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Review

Cite this article: Sakakibara A, Ando R, Sapir T, Tanaka T. 2013 Microtubule dynamics in neuronal morphogenesis. Open Biol 3: 130061.
http://dx.doi.org/10.1098/rsob.130061

Received: 7 April 2013
Accepted: 7 June 2013

Subject Area:
cellular biology/developmental biology/neuroscience

Keywords:
microtubules, polarity, centrosome, axon, migration, neuron

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Microtubule dynamics in neuronal morphogenesis

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1. Summary

Microtubules (MTs) are essential for neuronal morphogenesis in the developing brain. The MT cytoskeleton provides physical support to shape the fine structure of neuronal processes. MT-based motors play important roles in nucleokinesis, process formation and retraction. Regulation of MT stability downstream of extracellular cues is proposed to be critical for axonogenesis. Axons and dendrites exhibit different patterns of MT organization, underlying the divergent functions of these processes. Centrosomal positioning has drawn the attention of researchers because it is a major clue to understanding neuronal MT organization. In this review, we focus on how recent advances in live imaging have revealed the dynamics of MT organization and centrosome positioning during neural development.

2. Introduction

Neuronal migration and polarization are key activities in brain morphogenesis, and both rely on microtubule (MT) function [1–8]. MTs have intrinsic polarity based on the asymmetry of the αβ-tubulin heterodimer. MTs exhibit two distinct ends: a slow-growing minus end at which α-tubulin subunits are exposed, and a fast-growing plus end at which β-tubulin subunits are exposed [9,10]. MT network polarity within a cellular process affects not only its dynamic nature but also directed transport along MTs [3,6,9,10]. Formation of cytoplasmic MTs is initiated by binding of αβ-tubulin heterodimers to the γ-tubulin ring complex on the surface of an MT organizing centre such as the centrosome [9,10]. MT elongation through addition of tubulin heterodimers to the plus end forms a polarized cytoskeleton. Forming fibres undergo cycles of growth and shortening, a behaviour known as dynamic instability [11,12]. Neurons form large cellular protrusions such as leading processes, axons and dendrites, which function in neuronal migration and circuit formation. These processes contain an MT cytoskeleton, and dynamic changes in MTs underlie their extension and retraction [13–20]. Furthermore, the MT cytoskeleton is critical to maintain integrity of neuronal processes in the developing brain [21]. The highly polarized MT structure provides tracks for MT-based motors to enable directional movement of intracellular cargos within processes [22]. The minus-end-directed dynein motor complex plays a pivotal role in nucleokinesis in migrating neurons [23,24]. Kinesin super family proteins (KIFs), most of which are plus-end-directed motors, show multiple effects on MT dynamics and neuronal morphogenesis [22]. For example, kinesin-2 (KIF3) reportedly polarizes the Par3 complex leading to axon

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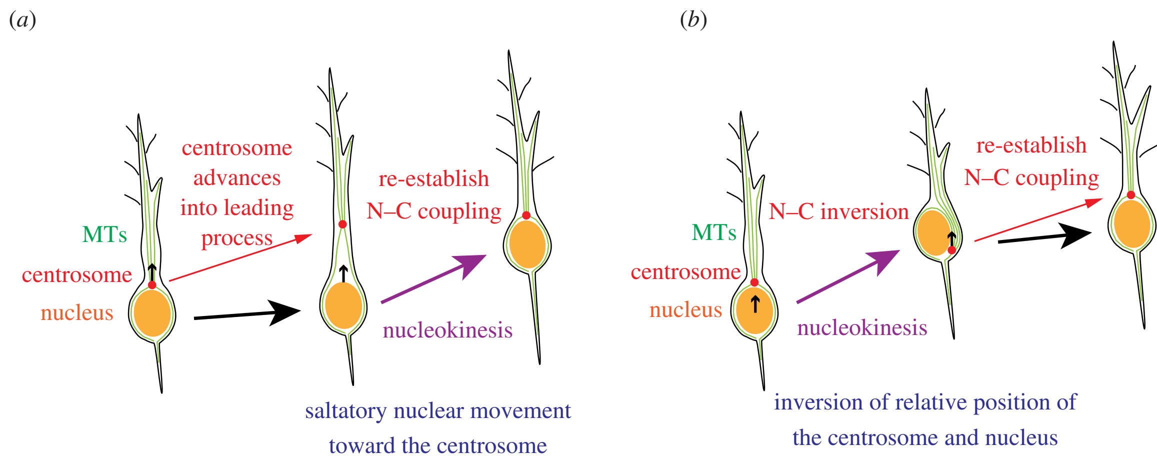

Figure 1. Potential function of MTs in nucleokinesis. (*a*) The dynein-dependent nucleokinesis model. In migrating neurons, the centrosome is positioned in front of the nucleus (N–C coupling). Cytoplasmic MTs derived from the centrosome surround the nucleus, and after the leading process elongates, the centrosome uncouples from the nucleus and advances into the leading process. Saltatory forward movement of the nucleus occurs using a minus-end-directed dynein motor along cytoplasmic MTs. (*b*) Occasionally, nucleokinesis occurs prior to centrosome movement, inverting the relative position of the centrosome and nucleus (N–C inversion). Centrosome positioning in front of the nucleus is then recaptured. This type of nucleokinesis cannot be explained by dynein-dependent pulling of the nucleus along cytoplasmic MTs. Small black arrows within the cells indicate the moving direction of centrosome or nucleus.

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specification [25,26], whereas kinesin-1 (KIF5) promotes axon formation and elongation via transporting cargos such as membrane vesicles and the CRMP2–tubulin complex [27–29]. The mitotic MT-associated motor proteins kinesin-5 (Eg5, KIF11) and kinesin-12 (KIF15) negatively regulate short MT transport, limiting both axonal growth and neuronal migration [30–32]. Kinesin-6 (CHO1, MKLP1, KIF23) and kinesin-12 (HKLP2, KIF15) reportedly regulate MT organization in axons and dendrites [33]. Other kinesin family members, such as kinesin-8 (Kip3) and kinesin-13 (MCAK), are known to control dynamic instability by promoting MT catastrophe [34,35].

Defects in MT-related genes cause human diseases ranging from severe brain malformations to mental disorders [36–41]. Point mutations in genes encoding tubulin α- or β-subunits alter MT dynamics, and cause aberrant neurogenesis, migration and circuit formation [42–44]. The *LIS1* gene encodes a key regulator of the dynein complex [45–48]. Heterozygous mutations in *LIS1* or alterations in its normal dosage cause a range of developmental abnormalities, the most severe of which is lissencephaly (also known as smooth brain) [49,50]. Mutations in the X-linked *doublecortin* (*DCX*) gene, which encodes an unconventional MT-associated protein, underlie double cortex syndrome in humans [51–55]. The MT lattice-binding protein Tau is implicated in human intellectual disability and neurodegenerative tauopathies, among which are Alzheimer's disease, frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) [56,57].

3. Microtubule function in the neuronal migration

Neuronal migration is central to proper neuronal alignment in the developing brain and requires orchestrated activity of cellular components, including cytoplasmic MTs [1,4,23]. During the development of cerebral cortex, three modes of migration have been described for excitatory neurons that are born in deep layers of brain, and then migrate radially towards the brain surface [2,4]. Those modes include somal translocation, multipolar migration and glial-guided radial migration or locomotion. Radial migration itself consists of three sequential steps: (i) leading process extension, (ii) nucleokinesis (i.e. nuclear translocation into the leading process) and (iii) retraction of the posterior end of the cell. In migrating neurons, the centrosome of migrating neurons is located in front of the nucleus (a phenomenon called N–C coupling). This configuration is considered crucial for nucleokinesis, as an N–C coupling is perturbed in migration-defective neurons [58–63].

3.1. Limitations of the dynein-based nucleokinesis model

Based on the observation that the nucleus of migrating neurons is surrounded by a centrosome-derived MT cage [64], researchers have proposed a dynein-based nucleokinesis model [23]. According to this model, nucleokinesis consists of two steps: (i) centrosome uncoupling from the nucleus and advancement into a proximal 'swelling' in the leading process, and (ii) translocation of the nucleus to restore N–C coupling (figure 1*a*). The minus-end-directed motor, dynein, provides pull forces but cannot push the nucleus in this configuration. Importantly, centrosome-derived MTs orient their minus ends towards the centrosome and their plus ends towards the periphery. Thus, to explain this type of nucleokinesis by a dynein motor-based driving force, one must assume that forward movement of the centrosome always precedes nucleokinesis.

However, transient overtaking of the centrosome by a translocating nucleus has been observed in many types of migrating neurons (N–C inversion; figure 1*b*). Umeshima *et al.* [65] showed that nucleokinesis in migrating cerebellar granule cells is independent of centrosomal positioning. Distel *et al.*, report that the translocating nucleus is not consistently led by the centrosome in migrating zebrafish tegmental hindbrain neurons [66]. Furthermore, we observed similar inversion of relative positions of the leading centrosome and following nucleus in locomoting neocortical neurons in the developing mouse cerebrum [67]. In these cases, the migration cycle consists of two steps: (i) forward nuclear translocation into the leading process associated with overtaking of the

centrosome, and (ii) re-establishment of the centrosome position in front of nucleus (figure 1*b*). Because the dynein-based nucleokinesis model cannot account for nuclear overtaking of the centrosome, a non-MT-based motor probably functions in this type of nucleokinesis.

3.2. Function of the actomyosin system in nucleokinesis

To explain mechanisms underlying the N–C inversion, the actomyosin system is an attractive candidate as a force generator [4,24]. Intracellular localization of actin filaments changes dynamically during nucleokinesis. Some investigators propose that a concentrated actomyosin system in front of a translocating nucleus pulls the nucleus [68], whereas others posit that actomyosin squeezes a translocating nucleus from the rear [69–71]. It is also plausible that cooperative force generation by dynein and actomyosin is used in nucleokinesis.

4. Microtubule regulation of neuronal polarization

Formation of functionally and structurally differentiated compartments such as axons and dendrites is a characteristic feature of neurons. Axons and dendrites emerge during a process known as neuronal polarization [26,72–74]. Each compartment contains distinct and highly organized MT-based networks whose roles in neuronal polarization have been investigated in various studies [2,3,5,6].

4.1. Function of microtubules in the spatial regulation of polarization signals

Axonogenesis, the extension of a single immature process and its differentiation into an axon, is the initial morphological event that defines neuronal polarization. Axonogenesis depends on extracellular cues that initiate signalling cascade leading to asymmetric distribution of components, including the polarity regulator, Par complex and phosphatidylinositol-triphosphate [25,26,75–77]. During the polarization process, MTs serve as tracks for translocation of polarity-regulating molecules. The plus-end-directed motor, kinesin-1, regulates spatially restricted specification of axons via its polarized transport activity [27,29]. CRMP-2, which binds to αβ-tubulin heterodimers, facilitates axon formation by transporting those heterodimers to the distal part of the axon via interaction with the kinesin-1 motor [28,78]. Local inactivation of GSK-3β by polarity signals reportedly regulates axonogenesis by controlling CRMP-2 affinity to tubulin [79]. The kinesin-3 motor protein GAKIN/KIF13B promotes axon specification by transport of phosphatidylinositol-triphosphate, which is also important for spatial restriction of axonogenesis signals [80]. These observations support the notion that plus-end-directed kinesin motors mobilize polarity signals in an emerging axon.

4.2. Regulation of microtubule stability in axon formation

Axons are typically thin, elongated processes that contain MTs with distinct organization and dynamics [3,5,6]. Altered MT stability underlies the dynamic nature of growth/retraction processes, as well as MT-based motor function. In axonogenesis, multiple protein kinases probably regulate MT remodelling in response to extracellular cues [62,63,81–85]. Kishi *et al.* [82] first found that SAD kinases phosphorylate Tau-1 S262, which is highly phosphorylated in dendrites but not in axons. Double knockout of genes encoding SAD-A and SAD-B kinases impairs axonogenesis, suggesting that initial axon formation is regulated by local inactivation of these kinases. LKB1 reportedly phosphorylates SAD kinases, and LKB1 loss of function promotes axonogenesis defects [83,84]. Others reported that local MT stabilization downstream of SAD kinases is important for axon specification in hippocampal neurons [86]. MARKs/Par-1, a kinase downstream of the aPKC/Par complex, phosphorylates DCX and reduces its MT-binding affinity [1,62,63,87,88]. The DLK–JNK pathway is implicated in axonogenesis via phosphorylation of the MT regulators DCX, MAP1B, MAP2 and SCG10/stathmin-2 [81,85,89,90], whereas others reported that local inactivation of stathmin/Op18 downstream of the DOCK7-Rac pathway regulates MT stabilization in axons [91]. Taken together, these observations suggest that regulation of MT stability by extracellular polarization signals is a key step in axonogenesis.

4.3. The role of centrosome translocation in axonogenesis

Centrosome translocation is observed during polarization of several types of neurons, but the significance of the dynamic nature of centrosomal positioning is unclear. Lefcort & Bentley [92] observed cytoskeletal organization of grasshopper pioneer neurons during axonogenesis and found that the axonal growth cone emerged preferentially at the opposite side of the cleavage point after the final cell division. The position of the centrosome after cell division corresponded to the axon emergence site, suggesting that centrosomal positioning determines axon orientation. Similar correlations of centrosome localization to the axon formation site have been observed during the early phase of axonogenesis in several types of neurons (figure 2*a*) [93–95]. These studies support the idea that centrosomal positioning plays an instructive role in determining axon orientation. However, there is a different conclusion in a recent manuscript from Dotti’s laboratory reporting that localization of N-cadherin rather than centrosomal positioning specifies the first asymmetry in developing neurons [96].

By contrast, using zebrafish retinal ganglion cells, Zolessi *et al.* [97] observed a form of axonogenesis during which an axon emerges from the basal side of the cell body after a cell delaminates from the apical domain of the neuroepithelium/ventricular zone (figure 2*b*). In this case, the centrosome remained in the apical cytoplasm opposite the site of axon formation. Distel *et al.* [66] also observed the formation of thin axon fibres from the basal side of migrating tegmental hindbrain neurons in zebrafish. Forward extension of axon was initiated from the basal side of soma, whereas the centrosome was located apically in the trailing process.

Recently, we found that mouse neocortical excitatory neurons show two distinct modes of axonogenesis (figure 2*c*) [67]. The morphology of migrating neocortical neurons changes from multipolar to bipolar as cells migrate towards the brain surface [2,4]. Interestingly, we observed that centrosomes in

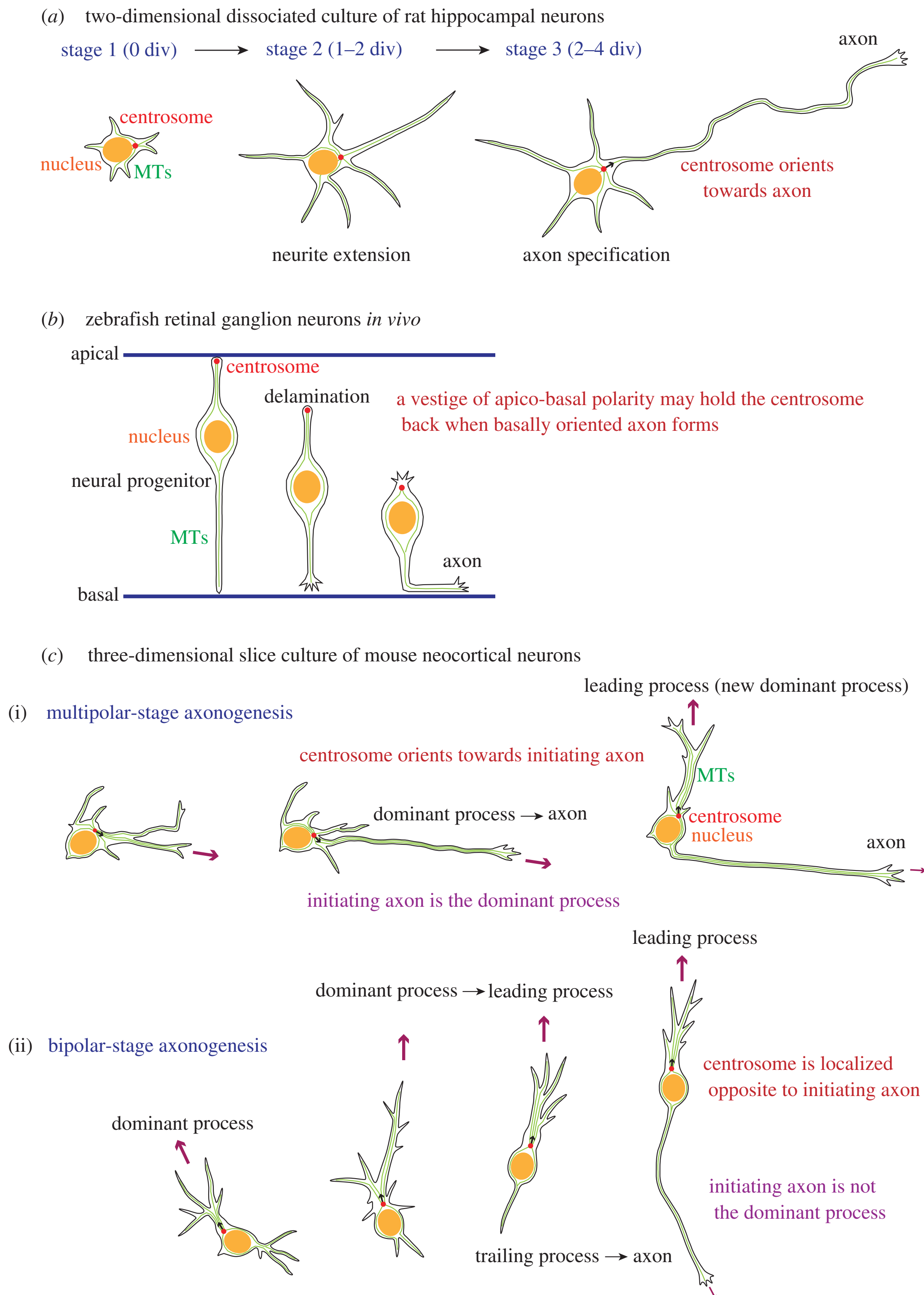

Figure 2. Centrosome positioning during neuronal polarization. (*a*) Rat hippocampal neurons cultured *in vitro* first extend multiple neurites (stage 2; 1–2 days *in vitro* (div)), and then one process differentiates into an axon (stage 3; 2–4 div). After axon specification, only the axon continues to extend, whereas other processes remain dynamic but short. In that case, the extending axon is likely to be the dominant process and attracts the centrosome (small black arrow). (*b*) In zebrafish retinal neural progenitors, the centrosome locates near the apical surface. After delamination, differentiated retinal ganglion neurons retain their bipolar shape along the apicobasal axis, and the centrosome stays on the apical side of the trailing process. The centrosome remains in the apical domain when an axon forms by extension of a basally oriented process, suggesting that a trace of apicobasal polarity may tether the centrosome. (*c*) Slice-cultured mouse neocortical excitatory neurons exhibit two modes of axonogenesis. (i) During multipolar (MP)-stage axonogenesis, an axon forms by extension of a dominant growing process (large purple arrows) targeted by the centrosome (small black arrows). (ii) Bipolar (BP)-stage axonogenesis is observed in neurons migrating radially towards the brain surface. An axon forms by extension of a thin trailing process from the rear of the cell, whereas the centrosome is located in front of the nucleus. In this case, the leading process (large purple arrow) rather than the initiating axon (small purple arrow) is likely to be the dominant process towards which the centrosome orients.

these neurons tend to move towards the most dominant growing process. Multipolar migrating neurons form an axon by extending a dominant process towards which the centrosome orients. In bipolar locomoting neurons, the axon extends from the rear opposite the dominant leading process. During this mode of axonogenesis, the centrosome remained at the

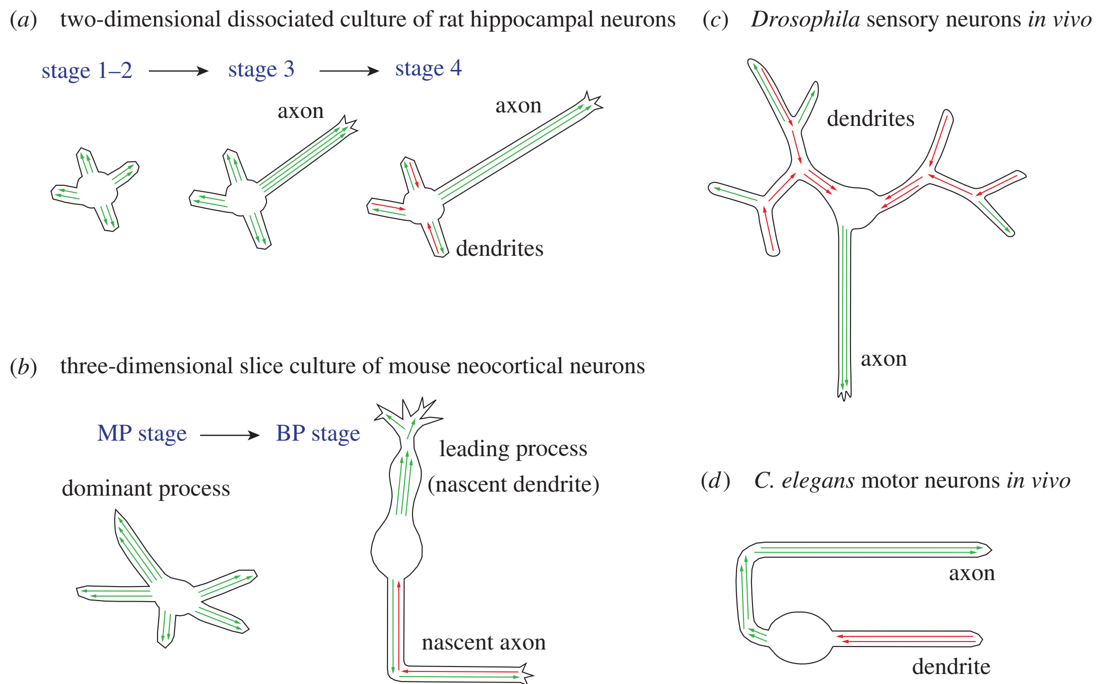

Figure 3. MT organization in axons and dendrites. (*a*) In the nascent process of *in vitro*-cultured rodent hippocampal neurons during polarization (stage 1–2), MT polarity is mostly plus-end-distal (green arrows). MTs are aligned in this manner in axons (stage 3–4). Minus-end-distal MTs (red arrows) increase in differentiating dendrites after stage 4. (*b*) Distal-oriented MT growth is predominantly observed in processes of MP-stage mouse neocortical neurons. The dominant process probably contains a greater number of MTs than do other processes and attracts the centrosome. Growing MTs are enriched in the leading process of BP-stage neurons, whereas bidirectional movements of MT plus ends are observed in the trailing axon. (*c*) *Drosophila* DA neurons exhibit highly branched sensory dendrites and a projecting axon. MTs are uniformly aligned in a plus-end-distal manner in the axon. MT polarity in dendrite shafts near the cell soma is mostly minus-end-distal, whereas short branches contain more plus-end-distal MTs. (*d*) In *C. elegans* motor neurons, polarity orientation of axonal MTs is mostly plus-end-distal. In dendrites, minus-end-distal alignment of MTs is predominant.

base of the leading process and did not target the initiating axon. Because we found that the centrosome in migrating neurons tends to move towards the most actively extending process, we concluded that centrosome positioning reflects relative protrusive activities of processes and that, in these cases, centrosome translocation during axonogenesis is likely to be a passive rather than an instructive event in orienting the axon.

5. Microtubule organization in axons and dendrites

MT polarity within neurons affects not only process morphology but also motor protein-mediated transport, both of which have a profound effect on neuronal function [3,5–8]. Early electron microscopy studies using the *in situ* MT hook assay revealed that axonal MTs orient their plus ends towards the distal tip [8,98,99]. In analysing rat hippocampal neuron polarization in *in vitro* cultures, Baas *et al.* [100,101] further showed that MT polarity in neuronal processes dynamically changes as processes differentiate (figure 3*a*). In nascent neuronal processes, most MT plus ends are distally oriented. After dendritic processes mature, bidirectional MT alignment is observed, whereas MT polarity in the extended long axon remains mostly in a distal plus orientation [101]. By contrast, MT plus ends in the trailing axon of migrating granule cells in the developing cerebellum show mixed polarity, whereas MTs are uniformly aligned towards the growing tip of the leading process [102].

Progress in live imaging technology now allows analysis of the orientation of growing MT plus ends in living neurons. Stepanova *et al.* [103] first used EB3-EGFP to specifically label growing MTs plus ends in rodent hippocampal neurons cultured *in vitro*. They observed alignment of plus-end-distal MTs in axons and mixed-polarity MTs in dendrites is in agreement with results reported earlier using the MT hook assay [100]. Several groups have applied live imaging of MT plus ends to monitor MT polarity in neurons migrating *in vivo*. Tsai *et al.* [59] first reported that MT plus ends in the leading process of locomoting neurons point primarily towards the tip of the process. Recently, we further showed that the trailing axon of locomoting neurons in mouse embryonic cerebral slices contains mixed polarity MTs (figure 3*b*) [67]. These observations are consistent with observations of MT organization made in cerebellar granule cells *in vivo*, but not in hippocampal neurons *in vitro* [100,102]. Taken together, these results suggest that MTs show unique organization in two structural components of migrating neurons—namely, a major leading process in which organization is relatively uniform and a thin trailing axon in which MTs display mixed polarity.

MT organization patterns in *Drosophila* peripheral nervous system neurons differ from those seen in vertebrate neurons [7]. In *Drosophila* dendritic arborization (DA) neurons, MTs orient plus ends distally in the axon, whereas most growing MTs in dendrites orient plus ends proximally (figure 3*c*) [104]. In *Caenorhabditis elegans* sensory and motor neurons, most axonal MTs grow towards the distal tip, whereas retrograde MT growth towards the cell body predominates in dendrites (figure 3*d*) [105,106]. Thus, MT organization patterns in nematode neurons resemble those seen in insect neurons.

In *Drosophila* DA neurons, formation of uniformly oriented MTs in axons depends on dynein function [107], and

morphogenesis of dendritic branches requires MT-based transport of Rab5-endosomes by dynein and kinesin [108]. Longer dendrites reportedly contain more retrogradely growing MT plus ends than do short branches, and MT organizing centres on Golgi outposts function in nucleation of MT polarity [109]. Overall, these findings suggest that an interplay between MTs, motor proteins and membrane organelles is critical for MT organization and neuronal process formation.

In mammalian hippocampal neurons, Golgi outposts are localized at branching points in dendrites [110], yet MT orientation nucleated from Golgi outposts has not been analysed. In slice-cultured mouse neocortical neurons, we observed generation of retrogradely growing MTs after retraction of the process tip [67], suggesting that MT severing and/or catastrophe near the tip of processes contributes to nucleation of mixed-polarity MTs [111].

Interestingly, cytoplasmic MTs can be organized independently of the centrosome in fly neurons [112,113]. It has also been demonstrated in rodent hippocampal neurons *in vitro* that once a nascent axon forms, a functional centrosome is dispensable for further axon extension [114]. These findings suggest that non-centrosomal MT nucleation functions to organize neuronal cytoplasmic MTs. In addition, transport of short MTs also participates in MT organization [33,115]. Taken together, multiple mechanisms probably regulate complex neuronal MT polarity. Further analyses of MT polarity in different neuronal subtypes and in diverse environments are needed to properly understand MT function in neuronal morphogenesis *in vivo*.

6. Concluding remarks

MTs, which are essential for cellular polarization and migration, exhibit unique structural and physical properties. Modulation of their structure is important for differentiation of immature processes into axons or dendrites. MTs serve as tracks for directed transport and as transducers of force generated by molecular motors, which control neuronal morphology and function. The importance of MT structure is highlighted by identification of mutations in tubulin genes and genes encoding MT-related proteins in patients showing brain defects or disorders. MT polarity within structural components of migrating neurons and polarized neurons has recently been described, yet how this organization is controlled requires further study. The centrosome, the main cellular MT organizing centre, displays dynamic behaviour during neuronal polarization and migration. Comparison of multiple cellular systems is currently promoting re-evaluation of its instructive function.

Acknowledgements. We thank Namiko Noguchi for her excellent illustrations. We also thank Orly Reiner for valuable comments.

Funding statement. A.S. was supported by JSPS KAKENHI (21890096, 23500410), MEXT KAKENHI (23113507), the Nitto Foundation and the Daiko Foundation. T.S. was supported in part by the Israel Science Foundation (grant no. 47/10), Minerva foundation with funding from the Federal German Ministry for Education and Research, a grant from the Chief Scientist Office at the Israeli Ministry of Health, under the frame of ERA-Net NEURON (DISCover, IMOS 3-00000-6785), the Fritz-Thyseen Foundation (grant no. Az. 10.11.2.161), the Benoziyo Center for Neurological diseases, the Kekst Family Center for Medical Genetics and the David and Fela Shapell Family Center for Genetic Disorders Research.

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