Microtubule assembly, organization and dynamics in axons and dendrites

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Abstract | During the past decade enormous advances have been made in our understanding of the basic molecular machinery that is involved in the development of neuronal polarity. Far from being mere structural elements, microtubules are emerging as key determinants of neuronal polarity. Here we review the current understanding of the regulation of microtubule assembly, organization and dynamics in axons and dendrites. These studies provide new insight into microtubules' function in neuronal development and their potential contribution to plasticity.

Cell cortex
The region of a cell that lies beneath the plasma membrane and contains a network of actin filaments and associated proteins.

Microtubule capture
The interaction between microtubules and components of the cell cortex that results in stabilization of the microtubules.

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Neurons are highly polarized cells with two molecularly and functionally distinct domains that emerge from the cell body: a single thin, long axon, which transmits signals, and multiple shorter dendrites, which are specialized to receive signals. The ability of neurons to polarize is crucial for synaptic transmission, and knowledge of the mechanisms that govern neuronal polarization is fundamental to our understanding of neural development, plasticity and neurodegenerative diseases. Over the past three decades, many studies have explored the cellular and molecular mechanisms that underlie neuronal polarization using cultured hippocampal neurons[1]. Remarkably, these neurons polarize in the presence of the trophic factor insulin-like growth factor 1 (IGF1)[2] and in the absence of cell-to-cell contacts or extracellular matrix molecules, to generate a single axon and multiple dendrites, which exhibit the molecular features that distinguish axons and dendrites *in vivo*. Using this model system it has been possible to identify proteins that control the establishment of neuronal polarity, including kinases, phosphatases, small GTPases and scaffolding proteins[3]. In addition, more recent studies have demonstrated that regulation of the actin cytoskeleton by phosphoinositide 3-kinase (PI3K), the Rho family of small GTPases, the Par complex and their downstream effectors is crucial for neuronal polarization[3–9]. It has also become clear that loss of polarity correlates with characteristic changes in microtubule organization and dynamics[10–12]. Thus, it has now been established that local microtubule assembly and stabilization in one neurite is a physiological signal that specifies neuronal polarization, and that changing microtubule dynamics is sufficient to alter axon and dendrite specification and development. Here we review current evidence regarding the regulation of microtubule assembly, organization and dynamics during neuronal polarization and briefly discuss microtubules' potential roles at synapses.

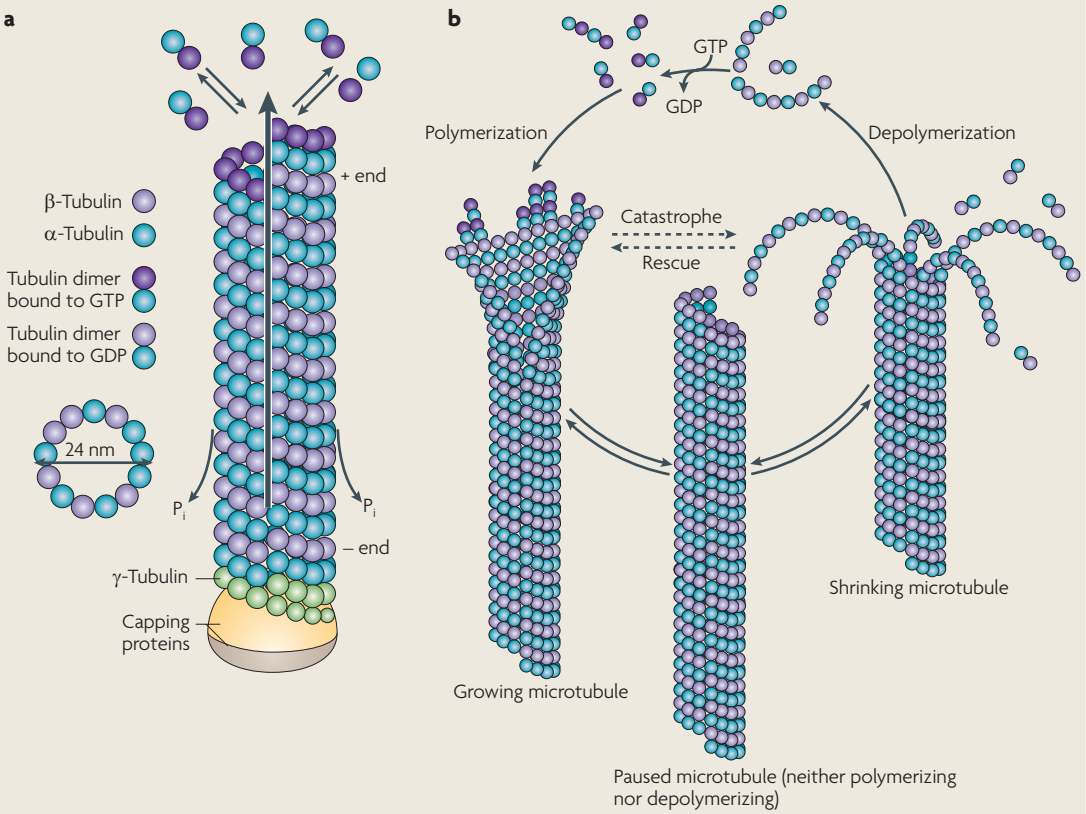
Microtubule dynamics and cell polarity

A characteristic property of microtubules (BOX 1) is their ability to undergo cycles of rapid growth and disassembly. This is known as dynamic instability and has been observed both *in vitro* and *in vivo*[13–15]. Thus, individual microtubules do not reach a steady-state length, but exist in either polymerization (growth) or depolymerization (shrinkage) states. Conversion from growth to shrinkage is termed 'catastrophe', whereas the switch from shrinkage to growth is called 'rescue'. This non-equilibrium behaviour depends on the binding of GTP at the nucleotide exchangeable site (E site) on β-tubulin during polymerization (see the figure in BOX 1). The polymerization dynamics of microtubules are central to their biological functions: they allow microtubules to rapidly reorganize, to differentiate spatially and temporally in accordance with the cell context[16], and to generate pushing and pulling forces during polymerization and depolymerization, respectively[17]. Dynamic instability allows microtubules to search the cell's three-dimensional space in order to find specific target sites on the cell periphery[18,19]. In the selective stabilization model, proposed by Kirschner and Mitchison[16], environmental signals or factors located at the cell cortex capture and stabilize a select population of dynamic microtubules searching the cellular space, and this leads to cell asymmetry. Since this model was first proposed, cell polarization mediated by selective stabilization of microtubules has been described in many cell types, including neurons. Direct evidence for microtubule capture has been obtained by observing changes in the dynamic behaviour of microtubules at

Microtubule severing
A process through which enzymes break the lattice of the microtubule to generate multiple short microtubules that have the potential to move and reconfigure.

cell cortical sites, and components of the microtubule capture machinery that is required for polarized growth have been identified[20,21]. Microtubule assembly and dynamics are further regulated by other components, including microtubule assembly-promoting factors, microtubule stabilizing factors (such as structural or classical microtubule-associated proteins (MAPs)), microtubule destabilizing factors, microtubule severing proteins and microtubule-based motors of the kinesin and dynein superfamilies.

Box 1 | **Microtubules: the basics**



Microtubules are non-covalent cytoskeletal polymers found in all eukaryotic cells that are involved in mitosis, cell motility, intracellular transport, secretion, the maintenance of cell shape and cell polarization. They are polarized structures composed of α- and β-tubulin heterodimer subunits assembled into linear protofilaments. A single microtubule is comprised of 10–15 protofilaments (usually 13 in mammalian cells) that associate laterally to form a 24 nm wide hollow cylinder (see the figure)[13,186–188]. The head-to-tail association of the αβ heterodimers makes microtubules polar structures, and they have different polymerization rates at the two ends. In each protofilament, the αβ heterodimers are oriented with their β-tubulin monomer pointing towards the faster-growing end (plus end) and their α-tubulin monomer exposed at the slower-growing end (minus end). The lateral interaction between subunits of adjacent protofilaments has been described as a B-type lattice with a seam (long arrow, part **a** in the figure). A third tubulin isoform, γ-tubulin, functions as a template for the correct assembly of microtubules[189,190]. On addition of a new dimer at the plus end, the catalytic domain of α-tubulin contacts the nucleotide exchangeable site (E site) of the previous β-subunit and becomes ready for hydrolysis; the plus end generally has a minimum GTP cap of one tubulin layer that stabilizes the microtubule structure. When this GTP cap is stochastically lost, the protofilaments splay apart and the microtubule rapidly depolymerizes. During or soon after polymerization, the tubulin subunits hydrolyse their bound GTP and become non-exchangeable. Thus, the microtubule lattice is predominantly composed of GDP–tubulin, with depolymerization being characterized by the rapid loss of GDP–tubulin subunits and oligomers from the microtubule plus end. At the minus end, contact is made between the E site of the new dimer and the catalytic region of the last subunit at the end; therefore, no GTP cap should be present.

The properties of microtubules depend on the tubulin isoforms they are made up of — there are three α-tubulins (α1, α2 and α4) and five β-tubulins (βI, βII, βIII, βIVa and βIVb) — and on how they have been altered by various forms of post-translational modification, including tyrosination, detyrosination, acetylation, polyglutamylation, polyglycylation, phosphorylation and palmitoylation[14,191–193]. Except for tubulin tyrosine ligase, the enzyme that adds a tyrosine to non-assembled α-tubulin, most of the modifying enzymes act preferentially on tubulin subunits that are already incorporated into microtubules[192,193]. Post-translational modifications of tubulin subunits mark subpopulations of microtubules and selectively affect their functions. Although they are not directly involved in determining the dynamic properties of microtubules, post-translational modifications of tubulin, such as the sequential tyrosination-detyrosination-acetylation, correlate well with the half-life and spatial distribution of microtubules[194–196].

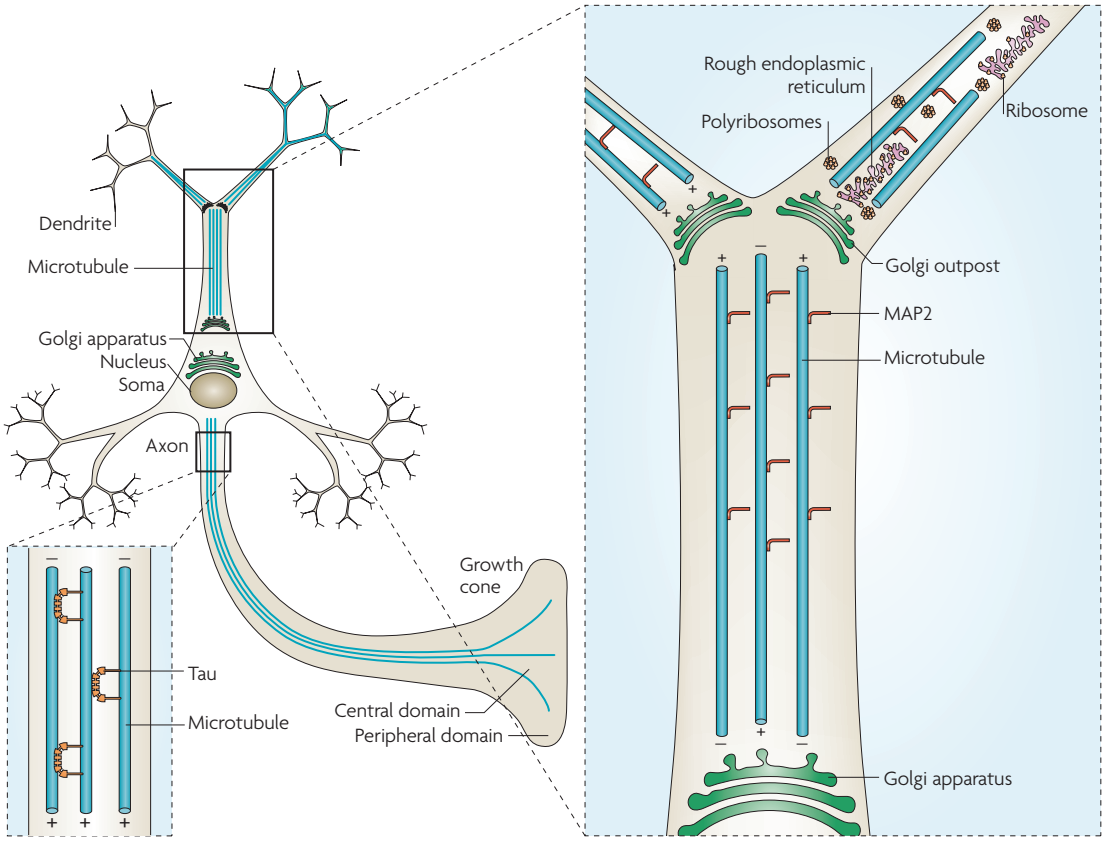


Figure 1 | **Microtubule organization and organelle distribution in axons and dendrites.** Axons have tau-bound microtubules of uniform orientation, whereas dendrites have microtubule-associated protein 2 (MAP2)-bound microtubules of mixed orientation. Dendrites also contain organelles that are not found in axons, such as rough endoplasmic reticulum, polyribosomes and Golgi outposts.

Neuronal microtubules

Microtubules form dense parallel arrays (bundles) in axons and dendrites that are required for the growth and maintenance of these neurites. This was initially inferred from ultrastructural analyses of microtubule organization in axons[22], from pharmacological studies which demonstrated that microtubule depolymerizing drugs inhibit the growth of neurites and cause their retraction[23,24], and from biochemical studies which showed that microtubule mass increases in parallel with axonal and dendritic elongation[25,26]. However, microtubule organization differs between axons and dendrites in at least two major aspects (FIG. 1). First, orientation: axonal microtubules have uniform orientation, with their plus ends facing the axon tip, whereas dendritic microtubules have mixed orientation, with their plus ends facing either the cell body or the dendritic tip. Second, microtubules differ in their complement of MAPs: for example, MAP2 is found mostly in dendrites and tau is found mainly in axons.

Neuronal microtubules are nucleated at the centrosome[27], rapidly released by the action of the microtubule severing protein, katanin[28–30], and then transported as short polymers into neurites by molecular motors[31–33]. Antibody blockade of the γ-tubulin isoform or katanin causes inhibition of axon outgrowth[28,34]. Furthermore, ninein, a protein that is present in the pericentriolar material and is involved in microtubule capture, is downregulated during neuronal development[35]. It has been proposed that the capacity of microtubules to move along axons is inversely proportional to their length, with only the shortest microtubules displaying rapid concerted motion[36]. Evidence for the transport of non-polymerized tubulin has also been obtained[37,38], but the contribution of microtubule and tubulin transport to axonal microtubule organization is still a matter of debate.

Microtubule stabilization and axon specification. Ultrastructural and quantitative immunofluorescence analyses of the composition and dynamic properties of microtubules in cultured neurons have revealed that individual axonal microtubules consist of two distinct domains that differ in their content of post-translationally modified α-tubulin subunits and in their stability[39,40] (BOX 2). One domain that stains poorly for tyrosinated tubulin is enriched in detyrosinated tubulin (Glu-tubulin) and acetylated α-tubulin, is resistant to the microtubule depolymerizing agent nocodazole and has a long half-life ($t_{1/2}$>2 h). The other domain stains strongly for tyrosinated tubulin, rapidly depolymerizes with nocodazole and has a short half-life ($t_{1/2}$<5 min). The tyrosinated tubulin-rich domain is situated at the plus end of the tyrosine-poor domain, and the transition

Nucleation
The formation of new microtubules from α- and β-tubulin heterodimers. It requires γ-tubulin protein complexes.

Box 2 | **Assessing microtubule dynamics in neurons**

Several methods have been used to asses the dynamic properties of neuronal microtubules. Stable and dynamic microtubules have been identified on the basis of their sensitivity to microtubule depolymerizing agents, such as nocodazole. In these and related experiments, stable and dynamic microtubules were distinguished through their content of post-translationally modified α-tubulin. Microtubules that are rich in tyrosinated, detyrosinated or acetylated α-tubulin were visualized using specific antibodies and immunofluorescence or immunoelectron microscopy[39,40]. In some experiments, quantitative fluorescence techniques were used to measure the ratio of tyrosinated to detyrosinated or acetylated α-tubulin in the cell body and along neurites using detergent-extracted cytoskeletons[41–43]. In other studies, the dynamic behaviour of microtubules was visualized in living or fixed cells by conventional fluorescence microscopy after microinjection of fluorescein- or biotin-labelled tubulin[43]. More recently, fluorescence speckle microscopy (FSM), an epifluorescence microscopy technique developed by Waterman-Storer in the late 1990s, has been used to analyse the dynamic properties of neuronal microtubules[197]. This technique is particularly useful for visualizing the movement, assembly–disassembly dynamics and turnover of macromolecular assemblies in living cells. In this method, low concentrations of fluorescently labelled tubulin subunits assemble with endogenous unlabelled subunits to generate structures with a speckled appearance. Changes in the pattern of fluorescence speckles correspond to the dynamics and movement of the system under analysis and can be quantified in space and time. In combination with total internal reflection fluorescence microscopy, another epifluorescence optical technique that is used to observe single molecule fluorescence at surfaces and interfaces, FSM has been used to analyse the dynamics of microtubule and actin filaments in neuronal growth cones.

between these two domains is quite abrupt. It is now accepted that the tyrosinated tubulin-rich domain assembles locally in the axon by elongating from the plus end of the stable domain. Spatial variations in the relative content of tyrosinated and acetylated α-tubulin along individual microtubules reflect differences in their dynamic properties. In growing axons, the more dynamic polymer is highly concentrated at neuritic tips, whereas the more long-lived polymer predominates in the proximal axon[41–43] (FIG. 2).

Attempts to correlate these observations with a specific role for selective microtubule stabilization in the establishment of neuronal polarity have been controversial until now. Early studies that analysed the distribution of dynamic and stable microtubules at the onset of neuronal polarization — when cells exhibit a symmetrical array of short neurites or minor processes (stage 2)[44], before one of them becomes specified as the axon (stage 3)[44] — yielded contrasting results[45–47]. A recent study[48] assessed the ratio of stable to dynamic microtubules in minor processes of stage 2 hippocampal neurons. It found that one neurite exhibited a significantly higher ratio of stable to dynamic microtubules than the other neurites. These results suggest that, in morphologically unpolarized cells, microtubule stabilization in one neurite precedes axon formation[48,49]. Microtubule stabilization was sufficient to induce axon formation, as neurons treated with low doses of taxol extended axon-like neurites that displayed a high ratio of acetylated to tyrosinated tubulin, a proximo-distal tau gradient and an absence of MAP2 (REF. 48). Moreover, selectively stabilizing microtubules in one neurite of unpolarized neurons by locally activating a membrane-permeable caged form of taxol at the growth cone or at the region adjacent to it resulted in axon formation[48]. Interestingly, photoactivation of caged taxol not only induced local microtubule stabilization, it also promoted the extension of newly polymerized microtubules to the distal part of the neurite, including the growth cone.

In cultured hippocampal neurons, a major change that accompanies the transformation of a minor neurite into the future axon occurs at the growth cone. It involves an increase in growth cone size, the expansion of the peripheral lamellipodial veil, a shortening of actin ribs, increased actin dynamics, an increase in the number and length of recently assembled dynamic microtubules, and penetration of microtubules into the central and peripheral domains of the growth cone[50–52]. Increased membrane traffic has also been observed[50]. These stages of axon outgrowth have also been described as protrusion, engorgement and consolidation[11,53].

It is likely that stable microtubules are at the heart of these modifications. For example, stable microtubules might act as nucleation seeds for the microtubule assembly and protrusion that is required before and during axon outgrowth. They might also provide tracks for the preferential binding of microtubule-based motors transporting membrane-bound organelles and regulatory macromolecular complexes[54–56]. In agreement with this latter suggestion, kinesin 1 preferentially binds to acetylated microtubules[56], and the kinesin 1 motor domain (when overexpressed) accumulates in the future axon before morphological polarization occurs[57]. These observations are important given that a complex consisting of kinesin family member 5C (KIF5C), a heavy chain of kinesin 1, kinesin light chain 1 (KLC1) and collapsin response mediator protein 2 (CRMP2; also known as DPYSL2) is involved in transporting tubulin heterodimers and oligomers to the tip of the growing axon[38]. CRMP2 is enriched at the distal part of growing axons[58], where it promotes the assembly of tubulin subunits onto the ends of existing microtubules and is required for axon formation[59] (FIG. 2). PI3K signalling also plays an important part in neuronal polarization[2,60,61]. Phosphatidylinositol-3,4,5-trisphosphate-containing vesicles interact with guanylate kinase-associated kinesin (GAKIN; also known as KIF13B) and accumulate at the tip of the future axon[62]; the anterograde translocation of this kinesin family member might also involve a preferential interaction with stable (acetylated) microtubules. Finally, stable microtubules themselves may help to establish a landmark that is required for maintaining axonal identity in functionally polarized neurons[63].

Dynamic microtubules in growth cones. Axonal microtubule bundles splay on entering the growth cone, where individual dynamic microtubules display highly complex behaviours including splaying, looping, bending and bundling (FIG. 2). This remodelling and reorganization of dynamic microtubules in the growth cone is required for persistent growth cone advance and axonal elongation, as well as for the recognition of guidance cues[64,65]. For example, low doses of vinblastine that do not affect the integrity of the existing axonal microtubule array, but that decrease microtubule dynamics, abolish

Taxol
A microtubule stabilizing agent and mitotic inhibitor used in cancer therapy.

Lamellipodial veil
A flattened and highly dynamic cell expansion that contains abundant branched and cross-linked actin filaments. It is particularly prominent at the leading edge of migrating cells and growth cones.

Actin ribs
Short actin filament bundles that are radially oriented and abundant in small growth cones.

Vinblastine
A natural alkaloid that binds to tubulin and inhibits microtubule formation.

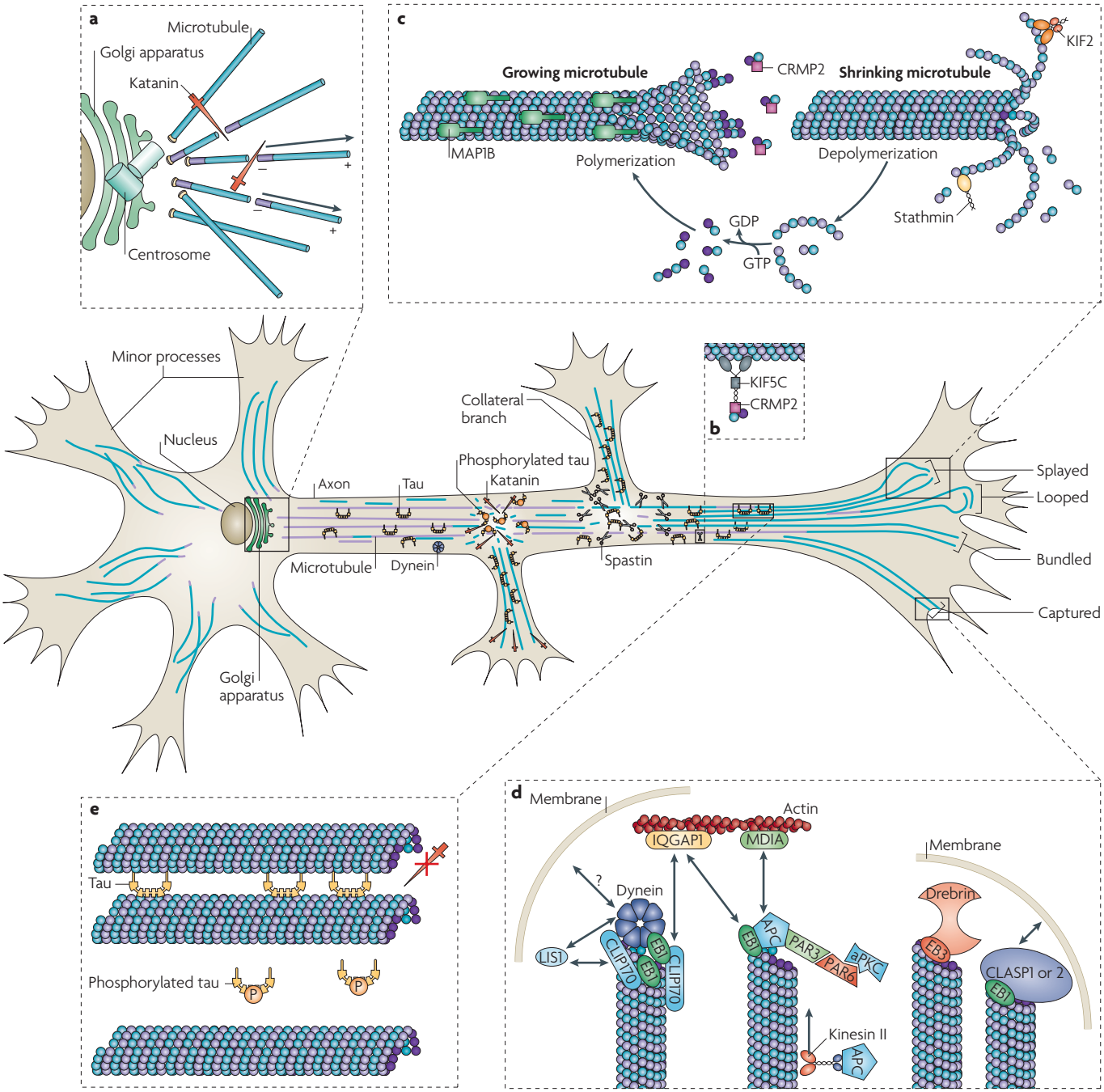


Figure 2 | **Microtubule organization in developing axons.** The organization and regulation of microtubules in a stage 3 hippocampal pyramidal neuron. Dynamic (blue) microtubules predominate in minor processes (short, unbranched neurites) and at the distal end of the axon and collateral branches, whereas stable (purple) microtubules are enriched in the proximal part of the axon. Inset **a** shows katanin-mediated release of microtubules nucleated at the centrosome. Short polymers are transported along microtubules by motors such as dynein. Inset **b** shows the transport of tubulin dimers or oligomers to the growth cone by a complex of kinesin family member 5C (KIF5C) and collpasin response mediator protein 2 (CRMP2; also known as DPYSL2). On entering the axonal growth cone microtubules splay, bend, loop, bundle or get captured at the cell cortex. Inset **c** illustrates the dynamic behaviour of splayed microtubules. Proteins such as CRMP2 promote microtubule assembly, whereas microtubule-associated protein 1B (MAP1B) contributes to the maintenance of microtubule dynamics and KIF2 and stathmin induce microtubule depolymerization. Inset **d** shows the protein machinery that is involved in microtubule capture (see also Supplementary information S1 (table)). Inset **e** shows how tau protects microtubules from katanin-induced severing, thereby contributing to microtubule stabilization and preventing excessive collateral branching. In the axon shaft, the formation of collateral branches is regulated by the action of microtubule severing proteins such as spastin. APC, adenomatous polyposis coli; aPKC, atypical protein kinase C (also known as PRKCI); CLASP, cytoplasmic linker associated protein; CLIP170, CAP-GLY domain containing linker protein 170 (also known as CLIP1); EB, end-binding protein; IQGAP1, IQ motif containing GTPase activating protein 1.

axonal elongation; the microtubules remain locked in a non-dynamic array that does not proceed through the subsequent step of microtubule bundling, which is essential for the formation of a new axonal segment[65]. It was proposed that a population of pioneer dynamic microtubules that can invade the growth cone lamellipodial veil acts as a guide for further invasion by microtubules carrying organelles; this in turn would allow further consolidation of the axon. Analysis of microtubule behaviour in living growth cones has revealed that the generation of the various microtubule configurations seems to reflect the relationship between the rate of microtubule extension and the rate of growth cone advance[64]. For example, when microtubules are splayed the amount of polymer in the growth cone seems to remain constant and the rate of polymer translocation matches the rate of growth cone advance. By contrast, when microtubules become looped the rate of polymer translocation exceeds the rate of growth cone advance and in many cases this looped morphology is observed when growth cones are in a paused state. Microtubule looping could result from the continuous polymerization of tubulin in a paused growth cone, which could cause microtubules to bend backwards when they reach the leading edge, or from a failure to get captured at the growth cone cell cortex. Microtubule bundling can occur through the coalescence of splayed microtubules or through the extension of a pre-existing bundle or loop of microtubules; during microtubule bundling the rate of growth cone advance exceeds that of microtubule translocation[64]. Finally, it is important to consider that a major function of microtubules in growth cones may be to act as sensors of cellular conditions rather than as mere structural scaffolds. By extending in various directions in the actin-rich peripheral domain, some microtubules interact with components of the cell cortex to activate signalling pathways required for regulating actin dynamics and axonal growth.

Regulation of microtubule dynamics

The observations described above strongly suggest that microtubule dynamics should be a major target of signalling pathways that regulate neuronal polarization. In fact, an increasing number of organelles and molecular systems have been identified that may allow for a precise spatial and temporal regulation of neuronal microtubule dynamics.

The centrosome. In several non-neuronal cells the centrosome and the Golgi apparatus (GA) are reoriented to a position between the leading edge and the nucleus during polarized migration[66,67]. This reorientation may be required for establishing a polarized microtubule network, as well as for the directed and polarized flow of membrane and polarity proteins. Early studies by Dotti and Banker revealed no correlation between the positioning of the centrosome and GA and the site of origin of the axon[47]. Live-cell image analyses of retinal ganglion cells in zebrafish confirmed this observation[68]. However, studies in cultured granule cells[69] and in pioneering neurons of the grasshopper[70] demonstrated a correlation between centrosome positioning and the site of axon origin. Recently, Dotti and co-workers showed that blocking the cytokinesis of neuroblasts present in hippocampal cultures from embryonic day (E) 16 rat embryos with cytochalasin D, which induces two centrosomes, led to the formation of neurons with two long axon-like neurites[71]. Conversely, microchromophore-assisted laser inactivation of the centrosome in *Drosophila melanogaster* neurons impaired axon formation[71]. Thus, appropriate positioning of the centrosome–GA complex might have an instructive, facilitatory role in axon specification. However, when neurite outgrowth is generated several hours after the last mitosis, the positioning of the centrosome may not be important for initial neuronal polarization. In the absence of cell division orientation mechanisms, axon formation might proceed at a much slower rate, which would explain the prolonged 'tug of war' among minor neurites that has been described in stage 2 hippocampal neurons obtained from E18 embryos[72]. Although no mechanisms for centrosome reorientation have been described in early postmitotic neurons, it is possible that it could involve microtubule capture events similar to those described in budding yeast and migrating cells. An obvious candidate to anchor microtubules and generate pulling forces on them that could reorient the centrosome is the minus end-directed motor complex dynein and its regulators (reviewed in REF. 67). If dynein did have this role, such a mechanism would probably rely on plus end-associated protein complexes tightly controlling microtubule dynamics at the cell cortex.

Microtubule plus end tracking proteins (+TIPs). +TIPs are proteins that associate and specifically accumulate at the plus ends of microtubules, allowing control of microtubule dynamics, growth directionality and interactions with components of the cell cortex (Supplementary information S1 (table))[73–85]. Recent studies have shown the importance of +TIPs in neurite extension and axon outgrowth (FIG. 2d). In cultured hippocampal pyramidal neurons, adenomatous polyposis coli (APC) initially localizes to the growth cones of all minor neurites but then becomes enriched at the tip of the future axon, before this neurite grows considerably longer than the others[86]. APC localization to the tip of the prospective axon is required for the proper targeting of PAR3 to the axonal growth cone, where its interaction with PAR6 and atypical protein kinase C (aPKC; also known as PRKCI) is required for neuronal polarization[87]. Expression of APC or end-binding protein 1 (EB1; also known as MAPRE1) mutants, which results in sequestration of APC from the axon tip, or APC depletion by RNA interference (RNAi) markedly inhibits axon outgrowth in cultured neurons, possibly by interfering with microtubule capture mechanisms at the cell cortex[88,89]. However, it should be noted that APC might not be required for polarization in all neurons: complete knockdown of APCs in *D. melanogaster* neurons does not affect axon outgrowth or targeting[4,90].

EB1 and EB3 have been found throughout neurons on microtubule plus ends. EB1 is upregulated during

Cytochalasin D
A cell-permeable and potent inhibitor of actin polymerization.

axon formation[91] and there is some evidence which suggests that it is required for axon elongation in hippocampal neurons[92]. A recent study showed that the actin binding protein drebrin binds to EB3 but not EB1 and is enriched in growth cone filopodia[93]. When the EB3–drebrin interaction was disrupted, growth cone formation and neurite extension were impaired. These results suggest that drebrin targets EB3 to coordinate filamentous actin–microtubule interactions during neurite formation[93].

LIS1 (also known as PAFAH1B1) has also been implicated in neuronal and non-neuronal migration[94,95] and axon formation[96]. RNAi-mediated reduction of LIS1 or injection of antibodies against LIS1 delays the onset of polarization, alters growth cone shape and decreases microtubule bundling. Although LIS1 inhibition has no effect on microtubule assembly, it does reduce the ability of microtubules to penetrate into the growth cone peripheral domain and increases microtubule pauses. It has been proposed that in rapidly growing axons LIS1, acting in concert with dynein, accumulates at the cell cortex, where it interacts with assembling microtubules to allow them to resist retrograde actin flow and penetrate the growth cone peripheral domain[96].

Doublecortin (DCX), a MAP that is enriched at the tip of growing neurites, has also been implicated in microtubule bundling[97] and axon formation. *Dcx* deletion in mice results in excessively branched axonal shafts in developing neurons[98,99]. Branching is initiated at the axonal growth cone and shaft when microtubules splay apart, allowing shorter microtubules to invade nascent actin-rich areas. Therefore, it is likely that failure to maintain bundled microtubules underlies the excessive branching that is observed in DCX-knockout animals. DCX interacts with spinophilin (SPN; also known as PPP1R9B), an actin binding protein that can bundle actin filaments and target protein phosphatase 1 (PP1) to the transition zone between the axon shaft and the growth cone, where it dephosphorylates and activates DCX[100]. It has been proposed that DCX, SPN and PP1 are part of the machinery that is involved in the transition from splayed to bundled microtubules and that has a role in the consolidation phase of axon formation.

Microtubule severing proteins. Another way in which neuronal microtubules could be remodelled during axonal growth is by severing, which is prominent at the centrosome[27] but has also been detected at the growth cone[101] and at axonal branch points[102]. Katanin, which consists of a P60 and a P80 subunit, severs microtubules from the centrosome but, as it is widely distributed throughout the neuron, probably also severs microtubules elsewhere. Levels of katanin are high during axon growth but diminish when axons reach their targets[29]. Expression of dominant-negative p60 katanin inhibits microtubule severing and impairs axonal outgrowth in cultured neurons; conversely, overexpression of p60 katanin results in excess microtubule severing, which is also deleterious for axonal growth[29]. Interestingly, overexpression of p80 katanin caused an increase in process number, suggesting that a mild increase in microtubule severing reconfigures the microtubule array to promote the formation of more axons; however, collateral branching is unaltered[30]. This last observation is intriguing, as serial electron microscopic and live-cell imaging studies have provided evidence for local microtubule severing at axonal branch points[101,102]. Spastin is another microtubule severing protein; its function is primarily related to microtubule reconfiguration at branch points[103–105]. Spastin overexpression dramatically enhances branch formation, whereas depletion of spastin results in a fairly modest decrease in axon length but a dramatic reduction in collateral branch formation[106]. Interestingly, katanin's microtubule severing activity and associated axonal branching have been reported after tau depletion[107]. This suggests that there are at least two modes of branch formation by microtubule severing, one based on the local concentration of spastin and another based on the detachment of tau (which normally protects microtubules from the severing activity of p60 katanin) from microtubules (FIG. 2).

Microtubule destabilizing proteins. Proteins that act as microtubule destabilizers have also been identified in neurons. Among these are stathmin, superior cervical ganglia neural-specific 10 protein (SCG10; also known as STMN2) and SCG10-like protein (SCLIP; also known as STMN3)[108]. Although these three proteins are highly related in structure, stathmin has a cytosolic distribution whereas SCG10 and SCLIP are found in the Golgi area, along neurites and enriched in growth cones[108,109]. Stathmin and SCG10 increase the rate of microtubule catastrophes, either through tubulin sequestration or by acting at microtubule ends[108,110]. It has been proposed that the growth cone localization of SCG10 might antagonize microtubule stabilizing factors, such as structural MAPs, to promote microtubule dynamics. Indeed, recent studies have shown that loss of SCG10 function reduced neurite extension and growth cone motility, phenomena that are associated with growth cone expansion and a greater proportion of looped microtubules[111,112]. By contrast, SCLIP downregulation specifically enhanced axonal branching, independently of SCG10[112]. Following SCLIP RNAi, neurons display filopodia extending from the axonal shafts that contain fragmented acetylated microtubules at their bases and are filled by splayed dynamic microtubules. The mechanisms that underlie the different effects of SCG10 and SCLIP in neurite formation are currently not well understood, but they may reflect interactions with different partners or differential phosphorylation[112].

Another microtubule destabilizing protein that is involved in regulating axonal collateral branching is KIF2A, a member of the kinesin 13 family[32]. Originally identified as a plus end molecular motor that is expressed in juvenile brain tissue, enriched in growth cones and associated with a subpopulation of non-synaptic vesicles, it was later demonstrated that members of this kinesin superfamily depolymerize microtubules using the energy from ATP hydrolysis (reviewed in REF. 32). Interestingly, KIF2A-null mice display aberrant axonal growth due to overextension of collateral branches and decreased microtubule depolymerization at growth

Retrograde actin flow
Myosin-driven, retrograde (relative to the substratum) movement of actin filaments in lamellipodia; it has a role in cell motility and growth cone advance.

cones[113]. It was postulated that KIF2 might depolymerize individual microtubules and control their dynamics at the growth cone edge (FIG. 2).

Structural MAPs. MAP1B and tau proteins (reviewed in REFS 114,115) were among the first proteins to be implicated in regulating microtubule organization and dynamics and neuronal polarization[116–118]. Despite the controversy that arose from early studies[116–121], a consensus finally emerged that both MAP1B and tau are required for axon formation, but that there is functional redundancy between them[122,123]. Structural MAPs have other important functions beyond their ability to directly stabilize microtubules. For example, tau protects microtubules from katanin-induced microtubule severing[107] and interacts with components of the actin subcortical cytoskeleton[124]. In the case of MAP1B, there is evidence suggesting functional redundancy between phosphorylated MAP1B and EB1 (REF. 92), an interaction with tubulin tyrosine ligase[125] and involvement in the maintenance of a dynamic population of growth cone microtubules[49,126].

Kinases. It has now been demonstrated that a signalling pathway that is locally activated at the growth cone and controlled by PI3K is involved in axon specification[2,3,60]. Glycogen synthase kinase 3 (GSK3) is a downstream effector of PI3K that phosphorylates proteins involved in regulating microtubule dynamics and microtubule-based transport. Experimentally induced alterations of GSK3 profoundly affect neuronal morphology in culture. These studies have yielded contradictory results, with some showing that inhibition of GSK3 activity impaired axon outgrowth[87,88,127–129] and others showing that it enhanced axon formation[88,130–132]. Two recent studies have stressed the importance of dosage, targeting, substrate and timing for reconciling this controversy[133,134]. Strong knockdown of both GSK3 isoforms (GSK3α and GSK3β) markedly reduced axon growth in dissociated cultures and slice preparations, whereas localized inactivation of both GSK3 isoforms at the distal axon led to efficient axon elongation and partial inactivation of axon branching[133]. Some GSK3 substrates, like APC and CRMP2, require a priming kinase and are inactivated by GSK3-mediated phosphorylation, whereas others, like MAP1B, do not require a priming kinase and are activated by GSK3-mediated phosphorylation. Thus, when GSK3 is locally inactivated near the growth cone, presumably by PI3K-mediated phosphorylation, APC and CRMP2 bind to microtubule ends or tubulin subunits, promoting microtubule capture and assembly; this local inactivation preserves GSK3's effects on MAP1B, favouring microtubule dynamics[133]. By contrast, strong global GSK3 inhibition may produce excessive microtubule stability all along the axon owing to inhibition of MAP1B and redistribution of APC to the axonal shaft. However, additional studies are required as this scheme does not take into account other important substrates or regulators of GSK3, such as the kinesin light chain[135] and the phosphatase PTEN[130].

Many other kinases are important for regulating microtubule dynamics and neuronal polarization. Two recent studies have shown that accumulation of the serine and threonine kinase LKB1 (also known as STK11) and its interacting partner STRAD in a minor neurite predicts axonal fate[136] and that downregulation of either LKB1 or STRAD prevents axon differentiation, whereas their overexpression leads to multiple axon formation[136,137]. One mechanism by which LKB1–STRAD could regulate neuronal polarization involves phosphorylation and activation of SADA (also known as BRSK2) and SADB (also known as BRSK1)[138], two PAR1 (also known as MARK2)-related kinases that are required for neuronal polarization and that phosphorylate several MAPs, including tau[139]. Another mechanism might involve modulation of aPKC phosphorylation and inactivation of GSK3β at the growth cone[136], or inhibition of PAR1 (REFS 139,140).

The JUN N-terminal kinase (JNK) family of mitogen-activated protein kinases (MAPKs) is highly enriched in the nervous system and has been implicated in regulating brain development. JNK1 (also known as MAPK8)-deficient mice display a progressive loss of axonal and dendritic microtubules. In addition, MAP1B and MAP2 are hypophosphorylated in JNK1$^{-/-}$ brains, which compromises their ability to bind to microtubules and promote their assembly and stability[141]. More recently it has been shown that JNK1 phosphorylates SCG10 and negatively regulates its microtubule destabilizing activity[142]. Thus, by regulating SCG10 activity, JNK1 may play an important part in maintaining microtubule stability and efficient neurite elongation.

Microtubule–actin interactions and Rho GTPase signalling. There is mounting evidence that actin filaments significantly influence the dynamic properties of microtubules, and that reciprocal interactions between these cytoskeletal elements are important for axon specification, guidance and elongation[3,11]. Studies in *Aplysia* growth cones using fluorescence speckle microscopy and total internal reflection fluorescence microscopy (TIRFM) have allowed the direct visualization of actin filament and microtubule dynamics, and established a crucial role for actin dynamics and myosin-dependent contractility in regulating microtubule bundling during growth cone advance[143,144] (FIG. 3). These studies showed that laterally moving actin arcs interact with growing microtubules, moving and compressing microtubules from the sides of the growth cone into the central domain for initial bundling. After myosin II inhibition, microtubules spread out and unbundle, suggesting that actin–myosin II contractility does not cause stable microtubule crosslinking[144] but rather is an initial step required for subsequent MAP-mediated stable microtubule bundling in the growth cone neck during consolidation of the axon shaft[100].

Members of the Rho family of small GTPases, including Rho, Rac and CDC42, seem to be major players in regulating the crosstalk between actin and microtubules in developing neurons. For example, the Rho–Rho kinase (RHOK; also known as GRK1) signalling pathway is directly involved in promoting actin contractility[145] and myosin II-driven microtubule bundling

Actin arcs
Arc-shaped actin filament bundles that are found at the transition zone between the central and peripheral domains of growth cones. Actin arcs undergo myosin II-driven contraction to compress and bundle microtubules in the growth cone central domain.

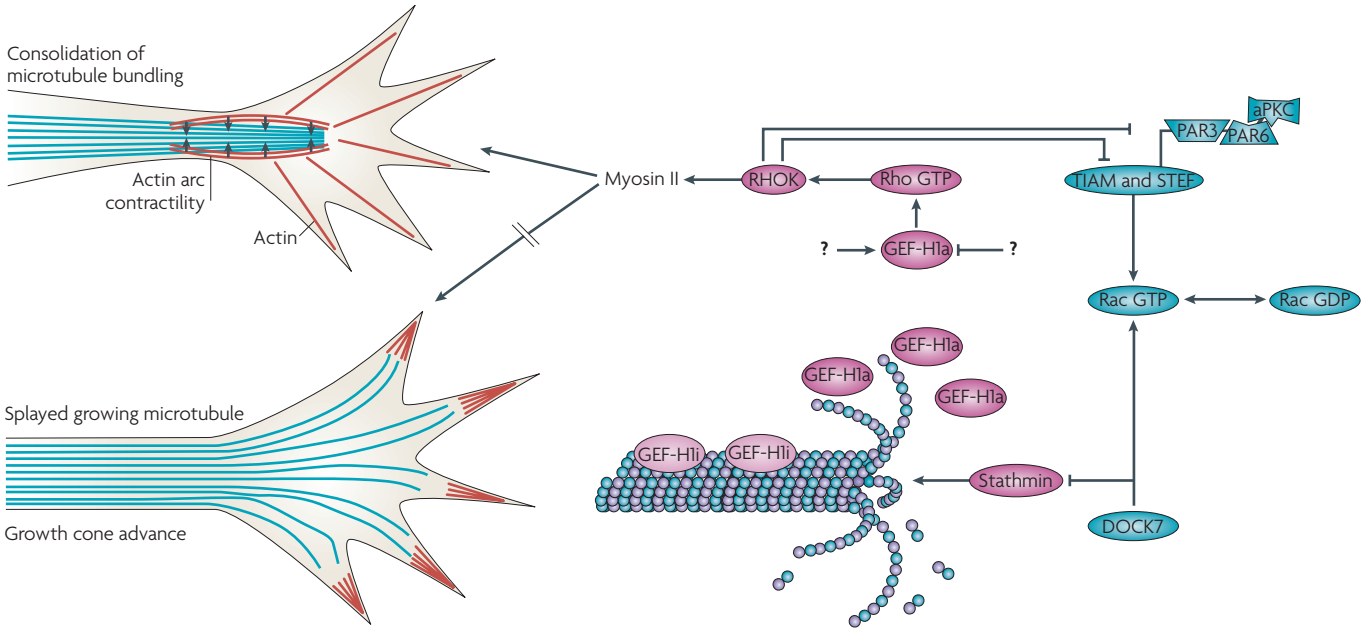


Figure 3 | **Microtubule–actin interactions in axonal growth cones.** A simplified microtubule–actin feedback loop regulating axon consolidation and growth cone advance. During consolidation, a Rho–Rho kinase (RHOK; also known as GRK1) signalling pathway phosphorylates myosin II, promoting actin arc contractility and microtubule bundling. RHOK phosphorylates and inactivates PAR3, disrupting the Par polarity complex as well as the activity of the Rac guanine nucleotide exchange factors (GEFs) TIAM1 and STEF (also known as TIAM2). Conversely, DOCK7-mediated Rac activation leads to phosphorylation and inactivation of stathmin; this could prevent GEF-H1 (also known as ARHGEF2)-induced activation of the Rho–RHOK signalling pathway and break this loop, tipping the balance in favour of Rac[150,151]. This inhibition of Rho–RHOK signalling promotes the formation of the PAR3–PAR6–atypical protein kinase (aPKC; also known as PRKCI) polarity complex, which in turn could activate TIAM1 or STEF, further enhancing Rac activity, growth cone lamellipodial expansion, microtubule penetration and splaying, and growth cone advance[3]. Thus, control of the on–off switching and direction of this loop (towards either Rho or Rac) could allow switching from growing to consolidation phases during neurite extension, and vice versa. Pointed arrows indicate positive regulation; blunt arrows indicate negative regulation.

during consolidation[144]. During the initial stages of neurite extension, inhibition of the Rho–RHOK pathway might promote growth by reducing the actin barrier that prevents microtubule advance towards the growth cone peripheral domain[146]; indeed, RHOK inhibition induces multiple axon formation in hippocampal neurons[146]. It has also been shown that RHOK phosphorylates and inhibits STEF (also known as TIAM2) and TIAM1, two guanine nucleotide exchange factors (GEFs) for Rac that associate with microtubules and are crucially involved in axon formation[52,147–149], as well as PAR3, disrupting the polarity complex[149]. GEF-H1 (also known as ARHGEF2) is a Rho GEF that associates with microtubules and becomes active on microtubule depolymerization[150]; in cultured hippocampal pyramidal neurons, suppression of LFC, the murine homologue of GEF-H1, enhances axon formation (C.C., C.H. Sung & A.C., unpublished observations). DOCK7, another GEF for Rac, induces multiple axon formation when overexpressed and prevents axon formation when it is knocked down[151]. DOCK7-mediated Rac activation leads to phosphorylation and inactivation of stathmin[151]. Thus, a feedback loop involving the opposing effects of Rho and Rac (acting through microtubule-associated GEFs) and the regulation of microtubule and actin dynamics could be crucial during axon specification (FIG. 3).

GTPase activating proteins (GAPs) also participate in neuronal polarization. Tuberous sclerosis complex 2 (TSC2) is a GAP for the RHEB GTPase, which is an activator for mammalian target of rapamycin (mTOR; also known as FRAP1) and associates with TSC1. Overexpression of TSC1–TSC2 suppresses axon formation, whereas a lack of the TSC complex produces ectopic axons in cultured neurons and *in vivo*. Inactivation of TSC1–TSC2 promotes axonal growth by upregulating the activity of SAD and tau phosphorylation[152]. It is worth noting that active PI3K and AKT phosphorylate and inactivate TSC2. Thus, TSC2 might mediate the effect of trophic factors, such as IGF1 (REF. 2), on neuronal polarization[152]. TCTEX-1 (also known as DYNLT1), a dynein light chain, has also been proposed to act as a linker between microtubules and the actin cytoskeleton, promoting Rac activation and axon outgrowth by as yet undefined mechanisms[153].

Regulation of microtubules in dendrites

So far, we have mostly discussed the roles of microtubules in axons; now we turn our attention to the distinct features of microtubules in dendrites and their contribution to dendritic development. As mentioned above, the orientation of microtubules in dendrites is one of the key features that distinguish them from axons. The presence

of distal microtubules oriented so that their minus ends point to the dendritic tip correlates with the acquisition of many distinctive features of dendritic morphology and organelle distribution, suggesting that there is a mechanistic link between microtubule organization and the distinct features of dendrites. Several studies have raised the possibility that neurons use motor-driven transport events to differentially organize the orientation of axonal and dendritic microtubules[154–156]. The mitotic microtubule-based motor CHO1 (also known as KIF23 and MKLP1) is enriched in dendrites and is required for the establishment of the dendritic microtubule array[154]. More importantly, suppression of CHO1 using antisense oligonucleotides inhibited dendritic differentiation, with arrested dendrites acquiring axonal features and microtubules of uniform polarity[154,155]. The microtubule minus end-based motor dynein has also been shown to have a crucial role in organizing dendritic arbors and the uniform orientation of axonal microtubules[156]: mutations in components of the dynein complex in *D. melanogaster* dendritic arborization (da) neurons cause a proximal shift in the distribution of branch points and dendritic organelles (such as Golgi outposts). These mutations cause dendritic cargo to be mislocalized to axons and lead to a mixed orientation of axonal microtubules.

Structural MAPs, such as MAP2 and MAP1A, have also been implicated in dendritic differentiation and maintenance. High molecular weight (HMW)-MAP2 preferentially associates with dendritic microtubules, as a result of differential protein sorting, stability and local synthesis[114,157]. MAP2 binds along the sides of microtubules and contributes to their stabilization by crosslinking adjacent microtubules[157]. Overexpression of HMW-MAP2 induces the extension of dendrite-like processes in non-neuronal cells, whereas MAP2 depletion in primary cultured neurons prevents dendritic differentiation (reviewed in REF. 157). In accordance with this, MAP2-deficient mice display a reduction in dendrite length[158], and deletion of the first 158 amino acids from the projection domain of HMW-MAP2 alters dendritic morphology in hippocampal neurons[159]. Although these phenotypes may reflect a loss of dendritic microtubule stability, adult MAP2-deficient mice showed only a moderate reduction of microtubule density[158]; this is not surprising given the functional redundancy that exists between MAP2 and MAP1B[160]. Other studies suggested that HMW-MAP2 might have additional functions at the surface of microtubules, by interacting with signalling proteins involved in dendritic remodelling and synaptic plasticity (reviewed in REF. 157). HMW-MAP2 might also contribute to segregating organelles, such as rough endoplasmic reticulum, to dendrites[161]. Kinases that phosphorylate MAP2, such as JNK1, are also crucially involved in dendritic differentiation[141,157].

MAP1A, which is enriched in mature dendrites and is structurally related to MAP1B, is also important for dendritic growth and activity-driven dendritic remodelling[162]. MAP1A depletion does not affect the elongation of primary dendrites, but it does significantly inhibit activity-induced dendritic branching and results in dendritic retraction. Although the mechanisms that underlie these effects are not fully understood, it is worth noting that MAP1A mediates interactions between microtubules and actin, and also binds components of the postsynaptic density (reviewed in REF. 115). Finally, downregulation of both the expression levels and the activity of the microtubule destabilizing protein stathmin is important for normal dendritic arborization in cultured Purkinje cells; stathmin downregulation is mediated by neuronal activity and calcium/calmodulin-dependent protein kinase II (CAMKII) phosphorylation[163]. Interestingly, CAMKII also phosphorylates MAP2, increasing its binding to microtubules[164]. Thus, stathmin and MAP2 could both mediate the regulation of microtubule stability by CAMKII that is required for dendritic growth and branching.

Regulation of microtubules at synapses

The regulation of microtubule organization and dynamics has been extensively studied during axon and dendrite formation and maintenance. By contrast, much less is known about the regulation of microtubule dynamics in synaptic terminals. Most of our current knowledge derives from studies of the *D. melanogaster* neuromuscular junction. Data from this model system have established that the conversion of a motile growth cone into a presynaptic terminal is associated with the appearance of a hairpin microtubule loop in the growth cone[165] (FIG. 4). Microtubules are found in stable synaptic boutons. Several MAPs and signalling pathways have been shown to regulate microtubule loop formation during synaptogenesis. For example, the cytoskeleton-associated *D. melanogaster* *futsch* protein associates with microtubules and is necessary for the organization of synaptic microtubules (FIG. 4). Two homozygous mutations in *futsch* alter synaptic growth and disrupt both microtubule localization and loop formation[165,166]. Bouton division and subsequent terminal branching induced by external factors also require *futsch* expression[165]. *futsch* seems to be under the control of the Bazooka (the *D. melanogaster* homologue of PAR3)-PAR6-aPKC complex[167] that, in mammalian neurons, is required for axon specification[60]. In fact, expression of a dominant-negative mutant of *D. melanogaster* aPKC (DN-PKM) resulted in a phenotype similar to that of *futsch* mutants; more importantly, the effect of DN-PKM was dependent on *futsch* expression, as presynaptic PKM overexpression did not rescue the increased microtubule fragmentation that is observed in *futsch* mutants[167]. This study also showed that aPKC mutants have alterations in synaptic efficacy, and that the synaptic localization of Bazooka and PAR6 depends on aPKC expression. A signalling pathway involving WG (also known as WNT) and SGG (also known as GSK3) has also been shown to regulate *futsch* protein binding to microtubules and the assembly of the neuromuscular junction. Loss of either *wg* or *sgg* leads to changes in *futsch* protein localization and to a decrease in splayed and looped microtubules at synaptic boutons[168,169] (FIG. 4). A similar pathway regulating presynaptic remodelling has been described in the zebrafish retinotectal projection and in mammalian cerebellar mossy fibres (reviewed in REF. 170).

Hairpin microtubule loop
A looped portion of microtubule in a stable synaptic bouton. Disassembly of hairpin microtubule loops is associated with boutons undergoing division or with sites of sprouting.

Synaptic bouton
A button-like terminal enlargement of an axon that contains synaptic vesicles filled with neurotransmitters.

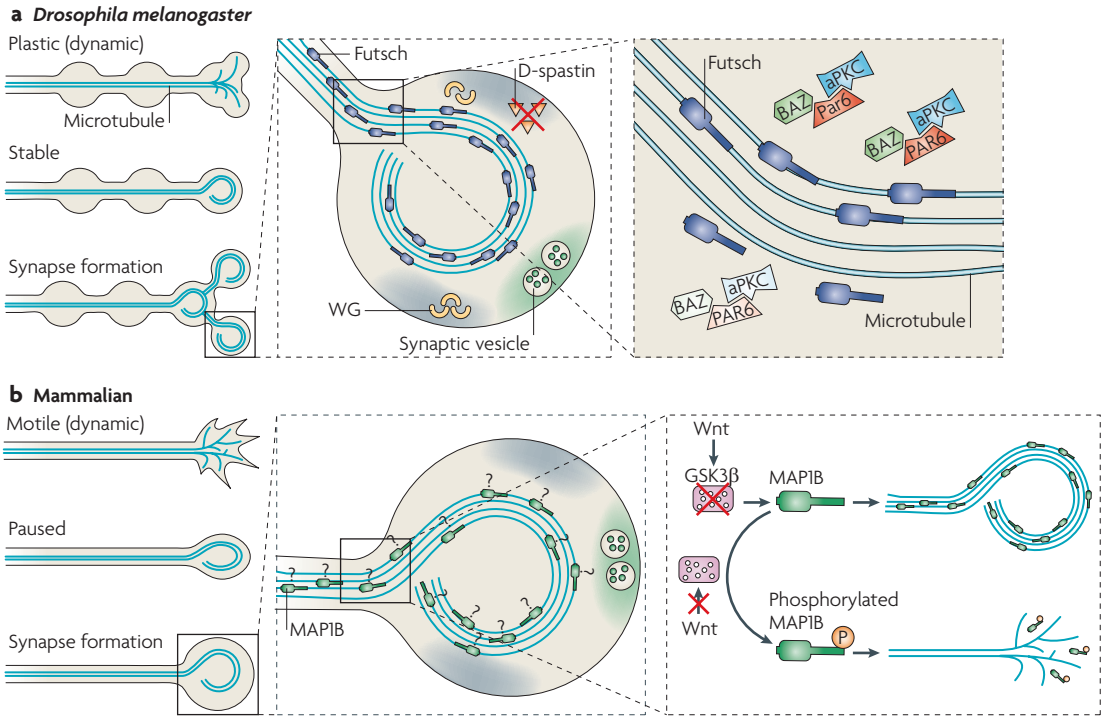


Figure 4 | **Regulation of the synaptic microtubule cytoskeleton.** A regulated microtubule architecture seems to be an essential element in the control of growth cone morphology and motility as well as synapse formation. **a** | During synaptic bouton formation at the *Drosophila melanogaster* neuromuscular junction, the irregular and highly variable branched microtubule array is indicative of an actively growing (plastic or dynamic) terminal. By contrast, the assembly of microtubule loops correlates with an arrest in synaptic bouton division (panels titled 'stable' and 'synapse formation'). The enlargement shows a bouton during synapse formation, illustrating that Futsch co-localizes with microtubules. Futsch is necessary for organizing and stabilizing synaptic microtubule loops. The active zone domain contains the machinery for neurotransmitter release, including synaptic vesicles. Peri-active zones contain proteins that play a part in modulating synaptic microtubule stability (for example, WG and D-spastin). The BAZ–PAR6–atypical protein kinase C (aPKC; also known as PRKCI) complex regulates microtubule stability by promoting the association of Futsch with microtubules. **b** | In the mammalian growth cone, similar microtubule loops (panels titled 'paused' and 'synapse formation'), which could be involved in synapse formation, have been observed[165,166]. The control of microtubule organization by microtubule-associated protein 1B (MAP1B) might represent a mechanism for regulating growth cone motility and synaptic growth. Phosphorylation of MAP1B by glycogen synthase kinase 3β (GSK3β) seems to maintain microtubules in the dynamic state that is necessary for growth cone motility and advance. As in *D. melanogaster*, the Wnt signalling pathway has been shown to modulate microtubule dynamics: Wnt signalling inhibits GSK3β to maintain microtubules in a stable and looped conformation.

Another protein that regulates synaptic microtubule stability at the neuromuscular junction is spastin. *D. melanogaster* Spastin (D-Spastin) is enriched in axons and is highly abundant in presynaptic terminals. RNAi suppression of D-Spastin causes a severe reduction of synaptic area, enhances synaptic strength and leads to an increase in stable and looped microtubules at synaptic terminals; by contrast, overexpression of D-Spastin reduces synaptic efficacy and causes loss of acetylated (stable) microtubules[171] (FIG. 4).

At postsynaptic spines the role of microtubules has been largely disregarded, as most light and electron microscopic studies have failed to detect their presence or that of MAPs here[172,173]. However, a few electron microscopic studies have provided evidence for the presence of microtubules[174–176] and MAP2 (REF. 177). Although some of these observations have been considered possible artefacts, three recent studies using live confocal imaging and TIRFM have reported that dynamic microtubules rapidly invade dendritic spines[178–180]. They showed that increasing neuronal activity enhanced both the number of spines invaded by microtubules and the time that the microtubules spent in the spines[179], and that EB3 knockdown significantly reduced the number of spines in cultured hippocampal neurons[178,180]. Together, these observations show that synaptic architecture and function are sensitive to changes in microtubule dynamics. Additional studies are required to understand the relationship between microtubule dynamics, synapse formation and functional plasticity in mammalian neurons[31,32,181].

Conclusions and future directions

During the past decade enormous advances have been made in our understanding of the basic molecular machinery involved in the development of neuronal polarity. However, most of these studies have been carried out in cultured neurons. Regulators of microtubule and actin dynamics have emerged as crucial players in this phenomenon. One of the major challenges for future studies will be to assign specific functions to the

proteins and signalling pathways involved in neuronal polarization. Most of the studies to date have used simple read-outs (such as suppression of axon formation versus extension of multiple axons) rather than characterized specific events underlying axon or dendrite formation. Methods are needed to quantitatively analyse cytoskeletal dynamics and its regulation by polarity regulators in living neurons. In addition, little is known about the machinery that is involved in regulating membrane addition[182] and its relationship to the signalling pathways that control cytoskeletal dynamics[183]. Finally, it will be essential to test whether the events and mechanisms described in cultured neurons also apply to neurons developing *in situ*[4,184]. The use of genetically modified animals and *in vivo* imaging with two-photon microscopy[185] will certainly help in this task.

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DATABASES
Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
aPKC | CDC42 | CHO1 | CRMP2 | DCX | DOCK7 | EB1 | *futsch* | GAKIN | GEF-H1 | GSK3α | GSK3β | JNK1 | KIF2A | KIF5C | KLC1 | LIS1 | LKB1 | MAP1A | MAP1B | MAP2 | mTOR | ninein | PAR1 | PAR3 | PAR6 | RHEB | RHOK | SADA | SADB | SCG10 | SCLIP | SGG | Spastin | SPN | stathmin | STEF | STRAD | TIAM1 | TSC1 | TSC2 | tau | WG

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