EB1/APC, is required to stabilize dynamic microtubule tips during cell migration [Palazzo et al., 2001; Gundersen, 2002; Wen et al., 2004]. mDia also interacts with Ena/VASP proteins that have been implicated in many processes requiring actin remodeling downstream of Rho, including neuronal polarization [Grosse et al., 2003; Shakir et al., 2006; Fleming et al. 2010].

The Rac Family (Rac1, Rac2, Rac3)

Rac family members have also been implicated in several aspects of neuronal morphogenesis, ranging from proliferation and migration to synaptic plasticity. There are three *Rac* genes in vertebrates encoding the Rac1, Rac2, and Rac3, proteins that share 88–92% sequence identity [Corbetta et al., 2005, 2009]. While Rac1 is ubiquitously expressed, Rac3 is neuron-specific and developmentally regulated [Malosio et al., 1997; Albertinazzi et al., 1998; Bolis et al., 2003]; by contrast, Rac2 is expressed in hematopoietic cells [Didsbury et al., 1989]. The Rac isoforms exhibit their highest amino acid divergence at their C-terminal region [van Hennik et al. 2003; Haataja et al. 1997]. It has been described that Rac1 and Rac3 are also differentially expressed in brain regions, such as the cerebellum [Bolis et al., 2003]. Besides, while Rac3 localizes

to the plasma membrane independently of its activation state, inactive Rac1 mainly localizes to the cytoplasm, and is translocated to the membrane after activation [Wennerberg and Der, 2004].

Most studies related with the functioning of Rac isoforms in vertebrate brain tissue have focused on Rac1; less effort has been devoted to Rac3 despite its neuronal-specificity, co-expression with Rac1 and developmentally regulated expression pattern [Hajdo-Milasinovic, 2007, 2009]. A genetic approach has now established that Rac1 and Rac3 are both critical for brain development, shedding light on previous discrepancies [Corbetta et al., 2009] (see below).

Pioneering work in flies showed the importance of *Drosophila* Rac (DRac) for axon elongation [Luo et al., 1994], and subsequent studies in mammalian brain demonstrated that it also participates in many other aspects of neuronal morphogenesis. For example, a recent study suggests that Rac1 regulates survival of neuronal progenitors [Leone et al., 2010]. It is likely that Rac1 acts in concert with Cdc42 to regulate corticogenesis serving distinct but complementary functions: Cdc42 is required for the maintenance of polarity and normal proliferation in radial glial cells of the ventricular zone (VZ) [Capello et al., 2006], whereas Rac1 regulates survival of cortical progenitors, including those located in the subventricular zone (SVZ) [Leone et al., 2010].

Rac1 has also been implicated in neuronal migration. Initial studies using *in utero* electroporation of DN Rac1 or the GEFs, Tiam1/Tiam2, into VZ progenitors produced an almost complete inhibition of radial migration of cortical neurons [Kawauchi et al., 2003]. By contrast, conditional deletion of the Rac1 gene using Foxg1-Cre mice produced mild defects in cortical radial migration [Chen et al., 2007]. These authors showed that the inside-to-outside sequence of neurogenesis and migration is preserved in the Rac1-deficient cerebrum; however, tangential migration of ventral telencephalic neurons was considerably compromised. The different severity of migration defects between these two approaches was interpreted as indicative that overexpression of DN mutants likely altered the activity of other RhoGTPases.

Rac1 is present in growth cones where it regulates adhesion and navigational activities [Lowery and van Vactor, 2009]. Not surprisingly, the participation of Rac1 in neurite outgrowth, axon formation, elongation, branching, guidance and pathfinding has been under intense investigation [Hall and Lalli, 2010]. The study of Rac1 functions in nerve cells with DN or CA mutants is a clear example of how results derived from these approaches should be interpreted with caution [Kuhn et al., 1998]. The initial studies of Luo's laboratory established that DN DRac1 prevents axonal outgrowth; it was later shown that this phenotype was the consequence of a progressive inactivation of DRac1, DRac2 and the related protein Mtl. This phenomenon leads to dose-dependent cumulative phenotypes involving firstly defects in axonal branching, and then in guidance, and finally in outgrowth [Luo et al., 1994; Luo, 2000; Ng et al., 2002; Chen et al., 2007]. In agreement with this, loss of function mutations in Drac1, DRac2, and Mtl produced abnormalities in axonal growth and guidance; it was also demonstrated that the GEF Trio is essential for DRac functioning in axon growth and guidance [Hakeda-Susuki et al., 2002].

A role for Rac1 in neuritogenesis and axon outgrowth has also been demonstrated in mammalian neuronal cell lines or primary cultures. Thus, PC12 cell differentiation requires Rac1 activation, by a mechanism involving RhoA translocation from the plasma membrane to the cytoplasm and inactivation [Nusser et al., 2002]. Activation of Rac1 by ectopic expression of Tiam1 promotes growth cone lamellipodial expansion and neurite sprouting in neuroblastoma cells [van Leeuwen et al., 1997]. A similar mechanism may operate in cultured hippocampal pyramidal neurons; ectopic expression of Rac1 or Tiam1/2 enhances axonal elongation and also induces the extension of multiple Tau-1 + axon-like neurites [Kunda et al., 2001; Nishimura et al., 2005; Chuang et al., 2005]. Suppression of Tiam1 also prevents expansion of the growth cone lamellipodial veil and the increase in actin dynamics that marks the transformation of a minor process into an axon [Bradke and Dotti, 1999; Kunda et al., 2001]. Interestingly, Tiam1 interacts with MAP1B [Montenegro et al., 2010] in axonal growth cones and may serve to link microtubules with Rho-GTPase signaling and the actin cytoskeleton. Tctex-1, a dynein light chain also stimulates axonal outgrowth and elongation, by a Rac-dependent mechanism that involves inhibition of RhoA [Chuang et al., 2005; Conde et al., 2010].

Three studies have used a conditional-gene targeting approach to study the function of Rac1 in mammalian neurons. The first study used Foxg1-Cre mice to suppress Rac1 in the VZ of telencephalon and Dlx5/6-CRE-EGFP mice to eliminate Rac1 from the SVZ of the ventral telencephalon [Chen et al., 2007]. The results obtained showed that deletion of Rac1 in VZ progenitors did not prevent axonal outgrowth of telencephalic neurons. However, several major axonal tracts display desfasiculation or projection defects. These results were interpreted as indicative that Rac1 controls axon guidance rather than neuritogenesis [Chen et al., 2007]. Functional redundancy is one possibility for explaining why the absence of Rac1 does not affect axon outgrowth in the KO animals, and one likely candidate is Rac3. Thus, conditional deletion of Rac1 in neurons combined with KO of Rac3 impairs development of dentate granule cells altering mossy fiber formation, and therefore hippocampal circuitry; besides, lack of both genes causes behavioral and motor defects, as well as premature death of mice. This fundamental study provides a set of evidence showing that Rac1 and Rac3 act

synergistically, with complementary functions [Corbetta et al., 2009]. This study also showed that single *rac1* or *rac3* deletions produce minor phenotypes. One possible explanation for these mild phenotypes is that the GTPase RhoG [Katoh and Neguishi, 2003] shares a high amino acid sequence homology (72%) with Rac1 and is also involved in neurite outgrowth, at least in PC12 cells [Katoh et al., 2000]; this protein may partially compensate for Rac1 and/or Rac3 functions in the nervous system. Other explanations are possible.

A third conditional KO deleting Rac1 in the whole brain has also been generated [Tahirovic et al., 2010]. These authors analyzed cerebellar granule cells, which are devoid of other Rac isoforms, and found that deletion of the *rac1* gene impairs migration and axon formation. This study also showed absence of Wiscott-Aldrich syndrome protein (WASP) family velprolin homologous protein (WAVE) from the plasma membrane of Rac -/- granule cells; interestingly, the axon growth defect of these neurons could be partially rescued by expression of a WAVE mutant that targets to the plasma membrane. The results of this study suggest that regulation of axon growth by Rac1 isoforms may be neuron-type specific [Tahirovic et al. 2010].

The study by Corbetta et al. [2009] also showed that spine development is strongly hampered in hippocampal neuronal cultures derived from the $Rac1^{-/-}/Rac3^{-/-}$ mice [Corbetta et al., 2009]. These alterations may underlie the epileptic phenotype found in the Rac1/Rac3 double KO animals [Corbetta et al., 2009].

Rac Regulators and Effectors

One of the most important GEFs in the nervous system is the T-lymphoma invasion and metastasis 1 factor (Tiam1) [Habets et al., 1994; Mertens et al., 2003]. Translocation of Tiam1-family members to the plasma membrane is crucial for inducing Rac-mediated membrane ruffles and activation of c-Jun N-terminal kinase (JNK) [Michiels et al., 1997; Stam et al., 1997]. Pioneering work from Collard's laboratory with N1E-115 cells showed that Tiam1 induces cell spreading, expansion of the growth cone lamellipodial veil and increases neurite sprouting [van Leeuwen et al., 1997]. Tiam1 has a closely related member known as Tiam2 or STEF that also participates in NIE-115 differentiation [Matsuo et al., 2003]. Tiam1 colocalizes with actin filaments and a subset of tyrosinated microtubules present in axonal growth cones [Kunda et al., 2001]. MAP1B regulates microtubule tyrosination by interacting with the enzyme tubulin tyrosine ligase (TTL) [Utreras et al., 2008]. Tiam1/STEF suppression inhibits axon growth, while overexpression induces multiple axon formation [Kunda et al., 2001; Nishimura et al., 2005], a phenomenon that involves interaction with Par3 [Nishimura et al., 2005]. Recent observations have shown that Tiam1 interacts with MAP1B favoring its localization at the distal axonal end [Tortosa et al., 2010]. In non-neuronal cells, Tiam1-dependent Rac1 activation requires the participation of the Arp2/3 complex [Ten Klooster et al., 2006]. Although the significance of this interaction has not been explored in neurons, it is plausible that Tiam1-promoted axonal elongation could require the participation of the Arp2/3 complex.

Current evidence suggests that Tiam1/STEF activity could be down regulated by a signaling pathway involving RhoA-ROCK [Matsuo et al., 2003]. ROCK phosphorylates STEF at Thr1662 diminishing its ability to activate Rac, presumably by altering its interaction with MAP1B or other molecules [Takefuji et al., 2007]. For example, Par-3 interacts directly with Tiam1/STEF, which is then assembled into the polarity complex aPKC-PAR-6-Cdc42-GTP to drive Rac activation. It has been reported that RhoA-ROCK phosphorylates Par3, disrupting its interaction with Par6-aPKC and suppressing Par3-mediated Rac activation [Nakayama et al., 2005].

Trio is another GEF for Rac, which is highly expressed in the brain [Debant et al., 1996]. A special feature of Trio is the presence of two different DHPH domains. The first domain is involved in both Rac and RhoG activation, whereas the second stimulates RhoA [Bellanger et al., 1998]. Trio appears to be a key component of the intracellular signaling pathway that regulates axonal guidance and cell migration in the nervous system [Liebl et al., 2000], a process which appears to involve regulation of Filamin, an actin binding protein [Fox et al., 1998]. A role for Trio in neuronal morphogenesis is further supported by other observations. Solo/Trio8, a short membrane-associated isoform of Trio selectively expressed in Purkinje cells, regulates neurite morphology in primary cultured neurons by activating endosome recycling [Sun et al., 2006]. Other Trio isoforms containing a GEFD1 domain also modulate neurite growth [Portales-Casamar et al., 2006].

Kalirin family members are highly homologous to Trio. The DHPH1 domain of Kalirin is similar to the first DHPH domain of Trio, which activates Rac1 [Debant et al., 1996], whereas Kalirin DHPH2 is most similar to the second DHPH domain of Trio, and therefore it can also activate RhoA. A functional analysis of Kalirin DHPH domains in primary cultured cortical neurons showed that DHPH1 reduced axonal length [Penzes et al., 2001]. Interestingly, this GEF domain binds and activates more strongly RhoG than Rac1. Kalirin-9 and Kalirin-12 also promote axonal elongation in cultured rat superior cervical ganglion neurons, presumably by activating RhoG [May et al., 2002].

Vav family members (Vav1, Vav2, and Vav3) are prototypical Dbl GEFs for Rac and Cdc42 [Bustelo, 2000]. Vav1 is exclusively expressed in hematopoietic cells, while Vav2 and Vav3 are ubiquitously expressed [Katzav et al.,

1989; Schuebel et al., 1996; Movilla and Bustelo, 1999]. Tyrosine phosphorylation of Vavs [Lopez-Lago et al., 2000] abrogates N-terminal auto inhibition leading to an open configuration with an exposed DH domain that triggers its GEF activity [Aghazadeh et al., 2000; Yu et al., 2010]. Expression of the three *vav* genes has been identified in some areas of the CNS [Movilla and Bustelo, 1999; Betz and Sandhof, 2003] and Vav proteins are implicated in axonal targeting/guidance in flies and mice [Cowan et al., 2005; Malartre et al., 2010]. ALS2/Alsin is a GEF that contains a central DH/PH domain responsible for Rac1 activation. It colocalizes with Rac at neuronal growth cones, and regulates neurite outgrowth and growth cone motility [Tudor et al., 2005]. The PAK-interacting exchange factor PIX, a GEF for Rac has an important role in regulating dendritic spine formation [Zhang et al., 2003].

Another protein that activates Rac is the Dock180-related GEF [Cote and Vuori, 2007]. Studies in mammalian cells have demonstrated that Dock180 is a GEF for Rac1 implicated in multiple activities ranging from cell migration to axonal outgrowth [Cote and Vuori, 2007]. Dock180 interacts with ELMO1, a highly evolutionarily conserved PH-domain containing protein. It has been proposed that the ELMO-PH domain interacts in *trans* with a Dock180:Rac1 complex, eliciting activation of the GTPase [Lu et al., 2004]. The C-terminal region of ELMO1 is necessary and sufficient to interact with Dock180, functioning as a bipartite GEF, promoting Rac GTP activation, membrane ruffling and spine morphogenesis [Miyamoto and Yamauchi, 2010].

Dock7 is an atypical GEF member of the Dock180 family that activates Rac and induces axon formation in primary culture neurons [Watabe-Uchida et al., 2006a,b]. Dock7-induced axonal growth involves phosphorylation and inactivation of the microtubule destabilizing protein stathmin/Op18 [Watabe-Uchida et al., 2006a,b; Conde and Caceres, 2009]. MOCA [modifier of cell adhesion) or Dock3 was described to be specifically present in neurons where it enhances Rac1 activity. Interestingly, MOCA farnesylation is required for its localization to the growth cone subcortical cytoskeleton and Rac activation [Namekata et al., 2004]. A recent study has shown that Dock3 acts downstream of brain derived neurotrophic factor (BDNF) to promote axonal outgrowth. It is worth noting that Dock3 binds and inhibits GSK3-β, increasing the non-phosphorylated form of CRMP2, which stimulates microtubule assembly and axonal growth [Namekata et al., 2012].

GEFT is another GEF, which is enriched in both hippocampus and cerebellum [Bryan et al., 2004] and activates RhoA, Rac and Cdc42. However, in neuroblastoma and primary neurons, its function has been primarily linked with Rac1 activation, and the control of neurite and dendrite remodeling [Bryan et al., 2004, 2006; Mitchell et al., 2011]. GEFT-mediated activation of Rac1 has been associated with downstream regulation of PAK1 and PAK5 [Bryan et al., 2004, 2006].

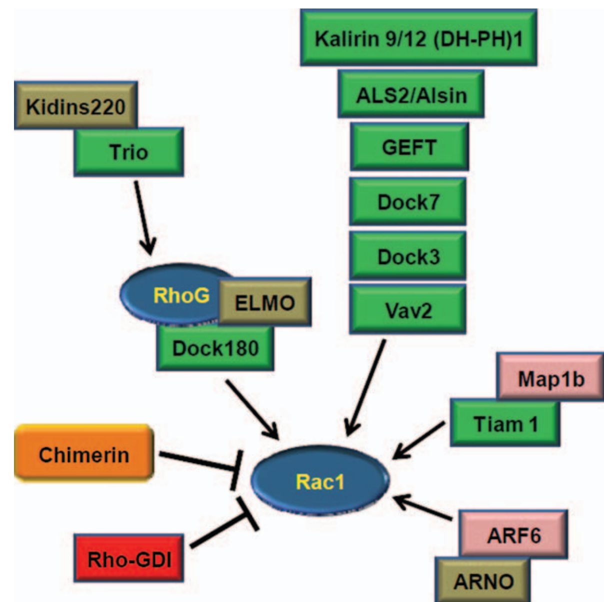

Fig. 5. Rac1 regulators involved in the development of the axon. The participation of Rac1 in the development of neuronal polarity is tightly regulated by several proteins acting as positive or negative regulators of Rac1 activity. The figure shows the GEF proteins that stimulate Rac1 activity (in green), and the GAP proteins that lead to inactive Rac1 (in orange). The role of Rho-GDI is also indicated (in red). Two scaffold proteins are relevant to control Rac1 activity involved in axonal elongation. Thus, ELMO and ARNO are showed in brown. Finally, Arf6 and MAP1B are proteins involved in the regulation of factors that control Rac1 activity (shown in pink).

Rac1 is also controlled by specific GAPs, such as α-chimaerins. They occur as α1 and α2 isoforms, the latter, an alternatively spliced isoform, contains an SH2 domain. These GAPs target primarily Rac1 activation and to a lesser extent Cdc42 [Hall et al., 1993b; Kozma et al., 1996]. The expression of α2-chimaerin mRNA is high in the developing nervous system, suggesting a role in neuronal differentiation [Lim et al., 1992; Hall et al., 2001]. α2-chimaerin promotes neuritogenesis in N1E-115 neuroblastoma cell lines, an effect that requires an intact SH2 domain [Hall et al., 2001] (Fig. 5). Another study has shown that ectopic expression of α1-chimaerin induces pruning of dendritic branches and spines [Buttery et al., 2006].

Rac regulates actin dynamics through two main downstream effector pathways, the p21-activated kinase (PAK) [Manser et al., 1994] and WAVE [Miki et al., 1998]. They induce changes in the actin cytoskeleton through the PAK-cofilin [Ng and Luo, 2004] and the WAVE-Arp2/3 pathways [Takenawa and Miki, 2001; Ng and Luo, 2004].

PAK1, is a serine/threonine kinase involved in axon development in flies and mammals [Hing et al., 1999; Jacobs et al., 2007]. It regulates actin dynamics by activating a LIMK-cofilin pathway implicated in neurite extension and growth cone motility [Endo et al., 2003; Rosso et al., 2004], linking the Rac-PAK module to the mechanisms controlling axonal guidance and branching.

The p35/Cyclin-dependent kinase 5 (Cdk5) [Tsai, 2001] is also a Rac1 effector. p35, the neuron-specific regulator of Cdk5 associates with Rac in a GTP-dependent manner, hyperphosphorylating Pak1 and inhibiting its activity [Nikolic et al., 1998]. Since the Cdk5-RacGTP-Pak1 complex localizes in the growth cone periphery, it is likely to have an impact on the dynamics of the actin cytoskeleton, thus promoting neuronal migration and neurite outgrowth [Paglini et al., 1998b; Tsai, 2001; Rashid et al., 2001]. Experiments with Rac1-deficient cerebellar granule neurons have shown that WAVE is absent from the plasma membrane; since loss of WAVE inhibits axon extension, these observations strongly support the view that the Rac1-WAVE pathway is necessary for axonal growth [Tahirovic et al., 2010].

The Cdc42 Family (Cdc42, Tc10, and TCL)

Cdc42 and the closer related members, teratocarcinoma 10 (TC10) and teratocarcinoma 10 like (TCL) [Drivas et al., 1990], share the ability to induce the formation of filopodia in cells [Neudauer et al., 1998; Vignal et al., 2000; Wennerberg and Der, 2004].

Current evidence favors the view that Cdc42 plays a central role in the establishment of cell polarity in a wide variety of cells, ranging from yeast to neurons [Etienne-Manneville, 2004]. In the case of nerve tissue, Cdc42-mediated polarization arises early in brain development. For example, the neuroepithelium (NE) has an apparent apical-basal polarity marked by the position of adherens junctions from neural progenitors facing the ventricular lumen (apical pole) and their ascending projections on radial glia towards the pial surface (basal pole). While the signaling pathways underlying NE polarization are poorly understood, it is known that Cdc42 plays a critical role [Chen et al., 2006]. Conditional deletion of Cdc42 abolishes the apical distribution of Par3, aPKC, E-cadherin, β-catenin, and Numbs. Additionally, Cdc42 deletion severely impairs the extension of nestin-positive radial glia. Interestingly, neural progenitors fail to anchor with the apical surface and become extensively intermingled with nascent neurons throughout the entire depth of the NE. Consequently, the Cdc42-deficient telencephalon fails to separate resulting in holoprosencephaly [Chen et al., 2006].

Cdc42 and the Par3-Par6-aPKC complex also have a role in neurite outgrowth and polarization in cultured hippocampal pyramidal cells [Wiggin et al., 2005; Arimura and Kaibuchi, 2007; Hall and Lalli, 2010]. Par3 and Par6 determine cell polarity in epithelial cells, and also in worms and flies. In cultured neuronal cells, the transformation of a minor neurite into an axon is preceded and accompanied by the translocation of Par3 and Par6 to the growth cone of the nascent axon [Shi et al., 2003]. Moreover, ectopic expression of Par3 or Par6 alters axon formation. These neurons fail to elaborate a single axon; instead they extend two or more axon-like neurites. It was also shown that the correct localization of Par3-Par6 and aPKC to the tip of the future axon is triggered by activation of a signaling pathway involving a receptor tyrosine kinase and phosphatidyl-inositol 3 kinase (PI3K) [Shi et al. 2003, 2004; Menager et al., 2004]. A molecular mechanism linking Cdc42 and the polarity complex is dependent on the GTP-Cdc42 interaction with the Par3/Par 6 complex [Nishimura et al., 2005]. Interestingly, a fast cycling mutant of Cdc42 mimics Par3 ovexpression inducing the extension of multiple axon-like Tau-1 + axon-like neurites [Sosa et al., 2006]; on the other hand, RNAi suppression of Cdc42 prevents axon formation [Schwamborn and Puschel, 2004]. Besides, IGF-1 a trophic factor required for axon formation in cultured hippocampal pyramidal [Sosa et al., 2006] and cortical motor projection neurons [Ozdinler and Macklis, 2006] acts through a signaling pathway involving a tyrosine kinase receptor (β-gc, a growth cone-enriched variant of the IGF1 receptor [IGF1R]; [Mascotti et al., 1997], PI3K and Cdc42 [Sosa et al., 2006]). The PI3K-Cdc42-Par3-Par6 signaling module is reinforced by the action of Tiam1/Tiam2, by direct interaction with Par3-Par6-aPKC and GTP-bound Cdc42 that leads to Rac activation [Nishimura et al., 2005]. Since Rac-GTP also activates PI3K [Tolias et al., 1995; Conde et al., 2010], a positive feedback loop is generated that drives the continued activation of PI3K and targeting of the polarity complex to the axonal growth cone. Major downstream effectors of this regulatory system are components of the microtubule cytoskeleton required for growth cone protrusion, engorgement and advance during the formation of a new axonal segment [Conde and Caceres, 2009]. Proper regulation of microtubule organization and dynamics could provide an additional positive feedback loop to further ensure Rac-Cdc42 activities [Waterman-Storer et al., 1999; Gonzalez-Billault et al., 2001; Montenegro et al., 2010].

On the other hand, the Cc42-Tiam-Par3-Par6-aPKC-Rac module could be a major target for a RhoA-ROCK inhibitory signaling pathway during axon formation; thus, ROCK phosphorylates and inhibits Tiam1/Tiam2 and Par3 disrupting the polarity complex and preventing Rac activation [Nakayama et al 2008; Caceres et al., 2010]. A genetic knock-out strategy used to ablate Cdc42 in mouse brain also revealed that loss of Cdc42 strongly suppressed axon formation *in vivo* and in culture and that cofilin is an important downstream effector in this process [Garvalov et al., 2007].

Studies in the Drosophila nervous system showed that Cdc42 is implicated in multiple aspects of dendritic morphogenesis [Scott et al., 2003]. The results of this work revealed that despite having a grossly normal dendritic arbor, Cdc42 mutant MB neurons display a number of key alterations in dendritic morphology, including 1) loss of proximo-distal dendritic tapering, 2) loss of stereotyped branching and 3) a drastic (50%) reduction in spine number.

A more recent study examined Cdc42 activity in single dendritic spines during LTP using two-photon fluorescence lifetime imaging (2pFLIM) [Murakoshi et al., 2011]. The results of this very innovative and fascinating study revealed that when spine volume increases, activated Cdc42 remains within the stimulated spine, while RhoA diffuses out of the spine into the shaft; interestingly, inhibition of the Rho-ROCK pathway preferentially inhibited initial spine growth, whereas inhibition of the Cdc42-PAK pathway blocked the maintenance of sustained structural plasticity [Murakoshi et al., 2011]. It is worth noting that PAK3, implicated in mental retardation, regulates spine morphogenesis through a Cdc42-dependent pathway [Kreis et al., 2007].

Another Cdc42-related GTPase involved in neuronal morphogenesis is TC10. For example, it was shown that TC10 induces neurite outgrowth in PC12 and N1E-115 cells [Abe et al., 2003], as well as in cultured dorsal root ganglia [Tanabe et al., 2000]. However, another study failed to demonstrate the participation of TC10 in NGF-induced neurite formation [Murphy et al., 1999, 2001]. Axonal elongation, a hallmark of neuronal polarization, requires membrane addition (exocytosis of plasma precursor vesicles or PPVs) at sites of active growth, such as the axonal growth cone [Pfenninger, 2009; Ory and Gasman, 2011]. This phenomenon has to be tightly coupled with cytoskeletal assembly to ensure neurite extension. In a recent study, Dupraz and colleagues showed that a signaling cascade involving IGF1-R (βgc) and PI3K activate TC10 to regulate membrane addition during axon formation. Activated TC10 triggers translocation of the exocyst component exo70 to the plasma membrane in the distal axon and growth cone. Moreover, silencing of either TC10 or exo70 inhibits axon formation by hindering the insertion of the β-gc subunit of IGF1R [Sosa et al., 2006; Dupraz et al., 2009]. Thus, by activating two highly related RhoGTPases, namely Cdc42 and TC10, PI3K controls two critical events underlying axon growth, namely cytoskeletal assembly and membrane addition.

Cdc42 Regulators and Effectors

One important set of factors for regulating Cdc42 are RhoGDIs. RhoGDIα, which is ubiquitously expressed and strongly interacts with Cdc42 [Zalcman et al., 1996] induces relocalization of Cdc42 to the cytoplasm and nucleoplasm, an effect which is not found with TC10 [Murphy et al., 2001]. Post-translational modifications can alter RhoGDIα functions, since phosphorylation at Ser34 might interfere with the hydrophobic interaction between Cdc42 and RhoGDI [Hoffman et al., 2000; Shin et al., 2009]. Suppression of RhoGDIγ, stimulates differentiation of neural stem cells [Lu et al., 2008]. It will be now of considerable interest to address the role of different RhoGDI-Cdc42 complexes in regulating the spatio-temporal activation of Cdc42, and their significance for axon specification, elongation and guidance.

Cdc42 and Rac share several regulators. Thus, some members of the Dock6 family activate Rac1 or Cdc42 through the catalytic Dock Homology Region-2 (DHR-2) and induce actin-based morphological changes both *in vitro* and *in vivo* [Miyamoto et al., 2007]. The expression of Dock6 increases during differentiation of NIE-115 cells, whereas Dock6 suppression inhibits neurite outgrowth [Miyamoto et al., 2007]. Vav proteins can also promote Cdc42 activity. In NGF-treated PC12 cells, Cdc42 and Rac1 are activated mainly through a signaling pathway involving Vav2/3 and PI3K [Aoki et al., 2005]. The transmembrane protein CD47 also regulates Cdc42 during neurite extension and filopodial formation in N1E-115 cells [Miyashita, et al., 2004] by a mechanism that involves Src kinase-mediated phosphorylation of Vav2 [Murata et al., 2006]. A specific GEF for Cdc42 is the protein hPEM-2. Functional assays with this protein suggest that it is necessary for filopodia formation and actin remodeling in non-neuronal cells. While little is known about its function in nerve cells, it is worth noting that it is highly enriched in rodent brain [Reid et al., 1999]. A recent high throughput analysis performed to uncover global proteomic changes occurring during N1E-115 differentiation revealed a Rac-Cdc42 compartmentalized signaling network that operates in conjunction with multiple GEFs and GAPs to control neurite outgrowth. In this study, RNAi experiments implicated the Cdc42-specifc GEF Dock10 and Cdc42 GAP ArhGAP17 in neurite extension [Pertz et al., 2008].

Another interesting specific regulator of Cdc42 is NOMA-GAP, which belongs to a new family of multi adaptor proteins with RhoGAP activity. This GAP is essential for NGF-stimulated neuronal differentiation and for regulation of the ERK5 MAP kinase and the Cdc42 signaling pathways [Rosario et al., 2007]. RICS and PX-RICS are splicing variants of a Cdc42 GAP which is highly enriched in brain. RICS was shown to be important for neurite extension [Nasu-Nishimura et al., 2006] and recycling of NMDA receptors [Okabe et al., 2003]. Mice lacking RICS showed increased Cdc42 activity in both hippocampal and granule cerebellar neurons [Nasu-Nishimura et al., 2006]. PX-RICS, a splicing variant with lower GAP activity, is the predominant isoform found during nervous system development and it has been

proposed that PX-RICS controls protein trafficking to the plasma membrane [Hayashi et al., 2007; Nakamura et al., 2008] (Fig. 6).

Cdc42 also shares with Rac1 some effector proteins, such as PAKs. In this regard, it is interesting to note that PAK3 appears to be preferentially activated by Cdc42. Cdc42-dependent-PAK3 activation had been proposed to modulate dendritic spine formation and synaptic plasticity [Kreis et al., 2007]. Importantly, PAK3 is another GTPase effector linked to mental retardation disorders, due to either loss of PAK3 protein or loss of its kinase activity [Allen et al., 1998].

Antagonistic Influences of RhoA vs. Rac/Cdc42 During Migration and Neuronal Polarity

As described in previous sections, it is now quite evident that RhoA, Rac and Cdc42 family members actively participate in several events during the neuronal morphogenetic program. One conclusion of many studies is that RhoA acts as an inhibitory regulator, as opposed to Rac and Cdc42 that serve as positive ones. However, this general idea could be misleading and is challenged by several experimental observations.

One example is cell migration, where the current view indicates that Rac regulates protrusion at the leading edge and Rho mediates actin-based contractility at the trailing side. However, studies using wounded fibroblast monolayers or LPA-stimulated serum starved fibroblasts have shown that microtubule reorientation and stabilization at the leading edge, required for direct cell migration, are dependent on a RhoA-mDia signaling pathway [Cook et al., 1998; Palazzo et al., 2001; Gundersen, 2002, 2008; Wen et al., 2004]. The development of biosensors to monitor

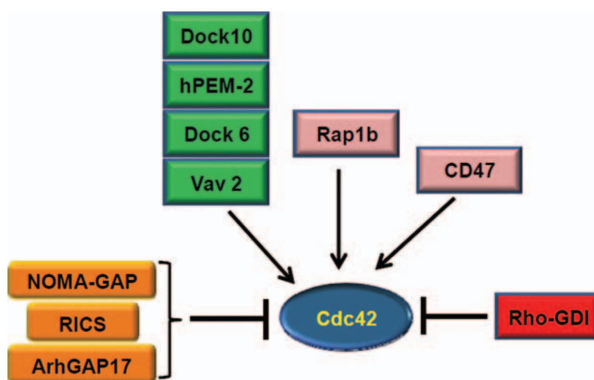

Fig. 6. Cdc42 regulators involved in the development of the axon. The participation of Cdc42 in the development of neuronal polarity is tightly regulated by several proteins acting as positive or negative regulators of cdc42 activity. The figure shows the GEF proteins that stimulate cdc42 activity (in green), and the GAP proteins that lead to inactive cdc42 (in orange). The role of Rho-GDI is also indicated (in red). Two important upstream proteins regulating Cdc42 are indicated in pink.

the spatio temporal activation of RhoGTPases has also revealed a more complex picture of the relationship between RhoA and Rac-Cdc42 during cell migration [Pertz, 2010]. Using FRET probes that report RhoA activity, two studies demonstrated that the bulk of RhoA activation occurs at the leading edge of migrating cells [Pertz et al., 2006; Machacek et al., 2009]. When used in combination with multiplexing techniques and biosensors for RhoA, Rac and Cdc42, this approach allowed measurements of the time and location of RhoGTPase activation during cell migration [Machacek et al., 2009]. The striking results of this study showed that RhoA activation occurs directly in the leading edge at the onset of protrusion, while Rac and Cdc42 are switched on later and remain active during retraction. It is likely that this behavior will apply to other RhoGTPases, and/or cellular events and/or contexts [Pertz, 2010].

With the exception of Murakoshi and colleagues [Murakoshi et al., 2011] that used 2p-FLIM to analyze the spatio temporal kinetics of Cdc42 and RhoA activation during synaptic plasticity, no experimental data is available about the spatio temporal patterns of Rho-GTPase activation during neuronal polarization. However, it is likely that this phenomenon is more complicated than currently envisioned and that the cellular context is a major factor to consider. For example, in cerebellar granule cells, a neuronal cytokine designated as Stromal-cell derived factor 1α (SDF1α) is a physiological ligand that can promote axonal elongation by a RhoA-mDia signaling pathway; interestingly, a high dose of SDF-1α produces axon retraction by a pathway involving RhoA-ROCK [Arakawa et al., 2003]. A recent study by Dotti and colleagues [Pollarolo et al., 2011] has followed the time course of appearance of a neuron's first neurite, an early polarization event that ultimately defines the growth axis. The results of this study suggest that the asymmetrical distribution of RhoA and Aurora kinase could be linked to the generation of the cleavage plane during the last mitotic division, which ultimately determines the site for the emergence of the axon.

RhoGTPase activity needs to be precisely controlled to ensure proper functioning among different cell types and intracellular compartments. In fact, there is a large group of accessory proteins which form a delicate and exquisite regulatory system to dynamically control the function of RhoGTPase family members.

Conclusions and Perspectives

Morphological changes occurring during neuronal differentiation are supported by dynamic changes of the cytoskeleton. Small Rho GTPases cycle between active and inactive states which are mutually exclusive. This property allows a local and fine regulation of their cellular functions. After activation, Rho GTPases control several neuronal morphogenetic and polarization events, including

cell division, migration, neurite sprouting, axon outgrowth and elongation, as well as regeneration and synaptic plasticity. Although it has been proposed that there are mutually exclusive and opposite roles for Rho and Rac/Cdc42 family members, this vision has started to change with the development of biosensors to monitor spatio/temporal changes in GTPase activity. Evidence derived from the use of FRET-based biosensors suggests that there is a coordinated fine-tuning of Rho GTPase activity that must be locally regulated with great precision. The mechanisms that control the activation of GTPases are based on protein-protein interactions involving positive and negative regulators. Some of them may serve not only to control GTPase functions, but also to allow interactions with other sub-cellular compartments, such as the microtubule lattice, the plasma membrane and organelles of the secretory and endocytic pathways. It is likely that new regulators will be discovered and characterized in the immediate future. We envision an exciting field for cell/molecular biology studies, which would consider: a) local and temporal changes of Rho GTPases, most likely defining subcellular domains; b) concurrent changes of GTPase regulators by signaling cascades; c) concurrent mechanisms controlling the activity of Rho GTPase effectors; and d) the integration of multiple extracellular cues into discrete signaling pathways affecting the function of Rho GTPases. Most of these questions will serve to understand the physiological roles of Rho GTPases in different cellular contexts and events, opening the possibility to address their contribution to pathological conditions due to loss- or gain-of-function.

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