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Current Opinion in Neurobiology

The role of the cytoskeleton during neuronal polarization

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The formation of an axon and dendrites, neuronal polarization, is a prerequisite for neurons to integrate and propagate information within the brain. During the past years progress has been made toward understanding the initial stage of neuronal polarization, axon formation. First, the physiological role of some candidate regulators of neuronal polarity has been affirmed, including Sad kinases, the Rho-GTPase Cdc42, and the actin regulators Ena/VASP proteins. Second, recent studies have revealed microtubule stabilization as a mechanism complementary to actin dynamics underlying neuronal polarization. Moreover, stable microtubules in the axon may form a landmark to confer identity to the axon. This review highlights the recent advances in understanding the intracellular mechanisms underlying neuronal polarization and discusses them in the context of putative cytoskeletal effectors.

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Current Opinion in Neurobiology 2008, **18**:479–487

This review comes from a themed issue on
Neuronal and glial cell biology
Edited by Peter Scheiffele and Pico Caroni

Available online 25th October 2008

0959-4388/$ – see front matter
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DOI 10.1016/j.conb.2008.09.019

Introduction

Differentiated neurons contain several compartments of distinct molecular composition and function. This polarized arrangement forms the basis for unidirectional signal propagation because it enables neurons to segregate signal reception, integration, and propagation to distinct sites.

Despite this sophisticated polarization at its mature stage, developing neurons start out as simple, rather symmetric spheres. The formation of the axon is one of the initial steps in breaking cellular symmetry and the establishment of neuronal polarity. In recent years, there has been a substantial accumulation of exciting data that helps to unravel the mechanisms behind neuronal polarization. Recently, the candidate regulators and signaling aspects of neuronal polarity have been the subject of a number of comprehensive reviews [1,2] and will thus not be the main focus here. Later stages of neuronal polarization that include dendritic growth and differentiation as well as synapse formation cannot be covered owing to length restraints but are discussed in depth elsewhere (reviewed in [3–5]). Moreover, we refer the reader, who is particularly interested in the culture systems to study neuronal polarity, including hippocampal neurons in cell culture, to previous reviews [6,7].

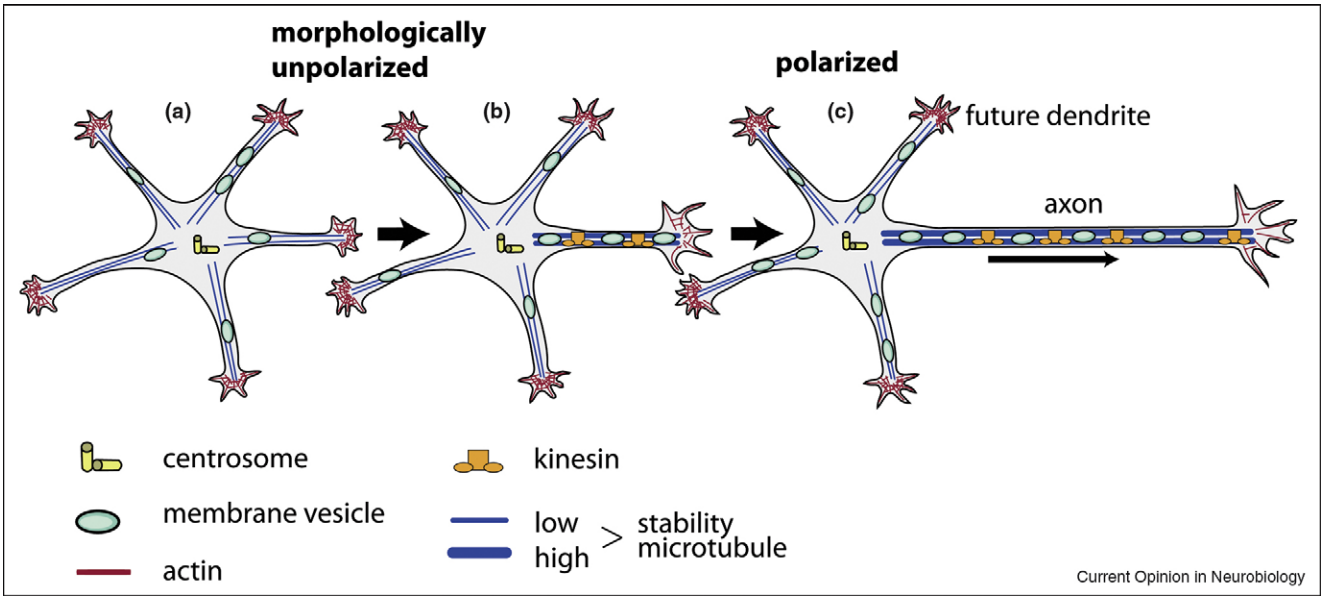
Here we will focus on discussing recent advances in understanding initial neuronal polarization. In particular, Cdc42, Sad kinases, and the Ena/VASP family of proteins have been identified as physiological key regulators of neuronal polarity. We will describe the intracellular mechanisms as well as involved effectors that underlie initial neuronal polarization, focusing on the role of actin dynamics and microtubules in this process (Figure 1). We will then lay out how neurons might sustain axon growth on the basis of our current knowledge of putative reinforcing mechanisms on the effector level. Finally, we will highlight future challenges in the field. Since the regulation of microtubule dynamics is essential during neuronal polarization, one challenge of particular interest is to depict whether the centrosome as microtubule organizing center (MTOC) is involved in axon growth. Finally, we propose that the field needs to assess whether cytoskeletal effectors can function as direct regulators of neuronal polarity.

Local actin instability and neuronal polarization

Actin dynamics contribute a key regulatory role during neuronal polarization. One hallmark of the future axonal growth cone in unpolarized neurons is a decreased stability of its actin cytoskeleton (Figure 1) [8]. Such local actin instability may cause reduced obstruction of microtubule protrusion and, consequently, of neurite outgrowth as suggested by the formation of multiple axons upon pharmacological actin depolymerization [8–10]. Importantly, several molecular counterparts mediating actin destabilization in axonal growth cones have been revealed. Multiple studies have emphasized the importance of different Rho-GTPases as well as their downstream targets including Rho kinase (ROCK) and profilin (Figure 2) in the regulation of actin dynamics during neuronal polarization [8–11].

Currently, it is hard to predict how many of the numerous actin regulators are involved in governing neuronal polarization. Indeed, various signaling pathways and actin regulatory proteins might affect polarization as changes

Figure 1



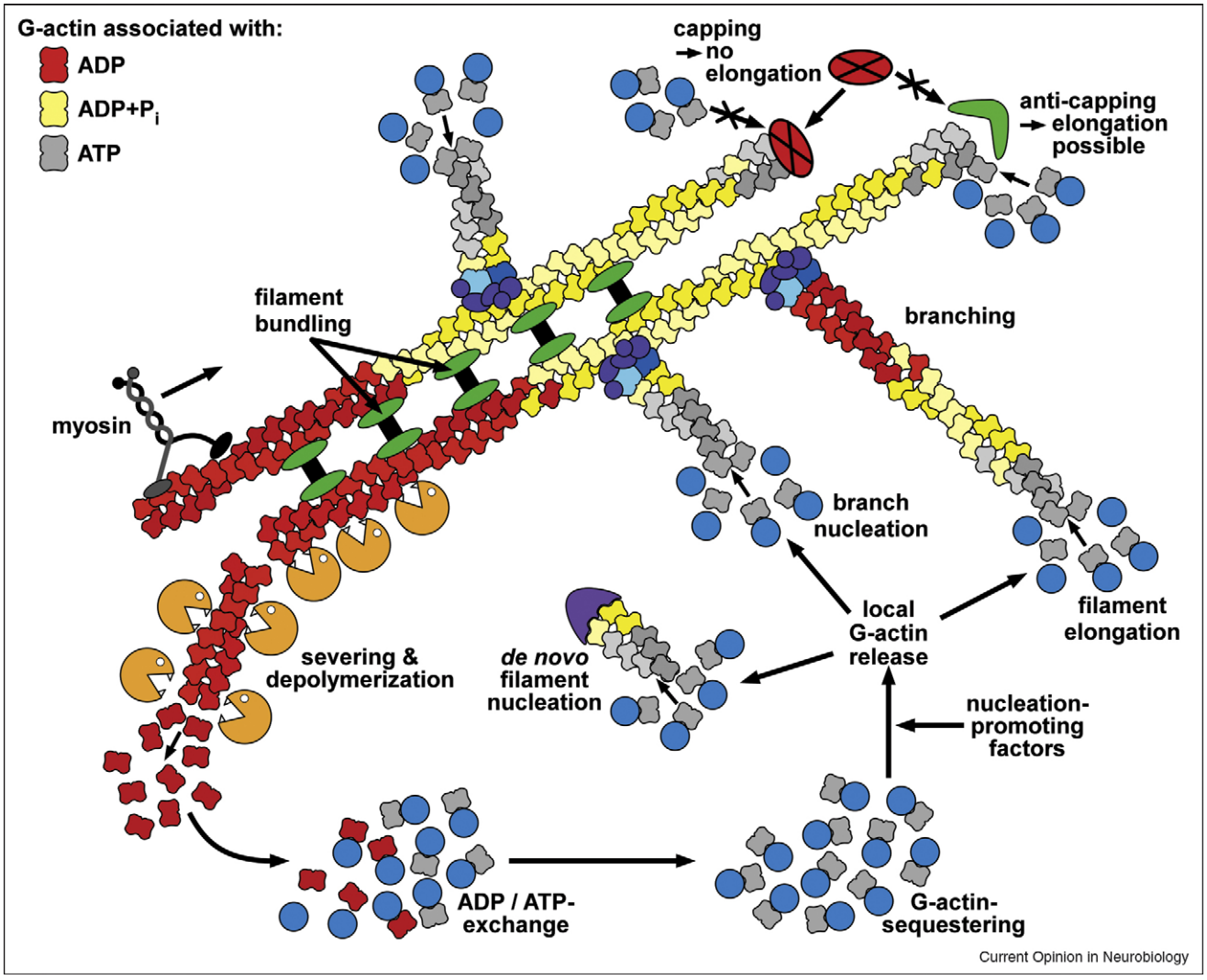
Mechanisms of neuronal polarization. In the course of axon formation, the neuronal cytoskeleton undergoes intense rearrangements. Initially, all neurites are assumed to be equal **(a)**. Changes in cytosketal dynamics then occur before morphological polarization **(b)** and are retained in the axon **(c)**. The actin cytoskeleton becomes more dynamic in the growth cone of one neurite (b) and remains dynamic in the axonal growth cone (c). Similarly, polarization of microtubule stability occurs in one neurite before axon formation (b) and is pronounced in the axon of morphologically polarized neurons (c). The motor domain of kinesin-1 preferentially localizes to the future axon (b) and the axon after polarization (c). The preference of motor proteins for microtubules with increased stability or specific posttranslational modifications may mediate directed trafficking into the axon. Possibly, the centrosome is positioned at the basis of the future axon early in development and promotes early polarization of the microtubule cytoskeleton.

in structure and dynamics of the actin network can be achieved by very different molecular means (Figure 2). For instance, the globular actin (G-actin) binding protein profilin stimulates the ADP/ATP exchange of G-actin, thus refilling the pool of ATP-associated G-actin that in turn promotes actin polymerization. During neuronal polarization, the inhibition of profilin IIa via RhoA and its downstream effector ROCK indirectly promotes a destabilization of the actin cytoskeleton [11] that allows neurite formation. Similarly, inhibition of the branch nucleator ARP2/3 enhances growth, providing further evidence for the idea that a dense actin network prevents protrusive activity of microtubules [12].

While many studies carried out in cultured neurons have granted valuable insights into the regulation of neuronal polarity, these mainly rely on the knockdown or overexpression of candidate genes. More recently, the field is heading to additionally analyze candidate regulators of neuronal polarity under physiologically more stringent conditions using mouse knockout approaches. For example, knocking out Nck-associated protein 1 (Nap1), an adaptor protein of the WAVE complex thought to modulate actin nucleation (Figure 2), restrains neuronal differentiation [13]. Conversely, premature expression of Nap1 reduces migration and promotes axon formation [13]. Other studies showed that knocking out all three murine Ena/VASP proteins, which antagonize actin filament capping and promote filament bundling (Figure 2) [14], causes the loss of axonal tracts *in vivo* as well as a failure to form neurites in cell culture [15••,16]. This function of the Ena/VASP proteins in neurite formation appears to be conserved across species as MIG-10/Lamellipodin, a regulator of lamellipodia dynamics acting through Ena/VASP [17], specifies axon formation in response to directional netrin cues in *C. elegans* [18••].

The knockout approach is currently being used to reveal how putative molecular orchestrators of neuronal polarity regulate effectors of the cytoskeleton. For instance, cell division cycle 42 (Cdc42), a member of the Rho-family of GTPases has turned out to be crucial for neuronal polarization. Mice deficient in Cdc42 in the nervous system fail to form axonal tracts [19•]. Concomitantly, in Cdc42 knockout neurons the actin depolymerizing factor cofilin (Figure 2) shows an increased inactivation by phosphorylation suggesting that cofilin is a physiological downstream effector of Cdc42. In line with this finding, the active form of cofilin was found to be enriched in axonal growth cones [19•]. Surprisingly, other effectors of Cdc42 that had been implicated in neuronal polarization show a change neither in activity nor in expression, including Rho, ROCK, and Par-3/Par-6 [19•]. Arguing again for conservation of

Figure 2



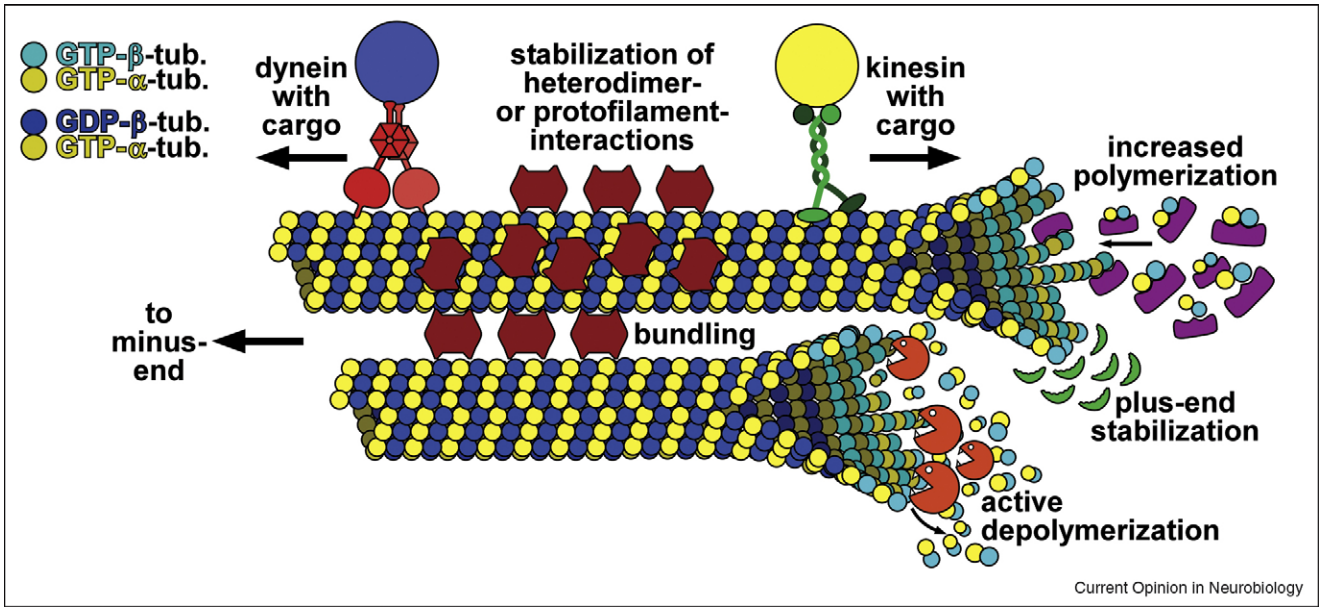
The regulation of actin dynamics. ATP-bound globular actin (G-actin) polymerizes to form helical actin filaments (F-actin), the basis of the actin cytoskeleton. In F-actin, hydrolysis of the bound ATP and subsequent dissociation of inorganic phosphate occurs. During axon formation, actin dynamics are modulated by various means. For example, actin filaments branch (initiated by the Arp2/3 complex) and can be bundled, for example, by fascin, or crosslinked (not depicted). Actin dynamics are increased by filament severing and depolymerization, for example, by cofilin. Released G-actin is set for repolymerization by profilin-mediated ADP/ATP exchange. To avoid its uncontrolled polymerization, G-actin is sequestered, for example, by profilin or thymosin β4. Elongation of existing or newly nucleated filaments occurs upon local release of G-actin from profilin. The release is triggered by nucleation-promoting factors, such as WASP-family proteins, for example, upon external signals. Capping proteins such as CapZ prevent filament elongation while anti-capping proteins, for example, of the Ena/VASP family, prevent the binding of capping proteins and thereby allow filament elongation. Myosin, an actin-based motor protein mediates transport and actin contractility.

polarity pathways across species, axon formation in *Drosophila* is regulated downstream of Cdc42 by cofilin [20] but not by the Par-3/Par-6 polarity complex [21]. Although it cannot be ruled out that the activity assays used are not sensitive enough to detect subtle differences, it might mean that, under physiological conditions, the involved pathways and effectors regulating polarity may be less complex than previously anticipated. Future studies assessing the knockouts of proposed effectors will help to clarify this issue.

Microtubule stabilization and axon formation

In line with their importance for the reorganization of cellular shape in various cell types, microtubules have been found to play an active role in axon specification, complementary to the actin network. Axonal microtubules show increased stability, and microtubule stabilization is sufficient to induce axon formation in dissociated unpolarized hippocampal neurons (Figure 1) [22••]. Similar to the wide variety of processes modulating actin dynamics, locally restricted, that is, polarized microtubule

Figure 3



The regulation of microtubule dynamics. Microtubules are hollow cylinders formed by usually 13 protofilaments of polymerized α/β-tubulin heterodimers. While α-tubulin is always GTP-bound, β-tubulin hydrolyzes GTP shortly after incorporation into the microtubule. Neurons possess various means to stabilize microtubules during axon formation. For instance, MAPs stabilize heterodimer interaction within a protofilament (e.g. Tau and MAP2) or link neighboring protofilaments (e.g. doublecortin). Specific MAPs, for example, MAP2c also facilitate microtubule bundling. Microtubule elongation is favored by factors that bind α/β-tubulin heterodimers and increase polymerization (e.g. CRMP-2) or by plus-end binding proteins (including APC, EB1, and EB3) that stabilize the dynamic plus-end. The inhibition of active destabilization at the plus-end, for example, the inhibition of the microtubule destabilizer stathmin via DOCK7, is another means to achieve increased microtubule stability during neuronal polarization. Microtubule-based motor proteins, kinesins, and dyneins mediate transport to the plus-ends and minus-ends of microtubules, respectively.

stabilization can be achieved by various means, including active stabilization of existing microtubules, increased polymerization, a reduction of microtubule destabilization and possibly microtubule bundling (for review see [23]) (Figure 3). Such stabilization probably allows microtubules to protrude with their dynamic ends more distally, thereby promoting axon formation. A growing number of studies have indeed identified regulators of neuronal polarity that function, at least partly, by modulating microtubule dynamics. The Rac activator DOCK7 (dedicator of cytokinesis 7) indirectly promotes microtubule stabilization by reducing the depolymerizing activity of stathmin from microtubule plus-ends in cultured hippocampal neurons (Figure 3) [24]. Instead, the microtubule regulator collapsin response mediator protein 2 (CRMP-2) binds tubulin heterodimers and promotes increased microtubule assembly during polarization (Figure 3) [25].

Glycogen synthase kinase-3β (GSK-3β) regulates the activity of CRMP-2 and the microtubule affinity of microtubule associated proteins (MAPs) including adenomatous polyposis coli protein (APC) and MAP1B by phosphorylation [26,27]. Indeed, the pharmacological inhibition of GSK-3β promotes the formation of multiple axons [26,27] by microtubule stabilization [22••]. The physiological relevance of these findings, however, is still unclear as mice deficient in GSK-3β show a normal polarization pattern during neuronal development [28••]. Knocking out all forms of GSK-3 will pinpoint a putative functional redundancy during neuronal polarization. Along those lines, double knockouts of the Par-1 homolog synapses of amphids defective (SAD) kinases A and B, show a distorted neuronal polarity [29••] while single knockouts appear normal. SAD A/B knockout neurons do not show a single axon, but multiple processes of undefined axonal-dendritic identity [29••]. Furthermore, unlike axons and dendrites these processes are indistinguishable in terms of their microtubule stability [22••].

Intriguingly, characterization of an upstream regulator of SAD kinases, LKB1, allows a first glimpse on how neuronal polarization could be regulated from an external cue down to the cytoskeleton. LKB1 regulates SAD kinases in response to brain-derived neurotrophic factor (BDNF) via TrkB and cAMP-dependent protein kinase (PKA) [30•,31•]. This in turn possibly changes SAD kinase-regulated MAP binding to microtubules resulting in changes in microtubule dynamics. A similar picture emerges from the analysis of WNT signaling, another pathway involved in polarization. In *C. elegans*, WNT signaling determines the polarity of a subset of mechanosensory neurons along the anterior–posterior body axis

[32,33]. Downstream of the WNT-receptor Frizzled, the scaffold protein Dishevelled mediates microtubule stabilization via the inhibition of GSK-3β together with JNK [34]. The aforementioned axon specification by other directional cues such as netrin [18••] may similarly be transduced, at least partly, via microtubule stabilization, for example, via phosphoinositide 3-kinase (PI3K), Akt, and GSK-3β [35] or Rac-mediated reduction of stathmin-activity [24].

Taken together, these studies suggest that several different signaling pathways that regulate neuronal polarity converge downstream by affecting microtubule stability. It will be interesting to follow whether future studies will provide a direct link between regulators of microtubule dynamics, microtubule stability, and axon formation.

Feedback loops and neuronal polarization

One interesting feature of the polarization of multipolar neurons is that in the absence of external cues, as in the culture situation, all neurites are assumed to be equal, competing to become the axon described as intracellular 'tug of war' [7]. At one point, one of the neurites is then singled out to rapidly grow and form the axon. A model describing this behavior postulated that a positive feedback loop – once triggered – reinforces growth in one neurite to become the axon while internal inhibitory cues prevent the growth of the remaining neurites [36]. Interestingly, brief local microtubule stabilization [22••] or actin depolymerization [8] is sufficient to bias the fate of a neurite of yet unpolarized cells to become an axon.

This suggests that a transient manipulation of either the actin or microtubule cytoskeleton is sufficient to promote axon specification, probably activating a cascade of reinforcing events that promote sustained axonal outgrowth. Indeed, actin and microtubules mutually influence each other through molecular interactors that have not only been analyzed in fibroblasts (for review see [37]) but also in neuronal growth cones [38,39••]. Thus, actin dynamics and microtubule stabilization might jointly orchestrate neuronal polarization. In this regard, microtubule plus-end binding proteins (Figure 3), including cytoplasmic linker protein of 170 kDa (Clip-170), APC, end binding protein 1 (EB1) and EB3 are prime candidates to mediate a putative interaction between the actin cortex within the growth cone and protruding microtubules [40]. However, their exact role in polarity is currently unresolved.

A reinforcement for microtubule-driven polarized growth might be further provided by vectorial membrane and cytoplasmic flow into the future axon preceding axon formation [41]. If this cargo contains limiting factors for axon growth, for example, microtubule stabilizers or actin regulators, the axon would enrich in those factors while the minor neurites would become depleted of them. Interestingly, specific microtubule-dependent motor proteins, including kinesin-1, transport vesicles preferentially on stable microtubules [42]. The overexpressed kinesin-1 motor domain accumulates in the future axon already before morphological polarization [43••]. Hence, it may link vectorial traffic to stable microtubules predominantly present in the axon [22••]. Posttranslational modifications of microtubules might provide a simple means to channel transport [44] to the future axon, thereby linking initial neuronal polarization with molecular segregation during later stages of polarization.

To conclude, a number of different processes could reinforce a transient change in cytoskeletal dynamics to sustain axon growth. By contrast, the downregulation of such processes should have the capability to limit the growth of neurites and thus possibly axon formation. The molecular elements of these potential feedback loops remain to be characterized and it has to be shown whether these potential feedbacks function during polarization.

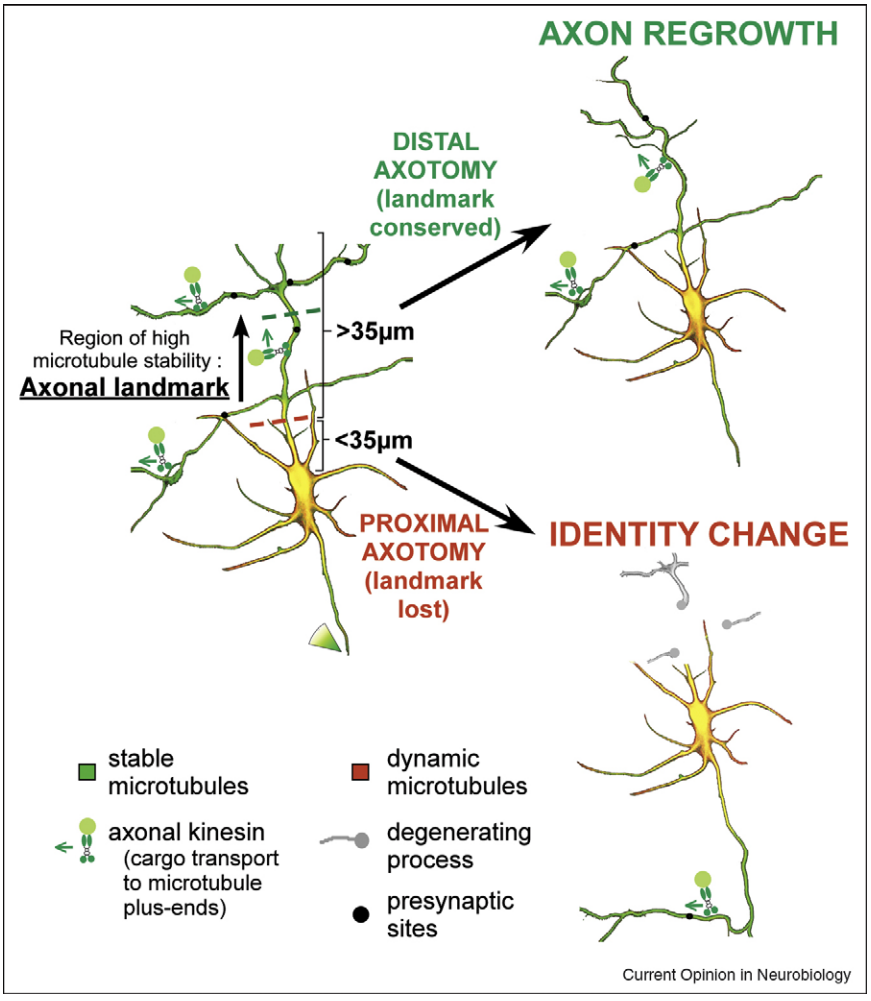
Axonal identity—the landmark hypothesis

A classical finding closely linked to the discussed feedback loops is that developing hippocampal and cortical neurons can change a future dendrite to an axon by cutting the original axon close to the cell body [45–48]. This demonstrates that all neurites have the potential to acquire axonal identity. This fate, however, seems inhibited in future dendrites of uninjured cells, presumably by the aforementioned feedback loops.

Importantly, plasticity in polarity does not end during early neuronal development. Even neurons integrated in a neuronal network, either in cultures of dissociated neurons or in organotypic hippocampal slices can respond to axonal injury by converting a mature dendrite to a new axon [49••]. Interestingly, both mature and developing neurons mainly undergo such a dendro-axonal fate change when the original axon is cut closer to the cell body than a crucial length of approximately 35 μm (Figure 4) [45,46,49••]. In addition, the time course of axon regrowth and identity change is surprisingly similar between undifferentiated and mature neurons. Hence, these data argue that the fundamental setup implementing axon-dendrite polarity in undifferentiated and functionally mature neurons is probably identical, despite the radical differences in their degree of polarization.

Stable microtubules that are enriched in the distal axon in morphologically [22••] but likewise in functionally polarized neurons [49••] appear to be part of a kind of landmark implementing axonal identity (Figure 4). Stable microtubules in the distal axon could, as part of the discussed positive feedback loops, trigger axon regrowth upon a distal axon cut. By contrast, a loss of stable microtubules, the putative axonal landmark, by a proximal axon cut seems to restart the 'axon lottery' and would in many cases allow an axon to grow from an existing dendrite. As

Figure 4



The landmark hypothesis: Microtubules may act as a landmark to specify the axon. The distribution of stable (green) and dynamic (red) tubulin along the axonal and dendritic processes is shown in the colored model neuron. Approximately 35 μm away from the cell body, the proportion of stable axonal microtubules is increased (axonal landmark). Distal axotomies (>35 μm) conserve the landmark region enriched in stable microtubules and axon regrowth is induced. Upon axon regrowth, the axonal transport, expression of axonal markers, and synaptic activity are recovered. After proximal axotomies (<35 μm) the proportion of stable and dynamic microtubules in the remaining axonal stump is similar to the one in the dendrites, and the landmark region in the distal axonal shaft, enriched in stable microtubules, is lost. The axonal process that arises from the dendrite by identity change elongates, stabilizes its microtubules further, and acquires axonal characteristics. After some days, the transformed axons will mature and become functional. Figure modified with permission from reference [49••].

postulated for early neuronal development, stable microtubules could provide a cue for axon-specific kinesins to transport their cargo into the newly formed axon. Hence, the outgrowth of a new axon could invariably be linked to transport of axonal cargo, a prerequisite for segregation of axonal and dendritic proteins. Consistently, pharmacologically induced stabilization of microtubules induces axon growth from mature dendrites [49••]. It will be now interesting to see whether this regenerative response can also be induced *in vivo*.

The centrosome and axon formation

Given that microtubule dynamics play a key role during axon formation [22••], their regulation is likely to be an important part of the neuronal polarization program. In this respect, it is currently under heavy debate whether the centrosome, one of the main microtubule organizing centers in many cell types, is actively involved in the regulation of axon growth (Figure 1) [50]. In cultured cerebellar granule neurons, the centrosome was found at the base of both the initial and the secondary axon during their emergence [51]. Similarly, the centrosome indicates the future site of axon formation in hippocampal neurons *in vitro* after the last round of precursor division [52•]. Hence, its position may indicate an instructive role for the centrosome in axon formation. However, fruit flies lacking centrioles and a resulting impairment in centrosome replication develop a largely

normal nervous system and are mainly defective in cilia formation [53]. Consistently, a localization of the centrosome to the site of axon formation is not correlated to the emergence of the axon in retinal ganglion cells in zebrafish [54•]. In this context, it is noteworthy that the position of the centrosome itself is controlled by regulators of neuronal polarity including PI3K and Cdc42 [1]. Hence, the specific centrosome localization may not be the cause for axon formation but rather a result and byproduct of axon-inducing cues [1]. Future studies in mammalian neurons will be necessary to reveal the relation between centrosomal function and axon formation.

Conclusions and future perspectives

We now understand key mechanisms regulating neuronal polarization, including actin dynamics, microtubule stabilization, and polarized membrane traffic occurring in the future axon. Candidate regulators of neuronal polarity, including profilin, cofilin, Nap1, and Ena/VASP, could affect those mechanisms to govern neuronal polarization. Still, we know relatively little about how effectors of the cytoskeleton regulate neuronal polarization. For example, we do not know whether specific effectors govern neuronal polarity simply by stabilizing microtubules. Therefore, here we intended to convey which actin and microtubule modulators have the potential to regulate polarization and how they could converge on the level of cytoskeletal dynamics. In future work, the contribution of these candidate factors has to be tested experimentally. Second, while we understand a great deal about candidate signaling pathways involved in neuronal polarization in cultured neurons, we are just starting to analyze which of those pathways are relevant under physiological conditions. Indeed, Cdc42, SAD kinases, and LKB1 emerged as physiological regulators of neuronal polarization, and it will be exciting to see which other signaling cascades affect polarization, and through which effectors. Third, one enigma in the polarity field is how neurons achieve to sustain the growth of a single axon. While it is likely that the underlying elementary processes have now been discovered, the challenge for the future will be to understand how they act onto each other on a molecular level.

Interestingly, non-growing neurites and injured axons of the central nervous system (CNS) may share mechanisms that prevent axon growth. Disorganized microtubules at the tip of the injured axon fail to support axon regeneration after spinal cord injury while microtubule stabilization enables CNS axons to grow *in vitro* on CNS myelin, a substrate inhibitory to axon growth [55•]. Since we can now change a non-growing neurite into a growing axon by pharmacological manipulations it will be exciting to see whether we could activate similar processes in pathological states to induce axon regeneration, for example, after spinal cord injury.

Acknowledgements

We would like to thank Robert Schorner for help with the preparation of Figure 1, Dr Susana Gomis-Rüth for providing the template for Figure 4, Dr Kevin Flynn for discussions and Jonathan MacKinnon for critically reading the manuscript. Frank Bradke is a recipient of a Career Development Award from the Human Frontier Science Program. This work was supported by SFB 391.

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