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ACTIN CYTOSKELETON REGULATION IN NEURONAL MORPHOGENESIS AND STRUCTURAL PLASTICITY

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■ **Abstract** The actin cytoskeleton plays a major role in morphological development of neurons and in structural changes of adult neurons. This article reviews the myriad functions of actin and myosin in axon initiation, growth, guidance and branching, in morphogenesis of dendrites and dendritic spines, in synapse formation and stability, and in axon and dendrite retraction. Evidence is presented that signaling pathways involving the Rho family of small GTPases are key regulators of actin polymerization and myosin function in the context of different aspects of neuronal morphogenesis. These studies support an emerging theme: Different aspects of neuronal morphogenesis may involve regulation of common core signaling pathways, in particular the Rho GTPases.

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INTRODUCTION

Neurons unarguably possess the most complex and diverse shapes of all cell types. For example, axons of motor neurons that innervate a giraffe's toe must travel through long distances of complex environment to reach their targets. The elaborate patterns of dendrites overshadow the most complex tree in nature, with the dendritic tree from an individual human Purkinje cell possessing thousands of branches and receiving input from 50,000 to 150,000 different axon fibers. Deciphering how neurons acquire such complex morphologies during development, as well as how learning and experience can influence morphological changes that alter the functional connectivity between a neuron and its target in adult animals, is key to our understanding of how the nervous system is built and functions. At the same time, these studies provide a rich system for, and may offer unique insight into, a key problem in cell and developmental biology: how extracellular signals regulate the cytoskeleton that eventually alters the shape of a cell.

Morphological Changes in the Lives of Neurons

Figure 1 summarizes the morphological changes in the life of a representative neuron. Neurons extend two types of cytoplasmic processes: axons and dendrites. These developing processes are led by a specialized end known as the growth cone. Growth cones are composed of finger-like filopodia and veil-like lamellipodia (Figure 2) that exhibit amoeboid movement as the growing process explores its environment. Axons and dendrites are both functionally and morphologically distinct. Axons often travel long distances, making stereotypical turning decisions along their paths. Upon reaching their targets, axons produce terminal branches, and their growth cones are converted into presynaptic terminals. Some axons branch in characteristic locations along their paths, connecting with multiple targets in different regions of the nervous system. Dendrites usually do not extend over as long a distance away from the cell body as axons but often branch extensively, giving rise to dendritic trees characteristic of a given neuronal type. Upon proper contact with axons, postsynaptic specializations form on dendrites to create functional synapses with the presynaptic terminals of axons. In addition, some neurons generate small protrusions on their dendritic trees called dendritic spines, which are the site of most excitatory synapses in the mammalian brain and are believed to play important functions in learning and memory.

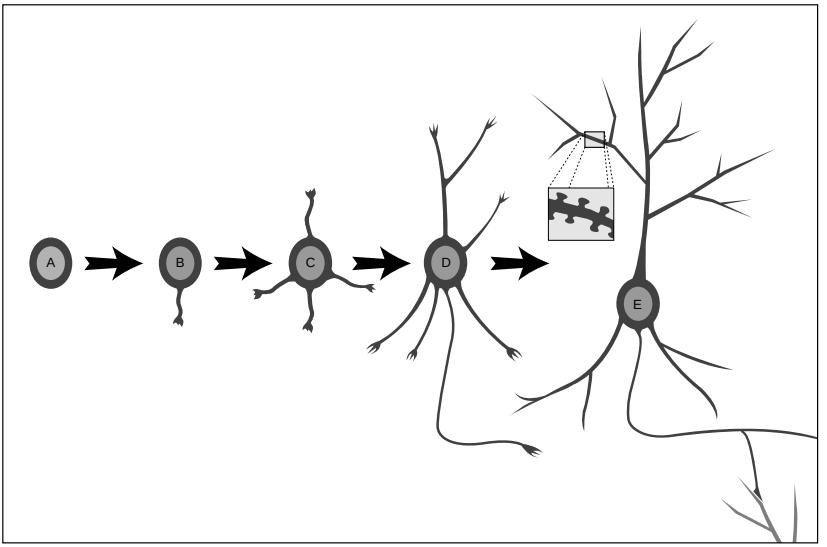

Figure 1 Schematic of morphological changes in the life of a neuron. (*A–E*) represent different stages of neuronal development. Inset in (*E*): high magnification schematic of a dendritic segment showing dendritic spines. The axon is the process projecting directly below the cell body, which usually develops first in vivo. See text for detail.

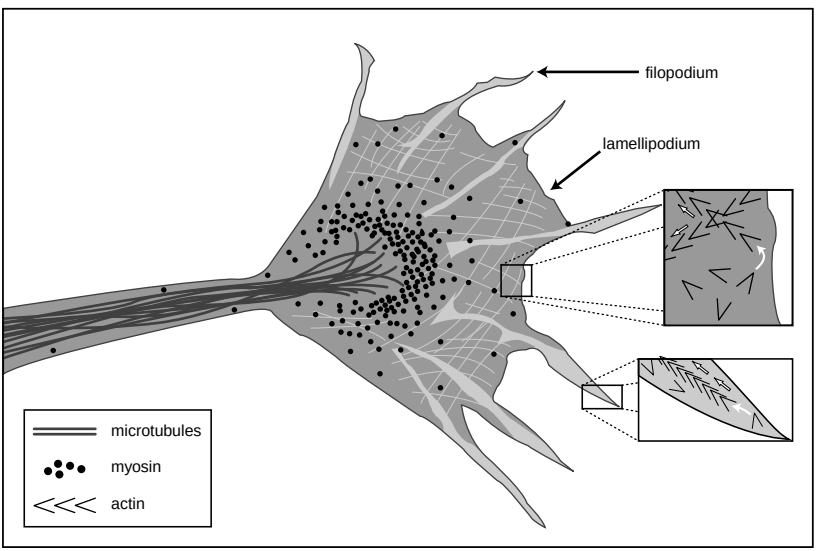

Figure 2 Cytoskeletal organization of a growth cone. In lamellipodia and filopodia, light gray represents F-actin, which is also represented by ⋘ in the insets representing the polarity of F-actin (− end ⋘ + end). In the insets, white arrows represent actin polymerization at the leading edges, and open arrows represent retrograde F-actin flow.

The formation of initial synaptic connections does not mark the end of the morphological development of neurons. During development there is a regressive phase after the initial progressive phase, in which neurons refine their connections by selectively removing projections to incorrect targets. Growth and pruning of neuronal processes, addition and removal of synapses, and changes of synaptic size and shape continue in the life of a neuron as a result of learning and experience.

The morphological changes neurons undergo during their lifetime are essential for the proper wiring of the brain during development and for the function of the brain in adults. Understanding the mechanisms that control these events may give clues as to how neurons become dysfunctional with age or disease.

Scope and Organization of this Review

Morphogenesis of neurons, like shape changes in all cells, is based on changes of the underlying cytoskeleton and its interaction with the plasma membrane. For instance, to generate a membrane protrusive structure such as an axon, there need to be coordinated changes in the organization of the actin and microtubule cytoskeleton, as well as polarized exocytosis to add new plasma membrane to the expanding end of the cell.

The actin cytoskeleton is poised to play a particularly important role in neuronal morphogenesis. In growth cones of developing neurons, for instance, rapidly extending and retracting filopodia are mostly composed of bundled F-actin fibers, whereas lamellipodia are composed of a cross-linked actin meshwork (Figure 2) (e.g., Yamada et al. 1971, Letourneau 1983, Lewis & Bridgman 1992). Actin is also highly enriched in mature synapses (see below). This review focuses on the function of the actin cytoskeleton and its regulation in neuronal morphogenesis and structural plasticity. The roles of microtubule in neuronal morphogenesis have been discussed in other reviews (e.g., Mitchison & Kirschner 1988, Tanaka & Sabry 1995, Suter & Forscher 2000).

This review is divided into three sections. The first section summarizes the various roles the actin cytoskeleton plays in the morphological changes of neurons. In the second section, I discuss the signaling pathways that regulate actin polymerization and myosin activity responsible for some of the morphological changes in neurons, focusing on the roles of the Rho family of small GTPases. In the third and final section, I review recent evidence supporting an exciting theme that is emerging: Different aspects of neuronal morphogenesis may involve regulation of common core signaling pathways, in particular the Rho GTPases. These new findings begin to make connections to the phenomenology described in the first section.

ACTIN IN NEURONAL MORPHOGENESIS AND STRUCTURAL PLASTICITY: PHENOMENOLOGY

Axon Initiation

As a Chinese saying goes, a one thousand-mile journey starts with the first step. Surprisingly little is known about the cell biological mechanisms of the first step of

the morphological development of a neuron: the initial budding of the axon from the cell body. We originally hypothesized that the mechanisms used to initiate a neuronal process might be similar to those used in polarized growth of a bud from budding yeast. This hypothesis led us to test the role of genes homologous to key genes that control yeast budding such as the small GTPase Cdc42 (Luo et al. 1996b). While this line of investigation, among others, did lead to the finding that Rho family GTPases are important in many different aspects of neuronal morphogenesis (see below), it has not provided much insight as to how the polarized growth of neuronal processes is initiated.

Dissociated neuronal culture has been used to study how axon is determined from a number of primitive neurites. For instance, embryonic hippocampal neurons dissociated and cultured in vitro undergo characteristic stages of development. These cultured neurons initially send out several primitive neurites termed minor processes. One of these processes then exhibits rapid growth and takes up the fate of the axon (Dotti et al. 1988). If the axon is experimentally severed, cultured neurons will recover by generating one and only one new axon either from the severed axon or from another minor process (Dotti & Banker 1987). How does the neuron determine which process results in an axon? Recent experiments, utilizing retrospective video microscopy, found that growth cones on neurites destined to become future axons exhibit a significant increase in actin dynamics and instability. In addition, destabilizing the actin cytoskeleton of a single growth cone with the administration of cytochalasin was sufficient to convert an otherwise minor neurite into an axon-like process (Bradke & Dotti 1999). Thus, in this in vitro system, determination and subsequent rapid growth of the axon is associated with the dynamics or instability of the actin cytoskeleton. However, since at the onset of this polarized growth these cultured neurons already possess minor processes, these studies still have not addressed the equivalent of axon initiation in vivo.

Axon Growth

The role of the actin cytoskeleton in neurite growth itself has been the subject of numerous studies. For instance, early studies showed that cytochalasin treatment of cultured chick dorsal root ganglion neurons in vitro resulted in retraction of filopodia and cessation of neurite elongation (Yamada et al. 1970). However, it was later reported that in neurons similarly treated with cytochalasin, under conditions in which filopodial and lamellipodial activities were not detectable, neurite extension persisted, albeit in a highly abnormal manner (Marsh & Letourneau 1984). These contradictory observations were hypothesized to be due to the type of substrata used: More adhesive substrata support neurite extension when actin polymerization is disrupted (Marsh & Letourneau 1984). Similar observations were reported in grasshopper pioneer sensory neuron growth in explant cultures, in which axons extended (albeit in a disoriented manner, see below) in the absence of filopodia caused by cytochalasin treatment (Bentley & Toroian-Raymond 1986). Thus, morphologically visible filopodia are not required for axon elongation.

Cytochalasin treatment coupled with high-resolution video microscopy of the giant *Aplysia* growth cones (Forscher & Smith 1988) provided insight into the function of the actin cytoskeleton in the inner workings of the growth cone. In filopodia and lamellipodia of these giant growth cones, there is a constant retrograde flow in which substances move backward away from the leading edge. Cytochalasin treatment suggested that this movement is based on retrograde flow of filamentous actin (F-actin), and this was later confirmed with actin fluorescence photobleaching experiments (Lin & Forscher 1995). After recovery from cytochalasin treatment, F-actin first appears in the periphery adjacent to the membrane, suggesting that this is the site of actin polymerization in the growth cone (Forscher & Smith 1988). Actin polymerization at the leading edge coupled with retrograde F-actin flow has been documented in many cell types (reviewed in Mitchison & Kirschner 1988) and has been observed in other neuronal types (for a recent study, see Mallavarapu & Mitchison 1999). A general picture emerges from these studies regarding the control of filopodial extension (Figure 2): Actin monomers assemble at the leading edge, which causes the filopodia and the lamellipodia to extend; at the same time, there is a net F-actin flow away from the leading edge that causes them to retract. In other words, the net rate of filopodial and lamellipodial growth may be regulated by modulating the rate of actin polymerization, the rate of retrograde flow, or both rates because they are kinetically independent processes (Lin et al. 1996) (Figure 2). For instance, in neuroblastoma cell lines, filopodia have a relatively constant retrograde flow rate, such that the filopodia behavior (extension, immobilization, or retraction) is mostly controlled by the rate of F-actin assembly at the leading edge (Mallavarapu & Mitchison 1999). In contrast, in *Aplysia* growth cone-target interaction, the rate of retrograde flow is inversely correlated with the rate of growth cone extension (Lin & Forscher 1995). Furthermore, it has been shown by dominant negative and pharmacological intervention that myosin(s) drive the retrograde F-actin flow in the *Aplysia* growth cone (Lin et al. 1996).

A growth cone may possess many filopodia and intervening lamellipodia extending in different directions. Do the mechanisms of filopodial extension discussed above apply to the extension of the entire growth cone and hence regulate the growth of the entire axon? At least in the case of *Aplysia*, actin polymerization at the leading edge and retrograde F-actin flow is also observed in the large lamellipodia (Lin & Forscher 1995) whose movement does control growth cone extension (see O'Connor et al. 1990 for a similar example in grasshopper sensory neurons). Normally, retrograde F-actin flow is isotropic—the speed is similar in all directions. However, when the growth cone is engaged in target interaction, retrograde flow slows down only in the direction of growth cone extension (Lin & Forscher 1995). So at least in this *Aplysia* example, retrograde F-actin flow is critical to the overall advance of the growth cone. It remains to be determined whether the same principle applies to more complex and motile growth cones in vivo. Regulating filopodial and lamellipodial extension/retraction also plays an important role in the arena of axon guidance.

Axon Guidance

One of the most fascinating features of the axon is its ability to follow a precise path leading to its target. From a cell biological point of view, this ability to navigate through its environment can be reduced to directed turning of growth cones toward sources of attractants and away from sources of repellents. These attractive and repulsive cues can be either cell surface bound or diffusible molecules that form concentration gradients to guide the growth of axons (reviewed in Tessier-Lavigne & Goodman 1996).

Axon guidance is exquisitely sensitive to perturbation of the actin cytoskeleton. For instance, pioneer sensory axons growing from grasshopper explants lose their pathfinding capability upon cytochalasin treatment but still maintain their ability to grow (Bentley & Toroian-Raymond, 1986). Under these conditions sensory neuron growth cones are also devoid of filopodia, suggesting that filopodia play important roles in axon guidance. Real-time observations of sensory growth cone advance in situ revealed that contact of a single filopodium with guidepost cells can cause dramatic growth cone turning. Upon contact with the guidepost cell, the filopodium dilates and becomes a nascent growth cone that repositions the direction of the growing axon toward the guidepost cell. Turning can also be achieved by selective advance of lamellipodia in between the filopodia on one side of a growth cone where contact with a positive cue is made. This leads to asymmetric advance of the growth cone, which eventually allows for a turning of the growing process (O'Connor et al. 1990). Analogous experiments have also been reported in vertebrate neurons. For instance, application of cytochalasin to an "open brain" preparation of *Xenopus* embryos resulted in elimination of filopodia in retinal neurons extending in situ and caused growth cones to ignore turning points. These two events exhibited a similar dose-response curve (Chien et al. 1993).

In addition to these observations of growth cone turning in situ, which are likely to be guided by contact-mediated cues, filopodia also respond to diffusible chemoattractants. For instance, growth cones of *Xenopus* spinal neurons in culture are attracted to a gradient of glutamate presented from a micropipette. An increased number of filopodia appear on the side of the growth cone facing the glutamate source. Elimination of filopodia by low-dose cytochalasin treatment, while not affecting axon growth, abolished the turning response of the axons (Zheng et al. 1996).

Just as attraction may depend on increasing the stability of filopodia and lamellipodia, repulsion may be mediated by their destabilization. For instance, application of growth cone collapsing factors induces a net loss of leading edge F-actin (Fan et al. 1993). When these F-actin disrupting factors are introduced so that only a subset of filopodia of the growth cone are exposed, selective collapse of the exposed filopodia results. The growth cone continues to extend away from the source of the collapsing factors, thus leading the axon away from the growth cone collapsing factors (Fan & Raper 1995).

Taken together, these observations suggest an appealing model: Growth cone turning is mediated by the asymmetric presence of negative or positive cues across a growth cone. The growth cone turns as a result of enhanced stabilization and expansion of filopodia and lamellipodia in the presence of an attractive cue, or selective destabilization of filopodia and lamellipodia in the presence of a negative cue, or a combination of both (Figure 3).

Axon Branching

Axons not only follow a precise path through their environment, but they also branch at stereotypical positions in vivo in order to innervate multiple and

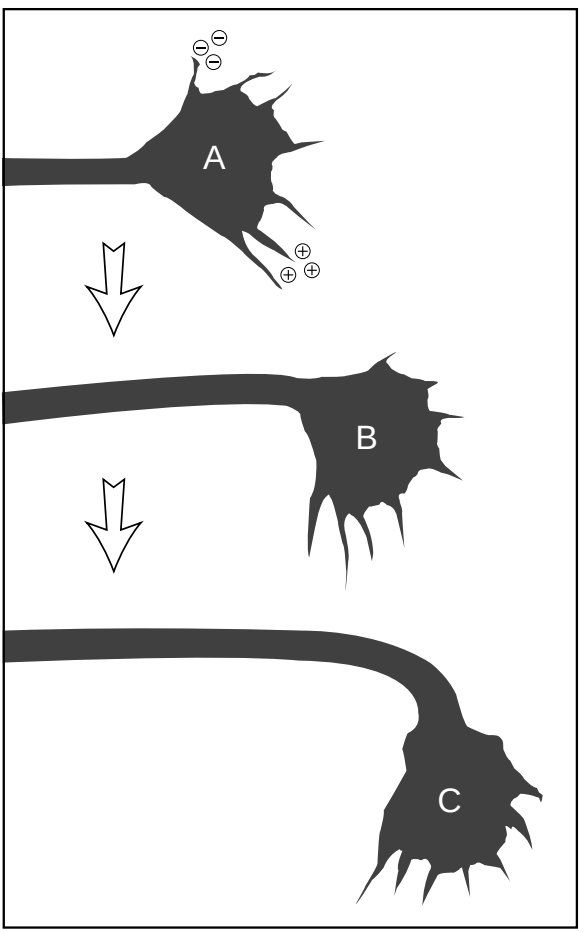

Figure 3 Schematic for growth cone turning. + and − represent attractive and repulsive cues, respectively. See text for detail.

sometimes distant targets. Branching can occur through two distinct cellular mechanisms. In principle, a growth cone can split at the branching point, thereby generating two sister branches; alternatively a new branch can emerge from the middle of an axon trunk, a process referred to as interstitial branching (Figure 4). Both of these branching mechanisms depend on proper cytoskeletal dynamics.

Interstitial branching occurs by local destabilization of the microtubule and actin cytoskeleton, followed by extension of a single filopodium, which is subsequently stabilized by microtubule invasion (e.g., Bastmeyer & O'Leary 1996, Gallo & Letourneau 1998; reviewed in Acebes & Ferrus 2000, Scott & Luo 2001). So it is not surprising that interstitial axon branching is sensitive to perturbation of actin dynamics. For instance, in cultured neurons, treatment with latrunculin and cytochalasin resulted in a reduction of interstitial branching under conditions in

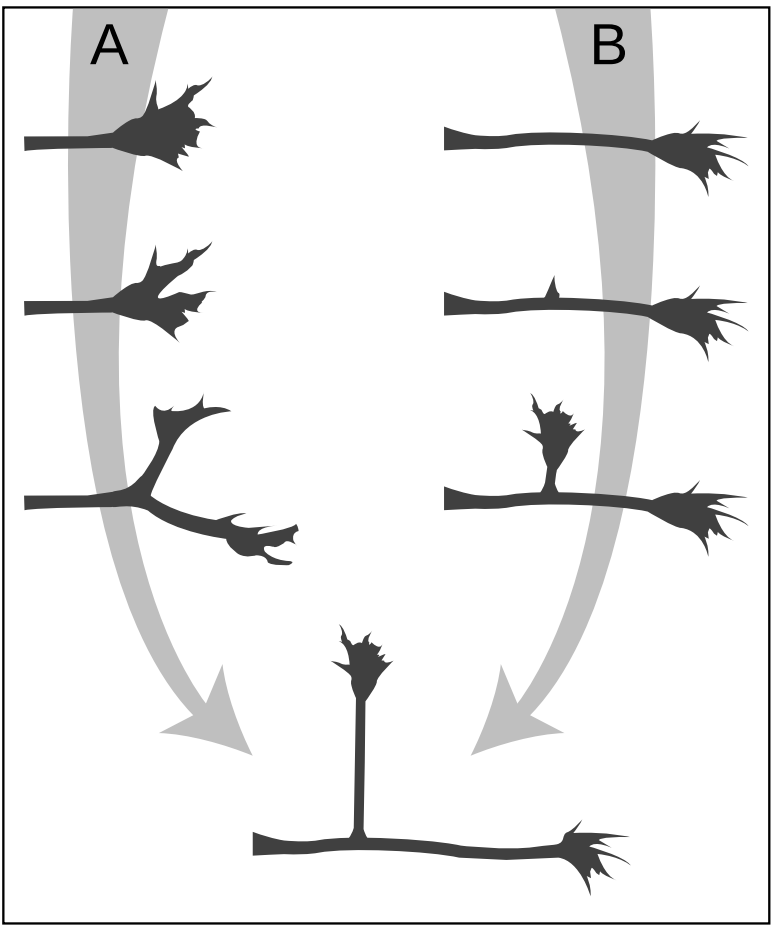

Figure 4 Two different mechanisms for axon branching. (*A*) Growth cone splitting. (*B*) Interstitial branching.

which axon growth was not perturbed (Gallo & Letourneau 1998, Dent & Kalil 2001).

What cell biological mechanisms could allow for growth cone splitting? A recent study on *Helisoma* giant growth cones in vitro offers some insight. In the growth cones of these neurons, loss of actin bundles correlates with repulsive axon guidance (Zhou & Cohan 2001). Local application of a myosin light chain kinase inhibitor resulted in local disruption of actin bundles, and subsequently the growth cone turned away from the source of the inhibitor. However, when the same inhibitors were applied to the middle of the growth cone leading edge, the growth cone often responded by splitting into two; each continued to grow away from the site of the inhibitor, thus forming two axon branches (Zhou et al. 2002). It remains to be determined whether analogous mechanisms could apply to other neurons with smaller growth cones and to neurons in vivo. A recent genetic study suggests that mushroom body neurons of the *Drosophila* brain might generate branched axon projections through growth cone splitting based on mutant phenotypes of the cell adhesion molecule Dscam. Each neuron normally generates a dorsal and a medial branch. In *Dscam* mutants, sister branches sometimes both extend either dorsally or medially. Sometimes more than two sister branches can be found in a single neuron extending in a parallel fashion. These observations suggest that Dscam is required for segregating axonal branches to separate paths and for suppressing the formation of extra branches, possibly by mediating adhesion of sister branches (Wang et al. 2002).

Dendritic Growth, Guidance, and Branching

Dendrites are at least as important in contributing to synaptic connections as axons and play additional functions to integrate the multiple input signals a neuron receives. In comparison to our knowledge of axonal development, the development of dendrites is much less well understood, as dendrites are morphologically more complex and are experimentally less accessible than axons. Recent technical advances in imaging and genetic manipulations of single dendritic trees have allowed rapid advances in the study of dendritic development (reviewed in Cline 2001, Jan & Jan 2001, Redmond & Ghosh 2001, Scott & Luo 2001). The cytoskeletal requirements of dendritic growth, guidance, and branching have not been examined as extensively as those of axons (or neurites in cases of cultured neurons where axonal/dendritic differentiation is not apparent). Given the similarities in the basic cell biology of axons and dendrites as neuronal processes, it is probably reasonable to assume that certain aspects of the cytoskeletal basis for growth, guidance, and branching of axons are generally applicable to dendrites. Indeed the majority of molecules that regulate dendritic morphogenesis play analogous roles in axons (reviewed in Scott & Luo 2001). However, dendrites and axons do differ in important aspects, such as growth rate and microtubule polarity (reviewed in Craig & Banker 1994), so there may be some inherent differences in their cytoskeletal regulations.

Perhaps the most distinctive features of dendrites are their characteristic branching patterns, which can be extremely complex. Interstitial branching from dendritic shafts, rather than splitting of dendritic growth cones, appears to be the mechanism for dendritic branching in vertebrate as well as *Drosophila* neurons (e.g., Dailey & Smith 1996, Gao et al. 1999, Wu et al. 1999). For instance, in vivo imaging of *Xenopus* optic tectal neurons shows rapid addition and retraction of branches along the primary dendrites (Wu et al. 1999). Dentritic elaboration occurs by the stabilization of interstitial branches and subsequent branch addition from stabilized branches. Similarly, live imaging of developing pyramidal neurons in hippocampal slices revealed that dendritic shafts constantly extend and retract filopodia. During early development some of these filopodia are stabilized into new dendritic branches, whereas later in development these dynamic filopodial extensions can develop into dendritic spines (Dailey & Smith 1996).

Dendritic Spine Formation, Stability, and Rapid Motility

Dendritic spines are small protrusions (a few microns in length) along dendritic shafts that are the postsynaptic sites of the majority of excitatory synapses in mammalian brains. It has been widely accepted that chemical and structural modifications in dendritic spines underlie much of the plastic changes in the brain in response to learning and experience (for recent reviews on dendritic spines, see Yuste & Bonhoeffer 2001, Hering & Sheng 2001). For instance, electrical stimulation that results in a long-lasting increase of synaptic transmission (long-term potentiation) also leads to the formation of new dendritic spines (Engert & Bonhoeffer 1999, Toni et al. 1999).

What controls the development and stability of dendritic spines? The actin cytoskeleton has long been suspected to be crucial, as a combination of electron microscopy, myosin decoration, immunohistochemistry, and recent actin-GFP fusion experiments demonstrates that dendritic spines are highly enriched for actin (Fifkova & Delay 1982, Matus et al. 1982, Landis & Reese 1983, Fischer et al. 1998).

Recent live imaging of neurons in primary culture and in brain slices also revealed that dendritic spines twitch over a timescale of seconds (Fischer et al. 1998, Dunaevsky et al. 1999), a movement hypothesized by Crick 20 years ago to encode ultrashort memory (Crick 1982). While the actual significance of the twitching of dendritic spines remains unclear (Bonhoeffer & Yuste 2002), it has been demonstrated that actin dynamics have much to do with this movement, as treatment with cytochalasin blocks this twitching (Fischer et al. 1998, Dunaevsky et al. 1999).

Synapse Formation and Stability

F-actin is highly concentrated in presynaptic terminals as well as in postsynaptic structures such as dendritic spines. Two recent studies examined the roles of actin in synapse formation and maintenance. In the first study, the role of F-actin

in synapse formation and maintenance was examined in cultured hippocampal neurons by treating the culture with latrunculin, an actin polymerization inhibitor, and assaying synapse formation and stability. In young cultures, formation of new synapses is absolutely dependent on actin dynamics. As synapses mature, their stability and function are increasingly independent of actin dynamics, as they are not perturbed by latrunculin treatment (Zhang & Benson 2001). In a second study, hippocampal neurons were cultured on silicon-coated dishes so that they could be locally stimulated by photoconduction. Repeated tetanic stimulation of the neuron could cause remodeling of presynaptic actin, as assayed by the localization of an actin-GFP reporter, and could result in the formation of potential new synapses capable of active vesicle recycling over the course of 2 h. Strikingly, a single tetanus resulted in rapid translocation of actin in both presynaptic and postsynaptic compartments: Presynaptic actin advanced toward and postsynaptic actin moved away from stimulated synapses (Colicos et al. 2001). It will be very interesting to determine whether these movements contribute to altering synaptic function in the short term and structural remodeling over a longer period.

Axon and Dendrite Retraction

So far I have discussed the constructive steps of neuronal morphogenesis. Neurons also have internal programs to remove their existing axons or dendrites, often referred to as axon or dendrite pruning. Pruning is important for the following reasons. First, during development, neurons often have exuberant axonal and dendritic projections making more connections than are finally retained. A refinement process is then activated to remove these exuberant processes. There seem to be two different mechanisms for process refinement: (*a*) removal of terminal axonal branches, best understood at the vertebrate neuromuscular junction (reviewed in Sanes & Lichtman 1999); and (*b*) pruning of collateral axon branches from long-distance projections, for example, in the mammalian CNS (O'Leary & Koester 1993, Weimann et al. 1999). Axonal and dendritic refinement is also observed in insects during the reorganization of their nervous system between larval and adult life (e.g., Truman & Reiss 1976, Technau & Heisenberg 1982, Lee et al. 2000a). Second, pruning also occurs in mature neurons. One example of this comes from experiments where repeated imaging of single identifiable neurons reveals that dendritic branching patterns are significantly changed over the course of a few months in adult mice (Purves et al. 1986). These alterations may reflect changes of neural circuits in response to learning and experience, and they must involve both destructive and constructive mechanisms. Third, many neurological diseases (such as Alzheimer's and Parkinson's disease) exhibit atrophic neuronal processes. It is possible that internal programs utilized for normal refinement of neuronal processes are misregulated in these disease states, thereby contributing to the pathology of these diseases.

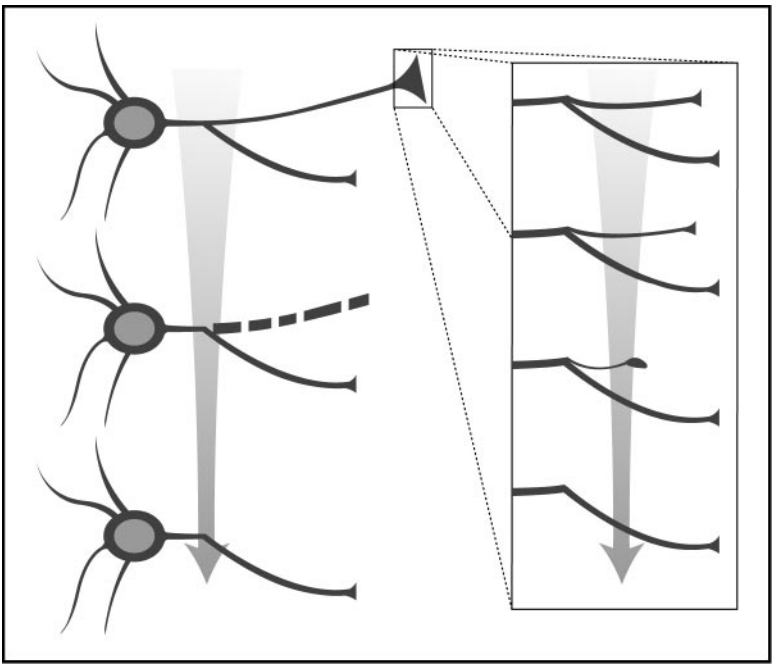

Figure 5 Two different mechanisms for axon pruning. Main figure: local (Wallerian) degeneration of an axon branch. Inset: retraction of an axon branch.

Cell biologically, at least two distinct mechanisms have been observed for process elimination (Figure 5). The first is process retraction, which has been demonstrated in the refinement of the vertebrate neuromuscular junction (Keller-Peck et al. 2001). The second is known as Wallerian degeneration, in which axons swell locally before undergoing fragmentation and phagocytosis (Waller 1850). Wallerian degeneration is usually observed under conditions of neuronal injury, and the cell biological mechanism is poorly understood (e.g., see Finn et al. 2000). The cytoskeletal requirements of axon retraction have been studied using neuronal culture as a model system. For instance, prolonged treatment of cultured neurons with drugs interfering with microtubule polymerization (Solomon & Magendantz 1981) or inhibiting microtubule motors (Ahmad et al. 2000) cause axon retraction. Intriguingly, inhibiting actin polymerization prevented axon retraction, suggesting that axon retraction is an active process that requires actin dynamics (Solomon & Magendantz 1981, Ahmad et al. 2000). Axon retraction could also be prevented by inhibiting myosin motors, suggesting that myosin-mediated forces on an F-actin array may be important in mediating axon retraction (Ahmad et al. 2000).

SIGNALING TO THE ACTIN CYTOSKELETON: REGULATION BY RHO GTPases

General Considerations

The actin cytoskeleton is central to every step of the morphologic changes of neurons during development and in adults. A common conceptual theme across all these morphogenic events is that the extracellular signals that direct these morphological changes must be interpreted by cell surface receptors and their downstream signaling pathways, eventually regulating the dynamics of the underlying actin cytoskeleton. Important questions arise. Do different neuronal morphogenic processes share similar mechanisms, or do they use distinct mechanisms to regulate the actin cytoskeleton? For instance, do signaling mechanisms that regulate axon growth, guidance, and branching differ completely, or do they use the same components in different ways? What are the important variations in the signaling pathways that set them apart? Are these mechanisms also used to construct a dendritic spine or change its shape in response to electrical activity? Are the cellular mechanisms used for axon growth simply the opposite of those used in axon retraction?

The rest of this article focuses on these unresolved questions. Given the magnitude of the morphogenic events covered and the multitude of signaling pathways from the cell surface to the actin cytoskeleton, I limit the discussion to recent advances in our understanding of how Rho GTPase signaling pathways are involved in neuronal morphogenesis. There are other important pathways leading from receptors to the cytoskeleton (for a recent comprehensive review, see Song & Poo 2001). For example, profilin is a central regulator of actin polymerization, which is also required for axon development (Wills et al. 1999). Profilin binds to Ena/VASP family proteins, which have been implicated in transducing signals from a number of cell surface receptors during axon guidance (reviewed in Lanier & Gertler 2000). Recent cell biological studies further implicate Ena/VASP in regulating cell motility by interacting with barbed ends and shielding them from capping proteins (Bear et al. 2002).

The focus on Rho GTPase reflects our research interests but is also justified for the following reasons: (*a*) Rho GTPases are key regulators of the actin cytoskeleton in many cell types studied so far (reviewed in Hall 1998, Van Aelst & D'Souza-Schorey 1997). (*b*) Rho GTPases are important regulators of actin polymerization and myosin activity, two major driving forces for all the actin-based motility discussed in the previous section. (*c*) Rho GTPases have been shown to play important roles in many aspects of neuronal morphogenesis (recently reviewed in Dickson 2001, Luo 2000, Redmond & Ghosh 2001). Therefore, the Rho pathway represents an example of how a central signaling pathway can be utilized in different fashions to regulate different aspects of neuronal morphogenesis. This multitasking role of the Rho pathway may also apply to other signaling pathways that connect extracellular cues to the regulation of the actin cytoskeleton throughout the lifetime of a neuron.

Rho GTPases and Regulation of Actin Polymerization

Rho GTPases act as intracellular molecular switches that cycle between an active GTP-bound form and an inactive GDP-bound form (Figure 6). Guanine nucleotide exchange factors (RhoGEFs) facilitate the conversion from GDP-bound to GTP-bound form and thus are activators. GTPase activating proteins (RhoGAPs) enhance GTP hydrolysis and are thus negative regulators. RhoGEFs and RhoGAPs are regulated by upstream signals. When bound to GTP, Rho GTPases bind to downstream effectors that transduce signals to regulate the actin cytoskeleton

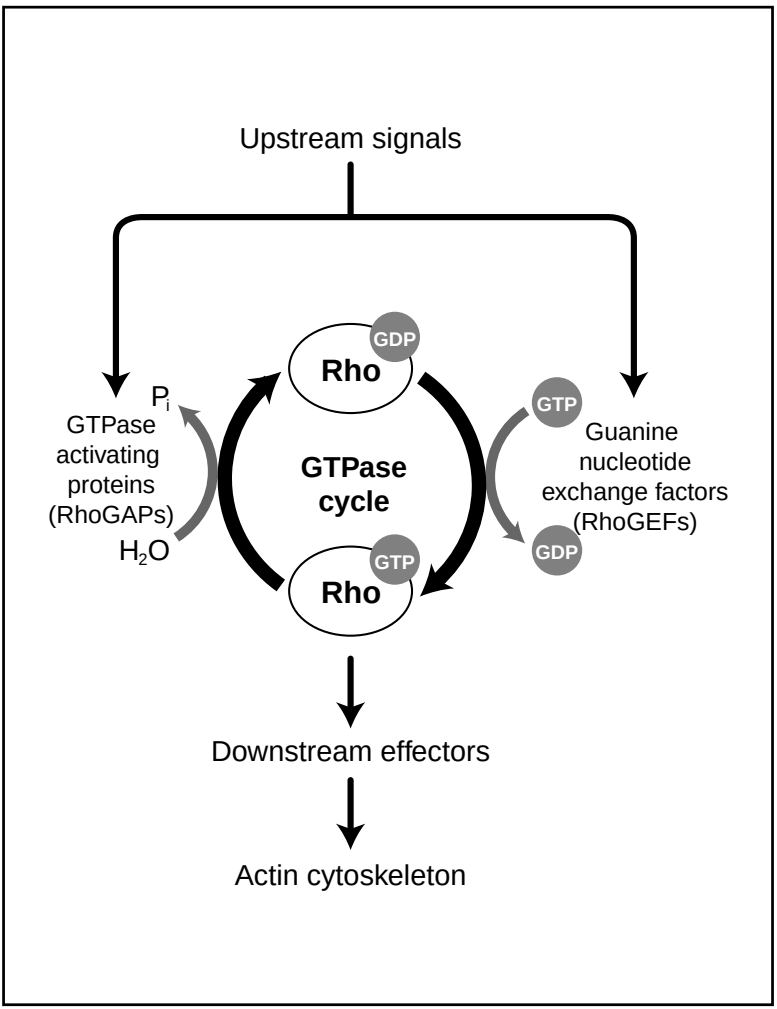

Figure 6 The Rho GTPase cycle. See text for detail.

(Figure 6). The three best-studied Rho GTPases are RhoA, Rac, and Cdc42, which in fibroblast control the formation of distinct F-actin-based structures such as the stress fiber, lamellipodia and filopodia, respectively (reviewed in Hall 1988). How does each member of the Rho GTPase family regulate actin dynamics?

DE NOVO ACTIN POLYMERIZATION AND Cdc42 One of the most exciting recent findings in the field of the actin regulation is the illustration of a mechanism for de novo polymerization through the Arp2/3 complex (reviewed in Mullins & Welch 2002, in this volume; Pollard et al. 2000). Of particular interest is the identification of a key signaling pathway that controls Arp2/3 activity through N-WASP and Cdc42. N-WASP is closely related to WASP **(**Wiskott-Aldrich syndrome protein), binds to Cdc42 in a GTP-dependent manner, and is required for filopodial formation in fibroblasts (Miki et al. 1998a). Activated Cdc42, as well as a lipid messenger $PI(4,5)P_2$, is able to bind to the N-terminal domain of N-WASP to unlock the auto-inhibitory conformation of N-WASP, thereby releasing the active C-terminal domain of N-WASP and stimulating the activity of Arp2/3 for de novo actin polymerization (Rohatgi et al. 1999; see also Higgs & Pollard 2000). (Figure 7). These findings give a biochemical explanation for how Cdc42 could control filopodial formation as observed in fibroblasts (Kozma et al. 1995, Nobes & Hall 1995). However, how Cdc42 activation leads to formation of bundled F-actin fibers in the filopodia remains a mystery.

Regulation of Cdc42 function in neuronal signaling has been demonstrated in a recent study of Slit/Robo signaling in the context of neuronal migration. Slit encodes a secreted protein that is a ligand for the Robo transmembrane receptor. Slit and Robo regulate repulsive axon guidance (reviewed in Yu & Bargmann 2001) as well as cell migration (e.g., Wu et al. 1999, Kramer et al. 2001). In this most recent study (Wong et al. 2001), a Robo-binding protein has been identified as a GTPase activating protein for Rho-family GTPases (RhoGAP). RhoGAPs downregulate Rho GTPase signaling by enhancing the rate of GTP hydrolysis (Figure 6). The presence of Slit enhanced the binding of this RhoGAP (termed srRhoGAP) to Robo and led to downregulation of Cdc42 activity in migrating neurons in culture. Expression of dominant negative srRhoGAP or activated Cdc42 in migrating neurons blocked their ability to respond to the repellent activity of Slit. Given that Cdc42 positively regulates de novo actin polymerization, this downregulation of Cdc42 activity is consistent with Slit's role in repulsive cell migration and perhaps axon guidance. High concentrations of Slit could reduce local actin polymerization by reducing Cdc42 activity, forcing the leading edge of migrating cells or growth cones of axons to turn elsewhere, leaving areas of high Slit concentration (see Figure 3).

Given the key roles of Cdc42 in regulating filopodia (Kozma et al. 1995, Nobes & Hall 1995), in regulating de novo actin polymerization (Rohatgi et al. 1999, Higgs & Pollard 2000), and now in mediating Slit/Robo signaling (Wong et al. 2001), one would expect that loss-of-function mutations of these signaling proteins in multicellular organisms would lead to dramatic disruption of numerous

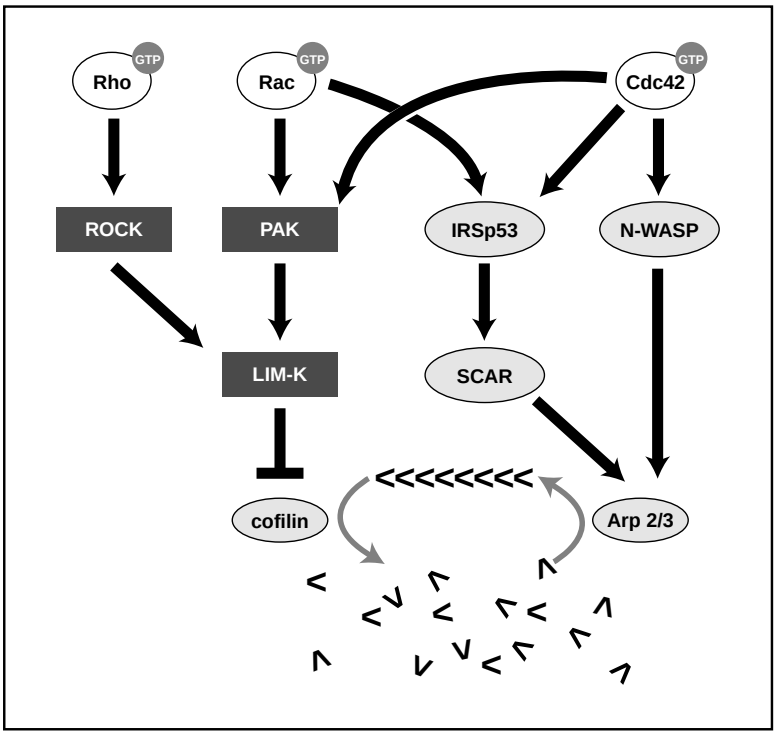

Figure 7 Signaling pathways from Rho, Rac, and Cdc42 to the regulation of actin polymerization and depolymerization. See text for detail. Abbreviation: ROCK, Rho associated coiled-coil containing kinase, PAK, p21 activated kinase; LIM-K; LIM domain containing kinase; IRSp53, insulin receptor substrate p53.

developmental processes. However, *Drosophila* homozygous for a null mutation in the only gene encoding a WASP-family protein (including WASP and N-WASP in mammals) exhibit no gross morphological defects other than cell-fate determination regulated by the Notch pathway (Ben-Yaacov et al. 2001). Likewise, animals homozygous mutant for the only *Cdc42* gene in *Drosophila* did not exhibit detectable phenotypes in many developmental processes including embryonic nervous system wiring (Genova et al. 2000), whereas *slit* and *robo* both have severe midline guidance phenotypes (Seeger et al. 1993). These findings suggest that perhaps some alternative pathways exist that can compensate for the loss of the Cdc42/N-WASP pathway in regulating actin polymerization.

RAC AND ACTIN POLYMERIZATION The closest relatives of Cdc42 are the Rac small GTPases, which in fibroblasts regulate a distinct feature of the actin cytoskeleton (lamellipodia) compared with Cdc42 (filopodia) (Ridley et al. 1992, Nobes & Hall 1995). The phenotypes caused by expressing dominant negative or

constitutively active Cdc42 or Rac, however, are generally more similar in neuronal cells (Luo 2000). These observations perhaps reflect the fact that in neuronal cells, dominant negative Rac or Cdc42 interfere with similar guanine nucleotide exchange factors (RhoGEFs), their upstream activators (Figure 6), or share a subset of common effectors in neurons (Luo 2000). It may also reflect the indispensable nature of filopodia and lamellipodia as two crucial structures of the neuronal growth cone.

Could Rac regulate actin polymerization independent of the Cdc42/N-WASP pathway? A protein distantly related to WASP, SCAR/WAVE, has been shown to be involved in Rac-mediated actin reorganization (Miki et al. 1998b) and to regulate Arp2/3 activity (Machesky et al. 1999). Recent genetic analysis in *Drosophila* indicated that SCAR is required in numerous actin-based morphogenetic events, including patterning the axons of the embryonic nervous system. In many processes examined, there are striking similarities between the phenotypes resulting from the SCAR and Arp3 mutants, suggesting that SCAR is a major regulator of Arp2/3 complex (Zallen et al. 2002). How is SCAR linked to Rac? SCAR/WAVE binds to a protein called IRSp53 (Miki et al. 2000), which in turn binds to both Rac (Miki et al. 2000) and Cdc42 (Govind et al. 2001) (Figure 7). The physiological significance of these connections, particularly in neurons, awaits further investigation.

RHO GTPases AND ACTIN DEPOLYMERIZATION The best-understood protein to mediate actin depolymerization is cofilin/ADF (actin depolymerization factor). Cofilin facilitates depolymerization of F-actin at the minus end and can also sever F-actin (reviewed in Bamburg 1999). Cofilin activity is regulated by phosphorylation at serine 3 in multicellular organisms; phosphorylation at this residue inhibits its activity (Bamburg 1999). A somewhat specific kinase that phosphorylates serine 3 was identified as LIM-kinase (Arber et al. 1998, Yang et al. 1998). Additionally, there is also a specific cofilin phosphatase, Slingshot, which is conserved from *Drosophila* to human (Niwa et al. 2002). While signal transduction pathways that regulate Slingshot await further study, LIM-kinase can be phosphorylated by the Pak kinase at a key amino acid (threonine 508) that greatly stimulates its kinase activity toward cofilin (Edwards et al. 1999). Pak is one of the best-characterized downstream effectors for Rac and Cdc42 (Manser et al. 1994). Thus, activation of Rac or Cdc42 activates Pak, which in turn activates LIM-kinase, leading to the downregulation of cofilin activity and inhibition of actin depolymerization (Figure 7).

What is the evidence that these signaling pathways are relevant in neurons? Cofilin is highly enriched in the growth cones of cultured neurons (Bamburg & Bray 1987). Overexpression of cofilin increases neurite outgrowth (Meberg & Bamburg 2000). The function of LIM-kinase in regulation of cofilin phosphorylation has also been reported recently in the context of growth cone collapse induced by Semaphorin 3A, a repulsive axon guidance cue and a collapsing factor, in primary neuronal cultures (Aizawa et al. 2001). Semaphorin 3A application induces rapid phosphorylation of cofilin at the growth cone. Both dominant-negative LIM-kinase and a peptide mimicking phospho-cofilin block Semaphorin 3A–induced growth

cone collapse. These studies implicate the phosphorylation of cofilin by LIM-kinase in mediating Semaphorin 3A signaling. However, how phosphorylation of cofilin (leading to its inactivation and hence stabilization of the actin cytoskeleton) paradoxically contributes to growth cone collapse remains to be determined.

Genetic analyses in *Drosophila* have further identified Pak as a regulator of axon guidance in photoreceptor neurons (Hing et al. 1999, Newsome et al. 2000). In *Drosophila* photoreceptor axons, Pak appears to receive input from two distinct upstream pathways: Rac GTPases, which appear to be activated by a RhoGEF member Trio (Newsome et al. 2000), and an SH3-SH2-domain-containing adaptor Dock (Garrity et al. 1996, Hing et al. 1999, Newsome et al. 2000). Dock in turn binds to the cell adhesion molecule Dscam, which serves as an axon guidance receptor (Schmucker et al. 2000). These axon guidance molecules thus could function by regulating Pak and thus actin polymerization (Figure 7).

In addition to being activated by Pak, LIM-kinase can also be phosphorylated at the same activating residue by Rho-associated kinase/ROCK (Maekawa et al. 1999, Ohashi et al. 2000), a multidomain serine/threonine kinase that was identified as a downstream effector of the small GTPase RhoA (Figure 7). However, RhoA/ROCK appears primarily to regulate myosin function, as discussed in the next section.

Rho GTPases and Regulation of Myosin Activity

As discussed in the first section of this review, myosin is implicated in at least several morphogenetic processes. Regulation of myosin can control retrograde F-actin flow in the growth cone, which may be important for axon growth and guidance. Myosin can also regulate the interaction of microtubules and the actin cytoskeleton contributing to axon retraction. How do myosins contribute to neuronal morphogenesis?

MYOSIN AND THE GROWTH CONE Non-muscle myosins constitute a superfamily with at least 14 classes (Mermall et al. 1998). This superfamily includes myosin II that is most similar to skeletal muscle myosins and highly concentrated in growth cones (Miller et al. 1992). In addition to myosin II, other nonconventional myosins such as myosin I and myosin V are also present at the tips of growth cones (Espreafico et al. 1992, Miller et al. 1992). Indeed, chromophore-assisted laser inactivation (CALI) experiments suggested that myosin V is essential for filopodial extension in cultured chick dorsal root ganglion (Wang et al. 1996). While myosin I and myosin V likely regulate vesicle trafficking (Mermall et al. 1998), myosin II is implicated in actin cytoskeleton assembly and is by far the best studied.

The primary function for myosin II in nonmuscle cells is to generate contractile forces essential for cell integrity, migration, and cytokinesis. Recently, perturbation experiments have shown that myosin II also regulates growth cone behavior. Treatment with antisense oligos against myosin IIB (one of the two myosin II genes in mammals) reduced neurite extension in neuroblastoma cells (Wylie et al. 1998). This finding of reduced growth with loss of myosin IIB function was

confirmed in recent studies using primary cultured neurons derived from myosin IIB knockout mice (Bridgman et al. 2001). In myosin IIB knockout neurons, growth cones spread less, and actin bundle density in the central growth cone is reduced, as is the traction force as measured by the displacement of beads in a soft gel when growth cones pass through (Bridgman et al. 2001). The fact that myosin IIA is also expressed at the growth cone may explain the mild phenotype of myosin IIB knockout neurons.

What are the likely mechanisms of myosin II function in growth cones? The localization of myosin II provides a hint. Although myosin II was originally described to be uniformly distributed throughout neurites and growth cones (Miller et al. 1992), close examination reveals that both myosin IIA and IIB are highly concentrated at the interface between the central growth cone and the actin rich lamellipodia (Rochlin et al. 1995) (Figure 2). This distribution is remarkably similar to myosin distribution in migrating cells (such as epidermal keratocytes), where the highest myosin concentration is found in the transition zone between the leading lamella and the bulk of the cell body (Svitkina et al. 1997). High-resolution microscopic studies of actin and myosin in migrating keratocytes suggest a dynamic network contraction model. Bipolar myosin II fibers, when attached to the criss-cross F-actin fibers, cause contraction of the actin fiber, forcing them to form actin bundles; in so doing, this process provides the driving force for forward translocation of the cell body (Svitkina et al. 1997). Recently, similar bipolar myosin II filaments have also been reported in neuronal growth cones, albeit at a much lower density (Bridgman 2002). Hence, it is conceivable that myosin II in the growth cone plays an analogous role by forcing criss-cross actin fibers in the lamellipodia to form actin bundles driving the growth cone forward. However, the fact that activation of myosin II also results in axon retraction (see below) suggests that myosin plays additional roles.

REGULATION OF MYOSIN II BY RHOa, ROCK, AND MRLC PHOSPHORYLATION How is myosin II activity regulated? Myosin II is composed of two heavy chains that harbor the motor domain along with two essential and two regulatory light chains. The motor function of myosin is positively regulated by phosphorylation at a key amino acid (ser 19 in mammals) of myosin regulatory light chain (MRLC) (Tan et al. 1992). MRLC (and in particular the sequences near the phosphorylation site) is highly conserved from amoebae to mammals (Tan et al. 1992). Ser 19 of MRLC is phosphorylated by myosin light chain kinase, which in turn is subject to regulation by Ca^{++}/calmodulin (Tan et al. 1992) and Pak (Luo 2000). The same residue is also phosphorylated by Rho-associated kinase/ROCK (Amano et al. 1996). Interestingly, ROCK can positively regulate MRLC in an indirect manner by an inhibitory phosphorylation of MRLC phosphatase (reviewed in Kaibuchi et al. 1999). Thus, ROCK positively regulates MRLC phosphorylation via two independent mechanisms (Figure 8).

ROCK phosphorylates many substrates besides MRLC and MRLC phosphatase (Kaibuchi et al. 1999). How much of ROCK's function is devoted to regulation

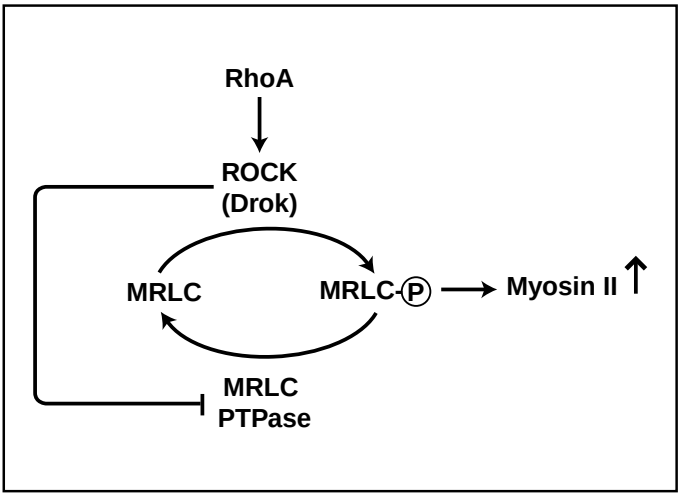

Figure 8 Signaling pathways from RhoA to the regulation of myosin activity. RhoA activates ROCK, which can either directly phosphorylate MRLC or phosphorylate and inactivate MRLC phosphatase (PTPase), leading to increase of MRLC phosphorylation and hence increased activity of myosin II. Abbreviation: ROCK, see Figure 7, Drok, *Drosophila* Rho-associated kinase; MRLC, myosin regulatory light chain.

of MRLC phosphorylation? Recent in vivo data from *Drosophila* provide evidence that regulating MRLC phosphorylation is a major function of Drok, the *Drosophila* homolog of ROCK (Winter et al. 2001). Homozygous *Drok* mutants all die as early larvae. Introduction of a transgene expressing a phosphomimetic mutant of MRLC in the key phosphorylation sites (thr20/ser 21 in *Drosophila*, equivalent to thr18/ser19 in mammals) rescues the lethality of *Drok* mutants such that about 5% of flies emerge as viable adults. In addition, defects in the polarization of F-actin-based wing hair in *Drok* mutants were completely rescued by the phosphomimetic MRLC transgene (Winter et al. 2001). This is surprising given that the phosphorylation status of this phosphomimetic MRLC can no longer be dynamically regulated by the absent Rho/ROCK pathway. Nevertheless these results demonstrate that regulating MRLC phosphorylation is a primary function of ROCK in vivo.

RhoA has many effectors besides ROCK (Kaibuchi et al. 1999). What proportion of the RhoA signals go through ROCK? It appears that ROCK is the principal effector of RhoA in the context of neuronal morphogenesis. The best-studied example of RhoA function in neurons is the regulation of axon and dendrite retraction. RhoA activation has been shown to cause cell rounding and process retraction in PC-12 (Jalink et al. 1994) and neuroblastoma cells (Kozma et al. 1997, Hirose et al. 1998) and to prevent axon outgrowth in primary neurons (Bito et al. 2000). In more physiological settings, RhoA activation also causes dendritic process

elimination of hippocampal pyramidal neurons in organotypic slice cultures (Nakayama et al. 2000), and RhoA-deficient neurons overextend their dendrites in vivo (Lee et al. 2000b). In all cases examined so far, inhibiting ROCK activity with a rather specific inhibitor (Uehata et al. 1997) prevented RhoA-induced process retraction (Hirose et al. 1998, Bito et al. 2000, Nakayama et al. 2000), indicating that ROCK function is necessary for this output of RhoA signaling. Activated ROCK variants could also cause phenotypes similar to RhoA activation, suggesting that ROCK activation alone is sufficient to mimic the activation of RhoA and prevent axon outgrowth (Hirose et al. 1998) and to cause dendritic (Nakayama et al. 2000) or axonal (Billuart et al. 2001) retraction. Thus, ROCK appears to be necessary and largely sufficient in mediating the process retraction caused by activation of RhoA.

What is the cell biological mechanism for neuronal process retraction that occurs when myosin II is activated? One possibility is that myosin II is the motor for retrograde flow in filopodia and lamellipodia (Figure 2). Activating myosin through MRLC phosphorylation would thus increase the speed of retrograde flow. Since actin polymerization at the leading edge could no longer keep up, the net outcome would be retraction of filopodia, lamellipodia, and perhaps the wholesale retraction of the neuronal process. Another hypothesis assumes that myosin-driven forces on the actin cytoskeleton generate tension within the axon, which is usually counterbalanced by the microtubule system. For instance, myosin inhibition has been shown to prevent axon retraction caused by inhibiting microtubule polymerization or dynein inactivation (Ahmad et al. 2000). Hyperactivation of myosin activity, on the other hand, could lead to an imbalance of the two systems such that the retractive effects of the actin/myosin system would overwhelm the extensive effects of microtubules. Further mechanistic insights await future cell biological studies.

VARIATIONS ON A COMMON THEME: VERSATILE ROLES OF RHO GTPase SIGNALING

Rac GTPases Control Axon Growth, Guidance, Branching, and Beyond

Given that there are many different ways to regulate the actin cytoskeleton, it is conceivable that different morphological processes such as axon growth, guidance, or branching may be regulated by distinct mechanisms. A simpler alternative is that all morphogenetic processes are variations on a common theme—one or a few key pathways play fundamental roles in regulating directed actin rearrangement. To achieve the variety of morphogenetic end points in the life of a neuron, the central pathways for regulating the structure of the cell may simply be activated via different receptors; these could give rise to differential signal strengths or use auxiliary pathways to achieve a range of different cellular responses. If there is any central pathway that can modulate all these diverse responses, the Rho GTPases, in

particular Rac, are good candidates for central pathway components. Expression of dominant negative or activated Rac GTPases in neurons has demonstrated that Rac is involved in axon growth, guidance, and dendritic branch dynamics, as well as dendritic spine morphogenesis (Luo 2000). Recent studies using loss-of-function mutants in *C. elegans* and *Drosophila* provide new insights into the functions of Rac GTPases in neuronal development.

In *Drosophila* and *C. elegans*, there are three Rac-like proteins. Two of them, Rac1 and Rac2, are highly related to mammalian Rac1 and Rac2. The third pair, Mig-2 in *C. elegans* and Mig-2-like (Mtl) in *Drosophila*, are more related to each other than to any mammalian counterpart (Newsome et al. 2000). In *C. elegans*, combination of *Rac1* (*ced-10*), *mig-2* mutations, and *Rac2* RNAi (double-stranded RNA interference) in the same cells results in defects in axon growth and guidance (Lundquist et al. 2001). Likewise, *Drosophila* CNS and PNS neurons homozygous mutant for various combinations of *Rac1*, *Rac2*, and *Mtl* genes exhibit defects in axon growth, guidance (Hakeda-Suzuki et al. 2002, Ng et al. 2002), and branching (Ng et al. 2002). All three proteins contribute to the fidelity of axon development in these systems, indicating considerable genetic redundancy. The guanine nucleotide exchange factor UNC-73 in *C. elegans* and its *Drosophila* homolog Trio appear to be key regulators of Rac GTPases in multiple aspects of axon development (Hakeda-Suzuki et al. 2002, Lundquist et al. 2001). Interestingly, when different combinations of mutants of these Rac genes were compared in the same cell type, a differential requirement appeared for the total amount of these Rac proteins for axon growth, guidance (Hakeda-Suzuki et al. 2002, Ng et al. 2002), and branching (Ng et al. 2002). For example, when *Rac* genes are progressively removed in *Drosophila* mushroom body neurons, defects in axon branching are the first to be detected, followed by defects in axon guidance. Only in neurons homozygous mutant for all three Rac genes did axon growth defects become significant.

These studies demonstrate the multifunctionality of Rac in these seemingly different processes (see the first section) and suggest that different levels of Rac activation are needed to instruct growth cones to advance, to turn, and to branch. How could different amounts of Rac activation regulate different downstream processes? Experiments in which *Rac* mutant axons were assayed for rescue by transgenic expression of effector domain point mutants suggested that different downstream pathways are used for regulation of axon branching, guidance, and growth. For instance, expression of a Rac mutant (RacY40C), which cannot bind to a large class of effector proteins including the Pak kinase, is nonetheless capable of rescuing the axon growth defect. However, RacY40C can only partially rescue the guidance defects and cannot rescue the branching defects (Ng et al. 2002). These data suggest that Rac may engage different downstream effector pathways (or different combinations of downstream pathways) to regulate axon growth, guidance, and branching and that differential Rac activation may specifically engage these distinct pathways. These results are reminiscent of earlier experiments in which axon growth and guidance were differentially affected by cytochalasin; axon

guidance was much more sensitive compared with growth (see the first section). Whether there is a mechanistic link between these two observations remains to be tested.

The differential requirements of Rac for axon growth, guidance, and branching also suggest the intriguing possibility that extracellular cues specifying these events activate Rac GTPases to different levels. Many extracellular cues/receptors regulating axon guidance and synaptic plasticity have been linked to Rac signaling. These include (*a*) Netrin through its receptor DCC and unknown intermediates (X. Li et al. 2002); (*b*) Ephrin A through its receptor EphA, which binds directly to a GEF called Ephexin that modulates Cdc42, Rac, and RhoA activity (Shamah et al. 2001) (also see below); (*c*) Semaphorin through its receptor Plexin, which binds directly to Rac-GTP and competes with Rac effectors for Rac binding (Hu et al. 2001) (Figure 9). It is possible that different extracellular cues, by coupling to specific activators/inhibitors of Rac signaling, activate different amounts of Rac

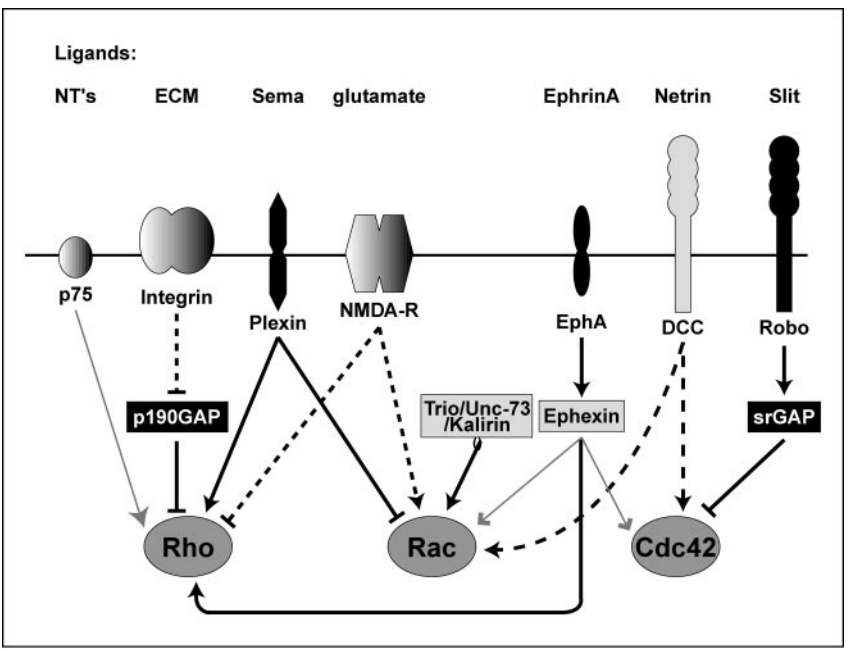

Figure 9 Links between various extracellular cues that affect neuronal development and plasticity to Rho GTPases. The receptors are directly below their corresponding extracellular ligands. Solid arrows represent direct interactions (physical binding of two proteins); dashed arrows represent physical links yet to be established. Gray arrows from p75 to Rho and from Ephexin to Rac and Cdc42 represent those activations that are actually reduced upon ligand binding. See text for detail. Abbreviations: NT, neurotrophin; ECM, extracellular matrix; Sema, Semaphorin; p75, p75 neurotrophin receptor.

GTPases, for different durations, or at different subcellular locations, thereby specifying distinct downstream pathways or combinations thereof. These differences eventually lead to the actin cytoskeletal rearrangements appropriate to specific forms of neuronal morphogenesis.

In extreme cases, it is possible that a single extracellular cue can regulate different aspects of neuronal morphogenesis by activating Rac GTPases at different levels depending on the context of the presentation of the cue. Indeed, multifunctionality of extracellular cues has become more of a rule than an exception. For instance, Slit is typically a repulsive axon guidance cue (see above), but it can also stimulate the growth and branching of axons and dendrites (Wang et al. 1999, Whitford et al. 2002). Likewise, Ephrins/Eph receptors are used for both repulsive axon guidance (Yu & Bargmann 2001) and synaptic plasticity including regulation of dendritic spine morphogenesis (Ethell et al. 2001). For a number of years, Rac has been suggested to be a key regulator of dendritic spine morphogenesis (Luo et al. 1996a, Nakayama et al. 2000, Tashiro et al. 2000). RacGEF Kalirin, the vertebrate ortholog of Trio/unc-73, appears to be a key regulator of Rac in dendritic spine morphogenesis (Penzes et al. 2001). It is conceivable that a similar pathway may be employed for Rac to regulate the rapid dendritic branch addition and retraction seen in a number of different types of developing neurons (Li et al. 2000, Wong et al. 2000), leading to its genetic requirement for dendritic growth and branching (Ng et al. 2002).

RhoA Signaling is Common to Axon Repulsion and Axon/Dendrite Stability

Two well-characterized repulsive axon guidance cues have been shown to engage the RhoA pathway. Ephrin A repels axons by binding to the Eph receptor tyrosine kinase. Ephrin activation results in robust RhoA activation as assayed biochemically by an effector pull-down assay (Wahl et al. 2000). Recent studies have found that EphA receptor binds to a RhoGEF, Ephexin, which activates Rho, Rac, and Cdc42 in vitro. Interestingly, Ephrin A binding stimulates Ephexin's activity toward RhoA but inhibits its activity toward Rac and Cdc42 (Shamah et al. 2001). The second repulsive cue recently linked to the Rho pathway is Semaphorin. Semaphorin repels axons using its receptor Plexin (Yu & Bargmann 2001). The cytoplasmic domain of both vertebrate and *Drosophila* Plexin binds directly to Rac-GTP (Driessens et al. 2001, Vikis et al. 2000). *Drosophila* Plexin also binds to RhoA via a second domain (Hu et al. 2001). Genetically, the effect of Semaphorin-induced axon repulsion through Plexin can be suppressed by reduction of Rac dose and enhanced by reduction of RhoA dose, consistent with the idea that Plexin activates RhoA and inhibits Rac (Hu et al. 2001). Thus, in the case of both of these repulsive cues, activation of RhoA (and downregulation of Rac) appears to mediate axon repulsion.

Given the model that growth cone turning is a result of selective stabilization and destabilization of filopodia/lamellipodia in a spatially regulated manner (Figure 3), it is conceivable that upon contacting repulsive guidance cues, or at the

higher end of a concentration gradient of secreted repulsive cues, RhoA activation leads to myosin II activation via ROCK phosphorylation of MRLC (Figure 8). Then either by an increase in retrograde flow or by an increase in contractile forces, as discussed earlier in this review in the context of axon retraction, filopodia selectively retract, and consequently the growth cone turns away from the source of repulsive cues. Thus, the difference between repulsive axon guidance and retraction could be explained by whether the RhoA signaling pathway is activated locally (part of the growth cone) or globally (the whole growth cone or the entire process).

Interestingly, mature neurons with extensive axons and dendrites appear to have an intact retraction signaling pathway from Rho to myosin activation that is under strict negative control. This notion has been suggested by previous studies (e.g., in dendrites of hippocampal pyramidal neurons; Nakayama et al. 2000) and was recently demonstrated definitively in the context of *Drosophila* mushroom-body neurons. In these neurons, genetic deletion of RhoA or ROCK does not lead to overt axon phenotypes (Lee et al. 2000a, Billuart et al. 2001), suggesting that under physiological conditions the pathway is minimally activated. However, it appears that this pathway is normally kept in check by a negative regulator of RhoA, p190 RhoGAP. Inactivation of p190 RhoGAP causes severe retraction of MB axons. This phenotype is mimicked by activation of RhoA or ROCK and is modulated by the level and phosphorylation state of MRLC (Billuart et al. 2001).

Why is this potentially destructive pathway kept intact in the developed neuron? One attractive hypothesis is that this pathway is utilized for structural plasticity of neurons. It is conceivable that local derepression of the RhoA pathway could lead to rapid changes of neuronal connections in response to experience and learning. Intriguingly, two potential upstream negative regulators for p190 RhoGAP in *Drosophila* MB neurons are integrin and the tyrosine kinase Src. Integrin and Src have indeed been implicated in neural plasticity in MB neurons and other systems (references cited in Billuart et al. 2001).

Morphological Development and Structural Plasticity: Why Reinvent the Wheel?

Many of the mechanisms that regulate structural changes of neurons have been studied in early development, in particular in axon growth and guidance. Given the shared importance of regulating the actin cytoskeleton for initial morphogenesis of neurons and for later changes of neuronal structure in response to experience and learning, it would make sense that similar mechanisms and signaling pathways are employed. Modulation of RhoA signaling in controlling axon and dendrite branch stability in mature neurons provides one such example (Billuart et al. 2001). Future experiments are required to test whether structural changes in neurons that involve reorganization of entire dendritic or axonal branches could contribute to learning and experience-dependent changes in neuronal connectivity in the adult brain.

Neurotrophins are a family of proteins implicated in mediating activity-dependent plasticity, including changes in neuronal structure (for a recent review, see Poo 2001). Neurotrophins are potent regulators of dendritic morphogenesis as are Rho GTPases (reviewed in McAllister et al. 1999, Scott & Luo 2001). A direct link has been established between these two classes of proteins: The p75 neurotrophin receptor binds directly to RhoA and activates it. Binding of neurotrophin to p75 abolishes this association and thus downregulates RhoA activity presumably to allow for the observed neurite outgrowth (Yamashita et al. 1999). It would be interesting to test whether this mechanism contributes to the structural plasticity induced by neurotrophins.

Learning and experience induce electrical impulses and the activation of neurotransmitter receptors. If structural changes result, they should activate signaling pathways leading to actin cytoskeletal reorganization. A link between activation of the NMDA subtype of glutamate receptor and modulation of RhoA activity has been suggested (Li et al. 2000). Recent experiments using an in situ method to detect Rho GTPase activation demonstrated that electrical stimulation of retinal ganglion cells in the optic nerve of *Xenopus* altered the activity of Rho GTPases in their target neurons in the tectum (Z. Li et al. 2002). Visual system activity also leads to increased Rac activity and decreased Rho activity (Z. Li et al. 2002), as well as robust dendritic arbor growth (Sin et al. 2002). These changes were also dependent on functional NMDA receptors. Figuring out how NMDA receptor activation could lead to Rho GTPase activation (Figure 9) will now become an important topic, as it is a direct bridge between our understanding of neuronal development and neural plasticity and learning.

SUMMARY AND PERSPECTIVE

Actin has long been postulated to play important roles in neuronal morphologenesis based on (*a*) its high concentration in the leading edge of the growth cones of developing neurons and in synapses of mature neurons and (*b*) cell biological studies using drugs that interfere with actin polymerization and myosin function. Recent studies on signaling pathways that regulate the actin cytoskeleton not only verify its function in myriad neuronal morphogenetic processes, but also start to link the regulation of actin dynamics to extracellular signals known to regulate different aspects of morphogenesis, such as the growth, guidance, branching, and stability of axons and dendrites. In addition, as exemplified by studies on Rho GTPases discussed in the second and third sections of this article, an exciting theme emerges that neurons use variations of the same core signaling pathways to regulate different aspects of morphogenesis.

I expect that this theme will develop further in the near future in several different ways. First, with regard to Rho GTPase signaling, functional studies will be performed on more components of upstream regulatory and downstream effector pathways in neurons. These studies will give us more insight into how common signaling pathways could be used differently to regulate different morphological

events. Second, Rho GTPases and their signaling pathways will be studied in aspects of neuronal morphogenesis whose mechanisms, such as axon initiation and branching, dendrite morphogenesis and synapse formation and stability, are less understood. These studies will benefit from comparison with better-studied processes, notably axon guidance and retraction. Third, I expect that other signaling pathways regulating the actin cytoskeleton not covered in this article may also follow this theme. This would make a lot of sense from both cell biological and evolutionary principles.

Although the actin cytoskeleton plays a major role in regulating neuronal morphology, microtubules and membrane trafficking (such as exocytosis and endocytosis) are also clearly essential. Understanding how these events are coordinated to bring about the morphological changes of a neuron will be a major challenge in the future. Interestingly, Rho GTPases, although well known for their regulation of the actin cytoskeleton, are also implicated in regulating microtubules (reviewed in Wittmann & Waterman-Storer 2001) and membrane trafficking (reviewed in Ridley 2001), further expanding the versatility of these core signaling pathways.

These are exciting times for neurobiologists to reduce their favorite problems to mechanistic understanding in cell biological terms and for cell biologists to contribute their expertise to the understanding of brain wiring and function.

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