REVIEW

Small GTPases 5, e27974; March 2014; © 2014 Landes Bioscience

Involvement of Rho-family GTPases in axon branching

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Keywords: interstitial branching, axon sprouting, actin filaments, filopodia, microtubules, lamellipodia, actin patch, collateral branching, axonal protein synthesis, mitochondria

Development of the nervous system requires efficient extension and guidance of axons and dendrites culminating in synapse formation. Axonal growth and navigation during embryogenesis are controlled by extracellular cues. Many of the same extracellular signals also regulate axonal branching. The emergence of collateral branches from the axon augments the complexity of nervous system innervation and provides an additional mechanism for target selection. Rho-family GTPases play an important role in regulating intracellular cytoskeletal and signaling pathways that facilitate axonal morphological changes. RhoA/G and Rac1 GTPase functions are complex and they can induce or inhibit branch formation, depending on neuronal type, cell context or signaling mechanisms. Evidence of a role of Cdc42 in axon branching is mostly lacking. In contrast, Rac3 has thus far been implicated in the regulation of axon branching. Future analysis of the upstream regulators and downstream effectors mediating the effects of Rho-family GTPase will provide insights into the cellular processes effected, and shed light on the sometimes opposing roles of these GTPases in the regulation of axon branching.

Introduction

The function of the nervous system depends on the establishment and maintenance of synaptic circuitry. Each neuron generates a single axon, but makes synaptic contacts with many target neurons, often in different parts of the nervous system. The formation of axon branches from the main axon shaft is fundamental to the ability of neurons to establish such complex patterns of connectivity during development. In addition to their significant role in the developing nervous system, axon branches also emerge from intact and damaged axons in response to external injury or neurodegeneration resulting in remodeling and adaptive plasticity of the adult nervous system.[1,2]

Axon branches can arise from phenomenologically distinct methods (reviewed in 3). As the growth cone is navigating, it can split in half generating two separate growth cones independently leading two axonal branches, termed bifurcation of the axon. In contrast, axonal collateral branches (also referred to as interstitial branches or axon sprouting) emerge from protrusive filopodia and lamellipodia initiated along the shaft of the axon. In one form of this mechanism, the protrusive activity is reflective of sites along the axon's path at which the growth cone stalled, leaving behind a domain of protrusive activity, followed by resumption of its advance. In a related and more common form of collateral branching, protrusive activity is generated de novo, in the form of filopodia or lamellipodia, from the otherwise generally quiescent axon shaft.

The cytoskeletal mechanisms of axonal branching are orchestrated by Rho GTPases. RhoA, Cdc42 and Rac are the most studied mammalian GTPases. Cdc42 activity has not been implicated in branch formation, although Cdc42 is involved in axon formation and growth cone filopodia formation.[4] The role of RhoA in formation of axonal branches is complicated and not universal among neurons. In response to RhoA signaling, the actin cytoskeleton is rearranged to support growth cone collapse and axon retraction.[5] In some cases, RhoA has been shown to inhibit the emergence of protrusive activity from the axon, and thus negatively regulate axon branching.[6-8] In contrast, other studies revealed that RhoA activity promotes axon branching.[9,10] Similarly, Rac1 GTPase facilitates and prevents axonal branching in different systems, whereas Rac3 and RhoG have a positive effect in axonal branch regulation. This review will focus on the role of Rho-family GTPases in mechanisms of axon collateral branching by primary neurons (summarized in **Table 1**).

Overview of the Cytoskeletal Mechanism of Axon Collateral Branching

The actin and microtubule cytoskeleton is critical for collateral branching (**Fig. 1A**). The first step in the formation of a collateral branch involves the actin filament dependent initiation of axonal filopodia, and in some cases lamellipodia. As formation of axonal filopodia is the most common first step in branch emergence (reviewed in ref. 3), this review will largely focus on this issue. Unlike growth cones, the shaft of axons contains relatively low levels of actin filaments and protrusive activity.[11] However, the axon is still capable of generating filopodia and extracellular

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Submitted: 09/28/2013; Revised: 01/06/2014; Accepted: 01/23/2014;
Published Online: 03/11/2014
Citation: Spillane M, Gallo G. Involvement of Rho-family GTPases in axon branching. Small GTPases 2014; 5:e27974; PMID: 24936971; http://dx.doi.org/10.4161/sgtp.27974

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Table 1. Summary of reported roles of RhoA-Family GTPases in axon branching

		Pathway			
GTPase	**Branching**	**Upstream**	**Downstream**	**Neuron type**	**Reference**
RhoA	Promotes	Neuronal activity	N/A	Cortical in situ	10
	Promotes	N/A	N/A	Hippocampal in vitro	9
	Inhibits	NGF	Myosin II	Chicken sensory in vitro	6
	Inhibits	FAK-P190RhoGEF	N/A	Purkinje in vivo/in vitro	8
RhoG	Promotes	Kalirin-GEF1	N/A	Sympathetic in vitro	66
	Inhibits	N/A	ELMO-Dock180-Rac1	Hippocampal in vitro	74
Rac1	Promotes	NGF-PI3K	N/A	Chicken sensory in vitro	32
	Promotes	Branching induced by contact with repellent cell	N/A	Chicken retinal in vitro	5
	Promotes	N/A	Pak	*Drosophila* mushroom body neurons in vivo	60
	Promotes	VAV2 GEF	N/A	Xenopus spinal cord neurons in vitro	62
	Inhibits	N/A	N/A	Purkinje in vivo	59
	Inhibits	N/A	N/A	*Drosophila* giant fiber in vivo	58
Rac2	Inhibits (loss of function)	N/A	UNC-115	*C. elegans* sensory in vivo	61
	Promotes (constitutively active)	N/A	UNC-115	*C. elegans* sensory in vivo	61
Rac3	Promotes	N/A	N/A	Chicken retinal in vitro	65
Cdc42	Promotes	Branching induced by contact with repellent cell	N/A	Chicken retinal in vitro	5
	No role	NGF-PI3K	N/A	Chicken sensory in vitro	32

The column labeled "Branching" reflects whether the GTPase was found to promote or inhibit branching. The column labeled "Pathway" denotes whether up or downstream components were investigated. Branch-inducing signals are included in the upstream category. N/A indicates none addressed. The "Neuron type" column denotes the type of neuron investigated, and whether the study was in vitro, in situ (e.g., in a tissue slice), or in vivo. Unless otherwise noted in the "Neuron type" column, studies were performed in mammalian systems.

signals that promote collateral branching drive the formation of axonal filopodia. Direct evidence for the requirement of filopodia in collateral branching has been provided by filopodia elimination studies. Specific depletion of Enabled (xENA)/xVASP (vasodilator-stimulated phosphoprotein) proteins in *Xenopus* retinal axons in vivo results in severe impairments of terminal axon branching,[12] without major effects on axon extension and path finding.

Although axons generate multiple filopodia during the process of branching, only a subset of these filopodia mature into collateral branches. The maturation of filopodia into branches requires the invasion of the filopodium by axonal microtubules, which must then be stabilized in situ in order to allow the transformation of the filopodium into an axon branch (**Fig. 1A**). Microtubules can be targeted into axonal filopodia both through the dynamic instability of microtubule tips, or the transport of microtubules into the filopodia in a cell and/or context dependent manner.[3] The targeting of microtubules into filopodia is considered to allow for the directed transport of organelles and proteins into the filopodium, culminating in the transformation of the filopodium into a bonafide axon branch capable of continued extension.

Mechanism of the Formation of Axonal Filopodia

Filopodia are thin, finger-like extensions mainly composed of a bundle of parallel actin filaments and actin associated proteins. The orientation of actin filaments in filopodia is polarized. The rapidly polymerizing barbed ends of filaments are oriented toward the filopodial tip, generating force that pushes the membrane forward.[13] A fine balance between actin polymerization at filopodial tips, and the centripetal retrograde flow of the filaments away from the tip, determines filopodia dynamics. In order for filopodia to form, actin filaments must be nucleated, elongated and bundled into parallel fibers. Despite the apparent simplicity of filopodial structures, consideration of the literature indicates cell and/or context specificity regarding the specific mechanism involved in the formation of filopodia.[14]

The mechanism underlying the formation of axonal filopodia has been most extensively investigated in embryonic sensory axons, and these axons will be the focus of this discussion. As noted previously, the axon shaft behind the advancing growth cone exhibits low levels of actin filaments and minimal protrusive activity. The localized formation of filopodia is due to the submembranous formation of actin filament based precursor

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structures termed actin patches (**Fig. 1B**; reviewed in ref. 14). Actin patches are detected along axons in vitro and in vivo.[15,16] As revealed by platinum replica electron microscopy, actin patches consist of micron sized domains of filaments with a general organization similar to that observed in lamellipodial structures.[15] Live imaging of GFP/YFP-actin dynamics along axons has determined that actin patches are dynamic and transient. Patches appear along the axon, initially grow in size and intensity, and eventually dissipate. In vitro and in vivo, the mean duration of actin patches is in the order of 20–40 s. While axons make many patches per unit time, only a subset of these patches gives rise to filopodia before dissipating. However, most filopodia are noted to arise from actin patches. Interestingly, actin patches have also been described in the most proximal segment of the axon, termed the axon initial segment, where they have a role in capturing transport cargo that is not intended for delivery into the axon.[17] Actin patches in the distal axon may thus similarly serve to locally trap molecules or organelles relevant to branching.

Along distal sensory axons, the sites of actin filament patch formation are determined by localized microdomains of phosphoinositide 3-kinase (PI3K) activity, and the branching inducing signal nerve growth factor promotes the colocalization of sites of actin patch formation with stalled axonal mitochondria.[21] Furthermore, the location of formation of axonal filopodia, and ultimately branches, is determined by the presence of actively respirating mitochondria.[89] The rate of formation of axonal actin patches also exhibits a distal (higher) to proximal (lower) gradient along axons,[15] which is reminiscent of the distribution of mitochondria in distal axons.[21] In conjunction with the demonstration that mitochondria stalling along axons is required for the formation of axon branches and correlates with site for branch formation,[29,89] these considerations indicate that the positioning of axonal mitochondria has a fundamental role in determining sites of actin patch formation and subsequent formation of filopodia and ultimately branching.

The Arp2/3 complex is an actin filament nucleator that binds the side of an existing filament and nucleates a new filament emerging at an approximate 70° angle from the "mother" filament. Arp2/3 mediated filament nucleation is a major contributor to the formation and maintenance of lamellipodial structures. Arp2/3 subunits target to axonal actin patches, in both sensory and central nervous system neurons.[15,18] Inhibition of the Arp2/3 complex or upstream regulators decreases the formation of axonal actin patches, filopodia and branches.[15,18,19] Collectively, these studies identify Arp2/3 as a regulator of the earliest stages of axon branching. It seems likely that additional actin nucleators (e.g., formins or cordon bleu) also contribute to the formation of actin patches, probably through the generation of mother filaments required by Arp2/3 to establish branch filament arrays. Based on the requirement for Arp2/3, the convergent elongation (CE) model for filopodia formation[20] appears to be most relevant to the formation of axonal filopodia. In the CE model, the barbed ends of filaments nucleated by Arp2/3 are brought together into a bundle which then determines the site of filopodia formation. Consistently, platinum replica microscopy of actin filament organization in axonal filopodia reveals filaments converging from

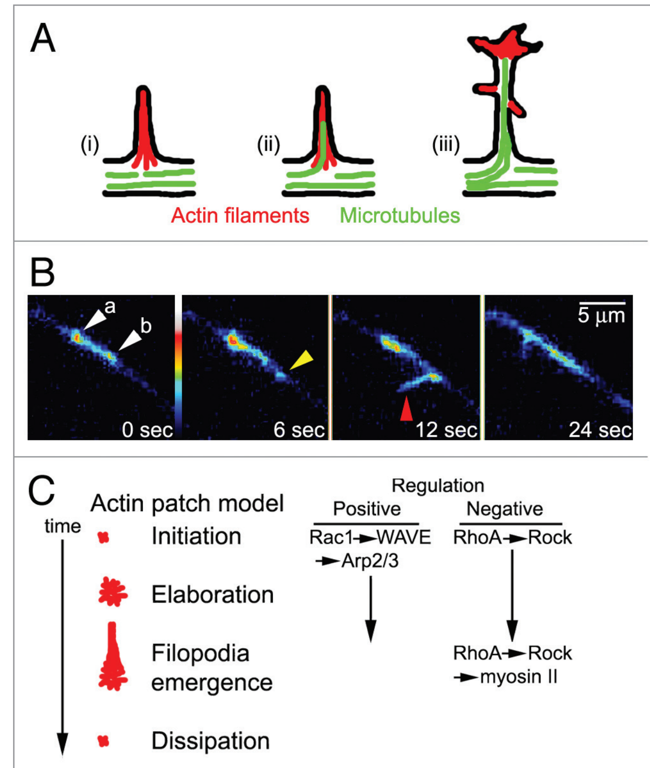

Figure 1. Collateral axon branching and axonal actin patch precursors to the emergence of axonal filopodia. **(A)** Schematic of the steps involved in axon collateral branching. (i) The axon forms a filopodium. (ii) The filopodium becomes invaded by axonal microtubules. (iii) The filopodium develops polarity and matures into a branch. **(B)** Example of axonal actin patches and filopodial emergence along a cultured chicken embryonic sensory axon expressing eYFP-β-actin. White arrowheads (a,b) show the presence of prexisiting patches which dissipate during the time-lapse sequence. At 6 s a new patch forms (yellow arrowhead) and by 12 s a filopodium emergences from the patch (red arrowhead). Between 6–12 s the actin patch that gives rise to the filopodium elaborates as reflected by the increase in fluorescence. The filopodium and patch have retarcted and disspipated, respectively, by 24 s. **(C)** Diagram of the current understanding of the role of Rho-GTPases in the regulation of axonal actin patches and filopodia emergence along embryonic sensory axons. The phases of actin patch development are shown as a function of time with assigned roles for Rho-GTPases as positive or negative regulators. For the roles of additional proteins in this mechanism, see reference 14.

the population within actin patches giving rise to filopodia.[15] The organization of actin filaments in axonal patches observed through platinum replica microscopy resembles that observed in lamellipodia (e.g., a meshwork of filaments at various orientations). Although not directly determined, this observation indicates that the filaments in patches are likely to have mixed polarity.

PI3K is a lipid kinase that generates PIP3 (phosphatidylinositol [3,4,5] triphosphate) from PIP2 (phosphatidylinositol [4,5] biphosphate) in the plasma membrane. PIP3 then recruits the Akt kinase to the plasma membrane, which in turn can inhibit GSK-3β signaling and activate other signaling pathways such as mTOR. These kinase systems have been implicated in collateral branching. In sensory neurons, NGF signaling induces filopodia

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and collateral branch formation through PI3K-Akt-mTOR by increasing the rate of formation of actin patches without affecting the probability that a patch will give rise to a filopodium.[21] Additionally, this study revealed that temporal and spatial development of actin patches correlate spatio-temporally with localized microdomains of PIP3 accumulation in the membrane. Genetic deletion of phosphate and tensin homolog on chromosome ten (PTEN), which has lipid phosphatase activity against PIP3 and thus antagonizes PI3K signaling, results in increased branching in vivo.[22] Similar effects on increased branching has been demonstrated by PTEN downregulation through Nedd4 (E3 ligase, part of the ubiquitin proteasome system) in *Xenopus* retinal ganglion cells in vivo.[23] Downstream of PI3K, Akt targets to the membrane through its plekstrin homology domain. The inhibition of Akt activity blocks F-actin patch and filopodia formation.[21] This is consistent with the increased branching that occurs in response to constitutively active Akt in sensory neurons.[24] Akt signaling inhibits GSK-3β, and studies in hippocampal neurons and cerebellar slices have shown that inhibition of GSK-3β activity or GSK-3β knockdown promotes axonal branching.[25,26] Additionally, cyclic GMP-dependent protein kinase 1 (PrKG1), which also inhibits GSK-3, induces collateral branching of rat embryonic DRG sensory neurons.[27] Other kinases that play a role in branching involve SAD kinases activated in response to NT-3. In response to NT-3, Raf/MEK/ERK pathway induces SAD kinase activity, which promotes axon branching of cultured DRGs and branching of proprioceptive sensory neurons in terminal fields within ventral spinal cord.[28] The related LKB1-NUAK1 kinase pathway regulates branching of cortical axons.[29]

Intra-Axonal Protein Synthesis in Collateral Branching

Localized protein synthesis in the developing and injured adult nervous system provides an efficient mechanism for the regulation of the axonal proteome. Multiple mRNAs coding for proteins that regulate the axonal cytoskeleton (β-actin, cofilin, GAP43, Arp2, cortactin, WAVE1, fascin, tubulin) are targeted into axons.[30-32] mRNAs are targeted into axons through motifs located within the 3′ untranslated region (3′UTR) or the 5′UTR (reviewed by refs. 33, 34). The transport and subcellular translation of mRNAs are regulated by neurotrophins, injury, and neuronal activity,[34] all of which have been involved in the regulation of axon branching.

PI3K signaling is a major determinant of axon branching and can regulate translation through the mammalian target of rapamycin (mTOR) pathway. A role for protein synthesis in collateral sprouting in vivo is suggested by the finding that intraperitoneal administration of rapamycin, an inhibitor of mTOR, results in a 50% decrease in sprouting of mouse dentate gyrus inter-neurons in induced status epilepticus.[35] In cultured chicken sensory axons in vitro, NGF-PI3K signaling promotes collateral branch formation dependent on intra-axonal protein synthesis of Arp2 subunit of the Arp2/3 complex, WAVE1, and the complex stabilizer cortactin.[32] In this study, inhibition of protein synthesis and PI3K-mTOR signaling in axons severed from their cell bodies impaired collateral branching induced by acute treatment with nerve growth factor. Additional evidence on the role of localized protein synthesis and collateral formation is provided by studies of the mRNA binding/localizing protein FMRP and its gene *fmr1*. Knockout of *fmr1* in mice is associated with extensive collateral formation from the granule cell axons in the hippocampus.[36] Loss of FMRP function and associated defects in axonal arborization are also observed in the *Drosophila* model of fragile X syndrome.[37] Motor neurons demonstrate increased branching in the absence of *dFmr1*, and decreased branching when dFMRP is overexpressed. These findings suggest that local protein synthesis is disrupted in fragile X syndrome. Injection of morpholino antisense oligonucleotides in zebrafish embryos to inhibit translation of target mRNA and knockdown of the survival motor neuron (SMN) protein significantly increases motor neuron branching.[38] SMN is involved in the formation of ribonucleoprotein complexes and its mutation/deletion results in spinal muscular atrophy.[39] Additionally, a possible role for axonal protein synthesis in branching is also suggested by the accumulation of β-actin and actin depolymerizing factor mRNAs at the base of branches in vitro.[40,41] Translation of axonal β-actin mRNA in cultured rat neurons and in vivo in sensory neurons of chicken spinal cord results in increased branching.[42] Furthermore, the mRNA binding proteins Vg1RBP and Hermes regulate axon branching in developing retinal ganglion cell axons.[43,44]

Microtubule Invasion of Filopodia and Branch Maturation

The second critical step of collateral branch formation requires the involvement of the microtubule cytoskeleton. Axonal microtubules are organized in bundles along the axon with the majority of their plus-end oriented toward the growth cone. The mechanisms that regulate the interaction of the actin and microtubule cytoskeleton in axonal branch formation are not well understood. Early steps of collateral branching involve the unbundling and splaying apart of microtubule bundles.[3,45] In cortical neuronal cultures, sites of collateral branches demarcated by growth cone pausing display disruption in microtubule bundling, exploration of axonal filopodia by microtubules, breakdown of bundles, and invasion of the nascent axonal branch by microtubules.[46] Localization of Septin-7 protein to the base of filopodia along the axon correlates with microtubule splaying apart and microtubule exploration of axonal filopodia in chicken sensory neurons.[47] The stabilization and continued extension of nascent branches correlates with microtubule invasion.[48] Invasion of axonal filopodia by microtubules requires microtubule dynamic instability and/or the transport of small microtubules.[3] Fragmented microtubules have been detected at axonal branching sites of hippocampal neurons.[49] Microtubule associated proteins that function in microtubule depolymerization and severing have also been shown to play a role in axon collateral branching. For example, kinesin superfamily protein 2A (KIF2A), implicated in microtubule depolymerization, functions downstream of phosphatidyl

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4-phosphate 5-kinase α to negatively regulate the length and the number of collateral branches longer that 50 μm in hippocampal neurons.[50] Microtubule severing at sites of branching is accomplished by microtubule severing proteins, katanin and spastin. The expression of these proteins are enhanced in hippocampal cultures treated with basic fibroblast growth factor (bFGF) which result in increased levels of short microtubules and promotion of axon branching.[51] The effect of katanin in branch enhancement is enabled by tau phosphorylation and subsequent dissociation from microtubules in response to bFGF.

Emerging Roles of Rho GTPases in Axon Branching

The most commonly studied Rho-GTPases that regulate the actin cytoskeleton in the mammalian system are RhoA, Rac1 and Cdc42. Investigation of Rho GTPase function in vivo by utilizing global animal knockout has been limited due to the embryonic lethality of RhoA, Rac1, and Cdc42 mutant mice.[52] Rho GTPases are monomeric G proteins that are switched on when bound to GTP, a process facilitated by guanine exchange factors (GEFs), and switched off when bound to GDP, a process promoted by GTPase-activating proteins (GAPs).[4] When bound to GTP, membrane-bound Rho GTPases initiate singaling cascades that reorganize the cytoskeleton. They are important for axonal growth, axonal morphology, pathfinding, and neuronal plasticity. Their functional implications are complex and display specificity toward neuronal cell type, context or developmental stage. The current understanding of the role of Rho-GTPases in the regulation of axonal actin patches and filopodia along embryonic sensory neurons is summarized in **Figure 1C**.

Overall, the current literature does not implicate Cdc42 function as a major regulator in axon branch formation. Consistent with a general lack of reported roles for Cdc42 in branching, relative to the growth cone the axon exhibits lower levels of Cdc42 activation,[53] and dominant negative Cdc42 has no effect on formation of axonal filopodia but it decreases the number of growth cone filopodia in cultured sensory neurons.[32] However, a role for Cdc42 in axon branching was suggested in a study investigating branching that coincides with the retraction of the axon tip following contact with repellent signals.[5] This form of branching may thus be different from the branching elicited by branch inducing signals such as neurotrophins. However, Cdc42 function has been associated with axonogenesis. Expression of constitutively active Cdc42 (V12) or hyperactivated Cdc42 (L28) in rat hippocampal neurons result in inhibition of neurite extension and formation of multiple axons, respectively.[54] This study revealed that Rap1B GTPase functions upstream of Cdc42, whereas PI3K functions upstream of both GTPases to determine axonal initiation. Additionally, Cdc42 signals through its downstream effectors Par6/aPKC.[4] Alternatively, Cdc42-mediated actin reorganization and filopodia formation can involve the activation of Wiskott-Aldrich-syndrome protein (WASP) and neuronal WASP (N-WASP).[55] WASP and N-Wasp are activators of the Arp2/3 complex which, as previously discussed, contributes to the formation of filopodia and lamellipodia. The subcellular functions of Cdc42 in the formation of filopodia remain poorly understood. The lack of a role for Cdc42 in axon branching may reflect the utilization of Arp2/3 activators of the WAVE family by branching signals, or a predominance of WAVE proteins along the axon shaft relative to the growth cone. Moreover, even along the same axons (e.g., sensory axons) Cdc42 can contribute to filopodia formation at the growth cone, but not along the axon shaft.[32]

The RhoA-Rock pathway has been associated with negative regulation of the axonal actin cytoskeleton. In chicken sensory neurons, RhoA-Rock signaling negatively regulates actin patch elaboration without affecting the rate of actin patch initiation.[6] Thus, patches form at normal rates but grow larger than in baseline conditions. These effects appear to be independent of myosin II contractility, a major effector downstream of Rock. However, through Myosin II activation RhoA-Rock suppresses axonal protrusive activity by negatively regulating the emergence of filopodia from actin patches. Consistently, inhibition of myosin II activity in this model system promotes axon branching (G. Gallo unpublished results). Semaphorin-3A is an inhibitor of axon elongation and branching. Semaphorin-3A signaling inhibits the formation of axonal actin patches through Rock.[56] RhoA signaling also negatively regulates axon branch formation in *Drosophila* mushroom body neurons. Inactivating p190RhoGAP, activating RhoA or its effector Drok/Rock all results in the retraction of branches into the main axon shaft.[7] These findings are generally consistent with the roles of RhoA-Rock-myosin II in mediating axon retraction in response to repellent signals.[56] RhoA also negatively regulates axon branching of Purkinje cells in the deep cerebellar nuclei.[8] In this study, cell specific ablation of FAK increased axon terminals in vivo. In vitro experiments revealed that FAK recruits and activates p190RhoGEF, which in turn activates RhoA in order to inhibit axon branching.

In contrast, RhoA activity has also been implicated in the promotion of axon branching. Inhibition of RhoA activity diminishes axonal branching in hippocampal neurons of embryonic mice, whereas expression of constitutively active RhoA has no effect of branching.[9] Activation and inhibition of RhoA in organotypic cortical slice cultures resulted in increased and decreased axon branching.[10] RhoA activity in these organotypic cortical slices is promoted by neural activity as demonstrated by inhibition of sodium channels and glutamate receptors. The role of RhoA in these central nervous system neurons may thus be related to their activity patterns and activity dependent mechanisms. A possible candidate downstream effector of RhoA that enables actin polymerization is the mammalian diaphanous formin protein 1 (mDia1). mDia1 contains a Rho-binding domain (RBD), which upon binding to activated Rho (A, B, or C) proteins is released from auto-inhibition.[55,57] The differences in the role of RhoA in branching between studies may reflect differences between cell types, culturing environment (or in vitro relative to in situ), or as suggested by the study by Ohnami et al.[10] the electrophysiological state of the neurons. Indeed, it will be of interest to determine further how neuronal activity regulates or orchestrates the function of GTPases.

Rac GTPases have been shown to have roles in the regulation of axon branching. Rac1 negatively regulates axon branching in the

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Drosophila giant fiber system.[58] Overexpression of dominant-negative Rac1 (N17) in the giant fibers is accompanied with enhanced axon branching. Purkinje cells of transgenic mice expressing the human constitutively active Rac1 (V12) display decreased axon terminals in the deep cerebellar nuclei.[59] In contrast to these roles of Rac1 in the suppression of branching, Rac1 has been shown to positively regulate axon branching in other neuronal systems. The activities of Rac GTPases (*Rac1*, *Rac2*, and related *Mig-2-like* (*Mtl*)) are critical for axon branching of mushroom body neurons in *Drosophila*.[60] Loss-of-function mutations of endogenous *dRac* genes revealed that compared with axon growth and guidance, axon branching displays a higher dependence on Rac GTPase activity, in which Rac1 function is more critical than Rac2. Moreover, the frequency of axon branching defects correlates with progressive loss-of-function induced by removal of wild-type copies of *Rac* genes. The axon branching defects seem to be independent of other observed defects in guidance and axon growth. Rac2/3 double mutations in *C. elegans* revealed abnormal and premature axon branching, indicative of the role or Rac GTPases in controlling axonal branching.[61] Expression of neuron-specific Rac also resulted in increased branching of axons. Additionally, this study revealed that UNC-115, an actin-binding protein, mediates the effects of Rac2 in regulating axonal morphology and pathfinding. Furthermore, the regulation of Rac1 GTPase activity by the Vav2 GEF positively regulates branch formation of *Xenopus* retinal ganglion neurons.[62] In spinal neurons, expression of Vav2 or constitutively active Rac1 promotes axonal branching on laminin substratum. Loss-of-function of Vav2 in neurons cultured on L1 substratum results in significant branch inhibition, despite the ability of L1 to promote branching. Similarly, Vav2 activity regulates branching of commissural interneurons as they approach the ventral spinal cord midline. In chicken sensory neuronal cultures, the activity of Rac1 is promoted by NGF-PI3K signaling and it induces formation of axonal actin patches and filopodia, which are precursors of collateral branch formation.[32] This effect of Rac1 is likely performed by activation of the downstream effector, WAVE. WAVE induces filopodia and axon collateral branching by promoting actin filament branching and polymerization through the Arp2/3 complex.[32,63]

Other GTPases implicated in axon branch formation are Rac3 and RhoG. Rac3 was first identified in chicken as cRac1B. It specifically expresses in the nervous system and it is developmentally regulated, with the highest expression correlating developmentally with axon branching.[64,65] Overexpression of cRac1B/Rac3 in cultured retinal cells promotes neurite branching.[65] Overexpression of the cRac1A/Rac1 in retinal cells had no effect on neurite morphology. Kalirin-GEF1 activates Rho GTPases and it has specificity for RhoG activation in postnatal sympathetic neurons.[66] Expression of Kalirin-GEF1 enhances multiple axon-like processes from the soma and branching, a phenotype consistent with expression of activated RhoG. In contrast to its role in postnatal sympathetic neurons, RhoG decreases the number of branches along the axons of embryonic hippocampal neurons.[74] In this cell system, ELMO-Dock180-Rac1 downstream of RhoG act to suppress the number of axon branches. However, the Rac and Cdc42 GTPases guanine exchange factor (GEF) αPIX induces axonal branching in embryonic hippocampal neurons.[67] Overexpression or depletion of αPIX or the associated protein GIT2 induce and impair axonal branching, respectively. Furthermore, inhibition of Rac1 and Cdc42 activity using cell permeable peptides in cultured retinal ganglion cells blocks the formation of axon collaterals in axons undergoing retraction following contact with repellent guidance signals.[5] The differences in the role of RhoG and downstream GTPases in branching between these studies may reflect a variety of differences (e.g., cell type, developmental stage, culturing substratum), including the possibility that different upstream activating mechanisms (e.g., the GEFs Dock180 and αPIX) which may drive a concerted response involving multiple effectors.

Ras family GTPases are also implicated in axonal morphology regulation by signaling through PI3K and the actin filament binding protein afadin (AF-6).[68] This study revealed that R-Ras induces axon branching in cortical neurons by translocating afadin to the cell membrane and by inhibiting GSK-3β through PI3K/Akt signaling pathway. Ras GTPases directly activates PI3K by interacting with the PI3K-p110 subunit.[69] Other GTPases functioning downstream of PI3K, such as Rac1–3, Cdc42, and RhoG are also capable of activating PI3K indirectly and inducing a positive feedback loop of PI3K activation. Whether positive feedback loops between GTPases and PI3K signaling contribute to axon branching remains to be elucidated.

Extracellular Signals and the Regulation of Axon Branches: Involvement of RhoA-Family GTPases

In vivo the formation and maintenance of axon branches in under bi-directional control by extracellular signals, cell-to-cell communication and neuronal activity patterns. While beyond the scope of this review, it is worth noting that these extrinsic regulatory signals have been described to act through RhoA-family GTPases. Neurotrophins are major regulators of axon branching in a variety of neural systems, and neurotrophin binding to Trk receptors regulates RhoA GTPases.[75] Similarly, netrins also regulate axon branching and signal through RhoA GTPases.[76-80] Interestingly, RhoA also acts to suppress the targeting of netrin receptors to the membrane,[81] revealing a multifunctional role for GTPases in this signaling system. Semaphorins also regulate axon branching, usually inhibiting branching (but see refs. 82, 92), and can activate RhoA GTPases.[83] Chondroitin sulfate proteoglycans (CSPGs) suppress axon branching in vivo and in vitro and also use RhoA-family GTPases to signal.[84-87] Finally, synaptic activity can also regulate RhoA-family GTPases,[88] emphasizing the notion that in electrically active neuronal cultures or in vivo circuits activity may further assist in the determination of GTPase activity or function. As noted by the multiple discrepancies in the literature regarding the roles of RhoA-family GTPases in axon branching, it will be important to further determine how the functions of GTPase signaling are determined in the context of additional signaling events induced by ligand binding to specific receptor systems and in different neuronal populations and contexts.

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Concluding Statement

The mechanisms of axon collateral branching are only partially understood. RhoA-family GTPases are key regulators of cytoskeletal reorganization, but specific roles for these GTPases in axon branching largely remain to be determined. The literature indicates complex roles for these GTPases in a neuronal cell type/context dependent manner. A major goal of future investigations will be to determine the upstream regulators and downstream effectors that determine the multi-functional nature of these GTPases. As an example, although the Rac1 GTPase is canonically considered to drive the elaboration of protrusive structures, it is also required for the loss of protrusive activity by growth cones in response to extracellular repellent guidance molecules. In this context, the function of Rac1 appears to undergo a switch from regulating protrusive activity to promoting endocytosis in response to repellent guidance molecules.[70] These types of observations underscore the importance of understanding the cellular roles of these GTPases in a context dependent manner. Ultimately, a unifying model for axon branching, beyond the basic sequence of cytoskeletal events (**Fig. 1A**), is not supported by the literature. Indeed, even the same kinases contribute to branching depending on the cell type they are expressed in in vivo, and also likely depending on which extracellular signals are regulating branching in that neuronal population.[29,89,90] Given that Rho-GTPases have been ascribed a variety of cellular functions, it will be necessary to determine their specific contributions to cytoskeletal dynamics, subcellular organization, organelle function, and membrane traffic, all components of axon branching.[3,29,91] Through regulation of the cytoskeleton, Rho-GTPases may also control aspects of somatic protein synthesis.[71] It will thus be of interest to determine if they may have such roles in axons in the context of axonal protein synthesis dependent axon branching. Furthermore, although microtubules have a fundamental role in axon branching, and some Rho-GTPases have been shown to regulate the microtubule cytoskeleton,[72,73] studies of these GTPases in the regulation of axonal microtubules during branching are lacking.

Finally, the majority of studies perform morphometric analysis and report on the number or length of axon branches, sometimes at single experimental time points. Differences in these morphometric variables can arise through the separate regulation of different aspects of the mechanism of branching. For example, an increase in the number of axon branches can arise from increased initiation of branches, and/or increased retention of nascent branches that would otherwise be retracted.[29,89] Thus, it is important to consider the dynamics of the branching process in the determination of the roles of specific molecules, and ultimately which aspects of the basic cytoskeletal events underlying branching are under regulation by GTPases. Future live imaging analysis of branch formation, and the underlying cytoskeletal dynamics, may shed light on some of the observed discrepancies between different studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported by NIH grant NS078030 (G.G.).

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