Microtubule Dynamics in Neuronal Morphogenesis

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A thesis

submitted in partial fulfillment of the

requirements for the degree of

Master of Science

University of Washington

2016

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Program Authorized to Offer Degree:

Biology

University of Washington

Abstract

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Microtubules in neurons provide structural stability, and also provide a transport route for cellular cargo carried by motor proteins. Exactly how microtubules are organized and maintained in neurons is not clear, but microtubule organizing centers (MTOCs) in neurons are not required as in non-neuronal cells. Assembly of microtubules within neurons is a major factor that affects neuronal morphology, and is regulated by tubulin availability and other nucleating factors. Maintenance of microtubules is determined in large part by post-translational modifications (PTMs), and regulation of disassembly factors. Consequences of perturbed microtubule dynamics include failure to specify the axon and dendrite of neurons, and can lead to neurological defects due to failed neuronal migration. Inhibited microtubule dynamics in neurons also lead to morphological defects and dampened synaptic plasticity. This review discusses the current state of knowledge regarding the effect of microtubule dynamics on neuronal morphogenesis.

1. Introduction: Significance of (neuronal) microtubule dynamics.

Structural support of cells derives in large part from formation and maintenance of cytoskeletal components. Actin based structures are often located at the periphery of cells. At the core of cells in nearly all organisms are microtubules composed of alternating tubulin subunits. In their best understood arrangement, α and β-tubulin dimerize and then build on a γ-tubulin ring complex to create a microtubule polymer. Equally important is the formation and maintenance of the spindle machinery in dividing cells, accomplished via microtubule dynamics that regulate attachment to and severance from centrosomes and kinetochores. Consequences of perturbed microtubule dynamics during cell division can be dire. In human germ cells, improper chromosome segregation can lead to mental retardation or non-viability[1], and in somatic cells aberrant genetic transfer can cause malignant cancers[2,3]. In a neuron, microtubules are invaluable; not only does the cell often migrate long distances for proper function, but also it must stretch and maintain the ability to change in response to various stimuli. Mutations in α-tubulin correlate highly with Lissencephaly and a small cerebellum in humans[4]. Thus complex regulation of microtubule dynamics on the cellular level supports healthy growth and development of an organism.

In the nervous system, it is clear that neuronal growth (axon/dendrite specification), migration, and morphogenesis (neurite arborization) are particularly dependent on proper microtubule dynamics. Whether a neuron achieves the proper patterns and reaches its proper targets depend on the reliable transport of cargo along microtubules. The mechanistic basis for neuron patterning is still under investigation, especially *in vivo*, but it is well established that activity of cytoskeletal proteins like actin and tubulin are crucial for proper neurite formation[5]. The role of actin in neuronal morphogenesis is not discussed further here. Rather, this review

describes our current understanding of how microtubule dynamics are regulated in neurons, whether these mechanisms are known to function in neuronal morphogenesis, and explores the relationship between microtubule-based processes and neuron function.

2. Microtubule organization in neurons: Intrinsic polarity of microtubules/neurons.

Microtubules have intrinsic polarity but their organization can and often does change. In a typical cell, α and β-tubulin dimers assemble from a "minus-end" that is capped by γ-tubulin ring complex (γ-TURC) designated as the microtubule organizing center (MTOC)[6]. α–tubulin occupies the position closer to the MTOC, whereas β-tubulin is added to the more dynamic microtubule "plus-end". The centrosome serves as the MTOC in most cells, including neurons, prior to axon and dendrite formation. In non-neuronal cells most microtubules originate from the MTOC[7] for the duration of the cell's life. In neurons however the role of a singular MTOC is not supported. In *Drosophila* larvae, the centrosome is dispensable for microtubule nucleation in mature non-ciliated sensory neurons and the centrosome is not required for maintenance of microtubule organization[8]. In mature hippocampal neurons, microtubules both detach from the centrosome and also nucleate from acentrosomal sites[9]. Rather than emanating only from the MTOC, most neuronal microtubule assembly correlates to Golgi structures, γ-TURC, or from other microtubule templates[10] (Figure 1).

Neurons also have intrinsic polarity which is established after an axon first forms. Stabilization of a subset of microtubules precedes axon formation and is detected by high levels of of acetylated α-tubulin (discussed in section 3.2)[11]. In cultured hippocampal neurons, axon formation correlates highly with centrosomal, Golgi and endosomal clusters, and centrosome motility is required for axon formation in the mouse neocortex[12,13]. However, in retinal ganglion cells of zebrafish, axon emergence does not correlate with the localization of centrosomes and

other apical determinants[14]. Similarly, after differentiation, centrosome-dependent microtubule organization is no longer essential to the growth of axons in culture[15]. In this respect, axon formation in multidendritic sensory neurons of the invertebrate nervous systems, as well as in multidendritic neurons of the mammalian CNS (hippocampal and Purkinje), have different centrosomal requirements. Whether bipolar or unipolar/pseudo-unipolar neurons also dispense with MTOC requirements has not been elucidated. Similarly, the microtubule organization in interneurons and motor neurons remain to be studied.

Across animals and neuron types, microtubule organization of the axon and dendrite differ[16,17,18]. One likely reason for this differential organization is so that compartments such as the axon initial segment (AIS) can form and the unidirectional information flow characteristic of neurons can be established[19]. The AIS contains a high density of voltage-gated Na^+ channels not found elsewhere, which are crucial to membrane depolarization and generation of an action potential[20]. Thus neuronal polarity achieved from axon/dendrite formation is essential to the function of a neuron.

3. Molecular mechanisms that regulate microtubule dynamics in neurons

Several types of mechanisms govern the growth and stability of microtubules in neurons. First, tubulin availability can preclude microtubule dynamics within a neuron. Post-translational modifications can stabilize or otherwise mark microtubules for growth/disassembly. Finally, severing enzymes and depolymerizing motors directly cause changes to microtubule length. All of these factors contribute to proper neuronal morphology and proper migration.

Tubulin chaperones such as tubulin-specific chaperone E (TBCE) are essential to facilitate dimerization of αβ tubulin subunits, and *Tbce* mutants display decreased microtubule density and subsequent degeneration of motor neuron axons[21,22,23]. Also essential to the proper

density of axonal microtubules are the availability of nucleation sites, which could be γ-tubulin and associated proteins, golgi outposts, or as of yet unidentified nucleation components. Microtubule assembly can also occur in neurites after microtubules are cut and transported, such as axonal microtubules that are moved by the minus-end motor Dynein[24]. Because microtubule plus ends are more dynamic than their minus counterparts, one possibility is that short microtubules are added to the growing plus end. Another possibility, not mutually exclusive, is that microtubules severed by Katanin or similar ATPases provide additional free ends that can be built upon[25]. It is not yet clear whether short microtubules of neurons are added at the ends, incorporated along the shaft, or a combination thereof.

The rate of polymerization at the plus end of microtubules also affects growth and stability, and is in large part determined by plus end tracking proteins (+TIPs) such as end-binding protein (EB1/EB3)[26] that track microtubules found in all neuronal compartments. Microtubule dynamics are also regulated by post-translational modifications (PTMs) such as tyrosination and acetylation of α-tubulin. Tyrosination alone promotes binding of +TIPs, cytoplasmic linker proteins, and motors to achieve rapid assembly or disassembly of the microtubule plus end[27, 28]. Other PTMs such as de-tyrosination, acetylation, and polyglutamylation are associated with microtubules that are less dynamic[29,30]. In particular, microtubule stretches are often acetylated and this correlates with stability.

Acetylated microtubules are more abundant in axons than in dendrites and are often decorated with microtubule associated proteins (structural MAPs such as Tau, MAP1 and MAP2) known to confer stability[31]. α-Tubulin acetylation also promotes recruitment of Kinesin-1, the main cargo-bearing motor responsible for deposition of ion channels that demarcate the AIS[32,33,34]. Local stabilization of microtubules causes axon formation and Kinesin-1 is required

for the initial microtubule movement to form an axon[35]. In this manner, cellular material can be transported long distances in neurons along the appropriate tracks within the proper axon or dendrite compartment. Paradoxically, acetylated microtubules are more prone to severing activity of Katanin than are deacetylated microtubules[36]. So systematic are PTMs that microtubule acetylation can mark microtubules over a certain age[37]. Thus, regulated dynamics at the microtubule plus end and appropriate PTMs along microtubule lengths are essential to development and maintenance of a neuron's cytoskeleton.

Other PTMs may also affect microtubule dynamics in neurons. For instance, polyglutamylation of microtubules promotes Spastin mediated severing of microtubules in HeLa cells[38]. As discussed, the number of microtubule ends affects microtubule dynamics. Polyglutamylation of microtubules is conspicuously high in centrioles, cilia, flagella, and neurons, but absent in other areas[39]. Indeed, loss of α-tubulin polyglutamylation coincides with abnormal KIF1A targeting and defective synaptic terminals[40]. In *Chlamydomonas* cilia, loss of long polyglutamate side chains disrupts flagellar function, which is dependent on inner-arm Dynein-microtubule interactions[41]. To what extent polyglutamylation of neuronal microtubules promotes Dynein mediated transport or movement is not yet known.

Assembly dynamics also occur at microtubule minus ends, although much more slowly. As most neuronal microtubules are not attached to a centrosome, microtubule minus ends are susceptible to Kinesin-13 (MCAK) mediated depolymerization[42]. The minus-end targeting proteins (-TIPs) such as Patronin protect microtubule minus ends from the depolymerase activity of some kinesins[43]. MAPs can confer stability to microtubules, at least upon challenge by depolymerizing agents, and motors can confer extra assembly. The dynamics of microtubule minus ends are not well understood, but Patronin seems to be a factor in regulation of neurite

arborization. An important research focus is to understand how and where these mechanisms function in individual neurons and also to understand if they differ between different neuron types and points during development.

4. Cellular consequences of perturbed neuronal MT dynamics in the nervous system

After birth, a developing neuron often migrates a great distance to mature in context with its substrate and targets. Several models describe the process through which a neuron migrates through the brain. For instance, migrating cells of the rodent medial ganglionic eminence are thought to achieve movement via nucleokinesis wherein the cell moves in the direction of the centrosome located at the leading edge[44]. Yet another mechanism observed is interkinetic nuclear migration with radial glial cells, wherein nuclei move along microtubules independent of centrosome movement but dependent on activity of the plus-end directed motor KIF1A (a Kinesin-3 family protein, known as Unc104 in *C. elegans*)[45].

Mutations that lead to disrupted nucleokinesis in mouse cerebellar granule neurons, such as loss of Lis1 and/or Doublecortin lead to severe neurological defects resembling smooth brained lissencephaly[46]. However, work in the same neuron type also shows that centrosome translocation is not essential for proper nuclear migration[47]. In many neurons, multipolar morphology allows the unique migration path through the cortex as development ensues[48]. To what extent centrosomes direct neuronal migration remains unresolved; however, both minus and plus end microtubule based processes play a signficant role in an individual neuron's migration and development.

A requirement for microtubule minus-end mechanisms in neurodevelopment has been described. For instance, expression of microtubule minus end associated ninein is regulated by the transcription facter Sip1, loss of which causes defects in axon guidance of mouse cortical

projection neurons and also correlates with defects in development of the corpus callosum[49]. Consistent with the idea that microtubule dynamics and not centrosome association per se affect migration of neurons is the finding that Patronin functions in parallel with NOCA-1, a protein in *C. elegans* with homology to vertebrate ninein, to assemble acentrosomal microtubules[50]. Future work should clarify whether or not Patronin is required for proper migration of neurons in vertebrate nervous systems, and if so, whether its role in migration is dependent on its capping or nucleation function at the microtubule minus end.

Recent evidence suggests that the state of microtubule PTMs within a neuron also affects its migration success. α-tubulin acetyltransferase (α-Tat1 or MEC-17), the enzyme primarily responsible for microtubule acetylation, is implicated in proper migration and morphogenesis of cortical projection neurons of the developing rat cerebral cortex[51]. Moreover, acetylation promotes microtubule severing, which is implicated in the tangential migration of cortical interneurons[52]. Thus, microtubule dynamics that influence PTMs are critical for proper migration of neurons and consequent brain function.

Ultimately, the success of neuronal migration depends on appropriate intracellular trafficking of material necessary for leading edge advancement and trailing edge retraction. Migrating cells depend on leading edge dynamics mediated by focal adhesion kinase (FAK) to stabilize microtubules[53]. Mutations in either α or β tubulin can result in migratory defects of neurons[54,55]. While microtubule minus end stability is important, a distinct role for the minus end lies in its role as a destination for the minus-end directed motors. For instance, Dynein complexes with Lis1 and thereafter trafficks to axons of migrating neurons[56,57]. During morphogenesis, KIF2 (also known as kinesin-13/MCAK) minus-end depolymerization regulates axon branching and also regulates axon pruning[58,59]. That KIF2 is required for proper positioning

of cortical granular neuron cell bodies is most likely due to its function restricting microtubule and subsequent axon length[60,61]. Thus the cargo bearing or depolymerizing activity of minus-end directed motors could separately or together contribute to development of neurons. While neuronal morphology and migration are connected, and KIF2 is important for the latter, the precise role of depolymerizing minus end motors in neuronal morphology is yet to be determined.

Once an axon growth cone has reached the central nervous system, diffusible signals such as Netrin direct it to the appropriate position in the central nervous system[62]. Roundabout receptors on commissural axons activated by the Slit ligand at the midline repel the axon, which then turns longitudinally along the CNS[63]. Roundabout receptors and Commisureless function in axon growth cones and are likely transported there via microtubule-based motors[64]. The extent to which diffusible signals direct growth and elongation of these axons is still being studied; however, it is clear the axons would not be in the general area were it not for the force produced by microtubule-based motors and for ability to traffic cargo continuously for long-range growth.

5. Cellular consequences of changed MT dynamics within a neuron

During and after migration of a neuron, both dendrites and axons must grow towards the appropriate direction at the proper time. As discussed above, the general morphology of a neuron can reflect its identity, age, and/or function. This relationship is especially pronounced and visibly apparent in multidendritic neurons. For instance, in class 4 dendritic arborization (C4da) sensory neurons of *Drosophila* larvae, pattern complexity increases along with size during development[65], and it is clear that full patterning is dependent on cargo provided by microtubule-based transport. In support of this hypothesis, mutant C4da neurons that lack function of dynein light chain, kinesin heavy chain, or Rab5 containing endosomes are greatly simplified with much reduced arborization[66,67].

Branching of dendrites involves nucleation of microtubules, and dendrite patterning may also be microtubule-based. For instance, self-avoidance behavior of sensory dendrites in *Drosophila* and in mammals is made possible through function of the Down syndrome cell adhesion molecule (Dscam) and/or protocadherins[68,69,70]. Recent work on *Drosophila* olfactory projection neurons demonstrates that Dscam genetically interacts with tubulin binding cofactor D gene (Tbcd)[71]. Stereotyped cell arrangements reminiscent of patterning also occur in distal medulla interneurons of the *Drosophila* visual system[72] but the mechanisms that underlie the complex arborization pattern of axons at the CNS are not fully understood. Future work will address the mechanisms of and the extent to which microtubule based processes guide self-avoidance behaviors of neurons, and this work promises to be an exciting avenue for exploration.

Not only dendrites but also axons display varied morphology, and in cases where axon morphology is not complex, the effect of microtubule dynamics can be overlooked. In nascent axons, microtubule growth labeled by EB1 signifies the presence of APC (adenomatous polyposis coli) that enhances microtubule stability[73]. Especially in developing axons, microtubule end numbers support growth and elongation[74]. Moreover, in *Drosophila* sensory neurons, axonal regeneration after ablation coincides with an increase of microtubule dynamics and reversed direction of microtubule growth[75]. Thus the regulation of microtubule assembly and microtubule dynamics are essential to axon development.

Due to the length of axons, a source of cellular energy at distal regions is vital. To this end, deposition of mitochondria at nerve terminals depends on microtubule-based, Milton-mediated transport via kinesin heavy chain[76]. Synaptic vesicle precursors that contain Rab3 depend on KIF1A-mediated transport through DENN/MADD association[77]. Further, axon specification in hippocampal neurons requires that Par proteins be properly segregated, which is

also a microtubule-dependent process[78,79]. While microtubule transport contributes to axon morphology, a distinct mechanism is that microtubule dynamics alone can affect axon morphology as well. In plated cortical neurons, distal axonal outgrowth and turning are inhibited after taxol or nocodazole induced disruption of microtubule dynamics, and conversely, growth cones turn towards local uncaging of photoactivateable taxol[80]. These results suggest that the role of microtubule dynamics may be distinct from the role of microtubule based cargo transport in neuronal morphogenesis.

Pruning of dendrites occurs during normal development, and is seen clearly during larval to pupal metamorphosis of *Drosophila*[81,82]. Recently calcium transients were shown to appear shortly prior to dendrite pruning during development[83]. Clearance of dendrites also occurs after laser ablation or other injury, which can be followed by regeneration[84]. Microtubule severing by Katanin is involved in regulation of dendrite maintenance and pruning; further evidence that regulation of microtubule severing controls neuron development lies in the finding that loss of Spastin function leads to hereditary spastic paraplegia[85,86]. Together, these findings suggest calcium signaling and microtubule dynamics are co-regulated, or at the very least correlated to control neuronal morphogenesis at the level of dendrite arborization in the peripheral nervous system.

Not unlike the phenomena in dendrites, axons also prune during metamorphosis and after injury[87]. Prior to local degeneration of *Drosophila* mushroom body axons during metamorphosis, synaptic and cytoskeletal markers are re-distributed, indicating a disruption of the microtubule cytoskeleton. Also, severed axons of mouse dorsal root ganglion neurons display Ca^{2+} mediated Calpain signaling prior to degeneration[88]. Because axon arborization is not as often readily accessible as dendrites, the relationship between microtubule dynamics and axon morphogenesis

requires further investigation. However, microtubule growth, organization, and maintenance likely inform axon arborization on some level. As axons relay information to post-synaptic partners, future studies should describe the connection between microtubule dynamics and axon targets.

6. Implications of modified microtubule dynamics for neuron function/disease

Integral to neuron function is the ability of post-synaptic dendrites to receive and integrate information. To this end, NMDA receptors transported into dendrites via KIF17 are required for signaling via neurotransmitters[89]. Further membrane proteins like the AMPA receptor must be trafficked and deposited on dendrites for proper ion channel signaling, and mouse models have shown a binding motif of Kinesin heavy chain to fulfill this role[90]. Thus, the microtubules may act as tracks for cargo supporting the localization of various membrane proteins associated with dendrites.

Also integral to neuron function is the ability of pre-synaptic axons to package and transmit neurotransmitters to targets. Loss of a Kinesin-3 family motor *immaculate connections* in *Drosophila* neuromuscular junction leads to a failure of synapse maturation, wherein growth cones can reach the proper area but not develop the electron dense membrane region that contains Ca^{2+} channels and vesicles characteristic of synaptic boutons[91]. Similarly, loss of KIF1A cause defects of mouse hippocampal synaptogenesis and learning enhancement induced by enrichment[92]. Taken together, these findings highlight the role of microtubules as transport tracks for components indispensable for neuron function. Certainly, microtubule associated proteins and microtubule PTMs contribute to the organization and stability of these tracks. Recent findings have highlighted the role of microtubules as more than mere tracks, but suggest that microtubules themselves could be regulators of neuronal function.

In mouse brain slices that contain hippocampal neurons, high frequency Schaffer Collateral stimulation induces a robust rise in excitatory post-synaptic potentials where long-term potentiation occurs[93]. Inhibition of microtubule dynamics after application of low-dose nocodazole abolishes this robust response and reduces the number of spines per dendrite, which suggests that microtubule dynamics regulate dendrite spine morphology and synaptic plasticity in hippocampal neurons[94]. The implication from this study that microtubule dynamics affects neuronal activity is correlative, as is the relationship between morphology and activity. Nevertheless, future studies to determine the extent of this relationship will surely be intriguing.

One of the most robust assays to monitor neuronal activity *in vivo* is through the use of genetically encoded calcium indicators[95]. Calcium transients via NMDA receptor signaling also induce microtubule polymerization, and microtubule based motor KIF17 is required for trafficking of NMDA receptor subunits[96,97]. Furthermore, in cultured hamster cortical neurons, microtubule organization that precedes growth cone elongation and turning requires calmodulin dependent protein kinase II[98]. That calcium signaling reports neuronal activity and may also affect microtubule dynamics could be an important clue for future research to determine to what extent microtubule dynamics affects, or is affected by neuronal activity.

Perturbed microtubule dynamics can clearly affect neuronal morphogenesis. While research on the role of microtubule dynamics to inform developmental neurobiology is an ongoing and important area, significant attention should also be paid to the role of microtubule dynamics in mature neurons. Importantly, classical and recent findings from cancer research should be reviewed in the context of microtubule dynamics in neurons. Studies on the latter should build off recent findings that different microtubule-targeting drugs (used to treat cancer) affect peripheral nerves differently[99]. An exciting discovery is that systemic application of

epothilone B in rats after CNS injury promotes axon regeneration and decreases fibroblast-induced scarring[100]. Better understanding of how microtubule-targeting drugs affect neurons can help mitigate the effect of chemotherapy-induced neuropathy. Secondarily, the efficacy of microtubule-targeting drugs that can cross the blood-brain barrier to treat neurodegenerative diseases merits attention to complement efforts to understand oxidative stress in neurons.

In extreme cases of disrupted microtubule dynamics, a neuron may fail to specify or extend the axon and dendrite. Migration could fail. Alternately a neuron could develop axons and dendrites that are malformed in shape or character due to disorganized microtubules. That disrupted neuronal morphology has negative consequences for the nervous system is obvious, but many questions remain. For instance, how microtuble dynamics affect a neuron's function is not well understood. Similarly it is not clear how the morphological defects that result from aberrant microtubule dynamics in one neuron affect other neurons. In sum, the understanding of microtubule dynamics in neuronal morphology is evolving; rather than focus on the neuronal centrosome, present and future studies will likely focus on how -TIP proteins and motors directed toward the microtubule minus-end affect the development and maintenance of neuronal morphology.

Acknowledgments

I wish to thank my thesis advisor, Dr. Jay Parrish for challenging discussions and thoughtful feedback, and Dr. Celeste Berg for helpful suggestions during preparation of this manuscript.

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Figure Legend

Figure 1. Microtubule organizing center (MTOC) or centrosome loses function in mature neurons. (A) *Left,* a typical cell with simple morphology uses the MTOC (light blue circle) to organize microtubules (dark blue lines) both early in development (A) and later in development (A'). **(B)** *Right,* the axon of a typical neuron is specified in the presence of a centrosome (light blue circle), but as the neuron develops, axonal microtubules detach from the centrosome and new microtubules nucleate from other loci.