Crucial polarity regulators in axon specification

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Abstract

Cell polarization is critical for the correct functioning of many cell types, creating functional and morphological asymmetry in response to intrinsic and extrinsic cues. Neurons are a classical example of polarized cells, as they usually extend one long axon and short branched dendrites. The formation of such distinct cellular compartments (also known as neuronal polarization) ensures the proper development and physiology of the nervous system and is controlled by a complex set of signalling pathways able to integrate multiple polarity cues. Because polarization is at the basis of neuronal development, investigating the mechanisms responsible for this process is fundamental not only to understand how the nervous system develops, but also to devise therapeutic strategies for neuroregeneration. The last two decades have seen remarkable progress in understanding the molecular mechanisms responsible for mammalian neuronal polarization, primarily using cultures of rodent hippocampal neurons. More recent efforts have started to explore the role of such mechanisms in vivo. It has become clear that neuronal polarization relies on signalling networks and feedback mechanisms co-ordinating the actin and microtubule cytoskeleton and membrane traffic. The present chapter will highlight the role of key molecules involved in neuronal polarization, such as regulators of the actin/microtubule cytoskeleton and membrane traffic, polarity complexes and small GTPases.

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Introduction

The polarization of neurons into axonal and dendritic compartments is a fundamental event for their development. Disruption of this process impairs the directional signalling between neurons, thus severely compromising their function. From the clinical point of view, studying the molecular mechanisms that underlie axon specification and growth is crucial if we hope to restore neuronal function in injury or neurodegenerative diseases. Indeed, recent studies in vitro have demonstrated the ability of old mature neurons to reactivate an intrinsic polarization programme favouring axon regrowth after lesion [1], an event that could be the key to regeneration in the nervous system after trauma, injury or in degenerative diseases.

Neuronal polarization relies on the fine regulation of cytoskeletal dynamics and membrane traffic. Over the last two decades many studies have investigated this process using cultured embryonic hippocampal neurons [2]. These cells develop through stereotyped stages, culminating in the clear compartmentalization of axon and dendrites (Figure 1). Immediately after plating, neurons are round and extend dynamic lamellipodia and filopodia (stage 1). This is followed by the extension of multiple short neurites (stage 2). Approximately half a day after plating (stage 3), one of these neurites starts to grow [3] and becomes the nascent axon. This will continue to elongate in the following 4–7 days while the rest of the neurites mature into dendrites (stage 4) and subsequently display spines (stage 5). In the present chapter I will focus on some molecular players crucial for axon specification, including regulators of the cytoskeleton, crucial signalling pathways and small GTPases. For more in-depth analysis of different aspects involved in neuronal polarization I refer the reader to excellent reviews [4–6].

Figure 1. Hippocampal neuron polarization in vitro
In stage 1, round neurons display dynamic lamellipodial and filopodial activity. In stage 2, they extend multiple immature neurites. In stage 3, one of these neurites starts to grow rapidly. Microtubule stabilization in the shaft (dark blue) and enhanced microtubule/actin dynamics at the growth cone of the future axon (red) play a key role in this crucial polarization step. In stage 4, the minor neurites mature into dendrites, whereas the axon extends and branches. Finally, in stage 5, dendrites display spines (yellow) to establish functional synapses.
The cytoskeleton

The cytoskeleton is a key player in neuronal polarization. A close interaction between actin filaments and microtubules (Figure 2, red and turquoise respectively) is observed in the growth cone, an important structure at the tip of the presumptive axon. Microtubules stabilize the nascent axon shaft and grow until the C-domain (central domain) of the growth cone (white background in Figure 2), helping the transport of vesicles and organelles towards the leading edge. In the growth cone peripheral area (also known as the ‘P-domain’, yellow background in Figure 2), filopodia (bundles of actin filaments) dynamically extend and retract, sensing the extracellular environment through receptors and providing directionality to axon growth. These filopodia are connected by thin lamellipodia, structures formed by an intricate actin filament meshwork [7]. Actin filaments forming filopodia and lamellipodia in the P-domain point their depolymerizing end towards the narrow region at the border between the C- and P-domains, the so-called T-zone (transition zone, purple background in Figure 2), whereas their fast growing ends are oriented towards the distal membrane. The ‘push’ of actin polymerization towards the periphery, together with the contractile action of the actin-driven motor myosin II anchored at the T-zone drives continuous movement of F-actin (filamentous actin) from the leading edge towards the centre of the growth cone. This process is called F-actin ‘retrograde

Figure 2. Schematic organization of the growth cone at the axon tip
Microtubules (turquoise) grow towards the C-domain of the growth cone (white background). During polarization, MAPs (yellow) stabilize the shaft of the future axon. CRMP2 (pink) can favour microtubule polymerization and endocytosis (see the text for details). +TIPs (blue) and APC bind to the plus-ends of microtubules, stabilizing them. They may also provide a link with the actin cytoskeleton (red). Actin arcs and depolymerization of actin filaments (red) favoured by cofilin (orange) are observed in the T-zone (purple background). Dynamic filopodia and lamellipodia are found in the P-domain (yellow background) together with some microtubules able to get through the T-zone and extend along filopodia.
flow’. Contraction of actin filaments by myosin II in the T-zone also leads to the formation of ‘actin arcs’, which are oriented perpendicular to the peripheral filopodia. Although actin arcs block microtubule growth into the P-domain [7], it is possible to observe some microtubules extending until the very end of filopodia [8]. Axon extension is favoured by actin depolymerization at the neck of the growth cone, which allows microtubule invasion of the C-domain and bundling, causing the consolidation of the proximal part of the growth cone and its transformation into a new segment of axon shaft. Such close interplay between the actin and microtubule cytoskeleton is critical for axon formation and needs to be precisely controlled.

**Actin dynamics**

Local actin depolymerization in one of the initial neurites is sufficient to promote or even regenerate axon growth [9]. Therefore, to allow microtubule extension, the actin cytoskeleton in the growth cone of the future axon has to be much more dynamic than in the minor neurites. Consistent with this idea, several regulators of actin dynamics are involved in neuronal polarization, at least in vitro: these include the WAVE [WASP (Wiskott–Aldrich syndrome protein) verprolin homologous] complex [10] and coflin [11]. The WAVE complex regulates actin polymerization in lamellipodia [12], whereas coflin binds to the minus ends of the actin filaments, favouring their severing and depolymerization. Expression of non-functional WAVE or inhibition of WAVE transport into the axon blocks axon growth in hippocampal and cerebellar granule neurons [10,13]. In addition, a conditional knockout mouse model for Cdc42 (cell division cycle 42), a small GTPase involved in the control of filopodia formation and polarity (see also below), displays strong neuronal polarity defects caused at least, in part, by increased levels of phosphorylated (inactive) coflin. Depolymerizing actin filaments rescued axon formation in Cdc42-depleted neurons, consistent with a requirement for a dynamic actin cytoskeleton in the establishment of polarity [11].

**Microtubule dynamics**

The initial growth of an axon relies on the consolidation and bundling of microtubules, so that the C-domain of the growth cone becomes part of the axon shaft. Microtubules consist of tubulin heterodimers, which polymerize with the plus-end oriented towards the axon tip. This location is characterized by ‘dynamic instability’, that is cycles of rapid microtubule assembly and disassembly (‘shrinkage’). The switch from growth to shrinkage is instead known as ‘catastrophe’ [14]. Importantly, microtubules have an instructive role in axon specification. In contrast with actin, microtubules in the axon are more stable compared with other neurites. Indeed, modest pharmacological microtubule stabilization that prevents catastrophe without completely blocking microtubule growth produces supernumerary axons in vitro [15]. Moreover, local microtubule stabilization of one minor neurite causes it to become an axon, a
remarkable phenomenon also observed with dendrites in mature neurons [1], suggesting that the potential to switch fate and extend as an axon is also maintained at later stages.

Post-translational tubulin modifications, such as detyrosination and acetylation, characterize stable microtubules and are found in the proximal section of the axon, in contrast with the C-domain of the axonal growth cone, where more dynamic microtubules prevail to quickly rearrange in response to extracellular guidance cues. The plus-end-directed motor KIF5 (kinesin-5) binds acetylated (stable) microtubules and preferentially accumulates in one neurite before morphological polarization, favouring transport of organelles and cargo into the nascent axon [16]. Its activity is required for axon specification, since dominant-negative KIF5 or KIF5 depletion impair neuronal polarization [17]. Although it is clear that regulation of microtubule dynamics is crucial for neuronal polarization, it will be important to investigate further how post-translational modifications of tubulin and motor proteins can be controlled to ensure microtubule consolidation and polarized transport in the perspective axon [18].

MAPs (microtubule-associated proteins)
Regulators of microtubule stability play an important role in axon specification, these include traditional MAPs belonging to the MAP2/tau family [19]. A crucial MAP in neuronal polarity is CRMP2 (collapsin-response-mediator protein 2). Overexpression of CRMP2 results in multiple axons, whereas dominant-negative CRMP2 impairs axon specification [20]. Importantly, overexpression of CRMP2 also causes a stage 4 dendrite to become an axon, suggesting that CRMP2 is sufficient to switch the fate not only of immature neurites, but also of mature dendrites [20]. CRMP2 may perform several important functions related to polarization. First, it carries tubulin heterodimers, favouring microtubule assembly. Secondly, it promotes transport of the actin-remodelling Sra-1 (specifically Rac1-associated protein 1)–WAVE complex along microtubules by linking it to the motor protein kinesin-1. Proper localization of the Sra-1–WAVE complex (which regulates actin stability during lamellipodia formation) is important for neuronal polarization since knockdown of Sra-1 or WAVE prevents CRMP2-dependent axon formation. Finally, CRMP-2 specifically accumulates in the C-domain where it binds Numb (a protein involved in clathrin-mediated endocytosis) and regulates endocytosis of L1, a neuronal cell-adhesion molecule important for axon growth [21].

+TIPs (plus-end tracking proteins)
+TIPs regulate microtubule dynamics by binding to the plus-end of microtubules where polymerization occurs. The +TIP CLIPs (cytoplasmic linker proteins) 170 and 115 favour the switch from microtubule catastrophe to polymerization, an important event in axon specification. Transfection of dominant-negative CLIPs impairs neuronal polarization, whereas overexpression of their microtubule-binding domains produces supernumerary axons, suggesting that
CLIPs are sufficient for polarization [22]. Why multiple homologues of CLIPs exist, how their function is regulated and whether their role is conserved in vivo remains to be investigated.

Similar to CLIPs, the APC (adenomatous polyposis coli) protein also accumulates at microtubule plus-ends and concentrates at the tip of the presumptive axon in stage 3 neurons, increasing microtubule stability. Overexpressing truncated forms of APC or depleting APC impairs neuronal polarization in mammalian neurons in vitro [23,24]; however, work in Drosophila suggests that APC may be dispensable for axon specification [25]. More studies will be needed to clarify whether APC is necessary for neuronal polarization in vivo. Interestingly, CLIPs and APC contain a domain able to directly interact with the actin cytoskeleton [26,27], potentially mediating cross-talk between the growing microtubule ends and the actin cortex. Indeed, recent observations raise the possibility that the mutual influence of the actin network and the microtubule cytoskeleton are part of a positive-feedback loop involved in establishing an axon by controlling growth cone dynamics [5]. The molecular nature of the microtubule–actin relationship in the growing axon still has to be completely elucidated. Such a relationship, however, is very likely to also maintain a central role in neuronal polarization in vivo.

Intracellular signalling

PI3K (phosphoinositide 3-kinase)/Akt/GSK3 (glycogen synthase kinase 3)

The lipid kinase PI3K, an effector of the small GTPase Ras, produces localized enrichment of PtdIns(3,4,5)P$_3$ at the membrane. Several studies indicate that PI3K activity is both necessary and sufficient for axon formation, since PI3K inhibition prevents axon specification, whereas overexpression of a constitutively active catalytic subunit of PI3K causes multiple axons [28–30]. PI3K activity is counteracted by PTEN (phosphatase and tensin homologue deleted on chromosome 10), a protein and lipid phosphatase that dephosphorylates PtdIns(3,4,5)P$_3$ into PtdIns(4,5)P$_2$, thus counteracting PtdIns(3,4,5)P$_3$ signalling. Overexpression of PTEN prevents axon specification [28], whereas PTEN depletion leads to multiple axons [31], thus demonstrating the requirement of tightly controlled PtdIns(3,4,5)P$_3$ levels for axon specification.

PI3K triggers a cascade of effects (Figure 3, right-hand side), such as activation of Rap1b [32], a small GTPase necessary and sufficient for neuronal polarization (see below) [33] and of the protein kinase Akt, recruited by its PH (pleckstrin homology) domain to PtdIns(3,4,5)P$_3$-enriched sites. Akt can subsequently phosphorylate and inhibit GSK3, a key regulator of neuronal polarization [31]. The most important effect of GSK3 inhibition is the activation of microtubule-binding proteins normally suppressed by GSK3, such as CRMP2, APC, MAP1b and tau [21], resulting in microtubule stabilization. How the action of these multiple GSK3 effectors is then co-ordinated during

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axon specification remains to be understood. Surprisingly, double knock-in mice lacking the Akt phosphorylation sites in both GSK3 isoforms exhibit normal axon polarity in vivo [34], suggesting the existence of different modes of GSK3 inhibition not requiring Akt-dependent phosphorylation. These may include the Wnt signalling pathway, which can indirectly inhibit GSK3 via the Frizzled receptor and Dvl (Dishevelled). Interestingly, Wnt5a activates Dvl, which in turn binds and activates aPKC (atypical protein kinase C) [35]. The final result is aPKC-dependent phosphorylation and inhibition of the microtubule-destabilizing protein MARK (MAP-regulating kinase) 2/PAR (PARtitioning defective)-1 [36] (see below), which favours growth of the future axon.

**PAR family members**

The six PAR genes were originally identified in a genetic screen to identify regulators of asymmetric cell division in Caenorhabditis elegans [37] and are known
to play a pivotal role in many polarization events, such as asymmetric mitotic spindle positioning, polarized localization of fate determinants and epithelial cell polarization throughout metazoans [38]. Some PAR family members are also involved in neuronal polarity: they include the serine/threonine kinases PAR-1 and PAR-4, and the scaffold proteins PAR-3 and PAR-6 (Figure 3, left-hand side).

**LKB1 (liver kinase B1)/PAR-4 and MARKs**

LKB1 (the mammalian orthologue of the PAR4 gene) is a key regulator of neuronal polarization, both in vitro and in vivo [39,40]. Once activated by binding to its necessary co-activator Strad and by phosphorylation on Ser\(^{431}\) by PKA (protein kinase A) or p90\(^RSK\) (p90 ribosomal S6 kinase) kinases [39,40], LKB1 phosphorylates a variety of substrates including the mammalian orthologues of PAR-1 (MARK1–4) [41]. Among these, MARK2 may act as a negative regulator of neuronal polarization since its depletion causes multiple axons [36]. LKB1-activated MARK2 phosphorylates a range of MAPs, causing them to detach from microtubules and leading to microtubule destabilization. LKB1 may favour MARK2 activation to promote local microtubule instability in the growth cone of the nascent axon, whereas other factors may inactivate MARK2 to stabilize microtubules in the axon shaft [41]. LKB1 also activates SAD (synapses of the amphid defective)-A/B kinases, which phosphorylate and modulate the microtubule-binding affinity of several MAPs, including MAP2, MAP4 and tau [4,41]. These effects ultimately modify microtubule organization to favour axon formation. Depletion of SAD kinases partially blocks the multiple axon phenotype caused by LKB1 overexpression, showing that LKB1 can influence axon specification, at least in part, by phosphorylating SAD kinases [39]. Besides affecting MAPs, it is tempting to speculate that SAD kinases may also function in promoting directed vesicular traffic along the axon, given their ability to promote presynaptic vesicular clustering in *C. elegans* [42].

**The PAR-3–PAR-6–aPKC complex**

PAR-3 and PAR-6 localize at the tip of the nascent axon in stage 3 neurons and their depletion inhibits polarization, whereas overexpression of PAR-3 causes multiple axon formation [28]. PAR-3 and PAR-6 associate with aPKC and inhibit its activity. However, localized activation of the small GTPase Cdc42 (see below), which binds to PAR-6, relieves this inhibition, achieving local and temporal control of aPKC activity [4]. aPKC subsequently phosphorylates some crucial targets, such as Lgl1 (lethal giant larvae 1), a PAR-3/6 binding partner involved in various polarization events, such as asymmetric cell division and polarized membrane traffic in neuroblasts and epithelia. Lgl1 in turn activates Rab10, a Rab GTPase promoting polarized membrane insertion, a process required for axonal specification in vitro and in vivo [43].

aPKC in complex with PAR-3–PAR-6 can also phosphorylate and inactivate MARKs, such as MARK2, leading to MAP dephosphorylation and microtubule
stabilization in the nascent axon [36]. Finally, the active Cdc42–PAR–aPKC complex also acts as an important scaffold platform to locally recruit activators of small GTPases such as the Rac GEFs (guanine-nucleotide-exchange factors) Tiam1 (T-cell lymphoma invasion and metastasis 1) and STEF (Sif and Tiam1-like exchange factor), ultimately influencing microtubule and actin dynamics at the axonal tip [44] (also see below). Interestingly, in Drosophila the PAR complex does not appear to be needed for axonogenesis [45]. This could be due to a species-specific requirement, even though formal confirmation of a role for PAR-3 and PAR-6 in neuronal polarization in vivo is still needed in mammalian systems.

Small GTPases

Several GTPases control neuronal polarization through their ability to influence cytoskeletal dynamics, protein translation and membrane traffic [46]. They are molecular switches, cycling between an active GTP-bound state, and an inactive GDP-bound state. GEFs activate GTPases by mediating the exchange of GDP for GTP, whereas GAPs (GTPase-activating proteins) inactivate them by stimulating their intrinsic GTPase activity. Interaction with specific effectors and a tight spatial/temporal control of GTPase activation can be achieved by the localized action of GEFs and GAPs, which is also observed in neuronal polarization.

Growth factor signalling and extracellular matrix signals can activate several members of the Ras GTPase family, including H-Ras, R-Ras, K-Ras and N-Ras (Figure 3). Polarized localization of R-Ras in one neurite is already visible in stage 2 [5,47], and is maintained by a positive-feedback loop involving the Ras effector PI3K [48]. This produces PtdIns(3,4,5)P_3 in the nascent axon and, as a consequence, leads to activation of kinases such as integrin-linked kinase and Akt, both of which are able to inactivate GSK3, leading to microtubule stabilization (see above). In addition, PtdIns(3,4,5)P_3 helps to localize the Rac GEF DOCK (dedicator of cytokinesis protein) 7 to the membrane of the nascent axon via its DHR-1 (DOCK homology region 1) domain. Activation of the small Rho GTPase Rac regulates local actin dynamics and lamellipodia formation, but can also lead to phosphorylation and inhibition of stathmin, a microtubule-destabilizing molecule, thus favouring axon growth [49]. In addition, Rac participates in a positive-feedback loop by activating PI3K [21], which would reinforce a localized signalling pathway promoting axonogenesis at the tip of the future axon. Here, active Cdc42, together with the PAR–aPKC complex, promotes local activation of Rac thanks to the ability of PAR-3 to directly interact with and recruit the Rac GEFs Tiam1 or STEF. Indeed, overexpression of Tiam1 leads to multiple axons, whereas its depletion impairs neuronal polarization [44]. Overall, these observations support an important role for Rac in axonogenesis; however, Rac1 RNAi (RNA interference) in both Drosophila and mammalian neurons does not seem to affect neuronal polarity [4]. It is likely that other Rac isoforms, such as Rac3, may be able to compensate for Rac1 depletion.
Another Ras family member, the small GTPase Rheb, and its target mTOR (mammalian target of rapamycin) also operate downstream of PI3K [50] by increasing translation of the Ras-related GTPase Rap1b in the nascent axon. Rap1b plays a pivotal role in neuronal polarity in vitro, since its depletion impairs axonogenesis, whereas its overexpression leads to multiple axons [33]. The small GTPase Cdc42, a master polarity regulator, also has a central role in axon specification. Both dominant-negative and constitutively active Cdc42 versions block axon specification, whereas transfection of a ‘fast-cycling’ mutant, which autonomously cycles between a GDP- and a GTP-bound state, produces a multiple axon phenotype and rescues the loss of axons due to Rap1b depletion, placing Cdc42 downstream of Rap1b [33]. This highlights the importance of a tight regulation of GTPase cycling in the nascent axon. The necessary role for Cdc42 in neuronal polarity has been shown in vivo with a conditional knockout mouse model displaying severe axonogenesis defects [11]. These may be due to alteration in actin dynamics, as suggested by abnormally high levels of phosphorylated cofilin, a substrate for LIM kinase, which can be activated by the Cdc42 effector Pak-1 (p21-activated kinase-1). Pak-1 is enriched in the presumptive axon in stage 3 neurons and is necessary and sufficient for neuronal polarization [51]. In addition to regulating actin dynamics, Cdc42 favours polarization by binding to PAR-6 and activating the PAR–αPKC polarity complex at the axon tip, triggering a series of downstream events culminating in microtubule stabilization (see the previous section on PAR).

Finally, the Ras-like GTPase RalA regulates neuronal polarity through the exocyst, a protein complex involved in several polarization events, such as bud growth in yeast, basolateral membrane delivery in epithelial cells and directed cell migration [52]. RalA is likely to act downstream of Rap1b, since Rap can activate RalGEFs in non-neuronal cells. Similar to the PAR complex, the exocyst accumulates at the tip of the future axon in stage 3 neurons. Importantly, biochemical evidence shows a progressive interaction between the exocyst and the PAR complex as polarization occurs, and such interaction depends on RalA activation [53]. Although the nature of this interaction still has to be clarified, it is interesting to note that depletion of either RalA or multiple exocyst subunits impairs the polarized localization of the PAR complex, and, as a consequence, inhibits neuronal polarization.

**Conclusion**

The last two decades have highlighted the role of many key players and signalling pathways in neuronal polarity. We are now left with a number of unanswered questions concerning the validity of these findings in a more physiological context. Do such signalling cascades also control neuronal polarity in vivo? Genetic manipulation, together with the latest advances in time-lapse imaging technology will help to answer this question. Also, how can extracellular cues and intrinsic cell polarity pathways integrate to achieve spatial and temporal
control of polarization in different neuronal cell types? And finally, how is the interplay between cytoskeletal dynamics and membrane traffic co-ordinated at a molecular level? Tackling these challenging questions will clarify not only how a neuron develops its polarized morphology, but also how this process may be exploited in regenerative strategies.

**Summary**

- Neuronal polarity relies on the co-ordinated interplay between the actin and microtubule cytoskeleton, polarized membrane traffic and regulated protein translation.
- Polarized activation of PI3K and local enrichment of its product PtdIns(3,4,5)P3 are required for axon specification.
- Inhibition of GSK3 is a key event in neuronal polarity, leading to microtubule stabilization in the growing axon.
- The Cdc42–PAR-3–PAR-6–aPKC complex acts as a central signalling scaffold contributing to the control of actin/microtubule dynamics and membrane traffic in the perspective axon.
- LKB1 (PAR-4) is a crucial regulator of neuronal polarization by phosphorylating SAD-A/B kinases and MARK2 (PAR-1), ultimately affecting microtubule dynamics in the nascent axon.
- The small GTPase Ras may act upstream of signalling cascades involving other GTPases, such as Rap1b, Cdc42, RalA, Rac and Rheb, all required for axon specification.

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**References**


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