 J. Biochem. **134**, 315–319 (2003)
DOI: 10.1093/jb/mvg147

Dynamic Control of Neuronal Morphogenesis by Rho Signaling

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Received August 8, 2003; accepted August 15, 2003

Polarization of the neuronal cell body and initiation of the first neuritic process represent the starting point of a series of dynamic metamorphic events by which the newly acquired identity of a group of neurons can be translated into a morphologically complex web of three-dimensional neuronal circuit. Despite the critical importance of these events, little is known about the molecular signaling mechanisms that either regulate the temporal sequence of these steps or ensure the accuracy and the spatial consistency of the resulting circuits. In this review, based on recent findings from our group and others, we present a working model on how the initial events in neuronal morphogenesis in the CNS may be controlled by multiple Rho pathways.

Key words: actin, axon outgrowth, cerebellar granule neurons, mDia1, ROCK.

Almost a century ago, Ramon y Cahal realized the immense potential that the mammalian central nervous system (CNS) acquired during development by virtue of connecting many neuronal cell types with extremely diverse morphology. Since then, a flurry of knowledge has been obtained, both at the physiological and biochemical levels, about the nature of the brain and neurons. Recent progress in cellular genetics makes it even possible to now envisage therapeutic usefulness for re-engineered neural stem cells in the fight against many debilitating diseases of the CNS.

During its lifetime, a neuron has to undergo numerous steps through which it ultimately distinguishes itself from all the other cells of the body (*1*). The first critical series of steps concerns the end of self-renewal whilst being a neuronal progenitor, then its exit from cell cycle, closely followed by its final cell fate choice. Recent advances have established that a complex network of transcription factors and trophic determinants controls the temporal sequence and the spatial spreading of these events. Taking advantage of these findings, several groups have now reported the successful propagation and derivatization *en masse* of various types of neurons out from embryonic as well as neural stem cells.

Astonishing, however, still remains our lack of insights concerning the molecular mechanisms controlling a second critical series of steps in neuronal development, namely the nascence of the first neuritic processes in a central neuron (*2*, *3*). Indeed, few studies have focused on the molecular events critical for understanding how and when an axon is formed. In contrast, once an axon is born, work from a number of laboratories have identified an essential role for molecular gradients formed by instuctive cues such as netrins, slits, semaphorins, ephrins, neurotrophins and chemokines during the guidance of this axon (*e.g. 4–8*).

Essential role for Rho-family GTPases in neuronal morphogenesis

How does a neuron shape itself? A dynamic morphological alteration is initiated during the acquisition of neuronal polarity and must continue till the completion of synaptogenesis. Rearrangement of actin and microtubule cytoskeleton clearly lies at the heart of such neuronal morphogenesis (*9*, *10*). This is also a critical moment in neuronal network generation since process generation and cell body migration must be spatially and temporally orchestrated in order to achieve patterned formation of neuronal cell layers and appropriate wiring through synapses (*1*, *4–6*).

Recent findings from work in cultured cells and in intact organisms indicate an important role for the antagonism between Rac and Rho GTPases during these steps (*11–16*) (Fig. 1). However, to date, a clear understanding regarding what specific effectors of the small GTPases contribute to each of these opposing signaling events is still missing. Furthermore, whether Rho always antagonizes with Rac remains controversial (see *e.g. 17*, *18*). In keeping with the critical role for the small GTPases, in humans, several hereditary forms of mental retardation or cognitive dysfunction have been linked to molecular components of the Rho signaling such as the RhoGAP oligophrenin-1 (*19*), the RhoGEF ARHGEF6 (*20*), or downstream effectors in the small G protein pathways PAK3 (*21*), LIMK-1 (*22*, *23*), or FMR1 (*24*). However, a clear molecular picture as to the exact role of each different small GTPase pathway is not yet established. As a matter of fact, most small GTPases are abundantly expressed early during development, especially in the brain and are known to have an extensive cross-talk between each other, thus rendering it challenging to crack the fine details of their combinatorial code and hierarchal cascade during any morphogenetic processes.

Control of earliest neuritogenesis by the Rho/ROCK pathway

In recent years, a number of studies have examined the morphological phenotypes associated with overexpression of Rho and related small GTPases in various

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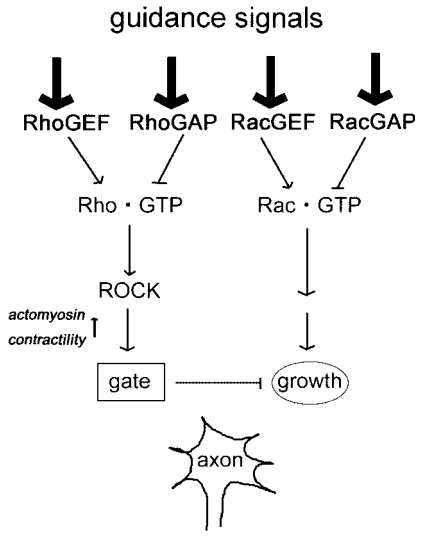

Fig. 1. **Classical view for the role of Rho family GTPases in axon growth.** Recent evidence shows that most guidance molecules are able to modulate small G proteins by acting either on a GEF or a GAP protein. ROCK, a Rho-associated kinase, invariably lies downstream of Rho and gates the ability of Rac-dependent signals to promote axonogenesis and axon growth.

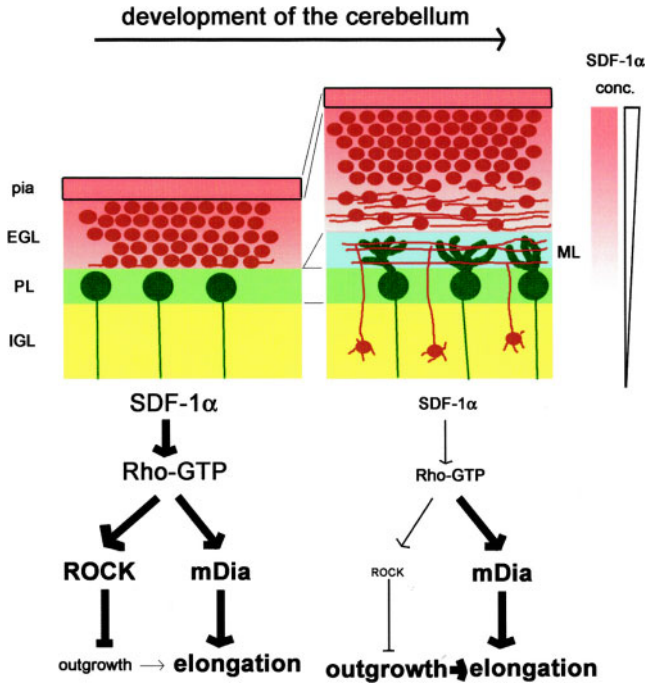

Fig. 2. **A schematic diagram illustrating how the balance between two opposing Rho effectors, ROCK and mDia1, could, in principle, help coordinate formation and elongation of bipolar axons at the utmost inner layer of the external granule cell layer (EGL) in the cerebellum.** Early in the development of the cerebellum when the EGL is still thin, SDF-1α which is heavily expressed in the pia mater (pia), provides a potent Rho-activating signal that prevents axonogenesis via strong activation of ROCK. However, as the EGL expands in size, the SDF-1α concentration near the Purkinje cell layer (PL) becomes more and more reduced, thereby allowing activity of Rho and ROCK to fall within a range where initiation of the first and second axons can occur. However, the residual Rho activity is likely to be sufficient to maintain a high level of mDia1 activity. Such condition would be ideally suited to promote coordinated outgrowth and elongation of parallel fibers, while still keeping the number of axons up to two.

types of neuronal cells, *in vitro* and *in vivo*. Resulting perturbations in neuronal morphogenesis as well as differential effects on actin, myosin, microtubules, or membrane trafficking were then usually attributed to the specific overexpressed small GTPase type. In central neurons, it has now become clear that up- and downregulation of the Rho or ROCK, one of its downstream effectors, dramatically alters axon outgrowth (*25*). In cerebellar granule neurons, strong activation of Rho or ROCK, by overexpression of their dominant active mutants or applying a high dose of an endogenous Rho activator such as SDF-1α, opposes and delays axonogenesis (*25*, *26*). Conversely, downregulation of Rho or ROCK immediately promotes initiation of neuritogenesis and promotes axon outgrowth (*25*, *27*, *28*), presumably by reducing the stability of the cortical actin network in the round neuron (Fig. 1).

Interestingly, the number of axons in cerebellar granule neurons is physiologically set at *two*, a figure that is just in between the number of axons reached with maximal Rho activity (*zero* axon) or minimal Rho activity (*four* or *five* axons). This is in keeping with the finding that constitutive Rho activity level remains relatively high in neurons throughout neuritogenesis (*29*). The elevated level of basal Rho and ROCK activity promotes a high degree of tonic actomyosin contractility (*30*, *31*), thereby setting an efficient gate that may help constrain the timing and the number of axonal outputs coming out from the cell body (Fig. 1), especially in neurons positioned most closely to the pia mater that contains the highest amount of the chemokine SDF-1α. Distancing away from the source of the chemokine gradient during the postnatal expansion of the cerebellar layers may *per se* play a significant part in triggering axonogenesis *in vivo* selectively at the utmost inner layer of the external granule cell layer (Fig. 2, upper panel).

Coordination of axon elongation via ROCK and mDia1

The significance of Rho pathway may not be restricted to negative regulation of axonogenesis. Additionally, ROCK critically controls the motility of axonal growth cones at the tip of extending axons (*25*, *32*). This process was suggested to be mechanistically somewhat distinct from the elongation of the newly formed axons, at least in the context of cerebellar granule neurons, as the latter was significantly facilitated (rather than repressed) in the presence of SDF-1α, a physiological Rho activator in the culture medium (*26*). Curiously, this facilitation correlated with an increase in Rho, rather than Rac activity, and was blocked by the Rho-inhibiting exoenzyme C3. Thus, the existence a Rho-dependent axon elongation mechanism was unexpectedly suggested in central neurons. And the Rho effector critical for mediating this effect was shown to be mDia1, an adaptor protein (*33*, *34*) enriched in cerebellar granule neurons (*26*, *35*).

This raises a rather interesting cell biological question: how can Rho in fact mediate *both* stimulation *and* inhibition of axon outgrowth, via two functionally antagonizing

effectors mDia1 and ROCK (*26*, *36*, *37*)? One factor to consider is the presumed distinct localization of these two Rho effectors within a neuron, as ROCK seems to be expressed diffusely in a cell, while mDia1 was concentrated in the growth cones (*26*). Additionally, the two effectors may differ in their responsiveness to intracellular Rho activity: indeed, it was shown that mDia-based axon elongation was induced by a SDF-1α concentration lower than that required for eliciting ROCK-based inhibition of axon outgrowth, at least in cerebellar granule neurons (*26*). As mDia-Rho binding domain (RBD) bound Rho-GTP tighter than did ROCK-RBD (*38*), one might speculate that the biphasic phenotype could, in principle, result from distinct Rho-GTP affinity of the two effectors (Fig. 2, lower panel).

Rho/mDia1-dependent remodeling of actin structures and microtubules may be crucial in the assembly of cytoskeletal scaffold required for axon outgrowth

How does mDia1, an adaptor protein, exert its effect on axon elongation? mDia1 is a prototypical member of a class of adaptor proteins called formins that contain multiple formin homology domains FH1, FH2 and FH3 (*33*, *34*, *39*). Mutations in various formin proteins in yeasts, flies and worms result in aberrant cell polarity and cytoskeletal remodeling during heavy metamorphic events such as cytokinesis or budding (*40–46*), suggesting that formins exert their effects via control of the actin and microtubule network. Indeed, in Saccharomyces cerevisiae, the Diaphanous homolog Bni1p was shown to be critically involved in controlling actin assembly and the formation of actin cables required for establishment of cell polarity and directed growth (*45*, *47*, *48*). Most remarkable, however, is the recent revelation that the FH2 domain of mDia and other ortholog proteins acts as potent and direct actin nucleators *in vitro*, in an Arp2/3-independent manner. FH2 domain seems to facilitate growth of nucleated actin filaments from the barbed ends, where it remains physically bound without blocking actin polymerization (*49–53*). As a result, continued elongation of unbranched microfilaments is obtained. The actin structure that formins organize seems to be distinct among species: Bni1p assembles actin cables in budding yeast, while mDia1 facilitates formation of stress fibers in mammalian fibroblasts.

Notwithstanding these differences, mDia1, like Bni1p in the bud of the yeast, localizes to the growing end of the cellular cortex in the round neuron and also to the growth cones. Thus it is likely that mDia1 helps tether the actin filaments it nucleates in a polarized manner, through its binding to the barbed ends of the nucleated filaments (*54*). Such mDia1-based polarization of assembled actin microfilaments may play a crucial role in the organization of an early cytoskeletal scaffold required for axon outgrowth.

In yeast, the polarized actin structures induced by Bni1p serve as track for type V myosin that migrates towards the barbed ends to transport secretory vesicles that are needed for budding and polarized growth (*54–57*). This mechanism also seems to be involved in forming the proper orientation of the microtubule organization, as the myosin V (myo2p) was shown to move microtubules along the polarized actin cables, via its binding to Kar9/Bim1 complex (*54–57* and references therein and Fig. 3, upper panel). APC and EB1 have been postulated as mammalian orthologs for Kar9 and Bim1, respectively, and while the final proof has yet to come, it is expected that similar kinds of actin-based microtubule organization strategies are employed, under certain contexts, in highly polarized mammalian cells such as neurons, as well. Consistent with this notion, mDia1 affects orientation and the stability of microtubules in HeLa cells and has been shown to spatially coordinate actin polymerization and the stability of microtubule structures (*58*, *59*).

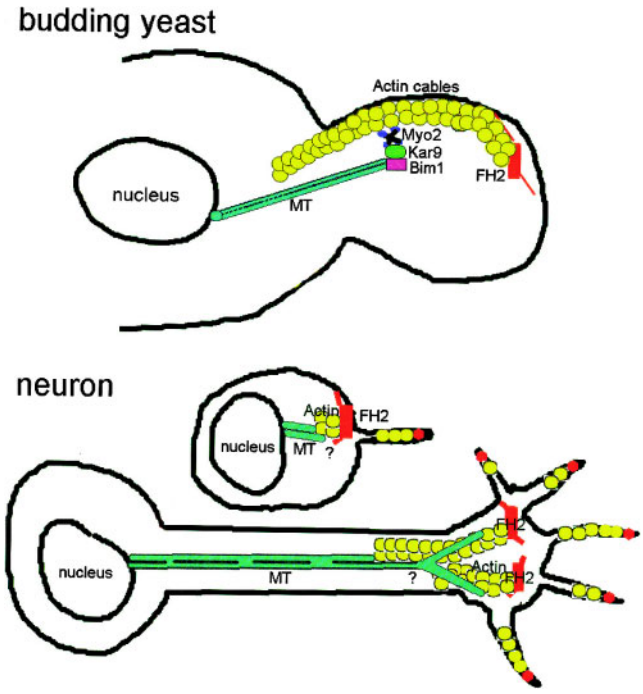

Fig. 3. **mDia may have a privileged role in assembling a cytoskeletal scaffold that links actin and microtubule structures, both in the yeast buds and in neuronal axons.** Upper panel: In the yeast, the FH2 (red rectangle)-containing formin Bni1p nucleates and assembles actin (yellow circles) cables which serve as track for myosin V motors. One of the cargo that the myosin V (Myo2) transports is the Kar9/Bim1 complex, which then is able to recruit microtubules (green rods) into the buds. Lower panel: FH2 domain (red rectangle) of mDia1 may exert a similar effect at the neck of an initiating axon or inside the growth cones. mDia1 nucleates and assembles actin (yellow circles) microfilaments, while probably also regulating microtubule (green rods) stability in a spatially coordinated fashion. However, the molecular identity of the scaffold that hinges together actin and microtubule structures remains to be identified. Furthermore, actin structures in the filopodia and lamellipodia present at the edge of a growth cone are likely to be assembled in a separate manner by distinct nucleators (red hexagone) such as e.g. Arp2/3.

As mDia proteins are most concentrated at the middle portion of the growth cones, an area where actin and microtubule cytoskeletons dynamically interact (*60*, *61*), it is tempting to speculate that mDia-driven remodeling of new F-actin bundles and coordinated microtubule arrays directs axon growth in a Rho-dependent manner, in a way that remains largely independent of the Arp2/3 complex-dependent lamellipodia and filopodia regulation

(*62*), at the tip and edge of the growing growth cones (Fig.3, lower panel). Further elucidation of the exact nature of the mDia-based neuronal cytoskeletal structures will certainly provide new insight to better understanding the molecular machinery allowing neurons to faithfully translate extracellular signals into orchestrated morphogenesis and faithful patterning that are critical for establishing a functional neuronal circuit.

My utmost thanks go to S. Narumiya, T. Furuyashiki, and Y. Arakawa, who were major contributors for the studies from which the model presented in this review was inspired. I apologize to the many authors whom I could not reference due to space limitations. I am also grateful to T. Kinbara for secretarial assistance. This work was supported in part by grants-in aid from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour and Welfare of Japan, and grants from PRESTO-Japan Science and Technology Corporation, the Tanabe Medical Frontier Conference, and Human Frontier Science Program.

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