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Signal-Regulated ADF/Cofilin Activity and Growth Cone Motility

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Abstract

It is becoming increasingly evident that proteins of the actin depolymerizing factor (ADF)/cofilin family are essential regulators of actin turnover required for many actin-based cellular processes, including motility. ADF can increase actin turnover by either increasing the rate of actin filament treadmilling or by severing actin filaments. In neurons ADF is highly expressed in neuronal growth cones and its activity is regulated by many signals that affect growth cone motility. In addition, increased activity of ADF causes an increase in neurite extension. ADF activity is inhibited upon phosphorylation by LIM kinases (LIMK), kinases activated by members of the Rho family of small GTPases. ADF become dephosphorylated downstream of signal pathways that activate PI-3 kinase or increase levels of intracellular calcium. The growth-regulating effects of ADF together with its ability to be regulated by a wide variety of guidance cues, suggest that ADF may regulate growth cone advance and navigation.

Index Entries: Actin depolymerizing factor; cofilin; growth cone; actin filaments; phosphorylation; LIM kinase; Rho family GTPases.

Introduction

Over the past few years, tremendous advances have been made in elucidating signal pathways that regulate actin dynamics as well as the actin-binding proteins that regulate actin-based cell motility in nonneuronal cells. Intracellular signals that regulate actin dynamics in growth cones are similar to those functioning in motile nonneuronal cells. For example, the Rho family of GTPAses (Rho, Rac, and Cdc42) were initially found essential for the formation of lamellipodia, filopodia, and actin stress fibers in nonneuronal cells *(1)*, and have been found to mediate not only lamellipodia and filopodia formation in primary neurons, but also responsiveness to certain guidance cues *(2–6)*. In neurons, significant progress has been made not only in identifying guidance

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cues, but also the receptors and downstream signal pathways that control growth cone motility *(7).* Less is known of which specific cytoskeleton-regulating proteins are effectors for these signals, but several likely candidates are emerging, including proteins of the ADF/cofilin family. This review will briefly summarize the characteristics of ADF/cofilin, then discuss evidence that suggests an integral involvement of ADF/cofilin in the regulation of growth cone motility. Other recent reviews provide more in-depth discussions of the biochemical properties of ADF and its function in nonneuronal cells *(8–10).*

Actin-Based Motility of Growth Cones

Actin polymerization supplies the protrusive force responsible for process extension in many cell types *(11).* In growth cones, actin filaments are concentrated at the periphery and extend into filopodia *(12).* Growth cone motility is reliant on actin polymerization *(13,14),* as is navigation in response to certain extracellular cues. Treatment of neurons with cytochalasins, which inhibit actin polymerization, eliminates most growth cone structure, alters the rate of neurite outgrowth *(15),* and impairs growth cone navigation *(16).* F-actin preferentially accumulates in growth cone regions that contact positive guidance cues *(17,18),* whereas F-actin concentrations in growth cones are often decreased by inhibitory factors *(5,19,20).* However, excessive accumulation of F-actin in growth cones may also inhibit growth cone advance *(2,4).* These findings indicate that actin dynamics are central to the regulation of growth cone morphology and movement. Therefore, determining which signal-regulated proteins bind actin and regulate actin dynamics in response to extracellular cues will be essential for fully understanding growth cone navigation to appropriate targets during development.

Basic Components Required for Actin-Based Motility

The basic mechanisms of actin-filament turnover are highly conserved and utilized for motility in a wide variety of cell types and organisms, from mammalian neuronal growth cones to fish keratocytes to pathogens traveling within eukaryotic cells. At steady-state concentrations of actin in cells, actin monomers are preferentially added at the barbed end of filaments and lost from the pointed ends. In motile cells, incorporation of monomer occurs predominately at the leading edge *(21),* creating a propulsive force that theoretically is capable of pushing lamellipodia and filopodia forward in the absence of myosin contractility *(11,22).* In growth cones, most fast-growing barbed ends of actin filaments are oriented towards the membrane *(12),* and membrane protrusion is regulated by assembly of actin filaments at the leading edge and disassembly at the central domain *(14).* This same treadmilling machinery used for filopodia and lamellipodia extension in neurons is exploited by certain pathogens, such as *Listeria monocytogenes,* for propulsion inside eukaryotic cells *(22–24).*

A recent study has identified minimal components required for actin-based motility in vitro using purified components *(25).* Actin treadmilling and propulsion of bacteria require only the Arp2/3 complex, an activator of Arp2/3 (*Listeria* ActA or activated N-WASP), capping protein, and ADF *(25).* In this model activated Arp2/3 promotes actin polymerization by crosslinking and nucleating new filaments and capping pointed ends, capping protein blocks actin monomer addition at barbed ends outside the active zone, and ADF promotes barbed-end polymerization and treadmilling by recycling monomers from pointed ends. Other proteins that enhanced motility in this system, but were not essential were profilin, α-actinin, and VASP.

Because *Listeria* can travel from peripheral nerve endings to the brain within axons, these

basic components are certainly present in neurons and likely also utilized for growth cone motility. Therefore the essentiality of these proteins for regulating membrane protrusion in growth cones should be investigated, as well as the potential for these proteins to serve as convergence points for a wide variety of growth-regulating signals. Profilin, Ena/VASP family members, and N-WASP have all recently been found essential for normal axonal outgrowth *(26–28).* Based on their localization in growth cones, the Arp2/3 complex and α-actinin may be essential as well *(24,29).* Recent evidence also suggests that ADF is a central component for signal-regulated growth cone motility.

ADF Regulation of Actin Dynamics

ADF and cofilin are actin-monomer sequestering, F-actin depolymerizing proteins that are closely related in sequence, have similar activities and can be considered isoforms *(30).* ADF/cofilins are ubiquitously expressed in eukaryotes, with activities conserved across phyla. For example, cofilin mutations in yeast are lethal, but rescued by addition of mammalian ADF *(31).*

Both ADF and cofilin are expressed in neurons, but ADF levels appear much higher than cofilin. For example, ADF levels are more than twofold greater than cofilin in embryonic chick brain *(32).* Colocalization of ADF/cofilin and actin to ruffling membranes and other regions of high actin-filament turnover first suggested that ADF/cofilin regulated actin dynamics during membrane extension. ADF binds to actin filaments at the leading edge of lamellipodia, especially in regions where actin filament disassembly occurs *(33–35),* and is highly expressed in both axonal and dendritic growth cones *(36,37).* Relative to total protein, ADF is enriched 5–10-fold in growth cone particle extracts compared to whole-brain extracts from embryonic chicks *(4).*

An important characteristic of ADF/cofilin is a greater affinity for ADP-actin than ATP-actin *(9,10).* Most actin monomers in vivo bind ATP, but incorporation at the barbed end of filaments is followed by conversion to ADP-actin. ADF therefore binds preferentially to actin subunits nearer pointed ends and ADF binding increases the rate of ADP-actin removal. Subsequent nucleotide exchange, enhanced by profilin, then increases the concentration of ATP-actin monomers available for polymerization, speeding growth at barbed ends. Hence ADF/cofilin increases steady-state treadmilling of filaments and accounts in great part for the enhanced rates of actin turnover and membrane protrusion observed in vivo, compared to that observed for pure actin in vitro *(9,10).* ADF/cofilin can also speed polymerization by severing actin filaments and thereby providing additional uncapped, free barbed ends for polymerization *(30,35,38).* In summary, the name "actin depolymerizing factor" is misleading, because ADF/cofilins do not simply depolymerize and reduce levels of F-actin, but rather increase F-actin turnover.

Regulation of ADF Activity

A major mechanism for regulating the activity of ADF/cofilin proteins is by phosphorylation *(30).* Phosphorylation at a single site (ser3) inhibits actin-binding and F-actin depolymerization *(39,40).* In vivo studies confirm the importance of ADF/cofilin phosphorylation. Cytokinesis, an actin-dependent process, is blocked in Xenopus blastomeres by injections of ADF/cofilin mutants that cannot be phosphorylated *(41),* but not by injections of wild-type ADF/cofilins *(42).* ADF/cofilin depolymerizing activity is also inhibited by PIP_2 and tropomyosin, and enhanced by increased pH in biochemical assays, although the physiological relevance of these characteristics has not yet been confirmed *(30).*

Much of ADF/cofilin in cells is in the phosphorylated, inactive form. Circumstantial evidence from many cell types suggests that signal-induced ADF/cofilin dephosphorylation is important for regulating actin dynamics

and altering cell morphology *(30,43).* ADF/cofilin is also the only in vivo substrate for LIM kinase-1 *(44,45).* Hemizygous deletions of the LIMK-1 gene have been implicated in the abnormal cortical development associated with Williams syndrome *(46),* although this finding remains controversial *(47).* This suggests that even a limited disruption of ADF phosphoregulation can result in developmental brain defects.

Effects of Guidance Cues and Downstream Signal Pathways on ADF Activity

A major pathway for regulating ADF/cofilin in growth cones is via activation of Rho family small GTPases, because Cdc42, Rac1, and Rho activate LIM kinases *(44,45,48–50).* LIM kinases are activated downstream of Rho family GTPases by phosphorylation at serine 508 by Pak1 *(48)* or the Rho-associated kinase ROCK *(51).* Rac1 is an important regulator of actin dynamics that is essential for axonal outgrowth *(2)* and growth cone responses to certain extracellular cues, such as Sema3A *(3,5).* Because Rac1 activates LIMK-1, some of the effects of Rac on neuronal morphology are likely due in part to modulation of ADF activity. The small GTPases Rho and Cdc42 are also important modulators of neurite outgrowth *(5,6,52),* and both activate LIMK-2 and increase ADF/cofilin phosphorylation *(49,50).*

A wide variety of intracellular signal pathways promote the dephosphorylation (activation) of ADF/cofilin in neurons *(33).* When $[Ca^{2+}]_i$ is raised to levels that induce filopodia extension using the calcium ionophore A23187, ADF is dephosphorylated in a calcineurin-dependent manner. Increased cAMP levels also promote ADF dephosphorylation, but in a calcineurin-independent manner. Basal dephosphorylation of ADF in unstimulated cells is greatly attenuated by inhibitors of protein phosphatases 1 and 2A, but minimally affected by calcineurin inhibitors. Inhibition of PI-3 kinase also blocks most ADF dephosphorylation in cultured neurons. The identity of this primary phosphatase for ADF, stimulated downstream of PI-3 kinase, has not been discovered, but may be a novel phosphatase based on certain experimental results *(30,33).* Because the PI-3 kinase pathway and the second messengers Ca^{2+} and cAMP are common downstream effectors of a wide variety of growth cone guidance cues, including neurotransmitters, growth factors, adhesion proteins, netrins, and MAG *(7),* ADF/cofilin may be a common target of all these cues (Fig. 1). In support of this, both glutamate *(37)* and NGF *(33)* decrease levels of phosphorylated ADF/cofilin in neurons.

Studies of signal-induced changes in ADF phosphorylation in response to growth-regulating cues suggested that increased ADF activity promotes lamellipodia and filopodia extension in neurons, as well as growth cone advance *(33).* For example, when serum-deprived PC-12 cells are treated with NGF, levels of phosphorylated ADF and cofilin decrease by more than 50% after 10 min, and ADF and cofilin accumulate and co-localize with actin at the tips of lamellipodia and some filopodia. Decreased phosphorylation of ADF and cofilin is maintained for more than an hour, during which time extensive membrane ruffling and neurite extension occur. NGF-induced dephosphorylation of ADF and cofilin may be downstream of PI-3 kinase and Rac activation, because PI-3 kinase is rapidly activated by NGF and required for neurite outgrowth in PC-12 cells *(53),* and growth factor and ras-mediated activation of Rac1 that leads to membrane ruffling is mediated by PI-3 kinase *(1,54).* In contrast, treatment of cortical neurons with lysophosphatidic acid (LPA), a cerebrospinal fluid constituent that inhibits neurite outgrowth in a Rho-dependent manner *(55),* decreases ADF activity by increasing levels of phosphorylated ADF. Interestingly, LPA-induced neurite retraction is prevented by dibutyryl-cyclic AMP treatment *(56),* a treatment which decreases levels of phosphorylated ADF either by activating a phosphatase

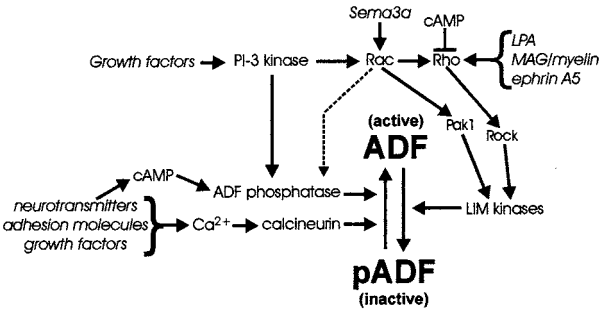

Fig. 1. Major signal pathways linking growth-cone navigational cues to changes in ADF activity. Selected major classes of growth-regulating signal molecules together with some specific extracellular cues are shown. Arrows indicate stimulatory pathways. Major bifurcation points for simultaneous stimulation of both the ADF phosphatases and kinases are at PI-3 kinase, and possibly Rac. Based on inhibitor results, the "ADF phosphatase" may be of protein phosphatase type 1 or 2A. Cyclic AMP may either stimulate ADF dephosphorylation or inhibit Rho. Additional details and descriptions are provided in the text (for simplicity, many intermediate signaling molecules are not shown). Cdc42 can also activate Rac1 and Pak1, but is not shown.

or inhibiting Rho, and therefore LIMK activity *(33)*.

That ADF/cofilin becomes phosphorylated downstream of both Rac and Rho activation may appear inconsistent with the hypothesis that increased ADF/cofilin activity spurs neurite growth or lamellipodia extension, because lamellipodia extension is promoted by Rac but inhibited by Rho. Although Rac1 activation does increase ADF/cofilin phosphorylation, as measured by enhanced ^{32}P incorporation *(44,45)*, no measures were reported of *net* phosphorylation levels. Rac1 activation may also increase ADF/cofilin dephosphorylation, as reported after injections of constitutively active Rac1 into Xenopus oocytes *(57)*. How Rac may activate the ADF phosphatase is presently unclear. Related to this, epidermal growth factor (EGF) treatment of serum-starved fibroblasts, which activates Rac and induces rapid lamellipodia extension *(58,59)*, greatly reduces the phosphate half-life on ADF/cofilin without affecting net phosphorylation levels *(33)*. Thus, both ADF phosphorylation and dephosphorylation are stimulated by EGF. Because EGF-stimulated lamellipodia extension appears to depend on ADF/cofilin *(35)*, this enhanced phosphate turnover without net phosphorylation change could be sufficient for altering F-actin dynamics in two related ways. First, spatially distinct locations for phosphorylation and dephosphorylation would allow for ADF/cofilin activation and enhanced actin dynamics in only certain, discrete cellular domains. Second, dephosphorylation would allow monomer removal from pointed ends, whereas transient phosphorylation would release ADP-actin monomers bound to ADF, enhancing nucleotide exchange and ATP-actin addition to barbed ends, and therefore actin treadmilling *(10)*.

Increased ADF/Cofilin Activity Enhances Neurite Outgrowth

To test directly if changes in ADF activity would increase growth cone advance, ADF/cofilin activity was increased in primary cultured cortical neurons using recombinant adenovirus-mediated expression of Xenopus ADF/cofilin (XAC) *(60)*. Increased ADF/cofilin expression increased neurite lengths by more than 50% compared to uninfected controls or controls infected with inactive, phosphorylation-mimic XAC(E3) mutants. The magnitude of this effect is rather surprising, considering it is a single actin-binding protein and not a signal molecule that regulates multiple substrates or signal-transduction pathways. In addition, based on expression and phosphorylation levels, expression of XAC(wt) increased levels of active (unphosphorylated) ADF/cofilin by <20%. Interestingly, wild-type XAC, which was over 50% phosphorylated, increased neurite lengths to a greater extent than the constitutively active XAC(A3) mutant which cannot be phosphorylated. Even though A3 mutants have only half the binding affinity of wild-type ADF *(40)*, expression of XAC(A3) would be

predicted to supply at least the same level of net ADF/cofilin activity. Taken together, these results suggest that even relatively modest changes in ADF phosphorylation may have significant effects on neurite outgrowth, and regulated phosphate turnover on ADF/cofilin may be of great importance.

Despite having considerable impact on neurite extension, ADF/cofilin overexpression has relatively minimal impact on growth cone morphology *(60).* Growth cone lamellipodia and filopodia remained highly motile and no qualitative differences were observed after ADF/cofilin overexpression. Quantitative analysis revealed slight increases in growth cone area (14%) and numbers of filopodia (12%), while filopodia number on neurite shafts proximal to the growth cones decreased (11%). F-actin levels in growth cones also decreased, but only slightly. However, F-actin turnover appeared to increase, based on growth cone collapse and rates of F-actin disappearance after treatment with drugs that block actin polymerization. Tubulin immunoreactivity was also slightly higher in growth cones, suggesting increased invasion of microtubules into the growth cones.

How Does ADF/Cofilin Activity Affect Growth Cone Motility?

The appreciable effect of relatively small changes in ADF/cofilin activity provides a compelling argument for an integral role of ADF/cofilin in regulating growth cone motility. However, whether ADF/cofilins set a base rate of actin turnover in growth cones that affects only a default rate of advance, or whether changes in ADF/cofilin are also required for growth cone turning in response to navigational cues, remains to be determined. The mechanism of the growth-promoting effect also remains to be elucidated, and this mechanism may illuminate potential ways of affecting growth cone navigation.

The effects of increased ADF/cofilin on growth cone advance could be accounted for by three interrelated mechanisms. Increased rates of growth cone advance may be achieved by either a decrease in the rate of retrograde actin flow, or increases in the rate of actin polymerization at the leading edge, two processes that may be molecularly independent *(23).* If ADF were to increase the rate of actin filament treadmilling without the rate of retrograde flow changing, then lamellipodia extension and growth cone advance should increase. This first mechanism would be similar to array treadmilling models described for motile nonneuronal cells and *Listeria* propulsion *(22).*

A second potential mechanism would be similar to models of "treadsevering" *(22,35).* If the ends of filaments are capped, ADF/cofilin may sever filaments, providing free barbed ends for polymerization. The end result would again be an increase in actin-filament turnover, because additional pointed ends would also be available for depolymerization. For example, EGF treatment of MTLn3 cells stimulates lamellipodia extension and an increase in free barbed ends, and both these effects are blocked by inhibition of cofilin *(35).* Because ADF/cofilin can sever or depolymerize filaments, its precise function in growth cones may not be easily predicted. Even within individual cells, an array treadmilling model may best describe lamellipodia extension, while treadsevering may occur in filopodia *(22).* Severing of filaments has been noted in actin bundles that form growth cone filopodia, even while retrograde flow predominates in lamellipodia of the same growth cone *(61).* In other types of neurons, filopodia extension appears dependent on actin treadmilling, albeit at slower turnover rates than observed in lamellipodia *(62).*

The third potential mechanism for ADF/cofilin increasing growth cone advance may relate to the potential of actin filaments to prevent microtubule invasion into growth cones *(15).* If ADF/cofilin increases actin filament depolymerization, this would allow increased microtubule extension into growth cones. It has been suggested that contact with targets, which increases F-actin at contact sites but also depletes actin filaments at proximal

regions, allows microtubule invasion that promotes and maintains accelerated growth *(17).* Also related to this, the formation of axons, which are much faster growing than dendrites, is accompanied by an enlargement of the growth cone and an increase in actin instability that may allow increased microtubule invasion and delivery of cellular organelles *(63).* Both growth cone size and F-actin instability are increased by ADF/cofilin overexpression *(60).*

These three mechanisms for ADF/cofilin effects on growth cone motility are obviously not mutually exclusive and all are consistent with the effects of ADF/cofilin overexpression on growth cone morphology as well as F-actin and tubulin content in growth cones. The actual function of ADF/cofilin in relation to growth cone motility still needs to be determined experimentally. The relative involvement of each mechanism may very well differ depending on the neuronal cell type involved, signal-transduction pathways engaged, and other actin-binding proteins present. In addition, growth cone motility is obviously regulated by other mechanisms as well, such as myosin-dependent changes in retrograde actin flow after contact with guidance cues *(23).* ADF/cofilin is unlikely to be involved in this process, unless it disrupts actin networks that are normally stabilized by substrate adhesion.

Does ADF/Cofilin Play a Role in Growth Cone Navigation?

Spatial and temporal differences in ADF/cofilin activity within growth cones may contribute to growth cone navigation by affecting the location and degree of actin assembly/disassembly. Rates of actin assembly and turnover can vary independently even among filopodia within single growth cones *(62).* Any of the three aforementioned mechanisms of ADF function may serve to promote growth cone turning after asymmetric ADF activation or inactivation in growth cones, especially if bifurcating signal pathways affecting phosphorylation and dephosphorylation differ in their spatial extents. For example, if the ADF phosphatase is only activated in the growth cone at sites contacting guidance cues while LIMK is activated throughout the growth cone, filament turnover and barbed-end polymerization would be greatest nearer the cue and allow for asymmetric lamellipodia and filopodia extension, and subsequent growth cone turning. A similar response could occur with diffusible factors. Asymmetric exposure to neurotransmitters causes a Ca^{2+}-dependent turning of growth cones toward the side of highest concentration *(64,65),* and inhibition of calcineurin decreases filopodia extension *(66).* These calcium-dependent processes are likely to affect ADF/cofilin phosphorylation in growth cones, with the asymmetric activation of ADF/cofilin then contributing to growth cone turning.

ADF/cofilin could certainly also be involved in growth cone turning away from inhibitory cues, such as Sema3A, ephrin A5, MAG, and myelin, because these all appear to signal through Rac and/or Rho *(3,5,67,68),* activators of LIM kinases. If LIM kinases reduce ADF activity near the site of inhibitory cue contact, the resulting asymmetry of ADF/cofilin activity across the growth cone could result in a turning away from the cue. In contradiction to this prediction, it has been suggested that ADF activity may actually be increased by Sema3A, because Sema3A appears to increase ADF immunoreactivity in growth cones without obvious changes in phosphorylated ADF/cofilin (pAC) immunolabeling *(20).* However, as discussed earlier, Rac may activate the ADF phosphatase in addition to LIM kinase (Fig. 1), with ADF activity being enhanced at more distal growth cone regions, while being decreased in regions nearer the cue. Alternatively, if ADF activity is increased at the same time that barbed ends are capped, F-actin levels would decrease and growth cone collapse could occur. Further experiments are needed to elucidate the potential role of ADF/cofilin in growth cone responses to these cues.

Even with an antibody specific for pAC *(33)* it will be difficult to assay changes in ADF phosphorylation within growth cones after contact with guidance cues, especially phosphorylation differences across growth cones. Long fixation times are required in order to retain pAC immunoreactivity after subsequent permeabilization (Meberg and Bamburg, unpublished observations), an indication that pAC not bound to actin can readily diffuse throughout the growth cone for some time before being crosslinked and later visualized. In addition, as noted for EGF, even without measurable net changes in phosphorylation, increased phosphate turnover on ADF/cofilin may serve to promote actin turnover. Therefore disruption of phosphoregulation may be a better approach for studying the role of ADF in growth cone responses to guidance cues.

ADF/Cofilin and Neurite Degeneration

Certain guidance cues promote rapid F-actin loss and growth cone collapse. It is unlikely that dephosphorylation and increased ADF/cofilin activity alone is directly responsible for this rapid F-actin loss, based on its functional characteristics described earlier. However, certain pathological signals may very well work through ADF/cofilin to inhibit neurite outgrowth in an unusual manner. ATP depletion, oxidative damage, or high glutamate concentrations can induce a marked dephosphorylation of ADF and formation of long-lasting rod-like inclusions in neurites that contain ADF and actin *(37).* These ADF-actin rods might block axonal transport and decrease growth cone actin levels, thereby preventing outgrowth. Because increased ADF/cofilin activity normally appears to increase neurite outgrowth, formation of these rods likely requires more than ADF dephosphorylation alone. Rod formation has been proposed to occur by ADF dephosphorylation in concert with decreased pH, lack of ATP, or insufficient profilin available to stimulate nucleotide exchange *(37).* These conditions would promote the formation of ADF-ADP-actin complexes at concentrations high enough to coalesce into rods. It should be noted that phalloidin does not bind to these actin rods *(37),* and ADF binding to F-actin also blocks phalloidin binding *(69).* Because of this, decreased F-actin levels determined by phalloidin staining after cell fixation may reflect increased ADF/cofilin activity, rather than actual decreases in F-actin levels.

Conclusions

ADF/cofilin is an essential regulator of actin-based motility in a wide variety of systems. In neurons, an increase in ADF/cofilin activity may speed the advance of growth cones by increasing actin polymerization at the leading edge, or possibly by altering F-actin-microtubule interactions. Because ADF/cofilin activity is regulated by many signal pathways that regulate growth cone motility, ADF/cofilin is likely a downstream target of many extracellular guidance cues. However, the relative importance of ADF/cofilin for growth cone navigation remains to be determined. This can be tested by disruption of ADF/cofilin phosphoregulation, using mutant forms of LIMK and ADF/cofilin, and observing if growth cone responses to certain guidance cues are attenuated. It also needs to be determined if ADF/cofilin is essential for growth cone motility, or if other actin-regulating proteins in growth cones are sufficient.

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